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# **Effects of burns and vasoactive drugs on human skin,**

- Clinical and Experimental studies using microdialysis

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**To Annika, Karin and Erik**

**“I started out with nothing and I still got most of it left”**

**Seasick Steve**

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## Abstract

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Patients who require critical care, including those with burns, are affected by a systemic inflammatory reaction, which at times has consequences such as multiple organ dysfunction and failure. It has become increasingly evident that other factors important in the development of organ dysfunction are disturbances at the tissue level, in the microcirculation. Such disturbances activate cascade systems including stress hormones, all of which have local effects on organ function.

Despite this knowledge, monitoring and treatment in critical illness today relies mainly on central haemodynamics and blood sampling.

Microdialysis is a minimally invasive technique that enables us to study the chemical composition and changes in biochemistry in the extracellular, extravascular space in living tissues. Most of our current experience is from animal models, but the technique has also been used in humans and has become routine in many neurosurgical intensive care units to monitor brain biochemistry after severe injury. In skin, this experience is limited.

During the first half of this thesis we studied the injured and uninjured skin of severely burned patients. The results show that there are severe local metabolic disturbances in both injured and uninjured skin. Most interesting is a sustained tissue acidosis, which is not detectable in systemic (blood) sampling. We also recorded considerable alterations in the glucose homeostasis locally in the skin, suggesting a cellular or mitochondrial dysfunction. In parallel, we noted increased tissue glycerol concentrations, which indicated appreciable trauma-induced lipolysis.

We also examined serotonin kinetics in the same group of patients, as serotonin has been claimed to be a key mediator of the vasoplegia and permeability disturbances found in patients with burns. We have shown, for the first time in humans to our knowledge, that concentrations of serotonin in skin are increased tenfold, whereas blood and urine concentrations are just above normal. The findings support the need for local monitoring of substances with rapid local reabsorption, or degradation, or both. The results also indicate that serotonin may be important for the systemic response that characterises burn injuries.

In the second half of the thesis we evaluated the effects of microdosing in skin on metabolism and blood flow of vasoactive, mainly stress-response-related, drugs by the microdialysis system. The objectives were to isolate the local effects of the drugs to enable a better understanding of the complex relation between metabolic effects and effects induced by changes in local blood flow. In the first of these two studies we showed that by giving noradrenaline and nitroglycerine into the skin of healthy subjects we induced anticipated changes in skin metabolism and blood flow. The results suggest that the model may be used to examine vascular and metabolic effects induced locally by vasoactive compounds. Data from the last study indicate that conventional pharmacodynamic models ( $E_{max}$ ) for time and dose response modelling may be successfully used to measure the vascular and metabolic response in this microdosing model.

We conclude that the microdialysis technique can be successfully used to monitor skin metabolism and isolate a mediator (serotonin) of the local skin response in burned patients. It was also feasible to develop a vascular model in skin based on microdialysis to deliver vasoactive substances locally to the skin of healthy volunteers. This model provided a framework in which the metabolic effects of hypoperfusion and reperfusion in skin tissues could be examined further.

## List of original papers

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

- I. Samuelsson A, Steinvall I, Sjöberg F. Microdialysis shows metabolic effects in skin during fluid resuscitation in burn-injured patients. *Critical Care* 2006; 10(6):R172.
- II. Samuelsson A, Abdiu A, Wackenfors A, Sjöberg F. Serotonin kinetics in patients with burn injuries: A comparison between the local and systemic responses measured by microdialysis – A pilot study. *Burns* 2008; 34: 617-622.
- III. Samuelsson A, Farnebo S, Magnusson B, Anderson C, Tesselaar E, Zettersten E, Sjöberg F. Critical Care implications of a new microdosing model administering vasoactive drugs (noradrenalin/nitro-glycerine) by microdialysis to human skin. *Submitted*.
- IV. Folkesson T, Tchou K, Samuelsson A, Tesselaar E, Dahlström B, Sjöberg F. Assessment of a microdialysis method using urea clearance as a marker of drug induced changes in dermal blood flow in healthy volunteers. *Submitted*.

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## Abbreviations

ACN	Acetonitrile
CO <sub>2</sub>	Carbon dioxide
ED <sub>50</sub>	Maximal effective dose
ELISA	Enzyme-linked immunosorbent assay
E <sub>max</sub>	Maximum effect
H <sup>+</sup>	Hydrogen ion
HPLC	High-performance liquid chromatography
ICU	Intensive care unit
IL	Interleukin
LDF	Laser Doppler flowmetry
LDPI	Laser Doppler perfusing imaging
MAO	Mono amino oxidase
MD	Microdialysis
MODS	Multiple Organ Dysfunction Syndrome
NA	Noradrenaline
NGT	Nitroglycerine
NIDDM	Non insulin dependent diabetes mellitus
NIRS	Near-infrared spectroscopy
NO	Nitric oxide
NPY	Neuropeptide Y
O <sub>2</sub>	Oxygen
OPS	Orthogonal polarization spectral imaging
PMN	Polymorphonuclear neutrophilic leukocyte
ROI	Region of interest

ROS	Radical oxygen species
SIRS	Systemic Inflammatory Response Syndrome
SkBF	Skin blood flow
TBSA	Total burned body surface area (%)
TNF- $\alpha$	Tumor necrosis factor
VAP	Vasopressin
VIP	Vasoactive intestinal peptide
VOP	Venous occlusion plethysmography

# Introduction

## Background

Most patients admitted to the ICU are treated for life threatening organ dysfunction. Organ failure is most often a consequence of the Systemic Inflammatory Response Syndrome (SIRS), a condition characterized by rapid and severe deterioration of physiological functions and it is defined by at least 3 of the following criteria: fever or hypothermia, tachycardia, tachypnea and elevated or low leukocyte counts. Most severe illnesses can elicit SIRS but it's usually associated with infection or trauma, including burns [1]. Excessive SIRS leads to shock, distant organ damage and multiple organ failure a conditions which is also characterized by inadequate oxygen delivery to the tissues [2]. Concomitant metabolic changes due to metabolic stress are seen such as hypermetabolism with enhanced energy expenditure and insulin resistance [3].

The treatment of SIRS and shock is based on the cornerstones, source control and restoration of oxygen delivery to tissue by aggressive fluid therapy and if needed vasopressors and inotropic drugs [4].

## SIRS/MODS

The pathophysiology of SIRS and Multiple organ dysfunction syndrome (MODS) is claimed to be based on a dysfunctional inflammatory response following shock and reperfusion. Mechanisms that are not completely understood, but includes the release of cytokines, pro-inflammatory lipids and proteins acting on polymorphonuclear neutrophils (PMN) [5]. PMN's are mobilized and migrates from the systemic circulation to end organs, where they cause direct local cytotoxic cellular effect by degranulation, release of nitric oxide (NO) and

reactive oxygen species as well as adhesion molecules [6]. There is also a remote systemic effect, by circulating systemic pro-inflammatory mediators such as IL-8, IL-6 and TNF- $\alpha$ . In parallel, in the pathophysiology, there are compensatory anti-inflammatory actions induced. The inflammatory reaction also involves the complement and coagulation systems as well as the release of bioactive amines [7]. Burn injury is known to elicit a prominent inflammatory response, which is elicited at a defined time and with a reaction that is proportional to the size of the burn injury [8]. Further, burn injury is easily accessible for studies and analysis and thus is frequently used as a SIRS/MODS model, not least in animal studies [9].

## **Treatment**

The underlying cause of the inflammation is therefore to be treated as soon as possible. It has been repeatedly shown that the extent of the inflammatory response is proportional to the risk of developing MODS. A short time to the correct institution of adequate antibiotics in sepsis, as well as rapid resuscitation of blood volume deficits and early surgery in trauma, have all proven to decrease the inflammatory response and thus, reduced the risk of sequential organ failure [10]. In burns early excision of injured skin have revolutionised the care and significantly improved morbidity as well as mortality [11].

Independent of the aetiology of SIRS, a consequence is global hypo-perfusion due to vasoplegia [12] and fluid loss from the circulating blood volume to the interstitial space as a result of increased permeability [13]. Rapid restoration of circulating blood volume is essential to maintain adequate oxygen delivery [14]. An obvious risk during permeability disturbances is over resuscitation with increased tissue oedema, impairing oxygen diffusion and local tissue blood flow. To guide fluid volumes, clinical parameters as urinary output and skin temperature is often used. In the ICU setting measurement of central hemodynamic parameters such as i.e., central venous pressure, cardiac output, stroke volumes and vascular

resistances or visualisation of cardiac function by ultrasound techniques is used [15]. Increasing awareness of the importance of adequate titration of the resuscitation fluid volumes has led to the development of numerous technical solutions aiming at examining more and more circulation parameters. To ensure proper tissue oxygenation, systemic lactate concentrations has been proven a good marker. Even more sensitive is a timely clearance of lactate, where a rapid normalisation is associated with better survival [14]. Oxygen delivery can also be measured by central or mixed venous saturation, which therefore has been increasingly used and advocated [16]. Systematic use of combined and fixed endpoints for optimizing tissue oxygenation and resuscitation has been proven to reduce morbidity and mortality considerably and has led to the introduction of internationally accepted guidelines in e.g., treating sepsis [10]. Still, it has to be recognised that measurement of blood lactate is a mean estimate of the perfusion in all organs and that hypoperfusion and oxygen deficit or depletion may persist locally in any of the tissues [17].

Burn injuries have unique characteristics in terms of resuscitation needs. The loss of barrier function of the skin together with local reactions in skin creating a negative interstitial pressures, so called “negative imbibition pressure”, in addition to SIRS associated changes, cause an enormous loss of fluid and effects on circulating volume giving rise burn shock and an concomitant massive oedema [18]. These changes, which are transient and most pronounced during the first 3-6 hours, are almost over in 24 hours. The fluid need is proportional to the size of the burn injury. Current strategies, which were established in the late sixties and early seventies, are aimed at providing sufficient fluid to ensure organ perfusion and at the same time minimise tissue oedema [19, 20]. Blood pressure and urinary output have remained as the relevant endpoints albeit that, more advanced monitoring for circulatory optimization have been suggested [21, 22]. Despite adequately fulfilling such

endpoints, also for burns severe tissue disturbances such as local acidosis in skin are found [23].

### **Microcirculatory changes**

Microcirculatory function is essential for adequate tissue oxygenation and organ function. It consists of the smallest blood vessels, arterioles, capillaries and venules. Correct function is dependent of driving pressure, arterial tone, rheology and capillary blood flow, and structure as well as function is heterogeneously distributed both within and between different organs [24]. Regulation of microvascular perfusion depends on several intrinsic systems. Myogenic sensors assesses stress were as, metabolic ones react on changes in  $O_2$ ,  $CO_2$ , lactate and  $H^+$ . These systems together with neurohumoral signalling regulate blood flow to meet the oxygen demands in tissue. Endothelial cells, lining the capillary walls, play a central role in this signalling and they are also important in controlling coagulation and immunology [25]. During SIRS and shock microcirculatory dysfunction is characterized by heterogeneously distributed abnormalities with areas of under perfusion whilst other areas are over or normally perfused [26]. This dysfunction is not clearly manifested in systemically monitoring techniques, such as e.g., mean arterial pressure and cardiac output variables. During SIRS and shock endothelial cells lose their regulatory capabilities [25]. Further, the nitric oxide (NO) system is often severely affected altering normal vasodilatation. Smooth muscle cells in the arteriolar wall lose their sensitivity to adrenergic stimuli and vasoconstrictive capacity [12]. Circulating red blood cells becomes less deformable and aggregates with effects on NO release [27]. Additionally, PMN's are activated locally causing direct vascular trauma by release of reactive oxygen species. Furthermore, they cause disruption of junctions between cells increasing risk of tissue oedema [13]. Activated coagulation reactions cause microthromboses, which may further impair microcirculation. Platelets are activated and known to

release serotonin to induce vasodilation and in order to prevent intravascular thrombosis. Serotonin has a strong vasodilation effect and also affects capillary permeability. In animal burn models, blocking serotonin has shown an attenuated vasodilatation response and permeability change secondary to the burn. In humans corresponding data is lacking. Sustained inflammation has also been shown to affect mitochondrial function, where uncoupling of the oxidative capacity remains despite adequate blood flow. This resulting in energy deficiency and dysfunctional energy dependent processes, such as substance transport, against concentration gradients [28, 29]. The net result is disturbances in substrate utility, and acidosis due to lactate formation.

From a clinical point of view a recent and important finding is that NA, the most frequently used catecholamine for shock treatment, does not correct microcirculatory alterations despite an improvement of central hemodynamic data [30]. Further, administered NA enhances the metabolic stress and induces increased levels of radical oxygen species, which may deteriorate mitochondrial function [31, 32]. Still, NA (or epinephrine) is widely recommended to treat vasoplegia in shock [33]. Very little is however known of the metabolic consequences of vasopressors, not least the more recently introduced i.e., vasopressin.

## **Skin**

The skin is the largest organ in the body, covering its entire surface. Its main function is to act as a barrier, sensory organ and it is of major importance for thermoregulation, all, functions that are essential for survival. Skin also has a pivotal role in the immune regulation of the body [34]. Anatomically the outermost layer (epidermis) consists mainly of keratinocytes that emerges from rete cells connecting the epidermis to the underlying dermis via the basement membrane. Epidermis also contains melanocytes for pigmentation, Merkel cells as sensory organs and immunological active Langerhans cells [34].



Most of the skin microvasculature is contained in the papillary dermis 1-2 mm below the epidermal surface. It comprises two horizontal plexuses. The upper, contains terminal arterioles from which capillary loops arise. These are always composed of an ascending limb, an intra intra-papillary loop with a hairpin turn and a descending limb, connecting to a post capillary venule. The single capillary loops per papilla have an intra and an extra papillary loop portion. The lower plexus is formed by perforating vessels from underlying muscle and subcutaneous fat and is connected to the upper horizontal plexus through arterioles and venules in a step angle also providing blood supply to glands in the reticular dermis. The character of the vessels in the lower horizontal plexus is similar to those in fat or muscle tissue. Generally arterioles and venules in skin run in parallel constituting a counter current mechanism of importance in thermoregulation [35], figure 1.

Skin is one of the most dynamic organs in the body in respect of blood flow changes. During normal baseline conditions the skin blood flow (SkBF) constitutes about 5% of cardiac output. However, skin circulation can vary from almost zero during maximal vasoconstriction to about 60 % of cardiac output in hyperemia or hyperthermia states [36]. Blood flow regulation in skin has been extensively investigated but is not fully understood.

