To Carina,
Paul, Petter and Hugo
Lower limb ischaemic rest pain, ulcers or gangrene are late consequences of advanced peripheral arterial disease, one of the facets of systemic atherosclerosis. The majority of patients will meet current criteria for critical limb ischaemia (CLI). Life expectancy in these patients is severely limited mainly due to coexisting coronary heart disease. Once believed to unavoidably end in amputation unless treated, CLI was long considered to imply imperative vascular reconstruction. However, as extensive bypass surgery with significant morbidity is often needed and a spontaneous one-year limb salvage rate of 50% can be expected, the question has been raised if not some of these patients would fare better with other treatment strategies. To date no methods have been presented that with any certainty predict the fate of the limb of an individual patient with severe limb ischaemia.

Identification of metabolic alterations preceding tissue necrosis in chronically ischaemic limbs could be a means to improve patient selection for reconstructive surgery. Lactate is a sensitive marker of tissue ischaemia and could possibly be used for this purpose.

Lactate levels were determined with the microdialysis technique, first in 9 healthy subjects performing one-legged knee extension exercise under a variable degree of skeletal muscle ischaemia in a pressure chamber to validate its capacity to grade ischaemia. There was a good correlation between microdialysate lactate levels and the degree of ischaemia.

Microdialysis lactate determinations were then used in 10 patients with CLI. Foot and lower leg subcutaneous adipose tissue and anterior tibial muscle were studied. Patients with CLI showed a heterogeneous metabolic response to apparent severe ischaemia but there seemed to be a connection between ischaemic pain and lactate levels. There was no clear correlation between lactate levels and the degree of ischaemia using ankle or toe pressure or transcutaneous oxygen tension. As lactate determination even with the minimally invasive microdialysis technique was not considered ideal in a larger patient cohort we sought an experimental model of long-lasting ischaemia to allow validations of other methods.

A rat model of unilateral resting limb ischaemia was modified and evaluated. Perfusion measured by laser Doppler imaging was significantly decreased for the full eight-week follow up. Decreased femoral artery volume blood flow and histological signs of ischaemia were seen for up to four weeks. During the early phase, resting lactate levels were increased.

Magnetic resonance (MR) T₂ characteristics of the tissue are determined by body water content and the composition of tissue fluid. A link between MR T₂, tissue fluid composition and lactate concentration has been described, suggesting the possibility of using T₂ relaxation time as an indirect marker of tissue lactate concentration. In the rat model, T₂ levels showed a strong correlation to clinically observed ischaemia score at one day and intramuscular lactate concentrations at one day and one week. T₂ levels gradually returned to baseline levels over a period of two months.

Exploring new methods to potentially improve the definition of CLI to safer select patients to treatment has been the overriding aim of this thesis. Both lactate and MR T₂ changes appear to correlate to symptoms and degree of ischaemia in patients and under experimental conditions. Further studies are needed to elucidate to what degree such findings are related to prognosis in CLI.

Keywords: limb ischaemia, diagnosis, metabolism, skeletal muscle, subcutaneous adipose tissue, microdialysis, muscle biopsy, lactate, perfusion, lower body positive pressure, human, rat, MR, T₂, relaxation time
LIST OF PUBLICATIONS

I  Lundberg G, Olofsson P, Ungerstedt U, Jansson E, Sundberg CJ.  
Lactate concentrations in human skeletal muscle biopsy, microdialysate and venous blood during dynamic exercise under blood flow restriction.  

II  Lundberg G, Wahlberg E, Swedenborg J, Sundberg CJ, Ungerstedt U, Olofsson P.  
Continuous assessment of local metabolism by microdialysis in critical limb ischaemia.  

III  Lundberg G, Luo F, Blegen H, Kalin B, Wahlberg E.  
A rat model for severe limb ischemia at rest.  

IV  Lundberg G, Olofsson P, Wahlberg E, Sundberg CJ, Klason T, Bjelke B.  
MR T2 relaxation time correlates to the ischaemia level in resting skeletal muscle.  
*Manuscript*

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### ABBREVIATIONS

<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABI</td>
<td>Ankle brachial blood pressure index</td>
</tr>
<tr>
<td>AP</td>
<td>Ankle blood pressure</td>
</tr>
<tr>
<td>CLI</td>
<td>Critical limb ischaemia</td>
</tr>
<tr>
<td>LD</td>
<td>Laser Doppler</td>
</tr>
<tr>
<td>LDPI</td>
<td>Laser Doppler perfusion imager</td>
</tr>
<tr>
<td>MR</td>
<td>Magnetic resonance</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>PAD</td>
<td>Peripheral arterial disease</td>
</tr>
<tr>
<td>PTA</td>
<td>Percutaneous transluminal angioplasty</td>
</tr>
<tr>
<td>SPP</td>
<td>Skin perfusion pressure</td>
</tr>
<tr>
<td>$T_2$</td>
<td>Transverse relaxation time</td>
</tr>
<tr>
<td>$TcPO_2$</td>
<td>Transcutaneous oxygen tension</td>
</tr>
<tr>
<td>TP</td>
<td>Toe blood pressure</td>
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INTRODUCTION

Critical limb ischaemia – the clinical problem

Lower limb rest pain, ulcers or gangrene as a consequence of poor blood supply can be estimated to afflict 5000 to 10000 new patients yearly in Sweden and the majority will be expected to meet current criteria for critical limb ischaemia (CLI)\textsuperscript{199}. CLI, though initially assumed to equal limb loss unless successful vascular surgery could be performed, is now known to sometimes remain stable for long periods of time or even improve. This is illustrated by the fact that amputations during the first year were needed in only 50% of patients with CLI who did not undergo vascular surgery\textsuperscript{103,119}.

CLI is the local presentation of a multiorgan atherosclerotic disease often in a late phase in life with a frequent involvement of coronary and cerebral circulation. Life expectancy is profoundly reduced mainly due to cardiac events\textsuperscript{95,212}. Patients with rest pain, ulcers or gangrene have widespread vascular disease and often face extensive bypass surgery with significant morbidity to get symptom relief. In spite of this, vascular surgery to overcome the local problem in the limb can be performed with low perioperative mortality and often good results regarding graft patency and limb salvage rate. Lacking effective pharmacological treatment for CLI and considering the alternative, often a major amputation, an aggressive policy of revascularisation whenever possible has since long been the adopted policy of vascular surgeons.

There is however also growing awareness of the often long rehabilitation period facing these patients even after a successful operation. Persisting pain, postoperative leg swelling, wound problems, delayed healing of ulcers, need for minor amputations and reoperations to maintain graft patency are commonly seen in the postoperative phase that can stretch for months or even years. Successful limb salvage ideally allows the patient to carry on an independent life at best with two pain free legs. At worst, pursuing limb salvage at all cost can mean unnecessary suffering and effort during the last few months or years of a critically ill patient’s life.

This makes patient selection to treatment crucial. The palliative nature of any therapy in critical limb ischaemia must always be remembered and vascular surgery should be performed whenever it is the best option for the patient, but only then.

Exploring new methods to potentially improve the definition of CLI to safer select patients to treatment has been the overriding aim of this thesis.

Atherosclerosis – a systemic disease

The common ground of the different clinical manifestations of arterial occlusive disease in man is atherosclerosis. The concept of a systemic vascular disease induced by the action of a variety of noxious stimuli on the vessel wall has gained rapid support and increased understanding during the last few decades\textsuperscript{122}. A complex interplay of genes and environmental factors determines the risk of initiation and progression of the process entailing deposition of lipids in the vessel wall, activation of adhesion molecules attracting leukocytes from the blood stream, migration of leukocytes into the vessel wall, activation of smooth muscle cells and reorganisation of the matrix – the supporting structure surrounding the cells. The resulting plaque formation – i.e. a localized fibrous thickening of the arterial wall – can cause symptoms in many principally different ways, by narrowing of the lumen or by ulceration or rupture of the plaque. The extent of narrowing – or stenosis – of the involved artery is determined not only by the size of the atherosclerotic plaque. Adaptive mechanisms aiming at maintaining flow promote
expansive or positive remodelling – i.e. rebuilding of the arterial wall – resulting in increased cross-sectional area of the artery and a lessened impact on the lumen area by the plaque. Absence of remodelling or sometimes constrictive remodelling results in aggravated stenosis. Ulceration or rupture of a plaque is more likely to happen when the plaque has a large lipid-rich core and the fibrous cap is thin. Plaque rupture may be asymptomatic but often trigger blood clot formation resulting in occlusion at the site of plaque rupture or distal spread of clot material – embolisation.

As long as blood flow through the diseased artery is not significantly decreased – and occlusion or embolisation has not yet occurred – the lesion is asymptomatic. Symptoms when they do occur vary depending on the organ supplied by the affected artery; angina pectoris or myocardial infarction in coronary heart disease, transient ischaemic attack or stroke in cerebrovascular disease and leg dysfunction or rest pain, ulcers or gangrene – often qualifying as CLI – in peripheral arterial disease (PAD).

**Peripheral arterial disease**

**Incidence and prevalence**

It is hard to assess the incidence and prevalence of critical limb ischaemia in the population. Attempts can be made from amputation rates, from hospitalisations for CLI and from the calculated risk for patients with intermittent claudication to develop CLI. All these methods were used by Catalano et al in northern Italy and showed an incidence of 450 to 600 per million per year. In Great Britain and Ireland the incidence was calculated to be 400 per million per year after a national survey sent to vascular surgeons. If patients with CLI not having had revascularisations or amputations are accounted for as well, an estimate of 500-1000 new cases of CLI per million per year can be made.

Substantially more research has been directed towards clarifying the prevalence of milder forms of PAD. Asymptomatic atherosclerosis could be described as an integral part of normal ageing at least in the western world. Autopsy studies of iliac arteries in adults have shown almost invariable occurrence of atherosclerosis. A simple and yet highly specific means of finding asymptomatic atherosclerotic disease is by use of the ankle brachial index (ABI) (Page 24). An ABI of <0.9 is highly specific and sensitive for angiographically diseased arteries (>50% stenosis) and is often used as an arbitrary cut off ratio for asymptomatic peripheral arterial disease.

The prevalence of PAD is strongly correlated to the age of the examined population. In the Swedish prospective population study “Men born in 1914” 14% of 68 year old men had ABI <0.9. Criqui et al found PAD by the same definition in 2-3% of 50 year olds and 20% in persons aged >75. In the Edinburgh Artery Study the prevalence of asymptomatic PAD was 8% in men and women aged 55-74 years. The prevalence of intermittent claudication is lower than that of asymptomatic disease and is influenced by the population studied, methods and definitions used. Often combinations of questionnaires and non-invasive methods are employed. In Scotland the prevalence was 2.8-4.6% in men and women 55-74 years old and in the Swedish Vadstena study 2.2-3.3%.

**Clinical presentation**

When blood flow to a limb is insufficient to meet tissue demand already at rest the patient will perceive ischaemic rest pain or develop ulcers or gangrene. A substantial proportion of these patients will have a high probability of limb loss unless revascularisation can be achieved and many will meet the criteria for CLI. CLI should refer to a chronic state and be separated from acute limb ischaemia. Acute limb ischaemia is defined as a state of blood flow reduction to a limb, posing a potential threat to the viability of the limb, with acute or semi-acute onset normally with less than two weeks duration and is most commonly caused by a cardiac embolus or acute thrombosis occluding a large artery.

In CLI, the pain is recurrent in nature and often worse at night when systemic blood pressure drops and the supine position reduces hydro-
static pressure in the limb arteries resulting in an inadequate perfusion pressure. The site of the pain is typically the toes or the sole of the foot. A strong indicator of ischaemic origin of any foot pain is when the pain disappears or is alleviated when the patient is hanging the foot out of the bed at night. Ulcers most often affect the toes or the heel. These sites are also most prone to the development of gangrene.

As blood flow and oxygen consumption in a resting limb is very low there is a high probability that progressive arterial narrowing in PAD will first cause no symptoms, or symptoms only during exercise, e.g. when walking. Stenosis of no importance at rest can significantly limit blood flow during exercise when normally blood flow is expected to increase several-fold. The symptoms are often described as pain, heaviness or fatigue in the affected muscle group. The location and extent of arterial disease determines the muscle group involved – calf, thigh or buttock – and the severity of symptoms. Typically, a few minutes rest alleviates symptoms and the patient can resume walking. This clinical entity of muscular pain or fatigue brought on by a reproducible workload and relieved by rest within minutes is known as intermittent claudication.

The perception of PAD development as a gradual decline from an asymptomatic state through intermittent claudication and with gradually aggravating stenosis – as reflected by falling distal blood pressure levels – to CLI with rest pain and tissue loss can be questioned. Dornandy et al found that 55% of patients amputated for CLI were asymptomatic six months prior to when they were diagnosed as having CLI. In another study 37% of CLI patients never had experienced claudication when they were referred for CLI. In the latter study patients without previous claudication were more likely to present with ulcers or gangrene whereas patients with a history of claudication more frequently only had rest pain as presenting symptom. In a study on 154 patients with major lower extremity amputations – 87% as a result of CLI – only 30% had experienced claudication prior to the onset of CLI. These findings are in appealing accordance with experiences from the pathophysiologic events preceding myocardial infarction. Angiographic studies six months prior to a subsequent myocardial infarction showed little correlation between degree of stenosis and later cardiac events in the region supplied by the stenosed artery.

In a postmortem study on culprit lesions in coronary arteries after fatal myocardial infarction it was found that these lesions often consisted of large plaques accompanied by vessel enlargement, the vessel enlargement possibly explaining the limited effect on lumen diameter of these plaques. The importance of plaque distribution and morphology in limb arteries and its potential role in the development of CLI is poorly understood.

Risk factors

The primary major risk factors for development of PAD are similar to those for atherosclerosis in other parts of the body; physical inactivity, smoking, hyperlipidaemia, obesity, diabetes and old age. Intermittent claudication generally occurs at an older age than angina pectoris, the difference being around ten years in the longitudinal Framingham Study. Smoking has been suggested as the single most important risk factor for the development of PAD. In these studies diabetes, hypertension and hyperlipidaemia (total cholesterol) were other strong predictors of PAD. In a subgroup analysis from the UK Prospective Diabetes Study (UKPDS) an elevated HbA1c by 1% increased the risk of developing intermittent claudication by 28%. Renal impairment increases the risk for PAD. Homocystein – an intermediary of amino acid metabolism – is an independent risk factor for the development of atherosclerosis and possibly in particular for PAD. Several studies have shown a strong influence of genetic factors predisposing to the development of atherosclerosis. Elevated fibrinogen levels have long been recognized as a risk factor for cardiovascular disease and PAD and ...
as understanding of the importance of inflammation in atherosclerosis has led to the search of novel risk factors and potential markers for disease and e.g. hs-CRP (high sensitivity CRP)\textsuperscript{171} and s-ICAM-1 (soluble intercellular adhesion molecule)\textsuperscript{165} have both been demonstrated to be independent markers for the development of PAD. Infection has been suggested as a possible causative agent and Chlamydia pneumoniae is the pathogen most commonly connected to the development of atherosclerosis\textsuperscript{149}. Recently a link between periodontal disease and PAD has been demonstrated, possibly related to other pathogens\textsuperscript{96}.

