On microvascular blood flow assessment with the new microdialysis urea clearance technique

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Linköping 2010
To

Lovisa, Lydia and Svante
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Abstract

The aim of this thesis was to develop and evaluate a new way of monitoring blood flow with microdialysis. A thin catheter consisting of a semipermeable membrane is implanted in the tissue being studied. The catheter is perfused by a solution that closely resembles interstitial fluid, and small water-soluble substances are allowed to diffuse passively through the pores of the membrane with the aim at reaching equilibrium with the surrounding tissue. The minimally invasive character of microdialysis, and its ability to sample from the organ being studied, make microdialysis attractive in most research settings as well as for clinical surveillance. It has, however, become increasingly evident that microdialysis under conditions of non-equilibrium - for example, fluctuating regional blood flow, will alter the results gained. We have therefore aimed to explore the possibilities of developing a new marker of blood flow that will yield information about changes in blood flow that occur in the area of the microdialysis catheter itself.

We hypothesised that the changes in the diffusion of exogenous urea could be used as markers of changes in tissue blood flow. The theoretical basis for this approach is that the mass transfer of urea will increase across the dialysis membrane secondary to increased blood flow. As removal of urea from the vicinity of the dialysis membrane increases with increased blood flow, the concentration gradient of urea between the perfusate and tissue will also increase. This in turn will result in a greater loss of urea from the perfusate. The changes noted in retrieval of urea from dialysate by the system are therefore thought to be inversely related to changes in blood flow. We tested our hypothesis in two species of animal (rat and pig) and in man, and in three organ systems (muscle, liver, and skin), and present four papers that indicate that the urea clearance technique provides reliable and reproducible results. The technique was evaluated against conventional metabolic markers (lactate and glucose), the ethanol clearance technique (microdialysis), laser Doppler perfusion imaging (LDPI), and polarisation light spectroscopy (TiVi).

We present evidence that the urea clearance technique can be used to assess blood flow in the organs studied reliably and reproducibly with microdialysis. The microdialysis technique is minimally invasive and safe for the recipient, and catheters can easily be implanted during operation to monitor organs at risk. Urea is easily analysed as a standard assay among other “basic” metabolic markers (in a standard microdialysis kit) and has favourable characteristics with a standardised measurement system that is routinely used for monitoring metabolites in the clinic. The technique is also effective when used at lower perfusate flow rates (<1 μl/minute), which is advantageous as the recovery of metabolic markers increases at low perfusate flow rates.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ADH</td>
<td>alcohol dehydrogenase</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>CV</td>
<td>coefficient of variation</td>
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<tr>
<td>CI</td>
<td>confidence interval</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>He-Ne</td>
<td>helium-neon</td>
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<tr>
<td>ISF</td>
<td>interstitial fluid</td>
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<tr>
<td>IL-6</td>
<td>interleukin-6</td>
</tr>
<tr>
<td>IL-8</td>
<td>interleukin-8</td>
</tr>
<tr>
<td>LDPI</td>
<td>laser Doppler perfusion imaging</td>
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<tr>
<td>LDF</td>
<td>laser Doppler flowmetry</td>
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<td>MD</td>
<td>microdialysis</td>
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<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
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<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>NA</td>
<td>noradrenaline</td>
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<tr>
<td>NAD⁺</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide (reduced form)</td>
</tr>
<tr>
<td>NG</td>
<td>nitroglycerine</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
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<tr>
<td>RBC</td>
<td>red blood cell</td>
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<tr>
<td>ROI</td>
<td>region of interest</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>TiVi</td>
<td>tissue viability index</td>
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<tr>
<td>TNFα</td>
<td>tumor necrosis factor alpha</td>
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# Definitions

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tr>
<td><strong>Perfusate</strong></td>
<td>The fluid perfused through the microdialysis catheter</td>
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<td><strong>Dialysate</strong></td>
<td>The fluid passing through the microdialysis membrane that includes substances from the interstitial sample (= microdialysis sample)</td>
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<tr>
<td><strong>Perfusate flow rate</strong></td>
<td>The velocity of the perfusate through the microdialysis system</td>
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<tr>
<td><strong>Recovery</strong></td>
<td>The dialysate:interstitial concentration ratio for a particular substance expressed as a percentage</td>
</tr>
<tr>
<td><strong>Cut-off</strong></td>
<td>Size of pores in the microdialysis membrane</td>
</tr>
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List of original papers

This thesis is based on the following papers, which will be referred to by their roman numerals:

I  S. Farnebo, L-E. Karlander, I. Steinwall, F. Sjöberg
   Continuous assessment of concentrations of cytokines in experimental injuries of the extremity

II S. Farnebo, A. Samuelsson, J. Henriksson, L-E. Karlander and F. Sjöberg
   Urea clearance: a new method to register local changes in blood flow in rat skeletal muscle based on microdialysis

III S. Farnebo, A. Winbladh, E.K. Zettersten, P. Sandström, P. Gullstrand, A. Samuelsson, E. Theodorson and F. Sjöberg
   Urea clearance - a new technique based on microdialysis to assess liver blood flow studied in a pig model of ischemia/reperfusion
   European Surgical Research (2010) 45, 105-112

IV S. Farnebo, E.K. Zettersten, A. Samuelsson and F. Sjöberg
   Assessment of blood flow changes in a new pharmacological model of microdosing in human skin by microdialysis urea clearance
   Submitted

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List of original papers
Introduction

Background and present techniques

All cells in the human body are interconnected by an advanced network of vascular pathways through which nutrients and oxygen are supplied and metabolic products are removed. Chemical messengers are transported long distances and between cells, through this network to allow integrated activity. This closed system is referred to as the circulation, and its smallest parts - the arterioles, capillaries, and venules - are collectively referred to as the microcirculation. The microcirculatory status reflects cell and organ homeostasis and may therefore often be of more relevance to a specific organ than the global circulatory condition.

Ways to investigate local microvascular function have therefore attracted increasing interest, particularly in various vascular diseases, such as diabetes, heart failure, and peripheral vascular disease, and for the understanding of tissue morbidity, as for example, after transfer of a free flap, revascularization of limbs, and in neurointensive care. Many methods have been published including imaging methods such as PET and MRI, plethysmography, laser Doppler flowmetry, Xenon clearance, and intravital microscopy, direct measurements, and microspheres.

Every method, however, has advantages and disadvantages depending mainly on whether they are done in vitro or in vivo. For example, possible systemic effects are difficult to distinguish from effects on the studied organ in many in vivo experiments. In vitro experiments, such as isolated vessel preparations or macroanatomical preparations including the hindlimb perfusion model, can therefore be used to exclude confounding systemic factors. These data must however be analysed cautiously as it is difficult to comment on their behaviour in vivo.

Methods used in experimental animals are generally less applicable to patient-based research because they are invasive or use radioactive isotopes. Much effort has therefore been put into the development of techniques that are safe, easy to handle, and are not invasive. Microdialysis enables studies on local metabolic events and perfusion in vivo without taking the whole organism into account, which enables a unique
means of investigation. Studies on virtually all organ systems can be made, and this is of particular value when estimates of blood flow are to be made in tissues in which such measures are particularly difficult, such as in the liver.

**Microdialysis: theory and practice**

Microdialysis is a well-established technique for continuous sampling of small water-soluble molecules within the extracellular fluid space in vivo. The first papers on membrane-based in vivo sampling of interstitial compounds were published by Bito et al. in 1966, followed by Delgado et al. and Ungerstedt et al. in 1974, who presented the first attempt to use a membrane similar to the one we use today in microdialysis. Since then, about 13,000 scientific papers have been published with the technique — among them some 2000 clinical investigations.

The basic principle of the technique is to mimic the passive function of a capillary blood vessel. The microdialysis probe is continuously perfused with a solution (perfusate) that closely resembles the interstitial fluid. Movement of solute across the dialysis membrane will aim at reaching equilibrium with its surroundings by diffusion in both directions. The concentration gradient of the interstitial fluid and the perfusate are the driving forces in this process.

The microdialysis sample (termed dialysate) is collected at the end of the outlet tubing in a vial suitable for a small volume (Figure 3). The molecular weight of
the substances being sampled is limited by the pore size of the microdialysis membrane (cut-off). With a various range of microdialysis membranes it is possible to sample molecules in sizes ranging from a few hundred Daltons up to about 30,000 Daltons; this makes it feasible to monitor energy metabolites, blood flow, neurotransmission, amino acids, markers of inflammation, and concentrations of certain drugs in target tissues in a reliable manner.

Some of the main advantages of microdialysis are therefore that: (1) It enables studies of local regulation of pathophysiological processes in the tissue being studied, in the absence of perturbations to the organism as a whole. (2) Pharmacologically-active drugs can be delivered locally through the probe, and endogenous target compounds can be collected simultaneously to assess their effects on the tissue. (3) Unique markers can be identified and used for early diagnosis of secondary complications and diseases, and free fractions of the drug can be collected in pharmacokinetic and pharmacodynamic studies. (4) The commercial introduction of catheters (MW 100kDa) that allow sampling of molecules of greater molecular weight, has expanded the scope of investigation to include inflammatory mediators. Such data will theoretically add an important dimension to the understanding of the pathogenesis of different diseases and subsequent reparative processes, and be an aid in monitoring organs at risk of failure.