In glabrous, non hairy regions i.e., palms and lips, vasoconstriction is dependent solely on noradrenergic vasoconstrictive nerves [36]. In hairy regions, the major part of the body, SkBF is mediated by two branches of sympathetic nerves: noradrenergic for vasoconstriction and cholinergic for vasodilatation, - a system unique for humans [37]. During baseline conditions the neurogenic activity is close to zero, thus altering effects between cholinergic and sympathetic stimulation may be seen, which leads to the effect of “vasomotion” [38]. Vasoconstriction is dependent on mainly  $\alpha_1$  and  $\alpha_2$  receptors. The response is also modulated by  $\beta$ -receptor mediated vasodilatation, possibly protecting tissue from ischemia during adrenergic provocations. Release of neuropeptide Y (NPY) concomitant to noradrenalin is

well established but the exact role of NPY is unclear, but a role as co-transmitter is most likely [36]. New insights to mechanisms of skin vasoconstriction have revealed that reactive oxygen species (ROS) from mitochondria in vascular smooth muscle mediate vasoconstriction [39]. The effect is mediated by translocation of  $\alpha_2$  receptors from the trans-Golgi apparatus thus increasing the density of receptors at the cell membrane and increasing the sensitivity to catecholamines [39]. The latter mechanism is only established in animal models and its occurrence in humans is still to be demonstrated.

Mechanisms of vasodilatation in skin remain to a large extent enigmatic, despite many investigations. Cholinergic activation by acetylcholine is of major importance but not sufficient to induce full vasodilatation. Several neurotransmitters have been suggested but at present the most likely and most important substance in early vasodilation is vasoactive intestinal peptide (VIP) and the better described nitric oxide (NO) dependent mechanism for continued vasodilation, especially related to thermoregulation [36]. A finding which is of interest from a critical care perspective as NO donors have been claimed to be of value in reestablishing tissue blood flow in shock [40].

Metabolism in skin is poorly described, not least during changes in SkBF. Most interesting is that the reactivity of the skin microvasculature to ROS indicate that skin is adapted to a more or less permanent state of low or hypo-perfusion which may also be important for other states of more pronounced vasoconstriction such as is seen in shock, hypovolemia, hypothermia or subsequent conditions with microcirculatory disturbances. Investigations of skin in the normal state reveals that skin then shows increased lactate levels, as compared to other tissues, indicating a normal, partly non-oxidative metabolism [41]. This indicates that there is a considerable non-nutritive blood flow present, and a relative capillary perfusion deficit.

In burn injury, independent of mechanism, the insult is directed primarily towards the skin. It has been recognised since decades that the local skin response is the motor of both local and systemic immunological responses that characterize burns[8]. The complex nature and interactions of these inflammatory mediators are not fully understood but certain systems are believed to be of major importance. Most investigated are the arachnidonic acid-, kallikrein-bradykinin-, complement-, coagulation/fibrinolytic cascade systems together with bioactive amines and catecholamines [42]. The local reaction affects and activates a generalised systemic response and cause subsequent microcirculatory disturbances, which promotes complications such as multiple organ failure [8]. The challenge in early burn care is to titrate fluid therapy to avoid both hypovolemia with further ischemic insult to tissue and over-hydration where oedema impairs gas and nutritive exchange at the tissue level [8].

## **Models**

As methods for local monitoring of human skin “in vivo” has been lacking, most knowledge on burn pathophysiology is derived from animal models [9]. Most of what is known of the inflammatory response to burns is gained from studies in primarily mice, rats and pigs. Even if such data has been fundamental for the understanding of the pathophysiology and enabled testing of therapeutic interventions, it has become increasingly evident that animal models have shortcomings. Small mammals are hairy, have thin dermis and epidermis and wound healing is by contraction rather than re-epithelialisation [9]. Larger animals like pigs and dogs are generally more like humans in both anatomy and in response to trauma. Further, there are substantial differences in biochemistry as many results from therapeutic interventions in animals have been difficult to reproduce in man, suggesting that many correlations are not representative in humans. There is also an ethical dilemma that can't be overlooked in inducing severe burn injury to animals.

## Serotonin

Serotonin is a biogenic amine most noted for its role as neurotransmitter. Over time it has become evident that serotonin is also important in a variety of functions outside the central nervous system. Example of such is significant effects of importance in the regulation of vascular tone, enhancement of platelet aggregation and involvement in the pathophysiology of emesis, irritated bowel syndrome and systemic and pulmonary hypertension. Synthesis of serotonin in humans outside the central nervous system is predominantly in the enterochromaffin cells of the gastrointestinal tract. Other quantitatively important stores are found in platelets and a small amount at nerve endings [43]. Platelets readily take up serotonin from plasma leaving very low concentrations in circulating plasma [44]. A minor quantity of its metabolism occurs outside the serotonin containing in the lungs, liver and kidneys.

At current, there are seven subtypes of receptors 5HT<sub>1-7</sub>. Most subtypes exhibit heterogeneity and are further divided into subtypes as e.g., 5-HT<sub>1A</sub>, 5-HT<sub>1B</sub> [45]. The effects and interaction of serotonin are complex and dependent on receptor type as well as receptor density in the target organ. The main vascular effects of serotonin released from platelets are vasoconstriction of large arteries, veins and venules [46]. Furthermore, serotonin indirectly contributes by amplifying the effect of NA, angiotensin and histamine [47, 48]. The vascular response may also involve vasodilatation and it is then linked to release of nitric oxide and dependent on the activation and integrity of the underlying endothelium [47, 48].

Additionally, activation of serotonergic receptors on adrenergic nerve endings reduces the release of noradrenalin [48].

Tissue destruction, such as burns, exposes sub-endothelial structures and circulating platelets react with exposed collagen, adheres and aggregates and releases their content including serotonin [49]. It has been recognised in animal burn models since decades that serotonin is a key mediator in burn injured tissue where it locally causes vasoplegia and a pronounced

increase in capillary permeability [50]. Serotonin is also about 200 times more effective in this aspect than is histamine [51]. Even so, data are conflicting as serotonin, post burn is increased in rats, but not rabbits, suggesting a significant species difference [51]. Other important differences includes that rats store and release serotonin from mast cells which is not the case in humans [52]. The kinetics of serotonin turnover is also different between animals and humans[53]. Despite conflicting results between species, pharmacological interventions blocking 5HT systemically have been successful. In dogs, the post burn blood flow increase was abolished and oedema formation decreased [54, 55]. In rabbits, the same 5HT - blocker closed functional shunts and reduced blood flow and redirected it from non-nutritive to nutritive areas of the skin, resulting in preserved protein kinetics and a reduction of oedema [56].

Even if serotonin effects and kinetics is thoroughly investigated the knowledge of its role in human burns is lacking. The only publication available is from 1960 and the study showed increased levels of 5HIAA in urine[51]. Tissue concentration effects would thus be expected to be more effected. From these observations it's clear that there is a definite need for more knowledge regarding the role of serotonin in burn induced vascular changes in humans.

## **Noradrenalin**

Vasoconstriction in skin is, as mentioned, mainly dependent on NA [36]. NA is synthesised from tyrosine and actively transported to the postganglionic sympathetic nerve endings. NA is stored within large vesicles also containing calcium, binding proteins and a variety of peptides and ATP. There seems to be both an actively re-circulating population of vesicles as well as a population only released on extensive stimulation. Ten % of the stored NA is readily available, approximately 1 % at each depolarization. Inactivation of NA is mainly by reuptake in vesicles for reuse. Smaller amounts, not recycled are deaminated by Mono Amino Oxidase

(MAO). Peripheral vessels almost lack re-uptake mechanisms whereas these are extremely effective in the heart [57].

Systemically, NA is derived from the adrenal medulla in which it constitutes about 10-20 % of the catecholamine content and which is released in conjunction to a stress response. NA is present in plasma in small concentrations and is rapidly, ( $T_{1/2}$  (half life) is less than a minute) cleared. Twenty-five % is removed by the lungs, the remaining amounts are degraded by MAO or catechol-O-methyl transferase in blood, liver and kidneys [57].

Post-synaptically, the effect of NA is exerted through binding to  $\alpha$  and  $\beta_1$  receptors. The main effects are increased heart rate, elevated blood pressure through vasoconstriction, mostly in the skin, gut, kidney, liver and an increased contractility of the heart. Effects on the peripheral circulation is more pronounced than that of adrenaline. Adrenalin is known to have profound metabolic effects with increased oxygen consumption, altered glucose homeostasis both mediated by adrenal receptor effects on insulin secretion and direct effects on cell metabolism [3]. Adrenalin and NA have been extensively compared and in general NA is less effective as a hormone compared to adrenalin [57]. Furthermore, NA per se has experimentally been proven to inhibit cellular energy metabolism, especially after sustained stimulation. These effects are at least to some extent mediated by oxidative stress with production of radical oxygen species (ROS), which are known to impair efficiency of mitochondrial respiration [31]. Given that vasoconstriction in skin is mainly dependent on NA [36] and that skin already at baseline is characterized by a partly non oxidative metabolism [41], suggests that skin might be especially susceptible to high doses of NA. The fact that ROS also induces a prolonged vasoconstriction underlines the need for further insights into the mechanisms of vasoconstriction and the concomitant metabolic effects NA in skin. It might be speculated that the lack of systemic effects is due to the fact that most investigations is examining systemic changes and given the low contribution from skin at rest, even a

significant increase locally may pass undetected. Based on the experiences from burns, in which the massive immunological activation in skin is considered fundamental in the pathophysiology of SIRS and MODS [8], the local effects on NA given systemically may also be important and needs examination. In order to examine local skin metabolic effects of NA, it is important to eliminate the consequences of the parallel systemic effects. This then calls for methods based on local administration, dosing and measurements.

### **Tissue metabolism**

The adequacy of tissue oxygenation is dependent on balance in oxygen delivery and tissue consumption. During balanced conditions glucose is completely oxidised in the mitochondria yielding 36 ATP per mole of glucose. In states when tissue needs exceeds oxygen delivery mitochondrial capacity to reoxidate NADH is impaired and NADH is reoxidised by reducing pyruvate to lactate. The subsequent metabolism of lactate yields 2 ATP per mol glucose which significantly limits energy production. The consequence is that much more glucose must be oxidised under anaerobic conditions to meet tissue energy demands.

Oxygen deficit is present in states of shock as a result of both increased metabolic demands and decreased oxygen delivery secondary to hypovolemia or microcirculatory disturbances [58]. SIRS and MODS can also result in mitochondrial oxygen utilization defects which may be present despite adequate blood flow and oxygen delivery [28, 29].

Lactate or lactate pyruvate ratio have been widely used in the critical care setting to monitor tissue ischemia [59]. The concomitant glucose decrease have been less used and described even if there is a few studies indicating that it may be a sensitive marker of ischemia as well [60].

From the critical care perspective these features are of central importance. Increased lactate levels have been found to correlate to increased morbidity and mortality rates among ICU

patients [14]. The prognosis in sepsis is dependent on rapid lactate normalization [61]. An impaired glucose homeostasis is also a significant sign in shock and sepsis where glucose intolerance and insulin resistance are key manifestations [62]. The underlying mechanisms are obscure and complex but a close link to effects of catecholamines has been suggested [3]. The importance is also demonstrated by the successful implementation of tight glucose control by means of aggressive insulin treatment, lowering mortality and morbidity in ICU patients [63]. There is therefore a definite need to better understand the mechanisms underlying peripheral insulin resistance and glucose homeostasis in critical illness.

## **Tissue monitoring**

The awareness of microcirculatory disturbances as a key factor in the development of SIRS and MODS, and its potential effects on patient outcome, have spurred the development of new tissue imaging techniques. The purpose has been to develop tools to early in the time course alert clinicians of a deterioration in e.g., the tissue oxygen supply. Ideally this information should be gathered early, before organ damage or a systemic response has been manifested [64]. A major difficulty in tissue monitoring is the heterogeneity in blood flow not only between organs, but also within the same organ [64]. Furthermore, most available techniques examine superficial tissue and within only a limited volume and at only one site. These measurements may therefore not be representative even for the whole organ examined and even less for other organs in the body. Another general limitation with currently available techniques is that none, yet offers information on both changes in tissue blood flow and metabolism [29]. Nevertheless some techniques have shown some success in demonstrating usability in clinical tissue monitoring. Among these one very important is gastric tonometry (pH<sub>i</sub>-tonometry) [64-67]. This technique examines indirectly tissue pH in vivo in the gut,

which has been claimed an early marker of intestinal hypoperfusion. Another non-invasive technique is Venous occlusion plethysmography (VOP), which may be applied to humans and which has shown value in detecting vascular permeability changes during sepsis and MODS [68]. Near-infrared spectroscopy (NIRS) is a technique suitable for measurement of changes in tissue oxygen content over time [64]. Clinically, NIRS has mainly been used for brain tissue monitoring, mostly during brain, vascular and cardiac surgery and in neonatology [69]. The last ten years NIRS have been applied for the skin saturation determinations and several studies have demonstrated applicability in monitoring disturbances in tissue oxygenation and effects of interventions during critical illness [70].

### **Orthogonal polarization spectral imaging (OPS)**

Another method, which is new and interesting, is OPS, in which the microcirculation can be visualized in humans “in vivo”. The instrument consists of a small endoscopic light probe with optic filters. The tissue is illuminated with polarised light which is scattered; depolarised and reflected; enabling video images of high resolution of the microcirculation. It provides measures of functional capillary density, vessel type and diameter, blood flow velocity and the images can also be analysed semi quantitatively [64]. Clinically, the method has been successfully applied in studies of microcirculation preferably in tongue, gingiva, vaginal mucosa, but also in burn wound, the liver and brain. OPS have also proven useful to monitor changes after therapeutic manoeuvres during critical illness [71]. Major limitations are; only tissue with thin epithelial layers can be examined and the results are most often user dependent. In the critical care setting blood and saliva has been shown to limit good visualisation of microcirculation orally. The method is currently also limited by that blood flow velocity and semi-quantitative analyses only can only be performed off-line [64].

