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DIABETES AND PERIPHERAL ARTERIAL DISEASE

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‘Every explanation is after all an hypothesis.’

Ludwig Wittgenstein, *Philosophical Occasions*  
1912-1951, p 123
To Lisa, Saga and Tore
**ABSTRACT**

**Background:** Diabetes mellitus increases the risk for peripheral arterial disease (PAD) early in life and the disease is likely to progress to advanced stages. Mechanisms responsible for premature PAD in diabetes are partly unknown. Leg ischaemia from PAD, together with other diabetic complications, is the key player in the pathway from ulceration to gangrene and infection, which ultimately results in major amputation. Infragingual bypass surgery (IBS) is carried out to restore leg perfusion and avoid amputations. Whether outcomes for this procedure are less favourable in patients with diabetes than in patients without diabetes is unclear.

**Aims:**
- To explore the impact of hyperglycaemia on outcome after IBS in patients with diabetes.
- To assess amputation-free survival (AFS) after IBS for critical limb ischaemia.
- To assess amputation-free survival (AFS) in patients with diabetes but without PAD during long term follow up.
- To investigate if receptor for advanced glycation end products (RAGE) and advanced glycation end products (AGE) are increased in plasma and vein grafts in diabetes patients.
- To investigate if the AGE-RAGE system predicts AFS and development of PAD, and if it is associated with AFS after IBS in patients with diabetes.

**Results:** In *Paper I*, we demonstrated an association between hyperglycaemia the first 48 hours after IBS and increased risk for wound complications, graft occlusion and amputation or death during the first 3 months in 91 patients with diabetes. Patients in the highest quartile of glucose exposure had an odds ratio of 13–14 in multivariate logistic regression.

In *Paper II*, we performed a nationwide, population-based cohort study and compared postoperative AFS in patients with and without diabetes. The analysis included data for 1,840 patients from the Swedish Vascular Registry who, during 2001–2003, underwent their first unilateral, below-knee, IBS procedure for critical limb ischaemia. Of these patients, 742 had diabetes and 1,098 did not. Patients were followed up until the end of 2005. Overall, 446 and 558 patients with and without diabetes, respectively, had undergone ipsilateral amputation or died by the end of the follow-up period. Patients with diabetes had a shorter AFS than patients without diabetes (2.3 years, 95% CI 1.9–2.8 years versus 3.4 years, 95% CI 3.1–3.7 years). The hazard ratio and incidence for ipsilateral amputation or death in patients with diabetes, adjusted for age, sex, smoking and other confounding variables, was
1.46 (95% CI 1.26–1.69) and 30.2 events per 100 person-years respectively. The incidence of amputation or death was 2.8 per 100 person-years, (95% CI 2.0 to 3.7) in the cohort of patients with type 2 diabetes who were free from PAD at start of follow up.

In Paper III and IV we showed that S100A12, a ligand to RAGE, is associated with AFS after IBS in patients with (n=38) and without (n=30) diabetes, and with AFS as well as development of PAD in a prospective longitudinal (10-year) population-based cohort (n=146) of patients with type 2 diabetes, free from signs of PAD at inclusion. Presence of AGE, RAGE and S100A12 were demonstrated in saphenous vein tissue with no difference between patients with and without diabetes.

Conclusions: Postoperative hyperglycaemia is associated with unfavourable outcome after IBS in patients with diabetes. Diabetes is associated with lower AFS after IBS for critical limb ischaemia. Plasma levels of S100A12 and RAGE components are elevated in PAD disease and markers of RAGE and its ligands are found in vein tissue used for bypass. This is consistent with a role for S100A12 in PAD complications by activation of the RAGE system. Higher plasma levels of S100A12 and the combined effect of RAGE components seem to be associated with AFS in patients with diabetes. Further study is needed to find methods of reducing this excess risk and prolonging AFS.
LIST OF ORIGINAL PAPERS

This thesis is based on the following studies, which will be referred to by their Roman numerals.

I  Malmstedt J, Wahlberg E, Jörneskog G, Swedenborg J.  
Influence of perioperative blood glucose levels on outcome after infrainguinal bypass surgery in patients with diabetes.  

II Malmstedt J, Leander K, Wahlberg E, Karlström L, Alfredsson L, Swedenborg J.  
Outcome after leg bypass surgery for critical limb ischaemia is poor in patients with diabetes: a population-based cohort study.  

The Receptor for Advanced Glycation End products (RAGE) and its Ligands in plasma and infrainguinal bypass vein.  
*In manuscript*

IV Malmstedt J, Kärvestedt L, Brismar K, Swedenborg J.  
The Receptor for Advanced Glycation End products and development of peripheral vascular disease in type 2 diabetes.  
*In manuscript*
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<td>AFS</td>
<td>Amputation Free Survival</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced Glycation End products</td>
</tr>
<tr>
<td>AUC</td>
<td>Area Under the Curve</td>
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<tr>
<td>CI</td>
<td>Confidence Interval</td>
</tr>
<tr>
<td>CII</td>
<td>Continuous Insulin Infusion</td>
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<tr>
<td>CLI</td>
<td>Critical Limb Ischaemia</td>
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<tr>
<td>CML</td>
<td>Ne-(carboxymethyl)lysine</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular Disease</td>
</tr>
<tr>
<td>DCCT</td>
<td>Diabetes Control and Complications Trial</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>esRAGE</td>
<td>Endosecretory form of Receptor for Advanced Glycation End product, a splice variant found in plasma</td>
</tr>
<tr>
<td>ESRD</td>
<td>End Stage Renal Disease</td>
</tr>
<tr>
<td>$\text{HbA}_{1c}$</td>
<td>Glycated haemoglobin ($\text{HbA}<em>{1c} = 0.923 \times \text{HbA}</em>{1c}\text{max} + 1.345$)</td>
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<tr>
<td>HR</td>
<td>Hazard Ratio</td>
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<tr>
<td>IBS</td>
<td>Infrainguinal Bypass Surgery</td>
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<td>IFG</td>
<td>Impaired Fasting Glucose</td>
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<td>IGT</td>
<td>Impaired Glucose Tolerance</td>
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<tr>
<td>IH</td>
<td>Intimal Hyperplasia</td>
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<tr>
<td>IR</td>
<td>Insulin Resistance</td>
</tr>
<tr>
<td>MG</td>
<td>Methylglyoxal or 2-oxopropanal, CH3–CO–CH=O</td>
</tr>
<tr>
<td>NADPH-oxidase</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate-oxidase, generates ROS</td>
</tr>
<tr>
<td>NF–κB</td>
<td>Nuclear Factor kappa-beta</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>OGTT</td>
<td>Oral Glucose Tolerance Test</td>
</tr>
<tr>
<td>OR</td>
<td>Odds Ratio</td>
</tr>
<tr>
<td>PAD</td>
<td>Peripheral Arterial Disease</td>
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<tr>
<td>RAGE</td>
<td>Receptor for Advanced Glycation End-products</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>S100A12</td>
<td>S100A12 protein also ENRAGE or Calgranulin</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>sRAGE</td>
<td>shed Receptor For Advanced Glycation End Product, found in plasma</td>
</tr>
<tr>
<td>Swedvasc</td>
<td>The Swedish National Registry for Vascular Surgery</td>
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<tr>
<td>TG</td>
<td>Triglycerides</td>
</tr>
<tr>
<td>UKPDS</td>
<td>United Kingdom Prospective Diabetes Study</td>
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<tr>
<td>VSMC</td>
<td>Vascular Smooth Muscle Cells</td>
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PREFACE

Why did I do this?

My contact with diabetes and peripheral arterial disease started during my training in surgery at a district hospital in northern Sweden, 15 years ago. I met patients with diabetes and foot ulcers, at the multidisciplinary foot clinic. I realised that these patients differed from those without diabetes but with lower limb ischaemia in several ways. Not only was it hard to diagnose and grade ischaemia in the patient with diabetes, but even harder to predict who would heal his or her ulcer without revascularisation and who was in need of revascularisation. Many clinical findings were unreliable and unpredictable. Ankle pressures could always be suspected to be falsely high and any pressure between circa 30 to 80 mmHg pushed me into the dilemma of performing unnecessary surgery vs doing it too late (or not at all). The surgery was even more challenging. These patients often had multilevel disease requiring distal bypass, sometimes so distal that the great saphenous vein was too short. Their arteries were ‘glued’ to the surrounding tissue and heavily calcified, making both dissection and anastomosis demanding. Postoperative complications seemed to be more frequent and many ulcers took a long time to heal or did not heal despite successful revascularisation. Many patients made a long journey, which ultimately ended with a major amputation despite all efforts from the foot team.

The question was – could we do this better, did we provide substandard care and surgery? The picture I had from reading textbooks in vascular surgery and publications from centres of excellence described another position of the land. First, the results were remarkably good – 80 % limb salvage at 5 years. Furthermore, bypass surgery seemed to be possible in the majority of patients with diabetes in the hand of an expert. Poor outflow? Just move distal – ‘the floor is the limit’. Not enough vein length? Use arm veins and splice.

Learning to master distal bypass was one of the goals when I was offered a residency in vascular surgery at Karolinska. After a year I realised that results were quite the same at an academic clinic and that pedal bypass was not so frequently performed.

When I was asked which research area I had interest in, I brought up diabetes and PAD. Was it true that results after bypass surgery in patients with diabetes was as good as in those without? If not, why? Could to the difficulty mastered postoperative hyperglycaemia increase the complication rate?
AIMS

The main aim of this thesis was to study lower limb peripheral arterial disease in diabetes regarding: outcome and complications after bypass surgery, mechanisms for intimal hyperplasia, and disease progression.

The specific aims were

I. To study if high perioperative glucose levels are independently associated with increased complications after infrainguinal bypass surgery in patients with diabetes.

II. To compare amputation-free survival, after leg bypass surgery for critical limb ischaemia in patients with and without diabetes.

III. To establish population based estimates of the incidence of amputation and death after leg bypass surgery for critical limb ischaemia, in patients with and without diabetes.

IV. To investigate if the receptor for advanced glycation end products (RAGE) and advanced glycation end products (AGE) are increased in plasma and vein grafts in patients with diabetes.

V. To assess amputation-free survival during long term follow-up of patients with diabetes but without peripheral arterial disease.

VI. To study if plasma levels of AGE and RAGE are associated with amputation-free survival or development of peripheral arterial disease in diabetes.
INTRODUCTION

Prevalence of diabetes is increasing worldwide (Danaei et al. 2011). Diabetes is a common component in cardiovascular disease and the coexistence of these two diseases has a profound effect on morbidity and mortality (World Health Organization).

HISTORICAL PREAMBLE

‘Sometimes the extremities become gangrenous...you must cut off that limb as far as the disease has spread, so that the patient may escape death or greater affliction, greater than the loss of the limb.’

Albucasis, c. A.D. 1000

Lower limb complications to diabetes have explicitly been recognized at least since the 19th century. Sir Bell (1791) advocated amputation as a last resort for gangrene in his book ‘a system of surgery. The enlightenment era brought interest in statistics as a mean of evaluating surgical procedures (Alanson 1782). Amputation was one of the most common procedures during the 17th and early 18th century, but the indication was with few exceptions civil or military injuries (Robinson 1991).

Textbooks of surgery from middle or late 19th century often stated diabetes as an absolute contraindication to surgery and the general opinion was that amputations for diabetic gangrene were of no benefit and should be avoided:

‘Diabetic gangrene of a limb is scarcely within the scope of surgical measures. An amputation in such a condition is almost invariably fatal.’ Erichsen and Ashhurst (1869).

The series of 140 amputations performed in Massachusetts general hospital until 1850, contain some cases of gangrene but the publication does not state diabetes status (Hayward 1850). It is however evident that mortality associated with amputations was of great concern.

The advice not to operate diabetic gangrene seems to be questioned from the early 1890ies when several lectures and case reports on diabetic gangrene appear. In 1892 Spencer reported four cases and summarised other reports of successful amputations in patients with diabetes, but he also discussed the aetiology of diabetic gangrene and stated that ‘[...] a large majority of such cases have arterial sclerosis, affecting especially the vessels of the leg[...]’(page 399) Moreover, several surgeons pointed out that presence of lower limb arteriosclerosis confer a poor prognosis, for example in this correspondence regarding amputations in diabetes:
‘whilst the presence of arterial degeneration makes the case much less promising, that of peripheral neuritis has not much to say to the matter. Gangrene starting from a perforating ulcer is a much more dangerous affection if the vessels be sclerosed than if they be healthy, but senile gangrene in a diabetic is not a more serious affection in a man with peripheral neuritis than in one without it.’

Godlee (1892)

Godlee makes reference to several publications (Frerichs 1884; Israel 1882; Winiwater 1879) dealing with diabetes as a risk factor for lower limb ischaemia ‘glycosuria is a very potent predisposing cause of atheroma’ (Godlee 1893).

Several authors describe the arterial pathology associated with diabetic gangrene (Godlee 1892), e.g. Spencer as shown below

Remarks by Mr. SPENCER.— In the above case arterio-sclerosis and thrombosis were well marked, indicating the necessity of a high amputation, so that the stump might depend for its blood-supply directly upon the branches of the deep femoral artery, the limited sloughing of the flap being connected with the thrombosis of superficial femoral artery above the level of amputation. It has been thought that perforating ulcers are the result of a previous neuritis in diabetic cases, but an examination of the nerves of the amputated limb did not support this view. The history that the patient gave was that of septic extension to the foot from a small crack on the toe, rather than of a preliminary perforating ulcer. The arterial changes were as extensive as those met with in cases of gangrene. That this did not happen may be put down to the otherwise good state of the patient’s circulation.

Spencer (1902)

The almost absolute contraindication to surgery in patients with diabetes gradually changed with the adoption of aseptic technique and with publications of results after general surgery on patients with diabetes (Witherbee 1907). In 1902 Phillips’ review the ‘Surgical aspects of glycosuria and diabetes’ in the Lancet, and described an overall ≥25% postoperative mortality mainly due to sepsis or coma in published series of general surgery in patients with diabetes, which at that time was regarded as an improvement (Phillips 1902). Regarding diabetic gangrene, Philips clearly stated the importance of vascular assessment:

‘The condition of the arteries should be carefully investigated on both sides. If the pulse be distinctly present in the anterior and posterior tibials and in the dorsalis pedis local removal of the diseased parts will suffice. If, however, the pulse be not perceptible there, but be well marked in the popliteal, amputation below the knee may be performed, provided that the gangrene has not spread beyond the dorsum of the foot and that the leg is free from phlebitis and lymphangitis. If, however, these conditions be not fulfilled
or the popliteal pulse be not felt, then amputation must be performed through the thigh. If gangrene has spread beyond the corium of the foot, or if the urine contains acetone or aceto-acetic acid, or if stator hiccoum is present, the prognosis is necessarily bad.’ Phillips (1902).

In the beginning of the 20th century, surgeons debated whether diabetic gangrene’s best treated conservatively or by amputation (Schlesiger 1907). Schlesiger quoted the results of Wolfe, comparing mortality in conservatively treated patients with diabetes and gangrene (45%, 50/110) with those who were amputated (37%, 28/75) (Wolf 1901). The conclusion was that amputation should be done if gangrenes progressed or if signs of general infection appeared (Schlesiger 1907). In the same period Lockwood stressed the importance of hypoxia: ‘This lack of oxygen plays a very profound part in the causation of diabetic gangrene’. He also for the first time reported on the beneficial effect of oxygen treatment and was well aware of arterial calcification and insufficiency, albuminuria and neuropathy (Lockwood 1912).

The link between lipidemia, cholesterol accumulation in vascular tissue and premature atherosclerosis in diabetes was proposed by Warren and Root already in 1926:

> Here then are five persons from 6 to 33 years of age, with lipoids in the blood that are taken up extensively by the cells of the reticulo-endothelial system, and all five show vascular changes. There may well be a real danger in permitting patients to live on a diet of high caloric value with the aid of large amounts of insulin. The considerable intake of food above energy requirements, as shown by marked and often rapid gain in body weight, must result in large increase in the amount of lipoid in the circulation. While it is not proved that lipemia is harmful, these few cases suggest that there may be some relationship between it and arterial disease. In a condition such as diabetes, which seems to predispose toward atherosclerosis, any factor which might tend to bring about vascular change should be avoided.

‘These cases of young individuals with extensive atherosclerosis, or perhaps better, atherosclerosis, emphasize the danger which arterial disease presents to diabetics and forcibly calls our attention to the need for considering the lipid, as well as the carbohydrate, metabolism of the diabetic’ (Page and Warren 1929)

The clinical and pathophysiological relationship between glucose- and lipid metabolism, and atherosclerosis evolved during the 50-ies and 60-ies (Fredrickson et al. 1967). The high percentage of prediabetes or undiagnosed diabetes was described 1958 by a rarely cited article by Bartels and Rullo in NEJM, where they found diabetes or abnormal response to glucose tolerance test in 67 of 100 patients with PAD but without known diabetes.

The discovery of insulin by Banting et al. (1922) and understanding of diabetic coma entirely changed the scene. The death rate from diabetes declined (Joslin 1936), while the incidence of gangrene was unchanged and accounted for a larger percentage of the nor-
tality in diabetes (39%) (Rabinowitch 1927). The publications from Joslin and Montreal General Hospital on diabetic gangrene both outlined the rare occurrence before the age of 50, the correlation to diabetes duration and the higher risk among men (Joslin 1934; Rabinowitch 1927). In 1928, the Mayo Clinic reported postoperative mortality of 6.8% among 233 cases of surgery in patients with diabetes (Roberts 1928). The earlier emphasis on atherosclerosis as the primary aetiology in gangrene seems to be challenged at this time, as the concept of microvascular disease emerges (Mason 1927). It also seems that the main responsibility for treating patients with diabetic gangrene was shifted over from surgeons to diabetologists, as greater chances to treat diabetes and prevent complications became available.

The introduction of penicillin gave further means of treating diabetic foot lesions with limb saving trans metatarsal amputations during the 40-ies, and the mortality is at this time reported to be about 5–7% (Cranley et al. 1969; McKittrick et al. 1949; Root 1948).

Finally, lower limb revascularisation by venous bypass graft surgery, first described by Kunlin 1951, gained increasing popularity the following decades (Darling et al. 1967; Hall 1962; Reichle et al. 1979), and has been the established treatment for lower limb ischaemia for the last 20 years (Pomposelli et al. 1990).

**DIABETES AND VASCULAR DISEASE.**

Vascular complications of diabetes have traditionally been divided into micro- and macrovascular complications, the latter affecting larger conduit arteries in the form of atherosclerosis, which is the aim of this thesis.

Diabetes mellitus and atherosclerosis are important and common diseases. They are accompanied by considerable mortality and morbidity and interact at various levels by complex mechanisms.

