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Degree MD Year 2006 Name of Author Youssef Fahed

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THE EFFECTS OF LIPID LOWERING TREATMENT ON THE
ARTERIAL WALL AND RENAL FUNCTION IN PATIENTS WITH
PERIPHERAL ARTERIAL DISEASE

By

Fahed Adel YOUSSEF, MB BS, MSc, FRCS Ed, FRCS (Glasg)

MD THESIS
2005

University Department of Surgery & Department of Clinical Biochemistry,
Royal Free and University College Medical School, University College London,
UNIVERSITY OF LONDON
Abstract

Background
Peripheral arterial disease (PAD) is a common condition and associated with an increased vascular risk and impaired renal function. Studies have shown a reduction in vascular risk following lipid lowering with a statin in patients with PAD, which may be attributed to a decrease in intima media thickness (IMT) and regression of atherosclerosis.

Aims
To study the effect of lipid lowering treatment with statin on patients with peripheral arterial disease. This included monitoring carotid and femoral IMT, renal function and distribution of the endothelin-1 (ET-1) and its receptors in the arterial wall of patients with advanced PAD.

Materials and Methods
Duplex ultrasound was used to measure the arterial IMT and renal function and renal blood flow (RBF) indices in hyperlipidaemic claudicants before and after short-term atorvastatin treatment. Radioimmunoassay and immunostaining were applied to study ET-1 receptors in segments of popliteal arteries obtained from amputated legs.

Results
There was regression in the carotid and femoral IMT and an improvement in both serum creatinine and urate levels after eight weeks of atorvastatin 20 mg/day (n=25). This improvement in renal function was confirmed after treatment with a different statin
(simvastatin) (n=103). Cystatin C, a more sensitive marker, decreased in addition to creatinine and urate in a prospective study (18 patients) after two months of atorvastatin treatment. However, the RBF indices did not change. The distribution of ET-1 receptors in the popliteal arteries from both normo- and hyperlipidaemic patients with advanced PAD (n=12) was also documented.

Conclusions
I am presenting evidence that statin can cause early regression in the IMT and protect renal function. This treatment may also reduce the inflammatory markers in atherosclerotic popliteal arteries.
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<td>ABPI</td>
<td>Ankle Brachial Pressure Index</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin-Converting-Enzyme</td>
</tr>
<tr>
<td>ARAS</td>
<td>Atherosclerotic Renal Artery Stenosis</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>CABG</td>
<td>Coronary Artery Bypass Graft</td>
</tr>
<tr>
<td>CCA</td>
<td>Common Carotid Artery</td>
</tr>
<tr>
<td>CFA</td>
<td>Common Femoral Artery</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary Heart Disease</td>
</tr>
<tr>
<td>DM</td>
<td>Diabetes Mellitus</td>
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<tr>
<td>eNOS</td>
<td>Endothelial Nitric Oxide Synthase</td>
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<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
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<tr>
<td>FAK</td>
<td>Focal Adhesion Kinase</td>
</tr>
<tr>
<td>FBG</td>
<td>Fasting Blood Glucose</td>
</tr>
<tr>
<td>FH</td>
<td>Familial Hypercholesterolaemia</td>
</tr>
<tr>
<td>FPP</td>
<td>Farnesyl Pyrophosphate</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular Filtration Rate</td>
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<tr>
<td>GGPP</td>
<td>Geranylgeranyl Pyrophosphate</td>
</tr>
<tr>
<td>HDL-C</td>
<td>High Density Lipoprotein Cholesterol</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and Eosin</td>
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<tr>
<td>HMG-CoA</td>
<td>3-Hydroxy-3-Methylglutaryl Coenzyme A</td>
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<td>HRT</td>
<td>Hormone Replacement Therapy</td>
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<tr>
<td>hs-CRP</td>
<td>High-Sensitivity C - reactive protein</td>
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<tr>
<td>IFG</td>
<td>Impaired Fasting Glucose</td>
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<tr>
<td>IC</td>
<td>Intermittent Claudication</td>
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<tr>
<td>IMT</td>
<td>Intima Media Thickness</td>
</tr>
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<td>LDL-C</td>
<td>Low Density Lipoprotein Cholesterol</td>
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<td>LOX-1</td>
<td>Lectinlike Oxidized LDL Receptor</td>
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<td>Lp (a)</td>
<td>Lipoprotein (a)</td>
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<td>MI</td>
<td>Myocardial Infarction</td>
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<td>MRI (3D)</td>
<td>Three Dimensional Magnetic Resonance Imaging</td>
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<td>mRNA</td>
<td>Messenger RNA</td>
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<td>Ox LDL</td>
<td>Oxidized Low Density Lipoprotein</td>
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<td>PAD</td>
<td>Peripheral Arterial Disease</td>
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<td>PAP</td>
<td>Peroxidase-Anti-Peroxidase Conjugate</td>
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<td>PDGF</td>
<td>Platelet-Derived Growth Factor</td>
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<td>RBF</td>
<td>Renal Blood Flow</td>
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<td>RF</td>
<td>Radio Frequency</td>
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<td>RIA</td>
<td>Radioimmunoassay</td>
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<td>TC</td>
<td>Total Cholesterol</td>
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<td>TG</td>
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<td>TGF</td>
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<tr>
<td>TGRLP</td>
<td>Triglyceride-Rich Lipoproteins</td>
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<td>VLDL</td>
<td>Very Low-Density Lipoproteins</td>
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<tr>
<td>VSMC</td>
<td>Vascular Smooth Muscle Cell</td>
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## SELECTED ACRONYMS FOR THE STUDIES CITED IN THE THESIS

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<td>Asymptomatic Carotid Artery Progression Study</td>
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<td>ADMIT</td>
<td>Arterial Disease Multiple Intervention Trial</td>
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<tr>
<td>AFCAPS/TexCAPS</td>
<td>Air Force/Texas Coronary Atherosclerosis Prevention Study</td>
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<tr>
<td>ARIC</td>
<td>Atherosclerosis Risk In Communities</td>
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<td>BARI</td>
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<td>CARE</td>
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<td>ECST</td>
<td>European Carotid Surgery Trial</td>
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<td>ELSA</td>
<td>The European Lacidipine Study on Atherosclerosis</td>
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<td>GEDENI</td>
<td>Spanish Group of Ischaemic Nephrology</td>
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<tr>
<td>GREACE</td>
<td>GREek Atorvastatin and Coronary-heart-disease Evaluation</td>
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<tr>
<td>HERS</td>
<td>Heart and Estrogen/Progestin Replacement Study</td>
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<td>MIRACL</td>
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<td>MRFIT</td>
<td>Multiple Risk Factor Intervention Trial</td>
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<tr>
<td>NCEP</td>
<td>National Cholesterol Education Program</td>
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ACKNOWLEDGEMENTS

There are numerous people that I would like to thank for their help during this project. Without their support it would have been impossible to finish this work.

Firstly, I would like to thank Professor G Hamilton for his constant encouragement and continuous support in both my clinical and research training in vascular surgery. I am also very grateful for Dr DP Mikhailidis's day-by-day supervision and his significant contributions.

I thank Professor A M Seifalian, Mr D Baker and Miss F Myint for their help and advice.

I also thank Professor M Winslet and Professor BR Davidson for their support throughout my training. Many thanks to Dr M Dashwood for doing the immunostaining and to Miss J Tsui for her help in the endothelin study. Special thanks to the vascular studies team (Dr K Tinkler, A Boutin and M Barbour) for their cooperation. I am also grateful to the patients involved in the studies for their willingness to participate.

On a personal note, I would like to thank my family especially my mother, my wife (Nesrin) and little Adel. I would like to dedicate this work to my father who left us early and whose dream was to see me finishing this thesis.

This project was carried out with the help of an unrestricted grant from Pfizer Ltd and with financial support from the Academic Department of Surgery, Royal Free Hospital. Royal Free and University College Medical School.
ETHICS

The Ethics Committee of the Royal Free Hospital NHS Trust approved the studies included in this thesis when needed and a written consent was obtained from each patient.


Alnaeb E, Youssef F, Mikhailidis DP, Hamilton G. Short term lipid-lowering treatment with atorvastatin improves renal function but not renal blood flow indices in patients with peripheral arterial disease. Angiology 2006;57: 65-71
PRESENTATIONS

Youssef F, Seifalian AM, Mikhailidis DP, Hamilton G. The early effect of lipid-lowering treatment on patients with peripheral arterial disease.

Presented at the International Angiology annual meeting (Afro-Chap-1), Cairo, Egypt, (November 2001)

Youssef F, Seifalian AM, Jagroop IA, Myint F, Baker D, Mikhailidis DP, Hamilton G. The early effect of lipid-lowering treatment on carotid and femoral intima media thickness (IMT).

Presented at the XV Annual meeting of the European Society for Vascular Surgery (ESVS), Switzerland (September 2001)

Youssef F, Seifalian AM, Mikhailidis DP, George Hamilton. The effects of short-term treatment with statin on renal function in patients with peripheral arterial disease.

Presented at the VI Annual meeting of the Reno-vascular forum, St Thomas Hospital, London, (March 2002)

Alnaeb E, Youssef F, Mikhailidis DP, Hamilton G. Short term lipid-lowering with atorvastatin improves renal function but not renal blood flow in patients with peripheral arterial disease.

GRANTS AND PRIZES

Prize of the best paper presentation in the junior vascular surgeon session at the XV Annual Meeting of the European Society for Vascular Surgery (ESVS), Switzerland (September 2001).

Unrestricted grant from Pfizer (£35,000) to measure the early effects of atorvastatin treatment on the arterial intima media thickness (IMT).

Unrestricted grant from Pfizer (£28,000) to investigate the effects of statin treatment on the renal function.
HYPOTHESIS

Peripheral arterial disease is associated with a markedly increased vascular risk. Surgical and medical management of patients with peripheral arterial disease have improved leading to a decrease in morbidity and mortality. There is also evidence showing that lipid lowering treatment improves the symptoms associated with intermittent claudication. However, there is little information on the possible effect of statin treatment on arterial intima media thickness, renal function and arterial wall. This prompted me to investigate these variables in patients with more advanced peripheral arterial disease. This research project is based on the hypothesis that short-term statin treatment is associated with beneficial effects on the arterial wall intima media thickness in both the carotid and femoral arteries in these patients. In turn, any improvement in the arterial function/structure may translate in better renal function (e.g. increased renal blood flow). There is evidence that raised serum creatinine levels are predictors of the vascular risk. By improving renal function in patients with peripheral arterial disease, lipid lowering treatment with a statin may result in additional vascular risk reduction. These early effects of statin treatment may be related to changes in the local arterial endothelin-1 and its subreceptors and the other inflammatory markers. Such receptors may prove to be targets for future drug design.
STATEMENT OF ORIGINALITY

After obtaining the ethical committee approval, I conducted the work leading to this thesis including preparing the protocols for the different projects in this thesis. I also did all the recruitment and the follow up for the patients involved in the studies. I performed/attended the Duplex ultrasound scans for the intima media thickness and the renal blood flow measurements. I collected and prepared the samples from amputated leg arteries and participated in the immunostaining and histopathological analysis of the slides. I also collected the blood samples and analysed the results for the studies. I collated the data and performed the statistical analysis for the results. During the period of this research degree, the results were presented in different local, national and international surgical meetings and submitted and published most of my work in different medical journals.
PATIENT RECRUITMENT

Hyperlipidaemic claudicants were included in the different studies in this thesis. Patients were recruited during their first visit to the vascular clinic at the Royal Free Hospital where consent was obtained. The initial assessment includes a detailed history of the presenting symptoms and any related past medical history. General and especially full vascular examination is carried out in addition to an initial assessment for their vascular risk factors and appropriate advice will be offered. Different tests will be carried out including measuring the ankle brachial pressure index (ABPI) before and after exercise, measuring the walking distance on a treadmill and checking the fasting blood biochemistry including lipid profile and serum creatinine and urate levels. Duplex ultrasound for the carotid arteries; aorta and lower leg arteries will be arranged if indicated or if needed for the purpose of this research project. Patients will be referred to the specialist risk modification clinic (with the results of the different requested investigation) where a more detailed assessment of the patient’s different vascular risk factors will be carried out and recorded. The objective of the clinic is to modify the vascular risk factors with special interest in lipid profile, smoking, blood pressure and antiplatelet treatment. Dietary advice will be offered and patients will be referred to a supervised exercise program if needed. Smokers will be referred to a smoking cessation clinic for advice. Patients with diabetes mellitus (DM) and renal failure will be followed up in specialist clinics. There will also be a regular assessment in the vascular clinic to assess the need for any interventions during follow up. During my research period, I reviewed the majority of PAD patients when they first presented to clinic and at least once more during the follow up. Once a patient was
recruited for a study, I will be informed about the outcome of outpatient’s attendances, vascular interventions if needed and any vascular events. Copies of all correspondence were also kept. Patients involved in the studies had a direct contact with myself and with a specialist vascular nurse in case of any urgent queries.

Arteries for the ET-1 receptors assessment were obtained from patients who underwent leg amputations secondary to advanced PAD. The patients for this study were recruited and consented in the ward prior to surgery.

The inclusion criteria are detailed in each study, because some of the criteria have changed (mainly the upper age limit and the lipid targets).
CHAPTER 1

GENERAL INTRODUCTION
1.1 Pathology of Atherosclerosis

Atherosclerosis is a progressive systemic disease, which affects most of the body arteries (Dieter et al 2002). The presence of clinical evidence of atherosclerosis increases the risk of subsequent vascular events by fivefold to sevenfold over the next 5 to 10 years (Robinson et al 1994). There has been a major increase in our understanding of the pathogenesis of atherosclerosis. Over the last few years the theory that inflammation plays a key role in atherosclerosis is becoming more popular (Poredos 2002). Inflammatory cell infiltration is observed in atherosclerotic plaques at virtually all stages, from the fatty streak to the advanced atheromatous lesions. In addition, plaque disruption and thrombosis, platelets, macrophages, polymerphonuclear leukocytes, and vascular smooth muscle cells (VSMC) become major players at the site of endothelial injury (Sabeti et al 2005). These activated cells promote an inflammatory response with the synthesis of vasoactive molecules including the peptide ET-1. The most accepted hypothesis for atherosclerosis is the response-to-injury described more than two decades ago (Dimmeler et al 1997, Ross 1993).

1.1.1 The response-to-injury hypothesis

The injury

Endothelial injury is a chronic condition occurs as a result to different causes mainly:

1. Haemodynamic disturbances
2. Hypercholesterolaemia
3. Hypertension
4. Cigarette smoking
The response

This endothelial dysfunction leads to:

1. Increased endothelial cell turnover and vessel permeability
2. Upregulation of adhesion molecules, cytokines and growth factors
3. Trapping of lipoprotein in the arterial wall
4. Migration of different inflammatory cells between the endothelial cells
5. Formation of foam cells from monocytes and macrophages

The endothelium plays a central role in the vascular control. Therefore, endothelial dysfunction plays a role in the pathogenesis of atherosclerosis by causing an imbalance between relaxing and contracting factors, procoagulant and anticoagulant substances, and between pro-inflammatory and anti-inflammatory mediators (Poredos 2002, Duerrschmidt et al 2000). Subsequently, the proliferation and synthesis of collagen will lead to the formation of early fatty steaks, which progress to fibrofatty atheroma and may cause vascular complications. In advanced cases there will be neovascularization and growth of microvessels (Kamat et al 1987). There are many similarities between atherosclerosis and inflammation (Sabeti et al 2005), which indicates that atherosclerosis itself, may represent an inflammatory phenomenon.
1.2 Peripheral Arterial Disease

Peripheral arterial disease represents any manifestations of vascular disease outside the cardiac or cerebral circulation. Nevertheless, PAD is mostly understood as a chronic obstruction of the arteries supplying the lower extremities. The condition can easily be detected by clinical examination and measuring the ankle brachial pressure index during a routine clinic visit (Feigelson et al 1994). Epidemiologic and natural history studies have determined that PAD confers a consistent high risk of fatal and nonfatal vascular events (Criqui et al 1990). Subsequently, PAD was considered a marker and independent risk factor for generalized atherosclerosis and the associated vascular risk (Hirsch et al PARTNERS 2001). That is why the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) classified PAD as equivalent to coronary heart disease.

The awareness of the condition is still relatively low despite the well known fact that PAD is closely associated with coronary and cerebrovascular disease (Donnelly et al 2002), particularly with an increased risk of significant internal carotid disease (Cina et al 2002). One of the reasons of the low attention given to PAD is the fact that the high morbidity and mortality associated with PAD is largely from coronary and cerebrovascular events, which often overshadows the PAD itself (Burns et al 2002). Indeed, patients with severe PAD have a worse outcome after coronary artery bypass grafting (Loponen et al 2002).

This might, to some extent, explain why patients with PAD receive less intensive treatment than patients with CHD and the fact that the diagnosis is often overlooked until the patient presents with PAD related complications, such as limb-threatening ischaemia (Robinson et al 2001, Mehler et al 2003). Recent evidence from published trials and studies strongly
suggested that early diagnosis combined with appropriate medical management is an effective approach to improve the quality of life and reduce the overall vascular risk in PAD patients (Dawson et al 2001).

The commonest symptom of PAD is intermittent claudication, which is an exercise-induced muscle ischaemia in one or both legs and consists of an aching pain, numbness, weakness, or fatigue in the muscle groups of the lower extremities (Schainfeld 2001). Intermittent claudication afflicts and limits the activities of a significant number of patients (Robinson et al 1994). The intensity of symptoms depends on a discrepancy between oxygen supply, limited by the arteriopathy and oxygen demand by the muscles involved in walking (Gardner et al 1991). The majority of PAD patients remain stable for years. However, worsening claudication occurs in approximately 16%, with approximately 7% requiring interventions including surgical bypasses and approximately 4% will end up having amputation. More than half of the patients with PAD have leg symptoms, but only (11%) report classic claudication (Bird et al 1999).

There are differences, in the current management, between CHD and PAD. While PAD patients are referred to smoking cessation programs at a higher rate, they are less intensively managed for hyperlipidaemia; hypertension and less often prescribed antiplatelet therapies as compared to patients with CHD. Even lipid measurements are obtained less frequently from claudicants (Clark et al 1999, McDermott et al 1997). The national current lipid lowering guidelines recommended aggressive treatment for elevated LDL-C and TG in patients with PAD (Hirsch et al 1997, Burns et al 2002).
1.2.1 Arterial Wall Characteristics in Peripheral Arterial Disease

Stiffening and thickening of the arterial wall are two important components of atherosclerosis that identify high risk patients (Simons et al 1999). Our understanding of molecular biology, physiology, and pathology of the arterial wall has improved significantly. Studies have examined the degree of endothelial dysfunction in patients with PAD showing an impaired endothelial dependent vasodilatation (compared with controls), which was associated with the presence of vascular risk factors. For example, smoking was associated with a dose related increase in intima media thickening and endothelial dysfunction (Poredos et al 1999). However, the effect of risk factor modification on the endothelial function in patients with PAD remains unclear and needs further studies (Yataco et al 1999).

Impaired common carotid artery (CCA) and common femoral artery (CFA) viscoelastic properties and increased intima media thickness (IMT) are reported in PAD patients (Cheng et al 2002). The CCA-IMT is shown to be related to vascular risk factors in cross-sectional studies and to the occurrence of coronary heart disease (Chambless et al 1997 ARIC). In addition, ultrasound assessment of carotid atherosclerotic disease emphasized the relationship between CCA-IMT and different vascular risk factors (Sugo et al 1997). Some guidelines have suggested that measuring the IMT may provide further information about the vascular risk assessment. Therefore, carotid scan findings may influence the clinician's decision to intervene with therapy. The mechanism of the intima media thickening in atherosclerosis is still unclear. However, experimental studies suggested that VSMC might be stimulated and transformed from a contractile type to a synthetic one, which invade the intimal layer indicating involvement of VSMC proliferation in the intima thickening
(Igarashi et al 1997). It was also suggested that atherosclerotic lesions might progress without the reduction in luminal size; instead a dilatation of the arterial wall might occur. Therefore, an estimation of the extent of atherosclerosis requires a simultaneous measurement of the arterial wall thickness and the residual luminal size. This assessment is also useful to observe the effect of different treatments on atherosclerosis.

The carotid IMT is accurately measured and monitored non-invasively using B-mode Duplex ultrasound, which also provide excellent clinical information about the vessel wall in terms of characterization and blood flow pattern (Sidhu et al 1997).

The last decade witnessed the discovery of a number of endothelium-derived peptides that have been implicated in the vasomotor and structural changes to blood vessels associated with atherosclerosis. They include (ET-1, ET-2, ET-3), each containing 21 amino acids (Weissberg et al 1990). The most studied peptide is ET-1, which is potentially the strongest vasoconstrictor currently known and is thought to be involved in atherosclerosis. It was first isolated from the culture medium of porcine aortic endothelial cells (Yanagisawa et al 1988). Since then many studies investigated the role of this peptide and its two receptors known as ETₐ and ETₐ in the development of atherosclerosis. This role was supported by the identification of ET-1 receptors within the atherosclerotic lesions in human arteries (Lerman 1991; Timm et al 1995). The release of ET-1 from the endothelial cells is stimulated by thrombin, interleukin-1 (Yoshizumi et al 1990) and invading macrophages in diseased arteries and is enhanced in culture by the presence of oxidized low density lipoprotein (oxLDL) (Boulanger et al 1992). In addition, vascular potency of ET-1 is increased in the presence of monocytes or macrophages (Magazine et al 1994). ET-1 is also a mediator for circulating inflammatory cells including monocytes and macrophage, which are involved in atherosclerosis (Haller et al 1991).
High plasma ET-1 concentrations are associated with endothelial cell injury mainly caused by physical stimuli such as shear stress, hypoxia, vasoactive hormones (angiotensin II and adrenaline) and cytokines. These conditions are present in PAD patients (Jagroop et al 1999, Rubanyi et al 1994). Circulating endothelial cells may also represent a marker of the severity of PAD (Blann et al 1997).

The vasoconstrictor role of ET-1 is either direct by causing vasospasm (Kurihara et al 1989) or indirect via stimulating local mediators especially prostacyclin and platelet-activating factors, which cause vasoconstriction (Schiffrin et al 1998). It has been suggested that the reduced vasodilatation responses in CHD is due to a dysfunctional endothelium and is not mediated by changes in the NO signalling pathway of the smooth muscle (Wiley et al 2002). ET-1 also has a mitogenic action on various cell types, mainly VSMC (Hirata et al 1989). This "growth factor" property of ET-1 has led to the suggestion that it may play a proliferative role in atherosclerosis (Kowala 1997).