![Figure 3](image)

**Figure 3** Photographs displaying vial (left) and catheter (10 mm membrane) with outlet coupling for the vial (right).

**Recovery**

The analyte concentration of the microdialysis sample is dependent on the degree of equilibration between the perfusate and interstitial fluid. This relation is generally termed the relative recovery and is defined as the dialysate:interstitial concentration ratio and presented as a percentage. In addition to relative recovery, absolute reco
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Microdialysis and the metabolic state of a tissue

Microdialysis is often used to monitor the metabolic state of different tissues, by the measurement of the concentrations of three important markers of cellular energy metabolism (glucose, lactate, and pyruvate)\(^\text{46}\). Assessment of whether tissue hypoperoxusion or ischaemia, or both, are present is therefore dependent on alterations caused by changes in the redox state of cells. When the supply of oxygen and glucose is less than it requires, the production of ATP from the citric acid cycle decreases. This decrease is compensated for by an increase in the turnover of glucose in the anaerobic part of glycolysis. During this process pyruvate is converted into lactate, which causes an increase in lactate and
then also an increase in the lactate:pyruvate ratio (Figure 5), commonly used as a sign for tissue ischaemia. A change in lactate or the lactate:pyruvate ratio must, however, be interpreted cautiously, for example in states of cytopathic hypoxia when mitochondrial dysfunction creates a metabolic state resembling ischaemia despite normal or even high blood flow (as can be seen in sepsis and burns) \(^{47}\). Lactate alone is no good as a sole marker of ischaemia. It will, for example, increase in cases of increased metabolism, such as in stress induced \(\beta\)-adrenergically mediated activation of glycogenolysis, and then primarily signal a state of metabolic crisis rather than one representing tissue ischemia.

**Microdialysis and cytokines**

The release of inflammatory mediators plays an important part in various pathological conditions and wound healing \(^{48, 49}\). The release is orchestrated by inflammatory and resident cells on activation, or after trauma, and will act both locally and systemically \(^{50, 51}\). High systemic concentrations of IL-6 and IL-8, have been thought to originate secondarily to a local overflow, as they are constitutively present in the damaged tissue \(^{51}\). It has also been proposed that the release of local cytokines play an important role in microcirculatory changes locally as well as for the effects often seen on systemic circulation and homeostasis secondary to the trauma. The relation between the involvement of local and systemic cytokines and the local microcirculatory status (markers of metabolism) after standard tissue trauma has, however, not previously been elucidated, mainly because of the limited availability of minimally invasive methods of measurement. Microdialysis presents an opportunity to study the chronology of changes after trauma in a minimally invasive way without the drawbacks of plasma and tissue sampling, including taking multiple biopsy specimens. It is therefore an attractive technique for studies on local cytokine production *in vivo*, and the results from pioneering studies have been promising \(^{31, 32, 40, 52}\).

Most of the inflammatory mediators are, however, molecules with a high molecular weight and these studies therefore demand the use of large-pore membranes (100 kDa). The use of these membranes has however been limited, much due to technical problems, such as poor sample volume retrieval, difficulties to analyze the very
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small quantities retrieved by the system, and commonly a low recovery profile of the compounds 30, 31. The problems in the collection of samples are, however, fewer when the perfusate contains a colloid. Several studies have confirmed that Ringer’s dextran 60 balances the colloid osmotic pressure of the perfusate against the colloid osmotic pressure of the surrounding extracellular fluid, to give almost complete fluid recovery, which is a prerequisite for adequate interpretation of the results 30, 32. The recovery of cytokines is, however, much lower than with smaller molecules, such as lactate and glucose, which are often assessed with low MW (20kDa) catheters 31.

Rationale for developing a marker of blood flow

Several advantages, considering the above mentioned issues, are possible if blood flow measurements could be conducted through the microdialysis system. Information about the local blood flow and rationale for developing a marker for this becomes increasingly evident when we know the following:

(1) The interstitial concentration of a given substance is influenced by supply and removal through the blood stream. The presumption that recovery is dependent on blood flow necessitates parallel measurements of flow to make the correct interpretation of ongoing changes with the microdialysis technique (Figure 6). This is particularly important if the analyte of interest has a low recovery, such as with high MW compounds such as cytokines 30, 31. As local supply and clearance of the compound is altered, simultaneous changes will be seen in the concentration gradient over the membrane, and also in recovery.

Figure 6
Schematic figure illustrating the main principles of microdialysis. It is important to realize that the concentration in the dialysate not only depends on the perfusate flow rate through the catheter and the length of the membrane, but also upon the supply and removal of substances from the capillaries as well as uptake and release from the cells.
Introduction

Changes in blood flow are not necessarily related to changes in metabolic markers, such as lactate and glucose.

Concurrent data on interstitial nutritive blood flow would be provided through the perfusion estimate, together with other markers of local metabolism. This is of particular importance when an approximation is warranted in a tissue that lacks available techniques for such measurements. A good example is the liver, where blood flow measurements are particularly troublesome, partly because of its complex dual blood flow system and anatomical position.

Prerequisites for a new marker of blood flow and urea

There are some fundamental characteristics (Table I) that need to be fulfilled to be regarded as a clinically useful marker of blood flow by microdialysis. The tracer must be: safe to the recipient, samples easy to analyse and not metabolised in the tissue, and should not have an effect on local metabolism or blood flow. It is beneficial if the technique functions well at a low perfusate flow rate, which enables more exact measurements of metabolic variables (increased recovery) and less removal of substances surrounding the probe.

Urea has an important role in the metabolism of nitrogen-containing compounds in the body. It is the main disposal form of amino groups that are derived from amino acids, a process that takes place in the periportal hepatocytes and is referred to as the urea cycle. It is excreted by the kidneys, and this handling is vital for human metabolism. Besides its role as a carrier of waste nitrogen, urea is also plays a role in the counter-current exchange system of the nephrons that allows reabsorption of water and critical ions from the excreted urine. It is a substance known to be evenly distrib-
uted throughout the body fluid compartments and it is not metabolised in skeletal muscle tissue or skin, or known in itself to affect local metabolism or circulation. It is also regarded as a non-volatile, stable, and non-toxic compound, often used in microdialysis as an endogenous reference compound\textsuperscript{53, 54}. It is easily analysed, usually as a standard assay among other “basic” metabolic markers (in a standard microdialysis kit) and has shown favourable characteristics with a standard measurement system that is routinely used for monitoring metabolites bedside (CMA 600, CMA Microdialysis, Stockholm)\textsuperscript{55}. Transport of urea over the microdialysis membrane is bi-directional.

\textbf{Previous attempts to measure blood flow with microdialysis, and hypothesis}

\textit{In vivo} measurements of blood flow within a tissue are not easy. Restrictions in spatial and temporal resolution, and systemic effects, must be taken into account. The development of a microdialysis technique for this purpose would provide a minimally invasive, safe technique that could be used both in experimental animals and in humans. Blood flow can be assessed at a site of limited volume (catchment volume less than 1 cm\textsuperscript{3}) and data from the organ of interest is provided without making perturbations to the organism as a whole. Concurrent data on the metabolic status of the tissue are also achieved in parallel, and the results can be used to interpret the other microdialysis data better. Previous attempts to achieve this through the microdialysis system have been published. The most thoroughly evaluated technique to date is the microdialysis ethanol technique\textsuperscript{26}, but other tracers have been evaluated (for example, [$^{14}$C]ethanol and $^3$H$_2$O) with the presumption that they function as local tracers in the same manner as ethanol\textsuperscript{25, 27}.

The microdialysis ethanol technique is based on the principle that ethanol added to the perfusate diffuses freely through the semipermeable membrane of the microdialysis catheter and further into the interstitium. Ethanol diffuses faster, and is lost to a larger extent as its concentration gradient between the perfusate and the interstitium increases, with higher concentrations in tissue blood flow and so local clearance. The difference in ethanol concentration in the perfusate between the inflow and outflow part of the catheter system will be altered as a result of this, and the difference is inversely proportional to tissue blood flow\textsuperscript{26, 56-58}. The term clearance, under
these circumstances, is defined as the relative loss of added ethanol, as a result of changes in local blood flow. The technique has been validated against direct measurements of blood flow and also against 133Xenon clearance and it is currently regarded as the standard for measurements of tissue blood flow with the microdialysis system.

However, from a practical perspective there are shortcomings with the ethanol technique. Ethanol is a highly diffusible compound and even small amounts of it in the nearby environment, which is not uncommon (at least not in the clinical setting), cause samples to be polluted. The technique requires special analytical procedures, and the best results are usually obtained if the analysis is done in close conjunction to the actual time of measurement. Most importantly, the ethanol clearance technique is not suitable at low perfusate flow rates, because of the highly volatile nature of ethanol, which brings on an increase in loss of tracer from the interstitium with lower perfusate flow rates. The optimal perfusate flow rate for ethanol clearance is, however, not known, although practical experience argues that it is less applicable at perfusate flow rates below 2 µl/minute. Lower perfusate flow rates result in low volumes being sampled and, in parallel, significant increases in variability (coefficient of variation), which reduces its applicability as a result of decreased accuracy.

One of our aims of the thesis has, therefore, been to find out whether urea clearance has advantages over ethanol clearance in functional terms at low perfusate flow rates, because most microdialysis protocols will benefit from the use of a low perfusate flow rate, as most analytes have their best recovery profiles below 1 µl/minute. Optimal flow rates (good recovery) for the microdialysis system for glucose, lactate, and urea, are achieved at – for example, 0.16 µl/minute, 0.33 µl/minute, and 0.66 µl/minute, respectively. For other more complex analytes, complete recovery is harder to accomplish, even at really low perfusate flow rates. This emphasizes the need for a blood flow marker that is sensitive at low perfusate flow rates, if concurrent assessments are warranted.