Differences in risk factors depending on the anatomical distribution of PAD have been described with more impact of smoking on proximal disease, especially in women, and more influence of diabetes in distal disease\textsuperscript{83}.

In established PAD important predictors for aggravation of disease – i.e. development of CLI – are continued smoking\textsuperscript{50,105}, diabetes\textsuperscript{50} and a low ABI\textsuperscript{51}.

**Risk factor modification – life style**

Smoking cessation in patients with claudication decreases both the progression of limb symptoms and the risk for cardiovascular events\textsuperscript{105}.

Regular exercise training improve walking capacity in patients with claudication at least in supervised programs\textsuperscript{57} and has a beneficial effect on heart rate and lipid profile\textsuperscript{197}. Positive effect of exercise on cardiovascular mortality has been established in patients after myocardial infarction\textsuperscript{113} and is likely to exist in PAD patients but this has not been proven. Concerns have been raised about possible adverse effects of repeated ischaemia-reperfusion episodes accompanying exercise in PAD. Leukocyte infiltration and muscle damage have been demonstrated in animal models of claudication\textsuperscript{106}. In patients with claudication markers of ischaemia-reperfusion injury were elevated immediately after exercise\textsuperscript{203}, Three months of exercise training decreased this potentially harmful effect of exercise.

The impact of life style modification in established CLI is largely unknown.

**Risk factor modification – medication**

Despite the excessive cardiovascular mortality in CLI patients little direct information exists on the value of pharmacological risk factor reduction aiming at reducing cardiovascular events in patients with CLI. Most information must be extrapolated from data on patients with intermittent claudication or coronary heart disease.

Intensive treatment of type 2 diabetes reduced diabetes related death and myocardial infarction but not the risk for amputation in the UKPDS\textsuperscript{8}. Patients with PAD were included but to what extent is unknown why possibly there was an insufficient number to show an effect on PAD-related outcome.

In a meta-analysis of antiplatelet – mostly aspirin – treatment in high risk patients to prevent cardiovascular events the overall risk reduction was 23% in patients with PAD (mainly claudication), i.e. comparable to the effect in other high risk groups\textsuperscript{89}. In a Cochrane review on adjuvant antiplatelet treatment there was only a mild not significant effect on overall cardiovascular outcome\textsuperscript{48}. The majority of the patients had CLI and it is hypothesized that the advanced state of disease might have limited the effect of antiplatelet therapy. In the CAPRIE study clopidogrel had a greater effect than aspirin in the prevention of cardiovascular events in the subgroup of patients with PAD (intermittent claudication)\textsuperscript{192}.

Lipid lowering therapy in patients with PAD appear to reduce cardiovascular deaths to a similar extent as in other high risk groups\textsuperscript{1}. Additionally, there may be a benefit for limb symptoms; a reduction of 38% in new or worsening claudication\textsuperscript{113} and better leg function-ing in patients with (intermittent claudication) and without PAD\textsuperscript{113} has been re-reported. In the latter study the effect was unrelated to lipid levels suggesting a non lipid lowering – possibly inflammation related – statin effect.

Recently, monitoring not only lipid levels but also hs-CRP levels to gauge the effect of statin treatment in patients with acute coronary syndromes has been suggested\textsuperscript{172}. 

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**Göran Lundberg**

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Aggressive antihypertensive treatment is recommended to decrease the risk of cardiovascular events in patients with PAD as in other high risk groups, though the specific information on this patient group is limited. Even less is known of the value of treating hypertension in CLI patients where theoretically a lower systemic blood pressure could risk aggravating limb symptoms.

ACE-inhibitor ramipril significantly decreased cardiovascular death, stroke and myocardial infarction in patients at high risk and the effect in the subgroup of PAD patients was similar. The mechanism was probably not a decreased blood pressure (only 2-3 mm Hg) but possibly a reduction of deleterious effects on the vasculature and the heart by the renin-angiotensin system.

Pain management

Pain control is by definition part of any CLI treatment regimen. Regular paracetamol is administered and frequently opioids are necessary. If non-steroidal anti-inflammatory drugs are used the risk for adversely affecting renal function must be carefully considered. There might also be ground for the use of agents aiming at handling possible neuropathic pain components.

Pathophysiology

Atherosclerosis of large arteries is the underlying cause of all PAD. The general principles discussed earlier – plaque formation, vascular remodelling, plaque ruptures, thrombosis and embolism – have been studied mainly in the coronary circulation but are likely to apply also in the peripheral arteries. However, the exact mechanisms responsible for deterioration of the macrocirculation in PAD are not fully understood.

In a post mortem study on cross sections from atherosclerotic femoral arteries markers of plaque vulnerability – more inflammatory cells, more atheroma, less collagen and fewer smooth muscle cells – correlated to larger plaque area and larger vessel area, i.e. features known from the coronary circulation to predict plaque rupture.

It could be hypothesized that if plaque vulnerability and positive remodelling coincide also in the peripheral circulation, part of the observed lack of correlation between impaired blood flow, as assessed by e.g. ankle pressure, and prognosis in CLI could be explained.

A physiological mechanism to compensate for the effect of stenosed or occluded arterial segments is the growth of pre-existing arterioles into more effective natural bypass vessels – collateral vessel development. Stimuli to development of collateral vessels appear to be local inflammation, release of growth factors and cytokines. An inadequate collateral formation may be a factor in the progression of disease in patients with PAD.
of capillaries caused by oedema result in further decreased nutritive flow. Loss of effective capillaries and oedema increase oxygen and nutrient diffusion distance leading to tissue ischaemia with increased susceptibility to infection and impaired wound healing capacity\textsuperscript{136}. Ultimately rest pain, ulcers and gangrene develop, though the exact mechanisms are not fully understood.

**Past and present definitions of critical limb ischaemia**

The term critical limb ischaemia is frequently used in an attempt to define a state of chronic limb threatening ischaemia implying an urgent need for vascular reconstruction to avoid amputation. Finding a useful definition of CLI in this sense has proven to be difficult due to the lack of prognostic factors able to forecast the prognosis of individual patients. The natural history of severe potentially limb-threatening ischaemia cannot be studied in an unselected patient population, as the majority of patients will be subjected to different kinds of treatment. However, some conclusions can be drawn from patients who for different reasons have not undergone revascularisation. Lepäntalo \textit{et al.} identified retrospectively 105 patients with 136 legs with critical limb ischaemia according to the current definition at the time of the study\textsuperscript{2, 119}. The motivations for not undertaking revascularisations were: technical reasons and operative risk 54%, operative risk alone 33%, borderline CLI 6% and patient preference in 7%. After one year 46% of the patients were alive while limb salvage rate in this selected - probably higher than average risk - population was 54%. Jivegard \textit{et al.} found a limb salvage rate of 45% at 18 months in the control group in a study comparing spinal cord stimulation with no treatment\textsuperscript{103}. In the latter study 88% of the patients complied with the definition of CLI cited above\textsuperscript{2}. A spontaneous one-year limb salvage rate of 50% raises the question if not some of the patients with CLI would be better off with other treatment strategies than revascularisation. The remaining problem is then how to identify these patients at lower risk for progression of disease.

Several attempts have been made over the years to define CLI (Table 1). The term limb salvage has been used extensively in the vascular surgical literature. Besides the meaning of a successful outcome the term has been used as a definition of a cohort of patients subjected to surgical procedures performed in order to save the limb, inferring the likely loss of the limb without a surgical procedure. A broad spectrum of often ill-defined patients has been included making interpretation of the reported results difficult. Many of these reports also include ischaemia secondary to vascular trauma and patients with acute ischaemia further adding to the problem. Critical limb ischaemia is the term preferred by most recent bodies that have attempted to find a useful definition of the condition\textsuperscript{2, 3, 199}.

Repeatedly each attempt has been questioned and challenged mainly on the ground that no definition have appeared to with any certainty predict the fate of an individual patient regarding limb survival in the absence of active treatment\textsuperscript{28, 205, 217}.

Some confusion may exist over the intended use of the concept of CLI. There is an obvious need for reproducible reporting standards when comparing e.g. surgical outcome and ideally the definition would also have the ability to assist in the selection of patients to potentially harmful surgical procedures.

Fontaine \textit{et al.} reported in 1954 on the treatment of 378 patients with arteriosclerosis in the lower limbs\textsuperscript{67}. With the specific aim of permitting a correct interpretation of late results the patients were divided into four groups. This classification is still in frequent use and Stage III and IV are commonly interpreted as inferring limb-threatening ischaemia. Often the Fontaine classification is referred to as clinical, which might be true in that it entails no other parameters than the patients’ clinical presentation. However, the intended use was not as a clinical prognostic tool but as a reporting standard.

Possibly the labelling of a patient as suffering from CLI diverts the attention of the vascular surgeon from other important factors – co-morbidities, extent of tissue loss, level of
Table 1 Classification of limb ischaemia and definitions of limb threatening ischaemia 1954-2000.

<table>
<thead>
<tr>
<th>Fontaine – 54⁶⁷</th>
<th>Working party of the International Vascular Symposium – 82²⁸</th>
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<tbody>
<tr>
<td>I</td>
<td>Severe rest pain &gt;4 weeks duration</td>
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<tr>
<td></td>
<td>and ankle systolic blood pressure &lt;40 mmHg</td>
</tr>
<tr>
<td>II</td>
<td>or ankle systolic blood pressure &lt;60 mmHg in combination with superficial tissue necrosis of the foot or the base of a phalanx</td>
</tr>
<tr>
<td></td>
<td>Patients with diabetes were excluded from the definition.</td>
</tr>
<tr>
<td>III</td>
<td>SVS/ISCVS – 86⁷</td>
</tr>
<tr>
<td></td>
<td>Grade Category Clinical diagnosis Objective criteria</td>
</tr>
<tr>
<td>II*</td>
<td>4 Ischaemic rest pain Ankle pressure &lt;40 mmHg or toe pressure &lt;30 mmHg</td>
</tr>
<tr>
<td>III*</td>
<td>5 Minor tissue loss Ankle pressure &lt;60 mmHg or toe pressure &lt;40 mmHg</td>
</tr>
<tr>
<td></td>
<td>6 Major tissue loss Ankle pressure &lt;60 mmHg or toe pressure &lt;40 mmHg</td>
</tr>
<tr>
<td></td>
<td>*Grade II and III equal chronic critical ischaemia in revised version 1997¹⁸⁰.</td>
</tr>
<tr>
<td></td>
<td>Second European Consensus Document – 92²</td>
</tr>
<tr>
<td></td>
<td>Recurrent rest pain for more than 2 weeks</td>
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<tr>
<td></td>
<td>or Ulceration or gangrene of the foot or toes</td>
</tr>
<tr>
<td></td>
<td>and Ankle pressure &lt;50 mmHg or toe pressure &lt;30 mmHg</td>
</tr>
<tr>
<td></td>
<td>TASC TransAtlantic Inter-Society Consensus – 00¹⁹⁹</td>
</tr>
<tr>
<td></td>
<td>Clinical:Chronically recurrent ischaemic rest pain, ulcers or gangrene secondary to demonstrated arterial occlusive disease. Most would be expected to require a major amputation within the next 6 months to a year</td>
</tr>
<tr>
<td></td>
<td>For research:Ankle pressure &lt;50-70 mmHg or Toe pressure &lt;30-50 mmHg or TcpO₂ &lt;30-50 mmHg</td>
</tr>
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independence, mobility etc. – when deciding on treatment145.

The latest work in this field (TransAtlantic Inter-Society Consensus Document on the management of peripheral arterial disease), has suggested a broad inclusive definition for clinical purposes and a stricter more exclusive definition for reporting results scientifically199. The implication for clinical practice is that even more patients will suffer from CLI underlining again the importance of finding better tools to select patients among them to treatment.

**Treatment options**

Randomised trials have been difficult to justify in a field where the alternative to surgical revascularisation is assumed to be amputation. Consequently, there are no randomised trials in patients with CLI comparing surgical or endovascular revascularisation with no treatment, pharmacological treatment or primary amputation.

**Revascularisation**

**Surgery** Two main open vascular surgical options are available in the treatment of patients with CLI; a vein or artificial conduit can be used to bypass the diseased vessel or alternatively, stenosing or occluding plaques can be surgically removed - thrombendarterectomy. The extensive vascular disease in CLI generally precludes more limited interventions why a bypass procedure is by far the most common surgical operation performed.

Anatomical distribution of disease determines the type of bypass used, habitually described by its inflow and outflow vessels. The inflow vessel can be the aorta or the iliac, femoral, popliteal or sometimes axillary arteries. The outflow vessel is in general the most proximal site distal to the diseased segment with preferentially an interrupted connection to the foot arteries; the femoral, popliteal, crural or even pedal arteries. Sometimes a bypass at one anatomical level e.g. aortofemoral bypass can increase perfusion in the foot enough to put an end to a state of CLI despite coexisting arterial disease at lower levels i.e. femoral, popliteal or crural arteries.

Increasingly, a combination of endovascular (see below) and open surgical methods are being used.

**Endovascular procedures** The percutaneous puncture of an artery and successive dilatations of the arterial puncture site allows the introduction of long catheters into the vessel lumen and a range of so called endovascular procedures to be performed. The common femoral or sometimes the brachial arteries are used as entry points. Balloon dilatation or percutaneous transluminal angioplasty (PTA) with or without supporting metal nets – stents – is the method used to dilate or open up stenosed or occluded vessels. In the treatment of stenosis in the iliac arteries, PTA is a universally accepted first-line treatment. Long occlusions with poor distal vessel run off, as often seen in patients with CLI, are features historically correlated to poor results of PTA.

**Results after revascularisation** In patients with CLI results after revascularisation are often reported in terms of graft patency, limb salvage, amputation rate and survival. There is a wealth of reports using these parameters on patients with varying proportion of CLI, with different definitions of CLI, diverse conduits used for the bypass, poor or no characterisation of distal runoff etc. making comparisons and generalisations hopelessly difficult. In addition, Jensen et al have demonstrated the low reliability of patency and limb salvage life table data after femorodistal bypass surgery, explained by the impact of a much higher rate of graft occlusion and death among patients lost to follow-up102. With complete follow-up limb secondary patency rate fell from 90% to 63%, limb survival from 97% to 77% and patient survival from 95% to 85%.