Advanced cases of atherosclerosis are characterised by neovascularization where microvessels grow from the native vasa vasorum between the adventitia and the intima of the atherosclerotic vessels (Kamat et al 1987). The vasa vasorum provides the media with oxygen and nutrients. Proliferation of the vasa vasorum in advanced atherosclerotic plaques leading to pronounced neovascularization corresponded with the severity of atherosclerosis (Kamat et al 1987). There is a potentiating role for ET-1 on neointimal formation, in this aspect, ET-1 is also thought to be involved in remodelling processes, such as that seen in vein graft failure following coronary artery by-pass graft (CABG) surgery, resulting in rapid occlusion of the graft caused by neointimal formation, which was supported by experimental study on pigs coronary arteries (Dashwood et al 1999). Different endothelial
cell markers were also found to be high in patients with vascular disease as compared to matched control, which was related to the extent of the disease.

Raised circulating and tissue levels of ET-1 have been described in advanced atherosclerosis in the presence of different vascular risk factors, such as diabetes, hypercholesterolaemia, and cigarette smoking. However, the extent of the relation between ET-1 and different vascular risk factors and the impact of risk factors modification is not yet known (Haynes et al 1993). Nevertheless, pravastatin treatment in claudicants modified the inflammatory response associated with atherosclerosis including the endothelial cells, platelet, and inflammatory markers (Blann et al 2001).

The overall result of ET-1 action is to increase blood pressure and vascular tone (Agapitov et al 2002). Therefore, ET-1 antagonists may play a role in the treatment of cardiac, vascular and renal diseases associated with chronic vascular conditions leading to atherosclerosis (Shiosaki 1994).

Experimental hypercholesterolaemia is associated with pro-inflammatory changes and impaired renal cortical perfusion regulation (Bentley et al 2002). Statin treatment resulted in regression of the coronary arterial lesions in patients with cardiovascular disease. Similarly, in a double-blind, randomized clinical trial, positive effects of pravastatin treatment on the carotid IMT were associated by a significant improvement in the outcome when compared with a placebo group (Byington et al 1995). The changes in IMT after statin treatment may be attributable to an anti-inflammatory effect. By increasing the bioavailability of NO and decreasing IL-6 synthesis in human VSMC, statin can modulate the immune system and inflammatory processes (Crisby 2003). In addition, treating dyslipidaemia with a statin reduces plasma ET-1 and subsequently should have beneficial effects on atherosclerosis (Martin-Nizard et al 1991, Nakamura et al 2001).
1.2.2 History of Peripheral Arterial Disease

There have been major advances in the management of PAD in recent years. One of the greatest advances is the development of arteriography. This was made possible by the observation of Seldinger in 1953 that "a catheter could be threaded through a peripheral vein into the right heart with the possibility of injecting a contrast agent through the catheter for imaging purposes". In the year 1952 DeBakey reported the replacement of an aortic aneurysm with a homograft (DeBakey et al 1954). Vorhees reported the first application of artificial prosthetic devices for arterial replacement in 1952 (Dubost et al 1952). This led to the development of other prosthetic materials with an improvement in long-term survival using grafts for bypassing areas of occlusion. At the same time the use of autogenous tissue to bypass areas of occlusion using the saphenous vein as a conduit was reported (Linton et al 1967). There were parallel advances in non-invasive diagnosis methods including ultrasound, which was introduced few decades ago (Strandness et al 1967). Duplex ultrasound is currently used to study and follow-up the arteriosclerotic disease of the carotid, abdominal aorta and its branches including the renal and arteries distal to the inguinal ligament. Assessment of blood supply by measuring the systolic pressures in the upper and lower limbs is expressed as ankle brachial pressure index (ABPI), soon became the best single evidence available concerning the prevalence of arterial occlusive disease and its severity (Criqui et al 1996). Less invasive interventions were also introduced including the use of balloon angioplasty and in more recent times the addition of stenting. Intra-arterial stents have been developed for use in almost every area. This field is moving very quickly. However, one of the most important developments is the increase in our understanding of PAD risk factors especially the devastating impact of
cigarette smoking. In addition, hyperlipidaemia and diabetes were recognized as major contributing risk factors to PAD. The development of low-molecular-weight heparins coupled with the introduction of powerful antiplatelet agents changed our therapeutic approach to both arterial and venous disease. Currently there is a lot of research into the pharmacotherapy of intermittent claudication with for example; cilostazol is approved for the treatment of claudicants with stable PAD. The role of gene-based therapy to improve collateral circulation to the limbs is a further clinical research area on interest.

1.2.3 The Incidence of Peripheral Arterial Disease

The more we look for PAD the more we find! Symptomatic PAD has been studied in vascular risk surveys but only limited information is available on the asymptomatic form. One in five of the middle aged adults in the United Kingdom have evidence of PAD disease on clinical examination, although only a quarter of them are symptomatic. The disease also affects approximately 8 to 12 million individuals in the United States. The prevalence of PAD in primary care practices is high and the condition is common in both sex with a predilection for men and an increasing prevalence with age and in lower social class (Hirsch et al 2002). Therefore, the incidence and prevalence of symptomatic PAD depends on the population studied and the diagnostic criteria and instruments used. For example, the prevalence of intermittent claudication in older adults by questionnaire was <5% whereas, non-invasive testing detected 2-4 folds more claudicants (Newman et al 2001). In the Edinburgh Artery Study, 1,592 men and women aged 55-74 years were selected at random from general practices in Edinburgh, Scotland and were followed up for 5 years. The
investigators reported IC in 4.5% (95% confidence interval (CI): 3.5%-5.5%), major asymptomatic disease causing a significant impairment of blood flow in 8.0% (95% CI: 6.6%-9.4%) and in a further 16.6% (95% CI: 14.6%-18.5%) there was criteria considered abnormal in clinical practice: 9.0% had ABPI less than 0.9 (Fowkes et al 1991). Moreover, PAD was found in 29% of patients aged >70 years or aged 50-69 years with history or cigarette smoking or diabetes in primary care clinics. Interestingly, 13% of them did not have any manifestation for CHD (The Minnesota Regional Peripheral Arterial Disease Screening Program 2001).

Non-invasive studies using Duplex ultrasound showed femoral artery plaque in 64% of a random sample of men and women aged 56-77 years, which increased with age and was more common in men (Leng et al 2000). However, femoral artery plaque was present in only 44% of a younger population (Karnegis et al 1992). A retrospective analysis of a hospital-based geriatrics practice, included a total of 467 men (mean age: 80.8 years) and 1,444 women (mean age: 81.8 years), found symptomatic PAD in 20% of the men and 13% of the women (Ness et al 2000). Here comes the importance of screening for PAD because, despite the fact that 83% of patients with prior PAD were aware of their diagnosis, only 49% of physicians were aware of this diagnosis (Bell et al 2002).

It is well documented that the real prevalence of PAD is considerably underestimated if only symptomatic patients are taken into account (Cimminiello 2002). Over the next 20 years, the total number of patients affected is expected to increase significantly due to anticipated demographic changes (Schmieder et al 2001).
1.2.4 Risk Factors for Vascular Disease Especially Peripheral Arterial Disease

In patients with severe PAD referred for percutaneous transluminal angioplasty, smoking, HDL-C (inverse relationship), LDL-C, TG and fasting blood glucose (FBG) were strong predictors of PAD when these patients were compared with age-matched healthy controls (Drexel et al 1996). The vascular risk factors are divided into correctable (i.e. smoking) or non-correctable (i.e. age). The most important risk factors for vascular disease are:

Age

As we grow older we are at increased risk of developing vascular diseases, a fact accepted by everyone including the general public. Age is one of the most powerful independent risk factors for vascular disease and the increased prevalence of PAD with aging was demonstrated in several studies (Ness et al 2000, Meijer et al 2000, Newman 2000). With the aging of the population, the prevalence of PAD is expected to increase, such that at least 12% of community-dwelling adults aged 65 and older will have significant disease on non-invasive testing, most of them are asymptomatic (Newman et al 1999). The age relation of PAD was best shown in the Honolulu Heart Program, which included 3,450 ambulatory elderly Japanese American men, the prevalence of PAD increased from 8.0% in those aged 71-74 years to 27.4% in those 85 to 93 (Curb et al 1996).
**Smoking History (current or former)**

Smoking is a powerful vascular risk factor. The tobacco producing companies who have to demonstrate this clearly on their products reluctantly accepts this fact! The risk of events correlates with the number of cigarettes smoked (Price et al 1999 Edinburgh Artery Study). Interestingly, after 7 years follow up for claudicants, ischaemic rest pain occurred in 16% of current smokers as compared to none of those who stopped smoking. Moreover, heavy smokers have a significantly higher amputation rate after arterial reconstruction (Jonason et al 1987). Although cigarette smoking is an established cause of atherosclerosis, the exact mechanism is unclear, but it affects the clotting factors thus, increases the risk of CHD (Meade et al 1987). Smoking also involves various inflammatory events such as:

- Granulocyte activation
- Increased platelet volume
- Raised plasma fibrinogen levels
- Endothelial injury

Arterial thrombogenity appears to be enhanced by heavy smoking in PAD patients (Lassila et al 1988). Atherosclerosis may also be initiated, or indeed, accelerated, by smoking via the upregulation of cellular adhesion molecules, which facilitate the binding of leukocytes and platelets to the activated endothelium. The effect of smoking on lipids is well illustrated by the findings of the Munster Heart Study (Cullen et al 1998). This prospective study included 20,696 men and 10,212 women. Smokers had higher TC, LDL-C (1.5%) and TG (13%) levels together with lower HDL-C (6.5%) as compared to non-smokers, which represents a considerable difference in terms of vascular risk.
Hyperlipidaemia

Current reports contribute to our growing knowledge of the predictive power for major coronary events of non-HDL-C lipids. There is a significant correlation between LDL-C and coronary events in patients with CHD (CARE study). In a meta-analysis of a large number of epidemiological studies, TG levels carried an independent predictive power (Austin 1998). Hyperlipidaemia was found in (82%) of patients with CHD as compared to (77%) in PAD patients. This issue is discussed in more detail in the next chapter.

Diabetes Mellitus (DM)

Diabetics have an increased prevalence of PAD. Up to 20% of patients with symptomatic PAD are diabetic; nearly 50% are undiagnosed at presentation. Moreover, the condition is more severe and rapidly progresses in diabetics (Hittel et al 2002). Diabetes is associated with an increased risk of macro-and microvascular disease and is a powerful risk factor for progression to critical limb ischaemia (Zander et al 2002). In addition, revascularization in diabetic patients is associated with poorer outcome (Hittel et al 2002). The associated vascular risk is closely related to the duration of diabetes, the waist: hip ratio (obesity) and LDL-C and TG levels (Planas et al 2001). Diabetics, especially those with type 2 diabetes, should have their vascular risk aggressively modified (Papadakis et al 2001).
Hypertension

Systolic blood pressure >140 mmHg or diastolic bloods pressure >90 mmHg increases the risk of vascular events, especially cerebrovascular events (Ascione et al 2002). Almost half PAD patients who had angiography were hypertensive. In PAD patients with diabetes, intensive blood pressure lowering resulted in a marked reduction in vascular events (Mehler et al 2003, Lindholm et al 2002 LIFE).

Renal Insufficiency

Serum creatinine is considered independent risk factor for vascular events (Zanchetti et al 2001, HOT study). Increased vascular risk and premature vascular disease even with mild renal failure has been described (Mann et al 2001, HOPE trial). It is also known that elevated urate contributes to the prediction of vascular risk (Puddu et al 2000). Recently microalbuminuria (MA) is reported to be associated with a higher vascular risk and cardiac damage in addition to indicating generalized atherosclerosis (Wachtell et al 2002). PAD is common in patients with end-stage renal disease who are usually smokers (Lhotta et al 2002) with a degree of dyslipidaemia (O’Hare et al 2001).

Obesity

Obesity, particularly central obesity, is associated with an increase in all-cause mortality, including vascular mortality. Obesity is also an independent vascular risk factor and is
associated with dyslipidaemia, which may be mediated by increased secretion of proinflammatory cytokines by adipose tissue (National Task Force on the Prevention and Treatment of Obesity, 2000). Vascular risk factors and inflammatory markers improved in obese women after significant weight loss (Esposito et al 2003).

**Post-Menopausal Status**

PAD affects up to 25% of women aged 55-74 years, which results in a significant increase in vascular morbidity (Gerhard et al 1995). Moreover, premature menopause is associated with a bigger risk. In this context, there is a considerable ongoing debate as to whether hormone replacement therapy (HRT) in post-menopausal women reduces the risk of CHD-related events (Westendorp et al 2000, Price et al 2002). The situation with PAD is not clear, largely because of the lack of trial-based evidence. For example, in the placebo controlled Heart and Estrogen/Progestin Replacement Study (HERS) (n= 2763) there was no evidence of protection from PAD after treating women with clinical CHD with conjugated equine estrogens and medroxyprogesterone acetate over a mean follow-up period of 4.1 years (Hsia et al 2000 HERS). In contrast, in the population-based Rotterdam study, HRT for one year or more was associated with a significant decrease (25%) in the risk of PAD (odds ratio, 0.48; 95% confidence interval = 0.24N0.85). This study included 2196 naturally menopausal women aged 55 N 80 years (Westendorp et al 2000). One reason for this confusing picture may be that different forms of HRT do not exert the same effect on the lipid profile especially after long-term treatment (Prelevic et al 2002).
**Hyperhomocysteinaemia**

Elevated plasma homocysteine levels have been reported in patients with PAD (Rassoul et al 2000) especially in association with dyslipidaemia (Landray et al 1999). There is emerging evidence that hyperhomocysteinaemia is associated with an increased risk of symptomatic PAD progression and may predict the vascular death (Taylor Jr et al 1999). High-dose simvastatin decreased plasma homocysteine (Lütjohann et al 2001).

**Lipoprotein (a)**

There is an association between lipoprotein (a) Lp (a) and the presence and severity of PAD (Dionyssiou-Asteriou et al 2000), especially in higher risk patients with dyslipidaemia and proteinuria. In addition, Lp (a) may be an independent predictor of PAD in patients with and without diabetes (Prior et al 1995, Bhatnagar et al 1995). Interestingly, patients who developed restenosis after an infrainguinal graft had a higher Lp (a) levels (Cheshire et al 1996).
1.3 MANAGEMENT OF PERIPHERAL ARTERIAL DISEASE

1.3.1 Diagnosis and Survey

Only small proportion of patients with PAD present with classical claudication, so clinicians who focus on a classic history of claudication alone are likely to miss up to 85% to 90% of the affected patients.

**History and clinical examination**

The cornerstone of patient evaluation is history and physical examination, including a detailed vascular risk-factors assessment. In the differential diagnosis of IC, clinicians should consider aetiologies such as arthritis, spinal stenosis, radiculopathy, venous claudication, or inflammatory processes.

**Leg symptoms**

The reported incidence is a conservative estimate, because many patients often attribute symptoms of PAD to "normal aging" and may not report to their physician. Additionally, physicians may miss the diagnosis if a comprehensive history and vascular examination are not a routine part of their assessment. In the UK, 5% of people aged 55-74 years are claudicants, which puts them at high risk of serious vascular risk (Leng et al 2000). Functional impairments, adversely affects quality of life, are found to different degrees in every PAD symptom group and limit their ability to perform daily activities depending on the type of leg symptom (Doyle et al 2003). Any symptoms of aching pain, numbness,
weakness, or fatigue in the muscle groups of one or both legs while walking should be investigated. Young adults with PAD present a diagnostic challenge and are typically smokers with multiple risk factors and a strong family history of CHD. They typically have chronic symptoms of claudication at diagnosis either not reported in a timely manner or attributed to other common causes. More than 70% of young claudicants have angiographic evidence of severe aortoiliac disease (Levy 2002).

**Physical examination findings**

Physical findings include trophic signs of ischaemia, vascular bruits and peripheral pulse deficits and ischaemic ulcers (Halperin 2002). Moreover, in >80% of all patients, it is possible to locate the responsible arterial segment by combining the location and severity of pain with a pulse examination.

**Measuring the ankle brachial pressure index and exercise test**

A simple ankle brachial pressure index measurement with a hand-held Doppler velocity meter identifies a large number of patients with previously unrecognized PAD (Feigelson et al 1994). Ancillary diagnostic modalities begin with measuring the ABPI; a value of <0.90 is a marker of impaired functional status and carries a significant prognostic implications in nondiabetic patients (McDermott et al 2001). More precise assessment in the non-invasive vascular laboratory may involve a combination of segmental limb pressure measurements and in north America, pulse volume waveform recording, which is over 90% accurate for predicting the level and extent of the disease (Halperin 2002). Exercise testing enhances the value of the clinical observations and could identify more than 50% of patients with PAD who might have pain on exertion without typical leg symptoms (Vogt et al 1993).
**Duplex Ultrasound**

The use of Duplex ultrasound enables diagnosis in more cases, especially early or asymptomatic PAD (Leng et al 2000). Non-invasive diagnostic studies can determine the level and severity of PAD unmask haemodynamically significant disease and are useful in the follow-up after conservative or interventional treatment.

**CT Scan and magnetic resonance imaging (MRI)**

New CT scan images with arteriography are now available. Contrast-enhanced three-dimensional MRI Angiography (3D MRA) is starting to emerge as powerful non-invasive imaging modalities for the assessment of patients with PAD. MRA is also emerging strongly to characterize the arterial wall and atherosclerotic lesions. Its clinical utility using current technology has already been well established, and the continuous development of hard and software will likely result in a significantly improved performance (Goyen et al 2002).

**Angiography**

The diagnosis of PAD does not generally require invasive techniques, and most patients with PAD do not require contrast angiography (Halperin 2002). However, angiography is indicated for mapping of the extent and location of arterial pathology prior to revascularization especially when the decision for revascularization (radiological or surgical) has already been made (Schmieder et al 2001).
1.3.2 Treatment of Peripheral Arterial Disease

The ultimate aim of PAD treatment is to stop or slow down the progression of atherosclerosis, induce stabilization and promote regression of atheromatous plaques. Therefore, the main goals of treatment are:

1) Maintaining or improving the functional status

2) Reducing or eliminating vascular ischaemic symptoms and events

3) Preventing disease progression

Medical therapy to relieve symptoms and prevent complications forms the mainstay of treatment for patients with uncomplicated PAD (Donnelly et al 2002). In addition, it is mandatory to treat PAD-specific symptoms aiming to decrease the functional impairment and thereby improve the quality of life and decrease the rates of amputation (Luther et al 1996). Exercise rehabilitation is widely accepted as part of the management of patients with PAD. Interestingly, exercise program not only improved functional performance but also resulted in a fall in TC and LDL-C by 5.2% (p< 0.005) and 8% (p< 0.01), respectively. Systolic blood pressure also fell by 5.7% (p< 0.05) (Zquierdo-Porrera et al 2000). Supervised exercising can produce a significant and clinically meaningful increase in walking distance and enhance the quality of life in most claudicants who adhere to it (Schmieder et al 2001). The exact mechanisms by which exercise leads to clinical improvement have not been precisely defined.
1.3.2.1 Best Medical Therapy (BMT)

Many patients with PAD do not receive an optimum package of secondary prevention, which aims at reducing direct or indirect vascular complications, e.g. amputations or vascular events. National recommendations mandate aggressive lowering of serum LDL-C as a primary treatment goal in all patients with overt arteriosclerosis, as 'CHD risk equivalent' syndromes (Hirsch et al 2002). BMT resulted in an improvement in PAD symptoms and provided patients with a substantial protection against future vascular events, yet it is poorly used in this group of patient as compared to patients with CHD. Previous data was largely restricted to patients with CHD and their relevance to PAD has been extrapolated. However, data from PAD patients is now emerging, such as the Heart Protection Study, with data specific to PAD patients (Burns et al 2002). Nowadays, invasive interventional treatments are considered only after BMT has been instituted and given sufficient time to take effect, as most patients will improve to a point where invasive intervention is no longer needed (Leng et al 1998).

**Best medical treatment includes:**

**Smoking Cessation**

Randomized controlled trials have shown that nicotine replacement approximately doubles the cessation rate in unselected smokers so every claudicant should be offered nicotine replacement treatment in the first instance. There is a significant \((p < 0.001)\) reduction in the need for major amputations from 21% of heavy smokers to only 2% in current moderate smokers. Moreover, smoking cessation not only slows the progression to critical leg
ischaemia, but also reduces the vascular risk and the severity of claudication by increasing the maximum walking distance (Girolami et al 1999). A further benefit of smoking cessation is slowing down the progression of renal disease and the need for dialysis in patients with additional primary renal disease (Schiffl et al 2002, Lhotta et al 2002). Smoking was associated with adverse changes in several risk factors that included a lower HDL-C and increased TG and plasma fibrinogen. After multivariate adjustment, the influence of smoking on conventional risk factors only accounted for part of the adverse effect on PAD and CHD. It follows that smoking cessation is an important preventive measure in patients with PAD (Quick et al 1982). Quitting will result in beneficial effects including an improved lipid profile and a reduction in plasma fibrinogen levels (Milionis et al 2001). Within 3-4 years of smoking cessation there is a well-reported significant reduction in vascular risk (Gensini et al 1998). There is also evidence from the major statin trials showing that the risk of events in non-smokers on placebo is similar to that of smokers taking statin treatment (Milionis et al 2001). All these reasons may explain why after smoking cessation there can be an immediate improvement in claudication and a reduction in the over all vascular risk.

**Single or combination anti-platelet therapy**

Antiplatelet treatment (usually aspirin) reduces the risk of fatal and non-fatal vascular events in patients with any manifestation of atherosclerotic disease by about 25% and is equally effective in patients who present with CHD or PAD (Antithrombotic Trialists collaboration 2002). Thus it is strongly recommended for secondary disease prevention and aspirin should be given to every PAD patient if there are no contraindications (Hennekens
et al 1989). Clopidogrel is at least as effective, and possibly more effective, than aspirin and should be considered in PAD patients if intolerance to aspirin develops or in patients with worsening symptoms or considerably higher risk. In a study by Duprez et al, aspirin was used in 48% of the total treated population, other antiplatelet therapies are used in 3%, but 49% were not using any antiplatelet medication (Duprez et al 2003). Antiplatelet treatment reduces the need for surgery in PAD and indirect evidence shows that antiplatelet agents improve walking distance (Goldhaber et al 1992 Physicians' Health Study). Cilostazol, a new antiplatelet, antithrombotic agent, has been shown to reduce claudication symptoms and significantly increases the walking distance, but the precise role of cilostazol remains to be defined (Schainfeld 2001).