The main hypothesis of this thesis was therefore that net loss of urea added to the microdialysis perfusate mirrors changes in local blood flow in the same way as the ethanol technique. Urea was chosen as the tracer because it is regarded as a non-
volatile, stable, and non-toxic compound, often used in microdialysis as an endogene-
ous reference compound. It is easily analysed as a standard assay among other “ba-
sic” metabolic markers, and is present in a standard measurement system such as the
CMA 600. Transport of urea over the microdialysis membrane is bi-directional, urea is
known to be evenly distributed throughout the compartments of body fluids, and it is
not metabolised in skeletal muscle tissue or skin, and is not known to affect local me-
tabolism or circulation53, 54.

Other methods used for validation of the measurements of blood flow
Numerous methods have been published for assessment of regional blood flow, some
of them mentioned previously in the introduction. There are however few methods
which can be used in humans with the objective to assess indirect measures of nutriti-
tive blood flow without being excessively invasive, require use of radioisotopes, are
expensive or are dependent on advanced equipment. We chose to do parallel meas-
urements with two non-invasive techniques that previously have gained acceptance
for assessment of indirect blood flow measurements; laser Doppler and polarisation
light spectroscopy.

Laser Doppler perfusion imaging
Laser Doppler perfusion imaging (LDPI) has been used to measure superficial skin
blood flow since the mid-90s 61. It does not involve contact with the patient and is
based on a continued development of the laser Doppler flowmetry technique (LDF) 14,
61, 62. A low power He-Ne laser is used (670nm) to scan the tissue of interest.

The laser beam is directed through a mirror system to the skin, where it forms a light
spot of about 1 mm². As the beam is moved in discrete steps over the skin surface, the
backscattered light is collected by a photo-detector inside the scanner head. Moving
blood cells will reflect some of the light back to the instrument where the change in
wavelength and the so-called “Doppler shift” is recorded (Figure 7). The detected Dop-
pler components are processed to generate an output signal that is generally regarded as being linear with tissue perfusion that is proportional to the product of the blood cell average speed and concentration. A value of perfusion, given in arbitrary units is provided together with a colour-coded perfusion image that gives the investigator an informative overview of the perfusion of the tissue that is studied (Figure 8). The main advantage, compared to LDF, is the better spatial characteristics and the ability to present the tissue perfusion as an image rather than as a single value recorded at one point only. The temporal resolution is, however, lower depending on the time it takes to scan the area of interest. This technique has a few drawbacks, including a limited ability to record decreases in blood flow below normal ranges and being highly sensitive to motion artifacts and ambient light. LDPI has been extensively used to measure skin perfusion in various models and specifically during the delivery of vasoactive compounds by microdialysis.

Polarisation light spectroscopy

Tissue viability imaging (TiVi) technology is a relatively new technique based on polarisation spectroscopy that has proven to be increasingly valuable in different research settings, mainly due to its possibility to indirectly measure blood flow changes below the resting state (changes in red blood cell concentration during vasoconstriction). Its functional unit is a standard “off-the-shelf” digital camera that is equipped with polarisation filters that are perpendicularly placed over the camera flash and lens (detector) (Figure 9). The flashlight is linearly polarised as the camera takes a picture. A portion of this light will reflect directly towards the lens as it hits the skin. This fraction of back-scattered light will, however, not pass through the lens filter (second filter in front of the detector), as it will retain its original polarised state. Most of the light will continue into the tissue and become randomly scattered. A portion of the light will therefore be
able to pass through the lens filter as it is depolarised. Each picture is subject to a specific computerised algorithm that divides the image into different colour planes (red, green, and blue). This separation and algorithm enables the computer to estimate the concentration of red blood cells (RBCs) in the tissue, as it is known that RBCs in the microcirculation absorbs green light to a much higher extent than red light, which is different from the absorption properties in the structures surrounding the vasculature. This feature makes it possible virtually to “see through” the top layer of the skin and probe the underlying dermal layer for indirect information about the microvascular status. The computer provides a measure of the concentration of RBCs referred to as the TiVi index, which correlates with the concentration of blood cells in the actual tissue. It has recently been validated as a method for assessment of microvascular reactivity in the skin. A recent study with laser Doppler and TiVi has also shown that perfusion and concentration of RBCs are related, and that TiVi has advantages in terms of reproducibility, sensitivity, and ease of use, compared with laser Doppler flowmetry, which makes it a possible option for minimally invasive tissue monitoring.

**Microdosing: a new approach to altering tissue blood flow in vivo**

Pharmacological provocations are often used in isolated vessel preparations and intravital microscopy models to study interactions between drugs and the microcirculation. The rationale for measuring responses to provocations is that the inherent temporal and spatial variability is less than in measurements on unprovoked, baseline flow, and responses can be related to specific pharmacological provocations instead.

The translation from *in vitro* models into microdosing models *in vivo* in humans has been complicated, but has been shown to be feasible when combined with vasoac-
tive substances given transdermally, either topically or by iontophoresis. A shortcoming with iontophoresis, however, is that the actual dose delivered can only be estimated and not measured directly, which limits its use in pharmacological experiments. Microdialysis, on the other hand could be used for administration of predefined microdoses of pharmacological substances through ex tempore perfusate preparations. Reliable vascular models could possibly be setup with the aim to test if a concept of pretreatment could produce a more unified experimental setting and reduce inter subject variability by predilatating the vascular bed before vasoconstriction.
Aims of the thesis

General

To develop the urea clearance technique as a method for assessment of blood flow through microdialysis.

Specific aims – study I-IV

I – To evaluate the use of microdialysis with high molecular weight (100 kDa) catheters, and to describe the inflammatory responses to trauma in an animal model, as assessed by measurements of inflammatory cytokines (IL-6, IL-8, TNFα) in skeletal muscle distal to a standard vascular contusion. To use measurements in the opposite (uninjured) leg as control and as an indirect measurement of the systemic response.

II – To assess the feasibility of including urea in the perfusion medium to assess pharmacologically (noradrenaline) altered blood flow in rat skeletal muscle. To examine if the resolution of the technique is better at lower microdialysis perfusate flow rates.

III – To find out if the microdialysis urea clearance technique is applicable for blood flow assessment in an established porcine ischaemia/reperfusion liver model.

To validate the microdialysis urea clearance technique against the established microdialysis ethanol technique in the assessment of blood flow at a higher perfusate flow rate (2 µl/minute).

IV – To compare the urea clearance technique with laser Doppler perfusion imaging and polarisation light spectroscopy in a new human skin model in which the tissue blood flow is altered pharmacologically with noradrenaline and nitroglycerine.
Aspects of the methods

**Subjects and microdialysis**

Experiments were conducted in three different organ systems (Table II): muscle (paper I and I), liver (paper III), and skin (paper IV), and three different species: rats (paper I and II), pigs (paper III), and humans (paper IV).

<table>
<thead>
<tr>
<th>Study</th>
<th>Catheters No</th>
<th>Species</th>
<th>Organ</th>
<th>Cut-off</th>
<th>Membrane length</th>
<th>Perfusate flow rate</th>
<th>Perfusion fluid</th>
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<tbody>
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<td>I</td>
<td>16</td>
<td>Rat</td>
<td>Muscle</td>
<td>100 kDa</td>
<td>10 mm</td>
<td>0.3 µl/minute</td>
<td>Ringer’s Dextran 60</td>
</tr>
<tr>
<td>II</td>
<td>15</td>
<td>Rat</td>
<td>Muscle</td>
<td>20 kDa</td>
<td>10 mm</td>
<td>0.4 µl/minute and 0.6 µl/minute</td>
<td>Ringer’s acetate</td>
</tr>
<tr>
<td>III</td>
<td>6</td>
<td>Pig</td>
<td>Liver</td>
<td>20 kDa</td>
<td>10 mm</td>
<td>2 µl/minute</td>
<td>Ringer’s acetate</td>
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<tr>
<td>IV</td>
<td>15</td>
<td>Human</td>
<td>Skin</td>
<td>20 kDa</td>
<td>10 mm</td>
<td>0.5 µl/minute</td>
<td>Ringer’s acetate</td>
</tr>
</tbody>
</table>

Table II

Brief description of the catheters, species, and organs studied in the respective papers.

All catheters consisted of polyurethane tubing and a polyamide dialysis membrane (CMA, Solna, Sweden). The catheters are needle-shaped with a double lumen cannula and a hollow fibre membrane at the tip (distal part, Figure 2). The molecular cut-off for the dialysis membranes was 100 kDa in paper I, and 20 kDa in studies II-IV.

Ringer’s dextran 60 was used as perfusion fluid in paper I, as it has been shown to balance the colloid osmotic pressure of the perfusate against the colloid osmotic pressure of the surrounding extracellular fluid, and so provides almost complete fluid recovery during microdialysis with high molecular weight catheters.

*Ex tempore* preparations of Ringer’s acetate (Braun Medical, Bromma, Sweden) were used in papers II-IV. These were made by Apoteksbolaget, Umeå, Sweden, to make sure that the amounts of urea in the perfusate fluid were correct (20 mmol/L, paper II-IV). Ethanol (5 mmol/L, paper III), noradrenaline (0.5 mg/L, papers II and IV), and nitroglycerine (0.5 mg/ml,
paper IV) were added at the local (Linköping University Hospital) ex tempore unit on the morning of each experiment, to make sure that the concentrations of the given substances in the perfusate fluid were correct.

