AN INVESTIGATION OF

THE MICRODIALYSIS TECHNIQUE

TO MONITOR METABOLISM

IN HEALTHY AND BURNED HUMAN SKIN

by

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ABSTRACT

Introduction: Burn patients are currently monitored by, and their treatment based on, systemic parameters. Although much of their outcome depends upon wound healing, very little work has previously been done to examine the possibility of monitoring the local wound environment. Microdialysis is a technique established in other specialties to monitor local tissues. This thesis investigates the use of the microdialysis technique to provide a method of continuously monitoring the burn wound. Markers of ischaemia, and nitric oxide metabolism were investigated.

Methods: Ten healthy volunteers and 10 patients with acute burns were recruited. Microdialysis of the dermis was undertaken to establish a normal range in the volunteers. Microdialysis probes were placed in the zones of coagulation, and stasis (as per Jackson's burn wound model) and in the unburned skin of the burn patients, and samples taken half hourly until 36 hours following the burn. An additional 2 patients with major burns were recruited. Microdialysate was examined for glucose, lactate, pyruvate, glycerol and urea in both groups. Amino acids were also measured in the burn groups. Time-related trends were compared with LDI images taken at the start and completion of the study period.

Results: From the volunteer studies, the technique was validated, and a normal range for healthy dermis was calculated. There was no significant change with increased ambient temperature. In the burn studies, there were several significant differences in metabolites between zones and with time. Glycerol in the zone of coagulation was significantly raised at 6-12 hours following burn compared to the remaining time periods. Glucose in the zone of stasis and in unburned skin was significantly lower at 6-12h, and increased with time to

30-36h post-burn. LPR was significantly higher in unburned skin at 6-12h post burn and declined with time. There were significant differences in glucose, lactate, pyruvate, LPR and glycerol between zones.

Patients undergoing a theatre episode during their treatment showed a significant peak of glycerol in the zone of stasis during this time. Aspartate showed significant changes with time and probe location. There was an elevated level of arginine in the burn tissues compared with the literature on surgical wounds, and it was significantly higher in the unburned skin, and lowest in the zone of coagulation. Ornithine showed the reverse. Orn/Arg ratio was significantly affected by probe location, with highest levels in unburned skin. Burn wound progression was evident in only one wound at the site of the zone of stasis probe.

Conclusion: This thesis has produced normal values for metabolites in the dermis of healthy volunteers. The Microdialysis technique was shown to be safe, well tolerated and effective as a method of monitoring the burn wound environment in patients. It was possible to monitor changes in biochemistry in the burn wound over time and between zones. Correlating biochemical changes with burn wound progression was not possible since all but one burn wound in this series showed no progression in the area monitored.

DEDICATION

In loving memory of my grandfather,

Robert Geoffrey Popple

(1914 - 2013)

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FIGURES

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1 INTRODUCTION

1.1 Burns – the historical perspective

1.1.1 The history of burn treatment

Documentation of the treatment of burns can be traced as early as 1500BC. Treatments constituted various topical agents from honey and resin, to rendered pig fat and vinegar soaks. Cold water was noted to relieve the pain from burns at an early stage (1) However there was a persistently high mortality from major burns until the latter part of the 20th Century. In 1954, Bull and Fisher, in Birmingham, published the mortality rate of patients in the burns unit between the years of 1942 and 1952. The Lethal Dose causing 50% mortality (LD50) was 49% Total Body Surface Area (TBSA) burn in children aged 0-14 years (2). Following refinements in fluid resuscitation, and introduction of topical antimicrobials in the 1950s, the LD50 had increased to 64% TBSA by 1971 (3). A move to early excision of large burn wounds gave further improvements in survival which led to an LD50 of 95% TBSA for children, reported in 1987 by Herndon et al, in Galveston (4).

1.1.2 The current challenge

As a consequence of the increased survival rates, burn wound management and subsequent functional and aesthetic outcome has become the next major challenge in burn care.

Burn wound healing occurs by migration of new epidermal cells from undamaged skin edges, or from adnexal skin structures. The deeper the burn wound, the fewer adnexal structures remain, and the longer it takes to heal. In superficial partial-thickness burns, most adnexal structures are undamaged, and re-epithelialisation can occur within 2 weeks, with little or no scarring. In full-thickness injury, no adnexal structures remain, and the natural history of the wound is to heal by contraction and epithelialisation from the edges, leading to thickened, shortened scars with a disfiguring and often functionally disabling outcome (5). Early excision and skin grafting shortens wound healing time, which has been shown to decrease hypertrophic scar formation (6) with its associated contractures and poor aesthetic outcomes. Mid-dermal burns present a challenge. They may heal with little scarring, if conditions are optimal, but the depth of the burn may increase (wound progression) for reasons which are discussed below. Treatment of these burns would ideally centre upon preventing wound progression. Monitoring the cellular activity in the wound would allow adjustment of management to improve conditions in the wound, and would also allow surgical decisions to be made early in those patients for whom wound progression may be inevitable.

1.2 The Burn Wound

1.2.1 Burn wound descriptions

1.2.1.1 Percentage Total Body Surface Area

Percentage of total body surface area (TBSA) is a measurement of the size of the burn as a proportion of the total surface area of the skin. This classification of the severity of a burn allows the systemic effects of the burn to be predicted. Burns

under 15% TBSA in an adult can usually be adequately treated with oral rehydration, and wound care. Burns over 15% TBSA routinely require additional intravenous fluid and thermal supportive care. Burn wounds over 30% TBSA result in burn shock. This is due to a combination of hypovolaemia and the release of inflammatory mediators from the burn wound. It is not entirely corrected by fluid resuscitation alone, as systemic effects on the microcirculation cause capillary leak and oedema.

1.2.1.2 Mechanism of injury

Description of the mechanism of injury is important, giving an idea of the severity of the likely injury, or of any coexisting trauma. A flash burn is unlikely to cause a deep burn, but a flame or contact burn may well cause full thickness injury. A fire in an enclosed space leads the clinician to suspect inhalational injury, while an explosion prompts one to consider fractures due to the patient being thrown some distance by the force. Mechanism of burn does not usually lead to an alteration in the treatment given, except in the case of chemical burns where thorough irrigation or neutralisation of the substance must be undertaken to prevent further injury.

1.2.1.3 Burn depth

Burns are also routinely classified by the depth of the dermis affected. Superficial burns involve the epidermal layer only, and will therefore heal within 2 weeks without scarring. Full thickness burns, at the other extreme, affect the entire

thickness of the dermis, and sometimes the tissue beneath. No viable epithelial structures remain. When left untreated, the wound becomes necrotic, then heals slowly by contracture and epithelialisation from the edges. This can take many months, and the patient is at risk of life-threatening infection, severe contractures, and the high metabolic demand of a healing wound. To reduce the burden of inflammatory mediators, speed up wound healing, and avoid functionally disabling contractures, full thickness wounds are excised and grafted, as early as possible.

In between these two extremes are burns which affect part of the dermis. They can be classified as superficial partial thickness and deep partial thickness. Their different clinical characteristics are summarised in Table 1. It is the deep partial thickness burns that can be the most difficult to predict in terms of their likely course and outcome. They can sometimes heal without treatment within 2-3 weeks, or they can progress to become deeper over the following few days, requiring excision.

Table 1: Characteristics of burn wound by depth

Burn Depth	Colour	Sensation
Superficial Partial Thickness	**Pink; blanches to touch**	**Painful**
Deep Partial Thickness	**Pink/red; fixed staining**	**Sensate to pinprick**
Full Thickness	**White**	**Painless**

1.2.2 Diagnosis of the Depth of Burning – Douglas Jackson, 1953

Our current understanding of the pathophysiology of the burn wound is based around Douglas Jackson's description of the burn wound in the British Journal of Surgery in 1953, entitled 'Diagnosis of the Depth of Burning' (7). In this paper, he explains why it can initially be difficult to assess burn depth, because of the potential for wound progression. He divides the wound into three zones each displaying particular clinical and histological characteristics, and each having a different course and outcome:

1.2.2.1 The Zone of Coagulation

The central zone consists of the tissue damaged irreversibly by the initial insult. The clinical appearance is that of white, coagulated skin which, on microscopy, reveals obliteration of the lumina of the subpapillary plexus and capillary loops.

1.2.2.2 The Zone of Stasis

This zone surrounds the zone of coagulation in both peripheral and deep directions. Clinically it is red and blanches on pressure. Extended application of a tourniquet proximal to the burn on a limb causes the unburned skin to become cyanosed and the zone of stasis to remain red. This indicates a failure of metabolism within the zone of stasis to produce deoxygenated haemoglobin. At 24 hours following injury, this zone shows complete stasis of blood flow with no blanching to pressure, and microscopically, dilated capillaries packed with

erythrocytes. Between three and seven days after the burn, the zone turns white and is indistinguishable from the zone of coagulation. This is due to the superficial dermis being necrotic, and the red cells previously present having been haemolysed.

1.2.2.3 The Zone of Hyperaemia

Lateral and deep to the zone of stasis lies the zone of hyperaemia. Initially red and blanching to pressure, this zone becomes cyanotic along with the rest of the limb on application of a proximal tourniquet. This indicates that both circulation and metabolism are intact. On biopsy, the epidermis is lost, but without structural damage to the dermis. By the seventh day this zone is completely healed.

1.2.3 Current knowledge of the burn wound

Much work has been done on investigating the course of the burn wound, and in trying to prevent the progression of the zone of stasis into necrotic tissue.

1.2.3.1 Diagnosis of Wound Progression

Histologically there has been some difficulty in identifying the zone of stasis as a distinct area as there are several variables to consider for any one burn. Not only does each burn vary in degree of intensity and depth of penetration through the dermis, even a standardised burn affects different dermal components at different depths of the dermis. In addition, a burn injury progresses with time, so in this

already complex picture, each element displays its individual course with time. In a full thickness burn, the intensity may have been so severe, that all changes could be seen in all structures and cells throughout the dermis, the zone of stasis (in its deep aspect) being located in an entirely different tissue, and therefore having a different set of histological features specific to that area, whether it be subcutaneous fat, muscle or bone. It is also important to note that while Jackson's model described the zone of stasis as a 3-dimensional area extending beyond the zone of coagulation, the data from the histological studies cited below focus on the deep aspect only, comments on the lateral extension of the zone of stasis in the literature being notably absent.

1.2.3.1.1 Dermal components

Each dermal structure or cell type (collagen, epithelial and endothelial cells) exhibits a characteristic change when exposed to thermal injury, and some components are more sensitive than others to injury. Using a standardised partial thickness burn wound model, and a standardised time post-burn, Singer et al have shown that collagen is the least sensitive indicator of the depth of heat damage, with changes to a relatively superficial level. Epithelial cells are more sensitive than collagen to thermal damage, with cellular injury extending past the deep margin of collagen damage. Endothelial cells are the most sensitive to the burning insult; damaged endothelial cells can be seen alongside undamaged epithelial cells at the deepest surface of the burn wound. This

structural pattern of injury holds true for different standardised burn depths (8).

1.2.3.1.2 Progression with time

With a standardised partial thickness wound model, individual dermal components have been observed to progress at different rates over the course of 4 days following thermal injury (9). These changes are described in

Table 2**.** Of particular note is that, while the superficial dermal vasculature is irreversibly damaged and shows progressive signs of necrosis with time, the deeper dermal capillaries, which show evidence of stasis until 8 hours post injury, begin to regain their patency from 24 hours onwards. In clinical studies, the presence of patent vessels within the superficial capillary plexus has been shown to be of importance in predicting wound progression, with those wounds having no viable vessels within the superficial 20% of the dermis, evidencing wound progression over 48 hours (10).

Table 2. to show progression of a standardised partial thickness burn wound in the guinea-pig, as described by deCamara et al (9) (RBC = red blood cell; PMN = polymorphonuclear neutrophil)

Time Post-burn	Epidermis	Collagen bundles	Appendages	Blood vessels	Nerves
2h	Signs of thermal injury to keratin, but keratin layer present. Extensive epidermal destruction. Some cell outlines visible	Oedema fluid spaces between collagen bundles in superficial dermis. Deep dermis unaffected	Most hair follicles preserved	Endothelial cell damage to subepidermal capillaries with occlusion of lumina. Crenation of RBCs. Deeper dermal capillaries packed with RBCs but structure of vessels intact	Axons swollen
8h	Wide separation of keratin from dermis. Coagulative necrosis of all epithelial cells. Loss of basement membrane continuity	Oedematous collagen bundles which are displaced by oedema fluid		Disruption of superficial capillaries and haemorrhage into superficial dermis. Deep dermal vessels show signs of stasis.	Fragmentation of myelin
24h	Keratin attached to dermis by debris. Basement membrane discontinuous	Collagen more tightly packed		No new capillary changes. Some dermal vessels acquired lumina	Progressive demyelination
96h	Sloughing of keratin and debris. Basement membrane not identifiable	Superficial dermal collagen necrotic. Infiltration of PMNs	Few epidermal cells around follicles	Subepidermal capillary plexus obliterated. Deep vessels patent	Swelling diminished. Progressive demyelination

1.2.3.1.3 Cell viability

When diagnosing wound progression by viability of mesenchymal cells (fibroblasts, melanocytes, Langerhan cells), wound progression is evident over a 3-5 day period in human and animal models (11)

1.2.3.2 Causes of Wound Progression

1.2.3.2.1 Vascular occlusion

As described above, several studies have shown a link between capillary patency in the dermal vasculature and wound progression, suggesting that capillary patency may have a direct role in the progression of the wound (9,10,12). Three zones of the burn have been described, based on vascular patency alone. A zone of complete capillary occlusion, a zone of partial occlusion and a zone of capillary dilation, which correspond to Jackson's zones of coagulation, stasis and hyperaemia, have been described in the mouse ear. The area of complete occlusion, or coagulation, in this experiment, increased in size by a factor of 10 over 48 hours, all of which became necrotic (13).

1.2.3.2.2 Leukocyte infiltration

Neutrophils infiltrate the burn wound early in the inflammatory process, and their role is to ingest and kill bacteria. They produce proteases and defensins

within granules which are discharged by exocytosis into phagocytosed bacteria, or by degranulation into the interstitium. NADPH oxidase is activated on the cell membrane, producing toxic oxygen metabolites (14). Although effective against bacterial invasion, there is also evidence to suggest that neutrophils may contribute to wound progression by damaging cells within the zone of stasis (15).

The course of inflammatory infiltrates has also been studied by Tyler et al. Superficial partial thickness burns and deep partial thickness burns show different patterns of leukocyte infiltration. An early neutrophil influx appears in all burns, which decreases by 48 hours in the superficial partial thickness burn, but persists in the deeper burn. A later infiltrate of monocytes/macrophages is present in both depths of burn, but is delayed and of a lesser magnitude in the deep partial thickness burn. A rapid lymphocyte influx occurs in superficial partial thickness burns which does not occur in deep partial thickness burns (12). Tyler proposes that it is the patency of the superficial plexus which allows or prevents the extravasation of lymphocytes, which in turn limits or fails to limit burn wound progression.

1.2.3.2.3 Wound Dehydration

Dressing of the wound has a role to play in the progression of the wound depth. Uncovered, debrided wounds have been shown to fare worst in a guinea-pig model, with full thickness necrosis ensuing, and no surviving

hair follicles at 1 week following a partial thickness injury. Wounds with intact blisters, or occlusive dressings, show less progression with no necrosis and a slow re-epithelialisation. Biological dressings show best results with complete healing in 1 week (16). The same author showed two groups of wounds treated either without dressing, or with porcine split skin graft (SSG), both with complete stasis in vessels to below the level of the deepest hair follicle between 16 and 24 hours post-burn. From this point on, the biologically dressed burn showed restoration of the dermal circulation, starting at the deep surface 2 days after the injury, then progressing more superficially, and being completely restored by day 7. In contrast, the untreated burns showed some return of circulation, but this reached its maximum by day 2, and never reached the deepest hair follicles. Water content was also assessed by weight of biopsy before and after drying for both wounds. The porcine split skin graft (SSG) treated group did not show any evidence of dehydration, whereas the untreated burn became dehydrated by 16 hours, reaching a maximum by 2 days post-burn.

1.2.3.2.4 Capillary Permeability, Oedema and Raised Interstitial Pressure

Increased capillary permeability, part of the inflammatory process, allows extravasation of plasma proteins and leukocytes, leading to an altered osmotic gradient across the capillary wall. Fluid therefore accumulates in the interstitial space, causing oedema. This oedema raises the interstitial

pressure which in turn reduces capillary flow, and thus reduces the diffusion of nutrients to, and waste products from, the cell. The escape of a protein-rich fluid into the interstitial space was studied in a guinea-pig model by Sevitt. He found that there was a two-phase response of the capillaries to burn injury of varying severity. All burns produced the delayed type of permeability, after 1-2 hours. An immediate permeability (within 1-2 minutes of burn) was also displayed following burns of moderate and major severity. The delayed response (1-2 hours post injury) was confined to the dermis for superficial injuries, or affected the subdermal plexus or panniculus carnosus for more severe burns. In these most severe burns, the two phases of capillary leak became a continuum, but the deeper capillaries were not affected until later (17). Sevitt postulated a separate mechanism for the two phases of leak, the first being due to a direct structural change caused by heat, and the second of chemical or neurochemical origin, and therefore potentially reversible. Studies in the mouse ear have shown oedema to be maximal at 6 hours following injury, remaining significantly greater than control until 9 hours, then to have returned to just under pre-burn levels by 48 hours. On the contralateral, unburned ear, oedema is significantly higher (9% greater) than normal levels at 2 hours following burn, but returning to normal by 9 hours, indicating a systemic component to the oedema process (13).

1.2.3.2.5 Apoptosis

Apoptotic cells have been found in superficial and deep dermal burn wounds. Both epithelial and dermal fibroblasts are affected. The rate is much higher (average 45% of dermal fibroblasts) in deep partial thickness injury than in superficial dermal injury (6%) and the affected cells tend to be grouped in clusters in the deeper burns (18). It is likely that either the initial injury, the persistent ischaemia from the stasis in the deep dermal vasculature or a combination of factors, is responsible for the cells undergoing apoptosis, and eventual necrosis.

1.2.3.2.6 Vasoconstriction

An initial intense vasoconstriction is evident within the arterioles of the burn wound, as a direct response to heat, with arterioles constricting to 20% of their original diameter for approximately 30 minutes (13). A more prolonged, pharmacologically induced vasoconstriction has been investigated, by administering an intermittent infusion of epinephrine over a 48 hour period. This led to progression of the necrotic area of the burn in a rabbit model (19).

1.2.3.2.7 Free Radicals and Ischaemia-Reperfusion Injury

It has been postulated that free radicals form part of the intermediary process of wound progression. Free radicals are known to be produced as

part of normal cellular activity, such as oxidative phosphorylation in the mitochondria, where approximately 1% of oxygen is converted to superoxide radicals (20). Free radicals, e.g. superoxide radical ($O^{\cdot}$) and hydroxyl radical ($OH^{\cdot}$), are also produced by activated neutrophils. They are bacteriocidal and chemotactic for neutrophil recruitment and activation. Neutrophils generate free radicals via the nicotinamide adenine dinucleotide phosphate (NADPH) dehydrogenase pathway, and in xanthine oxidase activity (20,21) and the prostaglandin system. Cellular damage by free radicals occurs most commonly to lipids in the cell wall (where lipid peroxidation proceeds as a chain reaction). Superoxide radicals can generate hydroxyl radicals, both of which are extremely reactive, and damage endothelial cells, increasing the permeability of capillaries. They can also inactivate antiproteases, leading to unopposed protease activity, and further propagation of the inflammatory response. They are also formed during the auto-oxidation of compounds such as ascorbic acid, glutathione, cysteine, adrenaline and flavin coenzymes, where transition metal ions such as free iron play an enhancing catalytic role. Defence against free radicals comes firstly from close control of biologic enzymatic reactions, and from chelation of metal ions which prevents their catalysis and further free radical generation. Secondly, there are specific free radical scavenging pathways, some of which are enzyme systems, such as superoxide dismutase (catalysing the downgrading of superoxide to hydrogen peroxide), catalase and glutathione peroxidase (reducing hydrogen peroxide to water and oxygen). Alpha-tocopherol (Vitamin E) intercepts lipid peroxyl radicals,

and ascorbic acid, uric acid and glutathione have scavenging properties (22). Free radicals are rendered 'safe' when they are reduced by addition of an electron donated by the free radical scavenger: the latter becoming oxidised. Oxidised free radical scavengers are regenerated by addition of an electron. In pathological states, a combination of increased free radical production and reduced energy supplies (ATP) preventing free radical scavenger regeneration, causes an imbalance in the system leading to free radical mediated damage to tissues.

In the burn wound, in addition to free radical damage due to thermal injury, local ischaemia prevents energy dependent regeneration of the natural scavenging of the free radicals, which exacerbates local damage. During reperfusion of burn tissue, where energy supplies are depleted, oxygen delivery leads to increased production of the superoxide radicals which are converted to hydroxyl radicals, the most reactive radical species, causing a further episode of tissue damage. Unbridled free radical activity increases capillary permeability, leading to increased oedema and tissue pressure, decreased capillary flow and another cycle of ischaemia (23).

1.2.3.3 Prevention of Wound Progression

1.2.3.3.1 Maintenance of vascular patency

Heparin has been shown to delay both the lumen obliteration and the progression by more than 4 hours in a full thickness burn model in rats when given as a pre-treatment prior to the burn injury. However there was

no similar delay when heparin was given post-burn only (24). A single study by Saliba et al treated a series of 7 patients, with 15 – 60% TBSA deep dermal and full thickness burns, with high doses of heparin intravenously, subcutaneously and topically. They found no wound progression, no oedema or shock, reduced fluid requirements, and reduced pain. Healing occurred spontaneously even in full thickness burns, without contractures (25). No other studies have confirmed these findings. Baskaran (26) used a mouse back skin flap model in order to visualise directly the dermal vasculature. Full-thickness burn wounds were created, and labelled red cells injected into the animal to visualise capillary blood flow. Animals treated with Poloxamer-188, a surfactant, revealed absence of a zone of stasis, and a reduction of necrosis (judged by fluoroscein injection at 24h) of 40% when compared to controls. Poloxomer-188 is known to be fibrinolytic and to increase blood clot permeability as well as inhibiting leukocyte chemotaxis, adhesion and migration.

1.2.3.3.2 Inhibition of leukocyte adhesion

Topical methyl-prednisolone has been shown to prevent progressive dermal ischaemia in partial thickness burn wounds in guinea-pigs when compared to emollient treated controls, and those treated with only intraperitoneal methyl-prednisolone (27). This may be due its properties of preventing leukocyte adhesion or due to its inhibition of prostaglandin synthesis. The leukocyte- endothelial cell adhesion mechanism was investigated with relation to zone of stasis in a rabbit model by Mileski et al. Monoclonal antibodies to intercellular adhesion molecule 1 (ICAM-1), the endothelial

ligand bound by the neutrophil, and to the CD18 adhesion complex on the neutrophil were given prior to or 30 minutes following partial thickness burn injury. Progression of the burn as measured by laser Doppler flowmetry, did not occur at 72 hours post injury in those animals treated with either antibody, whether administered before or after injury. Control animals displayed significant wound progression (28). Similar results were found in a rat model, where animals which received antibodies to CD18 post burn demonstrated maintained vessel patency, prevention of neutrophil migration and prevention of progression as measured by laser doppler, latex vascular casts and histology (15).

1.2.3.3.3 Prevention of dehydration

As discussed above, wound cover with porcine split skin dressing has been shown to reverse capillary stasis and prevent progression to necrosis in a guinea-pig partial thickness wound model (16).