An equally important issue is perhaps if these traditional outcome measures really gauge what is making a difference to the patients’ life. In a large multi-centre trial on the efficacy of adjuvant iloprost treatment after femorodistal bypass surgery it was noted that 13% of patients had improved clinically and 35% had avoided major amputation despite graft occlusion144. The percentage of patent grafts was also significantly
higher than the relief of rest pain and ulcer healing especially early after surgery.

It is probably safe to say that outcome reporting in vascular surgery has been centred too much on the fate of the operation and the bypass as compared to the fate of the patient. A clinically more relevant set of outcome end points have been proposed\(^{199}\). These include relief of rest pain, ulcer healing, cardiovascular morbidity and total mortality as primary end points while quality of life assessment and graft patency are suggested as secondary end points.

In accordance with these suggestions there is a growing number of studies reporting functional outcome in CLI treatment. An ideal post intervention result – defined as uncomplicated surgery, symptom relief, maintained level of function and the absence of any reoperation - was achieved in only 14\% of 112 patients operated on for limb salvage (ulcer or gangrene 66, rest pain 30, acute ischaemia 44)\(^{146}\). In another study on 318 patients undergoing infra-inguinal bypass surgery the indication was CLI (rest pain or tissue loss) in 72\%. Of those, 49\% were subjected to reoperation within 6 months and the time to heal ulcers exceeded three months in 54\%\(^{79}\). Ten to 15\% of patients never healed their ulcers, many of these patients had congestive heart failure.

Results appear, perhaps not surprisingly, to be influenced by the presenting symptom with a worse outcome in patients with gangrene than rest pain alone and possibly these two patient groups should be treated differently and reported separately\(^{143, 202}\).

During the last few years several non-randomised studies have been published on the use of PTA in patients with CLI. In a study on 133 limbs in 110 patients with CLI, technical success was demonstrated in 105 (79\%) limbs\(^{141}\). One-year limb salvage rate was 88\% (95\% in those with initial success). The patients with an initial technical failure had a very poor outcome. The authors report PTA being the first-line treatment for revascularisation in patients with CLI in their institution during the study period, of a total of 208 limbs with CLI only 21 had a bypass operation.

**Pharmacological treatment**

Pharmacological treatment aiming at affecting the systemic consequences of atherosclerosis has been discussed previously (Page 16).

**Prostanoids** Though the mode of action is not known in detail prostanoids are believed to exert a number of beneficial effects on the microcirculation in CLI e.g.; inhibitory effect on activation of neutrophils and platelet function, increased fibrinolytic activity, increased erythrocyte deformability, reduction of monocyte adhesion, and vessel dilatation\(^{169}\). The prostacyclin analogue – iloprost – is the drug used in most studies on patients with CLI. Intravenous treatment during two to four weeks reduced rest pain and ulcer size in six trials comparing iloprost with placebo\(^{147}\). In studies with more than three months follow-up a reduction in amputation rate and mortality could also be seen\(^{1}\).

**Anticoagulation** Long-term treatment with low molecular weight heparin (LMWH) appears to have a positive effect on healing of ischaemic ulcers and possibly amputation rate in patients with diabetes\(^{108}\).

**Sympathectomy**

Before the era of reconstructive vascular surgery sympathectomy was the treatment of choice for symptomatic PAD\(^ {37}\). In the material presented by Fontaine et al in 1954 nearly all patients were treated with various forms of sympathectomies or amputations\(^ {57}\). Sympathectomy can be achieved surgically, by section or removal of the lumbar sympathetic chain, or chemically by percutaneous injections of e.g. phenol, guided by X-ray or computed tomography (CT)\(^ {90}\). The mode of action is uncertain; sympathetic denervation of the limb arteries has been shown to cause increased flow in arterio-venous shunts. Why this would be of benefit to CLI patients remains unclear. Sympathectomy is a technique still in use and based on uncontrolled studies it has been claimed to have a possible benefit over conservative treatment in patients with CLI\(^ {92}\). However, no randomised studies are available.
Spinal cord stimulation (SCS)
Invasive electrical stimulation of the spinal cord through implanted epidural electrodes has been employed in a variety of chronic pain disorders. The mechanisms of action involved are complex and not fully understood. In CLI, a multitude of beneficial effects have been described as summarised in a Cochrane review of six controlled non-randomised studies comprising a total of 450 patients. The reviewers conclude that SCS causes improvement in the microcirculation leading to increased pain relief and limb salvage rate. No effect on ulcer healing was seen and it was added that cost and complications must be considered. Complications were; implantation problems (8%), need for surgical re-intervention (12%) and infection (3%). The reviewers’ conclusion has been challenged by the authors of one of the included studies and it is finally stated, by both sides, that positive effects if they exist are small.

Amputation
Vascular disease accounts for about 90% of the total number of major amputations in the community. Perioperative mortality rates of 10% - 17% have been reported i.e. considerably higher than after reconstructive surgery though obviously case mix is likely to be different. Major amputations are generally performed above knee or below knee and the ratio between the two when performed for limb ischaemia is usually around one.

Two contradicting aims in the management of vascular patients facing amputation can be noted. On one hand the urge to maximise the probability of ambulation, which is far greater with a below knee amputation, on the other hand to minimise time to wound healing and wound complication rate which is more readily accomplished after an above knee amputation.

A London study of 440 amputations for vascular disease revealed that 10-15% of the patients achieved limited mobility on a prosthesis but only 5% became independent of their wheelchair. In a more recent study Nehler et al found that 50% of vascular amputees were ambulatory at 10 and 17 months follow-up but only half of these ambulated outdoors. Despite this, the great majority of patients who had been living in the community prior to the operation continued to do so postoperatively, only now with a wheelchair. Both authors recommend more attention be paid to the palliative nature of the operation, to prioritize rapid healing and minimising surgical complications and to reserve aspiration for prosthesis and independent ambulation to selected good risk candidates.

Cost aspects
Diagnostic work up, treatment and long term care for patients with CLI demand increasing resources in an ageing population. Many attempts have been made to estimate the costs involved in available alternative treatment strategies. It has been suggested that major amputation in these patients often leads to extended length of stay and institutional care to much higher cost than advanced and even repeated vascular surgery. However, case mix is likely to be very different and no randomized trials are available.

An overview of studies comparing distal bypass with amputation as the primary procedure found similar long-term costs in the two groups. Secondary amputations after a failed bypass carried the highest costs. Luther et al analysed only additional costs of the CLI disease taking into account the preoperative level of care and independence of each patient. Reconstruction and primary amputation were equally expensive in potentially mobile patients living independently while primary amputation in institutionalized patients caused only little additional costs. Again, a failed reconstruction with secondary amputation carried the highest cost.

Quality of life
Chetter et al assessed quality of life prospectively in 55 patients following infrainguinal reconstruction in patients with CLI using the Short Form 36 (SF 36). SF 36 evaluates eight quality of life dimensions. The questionnaire was completed preoperatively and at one, three, six and 12 months after surgery. As expected patients scored low when compared to age-
matched controls. A patent graft at 12 months was associated with improved score in all domains except general health and role limitations due to emotional problems. More surprisingly perhaps, patients with an occluded graft and secondary amputation scored even higher than patients with functioning grafts regarding general health, vitality, mental health and emotional limitations. These areas were also significantly increased when compared to preoperative data in this group. Pain score was improved in both groups to an equal extent while as could be expected the amputated patients scored lower in physical functioning. However, in no domain did amputated patients seem to do worse than before surgery.

In a similar study, 52 patients were divided in three groups; revascularisation, primary amputation and conservative treatment and evaluated on admission, at six and 12 months. At six and 12 months there was no difference in quality of life measured by SF36 as compared to baseline, though all groups had significantly worse and declining results compared to a control population. Abou-Zamzam et al. studied the importance of the preoperative functional state on outcome in 513 patients with infrainguinal bypass performed for limb salvage. Only 4% of patients who were not ambulant prior to surgery became ambulant after surgery and among patients who were not living independently only 21% of patients did so after surgery.

The importance of studying changes in quality of life over time in individual patients is underlined by a study in 112 patients with rest pain or tissue loss who underwent femorodistal reconstruction or primary amputation. Social functioning, emotional disorder and mobility were studied. A postal self-assessment protocol at one postoperative occasion was used (median 16-18 months postop). Patients were classified into four groups according to outcome as having primarily patent or secondarily patent grafts, or having undergone a primary or secondary amputation. Quality of life score was identical in patients with patent grafts and significantly lower in the amputated groups regardless of whether the amputation was primary or secondary. It was concluded that femorodistal bypass surgery leads to a better quality of life even when there is a need for secondary procedures. However, no baseline data is given on the subject of quality of life before intervention, which obviously can be expected to differ as amputation was chosen as primary treatment in patients with extensive tissue loss, premorbid inability to ambulate or severe dementia. It is entirely possible that the quality of life for these particular patients improved following amputation. As it is equally possible that quality of life decreased during the postoperative period in some of the patients who needed repeat interventions to maintain graft patency.

Concerns have been raised about the ability of SF-36 to cover all areas important for patients with CLI and better instruments might become available in the future.

From these studies it appears plausible that individually tailored treatment, revascularisation in many patients and amputation in some, is a viable route to improve quality of life in patients with CLI, stressing the importance of properly selecting patients to the different treatment options available.

Non-invasive methods in the assessment of patients with CLI

The various definitions of CLI presented to date (Table 1) have rested on clinical symptoms and signs with the addition of objective assessment using one or more of a number of non-invasive methods.

Ankle blood pressure (AP) and Toe blood pressure (TP)

Ankle blood pressure is the external cuff pressure required to arrest flow by compressing the arteries in the distal lower leg. Absolute ankle pressure or the ankle-brachial index (ABI) can be used. The systolic blood pressure at the ankle divided by the brachial systolic pressure gives the ankle-brachial index or ABI. In healthy individuals the ankle pressure equals or slightly
exceeds the brachial pressure giving an ABI of 1-1.1. ABI is especially valuable in the assessment of an individual patient over time e.g. to judge the effect of an intervention. Absolute ankle pressure has been included in most of the cited definitions of CLI (Table 1).

Calcified vessels – common in diabetes and renal insufficiency – sometimes prevent a reliable pressure reading. Raised ABI as an indicator of increased resistance to cuff pressure was found in 18.3% of patients with insulin dependent diabetes, 4.5% in non-insulin dependent diabetes and 2.8% in non diabetic patients. In patients with varying degrees of renal impairment an ABI >1.3 indicating medial calcification was found in 20-40% and in 3.4% in a control group. The problem with stiff or incompressible arteries can be overcome to a large extent by the use of toe pressure (TP) or toe-brachial index (TBI) measurements. Another theoretical advantage of using TP is that arterial disease also distal to the ankle will influence results.

Completely incompressible arteries make use of the ankle pressure method impossible as the flow signal never ceases and no pressure can be recorded. Probably this is not an on or off phenomenon with either soft easily compressible arteries or stiff calcified tubes, but rather a continuum from one end of the spectrum to the other. Somewhat contradicting this notion Brooks et al demonstrated that as long as the ABI was <1.3, TBI was consistently 0.4 lower than ABI in both controls and patients with diabetes and hence there was a good agreement between the methods in the absence of abnormally elevated ABI. When ABI was >1.3 the TBI fell.

**Pole test**

Another way of approaching the problem with incompressible arteries is the pole test. A Doppler probe is used to monitor flow in the dorsalis pedis artery. With the patient in the supine position and the probe in place the limb is elevated from the couch. The perpendicular distance from the level of the heart to the probe

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**Fig. 1.** Simplified schematic view of cellular ATP production under anaerobic conditions when aerobic ATP production in the mitochondria (grey ellipse) is limited.
when the Doppler signal ceases is measured and gives the pressure in cm H2O in the vessel. The method is appealing by its simplicity though the patient’s hip mobility and leg length are limiting factors.

**Laser Doppler fluxmetry**

Ischaemia assessment with laser Doppler fluxmetry can be performed as baseline recording or more commonly as a reactive hyperaemia test. The laser light penetrates the tissue to a depth of about 0.5-1 mm, which will mean that not only nutritive capillaries will be assessed but also arterioles and arterio-venous shunts.

**Transcutaneous oxygen tension (TcpO2)**

The use of an oxygen sensor to determine the partial pressure of oxygen that diffuses through the skin has been used to assess the degree of ischaemia and to predict the future need for reconstructive surgery and limb loss. Cut off values were 30 mmHg in the former and less than 10 mmHg increase on breathing oxygen in the latter study. The probability of ulcer healing was investigated in patients with diabetes and ischaemic ulcers and was found to be low when TcpO2 was below 25 mmHg.

**Capillary microscopy**

With capillary microscopy the nutritive capillaries in the skin can be directly inspected usually at the nail folds of the toes. The appearance of the capillaries has been used to predict tissue necrosis in CLI. Capillary reactive hyperaemia is assessed in dynamic capillary microscopy.

**Skin perfusion pressure**

Skin perfusion pressure (SPP) can record a blood pressure closer to the tissue of interest in CLI as compared to ankle or toe blood pressure. Through direct pressure on the skin by a cuff, circulation in skin arterioles is arrested, release of the cuff will at a given pressure – the skin perfusion pressure – allow flow to resume. Flow can be detected with e.g. Xenon washout technique, laser Doppler or photoplethysmography.

The use of SPP to predict healing after amputations and the severity of ischaemia by forecasting future treatment in CLI has been attempted.

None of these methods have yet changed vascular surgical practise. A combination of nailfold capillary microscopy, TcpO2 and LD perfusion at rest and during reactive hyperaemia was used in patients with non-reconstructible CLI to create a good (capillary density >20/mm2, present reactive hyperaemia and TcpO2 >30 mmHg) and a poor microcirculatory group. The cumulative limb survival after 12 months was 88% in the good group and 17% in the poor microcirculatory group. Possibly the combination of several different methods as in this study will prove to be a way to better predict prognosis in CLI patients.

Most of these methods assess skin circulation while muscle tissue is harder to access non-invasively.

**Metabolic markers of ischaemia**

Assuming that inadequate tissue perfusion and resulting ischaemia is of vital importance in the pathophysiological process leading to loss of cellular integrity and eventually cell death in CLI, the occurrence of metabolic markers of ischaemia could be expected to precede a complete breakdown of metabolism. Identifying such potential markers in patients with CLI would be the first step in attempting their use as diagnostic or prognostic adjuncts.