## **Microdialysis (MD)**

MD is a semi/minimally invasive technique allowing sampling of compounds from the interstitial compartment. The method was introduced 1966 [72] and was initially designed and successfully used to investigate neural tissue in living animals, which still is the major field of use. First use in human was in 1987. Throughout the years most organs and species have been investigated and many substances have been successfully sampled and examined [73]. Clinically, MD is used routinely in neuro intensive care to monitor ischemia after traumatic brain injury [74]. Experimentally, the technique has been successful in monitoring ischemia, in i.e., skin flaps after microsurgery, in limbs pre- or intra-operatively in a variety of surgical settings. In sepsis, differences in tissue metabolism between e.g., septicaemia and cardiogenic shock have been shown by the use of the technique, a finding which supports the concept cellular dysfunction in sepsis [75]. MD has been increasingly used for pharmacological studies, measuring tissue concentrations of systemically or topically administered drugs or in micro dosing experiments, where also the drug has been administered through the MD probe [76].

The MD system mimics the function of a capillary. It consists of a probe with an inlet and outlet tubing connected to a semi permeable membrane. A physiological solution is pumped through the system allowing the fluid to pass the dialysis membrane and to collect substances which pass through the membrane for subsequent analysis. The technique is based on passive diffusion of compounds along their concentration gradient over the dialysis membrane to or from the dialysate depending on tissue concentration. This process will be affected by the characteristics of all involved compartments, i.e., the perfusate, membrane characteristics and the tissue specifics. The fraction of the substance retrieved through the MD system is referred to as recovery (extraction fraction or probe efficiency) [73].

A major determinant of recovery is the perfusion flow rate which has been demonstrated to be inversely proportional to recovery [77]. Only at extremely low perfusion velocity rates  $<0, 1\mu\text{l}/\text{min}$  a near 100% recovery can be achieved [78]. Use of such low rates only provide very small sampling volumes or demanding long experimental times impairing temporal resolution or demanding high sensitivity in the analysis methods. To enable meaningful data sampling the relative recovery is used instead. This is done by characterization of the system specific performance for collecting the substance in question and allows calculation of the true tissue concentration. Commonly, this is achieved by in vitro calibration or use of tracer substances in vivo.

Tissue properties are also of importance for the adequacy of MD results. Main determinants are lower fluid volumes, increased diffusion paths and binding to cell surface proteins [73]. It has become evident that tissue clearance of substances greatly influences the recovery. Consequently changes in blood flow have been demonstrated to greatly alter the recovery, similar to changes in perfusion rate [79, 80]. This is likely to be of less importance in experimental settings where sampling is made during steady state conditions in a standardized environment. However in clinical settings, not least critical illness, blood flow changes can be expected to be large and significantly influencing sampling recovery. Most investigators have in the clinical studies used recovery data retrieved from experimental settings and studied only the relative changes over time. An obvious shortcoming is the lack of insight in how blood flow changes affect the results even in the cases of the basic metabolic parameters. MD has been extensively used for studies of human skin. Methodology [41, 81], baseline metabolism [82], insertion trauma and inflammation have been thoroughly described [83] and investigated. It has also been used to examine changes in metabolites in pig skin after experimental burns [84]. Furthermore effects of blood flow changes on recovery have been studied in human skin using mainly NA for vasoconstriction and nitro-glycerine for

vasodilatation [79, 80]. Results have demonstrated that clearance is directly proportional to changes in tissue blood flow. Unfortunately, these experiments have targeted physiological or pharmacological effects, whereas the metabolic consequences have not been examined.

The characteristics of the MD system seem to support the technique as a valuable tool in monitoring metabolism in skin of burn victims. Furthermore, the well established sampling of neurotransmitters would enable characterization of tissue response of central burn induced mediators such as serotonin [85]. The possibility of continuous sampling is likely to increase the understanding of the local dynamics over time in the tissue response in burn injury.

To fully understand the metabolic response there is a need to investigate correlations between metabolites and changes in blood flow. It is likely that methods used in pharmacology exposing skin to vasoactive drugs [79, 80] are applicable to study metabolic responses as well. Most warranted is to study local effects of NA. Most interestingly, micro dosing of NA in “in vivo”, in humans with iontophoresis, has demonstrated that blood flow dose and time dependence may be modelled [86]. This supports that also time and dose modelling may be feasible using the microdialysis if tissue blood flow may be assessed or measured in parallel.

### **Laser Doppler flowmetry and laser Doppler perfusion imaging (LDF and LDPI)**

Are non invasive techniques permitting real-time measurements of microvascular blood flow. These methods are based on that a laser light penetrates the surface of the tissue and interacts with moving cells. Due to the Doppler effect, photons undergo a frequency shift that is proportional to the concentration and speed of the moving cells and allowing calculation of blood flow. LDPI, in contrast to LDF, uses moving mirrors allowing two dimensional colour coded images of the skin perfusion. This enhances the area measured and gives a better spatial resolution of blood flow. A main shortcoming is that results are expressed in arbitrary perfusion units (PU) and not as e.g.  $\text{ml} \times \text{min}^{-1} \times 100 \text{ g}$ . The technique is also sensitive to

light, changes in skin temperature and tissue motion [87]. Laser Doppler has been mostly used for experimental conditions, but there is some experience also from clinical use, most often skin tissue but intestinal mucosa and brain tissue has also been examined [64]. The latter locations thus need surgery to become available for measurements limiting its clinical use. LDF/LDPI is very valuable in provocation/stress experiments examining effects of e.g., temperature changes or response to drugs delivered by iontophoresis [68, 86].

## **Aims of the study**

The overall aim of this thesis was to: investigate the applicability of microdialysis in burn injuries to monitor skin metabolism and mediators of the local skin response and to for comparison, develop a skin vascular model using microdialysis to investigate metabolic effects of ischemia/reperfusion induced by local administration of vasoactive drugs (NA/NGT/Vasopressin) in healthy volunteers. The specific objectives of this thesis and its separate projects were to:

1. Evaluate the applicability of microdialysis, during the time course of conventional fluid resuscitation, in assessing skin metabolism in injured and un-injured skin in patients with major burns.
2. Investigate the kinetics of serotonin in skin, plasma and urine in patients after major burns.
3. Evaluate the local effect on skin blood flow and metabolism of micro dosing (NA and NGT) by microdialysis in skin of healthy volunteers.
4. Investigate if time and dose response models can be applied to data (tissue blood flow and metabolism) obtained from micro-dosing of NA and Vasopressin by microdialysis in skin of healthy volunteers.

## Material & methods

All participants in the studies, healthy volunteers and patient or relatives gave their written consent, before entering the studies. All studies were reviewed and approved by the Local Ethics Committee at the Faculty of Health Sciences, Linköping University, Sweden. Procedures were in accordance with institutional and international guidelines. Healthy subject were recruited mainly among students at Linköping University and hospital staff. Patients were consecutively included in the studies during their clinically indicated hospital stay. No complications were observed that could be attributed to the microdialysis experiments in any of the healthy subjects or patients. Detailed inclusion, exclusion criteria's and demographics are presented in each study paper.

**Table 1. Summary of study demographics, technique and interventions.**

Note that patients and healthy volunteers (HV) are the same in study I and II.

Study	Patients n	HV n	Gender F/M	Age (mean $\pm$ SD)	MD probe	Perfusion rate	Nr of probes/individual	Drug intervention
I	6		1/5	30,6 ( $\pm$ 11,5)	CMA 70	0,5 $\mu$ L/min	2	None
I		9	4/5	29 ( $\pm$ 7,2)	CMA 70	0,5 $\mu$ L/min	1	None
II	6		1/5	30,6 ( $\pm$ 11,5)	CMA 70	0,5 $\mu$ L/min	2	None
II		5	3/2	29 ( $\pm$ 6,6)	CMA 70	0,5 $\mu$ L/min	1	None
III		9	3/6	28 ( $\pm$ 5,6)	CMA 70	2 $\mu$ L/min	2-3	NA/NGT
IV		12	6/6	23,2 ( $\pm$ 2.3)	CMA 66	0,5 $\mu$ L/min	4	NA/AVP

### **Subjects (Study I-IV)**

**Patients (Study I-II);** Six consecutive patients with major burns admitted to the burn unit at Linköping University Hospital were included in the study. Patients were treated according clinical routines at the unit, which is in line with international guidelines [88] Summarizing: oxygen was supplied to maintain a SaO<sub>2</sub> above 90%. Resuscitation was based on total burn surface area % (TBSA %) and given according to the Baxter formula 2-4 ml/kg/TBSA %/24h. Crystalloids provided were adjusted to maintain a urinary output of > 0,5 ml/kg/h and a mean

arterial pressure > 70 mm Hg. Blood transfusions were administered to maintain haemoglobin concentrations above 9g/dl. All patients fulfilled preset endpoints.

**Healthy volunteers (Studies I-IV);** A total of 30 individuals were recruited. All subjects were screened and found healthy with no concomitant medication. Subjects in papers I and II (the same subjects) had microdialysis probes implanted continuously for 3 consecutive days. No restrictions in daily life were imposed except strenuous exercise. Subjects in paper III and IV were investigated in a research laboratory. Room temperature was kept stable at 20-23°C. Subjects were, during the experiments comfortably resting in a half supine position and the investigated arm/arms positioned at the level of the heart.

### ***Microdialysis***

Probes; in all the studies probes with a 10 mm membrane and 20 kDa molecular cut off was used. In paper I-III CMA 70 (CMA microdialysis AB, Solna, Sweden) a traditional probe with the membrane at the end of the tubing, inlet entering the same side of the membrane as the outlet tubing was used. In paper IV a linear probe CMA 66 (CMA microdialysis AB, Solna, Sweden), with an inlet tubing attached to one side of the membrane and outlet attached to the other side of the membrane was used.



CMA 70



CMA 66

Pictures © CMA microdialysis

## Microdialysis pumps

In paper I a CMA 102 (CMA microdialysis AB, Solna, Sweden) precision pump was used. This pump uses 2 parallel mounted 1 ml micro syringes and enables adjustable perfusion rates between 0,1 - 20  $\mu\text{L}/\text{min}$ . In healthy volunteers CMA 107 (CMA microdialys AB, Solna, Sweden) pumps were used. This pump is small; battery operated and allows adjustable perfusion rates between 0,1 - 5  $\mu\text{L}/\text{min}$ .



CMA 102



CMA 107

## Perfusion fluid

Sterile Ringer's solutions were used in all studies. In paper III NA (0,5 and 5  $\mu\text{g}/\text{ml}$  in Ringers solution) and NGT (0.5 mg/ml in Ringers solution) was added to induce vasoconstriction and dilatation, respectively. Urea (20 mmol/l) and ethanol (5 mmol/l) was added to the Ringer's solution as markers for tissue blood flow estimations [89, 90]. All solutions were prepared by Apoteksbolaget AB. In paper IV, four different concentrations of NA and vasopressin (VAP) (0,3, 1,0, 3,0 and 10.0 $\mu\text{g}/\text{ml}$  and 0,1, 0,3, 1,0, and 3,0 mU/ml, respectively) were added for vasoconstriction and urea (20 mmol/l) for blood flow measurements. All solutions were prepared by Apoteksbolaget AB.

Perfusion rates were 0,5  $\mu\text{L}/\text{min}$  in studies I, II and IV. In study III, 2  $\mu\text{L}/\text{min}$  was used.

## **Sampling**

Sampling in all studies was preceded by a time for stabilization after insertion (60-180 minutes). In all studies capped micro vials was used to avoid evaporation and loss of sampled fluid. In studies I and II vials were kept on ice and covered for light. Sampling times varied from 10 min in the experimental set ups to 180 minutes in patients and controls in studies I and II.

### ***Study I***

Patients had one microdialysis probe inserted intra-dermal in an area with second degree burn and one probe inserted in adjacent uninjured skin. Controls had one probe inserted intra-dermally and in the para umbilical region. All probes were perfused with sterile ringer's solution and a perfusion rate of 0,5 $\mu$ l/min. Perfusion fluid from both patients and controls were sampled every third hour, with interruptions for clinical procedures in patients and sleep for controls. Patients were investigated until mobilization was initiated, usually at day five. Samples from the controls were collected continuously for three days. Samples were, in both groups immediately frozen (-20°C) and kept in the freezer until analysed. All samples were analysed within three month.

### ***Study II***

Plasma samples were collected twice daily (days 2-4) and mean value used for analysis. Urine samples were taken from a 24 hour urine collection bag day's 2 - 4 post burn. Microdialysis samples from days 1-3 was sampled but statistics calculated only on data from days 2 and 3, due to few observations day 1. As several samples per day were obtained mean values were calculated and grouped per day. In controls no time dependency was anticipated why mean values were calculated and used as one group.

### ***Study III***

Nine healthy volunteers participated and received each two (LDPI measurements) or three (urea clearance measurements) microdialysis probes intra-dermal in the volar surface of the lower arm. After a 90 minutes stabilization period NA (LDPI-measurements) or NA, urea (20 mmol/l) and ethanol (5 mmol/l) (urea clearance) was added to the perfusate. In 16 probes the NA dose was 5 µg/ml and in seven probes the dose was 0,5 µg/ml. perfusion with NA continued for 60 minutes. This phase was followed by an equilibration period of 60 minutes. A final drug provocation with NGT 0,5mg/ml was performed during 60 minutes. The experiment ended with a 20 minute equilibration phase with ringer's solution. Sampling was made every 10 minute during the whole experiment. In four subjects LDPI measurement of blood flow changes was done, in remaining subjects blood flow changes was determined by changes in urea. All samples were analyzed for glucose, lactate, pyruvate and urea continuously. Samples were frozen at -70°C and ethanol was analyzed the day after the experiment and NA within a month.

### ***Study IV***

Twelve healthy volunteers were included. Each individual had four probes inserted, two in each volar surface of the lower arm. All probes were perfused for 60 minutes before sampling. During 45 minutes probes were perfused with ringer's solution with 20 mmol/l of urea and sampling was done every 15 minutes, these values were used as baseline. Thereafter the subjects were divided into two groups, one receiving NA, the other VAP. Subjects were initially in each probe exposed to the four doses and of the chosen drug, one dose in each probe, respectively, added to the ringer's solution containing urea during 75 minutes. In the probe with the lowest dose, perfusion continued, repeatedly with the next higher dose for 75 minutes and so on. Sampling continued every 15 minutes throughout the experimental period. The experiment generated 568 samples in total.