**Maximum Cholesterol Reduction**

The beneficial effects of cholesterol lowering therapy in primary and secondary prevention of CHD have been conclusively demonstrated in large-scale clinical trials (CARE, MRFIT, 4S, WOSCOPS). The reduction in vascular events and total mortality is directly proportional to the reduction of plasma cholesterol. Different lipid lowering treatments ranging from diet to drug treatments in PAD patients reduced the overall mortality to 0.7% as compared to 2.9% in the placebo group. Furthermore, in the Heart Protection Study (HPS) simvastatin treatment was associated with a significant decrease in major vascular events in PAD patients who did not have clinically obvious CHD when TC and LDL-C levels were lowered by 25%. This was irrespective of age, sex, or baseline cholesterol concentration. This was associated with a significant improvement in PAD symptoms and a decrease in new and worsening claudicants (Pedersen et al 1998).
The Program on the Surgical Control of the Hyperlipidaemias (POSCH) included patients with a single documented myocardial infarction (Buchwald et al 1998 POSCH). A partial ileal bypass was used to lower cholesterol levels. After a five-year follow up, effective cholesterol reduction led to a significant (p= 0.049) reduction in overall mortality as well as mortality from CHD (p= 0.03) in the intervention group. The onset of PAD was also significantly (p= 0.049) decreased in the treatment group. Therefore, the POSCH study suggests that non-pharmacological lipid lowering can also reduce the likelihood of developing PAD.

Statins are the most effective of current treatments in lowering LDL-C, and have a proven efficacy in secondary prevention. The use of statins in high-risk groups such as PAD patients could prove particularly beneficial in reducing vascular morbidity and mortality and therefore merits prospective clinical investigation (Hirsch et al 2002).

**Diagnosis and Treatment of Diabetes Mellitus**

Diagnosis of diabetes, or its exclusion, is important in patients with PAD. A threshold of FBG <7.0 mmol/l is recommended. Intensive control of blood glucose prevents the microvascular complications of diabetes and reduces the overall vascular risk, but its effect on the macrovascular circulation is not certain (Diabetes Control and Complication Trial 1995). However, there was no significant reduction in the risk related to PAD itself (UKPDS 1998). Diabetic patients respond less to surgical intervention but gain a greater benefit from medical treatments for cardiovascular disease.
Treatment of Hypertension

The benefit of treating hypertension in terms of reducing stroke and coronary events is well accepted; data indicate a target of less than 140/85 mmHg, but it is not known if aggressive blood pressure control alters the progression of PAD (PROGRESS Collaborative Group 2001). Some treatments (Beta-adrenergic-antagonist) are reported to have unfavourable affects on PAD (Solomon et al 1991), but other studies showed this to be only true in those with severe PAD (Radack et al 1991). On the other hand, angiotensin-converting-enzyme (ACE) inhibitors reduce the risk of vascular event in patients with PAD in secondary prevention (HOPE Study 2000). Most patients with PAD would benefit from 25% reduction in cardiovascular morbidity and mortality if they take ACE inhibitor, if that treatment is not associated with a deterioration of renal function due to occult renal artery stenosis.

1.3.3.2 Interventional Treatment

In general, most claudicants can successfully decrease their exertional limb symptoms via a combination of exercise (preferably supervised), secondary prevention and pharmacotherapeutic interventions (Hirsch et al 2001). Interventions (surgical or radiological) are indicated to relieve disabling symptoms when medical therapy had failed, for treatment of symptoms of limb-threatening ischaemia, including rest pain, ischaemic ulceration and gangrene, and to remove or bypass sources of thrombo-embolism. It is been reported that the utilization of PAD-related invasive procedures gradually increases over the first 8 years, and rises sharply in the ninth and tenth years after the initial diagnosis
(Zachry et al 2001). Knowing the anatomic detail of a lesion allows the clinician to determine whether and what type of intervention is feasible.

The criteria of appropriate patient referral to vascular surgeon are:

- The primary care team is not confident of making the diagnosis or in monitoring best medical treatment
- Non-specific symptoms
- Progressive disease and severe symptoms despite best medical treatment
- The patient has weak or absent femoral pulses indicating aortoiliac disease
- Suspicion of other vascular conditions (abdominal aortic aneurysm, carotid disease)
- Rest pain or ischaemic gangrene indicates severe, often multilevel arterial occlusive disease needing aggressive management, which usually includes angiography and revascularization by percutaneous angioplasty or surgery

Endovascular revascularization currently serves as an effective therapy for patients with high-grade stenosis of the proximal limb arterial segments, (e.g. the distal aorta, common iliac artery, or external iliac artery, and occasionally the proximal common femoral artery). There is no convincing evidence in support of the use of percutaneous balloon angioplasty or stenting in patients with uncomplicated PAD (TASC Working group 2000). Experimental studies showed that atorvastatin reduced in-stent stenosis in both normo- and hypercholesterolaemic rabbits (Herdeg et al 2003). Randomized controlled trials have shown that although successful percutaneous balloon angioplasty may lead to a short-term improvement, in the longer term, best medical treatment and supervised exercise is superior
to percutaneous angioplasty in terms of walking distance and quality of life (Whyman et al 1997, Perkins et al 1996).

Invasive revascularization procedures can be considered for patients with critical limb ischaemia or when BMT fails. Surgical revascularization is reserved for patients who present with severe aortoiliac disease in whom long-term patency is likely to be achieved and who have a low cardiovascular perioperative ischaemic risk. Patients who have predominantly aortoiliac (suprainguinal) disease seem to benefit more from surgical or radiological intervention (McCarthy et al 2000). Patients who undergo successful revascularization are likely to benefit from exercise rehabilitation programs and secondary prevention (Gardner et al 1995). The results of surgical revascularizations in young adults in the long term are inferior to those reported in older patients (Valentine et al 1999). Moreover, younger adults typically require multiple revascularizations with relatively high amputation rate, so symptomatic young adults with multiple risk factors should have aggressive medical treatment (Levy 2002).

In the UK, bypass surgery is infrequently performed for intermittent claudication because the risks of surgery are generally believed to outweigh the benefits, especially in patients who improve on best medical treatment.
1.3.3 Prognosis of Peripheral Arterial Disease

Analysis of the natural history of PAD demonstrates that the risk of vascular morbidity and mortality far exceeds that of severe limb ischaemia or limb loss. Recent results suggest that PAD-related studies should consider the progression of the disease past the fifth year after the initial inpatient visit (Zachry et al 2001). The onset of symptomatic PAD at a young age, before the age of 45, has been associated with rapid progression, bypass graft failure, and high amputation rate. Patients with premature PAD have objective evidence of advanced disease, 60% are expected to remain stable and approximately 40% will require multiple interventions (Valentine et al 1999). These patients are usually smokers with multiple risk factors and typically exhibit distinct lipid abnormalities, including low HDL-C levels (Chapman et al 2004).

1.3.3.1 Symptom Progression

Claudication symptoms may remain stable for years. However, there is a risk of worsening claudication requiring different interventions or major amputations. Most of lower-extremity amputations are secondary to vascular disease and the majority of patients are diabetic with or without chronic renal failure.

Old studies have reported 25% of PAD patients with symptomatic PAD to have a worsening in their symptoms with 5% needing amputation in five year follow up period (Imparato et al 1975). Critical limb ischaemia resulting in amputation has a perioperative mortality rate of 5-10% for below-knee and up to 50% for above-knee amputation. This
high risk is mainly related to the associated co-morbidities (Halperin 2002). At an average follow-up of 10.8 months, for patients who underwent major amputations, only 65% were still alive (Toursarkissian et al 2002).

1.3.3.2 Risk of Vascular Events

By now, PAD is well recognized as a marker of systemic atherosclerosis. Moreover, life expectancy is approximately 10 years less than that of an age-matched cohort (Schmieder et al 2001). Twenty year life table analysis showed a reduced survival (54%), in comparison with normal population (77%) (Poulia et al 1992). The incidence of vascular events in secondary prevention practice during 3.4 years follow up was 21% including 9% vascular related death rate (Kaplan et al 2002). Moreover, high incidence of myocardial infarction is associated with advanced PAD (Rossi et al 2002). Another study reported 20% event rate, 60% were fatal during 48.5 months follow up of 397 patients with PAD. Patients with PAD have a 5 years mortality rate of approximately 30% most of the morbidity is related to CHD. Those presenting with critical leg ischaemia and the lowest ABPI are reported to have an annual mortality rate of 25% (Dormandy et al 1999).
1.4 ATHEROSCLEROTIC RENAL DISEASE

Chronic kidney disease is defined according to the presence or absence of kidney damage and the degree of the remaining kidney function. Five stages were described based on glomerular filtration rate (GFR). Ranging from mild kidney damage and a normal GFR >90 ml/min in stage one to renal failure with a GFR <15 ml/min in stage five (Levey et al 2005, Williams et al 1988).

Atherosclerosis accounted for 90% of cases of renal artery stenosis in an autopsy study and usually involves the proximal third of the main renal artery (Sawicki et al 1991). In advanced cases, segmental and diffuse intrarenal atherosclerosis may also be observed, particularly in patients with ischaemic nephropathy. The prevalence of atherosclerotic renal artery stenosis (ARAS) increases with age (Tollefson et al 1991).

Duplex ultrasonography can provide images of the renal arteries and assess blood-flow velocity and pressure waveforms, but there is a 10% to 20% failure rate (Hansen et al 1990). Chronic atherosclerotic renal disease is characterized by a progressive loss of renal function resulting in an end-stage renal failure, so predicting future decline in renal function is important for subsequent therapeutic decisions. Risk factors for a more rapid decline in renal function include hypertension, proteinuria and hypercholesterolaemia, in addition to the severity of the impairment at the time of diagnosis, male gender, and age (Alberti et al 1993). Progressive chronic renal disease probably reflects a nonspecific renal scarring process involving all renal components and characterized by interstitial fibrosis, loss of capillaries and glomeruli, resulting in a reduction in the number and area of renal vessels (Ruillope et al 1994). Renal scarring ultimately leads to a reduction in the intrarenal vessel area, which in turn may be responsible for increased intrarenal vascular resistance.
(Aikimbaev et al 2001). Assessing the intrarenal vascular resistance may therefore be helpful in determining the degree of renal damage. The contribution of different renal vascular beds (preglomerular vessels, glomerular capillaries, and postglomerular vessels) to the raised resistance index is unclear (Bader et al 1980). Increased renal resistance can be measured by Duplex ultrasonography, which may help predict the subsequent function and prognosis of the kidney. Doppler indices of renal vascular resistance, resistive index (RI) and pulsatility index (PI) are usually determined as pulsed-wave Doppler of downstream renal artery or interlobar artery resistance. Doppler indices are suggested to be an informative technique in assessing the renal function, but little information is available on their correlation to the renal function parameters and haemodynamic (Petersen et al 1995).

Another way of assessing renal function is measuring cystatin C, which is an independent indicator of renal function and is shown to give a better estimate of the GFR (Laterza et al 2002, Simonsen et al 1985) and it reflects the vascular risk (Koeing et al 2005). Its measurement is generally recommended where it is not possible, for different reasons. Therefore, cystatin C determination preferred to creatinine.

It is important to study the effects of lipid lowering drugs in patients with renal disease, because abnormalities of lipid metabolism are common in these patients. (Wheeler et al 1994). Various lipid lowering drugs currently available are safe and efficient. In addition, Massy et al 1995, in a meta-analysis compared various lipid lowering therapies in patients with renal insufficiency and nephrotic syndrome, as well as those on various forms of renal replacement therapy. Their recommendations were that diet in combination with statins should be considered as the first step in the management of hyperlipidaemia in patients with nephrotic syndrome in whom lowering of LDL-C levels is the primary goal of therapy.
CHAPTER II

LIPID LOWERING TREATMENT AND PERIPHERAL ARTERIAL DISEASE
The concept of lipid lowering treatment is not new. The management of dyslipidaemia ranged from conservative with diet, weight loss to the invasive surgical partial ileal bypass and include pharmacological drugs (fibrates and statins).

2.1 STATINS

Statins (3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors) are a breakthrough in the treatment of elevated blood serum cholesterol, especially LDL-C. Statins decrease LDL-C by inhibiting the rate-limiting enzyme in cholesterol biosynthesis and interfering with the biosynthesis of farnesyl pyrophosphate (FPP), which not only is a precursor of cholesterol, but also is required for the post-translational lipidation of Ras. Furthermore, FPP condenses with isopentenyl pyrophosphate, whose synthesis is also blocked by HMG-CoA reductase inhibitors, to form geranylgeranyl pyrophosphate (GGPP), which is required for the post-translational lipidation, membrane localization, and function of RhoA. The cholesterol lowering effect of statins is also due to an increase in the uptake of cholesterol by the liver as a result of intracellular cholesterol depletion and subsequent enhanced expression of LDL-C receptors (Crisby 2003). Lovastatin was the first product to be licensed. Since then other similar products were made available including cerivastatin (this was withdrawn for safety reasons), simvastatin, fluvastatin, pravastatin, atorvastatin and more recently rosuvastatin. Statins are licensed to reduce cholesterol levels and some are licensed for both primary and secondary prevention. Cholesterol lowering with a statin is both safe and effective in high-risk patients (Massy et al 1995, Attman et al 1993).
2.2 STATINS AND VASCULAR DISEASE PREVENTION

The introduction of statins occurred about the same time as the initiation of the NCEP ATP II report (Adult Treatment Panel II 1993), which is a national effort to increase public and professional awareness of the dangers of high serum cholesterol and to emphasize the benefits of reducing serum cholesterol concentrations. This was followed by the third ATP report (Adult Treatment Panel III 2001), which classified the risk associated with PAD as equivalent to the risk of CHD. This is not surprising in view of the high risk of vascular events associated with PAD (Murabito et al 1997).

Several clinical trials demonstrate that statins can substantially reduce both morbidity and mortality from CHD and are indicated in the management of high-risk patients in both primary (WOSCOPS, AFCAPS/TexCAPS) and secondary prevention (4S, CARE, TNT). There is also evidence that statins reduce the risk of stroke (Rizos et al 2001). Of note, none of the reported trials specifically addressed the optimal goals for LDL-C lowering therapy. Therefore, the quantitative relation between serum cholesterol levels and coronary events has been a topic of interest. Earlier studies suggested a small change in the risk of new-onset CHD up to a level of total cholesterol 5.0 mmol/l, which corresponds to an LDL-C level of about 3.3 mmol/l; above this threshold level the vascular risk apparently, began to rise significantly (Kannel et al 1971, Goldbourt et al 1985). On the other hand, recent studies found a different relationship, i.e. the MRFIT study by greatly expanding the population base found a curvilinear relation between serum cholesterol levels and the risk of CHD, but no evidence for threshold level was observed (Stamler et al 1986). In the 4S (a secondary prevention trial) treatment with simvastatin resulted in a 35% reduction in serum
LDL-C levels, which was accompanied by a 34% reduction in major coronary events. Moreover, greater cholesterol reductions gave continuous but progressively smaller decrements in CHD risk. In the CARE trial, pravastatin therapy lowered LDL-C and decreased major coronary events by 24% and significantly prevented recurrent coronary events in patients with average cholesterol levels who had experienced myocardial infarction. The CARE results reveal no further risk reduction when LDL-C falls below 3.0 mmol/l. Conversely, the 4S results suggested continuing benefit below this level, but with diminishing returns. In the WOSCOPS trial (a primary prevention trial in high-risk patients), LDL-C reduction was accompanied by a 31% decrease in major coronary events (WOSCOPS 1998).

Although LDL-C is widely recognized as the major atherogenic lipoprotein and the primary target of lipid lowering therapy, other lipoprotein fractions appear to be involved in atherogenesis including very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL) and triglyceride-rich lipoproteins (TGRLP). This view led to the designation of LDL+IDL+VLDL cholesterol (called non-HDL-C) as "atherogenic cholesterol" and to identify them as a secondary target of therapy (O'Brien 1994).

It is now well accepted that the lower the serum LDL-C level the better; consequently, reducing TC levels to reach \( \leq 2.5 \) mmol/l, "as a new set target" could be advantageous.

There is overwhelming evidence that low HDL-C levels strongly predict vascular risk (Mikhailidis et al 2000). This lipoprotein is involved in the removal of cholesterol from cells in the vascular wall. Multiple lines of evidence and major epidemiological studies showed that HDL-C protects against CHD (Chapman et al 2004). Experiments in transgenic mice provide proof that increased HDL-C secretion protects against atherosclerosis caused by an atherogenic diet or genetic hyperlipidaemia (Rubin et al 1991).
This compelling scientific evidence thus justifies HDL-C as a target to reduce vascular risk (Rizos et al 2001). The European Consensus Panel recommends that the minimum target for HDL-C should be 1.0 mmol/l in high risk patients (Chapman et al 2004). Thus, therapeutic intervention aimed at raising HDL-C, within the context of reducing global cardiovascular risk, would benefit such patients. Indeed, when HDL-C level was raised by drug therapy, coronary atherosclerosis decreased and CHD events lessened (Nyman et al 2002 VA-HIT), which also was independently correlated with coronary angiographic findings. Therapeutic options for patients with low HDL-C include treatment with statins either as a monotherapy or in combination with fibrates.

Elevated TG is common in patients with PAD and increases the vascular risk (Cullen et al 2000, Haim et al 1999). When all available data are taken into account, a high plasma TG level increases the vascular risk independently of the other risk factors. Clinical trials are needed to determine the impact of lowering plasma TG levels on the subsequent vascular risk. Higher doses of statins do exert a more substantial TG-lowering action (Mikhailidis et al 1998).
2.3 LIPID LOWERING TREATMENT IN PATIENTS WITH PERIPHERAL ARTERIAL DISEASE

After multivariate analysis (Ridker et al 2001), the most reliable lipid predictor and marker for PAD is the TC/HDL-C ratio. In addition, HDL-C was inversely related with PAD (odds ratio= 0.70; 95% confidence interval: 0.50 N 0.80) as shown in a prospective study that included 14,916 initially healthy USA physicians (aged 40 to 84 years). Of them, 140 developed symptomatic PAD during an average follow up of nine years. When compared to matched (including for smoking) controls the relative risk ratio (RR) of the highest to the lowest HDL-C quartile was 3.9 (95% confidence interval: 1.7 N 8.6) (Ridker et al 2001).

Familial hypercholesterolaemia (FH) is associated with an increased risk of both PAD and CHD (Kroon et al 1995). In a case control study, premature PAD was present in 31% of FH patients (n= 68; age: 45.8±11.6 years) as opposed to 3.7% of the matched control subjects (n= 27). PAD was demonstrated in 50% of FH patients who also had clinical evidence of CHD. Interestingly, in 19% of them, PAD was the first manifestation of vascular disease (Kroon et al 1995).

2.3.1 Reduction in Vascular Events

Non-HDL-C is shown to independently predictor nonfatal MI (multivariate relative risk, 1.049 [95% confidence intervals, 1.006 to 1.093] for every 0.26 mmol/L increase) (Bittner et al 2002). Experimental studies showed that statins reduce the extent of myocardial necrosis in normocholesterolamaic rats after acute ischaemia/reperfusion injury by
increasing myocardial eNOS activity, indicating that statins may protect the heart not only by reducing the incidence of ischemic events, but also by limiting myocardial cell damage during the course of acute MI (Wolfrum et al 2003). Similarly, statin therapy reduced the size of brain infarct and improved the neurological outcome in experimental model of ischaemic stroke and directly upregulated brain eNOS (Vaughan 2003). Several large clinical trials documented conclusively that statins reduce the risk of ischaemic vascular events in secondary prevention by at least one third (4S, CARE, LIPID, GREACE, ALLIANCE). The recent TNT study included a bigger number (1,0001) of patients with CHD followed up for 4.9 years. Further serum LDL-C reduction with a higher dose of atorvastatin (80 mg/day compared with 10 mg/day) was advantageous (LaRosa et al 2005, Ridker et al 2005). Overviews of randomized trials showed that statin treatment reduce the risk of stroke and total mortality (Hebert et al 1997).

A Cochrane review of the lipid lowering trials in PAD, which included 698 patients in seven appropriately designed trials showed that treatment was associated with a marked reduction in mortality (odds ratio= 0.21; 95% confidence interval: 0.03±0.17), but this did not achieve statistical significance (Leng et al 2000 Cochrane review), which is not surprising in view of the relatively small number of patients included in this analysis.

A double-blind randomized study included 919 hyperlipidaemic with CHD, showed a significant (p= 0.001) regression in the carotid IMT and a significant reduction in vascular events and deaths (p= 0.04 and p= 0.02), respectively after 12 months of treatment with 20-40 mg/day of lovastatin (Furberg et al 1994 ACAPS). In the HPS study, which is a large study (20,536 patients) there was a significant decrease in major vascular events in patients with PAD even without manifestation of CHD. On an intention to treat analysis, the RR in those taking simvastatin (40 mg/day) was reduced compared to placebo (Heart Protection
Study 2002). This result has to be interpreted against an increasing background of treatment with statins in the placebo group as the HPS trial progressed over a period of five years. It follows that the real result is even more significant than what was reported. During 5.4 years follow up of hyperlipidaemic claudicants (n= 660); simvastatin 20-40 mg/day significantly (p<0.0001) reduced the incidence of new coronary events to 48% compared to 73% in the no treatment group (Aronow et al 2002). This is probably because a change in LDL-C, HDL-C and possibly TG offers protection from risk associated with PAD (Buchwald et al 1996 POSCH).