**Lactate**

Under normal physiological conditions aerobic breakdown of glycogen, glucose and free fatty acids provide the majority of the energy required for cell functioning and maintenance of cellular integrity. Pyruvate is the common endpoint of glycogen and glucose degradation and in turn enters the citric acid cycle to produce large amounts of adenosine triphosphate, ATP. Under anaerobic conditions - like ischaemia or during high intensity exercise causing regional hypoperfusion - lactate formed by the reduction of...
Pyruvate is a temporary metabolic end product (Fig. 1). The reduction of pyruvate to lactate is essential to regenerate the NAD$^+$ necessary to maintain the anaerobic part of glycolysis. The lactate produced will be exported from the cell or stored in the cytoplasm and later oxidized to pyruvate under aerobic conditions. Circulating lactate is mainly metabolized in the liver where it is used in gluconeogenesis. Brain, cardiac muscle and kidney are other organs that can be net consumers of lactate. Anaerobic glycolysis and lactate production is a normal physiological event and is part of regular muscular activity.

Lactate production allows the tissue to “buy time” and maintain vital ATP production until oxygen is again available. Increasing lactate concentration and accompanying hydrogen ions will lower pH and contribute to deteriorating cellular function. The exact consequences of lactate accumulation are however under debate. Nevertheless, cell death will not ensue as long as ATP production is sufficient.

In patients with intermittent claudication lactate in muscle biopsies has been shown to increase during exercise, in parallel with a reduced oxygen partial pressure in the tissue. In acute limb ischaemia muscle biopsy lactate levels were found to be elevated and of prognostic importance for limb survival. Increased lactate release at rest in venous blood from the symptomatic limb has been demonstrated in patients with rest pain or ischaemic ulcers while patients with claudication had normal levels. Contrary to these findings, in another study similar resting lactate levels in femoral venous blood in healthy controls, patients with claudication and CLI respectively were found.

**Pyruvate**

The lactate to pyruvate ratio has been proposed to be a better marker for ischaemia than an isolated lactate increase. In states of hypermetabolism, e.g. sepsis, lactate can increase as a consequence of an abundance of pyruvate secondary to a high rate of glycolysis. In such a case the lactate to pyruvate ratio can be assumed to be unaltered while during ischaemia the lactate to pyruvate ratio is expected to increase. Under experimental conditions the lactate to pyruvate ratio increased in response to addition of adrenergic agonists to the perfusion fluid interpreted as a stimulus of glycogenolysis. When vasopressin was added, decreasing blood flow resulted in a sharp increase in lactate concentration while pyruvate remained unchanged. In skeletal muscle and subcutaneous adipose tissue when blood glucose is kept relatively stable and no sepsis is at hand it is probably of little difference if lactate or lactate to pyruvate ratio is used as a measure of ischaemia.

**Oxygen (O$_2$)**

Oxygen is required to maintain cellular energy production from oxidative phosphorylation. At the cellular level, tissue oxygen tension (pO$_2$) is the determinant of oxygen availability and in turn dependent on blood flow, arterial pO$_2$ and diffusion conditions from capillary to cell. Oxygen requirement vary widely between tissues and current metabolic rate, why metabolism can switch to anaerobic routes at very different pO$_2$ levels. Transcutaneous detection of oxygen partial pressure (TcpO$_2$) has been discussed above.

Invasive determination of tissue oxygen is possible by the use of a polarographic oxygen probe. Invasive measurement of tissue pO$_2$ has been used in a rat model of limb ischaemia where a significantly decreased pO$_2$ could be registered for 40 days and in a model of compartment syndrome where it was shown to better predict muscle necrosis than did compartment pressure.

Near infrared spectroscopy (NIRS) is an alternative method so far used mainly in research to assess the availability of oxygen in the tissue. Near infrared light is absorbed differently by oxygenated and deoxygenated haemoglobin and myoglobin respectively. This difference in absorption is utilized to give a relative measure of tissue oxygenation.

The hypoxia marker EF5, first described in 1993, is one of many drugs originally designed to act as radiosensitisers i.e. to increase the sensitivity of hypoxic cancer cells to radiation therapy. One key feature of these molecules is
their ability to bind to intracellular compounds in hypoxic cells. This is also the basis for the spin off effect of using the drugs as markers of hypoxia. Numerous trials have shown the potential to use EF5 to demonstrate hypoxia in a wide range of species and organs, e.g. cardiac muscle. In histological sections the resolution of the method allows the differentiation of hypoxic regions on a cell to cell level from areas with normal oxygenation.

**Carbon dioxide (CO₂)**

Under aerobic conditions CO₂ is produced by the citric acid cycle. A decreased perfusion leads to a reduced rate of CO₂ removal and a resulting increase in tissue CO₂ concentration. The magnitude of this increase is limited by the availability of oxygen. A switch to anaerobic metabolism is followed by the production of H⁺-ions. The H⁺-ions are buffered by HCO₃⁻ to H₂O and CO₂. This conversion of hydrogen ions is the major source of CO₂ during anaerobic conditions. Carbon dioxide has been shown to increase in skeletal muscle, subcutaneous adipose tissue, skin and cardiac muscle. In a pig model of complete skeletal muscle ischaemia tissue pCO₂ was found to correlate closely to lactate concentration increase and to precede phosphocreatine and ATP depletion.

**Phosphocreatine, ATP and purine metabolites.**

Cellular integrity and function rest on the continuous supply of energy in the form of adenosine triphosphate (ATP). ATP is needed not only to allow skeletal muscle contraction but also to maintain function of ion pumps and synthesis of cellular components. In skeletal muscle ATP can be derived through four main routes. First, free ATP is available in small quantities within cells. Second, the conversion of stored phosphocreatine to creatine generates one ATP molecule from one ADP. As an example, free ATP in muscle fibres can support vigorous contraction for about one second and the conversion of phosphocreatine to creatine gives ATP lasting another four seconds. Third, via anaerobic glycolysis through breakdown of glycogen or glucose when each glucose molecule yields three ATP molecules. Fourth, under aerobic conditions the citric acid cycle and the respiratory chain can produce ATP from pyruvate or free fatty acids when e.g. each glucose molecule will give about 38 molecules of ATP. Phosphocreatine has been proposed to be an early and sensitive indicator of tissue ischaemia in skeletal muscle, cardiac muscle, small intestine and aorta. Phosphocreatine and ATP-depletion precede tissue necrosis in skeletal muscle. In human skin ATP and phosphocreatine levels have been determined using biopsies and magnetic resonance spectroscopy (MRS). The depletion of ATP under severe ischaemia is accompanied by a continuous breakdown of purine metabolites. As ATP cannot be regenerated from ADP, ADP is instead converted to uric acid via inosine, hypoxanthine and xanthine. Elevated levels of these purine metabolites have been linked to the degree of skeletal muscle ischaemia both in human muscle transplants and in rat.

**Microdialysis**

With the introduction of the microdialysis technique it has become possible to – in a minimally invasive fashion - continuously sample and analyse substances from the interstitial space in e.g. brain, heart, skeletal muscle, subcutaneous adipose tissue and skin in animals and humans. The microdialysis technique is based on the principle that a perfusion fluid is equilibrated with the interstitial fluid by diffusion through a semi-permeable membrane (Fig. 2). Small molecules diffuse freely, larger molecules, especially when charged, with increasing difficulty to the cut-off level of the membrane used, determined by the pore size. The degree of equilibration over the membrane determines the concentration of a given solute in the microdialysate in relation to the concentration of the same substance in the interstitial fluid. This ratio – the concentration in the microdialysate divided by the concentration in the interstitial fluid – is expressed as a percentage.
and is termed the relative recovery of a substance.

Relative recovery increases with decreasing perfusion flow rate and increasing length of the probe membrane. Relative recovery is additionally influenced by size and charge of the substance of interest and diffusion characteristics in the tissue surrounding the catheter, and – as mentioned – over the membrane. Blood flow influences recovery in that a greater amount of blood borne substances will be available for removal by the microdialysis catheter. This will limit the effect of drainage of metabolites from the interstitial space by the microdialysis catheter that is another inherent feature of the microdialysis technique. E.g., microdialysate glucose concentration is the result of a balance between arterial supply, cellular uptake and removal of glucose by the microdialysis catheter. When blood flow changes, this balance will shift and a new equilibrium will be established. It has previously been shown that changes in blood flow dramatically affect microdialysate glucose concentrations176. Drainage can affect concentrations of solutes in the microdialysate and even affect “true” concentrations in the interstitium. To minimise the effect of drainage and other effects on the surrounding tissue the composition of the perfusate is normally kept as close as possible to the interstitial fluid. Exercise has also been demonstrated to influence relative recovery, possibly by altering diffusion conditions in the tissue130.

Absolute recovery is the amount in mmol of a solute that is removed from the interstitium per unit time. Absolute recovery increases with increasing perfusion flow rate and length of the probe membrane.

The concentration of substances obtained with the microdialysis technique normally does not equal true concentration in the interstitial fluid, as relative recovery is rarely 100%. However, with a very low flow rate and large dialysis membrane surface area relative recovery can approach 100%18,11,177. Under such circumstances microdialysis can be used to determine absolute concentrations of various substances.

Fig. 2. Schematic view of the principle of microdialysis. A perfusion fluid is equilibrated with the interstitial fluid as small molecules diffuse through a semi-permeable membrane of a percutaneously placed catheter. Illustration: CMA Microdialysis.

MR

The magnetic resonance (MR) and magnetic resonance imaging (MRI) techniques have had tremendous impact on modern medicine and continue to find new applications. In magnetic resonance imaging, a radiofrequent (RF) pulse of high intensity excites protons – mainly in water and fat – subjected to a strong magnetic field. An RF signal is generated, detected and after mathematical transformation displayed as an image. The decay rate of the RF signal is called the transverse or $T_2$ relaxation time. The decay rate of the spins from the excited state towards equilibrium is called $T_1$ – the longitudinal relaxation time. $T_1$ and $T_2$ are dependent on the magnetic field strength162,173.

Direct determination of lactate levels by proton magnetic resonance spectroscopy (MRS) has been used predominantly in the brain168, its use during aortic surgery17,11,139, in critical limb ischaemia pre- and peroperatively140 (Study II) and during prostacyclin treatment45.
in skeletal muscle is hampered by difficulty in separating the lactate derived signal from influence of surrounding adipose tissue. Phosphorous magnetic resonance spectroscopy (MRS) has been used to determine tissue concentrations in vivo of ATP and phosphate/phosphocreatine ratio in human muscle. In mainly myocardial and cerebral ischaemia research - and to some extent clinical practice - a number of proton imaging techniques have been used. In delayed contrast-enhanced imaging infarcted cardiac muscle appears hyperenhanced when compared to surrounding normal tissue. Diffusion weighted imaging has been used in the early detection of brain ischaemia. 

$T_2$ relaxation time changes in skeletal muscle have been studied extensively mainly under exercise conditions in healthy subjects but also in various pathological conditions. $T_2$ characteristics of the tissue are strongly influenced by body water content and the composition of fluid in the tissue. In many situations a link between tissue fluid composition and lactate concentration can be found suggesting the possibility of using $T_2$ relaxation time as an indirect marker for tissue lactate concentration.

A $T_2$ map is the graphical representation of calculated $T_2$ relaxation times in a cross-section of the studied tissue.

**Animal models of limb ischaemia**

Several species and techniques have been used in the pursuit of experimental animal models mimicking the consequences in peripheral tissue of PAD. The aims of such studies have been to find acceptable models in which to test new diagnostic or therapeutic modalities and to improve the understanding of pathophysiological events. Different species e.g. mice, rats, rabbits, and dogs have been used. Some models have been used predominantly for ischaemia-reperfusion experiments, others to represent claudication, and some to achieve substantial long-lasting ischaemia already at rest. We sought an animal model able to produce metabolic consequences of severe limb ischaemia of sufficient duration to allow studies of different markers of ischaemia and adaptive mechanisms. For this purpose we modified and evaluated a rat model of unilateral resting limb ischaemia. A two-stage surgical procedure entailing left femoral artery ligation preceded by interruption of branches originating from the infra-renal aorta and left iliac arteries was performed in rats. This and some of the other models will be described and discussed in detail later (Page 55).

**Human models of limb ischaemia**

**Tourniquet**

Near complete occlusion of the arterial and venous circulation in a limb can be achieved by the use of a cuff or tourniquet applied to the thigh and inflated to supra-systolic pressure. Some circulation will be maintained via intraosseous vessels. Such models have been used extensively to study mainly acute ischaemia-reperfusion phenomena.

**Lower body positive pressure**

Eiken and Bjurstedt developed an experimental model initially designed to study effects on central circulation and respiration. Later it was used to mimic the blood flow restriction encountered in patients with PAD. In this model, muscle ischaemia is induced by the application of positive pressure over the lower part of the body including a working leg. The surrounding atmospheric pressure does not influence arterial pressure but venous pressure is elevated to a level just exceeding the atmospheric pressure with a resulting decrease in perfusion pressure. With 50 mmHg applied, exercise blood flow is reduced by 15-20% . Venous lactate has been shown to correlate to the degree of blood flow restriction applied. Major advantages of this model are the capacity to vary the degree of blood flow restriction in a controlled fashion and the possibility to take muscle biopsies and use microdialysis under a relatively long time.
AIMS

To explore new methods to potentially improve the definition of CLI aiming at safer patient selection to treatment.

To investigate if various degrees of experimentally induced leg blood flow restriction can be monitored by microdialysate lactate concentrations in humans.

To correlate microdialysate, skeletal muscle and venous lactate concentrations under such conditions.

To study the feasibility of using microdialysis in working skeletal muscle in humans.

To describe metabolic alterations in skeletal muscle and subcutaneous adipose tissue in patients with critical limb ischaemia.

To correlate these alterations to the degree of ischaemia assessed with conventional methods.

To describe and evaluate an animal model for unilateral resting limb ischaemia to allow further studies on diagnostic methods and pathophysiology.

To evaluate if MR T₂ relaxation time alterations can be used to assess the degree of ischaemia in resting skeletal muscle in rats.
Göran Lundberg
METHODOLOGY

Subjects and animals

Healthy subjects

Study I
Nine healthy male volunteers took part in the study. Mean (range) age, height, weight and number of physical exercise hours/week were 24 (22-27) yrs, 182 (169-195) cm, 74 (63-90) kg and 3 (0-5) hours. The study was approved by the Ethics Committee of the Karolinska Institute. The experimental protocol was explained to all subjects and their consent was obtained before inclusion.

Patients

Study II
Ten non-diabetic patients with critical limb ischaemia according to the Second European Consensus Document definition i.e. rest pain or tissue loss and ankle pressure <50 mmHg or toe pressure <30 mmHg took part in the study. The study was approved by the Ethics Committee of the Karolinska Hospital. All patients received oral and written information and consent was obtained prior to inclusion.

Animals

Study III
Forty male Sprague-Dawley rats were divided into five groups and kept for different time periods after induction of ischaemia; one day, one week, two weeks, four weeks and eight weeks. These rats were used for laser Doppler perfusion assessment and for histology. The same rats were also used for microdialysis lactate determinations (unpublished data). Three different rats were subjected to arteriography. Another 14 were used to measure femoral artery volume blood flow and blood flow in muscles using fluorescent microspheres.