## **Metabolic markers**

We chose glucose, lactate, pyruvate, glycerol and urea as these are well validated both experimentally and clinically (although not skin) in reflecting tissue ischemia and disturbances in substrate cycling [91]. Technique for analysing these parameters is also readily available, easy to perform bedside and have low costs [92]. For analyses of the microdialysis samples, a bedside analyzer, CMA 600 analyzer (CMA Microdialysis AB, Solna, Sweden) was used. CMA600 uses enzymatic reagents and colorimetric measurements. A high-precision pipetting device handles the sample (0.2-0.5  $\mu\text{L}$ ) and reagent volumes (14.5-14.8  $\mu\text{L}$ ). For glucose, lactate, pyruvate and glycerol the rate of formation of the coloured substance quinoneimine is measured in a filter photometer at 546 nm. For urea, the rate of utilization of NADH is measured at 365 nm. Reagents used were obtained from CMA Microdialysis AB (Solna, Sweden) [92, 93].

## ***Blood flow measurements***

### **Laser Doppler Perfusion Imaging (LDPI)**

A laser Doppler perfusion imaging technique (PIM 1.0, Lisca Development AB, Linköping, Sweden) was used to monitor skin blood. The LDPI scanning system contains a low power He-Ne laser (1mW, 632 nm), in which the beam is moved by a step motor device, which provides the scanning procedure over the skin surface. Doppler shifts in the backscattered light are detected and processed to generate an output signal, which is linearly proportional to tissue blood perfusion in the upper 200-300  $\mu\text{m}$  of the skin. The scanner head was positioned at a distance of 16 cm above the skin surface and set to scan an area of  $3 \times 3$  cm at each experimental site and at each occasion. Each image format consisted of  $64 \times 64$  measurement sites (medium resolution, high scan speed) with a distance of about 1 mm between each measurement point. The approximate time required for such an LDPI image recordings was

approximately 1 minute. Measurements targeted the skin area overlying the microdialysis probes. Data analysis was performed using the manufacturer's software (LDPI win ver. 2.3, Patch Test Analysis 1.3). The average blood perfusion was calculated from the perfusion values recorded within the region of interest (ROI), positioned above the tip of the catheters in an area of approximately  $1 \times 0.5$  cm. For comparison the biological zero signal from the laser Doppler was recorded at the end of the experiment by a temporary (2 minutes) occlusion of the arterial circulation to the limb by a blood pressure cuff.

### **Urea clearance**

Urea in retro dialysis have been used by several investigators to calculate relative recovery [94]. The technique is based on that tissue conditions are at steady state and that changes in perfusion rate will affect the ratio of urea that will equilibrate during diffusion. In study III and IV retro dialysis of urea was performed but with a fixed flow rate and changes in dialysate concentrations was anticipated to instead reflect the changes in local tissue blood flow, i.e., the urea cleared from the vicinity of the microdialysis catheter by the tissue blood flow.

### **Serotonin (5HT) analysis**

Microdialysate, plasma, and urinary serotonin concentrations were measured with an ELISA technique using a standard competitive radioimmunoassay kit (Serotonin (e) Enzyme immunoassay, Immunotech, Marseille, France). The results were read by a micro plate reader (Lab systems Multiscan RC 405-414 nm filter). All analyses were made in duplicate and the mean value was used.

## **Noradrenalin analysis**

A HPLC-system consisting of a P680 HPLC pump (Dionex), an automated sample injector ASI - 100 (Dionex), an electrochemical detector DECADE (Antec Leydon) were used. The analytical column was an Aquasil C18 250 mm x 4.6mm, particle size 5  $\mu\text{m}$ , with a preceding matched guard column Aquasil C18 10 mm x 4mm x 5  $\mu\text{m}$ , both from Keystone Scientific. The column temperature was set at 23°C with an integrated oven from Dionex.

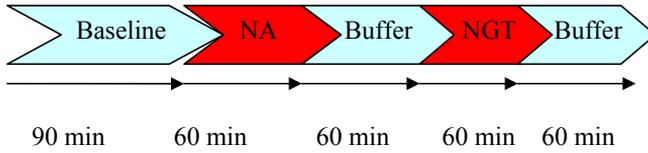
The mobile phase consisted of sodium 1-heptane-sulfonate 1mM, citric acid monohydrate 0.1 M, Na<sub>2</sub>-EDTA 0.05 mM and 5% acetonitrile (ACN), pH was adjusted to 2.7 with 1M NaOH before adding ACN. Flow rate was set at 1.0 mL/min, the runtime was set at 15 min and the detector was set at +750mV (nA range) versus the Ag/AgCl reference electrode. Injection volume was 10  $\mu\text{L}$  for both standards and samples.

Chromatograms were measured using Chromeleon software from Dionex. Quantitation was achieved by comparison of peak area generated from the standard curve.

## ***Drug protocols***

In paper III vasoconstriction was induced by NA (0.5 or 5  $\mu\text{g}/\text{ml}$  in Ringer's solution, Apoteksbolaget AB) and vasodilatation induced by NGT (0.5 mg/ml in Ringer's solution, Apoteksbolaget AB) administered by the microdialysis system. Below is a schematic presentation of the procedure, figure 2.

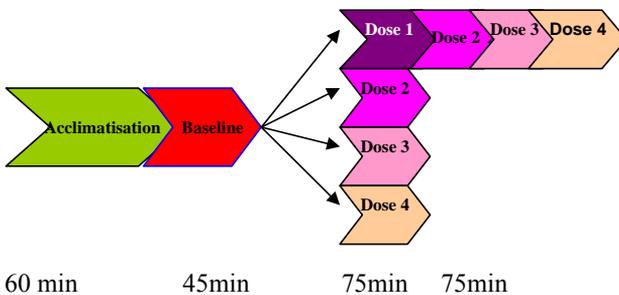
**Figure 2**



*Timeframes for drug interventions in study III. In 16 probes the NA dose was 5 $\mu$ g/ml and in seven probes the dose was 0, 5  $\mu$ g/ml. NGT concentration was 0,5mg/ml in all subjects*

In paper IV incremental doses of NA (0.003-10,0  $\mu$ g/ml in Ringer's solution, Apoteksbolaget AB) and VAP (0,1-30mU in Ringer's solution, Apoteksbolaget AB) was administered. NA doses used were (0.003; 0.01; 0.03; 0.1 in pilot subjects) 0,3; 1.0; 3.0; 10.0  $\mu$ g/ml. Vasopressin doses used were (0.1; 0.3 in pilot subjects) 1.0; 3.0; 10.0; 30.0 mU/ml. Schematic presentation of the procedure figure 3.

**Figure 3.**



*Dosage regimen in study IV. Subject were randomly divided into two groups, n=6 in each group. Each subjects had a total of four catheters inserted. NA was given to one group VAP to the other. Dose 1 was the lowest dose, dose 2 the second lowest, dose 3 the third lowest and dose 4 the highest dose.*

## ***Data processing and statistics***

In study I and II the same patients and controls were used. Time from injury to admittance varied depending on time for primary resuscitation and transport to the burn unit. This resulted in too few values for meaningful statistics day 1. In study I data from days 2 - 4 and in study II data from days 2 - 3 were used. Data showed a skewed distribution why median values are presented. Mann-Whitney U test was used to investigate differences between controls and uninjured and injured skin, respectively. Bonferroni corrections were performed. To investigate correlations in study I and II the Spearman rank correlation coefficient was used. Data in these studies are presented as median and range.

Statistics in study I and II were done using Statistica (version 7.0 Stat Soft, Inc, USA)

In study III to reduce anticipated inter-individual differences data was normalized and consequently data is presented as absolute changes over time. To examine changes over time we used a 2 - way repeated ANOVA measures for all parameters. Pearsons rank correlation analysis was used. .

In study IV we investigated whether a dose response model could be applied. Data was normalized by subtracting the mean values from 45 minutes baseline sampling from each observation, thus presenting absolute changes. Values from one probe with four incremental doses of NA or VAP and values from four different probes each with different dose was plotted over time. Dose response values were mathematically conformed to a sigmoid  $E_{max}$  model by fitting a non linear regression curve. The model enables estimation of the dose causing 50% of the vasoconstrictor response (i.e.  $ED_{50}$ ). Sum of square F-tests were used to reveal differences in best fit parameters for the curves induced by the different models for administration.

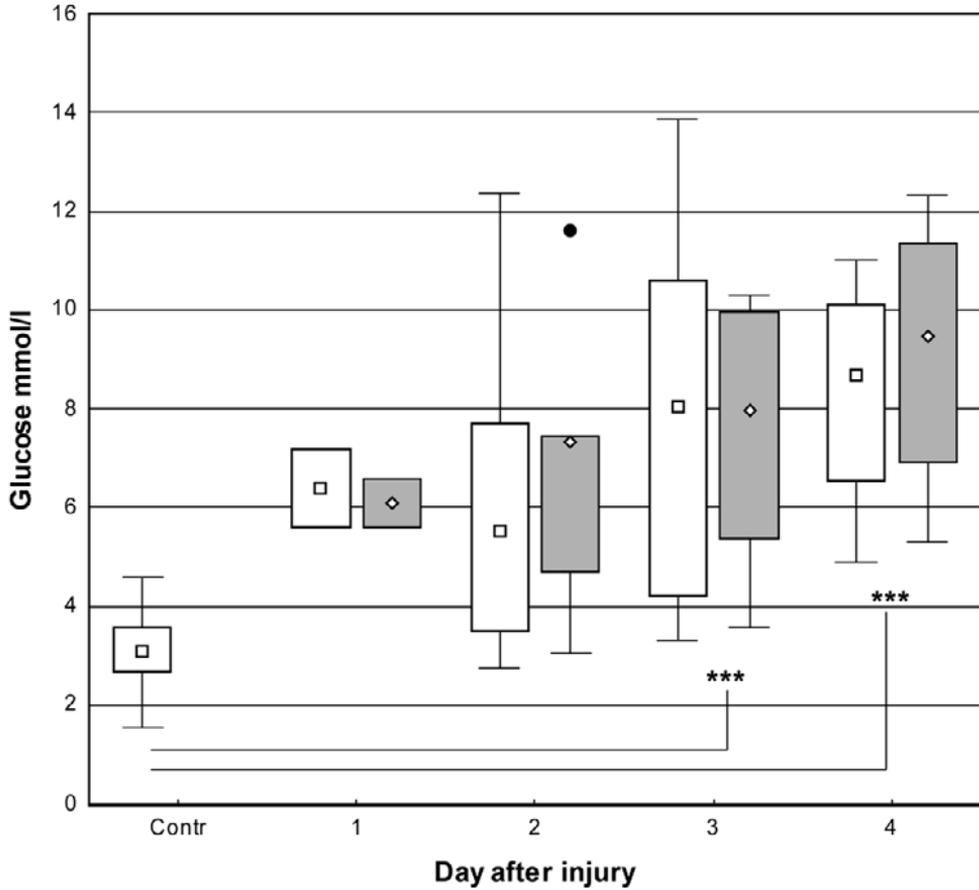
Statistics in study III and IV were calculated using GraphPad Prism version 5, 0 for Windows (GraphPad Software, San Diego California USA).

In all studies a probability of  $< 0, 05$  were considered significant.

# Results

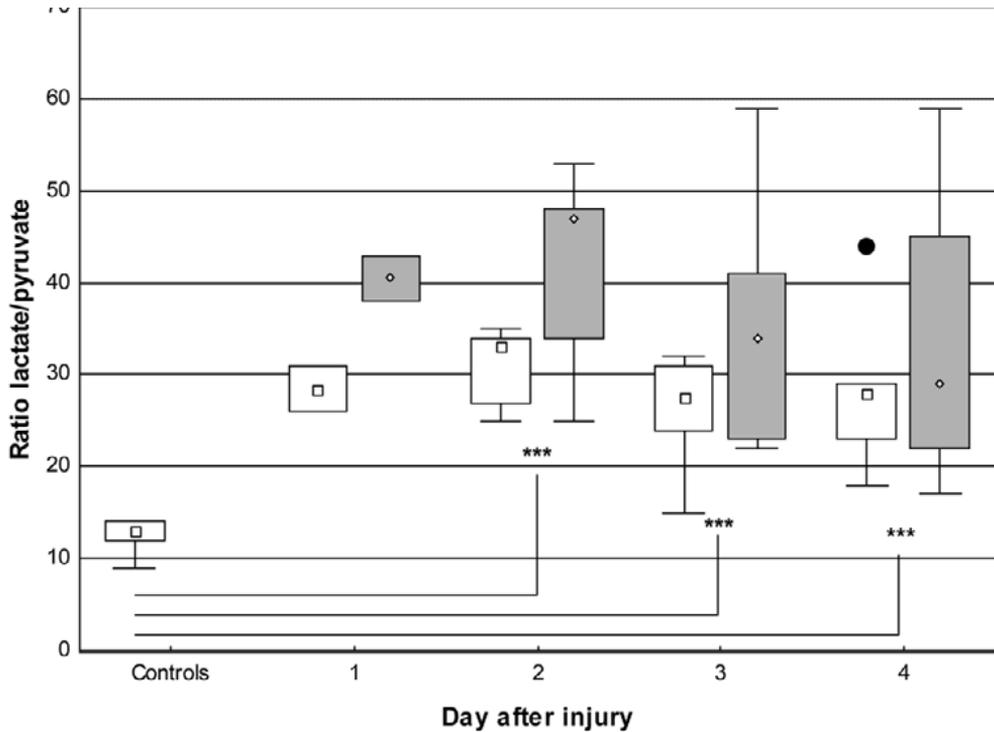
## Study I

Figure 4



Box-and-whisker plots showing median (interquartile) glucose concentrations in microdialysis days one to four. Open boxes indicate uninjured skin and controls; shaded boxes indicate burned skin. \*\*\*  $P < 0,001$ . Contr, controls

**Figure 5**



Box-and-whisker plots showing lactate/pyruvate ratio in microdialysis days one to four. Open boxes indicate uninjured skin and controls; shaded boxes indicate burned skin. \*\*\*  $P < 0,001$ .