There was no significant reduction in vascular events in the (LEADER) trial, which included 1568 men (mean age: 68.2 years; range: 35-92) blindy randomized to placebo or bezafibrate 400 mg/day. However, bezafibrate treatment reduced the non-fatal cardiac events in younger men age <65 years RR 0.13 (0.03 to 0.56). Also in these younger men, all events, fatal and non-fatal were 62% lower then those in the placebo group (0.38, 0.20 to 0.72) (Meade 2001). Stabilizing the arterial plaque and reducing thrombogenicity markers (matrix metalloproteinase, tissue factor and plasminogen activator-1) after 14 weeks of simvastatin may contribute to the vascular event reduction and may explain the early clinical benefits (Son et al 2003). However, these effects were independent of lipoprotein changes, as there was no correlation between lipoprotein levels and plaque stability or thrombogenicity markers (Son et al 2003).

These trials provide convincing evidence supporting the need to treat dyslipidaemic claudicants with or without CHD as outlined in the NCEP ATP III (Adult Treatment Panel III 2001) guidelines. Moreover, the vascular risk of vascular events is significantly reduced if patients with PAD are treated with a statin.
2.3.2 Improvement in Peripheral Arterial Disease Symptoms

The improvement in the symptoms associated with PAD, after treating dyslipidaemia, may relate to the contributions of dyslipidaemia to the abnormal vascular reactivity, which has been reported in PAD patients. Thus, the improvement in PAD symptoms may be related to lowering LDL-C or TG and increasing HDL-C levels (Meade 2001). Interestingly, a significant overall reduction in disease progression (as shown on angiography) has also been reported after lipid lowering treatment (Leng et al 2000). There were inconsistent changes in ABPI, but the trend was towards a general improvement in IC symptoms. The CLAS was a placebo-controlled randomized trial to assess the effect of treatment with colestipol-niacin in men with previous CABG, after treatment for two years there was significant benefits on coronary, carotid and femoral artery atherosclerosis (Blankenhorn et al 1991). During 5.4 years follow up of 4444 patients with CHD; the risk of new or worsening IC was significantly (p= 0.008) reduced by 38% when simvastatin was used to treat hyperlipidaemia (Pedersen et al 2000 4S study). However, this was a post-hoc analysis in patients recruited because they had CHD.

A recent trial blindly randomized (641 patients) with or without PAD to statin or no statin treatment; there was a significant (p<0.001) improvement in leg function in the statin group, which was independent of the cholesterol levels or degree of lipoprotein changes (McDermott et al 2003). The LEADER trial reported a significant (p= 0.02) improvement in the severity of PAD for up to three years when bezafibrate was used. However, this difference was only significant in the early stages of the study (p= 0.001) and turned not significant after 4-6 years (Meade 2001). In a survey included in this thesis, which included 200 hyperlipidaemic claudicants, there was a significant (p<0.0001) improvement in
walking distance after correcting dyslipidaemia mainly with a statin. These studies support the concept that treating dyslipidaemia results in a clinical improvement in claudicants.

2.3.3 Benefits of Lipid Lowering on Lipids and Non-Lipid Variables

2.3.3.1 Lipid Profile

Experimental evidence indicated that statins have direct vascular effects independent from LDL-C reduction. However, in humans there is not enough evidence yet (Tonolo et al 2000). Statins can exert differential effects on LDL-C, HDL-C and TG (Wierzbicki et al 2002). It follows that the choice of lipid lowering treatment may be relevant in patients with PAD where abnormalities in lipid fractions are common. Fibrates influence the HDL-C and TG levels and also alters the distribution of LDL-C fractions. Thus, after treatment with a fibrate there is a shift away from dense, highly atherogenic, LDL-C fractions towards more buoyant, less atherogenic, LDL-C fractions (Milionis et al 2000). Elevated TG is common in PAD patients (Pontrelli et al 2002). High doses of statins are recommended as they exert substantial TG lowering action (Mikhailidis et al 1998).

2.3.3.2 Inflammation

The relation between CRP and lipid levels is of interest because lipid lowering treatments correct dyslipidaemia and decrease serum CRP (Jialal et al 2001). Pravastatin and atorvastatin resulted in a significant reduction in CRP that were not related to the magnitude of lipid alterations observed, which further support the potential of nonlipid lowering effects of statins (Kent et al 2003). Another statin (simvastatin) reduced serological markers of inflammation and stabilized the arterial plaque, independent of
lipoproteins changes (Koh et al 2002). Adding CRP to the lipid variables significantly (p<0.001) increased the predictive value based on lipid screening alone. These findings are relevant as an elevated CRP level (measured as high-sensitivity CRP, hs-CRP) predicts the vascular risk including the high incidence of myocardial infarction in more severe cases of PAD needing revascularisation (Rossi et al 2002). Monitoring CRP is recommended because those patients who have lower CRP after statin treatment have lower vascular risk independent of their LDL-C values (Ridker et al 2001). A decrease in the CCA-IMT and CFA-IMT of patients with PAD occurred very rapidly (8 weeks) after treatment with atorvastatin 20 mg/day (Davis et al 2000, yousef et al 2002). These rapid responses probably reflect the anti-inflammatory properties of statins rather than resulting from a decrease in arterial lipid content (McDermott et al 2003). This finding is in line with the rapid changes in blood pressure that have been reported in other patient group after taking a statin (Borghi et al 2001). Statin treatment also improved the endothelial function, another marker of atherosclerosis and inflammation and also improved cutaneous microvascular responses (Khan et al 1997, Kirk et al 1999).

2.3.3.3 Angiogenesis

The relationship between lipid metabolism and angiogenesis is complex and not fully understood. Accumulating data indicate that many of statin effects are attributable to the cellular consequences of depletion of intermediates in the cholesterol biosynthetic pathway (isoprenoids). These molecules play fundamental roles in cell growth, signal transduction, and mitogenesis (Vaughan 2003). Statins may affect angiogenesis with possible important therapeutic implications (Park et al 2002). By inhibiting geranylgeranylation and membrane localization of RhoA, statins may interfere with angiogenesis as several signalling pathways
that play a role in angiogenesis are dependent on RhoA (Park et al 2002). Thus, statins interfere with new blood vessel formation both in vitro and in vivo models of angiogenesis (Walter et al 2004). Furthermore, it has previously been reported that the number of blood vessels in the atherosclerotic lesions of cholesterol-fed animals decreases after statin treatment compared with control animals, which is clinically relevant, because it indicates that prolonged statin treatment might play a role in the inhibition of angiogenesis. On the other hand simvastatin treatment promoted angiogenesis in normocholesterolaemic animals (Kureishi et al 2000).

2.3.3.4 Lipoprotein (a)

There is evidence that Lp (a) levels are relevant in diabetes patients with PAD (Wollesen et al 1999, Cheshire et al 1996). Elevated Lp (a) levels may increase the risk of stroke. However, there is no convincing trial-based evidence showing that lowering Lp (a) levels will result in a clinically relevant benefit. In this context, there is preliminary evidence that fibrates (Mikhailidis et al 1998) and long term statin (van Wissen et al 2003) treatment may reduce Lp (a) levels.

2.3.3.5 Platelet Activity

Platelets are hyperactive in patients with PAD. Thus, antiplatelet agents provide a significant reduction in the risk of vascular events (Robless et al 2001). Both statins and fibrates can reduce platelet hyperactivity (Milionis et al 1999).
2.3.3.6 Plasma Homocysteine

Elevated plasma homocysteine predicts the progression of CHD as well as ‘vascular’ death in patients with CHD, PAD or both conditions (Giral et al 2001). Mean plasma homocysteine level was significantly higher (p<0.001) in PAD patients as compared to age-matched controls. This abnormality occurred together with a low HDL-C and raised TG (Rassoul et al 2000). Therefore, it is relevant to consider the effect of lipid lowering drugs on plasma homocysteine. Fibrates (with the possible exception of gemfibrozil) appear to increase the plasma levels of serum homocysteine (Bostom 2001, Dierkes et al 1999). Statins do not have this effect (de Lorgeril et al 1999, Westphal et al 2001).

2.3.3.7 Renal Function

In general, fibrates raise creatinine levels whereas statins improve renal function (Giral et al 2001, Kakafika et al 2001). Microalbuminuria, which is considered as an indicator of generalized atherosclerosis and reflect the degree of renal impairment is associated with dyslipidaemia and is frequently found in patients with PAD (Velussi et al 1999). Both microalbuminuria and PAD were associated with an increased risk of cardiovascular mortality in a study involving 631 patients aged 50±75 years and followed-up for five years (Jager et al 1999). There is evidence of statin treatment decreasing microalbuminuria, although most of this research was carried out in diabetic patients and not in those with PAD (Nagai et al 2000, Fried et al 2001).
2.4 WHERE ARE WE IN PROVIDING LIPID LOWERING TREATMENT FOR
PERIPHERAL ARTERIAL DISEASE PATIENTS?

The morbidity and mortality associated with PAD creates a huge burden in terms of costs
both to the patient and to the National Health Service. Published PAD guidelines
recommend aggressive lipid lowering. However, there is a need to detect PAD patients at
an early stage.

The provision of appropriate lipid lowering treatment will undoubtedly vary considerably
even within the same country. However, there is evidence showing that clinician awareness
is poor and that the current standard of medical care is low in patients for PAD (Garg et al
2000, Nawawi et al 1999). Perhaps this reflects the lack of specialists, as well as training, in
vascular medicine. With the ever-increasing publication of trials relating to vascular disease
prevention, keeping up to date and providing optimal treatment has become a difficult task.

However, there is evidence showing that it is both feasible and safe to modify multiple
vascular risk factors, especially dyslipidaemia in patients with PAD (Aronow et al 2002,
McDermott et al 2003). That is why in January 1994 a specialist vascular risk modification
clinic is started at the Royal Free Hospital (Royal Free & University College Medical
School, University College of London).
2.5 CONCLUDING COMMENTS

Based on the NCEP ATP III (Adult Treatment Panel III 2001) guidelines and the Heart Protection Study results and other lipid lowering trials, it is clear that we must treat patients with PAD using the same lipid criteria as if they had CHD. Therefore, the main lipid target is LDL-C of 2.0 mmol/l or less (this goal may change in the future). In addition the ideal HDL-C value would be above 1.0 mmol/l. It goes without saying that the other nonlipid vascular risk factors (e.g. hypertension, hyperactive platelets, diabetes and smoking) must also be aggressively treated. PAD patients are likely to also take other drugs; for example, antihypertensives. Therefore, it is of interest that blood pressure lowering drugs may affect lipid and haemostatic variables (Yataco et al 1999). Since hypercholesterolaemia is a chronic condition, the long-term safety of statin treatment is important. The withdrawal of cerivastatin because of deaths from rhabdomyolysis, of which 25% were related to gemfibrozil-cerivastatin combination therapy, has focused attention on myotoxicity associated with statins and in particular with statin-fibrate combinations (Evans et al 2002). Thus, the choice of hyperlipidaemic therapy needs to be based not only on the outcome evidence and the cost-effectiveness analysis, but also on safety considerations for individual agents.
CHAPTER III

THE EARLY EFFECT OF LIPID LOWERING TREATMENT ON CAROTID AND FEMORAL INTIMA MEDIA THICKNESS
INTRODUCTION

Progressive carotid disease is common in PAD patients, which justifies screening for carotid stenosis even in asymptomatic PAD patients (Cina et al 2002). Both stiffening and thickening of the arterial wall reflects the extent of atherosclerosis. These variables reflect the atherosclerotic plaque burden thus offering one way to assess vascular risk (Taniwaki et al 2001). Furthermore, an increased CCA-IMT is associated with major cardiovascular risk and is a powerful independent predictor of cardiovascular events, particularly those associated with CHD (Zanchetti 1999, O'Leary et al 1996, Hulthe et al 1997). The CCA-IMT measured using non-invasive B-mode sonography has been shown to correlate with coronary plaque burden (Tang et al 2000, Montauban et al 1999). In addition, increased CCA-IMT was strongly associated with the risk of stroke (Chambless et al 2000 ARIC). Application of this technique may allow for more accurate prediction of risk in older persons. In 133 patients the severity of coronary disease, as shown by coronary angiography, was significantly correlated with the CCA-IMT measured by ultrasonography (Mack et al 2000). Even small changes in CCA-IMT were associated with clinically significant atherosclerosis in the peripheral arteries (Allan et al 1997, The Edinburgh Artery Study).

There are only few studies involving the CFA-IMT. In a multiple regression analysis of symptomatic PAD patients, reduced ABPI was associated with increased CFA-IMT (Taniwaki et al 2001). Other studies showed that the combined assessment of the carotid and femoral arterial walls provide a more accurate estimate of the atherosclerotic burden.
and risk prediction in patients with familial hyperlipidaemia (Wittekoek et al 1999). Both CCA-IMT and CFA-IMT were also increased in smokers even without other vascular risk factors (Van der Berkmortel et al 2000).

B-mode (two-dimensional) Doppler ultrasonography is increasingly used for non-invasive visualisation and monitoring of atherosclerotic changes in the vasculature (Willekes et al 1999). Its use in measuring the CCA-IMT is now well established (Stensland-Bugge et al 1997), especially after the automated computerized reading reduced the variability in IMT measurement (Wendelhag et al 1997, Schmidt et al 1999).

The above studies suggested that the arterial IMT might represent the sum of overall cardiovascular risk in an individual. Therefore, IMT is expected to increase in those with established vascular disease. Subjects with PAD have a significantly stiffer carotid and femoral arteries, which may reflect an increased cardiovascular load and may account for the higher mortality rate seen in these patients (Cheng et al 2002). IMT is a significant component that is independent of conventional risk factors, which suggest that IMT may be a better marker for the risk of future vascular events. Treating the associated cardiovascular risk is important and may have to be tailored on an individual basis according to the findings of the arterial wall mechanics.

Hyperlipidaemia predisposes to an increased CCA-IMT (Hodis et al 1997) and cholesterol-lowering treatment causes CCA-IMT regression (Davis et al 2000). Recent secondary prevention studies (4S, CARE, TNT) using statins have demonstrated a significant reduction in ischaemic strokes without increasing the incidence of haemorrhagic strokes. Statins probably reduce stroke by a variety of mechanisms, including modulation of precerebral atherothrombosis in the aorta and the carotid artery, thus preventing plaque disruption and artery-to-artery thromboembolism (Brown et al 1993). Cholesterol lowering
treatment effectively prevents the overall vascular events and reduces the mortality in patients with established CHD (Rizos et al 2001).

Therefore, we monitored the CCA-IMT and CFA-IMT in patients with PAD and raised serum cholesterol 4 and 8 weeks after treatment with atorvastatin. The aim of the study was to establish whether the IMT changes significantly within this short period. We also monitored the other risk factors and renal function during the same period.
3.2 METHODS

A total of 25 patients (14 men and 11 women), median age 69 years (range: 48 to 81) were recruited. Their pre-treatment characteristics are shown in (Table. 1). The study population consisted of patients who were referred to a university hospital vascular surgery clinic with stable PAD and who were found to have a serum TC ≥5.5 mmol/l and/or LDL-C ≥3.0 mmol/l. PAD was defined as stable uncomplicated IC with an ABPI <0.8.

3.2.1 Exclusion Criteria

- Critical leg ischaemia
- Significant carotid disease (>70% stenosis) or previous carotid surgery
- Significant femoral artery disease or previous femoral artery surgery
- Uncontrolled diabetes
- Poorly controlled hypertension (BP >150/85) at presentation
- Patients already receiving lipid lowering treatment

Blood samples and measurements

A fasting (12 h) venous sample was obtained to assess:

- Lipid profile
- Haematological and biochemical profile
- Plasma fibrinogen and factor XII levels (indices of coagulation system)
- Renal and liver function tests
- High sensitivity C-reactive protein (hs-CRP)
3.2.2 Carotid and Femoral Artery IMT Measurements

A variety of IMT measurement protocols, based on different ultrasound methods have been developed in order to obtain reliable and reproducible results. We used an automatic technique (Sidhu et al 1997) that has been validated against the conventional manual technique; there was a significant correlation between the two methods. This technique has been described elsewhere in detail (Willeks et al 1999); in brief, patients were scanned in the supine position and electrocardiography (ECG) leads were placed appropriately on the chest wall for ECG, R-wave triggered measurements. The patients were given 15 minutes break (after taking the blood samples). A longitudinal B-Mode image of the blood vessel was visualised using a Doppler ultrasonography scanner with a 7.5 MHz linear-transducer (Scanner 350, Pie Medical System, Maastricht, The Netherlands), then switched to echo M-mode with a high pulse-repetition frequency and a short activation pulse. The radio frequency (RF) signals were transferred to a computer with wall tracking software and the IMT was determined automatically (WTS ver 2.0, Pie Medical System, Maastricht, The Netherlands).

The IMT of the right carotid artery was measured at the posterior wall. The posterior wall was selected according to recommendations by previous studies as the trailing edge of the adventitia signal of the anterior wall will obscure the media and influence the measurement of this IMT. On the other hand the far wall is well visualized and likely to show the earliest atherosclerotic changes. The same locations of arteries were scanned pre-treatment as well as at 4 and 8 weeks after treatment with atorvastatin (Lipitor™) 20 mg/day. The post-treatment measurements were taken in the same room with the same ultrasound scanner in the same position to reduce the error.
The IMT measurements were taken 2 cm proximal to the carotid bifurcation and the results were expressed as the mean of six measurements taken at each visit; these were blindly analysed. The same method was used for the femoral artery measurements, which were taken 2 cm proximal to the origin of the superficial femoral artery.

Measuring the IMT using the automatic technique with wall tracking software is been used and validated in the department (Davis et al 2002).

**Ethics, data collection and statistical analysis**

The Ethics Committee of the Royal Free Hospital NHS Trust approved the study and written consent was obtained. The data was blindly analysed by giving random numbers to each measurement of IMT and each blood sample. Paired t test was used to check if the changes in IMT (pre-treatment vs. 4 weeks and pre-treatment vs. 8 weeks) and lipid profile (pre-treatment vs. 8 weeks) were significant. The multivariate ANOVA test was used to assess the trend of changes in IMT over the whole study period (i.e. 8 weeks). The significance of the changes in serum creatinine levels before and after treatment for 8 weeks was assessed using a paired Wilcoxon test. A p value of <0.05 was considered statistically significant. Correlation was assessed by deriving the Pearson’s coefficient.

The primary end point was to measure the changes in carotid and femoral IMT after 8 weeks of treatment. Secondary end points were the changes in the other variables measured in the blood.
Table 1
Baseline characteristics of patients for the IMT measurements

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>25</td>
</tr>
<tr>
<td>Age median (range) (years)</td>
<td>69 (48 - 81)</td>
</tr>
<tr>
<td>Gender (M,F)</td>
<td>14,11</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.5 (2.8)</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>145 (10)</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>87 (8)</td>
</tr>
<tr>
<td>No. of current smokers (%)</td>
<td>5 (20%)</td>
</tr>
<tr>
<td>No. of ex-smokers (%)</td>
<td>20 (80%)</td>
</tr>
<tr>
<td>No. of patients on aspirin (%)</td>
<td>18 (72%)</td>
</tr>
<tr>
<td>No. of patients on antihypertensives (%)</td>
<td>20 (80%)</td>
</tr>
</tbody>
</table>

Values are expressed as mean (SD) unless otherwise stated.

BMI= Body mass index
M= Male
F= Female
BP= Blood pressure (median of three measurements; at presentation, week 4 & 8 post treatment).
3.3 RESULTS

3.3.1 Biochemical Results

There was no statistical difference in the haematological profile between visits at 0 and 8 weeks (results not shown). There was no significant change in the pre- and post-treatment weight or BMI. There was a significant (p = 0.007) decrease in serum creatinine values. The patients were divided into two halves according to their pre-treatment creatinine levels, one with the highest serum creatinine levels (n= 12) and the other (n= 13) with the lowest serum creatinine levels. The decrease in serum creatinine was only significant (p = 0.008) in the ‘higher creatinine’ group. Similarly, there was a fall in serum urate values in the group with a higher creatinine.

There were no significant changes in liver function tests, creatine kinase, fibrinogen, and urea or factor XII levels (Table. 2).

3.3.2 Changes in Lipid Profile

After 8 weeks of treatment there was a significant reduction in total cholesterol (p = 0.0004) and in LDL-C (p = 0.0001). There was no significant change in the HDL-C, but the trend was to an increase in these levels. The triglyceride levels decreased significantly (p = 0.05) (Table. 3). After 8 weeks treatment all the patients had a LDL-C level <3.0 mmol/l, the UK guideline value for patients with vascular disease (Wood et al 1998).
3.3.3 Changes in Carotid and Femoral IMT

The ANOVA test for the trend was significant (p = 0.024) for the CCA-IMT and for the CFA-IMT (p = 0.0003) after atorvastatin treatment. The changes in CCA-IMT and CFA-IMT were not significant when the pre-treatment and 4 week values were compared. However, they were significant (p = 0.01 CCA-IMT; p = 0.005 CFA-IMT) when the pre-treatment and 8 week values were compared (Table. 4). There was no significant correlation between the carotid and the femoral IMT values pre-treatment or at the end of the 8 weeks treatment. A decrease of the CCA-IMT by >0.1 mm was noted in 10 patients (40 %) after 4 weeks and in 18 patients (72%) after 8 weeks of treatment. For the CFA-IMT the corresponding figures were 8 (32%) and 19 (76%) patients, respectively (Fig 1). There was no correlation between the changes in the lipid levels and the IMT changes.

Side effects

There were no side effects, vascular events or dropouts during this 8 week study.
Figure 1.

Overall changes in carotid and femoral IMT

Distribution of the common carotid (a) and common femoral (b) IMTs for the three measurements (baseline and after 4 and 8 weeks treatment). The horizontal line represents the mean value.

For statistical analysis please see (Table. 4) and text

PT = Pre-treatment IMT values

4WP = Four weeks post-treatment IMT values

8WP = Eight weeks post-treatment IMT values
**Table 2:**

**Changes in blood parameters after treatment with atorvastatin**

Changes in serum creatinine, serum urea, serum urate, plasma fibrinogen and plasma factor XII after 8 weeks of treatment with 20 mg/day of atorvastatin. The results are expressed as median and range.