The concentrations of glucose, lactate, pyruvate, and urea in the microdialysis dialysate samples were analysed on a CMA 600 microdialysis analyser (CMA, Solna, Sweden), using ordinary enzymatic methods.

The concentration of ethanol was calculated using a modification of the alcohol dehydrogenase method described by Bernt and Gutmann. Absorbance of NADH was measured at a wavelength of 334 nm in a spectrophotometer (Thermo Multiscan Spectrum) and a non-linear calibration curve was used to evaluate the data.

Cytokines were analysed using a high sensitivity enzyme linked immunosorbent assay (ELISA).

**Laser Doppler perfusion imaging**

We used a laser Doppler perfusion imager (PIM 2.0, Lisca Development AB, Linköping, Sweden) in paper IV to record changes in tissue perfusion. Data from 64 x 64 measured spots (pixels) are processed and numerical perfusion values presented in volts, but also as a colour-coded map where the area of most interest can be selected for numerical analysis. Small ink marks were used to ensure that the same region was investigated each time and the region of interest (ROI) was centred over each catheter and set to 1 x 2 cm. Resolution was set to “low” and speed “high”. LDPI maps were made at time 0 (directly after insertion of the microdialysis catheter) and then every 15 minutes until the end of the experiment (360 minutes). The ambient light level was kept to a minimum during recording, by turning lights off. The subjects were placed semisupine on a hospital bed during the entire protocol (paper IV) and were asked to move their arms as little as possible during the experiments, especially when the recordings with the LDPI were performed. The studied arms were placed on a light absorbing green cloth in order to maintain optimal background discrimination. An infrared camera was used to check that the temperature of the skin over the region of interest remained stable during the examination period, and a stable room temperature of 22° C was maintained.
Polarisation light spectroscopy

We used the TiVi600 (WheelsBridge AB, Linkoping, Sweden), which consists of a standard digital camera (Canon s80, Canon Inc., Japan) controlled by a personal computer through a USB-connected interface. The TiVi-system was positioned 15 cm above the volar aspect of the forearm on which the three microdialysis catheters were placed. The TiVi camera resolution was set to “small fine” (640 x 480 pixels), and the settings “flash on” and “macro” were used. ROI with a size of 1 by 2 cm was centred over each catheter and data were analysed using a graphical user interface (GUI, Matlab® (Mathworks Inc., Natick, MA, USA)) that controls the acquisition of cross-polarised images that displayed the calculated polarisation light spectroscopic images of the microcirculation.
Aspects of the methods
Review of the studies and main results

Study I: “Continuous assessment of concentrations of cytokines in experimental injuries of the extremity”

This study was designed to evaluate the use of microdialysis with high molecular weight (100 kDa) catheters in a standardised animal trauma model 2, 80-82, where a blunt trauma is directed towards the femoral vascular bundle in rats. Local effects were measured in skeletal muscle below the trauma and indirect measurements of the systemic effect were made from a control catheter in skeletal muscle in the opposite leg. Inflammatory cytokines IL-6, IL-8, and TNF-α were assessed in this study.

Experimental animals and operations

Sixteen male Sprague-Dawley rats were used. All animals were anaesthetised and given an identical blunt trauma towards the right femoral vessels in the groin, previously described in detail 2, 80-82 (Figure 10).

Experience from this model and previous papers have shown that most of the vessels remain patent despite the major contusion to the vessel wall and the mural damage seen in the microscope. Only a small, but reproducible, decrease in femoral blood flow and in muscular microcirculation indicated that the major effect of the trauma may not only be local, but also systemic, and we hypothesised that both local microcirculatory effects and systemic effects may be caused by an inflammatory response induced by the damage to the vessels.

High cut-off (100 kDa) microdialysis catheters were inserted into the middle part of the gracilis muscles both on the injured and the uninjured sides so that we could gain information both from the muscle below the injured feeding vessel (signalling local effects caused by the trauma) and from the control side (signalling possible systemic effects).
Metabolic markers such as urea, lactate, pyruvate, glucose, and glycerol (CMA 600) were measured in parallel to the inflammatory mediators IL-6, IL-8, TNF-α, and total protein (ELISA) (Table III). Data were evaluated by a forward, stepwise multiple regression model.

**Results and conclusions**

The response to the injury led to a significant and large increase in the concentrations of inflammatory cytokines (IL-6 and IL-8) in both the injured and control legs (Figure 11). Although there was a clear trend towards a difference between the trauma and control legs, the difference was not significant. The results indicated a general inflammatory response to the trauma. This may partly explain the systemic effects seen as patchy lung parenchyma and excessive amounts of pleural fluid after the experiments ended. There were no signs of ischaemia in either leg as estimated by the metabolic markers (lactate, pyruvate, and glucose). Concentrations of TNF-α remained stable throughout the experiment, which is in accordance with previous studies 83, 84.

![Blunt trauma](image)

**Figure 11:** IL-6 (left) and IL-8 (right) concentrations in control and trauma leg at three time-points after contusion trauma.
Loss of perfusion fluid was estimated as mean percentage volume recovery, through weighing of tubes before and after sampling. The weight after sampling minus the weight before sampling, divided by the expected multiplied by 100 was 94 (14) %, which led us to conclude that the fluid loss, was relatively small.

The results from paper I indicated that high molecular weight catheters may be useful in research of real-time measurement of concurrent changes in inflammation and metabolism \textit{in vivo}. The results also suggested that a generalised inflammatory response to trauma is elicited secondary to the trauma, and that these effects can be measured by a control catheter in the control leg. These systemic effects seem to be more important than the changes described in the local microvascular milieu. It is also possible that that the significant, but relatively small, reduction in femoral and microvascular blood flow that was shown in previous studies with the model, has relatively little effect on the muscle metabolism, as shown in the results reported in this paper. Data on volumes of perfusate confirm other studies in stating that the use of colloid prevents fluid loss over the dialysis membrane when high molecular weight catheters are used.
Review of the studies and main results

**Study IV: “Assessment of blood flow changes in a new pharmacological model of microdosing in human skin by microdialysis urea clearance”**

We aimed to introduce a new *in vivo* human skin model in which the tissue was pharmacologically provoked in a manner previously used only *in vitro*. The aim was also to compare the urea clearance technique with laser Doppler perfusion imaging and polarisation light spectroscopy (TiVi) to examine its usefulness as a method of measuring skin blood flow after a local provocation to the skin.

**Experimental subjects and procedures**

Fifteen microdialysis catheters were used. All catheters were inserted in the ventral forearm of healthy human volunteers. The catheters were perfused with an extempore preparation of urea (20 mmol/L) and Ringer’s acetate. The research protocol contained four phases (Table V), beginning with an initial stabilization period after preparation (phase I) of 90 minutes (urea perfusate only). Phase II (90 minutes) was induced by adding nitroglycerine (0.5 mg/ml) to the perfusate, followed by phase III (90 minutes), when noradrenaline (5 µg/ml) was added to the perfusate, and phase IV (90 minutes) when nitroglycerine (0.5 mg/ml) was again added. Baseline was defined as the mean value of the measurements taken at 75 and 90 minutes.

![Phase I, Phase II, Phase III, Phase IV]

<table>
<thead>
<tr>
<th>Phase I</th>
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<th>Phase III</th>
<th>Phase IV</th>
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<tbody>
<tr>
<td>90 min</td>
<td>Urea/NG</td>
<td>90 min</td>
<td>Urea/NG</td>
</tr>
<tr>
<td>Washout and Baseline</td>
<td>Vasodilatation</td>
<td>Urea/NA</td>
<td>Vasodilatation</td>
</tr>
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**Table V:**
The experimental design contained four phases. Urea was included (20 mmol/L) in the perfusate through all phases. Nitroglycerine (NG) was included in the perfusate in phase II to dilate the tissue prior to the vasoconstriction induced by noradrenaline (NA) in phase III. Hyperemia was induced by the second NG infusion in phase IV.

The dialysate was collected and analysed every 15 minutes. Polarisation light spectroscopy using the TiVi600, and a laser Doppler perfusion imager scanned the skin every 15 minutes until the end of the experiment after 360 minutes.
The correlation between urea and glucose concentrations was assessed using linear regression. Changes in the variables assessed over time were examined using ANOVA with a repeated measures design.

**Results and conclusions**

Noradrenaline infusions led to a significant reduction in blood flow, with metabolic changes indicating local ischaemia (a rise in the lactate:pyruvate ratio and lowered glucose concentration). Urea clearance varied accordingly with decreased diffusion of urea from the catheter into the interstitial fluid and a concomitant increase in percentage of basal values (Figure 12).

![Figure 12](image-url)

**Figure 12:**
Urea clearance presented as percentage of basal over time (left). An increase in urea retrieval by the catheter corresponds to a decrease in blood flow. Glucose and lactate changes over time (right). Black line depicts the period of Noradrenaline infusion.

As noradrenaline was excluded from the perfusion medium, skeletal muscle blood flow was allowed to recover, and the metabolic variables as well as the urea clearance (expressed as a percentage of the basal) stabilised and returned to baseline. The observed differences in the shapes of the curves between 0.4 and 0.6 µl/minute perfusate flow rate indicates that urea clearance might be functioning with increasing precision at lower perfusate flow rates. The simultaneous recovery of metabolites increased with the lower perfusate flow rate.