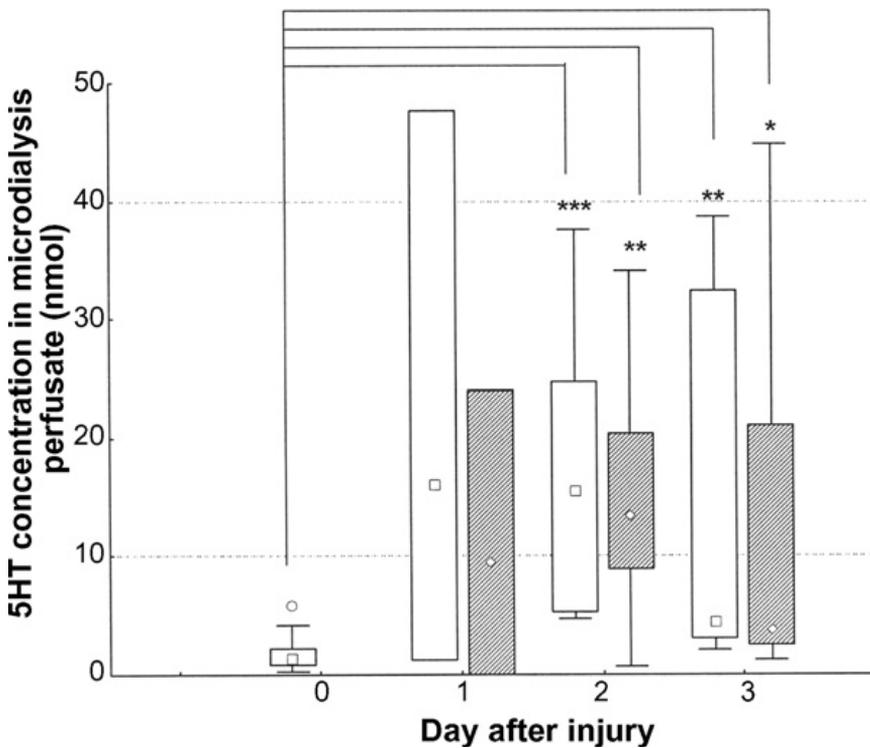
Contr, controls

The main results of study (I) were that in patients; trauma induced systemic hyperglycaemia that peaked on day no. two post burn, and it, gradually thereafter decreased days three and four. Locally in skin, extracellular glucose continued to increase throughout the study period with maximum concentrations registered on day four. Compared to controls, extracellular tissue concentration of glucose was significantly ( $p < 0,001$ ) higher in the skin of burn patients day three and four. There was no sign of acidosis in any systemic blood gas data from any of the patients during the study period. Arterial pH, Base excess and  $pCO_2$  were all in the normal range. Locally, in skin, lactate increased almost four times and lactate/pyruvate ratio showed a

twofold increase during all of the study days (two to four). These concentrations were significantly higher ( $p < 0,01-0,001$ ) in the skin of the patients as compared to controls. The change in extracellular skin glucose correlated significantly ( $p < 0,05$ ) to changes in lactate and pyruvate. Local skin levels in glycerol was significantly increased day three and four ( $p < 0,01$ ).

## Study II

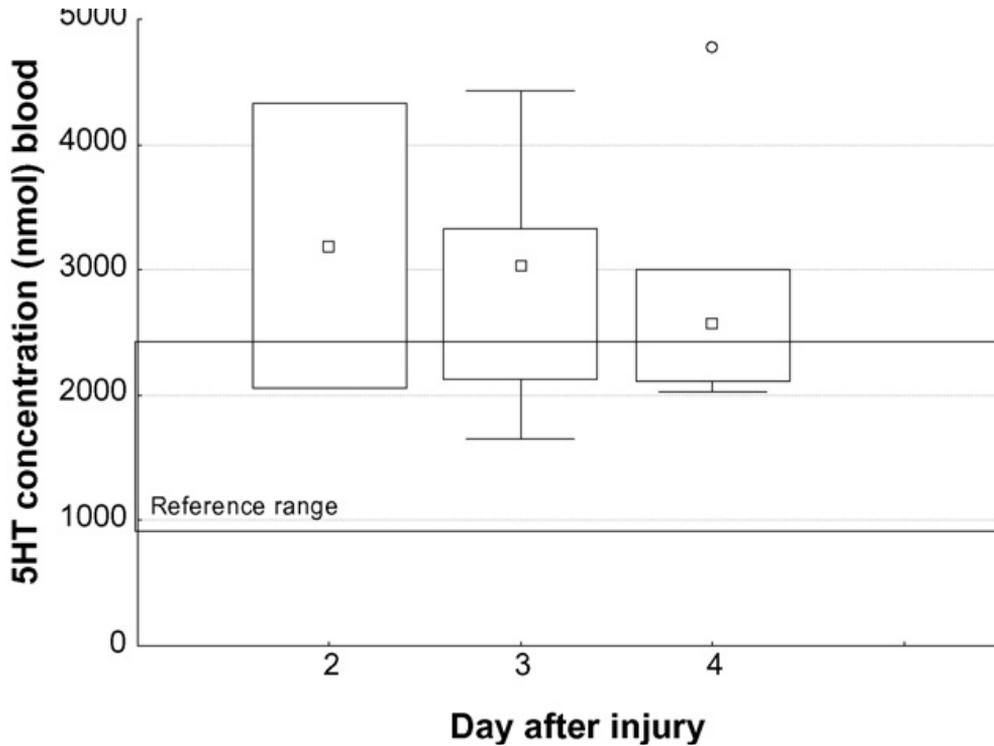
Figure 6



Box-and-whisker plots showing Serotonin(5HT) concentrations in microdialysis days one to three. Open boxes indicate uninjured skin and controls; shaded boxes indicate burned skin.

\* $P < 0,05$ , \*\* $P < 0,001$ , \*\*\* $P < 0,001$ . Contr, controls.

**Figure 7**



*Box-and-whisker plots showing Serotonin (5HT) concentrations in blood days two to four.*

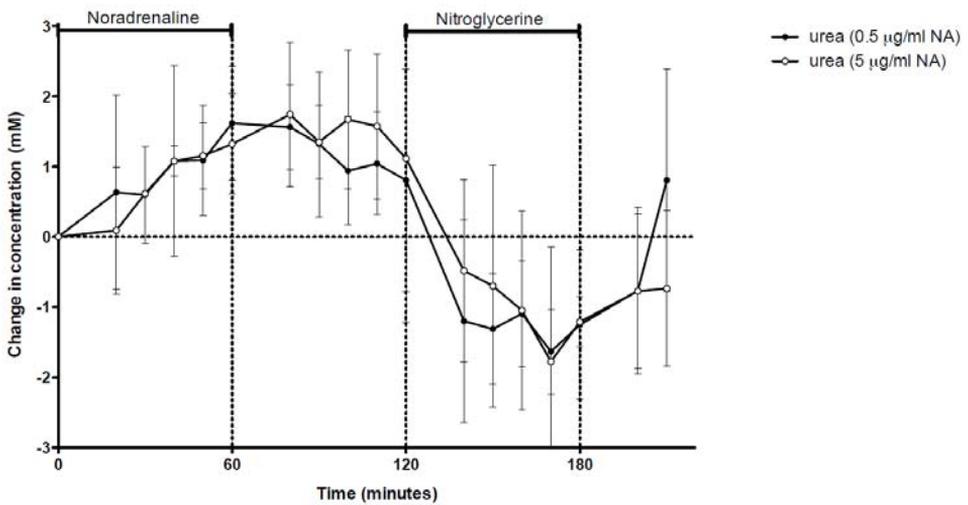
*Reference range is from the manufactures instruction.*

The results show that plasma serotonin was increased, 3189 nmol (median), twice the normal plasma value (1000 – 2500 nmol) on day 2 after burn. Thereafter, and gradually it decreased days 3 and 4, resulting in close to normal values on day 4, 2573 nmol (median). In urine, serotonin concentrations were considerably increased only on day 2, 1755 nmol (median) (normal values 900-1300). Furthermore, days 3 and 4 urinary serotonin levels were close to or within the normal range. In skin extracellular serotonin values were increased, close to or more than ten times compared to controls. In controls serotonin concentrations was 1,3 nmol (median). In patients uninjured skin serotonin concentrations was 16,1nmol (median) day 1 and 15,6nmol (median) day 2. In burn injured skin serotonin concentrations was 9,5nmol

(median) day 1 respectively 13,4nmol (median) day 2. Serotonin levels decreased on day 3 but remained three to four times that of the controls. Differences between controls and patients, both uninjured and burned skin was significant day 2 and 3 ( $p < 0,05$ ). Correlation between TBSA and serotonin was  $r = 0,8$  in uninjured and burned skin.

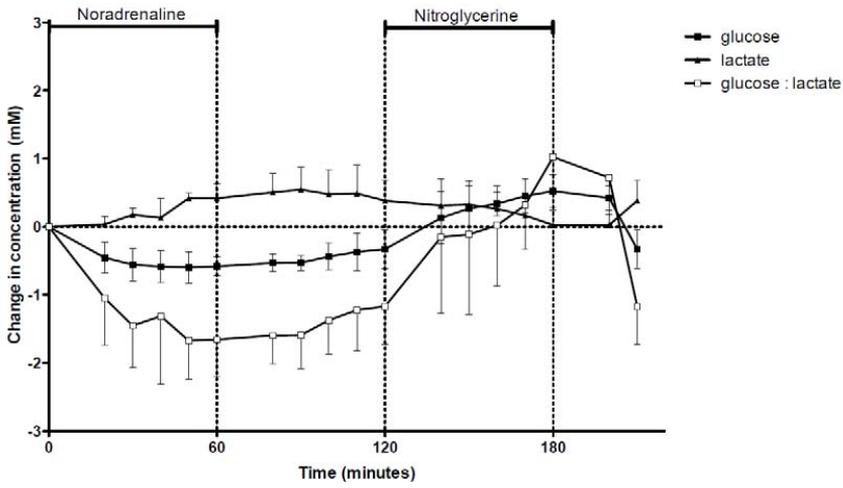
### Study III

Figure 8



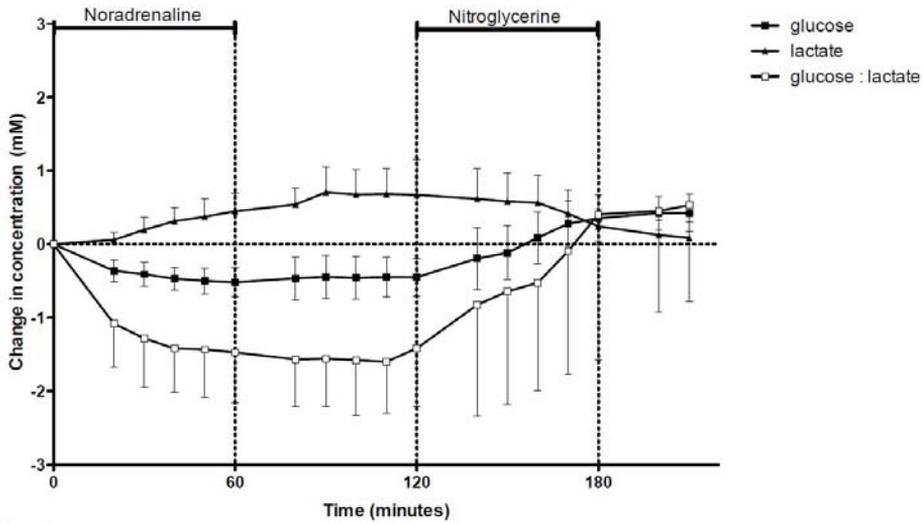
Changes in urea over time. Black boxes 0.5µg/ml, white boxes 5µg/ml. Data are normalized and changes represents absolute values.

**Figure 9**



Mean (SEM) absolute changes in metabolites: glucose=black boxes: lactate=black triangles; and glucose:lactate ratio=white triangles over time in subjects given 0,5µg/ml noradrenaline.

**Figure 10**



Mean (SEM) absolute changes in metabolites: glucose=black boxes: lactate=black triangles; and glucose:lactate ratio=white triangles over time in subjects given 5µg/ml noradrenaline.

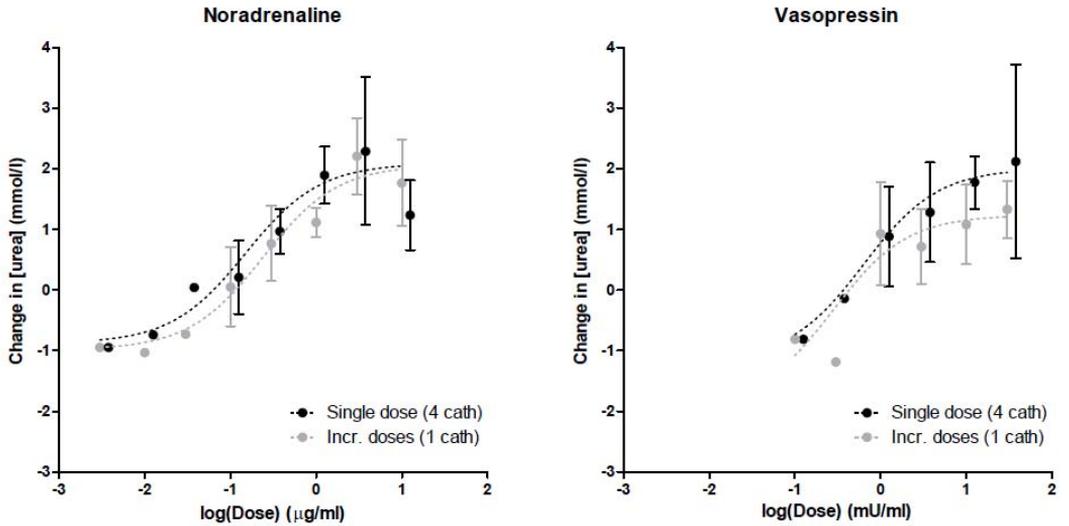
The result of the study (III) show that perfusing NA and NGT through the microdialysis probe induced significant and anticipated time dependent changes in all parameters, glucose, lactate and urea ( $p < 0,0001$ ). There was no significant difference between the NA doses in tissue response.

During NA LDPI showed a sudden drop to values equalling that normally registered during ischemia (biological zero) and remained there until 30 minutes of NGT perfusion had been undertaken. Urea values also increased rapidly at the onset of NA infusion and continued to increase through out the period of NA in to the equilibrium phase, stabilizing at a plateau in those receiving the higher dose ( $5\mu\text{g/ml}$ ) whereas it began to decline after 30 minutes for those with the lower dose ( $0,5\mu\text{g/ml}$ ). None of the groups reached baseline values during 60 minutes of ringer's perfusion. Perfusing the tissue with NGT induced an immediate and rapid decline in urea, independent of dose. The rapid plateau of glucose during vasoconstriction and the late improvement of lactate during vasodilatation, precluded analysis of correlations, for these parameters during this phase. Correlations to urea change in lactate and lactate/glucose during administration of NA was  $r = 0,8$  respectively  $r = -0,63$ . During NGT the urea change correlated to glucose  $r = -0,88$  and lactate/glucose  $-0,81$  these changes were significant ( $p < 0,03$ ).