<table>
<thead>
<tr>
<th>Blood test</th>
<th>Pre-treatment</th>
<th>8 Weeks</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Creatinine (µmol/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All Patients (n=25)</td>
<td>87 (67-114)</td>
<td>84 (64-112)</td>
<td>0.007</td>
</tr>
<tr>
<td>Patients with the highest 12 creatinine values*</td>
<td>98 (89-114)</td>
<td>91 (83-112)</td>
<td>0.008</td>
</tr>
<tr>
<td>Patients with the lowest 13 creatinine values*</td>
<td>77 (67-84)</td>
<td>77 (64-85)</td>
<td>NS</td>
</tr>
<tr>
<td>Serum Urate (mmol/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All Patients (n=25)</td>
<td>0.33 (0.17-0.43)</td>
<td>0.32 (0.19-0.40)</td>
<td>NS</td>
</tr>
<tr>
<td>Patients with the highest 12 creatinine values*</td>
<td>0.33 (0.24-0.43)</td>
<td>0.32 (0.22-0.40)</td>
<td>0.04</td>
</tr>
<tr>
<td>Patients with the lowest 13 creatinine values*</td>
<td>0.29 (0.17-0.40)</td>
<td>0.30 (0.19-0.35)</td>
<td>NS</td>
</tr>
<tr>
<td>Urea (mmol/l)</td>
<td>5.7 (1.9-9.6)</td>
<td>5.4 (1.8-9.1)</td>
<td>NS</td>
</tr>
<tr>
<td>Fibrinogen (mg/l)</td>
<td>323 (216-565)</td>
<td>304 (194-494)</td>
<td>NS</td>
</tr>
<tr>
<td>Factor XII (ng/ml)</td>
<td>1.7 (0.7-2.8)</td>
<td>1.2 (0.7-4.2)</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS= not significant

*pre-treatment
Table 3:

Changes in the lipid profile after 8 weeks treatment with atorvastatin

<table>
<thead>
<tr>
<th></th>
<th>Pre-treatment</th>
<th>8 weeks</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cholesterol (mmol/l)</td>
<td>6.0 (0.3)</td>
<td>4.3 (0.8)</td>
<td>0.0004</td>
</tr>
<tr>
<td>LDL-C (mmol/l)</td>
<td>3.7 (0.2)</td>
<td>2.2 (0.5)</td>
<td>0.0001</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>1.8 (0.6)</td>
<td>2.0 (0.5)</td>
<td>NS</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>1.3 (0.4)</td>
<td>0.8 (0.3)</td>
<td>0.05</td>
</tr>
</tbody>
</table>

LDL-C = low density lipoprotein cholesterol

HDL-C = high density lipoprotein cholesterol

TG = triglycerides
### Table 4:

Changes in carotid and femoral IMT after treatment

Right common carotid artery (CCA) and right common femoral artery (CFA) IMT before and after treatment with 20 mg/day atorvastatin.

The IMT (mm) is expressed as mean ±SD

<table>
<thead>
<tr>
<th>Arteries</th>
<th>Pre-treatment</th>
<th>4WP</th>
<th>8WP</th>
<th>*P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCA-IMT</td>
<td>0.79 (0.21)</td>
<td>0.75 (0.22)</td>
<td>0.64 (0.15)</td>
<td>0.024</td>
</tr>
<tr>
<td>CFA-IMT</td>
<td>0.83 (0.13)</td>
<td>0.80 (0.09)</td>
<td>0.69 (0.14)</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

*ANOVA test for the trend over the 8 week treatment period

4WP= Four weeks post-treatment

8WP= Eight weeks post-treatment
3.4 DISCUSSION

Achieving UK guideline values for LDL-C (<3.0 mmol/l) within 8 weeks using atorvastatin (20 mg/day) in patients with PAD was associated with a significant decrease in the CCA-IMT and CFA-IMT. These findings confirm those of our preliminary study where we only assessed the CCA-IMT under similar circumstances (Davis et al 2000).

We attributed this rapid improvement to anti-inflammatory changes because it is unlikely that in this short time span there could be a significant reduction in lipid deposits in the intima and media. Our conclusion is based on the findings of an experimental model of carotid injury. In this model, a decrease in intimal thickening, VSMC proliferation and infiltration by macrophages was noted after the administration of a statin for two weeks (Igarashi et al 1997). Furthermore, there is evidence that atorvastatin can significantly reduce the circulating levels of CRP in patients with combined hyperlipidaemia after treatment for 6 weeks (Jilal et al 2001). Improvement in endothelial function is also reported within one month of simvastatin treatment (O’Driscoll et al 1997, Dupuis et al 1999). Statin treatment reduces the risk of ischaemic stroke despite the fact that LDL-C is not directly associated with the risk of stroke. These observations lead to the investigation of the role of statins in inflammation and the immune system.

Observing a trend as early as four weeks after commencing atorvastatin supports the rapidity of the effect of lipid lowering treatment on the IMT. The four week IMT measurements were not significantly decreased when directly compared with pre-treatment values, but when they were included as part of the ANOVA analysis this was significant for both the CCA-IMT (p= 0.024) and CFA-IMT (p= 0.0003). This difference in significance suggests that the carotid and femoral arteries may not respond at the same degree to lipid
lowering with a statin. Indeed a greater decrease in the CFA-IMT compared with the CCA-IMT was reported after statin treatment for a period of one year (Ubels et al 2001). Others have proposed that initial lipid lowering therapy is associated with a greater improvement in the CFA-IMT and CCA-IMT than when this form of treatment was intensified in patients already taking statins.

Furthermore, in the Myocardial Ischaemia Reduction with Aggressive Cholesterol Lowering (MIRACL) study there was an unexplained significant (p= 0.045) and marked (50%) reduction in strokes in patients with acute coronary syndrome who were treated for 16 weeks with atorvastatin (80 mg/day). MIRACL was a placebo controlled, double blind, randomized study and it included >3000 patients (Schwartz et al 2001). Clearly there is a need to further investigate the rapid changes in the vasculature that follow lipid lowering treatment. This (unfortunately) should include animal-based studies so as to allow for the extensive histological and functional investigation of several arteries.

Cerivastatin treatment modulated the arterial wall biology by inhibiting the activation of neutrophils and macrophages, indicating a role for statin treatment in prevention (Nagashima et al 2002). Four weeks treatment with atorvastatin reduced VSMC proliferation and inflammation in stented vessels of rabbits with or without hypercholesterolemia (Herde et al 2003). In addition, cerevastatin prevented macrophage accumulation in the intima, which contributes to inhibiting the intimal thickening (Igarashi et al 1997). Lipid lowering treatment has positive effects on carotid and femoral artery wall stiffness and thickness. A decrease in carotid artery IMT with atorvastatin has been previously documented, but was of the order of 12±24 months (Smilde et al 2000). The patients had familial hypercholesterolaemia and the dose of atorvastatin was considerably higher (80 mg/day) compared to what we used in the present study (20 mg/day). In a trial
which randomized 919 hyperlipidaemic patient with asymptomatic carotid disease to
treatment with lovastatin 20-40 mg/day or placebo for 12 months there was a significant
(p= 0.001) regression in carotid IMT and significant (p= 0.04) decrease in both vascular
events and deaths (p= 0.02) in the lovastatin group (Furburg et al 1994).
The significant (p= 0.007) fall in serum creatinine levels was relatively unexpected.
However, we assessed that variable on the basis of anecdotal observations in patients with
PAD after correcting their dyslipidaemia. We interpret these findings as indicating an
increase in renal perfusion following lipid lowering treatment. This improvement was most
evident (p= 0.008) in the 50% of our study population with the highest pre-treatment serum
creatinine values. In this context, it is relevant that others have proposed that the
improvement in microalbuminuria (and blood pressure) after treatment with statins is
related to an increased production of nitric oxide (NO) in the kidney. NO is a vasodilator
and may well improve the renal blood flow (Tonolo et al 1997). It is also of interest that
microalbuminuria is associated with an increased carotid IMT (Mykkanen et al 1997). A
further possible effect of statin treatment is a reduction or stabilization of plaque in the
renal arteries. Localized renal atherosclerosis is probably present to a variable degree in
patients with PAD (Farmer et al 1998). The hypothesis that improved renal blood flow was
responsible for our observations require confirmation in larger placebo-controlled studies
with a randomized design. These studies should also include a more detailed assessment of
renal function. However, these changes may be restricted to a specific patient population.
Our findings have several limitations. Our study was not placebo controlled or randomized
and the number of patients was small (n= 25). We selected a more homogeneous group of
PAD patients by defining several exclusion criteria. However, future studies should also
consider further selection; for example by gender.
In concordance with some but not all other reports (Wierzbicki et al 2000, Song et al 2001), we did not observe a rise in plasma fibrinogen concentration after using atorvastatin. Equally, there was no rise in coagulation factor XII. There is also evidence that statins may differ in their effect on HDL-C elevation. However, in this study there was a non-significant 11.1% increase in HDL-C levels after treatment. This value is in line with what is expected with other statins (Mikhailidis et al 2000).

In conclusion, there was a rapid and significant decrease in the CCA-IMT and CFA-IMT in patients with PAD treated for 8 weeks with atorvastatin (20 mg/day). The trend for this change was visible after 4 weeks. In the same population there was a significant fall in serum creatinine values. This effect was restricted to the patients with the highest serum creatinine. This subgroup also had a small but significant fall in serum urate levels. There is a need to confirm these findings in larger placebo-controlled studies with a double blind randomized design. Our results support the findings of several studies showing a rapid (within a few weeks) improvement in indices of vascular function following the correction of a dyslipidaemia with a statin. On a speculative note the present findings, if confirmed, may provide a method for the rapid assessment of the effectiveness of lipid lowering therapy.
CHAPTER IV

THE EFFECT OF SHORT-TERM SIMVASTATIN ON RENAL FUNCTION IN PATIENTS WITH PERIPHERAL ARTERIAL DISEASE
Peripheral arterial disease is associated with a widespread atherosclerosis and a high vascular risk (Robless et al 2001). There is also a strong association between atherosclerotic renal artery stenosis (ARAS) and PAD. A recent study reported that 67% of patients with ARAS also had PAD (Alcazar et al 2001). An incidental finding of ARAS is not uncommon in PAD patients; the prevalence of ARAS is as high as 33% on angiography for PAD (Leertouwer et al 2001). However, angiography is only done in severe cases of PAD. Mild renal insufficiency predicts the vascular risk and the cardiovascular outcome in patients with a pre-existing CHD (Mann et al 2001). Furthermore, both serum urate and serum creatinine are considered independent predictors of vascular events (Puddu et al 2000, Lumley et al 2002). Moreover, hyperuricaemia is more pronounced in hypertensives complicated by PAD and is associated with worse functional status of the peripheral circulation (Langlois et al 2003).

Dyslipidaemia has been linked to the progression of renal disease (Elisaf et al 2002, Cappelli et al 1998). For example, clinical and experimental studies linked dyslipidaemia with microvascular segmental renal disease and glomerulosclerosis (Diamond et al 1988). It was also shown that diet-induced hyperlipidaemia is associated with an increased microvascular density in the renal cortex, which is an early sign of renal damage (Guijarro et al 1995). However, there is still some controversy about which lipoprotein cholesterol predicts the increased risk of renal failure (Muntner et al 2000). This potentially affects the intrarenal blood flow and renal disease progression (Bontley et al 2002). It is also suggested that lipid abnormalities in ARAS mirror that in other manifestations of other severe vascular disease (Scoble et al 1999). Moreover, correcting dyslipidaemia positively
influenced the GFR (Kasiske et al 1988). Also lowering blood LDL-C with a statin has additional positive effects including a reduction in blood pressure (Glorioso et al 1999) and 24 h urinary albumin excretion rate in type II diabetes (Tonolo et al 1997) or in patients with nephrotic syndrome (Rabelink et al 1990). Interestingly, atorvastatin treatment prevented glomerulosclerosis and renal endothelial dysfunction changes associated with hypercholesterolaemia in rabbits (Vazquez-Perez et al 2001). Statin treatment can slow the progression of renal disease in patients with nephrotic syndrome (Thomas et al 1993) and improve proteinuria in patients with well-controlled hypertension (Lee et al 2002). Furthermore, lovastatin retarded the progression of diabetic nephropathy (Lam et al 1995) and fluvastatin improved proteinuria in patients with nephropathy (Buemi et al 2000). The underlying mechanisms responsible for these effects remain unclear (Kakafika et al 2001). Nevertheless, some of the beneficial effects associated with the use of a statin were not seen with cholestyramine treatment despite achieving similar lipid targets (Tonolo et al 2000). Indeed, unlike statin, fibrates use is reported to causes an elevation in plasma creatinine, homocysteine and cystatin C levels, (de Lorgeril et al 1999, Landray et al 1999). In a previous study we reported a significant reduction in serum creatinine and urate levels in PAD patients after eight weeks of treatment with atorvastatin 20 mg/day (Youssef et al 2002). However, a literature review did not reveal any studies assessing the effect of statins on renal function in claudicants. Therefore, we conducted the present retrospective survey to assess the validity of our previous findings and to study the effects of lipid lowering treatment on renal function in patients with PAD. We included a greater number of PAD patients (103 vs. 25) who received a different statin (simvastatin vs. atorvastatin) for a longer period (3-4 months vs. 8 weeks).
4.2 PATIENTS AND METHODS

This is a retrospective analysis of a prospectively collected data. The study included 103 claudicants who were referred to a University Hospital vascular surgery and receive additional follow up in a vascular risk modification clinic as part of the comprehensive service offered to patients with established vascular disease.

The fasting lipid profile of all patients included in this study did not meet the United Kingdom guidelines LDL-C level (≤3 mmol/l) (Wood et al 1998). The study included stable uncomplicated claudicants with an ABPI <0.8. Dietary advice was given to all participants. All the patients were biochemically euthyroid. Their pre-treatment baseline characteristics are shown in (Table. 5). The treatment for high blood pressure was reviewed and changes in medications were necessary in 5% of patients after starting simvastatin treatment. All patients were started on simvastatin treatment 20 mg/day for 3 - 4 months.

Exclusion criteria

- Familial hypercholesterolaemia
- Diabetes
- Serum creatinine level above the reference range (120 µmol/l) at presentation
- Patients who were already receiving lipid lowering treatment at presentation
**Blood samples and measurements**

A fasting (at least 12 h) venous sample was obtained to assess the lipid profile as well as renal and liver function. The lipid profile included TC, LDL-C, HDL-C, TG and the calculated LDL-C/HDL-C ratio. All these lipid markers are useful predictors of vascular risk. After 3-4 months of treatment an identical assessment was carried out. It was documented if the patients actually took their treatment regularly. Patients who did not comply were excluded from the study.

**Data collection and statistical analysis**

The results were collected by referring to the patients’ notes and blood results. In the vascular risk modification clinic the protocol, for all patients, includes a record of the blood pressure measurements, weight and medication at baseline and during the follow up, especially changing the doses of existing medications or adding new treatment. The significance of the changes in serum creatinine, urate and lipids before and after treatment with simvastatin for 3-4 months were assessed using a paired t-test. A p value of <0.05 was considered significant. Correlation was assessed by deriving the Pearson’s correlation coefficient. Values are expressed as mean (±SD) unless otherwise specified. The results were divided into tertiles or halves to identify if there were any trends in the association between lipid and renal function changes.
Table 5:
Baseline patient characteristics for the retrospective renal function study

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>103</td>
</tr>
<tr>
<td>Age median (range) (years)</td>
<td>67 (51 N 83)</td>
</tr>
<tr>
<td>Gender (M, W)</td>
<td>57, 46</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.3 (3.1)</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>142 (15)</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>84 (7)</td>
</tr>
<tr>
<td>No. of current smokers (%)</td>
<td>26 (25%)</td>
</tr>
<tr>
<td>No. of ex-smokers (%)</td>
<td>66 (64%)</td>
</tr>
<tr>
<td>No. of patients on antiplatelet agent (%)</td>
<td>82 (80%)</td>
</tr>
<tr>
<td>No. of patients on antihypertensives (%)</td>
<td>74 (72%)</td>
</tr>
</tbody>
</table>

Values are expressed as mean (SD) unless otherwise stated

BMI = Body mass index
M= Men
W= Women
BP= Blood pressure
4.3 RESULTS

There were no significant changes in BMI or systolic and diastolic blood pressure post-treatment (Table. 5). Patients remained on 20 mg/day of simvastatin at the end of the study.

Biochemical investigations

Creatinine (Table. 6)

There was a 3.6% reduction in serum creatinine level, which was statistically significant (P< 0.0001). The 95% confidence interval of the difference was 1.8 to 4.1 µmol/l.

Urate (Table. 6)

There was a significant (P< 0.0001) fall in serum urate concentrations by 5.4%. The 95% confidence interval of the difference was 0.01 to 0.03 mmol/l.

Lipids (Table. 6)

As expected, there was a significant (P< 0.0001) reduction in both TC and in LDL-C. There was also a significant (P= 0.0004) increase in HDL-C levels. As a result the LDL-C/HDL-C ratio decreased significantly (P< 0.0001).

There was also a significant decrease (P< 0.0001) in TG serum levels.
Correlations between creatinine, urate and lipid variables

There was a significant correlation between creatinine and urate levels. There was a significant negative correlation between HDL-C and creatinine levels, which was more significant after treatment. There was also a correlation between the LDL-C/HDL-C ratio and serum creatinine post-treatment (Table. 7).

LDL-C goal

After 3-4 months treatment 70 patients (68%) had a serum LDL-C level \( \leq 3 \) mmol/l, the UK guideline target value for patients with vascular disease (Wood et al 1998).

Changes when patients were divided into three tertiles according to baseline serum creatinine levels

The patients were divided into tertiles according to their pre-treatment serum creatinine levels. There was a more significant decrease in serum creatinine in the tertile with the highest pre-treatment serum creatinine as compared to the second and third tertile (\( P = 0.0003 \) vs. \( P = 0.04 \) and \( P = 0.02 \)), respectively. The TC, LDL-C and TG values fell significantly in all three tertiles. The baseline and post-treatment values of these lipid variables were similar in all three tertiles. Baseline and post-treatment values of HDL-C were identical in the tertile with the highest pre-treatment creatinine values; this tertile also showed the greatest and most significant changes in creatinine. In contrast, in the other two tertiles HDL-C increased significantly after treatment (Table. 8).
The baseline and post-treatment HDL-C values were lower in the third tertile when compared with the first tertile (P< 0.016). This change is reflected in LDL-C/HDL-C ratio (Table. 8).

**Changes when patients were divided according to the extent of the fall in serum creatinine level after treatment**

The patients were divided into two groups according to the change in serum creatinine level. The fall in the urate values was only significant in the group with the greatest drop in creatinine levels. The TC, LDL-C and TG values fell significantly in both groups. The baseline and post-treatment values of these lipid variables were similar in both groups. The rise in HDL-C level was more significant (P= 0.0024 vs. P= 0.05) in the group with the greatest decrease in serum creatinine level (Table. 9).

**Changes when patients were divided according to the extent of the fall in serum urate level after treatment**

We divided the patients into two groups according to the fall in serum urate level. The fall in serum creatinine level was more significant in the group with the greatest drop in serum urate (P< 0.0001 vs. P= 0.0086). The TC, LDL-C and TG values fell significantly in both groups. The baseline and post-treatment values of these lipid variables were similar in both groups. There was no significant change in HDL-C in the patients with the best urate changes. However, the HDL-C rise was significant (P< 0.0003) in the other group (Table. 10).
Table 6:

Changes in renal indices and biochemical variables in all patients (n= 103)

<table>
<thead>
<tr>
<th></th>
<th>Pre-treatment</th>
<th>Post-treatment</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine (μmol/l)</td>
<td>87 ± 12</td>
<td>84 ± 12</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Urate (mmol/l)</td>
<td>0.37 ± 0.07</td>
<td>0.35 ± 0.07</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>TC (mmol/l)</td>
<td>6.6 ± 1.0</td>
<td>5.2 ± 0.8</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>1.4 ± 0.3</td>
<td>1.5 ± 0.4</td>
<td>p = 0.0004</td>
</tr>
<tr>
<td>LDL-C (mmol/l)</td>
<td>4.3 ± 1.0</td>
<td>2.8 ± 0.7</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>2.1 ± 1.0</td>
<td>1.7 ± 1.0</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>LDL-C/HDL-C</td>
<td>3.3 ± 1.1</td>
<td>2.1 ± 0.7</td>
<td>p &lt; 0.0001</td>
</tr>
</tbody>
</table>
Table 7:

The correlation between creatinine, urate and lipids

<table>
<thead>
<tr>
<th></th>
<th>Pre-treatment</th>
<th></th>
<th>Post-treatment</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Correlation</td>
<td>P value</td>
<td>Correlation</td>
<td>P value</td>
</tr>
<tr>
<td></td>
<td>coefficient</td>
<td></td>
<td>coefficient</td>
<td></td>
</tr>
<tr>
<td>Urate vs. creatinine</td>
<td>$r = 0.35$</td>
<td>$P = 0.0004$</td>
<td>$r = 0.42$</td>
<td>$P &lt; 0.0001$</td>
</tr>
<tr>
<td>HDL-C vs. creatinine</td>
<td>$r = -0.23$</td>
<td>$P = 0.023$</td>
<td>$r = -0.34$</td>
<td>$P = 0.0006$</td>
</tr>
<tr>
<td>HDL-C vs. urate</td>
<td>$r = -0.22$</td>
<td>$P = 0.031$</td>
<td>$r = -0.21$</td>
<td>$P = 0.035$</td>
</tr>
<tr>
<td>LDL-C/HDL-C vs. creatinine</td>
<td>$r = 0.18$</td>
<td>NS</td>
<td>$r = 0.42$</td>
<td>$P &lt; 0.0001$</td>
</tr>
<tr>
<td>LDL-C/HDL-C vs. urate</td>
<td>$r = 0.12$</td>
<td>NS</td>
<td>$r = 0.21$</td>
<td>$P = 0.032$</td>
</tr>
</tbody>
</table>

NS = not significant
Table 8:

Changes in the tertiles according to pre-treatment serum creatinine levels

<table>
<thead>
<tr>
<th></th>
<th>creatinine μmol/l</th>
<th>Urate mmol/l</th>
<th>TC mmol/l</th>
<th>HDL-C mmol/l</th>
<th>LDL-C mmol/l</th>
<th>TG mmol/l</th>
<th>LDL-C/ HDL-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pre Post p value n = 35</td>
<td>75 ± 4</td>
<td>0.33 ± 0.06</td>
<td>6.7 ± 1.3</td>
<td>1.5 ± 0.33</td>
<td>4.4 ± 1.2</td>
<td>3.2 ± 1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>73 ± 7</td>
<td>0.31 ± 0.06</td>
<td>5.0 ± 0.8</td>
<td>1.6 ± 0.41</td>
<td>2.8 ± 0.9</td>
<td>1.9 ± 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p = 0.02</td>
<td>p = 0.01</td>
<td>p &lt; 0.0001</td>
<td>p = 0.01</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>2</td>
<td>Pre Post P value N = 34</td>
<td>84 ± 2</td>
<td>0.36 ± 0.07</td>
<td>6.5 ± 0.8</td>
<td>1.4 ± 0.4</td>
<td>4.2 ± 1.0</td>
<td>3.2 ± 1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>83 ± 6</td>
<td>0.34 ± 0.06</td>
<td>5.2 ± 0.8</td>
<td>1.5 ± 0.4</td>
<td>2.9 ± 0.7</td>
<td>2.0 ± 0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p = 0.04</td>
<td>p = 0.01</td>
<td>p &lt; 0.0001</td>
<td>p = 0.004</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>3</td>
<td>Pre Post p value n = 34</td>
<td>100 ± 9</td>
<td>0.40 ± 0.07</td>
<td>6.6 ± 0.8</td>
<td>1.3 ± 0.3</td>
<td>4.3 ± 0.8</td>
<td>3.5 ± 0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>96 ± 11</td>
<td>0.38 ± 0.06</td>
<td>5.3 ± 0.7</td>
<td>1.3 ± 0.3</td>
<td>3.3 ± 0.7</td>
<td>2.5 ± 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p = 0.0039</td>
<td>p = 0.009</td>
<td>p &lt; 0.0001</td>
<td>p = 0.008</td>
<td>p &lt; 0.0001</td>
<td>p &lt; 0.0001</td>
</tr>
</tbody>
</table>

NS = not significant

Pre = pre-treatment

Post = post-treatment
Table 9:
Lipid and urate changes according to the change in serum Creatinine concentration after treatment with simvastatin

<table>
<thead>
<tr>
<th></th>
<th>Best creatinine changes n = 51</th>
<th>Worse creatinine changes n = 52</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Creatinine (μmol/l)</td>
<td>88 ± 12</td>
<td>81 ± 13</td>
</tr>
<tr>
<td>Urate (mmol/l)</td>
<td>0.37 ± 0.07</td>
<td>0.34 ± 0.07</td>
</tr>
<tr>
<td>TC (mmol/l)</td>
<td>6.6 ± 1.0</td>
<td>5.3 ± 0.7</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>1.5 ± 0.4</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>LDL-C (mmol/l)</td>
<td>4.2 ± 1.0</td>
<td>3.0 ± 0.7</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>2.1 ± 1.0</td>
<td>1.7 ± 0.9</td>
</tr>
<tr>
<td>LDL-C/HDL-C</td>
<td>3.2 ± 1.2</td>
<td>2.0 ± 0.7</td>
</tr>
</tbody>
</table>

+ = pre-treatment vs. post-treatment values

* No statistical analysis because this is the variable that defines the two patient groups

NS = not significant
Table 10:
Lipid and creatinine changes in-patients classified according to the change in serum urate after treatment

<table>
<thead>
<tr>
<th></th>
<th>Best urate changes (n = 51)</th>
<th></th>
<th>Worst urate changes (n = 52)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>P value +</td>
<td>Before</td>
</tr>
<tr>
<td>Urate (mmol/l)</td>
<td>0.40 ± 0.06</td>
<td>0.34 ± 0.06</td>
<td>*</td>
<td>0.34 ± 0.07</td>
</tr>
<tr>
<td>Creatinine (µmol/l)</td>
<td>86 ± 12</td>
<td>82 ± 12</td>
<td>p &lt; 0.0001</td>
<td>87 ± 13</td>
</tr>
<tr>
<td>TC (mmol/l)</td>
<td>6.6 ± 1.2</td>
<td>5.1 ± 0.9</td>
<td>p &lt; 0.0001</td>
<td>6.7 ± 0.9</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>1.3 ± 0.3</td>
<td>1.4 ± 0.4</td>
<td>NS</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>LDL-C (mmol/l)</td>
<td>4.3 ± 1.1</td>
<td>3.0 ± 0.9</td>
<td>p &lt; 0.0001</td>
<td>4.3 ± 0.9</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>2.1 ± 1.2</td>
<td>1.6 ± 0.9</td>
<td>p = 0.0024</td>
<td>2.0 ± 0.9</td>
</tr>
<tr>
<td>LDL-C / HDL-C</td>
<td>3.3 ± 1.3</td>
<td>2.3 ± 0.9</td>
<td>p &lt; 0.0001</td>
<td>3.2 ± 1.1</td>
</tr>
</tbody>
</table>

+ = pre-treatment vs. post-treatment values

* No statistical analysis because this is the variable that defines the two patients groups

NS = not significant
4.4 DISCUSSION

After treatment with simvastatin 20 mg/day for 3-4 months the UK guideline goal for LDL-C (≤3 mmol/l) was achieved in 68% of the patients with PAD. This improvement in lipid profile was associated with a small but significant (P < 0.0001) decrease in both serum creatinine and urate concentrations. The fall in creatinine was more significant in the tertile of patients with the highest baseline serum creatinine levels, which is in agreement with our previous findings after treatment with atorvastatin (20 mg/day) in similar PAD patients (n=25), but over an eight week period (Youssef et al 2002). Producing comparable results with a different statin suggests that these findings represent a class effect.

When the patients were divided according to the baseline serum creatinine level, the fall in TC, LDL-C and TG levels was similar in all tertiles after treatment with simvastatin. However, the HDL-C did not rise significantly in the third tertile unlike in the other tertiles. When the patients were divided into two groups according to the decrease in creatinine or urate concentrations, the fall in TC, LDL-C and TG levels was similar in both groups after treatment. However, the rise in HDL-C was only of borderline significance (p = 0.05) in those with the least fall in creatinine levels in contrast to a more significant rise (p = 0.0024) in those with the biggest fall in creatinine. This rise in HDL-C was not significant in those with the best fall in urate unlike in those with no decrease in urate levels (p = 0.0003). The post-treatment correlation between HDL-C and creatinine or urate may reflect an improvement in renal function. These findings suggest that the change in any of the measured lipid indices was not consistently associated with the fall in creatinine or urate concentrations.
We noted a correlation between the serum creatinine and urate levels before \((r = 0.35, P = 0.0004)\) and after \((r = 0.42, P < 0.0001)\) treatment with simvastatin. Furthermore, the baseline HDL-C value was significantly \((p < 0.016)\) lower in the tertile with the highest baseline creatinine values when compared with the tertile with the lowest creatinine. Both these findings validate our results because it is expected that creatinine and urate levels will be related and the HDL-C levels fall as renal function deteriorates (Barry et al 1990). The relationship between dyslipidaemia and renal function was also demonstrated by the correlation between creatinine or urate and HDL-C or the LDL-C/HDL-C ratio.

Lipid lowering treatment with a statin is safe even in patients with nephritic syndrome (Thomas et al 1993). Statins may improve renal function by restoring endothelium-dependent nitric oxide (NO) production, which could improve renal blood flow by exerting its vasodilator action (Anderson et al 1995). In a similar manner, it has been proposed that statins up-regulate eNOS, which may result in increased cerebral blood flow. Furthermore, in an experimental hypercholesterolaemic animal model, renal artery endothelium-dependent relaxation was attenuated (Lauufs et al 1998). This effect was attributed to the decrease in NO bioavailability because eNOS was almost undetectable and the relaxation of intact vessels to nitroprusside (a NO donor) was unaltered in the hypercholesterolaemic arteries (Lauufs et al 1998). This effect may be similar to the early improvement in myocardial perfusion as well as coronary and brachial artery blood flow that occurs soon after initiating statin treatment (Huggins et al 1998).

We showed a significant fall in carotid and femoral IMT after eight weeks treatment with atorvastatin \((20 \text{ mg/day})\) in patients with PAD. We attributed this rapid change in IMT to an anti-inflammatory action on the basis of histological findings in an animal model (Igarashi et al 1997). Other studies have shown that these drugs can ameliorate both
structural and functional changes in the glomeruli in experimental models of nephrotic syndrome (Dupuis et al 1999). Simvastatin may also inhibit human renal mesangial cell proliferation, beyond its cholesterol lowering effect (Harris 2002). There is also considerable evidence showing that statins exert an anti-inflammatory action. For example, these drugs lower the circulating levels of CRP, a marker of inflammation and vascular risk in clinical trials of patients with CHD (Ridker et al 2001).

Experimental hypercholesterolaemia is associated with pro-inflammatory changes and impaired regulation of tissue perfusion; this may lead to neovascularization. It is therefore, of interest there was an increased microvascular density in the renal cortex after a high cholesterol diet (Koh et al 2002). There was also some mild interstitial mononuclear infiltration and heavier immunostaining of endothelial growth factor. These findings may relate to renal disease progression (Grandaliano et al 1993).

It was suggested that aggressive lipid lowering might retard the progression of diabetic nephropathy (Lam et al 1995). Statins directly influence inflammatory mechanisms and may protect renal tissue from the effects of ischaemia-reperfusion injury and thus reduces the severity of acute renal failure. The chain of events may involve anti-inflammatory effects, with inhibition of mitogen-activated protein kinase activation (Gueler et al 2002). An increase in RBF would be anticipated after treatment with a statin. However, the actual underlying molecular and cellular mechanisms that may account for statin-induced effects on renal function remain unclear.

The present study has several limitations. Smoking was not considered in this retrospective analysis. The reason for this omission is that we did not have sufficient number of patients for a separate subgroup analysis. However, larger studies will need to address this issue because smoking could adversely affect the small interlobular renal arteries (Bentley et al
2002). Also in the main lipid lowering trials, smokers who take a statin tended to have a vascular risk that was similar to that of non-smokers on placebo (Milionis et al 2001).

The findings of the present study also need to be considered in the light of the emerging evidence that the plasma creatinine level is itself an independent predictor of cardiovascular risk (Milionis et al 2000). Perhaps this is because both renal and cardiac arterial diseases progress in parallel (Kaplan et al 2002). It follows that treatment-induced improvement in the function of one organ probably reflects similar changes in another. The advantage of assessing renal function is that it may be more amenable to routine biochemical testing using blood and urine samples. Some of these tests (e.g. cystatin C) may be more sensitive than creatinine or urate. Further studies are needed to define the clinical relevance of statin effects on the renal function and to establish whether they are limited to certain patient groups or some statins. These studies should preferably adopt a prospective design. It is also important to establish whether any fall in creatinine or urate is related to specific changes in the lipid profile and an increase in renal perfusion.

This and previous studies suggest that statins beneficially affect renal function. This effect may involve a complex action, which could include NO, the inflammatory process and/or the microvascular circulation in the kidneys.
CHAPTER V

SHORT-TERM LIPID-LOWERING WITH ATORVASTATIN IMPROVES RENAL FUNCTION BUT NOT RENAL BLOOD FLOW INDICES IN PATIENTS WITH PERIPHERAL ARTERIAL DISEASE
5.1 INTRODUCTION

The decline in renal function in healthy humans begins after maturity and is reflected in a fairly consistent decrease in glomerular filtration rate. An averages decline by 8 ml/min per decade in 40 to 60 year olds as determined by creatinine clearance (CrCl) has been reported (Lindeman et al 1985). Both renal vascular disease (as part of the generalised atherosclerosis) and ageing are associated with renal glomerulosclerosis (Kasiske et al 1988). Evidence based on animal experiments suggested that lipids are involved in the glomerular renal injury leading to glomerulosclerosis (Moorhead 1991). It is also suggested that both glomerulosclerosis and atherosclerosis share a common pathophysiology (Kasiske et al 1988).

Lipid abnormalities are common in patients with chronic renal disease. However, there is only limited data regarding the impact of lipid lowering treatment on the incidence of cardiovascular events or any other endpoints in patients with renal disease (Athyros et al 2004 GREACE, Massy et al 1995, Wheeler 2001). Nevertheless, these studies provided evidence about the safety and efficacy of various lipid-lowering drugs, especially statins on the renal function (Attman et al 1993). Statins appear to exert effects on endothelial function, plaque stability (Brown et al 1993) and platelet reactivity in addition to lowering plasma cholesterol (Tsiara et al 2003). This may provide a logical basis for using them in the management of PAD.

Studies on animal models have found favourable effects of lipid reduction on the progression of kidney disease, but most controlled trials on humans have been too small to make definitive conclusions (Wheeler et al 1994). Previous experimental and clinical
studies demonstrated that statins could attenuate endothelial function and both acute and chronic inflammatory processes (Ray et al 2004). For example, lovastatin ameliorated the development of glomerulosclerosis in experimental nephrotic syndrome (Harris et al 1990). Clinical studies demonstrated an improvement in coronary endothelial function one month after initiating simvastatin therapy (O'Driscol et al 1997). It became evident that statins had potent actions on the vascular endothelium that might be mediated by endothelial NO. The MRC/BHF (HPS) subgroup analysis of participants with or without diabetes showed that over a 4.6 years period, simvastatin-treated patients had significantly less increased serum creatinine values as compared to those on placebo (Collins et al 2003). In another study, 43 patients with idiopathic nephrotic syndrome were randomized to fluvastatin treatment or acted as controls (Gheith et al 2002). Fluvasstatin was associated with a significant improvement in proteinuria and CrCl. Interstitial fibrosis and renal fat deposits were less evident in the statin-treated group. The use of cerivastatin (a statin that has now been withdrawn) was associated with decreased microalbuminuria and both plasma and urinary endothelin (ET-1) levels in patients with microalbuminuria and type 2 diabetes, which may represent an amelioration of renal injury (Nakamura et al 2001).

Cystatin C is a protein produced by the majority of cells in the body and filtered out by the kidneys. Serum cystatin C has been proposed as a potentially superior marker of renal function than serum creatinine because it is believed to be produced at a constant rate and not to be affected by factors (e.g. muscle mass, age and gender) that influence serum creatinine level (Finney et al 2001, Knight et al 2004). However, there are limited data on factors that may influence serum cystatin C levels.

There is no accepted accurate non-invasive method to assess RBF. Duplex indices of renal vascular resistance have been proposed as a useful technique to assess the renal function in
patients with systemic sclerosis (Aikimbaev et al 2001). So far, little information is available on their value in chronic renal failure and their correlation with biochemical parameters of renal function (Petersen et al 1995). Moreover, there are no such studies in patients with established PAD.

The aim of the study was to extend our previous findings that lipid lowering with a statin improves renal function in dyslipidaemic claudicants. In addition, we evaluated the effect of atorvastatin treatment on serum cystatin C levels and Duplex indices of RBF.
5.2 STUDY DESIGN, PATIENTS AND METHODS

Patients were symptomatic claudicants aged between 40-85 years were recruited from the vascular out patient clinic. Their ABPI was between 0.5-0.9 and serum LDL-C concentration >2.0 mmol/l and/or TC >4.0 mmol/l. The study received local ethical approval and informed consent was obtained before enrolment.

All patients had their follow up in a specialist risk modification clinic and were started on 20 mg/day atorvastatin. The patients had two assessments at baseline before starting treatment and after 8 weeks.

Blood samples for fasting lipid profile, serum creatinine, urate and cystatin C were collected (at baseline and after 8 weeks). The CrCl was calculated using the Cockcroft and Gault formula (Drinka et al 1989). Serum creatinine and urate levels were measured by methods in routine use in the Department of Clinical Biochemistry, Royal Free Hospital.

5.2.1 Serum cystatin C assay

All samples were stored at -20° C until analysis. We used the Dako PETIA assay (Dako, Denmark), based on polystyrene particles coupled with rabbit anti-human serum cystatin C. This reacts with serum cystatin C causing a change in absorbance. This kit was adapted for use on an Advia 1650 (Bayer, Newberry, UK). Serum cystatin C concentrations are calculated by comparing the absorbance readings of unknowns to those from a previously stored standard curve using 5 cystatin C calibrators: 0.4 to 7.5 mg/L.
5.2.2 Duplex scanning for renal blood flow indices

The right kidney was identified in its full length and the right renal artery was assessed to rule out the presence of stenosis. The Duplex RBF indices, including PI and RI were based on measurements obtained from the middle pole parenchyma (reference range: PI: 0.8-1.5, RI: 0.55-.75).

A longitudinal B-Mode image of the blood vessel was visualised using a Philips HDI 5000 System Duplex scanning with a CS-2 MHZ probe. Hard copies of the scan were stored for documentation. For each visit the measurements were repeated three times and the median value was used. Accredited vascular technicians carried out the Duplex scan.

Statistical analyses

Samples for the serum cystatin C and the Duplex measurements were given random numbers for each sample. The matching of numbers to patients and visits was done once the data was completed. Prism 4 program (statistical software) was used for the analyses of the results. A two-tailed paired t-test was used to assess the differences between baseline and 8 weeks follow up for CrCl, serum creatinine, urate, lipids and serum cystatin C.

A p value < 0.05 was considered significant.
5.3 RESULTS

**Patient characteristics**

Eighteen patients were included in the study. Only nine patients had a complete Duplex studies (due to body habitus or lack of compliance). Of the total number (n=18) 10 were men (56%). The median age was 66.5 years (range 44-85). Baseline weight was 64.4 kg (range 44.5-87). Baseline body weight did not change after treatment (Table. 11). The patients were mild claudicants (median ABPI= 0.75; range: 0.65-0.9).

**Changes in the lipid profile**

As expected, there was a significant improvement in both TC and LDL-C values. The LDL-C target (< 2.6 mmol/l) was reached by 89% of the patients after eight weeks treatment with 20 mg/day of atorvastatin (Table. 11).

**Changes in serum creatinine and creatinine clearance**

There was a significant decrease in serum creatinine levels after treatment from 89 µmol/l (58-125) to 79 µmol/l (54-119) (p<0.0001). There was also a parallel improvement in CrCl from 72 ml/min (40-129) to 80 ml/min (47-138) (p<0.0001) (Table. 12).

**Serum cystatin C values**

There was a significant (p=0.0002) decrease in serum cystatin C values from 1.04 mg/l (0.57-1.56) to 0.90 mg/l (0.47-1.47) after treatment. There was no significant correlation between the serum cystatin C and both serum creatinine levels and CrCl.
6.3.4 Macrophages

In the atherosclerotic lesions studied, distinct areas of ET\textsubscript{B} receptors were found in cells subsequently identified as macrophages on haematoxylin and eosin stained sections, which were confirmed by CD 68 immunostaining. The median tissue macrophage was 11 cells/unit area in the no treatment group as compared to 8 cells/unit area in the treatment group. This difference was not significant. However, there was a significant correlation between tissue ET-1 and tissue macrophage (r=0.62, p=0.01).

6.3.5 Differences between the two groups according to achieving lipid targets

Of the total 12 patients, six were taking statin treatment to achieve LDL-C target (<3.0 mmol/l). There was no significant difference between the measurements in arteries from patients in the two groups, but the trend was in favour of those on lipid lowering treatment achieving targets (Table. 17). The correlation between serum LDL-C and plasma and tissue ET-1 levels was (r= 0.42, r= 0.52), respectively, but still not significant. Densitometric analysis of autoradiographic in different regions showed significant difference only in the regions of neovascularization (p=0.03) with less ET\textsubscript{A} receptor density in the treatment group (Table. 15). There was no correlation between plasma and tissue ET-1 values in the two groups. The difference between plasma ET-1 levels in the two groups was also not significant. However, the average tissue ET-1 (cells/unit area) was higher, but not significantly in those who did not have their risk factors adjusted.
Table 15:

The differences in $\text{ET}_A/\text{ET}_B$ density ratio in regions of the arterial wall

<table>
<thead>
<tr>
<th>Receptors (Radiodensometry)</th>
<th>Media</th>
<th>Plaque</th>
<th>Neovascularisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients $\text{ET}_A/\text{ET}_B$</td>
<td>2.5:1</td>
<td>2.9:1</td>
<td>2.1:1</td>
</tr>
<tr>
<td>On treatment $\text{ET}_A/\text{ET}_B$</td>
<td>2.25:1</td>
<td>1.85:1</td>
<td>1.55:1</td>
</tr>
<tr>
<td>No treatment $\text{ET}_A/\text{ET}_B$</td>
<td>2.5:1</td>
<td>2.2:1</td>
<td>2.9:1</td>
</tr>
<tr>
<td>P value between the two groups*</td>
<td>NS</td>
<td>NS</td>
<td>0.03</td>
</tr>
</tbody>
</table>

* Group one on lipid lowering treatment achieving UK targets
Table 16:

The changes in plasma and tissue variables in the two groups

<table>
<thead>
<tr>
<th></th>
<th>No treatment</th>
<th>On treatment</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cholesterol mmol/l</td>
<td>5.4 (4.2-7.8)</td>
<td>3.9 (3.1-4.5)</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>LDL-C mmol/l</td>
<td>3.9 (3.7-4.2)</td>
<td>1.9 (1.1-2.5)</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Plasma ET-1 fmol/ml</td>
<td>1.14 (0.19-8.9)</td>
<td>0.75 (0.02-3.93)</td>
<td>NS (P=0.08)</td>
</tr>
<tr>
<td>Tissue ET-1 cell/area</td>
<td>16.4 (1.2-20.8)</td>
<td>7.1 (2.4-14.)</td>
<td>NS</td>
</tr>
<tr>
<td>Macrophage cell/area</td>
<td>11 (4.2-14.4)</td>
<td>8 (0.6-22.4)</td>
<td>NS</td>
</tr>
</tbody>
</table>
Table 17:

The differences in immunohisopathology findings according to lipid status

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Patients on lipid Lowering treatment</th>
<th>Patients not on lipid lowering treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL-C mmol/dl</td>
<td>1.85</td>
<td>3.9</td>
</tr>
<tr>
<td>Tissue ET-1</td>
<td>7.85</td>
<td>13.4</td>
</tr>
<tr>
<td>Positive cell/area</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Macrophages cells/area</td>
<td>73</td>
<td>67.5</td>
</tr>
</tbody>
</table>

Values are expressed as average
Fig 2.

Histology of atherosclerotic popliteal artery

Representative examples of haematoxylin and eosin-stained transverse sections of human popliteal artery.

Left panel: Vasa vasorum within the adventitia

Middle panel: Paravascular nerve bundle.

Right panel: Section through the vessel wall, showing a region of atherosclerotic plaque, tunica media and thrombotic occlusion.