1.2.3.3.4 Restoration of capillary wall integrity and prevention of oedema

Tarnow et al (29) used an anti-inflammatory agent, D-myo-inositol-1,2,6-triphosphate (IP_3), in a full thickness rat burn model, measuring dermal blood flow with laser doppler. They showed a significantly increased wound perfusion in the IP_3 treated animals when compared to the burned, saline treated controls. They concluded that IP_3 prevents progressive dermal

ischaemia. While this study showed a slight increase in the wound perfusion, measured by LDI, in IP_3 treated patients, there was no attempt to quantify wound progression, either by measurement of the size of the wound, or by follow-up to healing.

An isomer of IP_3 has been shown to reduce oedema formation in burns in a rat model, when given post-injury, decreasing both water loss into tissues, and albumin extravasation, and also by reducing the negative interstitial fluid hydrostatic pressure (30).

1.2.3.3.5 Inhibition of apoptosis

Two studies from Australia have shown substances which decrease apoptosis, and promote wound healing, in animal models:

A peptide inhibitor of c-Jun (a transcription factor) reduced UV-induced apoptosis in a keratinocyte cell line in-vitro. When the peptide was applied topically to the burn in an in-vivo mouse burn model, the size of the eschar was smaller in treated animals compared to controls. There was also a lower intensity of cells undergoing apoptosis in the area immediately adjacent to the wounds in treated animals when compared to controls. Re-epithelialisation was also quicker in treated animals. (31)

Metallothionein-IIA, a cysteine-rich protein with antioxidant and metal sequestering properties, was used in a similar series of experiments. Its use increased cell viability, decreased apoptosis and promoted migration in an

in-vitro keratinocyte cell line when compared to controls. When administered topically in-vivo in a mouse model, healing time was reduced, and eschar diameter was reduced compared to controls. Apoptosis was not measured for the in-vivo part of this study (32).

1.2.3.3.6 Vasodilators and anti-inflammatories

Corticosteroids, indomethacin and acetylsalicylic acid (ASA) are known to inhibit prostaglandin (PG) synthesis. Topical methyl-prednisolone and systemic (intraosseous) infusion of indomethacin and ASA in a guinea-pig burn model have been shown to prevent the progressive ischaemia of a partial thickness burn when compared to control (33). Furthermore, histological determination of levels of PG have been found to be reduced in burns treated with topical methylprednisolone (34). This indicates that the prostaglandins have a role to play in the progression of dermal ischaemia. Imidazole, and two study compounds, UK38485 and U 63557A, all known to be specific thromboxane inhibitors were applied intraperitoneally (imidazole) and topically (UK38485 and U 63557A) to guinea-pig partial thickness burns. The rise in thromboxane levels seen in controls was not evident in any of the treatment groups. However PGE_2 and PGF_{2a} levels were not reduced in burnt animals (except in an additional group treated with methylprednisolone). At 3 weeks post-burn there was decreased tissue loss in the groups treated with UK38485 and U 63557A and imidazole, and at 6 weeks they were completely healed, in contrast to the control wound

which was still open and full thickness, and the methylprednisolone group which was partially healed.

1.2.3.3.7 Prevention of reperfusion injury

Nitric oxide, a labile nitroso compound, occurs naturally in the body during the conversion of L-arginine to citrulline by Nitric Oxide Synthase (NOS). It is a potent vasodilator and prevents aggregation and adhesion of platelets and neutrophils to endothelium, and to each other (35,36). Three isoforms of NOS exist in humans. Two are constitutive, and one is inducible (iNOS). The two constitutive forms are neuronal (nNOS) and endothelial (eNOS). nNOS produces NO in neuronal cells as part of the cell signalling pathway. eNOS produces NO in endothelial cells, as part of the vasodilatory control mechanism. iNOS is mainly involved in inflammation.

The production of NO in endothelial cells has been shown to be lacking following ischaemia, possibly contributing to reperfusion injury (37). Infusion of L-arginine prior to the ischaemic event has been shown both to increase the amount of Nitric Oxide (NO) available (measured as nitrite + nitrate from microdialysis samples), and to prevent muscle necrosis in an ischaemia/reperfusion muscle flap model (38). However there is a very limited time following reperfusion that addition of L-arginine can be successful in preventing neutrophil adhesion, suggesting that either the endothelial uptake of L-arginine, or the nitric oxide manufacturing system are damaged during the reperfusion injury (37). Despite having possible

beneficial effects locally within the ischaemic areas of the wound, systemic administration of NO has been shown to contribute to macrophage dysfunction, stimulating production of Tumour Necrosis Factor alpha (TNF-a) and Prostaglandin E2 (PGE2), potentially leading to suppressed lymphocyte function, and contributing to immunosuppression and Systemic Inflammatory Response Syndrome (SIRS). Macrophages in burnt mice have been shown to have increased levels of NO, TNFα and PGE2, together with increased expression of inducible Nitric Oxide Synthase (iNOS) (39).

Hypertonic saline dextran (HSD) resuscitation has been studied with regard to its effect on diene production, a result of lipid peroxidation from free radical injury. Pigs with 40% full thickness burns resuscitated using HSD showed a reduction in diene production during the 24 hour study period, compared with those resuscitated using Hartmann's by the Parkland formula (40). The concomitant improvement in haemodynamic status may mean that the initial vasoconstriction is less prolonged, thus leading to a lesser reperfusion insult. Alternatively there may be some inherent property of the dextran solution which attenuates the inflammatory response.

1.2.3.3.8 Maintaining oxygenation

A single study investigating the effects of eight-hourly hyperbaric oxygen (each treatment lasting one hour) on the partial thickness rat burn wound over 3 days, showed a minimal, but statistically significant reduction in skin appendage and basement membrane destruction, and a lesser degree of

leukocyte infiltration of the wound (41). These results may represent the benefit of increasing local tissue oxygen tension in preventing ischaemic necrosis, however the observation that some necrotic progression persists may be due to the increase in reactive oxygen species caused by the high levels of available oxygen, which in turn can cause cellular injury.

1.2.3.3.9 Antioxidants

Oxygen free radical scavengers often have a limited half-life and may be too large to enter the interstitial space. Initial experiments using antioxidants such as superoxide dysmutase (Mr 33,000Da; half-life 8-10mins), and catalase (Mr 250,000Da) were unsuccessful, probably for one of these reasons (23).

Kaufman et al used Compound-23, a free radical scavenger with a much smaller molecular weight (400Da) and stable for 12 hours, to treat deep partial thickness burn wounds and found an epithelialisation rate of 50% compared with 34% in controls, and the presence of 15 hair follicles per high power field, compared with 9 in controls (23). Glutathione has been used in ischemic wound models as an antioxidant, and has been shown to improve the healing time of ischaemic wounds, associated with a rebalance in the matrix metalloproteinase (MMP)/ tissue inhibitors of matrix metalloproteinases (TIMP) ratio (42).

1.2.3.4 Monitoring and Predicting Progression

Current routine clinical practice is to prevent wound progression by optimising resuscitation with carefully titrated intravenous fluids in patients with large burns, and preventing desiccation with dressings. Monitoring the progress of these treatments relies on measurement of systemic parameters such as urine output, pulse and blood pressure and serum lactate. There is no equivalent measurement of the local burn wound environment in current routine clinical use.

Parameters which could be measured, and may be linked to progression include blood flow or vascular patency, biochemical markers of ischaemia, nitric oxide production, interstitial pressure, leukocyte or cytokine concentration, hydration of the wound, free radical activity and apoptosis. Laser Doppler Imaging (LDI) is used in clinical practice to give information on the blood flow within the wound, but is only validated for its accuracy at 48h post burn, and is not ideal as a continuous monitoring tool as it would require frequent exposure of the wound, risking wound desiccation, and infection. Confocal microscopy (FOCI) has been used only as research tool, and again the technique does not lend itself to the concept of continuous monitoring. Microdialysis is a monitoring device with the potential for continuous use throughout the period of early burn management, and could be used to monitor biochemical parameters of ischaemia, products of nitric oxide metabolism or cytokines.

1.2.3.4.1 Laser Doppler Imaging

The Laser Doppler technique has been developed for monitoring differences in tissue perfusion. It is based on the principle that light is reflected from moving cells differently than from non-moving structures. Laser light undergoes a Doppler frequency shift when reflected from moving objects, and the change in the Doppler frequency shift can be measured from the return of light to photo-detectors inside the laser machine. This change in Doppler frequency shift, within a given volume of tissue, is termed 'flux' and is measured in arbitrary units. Early equipment (laser Doppler flowmetry) required direct contact between the probe and the skin, and could only assess a small area at once. The newest equipment (laser Doppler imaging, LDI) uses a scanning laser, a non-touch technique, and a computer system. The laser penetrates 1mm into the surface of the wound, and a colour-coded, two-dimensional image of the entire wound is produced, together with a high resolution photograph, for comparison. Flux is graded by the colours blue, green, yellow, pink and red, representing increasing perfusion.

In the burn wound, the depth of injury is related to the depth of patent capillary circulation. In the inflammatory phase, blood flow is increased in the burn wound. Superficial burns have lost the epidermal surface barrier, and have a higher blood flow than the surrounding normal skin. This is

measured by the LDI as high flux. Full thickness burns have no intact microcirculation, and thus show up as low flux areas.

LDI has been validated for clinical use in many studies. Kloppenberg et al investigated LDI flux values on days 1 and 4 following burn injury (43). They showed a significant difference in LDI average perfusion, between spontaneously healing wounds and non-healing wounds. McGill et al showed good correlation of LDI at 36-72h post –burn, with wound outcome (44). Riordan et al correlated LDI at 48 hours post burn with histological biopsy, and found that a LD perfusion index of >1.3 predicted a superficial injury with 95% sensitivity and 94% specificity (21,45). They also performed serial LDI and biopsy on ten wounds at 24, 48 and 72 hours, and showed progressive deepening over time in 90% of cases. Hoeksema et al studied 40 intermediate depth burns in humans (46). They compared LDI diagnosis to clinical evaluation on Days 0, 1, 3, 5 and 8 post-burn. LDI was shown to be significantly more accurate than clinical diagnosis on Days 3 and 5 post-burn, when predicting outcome as measured by time to healing or biopsy. LDI was at least as accurate as clinical evaluation on Days 0, 1 and 8. Accuracies from Day 3 onwards were >95%. On days 0 and 1, accuracies were lower (55%, 79.5% respectively) but comparison with final outcome measures before 48 hours does not take into account the possibility of wound progression.

In this study, LDI was used to assess the depth of the burn wound at entry into the study and after removal of the probes, in order to assess wound

progression. Wound progression was diagnosed if the total area of low flux had increased.

1.2.3.4.2 Fibre Optic Confocal Imaging

Fibre optic confocal imaging or microscopy (FOCI) enables fluorescence imaging of cellular structure and blood vessels below the surface of the skin. It is a method of directly visualising the changes within the vasculature, such as vasodilatation and vessel leakage, via imaging of FITC-dextran injected into the circulation. This has been shown to enable diagnosis of the different zones of a burn, and assess the level of vasoconstriction and of capillary integrity, having a high correlation with histological sampling (47).

1.2.3.4.3 Microdialysis

Microdialysis is a technique by which interstitial fluid can be continuously monitored. It is minimally invasive, and provides a window into the dynamic physiology of the burn wound. See section 1.3 below.

1.3 Microdialysis

1.3.1 History of Microdialysis

Microdialysis is a technique by which the chemical composition of interstitial fluid can be continuously sampled in vivo. In its present form, it was conceived by Ungerstedt et al in 1974 (48) who used it to monitor neurotransmitters in the rat brain. Since then it has proliferated as a research tool in physiological and pharmacological studies (49) (50). It has found uses in both in-vitro and in-vivo situations, and in animal and human studies. Recent uses have covered the fields of neurophysiology (51), dermatology (52), liver transplantation (53), and oncology (54). Clinically, basic metabolite monitoring has been shown to be effective in detecting cerebral ischaemia in neurointensive care (55), and warning of inadequate perfusion of skin and muscle flaps postoperatively in plastic surgery (56). Edsander-Nord showed low glucose (2mmol/l), high LPR (100) and high glycerol (1000umol/l) in free flaps during their warm ischaemic time, with a return to normal values after anastomosis. Nielsen and Birke-Sorensen showed accurate detection of flap ischaemia in their series of 78 intraoral flaps, suggesting an 'alarm'level of glucose <1mmol/l and lactate >10mmol/l (57). In the field of burns, it has been used to research histamine changes within experimental animal studies (21,58). A clinical study in human burns in 2006, described some biochemical characteristics of the burn wound during fluid resuscitation, with trends towards higher LPR levels in burned skin when compared to unburned skin and controls, which did not correlate with systemic measurements. The authors concluded that local metabolic processes were not fully appreciated when examined systemically and that microdialysis offered a new method of monitoring the local environment (59).

No study has yet assessed the biochemical differences between zones of the human burn wound.

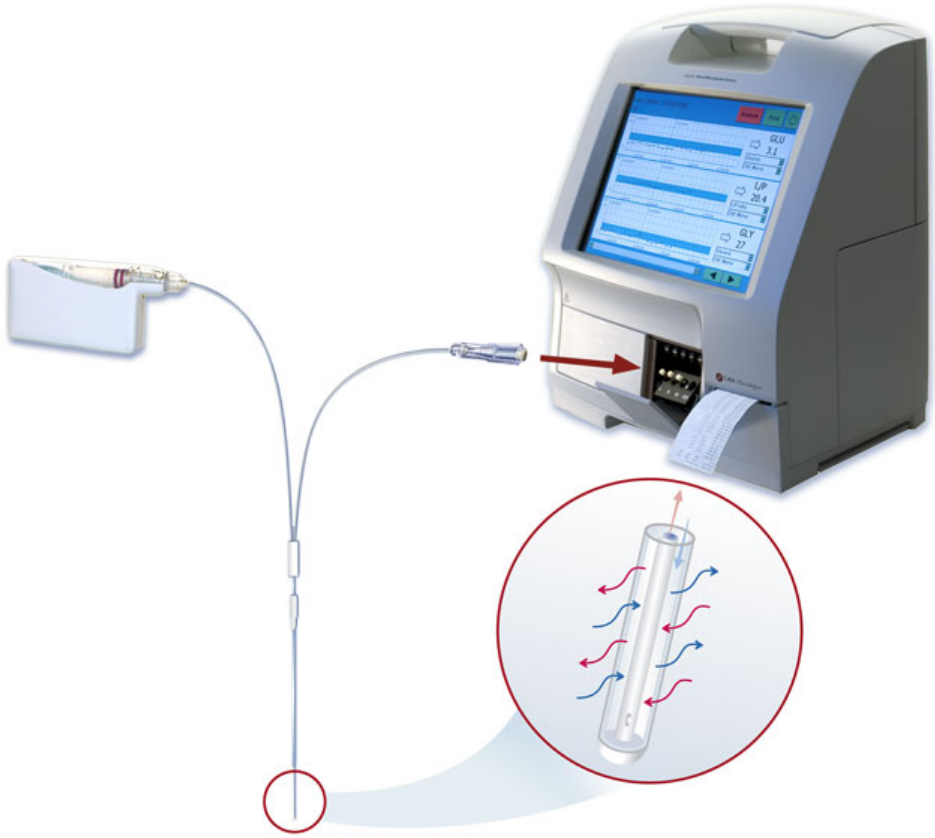


Figure 1: Microdialysis system, showing microdialysis pump on the left, microdialysis catheter (which is implanted in the tissue to be monitored) with membrane and double-lumen flow system (circled), collecting vial on the right. The collecting vial is taken and placed in the analyser (far right) (Courtesy of M Dialysis)

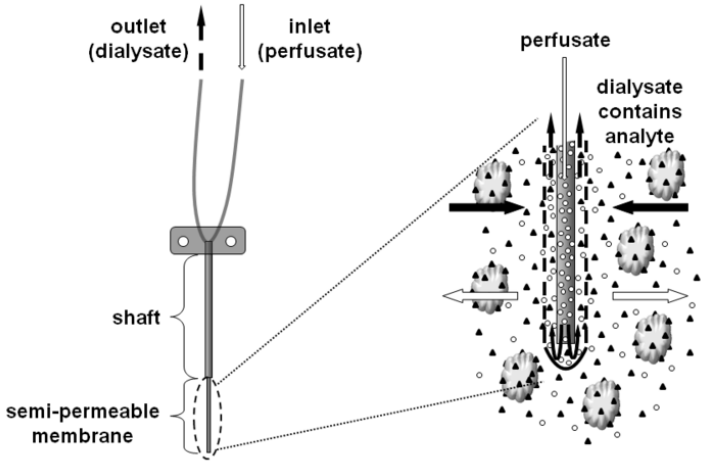


Figure 2: Diagram to show flow through the microdialysis probe. Perfusate is pumped through the core of the probe to the semi-permeable membrane (dotted line). Analytes perfuse freely across the semi-permeable membrane (large arrows). The fluid is then termed dialysate and exits the probe through the outer lumen (small arrows). (From www.ask.com/wiki/Microdialysis under Creative Commons CC-BY-SA license)

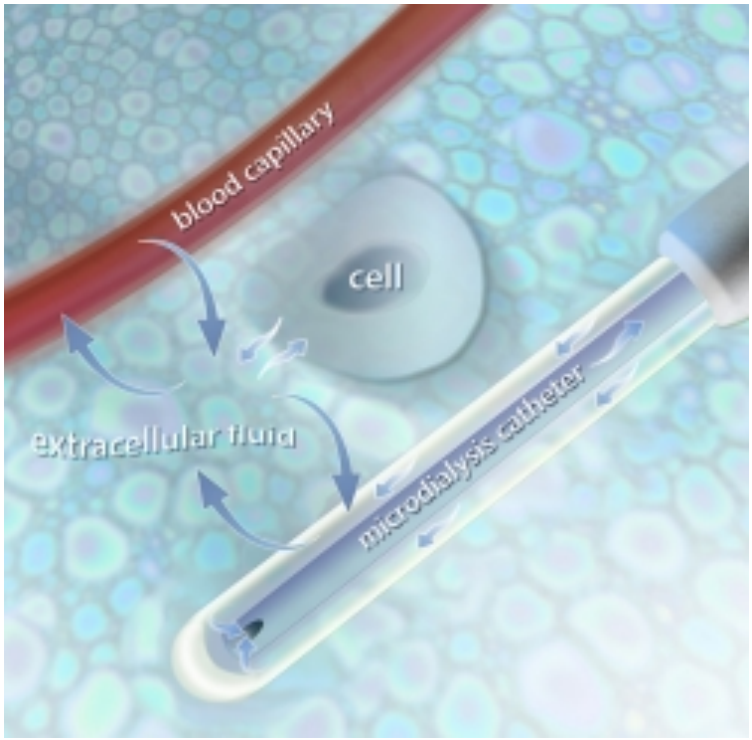


Figure 3: A schematic diagram showing the interaction between capillary supply to the tissues, the intra- and extracellular environment and the microdialysis probe (Courtesy of M Dialysis)

1.3.2 Principles of the technique

Microdialysis uses the principle of dialysis to obtain a fluid sample with similar concentrations of small molecules, to the tissue to be studied. It consists of a probe with a semi-permeable membrane, which is positioned in the tissue of interest, and a pump system to circulate fluid past the semi-permeable membrane, allowing small molecules to diffuse into the system. The resulting sample is representative of the concentrations of these small molecules within the interstitial fluid of the tissues being studied.

The microdialysis system is composed of a microdialysis pump (capable of very low flow rates, 0.1 - 5 μl/min), a microdialysis probe containing a segment of semi-permeable membrane of a predetermined pore size, and a microvial collecting system, all of which are connected by tubing (Figure 1). The probe is implanted into the tissue of interest using a hollow needle introducer. The needle is withdrawn, leaving the probe in place within the tissue. The probe has a double lumen tube attached to one end by which fluid enters and exits the probe (Figure 2). Fluid is pumped through the system, from a syringe which is preloaded in the pump. Before it enters the probe it is termed perfusate. As it enters the probe, it passes along the portion of tubing containing the semipermeable membrane. At this point, small molecules (relative to the pore size of the membrane) are free to pass between the interstitial fluid and the perfusate by diffusion (Figure 3). The slower the pump speed, the longer the time available for equilibration, and the closer to an equal concentration of these molecules in the dialysate and interstitial

fluid. Once it has passed the semi-permeable membrane, the fluid is termed dialysate. It continues to be pumped into the outer lumen of the tubing, and is collected in an exchangeable vial at the end of the system. So, the dialysate is a representative sample of the small molecule constituents of the interstitial fluid.

The proportion of a particular substance in the dialysate as compared to the actual concentration in the sampling medium is termed ‘relative recovery’, and is expressed as a percentage. Relative recovery is increased with a lower flow rate. Absolute recovery is the amount of substance collected in the dialysate per unit time and is increased with a higher flow rate.

Several different designs of probe are available with different arrangements for the flow pathway. Concentric (double-lumen) probes are the most common commercially available, with the membrane at the most distal point of the system. Fluid enters through the central portion of the tubing, which directs fluid to the tip of the membrane. The fluid then travels back past the semipermeable membrane, into the outer portion of the tubing and into a collecting vial. Thus only one entry point into the tissue is needed for a complete system. Another form of system is a linear probe, with the inflow connecting to the semipermeable membrane, which is then continuous in a linear fashion with the outflow tubing and collection vial. This means an entry and an exit point is needed, however of benefit is the improved retention of the probe in a mobile subject. The linear system was not available as a CE marked probe for clinical use at the time of these studies.

1.3.2.1 Factors affecting substance recovery (60,61)

1.3.2.1.1 Flow rate

Molecules transfer across the membrane passively, by diffusion. Since the probe is continuously perfused, equilibrium is never reached. Relative recovery is the dialysate concentration expressed as a percentage of the interstitial concentration. A faster flow rate increases the gradient for transfer, and gives a greater absolute recovery (mass of analyte recovered per unit time), but a lesser relative recovery, as the dialysate will be dilute. A high flow rate will also drain the surrounding tissue, altering the biochemical picture. A low flow rate gives the best relative recovery, and a dialysate which is most similar to the interstitial fluid itself. Therefore the lowest flow rate possible is accepted as being the best method of obtaining an accurate measure of the concentration of the tissue fluid in which the probe is situated (61) (21,55). Other factors to be considered in the determination of flow rate include the frequency of the sampling interval, and the volume needed for analysis of each sample. A flow rate of 0.3ul/min has been put forward by several authors as optimal, as this minimises the drainage of substances from the sampled tissue, but allows 9ul to be collected every 30 minutes, sufficient for analysis of a basic set of metabolites (62) (63) (55).

1.3.2.1.2 *Membrane Length*

The length of the semi-permeable membrane also has an effect on the uptake of a substance. The larger the surface area, the quicker the equalisation of concentration across the two sides of the membrane (Fick's law). The longest commercially available probe is 30mm long.

1.3.2.1.3 *Pore Size*

The semi-permeable membrane can be manufactured with a pre-determined pore size to select for uptake of particular sized molecules. Although in theory, any molecule with molecular mass below the size of the pores will diffuse across, in reality, molecules approaching the size of the pore will display very limited recovery. The molecule must be less than a quarter the pore size to provide acceptable recovery (61). Size of the following substances was taken into account when selecting pore size of the probe: Glucose 180 Da; Lactate 89 Da; Pyruvate 87 Da; Glycerol 92 Da; Amino acids 75-214Da.