Glucose was more rapid than the lactate to respond to NA administration. Glucose decreased and lactate increased in a sigmoid pattern. Glucose showed a plateau already during NA perfusion whereas lactate continued to increase and showed a plateau first after 30 min of equilibration. Neither glucose nor lactate returned to baseline. When perfusing the tissue with NGT, glucose normalized independently of dose, whereas tissue lactate values normalised only in those cases who had received the lower NA dose. Lactate in tissue remained high for those receiving the higher dose, and normalized first after NGT perfusion was instituted.

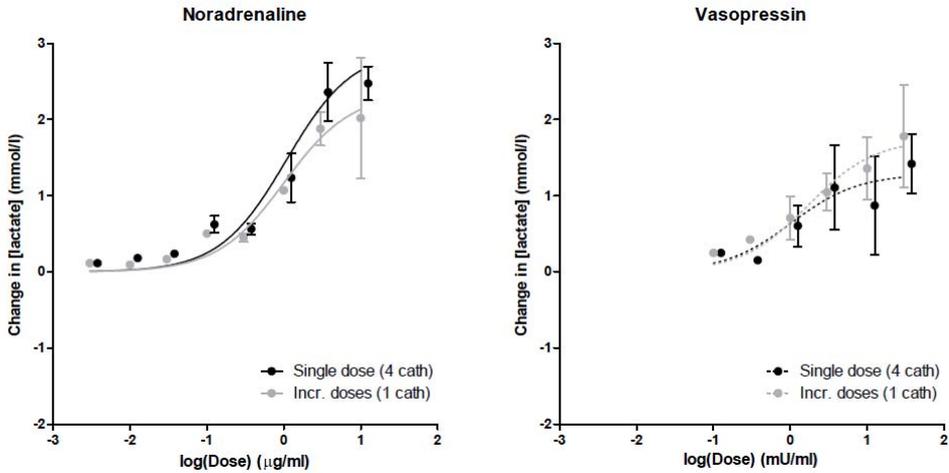
## Study IV

Figure 11



*Absolute changes from baseline (mean (SD) values) of urea in the dialysate for increasing doses of NA (left panel,  $n = 5$ ) and VAP (right panel,  $n = 4$ ). There was a significant, dose-dependent increase in urea clearance, but no difference between curves obtained from measurements in a single catheter and from measurements in four separate catheters.*

**Figure 12**



*Absolute changes from baseline (mean (SD) values) of lactate in the dialysate for increasing doses of noradrenaline (left panel,  $n = 5$ ) and vasopressin (right panel,  $n = 5$ ). There was a significant, dose- dependent increase in lactate concentration, but no difference between curves obtained from measurements in a single catheter or from measurements in four separate catheters. A significantly greater effect was recorded in the absolute change in lactate concentration in the noradrenaline experiments than in the vasopressin ones.*

The results showed that there was a gradual increase in lactate and urea and a concomitant decrease in glucose associated to the increment in dose of NA and VAP. In the individual dose a plateau was reached in 60 minutes. The response in all parameters was well fitted to the applied  $E_{\max}$  model  $r^2$  urea 0,83-0,99; glucose 0,66-0,96 and lactate 0,85-0,96. No difference was found between the different provocation protocols. Lactate concentrations was twice as high at the same degree of vasoconstriction in individuals exposed to NA compared to VAP.

## **Discussion**

### **Monitoring skin metabolism in burns**

Trauma to the skin, such as induced by burn injury, elicits severe effects on the human body not least the micro vascular bed. This effect has among several other mechanisms been claimed important in the development of subsequent multiple organ failure [95, 96]. Despite aggressive optimization of the central hemodynamics, using well known endpoints which have been proven successful in other states of shock [22], multiple organ failure is still a dominating cause for mortality in burns. This suggests, that techniques that monitors local tissue events may be of value in the resuscitation of burns [23, 71].

### **Microdialysis**

Microdialysis is well validated technique for experimental studies in skin and it has the advantage to most other methods for tissue monitoring, in that it offers not only information on ischemia, but also may depict concomitant changes in biochemistry [41, 81, 97]. For meaningful applications of the microdialysis technique in skin of burn patients there are several issues that have to be addressed. The validation of skin microdialysis has mainly been based on data obtained from experiments in healthy volunteers during stable experimental conditions [41, 81]. The results obtained from microdialysis are a product of the perfusate (composition and flow rate), membrane properties (length and pore size) and very importantly tissue factors. During non-steady state conditions in general and especially in critical illness, where there is a pronounced dynamic variability in many skin parameters one has to be cautious in interpreting the microdialysis results. This may also call for modelling experiments that further validates, confirms and explores the findings obtained from the clinical setting. This is the explanation for the experimental modelling experiments made in

healthy volunteers that constitute the last two studies (Study III and IV). In these models, especially the effects of skin blood flow alterations and the metabolic effects of stress hormones were further examined in the skin. [73].

## **Control groups**

To overcome the lack of reference values for the burn patients we chose to use a control group, based on healthy volunteers in which sampling was made continuously over three consecutive days during ordinary daily life (Studies I and II). Interestingly, using median values, there was no significant differences seen over time and the values were in the range of those obtained by others during experimental conditions and during steady state [41, 81, 82].

## **Metabolites**

We chose glucose, lactate, pyruvate, glycerol and urea as these are well validated both experimentally and clinically (although not in skin) in reflecting tissue ischemia and disturbances in substrate cycling [91]. A standardized measuring apparatus for analysing these parameters is also readily available, easy to work with bedside and is favourable also from an economical perspective. [92]. Furthermore, its precision and function has been critically examined [92, 93].

## ***Review Study I***

The aim of the first study (Study I) was to evaluate the applicability of the microdialysis technique to record metabolic events in both injured and uninjured skin of patients with major burn injury during the course of the initial fluid resuscitation. Local changes in glucose, lactate, pyruvate, glycerol and urea were measured. These changes were compared to the

values of the same parameters, examined in skin of healthy volunteers, retrieved during steady state conditions during ordinary daily life.

In burn patients we demonstrated that the microdialysis technique could be applied in the critical care setting and that local metabolism could be examined in skin during several days. Most interesting is the discrepancy between the systemic and local values indicating that severe local disturbances in tissue oxygenation, glucose and fat metabolism is present in the skin.

Despite the fact that all patients fulfilled resuscitation endpoint [19, 20] and no sign of ischemia was present in the systemic circulation, the microdialysis data revealed severe tissue acidosis locally, consistent with previous findings using other techniques [23]. Additional information was thus gained through the microdialysis methodology demonstrating acidosis to be caused by high tissue lactate levels and further supported by an increased lactate/pyruvate ratio [41, 98]. The cause of this acidosis may have several explanations: Burn resuscitation using a standard protocol usually leads to a controlled under-resuscitation initially, during which vasoconstriction in skin may be anticipated [21, 99]. Also, haemodilution is frequently seen during the aggressive crystalloid resuscitation used in burns. Furthermore, the permeability increase and the corresponding large resuscitation volumes that is provided leads to a pronounced tissue oedema well known in burns [18]. Both haemodilution and tissue oedema may cause a reduction in tissue perfusion with a concomitant acidosis. In the present study the patients received what were slightly “excessive” amounts of fluid during the initial 24 h period as compared to the Parkland formula. Although it has to be stressed that the present burn cohort had a large burn size and that the fluid amount is in line with modern fluid strategies, it is above the levels described in the original Parkland formula publication [100]. Interestingly, the course of the tissue acidosis

coincided with the development of the tissue oedema [101] favouring the argument that the oedema is of importance for the development of skin tissue acidosis. Furthermore, a SIRS reaction is present in all major burns. A prominent feature in this reaction is disseminated intravascular coagulation with loss of platelets due to formation of thromboses in the microvasculature [42]. This will also impair the microcirculation and further compromise tissue perfusion.

## **Glucose**

The glucose homeostasis followed an anticipated course, which is influenced by a well described trauma induced insulin resistance with increased blood glucose levels peaking on day two after the burn and thereafter gradually decreasing. Locally, in skin, glucose levels continued to increase despite the systemically improvement. This finding contradicts findings in ischemia models [91, 98], both experimentally and clinically in which glucose decreases as consumption exceeds delivery. Hence glucose/lactate ratio has been used to increase sensitivity in detecting ischemia using microdialysis [75].

The mechanisms underlying the trauma induced insulin resistance are complex and not fully understood. In skeletal muscle, low interstitial insulin concentrations have been demonstrated suggesting that the capillary wall is rate limiting [102, 103]. This concept would be consistent with the microcirculatory impairment secondary to burn injury as described above.

The timely occurrence of interstitially increasing glucose levels appearing and worsening after the burn chock period is as anticipated as the hyper metabolic syndrome is known to start after the fluid resuscitation period. Furthermore, no global signs of hypoperfusion such as systemic acidosis were recorded. These observations together with a normal urea, often used as a reference substance in microdialysis, contradict a generalized tissue hypoperfusion.

## **Cytopathic hypoxia**

Novel insights in cell metabolism during critical illness have demonstrated that cellular dysfunction, resulting in bioenergetic failure, independent of tissue perfusion and oxygenation is an important mechanism in the development of organ dysfunction [28, 104]. This condition is often referred to as cytopathic hypoxia and is previously described in trauma and septic patients. Underlying mechanisms are complex and not fully understood but a close relation to several factors present in severe inflammation have been claimed [29, 105]. The finding in this study is consistent with the model of cytopathic hypoxia and this has not previously been described in burn shock. The finding of high glucose interstitially may also be of importance in the development of burn oedema as an osmotic gradient towards the interstitial tissue is created during a period of disturbed permeability and a low systemic oncotic pressure. Similar findings have been described in the brain after stroke [106].

Correlation of the local skin disturbances to the general SIRS reaction rather than local hypoperfusion is also supported by the finding that the changes observed seemed global as there was no significant difference between the injured and non-injured skin.

## **Lipolysis**

A key manifestation associated to burn injury is the catecholamine induced lipolysis, which causes weight loss and affects outcome [107]. Treatment with beta-blocking agents have been demonstrated to reduce tissue catabolism and especially lipolysis, possibly improving outcome [108]. The finding of increased glycerol levels locally in the skin as a response to stress is consistent with other experimental microdialysis studies using sympathetic stimulation or insulin clamps [109]. We do believe that the finding of increased skin glycerol levels reflects lipolysis induced by the trauma response. Increases in glycerol are also present in ischemia, as a result of cell injuries, then representing membrane components. [74] The

increased skin glucose levels, in combination to normal urea levels, supports lipolysis as the source of the increased skin glycerol levels in this study.

Summarizing the findings we can conclude from the first application of microdialysis in skin of burn injured patients that microdialysis seems to reflect changes in lactate, pyruvate, glucose, urea and glycerol locally in skin, and appears to depict these continuously for several days. Some of these changes seem to be of local origin in skin and are not recognised in blood samples representing the central circulation. Most importantly, there seem to be a sustained acidosis in skin, which might be related to ischemia from insufficient blood flow and/or e.g., reduced diffusion effects of the fluid resuscitation. Also plausible is that it may be effects consistent with the concept of cytopathic hypoxia in which the acidosis is caused by a local metabolic cell dysfunction, despite an adequate blood flow, as is indicated by high interstitial glucose and normal urea concentrations locally. This assumption is also supported by the lack of differences between injured and non-injured skin, which favours changes seen as a result of a systemic reaction e.g., the inflammatory response, consistent with SIRS. Furthermore, the impaired glucose metabolism may create an osmotic gradient contributing to the tissue oedema seen in burn injury. The changes in glycerol also suggest that a trauma induced local skin lipolysis may be monitored successfully.

### **Methodological considerations**

A significant methodological shortcoming in this study is the inability to distinguish ischemia from cellular dysfunction, as blood flow was not measured in parallel. Therefore the addition of appropriate and concomitant measurements of changes in blood flow is most warranted for future studies in this model.

From a clinical point of view the microdialysis technique seems to offer an interesting tool to monitor metabolic changes in skin, and possibly, given further refinements it may develop into an aid in optimizing fluid treatment and for other experimental clinical interventions.

It has to be recognised that this study was conducted prior to the era of tight glucose control [63, 110] and that a more aggressive insulin therapy might have influenced the results.

## ***Review Study II***

The aim of the study was to investigate the distribution of serotonin in plasma, urine and skin tissue in patients after severe burn injury. Serotonin tissue concentrations were also examined in healthy controls for comparison. Samples from the patients and 5 of the controls from study I were used.

## **Serotonin in burns**

An important aspect of burn care and its treatment is the pathophysiology of the burn injury. This response is characterized by a rapid loss of homeostatic control, in both injured and non-injured tissue, as demonstrated in paper 1. This leads to loss of fluid from the circulation into the interstitial space, thus creating the significant tissue oedema, which is well known to burn injury. This oedema has in itself been claimed to cause further damage. The complete pathophysiology of these alterations is complex and not fully understood [18, 111]. A cornerstone, known since decades, is the activation of the inflammatory response by leukocytes, platelets and endothelial cells and the release of several cascades of chemical mediators, such as amines, proteases and cytokines [50, 112].

We chose to study serotonin turnover in burns since it has been claimed for decades to be of primary importance in the early burn response, including vasodilatation and increased permeability [18, 112]. According to the literature, we found that the role of serotonin, described in most textbooks and review articles on burn pathophysiology, is almost exclusively based on animal studies. We found only one study in humans, published in 1960 [51], which demonstrated only minor increased amounts of serotonin, locally in burn blister, although not in skin and increased 5HIAA levels in urine. Furthermore, it needs then to be appreciated that serotonin is released from mast cells in most animal species, but not in humans [52]. There are also differences in serotonin kinetics found between humans and animals and it has to be stressed that animal data are conflicting [51, 113]. Some investigators have found increased serotonin levels in skin of rats but not in rabbits. Others have been unable to document increased serotonin levels in rats, even after burns. Even so, the strongest evidence for serotonin as an important mediator in burns is the effect shown of serotonin blocking agents in an animal burn model. Metysergide, a 5HT blocker has been demonstrated to decrease both blood flow and oedema formation [54, 55]. In rabbits, metysergide reduced blood flow by closing functional shunts, redirecting blood flow from non-nutritive to nutritive areas of the skin, and thereby preserving protein kinetics [56]. No corresponding data exists for humans.