The present study showed that inclusion of urea in the perfusion medium can successfully be used to monitor changes in skeletal blood flow parallel to assessment of meta-
bolic variables. The properties of the urea clearance technique seemed to improve at lower perfusate flow rates (0.4 compared to 0.6 μl/minute), and that a temporal advantage to lactate:pyruvate ratio is gained, as urea clearance responds faster to reduced blood flow by noradrenaline than lactate.
**Study III: “Urea clearance – a new technique based on microdialysis to assess liver blood flow studied in a pig model of ischemia/reperfusion”**

We aimed to find out if the microdialysis urea clearance technique is applicable in an established porcine ischaemia/reperfusion liver model, and to validate the technique against the established microdialysis ethanol technique in the assessment of blood flow at a higher perfusate flow rate (2 µl/minute).

**Experimental animals and operation**

Six castrated male pigs were anaesthetised and operated on according to a new protocol for segmental liver ischaemia, previously described in detail (Figure 13). An extemperate preparation urea (20 mmol/L), ethanol (5 mmol/L), and Ringer’s acetate was used. Microdialysis catheters were inserted into segment IV of the left medial lobe of liver and perfused at 2 µl/minute. The research protocol was divided into three phases (see Table V). All pigs were subjected to an ischaemic phase (phase II) when segment IV was clamped by forceps and a towel clamp.

![Diagram showing the liver clamp in relation to the microdialysis catheter.](image)

**Table V:**
The experiment design contained three phases. Urea (20 mmol/L) and ethanol (5 mmol/L) was included in the perfusate through all phases. Ischemia was induced in the fourth segment in phase II. After 80 minutes the forceps were removed in order to allow reperfusion of the tissue (recovery phase, phase III). This phase was maintained for the rest of the trial period, 340 minutes. Dialysate was collected in capped microvials every 20 minutes.

The ethanol concentration was analysed using an enzymatic reaction with alcohol dehydrogenase (ADH) and NAD⁺. The oxidation product NADH is thought to be proportional to the amount of ethanol in the sample (dialysate) (Figure 14). NADH was measured on 96 whole
Review of the studies and main results

Microtitre plates in a spectrophotometer at 334 nm wavelength. Urea, glucose, lactate, and pyruvate concentrations were analysed in a CMA 600. All measurements were analysed using Friedman’s analysis of variance to assess the significance of differences over time. Pearson’s correlation test was used to compare ethanol with urea, and lactate with glucose.

Results and conclusions

A significant increase in urea was seen promptly after induction of the ischaemic phase (p<0.005) and an even greater rise in ethanol (p<0.005).

Figure 14:
Enzymatic reaction producing NADH, which was detected as an indirect measure of ethanol in the sample.

Figure 15:
Urea clearance and ethanol clearance presented as percentage of basal (top left). Corresponding changes in glucose and lactate (top right). Ischemic period, clamping, is depicted by black line. Correlation analysis between ethanol and urea (bottom left) and lactate and glucose (bottom right), including regression lines for the corresponding parameters (ethanol:urea r=0.77 and lactate:glucose r=0.98). Dotted lines show 95% confidence intervals.
Metabolic markers including lactate, pyruvate, and glucose varied accordingly in a manner well recognised as during tissue ischaemia. As reperfusion was allowed, all variables returned to their reference ranges (Figure 15, top left and top right).

There was a good correlation between the ethanol and the urea techniques \( (r=0.77) \) over all phases of the protocol and during reperfusion \( (r=0.77) \). Laser Doppler measurements confirmed the rapid restoration of blood flow as reperfusion was allowed.

We therefore confirmed that urea clearance works in a similar way to ethanol clearance, further indicating its validity as a marker for tissue blood flow. Urea clearance changes also followed known markers of metabolism in a manner well recognised in tissue ischaemia, and it seems faster in detecting such changes in the tissue. The results also suggested that urea clearance seems applicable even at a high perfusate flow rate (2 μl/minute) in the liver.
Study IV: “Assessment of blood flow changes in a new pharmacological model of microdosing in human skin by microdialysis urea clearance”

We aimed to introduce a new in vivo human skin model in which the tissue was pharmacologically provoked in a manner previously used only in vitro. The aim was also to compare the urea clearance technique with laser Doppler perfusion imaging and polarisation light spectroscopy (TiVi) to examine its usefulness as a method of measuring skin blood flow after a local provocation to the skin.

Experimental subjects and procedures

Fifteen microdialysis catheters were used. All catheters were inserted in the ventral forearm of healthy human volunteers. The catheters were perfused with an extempore preparation of urea (20 mmol/L) and Ringer’s acetate. The research protocol contained four phases (Table V), beginning with an initial stabilization period after preparation (phase I) of 90 minutes (urea perfusate only). Phase II (90 minutes) was induced by adding nitroglycerine (0.5 mg/ml) to the perfusate, followed by phase III (90 minutes), when noradrenaline (5 μg/ml) was added to the perfusate, and phase IV (90 minutes) when nitroglycerine (0.5 mg/ml) was again added. Baseline was defined as the mean value of the measurements taken at 75 and 90 minutes.

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Table V:
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The dialysate was collected and analysed every 15 minutes. Polarisation light spectroscopy using the TiVi600, and a laser Doppler perfusion imager scanned the skin every 15 minutes until the end of the experiment after 360 minutes.
All the variables were analysed using Friedman’s analysis of variance to assess the significance of differences over time. Pearson’s correlation analysis was used to determine the correlation between the different methods.

**Results and conclusions**

Following an initial perfusion with nitroglycerine, a modest increase in blood flow was detected by urea clearance and TiVi (p<0.05), but not by LDPI. The hypoperfusion with noradrenaline that followed was clearly shown by urea clearance and TiVi (p<0.05), but not by LDPI (p=0.27). A final perfusion of nitroglycerine resulted in extended hyperaemia (Figure 16), which was detected by all methods (p<0.05) with similar high degrees of correlation.
Review of the studies and main results

(LDPI-TiVi $r=0.95$, LDPI-urea clearance $r=-0.98$, TiVi-urea clearance $r=-0.94$). The overall correlation between LDPI-TiVi was $r=0.74$, TiVi-urea clearance $r=-0.70$ and LDPI-urea clearance $r=-0.31$.

The main results are that urea clearance with microdialysis and TiVi, in contrast to LDPI, seems to be able to detect small changes in cutaneous blood flow during vasoconstriction of the skin. The results also show that in vivo microdialysis can be used to alter the blood flow in the tissue studied.

We have presented a new in vivo microvascular pharmacological model in which local blood flow and metabolism are altered by giving microdoses of vasoactive substances through the microdialysis system.
General discussion

The studies presented show that urea clearance can successfully be used with the microdialysis technique in various tissues. A more informed interpretation of microdialysis results can be achieved as changes in local blood flow are detected. Considerations about the technique are addressed below.

General comments

The general usefulness of the urea clearance technique is based on its ability to monitor changes in blood flow within tissue over hours or even days parallel to changes in local metabolism, and changes in concentrations of larger molecules such as markers of inflammation. Unique ways to investigate the microvascular regulation surrounding the catheter are enabled, as microdoses of pharmacologically active substances can be given through the system.

Another advantage is that "deeper" tissues that are not accessible superficially, can be monitored by implantation of the catheter - for example, at time of operation - as a valuable tool for tissue monitoring and detecting organs at risk of failure as in neurosurgery, liver surgery, and reconstructive plastic surgery. As a clinically relevant example, recent data on 109 consecutive free flaps showed that microdialysis provided early diagnosis of failure of perfusion and helped to save flaps. It was estimated that if one or two flaps/year are saved as a result of more effective monitoring, the extra costs of using microdialysis are covered.

As our data have indicated that changes in urea concentration may be faster than changes in the lactate:pyruvate ratio in detecting decreases in local blood flow (that is commonly used for tissue monitoring), there may be extended temporal advantages if urea clearance was used for clinical monitoring in similar settings.

High molecular weight catheters and cytokines (paper I)

Recent advances in high molecular weight catheters have expanded the scope of microdialysis to include monitoring of larger molecular weight peptides and proteins such as cytokines. These factors are likely to be increasingly interesting for the pathophysiological understand-
ing of organs with challenged homeostasis. Information may also have an impact on clinical decision-making and finding organs at risk, as shown for example in liver surgery \(^{39}\), neuro-surgical intensive care \(^{38}\), and after free tissue transfers where there are indications that expression of certain pro-inflammatory cytokines differs depending on whether the injury originated on the arterial or venous side \(^{90}\).

Cytokines have also been shown to have a regulatory effect on vessel tone \(^{91}\) and may therefore be of significance in the impairment of the microcirculatory bed, as can be seen in critically ill patients, in surgical failures or after traumatic injuries towards feeding vessels. IL-6, for example, is a potent inhibitor of the \(\alpha\)-adrenergic-stimulated contraction of smooth muscle. It is thought to have a direct effect on the contractility of vessel walls, and has therefore been speculated to be one of the key mediators in the regulation of blood flow and blood pressure \(^{91}\). Trauma-induced IL-6 may therefore be a key factor in local morbidity after blunt vascular trauma.