Patients with diabetes have an increased risk for cardiovascular events. This risk is equal to the risk in patients without diabetes who have survived a myocardial infarction (Haffner et al. 1998). This excess risk is about two- to fourfold, independent of other risk factors (Sarwar et al. 2010; Zimmet et al. 2001). Diabetes is therefore considered a 'cardiovascular (CVD) risk equivalent' (Huxley et al. 2006; Ryden et al. 2007) a risk that can also be expressed as equivalent to the ageing of 15 years (Booth et al.), and is more pronounced in patients with PAD (Marso and Hiatt 2006). Men and women with diabetes thus enter the high-risk group at 41 and 48 years respectively (Booth et al. 2006) with relatively greater effect of diabetes in women (Hu et al. 2005). The increased CVD risk also translates into an increased mortality with approximately 60% of deaths in patients with diabetes caused by CVD (Henricsson et al. 1997; Joron et al. 1986; Rossing et al. 1996).

Hyperglycaemia and impaired glucose tolerance (IGT) are linked to adverse macrovascular outcomes, although clear evidence that lowering glucose decreases macrovascular events are still lacking (Boussageon et al. 2011). This may be explained by a lower glucose threshold level required to prevent macrovascular changes in diabetes, in contrast to microvascular effects.
Type 2 diabetes, in its later stages, is a disease with multi-organ involvement. Complications are almost always seen 15 years after the diagnosis. The most common complications are peripheral sensory and autonomic neuropathy, retinopathy, nephropathy and accelerated cardiovascular disease including lower limb ischaemia of various degrees.

The majority of lower limb amputations in the western world are performed on patients with diabetes, who at ages 45 years, 65 to 74 years and ≥75 years have an 8-fold (Johannesson et al. 2009), 23.5-fold and 8.6-fold relative risk respectively (MMWR 1998) for major amputation compared to those without diabetes in corresponding age groups.

Ischaemia is an etiologic factor in over 90% of major amputations in diabetes (Eskeleyen et al. 2004) and the combination of lower limb ischaemia and infection are the major etiologic factors (Armstrong et al. 1998; Carmona et al. 2005; GLEASG 2000).

The impact of diabetes on cardiovascular disease is greater than can be explained by higher prevalence of traditional atherosclerotic risk factors (Hu et al. 2005; Ryden et al. 2007). Several mechanisms coupled to hyperglycaemia have been shown to augment the atherosclerosis process (Brownlee 2001). Activation of alternative pathways in glycolysis by hyperglycaemia seems to be an important initial step leading to oxidative stress and endothelial dysfunction (Creager et al. 2003). Hyperglycaemia also induces formation of advanced glycation end products (AGEs) which have both direct vascular effects and indirect ones via the receptor for advanced glycation end products (RAGE). Diabetes is also characterized by distinctive atherogenic lipid disturbances, increased platelet activation and a hypercoagulable state (Creager et al. 2003). These are all potentially modifiable mechanisms, pointing to the possibility that improved glucose control and targeted therapies could improve prognosis in patients with diabetes.

**Epidemiology of Diabetes and Peripheral Arterial Disease**

Some 26% of people with symptomatic PAD also have diabetes (Selvin and Erlinger 2004), predominately type 2 (90%). The proportion of patients with diabetes increases with increasing severity of ischaemia (Feinglass et al. 2001; Goshima et al. 2004; Luther 1994; Nowygrod et al. 2006). The prevalence of diabetes in comparable age groups is approximately 10% (Eliasson et al. 2002; Laakso and Pyorala 1985; Wilson et al. 1986).

Diabetes is associated with earlier onset, more rapid and severe progression, and more distally located atherosclerosis of the lower extremities (Diehm et al. 2006; Strandness et al. 1964).

Duration of diabetes and degree of hyperglycaemia were associated with risk for PAD in the United Kingdom Prospective Diabetes study (UKPDS) (Adler et al. 2002). Each 1% increase in Hba1c was associated with a 28% excess risk for incident PAD at the end of 18 years.

The odds ratio (OR) for PAD were 3.8 and 4.3 for a diabetes duration of 10 to 19 years and over 20 years, respectively, in patients with type 2 diabetes (Zander et al. 2002).
Increasing age correlates with PAD in type 2 diabetes (Walters et al. 1992), for every 10 years, the OR of PAD was 2.6 in the Framingham study (Murabito et al. 2002).

The protective effect of female gender on PAD is blunted in women with diabetes (Dormandy and Rutherford 2000), and the increase in risk for PAD in women with, compared to women without diabetes is greater than the increased risk among men (Abbott et al. 1990).

**Incidence and prevalence of Peripheral Arterial Disease in Diabetes**

Our estimate 16 per 1000 person-years for incident PAD (Paper IV) is close to a similar study from Finland reporting new PAD in 21 of 107 patients followed for 11 years (i.e. \( \approx \) 18 per 1000 person-years) and the ARIC study with 14 per 1000 person-years (Kallio et al. 2003; Wattanakit et al. 2005). The UKPDS trial estimate for incident PAD during the first 6 years after diabetes diagnosis is 5 per 1000 person-years. This lower incidence can be explained by younger age and shorter diabetes duration in the UKPDS participants (Adler et al. 2002). In the Framingham study the incidence of PAD, defined as claudication, was 12.6 and 8.4 per 1000 person-years in men and women with diabetes. Corresponding rates in the non-diabetic populations were 3.3 and 1.1 person-years for men and women respectively (Kannel and McGee 1979). The prevalence of diabetes increased from 9% in people without PAD to 14% in people with asymptomatic PAD and reached 26% in those with critical limb ischaemia (CLI) in a Swedish population-based study (Sigvant et al. 2009). The prevalence of diabetes among patients with leg bypass surgery for CLI varies between 50 and 80% (Weiss and Sumpio 2006).

**OUTCOME OF INFRAINGUINAL BYPASS SURGERY IN PERIPHERAL ARTERIAL DISEASE AND DIABETES**

‘The memory is apt to be treacherous with regard to unfavourable cases, the successful ones are usually remembered, and too often published alone.’

Geo. Hayward, Mass Gen Hospital, 1850

Patients with CLI commonly require leg bypass surgery to distal arteries to prevent major amputation. The development of distal bypass techniques during the last 20 years has enabled bypass surgery to distal crural and pedal arteries in patients with atherosclerotic disease due to diabetes. Patients with distal arterial occlusive disease in this location were regarded unreconstructable prior to the 1980-ies.

Current knowledge from the results after distal lower limb bypass procedures in patients with diabetes is mainly based on data from single centres. It is unclear if the excess risk for amputation and death among patients with diabetes in general, is mirrored in patients undergoing vascular reconstruction. Population-based data is lacking in this group of patients.
Despite the known excess risk of death and amputation among people with diabetes, most reports in vascular literature conclude that diabetes does not negatively influence the long term outcome of lower limb revascularisation (Weiss and Sumpio 2006). This conclusion can be questioned since most published reports have problems with study design, inclusion criteria and outcome measures. One particular problem is the use of limb salvage instead of more patient oriented outcome measures (see methods section for a detailed discussion on outcome measures). The high concomitant mortality in this group of patients is ignored when limb salvage is used as outcome measure in survival analysis models. Censoring for death will most probably induce differential censoring patterns among patients with and without diabetes, as death is related to diabetes. Thus, the basic assumptions in survival analysis that censoring should be unrelated to exposure and outcome will not hold true.

Moreover, most studies include a wide variety of patients, with different degrees of limb ischaemia and different levels of occlusion: suprainguinal, supragenicular and infragenicular. These factors are all closely related to the prognosis after revascularisation.

Thus numerous observational studies have reported excellent results after leg arterial bypass surgery with no difference between patients with and without diabetes (Akbari et al. 2000; Panneton et al. 2000; Wolfe et al. 2003), whereas others have found results to be worse in patients with diabetes (Feinglass et al. 2001; Goshima et al. 2004; Taylor et al. 2006). Some centres (Akbari et al. 2000; Panneton et al. 2000; Pomporelli et al. 2003) report 70–80% patency and 80–90% limb salvage rate at 5 years with no difference between patients with and without diabetes. Most of these studies are series from referral hospitals regarded as centres of excellence, making the source population difficult to identify, and therefore these results are not generalizable.

Population-based studies on outcome after infrainguinal revascularisation in patients with diabetes and CLI are lacking. Indirect data from other population-based studies, including patients with various degrees of lower limb ischaemia and a broad range of vascular interventions, suggest either equal (Karlstrom and Bergqvist 1997; Virkkunen et al. 2004) or worse (Al-Omran et al. 2003; Hallett et al. 1997) outcome for patients with diabetes. The conclusion of most reports, however, is that diabetes does not negatively influence the long term outcome of lower limb revascularisation (Weiss and Sumpio 2006).

Data on this issue are important as they could promote the understanding of both diabetes and CLI, and provide further insight into the clinical course of patients with diabetes.

**Diabetes, hyperglycaemia and complications after vascular surgery**

Patients with diabetes have a higher risk for infections in general (Shah and Hux 2003). In vascular surgery, diabetes is reported to be a risk factor for incisional complications after infrainguinal bypass in some studies (Casey et al. 1983; Richet et al. 1991) whereas others have failed to find any association (Johnson et al. 1988; Schwartz et al. 1988). The influence of glycaemic control on outcome after lower extremity bypass in patients with diabetes is largely unknown. An increased risk for postoperative nosocomial infection in
patients with any postoperative B-glucose value exceeding 220mg/dl (12.2 mmol/L) the first postoperative day after general surgical procedures has been reported (Pomposelli et al. 1998). In a patient material consisting of a majority of non-diabetic patients undergoing various infrainguinal vascular procedures, postoperative hyperglycaemia during the first two postoperative days was found to be an independent risk factor for postoperative infections (Vriesendorp et al. 2004).

There is growing evidence that hyperglycaemia is one reason for the higher complication rate in patients both with and without diabetes undergoing cardiac surgery (Golden et al. 1999; Latham et al. 2001; McAlister et al. 2003; Zerr et al. 1997). The importance of glycaemic control is also illustrated by the fact that intensive blood glucose control in patients receiving postoperative critical care reduces mortality and morbidity mainly by reducing sepsis and multiorgan failure in cardiothoracic patients (van den Berghe et al. 2001). Focus was set on in-hospital glycaemic control by the study from van den Berghe, and strict blood glucose control for all patients has been proposed (Garber et al. 2004). On the other hand this has been questioned (Inzucchi and Rosenstock 2005), and a review suggested that further research was needed to define the importance of targeted glucose control and threshold levels of blood glucose for clinical outcome in diverse clinical settings, such as general medicine and surgery (Clement et al. 2004). Several RCTs (Griesdale et al. 2009; Wiener et al. 2008), from recent years, have failed to show benefit from strict glucose control, and this is discussed further in relation to Paper I at pp. 60-61.

ATHEROSCLEROSIS DEVELOPMENT AND PROGRESSION IN DIABETES

Diabetes is one of the three most important factors for development of PAD, as seen from a clinical and epidemiological perspective. This section will describe why diabetes is a strong risk factor for atherosclerosis and covers one of the proposed mechanisms, namely the AGE-RAGE system in more detail.

There is an apparent risk for oversimplification when trying to assign a few common pathways linked to hyperglycaemia and insulin resistance as responsible for cardiovascular effects of diabetes. The effects of the diabetic state on the cardiovascular system are characterised by their multitude, both regarding early versus late atherosclerosis and in different tissues and cell types.

Diabetes is coupled to generation, progression and instability of atherosclerosis. The link between accelerated macrovascular disease and diabetes is, however, not fully understood. Activation of innate immunity and inflammation is a common feature in diabetes and atherosclerosis.

Oxidative stress, linked to glucose and lipid metabolism, is another cause of vascular dysfunction that can induce inflammation (Brownlee 2005; Zhang et al. 2003).

In more advanced stages of atherosclerosis, plaques from patients with diabetes show increased macrophage infiltration and thrombus formation. Diabetic atherosclerosis is also
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characterised by structural changes, such as adventitial inflammation and plaque neovascularisation, leading to lipid core expansion and plaque instability (Moreno and Fuster 2004).

Once a plaque ruptures, thrombogenic changes may be more prominent in patients with diabetes. Several procoagulant factors are elevated in diabetes, including tissue factor, fibrinogen, vWF, factor VII, whereas the anticoagulants antithrombin III and protein C are decreased. PAI–1, a fibrinolysis inhibitor is also increased in diabetes (Carr 2001; Huszka et al. 1997). Platelets in diabetes are more prone to aggregate and activate (Li et al. 2001; Zhu et al. 2012). Taken together, increased platelet activation, procoagulant changes and diminished fibrinolysis increase the risk for atherothrombosis when plaque rupture occurs (Creager et al. 2003; Moreno and Fuster 2004).

Traditional risk factors vs diabetes specific factors

It is generally agreed that diabetes is linked to an approximately two-fold excess risk of vascular disease, independently from other conventional risk factors (Sarwar et al. 2010). Hence, increased frequency or severity of traditional risk factors for atherosclerosis (dyslipidaemia, smoking, hypertension, male sex and advanced age) do not explain the excess risk for atherosclerosis. Although patients with diabetes have a higher proportion of risk factors such as dyslipidaemia, hypertension and overweight, they do not account for the entire risk increment (Hanley et al. 2002). Thus, various mechanisms specifically related to diabetes have been proposed, in order to explain why subjects with diabetes are prone to develop atherosclerosis.

OVERVIEW OF MECHANISTIC FACTORS FOR INITIATION AND PROGRESSION OF ATHEROSCLEROSIS IN DIABETES

Diabetes is characterised by hyperglycaemia, insulin resistance and dyslipidaemia. These three are linked to each other in a complex interplay resulting in inflammation, oxidative stress and endoplasmatic reticulum stress.

Hyperglycaemia and Advanced Glycation End-products

Hyperglycaemia is a hallmark for diabetes and has both direct and indirect effects on the vasculature but the exact mechanisms and the relative importance of hyperglycaemia in atherosclerosis remain to be clarified. There is still no high level evidence for a beneficial effect of glycaemic control on cardiovascular outcomes, and several large studies have failed to establish the role of glycaemic control in the context of CVD (Boussageon et al. 2011). Indeed, the Action to Control Cardiovascular Risk in Diabetes trial (ACCORD) was terminated prematurely because of the negative effect of strict glucose control by insulin in high CVD-risk patients (ACCORD 2008). When added to conventional risk factors, fasting glucose concentration does not provide any prediction of vascular disease among persons without diabetes (Sarwar et al. 2010).
There is no clear explanation why glycaemic control fails to lower macrovascular complications in the same way as in microvascular complications. Glycaemic control in two large trials, (UKPDS and DCCT), were so closely linked to microvascular complications, that the modern diagnostic cut off values of HbA1c for diabetes are derived from these studies (DCCT 1993; UKPDS 1998; WHO/IDF 2006). One argument for the lack of effect on macrovascular complications is that a lower threshold might be required for macrovascular effects. Another proposed explanation is that glycaemic control has to be initiated at a very early stage of diabetes, as beneficial effects have been reported particularly in studies targeting subjects with newly diagnosed diabetes (Holman et al. 2008; Nathan et al. 2005). It is also possible that HbA1c poorly reflects the biological effects of hyperglycaemia on larger arteries. Indeed, glucose variability with short episodes of hyperglycaemia has been linked to increased risk of vascular complications in type 2 diabetes, but this pattern is not reflected by HbA1c (Nalysnyk et al. 2010). The paradox that tighter glucose control limits microvascular disease, while the effect on macrovascular outcomes to date have been unsuccessful, raises the possibility of different vascular effects of glucose based on vessel size.

To date, the only randomised controlled trial which has demonstrated effect on macrovascular events including amputations in type 2 diabetes is the Steno II study. This trial is unique in targeting multiple cardiovascular risk factors simultaneously in high risk type 2 diabetes patients relatively early in the disease (mean age was 55 years and diabetes duration 6 years). They achieved a 59% relative reduction in CVD events and a decrease in mortality by adopting a pharmacological and life style changing intervention programme targeting glucose control, hypertension, dyslipidaemia, overweight, physical activity, smoking and diet (Gaede et al. 2008; Gaede et al. 2003).

**Direct effects of hyperglycaemia: linked to vascular dysfunction but clear evidence is lacking**

A general concept proposed to explain how glucose induces cellular injury, suggests that the common cause is the inability to handle increased intracellular glucose levels, and the subsequent activation of pathways for cell injury and inflammation (Brownlee 2001).

There is no lack of studies showing deleterious effects of hyperglycaemia in vitro and in vivo, but direct effects on the extent and severity of atherosclerotic lesions in animal models are limited. One problem with currently used animal models of hyperglycaemia and atherosclerosis is the inability to differentiate the effects of hyperglycaemia from those of hyperlipidaemia (Goldberg and Dansky 2006). It is also uncertain if toxic or genetic procedures used to render mice diabetic also have other systemic effects related to vascular pathology (Hsueh et al. 2007). One of few studies showing direct macrovascular effects used mice with viral induced beta cell destruction. These mice had greater early atherosclerosis lesions even when fed on low-cholesterol diet, and correction of hyperglycaemia and insulin deficiency by insulin treatment reduced the size of early lesions (Renard et al. 2004). Still, this was only seen in early development, and not in more advanced and human-like atherosclerosis, where diabetic mice had more severe hyperlipidaemia and the direct effect
of hyperglycaemia was less apparent (Reaven et al. 1997). Further evidence for a lipid-
independent effect of glucose is provided by a study where diabetic non-dyslipidemic pigs
developed atherosclerosis more rapidly than similar pigs with hyperlipidaemia but without
diabetes (Gerrity et al. 2001).

There are no animal models which replicate the later stages of atherosclerosis as seen in hu-
mans. Thus, knowledge concerning mechanisms in plaque instability, rupture and healing
and the role of diabetes in this setting is even more limited. It is clear that mechanisms in
late atherosclerosis are different from those seen in early stages. Regulation of thrombosis
and fibrinolysis is also of greater importance for clinical manifestations of advanced athero-
sclerosis (Moreno and Fuster 2004).

Indirect effects of hyperglycaemia: a complex pattern promoting oxidative stress and
inflammation

The indirect effects of hyperglycaemia in the vasculature are mainly caused by modification
of proteins and lipids by glucose, a process called glycation. The products formed by this
process are called advanced glycation end products (AGE) and include a wide variety of
protein and lipid modifications, which occur during prolonged hyperglycaemia (Brownlee
et al. 1984; Cerami et al. 1985). AGEs exert their effect on the vascular wall by formation
of stable crosslinking in the extracellular space (Brownlee et al. 1988b) and by activation
of the receptor for AGE (RAGE) found on endothelial and vascular smooth muscle cells
(Wautier et al. 1996).

Glucose induces oxidative stress, monocyte adhesion and dysfunction in endothelial cells
direct and indirect via AGE

The direct effects of hyperglycaemia in endothelial cells involve intracellular glucose over-
load which directly can activate nuclear factor κ-beta (NF-κβ) (Piga et al. 2007). Activation
of NF-κβ results in altered gene expression patterns typical of endothelial dysfunction,
with increased adhesion of monocytes, permeability as well as reduced vasodilatation and
inflammation. These effects are probably also mediated by protein kinase C and increased
oxidative stress (Arcutia et al. 2010; Booth et al. 2006). High glucose levels can also de-
crease nitric oxide (NO) availability and prostaglandin I2 and increase vasoconstrictor sub-
stances such as prostanoids and endothelin–1 (Pernow et al. 2012).

Furthermore, effects of acute glucose overload (<12 h) induces chronic epigenetic changes
in NF-κβ resulting in increased expression of vascular cell adhesion molecule 1 (Acosta et
al. 2008), an example of the phenomenon known as metabolic memory.