Scale bar = 100μm for left and middle panels and 0.5 mm for right panel.
Endothelial cells and macrophages were identified using CD31 and CD68, respectively (see methods).

Scale bar = 0.5 mm.
Fig 3.

$\text{ET}_A$ and $\text{ET}_B$ receptors on human atherosclerotic popliteal artery

$\text{ET}_A$ and $\text{ET}_B$ immunostaining (red) on serial transverse sections of an atherosclerotic human popliteal artery.

Endothelial cells and macrophages were identified using CD31 and CD68, respectively (see methods).

Scale bar = 0.5 mm
Fig 4.

**ET\textsubscript{A} and ET\textsubscript{B} receptors on vasa vasorum of atherosclerotic human popliteal artery**

ET\textsubscript{A} and ET\textsubscript{B} immunostaining (red) in adventitial vasa vasorum of human segment of popliteal artery. There is moderate immunostaining for both receptors at the micro vessel wall, with ET\textsubscript{B} immunostaining of the luminal endothelium (arrows) (identified using CD31, left panel).

Scale bar = 100\textmu m
ETB receptor-staining is denser than ETA receptor-staining.

The immunostaining is present on the smooth muscle layer of the vessel that is included by the thrombus (?).

Scale bar = 230um
Fig 5.

Immunohistochemical identification of ET-1 and its receptors

ET-1, ET$_A$ and ET$_B$ receptors identified by immunohistochemistry (red staining). ET-1 immunostaining shows a similar distribution to that of macrophages (CD68, red staining) on an adjacent section.

ET$_A$ receptor immunostaining is denser than ET$_B$ receptor staining.

The immunostaining is present on the subintimal layer of the vessel that is occluded by thrombus (*).

Scale bar = 250µm
Fig 6.

**ET_\text{A}** and **ET_\text{B}** receptor immunostaining in atherosclerotic human popliteal artery

**ET_\text{A}** and **ET_\text{B}** receptors (red immunostaining) identified on serial sections of atherosclerotic human popliteal artery. Both receptors were associated, to varying degrees, with vasa vasorum (identified using CD31) and macrophages (CD68).

Endothelial cells were associated with vasa vasorum, or regions of neovascularisation, within the media (TM) (small arrows) as well as the vessel lumen (large arrows). Macrophages were identified within inflammatory regions of the media (arrows top right panel). The vessel was occluded by thrombus (Th).

Scale bar = 0.5 mm
Fig 7.

ET-1 and its receptors in paravascular nerve bundle

Representative examples of paravascular nerve bundle in serial sections of a human atherosclerotic popliteal artery showing immunostaining (red) for ET-1, ET$_A$ and ET$_B$ receptors associated with vasa vasorum and vasa nervorum.

An example of a negative control (section incubated in the absence of primary antibody) is shown in the top right panel (-ve).

Scale bar = 50µm
Fig 8.

ET-1 receptors in a paravascular nerve bundle of atherosclerotic popliteal artery

\( \text{ET}_A \) and \( \text{ET}_B \) immunostaining (red) is associated with vasa vasorum (large arrow) and vasa nervorum (small arrows) identified using CD31 (left panel).

Scale bar = 50\( \mu \text{m} \)
where binding is evident as white spots on a dark background.

E.g.: Determination of co-localized venom components in the microenvironment. There is
binding to the perineurium, surrounding the nerves, as well as to the veins vasa vasorum and
vasa nervorum (arrow).

Scale bar = 25 μm.
Fig 9.

Autoradiography of $\text{ET}_A$ receptor binding to nerve bundle, vasa vasorum and vasa nervorum

Left: High resolution autoradiograph showing $[^{125}\text{I}]-\text{PD151242}$ (ET$_A$-selective radioligand) to adventitial regions of atherosclerotic human popliteal artery (dark-field illumination where binding is evident as white grains on a dark background).

Right: Haematoxylin and eosin-stained tissue underlying the autoradiograph. There is binding to the perineurium, surrounding the nerves, as well as to the vasa vasorum and vasa nervorum (arrows).

Scale bar = $25\mu\text{m}$
Fig. 10

Immunostaining for ET-1 and its receptors in a paravascular nerve bundle of atherosclerotic human popliteal artery

ET-1 and its receptors ($E_{TA}$ and $E_{TB}$) associated with microvessels (vasa vasorum and vasa nervorum) of a paravascular nerve bundle. Red immunostaining on serial sections shows the same distribution as the microvessels identified using CD31.

Scale bar = 100μm
Fig 11.

Low resolution autoradiographs of ET\textsubscript{A} and ET\textsubscript{B} receptor binding to atherosclerotic human popliteal artery

Examples of autoradiographs generated on film (low-resolution) from transverse artery sections incubated in $[^{125}\text{I}]-$PD 151242 (ET\textsubscript{A}) and $[^{125}\text{I}]-$BQ 3020 (ET\textsubscript{B}).

An example of non-specific binding is illustrated where sections were incubated in the presence of excess (1\,\mu M) unlabelled ET-1 (NSB). Such images were used for densitometric analysis (see methods).

Scale bar = 2 mm
Fig 12: Autoradiographic autoradiography on chromogen-type slides showing the distribution of receptor binding at the microscopic level. The white areas on the thin sections indicate binding at the ultramicroscopic level (arrow). The right panel shows the control. This vessel was incubated with receptor blockers.

Scale bar = 2 mm for the left panels and 250 μm for the right hand panels.
Fig 12.

**Autoradiographic and immunohistochemical identification of ET$_A$ and ET$_B$ receptors on atherosclerotic human popliteal artery**

Left: Film autoradiographs of $[^{125}\text{I}]-$PD 151242 (ET$_A$) and $[^{125}\text{I}]-$BQ3020 (ET$_B$) binding to transverse sections of atherosclerotic human popliteal artery. Non-specific binding (NSB) is shown at the top.

Right: Immunohistochemical identification of ETA and ETB receptors (red staining) to an inflammatory region exhibiting dense patches of CD68 immunostaining (macrophages).

This figure provides a good example of how film autoradiographs illustrate the regional distribution of receptor binding and how immunohistochemistry is used to localise receptors at the microscopic level. The white arrows on the film autoradiographs indicate binding to the inflammatory regions shown (arrows) in the right panels. This vessel was occluded by thrombus (*).

Scale bars = 2 mm for the left panels and 250μm for the right hand panels.
Endothelial dysfunction causes impaired vasodilatory response in atherosclerotic vessels, (i.e. coronary arteries) with a decrease in eNOS expression at atherosclerotic sites (Mohamed et al., 2002; Bernier et al., 1995). This in turn leads to an imbalance in the production of vasoactive/vasoconstrictors factors such as ET-1 and the anti-inflammatory vasodilator NO suggesting NO as a physiological antagonist for the long-term vasomobilisation induced by ET-1. Both ET-1 and NO are continuously released from the endothelium via a constitutive pathway.
6.4 DISCUSSION

There is increasing evidence of the potential role for ET-1 in the pathogenesis of cardiovascular diseases and vasoconstriction (Miyauuchi et al 1999). Raised plasma ET-1 levels are reported in different vascular conditions including PAD (Levin 1995, Hocher et al 1997) and in conditions causing decreased tissue perfusion and oxygenation (Velasco et al 1994). In addition, augmented local ET-1 concentrations have been found in the atherosclerotic arterial plaque (Zeiher et al 1995).

Increased circulating ET-1 levels in hypertension promote Lectin-like oxidized LDL Receptor (LOX-1) mediated oxLDL-C uptake in human endothelial cells, which in turn, could promote endothelial dysfunction and atherosclerosis. This LOX-1 mediated oxLDL-C uptake may represent the missing link between hypertension and atherosclerosis (Kita 1999). Interestingly, both hypertension and raised plasma ET-1 levels have been described in patients with malignant endothelin-secreting tumour (hemangioendothelioma) that were normalised once the tumour was removed (Yokokawa et al 1991).

Endothelial dysfunction causes impaired vasodilator responses in atherosclerotic vessels, (i.e. coronary arteries) with a decrease in eNOS expression in atherosclerotic sites (Morawietz et al 2002, Butterly et al 1996). This in turn, leads to an imbalance in the production of vasoconstrictor/mitogenic factors such as ET-1 and the antimitogenic vasodilator NO suggesting NO as a physiological antagonist for the long-lasting vasoconstriction induced by ET-1. Both ET-1 and NO are continuously released from the endothelium via a constitutive pathway.
Raised concentrations of ET-1 have been reported in different regions of human atherosclerotic vessels including the regions of neovascularization and in the invading inflammatory cells in atherosclerotic human coronary arteries (Zeiher et al 1995). Moreover, the presence of ET-1 immunoreactivity within atherosclerotic segments of the popliteal arteries suggests a tissue source for the raised plasma levels of ET-1 described in PAD. This local stimulation and release of ET-1 synthesis within the atherosclerotic femoropopliteal arteries may contribute to vessel constriction and reduced blood flow (Dashwood et al 1993). Plaque-derived ET-1 will enter the circulation and act on microvessels “downstream”, including those supplying the calf muscles of patients with severe PAD (Tsui et al 2002). Additionally, hypoxia and shear stress in the popliteal arteries of PAD patients where stenotic lesions will cause turbulent flow through the lumen stimulate the release of ET-1 from the endothelium leading to additional reduction in oxygen supply to the peripheral tissue (Kourembanas et al 1991, Rubanyi et al 1994). Thus, ET-1 via its receptors identified on the popliteal artery may play a role in the ischaemia associated with PAD (Tsui et al 2003).

Distinct areas of ET-1 and \[^{125}\text{I}\] \text{ET-1} receptor binding were reported in macrophages, which provide further support to the theory of local ET-1 production by inflammatory cells, mainly macrophages lymphocytes and monocytes (Ehrenreich et al 1990). This was not observed in normal subjects (Krum et al 1994). We did not find a significant correlation between tissue and plasma ET-1 levels to support the theory of ‘spillover’. However, we found a correlation between tissue ET-1 and local macrophages indicated the local production of ET-1 by both macrophages and endothelial cells and suggesting that macrophage-derived ET-1 is associated with atherosclerosis/plaque formation.
Pro-atherosclerotic risk factors could potentate the increased vascular superoxide anion formation, increase ET-1 expression, release and augment $E_{TA}$ receptor in VSMC and macrophages in the later phases of atherosclerosis (Goettsch et al 2001, Griendling et al 2000). In agreement, we detected $E_{TA}$ receptors on the thinned media underlying atherosclerotic plaques, but were undetectable in the neointima. Moreover, $E_{TA}$ receptors were present in the contractile medial smooth muscle, with comparatively little $E_{TB}$ binding in this region. We also found intense ET-1 staining in the endothelium of the newly formed microvessels associated with atherosclerosis, including recanalization of organized thrombus. In addition, autoradiography revealed $E_{TA}$ receptors on the VSMC in regions of organized thrombus associated with arterial recanalization. $E_{TA}$ receptors were also prominent in the media and within the arterial plaque supporting this receptors involvement in atherosclerosis. Whereas $E_{TB}$ receptors were prominent in the endothelial cells of regions of neovascularization extending through the media, which might indicate a possible less atherosclerogenic or even favourable effects of $E_{TB}$ receptor. The above indicate a possible role for selective $E_{TA}$ receptor antagonists in the management of atherosclerosis. The presence of $E_{TB}$ receptors on the new microvessels may explain why these sites are resistant to $E_{TA}$ receptor antagonists in atherosclerotic arteries (Iqbal et al 2005).

Microvessels are the most important vascular bed affected in tissue ischaemia. Unlike large arteries, which are involved mainly in the transport of blood to the systemic circulation, microvessels (e.g. arterioles/capillaries) are associated with local regulation of tissue blood flow and oxygen supply. There is considerable interest in the effects of ET-1 on microvessels. In an experimental rat model microvessels exhibited $[^{125}\text{I}]$ ET-1 binding and were exquisitely sensitive to its constricting action (Pile et al 1991). In addition, muscle
ischaemia was caused by vasoconstriction resulting from activating \( \text{ET}_A \) receptor subtype in the calf muscles in claudicants due to locally released ET-1.

There were no alterations in proximal/distal \( \text{ET}_A \) or \( \text{ET}_B \) receptors distribution or density when studied during arterial constrictions (Dashwood et al 1998). Experimental studies showed that ET-1 receptor blockade resulted in regression of atherosclerotic lesions in apolipoprotein E-deficient mice treated with endothelin receptor blockers (Barton et al 1998). Therefore endothelin receptor blockade could be considered as an anti-atherosclerotic therapeutic concept (Bacon et al 1996). Moreover, in a study by (Kanse et al 1995), the \( \text{ET}_A \) antagonist BQ123 completely inhibited \(^{[125]}\text{I}\) ET-1 binding, and ET-1 induced mitogenesis in cultured human VSMC. In addition, PD156707 is another potential antagonist of endothelin-1 in human diseased coronary arteries and vein grafts (Maguire et al 1998). Therefore, it is possible that \( \text{ET}_A \) receptors are involved in an initial proliferative response in VSMC, but the progression of cells through successive divisions appears to be accompanied by down regulation of the receptors. Perhaps this down regulation accounts for the deficiency of \( \text{ET}_A \) receptors in the intimal layer of the arteries.

We found more \( \text{ET}_B \) receptors on the vasa vasorum and vascular nerves of popliteal arteries, which represent novel sites where ET-1 may play a role in claudication by causing direct or neurally-mediated microvascular ischaemia and contributing to the onset and severity of IC. In agreement with previous published data our results suggest that the distributions of \( \text{ET}_B \) receptors may indicate that these receptors do not contribute much to the ET-1-mediated constrictor response in these vessels (Maguire et al 1998).
We found differences in the distribution of ET-1 and its receptors corresponding to the presence of the endothelial cells at regions of neovascularization and the presence of inflammatory cells in diseased human popliteal arteries. The close relation between arterial plaque, inflammatory cells and ET-1 and its receptors in our data coupled with recently published studies suggest a potential therapeutic use for ET-1 antagonists in human atherosclerosis. Pharmacological intervention in the form of ET-1 receptor antagonists is currently being considered for the treatment of vascular disease (Iqbal et al 2005), making ET-1 receptors ET_A and ET_B an attractive new therapeutic targets for blockade in different vascular diseases associated with elevated ET-1 levels (Bohm et al 2005). This has thus led to the discovery of selective ET_A receptor antagonists as well as non-selective ET_A/ET_B antagonists (Honore et al 2004). Experimental and clinical studies have clearly established that these antagonists are effective in treating essential hypertension, renal failure (Lariviere et al 2003) and heart failure (Agapitov et al 2002). The orally active antagonist (Bosentan) is approved in pulmonary hypertension (Rubin et al 2002). A better understanding of the implications of these therapeutic strategies is likely to result from identification of cell types expressing ET-1 and characterization of ET-1 receptors within atherosclerotic plaques in different places of the vascular tree.

The results of the present study demonstrate in a semi quantitative manner an increase in the level of plasma and tissue ET-1 of patients with advanced atherosclerosis. Previous studies suggested a role for lipid lowering with a statin on endothelial function and inflammatory markers in PAD patients (Blann et al 2001). We failed to find a significant differences between the two groups of patients. That is because of the small number in each group, but the trend was obvious. However, we found that treatment with a statin resulted
in a non-significant reduction in tissue ET-1 and macrophages in human popliteal arteries obtained from patients with advanced PAD. This may provide a supporting evidence for the importance of statin treatment in reducing the inflammatory process and subsequent vascular events. Larger numbers are needed to evaluate the significance of theses observation.
CHAPTER VII

CONCLUSION AND SUGGESTIONS FOR FUTURE STUDIES
8.1 GENERAL CONCLUSION

Unrecognised PAD carries a high risk of vascular ischaemic events. The prevalence of vascular risk in PAD makes risk modification mandatory to reduce functional decline and subsequent vascular events and complications (Murabito et al 2002). Of interest, patients with a low ABPI (<0.78) carry an approximate 30%, 5-year risk of vascular events and death even without CHD. Subsequently, the use of evidence-based therapy was associated with a significant reduction of vascular events in claudicants (Mukherjee et al 2002). Statins are a breakthrough in the treatment of high serum cholesterol and can substantially reduce both morbidity and mortality from CHD complications (Grundy et al 2001). The beneficial effects of statins on clinical events involve nonlipid mechanisms that affect the endothelial function and inflammatory responses. This might explain the early clinical benefit observed in clinical trials, independent of lipoprotein changes (Koh et al 2001).

The targets for lipid lowering have changed a few times while carrying out this research suggesting more aggressive approach (Smilde et al 2001, NCEP ATP II&III). The LDL-C target is now down from 3.3 mmol/l to <2.0 mmol/l, with an optimal 1.8 mmol/l for high-risk patients.

This research was intended to study the changes in arterial wall in symptomatic claudicants. In the first study included in this thesis, the carotid and femoral IMT were measured after 8 weeks treatment with atorvastatin in 25 claudicants. There was a significant decrease in IMT, which was attributed to the other effects of statin treatment rather than lipid lowering. The decrease in IMT was in both the femoral and carotid arteries indicating a generalised effect. Achieving significant results after this short duration of treatment may support a role
for statins in acute vascular conditions. There was also an unexpected significant decrease in serum creatinine and urate levels in these patients, which prompted me to dedicate a significant part of the thesis to study the renal function after statin treatment (Youssef et al 2002, Youssef et al 2004, Alnaeb et al 2005). This improvement in renal function was confirmed in a retrospective analysis, which included a larger number of patients (n= 103) who received a different statin (simvastatin). Further support came from a prospective study (n= 18 patients) where additional specific indices of renal function, cystatine C and Duplex indices (RI and PI) were added to the measurements in the same time span. The findings confirmed the previous results and we for the first time clearly and convincingly documented the early improvement in renal function after statin treatment in claudicants. Subsequent publications from different groups also supported our observation. The RBF did not change perhaps as a result of the short duration of the treatment or the number of patients. However, the consistency of the Duplex assessment of RBF indices, especially RI and its significant correlation with CrCl makes it a possible useful index. More studies are needed to evaluation the Duplex measurements of the renal indices in PAD patients and in patients with more advanced renal failure.

We investigated the effect of statin treatment on the arterial wall characteristics at the cellular level as an attempt to produce objective proof of the local effects of these drugs. We studied the vasoconstrictive peptide ET-1 and its receptors ET_A and ET_B in amputated leg arteries obtained from patients with advanced PAD looking for changes in receptor distribution. No significant difference between the group on lipid lowering treatment and the group not on treatment was found, but the receptors distribution confirmed the possible involvement of ET-1 and ET_A receptor in atherosclerosis, whereas ET_B receptor binding around the neovascularisation area may indicates a possible favourable role in
atherosclerosis. More studies into selective ET-1 receptors antagonist in the treatment of atherosclerosis are needed.

Effective long-term care of patients with PAD will require increased diagnostic efforts and appropriate medical interventions in community-based, primary care settings to decrease limb-specific symptoms, improve quality of life and decrease the risk of vascular events. Appropriate lifestyle and pharmacological interventions within a primary care setting could presumably reduce the need for both expensive and potentially "high risk" forms of therapy. In this regard, it is hoped that basic research in the pathogenesis of atherosclerosis and the involvement of different risk factors will provide better understanding of this complex pathology.

Interested general practitioners and secondary care specialists in vascular medicine or surgery could oversee vascular prevention clinics, which would have clear and widely agreed policies for further investigations and referral to secondary care clinics. Such clinics would need additional funding in the short-term are likely to be cost neutral, or even beneficial, in the medium-and long-term preventing expensive vascular events such as strokes and amputations and possibly the need for surgical or radiological interventions.
8.2 SUGGESTIONS FOR FUTURE STUDIES

There are several areas of study, which I suggest should be pursued as a result of the finding reported in this thesis.

1. The findings of the IMT study need to be correlated to the clinical outcome in a greater number of patients and over a longer follow up period.

2. The changes in renal function needs to be studied in claudicants with advanced renal disease and in diabetics.

3. The changes in renal function after renal artery angioplasty in patients on or not on statin treatment are relevant.

4. Studies are needed to confirm the reliability of Duplex indices in the follow up for patients with renal disease.

5. Researchers should look for a less complicated non-invasive technique, which provides accurate assessment of the renal blood flow. Ideally, this could possibly be performed in out patient clinics.

6. Studying the ET-1 receptors in the proximal and distal arteries of patients undergoing anatomical lower limb bypasses will identify the receptors predominant at each level. This may help target treatment when specific ET-1 receptor antagonists become available.

7. The effects of statin treatment on arterial ET-1 receptors in patients with advanced PAD needing surgery will need to be investigated.

8. More studies are needed to assess the long-term benefits of statin treatment in young hyperlipidaemic claudicants.
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But-Cha


Cha-Cim


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**Duplex studies of renal blood flow**

The PI or RI indices were similar before and after treatment (Table. 13). There was no significant correlation between PI and serum creatinine, CrCl or serum cystatin C. There was negative correlation between RI and CrCl ($r = -0.61$), but this was not significant. However, there was a significant correlation ($r = 0.75$, $p<0.02$) between plasma serum creatinine level and the RI.