All protocols were approved by the Ethics Committee in Stockholm County. The volume blood flow and microsphere experiments were approved by the Committee on Animal Research, University of California, San Francisco, United States.

Study IV
Twenty male Sprague-Dawley rats were prepared as in Study III. Ten were used for a longitudinal study and underwent repeated MR scans at 1 day, 1 week and 2 months post surgery.

Ten other rats were used for an experiment correlating MR T₂ relaxation time to lactate levels. These rats were divided into two groups of five animals each. Five rats underwent MR scans at 1 day after the second operation and five rats at 1 week.

The experiments were approved by the Ethics Committee in Stockholm County.

Methods

Study I

Pressure chamber
The subjects performed one-legged knee-extension exercise in a pressure chamber (Fig. 3). The workload was selected individually during a familiarisation exercise, aiming at a constant workload that would give exhaustion at the end of the experiment when blood flow was restricted. This workload was then used for all exercise periods in each individual.

The protocol involved two different experiments, one with blood flow restriction (R) and one with non-restricted blood flow (NR). All 9 subjects participated in the R-experiment, where external application of pressure – 30 and 50 mmHg – over the working leg restricted blood flow during part of the experimental session. In this way exercise was performed under three different levels of blood flow (see below). Five of the subjects also took part in the NR-experiment.

Each experiment started with the insertion of two microdialysis catheters into the vastus lateralis muscle of the working leg. The subjects rested for one hour to allow equilibration of the metabolites of interest. Before the onset of exer-
exercise they were positioned in the chamber opening. Three 15-min exercise periods (Ex 1-3) were performed. Ex 1 was carried out under normal atmospheric pressure. In the R-experiment 30 and 50 mmHg supra-atmospheric pressure, respectively, was applied over the working leg during Ex 2 and Ex 3.

In the NR-experiment, normal atmospheric pressure was applied throughout the experiment including the exercise periods (Ex 1-3). The subjects rested for 10 min – under normal atmospheric pressure – between exercise periods. Microdialysate samples were collected every 5 min.

In the R-experiment four muscle biopsies were taken from the vastus lateralis muscle; immediately (5-10 s) after each exercise period and 10 min after the last exercise period.

Muscle biopsies
The percutaneous needle biopsy technique was used to obtain samples from the vastus lateralis muscle of the working leg. Biopsies were taken from a position 2-6 cm proximal to the tips of the microdialysis catheters. The biopsies were frozen in isopentane precooled with liquid nitrogen and stored at -70°C until later analysis. Muscle lactate was analysed in neutralized perchloric acid muscle extract by a fluorometric enzymatic method.

Microdialysis
We used a catheter with a diameter of 0.5 mm and membrane length of 30 mm (CMA 60, CMA, Solna, Sweden). The perfusion fluid had the following composition: Na⁺ 147 mM, K⁺ 4 mM, Ca²⁺ 2.3 mM, Cl⁻ 156 mM; osmolality 290 mosm l⁻¹. The pump used was a CMA 107, (CMA, Solna, Sweden). Flow rate was 2 µl min⁻¹.

Two microdialysis catheters were placed in the vastus lateralis muscle 2-3 cm apart, 10 cm proximal to the knee joint space at a 45° angle to the surface of the skin with the tip proximally. Mean concentration in the two vials corresponding to the last 5 min of pre-exercise rest, exercise 1-3 and post-exercise periods was used for statistical comparisons. The calculated time delay from catheter to vial was one min at the

![Fig. 3. Schematic view of the pressure chamber. Application of supra-atmospheric pressure over the lower part of the body allows one-legged knee extension exercise to be performed under blood flow restriction. Illustration: O. Eiken.](image-url)
flow rate used and this was compensated for when timing the collection of the microdialysate.

Microdialysate samples were stored at -20°C and subsequently analysed using the CMA 600 Microdialysis Analyser (CMA, Solna, Sweden) for glucose, lactate, glycerol and urea.

Study II

Model for ischaemia provocation

Patients with CLI and rest pain typically report recurrent rather than continuous pain. The pain is often worse in bed at night when systemic blood pressure falls and the contribution of gravity to the perfusion pressure in the foot is lost. To be able to demonstrate metabolic alterations with a relation to ischaemic symptoms we sought to imitate this physiological reduction of blood flow during the experiment. This was accomplished by elevating the symptomatic lower leg to a position 30 cm above the level of the heart (Fig. 4). This position was maintained for one hour (n=7) or until the patient experienced severe pain in the foot (n=3).

Microdialysis

The same equipment as in Study I was used. Prior to insertion of the catheters, the skin was anaesthetised with 0.5 ml bupivakain (Carbocain®, Astra, Södertälje, Sweden). Three microdialysis catheters were used. Two were placed subcutaneously, one on the lateral aspect of the dorsum of the foot and one on the anterior aspect of the lower leg, midway between the tuberositas tibiae and the lateral malleolus two cm lateral to the tibia. The third catheter was placed in the anterior tibial muscle, five cm proximal to the subcutaneous catheter at a 45° angle to the surface of the skin with the tip proximally. Microdialysate samples were collected in ten-minute fractions. Flow rate was 1 µl min⁻¹. A mean of the last two samples, i.e. a total sampling period of 20 minutes in the horizontal and elevated positions was used for statistical comparisons.

Microdialysis samples were stored as described above and analysed for glucose, lactate and pyruvate.

Fig. 4. Elevation of the symptomatic lower leg 30 cm above the level of the heart in order to further reduce blood flow in patients with critical limb ischemia. Microdialysis catheters and pumps, laser Doppler and TcPO2 probes in place. Photo: Anders Vigant.
TcpO$_2$

The TcpO$_2$ electrode was placed on the dorsal aspect of the forefoot between metacarpophalangeal joints I and II. Measurements were carried out at an electrode temperature of 44°C. Registration was done every 10 min through-out the experiment and a mean value was calculated corresponding to each leg position.

**Ankle (AP) and toe (TP) blood pressure**

Brachial systolic blood pressure was measured with a standard 12 cm cuff and a stethoscope. Ankle systolic blood pressure was measured with an identical cuff and a continuous-wave pen-Doppler over the dorsal pedal artery or, when this artery was impossible to insonate, the posterior tibial artery. Toe systolic blood pressure was measured with a two cm cuff on the first toe and a laser Doppler probe on the pulp of the first toe. The mean of two pressure readings that made laser Doppler flux reappear on release of cuff pressure was registered as the toe systolic blood pressure.

**Laser Doppler fluxmetry**

A computerized system (Perisoft®, Perimed, Stockholm, Sweden) for continuous registration of resting flux, expressed in arbitrary units, from four laser Doppler probes was used (Perimed 4001®, Perimed, Stockholm, Sweden). One probe was placed on the pulp of the first toe and was also used for toe blood pressure measurement. The remaining three probes were placed one next to each microdialysis catheter. A mean resting flux value from each probe was calculated corresponding to the horizontal and to the elevated leg positions.

**Study III and IV**

**Rat model (III, IV)**

The model we used is a two-stage procedure that creates ischaemia in the left hind limb whereas the right serves as control.

The rats were sedated by inhalation of methoxyflurane (Metofane®, Schering-Plough Animal Health Corp., Union, NJ, US) dispersed in a glass cylinder and anaesthetised with a combination of Hypnorm (fentanyl/citrate 0.315 mg/ml and fluanisone 10 mg/ml, 0.05 ml/100 g BW) and pentobarbital (60mg/ml, 0.05 ml/100 g BW) intraperitoneally.

The first operation was performed through a midline laparotomy. By the aid of a microscope all branches originating from the left side of the aorta distal to the renal arteries, a branch from the left renal artery and all branches from the left iliac artery were visualised, ligated with 6-0 resorbable suture and divided (Fig. 5). After a week the rats were again anaesthetised and a second operation was performed. Through a left inguinal incision, the femoral artery was ligated close to the origin of the superficial epigastric artery. The latter was also ligated and divided. From the femoral artery between the level of the inguinal ligament and its division arises the superficial circumflex iliac artery.

Cutting the superficial epigastric artery and preserving the superficial circumflex artery differs from the original procedure and was done to achieve slightly less severe ischaemia and to further simplify and standardise the operative procedure. Moreover, the spared artery serves as an ideal model of a developing collateral vessel and is easily harvested at a later time point.

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**Fig. 5. Schematic view of the rat model for unilateral resting limb ischemia demonstrating ligatures on all branches from the left side of the infrarenal aorta, the left iliac artery and the ligature on the femoral and superficial epigastric arteries.**
An analgesic (Temgesic®, buprenorphin 0.4 mg in 200 ml water, Reckit & Colman, Hull, England) was mixed into the drinking water both after the first and second operations.

Laser Doppler Perfusion Imager (LDPI) (III)
A moorLDI-VR (Visible Red Laser Doppler Imager, Moor Instruments Ltd, Axminster, England) was used to assess limb perfusion. The laser Doppler source is mounted on a desktop stand and a laser beam scans the tissue using a moving mirror. The laser beam reflected from moving red blood cells in nutritional capillaries as well as in arterioles and venules is detected and processed to provide a flux value. The information is colour coded to provide a map of tissue perfusion (Fig. 6).

Regions of interest corresponding to the plantar and dorsal aspects of the hind paws and the exposed anterior tibial muscles were marked manually using the Moor LDI Image Processing V 3.01 software. Mean flux values within the regions were then calculated to allow comparisons between the two sides.

Measurements were done under anaesthesia as described above. The rats were breathing room air spontaneously. Supplementary oxygen was delivered on demand through a facemask, maintaining the saturation at or above 90%. To reduce heat loss during measurements the rats were kept on a 37°C heating plate.

Haemoglobin oxygen saturation (III, IV)
The haemoglobin oxygen saturation was measured with a pulse oximeter (Nonin 8500V, Nonin Medical, Inc., Plymouth, MN, USA) using a flexible sensor (Sensor 2000 SA, Nonin) on a forepaw.

Angiography (III)
Three rats (one day, two weeks and four weeks) were anaesthetised and the proximal abdominal aorta was ligated through a midline incision. The aorta was cannulated distal to the ligature and the tip of the catheter was placed just above the bifurcation. The rats were positioned supine on an image plate (AGFA), directly on the collimator of a mobile X-ray system. First, 0.5 ml of papaverin (4mg/ml) was injected followed by 0.3 ml/100mg body weight of Omnipaque® (325 mg/ml) diluted to 75% in saline. Immediately after contrast injection an image was obtained. The developed films were blinded and then examined by one of the authors to identify missed branches and to compare differences in the presence of collateral vessels between the control and the ischaemic side.

Volume blood flow (III)
Volume blood flow was measured with transit-time technique (Transonic Instruments, Seattle, USA). Through skin incisions in both groins one mm probes were applied to the distal femoral arteries. Volume blood flow in ml/min was simultaneously measured in both limbs for ten minutes and the values recorded on a strip chart. The mean value during the last minute from the recording was used for comparisons.

Microspheres (III)
Analysis of blood flow using microspheres was done by one of the authors of Paper III. Six rats at one day of ischaemia, six at four weeks and two at eight weeks were used. The rats were ana-

Fig. 6. Example of LDPI scan of dorsal aspects of ischemic and control hindpaws in the rat model of unilateral limb ischemia.
esthetised and both carotid arteries cannulated through a midline neck incision. A catheter was placed via the right carotid artery with the tip in the left ventricular cavity and used for injection of microspheres. The left catheter was advanced to the aorta and later used to draw the reference sample and to measure arterial blood pressure between microsphere injections. The left carotid artery was used to conserve the leg arteries and distal aorta from a cannulation that might have interfered with perfusion of the legs.

Around 400 000 fluorescent microspheres (Fluospheres, Molecular probes Inc, Oregon, USA) were injected during 60 seconds. The reference blood sample was drawn at a constant rate of 0.39 ml•min\(^{-1}\) for 90 seconds starting before the injection of spheres. The procedure was repeated twice using spheres with different colors (blue-green and red) and the mean value was used for analyses.

The fluorescence intensity in the supernatant from each dissolved sample was measured using a spectrophotometer (Perkin-Elmer LS 50). The fluorescent signal from each tissue sample is proportional to the number of microspheres dissolved and blood flow in each sample was calculated by multiplying the fluorescent intensity and the reference sample withdrawal rate and dividing with fluorescent intensity in the reference sample.

For flow determination with microspheres the whole anterior tibial, soleus and gastrocnemius muscles and the proximal half of the excised adductor magnus and semimembranosus muscles and vastus lateralis were used.

**Histology (III)**
The anterior tibial muscles from both sides were carefully dissected en bloc and weighed wet. In a subset of three rats at one day, one week, four weeks and eight weeks this muscle was used for histologic examination. For comparison, samples were also taken from the adductor muscles (adductor magnus and semimembranosus). The samples were snap frozen in liquid nitrogen and stored at -80\(^\circ\)C. From each specimen, three 5 \(\mu\)m thick sections were cut using a cryostat (Miles) and placed on a glass slide.

The sections were stained (Hematoxylin and Eosin) and three muscle specimens from each side were randomly selected and used for descriptive histology performed manually under a light microscope at high-power magnification by one of the authors of Paper III in a blinded fashion. The findings were subjectively graded (0-3) regarding presence of ischaemic signs; oedema, inflammation and necrosis, (0 = no changes, 3 = pronounced changes) by the examiner.

**Microdialysis (III) (unpublished data)**
Microdialysis probes with a 10 mm long membrane, 0.5 mm in diameter (CMA 20, CMA Microdialysis, Solna, Sweden) were used. Probes were inserted in the anterior tibial muscles of both limbs after exposing the muscle via a skin incision. The probe was perfused with an isotonic perfusion fluid (Na\(^+\) 147 mM, K\(^+\) 4mM, Ca\(^{2+}\) 2.3 mM, Cl\(^-\) 156 mM; osmolality 290 mosm\(\text{L}^{-1}\)). A CMA 102 pump was used and flow rate set at 2 \(\mu\)l min\(^{-1}\). A one-hour equilibration period was allowed before sampling started.

**MR (IV)**
All experiments were performed on a 4.7 T/40 cm Biospec Avance system (Bruker, Karlsruhe, Germany) with a 40 cm bore horizontal magnet. The system was equipped with a 12 cm self-shielded gradient coil capable of producing gradients to a maximum of 200 mT m\(^{-1}\). Images were acquired using a 7.2-cm inner diameter birdcage coil. Prior to and during scanning the animals were anaesthetised by inhalation of Fluothane in oxygen. Immediately before and after scanning the haemoglobin oxygen saturation was measured. Saturation was kept at or above 92% in all animals.