1.3.2.1.4 *Perfusate composition*

The perfusion fluid usually contains an aqueous solution similar to the surrounding medium to prevent excessive washout of substances from the area adjacent to the probe. Perfusate can be altered to influence the diffusion pattern in a particular way. For example, facilitated transport has been

described by the addition of β-cyclodextrin to the perfusion fluid as a complexation agent for ibuprofen, increasing the relative recovery by 1.5 – 2 times (64). Lipophilic compounds or highly protein bound substances have also been recovered by altering the composition of the membrane or the perfusate (65), and pH has been used to influence recovery (66).

Perfusion Fluid T1™ (CMA microdialysis, Sweden) is a basic perfusion solution which prevents depletion of salts from surrounding tissues.

1.3.2.1.5 Tortuosity of sample matrix

Cellular content and viscosity of the extracellular matrix will delay diffusion of substances into the probe. Thus in-vivo recovery will never equal in-vitro recovery (67) (68). In a clinical study, the ratio of in-vivo to in-vitro recovery differed between substances, but was constant for the same substance (69).

1.3.2.1.6 Temperature

Molecules diffuse at different rates according to their radius, the viscosity of the suspending fluid, and the temperature (Stokes-Einstein equation). Thus microdialysis should ideally be undertaken at a constant temperature (61). However, where recovery approaches 100%, the effect of temperature on the relative recovery will be minimal.

1.3.3 Microdialysis and the Dermis

Microdialysis in the dermis has been used widely for over fifteen years to investigate pharmacokinetics of drug distributions from systemic administration and from topical preparations, and as a method of direct delivery of drugs into the skin. Other investigations can be categorised as follows:

1.3.3.1 Inflammatory mediators

Measurement of inflammatory mediators has been undertaken by several authors, investigating the skin's role in the allergic response. The role of histamine, substance P and codeine in allergic responses of the skin have been studied (70,71) (72). The topography of plasma extravasation in response to histamine stimulation was studied using plasmapheresis fibres with a 3000 kDa cut-off (73). Neurogenic inflammation, and the role of histamine and substance P, has been studied using the microdialysis technique as summarised by Schmelz and Petersen (74). The use of microdialysis for studying the vascular response of skin to inflammatory stimuli, particularly when paired with laser-Doppler imaging has been extolled (75).

1.3.3.2 Skin Metabolism

Measurement of metabolism in the skin has been another field of interest within the literature. Cellular energy production relies on the substrate glucose which is converted to pyruvate in the presence of oxygen. If the environment becomes anaerobic, glucose metabolism and energy production can continue for a time, by conversion of glucose to lactate. If the environment remains anaerobic for too long, cell death occurs, with the release of glycerol from the cell wall. Glucose levels indicate the delivery of substrate to the tissue from the blood, i.e. perfusion of the tissue. The ratio of lactate to pyruvate indicates the proportion of aerobic activity, i.e. the supply of oxygen. Elevated glycerol levels indicate cell death. A reduced blood supply would lead to a low glucose level, together with a rise in lactate/pyruvate ratio, and this would be followed by a rise in glycerol, if blood supply is not restored.

Glucose and lactate concentrations in the skin have been studied in normal subjects at rest during fasting and after glucose load (76-78) and during exercise (79).

Lactate and pyruvate was studied by Krogstad and Jansson, and the lactate/ pyruvate ratio (LPR) as a function of aerobic metabolism was introduced. These authors validated the technique of intradermal microdialysis, using ultrasonic measurement to ensure probes were accurately sited, and using various calibration techniques to allow absolute values to be estimated. They investigated changes in lactate and pyruvate in relation to the depth of

the probe within the dermis, and found both lactate and pyruvate levels to be higher the more superficial the probe. However they noted that the increase was equal for both substances, indicating an increase in metabolic rate in the more superficial layers, but no change in the aerobic status of the metabolism. They concluded that the LPR was unaffected by the depth of the probe in the dermis (52,76).

No studies could be found which measured the levels of glycerol in the dermis.

The levels of uric acid, an antioxidant, in the dermis and its decline with age has also been reported (80).

1.3.4 Microdialysis in Burns Research

Although microdialysis is an ideal tool with which to monitor the burn wound, very little research has been undertaken in this field to date. Work investigating the role of histamine in burn oedema has been undertaken in animal studies (58,81).

C3a levels were found to be elevated in the subdermal tissue in deep partial thickness burns, which decreased after 24 hours in younger patients, but persisted in patients over 60 years, and this correlated with numbers of thrombosed subdermal vessel after 24 hours (82).

The only other clinical study was a series of six patients with burns >25% TBSA who were studied with two microdialysis catheters, one in the burn wound and

one in normal skin. Samples were collected 3 hourly until day 5. The presence of dermal ischaemia was noted, together with hyperglycaemia and raised glycerol when compared to normal volunteers (59).

Tissue ischaemia is the final common pathway in most of the theories about burn wound progression, and therefore measurement of ischaemic parameters should reflect the likelihood of impending tissue death. Changes in glucose, LPR and glycerol using the microdialysis method have been shown to accurately reflect tissue ischaemia in free flaps. Another proposed mechanism of wound progression involves changes in nitric oxide, and, while nitric oxide itself is too unstable to measure directly, monitoring nitric oxide precursors and metabolites, such as arginine and citrulline, is another possible way of predicting progression.

2 HYPOTHESES

- The Microdialysis method can be used to detect changes in biochemistry within the burn wound with time.
- Different zones of the burn wound are characterized by different biochemical profiles, and these profiles change with time.
- Changes in the biochemistry of the zone of stasis can predict wound progression.

Proving these hypotheses would further the understanding of burn wound progression, and provide a useful tool for clinical research aimed at modulating this progression.

2.1 Research Plan

Two clinical phases of this study were performed. The first was to establish baseline values in normal skin in healthy volunteers using the microdialysis technique. The second phase was to characterize biochemical differences between zones of the burn wound and changes with time post-burn. During the second phase, laser Doppler imaging was used as a method of detecting wound progression in order to correlate outcome with biochemistry. Dialysate was tested for products of aerobic and anaerobic glucose metabolism (glucose, lactate and pyruvate), and a marker of cell necrosis (glycerol). Metabolites associated with nitric oxide synthesis (Arginine, ornithine and citrulline) were tested with an amino acid assay, and additional assays of the spectrum of amino acids were performed concomitantly. The clinical studies were supported by calibration studies to test the validity of the results.

3 MATERIALS AND METHODS

3.1 Microdialysis

3.1.1 Microdialysis Equipment

CMA60 Microdialysis catheter (CMA Microdialysis, Solna, Sweden)

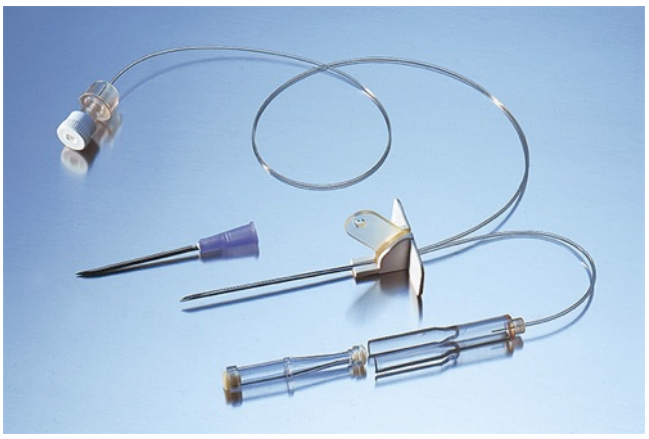


Figure 4: CMA 60 Microdialysis catheterwith needle introducer. Microvial also shown. Photograph courtesy of M dialysis, Solna, Sweden

Probe consisting of a semipermeable membrane of length 30mm, and diameter 0.6mm with a pore size of 20kDa. Inlet and outlet tubing connect the membrane to a pump and a collection vial.

CMA 107 microdialysis pump (CMA Microdialysis, Solna, Sweden)

Figure 5: CMA 107 Microdialysis pump. Photograph courtesy of M Dialysis, Solna, Sweden

A small, portable pump with adjustable flow rate from 0.1 – 5.0 µl /min. During this study a flow rate of 0.3µl/min was used.

CMA106 syringe (CMA Microdialysis, Stockholm, Sweden).

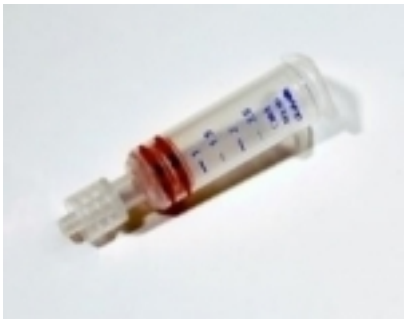


Figure 6: CMA106 Syringe. Photograph courtesy of M Dialysis, Solna, Sweden

A plastic syringe holding 2.5ml of T1 perfusion solution, placed inside the CMA107 pump and attached to the inflow tubing of the CMA60 microdialysis catheter.

Microvials (CMA Microdialysis, Stockholm, Sweden)

Figure 7: Microvials and Microvial rack. Photograph courtesy of M Dialysis, Solna, Sweden

Small plastic vials used to collect microdialysate from the outflow tubing of the CMA60 catheter. These are removable, and used to transfer the samples to the CMA 600 analyser for analysis. A plastic storage rack to minimise evaporation during freezing was also used.

T1 Perfusion Solution (CMA Microdialysis, Stockholm, Sweden)

Figure 8: T1 Perfusion Solution. Photograph courtesy of M dialysis, Solna, Sweden

A sterile, isotonic solution which is perfused through the microdialysis catheter. Concentrations of electrolytes are as follows: Na^+ 147 mmol/l (3.38 g); K^+ 4 mmol/l (156 mg); Ca^{2+} 2.3 mmol/l (92mg); Cl^- 156 mmol/l (5.59 g); Osmolality 290 mosm/kg; pH c.6

3.1.2 CMA600 Analyser

Figure 9: CMA 600 Analyser. Courtesy of CMA Microdialysis, Solna, Sweden

CMA600 (CMA Microdialysis, Stockholm, Sweden) is a kinetic enzymatic analyser using colorimetric measurements. Imprecision is documented in the product literature to be <6% Relative Standard Deviation. Reagents are commercially available for analysis of glucose, lactate, pyruvate, glycerol, urea and glutamate. In this study, all except glutamate were used. The analyser allowed analysis of up to four substances during any one 'run'. In these studies, glucose,

lactate, pyruvate and glycerol were studied in the first run. Samples were returned to the freezer before analysis of urea in a separate run. The order of analysis was glucose, lactate, pyruvate then glycerol. Therefore in the event of there being insufficient sample in a vial, the later analyses may have been missing. Consumption of sample was 0.2µl for lactate, and 0.5µl for each of glucose, pyruvate, glycerol and urea. A residual 2 µl was required in addition to the minimum volume for analysis, so total volume required for a full analysis was 4.2µl. Time for each complete analysis was approximately 4 minutes.

LabPilot Software (CMA Microdialysis, Stockholm, Sweden) was used to handle the data, including some of the graphical representations.

Calibrator A (CMA Microdialysis, Stockholm, Sweden) was used as a calibration solution, as an internal standard, and as a reference solution in which microdialysis was undertaken to detect percentage recovery of substances. Published concentrations of substances within this solution were as follows: Glucose 5.55mmol/l; Lactate 2.5 mmol/l; Glycerol 475 µmol/l; Urea 13.3 mmol/l; Pyruvate 250 µmol/l; Glutamate 25 µmol/l.

3.1.3 Summary of Technique

The CMA106 syringe was filled with T1 perfusion solution, and attached to the microdialysis catheter. The syringe was depressed until all air was expelled from

the microdialysis tubing, then the syringe was placed into the CMA107 pump, and a collecting vial was attached to the end of the catheter. The pump was turned on and the priming mechanism initiated. After the 5 minute automatic priming was completed, the pump returned to the preset perfusion rate of 0.3µl/min. The first collection vial was discarded and a new one attached. The microdialysis catheter was then placed either into a bath of calibration fluid (for calibration experiments, and normal volunteer study) or directly into the patient (for the clinical burn studies).

Probe insertion was undertaken after local anaesthesia of the area was achieved. For normal volunteers expediency was not an issue, so topical EMLA cream was applied 30-60 minutes prior to probe insertion. For burn patients, delay was minimised by injecting 5ml of 1% lidocaine into the dermis in the sensate areas (Zones B and C). Microdialysis probes were inserted into the dermis using the insertion needle provided with the catheter. Catheters were secured with a suture into the burned areas, or with a clear adhesive Tegaderm dressing (3M, Bracknell UK) onto intact skin. A dressing of tulle gras, gauze and crepe bandage was used to protect the burn where necessary, and to keep the probe in place, preventing accidental dislodging.

Samples were collected by exchanging each collection vial every 30 minutes. Each vial was labelled with a unique number, and placed into a storage tray. Once a complete tray of 12 vials was collected, this was placed into the freezer at -80^{o}C. Samples were stored for up to 3 months before first analysis. Just prior to analysis, samples were removed from the freezer and defrosted at room temperature. Samples were analysed for glucose, lactate, pyruvate and glycerol, and then

refrozen. Urea analysis and amino acid analysis were performed after defrosting a second time.

Before each batch was analysed, the CMA 600 ran an automatic calibration. For analysis of Study 1 Normal Volunteer samples, an internal standard was used by filling a collection vial with Calibrator A, and analysing this alongside the samples for each patient.

Data were downloaded from the CMA 600 analyser. LabPilot, (CMA Microdialysis, Solna, Sweden) and Microsoft Excel were used to produce the graphical images, and SPSS Version 15.0 was used for statistical analysis and some graphical images of the lactate, pyruvate, glucose, glycerol and urea results.

3.2 Laser Doppler Imager

Figure 10: moorLDI Laser Doppler Imager

Following enrolment into the study, and just prior to probe insertion, a laser Doppler image was taken. In order to provide a reference point for later calculations of area, a 5cm standard line was drawn on the skin next to the wound. The patient was given laser-protective glasses to wear, and the moorLDI laser Doppler imager (Moor Instruments

Ltd, Axminster, Devon) was turned on. The area to be scanned was cleaned of any debris and all dressing material was removed. The laser scanner was positioned 50-70cm from the wound, and the exact distance recorded. The patient was asked to remain still, and the scan was taken. On completion of the study, a second LDI image was taken using the same protocol. The scanner distance, and the orientation of the wound was matched with the first scan as closely as possible. Images were interpreted using the colour coding as standard to the moorLDI software. Areas of full thickness burn were calculated by tracing the areas of low flow (<200 perfusion units), using the moorLDI software. Wound progression was deemed to have occurred if this area had increased between the first and second images.

3.3 Amino Acid Analysis

For determination of amino acids, the High Performance Liquid Chromatography (HPLC) method was used. Insufficient sample volumes remained in the vials, after analysis with the CMA600, to analyse every sample vial individually for amino acids, so samples were pooled in consecutive groups of four. This provided an average result for every 2 hours throughout the study period, for each patient.

3.3.1 HPLC of amino acids

Amino acids were determined by HPLC with fluorometric detection of o-phthaldialdehyde derivatives, using a modification of the classical method of

Lindroth and Mopper (21). The system consisted of a ChromSpher ODS column (5μm, 150 x 3 mm, with guard column; Varian Chrompack, Walton-on-Thames, UK) with a CMA 280 fluorescence detector (maximum excitation, 340-360 nm; maximum emission 495 nm: CMA Microdialysis Ltd, Sweden). The derivatisation reagent was prepared by mixing 975μl of incomplete o-phthaldehyde reagent solution (Sigma, Poole, UK) with 25μl of 10% (v/v) mercaptopropionic acid in methanol (Sigma, Poole, UK).

Derivatisation of dialysis samples and standards, as well as injection, was carried out with a CMA 200 refrigerated (8^{o}C) autosampler (CMA Microdialysis Ltd, Sweden), which added 1.5 μl of derivatisation reagent to 5 μl of each sample or standard, and reacted for 60 seconds prior to injection. The mobile phase gradient consisted of 100mM sodium acetate buffer, pH 6.9, containing an organic modifier mixture (water; acetonitrile, methanol: 1:3:6 (v/v), increasing linearly from 8 to 48% (v/v) over a period of 46 minutes. This was delivered at a flow-rate of 0.5 ml/min, using a PM-80 twin-reciprocating pump with LC-26A vacuum degasser (BAS Technicol, Congleton, UK). Data were collected and analysed using EZChrom software (Aston Scientific, Stoke Mandeville, UK) after calibration with a range of standard amino acid solutions (10-80 μM) containing aspartate, glutamate, serine, glutamine, histidine, glycine, threonine, citrulline, β-alanine, arginine, alanine, taurine, GABA, tyrosine, ethanolamine, valine, methionine, tryptophan, phenylalanine, isoleucine, leucine, ornithine, lysine and putrescine. Other amino acids and primary amines were identified by co-elution with native compounds.

3.4 Calibration Studies

3.4.1 Accuracy of Analyser

A sample of calibration solution (Calibrator A, CMA Microdialysis, Solna, Sweden) was analysed with each run of analysis throughout the initial experiments, in order to assess the accuracy of the analyser.

3.4.2 Experiment 1: The effect of sample storage conditions on accuracy of analysis

Aim: To establish the effect of repeated freeze-thaw cycles or refrigeration of the microdialysis samples.

Method: Each experiment was performed in triplicate. On Day 1, microdialysis vials (CMA, Sweden) were prepared with 30µl of calibration fluid (Calibrator A, CMA, Stockholm Sweden). Vials were labelled A1-3, B1-3 and C1-3. Vials were analysed for Glucose, Lactate, Pyruvate, Glycerol and Urea using the CMA600 analyser (CMA, Sweden). After analysis, all vials were placed into microvial racks (CMA, Sweden). Vials A1-3 were put into the -80^{o}C freezer. Vials B1-3 were placed into a temperature controlled refrigerator at 5^{o}C. Vials C1-3 were kept at room temperature (23^{o}C). On Day 2, the vials were removed from their storage conditions and transported to the analyser. They remained at room temperature until analysis. Analysis was performed as above. Samples were then

returned to their respective storage conditions. On Day 3, samples were again removed from their storage conditions and analysed for the third time.

Graphs were created using the average of each triplicate reading for each time-point. Statistical calculations were performed using SPSS Version 15.0 for Windows. Results were analysed using two-way ANOVA, with a Post-hoc Tukey analysis.

3.4.3 Experiment 2: The effect of sample volume on accuracy of analysis

Aim: To establish the effect of the volume of the sample within the vial on the results of the analysis of glucose, lactate, pyruvate, glycerol and urea.

Method: Each experiment was performed in triplicate. On Day 1, microdialysis vials (CMA, Sweden) were prepared with calibration fluid (Calibrator A, CMA, Stockholm Sweden). Vials were labelled A1-3, F1-3 and G1-3. Vials A1-3 were filled with 30µl. Vials F1-3 were overfilled almost to the capacity of the vial. Vials G1-3 were filled with 10 µl. Vials were analysed for Glucose, Lactate, Pyruvate, Glycerol and Urea using the CMA600 analyser (CMA, Sweden). After analysis, all vials were placed into microvial racks (CMA, Sweden) and put into the -80^{o}C freezer. On Day 2, the vials were removed from the freezer and transported to the analyser. They remained at room temperature until analysis. Analysis was performed as above. Samples were then returned to the freezer. On

Day 3, samples were again removed from the freezer and analysed for the third time.

Graphs were created using the average of each triplicate reading for each time-point. Statistical calculations were performed using SPSS Version 15.0 for Windows. Results were analysed using two-way ANOVA, with a Post-hoc Tukey analysis.

3.5 Study Design

3.5.1 Power Calculations

A power calculation was performed based on the only clinical microdialysis study in burns available at the time. This was a study which showed a difference in histamine levels from different depths of burn in pigs (21). There were no other studies on biochemical parameters from different zones of burn wounds, or on human burns, available prior to the commencement of this study, so it was not possible to perform a power calculation with any greater accuracy.

The values used in the power calculation were based on Fig 2 of the Papp et al paper and are shown in Table 3. Power was calculated with SPSS for Windows using the method described by D'Amico et al (21). With a sample size of 5 in each group, the power to detect the estimated difference between the groups at the 5% significance level was over 95%.

Table 3: Estimated values of histamine (mmol/l) with time for Group 1(unburned skin), Group 2 (1second thermal burn) and Group 3 (9 second thermal burn) from Papp 2005

	1 hour	2 hours	6 hours	12 hours	24 hours
Group 1 mean	240	210	40	25	100
Group 2 mean	65	50	25	25	130
Group 3 mean	30	20	15	10	10
Standard deviation	60	60	10	10	40

This calculation showed a statistical difference could be shown in this group with a sample size of 5 patients. Since this study is not directly comparable to our study, it was decided to increase the sample size to 10 patients in each group, to allow for the possibility of smaller differences in the biochemical parameters measured in humans in this study.

3.6 Protocol for Clinical Study 1: Normal volunteers

3.6.1 Aims

- To establish a normal range of metabolites for healthy skin using the microdialysis technique
- To ascertain if the normal range alters with the raised temperature environment experienced by the burns patients during their treatment
- To validate probe insertion technique

3.6.2 Methods

3.6.2.1 Volunteer Recruitment

Ethics Committee approval was obtained from the Black Country Research Ethics Committee (Ref 05/Q2702/76). A Participant Information Sheet was produced (Appendix A) and the protocol was explained to the volunteers together with potential risks. Informed consent was obtained. Eleven healthy male volunteers were recruited. Volunteers were asked to refrain from drinking alcohol or caffeine during the six hours prior to the study, and during the study period.

3.6.2.2 Analgesia

Each volunteer had 5g EMLA cream (AstraZeneca, UK) applied to the skin on the dorsal surface of the non-dominant forearm, under an occlusive dressing (Tegaderm, 3M) for 60 minutes, prior to probe insertion.

3.6.2.3 Equipment set-up and calibration

The CMA60 microdialysis catheter was connected to a syringe containing T1 Perfusion Solution (CMA, Sweden), and connected to the CMA 107 microdialysis pump set at a flow rate of 0.3µl/min. After the initial flush sequence, the probe was placed into a sterile calibration solution, Calibrator A (CMA, Sweden), a sample of which was taken before probe insertion to act as an internal standard. The probe

was left in this solution and microdialysis undertaken for 60 minutes, sample vials being collected half hourly.

3.6.2.4 Probe insertion

Aim: to assess the reproducibility of the catheter insertion technique, and to ensure all microdialysis samples were taken from the dermal layer.

The EMLA cream was removed from the volunteer's arm, and the arm tested for anaesthesia. The CMA60 probe was then inserted intradermally into the dorsal forearm skin using the insertion needle provided. The needle was then withdrawn, and the catheter held in place by a Tegaderm dressing. The pump was loosely bandaged to the upper arm to allow free movement. Depth of the probe and total depth of the dermis was measured ultrasonically with a Sonosite 180Plus (SonoSite Inc, USA). Distance from epidermis to probe and distance from epidermis to junction of dermis and subcutaneous fat was measured (Figure 11). Probe depth was expressed as a percentage of the total depth of the dermis.