We used a well-established model for standard blunt vascular trauma \(^2, 80-82\) to examine the function of large molecular weight catheters in muscle distal to the trauma in paper I. Present knowledge about the inflammatory process after blunt vascular trauma is based mainly on studies of the effect of on inflammatory mediators on the central circulation. The local pattern of cytokine release at the tissue level had never been studied previously, and animal models have been asked for \(^{50, 51}\). Detection of cytokines through microdialysis with large pore membranes have however been hampered by technical difficulties. As the resistance to fluid fluxes through a single pore is partly related to its radius, microdialysis with large pore diameters has resulted in small amounts being retrieved because of an imbalance in pressure differences between the membrane and the surrounding tissue, which forces the perfusate out of the catheter. Recent data have shown, however, that this problem can be solved by using a push-pull pumping system \(^{31}\) or by the use of Ringer’s dextran as perfusate \(^{30, 32}\). We confirmed previous data, showing expected sample volumes, and verified a simple way to use microdialysis with high molecular weight catheters.

Insertion of the catheter has been an issue of interest. Although it is generally well tolerated, it is still undeniably invasive. The “equilibration period” with local metabolic events and neurogenic effects on blood flow and oedema after insertion has been well-documented \(^{92, 93}\). However, the temporal response of cytokines triggered by the insertion is
clearly much more complicated \cite{94,95}, and it is likely that it will differ depending on the tissue sampled, as inherent cytokine concentrations vary among different tissues \cite{51}. The variability in concentrations between subjects after insertion of the catheter has been shown to be substantial \cite{31}. We used the opposite leg as control, and for comparison between trauma-induced cytokine release and systemic effects. Considering the now known impact of insertion of the catheter, the use of sham experiments to exclude possible interfering factors would have strengthened our results, although comparison with other published data indicates that the insertion caused only a minor impact in relation to the large response caused by the blunt trauma.

Previous results on in vitro recovery of different cytokines at a low perfusion rate show poor recovery for all cytokines studied (IL-6 11.4 %, IL-8 16.4 % and TNFα 5.3 %) \cite{31}. Apart from interindividual differences, both insertion effects and inherent low recovery for larger molecules raise the question of the impact of changes in blood flow on the interpretation of results. Increased supply or removal by the blood will be detrimental to the data acquired, and a concurrent blood flow marker would therefore be of great value to account for the blood-flow-dependent recovery (see below). The increase or decrease in retrieval of a given substance after local microvascular alterations can be hypothesised to be mainly the result of decreased clearance of the substance from the interstitial pool by the blood, depending on which organ is studied. If the recovery of a studied compound is low as a result of limitations in technique, the impact of changes in blood flow is likely to increase. We therefore conclude in paper I that high molecular weight catheters are well suited to quantitative and qualitative studies on the chronology of cytokine production, but the trauma of insertion and changes in blood flow must be accounted for to enable proper interpretation of the data.

**Blood-flow-dependent recovery (papers I-IV)**

As previously mentioned there is increasing awareness that microdialysis recovery is altered during conditions of non-equilibrium in vivo, and that many factors influence data sampled by the technique. In vivo recovery of metabolites of the microdialysis catheter is a complex function of transport and metabolism in the sampled tissue, as well as transport across the dialysis membrane itself. The principle of passive diffusion over the membrane that describes the process of dialysis is similar to that governing the exchange of small hydrophilic
solute across the microvascular wall. This process may be described by the Fick equation, where the solute flux across the membrane is proportional to the diffusion coefficient of the solute, the area of diffusion, and the concentration gradient.

However, it is not only experimental conditions such as length of membrane, pore-size (often referred to as molecular mass cut-off), physicochemical properties of the solute, including its size and charge, and the chosen perfusion rate that will characterise the recovery of the microdialysis system. Various kinetic processes that control transport through and from the tissue, including tissue metabolism, capillary exchange, cellular uptake, diffusion through cellular membranes and tissue blood flow are of importance. These factors are, however, often assumed to be stable during microdialysis experiments. In reality this is not always true, particularly not in most clinical settings.

It is likely that metabolic processes are more important to recovery in tissues with slow extracellular to plasma exchange such as the brain, because of the blood-brain barrier. On the other hand, in tissues with high capillary exchange such as liver or muscle, changes in the metabolic rate do not alter recovery. Blood flow in these organs is likely to be a dominant factor in the recovery of the system. A few papers have previously addressed this issue and emphasized the major impact of changes in blood flow on recovery. In these pioneering studies, microdialysis was used to assess the distribution and clearance of transdermally-delivered compounds in human skin in vivo. It was shown that local modulation of the blood flow influenced the recovery of tracer substances. Local vasoconstriction was associated with increased extraction of tracer to the dialysate, which was proposed to result from a reduction in clearance of tracer from the interstitial pool by the blood and a subsequent increase in the interstitial concentration of solute in the vicinity of the catheter in a similar way as we have shown with our urea clearance model. Conversely, vasodilators induced an increase in local blood flow, and also a subsequent increase in removal of the tracer. Evidence was provided that it is a change in blood flow rather than the nature of the exchange surface that is most influential in the clearance of solute by the superficial microvascular bed in the skin. A tissue with a viable microvasculature is, thus, likely to have a significant impact on the dialysis extraction of small, freely diffusible solutes, and analysis without a concurrent flow marker may cause misinterpretation of the microdialysis results.
Urea as a tracer and endogenous urea (paper II-IV)

The main advantages of urea have been discussed in the introduction.

Urea is a slightly larger molecule than ethanol and $^3$H$_2$O (urea 60 Da, ethanol 46 Da, $^3$H$_2$O 22 Da, compared with glucose 180 Da), but the effective dialysis coefficient is only slightly higher than $^3$H$_2$O in rat muscle (urea 0.380 compared to $^3$H$_2$O 0.355) 99. The molecular mass has implications for the diffusibility of the molecules in the tissue. The Stokes-Einstein equation states that the diffusibility of a molecule is increased with decreased molecular weight, which is in accordance with the fact that complete equilibration for urea (0.66 μl/minute) is achieved at a higher perfusate flow rate than glucose (0.16 μl/minute).

A conceivable drawback when using a tracer that is normally present in the body is the possible influence of endogenous concentrations of that compound on the measurements. Urea is well known to be evenly distributed throughout all body compartments, including the vascular one. Localised changes in blood flow are therefore unlikely to cause changes in the urea concentration in the tissue per se. Nor will urea be produced or metabolised in the tissue (skin or muscle). Relatively large urea variations have, however, been shown over time, when studied in the brain of critically ill and catabolic patients, in neurointensive care. These changes are however slow, and may be explained by a generalised catabolic state of the patients. These alterations can also be corrected for if the technique is to be used over a longer period of time.

Changes in the regional blood flow may however cause changes in the recovery of the endogenous urea concentration in the tissue. Endogenous urea may thereby interfere with the changes in the exogenous urea added to the perfusate. We can speculate that in the case of vasoconstriction, such as induced by noradrenaline, the contribution of endogenous urea to the dialysate concentration is likely to decrease, while that of exogenous urea increases. However, during vasodilatation (increase of regional blood flow) the contribution of endogenous urea to the dialysate concentration increases and that of exogenous urea decreases, and the effect of endogenous urea may affect the final assessment of the change in blood flow, so the urea clearance method may be less sensitive to vasodilatation stimuli. It is likely that this effect will increase if high perfusate flow rates are used, and be less when relative recovery is increased with lower perfusate flow rates. Such effects has however not been examined in our experiments, and conclusions about the magnitude of the recovery
effects are hard to make as no quantitative measurements of the flow changes were made. This is something that needs clarification to appreciate fully the technique and enable conclusions to be made concerning the linearity in the measurements. To elucidate the contribution of endogenous urea to final urea concentration in the dialysate during vasoconstriction and vasodilatation a control experiment could be done in which no urea is added to the perfusate.

The fact that the retrieved urea concentration in the hypoperfusion phases of studies II-IV does not reach 20 mmol, which was the infused concentration, is important, and there are several possible reasons. Firstly, it seems theoretically unlikely that the retrieval of urea would reach the perfused amount even if ischaemic conditions in the tissue were reached. This is exemplified by the data from study III in which the studied liver segment was clamped and no blood flow allowed. Urea concentration reached 18 mmol, and it is likely that the 2 mmol discrepancy indicates the amount of urea that continues to diffuse into the surrounding tissue during the ischaemic phase. This is even though the global ischaemia prevented removal outside the functional catchment volume as the whole segment was under circulatory arrest.

In papers II and IV, on the other hand, local vasoconstriction was achieved by an infusion of noradrenaline. This implies that the vascular changes were limited to the area of dispersion of the vasoactive compound, which in turn may vary from tissue to tissue, for various tissue-specific reasons such as the tortuosity and inherent basal blood flow, and the structural arrangement of the microcirculation. One may therefore expect that the functional range of urea clearance (from complete ischaemia to hyperaemia) may vary between different tissues. It is therefore plausible that the urea molecules diffuse over a larger area than the molecules of vasoactive drug, and urea clearance may partly sample an area where the vasoactive drugs have no effect, and blood flow is normal with high tissue clearance. It is likely to be beneficial if the rate of flow of the perfusate is adjusted according to the inherent blood flow of different tissue compartments so that it better follows the physiological properties of the studied tissue. The perfusate flow rate should, according to these suggestions, be increased and the microdialysis membrane be shortened with an increased blood flow. A possible alternative to this would be to increase the concentration of urea in the per-
fusate - for example, in the brain if the technique is to be used there as it has a high basal blood flow.

In future experiments it is therefore important to define optimal technical properties further, including optimal perfusate flow rate, and length of membrane in the tissue of interest, rather than expecting that these properties will be constant for different settings or tissues.