The rats were placed in the supine position using a custom made cradle and the lower limbs were secured with adhesive tape to maintain an extended position in both hip and knee joints. Body temperature was maintained by a circulating warm air stream through the magnet bore during the experiment.

A fast gradient echo imaging sequence technique (SNAPSHOT) was used for scout images to allow correct positioning of the rat. Determination of \(T_1\) was done by a multislice multiecho
Data was acquired using a total of 32 spin echoes giving 32 images with echo time (TE) ranging from 6.13-196.16 ms. Quantitative T2 maps were generated by a non-linear least-squares fit to the normalised image intensity vs the TE values on a pixel-by-pixel basis for each slice using ParaVision software.

An image of a slice through the maximum diameter of the anterior tibial muscles was used for quantitative analysis. Regions of interest (ROIs) were marked manually with a cursor on the screen and an average T2 relaxation time value from one ROI in each muscle was automatically calculated. Care was taken to make sure the entire ROI was within muscle tissue excluding visible vessels and bone. In the group of rats that were sacrificed after completing scanning at 1 day and 1 week respectively, the anterior tibial muscles were harvested and snap frozen in liquid nitrogen for later analysis.

Clinical assessment (IV)
In both groups at all time points the status and behaviour of the rats were assessed by direct observation during five minutes by the author. This was done prior to the MR scan to minimise bias. A score on a four-graded arbitrary scale developed for this study was given for each of the following findings; cyanosis of the plantar aspect of the foot 0-3 (0=normal, 3= cyanotic), locomotion in the cage (0=normal, 3=static), use of the operated limb (0=normal, 3= foot never touches the ground).

Dark spots on the plantar aspects of the digits and on the prominent plantar pads on the sole of the ischaemic limb were noted in most rats between the first and fourth postoperative weeks. These spots likely to indicate superficial ischaemic gangrene were counted (0 = no spots, 1 = 1 spot, 2 = 2 spots, 3 = three or more spots). Finally, a sum of all variables (0-12) was calculated for each rat and correlated to T2 levels.

Statistics

Study I
For statistical comparisons, the mean of the microdialysate concentrations from the two simultaneously sampled vials was calculated. A two factor ANOVA was applied to compare differences between the R- and the NR-experiments (n=5). The interaction term was accepted as statistically significant at $p<0.05$ and in the subsequent contrast analysis Bonferroni correction was used. Corrected $\alpha$-level for repeated comparisons was set at $\alpha = 0.05/2$.

A one-way ANOVA was applied to compare differences between the three degrees of blood flow restriction in microdialysate lactate (n=9) and muscle biopsy lactate (n=8) concentrations. For post-hoc comparisons of means, Scheffé's test was applied. To analyse the relationship between the concentrations of muscle biopsy lactate, microdialysate lactate and venous blood lactate, simple linear regression was applied.

Study II
Wilcoxon signed-rank test was used for comparisons between the horizontal and the elevated position regarding microdialysate concentrations and pressure readings (AP, TP, TcPO2). The same method was used to compare microdialysate concentrations at the different catheter sites in both leg positions. Simple linear regression was used to correlate the microdialysate concentrations in the horizontal position, in the elevated position and the concentration changes between the two conditions with the degree of limb perfusion assessed by conventional methods (AP, TP, TcPO2, laser Doppler fluxometry) in the horizontal and elevated positions.

Study III
Wilcoxon signed-rank test was used to compare results from the ischaemic and the control sides. For comparisons of data over time ANOVA was used. For all analysis $p<0.05$ was interpreted to denote statistical significance.
Study IV
Wilcoxon signed-rank test was used to compare results between the operated and the control side. To assess the relation between T₁ relaxation time and lactate concentration and between T₂ and clinical ischaemia grade Spearman rank order correlation coefficient was calculated. For all analyses, p<0.05 was interpreted to denote statistical significance.
REVIEW OF RESULTS

Microdialysis for the assessment of graded leg ischaemia (I)

The main finding from this study was that intramuscular microdialysate lactate concentration increased with stepwise increments in the degree of blood flow restriction applied (Fig. 7).

Microdialysate glucose concentrations increased during exercise but with no significant difference between ischaemic and non-ischaemic exercise (Fig. 8).

Microdialysate lactate concentrations correlated strongly to lactate concentrations in venous blood (Fig. 9a-d) and tended to correlate to lactate concentrations in muscle biopsies (Fig. 10a-d).

We were able to demonstrate that microdialysis could be used in working skeletal muscle with acceptable <5% damage to the dialysis membrane.

![Graph](image_url)

Fig. 7. Microdialysate lactate concentrations (mmol/L) in five minute fractions during the experiment including exercise with blood flow restriction (R-experiment, n=9, □) and the control experiment with non-restricted blood flow (NR-experiment, n=5, ▲). Before, in between and after the three 15 min one-legged knee-extension exercise periods (Ex 1-3, shaded boxes) the subjects were resting. In the R-experiment, normal atmospheric pressure, 30 and 50 mmHg supra-atmospheric pressures were applied over the working leg during the Ex 1-, Ex 2- and Ex 3-periods, respectively, and ▲ denotes time points when muscle biopsies were taken. In the NR-experiment, normal atmospheric pressure was applied during all three exercise periods. Values are means +/- SE.
Fig 8. Microdialysate glucose concentrations (mmol L⁻¹) in five minute fractions during the experiment including exercise with blood flow restriction (R-experiment, n=9, □) and the control experiment with non-restricted blood flow (NR-experiment, n=5, △). Values are means +/- SE. For explanation of other symbols see Fig. 7.

Figs 9. a-d Correlations by simple linear regression between individual microdialysate and venous blood lactate concentrations at the end of exercise periods 1 (a), 2 (b), 3 (c) and post exercise (d). Each symbol refers to the same individual in all diagrams (Figs. 9 & 10). The correlation coefficient (r) and corresponding p-value is given in each diagram when correlation is significant.
Figs 10. a-d Correlations by simple linear regression between individual microdialysate and muscle biopsy lactate concentrations at the end of exercise periods 1 (a), 2 (b), 3 (c) and post exercise (d). Each symbol refers to the same individual in all diagrams (Figs. 9 & 10). The correlation coefficient ($r$) and corresponding p-value is given in each diagram when correlation is significant.
Microdialysis in patients with critical limb ischaemia (II)

Elevation of the lower leg caused a marked decrease in ankle and toe systolic blood pressures (AP and TP), TcpO₂ and distal laser Doppler flux levels (Fig. 11). This was interpreted as an achievement of the intended aggravation of ischaemia during the experiment.

Microdialysate glucose levels decreased significantly at all sites in the elevated position (Fig. 12). Microdialysate lactate levels increased in most patients in the anterior tibial muscle (Fig. 13). There was no clear correlation between microdialysate lactate levels or lactate level changes, neither at baseline nor in the elevated position and AP, TP, TcpO₂ or distal laser Doppler flux levels. This finding must be interpreted with care, as the number of patients was limited.

Three of the patients were unable to maintain the elevated position for the intended 60 minutes due to pain in the foot (Figs. 12, 13). These patients - although a small number – interestingly enough had among the highest lactate levels at baseline and among the highest increments following elevation.

Microdialysis in this setting was associated with some discomfort for the patients despite the use of local anaesthetics. However, no problems with healing, no infections or other complications caused by the microdialysis catheters were seen.

The metabolic consequences of what is generally perceived as limb threatening ischaemia were surprisingly limited in the majority of the patients.

- Fig. 11. Effect of leg elevation on ankle and toe systolic blood pressures and transcutaneous oxygen tension (TcpO₂) on the dorsum of the foot.
Review of Results

Fig. 12. Capillary blood glucose concentration before the start of the experiment (n=7). Microdialysate glucose concentration in the subcutaneous foot catheter (n=7), subcutaneous catheter on the lower leg (n=9) and in the anterior tibial muscle catheter (n=9). Horizontal and elevated position. Interrupted lines indicate patients who were unable to maintain the elevated position for one hour due to severe pain in the foot.

Fig. 13. Microdialysate lactate concentration in the subcutaneous foot catheter (n=7), subcutaneous catheter on the lower leg (n=9) and in the anterior tibial muscle catheter (n=9). Horizontal and elevated position. Interrupted lines indicate patients who were unable to maintain the elevated position for one hour due to severe pain in the foot.
The rat model for unilateral limb ischaemia (III)

We modified and evaluated a rat model of unilateral limb ischaemia attempting to mimic the consequences regarding perfusion and metabolism in peripheral tissue of blood flow reduction in severe PAD.

Perfusion measured by LDPI was significantly decreased in the dorsal aspect of the foot for the full eight-week follow-up (Fig. 14). Volume blood flow was decreased in the ischaemic limb still after four weeks (0.9 vs 4.2 ml min$^{-1}$ p=0.01).

At one day there was no difference between the two sides while at all other time points anterior tibial muscle mass was significantly lower on the ischaemic side as an indication of atrophy. Histologic signs of ischaemia – oedema, inflammation and necrosis – were seen for up to four weeks.

Angiography demonstrated collateral vessels on the ischaemic side after two weeks. Microsphere assessment was inconclusive due to technical problems.

Fig. 14. LDPI perfusion in the dorsal aspect of the foot (a), the plantar aspect of the foot (b) and the exposed anterior tibial muscle (c) in the control and in the ischemic limb at different time points after the second operation. Arbitrary perfusion units. Box plot showing median, 25th and 75th percentiles (box) and 10th and 90th percentiles (whiskers).
Microdialysate lactate levels from probes in the anterior tibial muscles were markedly increased after 24 h while the increase did not quite reach statistical significance after one week, followed by gradual normalisation at later time points (Fig. 15) (unpublished data).

Favouring of the control limb and cyanosis of the ischaemic limb was seen during the first week. Small dark spots were seen in most rats between one and four weeks. None of the rats showed signs of gangrene or major tissue loss and they also gained weight over time indicating that they sustained the procedures well.

**MR T₂, lactate and clinical assessment in experimental resting limb ischaemia (IV)**

The main finding was that severe ischaemia led to marked prolongations of T₂ relaxation time in resting skeletal muscle 1 day after surgery (Fig. 16). T₂ levels gradually returned to baseline levels over a period of two months (Fig. 17). T₂ levels showed a strong correlation both to clinically assessed degree of ischaemia at one day (Fig. 18) and to intramuscular biopsy lactate concentrations (Fig. 19). Minimal changes in both T₂ and lactate levels were seen in the control limb (Fig. 20). At one week, there was no correlation between clinically assessed degree of ischaemia and T₂ levels.

![Fig. 16. MR T₂ map from a cross section through the lower legs from one rat at 1 day. Ischaemic limb to the right in the picture. Region of interests were marked manually on the screen. Mean T₂ relaxation time within each region of interest was used for comparisons. The anterior tibial muscle was generally most affected.](image-url)
Fig. 17. $T_2$ relaxation time (msec) at one day, one week and two months in the operated and the control limb in individual rats in the longitudinal study group, $n=10$.

Fig. 18. $T_2$ relaxation time (msec) in the operated limb vs. clinically assessed degree of ischemia at one day in both study groups, $n=15$. The clinical assessment denotes a sum of cyanosis (0-3), locomotion in the cage (0-3), use of operated limb (0-3), dark spots on the plantar pads of the operated limb (0-3). On the vertical axis zero is omitted to adjust for an approximate base line $T_2$ level corresponding to the control limb level. The Spearman rank correlation coefficient and corresponding $p$-value is given in the figure.
Fig. 19. $T_2$ relaxation time (msec) vs. intramuscular lactate (mmol kg$^{-1}$) in the ischaemic limb in the lactate biopsy group. On the vertical axis zero is omitted to adjust for an approximate base line $T_2$ level corresponding to the control limb level. Solid squares denote one day and open squares one week, $n=10$. The Spearman rank correlation coefficient and corresponding p-value is given in the figure.

Fig. 20. $T_2$ relaxation time (msec) vs. intramuscular lactate (mmol kg$^{-1}$) in the control limb in the lactate biopsy group. On the vertical axis zero is omitted to adjust for an approximate base line $T_2$ level corresponding to the control limb level. Solid squares denote one day and open squares one week, $n=10$. The Spearman rank correlation coefficient and corresponding p-value is given in the figure.
GENERAL DISCUSSION

When facing patients with critical limb ischaemia the aim for modern vascular surgery should be to preserve ambulation and independence as long as this is the best option for the patient. Currently no methods are available that with any certainty can predict the fate of the limb — or life — of an individual patient with CLI.

Identification of metabolic alterations preceding tissue necrosis in chronically ischaemic limbs could be a means to advance the understanding of the pathophysiology of limb ischaemia and to improve patient selection for reconstructive surgery. Lactate is a sensitive marker for tissue ischaemia and could possibly be used for this purpose.

Metabolism in limb ischaemia

We first chose to use microdialysis for the determination of lactate as the method allows sampling close to the tissue of interest and so could be expected to be more specific than assessment of lactate release in e.g. venous blood. It is also a less invasive method than the alternative biopsy technique. Microdialysis had previously been used to monitor tourniquet-induced ischaemia in human subcutaneous adipose tissue and skeletal muscle. As microdialysis had not been used for grading of ischaemia we set out to test its applicability in doing so in healthy subjects (Study I).

When using microdialysis for lactate determination under ischaemic conditions we found a good agreement between microdialysate lactate concentration and the degree of blood flow restriction applied. This is in line with previous experience studying lactate release in femoral and ante-cubital venous blood using the same experimental setup.

We found a strong correlation between microdialysate lactate concentration and lactate in ante-cubital venous blood. This was interpreted as resulting from a rapid diffusion of lactate from the interstitial space to venous blood during exercise and the phase of increased blood flow post-exercise. These findings lended support to the use of microdialysate lactate concentration as an indicator of changes in interstitial lactate concentrations in lactate-producing tissues such as dynamically exercising or ischaemic limbs. In patients with PAD where blood flow is slow, changes in lactate concentrations are likely to be found somewhat earlier in peripheral tissue than in venous blood.

The correlation between microdialysate lactate concentration and lactate in muscle biopsies from the working leg did not quite reach statistical significance. The fact that a biopsy is obtained at one instance while the microdialysate is sampled continuously during a longer period of time — in the present study five minutes — may make correlations less valid. Also, the biopsies were taken not during but immediately after exercise when intramuscular lactate concentration might no longer be in a steady state. Moreover, lactate may not diffuse freely over cell membranes why there could be some delay in the flux of lactate from the production-site in the cytosol to the interstitial space and as biopsies consist mainly of intracellular material and microdialysis samples derive from the extracellular space this might be important. Finally, although biopsies were obtained from the same part of the muscle as close as possible to the site of the microdialysis catheters, regional differences in lactate concentration could have been present. This could also explain part of the variability seen between the two microdialysis catheters.