Throughout this experiment, air temperature measurements were taken adjacent to the forearm containing the probe using a calibrated thermometer (Comark, UK)

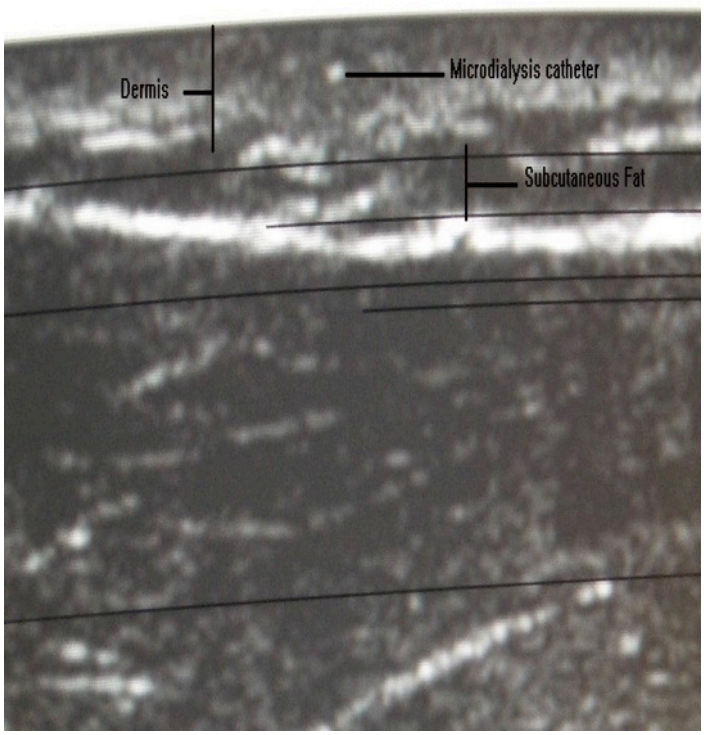


Figure 11: Ultrasound image of the skin and subcutaneous layers of the forearm following microdialysis probe insertion. Microdialysis probe, dermis and subcutaneous fat are indicated

3.6.2.5 Equilibration Period

Aim: To allow normalization of the probe environment and to assess the effect of insertion trauma.

The first 90 minutes was designated the equilibration period. The volunteer was asked to sit in a temperature monitored room, and was free to eat and drink, watch television or read. Microdialysis samples were collected at 30 minute intervals.

3.6.2.6 Study Period 1

Aim: To obtain a baseline reading at room temperature

The next 60 minutes was designated Study Period 1. Room temperature was maintained and a further 2 samples obtained at 30 minute intervals. Air temperature was measured.

3.6.2.7 Study Period 2

Aim: To assess the effect of raised temperature

Immediately following this the volunteer was entered into Study Period 2. The volunteer was asked to lie on the bed, and the Aragona CTC Thermal Ceiling (Aspira, Sweden) was switched on. Air temperature measurements were taken. Two further microdialysis samples were collected at 30 minute intervals. The presence of sweating local to the probe site was also noted.

3.6.2.8 Study Period 3

The volunteer was returned to room temperature for Study Period 3, with a further two samples collected at half hourly intervals. Air temperature was measured.

3.6.2.9 End of Study

At the end of Study Period 3, the probe was removed from the forearm and returned to the calibration solution for a further hour. The volunteer was then allowed to leave, and the probes were discarded.

3.6.2.10 Labelling and Storage of Samples

Labelling of sample vials is summarised in Table 4. Samples were stored at -80^{o}C before analysis, and were analysed within 12 weeks of collection.

3.6.2.11 Statistical Analysis

Repeated measures ANOVA was used to test for differences during the calibration period.

To allow tissues to respond to change in temperature and to allow for the slight delay in flow from probe to sample collection vial (approx. 2 mins), the second sample from each study period was used for statistical analysis. Repeated measures ANOVA was also used to detect differences between the three temperature study periods.

Analysis of the ambient temperatures between the study periods was performed using a paired T-test

Table 4: Labelling of samples relative to study protocol

Vial Code	Time (hours:minutes)	Description of study period
NV__C1	0:00	Calibration solution
NV__01	0:30	Probe in Calibration solution
NV__02	01:00	Probe in Calibration solution
NV__03	01:30	Probe in Volunteer; Equilibration Period
NV__04	02:00	Probe in Volunteer; Equilibration Period
NV__05	02:30	Probe in Volunteer; Equilibration Period
NV__06	03:00	Probe in Volunteer; Study Period 1 (Room Temperature)
NV__07	03:30	Probe in Volunteer; Study Period 1 (Room Temperature)
NV__08	04:00	Probe in Volunteer; Study Period 2 (Raised Temperature)
NV__09	04:30	Probe in Volunteer; Study Period 2 (Raised Temperature)
NV__10	05:00	Probe in Volunteer; Study Period 3 (Room Temperature)
NV__11	05:30	Probe in Volunteer; Study Period 3 (Room Temperature)
NV__12	06:00	Probe in Calibration solution
NV__13	06:30	Probe in Calibration solution
NV__C2	07:00	Calibration solution

3.7 Protocol for Study 2: Small Burns

3.7.1 Aims

The aims of this study were as follows:

- To characterize the different zones of the burn wound biochemically.
- To assess whether wound progression could be predicted by the local biochemical changes within the first 36 hours following injury.

3.7.2 Materials and Methods

3.7.2.1 Recruitment

Ethical approval was granted from the Black Country Research Ethics Committee (Ref 05/Q2702/76). A participant information sheet and consent form were prepared (Appendix A). Informed consent was obtained prior to enrolment. Potential participants were identified by the on-call burns doctor and the Researcher was contacted. The patient was approached as soon as possible after admission to the hospital.

Ten patients were recruited. Any patient admitted to the Burns Unit at Selly Oak Hospital with total burn <15% total body surface area (TBSA) was considered for inclusion (Table 5). Exclusion criteria are defined in Table 6.

Each patient fitting the inclusion criteria had the study explained, and was given a participant information sheet. Due to the nature of the study, it was

necessary that the study must be started as soon as possible after admission. Consent was sought immediately after the explanation was given. Where consent was given, enrolment ensued.

Table 5: Inclusion criteria

Criteria	Study 1	Study 2	Study 3
Age	18-80	18-80	18-80
Gender	Male	Male or Female (except during pregnancy)	Male or Female (except during pregnancy)
Size of Burn	N/A	<15% TBSA.	>30% TBSA.
Site of Burn	N/A	Includes arm or leg	Includes arm or leg
Time of Burn	N/A	Admitted within 6 hours of burn	Admitted within 6 hours of burn
Administrative	Volunteer able to understand trial	Patient able to understand trial	Patient able to understand trial

Table 6: Exclusion criteria

Criteria	Study 1	Study 2	Study 3
Current Co-morbidity	Ongoing medical condition or medication.	Ongoing medical condition or medication. Co-existing trauma	Ongoing medical condition or medication. Co-existing trauma
Past Medical History	History of skin disease	History of skin disease	History of skin disease
Alcohol and Caffeine	Alcohol/caffeine consumed within 12h		
Allergy	Allergy to polyurethane or polyamide	Allergy to polyurethane or polyamide	Allergy to polyurethane or polyamide
Other clinical trial	Treatment in other clinical trial within past 30 days	Treatment in other clinical trial within past 30 days	Treatment in other clinical trial within past 30 days

3.7.2.2 Equipment set-up

Once initial clinical assessment of the burn was completed, and any necessary therapy commenced (e.g. analgesia, debridement of blisters), the study was started. Details of the distribution and depth of the burns, were recorded for correlation with the results.

Three CMA60 probes were set up on a sterile trolley. T1 perfusion solution was drawn into the CMA106 syringe, attached to the CMA60 probe and inserted into the CMA107 pump. The pump was set to a flow rate of 0.3µl/min. The flush sequence was allowed to progress and was collected in a sample vial to check the correct functioning of the equipment. This sample vial was discarded on insertion of the probe.

3.7.2.3 Selection of Probe Location and Probe Insertion

Zone of coagulation was defined as an area of non-blanching, anaesthetic skin within the area injured. Zone of stasis was defined as an area of blanching skin, with epithelial loss, adjacent to the area defined as zone of coagulation. The area of unburned skin chosen for the insertion of the third probe was an area distant from the injured skin, if possible contralaterally in a matched area. If this was not possible, a site greater than 10cm from an injured area was chosen.

Where possible, probe insertion took place immediately following blister debridement and cleaning of the wound which took place under sedation with morphine and midazolam, or with morphine alone, as per unit protocol. Prior to insertion of the probes a total of 5ml Lidocaine 1% was injected intradermally at two of the three areas to be studied: the zone of stasis (as defined above) and the normal area of unburned skin. The zone of coagulation (centre of burn) is insensate, so anaesthesia was deemed to be unnecessary.

Once anaesthesia was attained, a microdialysis probe was placed intradermally at each of these sites using the insertion needle provided with the probe. Dressings were standardised: Jelonet, gauze and crepe. This was the standard dressing applied for the first 48 hours within the Burns Unit at the time of commencement of the trial.

3.7.2.4 Data collection

Microdialysate samples were collected every 30 minutes throughout the first 36 hours following injury. Time was measured as number of hours after reported time of burn. Patient activity, medical intervention and events such as theatre visits were noted throughout the study period.

Medical notes were reviewed subsequent to patient discharge, and events in relation to surgical procedures, skin grafting and wound healing were noted.

3.7.2.5 Laser Doppler Imaging

The Moor LDI Laser Doppler machine was calibrated and set up according to the manufacturer's instructions. The distance of the scanning laser from the patient was noted, and an initial scan of the burnt area chosen for microdialysis was taken before probe insertion. A second scan was taken of the same area, at the end of the study, just prior to removal of the microdialysis probe. The distance of the scanning laser from the patient was matched to that of the first scan. The graphics package supplied with the moorLDI machine was used to calculate the total area of low perfusion within the wound. Wound progression was deemed to occur if the area of perfusion coloured blue (<200 perfusion units) had increased between the first and second images.

3.7.2.6 End of Study

At the end of the 36 hour study period, the microdialysis probe was removed, and all other treatment for the burn continued as normal.

3.7.2.7 Analysis of samples – Glucose, Glycerol, Lactate, Pyruvate, Urea

Sample vials were placed into the freezer at -80^{o}C for storage until analysis. Analysis was undertaken within 3 months in all cases. Analysis of glucose,

glycerol, pyruvate and lactate was performed using the CMA600 analyser (CMA, Sweden) and glucose, lactate, pyruvate and glycerol reagents (CMA, Sweden). Samples were refrozen at -80^{o}C pending the Urea analysis which was undertaken as a batch analysis at the end of the entire study period using the same analyser, and urea reagent (CMA, Sweden).

3.7.2.8 Calculated Results

Lactate: Pyruvate Ratio was calculated by dividing the Lactate result (µmol/l) by the Pyruvate result (µmol/l) for each individual time point. This method provides an estimation of the aerobic state of the tissue and controls for the placement of the probe in different levels of the dermis (21). LPR <25 was interpreted as being predominantly aerobic, and LPR>25 predominantly anaerobic metabolism.

3.7.2.9 Analysis of samples – Amino Acids

Following analysis of glucose, glycerol, lactate, pyruvate and urea, the remainder of each sample was then pooled in groups of 4 consecutive samples to allow analysis of amino acids.

3.7.2.10 Statistical Analysis

SPSS Version 15.0 for Windows was used to analyse the results. Values were grouped into 6-hour time blocks from the purpose of analysis of time-

related trends. Generalised Estimating Equations were used to detect differences between time blocks and between zones. A subgroup analysis was performed for patients attending theatre during the study period.

For the amino acid analysis, a 2-way ANOVA (GraphPad Prism software, version 5) was used. Variables were time post-burn and probe location.

3.7.2.11 Late addition to protocol for measurement of serum urea

A late addition was made to the protocol for collection and microdialysis of serum urea. This was in order to attempt in vivo calibration using the endogenous reference method (See section 5.2). This was agreed by the Research Ethics Committee by way of a minor amendment. Serum samples, which had already been taken for routine clinical use, were collected from frozen storage in the hospital biochemistry laboratory, defrosted, and the excess serum used for microdialysis. Serum was drawn from the top of the tube into a 1ml syringe. One of the probes which had been used previously for the dermal microdialysis of the same patient was set up as previously described. The microdialysis probe was placed into the syringe of serum, and microdialysis was undertaken for three continuous 30 minute periods. Microdialysis samples were analysed in the usual way. This had to be undertaken after the completion of the dermal microdialysis in all cases because there was insufficient equipment (probes and pumps) in order for this to be done synchronously. Microdialysis results were compared to results from standard laboratory analysis and to zone C results, taken at the

closest time to the drawn blood. Only patients recruited after S06 were included as the blood samples of patients prior to this had been discarded by the laboratory.

3.8 Protocol for Study 3: Large Burns

3.8.1 Aims

The aims of this study were as follows:

- To characterize the different zones of the burn wound biochemically.
- To assess whether wound progression could be predicted by the local biochemical changes within the first 36 hours following injury
- To compare the biochemical behaviour of large burn wounds with that of small burn wounds

3.8.2 Materials and Methods

3.8.2.1 Recruitment

Ethical approval was granted for this study. A participant information sheet and a consent form were prepared, similar to those for Study 2 (Appendix A). In addition, an assent form was prepared to seek the agreement of relatives of any patient who was unable to give consent due to the severity of their injuries. Informed consent was obtained from all conscious patients

prior to enrolment. Assent was gained from the next of kin of any patient who was unconscious, prior to enrolment. Potential participants were identified by the on-call burns doctor and the Researcher was contacted. The patient or relative, where appropriate, was approached as soon as possible after admission to the hospital.

Recruitment of ten patients was planned. Any patient admitted to the Burns Unit at Selly Oak Hospital with total burn greater than 30% total body surface area (TBSA) was considered for inclusion (See Table 5). Exclusion criteria are defined in Table 6.

Each patient fitting the inclusion criteria had the study explained, and was given a participant information sheet. Due to the nature of the study, it was necessary that the study must be started as soon as possible after admission. Consent, or assent, was sought immediately after the explanation was given. Where consent, or assent, was given, enrolment ensued.

3.8.2.2 Equipment set-up

Once initial clinical assessment of the burn was completed, and any necessary therapy commenced (e.g. analgesia, intravenous fluid therapy, debridement of blisters), the study was started. Details of the distribution and depth of the burns, was recorded for correlation with the results.

Three CMA60 probes were prepared as for study 2 (See 3.7.2.2)

3.8.2.3 Selection of Probe Location and Probe Insertion

Probe site selection and probe insertion was performed as for Study 2 (See 3.7.2.3).

3.8.2.4 Data collection

Microdialysate samples were collected every 30 minutes throughout the first 36 hours following injury. Patient activity, medical intervention and events such as theatre visits, and the addition of vasoconstrictors such as noradrenaline, were noted throughout the study period.

Medical notes were reviewed subsequent to patient discharge, and events in relation to surgical procedures, skin grafting and wound healing were noted.

3.8.2.5 End of Study

At the end of the 36 hour study period, the microdialysis probe was removed, and all other treatment for the burn continued as normal.

3.8.2.6 Analysis of samples

All samples were stored and analysed as for Study 2.

3.8.2.7 Statistical Analysis

SPSS Version 15.0 for Windows was used to analyse the results. Generalised Estimating Equations were used to detect differences between time blocks and between zones.

For the amino acid analysis, a 2-way ANOVA (GraphPad Prism software, version 5) was used. Variables were time post-burn and probe location.

4 RESULTS

Results of the metabolic parameters glucose, lactate, pyruvate and glycerol, and the standard urea are presented in sections 4.1-4.4 and the results of amino acid metabolism, with particular reference to nitric oxide metabolism, are presented in section 4.5.

4.1 Results of calibration experiments

4.1.1 Error margin of CMA600 analyser

Results of analysing the calibration fluid are detailed in Appendix B, Tables 6-9 (Columns C1 and C2). Below is the mean and percentage error (standard deviation divided by the mean) for each substance.

Glucose: 5.68 mmol/l (+/- 4%)

Glycerol: 487 µmol/l (+/- 6%)

Lactate: 2.47 mmol/l (+/- 10%)

Pyruvate: 241 µmol/l (+/- 5%)

4.1.2 Results of calibration experiments – Storage conditions

Raw data are presented in Appendix B, Tables A1-A5, Columns A, B, C.

4.1.2.1 Glucose

Glucose results are shown in Table 7 and Figure 12. Statistically significant results are recorded below the table.

Table 7 to show mean and standard deviation of glucose results from microdialysis samples stored at different temperatures over 3 successive days

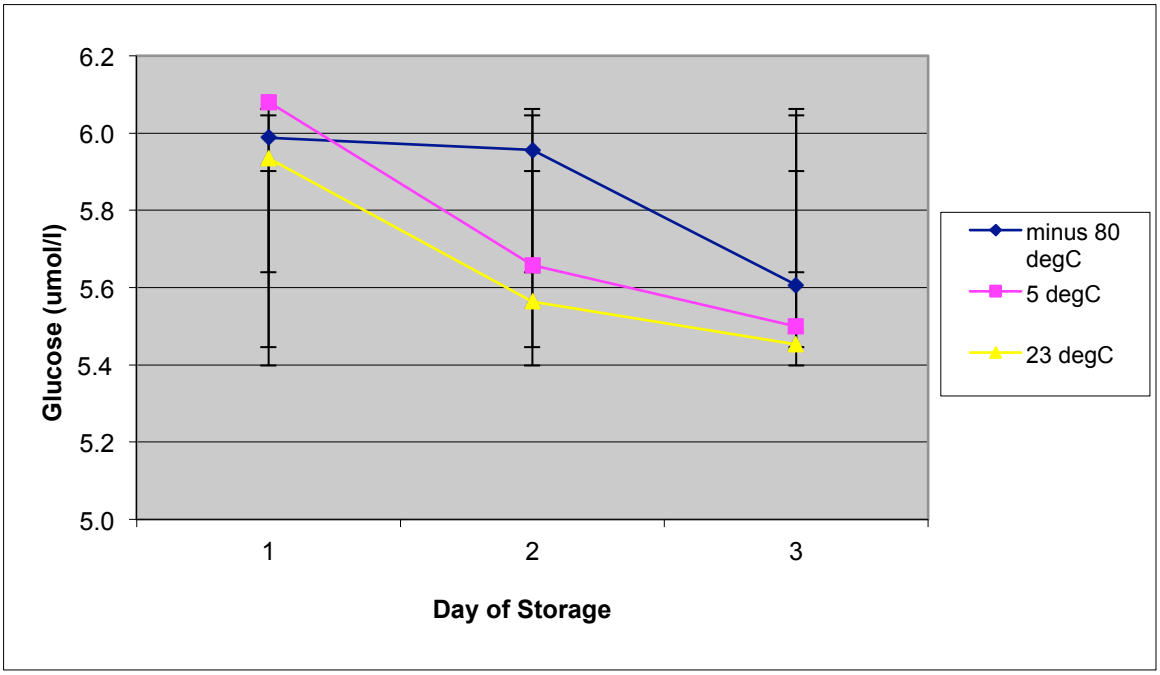
	Mean Glucose Concentration (SD) mmol/l		
	Freezer -80^{o}C	Fridge 2-5^{o}C	Room 22^{o}C
Day 1	5.99 (0.14)	6.08 (0.51)	5.93 (0.08)
Day 2	5.96 (0.17)[a]	5.66 (0.29)[b]	5.56 (0.03)
Day 3	5.61 (0.05)	5.50 (0.05)[c]	5.45 (0.08)[d]

a: On Day 2, samples stored in the freezer were more concentrated than those stored at room temperature (P=0.046)

b,c: For samples stored in the fridge, there was a significant decrease in concentration between Day 1 and Day 2 (P=0.033), and between Day 1 and Day 3 (P=0.005).

d: Samples stored at room temperature, became less concentrated with time, and this reached significance by Day 3 when compared to Day 1 (P=0.017).

Figure 12: to show mean and standard deviation of glucose results from microdialysis samples stored at different temperatures over 3 successive days



4.1.2.2 Lactate

Lactate results are shown in Table 8 and Figure 13. Statistically significant results are recorded below the table.

Table 8 to show mean and standard deviation of lactate results from microdialysis samples stored at different temperatures over 3 successive days

	Mean Lactate Concentration (SD) mmol/l		
	Freezer -80^{o}C	Fridge 2-5^{o}C	Room 22^{o}C
Day 1	2.72 (0.08)	2.72 (0.14)	2.73 (0.09)
Day 2	2.56 (0.04)[b]	2.60 (0.09)	2.55 (0.05)[e]
Day 3	2.72 (0.03)[a,c]	2.53 (0.02)[d]	2.53 (0.04)[f]

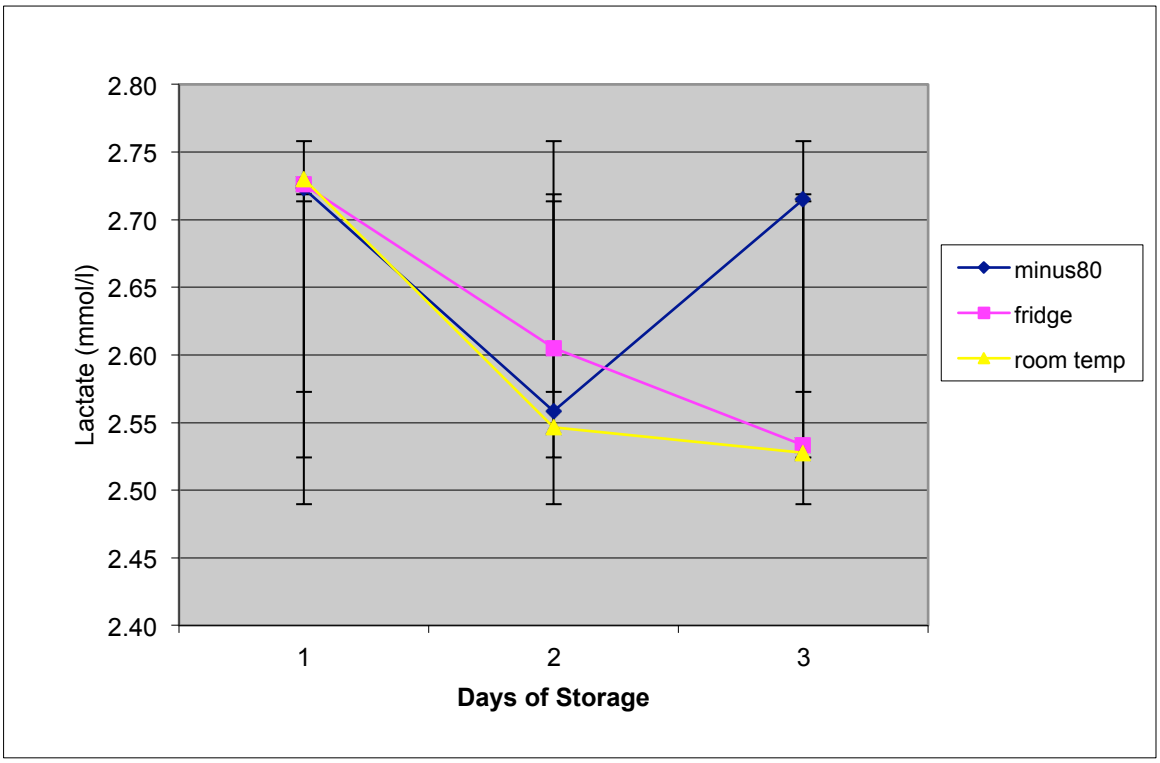
a: On Day 3, freezer-stored samples were more concentrated than room temperature (P=0.006), and fridge-stored samples (P=0.008).

b,c: For samples stored in the freezer, there was a significant drop in concentration between Day 1 and Day 2 (P=0.014), and an increase between Day 2 and Day 3 (P=0.018).

d: For samples stored in the fridge, there was a significant fall in concentration between Day 1 and Day 3 (P=0.005).

e,f: For samples stored at room temperature, there was a significant fall in concentration between Day 1 and Day 2 (P=0.007), and samples on Day 3 were also significantly less concentrated than Day 1 (P=0.004).