Although direct effects of glucose presumably play a role, indirect effects via stimulation
of RAGE by AGEs formed during hyperglycaemia are probably of greater importance.
Activation of RAGE on endothelial cells leads to several proatherosclerotic alterations. NO
availability decreases by inhibition of endothelial nitric oxide synthase, eNOS, and reactive
oxygen species (ROS) formation is promoted by activating NADPH oxidase (Yan et al.
1994). Figure 7 illustrates the diverse effects of the AGE-RAGE system in atherosclerosis.
Insulin Resistance

Insulin resistance (IR) has been defined as the inability of insulin to increase glucose uptake and utilisation, regardless of whether insulin is endogenous or exogenous. By the definition, insulin resistance can refer both to defective insulin receptor signalling or overstimulation of insulin receptor pathways by hyperinsulinemia (Brown and Goldstein 2008). In fact, there is a selective mechanism in IR, causing hyperglycaemia by defective glucose uptake in muscle and fat and inability of insulin to decrease liver glycolysis. At the same time the liver continue to produce triglycerides and free fatty acids in response to insulin. This results in the sinister combination of hypertriglyceridemia and hyperglycaemia (Brown and Goldstein 2008).

The role of insulin resistance as an important player in atherosclerosis – evidence from animal models

The complex effects of IR on endothelial and vascular smooth muscle cells and macrophages are not fully elucidated. Uncertainty also remains whether IR has different effects in early and late atherosclerosis. IR has been associated with decreased NO and increased vascular cell adhesion molecule (VCAM-1) expression in endothelial cells, thus inducing endothelial dysfunction involved in early arteriogenesis. In VSMC, IR may induce increased proliferation and migration, and later apoptosis, there-by promoting mid- and late atherogenesis and plaque instability, although the exact mechanisms for this remain unclear (Bornfeldt and Tabas 2011).

Insulin resistance in human studies: relation to hyperglycaemia

The strongest evidence supporting an independent role of insulin resistance in development of atherosclerosis comes from Quebec cardiovascular study. Plasma insulin levels predicted development of coronary artery disease (OR 1.6 per 1 SD increase) after controlling for other risk factors in this large case-control study (Despres et al. 1996). This association is questioned by other studies, which did not find any increase in CVD (Knatterud et al. 1978; 1998), or yielded conflicting data (Perry et al. 1996; Resnick et al. 2003) indicating that IR is a marker for the cluster of risk factors in the metabolic syndrome. This is in accordance with a recent large meta-analysis, showing that fasting glucose levels were only modestly and non-linearly associated with risk for vascular disease in patients without diabetes, and that information about impaired fasting glucose did not provide predictive value for CVD in addition to conventional risk factors (Sarwar et al. 2010).

Glycation is linked to insulin resistance

Glycation of proteins and precursors to such glycation (see pp. 33-34), has recently been linked to development of IR. Levels of AGEs correlated closely to IR in two studies in subjects free from diabetes (Tahara et al. 2012; Tan et al. 2011). This correlation was present even after adjustment for several conventional risk factors (Tahara et al. 2012).
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The mechanisms suggested to be responsible for this link involve direct glycation if insulin resulting in decreased glucoselowering capacity and indirect suppression of insulin signal transduction via glycated albumin (Song and Schmidt 2012). In vitro studies are also suggestive of AGE damage to pancreatic β-cells via oxidative stress and RAGE, thereby decreasing insulin production capacity (Lee et al. 2010; Zhu et al. 2011). Recently, Cai et al published convincing multigeneration studies which revealed development of IR, oxidative stress, inflammation and obesity in wild type mice fed isocaloric AGE-rich diet (Cai et al. 2012). The mice fed AGE-diet developed IR earlier in life for every generation pointing to possible epigenetic mechanisms. One of the mechanisms for IR was decreased function of insulin receptor 1 and 2. This is a model which reflects the increase of IR and metabolic syndrome in human populations and proposes a role for AGE in causing IR and type 2 diabetes (Cai et al. 2012). In conclusion, insulin resistance is associated with development of CVD, but there is no evidence in clinical studies for an independent role of IR.

Dyslipidaemia

Oxidative stress, a key process in vascular dysfunction is promoted by free fatty acid mobilisation

Several facts point towards a link between hyperglycaemia and oxidative stress with generation of ROS, which in turn causes vascular damage via several pathways, such as AGE-RAGE, NF-κB, Protein Kinase C and the hexosamine pathway (Brownlee 2001).

This increase in ROS generation is driven by mobilisation of free fatty acids free fatty acids from adipose tissue and diminished uptake in skeletal muscle. Instead, free fatty acids uptake into macrovascular endothelial cells is increased. These free fatty acids are oxidized intracellularly, generating electron donors, which in turn cause overproduction of ROS. The increase in ROS causes endothelial dysfunction, diminished levels of NO and prostacyclin, increased levels of vasoconstrictors (prostanoids and endothelin), which promotes plaque formation (Brownlee 2005).

Elevated triglycerides and low HDL is associated with atherosclerosis in diabetes

Increasingly, attention has focused on the importance of hypertriglyceridermia (Watanakit et al. 2005), low levels of high-density lipoprotein cholesterol (HDL-C), and inflammation as factors driving diabetic cardiovascular disease. Inflammation is a key process involved in promoting and augmenting atherosclerosis by various mechanisms. High triglycerides (TG), (Callaghan et al. 2011) and low HDL-C have been shown to be risk factors for CVD especially in diabetes, but there is still some debate about to which extent and if there is a causative relationship or if TG and HDL merely serve as risk markers (Chapman et al. 2011; Khera et al. 2011; Thompson et al. 2010). Mechanistic data support a role for TG in pathogenesis of atherosclerosis, mediated partly by an augmented inflammatory response in the vessel wall (Chapman et al. 2011; Mazzone et al. 2008; Ting et al. 2007; Wang et al. 2011).
Advanced glycation end products, common but not limited to diabetes

Advanced glycation end products (AGE), a heterogeneous group of stable compounds formed during hyperglycaemia or cellular stress (Brownlee et al. 1988a), have been proposed to play a role in both intimal hyperplasia (Aronson 2002) and atherosclerosis (Vlassara 1996).

*Glycation is a non-enzymatic process*

AGEs are formed intra- and extracellularly by a process called glycation, which refers to binding of glucose to amino acids on proteins, a process is driven by high glucose levels and oxidative stress. The glycation process involves several biochemical alterations of the protein. Early reactions in glycation produce intermediate forms of glycation which are reversible, but the end products of glycation are irreversible, hence the name advanced glycation end products.

Most proteins can be glycated, but there is some controversy regarding which AGEs are the most important in atherosclerosis. The glycoxidation product Ne-(carboxymethyl) lysine (CML), (figure 1) is one well characterised specific AGE which accumulates in both plasma and tissues in patients with diabetes (Dunn et al. 1991; Kilhovd et al. 2003; Reddy et al. 1995; Schleicher et al. 1997; Valencia et al. 2004) and has been associated with diabetic vascular complications (Brownlee et al. 1988a; Tan et al. 2002).

![Figure 1. Structure of Ne-(carboxymethyl)lysine (CML), one of the most common AGEs](image)

*Advanced glycation end products in humans is formed both endogenously and exogenously*

AGE formation was first described in diabetes, driven by longstanding hyperglycaemia (Brownlee et al. 1988a; Cerami et al.). The source of plasma AGE in patients with diabetes has been correlated to poor glycaemic control and decreased renal elimination (Tsukahara et al. 2003). The concept that hyperglycaemia is the major source of AGEs has been challenged in recent years. Several studies have found increased AGE levels in non-diabetic patients, and proposed at least three different sources:
First, recent research underscores the link between ingested AGEs and RAGE activation, and food-derived AGE seems to be an important source of AGEs (Negrean et al. 2007; Uribarri et al. 2007; Vlassara 2001). Ingestion of foods rich in AGE has been shown to increase AGE burden both in patients with diabetes (Cai et al. 2004; Koschinsky et al. 1997), and healthy individuals (Birlouez-Aragon et al. 2010; Tessier and Birlouez-Aragon 2010; Vlassara et al. 2009). Furthermore, foods rich in AGEs are of the same sort as proatherogenic food (Birlouez-Aragon et al. 2010; Goldberg et al. 2004). Second, a decreased elimination of AGE in patients with end stage renal disease (ESRD), results in elevated AGE levels. Third, smoking (Cerami et al. 1997; Dickerson and Janda 2002; Nicholl et al. 1998), and diesel exhaust particles can induce AGE generation (Kodavanti et al. 2011). This provides new pathways for activation of the RAGE axis by formation of ligands unrelated to diabetes.

Glycation by glucose involves several biochemical reactions – The Maillard reaction

The Maillard reaction, named after the French chemist and physician Louis Camille Maillard, is one common pathway with several steps for formation of AGEs from glucose reacting with proteins, both in vivo and in food.

![Figure 2. Louis Camille Maillard (1878 – 1936). French chemist and physician.](image)

This reaction is also called ‘the Browning reaction’ as the brown surface seen on food processed at high temperature consists of AGE. Foods rich in AGE are typically fried or grilled, and interestingly resemble food identified as atherogenic. The process requires heating up to at least 154 °C and involves a reaction between a carbonyl group from a reducing sugar and a nucleophilic amino group of the amino acid (Robert et al. 2011). Proteins with exposed lysine or arginine residues are the most common to be glycated.

AGE formation in vivo takes place in several steps with intermediate stable, but not irreversible forms of protein modification, called early glycation. The amino group first reacts with a carbonyl group (figure 3) on the sugar molecule, forming a Schiff
base, which in turn undergoes rearrangements to a more stable Amadori product (figure 4). HbA₁c, used for monitoring glycaemic control, is the best known Amadori product. It is however worth noting that HbA₁c is not an AGE. The last steps in AGE formation require further dehydration, rearrangement and fragmentations of the Amadori product, yielding the stable irreversible AGE (Westwood and Thornalley 1995).

Figure 3. The carbonyl group found in glucose derivatives, e.g. methylglyoxal.

Figure 4. Possible pathways in the formation of advanced glycation end products (AGEs). The initial interaction between the highly reactive aldehyde group of glucose with any free amine group on proteins creates a Schiff’s base, which spontaneously rearranges itself into an Amadori’s product. Subsequent, slower changes (not shown) are progressively less reversible, and ultimately lead to the formation of AGEs. In addition, a variety of highly reactive carbonyl intermediates such as 3-deoxyglucosone, glyoxal and methylglyoxal can be formed by glucose or Schiff’s base or Amadori’s product auto-oxidation, which can react again with free amine groups to form AGE products such as imidazolone, Nε-carboxymethyllysine (CML), Nε-carboxyethyllysine (CEL), glyoxal-lysine dimer (GOLD) and methylglyoxal-lysine dimer (MOLD). Reprinted with permission from Basta et al. (2004).

Glycation under normal physiological conditions is a slow process

This process is under normal physiological conditions a slow process, and glycation takes place over several months. Slow glycation of long lived proteins is a part of the normal ageing, seen especially in for example cartilage and nerve proteins. It has been proposed that glycation under normal conditions serves the purpose to date protein age, and thereby control protein turnover (Vlassara et al. 1985). Scavenger cells could then target old proteins by expression of scavenger receptors.

Both hyperglycaemia and hypoxia increase and speed up AGE formation

In contrast to the normal slow glycation, hyperglycaemia increases the amount and rate of AGE formation. Glucose is under normal conditions metabolised by glycolysis, which produce relative stable metabolites with few reactive carbonyl groups. During hyperglycaemia glucose is diverted to the alternative polyl pathway (figure 5), producing methylglyoxal (MG) (2-oxopropanal, CH₃–CO–CH=O) from glyceraldehyde-3-phosphate intermediates of glycolysis (Phillips and Thornalley 1993). MG is highly reactive and rapidly forms AGE by interacting with arginine or lysine residues (Westwood and Thornalley 1995). Thus, hyperglycaemia will directly speed up and increase AGE formation.

![Figure 5. The polyl pathway and formation of methylglyoxal. Adapted and reprinted with permission from (Thornalley 1996).](image-url)
Hypoxia increases the activity of the polyol pathway enzyme aldose reductase resulting in increased production of MG. Both endothelial cells and macrophages exposed to hypoxia show rapid generation of AGEs within minutes (Chang et al. 2008; Xu et al. 2010). Furthermore, animal models of ischaemia-reperfusion injury have demonstrated rapid increase of MG, shortly followed by detection of AGE epitopes after induction of ischaemia, even without diabetes (Aleshin et al. 2008; Bucciarelli et al. 2006; Xu et al. 2010).

Interestingly, activation of aldose reductase by hypoxia seems to be boosted by stimulation of RAGE by AGE as the effect of hypoxia could be inhibited or decreased by administration of soluble RAGE (Aleshin et al. 2008; Bucciarelli et al. 2006). These observations point to another positive feedback loop (Hallam et al. 2010).

AGE can also form by non-glucose dependent enzymatic pathways by inflammatory stimulation of monocytes, macrophages and neutrophils by the NADPH-oxidase pathway (Anderson and Heinecke 2003; Anderson et al. 1999).

**Oxidative stress enhances AGE formation by inhibiting antiglycation defence**

Rapid AGE formation is also seen under oxidative stress. Oxidative stress may decrease detoxification of the AGE precursor methylglyoxal (MG) by inhibition of glyoxalase 1 (glo1), the enzyme responsible for MG removal (Thornalley 2003). The glyoxalase system is the most important glycation defence system, and is responsible for handling of cellular MG (Thornalley 2003).

**Direct effects of AGE on vessels**

The crosslinking capacity of some AGEs alters the mechanical properties of the vessel wall towards non-compliance (Choi et al. 2009; Semba et al. 2009b). The decrease in arterial compliance seen both at old age and in diabetes is consistent with the finding of increased CML in arterial tissue with age and in diabetes (Schleichert et al. 1997). Furthermore, crosslinking of collagen increases the trapping of LDL particles, which in turn activates monocytes (Brownlee et al. 1985). AGEs can also function as chemo attractants, aiding in recruitment of monocytes into the vessel wall (Abordo et al. 1996; Kirstein et al. 1990). AGEs have also been linked to an increased production of extracellular matrix via transforming growth factor-β (Pugliese et al. 1997; Rumble et al. 1997). AGEs can activate certain matrix metalloproteases in atherosclerotic plaques, which are thought to promote plaque instability (Cipollone et al. 2003).

**Glycation may alter functional properties of extracellular proteins and regulate intracellular metabolism**

Research on protein glycation was initially focused on the direct effects on extracellular tissue induced by AGEs. Cross-links of matrix proteins (e.g. elastin) by AGEs could modify properties if the vessel wall (Paul and Bailey 1996) and AGE-degradation peptides could act in the same way (Vlassara 1996). The biological importance of such changes has been
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Challenged arguing that the extensive protein modifications used in vitro to show biological responses would rarely be present in vivo (Thorpe and Baynes 2003). Focus regarding the direct effects of AGE has shifted towards intracellular protein glycation, which occurs more easily in vivo and is thought to play a role in regulation of cellular metabolism (Puddu et al. 2010). As discussed in previous sections, intracellular glycation is not glucose dependent per se, and is also induced by oxidative stress (Naudi et al. 2012). Protein carbonyl content has been proposed as an overall marker of profound oxidative stress and protein dysfunction (Naudi et al. 2012), and intracellular AGEs make up part of the protein carbonyl content. An example of change in protein function by AGE is cardiomyocyte injury from inhibition of thioredoxin and change in albumin binding sites (Ahmed et al. 2005; Wang et al. 2010).

Quantification of AGE

The nonspecific glycation process produces a heterogeneous group of AGEs as nearly every protein has the potential to be glycated, and on different sites. Far from all AGEs have been characterised in detail, the most common AGE found and studied in humans is Nε-(carboxymethyl)lysine (CML) (Ikeda et al. 1996; Kislinger et al. 1999; Reddy et al. 1995; Takeuchi et al. 1999). The specific glycation structure of CML has been characterised in detail, enabling production of specific antibodies for detection of CML. For the majority of other known AGEs (about 25) there is insufficient detailed information to produce antibodies with adequate specificity. However, it may be more appropriate to consider AGEs as a group of biological active substances, rather than focus on individual AGE structures. Determination of CML levels could then serve as a measure of overall AGE burden. Table I provide some clinical follow up studies with associations between AGEs in plasma or serum and cardiovascular outcome.

Receptor for Advanced Glycation End-products (RAGE)

AGEs exert their main effects by binding to the receptor for advanced glycation end products (RAGE), a multiligand receptor in the immunoglobulin superfamily of cell surface molecules (Kislinger et al. 1999; Neeper et al. 1992; Schmidt et al. 1992). RAGE is expressed in vascular smooth muscle cells (VSMC) as well as in endothelial cells and monocytes, all relevant in atherosclerosis (Ritthaler et al. 1995; Schmidt et al. 1996).

There ample evidence from animal studies proving that stimulation of RAGE amplifies atherosclerosis progression, and that this effect can be reversed by inhibition of the AGE-RAGE system (Bucciarelli et al. 2002; Naka et al. 2004; Park et al. 1998; Wendt et al. 2006). This inhibition can take place at several levels. First, ligands can be reduced by lowering AGEs in food and treating hyperglycaemia. Secondly, treatment by scavenger molecules such as endocerytor RAGE (esRAGE) can lower levels of ligands available to the RAGE. Third, treatment with substances such as aminoguanide can block the RAGE. Animal studies have proven that all these concepts work to reduce diabetic vascular complications. Applicability in humans is restricted to dietary restriction and treatment of hyperglycaemia, and no treatment/medication specifically targeting the AGE-RAGE system is available yet.
The expression of RAGE is upregulated by binding of AGE (Schmidt et al. 1999; Tanaka et al. 2000) and is particularly abundant in tissues where the AGE burden is high such as vasculature from patients with diabetes (Rithaler et al. 1995).

RAGE consists of three extracellular C-domains and one V-domain (figure 6), linked to a short transmembrane domain with a short cytosolic signal transducing domain (Dattilo et al. 2007). The V-domain is responsible for the majority of ligand binding, (see next section). RAGE activation is most commonly mediated via downstream activation of NF-κβ, a central pathway for various inflammatory responses. Activated NF-κβ induces different effects in different cell types, VSMCs exhibit increased proliferation, migration and sensitivity for the renin-angiotensin-aldosterone system. Endothelial cell function is impaired with inhibition of NO-production and expression of receptors promoting adhesion. Activation of NF-κβ in monocytes and macrophages promote adhesion to the endothelium, and secretion of cytokines from these cells inside the vessel wall (Basta et al. 2002; Kislinger et al. 2001; Mohamed et al. 1999; Schmidt et al. 2000). It is proposed that RAGE act to boost and prolong an inflammatory process initiated by vascular injury (e.g. vascular surgery or PTA) or by lipid accumulation.