As microdialysis had not been used before in working skeletal muscle we used two microdialysis catheters in each subject to make sure valid results could be obtained from at least one of the catheters. This also gave us the opportunity to compare results from the two adjacent sites in each patient. Differences between the two catheters could be physiological, signifying different metabolic activity at the two sites, or implying a methodological problem. Concerning lactate and glucose, differences were small; the
mean relative differences between the two catheters in relation to the average concentrations were 8% and 7%, respectively.

All the substances studied showed marked increases during exercise – whether ischaemic or not – probably explained by an increase in relative recovery related to the exercise. As blood flow increases during exercise the supply of substances not locally produced in the muscle, e.g. glucose and urea, will increase and, as discussed earlier, shift the equilibrium between supply, cellular uptake and removal by the microdialysis catheter resulting in increased microdialysate levels.

Additionally, tissue diffusion characteristics have been suggested to be altered during exercise leading to an increased relative recovery. Both these mechanisms are likely to contribute to the observed differences in metabolite levels seen during exercise as compared to rest.

An increase in relative recovery during exercise is likely also to account for part of the lactate level increase seen during ischaemic conditions. However, only for lactate was there a significant difference between non-ischaemic and ischaemic exercise why the main explanation remains a true lactate concentration increase. Supporting this notion is the fact that lactate levels under non-ischaemic conditions tended to be least affected by exercise, as compared to e.g. glucose.

It could be speculated that the increased blood flow per se would rather tend to lower lactate levels as more lactate is removed from the tissue, that the above mentioned effect on diffusion would tend to increase microdialysate lactate levels and that some increase in local lactate production even during non-ischaemic exercise would tend to increase supply and thereby microdialysate lactate levels. The balance could well be, as observed, only a minimal positive and decreasing effect on microdialysate lactate levels of non-ischaemic exercise.

In the patients with CLI the lower leg was positioned horizontally on a padded support cushion, bringing the ankle to a level 30 cm above the level of the heart (Study II). The rationale for this set up was to create a situation in which perfusion would be challenged and metabolic correlates to symptoms and signs of ischaemia could be tested. As discussed previously patients with rest pain often experience an aggravation of pain at night. Improving distal perfusion pressure by hanging the foot out of the bed or getting up to take a few steps often alleviate symptoms. Jelnes et al. used Xenon washout technique to demonstrate this phenomenon and found a 37% decrease in forefoot skin blood flow at night as compared to during daytime in CLI patients. In that study there was a strong correlation to systemic blood pressure alterations.

We found in our patients that elevation of the lower leg did cause a marked decrease in ankle and toe systolic blood pressures, TepO2 and distal laser Doppler flux levels. This was interpreted as an achievement of the intended aggravation of ischaemia during the experiment.

In four of the patients lactate concentrations increased substantially at one or more sites during elevation. Two of these patients had non-measurable ankle pressures in the elevated position due to non-detectable flow in the foot arteries, for the other two ankle pressure data in the elevated position were not available. In general, lactate concentration increases were most pronounced in the intramuscular catheter.

Subcutaneous lactate levels increased markedly in only two patients in the lower leg position and in only one patient in the foot catheter.

Intramuscular lactate levels were generally higher and increased in all but one patient. This difference between the tissues examined could possibly be explained by the lower energy consumption in subcutaneous adipose tissue resulting in greater resistance to low perfusion. Microdialysate glucose levels decreased significantly at all three sites during elevation consistent with the expected effect of a decreased blood flow. A decreased blood flow might lower microdialysate glucose levels by at least two mechanisms. First, a decreased flow will result in a decreased glucose supply and second, by increased cellular uptake as anaerobic glycolysis needs relatively more glucose to maintain ATP production. Consequently a decreased microdialysate glucose level is expected as blood
flow decreases but does not in itself signify ischaemia.

The lactate to pyruvate ratio did not show any consistent changes in our patients. However, pyruvate was available in only two of the patients with the greatest increases in lactate concentration. One of these showed an increase also in lactate to pyruvate ratio while in the other patient the lactate to pyruvate ratio decreased. The latter patient was one of those who interrupted the elevation position early.

In view of an unaltered or even lowered lactate to pyruvate ratio other explanations to the observed lactate concentration increases than ischaemia have to be considered. One possible reason for the substantial increase in pyruvate in some of the patients could be pain mediated sympathetic activation of glycolysis. In a microdialysis experiment in rat skeletal muscle the addition of adrenalin to the perfusate led to an increase in lactate and pyruvate interpreted as a \(\beta\)-receptor mediated activation of glycolysis. An increased metabolic rate as in sepsis or hyperglycemia appear less likely to explain the lactate concentration increases under the present conditions.

The metabolic consequences of profoundly reduced blood flow during this experiment were surprisingly small in the majority of our patients. However, Metzsch et al when using microdialysis in patients with PAD (Fontaine II-IV) per- and postoperatively while undergoing infrainguinal bypass surgery did similar findings. Glucose levels decreased at all catheters sites (s.e. lower leg, anterior and posterior tibial muscles) and intramuscular lactate levels increased during surgery. Lactate levels increased already during preparation and anaesthesia possibly explained by the elevation of the leg, relative hypotension preoperatively and the prolonged horizontal position on the operating table. Lactate levels were consistently slightly higher than in our patients and in previously performed experiments aiming at determining absolute concentrations of muscle and adipose tissue lactate by Rosdahl et al. Considering differences in flow rate it is possible that absolute lactate concentrations also in our patients were elevated as compared to expected levels from the study by Rosdahl. This could imply a chronic reliance on anaerobic metabolism in our patients and perhaps lactate levels could not increase further. Another possible explanation to the limited lactate response in our patients is the fact that the microdialysis catheters – to maintain patient safety – had to be positioned at locations where healing could be expected and thus by definition in tissue where the local degree of ischaemia could not be expected to be critical.

No complications to the use of the microdialysis catheters were seen. However, the procedure was perceived as time consuming by the patients and did cause some discomfort. The protocol demanded two to three hours bed rest in a fairly immobile position, the insertion of the microdialysis catheters was made easier but not completely pain free by the use of local anaesthetics. We concluded that for research purposes the use of microdialysis in this setting was feasible but that other methods must be sought, for use in a wider clinical setting.

The heterogeneity of these patients in terms of the degree of ischaemia appears to be relatively comparable to the experience from the above mentioned study on similar patients and in line with the notion from clinical practice that patients with critical limb ischaemia represent a wide spectrum.

In summary, we found microdialysis to be a feasible method for grading of ischaemia in healthy subjects. In some of our patients ischaemic pain appeared to have a metabolic correlate, however, many patients with CLI according to current definition showed no metabolic signs of tissue ischaemia when subjected to markedly decreased blood flow and especially not so in subcutaneous tissue.

**Pain in limb ischaemia**

Initially, we attempted to evaluate the degree of ischaemic foot pain during elevation with a visual analogue scale (Study II). However, great difficulties arose when trying to distinguish ischaemic pain from other symptoms from the leg caused by the position on the couch. The only reliable measure of ischaemic rest pain was...
therefore considered to be the inability to maintain the elevated position for one hour. Three of the patients were unable to do this due to severe pain in the foot. In two of these patients the period in the elevated position was long enough to allow microdialysis sampling (27 and 40 min) while one patient was excluded, as sampling time was too short (5 min). Both these patients were among the four with substantial lactate level increase on elevation.

The origin of ischaemic rest pain is not known in detail. Pain in skeletal muscle has been linked to a number of substances e.g. bradykinin, serotonin, calcitonin, substance P, potassium ions, histamine and prostaglandin E₂ (PGE₂), while lactate has been shown to elicit pain only at supraphysiological concentrations.\textsuperscript{138}

In a microdialysis study on resting skeletal muscle in anaesthetised rats during four-hour tourniquet induced ischaemia followed by reperfusion an increase in hypoxanthine, potassium, PGE, and histamine was demonstrated.\textsuperscript{133} Hypoxanthine increase was interpreted as a measure of ATP-depletion and preceded potassium ion concentration increase. The latter probably an effect of dysfunctions membrane ion pumps as a sign of failing energy supply. The potassium ion concentration was lower than levels known to elicit pain but it was assumed that potassium could act as a pain sensitizer. Rosendal et al used microdialysis in trapezius muscles of patients with chronic work related muscular pain.\textsuperscript{179} Increased resting concentrations of lactate, pyruvate, serotonin and glutamate – another potential pain eliciting substance – were seen. Low-force exercise increased both lactate and pyruvate further.

In addition to nociceptive pain mechanisms neuropathic pain has been described in patients with CLI.\textsuperscript{121} Nineteen patients with CLI – rest pain or nonhealing ulcers since more than four weeks – were evaluated clinically and with electrophysiologic methods. The gravity of the neuropathy correlated to ABI. The most frequent symptoms were numbness, paresthesia and pain often described as burning. The presence of neuropathic pain may help to explain the often limited effect of opioids in the management of pain in patients with CLI.

### Tissue viability

We studied tissue lactate concentration increase as a potential measure of a threateningly low perfusion in still viable tissue. In cells, free ATP is available only in small quantities and normally ATP is constantly regenerated. During limited periods of relative oxygen shortage, ATP production can be maintained by anaerobic glycolysis, only then with a much lower yield per glucose or glycogen molecule utilized and with a concomitant accumulation of lactate and hydrogen ions. In skeletal muscle a substantial store of ATP in the form of phosphocreatine is equally available as a temporary energy source. Notwithstanding, muscle tissue is more sensitive to ischaemia than skin and adipose tissue as skeletal muscle energy consumption is higher also under resting conditions.

The link between the duration of ischaemia, metabolism and the chain of events leading to tissue necrosis has been studied experimentally in numerous models. Tissue hypoxia is an early and, at least in the absence of mitochondrial dysfunction, essential sign of tissue ischaemia. On the other hand a decreased pO₂ can still be adequate to meet the demand of the tissue why in isolation it does not equal ischaemia. In a hypoxic rabbit model, skin pO₂ was significantly reduced by severe hypoxaemia while skin pCO₂ was unaltered.\textsuperscript{182} Methodological and physiological explanations were discussed where the latter would be that aerobic tissue metabolism in skin could be maintained despite severe hypoxia, in accordance with the low energy demand of skin.

When oxygen supply is insufficient to maintain oxidative phosphorylation phosphocreatine is utilized, anaerobic glycolysis is activated and lactate starts to accumulate. An increase in phosphocreatine was shown to precede lactate concentration increase by some researchers, while others have found the lactate concentration increase to be an earlier event than phosphocreatine and ATP depletion. In a pig model of skeletal muscle ischaemia tissue pCO₂ was found to be an early indicator of ischaemia, paralleling and being closely correlated to lactate concentrations. In dog gracilis muscle until
ATP stores were depleted only minor muscle necrosis occurred and the decrease in ATP concentration was preceded by muscle glycogen and phosphocreatine depletion\textsuperscript{20, 21}. Using a similar model after 3, 4, and 5 hours of complete ischaemia the extent of muscle necrosis was 2, 30 and 90\%\textsuperscript{117}.

In patients with PAD the inorganic phosphate/phosphocreatine ratio in foot muscle in vivo has been determined with phosphorous MRS and was even found to correlate to the severity of symptoms\textsuperscript{84}. The ratio – as a measure of phosphocreatine depletion – was higher in patients with rest pain than in claudicants, and yet higher in the patients with ulcer or gangrene.

Interestingly, in some of the above mentioned studies it was noted that muscle necrosis was most pronounced centrally in the muscle - possibly explained by the distribution of muscle fibres with a larger part of more oxygen dependent ischaemia sensitive red fibres centrally - and that the perfusion matrix i.e. vessel architecture and vessel function was preserved even when muscle fibres were irreversibly damaged\textsuperscript{20, 53}. These findings call into question the clinical practice of peroperative external evaluation of potentially non-viable muscle tissue by appearance and presence of muscular bleeding as e.g. when fasciotomies are performed.

The viability of skin and subcutaneous adipose tissue in relation to metabolism and ischaemia is less well studied. In our rat model the skin overlying muscles with regional necrosis was intact and there were only the dark spots likely to represent minimal tissue loss to be noted distally (Studies III & IV). Growth factors (FGF-2 and VEGF) were not elevated in ischaemic hind paw skin as compared to the control side in another study using the present rat model\textsuperscript{184}, aiming at achieving unilateral resting limb ischaemia of long duration without significant tissue loss.

**Validity of the animal model**

Even with the minimally invasive microdialysis technique it was not considered feasible to move on to determine the potential prognostic value of interstitial lactate determinations in a larger cohort of patients with CLI. Therefore we sought an experimental model of long-lasting ischaemia to allow validation of other methods.

Animal models of limb ischaemia should produce ischaemia of sufficient severity and duration to allow studies with relevance to human peripheral arterial disease. Not least important, any animal model must also be ethically acceptable.

We modified and evaluated a previously described rat model\textsuperscript{184}, aiming at achieving unilateral resting limb ischaemia of long duration without significant tissue loss.

**Severity of ischaemia**

In rat simple arterial ligation - iliac\textsuperscript{99, 175} or femoral\textsuperscript{13, 87} - produces a moderate degree of blood flow reduction at rest. Common iliac artery ligation reduced blood pressure by 40 to 60\% distal to the ligature\textsuperscript{175}. Femoral artery ligation reduced gastrocnemius muscle blood flow at rest using Xenon\textsuperscript{133} injection technique by 50 \% at 1 week and by less than 10 \% 10 weeks following ligation\textsuperscript{13}. With few exceptions these models do not produce ischaemia at rest but require exercise or other stimulation e.g. electrical to result in
ischaemia. In iliac or femoral ligation models the contralateral leg can be used as control. Aortic ligation produces a variable degree of ischaemia depending on e.g. sex and the opportunity to use the contralateral leg as control is lost.

To achieve significant ischaemia at rest more complex models must be used. Pu et al described a rabbit model comprising ligation of the external iliac artery and excision of the entire femoral artery. A significant increase in resting venous lactate concentration after 10 days was reported, after 40 days the difference in lactate concentration was small. Distal blood pressure was reduced for up to 90 days. The effect of the operation on the limb is a concern as a varying degree of superficial skin necrosis was seen in a third of the animals and ten percent had non-functioning hind limbs. Twenty percent of the animals died during the study period. This model has been used extensively for studies of angiogenesis because collaterals in the thigh are clearly visualized by angiography.

An identical femoral artery excision model has been described also in mice. Perfusion by LDPI was reduced for 28 days. A drawback of the femoral artery excision models using either species is the rather extensive dissection in the thigh, which may affect the surrounding tissue during the procedure and influence tissue analyses at early time points.