Figure 13 to show mean and standard deviation of lactate results from microdialysis samples stored at different temperatures over 3 successive days



4.1.2.3 Pyruvate

Pyruvate results are shown in Table 9 and Figure 14 below. Statistically significant results are recorded below the table.

Table 9 to show mean and standard deviation of pyruvate results from microdialysis samples stored at different temperatures over 3 successive days

	Mean Pyruvate Concentration (SD) mmol/l		
	Freezer - 80°C	Fridge 2-5°C	Room 22°C
Day 1	233 (8)	233 (4)	246 (5)[a]
Day 2	247 (8)[d]	244 (11)	242 (4)[b]
Day 3	225 (4)[e]	226 (9)[f]	221 (11)[c]

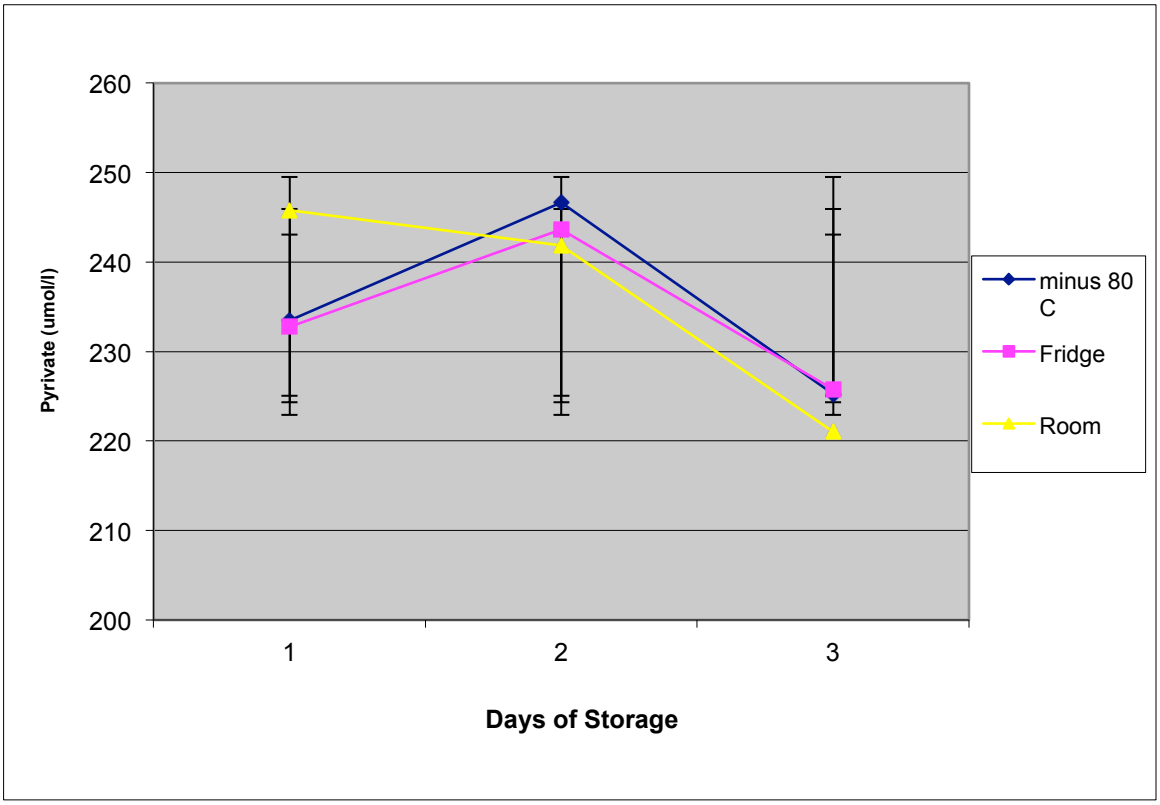
a: On Day 1, room temperature-stored samples were more concentrated than samples stored in the fridge (P=0.048).

b,c: For samples stored at room temperature, there was a fall in concentration between Day 2 and Day 3 (P=0.003), and between Day 1 and Day 3 (P=0.001).

d,e: For samples stored in the freezer, there was a rise in concentration between Day 1 and Day 2 (P=0.045), and a fall between Day 2 and Day 3 (P=0.003).

f: For samples stored in the fridge, there was a significant fall in concentration between Day 2 and Day 3 (P=0.009).

Figure 14 to show mean and standard deviation of pyruvate results from microdialysis samples stored at different temperatures over 3 successive days



4.1.2.4 Glycerol

Glycerol results are shown in Table 10 and Figure 15. Statistically significant results are recorded below the table.

Table 10 to show mean and standard deviation of glycerol results from microdialysis samples stored at different temperatures over 3 successive days

	Mean Glycerol Concentration (SD) mmol/l		
	Freezer -80^{o}C	Fridge 2-5^{o}C	Room 22^{o}C
Day 1	464 (12)	467 (4)	480 (3)[a,b]
Day 2	486 (8)[c,d,f]	463 (6)	464 (5)[j]
Day 3	459 (3)[e,g]	451 (8)[h,i]	445 (6)[k,l]

a,b: On Day 1, the room temperature stored sample group were more concentrated than the fridge-stored (P=0.029) and freezer-stored sample groups (P=0.008).

c,d: On Day 2, freezer-stored samples were more concentrated than room temperature (P=0.001), and fridge-stored samples (P<0.001).

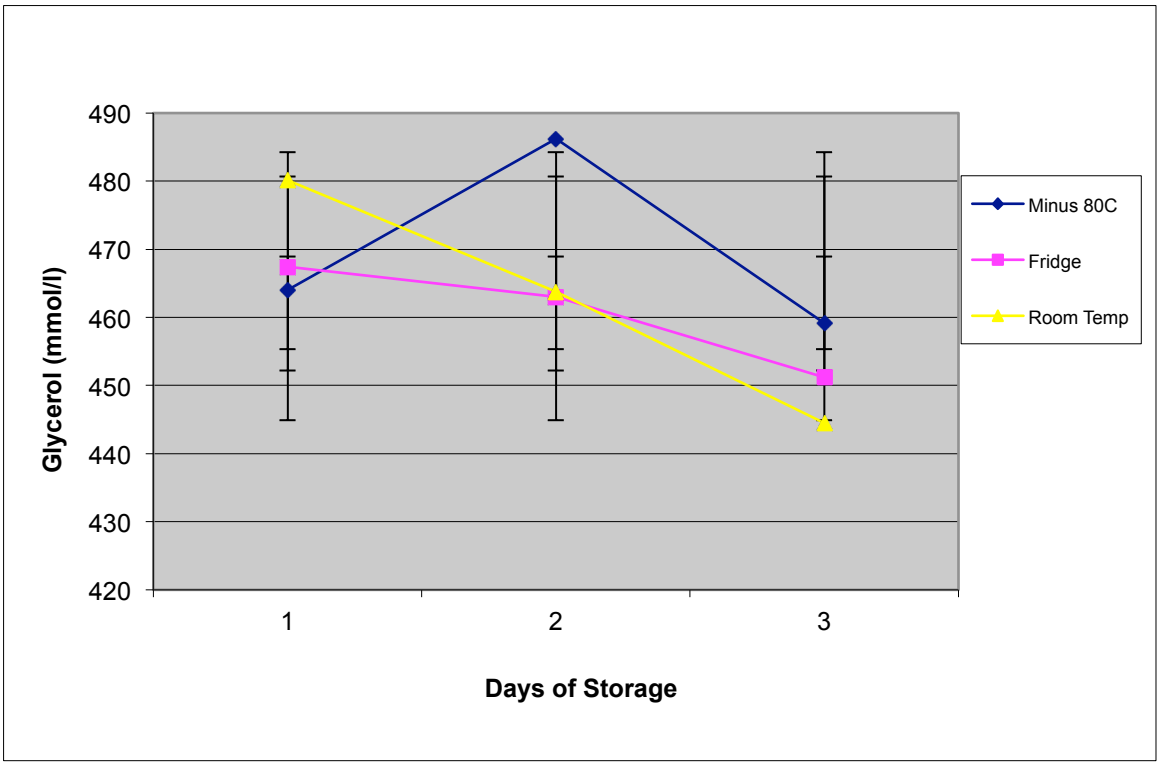
e: On Day 3, freezer-stored samples were more concentrated than room temperature stored ones (P=0.014).

f,g: For samples stored in the freezer, there was an increase between Day 1 and Day 2 (P=0.001), and a fall between Day 2 and Day 3 (P<0.001).

h,i: For samples stored in the fridge, there was a fall in concentration between Day 2 and Day 3 (P=0.041), and between Day 1 and Day 3 (P=0.008)

j,k,l: For samples stored at room temperature there was a fall in concentration between Day 1 and Day 2 (P=0.007), between Day 2 and Day 3 (P=0.002), and between Day 1 and Day 3 (P<0.001).

Figure 15 to show mean and standard deviation of glycerol results from microdialysis samples stored at different temperatures over 3 successive days



4.1.2.5 Urea

Urea results are shown in Table 11 and Figure 16. Statistically significant results are recorded below the table.

Table 11 to show mean and standard deviation of urea results from microdialysis samples stored at different temperatures over 3 successive days

	Mean Urea Concentration (SD) mmol/l		
	Freezer -80^{o}C	Fridge 2-5^{o}C	Room 22^{o}C
Day 1	13.9 (0.1)	14.1 (0.2)	14.0 (0.2)
Day 2	14.0 (0.1)	13.7 (0.1)[e]	13.7 (0.1)[g]
Day 3	14.4 (0.3)[a,b,c,d]	13.8 (0.1)[f]	13.7 (0.1)[h]

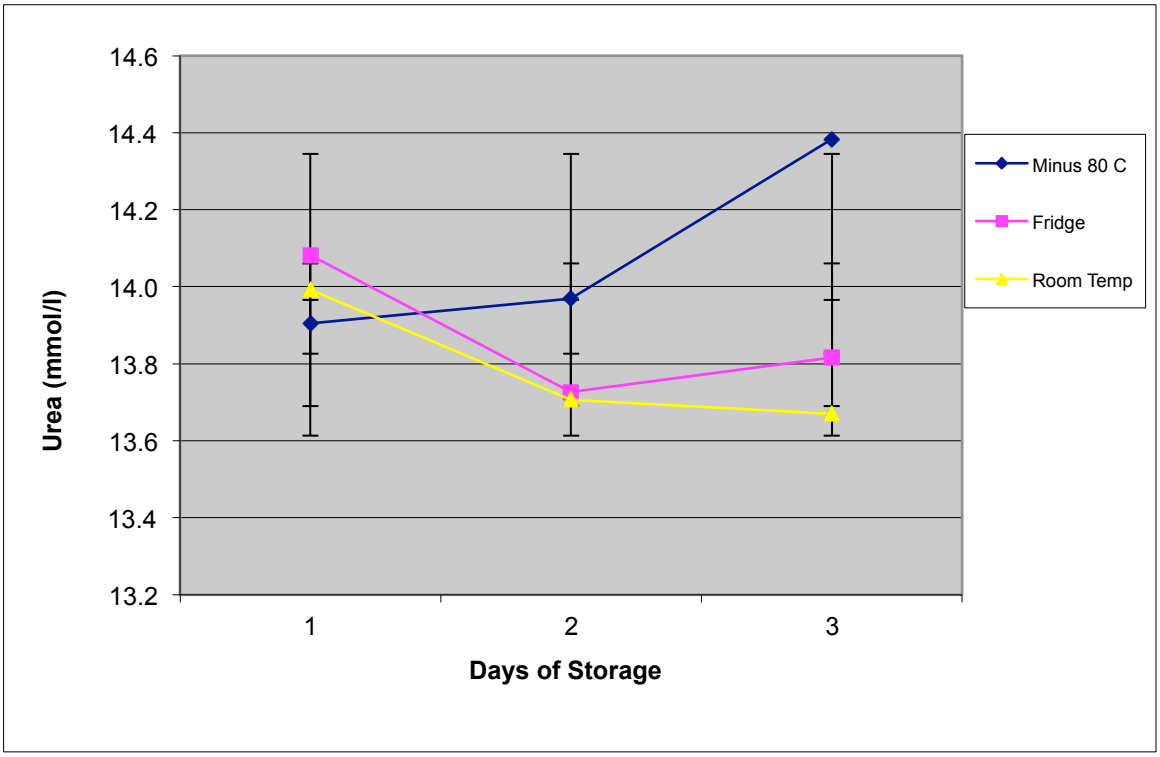
a,b: On Day 3, Freezer-stored samples were more concentrated than Room- ($P<0.001$) and Fridge-stored samples ($P<0.001$).

c.d: For samples stored at -80 ^{o}C, there was a significant rise in concentration between Day 1 and Day 3 ($P=0.001$), and between Day 2 and Day 3 ($P=0.004$).

e,f: For samples stored in the fridge, there was a fall in concentration between Day 1 and Day 2 ($P=0.011$), and between Day 1 and Day 3 ($P=0.049$).

g,h: For samples stored at room temperature, there was a fall in concentration between Day 1 and Day 2 ($P=0.037$), and between Day 1 and Day 3 ($P=0.020$).

Figure 16 to show mean and standard deviation for urea results from microdialysis samples stored at different temperatures over 3 successive days



4.1.3 Results of calibration experiments – Volume studies

Raw data are presented in Appendix B, Tables A1-5, Columns A,D,E. The following results were recorded:

4.1.3.1 Glucose

Results are shown in Table 12 and Figure 17. Statistically significant results are recorded below the table.

Table 12 to show mean and standard deviation of glucose results from microdialysis samples of different volumes stored in the freezer over 3 successive days

	Glucose Mean (SD) mmol/l		
	High Volume	Medium Volume	Low Volume
Day 1	5.97 (0.19)	5.99 (0.14)	5.94 (0.14)
Day 2	5.54 (0.12)[a,d]	5.96 (0.17)	5.80 (0.12)
Day 3	5.89 (0.14)[e]	5.61 (0.05)[b,c,f,g]	6.09 (0.23)[h]

a: On Day 2, High volume samples were less concentrated than Medium volume samples (P=0.006).

b,c: On Day 3, Medium volume samples were less concentrated than both High (P=0.047), and Low volume samples (P=0.002).

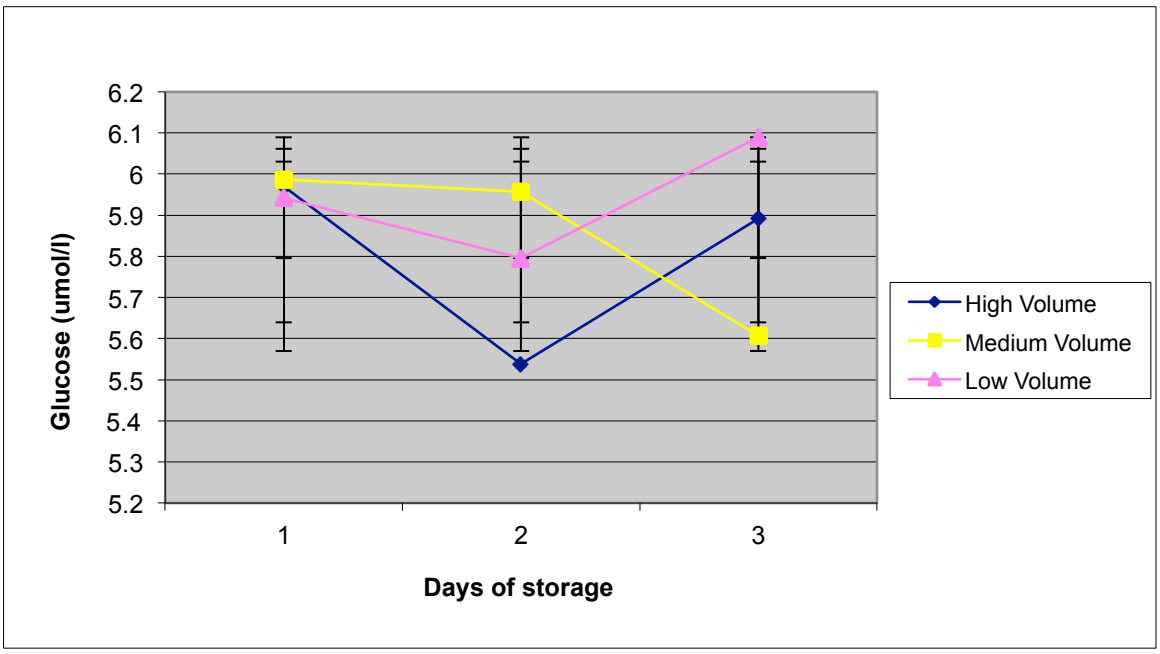
d,e: For High volume samples, the concentration dropped between Day 1 and Day 2 (P=0.005), but increased between Day 2 and Day 3 (P=0.017).

f,g: For Medium Volume samples, concentration dropped significantly between Day 2 and Day 3 (P=0.017), and it was significantly lower on Day 3 than Day 1 (P=0.011).

h: For Low volume samples, there was a significance rise between Day 2 and Day 3 (P=0.042).

The mean glucose value for all glucose samples, in volume and storage experiments, was 5.798mM. Standard deviation was 0.273mM. All except one glucose value were within 2 standard deviations of the mean.

Figure 17 to show mean and standard deviation of glucose results of microdialysis samples of different volumes, stored in the freezer over 3 successive days



Lactate

Results are shown in Table 13 and Figure 18. Statistically significant results are recorded below the table.

Table 13 to show mean and standard deviation of lactate results from microdialysis samples of different volumes stored in the freezer over 3 successive days

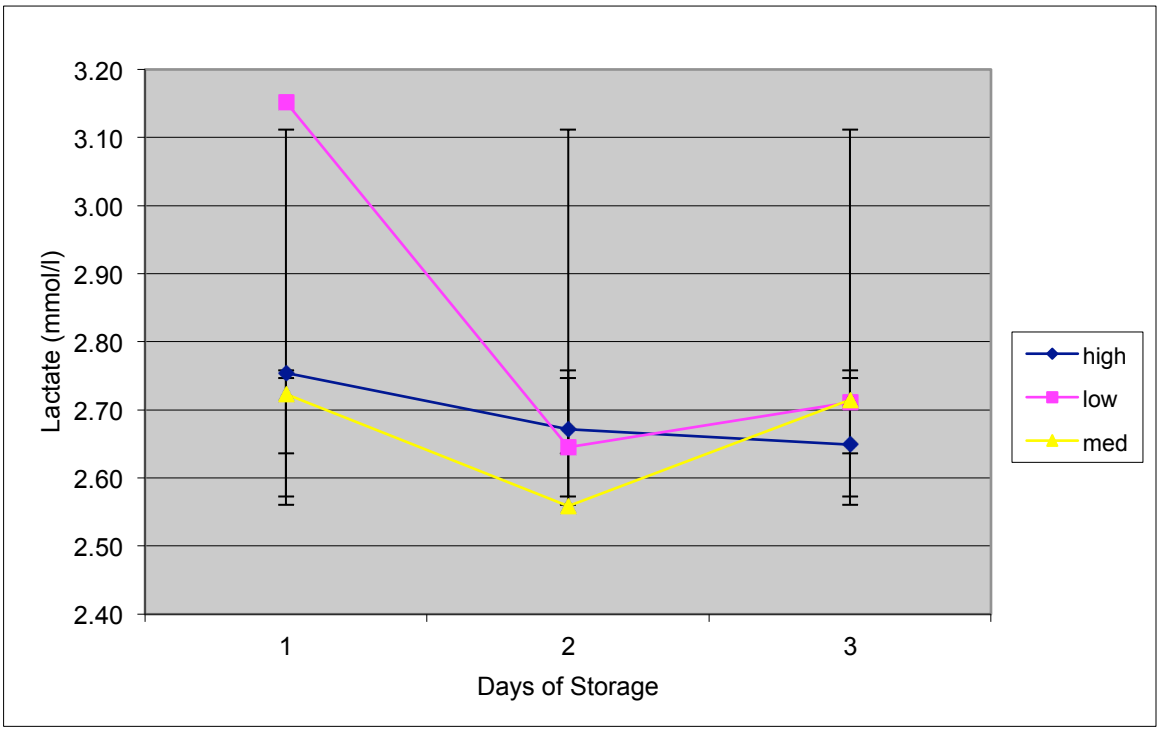
	Lactate Mean (SD) mmol/l		
	High Volume	Medium Volume	Low Volume
Day 1	2.75 (0.04)	2.72 (0.08)	3.15 (0.64)[a,b]
Day 2	2.67 (0.08)	2.56 (0.04)	2.64 (0.08)[c]
Day 3	2.65 (0.02)	2.72 (0.03)	2.71 (0.11)[d]

a,b: On Day 1, Low volume samples were more concentrated than High (P=0.041), and Medium volume samples (P=0.029). N.B. This mean value is skewed by one of the triplicate readings for low volume on day 1, which outlies the other results.

c,d: For Low volume samples, there was a significant fall in concentration between Day 1 and Day 2 (P=0.012), and between Day 1 and Day 3 (P=0.026).

The mean value for all lactate samples was 2.683mM. Standard deviation was 0.210mM. All except one lactate value were within 2 standard deviations of the mean.

Figure 18 to show mean and standard deviation of lactate results of microdialysis samples of different volumes, stored in the freezer over 3 successive days



4.1.3.2 Pyruvate

Results are shown in Table 14 and Figure 19. Statistically significant results are recorded below the table.

Table 14 to show mean and standard deviation of pyruvate results from microdialysis samples of different volumes stored in the freezer over 3 successive days

	Pyruvate Mean (SD) µmol/l		
	High Volume	Medium Volume	Low Volume
Day 1	233 (2)	233 (8)	248 (4)[a,b]
Day 2	250 (8)[e]	247 (8)[f]	250 (3)
Day 3	241 (6)	225 (4)[c,d,g]	245 (6)

a,b: On Day 1, Low volume samples were more concentrated than High (P=0.004), and Medium volume samples (P=0.005).

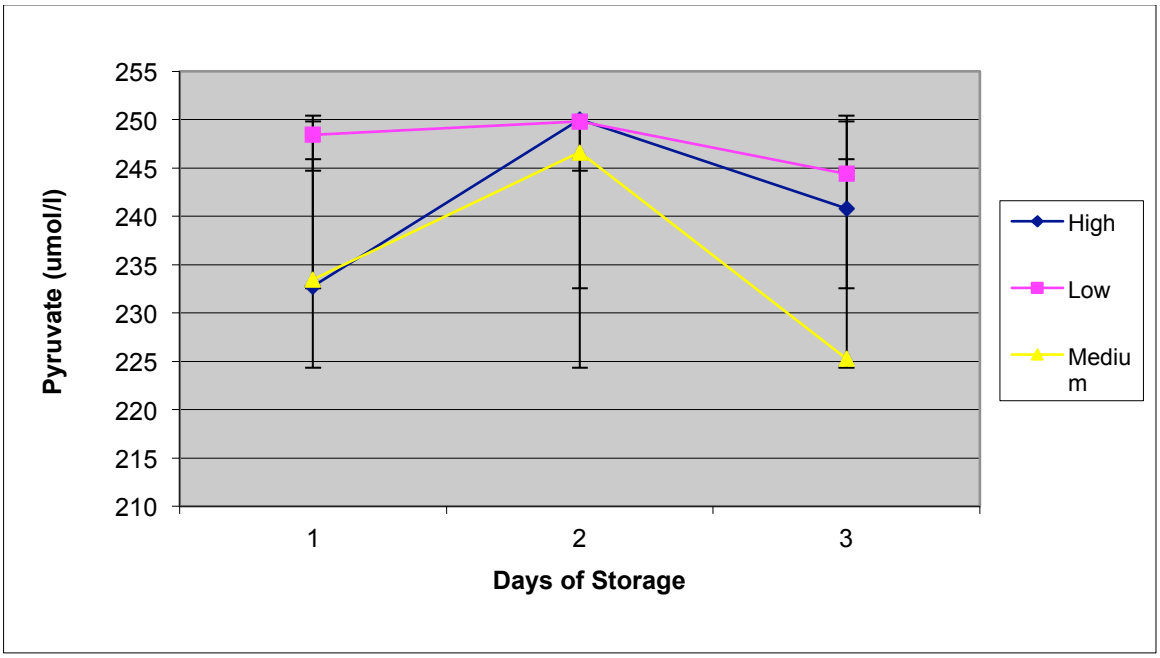
c,d: On Day 3, Medium volume samples were significantly lower than High (P=0.004), and Low volume samples (P=0.001).

e: For High volume samples, there was a significant rise in concentration between Day 1 and Day 2 (P=0.002).

f,g: For Medium Volume samples, there was a rise between Day 1 and Day 2 (P=0.012), and a fall between Day 2 and Day 3 (P<0.001).