**Equilibration time in measurements (paper II-IV)**

It is evident from our studies that the urea response is time-dependent, as there is a lag time from stimulation to stabilisation of clearance when the new rate of blood flow is established. That is, the value that most reliably indicates the new blood flow. This delay is mainly dependent on the actual time it takes for the equilibration of the marker between the catheter and the interstitium, and secondarily on the time-consuming sampling procedure in microdialysis, which in turn is based mainly on the perfusate flow rate used.

We think that the equilibration time ranges between 40-60 minutes; however, this issue needs further investigation. Interesting information could be gained in future studies if continuous infusions of noradrenaline and nitroglycerine were given at different rates of perfusion to produce the corresponding delay until a steady state is reached. We can see in our data a tendency for a decrease in blood flow to increase the volume of the accumulated interstitial urea around the catheter, in which steady-state concentrations were reached, in addition to increasing the steady-state concentration itself. Similarly, an increase in blood flow causes increased clearance from the immediate vicinity of the catheter, and reduction of the size of the accumulated urea, as well as the steady-state concentration. It is likely that these reciprocal changes in the volume of the pool, with the changes in blood flow, will account for the time it takes to reach a steady state, and further studies are needed to explore this issue.

Interestingly we see a difference in how urea clearance and the lactate concentrations vary over time, after a blood flow provocation. In papers II, III and IV, lactate showed a tendency towards a longer duration before the response reached a steady state. Glucose, on the other hand, responded in a similar way to urea clearance, although with a mirrored profile. This indicates that glucose may be used as an indicator of changes in blood flow. The
The rationale for using urea instead of glucose is, however, that it is not involved in tissue metabolism. Incorrect conclusions about changes in metabolic variables can then be avoided as they may be signalling a state of metabolic crisis rather than tissue ischaemia.

The lag time before a new steady state is reached after alterations in the blood flow may therefore be regarded as acceptable, as the response is still faster than that of conventional metabolic markers such as lactate.

**Comparisons with other techniques – validation (paper III-IV)**

When blood flow estimates based on different techniques are compared, one must be aware of the fact that they invariably use different approaches to depict changes in the microcirculatory blood flow indirectly. Apart from presenting data on different aspects of the actual blood flow (clearance from the interstitial compartment (MD), erythrocyte concentration (TiVi), and velocity (LDPI)), temporal and spatial resolution will be different, which makes direct comparisons between urea clearance, TiVi, and LDPI difficult.

The catchment volume should be considered when microdialysis is compared with other techniques. The exact volume drained by the microdialysis probe is still unknown. It has been estimated to be about 1 mm radius based on studies in the rat brain with microelectrodes positioned close to the microdialysis catheter. This estimation has been shown to fit theoretical predictions although visual effects, as noted in skin colour after perfusion of nitroglycerine and noradrenaline (in paper IV), suggested that the region of effect is somewhat larger. The regions of interest (ROI) for LDPI and TiVi measurements were therefore set to 1 x 2 cm (radius 0.5 cm from the midline of the catheter), which should cover the main area of provocation. This thesis does not, however, present proof that this area is not overestimated in relation to the provocation or data recovered from the microdialysis catheter, which theoretically could lead to an overestimation or underestimation of the skin provocations as recorded by LDPI and TiVi. One must realise that the catchment volume for microdialysis is partly dependent on whether the microvascular bed of the tissue is vasodilated or vasoconstricted, and that this may have consequences when compared with LDPI and TiVi, as the ROI with these measurements remained constant through all phases. We therefore decided that the ROI should be (1) constant in size, and (2) sufficiently large to capture changes that occurred during both vasodilatation and vasoconstriction. Importantly,
the measurement volume of the optical techniques LDPI and TiVi, are likely to vary despite
the ROI remaining constant. This is dependent on whether the capillary bed is vasodilated or
vasoconstricted in the sense that the optical properties of the skin will then be altered. It is,
for example, possible that the laser light (LDPI) during vasoconstriction will partly penetrate
through the capillary layer into deeper areas, which in turn will be unable to correspond to
the catchment volume of the other techniques.

The positioning of the microdialysis catheter in the skin is, furthermore, of interest in
relation to other measures. Catheters were inserted intradermally (paper IV), which corre-
sponds to a depth of about 100 µm. The placement of the catheters has previously and con-
sistently been confirmed to be in this segment of the skin, and this was also done initially as
we adopted the technique. The main proportion of the Doppler signal is thought to
originate from the upper 200-300 µm of the skin, and the sampling depth of TiVi is thought
to be at roughly 400-500 µm. This implies that all three measurements are made within the
papillary and reticular dermal compartment, which contains the rich capillary network that
constitutes the microcirculation of the skin. Even though we aimed at uniform analysis of
three aspects of the microcirculatory changes, these discrepancies of somewhat different
catchment volume must be kept in mind when we consider the correlation of the urea clear-
ance technique and LDPI and TiVi in paper IV.

These challenges were assumed to be less in paper III, in which comparisons were
made with the ethanol clearance technique that functions according to the same principles
as urea clearance. A minor difference in catchment volume can, however, be assumed be-
cause of differences in diffusibility between these substances.

The disadvantage of using ethanol clearance as reference is that it is not suitable at
microdialysis perfusate flow rates of less than 2 µl/minute. To enable such a comparison it
was therefore inevitable for us to use the higher flow rates, although our results in paper I
suggested that the functionality of urea clearance is increased with lower flow rates. The
ethanol clearance technique has several other drawbacks (discussed earlier), which we have
also encountered in the process of developing a valid protocol. The analysis procedure is
tedious and the risk of contaminated samples always present, as even small amounts of al-
cohol will contaminate the samples. The urea protocol lacks these disadvantages, and func-
tions with higher precision at lower perfusate flow rates (paper I). This is a great benefit
when a higher recovery of metabolites are warranted, and permits sampling of substances with low interstitial concentration such as cytokines. Further studies are, however, warranted to find out which perfusate flow rate is optimal for urea clearance in respective tissues, as diffusion properties will be different depending on whether the experiments are done in skin, muscle or liver.

**How are data processed? (paper II-IV)**

Clearance is traditionally referred to as the rate at which a given substance is eliminated from a body fluid, usually blood. The rate of elimination is dependent mainly on two factors: the function of the organ that does the eliminating (for example, the kidneys), and the current concentration of the substance in the body fluid being investigated. The term “clearance” in these instances has been used as a quantitative measure of the rate at which a substance is removed from the blood:unit of time, and this is often used as a reference of - for example - renal function. We refer to changes in clearance as the blood-flow-dependent removal of the interstitial urea concentration after continuous supply of a fixed amount through the microdialysis catheter (20 mmol/L). This is expressed as a ratio of dialysate concentrations and the basal urea concentration at specific time points, and is a modification of the technique introduced by Hickner. In Hickner’s original work he expressed ethanol clearance as a ratio of changes in inflow:outflow ratio to baseline, which is actually a ratio of a ratio.

We aimed to find a simplified way of making the estimation. As the inflow concentration in our studies is regarded as stable, calculations of inflow:outflow ratios are therefore regarded as unnecessary, as data are related to the individual baseline. It is, furthermore, beneficial if the urea clearance is presented as ratios rather than in absolute values, as inter-individual variations will be less and it enables better comparison between individuals. It is also well known that there is considerable spatial heterogeneity in the distribution of blood flow within most organs, particularly in skin and muscle where extrapolation of total blood flow from a single microdialysis probe may be hazardous. This factor becomes less relevant, however, when changes over time are related to the baseline recording.
Quantification? (paper II-IV)

The microdialysis urea technique must be regarded as being semiquantitative in its present state. To allow for better and quantitative measurements further studies are necessary, particularly of linearity and the detection limit of urea clearance in measurements of blood flow. This information can be gained only if the method is validated against direct measurements, such as in an in vitro model of a perfused organ where flow can be adjusted in defined steps. Such studies have been made on the ethanol clearance technique, but comparisons with direct measurements show that the relation between ethanol clearance and whole muscle blood flow is not linear 17. The outflow:inflow ratio, however, continues to decrease with increasing blood flow to about 100 ml/100 g/minute 17, and there is linearity in the measurements in the blood flow range of 4 to about 45 ml/100 g/minute. Our experimental setup, using urea clearance with the characteristics shown, is well able to detect flow when using the microdialysis system at low perfusate flow rates which also permits high recoveries of other solutes. In the clinical setting the latter has the potential for being more valuable than the ethanol technique, which has several practical shortcomings previously described in the thesis. It is, however, possible that the optimal detection range of the technique is limited, and further studies are warranted to find out these limitations.

New skin model (paper IV)

Urea clearance was also evaluated in a new skin model in which vasoactive agents were given through the catheter to modulate the tissue. The rationale for using microdialysis in the development of such a model was that most drugs exert their pharmacological effects on extravascular structures rather than in the bloodstream. The interstitial fluid of a target tissue may, thus, be regarded as the true anatomical “effect compartment” where pharmacological compounds effect local microcirculatory and cellular events in vivo. Microdosing with different vasoactive substances enables us to give precisely measured precalculated doses in a precise site. Organ-specific effects can therefore be monitored without the need for consideration of systemic upsets, and still provide in vivo data on the effects of drugs that previously has been studied only in vitro.