### **Serotonin and microdialysis**

The applicability of microdialysis to determine extracellular serotonin concentrations is demonstrated by a broad use in animal models to determine mainly serotonin *in vivo* in the brain [85]. In humans, serotonin concentrations in skeletal muscle have been successfully examined [114]. The use of microdialysis in burns is limited to only a few animal

experimental studies examining histamine and substance P turnover and their vascular effects [84].

Based on the claimed importance in burns and that it has been extensively studied before using microdialysis we thought that serotonin experiments would be ideal in a first “in vivo” pilot study of human burns using microdialysis

### **Serotonin kinetics**

The main storage and synthesis location of serotonin in humans is within the enterochromaffine cells in the gastro intestinal tract. This accounts for more than 80 % of the body content. Another quantitatively important store is in the dense granules of platelets [43]. Platelet reacts to exposed collagen, adheres and releases their granular content, including serotonin [49]. In burns there is a massive destruction of the vessels in the burn injured skin, exposing sub-endothelial collagen.

It seems likely, that this link between exposed collagen and platelet activation is the main source of the tenfold increase in serotonin concentrations that we found locally in the skin. As such exposure of collagen is likely to prevail for several days; it would also explain the sustained increase in serotonin that was registered in the study also after the first 2 days. This hypothesis, is further supported by the well documented decrease in platelets that is usually seen days, 2 - 4 post burn [115]. This hypothesis would also be consistent with the speculated DIC reaction as another possible cause of the acidosis found locally in skin in study I. However, these increased concentrations, may not only be dependent on release of serotonin from the platelets but may also be a result of a decreased uptake or clearing mechanism. An important clearing mechanism is re-uptake of serotonin in platelets. This is affected by reduced uptake and storage capability secondary to the low platelet counts and possibly an impaired platelet function. Normally occurring serotonin is rapidly eliminated from the

circulation by several organ systems, such as the lungs, liver, spleen and kidneys [52]. Failure in these organ systems, not infrequently seen in burns, may also be of importance for the clearance of serotonin and may contribute to the increased concentrations found in the study. It is reasonable to believe, that this mostly would affect the blood and urine serotonin concentrations.

The anticipated changes induced by serotonin is: increased vascular permeability and vasoplegia promoting the development of the characteristic burn induced oedema[18]. The oedema may also contribute to the increased concentrations of serotonin extracellular as diffusion may be expected to be impaired as well, reducing tissue clearance. If so, the deterioration in the tissue would get worse, possibly giving raise to further more endothelial damage and concomitant platelet activation.

The highest concentrations of serotonin measured extracellular coincided in time with the most pronounced metabolic disturbances registered (study I), suggesting that there may be a relationship. An important finding is also the generalized effects, demonstrated by the lack of significant differences between injured and uninjured skin, further supporting that serotonin might be an important mediator of the generalized vasoplegia and permeability disturbances seen in burns [18]. Interestingly, the concentration of serotonin correlated to the extent of the injury and possibly that reflects the extent of damaged endothelial cells by the burn. This finding also support that the changes in serotonin is induced by the burn injury rather than some other mechanism.

Summarizing, the study demonstrates, for the first time, that serotonin levels systemically and locally in skin is increased in burn injured humans. This finding suggests that serotonin may be of importance for the vasoplegia and oedema formation that is seen in burns. The finding of sustained increased serotonin levels after day 1 is interesting, as it suggests that patients could be treated after admission to the hospital even after a delay from the time of the injury.

The study also suggests that microdialysis can be applied in burn patients in the critical care setting to monitor local chemistry of substances in low interstitial concentrations over several days.

### ***Study III***

#### **Measurement of blood flow changes**

As shown by the results in paper I there is a local skin acidosis and there seem to be a lack of autoregulatory blood flow regulation in both injured and uninjured skin of burn victims.

These changes were in parallel to an altered glucose homeostasis with high tissue glucose levels, possibly as a result of cellular dysfunction [29]. These results suggests both that there may be a blood flow decrease causing tissue acidosis (lactate increase) and at the same time skin glucose levels are increased, which suggests normal blood flow or possibly decreased blood flow and a cellular glucose uptake defect. The underlying mechanisms appear complex, and difficult to understand. Also a coupling to high inflammatory activity and NO appears plausible [116]. NA, present endogenously in high concentrations in states of stress, and used pharmacologically to treat circulatory failure in the ICU, have also been demonstrated to have direct effects on mitochondrial metabolic function [32, 117]. These findings suggested that further studies should be made examining the effects of local blood flow on skin metabolism. Especially the effect of e.g., NA seemed warranted. Important for the use of microdialysis, it has also become increasingly evident that interstitial concentration of any given substance examined in the extracellular space is influenced by changes in local blood flow, affecting both supply and removal of the substance itself [118].

As we are aiming at using microdialysis in dynamic states, in the critical care setting where blood flow is rapidly changing we recognized early the need for methods measuring concomitant blood flow changes. Given the heterogeneity of blood flow in shock states

measurement should preferably be made within the same compartment as the metabolic measurements are made. The method should also be robust enough for clinical use bedside in the ICU.

## **Ethanol**

Experimentally, the golden standard for blood flow measurements using microdialysis is the ethanol clearance technique, in which the difference in inflow outflow concentrations from the microdialysis catheter is inversely proportional to the changes in tissue blood flow surrounding the catheter [119, 120]. The main limitations with the ethanol method are that samples are contaminated by even small concentrations of ethanol in the surrounding, making it difficult to apply it in a clinical settings. Furthermore, ethanol clearance is dependent on high perfusion rates, at least  $2\mu\text{l}/\text{min}$  which lowers the sensitivity and making sampling of i.e., amines, peptides and cytokines difficult or even impossible in skin were low concentrations are anticipated. Also, the ethanol analysis requires special analytical procedures and the results are strongly influenced by time to analysis. Ethanol is validated in skeletal muscle tissue which has a known high blood flow and where large changes are regularly seen. No corresponding investigations, as far as we know, are found for skin or other “low” flow tissues.

## **Urea**

Based on the same principle as ethanol clearance in microdialysis, we made the hypothesis that urea may replace ethanol as a blood flow marker in the microdialysis system. Urea, is small freely diffusible, non-toxic, and evenly distributed molecule in biologic tissue. Urea is neither expected to be metabolised or influence blood flow in itself. Urea has also been used

previously as an endogenous reference substance [94]. We have demonstrated, in a rat model, that blood flow changes induced by local administration of noradrenalin in skeletal muscle [90] seemed to be accurately reflected by changes in urea in retrodialysis. Importantly, urea in these studies seemed to reflect changes in blood flow at low perfusion rates and showed an enhanced sensitivity with lowering of the perfusion rate, which therefore would permit sampling of metabolites in low interstitial concentrations. Bedside standardised analysers (CMA 600) are also available, enabling direct and cost effective analysis of urea [92]. These findings and features suggested that urea might be applied to measure dermal blood flow in the microdialysis system.

### **Skin acidosis**

To be able to investigate the different components causing tissue acidosis (study I) knowledge on metabolism during physiological changes in blood flow is needed. It is also important to isolate the local vasoconstriction from effects of the systemic response to trauma. Despite extensive research on mechanisms for of blood flow changes, extremely little is known of co-occurring metabolic changes in skin [121]. This is surprising since skin is easily accessible and the metabolism at baseline shows partly a non-oxidative metabolism indicating that changes in blood flow for this organ is important [41].

### **Modelling vascular responses in skin**

Vasoconstriction in skin is mainly dependent on noradrenalin. This has been investigated in the pharmacological “in vivo” models where NA have been delivered through the microdialysis technique in what have been claimed as physiological doses [79, 80]. Even if these models have been extensively used and considered validated the doses used is 100 times

higher than the highest levels that can be experimentally elicited endogenously in vivo [122]. Vasodilatation mechanisms in skin are more complex and less standardised experimentally, most often used is NGT based on the central role of nitric oxide in skin dilatation, but other vasodilators as e.g., nitroprusside and adenosine have also been used [36].

The aim of study III was to develop a human “in vivo” skin model. Our hypothesis was that we, by delivering noradrenalin through the microdialysis system could induce a gradual change in vascular tone [79, 80]. Noradrenalin and nitro-glycerine was chosen both, based on their previous use in similar skin models, but also since the effects of both these drugs may be of interest in the context of critical care and particularly the findings in Study I. We anticipated concurrent changes in the skin metabolites (glucose, lactate, pyruvate and glycerol). Skin blood flow was to be examined in parallel by laser Doppler imaging [123] and urea clearance [90]

### ***Review study III***

We found, by using laser Doppler as a reference, that administration of noradrenalin and nitro-glycerine through the microdialysis system induces anticipated changes in skin blood flow. The accuracy of the model was suggested by the correlation between LDPI and changes in both lactate and glucose. The validity of the finding is further supported by the similarity to results obtained from previous pharmacological studies using microdialysis [79, 80, 124] as well as other methods to administer vasoactive drugs to the skin, such as iontophoresis [86]. A low sensitivity of laser Doppler for depicting vasoconstriction in the skin was also appreciated by the small changes that were registered during the vasoconstriction experiments.

The urea values registered through the retrodialysis changed considerably over time during the pharmacological interventions. The close correlations to alterations in metabolites (lactate, glucose and lactate:glucose ratio) supported the idea that urea adequately reflected the

induced changes in blood flow. Interestingly, during vasoconstriction urea continued to increase even after laser Doppler values had reached a plateau at a value equalling the biological zero value (obtained during tourniquet). The concomitant change in metabolites, also indicating a further reduction in blood flow, suggests that urea and the other metabolites examined by the microdialysis system are more sensitive detectors of vasoconstriction in skin, as compared with e.g., laser Doppler.

During vasodilatation with NGT, urea, independently of dose, indicated a rapid restoration of blood flow and hyperaemia. This finding is also supported by the correlation to the change in glucose. This is likely to represent nutritive blood flow and occurred in parallel to the ocular observation of flushing of the skin at the site of the probes. Laser Doppler did not reach values consistent with significant hyperaemia until 35 minutes after the introduction of NGT. Compared to the ethanol clearance technique, the changes in urea were detected at a low perfusion rates, indicating that urea may be used for blood flow measurements in skin with parallel sampling of substances at low perfusion rates which would permit sampling metabolites with low interstitial concentrations such as cytokines [83, 125]. This would be of interest for further use in clinical skin research.

Ethanol clearance data did not generate any meaningful results, consistent with low sensitivity at low microdialysis perfusion rates (<2.0 µl/min).

From the results in the study it was concluded that urea clearance seemed to offer a promising skin blood flow method that; is easily performed; is available by most standard bedside analyzers and may be operative at a low cost. The addition of skin blood flow measurements by the urea methodology in parallel is also likely to enable correction of the results that are a consequence of local blood flow changes.

## Glucose

Metabolic changes were anticipated to occur during the induced blood flow changes and ischemia. Most interestingly, glucose decreased rapidly during the early decrease in blood flow, but surprisingly the decrease stopped and there was a plateau in the skin glucose values at approximately 2 mmol/l. This is puzzling since we had anticipated a continuous consumption as more glucose is consumed during ischemia to keep up the energy production as e.g., demonstrated in other ischemia models using microdialysis [98]. Our finding may be parallel a recurring finding in muscle ischemia in non-insulin dependent diabetic (NIDDM) patients, where glucose uptake is claimed to be dependent on the integrity of the insulin receptor, which is energy dependent and acting on phosphorylation. In NIDDM patients a putative signalling pathway for glucose uptake is demonstrated during ischemia [126]. The result in this study, based on this explanation, suggests that this mechanism is exhausted, due to the energy depletion by ischemia. Another plausible explanation suggested by other investigators, is that the capillary wall is rate limiting for the insulin effect [103]. Given the effect of NA on local blood flow, this explanation also seems relevant.

Independent of the correct underlying mechanism for this finding, this model presents insights to the glucose metabolism that may be of importance for a better understanding of the pathophysiology of the glucose metabolism also in critical illness as exemplified by the glucose turnover findings in Study I.

During reperfusion under the vasodilatation, glucose, after a slow recovery, reached supra systemic values, consistent with the findings in study I. This is interesting as it seem to picture a situation demonstrating glucose delivery to tissues exceeding metabolic capacity and needs. Nitric oxide delivered by nitro-glycerine and ROS, likely to be present during ischemia, are both known play a central role in the pathophysiology of mitochondrial dysfunction [29] and the surplus glucose levels found may indicate effects on mitochondrial function already after

120 minutes of hypoperfusion/ischemia. High or increased glucose values in the tissues might also be of importance for the development of local tissue oedema, which is present in both burn injury and reperfusion injury. Locally in tissue, varying glucose levels heralds also a relative risk with tissue monitoring of glucose to guide systemic insulin treatment as is advocated by some investigators [127]. We have previously challenged this view and the findings in the present study further supports this position [127, 128].

## **Lactate**

Lactate has been extensively used to detect tissue ischemia in microdialysis studies [91]. The continuous lactate increase during NA provocation in the present study indicates a significant decrease in blood flow. This change correlated significantly to the changes in urea (blood flow), despite that a plateau phenomenon was observed for both the LDPI and glucose data. The changes recorded followed an anticipated course in which, a decrease in skin glucose preceded the increase in skin lactate. This indicated that in this blood flow model changes in tissue glucose is a fastest parameter to detect insufficient blood flow. Although it needs to be stressed that this advantage of glucose as a hypoperfusion/ischemia marker is limited for the early blood flow decrease and based on the findings of the present study it is insensitive to sustained and severe ischemia.

In the present model both lactate and glucose seemed to be sensitive markers to detect changes in tissue blood flow as compared to laser Doppler recordings. In comparison to the changes in urea, (as a blood flow estimate), lactate seems to be superior in detecting ischemia and whereas glucose seem to better pictures reperfusion. Combining these two (glucose and lactate) into a glucose lactate ratio increases the blood flow detection sensitivity and the ratio detects blood flow changes in both these aspects (ischemia/reperfusion) and support finding of other investigators, who advocates the use of this ratio in detection of tissue ischemia [60].

The usefulness of the ratio is also illustrated by: applying the ratio on the results in paper I in which the conclusion of ischemia would have been rejected.