Ligands

Most proteins have the potential to be AGE-modified, so why could a single receptor recognise a diversity of ligands, whose only shared structure is the addition of a reduced sugar? It has been shown that neither early glycation products nor single glycated amino acids are recognised by RAGE. Thus RAGE has high specificity for AGEs, (or at least CML as the interaction with this molecule has served as a model for RAGE-AGE interaction in most studies). Glycation have to be positioned at a side chain, (as opposed to within a fold of the protein), and the glycated amino acids have to be surrounded by strongly negatively charged areas of the AGE-protein in order to effectively bind to RAGE. Furthermore, the exact amino acid sequence or the extent of AGE modification seems not to be critical for recognition.

The interaction between single AGEs and an individual V-domain is thus specific but takes place with low affinity.

Effective binding with high affinity takes place when there are multiple AGE-modified domains available, and oligomerisation of RAGE provides multiple V-domains to bind the AGE-modified side chains (figure 6). This is the proposed mechanism for the pattern recognition ability of RAGE (Xie et al. 2008). Oligomerisation of RAGE units provides a basis for how sRAGE functions (Xie et al. 2008). sRAGE inhibits effective RAGE signalling by forming oligomers with the membrane bound RAGE (figure 6) (Koch et al. 2010).

RAGE effects on vessels

Ligation of RAGE in arterial vessels promotes endothelial dysfunction with increased permeability, adhesion and vasoconstriction. RAGE signalling induces matrix production and proliferation in VSMCs. These changes involve an array of different pathways, many
of which are mediated via activation of NF-κB. Prominent features are inhibition of endothelial nitric oxide synthase, eNOS, with decreased NO availability and increased free radical formation, expression of adhesion molecules and activation of macrophages (Basta 2008), all relevant for development of atherosclerosis and are summarised in figure 7.

**Figure 6.** The RAGE receptor. Schematic representation of full-length RAGE (orange and red) with the three extracellular domains, V is the ligand binding domain, C1 and C2 extracellular C-domains connected to a short transmembrane domain and a short cytosolic tail responsible for signal transduction. Models of RAGE Activation and Inhibition by sRAGE. (A) Model in which RAGE preassembles in the plasma membrane. Oligomerisation of RAGE provide multiple binding sites and is necessary for high affinity binding of AGE and signal transduction. Ligand binding to RAGE stabilizes oligomers, which then can bind a signaling adaptor protein (gray sphere) to the cytoplasmic region of RAGE. (B) Model showing action of soluble RAGE (sRAGE, green), which inhibits signal transduction by oligomerisation with membrane bound RAGE, limiting binding of intracellular adaptors and blocks signal transduction. Reprinted with permission from Koch et al (2010)
Splice variants (esRAGE) and shredded RAGE (sRAGE) modify RAGE signalling

The regulation of the AGE-RAGE system is not fully understood. Endocytotic RAGE (esRAGE), a beneficial form of the AGE receptor has been identified in plasma and vascular tissue (Cheng et al. 2005), and is thought to act as a scavenger for AGE. esRAGE is lacking the transmembrane domain of RAGE, and is produced by alternative splicing of RAGE mRNA (Yonekura et al. 2003). Free RAGE is also formed by ectodomain shedding of membrane bound rage and this form is denoted sRAGE (Kalea et al. 2011). The exact relationship between circulating RAGE and complications has not been established and results are conflicting. Some studies have found low levels of esRAGE associated with various vascular complications in both diabetic (Basta et al. 2006; Katakami et al. 2005; Koyama et al. 2006; Koyama et al. 2005) and non-diabetic patients with coronary artery disease (Falcone et al. 2005). Plasma levels of sRAGE is downregulated in patients with chronic hyperglycaemia, among its ligands, S100A12 protein, but not CML, appear to downregulate RAGE (Basta et al. 2006). On the contrary, others have shown esRAGE to be elevated in patients with vascular complications, probably as a sign of activated defence to vascular injury. For example, sRAGE remains elevated in serum 180 days post-PTCA (Basta et al. 2008).

S100A12 is a endogenous ligand to RAGE

The family of proinflammatory proteins called calgranulins has been identified as endogenous ligands to RAGE (Hofmann et al. 1999; Hsieh et al. 2004). One member of this family, S100A12, is elevated in diabetes and linked to mortality (Kosaki et al. 2004; Koyama et al. 2006; Nakashima et al. 2010). S100A12 is an intracellular protein proposed to be involved in specific calcium-dependent signal transduction pathways and its regulatory effect on cytoskeletal components may modulate various neutrophil activities. S100A12 is released extracellularly during apoptosis and has been related to various inflammatory and degenerative conditions (i.e. rheumatoid arthritis, Chron’s disease) (Chen et al. 2009; Foell et al. 2003). S100A12 binds to RAGE at a site separated from the site responsible for CML binding. Binding and activation of RAGE by S100A12 requires oligomerisation of S100A12, which in turn depends on presence of Ca²⁺ and Zn²⁺ ions (Moroz et al. 2009).

Interestingly, S100A12 seems to induce accelerated atherosclerosis and vascular calcification in transgenic S100A12/ApoE-null mice under the proinflammatory and proatherosclerotic environment (Hofmann Bowman et al. 2011). An animal model of osteogenesis in murine vasculature triggered by chronic kidney disease provides further evidence for a causative role of S100A12 in vascular calcification (Gawdzik et al. 2011). This may be an explanation of the increased vascular calcification seen in patients with diabetes.

There are conflicting data about the relation between diabetes status and S100A12 levels. Several papers have reported higher serum S100A12 levels in patients with diabetes (Kosaki et al. 2004). In contrast another study reported an association between mortality and S100A12 levels in patients with coronary heart disease but did not find any difference between patients with or without diabetes (Nakashima et al. 2010).
Figure 7. Implications for atherosclerosis of the vicious cycle RAGE–ligand axis. The AGE formation, as consequence of oxidative stress, hyperglycemia, inflammatory stimuli, determine a site for the amplification of inflammatory pathways. In endothelial cell RAGE–ligand interaction increases leukocyte adhesion molecules expression and the tissue factor procoagulant activity. AGE–RAGE interaction triggers a vicious cycle of cellular injury, by upregulating the RAGE itself and by attracting polymorphonuclear leukocytes, monocytes, and lymphocytes. S100A12 protein and amphoterin release from such cells triggers a new wave of cell perturbing molecules. It promotes the migration of circulating monocytes into the intima (chemotaxis), their conversion to activated macrophages, and their release of cytokines and proteases. Within the intima, activated macrophages increase their lipid uptake – mostly due to an increase of AGE-induced oxidized LDL receptors – leading to the formation of foam cells. Next, activated SMC migrates into the intima at sites of vascular lesions, and here proliferates, producing new extracellular matrix.

Reprinted with permission from Basta et al. 2008.

Introduction
It seems clear that S100A12 is not specific for diabetes. For example, a longitudinal study of haemodialysis patients followed for 5 years showed association with mortality and elevated S100A12 (Kalousova et al. 2012). Furthermore, S100A12 was overexpressed in non-diabetic subjects with premature coronary artery disease (Mahajan et al. 2009). A link to PAD has recently been reported in a cross sectional study in ESRD patients undergoing haemodialysis, where plasma S100A12 levels were associated with PAD prevalence (Shioitsu et al. 2011). For details see table I.

The presence of S100A12 in human vascular tissue has been studied in coronary atherosclerotic plaques, where S100A12 expression was associated with apoptotic smooth muscle cells and macrophages (Burke et al. 2004). Furthermore, patients with diabetes had greater expression of both S100A12 and RAGE compared to those without diabetes (Burke et al. 2004).

CONCLUDING REMARKS – DIABETES AND ATHEROSCLEROSIS VS AGE – THE HEN OR THE EGG?

It has recently been proposed that increase in AGES from environmental sources, mainly food, play a major initial role in the pathogenesis of both diabetes and atherosclerosis (Vlassara and Striker 2011). This new causative model (figure 8) involves depletion of host defences against oxidative stress and concomitant upregulation of inflammatory pathways resulting in further amplification of oxidative stress (Cai et al. 2012). The resulting dysvascular and inflammatory state is thought to contribute to insulin resistance, beta cell destruction and early atherosclerosis (Vlassara and Striker 2011). Exposure to elevated AGE levels in utero may render the offspring with epigenetically compromised defence against oxidative stress already from birth (Ling and Gioop 2009, Mericq et al. 2010).

Figure 8. Alternative causal pathways to diabetes complications. (A) traditional view with diabetes and hyperglycaemia as initial events. (B) proposed new causal pathway starting with AGE excess and epigenetic impairment of host defence against oxidative stress (ox).
<table>
<thead>
<tr>
<th>Author, year</th>
<th>Design</th>
<th>n</th>
<th>Study population</th>
<th>AGE-RAGE component</th>
<th>Mean</th>
<th>Association with</th>
<th>HR/OR and Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Klevraeva et al. 2012)</td>
<td>Longitudinal cohort, 5yr</td>
<td>261</td>
<td>ESRD, haemodialysis (33% diabetes) Healthy controls</td>
<td>Serum S100A12*</td>
<td>98 +/- 1176 ng/mL 87 +/- 193 ng/mL</td>
<td>Mortality, CVD mortality, Infectious-mortality</td>
<td>1.13, (0.96–1.33, 95% CI) 1.07, (0.82–1.34, 95% CI) 1.31, (1.06–1.60, 95% CI)</td>
</tr>
<tr>
<td>(Semba et al. 2009a)</td>
<td>Longitudinal 4.5 year cohort</td>
<td>559</td>
<td>Disabled women &gt;65 years, 15% diabetes</td>
<td>Serum CML*, sRAGE, sRA*GE</td>
<td>CML: 0.54 μg/mL (alive), 0.59 μg/mL (died) sRAGE: 0.33 ng/mL (0.24–0.45, IQR) (alive), 0.37 (0.20–0.51) (died)</td>
<td>CVD mortality</td>
<td>HR 2.31 (1.32–4.05) high Q vs 14–3% Q CML HR 1.52 (1.24–1.85) per 1 SD sRAGE</td>
</tr>
<tr>
<td>(Basta et al. 2008)</td>
<td>Longitudinal, before/after 180 days</td>
<td>81</td>
<td>PTCA</td>
<td>Plasma CML</td>
<td>–</td>
<td>Post-PTCA and stent area elevation persists up to 180 days</td>
<td></td>
</tr>
<tr>
<td>(Nin et al. 2011)</td>
<td>Longitudinal 12-year, cohort</td>
<td>339</td>
<td>Type 1 diabetes, 40–45 years</td>
<td>Plasma CML*</td>
<td>3.5 μmol/L</td>
<td>Fatal and non-fatal CVD, total mortality</td>
<td>HR 1.22 (0.96–1.56, 95% CI) CML fatal or nonfatal per 1 SD AGES-score</td>
</tr>
<tr>
<td>(Kihovd et al. 2005)</td>
<td>Longitudinal 18-year, population based cohort</td>
<td>1141</td>
<td>Non-diabetes, 45–64 years</td>
<td>Serum AGE,</td>
<td>–</td>
<td>Total and CVD-CHD-mortality in women</td>
<td>Crude HR 1.13 (1.05–1.20, 95% CI) for CVD mortality in women</td>
</tr>
<tr>
<td>(Choi et al. 2005)</td>
<td>6-month follow-up</td>
<td>203</td>
<td>Type 2 diabetes with coronary stenting after ACS</td>
<td>Serum AGE*</td>
<td>–</td>
<td>In-stent restenosis</td>
<td>Adjusted OR, high vs low AGE: 2.7 (1.4–4.9, 95% CI)</td>
</tr>
</tbody>
</table>

* Circulrex ELISA. * Crude Hazard Ratio (HR), 95% CI. Not independent of CRP.
HR, hazard ratio. IQR, interquartile range. Q, quartile.
THE PATIENT WITH DIABETES AND PERIPHERAL ARTERIAL DISEASE – SOME UNIQUE CONCEPTS

The atherosclerotic changes occur earlier and are accelerated among patients with diabetes, especially among those with poor glycaemic control. Critical ischaemia can also be the main cause of ulceration without trauma. Diabetes and lower limb ischaemia increase the susceptibility for infection by detrimental effects on infectious defence mechanisms.

It is believed that even mild ischaemia can be an important contributor to non-healing of diabetic foot ulcers in the presence of compromised biology in the diabetic foot. The pattern of atherosclerosis differs in patients with diabetes having a more distal localisation and relative sparing of femoral vessels and foot arteries (Strandness et al. 1964). The resulting lower limb ischaemia in combination with peripheral neuropathy poses the patient with diabetes at an extremely high risk for foot ulcer development, even with minor trauma.

Patients with PAD in its most severe form develop progressive tissue ischaemia with rest pain, tissue loss, gangrene and ultimately necessitating amputation. Patients with both diabetes and PAD have at least as high mortality as those with coronary artery disease and diabetes (Caro et al. 2005).

People with diabetes are at high risk for foot ulcers, even without the presence of severe lower limb ischaemia. This increased risk is due to several complications specific to diabetes and related to the hyperglycaemic state. Sensory neuropathy makes the foot susceptible to minor trauma, whereas the autonomic neuropathy cases arterio-venous shunting, foot deformities and reduced skin integrity due to loss of sweating. Structural changes at the microcirculatory level reduce the nutritive perfusion and oxygen delivery to tissues. Healing of an established foot ulcer is also impaired in diabetes via several mechanisms, e.g. diminished fibroblast function. Prothrombotic mechanisms and reduced infectious defence potential in diabetes also contributes to poor wound healing.

Ischaemia due to PAD is present in approximately 50% of patients with diabetes presenting with a foot ulcer (Jeffcoate et al. 2006; Prompers et al.). Addition of ischaemia to the compromised biology in the diabetic foot increases the risk for ulcer development and progression to an unsalvageable limb. The majority of amputations among patients with diabetes are preceded by severe limb ischaemia (Armstrong et al. 1998; Carmona et al.; Moulik et al. 2003). This is reflected in the commonly used grading systems for diabetic foot ulcers. Diabetic foot ulcers are classified according to depth (grade 0 – III) and presence of lower extremity ischaemia and/or infection (stage A to D) in University of Texas Wound Classification System (Armstrong et al. 1998). Having both ischaemia and infection (stage D) carried a 90 times higher risk for midfoot or higher amputation compared to a clean wound without ischaemia (stage A). The PEDIS system, (International Consensus on the Diabetic Foot PDIS system: perfusion, extent/size, depth/tissue loss, infection, and sensation), also classifies foot ulcers according to five categories: perfusion, extent/size, depth/tissue loss, infection and sensation (Schaper 2004). Acute infection in the compromised foot seems to be equally important in the pathway to major amputation. All these circumstances taken together result in an 12–20 fold higher risk for major amputation among patients with

Presence of PAD is also significantly associated with reduced survival in patients with diabetic foot ulcers, with several studies reporting 5-year survival below 50% (Boyko et al. 1996; Campbell et al. 2000; Moulik et al. 2003).

**INTIMAL HYPERPLASIA IS ACCELERATED IN DIABETES**

Autologous vein is the most durable conduit when performing bypass surgery for lower limb ischaemia, but 30% to 50% of vein grafts will fail within 1 to 5 years (Conte et al. 2006; Gupta et al. 1997; Taylor et al. 1990), resulting in relapse of symptoms or even severe ischaemia requiring amputation (Baldwin et al. 2004). Vein grafts are subject to various injury mechanisms that probably cause pathological changes in vein structure and function (Cox et al. 1991; Davies and Hagen 1995), a process known as intimal hyperplasia (IH). Excess neointima can cause stenosis and occlusion of the graft (Kaneda et al. 2006; Nielsen et al. 1997). The exact molecular mechanisms controlling neointima formation are not fully understood, but an inflammatory response to injury seems to be one important factor (Mitra et al. 2006). This inflammatory response is seen in experimental vein grafts during arterialisation and is characterised by cytokine signalling and infiltration of activated leukocytes (Hoch et al. 1994; Stark et al. 1997; Sterpetti et al. 1998; Zhang et al. 2004). One study has demonstrated marked expression of inflammatory cytokines in stenosed saphenous vein grafts from patients with coronary artery disease (Christiansen et al. 2004).

Intimal hyperplasia is underlying restenosis after stenting procedures and diabetes is one important factor associated with higher restenosis rate after coronary and carotid interventions (1996; Carrozza et al. 1993; Kornowski et al. 1997; Willfort-Ehringer et al. 2004). Much less is known about the role of diabetes in vein graft intimal hyperplasia but patients with diabetes do worse after coronary bypass grafting (Lawrie et al. 1986; Morris et al. 1991). It has also been shown that patients with diabetes have a lowered prostacyclin release from the saphenous vein indicating a dysfunctional endothelium (Brunkwall and Bergqvist 1992; Hicks et al. 1997). Human vascular smooth muscle cells (VSMC) of diabetic origin exhibit increased proliferative and migratory characteristics (Faries et al. 2002) in cell culture and diabetes augments intimal hyperplasia (IH) in experimental vein grafts in rabbits (Davies et al. 1995).

Hyperglycaemia promotes proliferation in human VSMC in vitro (Avena et al. 1998) further linking diabetes to increased intimal hyperplasia. Lorusso et al. demonstrated profound structural derangement and decreased endothelium-dependent relaxation in saphenous vein grafts from patients with diabetes compared to non-diabetics. These changes were also correlated to metabolic control with most severe abnormalities found in veins from patients with poor metabolic control (Lorusso et al. 2003).

As discussed earlier, RAGE is expressed in VSMC as well as in endothelial cells and monocytes, all relevant in intimal hyperplasia (Rithaler et al. 1995; Schmidt et al. 1996).
AGEs have also been linked to increased production of extracellular matrix via transforming growth factor-β (Pugliese et al. 1997; Rumble et al. 1997).

It has been proposed that the RAGE-AGE axis plays an important role in restenosis and IH in diabetes (Aronson 2002) and animal studies have found a link between RAGE stimulation and IH (Sakaguchi et al. 2003; Zhou et al. 2003).

A recent animal vein graft study directly links RAGE activation to increased intimal hyperplasia formation by proliferation of VSMC in vein grafts both in diabetic and non-diabetic mice (Li et al. 2012). Interestingly, both AGEs and increased blood pressure independently activated RAGE in this model (Li et al. 2012).

Chello et al (2009) suggest that elevated serum levels of AGE determine a prothrombotic state, which could explain the increased rate of vein graft occlusion. They found that AGEs activate expression of RAGE and enhance intracellular ROS formation in cultured endothelial cells from human vein (Chello et al. 2009). These results were found even in endothelial cells from patients with good glycaemic control, that is HbA$_{1c}$ <6.0%.