The mean value for all pyruvate samples was 238.83µM. Standard deviation was 10.87µM. All except one pyruvate value were within 2 standard deviations of the mean.

Figure 19 to show mean and standard deviation of pyruvate results of microdialysis samples of different volumes, stored in the freezer over 3 successive days



4.1.3.3 Glycerol

Results are shown in Table 15 and Figure 20. Statistically significant results are recorded below the table.

Table 15 to show mean and standard deviation of glycerol results from microdialysis samples of different volumes stored in the freezer over 3 successive days

	Glycerol Mean (SD) µmol/l		
	High Volume	Medium Volume	Low Volume
Day 1	481 (11)	464 (12)[a,b]	491 (6)
Day 2	483 (12)	486 (8)[e]	489 (18)
Day 3	474 (6)	459 (3)[f]	510 (6)[c.d,g,h]

a,b: On Day 1, Medium volume samples were less concentrated than High (P=0.048) and Low volume ones (P=0.003).

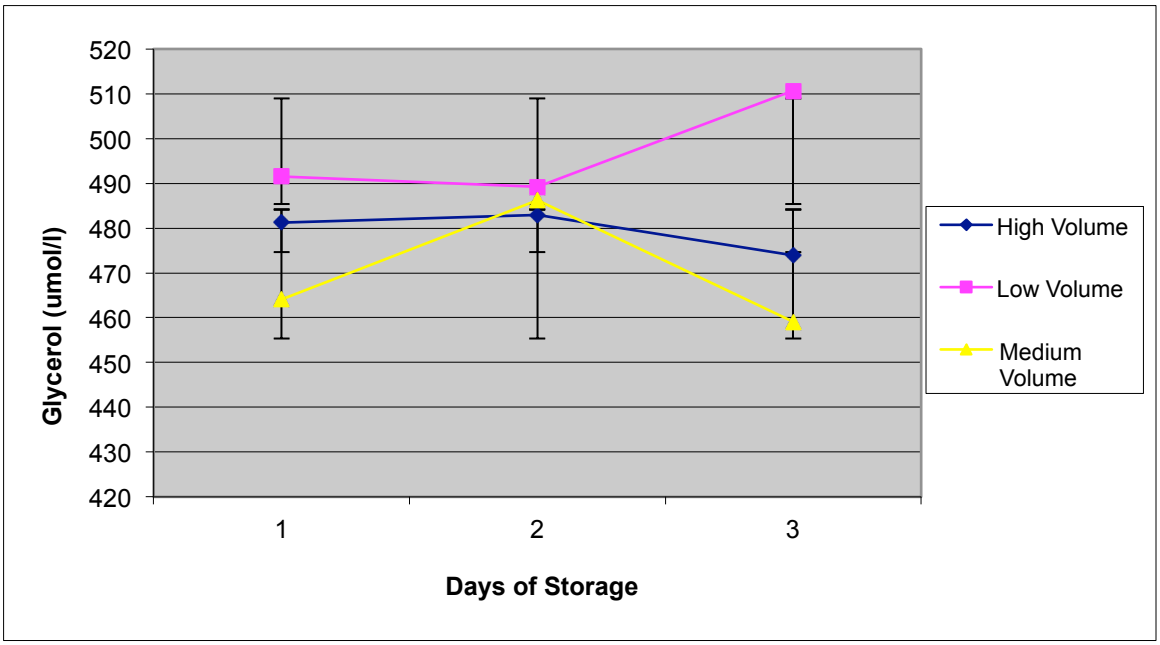
c.d: On Day 3, Low volume samples were more concentrated than High (P<0.001) and Medium volume samples (P<0.001).

e,f: For Medium volume samples, there was a rise in concentration between Day 1 and Day 2 (P= 0.014), and a fall between Day 2 and Day 3 (P=0.004).

g,h: For Low volume samples, there was a rise in concentration on Day 3 when compared with Day 1 (P=0.030) and Day 2 (P=0.017).

The mean value for all glycerol samples was 473.92µM. Standard deviation was 18.29µM. All except one glycerol values were within 2 standard deviations of the mean.

Figure 20 to show mean and standard deviation of glycerol results of microdialysis samples of different volumes, stored in the freezer over 3 successive days



4.1.3.4 Urea

Results are shown in Table 16 and Figure 21. Statistically significant results are recorded below the table.

Table 16 to show mean and standard deviation of urea results from microdialysis samples of different volumes stored in the freezer over 3 successive days

	Urea Mean (SD) mmol/l		
	High Volume	Medium Volume	Low Volume
Day 1	13.4 (0.5)[a]	13.9 (0.1)	14.3 (0.2)
Day 2	14.1 (0.1)[f]	14.0 (0.1)	14.7 (0.3)[b,c]
Day 3	13.1 (0.7)[d,e,g]	14.4 (0.3)	14.4 (0.2)

a: On Day 1, there was a significant difference between High and Low volume samples (P=0.002).

b,c: On Day 2, Low volume samples were more concentrated than both High (P=0.048), and Medium volume samples (P=0.016).

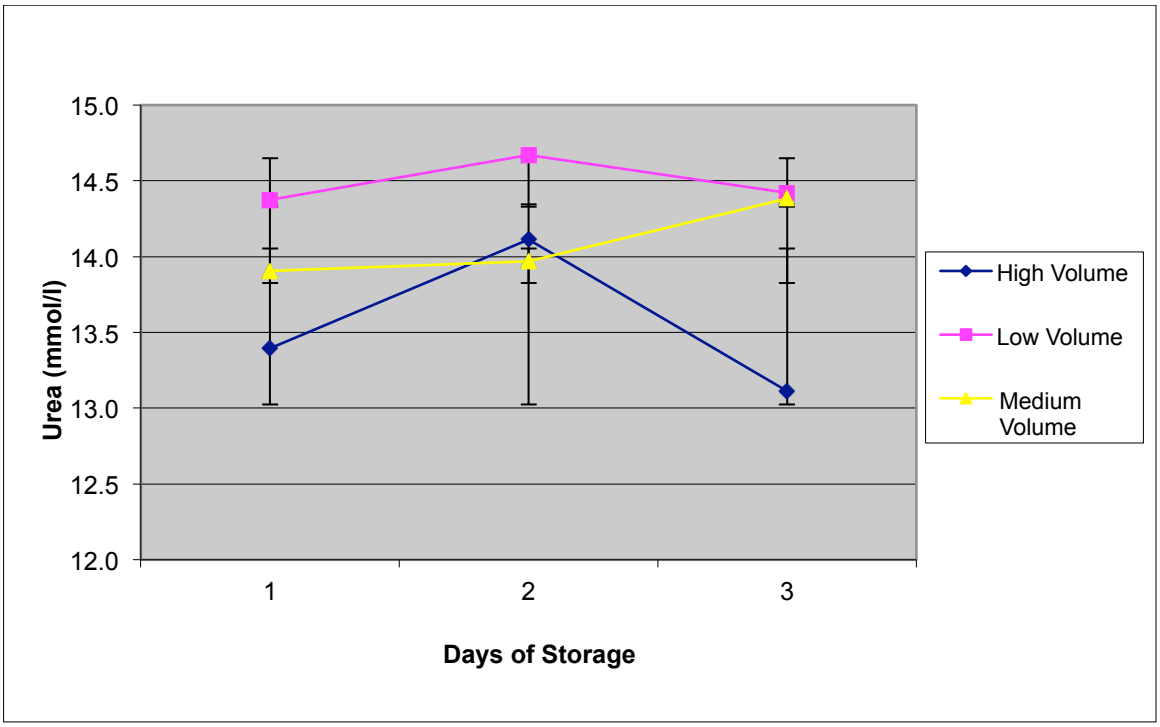
d,e: On Day 3, High volume samples were less concentrated than Low (P<0.001), and Medium volume samples (P<0.001).

f,g: For High volume samples, there was a rise in concentration between Day 1 and Day 2 (P=0.014), and a fall between Day 2 and Day 3 (P=0.001).

The mean value for all urea samples was 13.96mM. Standard deviation was 0.46mM.

All except four urea values were within 2 standard deviations of the mean.

Figure 21 to show mean and standard deviation of urea results of microdialysis samples of different volumes, stored in the freezer over 3 successive days



4.2 Results of Clinical Study 1 – Normal Volunteers

Eleven volunteers were recruited to undergo microdialysis of normal skin, to give a baseline for the burns studies. Volunteer characteristics are detailed in Table 17. All volunteers were male. Due to technical problems, all collection vials for NV01 were empty. Results for depth of probe insertion, and temperature readings were retained as the protocol was followed in full.

Table 17: Characteristics of volunteers recruited to Study 1

Patient number	Age	Ethnic Origin	Comorbidities/ Medications
NV01	37	Chinese	None
NV02	26	White British	None
NV03	33	White British	Mild asthma
NV04	28	Asian	Gilbert's syndrome
NV05	26	White British	None
NV06	40	White British	Pain/ Gabapentin 200mg bd
NV07	30	White British	Asthma/ Becotide 100mcg bd
NV08	41	White British	None
NV09	38	White British	None
NV10	41	White British	None
NV11	30	White British	Psoriasis

4.2.1 Ambient Temperature

Air temperature readings are shown in Table 18. Mean (SD) temperatures were 23.8 (0.89)^{o}C for Study Period 1, 33.3 (2.35)^{o}C for Study Period 2 and 25.3 (1.84)^{o}C for Study Period 3. There was no significant difference between Study periods 1 and 3, and a significant difference when comparing study period 1 with study period 2 ($P<0.01$), and comparing study period 2 with study period 3 ($P<0.01$).

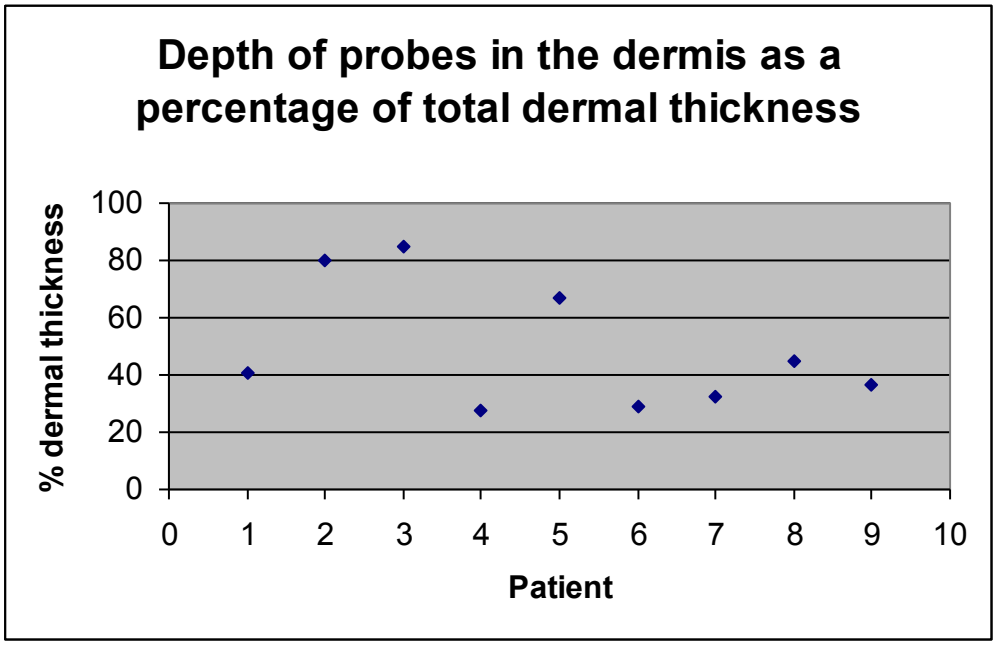
Table 18 to show air temperature during each study period, for each volunteer

Volunteer number	Study Period 1 Temperature, ^{o}C	Study Period 2 Temperature, ^{o}C	Study Period 3 Temperature, ^{o}C
NV01	22.8	37.1	25.4
NV02	23.3	31.6	23.7
NV03	22.6	35.2	26.3
NV04	23.5	31.7	23.1
NV05	24.5	31.6	24.8
NV06	24.7	32.8	25.4
NV07	25.5	36.0	28.5
NV08	23.5	32.1	
NV09	23.0	33.1	24.2
NV10	24.0	31.9	27.0
NV11	23.7	33.0	24.2

4.2.2 Probe Depth

Ultrasound measurements were collected for 10 out of 11 probes. The ultrasound machine was not available for NV10. In one patient, only the depth of the probe was measured, and not the thickness of the skin. As a result, this patient is not represented on the graph, however it had been noted that the probe was within the dermis throughout its length. In all cases the probe was sited within the dermis. Probe depths are represented in **Error! Reference source not found.**.

Figure 22: Scatter plot to show depth of microdialysis probe in the dermis as a percentage of total dermal thickness, for each patient



4.2.3 In-vitro probe calibration

Raw data calibration values for each probe are shown in Appendix B, Tables A6-A9. Recovery for each probe and for each substance was calculated as a percentage of the calibration solution value. The mean (SD) recovery was 99.5 (17.1) % for glucose; 107.3 (13.1) % for pyruvate; 108.2 (15.2) % for lactate; 113.7 (23.1) % for glycerol. There was no significant difference between recovery at the start and end of the experiment ($P>0.05$ for all analytes; Wilcoxon signed rank test).

4.2.4 Equilibration Period

Raw data are shown in Appendix B, Tables A10-A14.

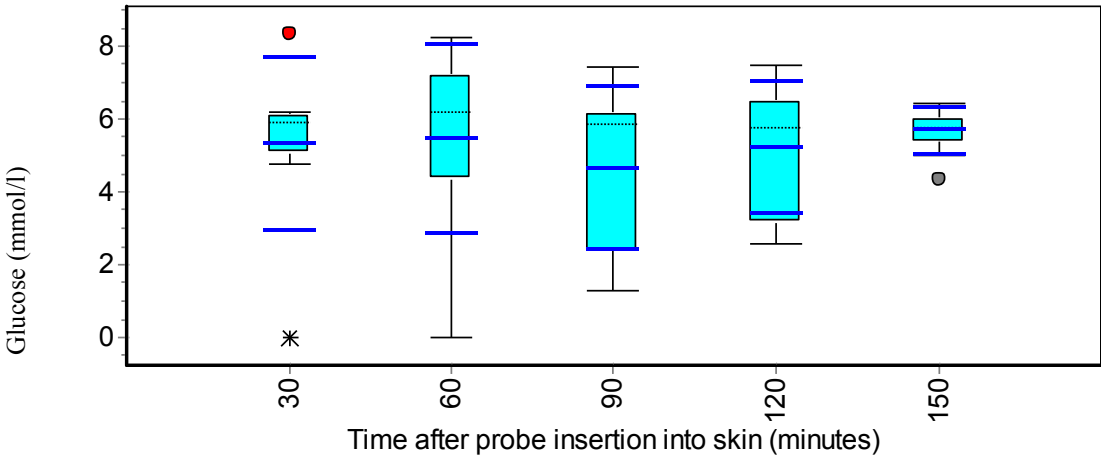
Mean and standard deviations are shown for glucose, lactate, pyruvate, lactate/pyruvate ratio (LPR) and glycerol in Figure 23,

Figure 24, Figure 25,

Figure 26, & Figure 27. There was no significant difference for lactate, pyruvate or glucose individually when comparing any of these time points with any other. For the calculated LPR, there was a significant difference between the 1st ($P<0.01$)

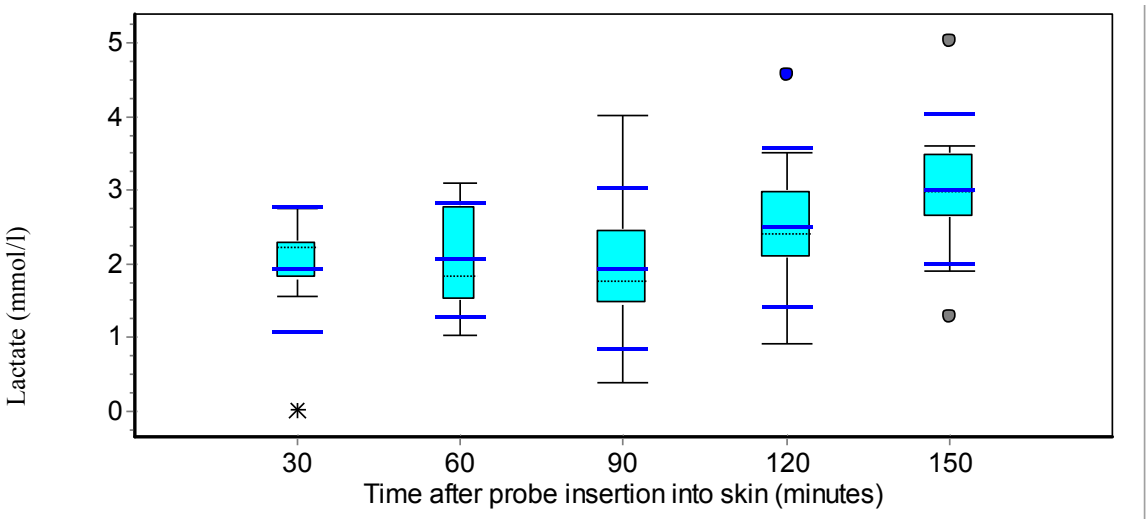
and 2nd ($P<0.05$) samples within the equilibration period when compared with the 5th sample. For Glycerol, there was a significant difference between the 1st sample and all other samples ($P<0.01$). This shows that insertion of the probe has no significant effect on results of glucose, lactate and pyruvate individually. There is a significant effect of probe insertion on LPR, lasting 1 hour, and on glycerol, lasting 30 minutes.

Figure 23: Boxplot to show dermal glucose (mmol/l) during each half hour period after probe insertion in healthy volunteers



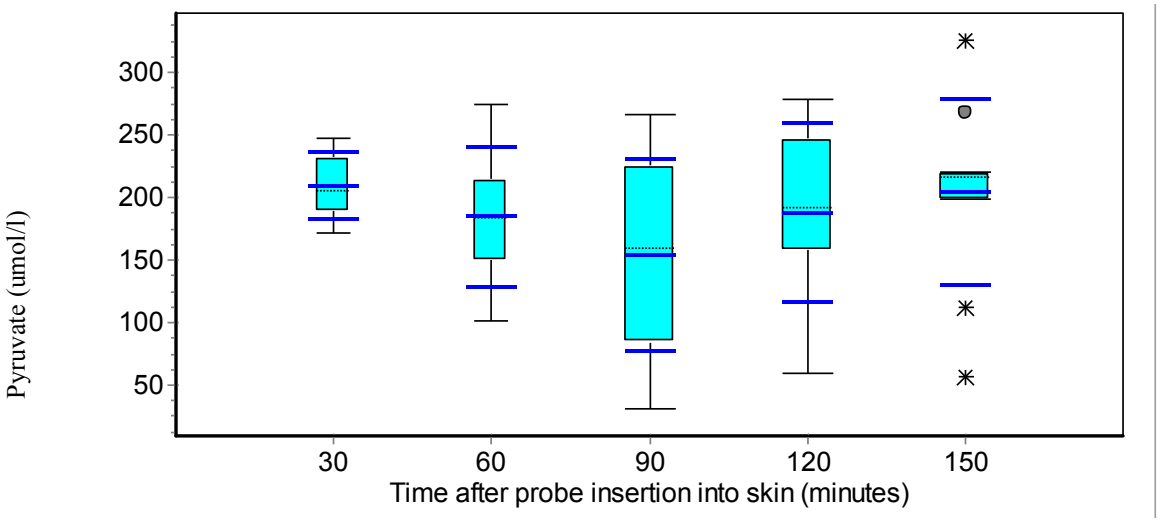
Median (dotted line), Interquartile range (boxplot), mean and standard deviation (blue bars), near outliers (o), far outliers (*)

Figure 24: Boxplot to show dermal lactate (mmol/l) during each half hour period after probe insertion in healthy volunteers



Median (dotted line), Interquartile range (boxplot), mean and standard deviation (blue bars), near outliers (o), far outliers (*)

Figure 25: Boxplot to show dermal pyruvate (µmol/l) during each half hour period after probe insertion in healthy volunteers



Median (dotted line), Interquartile range (boxplot), mean and standard deviation (blue bars), near outliers (o), far outliers (*)

Figure 26: Boxplot to show dermal LPR during each half hour period after probe insertion in healthy volunteers. 30 and 60 minute values are significantly lower then at 150 minutes (P<0.01, P<0.05 respectively)

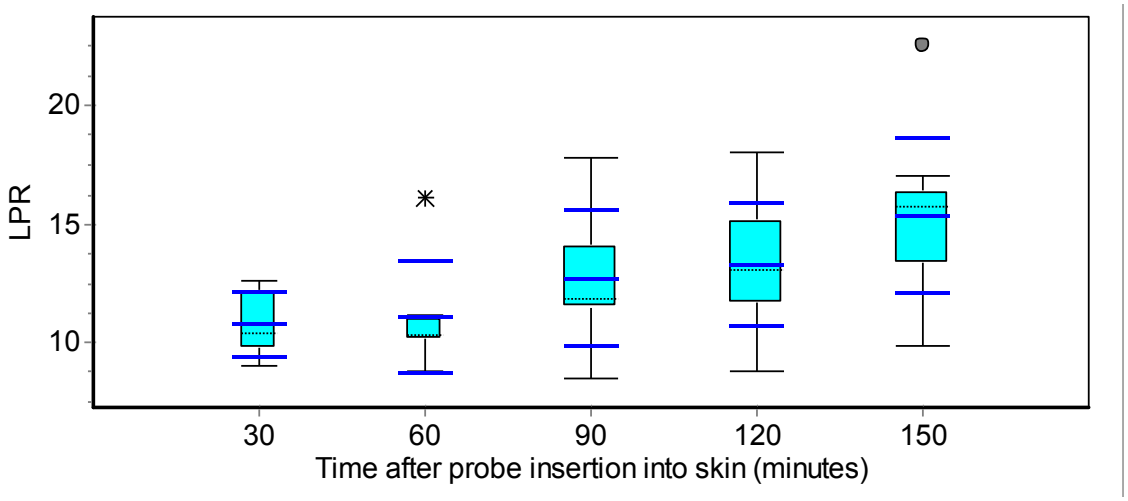
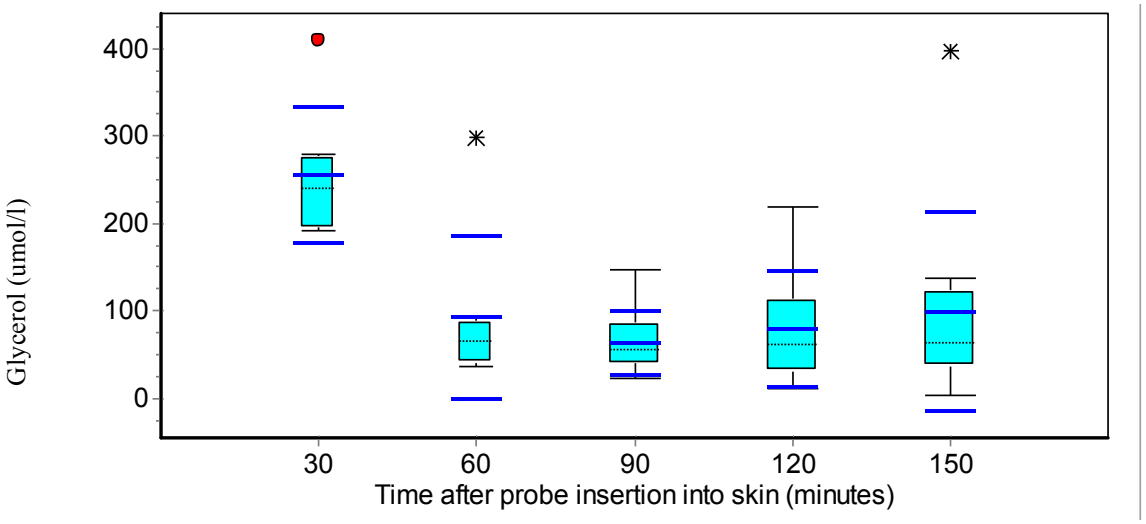


Figure 27: Boxplot to show dermal glycerol (µmol/l) during each half hour period after probe insertion into healthy volunteers. Values at 30 minutes are significantly higher than at the subsequent time points (P<0.01).