Seifert et al described a two-stage operation in rat where all branches from the left side of the aorta below the left renal artery and the left iliac artery were divided in the first operation. A week later, the left femoral artery just below the inguinal ligament was ligated. The effect was a reduction in resting blood flow using Xenon clearance to 33% after five days. Longer follow-up was not provided why it has not been known how long-lasting this model is.

We have used a modification of this model and evaluated it for eight weeks. Perfusion by laser Doppler imager was decreased in the ischaemic hindpaw for eight weeks. Resting lactate levels were elevated, though the difference did not quite reach statistical significance beyond one day (Fig 15) (unpublished data). The lack of significance at one week was explained by the substantial variability between animals, four rats still had elevated lactate levels in the ischaemic limb whereas four had not.

A moderate rate of muscle fibre necrosis accompanied by inflammatory cell infiltrates was found peaking at one day to one week (Study III and IV). This is in accordance with other models of severe limb ischaemia as e.g. the models of femoral artery excision both in mouse and rabbit. Seifert et al found a similar histological picture. In our rats necrosis and inflammation was most extensive in the anterior tibial muscles. Also others have found the anterior tibial muscle to be more sensitive to ischaemia than other muscle groups. It is hypothesized that muscle fibre type composition might in part explain the difference as the anterior tibial muscle in rat consists to a larger part of fibres with a higher oxygen demand.

Recently, it has been suggested that the presence of necrosis and inflammation in these animal models can act as powerful stimuli of angiogenesis and help explain the greater effect of angiogenic treatment in such animal models as compared to in patients with CLI. It could though be argued that little is known about the condition of skeletal muscle in CLI in humans regarding the presence and extent of inflammation and necrosis. Muscle atrophy is a well-known feature of human CLI. Hedberg et al found extensive replacement of muscle fibres by connective tissue in cross sections of limbs amputated for CLI when compared to limbs from patients with healthy vessels. If the loss of muscle fibres is a consequence of necrosis accompanied by inflammation or by active programmed cell death – apoptosis – is not known. Apoptosis has been suggested as the mechanism for skeletal muscle atrophy in patients with congestive heart failure, a finding though contradicted by others.

Our primary interest in the rat model in this work has been to describe the time course of ischaemia defined by perfusion data and histology (Study III) and MR findings, clinical
assessment and lactate levels (Study IV). In this context the presence of necrosis and inflammation does not negatively influence the results.

Dark spots, 1-2 mm in diameter, on the plantar aspects of the digits and on the prominent plantar pads on the sole of the ischaemic limb were noted in most of our rats between the first and fourth postoperative weeks. These spots were interpreted as superficial skin necrosis and have been described also by others\(^\text{156}\). They disappeared in all rats toward the end of the observation period. Most rats favoured the contralateral limb for the first few days and most limbs appeared cyanotic during the same period in line with what has been described by others\(^\text{166}\).

The level of ischaemia achieved using our model appears to have been similar to that in the original model\(^\text{184}\) but less severe when compared to the femoral artery excision model\(^\text{166}\). As opposed to in the latter model none of our rats had a persistently non-functional limb and no extensive tissue necrosis was seen.

**Onset and duration of ischaemia**

Any significant abrupt interruption of blood flow to a limb is by definition causing an *acute* ischaemic insult. Several authors have proposed the concept of using the phase after the acute event but before full recovery of blood flow as a representation of a chronic ischaemic state similar to that seen in patients with PAD\(^\text{23,166,213}\). The severity of ischaemia will then determine the resemblance of the model to intermittent claudication or CLI in man, with the important reservation for the different mode of onset of ischaemia. In this context our model can be described as an acute ischaemia model producing resting ischaemia for at least one week and significantly decreased perfusion level as assessed by LDPI for eight weeks.

An interesting recent contribution to available models is the presentation by Tang and co-workers of a method for subchronical induction of resting limb ischaemia in rat\(^\text{198}\). A slowly constricting ameroid is surgically positioned around the common iliac and the femoral arteries. The ameroid consists of a steel casing covering a hygroscopic casein material with an internal lumen fitted over the artery. Over time the casein absorbs water reducing the cross section area of the lumen and constricting the artery. After 7-17 days the blood flow reached its lowest level and then gradually recovered. Clinical ischaemia score was lowest at 40 days postoperatively. When compared to an acute ischaemia model earlier described by the same group\(^\text{156}\) recovery of blood flow was slower and less complete in the constrictor model. As pointed out by the authors the subacute onset of ischaemia in the range of one to two weeks is still a short time span as compared to the years or decades it would take in the clinical setting of PAD\(^\text{180}\).

**Variability**

In any ischaemia model using wild type animals a certain variability in the degree of ischaemia can be expected. Vascular anatomy is likely to differ between animals – as in man – and the capacity for collateral vessel development may vary. The extent of variability is only occasionally clearly reported in the literature. Seifert et al\(^\text{184}\) described identical histological signs of ischaemia in eight animals whereas the rate of cyanosis and favouring of the unoperated limb varied between animals\(^\text{184}\). Also Pu et al\(^\text{166}\) noted substantial inter-individual differences regarding the extent of tissue loss and functional impairment, while blood pressure and lactate levels appear to have varied less\(^\text{166}\).

In our rat model we observed a greater than expected variability in the degree of ischaemia. The modification of the original model comprised cutting the superficial epigastric artery and preserving the superficial circumflex artery. The rationale was to further simplify and standardise the operative procedure. It is possible that another effect of the modification was that the variability regarding the degree of ischaemia increased. We believe the main reason for the observed variability to be a biological diversity in these wild type animals with different capacity of the superficial circumflex artery and collateral formation. Differences also in metabolic aspects can have been of importance.
A large variability in terms of lactate and CO$_2$ increase in the previously discussed pig model of skeletal muscle ischaemia was reported\textsuperscript{115}. In this model ischaemia was induced by circulatory arrest as the animals were sacrificed prior to the measurements. This technique would be expected to leave little room for variations in perfusion but the differences are more likely explained by variable metabolism.

In the context that we used the ischaemia model a variable degree of ischaemia was not unwanted and could even be considered a prerequisite for the possibility to perform correlations to other methods for ischaemia assessment.

**Animal age**

In rabbits and mice age-dependent consequences of severe resting limb ischaemia as a consequence of femoral artery excision have been demonstrated\textsuperscript{174}. In old animals the effects of ischaemia were much more profound especially in mice where five of six animals suffered severe necrosis of the distal part of the ischaemic limb.

We chose to study relatively young animals (two months) with a probably better capacity to overcome the effects of the operations limiting the extent and duration of ischaemia thereby evading unacceptable consequences in terms of tissue loss.

**Other limitations**

Our model – as the vast majority of other models used – lack the influence of atherosclerosis and inflammation and common concomitant diseases seen in patients with PAD. This problem could possibly be overcome by the use of genetically modified animal strains as diabetic, hypercholesterolemic and hyperhomocysteinemic mice are available\textsuperscript{213}.

**MR T$_2$ and limb ischaemia**

We studied the feasibility of using T$_2$ relaxation time mapping of an ischaemic limb as a potential non-invasive measure of the degree of ischaemia in the rat model (Study IV). T$_2$ changes in exercising skeletal muscle have been studied extensively both under ischaemic\textsuperscript{14} and non-ischaemic conditions\textsuperscript{69} and in models with\textsuperscript{66} and without\textsuperscript{64} resulting muscle damage. In resting skeletal muscle T$_2$ has been used to demonstrate muscle necrosis both in animal models\textsuperscript{16, 89} and in patients\textsuperscript{220}.

The link from lactate to MR T$_2$

Activity induced T$_2$ relaxation time alterations are thought to reflect a flux of osmotically active substances into the extracellular space with a resulting increase in tissue water content and change in tissue fluid composition\textsuperscript{66}. Concentric low intensity exercise has been shown to cause a brief and reversible increase in T$_2$ relaxation time directly related to the exercise intensity\textsuperscript{63} and immediately after exhaustive concentric biceps exercise there was a marked increase in T$_2$ relaxation time\textsuperscript{87}.

Increase in extracellular fluid volume alone appears to be an insufficient explanation. Increased leg muscle cross-sectional area by 21% in normal human limbs subjected to external negative pressure resulted in only a minimal increase in T$_2$\textsuperscript{164}. Possibly the mechanism explaining T$_2$ level increase is not the altered amount of tissue water but rather changes in relaxation time of the tissue fluid determined predominantly by altered tissue protein concentrations secondary to fluid shifts\textsuperscript{162}.

Lactate is known to be one of the key substances contributing to the increased osmolality and accompanying fluid shifts during exercise\textsuperscript{65} and thus an increased intra- or extracellular lactate concentration could be part of this mechanism\textsuperscript{55}. Accordingly, T$_2$ levels were found to correlate to acidosis by pH measurements in human forearm exercise\textsuperscript{5}. Additional and interesting support for lactate as an important factor is given in a study on patients with McArdle’s disease i.e. patients with an inability to form glycogen and hence an impaired capacity of lactate formation through glycogenolysis\textsuperscript{63}. These patients showed no or only mild increase in T$_2$ relaxation time after ischaemic exercise as compared to healthy controls. In patients with chronic exertional compartment syndrome...
symptoms are believed to result from local ischaemia in the affected muscle\(^{167}\). In patients with this diagnosis a marked acute increase in \(T_2\) relaxation time after exercise has been observed\(^ {216}\) that was normalised after fasciotomy. In patients with claudication the \(T_2\) relaxation time before and after exercise has been studied\(^ {221}\). The symptomatic limbs showed a pronounced increase in \(T_2\) relaxation time following exercise. The effect of exercise on \(T_2\) levels diminished after successful treatment.

**\(T_2\) and necrosis**

It appears that apart from the mechanism discussed above \(T_2\) relaxation time in muscle is also increased as a result of muscle fibre damage and necrosis. Exhaustive eccentric exercise resulting in muscle fibre damage correlated to prolonged \(T_2\) relaxation time increase in healthy volunteers peaking at day 3, 5 and 10 respectively\(^ {186}\). In an animal model of skeletal muscle infarction \(T_2\) was increasingly prolonged for up to 24 hours\(^ {89}\) and in a rat model of limb ischaemia \(T_2\)-weighted images – reflecting prolongation of \(T_2\) – showed a gradually normalising hyper-intense area corresponding to the ischaemic region\(^ {16}\).

However, areas with an increased \(T_2\) do not seem to exactly match the area of infarction. In a dog model of myocardial infarction \(T_2\) weight images and dysfucntioning myocardium was found, while the infarct size was overestimated\(^ {82}\). The difference between \(T_2\) hyperintense area and the infarct area is believed to reflect ischaemic but salvageable myocardium\(^ {52,75}\).

We found prolonged \(T_2\) relaxation time in the ischaemic anterior tibial muscle correlating both to clinically assessed degree of ischaemia and to intramuscular lactate concentrations (Study IV). In our animal model areas of muscle necrosis were present in histologic sections from the anterior tibial muscle (Study III). We believe that lactate concentration increase and muscle necrosis have been of main importance in explaining the observed \(T_2\) relaxation time alterations.

Identifying patients with intramuscular lactate concentration increase at rest or subclinical muscle damage could be of great interest in attempting a more precise diagnosis in limb-threatening ischaemia.

**Future focuses**

Most attempts made to define CLI have focused on the existing clinical manifestations of disease – rest pain, ulcers, gangrene – and haemodynamic assessment of the current state of blood flow. Maybe markers of limb-threatening ischaemia should be sought not in such parameters or even in metabolic markers but rather in tissue or plasma markers predicting risk for further deterioration in existing disease or future development of limb-threatening disease. A correlation between risk for development of PAD requiring revascularisation and levels of inflammatory markers in plasma 16 years earlier – when all subjects were asymptomatic – has been demonstrated\(^ {56}\). Using these markers patients with CLI could not be separated from patients developing intermittent claudication. Other more sensitive and specific markers will perhaps be able to do so in the future.

Muscle atrophy is a well known feature of PAD and loss of a large proportion of muscle fibres have been demonstrated in CLI patients\(^ {88}\). Whether the demise of muscle fibres in CLI is explained by necrosis or apoptosis – active programmed cell death - has not been clarified. Apoptosis is known to contribute to skeletal muscle atrophy in muscle disuse by unloading\(^ {12}\) and suggested as a mechanism in muscle atrophy in cardiac failure\(^ {9}\). Skeletal muscle apoptosis in CLI could hypothetically simply be a consequence of muscle disuse or a way for the organism to diminish demand on limited blood flow resources by actively decreasing muscle mass. Perioperative biopsies from CLI patients during revascularisation or amputation as well as material from the rat model could be used and analysed for signs of apoptosis to increase the understanding of the pathophysiology involved in CLI development.

If pharmacological manipulation of pathways regulating apoptosis would become possible it could be of theoretical benefit in CLI patients to
block skeletal muscle apoptosis in an attempt to increase muscle mass and thereby graft outflow and possibly graft patency.

In CLI patients with disease not amenable to revascularisation, and when amputation is not considered the best option, control of pain is essential. Neuropathic pain has been described in CLI patients\(^{215}\) and there are good reasons to evaluate the addition of drugs aiming at controlling neuropathic pain in a randomised trial.

MR angiography is emerging as an alternative to routine intra-arterial contrast angiography in the preoperative evaluation of CLI patients. The addition of lower leg soft tissue evaluation with e.g. perfusion, diffusion and \(T_2\) relaxation time determinations could be done for research purposes. These parameters could then be correlated to the degree of ischaemia using current methods, symptoms and – in patients treated conservatively – prognosis.

The hypoxia marker EF5 (Page 26) has the potential of becoming a clinically useful diagnostic tool as the drug is non-toxic and thus can be given to patients and if marked with radioactive labelled fluoride (F18) traced non-invasively with e.g. PET\(^{60}\). To date human applications have mainly involved cancer research where EF5 has been used to separate tumour tissue according to degree of hypoxia, which in turn has prognostic implications. It has not yet been used in skeletal muscle and skin ischaemia and the animal model could be used for evaluation of its applicability in this setting.
CONCLUSIONS

In patients with critical limb ischaemia by current definitions there was no clear correlation between lactate levels in the symptomatic limb and the degree of ischaemia using current non-invasive methods.

In a small group of patients there seemed to be a connection between ischaemic pain and intramuscular and subcutaneous lactate levels.

Lactate levels in working skeletal muscle under ischaemic conditions could be determined with microdialysis.

Microdialysis lactate levels under such circumstances and the degree of ischaemia and lactate levels in venous blood correlated well.

A rat model could be used to monitor pathological changes and repair mechanisms following an ischaemic event in a limb.

MR T₂ relaxation time alterations could be used to obtain an estimate of the degree of ischaemia or ischaemic damage in skeletal muscle.
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