**SURGERY AND ACUTE HYPERGLYCAEMIA**

The combination of the surgical trauma, ischaemia-reperfusion injury and acute hyperglycaemia seems to interact in a complex way, ultimately resulting in disturbed function of several systems, i.e. innate immune system (Turina et al. 2005), the coagulation system (Carmassi et al. 1992; Carr 2001), endothelial function (Langouche et al. 2005), inflammatory mediators and cellular metabolism (Van den Berghe 2004; Vanhorebeek et al. 2005). The stress response overrides the normal cellular protection against hyperglycaemia causing intracellular glucose overload. This is thought to trigger impaired mitochondrial function with increased superoxide production causing cellular damage (Vanhorebeek et al. 2005). High intracellular glucose also induces enzymes that produce nitric oxide. Endothelial dysfunction in hyperglycaemia is in part caused by supernormal nitric oxide production leading to increased adhesion of leukocytes and inflammatory responses (Langouche et al. 2005). Other direct effects of hyperglycaemia include glycation of immunoglobulin G and complement factor C3 leading to impaired function of immunoglobulins and decreased opsonisation (Turina et al. 2005). Hyperglycaemia also affects leukocyte function with decreased phagocytosis, decreased bacterial killing and increased adhesion that may compromise extravasation (Alexiewicz et al. 1995; McManus et al. 2001; Perner et al. 2003). There is also growing evidence for a role of RAGE in infections (van Zoelen et al. 2011). Acute hyperglycaemia may thus contribute to each of the studied outcomes in Paper I and III via several different mechanisms. For example – hypercoagulability will increase the risk for graft failure, endothelial dysfunction and cellular damage increases the risk for cardiovascular events and death, and impaired host defence can accelerate infection in pre-existing foot gangrene ultimately resulting in limb loss and wound complications.
PATIENTS AND METHODS

DEFINITIONS

Peripheral arterial disease (PAD)

The term peripheral arterial (or artery) disease (PAD) is used in the literature both to denote a wide group of non-coronary arterial syndromes (Hirsch et al. 2006) and to denote the progressive narrowing of arteries in the lower extremities, due to atherosclerosis (2003; Norgren et al. 2007; Selvin and Erlinger 2004). The latter definition is used in this thesis and Paper I-IV.

Amputation

A major amputation was defined as an amputation above midfoot level, i.e. Syme’s or Lisfranc’s amputations were considered major amputations. With this definition all amputations resulting in need for prosthesis or reduced walking capacity is classified as major. Minor amputations were not studied, as these not necessarily represent a failure when treating diabetic foot ulcers.

Classification of diabetes and non-diabetes

Paper I and IV included only patients with established diabetes, i.e. clinically diagnosed diabetes with treatment. Patients with diabetes in Paper III include also newly diagnosed patients according to either oral glucose tolerance test (OGTT) (WHO/IDF 2006) or HbA\textsubscript{1c} (ADA 2010) performed before bypass procedure (table II). In Paper II diabetes status is based in Swedvasc registry data, and some misclassification of diabetes patients as non-diabetes patients can be expected.

Table II. Classification of diabetes according to OGTT and HbA\textsubscript{1c}.

<table>
<thead>
<tr>
<th></th>
<th>OGTT – P-Glucose (venous) mmol/L</th>
<th>HbA\textsubscript{1c} (MonoS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fasting</td>
<td>120 min</td>
</tr>
<tr>
<td>Healthy</td>
<td>&lt; 6.3</td>
<td>AND</td>
</tr>
<tr>
<td>IFG</td>
<td>6.3 – 6.9</td>
<td>–</td>
</tr>
<tr>
<td>IGT</td>
<td>&lt; 7.0</td>
<td>AND</td>
</tr>
<tr>
<td>Diabetes</td>
<td>≥ 7.0</td>
<td>OR</td>
</tr>
</tbody>
</table>
PATIENTS

The papers in this thesis include 1017 patients with diabetes and about the same number of patients without. All of the patients, except 146 in Paper IV, had PAD at inclusion and 8 of 10 had tissue loss. Patients with diabetes and PAD had a mean age of ≈ 70 years and 60–70% were male. Details are found in table III.

Table III. Characteristics of the study participants (Paper I–IV)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Paper I</th>
<th>Paper II</th>
<th>Paper III</th>
<th>Paper IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes</td>
<td>742</td>
<td>1098</td>
<td>38</td>
<td>30</td>
</tr>
<tr>
<td>Not diabetes</td>
<td>91</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>With diabetes %</td>
<td>100%</td>
<td>40%</td>
<td>56%</td>
<td>100%</td>
</tr>
<tr>
<td>Male % (n)</td>
<td>71% (65)</td>
<td>58%</td>
<td>49%</td>
<td>66%</td>
</tr>
<tr>
<td>Age (mean)</td>
<td>70</td>
<td>74</td>
<td>78</td>
<td>69</td>
</tr>
<tr>
<td>Type 1 diabetes</td>
<td>11% (10)</td>
<td>–</td>
<td>NA</td>
<td>–</td>
</tr>
<tr>
<td>Type 2 diabetes</td>
<td>89% (81)</td>
<td>–</td>
<td>NA</td>
<td>–</td>
</tr>
<tr>
<td>Design</td>
<td>Cohort, retrospective inclusion, prospective follow up.</td>
<td>Prospective cohort with nested case-control design</td>
<td>Prospective case-control</td>
<td>Prospective cohort</td>
</tr>
<tr>
<td>Source population</td>
<td>Consecutive hospital based</td>
<td>National population based, &gt;90% coverage</td>
<td>Non-consecutive hospital based</td>
<td>Representative population-sample</td>
</tr>
<tr>
<td>Proportion with tissue loss</td>
<td>77% (70)</td>
<td>82%</td>
<td>67%</td>
<td>79%</td>
</tr>
<tr>
<td>Renal impairment</td>
<td>22% (20)</td>
<td>20%</td>
<td>8%</td>
<td>10%</td>
</tr>
<tr>
<td>Main exposure</td>
<td>Hyperglycaemia 48 h postop</td>
<td>Diabetes vs no diabetes</td>
<td>RAGE/AGE/5100A12</td>
<td>RAGE/AGE/5100A12</td>
</tr>
<tr>
<td>Main outcome</td>
<td>AFS with a patient graft at 90 days</td>
<td>AFS</td>
<td>AFS</td>
<td>AFS</td>
</tr>
<tr>
<td>Other outcome(s)</td>
<td>Wound complications</td>
<td>Survival</td>
<td>NA</td>
<td>PAD</td>
</tr>
<tr>
<td>Follow up time</td>
<td>90 days</td>
<td>2.2 years</td>
<td>702 days</td>
<td>12 years</td>
</tr>
<tr>
<td>AFS</td>
<td>88% at 90d</td>
<td>68/100py</td>
<td>77/100py</td>
<td>83/100py</td>
</tr>
<tr>
<td>HR diabetes (without diabetes is ref.)</td>
<td>NA</td>
<td>1.49</td>
<td>1.63</td>
<td>NA</td>
</tr>
</tbody>
</table>

AFS, amputation-free survival. HR, hazard ratio. Py, person-years.
All patients in **Paper I–III** had infrainguinal bypass procedure as an inclusion criterion. Six of ten patients with diabetes had the distal anastomosis below the popliteal artery and 8/10 had a venous graft (table IV).

**Table IV. Surgical data for infrainguinal bypass procedures (Paper I–III)**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Paper I</th>
<th>Paper II</th>
<th>Paper III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diabetes</td>
<td>Diabetes</td>
<td>Not diabetes</td>
</tr>
<tr>
<td>n</td>
<td>91</td>
<td>742</td>
<td>1098</td>
</tr>
<tr>
<td>Outflow</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Popliteal</td>
<td>37%</td>
<td>35%</td>
<td>46%</td>
</tr>
<tr>
<td>Cural</td>
<td>58%</td>
<td>59%</td>
<td>52%</td>
</tr>
<tr>
<td>Pedal</td>
<td>4% (4)</td>
<td>5%</td>
<td>2%</td>
</tr>
<tr>
<td>Vein graft</td>
<td>86% (78)</td>
<td>61%</td>
<td>80%</td>
</tr>
</tbody>
</table>

**STUDY DESIGNS**

**Source populations and patient samples**

The patients in **Paper II** and **IV** can be regarded as a national population based sample in contrast to **Paper I** and **III** which consists of patients from a single academic vascular surgical centre, and may thus not be representative for patients with PAD undergoing lower limb bypass in general. The proportions of patients with tissue loss, male patients and the mean age in **Paper I** and **III** did not differ substantially compared to the population based sample of patients with PAD undergoing IBS in **Paper II**. Furthermore, the department of vascular surgery at Karolinska university hospital provided care for the population in defined geographical areas in the Stockholm county, compromising roughly 50% of the total population in the county (1.6 x 10^6 inhabitants) and did not serve as a referral centre for this group of patients.

The study sample in **Paper II** is representative of the Swedish population as the Swedvasc registry cover >90% of vascular surgery procedures (Bergqvist et al. 1998).

The cohort was restricted to first ever infrainguinal bypass procedure due to non-acute lower limb ischaemia with rest pain or tissue loss defined according to Rutherford category 4–6 (Rutherford et al. 1997). This restriction was made in order to study a well-defined clinically relevant sample, i.e. patients presenting with chronic critical limb ischaemia (CLI) and deemed suitable for infrainguinal bypass surgery. Accordingly, patients presenting with acute ischaemia, ischaemia due to aneurysm or trauma were not included. The restriction algorithm allowed for previous suprainguinal inflow procedures. For details, (figure 9).
Figure 9. Selection diagram for paper II. Detailed description of how the study cohort was extracted.
The patients with type 2 diabetes in Paper IV were recruited from a defined geographical area with 32487 residents, of whom 10156 were born 1927–1957 (40–70 years old). 456 patients had the diagnosis diabetes recorded in primary care records. After verification and classification (type 1 diabetes and Latent Autoimmune Diabetes in the Adult, LADA, were excluded), the final study population consisted of 156 subjects with type 2 diabetes, representing a 68% participation rate (figure 10). Non-participants were comparable in age, diabetes duration, glucose control and prevalence of diabetes complications except having more cardiovascular disease as judged by review of the medical records (Karvestedt et al. 2011).

The study population included 62 % males, mean age 62 years, diabetes duration 7 ± 5.7 (SD) years, body mass index 29.2 and HbA1c 6.4% ± 1.3 (SD) (MonoS, reference <5.2 %). They were investigated by questionnaires, clinical examinations, blood and urine sampling and review of medical records. Twenty-eight per cent of the patients were treated with diet.

Patients free from signs of PAD at inclusion were followed for 10 years in Paper IV in order to identify possible contribution to PAD development from the AGE/RAGE system. The identification of patients free from signs of PAD was based on clinical examination with palpation of foot pulses and medical history. The clinical examination was aimed at detecting foot at risk, a similar protocol has been validated and shown to predict development of foot complications (Abbott et al. 2002). Freedom from PAD required at least one palpable foot pulse in each foot, no history of major amputation or previous vascular intervention for PAD and no signs of foot ischaemia. Ankle brachial index measurements were not performed at baseline visit.

**Figure 10. Description of the study cohort in paper IV**
(reprinted with permisson from Kärvestedt L, unpublished).
Development of PAD during follow up required loss of palpable foot pulse or vascular intervention for PAD or major amputation due to ischaemia or ankle brachial index <0.9.

All papers used cohort designs. All were prospective, in the sense that inclusion in the cohort was defined first and patients followed from inclusion until an event occurred. **Paper II** used a nested case control design, with the control group consisting of patients inside the cohort without diabetes, whereas **Paper III** was designed as a cohort study with external healthy controls matched on age and gender.

**DATA VALIDITY**

Both Swedvasc and the Swedish National Inpatient register (IPR) have been validated before (Bergqvist et al. 1998; Ludvigsson et al. 2011; Troeng et al. 2008). **Paper II** included validation of all amputation procedures in the cohort found in IPR. We were able to validate all amputations registered in IPR, i.e. there were no false positive major amputations found. Swedvasc data regarding the variables found in table V were validated in a random sample of 140 patients from the study cohort. The accuracy was over 90% for all variables except cardiac disease, hypertension and pulmonary disease (table V). Examining the diabetes variable, we found that two hospitals accounted for 77/146 (53%) of missing values. Having checked the medical records relating to the hospitalisation for the bypass procedure of these 77 patients, 73 were considered not to have diabetes. The remaining 70 (3.8%) records left with missing values for diabetes status in the final cohort were assumed to be individuals without diabetes in analyses.

**Table V. Validation of Swedvasc Registry data**

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes</td>
<td>96.2</td>
<td>98.9</td>
<td>97.9</td>
</tr>
<tr>
<td>Sex</td>
<td>–</td>
<td>–</td>
<td>100.0</td>
</tr>
<tr>
<td>Indication, rest pain vs tissue loss</td>
<td>78.6</td>
<td>92.9</td>
<td>90.0</td>
</tr>
<tr>
<td>Correct date for operation within 7 days</td>
<td>–</td>
<td>–</td>
<td>97.1</td>
</tr>
<tr>
<td>Operated side (right/left)</td>
<td>98.7</td>
<td>98.4</td>
<td>98.6</td>
</tr>
<tr>
<td>Distal anastomosis, popliteal artery</td>
<td>100.0</td>
<td>98.9</td>
<td>99.2</td>
</tr>
<tr>
<td>Distal anastomosis, crural artery</td>
<td>100.0</td>
<td>99.3</td>
<td>99.2</td>
</tr>
<tr>
<td>Distal anastomosis, foot artery</td>
<td>85.7</td>
<td>99.2</td>
<td>99.2</td>
</tr>
<tr>
<td>Vein as graft material</td>
<td>92.6</td>
<td>94.7</td>
<td>92.9</td>
</tr>
<tr>
<td>Cerebrovascular disease</td>
<td>69.6</td>
<td>96.6</td>
<td>92.2</td>
</tr>
<tr>
<td>Cardiac disease</td>
<td>85.5</td>
<td>63.4</td>
<td>75.2</td>
</tr>
<tr>
<td>Hypertension</td>
<td>74.7</td>
<td>74.0</td>
<td>74.5</td>
</tr>
<tr>
<td>Renal insufficiency</td>
<td>93.3</td>
<td>95.2</td>
<td>95.0</td>
</tr>
<tr>
<td>Pulmonary disease</td>
<td>52.9</td>
<td>93.5</td>
<td>88.7</td>
</tr>
<tr>
<td>Smoking</td>
<td>82.8</td>
<td>91.1</td>
<td>93.6</td>
</tr>
</tbody>
</table>

Level of agreement between charts and registry data in a 7.6% random sample, n=140.  
Data are percentage (%). Sensitivity=TPx100/(TP+FN), Specificity=TNx100/(TN+FP).  
Accuracy (percentage agreement)=(TP+TN)x100/(TP+TN+FP+FN).  
TP=true positive, TN=true negative, FP=false positive, FN=false negative. All values calculated with chart data as standard and missing values assumed negative.
The risk for incorrect coding is inherent to all manual data extraction from medical records or other unstructured data sources (Brown 1987). Data in Paper I was not validated in a formal way with regard to incorrect coding. Inappropriate software (Microsoft Excel) was used for data collection due to lack of experience. In the validation study in Paper II and Paper III, EpiData software was used in order to increase internal validity (M 2005). We also performed double-entry of all the data in Paper III to assure that no values were coded incorrect. To avoid information bias, data for the main variable (blood glucose) were extracted separately from risk factors and outcome data, and entered into a separate database.

LABORATORY PROCEDURES

Regarding blood levels of CML, S100A12 and esRAGE there are some general methodological issues such as the use of plasma or serum samples and type of antibodies used. A further problem is the of established reference levels. We consequently used plasma, as this probably is the best estimate of physiological conditions, avoiding alterations caused by activated coagulation and platelet aggregation seen in serum samples (Yu et al. 2011).

Enzyme-Linked Immunosorbent Assays

In Paper III and IV plasma levels of esRAGE, S100A12, CML, and AGE were profiled using commercially available indirect enzyme-linked immunosorbent assay (ELISA) kits, B-Bridge esRAGE ELISA Kit (Yonekura et al. 2003) (Daiichi Fine Chemicals, Takaoka, Japan), CircuLex™ S100A12/EN-RAGE ELISA kit no. CY-8058, CircuLex™ CML/Nc-(carboxymethyl)-lysine ELISA Kit no. CY-8066 (MBL International Corporation, Woburn, USA) and ELISA Kit for Human Advanced Glycation End Product no. E1353Hu (Usn Life Science Inc, Wuhan, China). Measurements were performed following the manufacturer’s instructions. Background activity in the negative control wells was subtracted from the experimental wells in reporting the data. The positive controls were the recombinant protein standards, and the negative controls were the calibrator diluents. 96-wells plates were read on a microplate reader.

The ELISA used is specific to esRAGE and do not recognise sRAGE. This specificity is accomplished by the antibody directed to the N-terminal 16-amino-acid peptide which is unique to the splice variant as described by Koyama et al (2005). There is currently no specific ELISA for the sRAGE subfraction (Kalea et al. 2009).

Immunohistology

Histochemistry of Great Saphenous Vein Sections (Paper III)

Immunohistochemistry was performed for detection of AGE, RAGE, CML and S100A12. Each individual immunohistochemistry included a negative control. In short the procedure was carried out as follows:
Paraffin embedded transverse sections of great saphenous vein on glass-slides were rehydrated. Sections were washed in distilled water and epitopes retrieved by high-pressure boiling in Diva Decloaker. Samples were washed with Tris-buffered saline (TBS) and blocked with background sniper solution. Primary antibodies diluted in DaVinci green solution were applied to the sections and incubated at room temperature for 1 hour. After washing three times in TBS, a MACH3 probe-polymer system containing alkaline phosphatase was applied to the slides and detection was performed using Vulcan Fast Red. Haematoxylin was used for background staining. Samples were dehydrated in ethanol and xylene and then mounted. Nonspecific rabbit and mouse IgG antibodies were used for negative control. For negative Goat control, Da Vinci Green without addition of antibody was used. This method has routinely been used at our lab. In order to exclude the possibility of formation of AGE epitopes during retrieval by high-pressure boiling, we also used alternative methods performed at room temperature for retrieval, showing no difference from our standard method.

The anti-AGE antibody was positively specific for AGE-human albumin serum, AGE-bovine serum albumin, AGE-haemoglobin, AGE-collagen, AGE-lysine-derivatives, AGE-monoamino carboxylic acids and negative for early stages of the Maillard reaction. The anti-CML antibody was positively specific for N’-(carboxymethyl)-lysine (CML). The antibodies for S100A12 and RAGE were specific for humans.

Quantitative image analysis

We used automated quantification of the positively stained vein tissue area in order to minimise bias and maximise accuracy. Images from light microscopy at ×10 magnification of the vein specimens were acquired at constant background illumination with fixed exposure and gain. Fixed white balance setting, obtained from averaging several areas outside the specimens, was used in all images. We used the open-source software CellProfiler version 1.0.9717 for all processing and measurements (Lamprecht et al. 2007). First a pipe-line for processing the images was constructed and calibrated. The discrimination of foreground (tissue, cells or other structures) from background is central in quantitative image processing (Buchser et al. 2004). The aim is to identify objects and then quantify number, areas, shape and other multidimensional parameters. In Paper III the objective was to measure the positively stained area relative to the total tissue area. In brief, the each colour image was separated to three greyscale images representing the red, green and blue channels. In the next step these three greyscale images were combined to two greyscale images by applying different weights to the three images from the original colour image. The first of these two greyscale images was optimised to distinguish tissue from background. This separation was then performed by thresholding with the Mixture of Gaussian (MoG) method, and an outline of the tissue area was produced. This ‘tissue outline’ was used to crop out background from the other greyscale image to get a ‘tissue image’ which then was optimised for discrimination between positive staining and background staining using the Robust background method to find the threshold. Finally, the area of the integrated stained area was divided with the total tissue area to get the proportion of tissue stained. Details can be found at: www.cellprofiler.org/CPmanual/IdentifyPrimaryObjects.html. From every vein specimen, four images were quantified and averaged.
EXPOSURES

Hyperglycaemia

Hyperglycaemia can be estimated in several ways depending on laboratory analysis used, sample methods, sample frequency and nutritional state of the patient (i.e. fasting, random sample or post-glucose challenge).