Median (dotted line), Interquartile range (boxplot), mean and standard deviation (blue bars), near outliers (o), far outliers (*)

4.2.5 The Effect of Raised Temperature

Raw data are detailed in Appendix B, Tables A15-A19. Comparison of the means for the three groups revealed no significant difference for any of the substances tested (Glucose P=0.853, Lactate P=0.7281, Pyruvate P=0.8914, LPR P=0.5045, Glycerol P=0.6889), (Figure 28,

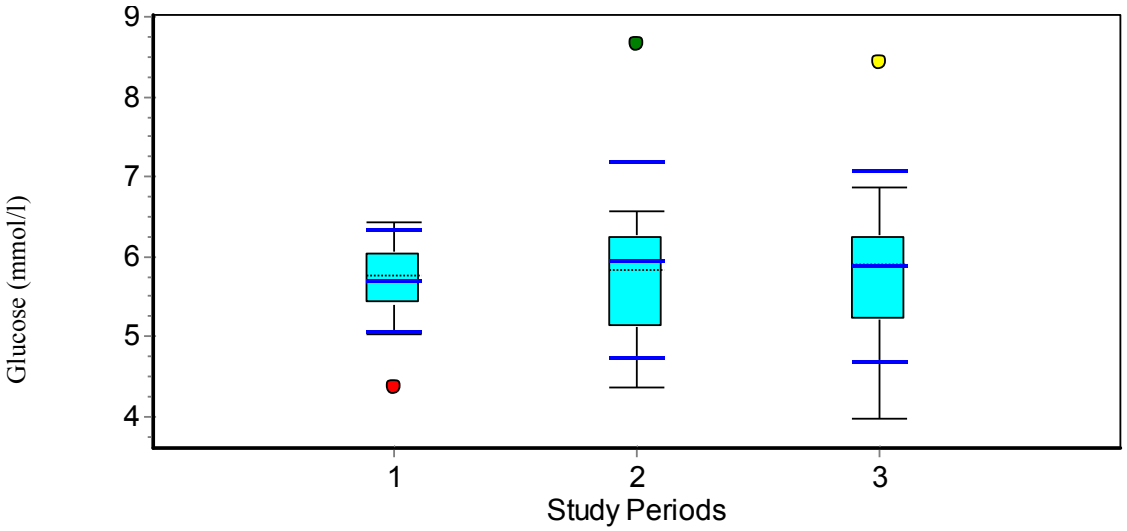
Figure 29,

Figure 30,

Figure 31,

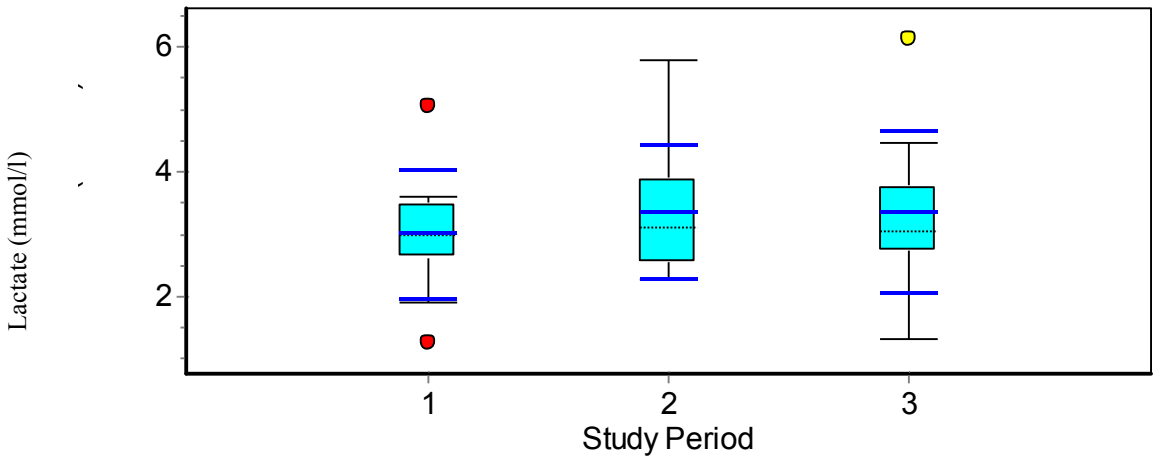
Figure 32). There was no evidence of any effect of raised ambient temperatures of up to 33^{o}C on glucose, lactate, pyruvate, glycerol or LPR microdialysis readings.

Figure 28: Boxplot to show dermal glucose in healthy volunteers under differing ambient temperatures (Study period 1=23°C, Study period 2=33°C, Study period 3=25°C)



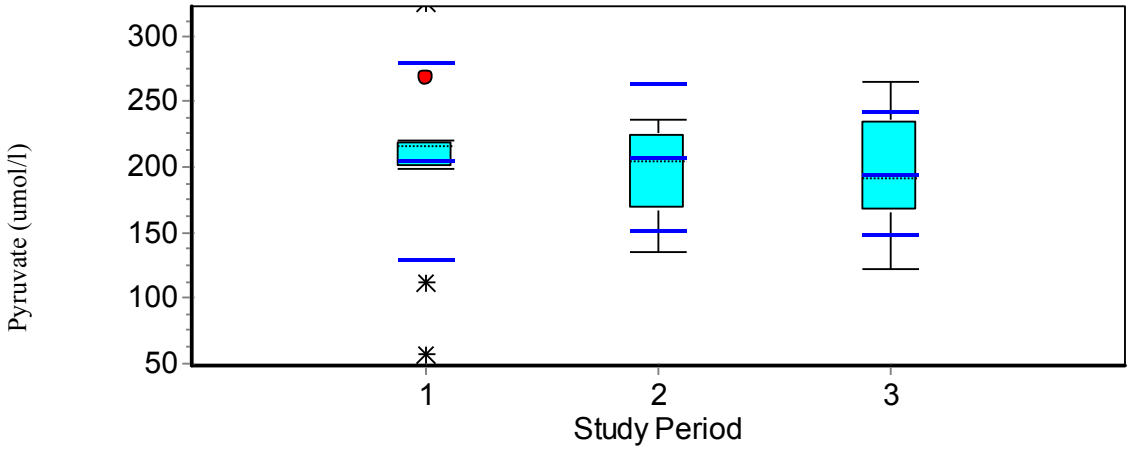
Median (dotted line), Interquartile range (boxplot), mean and standard deviation (blue bars), near outliers (o), far outliers (*)

Figure 29: Boxplot to show dermal lactate in healthy volunteers under differing ambient temperatures. (Study period 1=23°C, Study period 2=33°C, Study period 3=25°C)



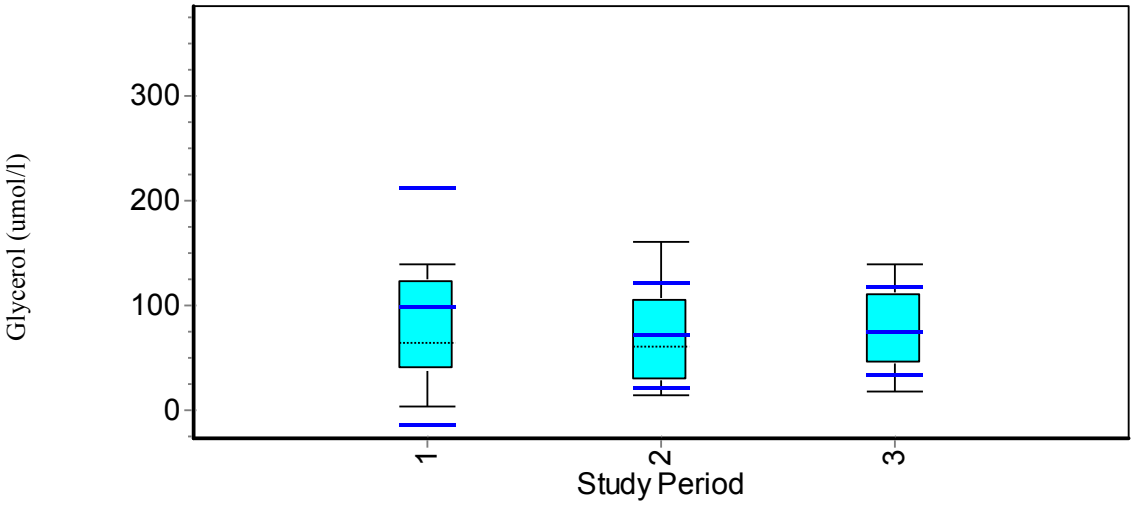
Median (dotted line), Interquartile range (boxplot), mean and standard deviation (blue bars), near outliers (o), far outliers (*)

Figure 30: Boxplot to show dermal pyruvate in healthy volunteers under differing ambient temperatures. (Study period 1=23°C, Study period 2=33°C, Study period 3=25°C)



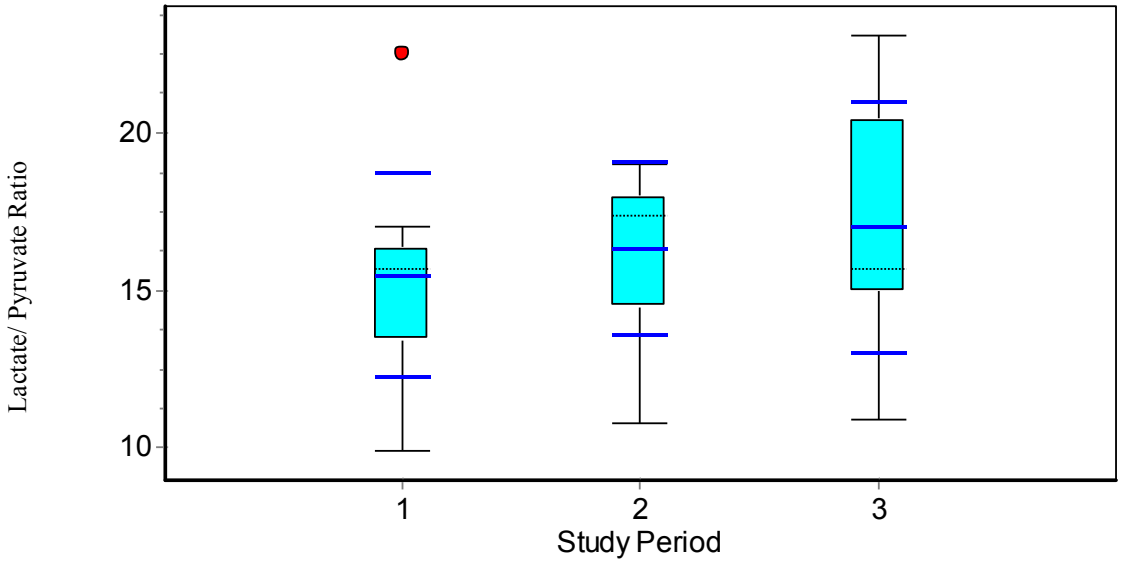
Median (dotted line), Interquartile range (boxplot), mean and standard deviation (blue bars), near outliers (o), far outliers (*)

F
igure 31: Boxplot to show dermal glycerol in healthy volunteers under differing ambient temperatures. (Study period 1=23°C, Study period 2=33°C, Study period 3=25°C)



Median (dotted line), Interquartile range (boxplot), mean and standard deviation (blue bars), near outliers (o), far outliers (*)

Figure 32 Boxplot to show dermal LPR in healthy volunteers at differing ambient temperatures. (Studyperiod 1=23°C, Study period 2=33°C, Study period 3=25°C)



Median (dotted line), Interquartile range (boxplot), mean and standard deviation (blue bars), near outliers (o), far outliers (*)

4.2.6 Normal Ranges

Normal range for each substance was calculated as the 95% Confidence Interval of the mean for all the results taken during the study period. These were as follows:

Glucose: 3.81-7.75 mmol/l

Lactate: 0.72-5.76 mmol/l

Pyruvate: 73.2-327 µmol/l

LPR: 10.1 – 21.3

Glycerol: 7-446 µmol/l

4.3 Results of Clinical Study 2 – Small Burns

Between 1st November 2006 and 31st October 2007, 184 patients were admitted to the burns unit with acute burns <15%TBSA. The following patients were unsuitable for the study: 91 were admitted more than 8 hours after injury; 2 had co-existing trauma; 1 had inhalation injury; 32 had no suitable area for insertion of a microdialysis probe (23 due to flash burns to the face without significant other injury; 9 had very small burns <1% TBSA with no area suitable for probe insertion). A further 10 were outside the 18-80 age group; 2 could not give full consent due to learning difficulties; 2 were prisoners. Of the remaining 44 patients, the majority had significant comorbidities. Twelve patients were made known to the researcher within 8 hours of burn injury, and fulfilled the inclusion criteria for the study. All of these were invited to take part. Two patients declined to participate having read the participant information leaflet. Ten patients were recruited. Patient characteristics are detailed in

Patient Number	Age	Sex	Burn %TBSA (FT/DD)	Time from burn to 1st sample	Mechanism	Co-morbidity	Pre-admission Medication
S01	50	F	6 (3)	10h	Contact	None	None
S02	36	M	4 (1)	7h	Flame	None	None
S03	43	M	6.5 (2)	7.5h	Hot fat/ flame	None	None
S04	19	F	9 (3)	9.5h	Flame	None	None
S05	19	F	11 (7.5)	6.5h	Flame	None	None
S06	42	M	7(3)	12h	Contact	Heavy facial bruising. Alcoholism. Depression	Chlordiazepoxide
S07	67	F	3(2.5)	7.5h	Flame	None	None
S08	29	M	5.5(4)	7.5h	Lightning	None	None
S09	59	F	8(4)	7h	Flame	IDDM, Bipolar disorder, Hypertension	Dosulepin, Simvastatin, Aspirin, Frusemide, Lisinopril, Adalat, Doxazosin, Insulin, Metformin
S10	68	F	12 (5DD)	9.5h	Scald	IHD	Tamoxifen, Diazepam, Lisinopril, Bendrofluazide

Table 19. Average Total Body Surface Area (TBSA) burn was 7.5% (Range 3-12%).

4.3.1 Deviations from protocol, missing and anomalous results

4.3.1.1 Deviations from protocol

The following patients had deviations from the protocol described in the methods section. Reasons are described below:

S02: Biobrane ™ applied to burn instead of Jelonet and gauze. This was requested by the consultant in charge of the patient's care, immediately after probe insertion and was not anticipated beforehand. LDI images were taken prior to Biobrane application, and it had been removed prior to the second LDI image.

S03: Probe C pulled out by patient at +21.30h (Sample 30).

S06: Co-morbidity and medication was not described by patient on admission. Facial bruising was present, but there were no fractures.

S07: Probe broken on insertion, but not identified until further into the study, so no values were acquired for probe 'C'.

S08: Study terminated early (+21h, Sample 29) as patient taken to theatre for excision of burn including site where probes located.

S09, S10: Patients taken to theatre, during study period, for excision and grafting at sites distant from probes. Probes remained in situ. Both patients were taken to theatre between 18 and 24 hours post-burn.

4.3.1.2 Missing Results

Data points were missed for one of three reasons:

1) insufficient volume in the sample vial for analysis was noted on occasion;
2) blood in the sample vial meant the vial had to be discarded since the large proteins would be detrimental to the analyser, and because red blood cells could undergo metabolism within the sample vial, and alter results;
3) sampling errors occurred very infrequently whereby a sample vial was not taken at the correct time, or was mislabelled. These missing results are detailed in Appendix C, Table 20.

Patient Number	Age	Sex	Burn %TBSA (FT/DD)	Time from burn to 1st sample	Mechanism	Co-morbidity	Pre-admission Medication
S01	50	F	6 (3)	10h	Contact	None	None
S02	36	M	4 (1)	7h	Flame	None	None
S03	43	M	6.5 (2)	7.5h	Hot fat/ flame	None	None
S04	19	F	9 (3)	9.5h	Flame	None	None
S05	19	F	11 (7.5)	6.5h	Flame	None	None
S06	42	M	7(3)	12h	Contact	Heavy facial bruising. Alcoholism. Depression	Chlordiazepoxide
S07	67	F	3(2.5)	7.5h	Flame	None	None
S08	29	M	5.5(4)	7.5h	Lightning	None	None
S09	59	F	8(4)	7h	Flame	IDDM, Bipolar disorder, Hypertension	Dosulepin, Simvastatin, Aspirin, Frusemide, Lisinopril, Adalat, Doxazosin, Insulin, Metformin
S10	68	F	12 (5DD)	9.5h	Scald	IHD	Tamoxifen, Diazepam, Lisinopril, Bendrofluazide

Table 19: Patient Characteristics

4.3.1.3 Anomalous Results

Results which were suspected of being anomalous have been highlighted, and the reason for the anomaly indicated, in Appendix C, Table 21. The majority of these samples were of very low sample volume, and showed very low results for all analytes. These results are indicated on the graphs below, and have been retained for purposes of statistical calculation. The few results which had been mislabelled were corrected before the graphs and statistical calculations were created. A note has been made under the relevant graph, and in Appendix C, Table 21.

4.3.2 Urea Standards and Recovery Calculations

The results of the laboratory analysis of serum urea, and the microdialysis analysis of the serum and dermal (Zone C) urea are detailed in Table 20. This part of the study was an addition to the protocol after recruitment of patient S05, by which time the serum samples of the previous patients had been discarded. Four patients had serum taken at the same time as dermal microdialysis (S02, S04(2), S05(2), S06(1)).

Table 20: to compare urea results of hospital laboratory analysis (lab) with microdialysis (MD) of serum and with microdialysis of skin. Urea measured in mmol/l. Sampling time is also recorded.

Patient		Urea serum/lab	Urea serum/MD	Urea Unburned skin/MD
S01		**3.3**		**4.96**
	Time	6/11/06 06:00		6/11/06 15:00
S02		**4.0**		**3.9**
	Time	9/11/06 11:58		9/11/06 11:45
S03		**5.6**		**6.135**
	Time	27/1/07 05:22		27/1/07 09:30
S04 (1)		**3.1**		**4.337**
	Time	30/1/07 20:10		30/1/07 22:00
S04 (2)		**3.4**		**4.09**
	Time	31/1/07 13:17		31/1/07 13:00
S05 (1)		**4.1**	**7.6; 6.9; 5.2**	**4.634**
	Time	4/4/07 19:30		4/4/07 22:30

S05 (2)		**2.9**	**5.2; 6.1; 3.8**	**4.4**
	Time	5/4/07 08:30		5/4/07 08:30
S06 (1)		**3.8**	**2.6; 3.3;**	**4.15**
	Time	20/5/7 14:00		20/5/7 14:00
S06 (2)		**5.2***	**3.1; 4.9; 5.7**	**7.482**
	Time	21/5/7 09:00		21/5/7 02:00
S07 (1)		**3.7**	**4.2; 3.2**	**4.238 (ZoneB)** **4.145 (ZoneA)**
	Time	23/5/7 14:00		23/5/7 15:30
S07 (2)		**7.7**	**4.8; 5.6; 7.6**	**8.986 (ZoneB)**
	Time	24/5/7 09:00		24/5/7 09:00
S08		**7.0**	**12.5**	**12.54**
	Time	1/6/07 01:00		1/6/07 13:30
S09 (1)		**6.3**	**4.9; 8.7; 8.5**	**7.842**
	Time	30/7/7 00:30		30/7/7 02:00
S09 (2)		**13.3**	**12.7; 5.9; 16.1**	**14.37**
	Time	31/7/7 13:00		31/7/7 07:00
S10 (1)		**6.3**		**7.492**
	Time	23/8/7 23:45		24/8/7 03:00
S10 (2)		**6.5**		**10.17**
		25/8/7 11:00		25/8/7 05:00

*small volume sample

4.3.3 Biochemical Characteristics by Zone

4.3.3.1 Trends with Time post-burn

No patients were recruited at less than 6.5 hours post-injury. Time-blocks were grouped as follows: Block 1: 6.5-12h; Block 2: 12.5-18h; Block 3: 18.5-24h; Block 4: 24.5-30h; Block 5: 30.5-36h.

Zone A = Zone of Coagulation. Zone B= Zone of Stasis. Zone C = Unburned skin.

4.3.3.1.1 Glucose.

Time-related trends for all zones are shown in Figure 33.