When all RI measurements ($n=18$) were considered, there was a significant correlation between RI and both serum creatinine and CrCl ($r=0.67$, $p=0.002$) and ($r=0.58$, $p=0.01$), respectively (Table. 14).
Table 11:

Patient characteristics for the renal blood flow study

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Baseline</th>
<th>Follow up</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex M/F</td>
<td>10/8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (median, range) years</td>
<td>66.5 (44-85)</td>
<td>68 (55-89)</td>
<td>NS</td>
</tr>
<tr>
<td>Weight (median, range) Kg</td>
<td>64.4 (44.5-87)</td>
<td>68 (55-89)</td>
<td>NS</td>
</tr>
<tr>
<td>Ankle brachial pressure index</td>
<td>0.75 (0.65-0.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.8 (4.3-7.1)</td>
<td>4.4 (3.7-6.0)</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Low-density lipoprotein cholesterol (mmol/l)</td>
<td>3.1 (2.3-4.2)</td>
<td>2.1 (1.5-3.8)</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>High-density lipoprotein cholesterol (mmol/l)</td>
<td>1.7 (1.2-2.5)</td>
<td>1.75(0.6-2.4)</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.9 (0.6-4.8)</td>
<td>1.2 (0.4-2.8)</td>
<td>P&lt;0.0001</td>
</tr>
</tbody>
</table>
Table 12:

Changes in renal function markers (n=18)

<table>
<thead>
<tr>
<th>Marker</th>
<th>Pre-treatment</th>
<th>Post-treatment</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum cystatin C (mg/l)</td>
<td>1.04 (0.57-1.56)</td>
<td>0.90 (0.47-1.47)</td>
<td>P=0.0002</td>
</tr>
<tr>
<td>Serum creatinine (μmol/l)</td>
<td>89 (58-125)</td>
<td>79 (54-119)</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>CrCl (ml/min)</td>
<td>72 (40-129)</td>
<td>80 (47-138)</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Urate (mmol/l)</td>
<td>0.30 (0.13-0.45)</td>
<td>0.26 (0.12-0.35)</td>
<td>P=0.0003</td>
</tr>
</tbody>
</table>

CrCl: Calculated creatinine clearance (Cockcroft Gault formula):

\[(140-\text{age}) \times \text{body weight (kg)} / \text{serum creatinine (μmol)} \times 0.8 \text{ for men (0.85 for women).}\]
Table 13:

Results from Duplex indices measurements

<table>
<thead>
<tr>
<th>Index</th>
<th>Pre-treatment</th>
<th>Post-treatment</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI (8 patients)</td>
<td>1.25 (1.0-1.93)</td>
<td>1.23 (0.97-1.77)</td>
<td>NS</td>
</tr>
<tr>
<td>RI (9 patients)</td>
<td>0.63 (0.62 N 0.79)</td>
<td>0.62 (0.56-0.77)</td>
<td>NS</td>
</tr>
</tbody>
</table>
Table 14:

Correlations between all PI, RI results and renal function variables

<table>
<thead>
<tr>
<th>Duplex index</th>
<th>Plasma serum creatinine</th>
<th>CrCl</th>
<th>Serum cystatin C</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI (n=16)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>RI (n=18)</td>
<td>(r=0.67, p=0.002)</td>
<td>(r=0.58, p=0.01)</td>
<td>NS</td>
</tr>
</tbody>
</table>
5.4 DISCUSSION

In hyperlipidaemic patients with established vascular disease the decline in CrCl over time further increases the risk for vascular events related to atherosclerosis (Collins et al 2003, Athyros et al 2004 GREACE). In contrast, patients on long-term aggressive statin treatment experience a significant increase in CrCl and a decrease in serum creatinine, which may contribute to the reduction of vascular risk. Studies have demonstrated the role of lipids and lipoproteins in the decline of renal function, with an emphasis on glomerulosclerosis (Guijarro et al 1995). In addition, in a meta-analysis comparing various lipid lowering therapies in patients with renal insufficiency, diet in combination with a statin was considered the first step in the management of hyperlipidaemia in these patients. Lowering LDL-C levels should be considered the primary goal of therapy (Massy et al 1995). We reported in a prospective study an early significant decrease in serum creatinine after treatment with atorvastatin (20 mg/day) (Youssef et al 2002). We also reported retrospectively an improvement in serum creatinine levels after treatment with a different statin (simvastatin) (Youssef et al 2004). The present study confirmed the previous findings. Moreover, in the present study we also assessed the effect of statin on arterial wall resistance in the kidney, which may result in an improvement in RBF. Other studies showed direct effects of statin treatment on the arterial wall structure resulting in endothelial-related vasodilatation (Davignon et al 1999). In this context, an early improvement in coronary blood flow has been documented after statin treatment (Huggins et al 1998). It is not known if this improvement applies to the flow to other organs. However, our results suggested that similar improvements do actually occur in the kidneys.
Regressions of atheromatous renal artery stenosis, regression of intimal hyperplasia in arcuate arteries, or reversal of hyaline arteriosclerosis in afferent arterioles are unlikely to be achieved as early as after eight weeks of treatment. Nevertheless, short-term hypercholesterolaemia modulates endothelium-derived hyperpolarizing factor-mediated relaxation in the rabbit renal artery (Honda et al 2001). This may be related to an impaired renal vascular endothelial function and a low bioavailability of NO in experimental hypercholesterolemia (Stulak et al 2001). Statins have been shown to have a protective effect on renal function, by diminishing the lipid contribution to glomerulosclerosis (O'Donnell et al 1993) and by upregulating the eNOS (Joyce et al 2001).

Serum cystatin C is a sensitive marker of the renal function because other studies confirmed a strong correlation to serum creatinine and other clearance substances (Laterza et al 2002). However, in this study we found no significant correlation between serum cystatin C and either serum creatinine or creatinine clearance pre-or post-treatment. One explanation for this discrepancy is that our patients had preserved renal function and the correlation between CrCl and cystatin C is weaker in this situation (Xu et al 2004).

Our hypothesis was that the effect of atorvastatin on arterial wall resistance in the kidney might increase RBF. Increased renal resistance can be measured by Duplex ultrasonography as reported years ago (Azar et al 1977). Duplex can assess renal blood flow velocity and pressure waveforms, but there is a 10-20% rate of failure due to the operator's inexperience or the presence of obesity or bowel gas (Hansen et al 1990). We had a similar percentage of failure. The RI seems to be closely related to parameters of renal haemodynamic and CrCl (Petersen et al 1995). The assessment of intrarenal vascular resistance (RI) may therefore be helpful in determining the degree of renal damage and may be useful in predicting subsequent renal function change. However, in the current study, the
PI index did not correlate with biochemical indices of renal function and did not change significantly after treatment. This may be because arterial compliance did not change to an extent that is detectable within our 8-week study.

Progressive renal disease is believed to reflect a nonspecific renal scarring process involving all renal components. In multivariate regression analysis RI was an independent predictor of declining renal function and identified patients at risk for progressive renal disease (Radermacher et al 2002). Atherosclerosis causes reduction in postglomerular capillaries and renal scarring, ultimately leading to a reduction in the intrarenal vessel area. Thus, increases intrarenal vascular resistance (Bader et al 1980). Assessment of intrarenal vascular resistance may therefore be helpful in determining the degree of intrarenal damage. How much the three renal vascular beds, preglomerular vessels, glomerular capillaries, and postglomerular vessels contribute to the raised RI is unclear. It is also unclear whether an increased RI indicates irreversible renal scarring or if there are strategies that could favourably influence this index and improve prognosis.

There are several limitations to our study. For example, the overall number of patients studied is small (n=18) and only nine patients had a successful Duplex assessment pre- and post-treatment. The study did not have a double blind placebo controlled design and it was of short duration (8 weeks). The potential effect of atorvastatin and other statins on renal function will need to be investigated extensively using more specific tests (e.g. urine collections for CrCl and assessing inulin or $^{51}$Cr-EDTA clearance).

In conclusion, there may be a need to use statins not just to treat hyperlipidaemia in claudicants but also to target the procession of atherosclerosis and the associated decline in renal function.
CHAPTER VI

DISTRIBUTION OF ENDOTHELIN-1 AND ITS RECEPTORS
IN HUMAN POPLITEAL ARTERIES FROM PATIENTS
WITH ADVANCED PERIPHERAL ARTERIAL DISEASE
6.1 INTRODUCTION

Atherosclerotic arterial disease remains the single most prevalent cause of death in the western world. One of its manifestations is PAD, which usually presents with IC and may progress to rest pain resulting in critical leg ischaemia (TASC 2000).

The peptide ET-1 has been implicated in the vasomotor and structural changes to body arteries associated with atherosclerosis and vasoconstriction (Yanagisawa et al 1988). ET-1 is secreted mostly by the vascular endothelial cells after endothelial damage (Lerman et al 1991, Jagroop et al 1999). In addition, cultured VSMC synthesize and secrete ET-1 (Resink et al 1990). High plasma ET-1 is described in acute myocardial infarction and in PAD suggesting a role for ET-1 in advanced atherosclerosis conditions (Rubanyi et al 1994, Miyauchi et al 1989).

Circulating levels of ET-1 are low, or non-detectable, in healthy man (Suzuki et al 1989) and because of its short half-life, high plasma levels of ET-1 must be related to a continuous production (de Nucci et al 1988). The theory of "spillover" from the damaged endothelium is also widely accepted (Heublein et al 1989). Vascular risk factors, such as diabetes, hypercholesterolaemia (Bath et al 1991), and cigarette smoking (Haak et al 1994), are associated with elevated plasma ET-1. The production of ET-1 was enhanced in culture by the presence of oxLDL and stimulated VSMC (Agapitov et al 2002).

The "growth factor" property of ET-1 suggested its important role in VSMC proliferation and neointimal formation, which are key features in the developing atherosclerotic plaque (Gibbons et al 1994). In addition, experimental studies supported a role for ET-1 in
neovascularization associated with early coronary atherosclerosis resulting from promoting new vessel growth and angiogenesis in atherosclerotic arteries. This action of ET-1 on neointimal formation has been clearly demonstrated in rats after carotid artery balloon angioplasty (Douglas et al 1994).

Endothelin-1 is a strong chemoattractant for circulating inflammatory cells. By stimulating the release of different cytokines such as interleukins (Helset et al 1994) and upregulating adhesion molecule expression (Stankova et al 1995, Ishizuka et al 1999), ET-1 may, indirectly cause vasoconstriction by suppressing the vasorelaxant action (Schiffrin et al 1998). The presence of ET-1-like immunoreactivity in the atherosclerotic plaques implicated a therapeutic potential of ET-1 antagonists in treating chronic vascular conditions including atherosclerotic renal disease (Weber et al 1996).

The targets for ET-1 are both ET_A and ET_B receptors.

ET_A receptors predominate in arteries prone to atherosclerosis and can cause vasoconstriction mediated by VSMC hypertrophy, hyperplasia and proliferation (Weissberg et al 1990). For example, ET_A receptors in atherosclerotic popliteal arteries represent sites where the vasoconstriction caused by ET-1 may reduce blood flow to the thigh and calf muscles and induce claudication (Tsui et al 2002).

On the other hand, ET_B receptors may mediate vasodilatation by releasing NO (Takayanagi et al 1991). However, it is been reported that ET_B receptor also mediates the upregulation of superoxide anion in human endothelial cells, which could promote the oxidative modification of LDL-C to oxLDL-C. In this way, ET-1 may promote endothelial dysfunction and atherosclerosis (Duerrscheidt et al 2000, Sawamura et al 1997).

Experimental hypercholesterolaemia was associated with increased ET-1 reinforcing the role of both LDL-C and ET-1 in atherosclerosis (Haak et al 1994). Oxidized lipoprotein
caused impairment of endothelium-dependent dilation in rabbit renal arteries, which may be mediated by oxygen-derived free radicals (Galle et al 1995). In some animal models, an upregulation of ETB receptors has been identified after high cholesterol diet (Elshourbagy 1993). OxLDL-C is another potent pro-atherosclerotic factor, which accumulates in the atherosclerotic plaques. ET-1 augments oxLDL-C uptake in human endothelial cells, promoting the development and progression of endothelial dysfunction, and impairs endothelial relaxation via a decrease in eNOS expression (Kugiyama et al 1990).

The effects of statins extend far beyond cholesterol reduction including stabilizing the atherosclerotic plaque and reducing thrombus formation (Veillard et al 2002). Statins also improve the endothelial function by increasing the bioavailability of NO, which orchestrates the paracrine antiatherosclerotic function of the endothelium (Vaughan 2003). In addition, statins inhibit interferon-gammag-induced expression of intercellular adhesion molecule-1 in vascular endothelium and VSMC and the expression of specific cell surface receptors on monocytes and integrin-dependent leukocyte adhesion (Chung et al 2002).

The purpose of this study was to qualitatively and semi quantitatively measure ET-1 and its receptors in human atherosclerotic arteries of patients with advanced PAD in view or investigating the impact of lipid lowering treatment with a statin on this distribution.
6.2 METHODS

6.2.1 Tissue Collection, Storage and Preparation

Following ethical committee approval at the Royal Free Hospital, patients' informed consent was obtained; segments of popliteal artery were collected from patients undergoing major leg amputations for critical limb ischaemia secondary to advanced PAD. Patients profile including the vascular risk factors, current medication especially lipid lowering treatment were recorded. The popliteal arteries were selected because of the greater mass of tissue available for ET-1 analyses. Ring segments were obtained from the amputated side of the limb. All tissue samples were collected at the time of surgery and samples were immediately snap-frozen in liquid nitrogen, and stored at \( -70^\circ \text{C} \) until use.

We used a combination of immunohistochemistry and in vitro autoradiography to identify the density and distribution of ET-1 and its receptor subtypes (\( \text{ET}_A \) and \( \text{ET}_B \)) and for semi-quantitative measurements of the alterations of ET-1 and its receptors.

6.2.2 ET-1 Immunohistochemistry

After removal from \( 70^\circ \text{C} \) storage sections were allowed to equilibrate to room temperature for 30 mins, post-fixed in acetone at \( 20^\circ \text{C} \) and incubated with normal 'blocking' horse serum followed by incubation with primary antibodies to identify endothelial cells (mouse anti-CD31, diluted 1:200 in PBS, Dako, Cambridge, UK), nerves (NF200, diluted 1:200 in PBS, Dako) and macrophages (CD68, diluted 1:500 in PBS, Dako). ET-1 and its receptors were identified on consecutive slides using a monoclonal anti-ET-1 antibody (1:500
dilution in PBS, Cambridge Research Biochemicals, Cheshire, UK) and anti-ET\textsubscript{A} and anti-ET\textsubscript{B} receptor antibodies (both diluted 1:200 in PBS, Alomone Labs, Jerusalem, Israel) used to identify the ET-1 receptor subtypes. Negative controls were generated in the absence of primary antibody. After a one hour incubation period, sections were rinsed with PBS and antibody staining detected with a standard ABC/alkaline phosphatase method (Vector labs, Burlingame, CA, USA). Immunostaining was achieved using a Vector Red alkaline phosphatase substrate kit (Vector Labs).

Following immunostaining, sections were counterstained with Mayer’s haematoxylin, prepared for microscopic examination and examined using an Olympus BX 50 microscope (Olympus, UK) and photographed where appropriate. Manual counting from immunostaining sections was performed for tissue ET-1, degree of atherosclerosis and macrophages distribution and number. The mean of five measurements from different sites for each value was considered for analysis. An accredited histopathologist performed the histopathology readings.

6.2.3 ET-1 Receptor Autoradiography

ET-1 was identified using \(^{125}\text{I}\)-ET-1. ET\textsubscript{A} and ET\textsubscript{B} binding sites were assessed using the subtype selective radioligands \(^{125}\text{I}\)-PD151242 and \(^{125}\text{I}\)-BQ3020, respectively (Dashwood et al 1994, Davenport et al 1994).

Slide-mounted cryostat-cut sections were pre-incubated in 50mM Tris HCl buffer, pH 7.4 for 15 minutes at room temperature to reduce endogenous peptide levels. Sections were then incubated in Tris HCl buffer (plus 5mM MgCl\textsubscript{2}, 0.2% bovine serum albumin and 100 k.i.u/ml aprotinin) containing 100 to 150 pM \(^{125}\text{I}\)-ET-1 (specific activity 2000 Ci/mmol/L, Amersham Pharmacia Biotech) or \(^{125}\text{I}\)-PD151242 to assess ET\textsubscript{A} and \(^{125}\text{I}\)-BQ3020.
(specific activities 2000 Ci/mmol/L, Amersham Pharmacia Biotech, UK) to assess ET_B binding sites, for 120 minutes at room temperature. The degree of non-specific binding for each radioligand was established by incubating alternate slides in the presence of an excess concentration (1 μM) of unlabelled ET-1 (Bachem Fine Chemicals, Switzerland). After incubation tissue was washed twice for 10 minutes in Tris HCl buffer at 4°C, dipped in ice-cold distilled water and dried in a stream of cold air.

Autoradiographs were generated by exposing incubated tissue to Hyperfilm [³H] (Amersham Pharmacia Biotech) in X-ray cassettes for 4 to 6 days at 4°C. Films were then processed according to the manufacturer's instructions and used for densitometric analysis of receptor binding.

Microscopic localisation (high-resolution autoradiography) of binding sites was performed by post-fixing tissue in paraformaldehyde vapour for 2 hours at 80°C and then dipping the slides in molten K2 nuclear emulsion (Ilford Ltd., UK) under darkroom conditions. Slides were then stored in lightproof boxes containing dessicant for up to 8 days at 4°C. Emulsion was then processed in D19 high contrast developer (Kodak, UK) and fixed (Hypam, Ilford Ltd.), following the manufacturer's instructions. Underlying tissue was stained with Mayer's haematoxylin and eosin for histological examination. Sections were viewed on an Olympus BX 50 microscope under bright and dark field illumination and photographed where appropriate.
6.2.4 Densitometric Analysis of Autoradiographs

Densitometric analysis of autoradiographs was performed using a Bio-Rad GS-700 imaging densitometer with Molecular Analyst Software (Bio-Rad Laboratories, USA). Binding was expressed in terms of radioactivity (disintegrations per min; dpm x 10³ per unit area (mm²)), calculated from standard curves generated by I²5I microscales (Amersham Pharmacia Biotech) that were co-exposed with tissue sections. Specific binding was calculated by subtracting non-specific from total binding.

6.2.5 ET-1 ELISA

Peripheral venous blood was taken from the upper limb from all patients. Blood samples were collected in chilled EDTA-tubes and placed on ice immediately. Samples were centrifuged at 3000g for 20 minutes at 4° C within 20 minutes of collection. Plasma was collected and divided into 4 aliquots to avoid repeated freeze/thaw cycles, and stored at -70° C until tested.

Plasma ET-1 concentration was determined using ELISA assay kits (Biomedica, Austria), consisting of a polyclonal capture antibody, a monoclonal detection antibody and the peroxidase detection method. Plasma samples were thawed at room temperature and reagents reconstituted with assay buffer according to the manufacturer’s instructions. 200 µl of standards, quality control samples and test samples were pipetted into the appropriate ELISA plate wells, which are precoated with the capture antibody. 50 µl of detection antibody was then added to all wells except for wells designated for blank controls, and incubated for 22 hours at room temperature. ET-1 present in the samples binds the capture and detection antibodies, forming a sandwich complex. Following incubation, the wells
were washed 5 times with washing buffer, before adding 200 μl of a peroxidase-conjugated antibody to detect the bound detection antibodies. After incubating for 3 hours at 37°C, the wells were washed 5 times with washing buffer to remove unbound conjugate. 200 μl of the substrate tetramethylbenzidine (TMB) was then added to the wells and incubated for 30 minutes at room temperature in the dark. The reaction was stopped with 50 μl of stop solution.

The peroxidase-catalysed reaction results in a change in colour of the TMB, which is directly proportional to the amount of ET-1 in the sample. ET-1 concentration was therefore quantified spectrophotometrically by measuring the optical density at a wavelength of 450 nm using a Multiskan MCC/340 MKII plate reader (ICN Flow, UK). A standard curve was constructed from the standards and the ET-1 concentration of each sample was determined using Titertek Titorsoft II software (ICN Flow). Samples were analysed in duplicate and the mean value calculated. The sensitivity of the assay was 0.05 fmol/ml, with intra-assay coefficient variation of 4.5% and inter-assay coefficient variation of 6.9%. Cross-reactivity with Big ET-1 was <1%.
6.3 RESULTS

A total of 12 arterial rings were obtained from 12 patients, all were men with a median age of 72 (range 60-86) years. Patients profile is shown in table

6.3.1 Description of the distribution of ET-1 and its receptors

Transverse sections of human popliteal artery were studied using haematoxylin and eosin-staining (Fig.2). Positive immunostaining of ET-1 was seen in the endothelial cells lining the remains of the luminal endothelium identified using CD31. Positive staining was also observed in the endothelial cells in microvessels and regions of neovascularization and the recanalisation extending through the media to the plaque (Fig.3,4). Moreover, discrete patches of ET-1 immunoreactivity were visualized in regions corresponded to inflammatory cells mainly macrophages (Fig.5). Positive staining was also localised to the vasa vasorum, the adventitial microvessels (Fig.6). In addition ET-1 immunostaining was present in vascular nerve bundles and neural microvessels (vasa nerorum) (Fig.7,8).

There were variable distributions in [$^{125}$I]-PD151242 (ET$_A$) and [$^{125}$I]-BQ3020 (ET$_B$) receptor binding, but followed a similar pattern of distribution as [$^{125}$I] ET-1 binding.

ET$_A$ receptors were localized to medial VSMC, but were lacking in the VSMC of the intimal layer (where ET$_B$ receptors were absent). ET$_A$ and ET$_B$ receptor binding was also demonstrated at the cellular level on macrophages identified by CD68 staining (Fig.8). However, ET$_A$ receptors were predominant (Fig.9). ET$_B$ receptors localized to neovascularization, microvessels and mainly to the perivascular nerves of the artery and were predominating on endothelial cells at regions of neovascularization (Fig.10).
6.3.2 Quantitative Receptor Autoradiography

Densitometric analysis of autoradiographic images was used to determine ET_A and ET_B receptor density and the ET_A/ET_B ratio in the arterial media, the atherosclerotic plaque and the areas of neovascularization. There was a higher ET_A receptor density in the arterial wall media with an ET_A/ET_B ratio of 2.5:1. This ratio was 2.1:1 in regions of revascularisation and 2.9:1 in regions of atherosclerotic plaque (Table. 15). ET_A receptors were predominant in the plaque regions, whereas ET_B receptor binding was better seen in the regions of neovascularization and around the vascular nerve bundles (Fig.11).

Autoradiographs of $[^{125}\text{I}]-\text{PD 151242}$ (ET_A) and $[^{125}\text{I}]-\text{BQ3020}$ (ET_B) binding were compared to immunohistochemical identification of ET_A and ET_B receptors. This clearly confirmed that film autoradiographs illustrate the regional distribution of receptor binding, whereas immunohistochemistry localised receptors at the microscopic level (Fig.12). However the receptors distribution was almost identical.

6.3.3 Plasma and Tissue ET-1

We compared patients' plasma ET-1 levels with a 13 matching age control group undergoing total knee replacement who did not have a history of PAD and a normal ABPI. The median control plasma ETNI level was 0.33 fmol/ml (range: 0.1-1.63) as compared to the patients 3.15 fmol/ml (range: 0.02-9.4) the difference was highly significant (p<0.0001). There was no correlation between all patients' plasma and tissue ET-1 values. Based on counting the immunopositive cells, the average tissue ET-1 was 10.25 positive cells/unit area.


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