The estimate chosen depended on the purpose. Diagnosis of diabetes require fasting plasma glucose or OGTT according to WHO definition (2006) or HbA1c according to the American Diabetes Association (2010).

Estimating acute exposure to hyperglycaemia by the area under the curve

There is no standard for estimation of acute exposure to hyperglycaemia, although most studies use the area under the curve (AUC), mean glucose or peak glucose. In Paper I we used AUC excluding the area under fasting level (figure 11) as a measure of exposure to hyperglycaemia over time (Wolever and Jenkins 1986) in order to avoid bias induced by sampling frequency of the blood glucose levels. The AUC was calculated as seen in figure 11 for each patient using Tai’s formula (Tai 1994).

Long term exposure to hyperglycaemia = HbA1c

HbA1c is the common used measure of treatment effect and long-term glycaemic control. All HbA1c measurements in Paper I, III and IV were quality assured by regular calibration with the HPLC Mono-S method, which was the Swedish standard at the time of these studies. Mono-S HbA1c values can be converted to the DCCT standard values using the formula: HbA1c_{DCCT} = 0.923 \times \text{HbA1c}_{\text{lab}} + 1.345, R^2 = 0.998 (Hoedel et al. 2004).

![Figure 11. Exposure to hyperglycaemia. Area under the curve (AUC) method for blood glucose values above 6.1 mmol/l. Exposure to hyperglycaemia is represented by the dark shaded area. The total AUC (dark plus light shaded areas) is used for comparisons between blood glucose levels.](image-url)
The AGE-RAGE system

We calculated a RAGE-score to estimate the compound effect of the AGE-RAGE-system on primary and secondary outcomes. We constructed the RAGE-score by averaging Z-standardized values, (Encyclopedia Britannica 2012) of plasma CML, S100A12 and esRAGE (RAGE-score = [Z-CML+Z-S100A12 – Z-esRAGE]/3) in order to evaluate the combined effect of the AGE/RAGE system. The reason for this was to avoid fitting models with too many variables in relation to sample size and the fact that the interplay between these plasma proteins is not defined. A similar score has been used by others (Nin et al. 2011).

OUTCOME MEASURES IN LOWER LIMB VASCULAR SURGERY

Outcome is predominately reported as the proportion of limbs without amputation (limb salvage) at different time points after the vascular procedure. This is a problematic, and not always relevant, measure in an elderly population with high mortality. It may well be that patients with the highest risk for amputation die shortly after vascular reconstruction, and diabetes per se will increase that risk. Thus, by focusing on the fate of the limb, the fate of the patient will be more or less neglected and is better evaluated by amputation-free survival.

The difference between limb salvage and amputation-free survival

All papers in this thesis used amputation-free survival (AFS) as outcome, and the motives for using AFS are discussed in this section. The choice of outcome measures and definition of endpoints has a profound principal impact on how results from clinical trials are interpreted. There are certain issues related to outcome in infrainguinal bypass surgery (IBS) that merits to be discussed. There are a vast number of clinical studies whose main outcome is measured as limb salvage (or patency) in the vascular surgery literature regarding PAD. The limb salvage concept is problematic in several ways. The most serious problem is related to competing risk. Patients with PAD have a short survival, and about 50% of patients with CLI are dead 4 years after a vascular reconstruction. Limb salvage is in essence a survival variable, i.e. survival of the limb. This can be quantified either as the proportion of (limbs) surviving at a defined time point or as the mean survival time of limbs in the populations studied. Using limbs as the primary unit in which outcome are measured, implies that the fate of the individual at end of follow up is of no interest, both in a strict statistical sense (Prentice et al. 1978), as well as in a clinical perspective. Successful outcome is merely defined as a preserved limb, and the only event of interest is amputation. A limb which has not been amputated at end of follow up will be classified as censored. The problem is that censoring can occur for at least three different reasons. First, a limb can be censored at the end of the planned follow up in the study. Second, a limb can be censored because it is lost to follow up (before the end of study). Third, a limb attached to a dead person will be regarded as censored. A prerequisite for a valid result in survival analysis is that any censoring is non-informative (Breslow 1979; Crowley and Breslow 1984). That is, the
reason for censoring should be completely unrelated to the risk for the outcome studied. In the case of limb salvage, one has to assume that the reason for lost to follow up is unrelated to lower limb problems and that the cause of death is unrelated to lower limb disease, which clearly not is the case. Thus, using limb salvage (or patency) in the CLI setting violates a fundamental prerequisite in survival analysis, and therefore the estimates will not have a meaningful interpretation and serious bias will occur (Peto 1974).

Bedside this major violation, other issues related to precision and discontinuous recording of events makes the standard ‘life-table’ method unsuitable for patency or limb salvage data, as shown by Underwood et al (1984).

Complications to Infringuinal Bypass Surgery

Complications are frequent in IBS and the most common types are related to infections or vascular complications. The 30-day mortality varies between 2–6% and the common cause of death is related to cardiovascular events (Nehler et al. 2003; Nicoloff et al. 1998). In Paper I we used two measures of postoperative complications. Wound complication was a composite of local complications (haematoma, skin necrosis at incisions, delayed healing of incisions and graft infection) and infectious complications (superficial or deep infection with redness, purulent discharge, swelling, antibiotic treatment). The reason for this combined endpoint was that the exact diagnosis of infection is hard to define and establish, and one can question the relevance for the patient in differentiating wound infections from wound complications. The other endpoint was similarly a composite of amputation, death or graft occlusion. Freedom from these events can be looked at as an ideal outcome for the patient (Goshima et al. 2004; Nehler et al. 2003; Nicoloff et al. 1998).

STATISTICAL METHODS

The analytic approach to the data has changed during the work with this thesis, mainly as a result of gained knowledge.

Confounder selection, from data-driven to causal reasoning

In Paper I we used the standard univariate screen, for detection of confounders which were then included in the subsequent multivariate analysis by the forward stepwise likelihood ratio method, i.e. data-driven confounder selection for multivariate analysis.

There are numerous problems using data-driven confounder selection, a method originally developed to construct the best predictive model, but not appropriate for a causal model (Groenwold et al. 2011). As the purpose of Paper I was to gain insight in the causal relationship between hyperglycaemia and outcome, causal reasoning with covariate selection based on subject matter knowledge had been the correct method.

Causal reasoning was used from Paper II and onwards, where the main analysis, evaluating the impact of diabetes on amputation-free survival, included age, sex, severity of limb
ischaemia, level of distal anastomosis, type of graft, pulmonary disease and smoking – factors clearly related to both exposure and outcome but not regarded to be in the causal pathway. Consequently we did not include cardiac disease or renal impairment, as these is regarded intermediate factors. Adjusting for intermediate factors in a causative model bias the estimates and is questionable. Another simple example is illustrated with directed acyclic graphs in figure 12 (Shrier and Platt 2008). The same principles apply to Paper III and IV.

With respect to this, our inclusion of Framingham score in the analysis in Paper IV may be erroneous depending on the causal model used (figure 12). Adjusting for the Framingham score could in fact introduce bias, if some of the factors (e.g. LDL-cholesterol) in the score are mediators of the effect of AGE (Greenland et al. 1999).

**Figure 12.** Illustration of two simple causative models with directed acyclic graphs. In (A) adjustment for the mediators hyperglycaemia and LDL-cholesterol would bias the estimate of AGE on PAD. In (B) adjustment for hyperglycaemia or LDL-cholesterol is necessary if the purpose is to estimate the effect of AGE on PAD.

**Significance testing, from P-values to confidence intervals**

We presented findings in Paper I with the standard dichotomous statistical significance level (p<0.05), a practice which has been increasingly questioned (Gardner and Altman 1986; Stang et al. 2010) since Berkson discussed this in 1942. In the following papers we try to follow the recommendation to report CI instead of P-values to provide information about the extent of associations and differences (i.e. effect size and precision) (Rothman 1978; Sterne and Davey Smith 2001). CIs for proportions and events rates were calculated according to Wilson without continuity correction (Newcombe 1998).

Adjustment for multiple significance testing has not been performed, as this practice has been questioned especially in epidemiology (O’Keefe 2003; Perneger 1998).
RESULTS AND DISCUSSION

PAPER I

The purpose of Paper I (Malmstedt et al. 2006) was to investigate the influence of postoperative hyperglycaemia on outcome in patients with diabetes undergoing IBS. As has been already referred to in the introduction section, the studies from van den Berghe et al on blood glucose control and intensive insulin therapy in critical ill patients had profound impact on postoperative glucose control for patients admitted to intensive care units (Garber et al. 2004; Van den Berghe 2004; van den Berghe et al. 2001). The concept of intensive glucose control with insulin was adopted even outside the patient group studied in the original study (i.e. mainly non-diabetic cardiothoracic surgery patients with postoperative stress hyperglycaemia, all requiring mechanical ventilation). A benefit in term of lower mortality was seen exclusively in patients staying in intensive care units for more than 5 days. Insulin therapy mainly reduced mortality due to sepsis. From this, it was clear that these results were not generalizable to the patient group of interest in this thesis (IBS in patients with diabetes), although the concept was promising for several reasons. First, IBS has a high morbidity, with infectious and vascular being the most common types of complications (Kent et al. 1996; Nam et al. 1999; Nicoloff et al. 1998). Second, postoperative hyperglycaemia is common as up to 50% of IBS patients have diabetes and additional stress from ischaemic and infected tissue (Soderstrom et al. 2009). Third, coexisting coronary heart disease is common and the landmark Diabetes Mellitus, Insulin Glucose Infusion in Acute Myocardial Infarction study (DIGAMI) had proven benefit of glucose lowering using insulin in patients with diabetes and myocardial infarction (Malmberg et al. 1999; Malmberg et al. 1995; Norhammar et al. 1999). Fourth, the proposed mechanisms responsible for the benefit were improved endothelial function (Langouche et al. 2005) and maintained immune function and infectious defence (Van den Berghe 2004).

The clinical reality for our patients with diabetes and IBS was however different, although glucose control was maintained with insulin infusion during surgery and the first 6–12 postoperative hours spent at intensive care units, it quickly declined.

Findings

We found exposure to hyperglycaemia, measured as area under the curve (AUC) for time spent with glucose over 6.1 mmol/L during first 48 hour after IBS, were predictive for both wound complications (table VI) and a general poor outcome (amputation, graft occlusion, death) even after adjusting for confounders (table VII). This result was evident although tight glucose control was aimed for during operation and in the immediate postoperative period.
Table VI Multivariate logistic regression analysis of the impact of perioperative hyperglycaemia (AUC for blood glucose the first 48 hours) on outcome major amputation or death or graft occlusion within 90 days

<table>
<thead>
<tr>
<th></th>
<th>P-value</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lower Upper</td>
</tr>
<tr>
<td>Blood glucose AUC first vs fourth quartile</td>
<td>.007</td>
<td>13.35</td>
<td>2.06 86.70</td>
</tr>
<tr>
<td>Infected foot ulcer at the time of surgery</td>
<td>.048</td>
<td>3.38</td>
<td>1.01 11.26</td>
</tr>
<tr>
<td>Renal insufficiency</td>
<td>.018</td>
<td>4.77</td>
<td>1.30 17.50</td>
</tr>
</tbody>
</table>

OR, odds ratio. CI, confidence interval. *P*-values were determined with the use of the Wald-test.

Table VII Multivariate logistic regression analysis of the impact of perioperative hyperglycaemia (AUC for blood glucose the first 48 hours) on outcome wound complication

<table>
<thead>
<tr>
<th></th>
<th>P-value</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lower Upper</td>
</tr>
<tr>
<td>Blood glucose AUC first vs fourth quartile</td>
<td>.001</td>
<td>14.45</td>
<td>2.86 73.12</td>
</tr>
<tr>
<td>Female gender</td>
<td>.032</td>
<td>3.49</td>
<td>1.11 10.94</td>
</tr>
<tr>
<td>Tissue loss as indication †</td>
<td>.059</td>
<td>3.30</td>
<td>.95 11.43</td>
</tr>
</tbody>
</table>

OR, odds ratio. CI, confidence interval. † P-values were determined with the use of the Wald-test.

In addition to the main results, we confirmed the clinical impression of poor glucose control among these patients, and with an AUC glucose representing a mean glucose level of 9.3 mmol/L, 2.1 (SD) the first two postoperative days (figure 13). Furthermore, the high complication rate described earlier was evident also in our study with half of the patients having a wound complication and one third having a general poor outcome at three months.

The main limitation of our study is the possibility of the reverse causal relationship, i.e. perioperative hyperglycaemia may be just a marker for poor long-term metabolic control in the patients before surgery, which may increase the risk for poor outcome.

Discussion – what this study adds and recent updates on postoperative glucose control

The main outcome variable in the present study, in addition to wound complications, was an ideal outcome for the patient, i.e. survival with an intact limb and patent graft. No previous study has addressed this endpoint for patients with diabetes. Vriesendorp et al (Vriesendorp et al. 2004) showed that poor glucose control was associated with an increased risk of postoperative infections in vascular surgical patients. Their results were based on mean glucose values during the first 48 h in patients with and without diabetes undergoing various infrainguinal procedures. We had the opportunity to avoid sampling bias in our study by calculation AUC for glucose exposure, which gives a more precise estimate of exposure to hyperglycaemia (Finney et al. 2003; Tai 1994; Wolever 2004). Our study is also the first
to exclusively include patients with diabetes and only primary infrainguinal bypass procedures providing a somewhat less heterogeneous cohort. Therefore we think the conclusions drawn can be justified in this group of patients.

Two meta-analysis of several randomised controlled trials (RCT) in patients admitted to surgical and mixed surgical-medical intensive care units could not confirm the positive effect on postoperative mortality found in the initial study by van den Berghe, but showed decreased frequency of septicemia, although to the cost of a five to six fold increase in hypoglycaemia (Griesdale et al. 2009; Wiener et al. 2008).

The only RCT in patients undergoing vascular surgery, comparing intensive glucose control with continuous insulin infusion (CII) vs subcutaneous bolus insulin first 48 h after surgery for abdominal aortic aneurysm (n=58), leg bypass (n=173) or amputation (n=5) in 242 patients, of whom 126 had diabetes, was published 2009 by Subramaniam et al. The CII group achieved better average glucose control 12–24 h postoperatively, although CII was associated with a greater number of hypoglycaemic periods in patients with diabetes. The CII group had lower risk for postoperative myocardial infarction or congestive heart failure (4 vs 15 patients), relative risk 0.29 (95% CI, 0.10 – 0.83), without having hypoglycaemia more frequently. The glucose lowering effect from CII was more pronounced in patients without diabetes (Subramaniam et al. 2009). However, the effect on mortality was not possible to address in this RCT as no patients died during the 30-day follow-up. Moreover, despite a sufficient number of events, no effects on wound and infectious complications were seen (Subramaniam et al. 2009). This study is presently the best evidence for some beneficial effects of postoperative tight glucose control even in non-critical ill patients undergoing vascular surgery, and is in line with our and Vriensornorphs findings (Malmstedt et al. 2006; Vriesendorp et al. 2004). The reason for the discrepancy between the fairly strong observational evidence for harmful effects of hyperglycaemia and the negative result from interventional trials aimed at lowering glucose, may be that other factors related to hyperglycaemia such as glucose variability is more important than mean glucose levels and that insulin infusion have negative consequences which outweigh the benefit from glucose lowering. In fact, the risk for myocardial infarction and death associated with rapid lowering of blood glucose by insulin was observed in 1930 (Blotner 1930).

![Figure 13.](image-url)

**Figure 13. Distribution of patients with wound infection or wound complications among quartiles for glucose exposure (area under the curve).** **P<0.001 and *P<0.043 versus lowest quartile** (Wald statistic, multiple logistic regression). Q1-Q4, quartiles with boundaries.
PAPER II

The purpose of this paper was to establish a population based estimate of amputation-free survival after infrainguinal bypass surgery for critical limb ischaemia (CLI) in patients with diabetes and compare this with the result in similar patients without diabetes. As already outlined in the introduction, IBS is regarded as an important treatment of CLI especially in patients with diabetes, but unbiased results from population based samples regarding AFS are scarce (Hinchliffe et al. 2012). The evidence quantifying efficacy of revascularisation in patients with diabetic foot ulcers and PAD is mostly based on single centre series from expert centres using different classification of both extent of PAD, wound severity and outcome (Hinchliffe et al. 2012) which makes results likely to be biased or difficult to generalise.

The particular question asked in Paper II, if results from revascularisation in patients with diabetes is comparable to those in patients without diabetes, has been answered with both yes and no depending on the following circumstances. First, in which era the answer was given. Second, the definition of outcome measure used to determine the results. Third on the characteristics of the groups compared, with regard to age, comorbidities, risk factors and degree of ischaemia and ulcer severity.

In the early period of IBS, the patient with diabetes and CLI was looked upon with a pessimistic attitude, influenced by the emphasis on microvascular or distal small vessel disease in diabetes, not amenable to bypass surgery. This view was successfully changed by the work from Boston Medical Deaconess center, by demonstrating that pedal outflow vessels in fact were present and could be revascularised (LoGerfo and Coffman 1984; Pomposelli et al. 1990; Pomposelli et al. 2003; Stonebridge et al. 1991).

As mentioned earlier, the most commonly used outcome measures in IBS has been limb salvage and patency, in addition to the standard 30-day mortality. Most studies and reviews state non-inferior results in patients with diabetes when these are used (Akbari et al. 2000; Karlstrom and Bergqvist 1997; Panneton et al. 2000; Weiss and Sumpio 2006; Virkkunen et al. 2004; Wolfe et al. 2003) and others have found results to be worse (Al-Omran et al. 2003; Feinglass et al. 2001; Goshima et al. 2004; Hallett et al. 1997; Taylor et al. 2006) in patients using different outcome measures.

Bearing in mind the generally elevated risk for amputation and mortality in patients with diabetes, we postulated that diabetes decreases the amputation-free survival after leg bypass surgery for critical ischaemia.

Findings

We performed a nationwide, population-based cohort study and compared postoperative amputation-free survival in patients with and without diabetes undergoing IBS for CLI, the majority of them having tissue loss (82%) (Malmstedt et al. 2008).

The analysis included data for 1 840 patients from the Swedish Vascular Registry who, during 2001–2003, underwent their first unilateral, below-knee, infrainguinal distal bypass
procedure for CLI. Of these patients, 742 had diabetes and 1 098 did not. Patients were followed up until the end of 2005.

Overall, 446 patients with and 558 without diabetes had undergone ipsilateral amputation or died by the end of the follow-up period. Patients with diabetes had a shorter amputation-free survival than patients without diabetes (2.3 years, 95% CI 1.9–2.8 years vs 3.4 years, 95% CI 3.1–3.7 years). The rate of ipsilateral amputation or death was higher in patients with diabetes, 30 per 100 person-years, than in patients without, 22 per 100 person-years (table VIII). Almost half of the patients with diabetes and a third of those without had lost their leg or were deceased two years after index procedure (figure 14).