Much of our present knowledge about vascular function is based on in vitro studies of isolated vessels. The important functions of nitric oxide on the endothelial cells were, for example, discovered this way 104, 105. Apart from the fact that they lack interaction with the
surrounding tissue, the shortcomings of the in vitro models are that they are often of uniform size and seldom of human origin. Our hypothesis was that vascular effects studied in a microdialysis model in vivo would be more adequate than the original in vitro models, as it incorporates the influences of structures and physiology surrounding the microvasculature. We used skin for the development of our model (paper IV), as it is often used as a representative organ for studies of microcirculatory function and dysfunction. An advantage of skin is that it is easily accessible and richly vascularised. However, as the upper dermis generally contains only few and slow moving RBCs, background flow is low and close to biological zero. This makes measurements of blood flow somewhat troublesome (LDPI and TiVi) in the normal state. The microvascular bed was therefore predilated to avoid the development of a floor effect when vasoconstrictors were applied. This is a technique that has been used often both in vitro and in skin models using iontophoresis. The main advantage of such an approach is that it normalises the sample population and increases contrast of the following provocations. We saw that the variability between subjects (SD/mean) of TiVi and LDPI was lower than that of urea clearance during all phases. A likely explanation of this is the larger area over which the perfusion was measured, because both the TiVi index and perfusion measured with laser Doppler were mean values obtained from thousands of image pixels from the chosen region of interest, while urea clearance was measured on a single site with a measurement radius of typically a few mm.

We found urea clearance to be a good and valid marker for blood flow in this model, and concluded that it has several advantages over LDPI, particularly in detecting reductions in blood flow when the basal perfusion of the organ was small. The changes in skin blood flow that we induced experimentally were similar in magnitude to those seen under normal physiological conditions. Thus, our findings will have important implications for the design and interpretation of studies in which local tissue blood flow may be subject to change. The development of this model has laid the foundation for further analysis of how urea clearance responds to graded provocation. Further refinement and characterisation of the urea clearance technique is needed to express the data in conventional dose response models. This will ultimately strengthen the method as a dose-response analysis for interpretation of results in well-defined pharmacodynamic measures. In future projects using this model it may be interesting to explore the impact of the clearance of drugs from the department by the
vasculature and the relation between local drug concentration and blood flow, or to evaluate optimisation of transcutaneous drug delivery through manipulation of local blood flow to facilitate (vasodilatation) or limit (vasoconstriction) distribution in the skin.
Conclusions and comments

I - We have shown that high molecular weight microdialysis catheters may be used to assess inflammatory mediators in microdialysis studies. The use of high cut-off catheters is however likely to be susceptible to influence by alterations in blood flow, and our experience further stresses the importance of concurrent blood flow registrations to appreciate fully the results of such studies.

II - The data presented have shown that inclusion of urea in the perfusate and clearance of urea reliably and reproducibly can be used to monitor changes in local blood flow. The simplicity of the technique is its main feature, as it functions parallel to routine microdialysis measurements with a standard microdialysis kit, which enables concurrent, reliable monitoring of metabolites with acceptable temporal characteristics. The urea clearance technique lacks the disadvantages of existing methods for monitoring blood flow with microdialysis, including the need for exposure to radiation for detection, advanced analytical procedures, and risk of contaminated samples. Based on the results presented it is likely that it may function with a higher precision at lower perfusate flow rates. This is a great benefit when greater recovery of metabolites is warranted, and permits sampling of substances with low interstitial concentration such as cytokines or other high molecular weight substances that may be of interest if the technique is used for advanced investigations in routine patient care.

III - The microdialysis urea clearance technique was tested in an established porcine ischaemia/reperfusion liver model and validated against the microdialysis ethanol clearance technique at a higher perfusate flow rate (2 μl/minute). Urea clearance had similar characteristics to ethanol clearance, and adequately followed known markers of metabolism and laser Doppler flowmetry in a manner well recognized as tissue ischaemia, and seems faster in detecting such changes in the tissue.

IV - We further compared the urea clearance technique with laser Doppler and polarisation light spectroscopy and showed reproducible and reliable results with the technique. We used new approaches to administer microdoses of drugs with microdialysis to modulate and test the local microcirculatory physiology. The new human skin model presented here enables manipulation of the local microcirculation in a manner previously not shown in vivo.
Future perspectives

The main finding of this thesis is that inclusion of urea in the perfusate can be used to assess tissue blood flow in various tissues and species. This new technique may be of use in several areas of microvascular research, and for clinical evaluation and surveillance of tissues with challenged homeostasis. Some issues, however, need further clarification; including the linearity of blood flow estimation, detection limits, and optimal perfusate flow rates and the optimal concentration of urea for the technique in the tissue compartment of interest.

The findings in paper IV open a new application for the microdialysis technique, where controlled modelling of an easily accessible tissue compartment (skin) can be used to examine and understand local physiological responses that can be related to the same individual's general microvascular function or dysfunction. Microvascular reactivity can be assessed after modulation by various means, such as local heat stress (nitric-oxide-dependent vasodilatation) and postocclusive hyperaemia as well as according to the described protocol in paper IV, and the method refined according to the suggestions above. Responsiveness to physiological doses of vasoactive substances can solely, or in combination with heat stress or a localised insulin clamp attained through the microdialysis system, be evaluated while nitric-oxide-dependent reactions and skin blood flow are controlled. Future experiments in healthy subjects, patients with impaired microvascular function (such as for example diabetes mellitus and peripheral vascular disease) as well as critically ill patients (such as burn victims) can thereby be conducted with the incentive to give an informed picture of microvascular status and possibly inform about risk of tissue morbidity.
Future perspectives
Summary in Swedish

Urea clearance – en ny metod för att studera lokala blodflödesförändringar i olika vävnader


Med tekniken kan man övervaka vävnader under stress, till exempel i hjärnan efter blödnings, eller lambåvävnader som används i rekonstruktiv plastikkirurgi, för att bilda sig en uppfattning om hur de mår.


Vår hypotes har varit att vi genom att tillföra ett överskott av urea i buffertlösningen som flödar genom mikrodialysetet kan mäta hur stor andel av densamma som borttransporteras av det lokala blodflödet. På detta sätt får vi ett indirekt mått på lokala blodflödet. Vi har testat tekniken i tre olika organsystem (muskel, lever och hud), i tre olika arter (råta, gris och människa) för att få stöd för hypotesen stämmer. I avhandlingen styrker vi våra hypoteser och konkluderar att urea clearance kan vara ett bra instrument att mäta lokala blodflödesförändringar i vävnader, dels för att följa vävnadens faktiska blodflöde, men också för att bättre kunna tolka data från andra markörer i mikrodialyse (såsom blodsockernivåer och inflammationsmarkörer). Analysen är enkel och kräver ingen avancerad utrustning vilket är en av dess styrkor.
Summary in Swedish
Acknowledgements

Folke Sjöberg – for never-ending enthusiasm and cheerful mind! When in doubt, you always made this project feel worthwhile and me feel like a winner. I know (some of) your tricks by now, and have come to understand how to manage this despite them. I have really enjoyed making this journey with you and look forward to being able to continue to bug you about future projects and being your friend. Thank you Folke - for believing in me and this thesis!

Lars-Erik Karlander – for inviting me to continue the work you started. For all your invaluable help at the beginning of this project and encouragement, both research wise and in hand surgery.

Erik Zettersten – superstudent, collaborator and good friend! For all the help and some memorable days in Cologne!

Anders Samuelsson – running mate and fellow researcher. Thank you for all support, pep talk and good discussions over a cup of coffee. You really made this journey more fun and personalises the research group I much lacked in the beginning of this.

Erik Tesselaar – for being such a genuinely nice guy! For all your invaluable help and support (!), superb discussions and fun talking about our future projects. I really look forward to what is coming and enjoy having you next door...

Johan Thorfinn – for your generosity, time and being the best possible room mate and friend. It has been great working with the bookchapter and endless discussions about various projects, technical gadgets and late night mail chats...

Thomas Hansson – boss, tutor and friend. For making this possible and being supportive in the process of both this and during my pursuits as hand surgeon.

Göran Nylander – former head of department, who believed in me and hired me some years ago. For your dedication to hand surgery, our clinic and for all support through the years.

Carin Rubenss – special thanks for encouragement through this process and for always being so cheerful and supportive!

All other super hand surgery collegues: Magnus Berggren, Ulf Larsson, Erika Nyman, Anna-Carin Lundin, and all the collegues att the Plastic Surgery Dept. – for being great collegues and friends, and for making our clinic a really good and fun place to work in!

Anders Winbladh, Jan Henricsson, Florence Sjögren and Ingrid Steinwall – co-authors without whom some of this work would never have happened...
Acknowledgements

Joacim Henriksson – sharing your knowledge and experience with TiVi and LDPI and good collaboration on the hyperaemia study.

All great reviewers out there – for challenging me and my papers and helping me becoming better at this.

Most importantly - my family:

Ulla and Lennart Malmgren and all of the wonderful “Malmgren clan” for great support and fun times together in our Kivik haven.

My brothers Filip and Jacob with families for remaining close although we see less of each other than I would have wanted!

and foremost my mother Gunnel and father Lars-Ove for always being there, and all help throughout the years – I would never have been here without your support and guidance! Thank you for being excellent grandparents to our kids and helping out when we needed.

Finally, the really loved ones, my little wonders:

Lydia and Svante who make my every day life fantastic – you are the very best a father could wish for! Thanks also for the “scribble” on the blackboard...

Lovisa - the love of my life! You make me happy, you are my best friend and true companion in everything! I have really enjoyed doing the “thesis race together” and believe we managed really well.
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