### **Autoregulatory escape**

The microcirculation is generally protected by an intrinsic system, balancing sympathetic tone (vasoconstriction) to locally elicited vasodilatation that maintains oxygen above critical values [25]. These systems have been demonstrated to protect other vascular beds such as in skeletal muscle and intestine during high sympathetic tone and/or pharmacological vasoconstriction [25, 129]. The finding that blood flow and metabolic disturbances continued after cessation of NA infusion in skin suggests that this protective mechanism operative in other tissues is either absent or dysfunctional in skin tissue, in the present model. In the high dose group the effect observed may also be explained by a possible and significant deposition of noradrenalin in the skin tissue. However, as there was also a remaining vasoconstriction in the low dose group, where no deposition of NA was discernible, suggests on the other hand that this may be due to that skin lacks an autoregulatory escape mechanism. The often, clinically observed phenomenon, in which patients in shock, who have had their central circulation restored and optimized as demonstrated by invasive measurements, but continues to be pale and cold in the skin, may be a clinical manifestation of a suggested lack of blood flow autoregulation mechanism in skin. This lack of autoregulation may also explain why there is a success in early introduction of nitro-glycerine in severely septic patients [130].

## **Dose**

The slow onset of the effect of the NA doses suggests a successive NA clearance decrease by the decreasing blood flow, increasing in succession the relative dose locally in the tissue. This finding is consistent with effects of a vasoconstrictive drug dose, previously also demonstrated in skin when delivering the dose by iontophoresis [86]. In the latter setup the model and approach enabled calculation of pharmacodynamics such as ED 50 and the corresponding Hill slope. We later applied these models also to our microdialysis data and were able to apply a sigmoid curve fit and to demonstrate differences in ED 50 and Hill slope as suggested by Gabrielsson and Weinerl [131].

The effect on blood flow as well as metabolism was almost identical with both doses and this suggests together with the findings in paper 4 that this dose level is supraphysiologic in this previously frequently used model [79, 80].

## ***Review Study IV***

The aim of the study was to evaluate a dose response model using microdialysis for administration of vasoactive drugs in skin of healthy volunteers. Dose effects on blood flow were estimated with urea clearance and metabolic effects on glucose and lactate measured. A second aim was to compare the effects of NA and AVP on tissue metabolism. The study was designed to address the question raised from the results in paper 3. Most interestingly those results suggested that microdosing by the microdialysis system may present a pharmacological model to evaluate dose response “in vivo” in humans. This may present advantages to current models to investigate microvascular function and effects of vasoactive drugs, which is mainly based on isolated vessels often of not human origin “in vitro”, or by e.g., intravital microscopy in animals [132]. Furthermore the dose effects sites for the pharmacological effect is mainly on extravascular structures rather than in the blood stream

itself [133]. The need for such methods is further advocated by e.g., differences in autoregulatory capacity between different tissues, as was demonstrated in paper 3 [25, 129]. To enable dose response calculations physiological doses of NA needed to be established for the present skin model to allow proper dose escalation schemes. A second aim was to investigate the concomitant effects on skin tissue metabolism by NA. As noradrenalin is known to have direct effects on tissue metabolism [32, 134] equipotent doses of vasopressin was chose for comparison. Urea clearance was chosen for the experiments to monitor blood flow changes. At the time the research group has gathered more data supporting its potential as a blood flow determining technique [125]. We also used two delivery protocols; a single catheter with increasing doses of the drug and with a flush sequence in between the doses to reduce equilibrium times and catheters where only one dose in each was provided.

## **Dose**

Based on pilot study data we chose eight doses of NA from 0,003-10 $\mu$ g/ml for the study. These doses were distributed to create a logarithmic dosing scale for the purpose to enable an even distribution when fitting the response data on each dose level to a sigmoid  $E_{max}$  model with the intent to determine  $EC_{50}$  and to do Hill slope calculations [135]. We also decided to use a lower perfusion rate (0.5  $\mu$ l/min) to increase recovery. Using precision pumps a flush sequence of 15  $\mu$ l/min for 5 minutes when also syringes were changed was undertaken. We did notice in the previous study that the result immediately after a flush was highly influenced by the procedure and these samples was therefore discarded.

Based on the results in paper 3 an initial dose interval between 0,003-0,1  $\mu$ g/ml of noradrenalin and 0,1-3mU/ml of vasopressin was examined in the first pilot experiments but the response was not sufficient and higher doses were needed. The final doses presented in the

study are therefore 0,3, 1,0, 3,0 and 10  $\mu\text{g}/\text{ml}$  for noradrenalin and 1,0, 3,0 10 and 30  $\text{mU}/\text{ml}$  for vasopressin. Interestingly similar doses have successfully been used in corresponding in vitro studies, thus supporting the relevance of the present “in vivo” study [136, 137]. This also suggests that the tissue dose is close to the concentration in the dialysate. To investigate the absolute tissue dose delivered, analyses of drug concentrations in the outflow, or e.g., inflow/outflow ratio should be undertaken. Interestingly, the very close agreement between dose ranges seen in vitro, compared to the present study “in vivo” is further supported by other human in vivo skin models such as iontophoresis [138].

### **Dose response modelling**

A new finding in this study was that urea clearance seemed adequate to assess vasoconstriction in skin, induced by increasing doses of vasoactive drugs administered by microdialysis system. Most interestingly we were also able to demonstrate the applicability of pharmacodynamic calculations based on the  $E_{\text{max}}$  model, enabling quantification of vasoactive effects of the drugs in terms of  $ED_{50}$  and  $E_{\text{max}}$  changes in both perfusion represented by changes in urea and in the metabolic markers, lactate and glucose. As expected from a physiological perspective, urea equilibrated faster than glucose and lactate and is also consistent with previous investigations [139, 140]. Even if the changes in urea was significant and resulted in adequate dose response curves, the absolute change from baseline is small, 2 - 3  $\text{mmol}$  at the highest doses. The absolute concentration difference in the dialysate of urea is also low, 8 - 12  $\text{mmol}$  compared to a perfusate concentration of 20  $\text{mmol}/\text{l}$ . The latter level is chosen not to influence the oncotic properties of the system more than necessary. This may be considered a shortcoming with the urea technique and has also been discussed by other investigators [90]. It may also be argued that the small change in urea indicate that blood flow

did not change substantially. However, we find this unlikely as the blanching of the skin and plateaus coincided with the highest doses. More likely is that the low urea concentrations are caused by the high diffusion capacity of urea in the extracellular space, possibly beyond that of the effect of the blood flow. Urea may be expected to diffuse easily and may diffuse over a larger volume reaching tissue not affected by the vasoactive drug and the blood flow change, thereby increasing removal.

## **Metabolism**

The results of the study suggest that glucose as well as lactate may be used to examine dose response effects in the present model. An disadvantage is the energy dependent uptake of glucose [126], reaching a plateau in ischemia, as demonstrated in paper 3, limiting dose response quantification to the period of ischemia preceding that threshold. Another confounder of importance is the anticipated temporal delay in the lactate compared to glucose and urea response.

Especially interesting from a critical care perspective is the finding that noradrenalin induced a higher skin lactate concentration compared to an equipotent dose of vasopressin. This is consistent with the well known direct metabolic effect of noradrenalin [141]. NA is still the drug of choice amongst vasoactive drugs in the critical care setting despite a growing evidence of the usefulness of vasopressin [16, 142].

## **Drug protocol**

When developing the urea technique it early became obvious from the experiments in the animal model [90] that a disadvantage with avoiding a flush sequence between changes in perfusate/dose was that it took up to 60 minutes for the urea concentration to stabilise. In

paper 3 we found that we had to exclude the values from the time closes to the flush sequence as there were obvious flush effects on the results.

In this study we found no changes in concentration of either urea or metabolites during each sampling period after a flush. This is important from a methodological perspective as this supports that the flushes may be used to eliminate long equilibrium times. The lack of significant differences in dose response or goodness of fit values between administering the different drugs repeatedly in one single catheter compared to one catheter for each dose is also an important finding for future studies. Using a setup with a single catheter is both easier to handle, cheaper and enables multiple observations in a limited number of catheters and subjects. It may also be suggested that a single catheter design is less likely to be affected by local condition differences between different skin sites. This would then possibly lower variability as has been suggested by other investigators [143].

# Conclusions

## General conclusions

In the studies we found that the microdialysis technique can be successfully applied to monitor skin metabolism and depict a mediator (serotonin) of the local skin response in burn patients. It was also feasible, for comparative purposes, to develop a skin vascular model, based on microdialysis to deliver vasoactive substances locally in the skin of healthy volunteers. This model provided a framework where the metabolic effects of ischemia and reperfusion elicited by local administration (NA/Vasopressin) could be examined.

## Specific conclusions.

1. Skin metabolic events both in injured and in non-injured skin tissue in burn injured patients may be examined during several consecutive days of conventional critical care and during the initial fluid resuscitation.
2. A significant serotonin increase, several fold higher than both plasma and urine levels, were registered in the burn injured skin in burn patients. This increase suggests that serotonin is an important mediator of the skin response to burns in humans.
3. Large and dose dependent local metabolic effects were detected after micro dosing of both NA and NGT by means of microdialysis in skin of healthy volunteers. This model may be used in studies examining vascular and metabolic effects of vasoactive substances such as NA or NGT.
4. Time and dose response modelling is feasible on data (tissue blood flow and metabolism) generated by microdialysis delivering vasoactive substances locally in skin of healthy volunteers.

## ***Future perspectives***

The main finding in the first part of this thesis was that local extracellular and extravascular biochemistry in the skin of burned patients can be investigated for several consecutive days using microdialysis. The local acidosis in the skin that was shown in both injured and non-injured skin in patients, and which was not illustrated in the blood gas analyses from their central circulation, suggests that microdialysis may become a valuable tool in the future when we design new fluid regimens to optimise tissue conditions. In the same experiment a most interesting finding was altered local glucose homeostasis. This suggests that the technique may also become useful for future investigations into trauma-induced insulin resistance.

The finding in paper II that described serotonin kinetics emphasises for the first time that serotonin may be a significant mediator of the burn-induced trauma response in humans. This finding supports the hypothesis that microdialysis may have future applications in the investigation of trauma-elicited changes in the response cascade system in humans, particularly at the tissue level. This may involve investigation of other mediators of importance for the physiological and pathophysiological responses and development of SIRS and MODS such as those mediated by cytokines, complement, coagulation, and bioactive amines. The local nature of microdialysis may increase our understanding of the local relations between the systems involved in the pathophysiology and interactions between tissues and different organ systems.

The findings in the second part of the thesis, in which the technique was useful in attempts aimed at understanding and modelling the tissue response after isolated provocation with separate drugs in the skin of healthy volunteers, suggests that such models may be used to increase the understanding of the tissue responses recorded in critical care.

It is also reasonable to think that the model and technique described will be used in the future in intensive care to characterise local vascular sensitivity to vasoactive compounds in different conditions.

Lastly, in a wider perspective, the addition of active compounds to the perfusate suggests that any substance that passes the microdialysis membrane may be used to provoke an isolated tissue response; this may also be examined by the response mediators that it releases, and sampled in parallel by the microdialysis system. This in turn enables “microdosing” of the tissues and depicts pharmacodynamic responses locally. From the point of view of critical care it would be interesting to use substances central to the inflammatory response such as endotoxins or exotoxins.

## ***Svensk sammanfattning***

Kritisk sjukdom, inklusive brännskador åtföljs inte sällan av svikt i ett eller flera organsystem. Detta utgör en betydande orsak till morbiditet och mortalitet. Parallella störningar i microcirkulationen i de olika organen vid dessa åkommor aktiverar ett flertal kaskadsystem, bl.a. inflammation- och koagulationssystemen, som visat sig vara av central betydelse. Också stresshormoner frisatta vid stora skador, har vid sidan av den positiva effekten för cirkulationsanpassning som behövs vid kritisk sjukdom, också direkta negativa effekter på vävnadscellernas ämnesomsättning.

Microdialys är en minimalt-invasiv teknik som använts sedan 70-talet för att studera den kemiska sammansättningen och förändringar i biokemin i levande vävnad. Tekniken bygger på att en tunn kateter försedd med ett semipermeabelt membran placeras i den vävnad man avser undersöka och katetern kan beskrivas som ett konstgjort litet blodkärl. Det sker en diffusion med koncentrationsgradienten från vävnaden in över membranet till katetern och vätska som finns i katetern samlas upp för mätning av dess beståndsdelar.

I denna avhandling presenteras forskning där mikrodialys tekniken appliceras i hud, hos såväl allvarligt brännskadade patienter som friska försökspersoner i syfte att bättre förstå hudens lokala reaktioner och dessas betydelse för systemreaktioner.

I avhandlingens första hälft studeras parallellt bränd och obränd hud hos människor med omfattande brännskador. Resultaten visar att hos de brännskadade patienterna, jämfört med friska kontrollpersoner så finns det betydande störningar i den lokala ämnesomsättningen i skinnet trots normala blodvärden. Dessa fynd med en mjölksyreansamling lokalt i huden hos dessa patienter är förenliga med tidigare kunskap förvärvat med andra tekniker. Fynden kan förklaras av en lokal syrebrist till följd av otillräckligt lokalt blodflöde i huden hos dessa patienter. Samtidigt påvisar vi också en kraftig förhöjning av de lokala sockervärdena i hudvävnaden. Detta fynd att vävnaden inte kan ta upp socker trots god tillgång lokalt, talar för

att det också skulle kunna föreligga en cellulär ämnesomsättningsstörning. Ett fenomen tidigare visat för patienter i chock efter trauma eller svår blodförgiftning, men inte tidigare beskrivet i samband med brännskador.

I det andra delarbetet av 4, studerade vi också omsättningen av serotonin, som i djurmodeller visat vara av betydelse för de fysiologiska förändringar som leder till det omfattande vätskebehov som kännetecknar brännskador. Motsvarande studier saknas för människa. Data visar, för första gången på människa, att det föreligger kraftigt förhöjda nivåer av serotonin lokalt i huden, trots att motsvarande blod och urinvärden är i det närmaste normala. Fyndet pekar på behovet av vävnadsmätningar för ämnen med snabb återresorption eller nedbrytning. Data från denna studie stöder också att serotonin är betydelsefullt i människans svar på brännskada

I den andra delen av avhandlingen studeras möjligheterna att använda microdialys för att tillföra läkemedel lokalt i vävnad och samtidigt studera dessas lokala effekter, genom så kallad ”mikrodosering”. Modellen tas fram för att lättare kunna isolera en del av de komplexa förlopp som studerats hos patienterna som brännskadats och att sedan genom att ge isolerade substanser till friska försökspersoner kunna pröva om modellhypoteserna är riktiga.

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