![Figure 14. Kaplan-Meier curves of amputation-free survival after leg bypass for critical limb ischaemia in patients with and without diabetes. Vertical bars show 95% confidence intervals at selected time points.](image)

The crude and age-adjusted hazard ratios (HR) for ipsilateral amputation or death in patients with diabetes were 1.32, 95% CI 1.17–1.59 and 1.55, 95% CI 1.37–1.77 (figure 15). Adjustment for sex, smoking, pulmonary disease, degree of limb ischaemia, type of graft and level of distal anastomosis did not substantially affect this finding, HR 1.46, 95% CI 1.26–1.69 (figure 15).

Several other factors in the causal pathway to diabetes complications were also related to ipsilateral amputation and death, including renal disease, cardiac disease, hypertension and cerebrovascular disease. Additional adjustment for these factors did not substantially influence the effect of diabetes, HR 1.36, 95% CI 1.39–1.56 (figure 16). The effect of diabetes on amputation-free survival was more pronounced in male patients, HR \(_{age\text{-adj}}\) 1.75, 95% CI 1.47–2.08 than in female HR \(_{age\text{-adj}}\) 1.35, 95% CI 1.11–1.64. Analysis of secondary endpoints showed consistently higher risk for amputation or death in patients with diabetes. Diabetes was associated with a higher risk for amputation of any leg in analysis of amputation-free survival including both ipsi- and contralateral amputations, HR \(_{age\text{-adj}}\) 1.57, 95% CI 1.39–1.78 (figure 15). Diabetes was also associated with a higher risk of death, HR \(_{age\text{-end}}\) 1.49, 95% CI 1.31–1.71 (figure 15).
Figure 15. Hazard ratios (Cox proportional hazards models) for outcomes (amputation, amputation or death, death, ipsilateral amputation, and ipsilateral amputation or death) after leg bypass surgery in patients with diabetes compared to patients without diabetes. Filled squares denote crude hazard ratios and unfilled adjusted. Confounders are age, sex, smoking, pulmonary disease, degree of limb ischaemia, type of graft and level for distal anastomosis. CI, confidence interval. CL, confidence limit. Amputation includes contralateral or ipsilateral major amputations.

<table>
<thead>
<tr>
<th>Covariate</th>
<th>Hazard ratio</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes (yes vs. no diabetes)</td>
<td>1.36</td>
<td>1.10–1.68</td>
</tr>
<tr>
<td>Confounders:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (per year)</td>
<td>1.04</td>
<td>1.03–1.05</td>
</tr>
<tr>
<td>Sex (female vs. male)</td>
<td>3.67</td>
<td>0.26–1.00</td>
</tr>
<tr>
<td>Degree of limb ischaemia (mild vs. moderate)</td>
<td>1.42</td>
<td>1.21–1.66</td>
</tr>
<tr>
<td>Distal anastomosis poplite artery (yes)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Coronary artery</td>
<td>1.08</td>
<td>1.13–1.47</td>
</tr>
<tr>
<td>Foot artery</td>
<td>1.04</td>
<td>0.74–1.46</td>
</tr>
<tr>
<td>Graft material other than autogenous vein</td>
<td>1.20</td>
<td>1.11–1.31</td>
</tr>
<tr>
<td>Pulmonary disease</td>
<td>1.31</td>
<td>1.08–1.57</td>
</tr>
<tr>
<td>Smoking</td>
<td>1.07</td>
<td>0.91–1.24</td>
</tr>
<tr>
<td>Intermediate factors:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>3.87</td>
<td>0.77–1.60</td>
</tr>
<tr>
<td>Renal insufficiency</td>
<td>2.61</td>
<td>1.70–2.34</td>
</tr>
<tr>
<td>Cerebrovascular disease</td>
<td>1.20</td>
<td>1.03–1.40</td>
</tr>
<tr>
<td>Cardiovascular disease</td>
<td>1.26</td>
<td>1.13–1.45</td>
</tr>
</tbody>
</table>

Figure 16. Full Cox proportional hazards model of time to ipsilateral amputation or death. CI, confidence interval. CL, confidence limit.
**Table VIII.** Crude incidence rates for events after leg bypass surgery in patients with and without diabetes

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Diabetes (n=742)</th>
<th>No diabetes (n=1098)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Rate* (95% CI)</td>
</tr>
<tr>
<td>Ipsilateral amputation or death</td>
<td>446</td>
<td>30.2 (26.6–34.2)</td>
</tr>
<tr>
<td>Ipsilateral amputation</td>
<td>156</td>
<td>10.6 (8.5–13.2)</td>
</tr>
<tr>
<td>All-cause mortality</td>
<td>486</td>
<td>27.7 (24.1–31.6)</td>
</tr>
<tr>
<td>Any amputation or death</td>
<td>463</td>
<td>32.5 (28.8–36.8)</td>
</tr>
<tr>
<td>Any amputation</td>
<td>192</td>
<td>13.5 (11.0–16.5)</td>
</tr>
</tbody>
</table>

*Rate is per 100 person years. CI, confidence interval.

**Discussion**

We observed that diabetes was associated with a 55% increase in the risk for major amputation or death after leg bypass surgery for critical lower limb ischaemia compared with patients without diabetes. This is the first nationwide population-based study of amputation-free survival after leg vascular reconstruction for CLI comparing patients with and without diabetes. We believe that our results are likely to be a proper estimate of amputation-free survival after leg bypass for the following reasons. First, we used nationwide data with complete coverage for identification of the cohort, thereby minimising selection bias. Referral and diagnostic bias are also almost completely avoided in a setting with a national health care service with free access to health care. Second, the diabetes diagnosis was found to have a sensitivity of 96% and a specificity of 99%. Third, we were able to achieve complete and accurate follow-up of amputation status and death. This was possible by record linkage to the nation-wide hospital patient and cause of death registry using high quality data covering the whole population in Sweden. Moreover, our cohort consisted exclusively of patients with critical ischaemia undergoing distal bypass surgery, thus avoiding confounding due to the inclusion of patients with less severe degree of ischaemia or more proximal procedures.

Our results seem plausible considering the substantial body of evidence of increased cardiovascular risk (Huxley et al. 2006) and markedly elevated risk for amputation (GLEASG 2000) in patients with diabetes. The difference between our results and those obtained from numerous case series (Weiss and Sumpio 2006) showing equal outcome in patients with and without diabetes is most probably explained by different outcome measures, i.e. limb salvage instead of amputation-free survival, by inclusion of patients with less severe degree of ischaemia and younger patients.

We found only one population-based study from Finland aiming at investigating outcome after vascular procedures for CLI in patients with and without diabetes (Virkkunen et al. 2004). This study also found higher amputation rates in patients with diabetes, but patients were followed only for 30 days.

Due to the scarce use of amputation-free survival as outcome measure in contemporary case series and trials, we found it difficult to compare our results with those from these
studies. The short amputation-free survival in our study is in accordance with the over-all amputation-free survival of the Bypass vs Angioplasty in Severe Ischaemia of the Leg (BASIL) trial (55% at 3 years) and in The Veterans Affairs National Surgical Quality Improvement Program (57% at 3 years), which recruited participants comparable to our cohort in terms of age distribution and proportion of patients with diabetes (Adam et al. 2005; Feinglass et al. 2001). The largest RCT including 1404 patients with infrainguinal reversed vein bypass for CLI, the PREVENT III trial, reported an overall 1 year AFS of 75% (354 events in 1404 patients) (Conte et al. 2006; Schanzer et al. 2008). AFS was not stated for patients with diabetes in the total cohort, but in the derivation set (n=953) used for development of the PIllie-score, patients with diabetes had a lower estimate of AFS (73%) than without diabetes (82%), equal to an 57% increase in risk for amputation or death, HR 1.57, 95% CI 1.17 – 3.00 (Schanzer et al. 2008). Furthermore, the group from Helsinki university hospital recently published results from 1425 patients with CLI undergoing either IBS or endovascular procedures, with a AFS of 67% at 1 year for all patients (Arve et al. 2010), which is almost exactly equal to our estimates (62% and 72% at 1 year for those with diabetes and without respectively). Both these studies included patients with similar baseline characteristics as our study. Approximately 80% had tissue loss, 60% diabetes and frequent cardiovascular comorbidities. There was a slight difference in age, with 73 years in the Helsinki study, 68 years in PREVENT III and 76 years in our study, which probably explains the higher AFS in PREVENT III.

Our results indicate that the impact of diabetes on amputation-free survival was more pronounced in male than in female patients. Other studies support this finding regarding PAD outcome (Al-Omran et al. 2003), amputations (van Houtum et al. 1996) and cardiovascular disease in general (Booth et al. 2006). Biological differences, with delayed onset of cardiovascular disease in women, may perhaps explain this effect. However, results from outcome studies on coronary bypass surgery suggest female gender to be associated with poor outcome (Richison et al. 2007). According to a recent population-based study on PAD in Sweden, the prevalence of CLI is higher among women (Sigvats et al. 2007). This higher prevalence is not reflected in our cohort suggesting a relative under-use of revascularisation in women. If true, a selection bias of healthier women for revascularisation could explain our observed better outcome in women.

There are limitations to this study that merit discussion. The observational design might have permitted uncontrolled confounding factors to affect the results. A second limitation involves our definition of diabetes, which is based on clinical overt disease reported to Swedvasc, and therefore did not identify people with undiagnosed diabetes. The risks for PAD (Gregg et al. 2007), amputation (Treweek et al. 1998) and death (Wild et al. 2005) are elevated in patients with undiagnosed diabetes, thus the possible misclassification of exposure would lead to falsely high estimates of amputation and death in our reference group without diabetes, biasing our results towards the null hypothesis. Lastly, we were unable to distinguish between type 1 and type 2 diabetes. As the risk for amputation (Chaturvedi et al. 2001) and the risk for cardiovascular death (Morrish et al. 2001) are approximately equal in type 1 and 2 diabetes, this limitation has probably not affected our results.
An additional explanation of our findings is the high proportion of tissue loss in patients with diabetes and lower limb ischaemia, which may indicate an undue delay in recognition of ischaemia (Apelqvist 2012). This delay is probably explained by masking of ischaemic symptoms from concomitant peripheral neuropathy (ADA 2003).

Our findings might have consequences for research and care of patients. Patients with diabetes presenting with limb ischaemia should be treated as having very high mortality risk. They require intense treatment of cardiovascular risk factors at earliest possible stage and revascularisation should probably be considered early, preferably before frank gangrene develops (Apelqvist and Lepantalo 2012; Lepantalo et al. 2011). In summary, diabetes diminishes the chances of survival and avoiding amputation in patients with critical lower limb ischaemia undergoing leg bypass procedures. Further studies in this group of patients should be directed to find ways to reduce this excess risk and a new approach to this group of patients has recently been proposed (Lepantalo et al. 2011).
PAPER III

The purpose of this study was to investigate the role of the AGE-RAGE system in patients with PAD undergoing IBS. We measured components of the AGE-RAGE system in plasma and vein tissue and compared levels in patients with and without diabetes undergoing IBS. We also investigated if these levels were associated with outcome after IBS. Our original hypothesis postulated that patients with diabetes would exhibit higher levels of AGEs and S100A12 in plasma and vein, low plasma levels of plasma esRAGE, and high expression of RAGE in vein used for bypass. If so, this may be part of the explanation why patients with diabetes have worse outcome after IBS compared to non-diabetics. Activity in the AGE-RAGE system can have both systemic effects with increased mortality by accelerating vascular dysfunction (Tan et al. 2002) and local effects rendering venous grafts susceptible to increased intimal hyperplasia formation (Chello et al. 2009; Choi et al. 2005; Park et al. 2011) and thrombosis (Enomoto et al. 2006; Hasegawa et al. 2002).

Findings

The results consist of three main findings. First, we found an association between elevated S100A12 plasma levels and reduced AFS after IBS. Second, patients with and without diabetes had higher plasma levels of S100A12 and CML compared to healthy controls. Third, we demonstrated expression of AGE, CML, S100A12 and RAGE in vein used for bypass graft.

This study included thirty-eight patients with and 30 without diabetes. Almost one third of patients with previously unknown glucose homeostasis disturbance had a pathological OGTT or HbA1c resulting in a total prevalence for impaired glucose homeostasis or diabetes of 62% in this group of patients. Mean age was close to 70 years and patients with diabetes had the diagnosis for at least 15 years. Patients with diabetes had more severe limb ischaemia as reflected by higher proportions of tissue loss (79%), distal anastomosis placed below the popliteal artery (63%) and low runoff score (80%) as compared to non-diabetic subjects. There was a higher proportion of smokers among non-diabetic patients (42%).

Amputation Free Survival

Forty-six patients survived with intact leg during follow up and one patient was lost to follow up due to migration. Seventeen died, 11 with and 6 without diabetes. Median survival time was 702 days (interquartile range, 188 – 899). Six patients with diabetes were amputated.

Having diabetes was associated with shorter AFS in crude analysis (figure 17), but this association was lost after adjustment for age, sex and tissue loss (figure 17).

Patients with high plasma levels of S100A12 were three times more likely to die or undergo amputation, comparing patients above (8.6 ng/mL) with patients below the 75th percentile S100A12 (figure 18). The increased risk was essentially unchanged after adjustment for
age, sex and diabetes. Adjustment for tissue loss yielded similar estimates (figure 17). Half of the patients in the 75th percentile of plasma S100A12 had died or lost their leg after 30 months, compared to 18% in the lower percentiles (figure 18).

A model with AGE/RAGE-score indicated a composite effect on AFS which after adjustment for age, sex and diabetes increased the estimate but yielded a wider confidence interval (figure 17). Other plasma markers of the AGE/RAGE system were not associated with the primary outcome in Univariate Cox regression (data not shown).

![Figure 17](image1.png)

**Figure 17.** Forest plot with estimated hazard ratios (Cox regression) for amputation-free survival in relation to diabetes, S100A12 and RAGE-score. Reference categories are: Without diabetes, RAGE-score per 1 standard deviation increase and S100A12 below 75th percentile in respective model. DM, diabetes mellitus. CI, confidence limit.

![Figure 18](image2.png)

**Figure 18.** Kaplan-Meyer estimate for cumulative probability of survival without major amputation in relation to plasma S100A12 levels. Groups are plasma S100A12 above (blue line) and below (green line) the 75th percentile (8.6 ng/mL).
Plasma levels of AGE-RAGE components in relation to diabetes and compared to healthy controls.

No differences in plasma AGE-RAGE components were found comparing patients with and without diabetes, with the exception of S100A12 which was doubled in patients with diabetes: 11.98 vs 5.01 ng/mL (figure 19).

![Figure 19. Plasma levels of esRAGE, CML and S100A12 in relation to diabetes status. Forest plot of mean with 95% CI for plasma concentrations in relation to diabetes status. CL, confidence limit.]

These findings lead us to compare plasma levels of CML, esRAGE and S100A12 in our patients with levels in age- and sex-matched healthy subjects without diabetes or CVD. Both CML and S100A12 were higher in patients whereas esRAGE levels did not differ between patients and controls (figure 20).

![Figure 20. Comparison between patients and healthy controls of plasma levels of esRAGE, CML and S100A12. Forest plot of mean with 95% CI for plasma concentrations in 68 patients and healthy controls. CL, confidence limit]
**Results and Discussion**

**Plasma Levels of esRAGE, AGE, CML and S100A12 versus Degree of Lower Limb Ischaemia.**

S100A12 was higher in patients with more severe ischaemia (figure 21). Plasma AGE and CML did not differ between ischaemic grades. There was a tendency to higher esRAGE levels in patients with tissue loss 0.43 (CI, 0.28 – 0.59) versus 0.29 (CI, 0.22 – 0.36) ng/ml.

![Figure 21. Plasma S100A12 in controls, patients with and without diabetes, and in relation to tissue loss, outflow and runoff score. Data points are mean plasma S100A12 and error bars are 95% CI for mean. Mann-Whitney U nonparametric 2 sided test.](image)

**AGE-RAGE in vein tissue**

There was a low variability in proportion of vein tissue stained for AGE, CML RAGE an S100A12, all these were found consistently among both patients with and without diabetes (see figures in Paper III) and were not influenced by smoking.

**Discussion**

Our study extends the results from previous animal and in vitro studies proposing a role for AGE-RAGE in intimal hyperplasia, where we demonstrate the presence of AGE-RAGE components in human saphenous vein tissue for the first time in man. Several animal studies have demonstrated evidence for a role of AGE-RAGE interaction in promoting intimal hyperplasia in response to arterial injury (Sakaguchi et al. 2003; Yu et al. 2012; Zhou et al. 2003) and recently also in an animal model of vein graft hyperplasia (Li et al. 2012). Chello and co-workers (2009) cultured human venous endothelial cells with AGE and found increased expression of RAGE and increased ROS formation. Our finding of expression of AGE-RAGE components in both patients with and without diabetes indicates a role for this system even in patients with PAD but without diabetes. RAGE expression was elevated in
arterial tissue obtained from autopsy of patients with PAD (Park et al. 1998), Lund et al (2011) recently found AGEs in aortic tissue from patients with and without diabetes.

Our finding of an association between AFS and elevated plasma levels of AGE-RAGE components is in agreement with other observational studies linking elevated plasma or serum levels of AGEs to in-stent restenosis after PTCA (Basta et al. 2008; Choi et al. 2005; McNair et al. 2010; Park et al. 2011). We did not have the opportunity to monitor development of intimal hyperplasia in our patients, which could provide a more direct link to activation of the AGE-RAGE system, especially if examination of grafts excised during revision for intimal hyperplasia had been available. These aspects need to be further studied in order to elucidate the temporal relationship between RAGE activation and IH, and to estimate the importance of RAGE-activation relative to other established factors for IH in humans such as sheer stress. It is also noteworthy that endothelial cells from arteries and veins have different embryonic origin and differ in function and response to injury (Eriksson et al. 2005; Lawson and Weinstein 2002; Szasz et al. 2007).

Thus, interpreted together with previous studies proposing a role of the AGE-RAGE system in intimal hyperplasia, progression of atherosclerosis and thrombosis, we propose that our findings may in part explain poor outcome after IBS in patients with diabetes.

A growing body of evidence has shown that the AGE-RAGE system has several adverse effects on the cardiovascular system in general, (Koyama et al. 2005; Mahajan et al. 2009; Ramasamy et al. 2012; Yamagishi 2011), e.g. in progression of atherosclerosis (Del Turco and Basta 2012) and by contributing to a prothrombotic state (Cai et al. 2006; Enomoto et al. 2006; Gawłowski et al. 2009; Zhu et al. 2012), which may underlie the association between decreased survival and AGE-RAGE components in our patients.

There are several limitations accompanying clinical research regarding AGE-RAGE. The lack of standardised methods for determination of AGEs in blood and tissue, and limited knowledge on which specific AGEs are clinically most relevant in humans makes comparison difficult. Furthermore, the complex interplays between AGEs, their different receptors and the regulation of pathways involved in formation and elimination of AGEs further complicates our understanding of this field. All these general limitations in this relatively new research field apply to our study. We used primarily the best characterised AGE – CML, but this may be a too limited marker of AGEs. However, the reliability of the ELISAs for CML and esRAGE have been validated (Sakurai et al. 2006; Takeuchi et al. 1999), in contrast to the ELISA employed for detection of a broader range of AGEs. Furthermore, we used a RAGE-score in order to estimate an integrated effect of the AGE-RAGE system.

The major limitations specific to this study revolve around the heterogeneity in our patient cohort and the multiple variables that may influence survival and amputation in this patient group. Especially, the inclusion of patients having IBS for claudication is problematic since both survival and amputation are less likely in this group. We tried to take this into account by adjusting the Cox model for the degree of PAD. Furthermore, due to the sample size we had to limit our multivariable analysis in order to avoid distorted estimates. We did not perform interaction analysis for the same reasons.
Results and Discussion

We used a simplified version of the *ad hoc* runoff score (Rutherford et al. 1997), giving each crural vessel 0, 0.5 and 1 points for occluded, stenosed and completely open vessels respectively. Low runoff scores were correlated both with amputation and survival. This is in agreement with earlier results from the Helsinki group showing that a score taking into account the entire length of the crural arteries is superior in predicting survival in IBS patients, in contrast to the *ad hoc* score using only the segment distal to the planned anastomosis (Alback et al. 2000; Biancari et al. 1999). Our scoring did not include the status of pedal vessels and we did not apply the resistance weighting as this was considered of limited extra value.

There are no direct clinical implications of **Paper III**, but further studies of the AGE-RAGE concept seem to be warranted given the proposed role of AGE-RAGE in several mechanisms important for IBS and our demonstration of the presence of AGE-RAGE components both in vein tissue and plasma in IBS patients.
PAPER IV

The primary purpose of Paper IV was to investigate the following two parameters in patients with type 2 diabetes: first, are plasma levels of the AGE-RAGE system associated with development of PAD? Second, to estimate 10-year AFS in a population based sample of relatively healthy patients without PAD at baseline. Population-based estimates of AFS and incidence of PAD for type 2 diabetes patients are scarce. The role of the AGE-RAGE system in development of PAD has not been studied previously, except for cross sectional studies in selected populations of type 1 diabetes and ESRD patients reporting an association with esRAGE (Catalano et al. 2008) and S100A12 (Shiotsu et al. 2011).

Findings

We found plasma levels of S100A12 and RAGE-score being associated with AFS and development of PAD, in 146 patients with type 2 diabetes, during a 10-year follow up period (figure 22 and 23). These findings were not influenced by age, sex or glycaemic control (figure 22 and 23). Including Framingham 10-year CVD-risk score attenuated the association (figure 22 and 23).

![Figure 22. Estimated hazard ratios (Cox regression) for amputation-free survival in relation to RAGE system components. HR for S100A12 is per 100 ng/mL and for RAGE-score per 1 unit (standard deviation). TG, plasma triglycerides. HbA1c, glycosylated haemoglobin. Framingham, Framingham 10-years CVD risk score.](image-url)

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**Results and Discussion**

![Figure 23. Estimated hazard ratios (Cox regression) for PAD free survival in relation to RAGE system components. HR for S100A12 is per 100 ng/mL and for RAGE-score per 1 unit (standard deviation). TG, plasma triglycerides. HbA1c, glycated haemoglobin. Framingham, Framingham 10-years CVD risk score.](image)

The incidence rate for amputation-free survival was 2.8 per 100 person-years, (95% CI 2.0 to 3.7). The incidence rate for PAD free survival was 3.6 per 100 person-years, (95% CI 2.7 to 4.8). The incidence for PAD (censoring for death) was 16 per 1000 person-years (95% CI, 10 to 24). The mean Framingham 10-year risk for CVD was 37%, (95% CI, 33 to 40%).

The mean duration of diabetes at baseline was 7.1 years, 95% CI 6.2 – 8.0 and 27%, 95% CI 21 – 35% had insulin treatment. The majority was relatively well controlled with respect to HbA1c, 6.4%, 95% CI, 6.2 – 6.6% (MonoS). Two thirds had peripheral neuropathy, one third retinopathy and 8.5 % nephropathy at baseline. Hypertension and body mass index above normal were common. Thirteen patients had undergone CABG and four had stroke prior to inclusion. Further details regarding baseline characteristics are found in the complete manuscript for **Paper IV**.

Univariate correlations between AGE-RAGE components and other variables were rare and weak (0.18 < R < 0.31) when found. None of the AGE-RAGE components were correlated with measures of glycaemia.
Discussion

The main importance of our results showing S100A12 and RAGE-score to be associated with AFS and PFS, combined with aforementioned mechanistic and clinical studies, is that the RAGE-system likely has an important role in PAD patients, and that the associations may represent causality. Our study is the first to investigate S100A12 in relation to outcome in a population based sample of type 2 diabetes patients. One previous study has shown that S100A12 is associated with mortality in ESRD patients in haemodialysis (Nakashima et al. 2010). Nin et al recently reported higher levels of CML being associated with incident fatal and nonfatal CVD as well as all-cause mortality in individuals with diabetes type 1 during long-term follow up (Nin et al. 2011). The reason why we were unable to confirm this in our cohort with type 2 diabetes may be that our patients were younger with relatively short diabetes duration, hence a longer follow up may be needed as CML in arterial tissue has been shown to increase with age and in diabetes (Schleicher et al. 1997). A second reason could be difference in study subjects, ours being representative for type 2 diabetes whereas those in the study by Nin et al were a selected (type 1 diabetes with haemodialysis) group (Nin et al. 2011).

There is scarce data on incidence rates for PAD based on population samples with low bias. Criteria used, length and intensity of follow up, and diabetes duration influences the estimates of PAD incidence. Our estimate for incident PAD (16 per 1000 person-years) is close to a similar study from Finland reporting new PAD in 21 of 107 patients followed for 11 years (i.e. = 18 per 1000 person-years) and the ARIC study with 14 per 1000 person-years (Kallio et al. 2003; Watanakkit et al. 2005). The UKPDS trial estimate for incident PAD during the first 6 years after diabetes diagnosis was 5 per 1000 person-years. This lower incidence can be explained by younger age and shorter diabetes duration (Adler et al. 2002).

The baseline characteristics of our study cohort are comparable to population data from the National Diabetes Register (Cederholm et al. 2008), further reinforcing that this cohort is a representative population based sample with appropriate external validity.

Inclusion restricted to patients with type 2 diabetes and not Latent Autoimmune Diabetes in the Adult or type 1 diabetes is also a strength. We believe that our results are valid and conclusions justified regarding the associations of S100A12 and RAGE components with amputation/PAD free survival, even if adjusted HR fall outside the commonly (mis)used significance limits (i.e. P<.05) (Stang et al. 2010). We report point estimates with confidence intervals in order to give a more precise interpretation of the study results regarding both effect size and precision.

An excess of the traditional risk factors for atherosclerosis in subjects with diabetes do not explain the increased risk for PAD or CVD compared to subjects without diabetes (Hanley et al. 2002; Sarwar et al. 2010). Prediction scores for development of cardiovascular disease are often derived from a combination of traditional risk factors, e.g. age, sex, smoking, lipid profile, diabetes and hypertension (D’Agostino et al. 2008; Wilson et al. 1998), with addition of diabetes specific variables, e.g. HbA1c and diabetes duration, in scores designed for diabetes populations (Cederholm et al. 2008; Elley et al. 2010; Hippisley-Cox et al. 2008; Stevens et al. 2001). We used the Framingham 10-year CVD-risk score to adjust for
the effect of established risk factors. The reason for using this score was that it is the only validated score estimating cardiovascular risk including PAD events (D’Agostino et al. 2008). Furthermore, the Framingham equations include the direct data on HDL and LDL measurements, instead of other indirect non-laboratory variables. We were also able to adjust for baseline HbA$_1c$, in addition to Framingham score, as scores specific to diabetes type 2 have shown HbA$_1c$ to be an important predictor of CVD and glycaemic control is likely to be an important causative factor (see introduction). High triglycerides (TG) and low HDL-cholesterol have been shown to be associated with CVD especially in diabetes, but there is still debate about to which extent and if there is a causative relationship or if TG and HDL merely serve as risk markers (Chapman et al. 2011; Rader and Tall 2012). Including TG in our regression models did not change the estimated effect of S100A12 and RAGE-score, although TG showed an association in univariate analysis (data not shown). HDL cholesterol is included in the Framingham risk score among LDL cholesterol. Including the Framingham score in our multivariate model may be erroneous as the AGE-RAGE system can be regarded as a very early event in the causal pathway to atherosclerosis preceding some of the elements in the score such as elevated lipids and hypertension (Vlassara and Striker 2011).

We used pre specified adjustment for possible confounders based on subject matter knowledge regarding causal factors for PAD instead of statistically driven covariate selection, i.e. ‘univariate screen’ (Groenwold et al. 2011).

The primary limitations of this study is the low incidence of events and wide confidence intervals in the measured plasma components making findings liable to partly be explained by confounding factors and measurement errors.

It should also be noted that the exclusion of PAD diagnosis at entry was based on foot examination including pulse palpation and medical history. Hence, there is a risk for bias by misclassification of subjects with a palpable foot pulse and ankle brachial index <0.9 as free from PAD. The contrary, i.e. excluding subjects without PAD due to false negative foot pulse palpation is less problematic as only two patients of 136 were diagnosed with PAD solely due to non-palpable foot pulse. Similar arguments can be made regarding the definition of development of PAD during follow up, although only 6 patients were diagnosed with PAD solely by loss of foot pulse. Furthermore, evaluation of foot pulses and medical history have been shown to have >90% sensitivity to detect PAD (Boyko et al. 1997).
GENERAL DISCUSSION

This thesis evolved from concerns regarding patients with diabetes and severe lower extremity arterial disease. It was hoped that this work would add knowledge contributing to improved care for these patients. If so, this is the time to conceptualise it.

The results from Paper II may add valuable information about the prognosis and aiding both the patient and surgeon in the decision if and when to revascularise. Given the dismal long term prognosis, a patient with diabetes has at least one year shorter expected survival with an intact leg, compared to a non-diabetic patient with comparable risk factors. Thus, in a fragile patient with gangrene and other pronounced comorbidities, diagnosis of diabetes should be viewed as an important extra risk.

If the decision is made to proceed with IBS, Paper I provides evidence that postoperative hyperglycaemia can be anticipated, which may increase the risk for poor outcome. Whether this should be intensively treated is still unclear, but at least Paper II supports the general recommendation to keep glucose levels below 10 mmol/L in patients admitted to hospitals (ADA 2012). Although Paper II does not provide high level evidence for the harmfulness of hyperglycaemia, it is reasonable to conclude that it is at least not beneficial. The findings in Paper III, together with several reports from animal studies, provide a plausible explanation of how the accelerated vascular complications may be linked to activation of RAGE and increased AGE burden initiated by hyperglycaemia and other pathophysiological disturbances common in diabetes. Furthermore, the findings in Paper III and IV underline that there are several factors other than hyperglycaemia that are important in the pathogenesis of peripheral arterial disease. The AGE-RAGE system may thus be important even without concomitant diabetes, and may be active despite good metabolic control. The studies from Uribarri et al (2007) showing food derived AGEs and an inflammatory state being able to activate the RAGE system, support our findings of elevated AGE-RAGE levels in patients without and with diabetes irrespective of glucose control (Paper III and IV).

In both Paper III and IV we found that the RAGE ligand S100A12 is particularly associated with poor outcome. This protein was also the only AGE-RAGE component observed to be higher in patients with diabetes compared to those without in Paper III. This may represent an increased inflammatory activity in patients with diabetes, and needs to be further explored.

There is a profound difference in amputation-free survival comparing patients undergoing IBS with the patients in Paper IV. This is of course expected as the patients in Paper IV were free from PAD, in contrast to the patients in Paper II who all had CLI, when follow up started. The clinical implication merely comes from the magnitude of the difference, 2.8 per 100 person-years vs 32 per 100 person-years, representing a more than 10-fold increase in incidence. This difference had probably been even more pronounced if patients
not offered IBS for critical ischaemia had been included in the estimate in Paper II. Paper I-III studied advanced PAD in older patients with multiple comorbidities. If real change in survival and amputations are to be achieved, prevention is mandatory. The aetiology to PAD in diabetes is clearly multifactorial, thus treatment and research must be multifactorial. The conclusion is that we need to implement the findings from the STENO study, to achieve effective prevention of mortality and morbidity from macrovascular complications in type 2 diabetes (Gaede et al. 2008; Gaede et al. 2003). Our findings in Paper III and IV encourage further research into the mechanisms for vascular damage in type 2 diabetes. There are several interventions directed to decrease AGE and inhibit RAGE that may be useful adjuncts to the established multifactorial intervention, shown to be effective in the STENO trial.
CONCLUSIONS

I. Perioperative hyperglycaemia is associated with unfavourable outcome after distal bypass surgery in patients with diabetes.

II. Type 2 diabetes is independently associated with an increased risk (55%) for poor outcome after infrainguinal bypass surgery compared to no diabetes.

III. The incidence of major amputations and death after infrainguinal bypass surgery for critical limb ischaemia is higher in patients with diabetes, 33 per 100 person-years, than in those without, 23 per 100 person-years.

IV. Components of the AGE-RAGE system are found in vein tissue and plasma from patients with peripheral arterial disease, regardless of diabetes or not.

V. Plasma S100A12 and a composite measure of the AGE-RAGE system are associated with unfavourable outcome after infrainguinal bypass surgery.

VI. Plasma S100A12 and a composite measure of the AGE-RAGE system are related to development of peripheral arterial disease and decreased amputation-free survival in patients with diabetes.

VII. The incidence of amputation or death in patients with type 2 diabetes since 7 years and initially free from peripheral arterial disease was 2.8 per 100 person-years during a 10-year follow up period.

The underlying theme in this thesis has been that patients with type 2 diabetes and peripheral vascular disease should be studied and cared for with respect to their unique situation regarding pathophysiology and prognosis. The main conclusion therefore is that both research design and treatment should be tailored according to this.
FUTURE

There is a need to improve the outcome after IBS, particularly in patients with diabetes. The discussion here will be restricted to patients with tissue loss, as this group is the ‘real challenge’ (Söderström 2011).

First, we need to use outcome measures relevant to patients, and these will at least include amputation-free survival and time to wound and ulcer healing, as has been proposed (Apeteqvist and Lepantalo 2012; Benoit et al. 2012; Hinchliffe et al. 2012; Schaper et al. 2012). Use of limb salvage and patency in patients with diabetes and critical limb ischaemia should be abandoned, not only as they are of low relevance to patients, but also because they are methodologically questionable as censoring is most likely to not be non-informative.

Given proper outcome assessment, we also need to define the patient population under study. Ideally, studies should not mix patients with and without tissue loss and if patients with and without diabetes are studied together, these groups need to be defined and contain sufficient numbers to be analysed separately. There is also need for a staging system regarding the severity of the leg, which accounts both for degree of ischaemia, extent and location of tissue loss, and infection.

New revascularisation techniques or adjunctive medical treatments will most certainly not substantially improve outcome alone. Endovascular interventions are promising, but there is still no evidence of significant improvement (Adam et al. 2005; Rana and Gloviczki 2012), and adjunctive pharmacological interventions to prevent thrombosis (Belch et al. 2010) or graft failure (Conte et al. 2006) has so far been disappointing. Therefore it seems advisable to orient clinical research to other aspects and integrate pre- and post-revascularisation treatments. First, the timing of revascularisation needs to be studied. Early correction of milder ischaemia in patients with minor ulcers could prevent progression to gangrene and infection. Second, interventions to reduce complications after IBS need to be addressed. We have contributed to initiate two trials, one trying to show if perioperative hyperglycaemia can be controlled in a safe way, and another evaluating if perioperative hyperbaric oxygen treatment can reduce complications (www.clinicaltrials.gov/ct2/show/NCT01002209 and www.clinicaltrials.gov/ct2/show/NCT00700154).

The research field concerning AGE and RAGE was focused mainly on hyperglycaemia and diabetes complications at the time we planned Paper III, but has expanded in recent years and is now relevant in a wider context regarding vascular disease. AGEs in processed foods may initiate a state of chronic oxidative stress, impaired host defence and insulin resistance, which precedes diabetes (Vlassara and Striker 2011). AGE may thus be both the cause of diabetes and macrovascular disease, and also contribute their progression. Research to test if primary prevention of diabetes and cardiovascular events can be achieved by restriction of AGE intake is desirable. Diet advice to restrict AGE ingestion is already possible to test without the need for expensive phase II trials (Uribarri et al. 2010).
**SUMMARY IN SWEDISH**

**Bakgrund:**

Den här avhandlingens syften var:
- att undersöka effekten av högt blodsocker på resultatet efter infrainguinal bypass kirurgi hos patienter med diabetes.
- att ge ett mått på amputationsfri överlevnad efter infrainguinal bypass kirurgi p. g. a. kritisk ischemi och jämföra amputationsfri överlevnad hos patienter med och utan diabetes.
- att uppskatta amputationsfri överlevnad och utveckling av perifer kärlsjukdom hos patienter med diabetes på lång sikt.
- att undersöka om receptorn för avancerade glykera slutfaktorer (RAGE) och avancerade glykera slutfaktorer, (”advanced glycation end products”), (AGE) är förhöjda i plasma och venvävnad hos patienter med diabetes.
- att undersöka om AGE och RAGE är kopplat till utveckling av perifer kärlsjukdom och resultat efter IBS.

**Resultat:**
I studie I, visade vi en koppling mellan hyperglykemi de första 48 timmarna efter IBS, och ökad risk för sårkomplikationer, graflockslusion och amputation eller dödsfall under de första 3 månaderna hos 91 patienter med diabetes.

I studie II jämförde vi amputationsfri överlevnad efter IBS för kritisk ischemi hos patienter med och utan diabetes. Studien var en populationsbaserad kohort med data från 1 840 patienter från Svenska kvalitetsregistret för kärlkirurgi, (Swedvasc), 742 med och 1098 utan diabetes. Dessa följdes avseende amputation och död via länkning till patientregistret och befolkningssregistret.

Patienter med diabetes hade kortare amputationsfri överlevnad än patienter utan diabetes (2,3 år, 95% konfidensintervall 1.9–2.8 år jämfört med 3,4 år, 95% konfidensintervall 3.1–3.7 år). Den relativ risken och incidenst för ipsilateral amputation eller död hos patienter med diabetes var 1.46 (95% konfidensintervall 1.26–1.69) och 30.2 per 100 personår.
Hos patienter med diabetes men utan initial perifer kärlljuckdom var incidensen för amputation eller död 2.8 per 100 personår, (95% konfidensintervall 2.0 till 3.7) när dessa följdes i genomsnitt 10 år.

I studie III och IV visade vi att plasmanivåerna av S100A12, en ligand till RAGE, var associerade med AFS efter IBS hos patienter med (n=38) och utan (n=30) diabetes (studie III). Markörer för AGE och RAGE förekom i plasma och vennävad både hos patienter med och utan diabetes.

Vidare var S100A12 associerat med AFS och utveckling av PAD vid långtidsuppföljning (10 år) av 146 patienter med typ 2 diabetes (studie IV).

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