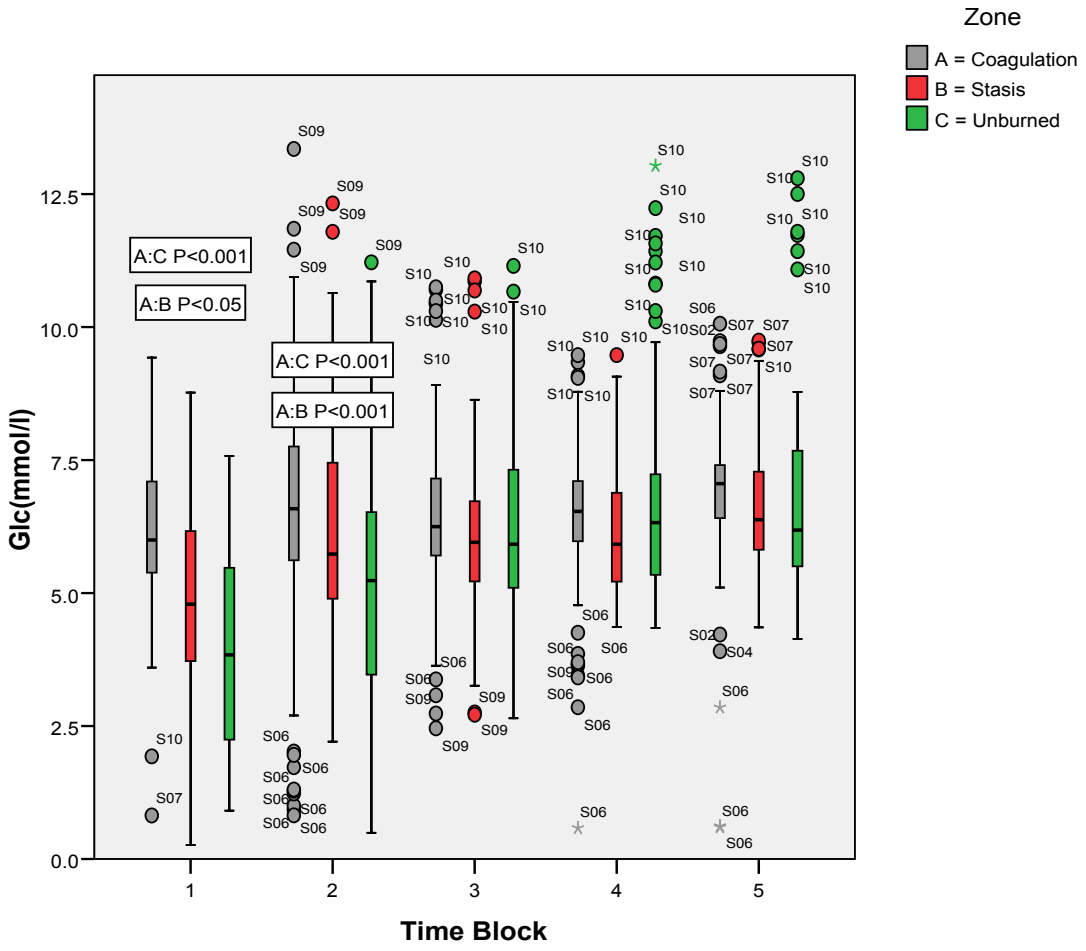
In Zone A, there were changes in glucose levels with time for some individual patients, but overall, glucose in Zone A was constant, with no statistically significant differences over time.

Glucose in Zone B showed an upward trend with time. Glucose in Block 1 was significantly lower than Block 2 ($P<0.001$), and Block 5 ($P<0.01$)

Glucose in Zone C showed the most marked upward trend. Glucose in Block 1 was significantly lower than in Block 2 and Block 5 ($P<0.001$). Glucose in Block 2 was significantly lower than in Block 3 and Block 5 ($P<0.001$). Glucose in Block 3 was significantly lower

than in Block 5 (P<0.05). In over half of the patients, glucose in Zone C was below the normal range (see Section 4.2.6) for the first 12 hours. Patient S09 exhibited swinging glucose levels. Patient S10 was hyperglycaemic for the majority of the time.

Figure 33: Boxplot to show trends in glucose with time for each zone



Time blocks refer to time post-burn as follows: 1=6.5-12h, 2=12.5-18h, 3=18.5-24h, 4= 24.5-30h, 5=30.5-36h. Boxplot shows minimum, 1st quartile, median, 3rd quartile and maximum. Near outliers (o) and far outliers (*) are annotated with patient number. Significant differences between zones are indicated for each time block.

4.3.3.1.2 Lactate

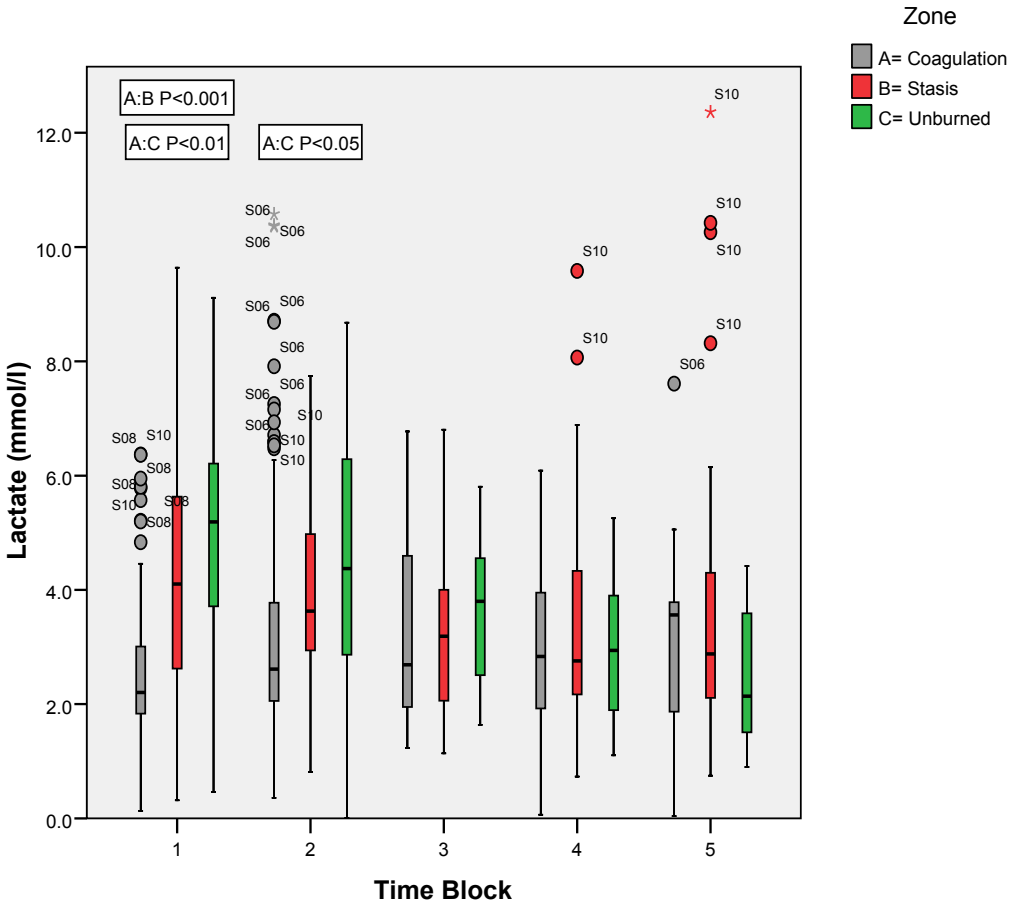
Time-related trends are illustrated in Figure 34.

For the majority of patients, lactate in Zone A was stable with time. There were no statistically significant differences between time blocks.

Lactate in Zone B showed an initial peak, above the normal range, over the first few hours in most patients. When analysed by time blocks, lactate showed a slight tendency to decline with time. There was a statistical difference between Blocks 3 and 5 with Block 5 being significantly lower than Block 3 ($P<0.05$).

Lactate in Zone C showed an initial peak, above the normal range, then a marked downward trend towards the end of the study period. Lactate in Blocks 1, 2 and 3 was significantly higher than in Block 5 ($P<0.001$). Lactate in Block 4 was significantly lower than in Block 3 ($P<0.05$).

Figure 34: Boxplot to show trends in lactate with time for each zone



Time blocks refer to time post-burn as follows: 1=6.5-12h, 2=12.5-18h, 3=18.5-24h, 4= 24.5-30h, 5=30.5-36h. Boxplot shows minimum, 1st quartile, median, 3rd quartile and maximum. Near outliers (o) and far outliers (*) are annotated with patient number. Significant differences between zones are indicated for each time block.

4.3.3.1.3 Pyruvate

Time-related trends are shown in Figure 35.

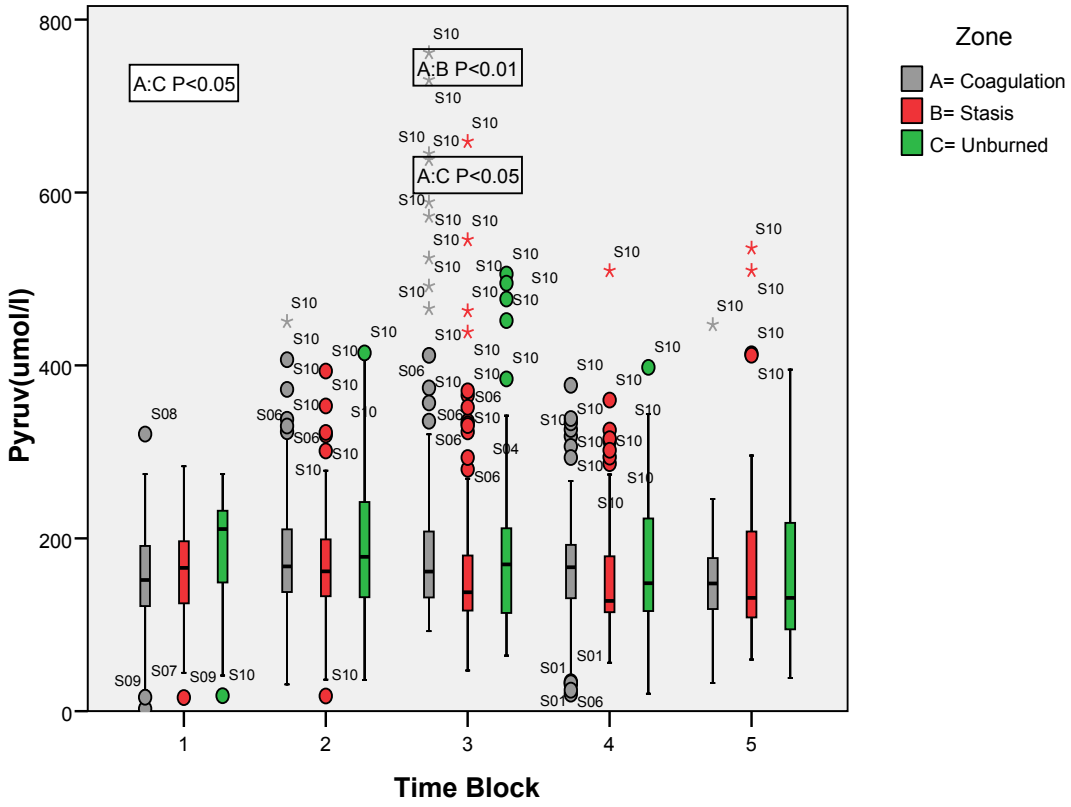
With one exception, S10, pyruvate in Zone A was stable with time. There were no statistically significant differences between time blocks.

Pyruvate in Zone B showed a slight upward trend to Block 2 then a slight downward trend. There was a small difference between Blocks 1 and 2 with Block 1 being significantly lower than block 2 ($P<0.05$). There was a difference between Blocks 3 and 4 with Block 3 being significantly higher than Block 4 ($P<0.01$).

Although there was a wide spread in individual results, overall pyruvate in Zone C was stable with time. There were no statistically significant differences between time blocks.

Patient S09 showed notable peaks of pyruvate in all zones, which were not matched by lactate levels.

Figure 35: Boxplot to show trends in pyruvate with time for each zone



Time blocks refer to time post-burn as follows: 1=6.5-12h, 2=12.5-18h, 3=18.5-24h, 4= 24.5-30h, 5=30.5-36h. Boxplot shows minimum, 1st quartile, median, 3rd quartile and maximum. Near outliers (o) and far outliers (*) are annotated with patient number. Significant differences between zones are indicated for each time block.

4.3.3.1.4 Lactate:Pyruvate Ratio (LPR)

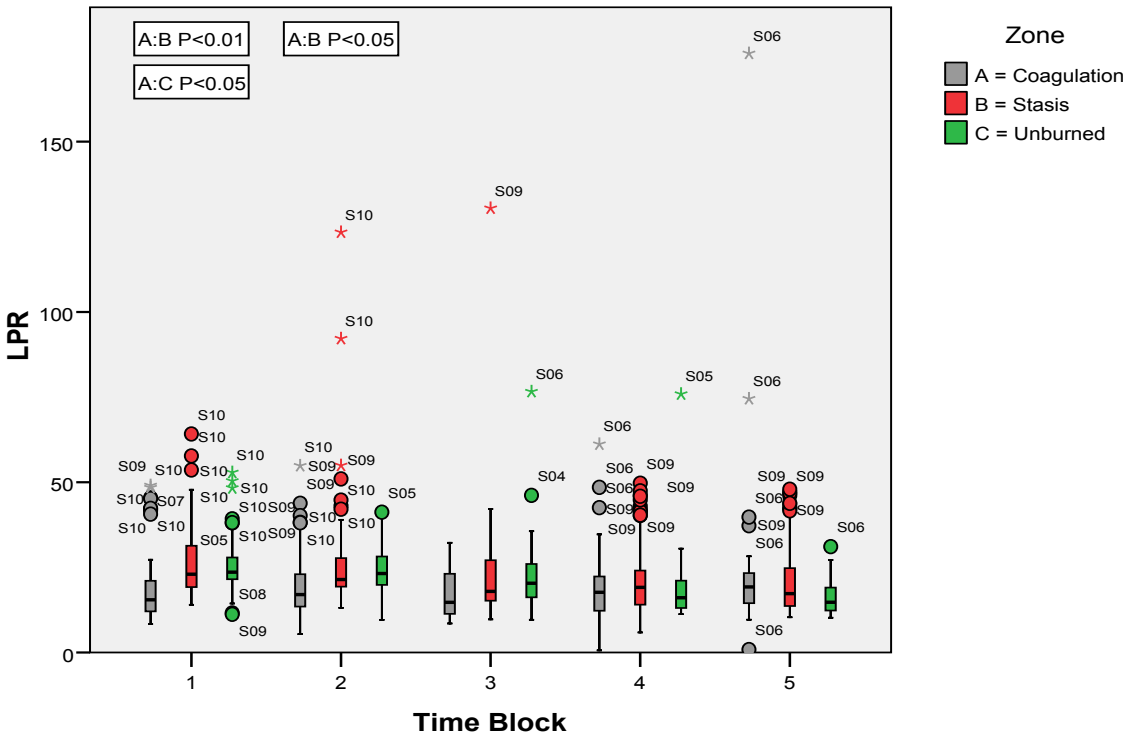
LPR trends are illustrated in Figure 36.

LPR in Zone A was stable with time. There were no statistically significant differences between time blocks. Most patients had LPR results consistently below 25, with the exception of S06, S09 and S10.

LPR in Zone B was also stable with time. There were no statistically significant differences between time blocks. More patients had LPR results above 25 for a significant period of time (S02, S05, S07, S08, S09, S10), than in Zone A.

LPR in Zone C showed a marked downward trend throughout the study period. LPR in Block 1 was significantly higher than in Block 5 ($P<0.01$). LPR in Blocks 2 and 3 was significantly higher than in Block 5 ($P<0.001$). Block 1 was significantly higher than Block 2. Block 2 was significantly higher than Block 3 ($P<0.05$), and Block 3 was significantly higher than Block 4 ($P<0.01$). Patients S02, S04, S05, S06 and S10 had LPR readings over 25.

Figure 36: Boxplot to show trends in Lactate/Pyruvate ratio with time for each zone



Time blocks refer to time post-burn as follows: 1=6.5-12h, 2=12.5-18h, 3=18.5-24h, 4= 24.5-30h, 5=30.5-36h. Boxplot shows minimum, 1st quartile, median, 3rd quartile and maximum. Near outliers (o) and far outliers (*) are annotated with patient number. Significant differences between zones are indicated for each time block.

4.3.3.1.5 Glycerol

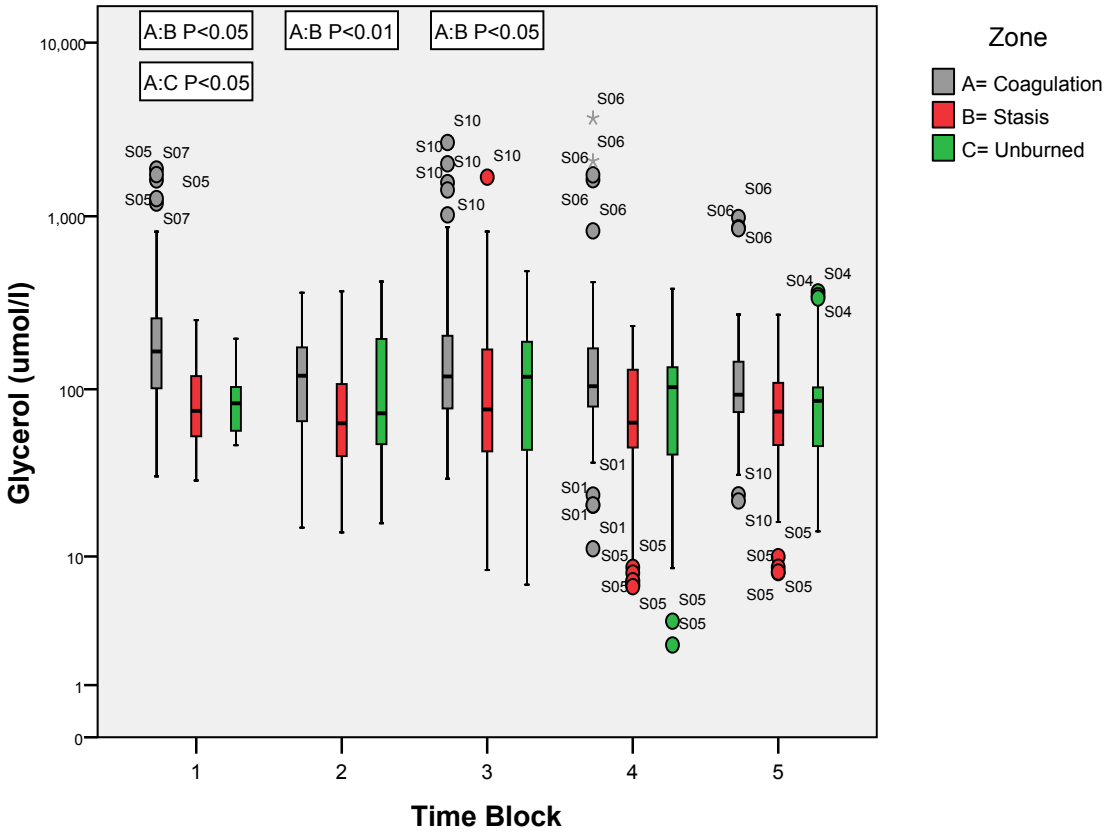
Glycerol trends are illustrated in Figure 37.

Glycerol in Zone A showed some variation with time. Block 1 was significantly higher than Block 5 ($P<0.01$). Block 2 was significantly higher than Block 5 ($P<0.05$). Patients S05, S07 and S08 showed extremely high peaks of glycerol in the early hours following reported burn injury. Patients S06, S09 and S10 showed high peaks later in the study period.

Glycerol in Zone B overall was stable with time. There was no statistical difference between time blocks. Only patients S09 and S10 showed a variation from this course, with a high peak at around 12 hours from burn injury.

Overall glycerol results in Zone C were stable, with a slight elevation in Block 3. Glycerol in Block 3 was significantly higher than in Block 4 ($P<0.001$). There were no extreme peaks of glycerol as in the other two zones, and all readings were under 500µmol/l. There was a downward trend in S06 and S10, and a cyclical pattern in S04, but the remainder showed a very stable course.

Figure 37: Boxplot to show trends in Glycerol with time for each zone



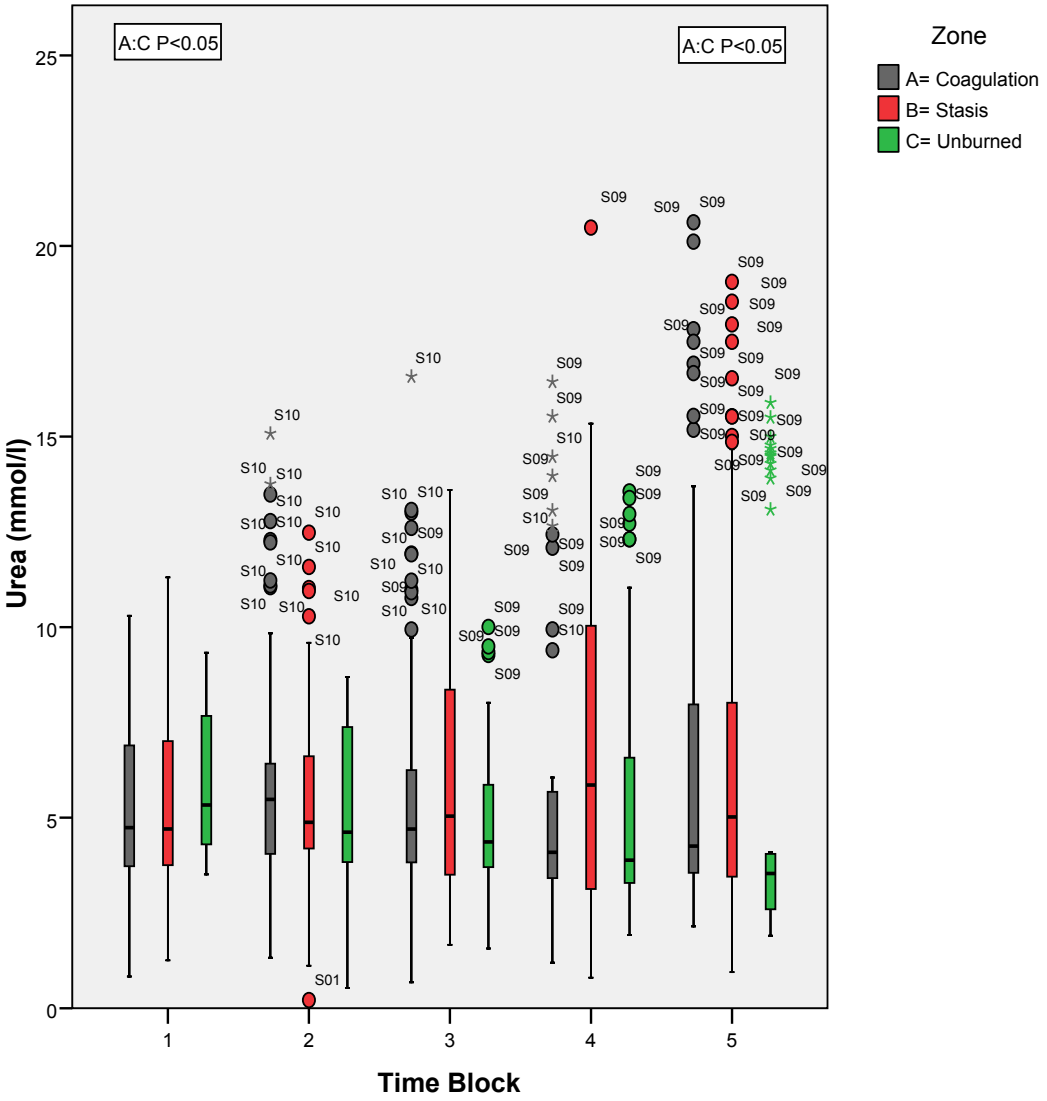
Time blocks refer to time post-burn as follows: 1=6.5-12h, 2=12.5-18h, 3=18.5-24h, 4= 24.5-30h, 5=30.5-36h. Boxplot shows minimum, 1st quartile, median, 3rd quartile and maximum. Near outliers (o) and far outliers (*) are annotated with patient number. Significant differences between zones are indicated for each time block.

4.3.3.1.6 Urea

Urea trends are illustrated in Figure 38.

There was no significant difference in urea between the time blocks for any of the three zones.

Figure 38: Boxplot to show trends in Urea with time for each zone



Time blocks refer to time post-burn as follows: 1=6.5-12h, 2=12.5-18h, 3=18.5-24h, 4= 24.5-30h, 5=30.5-36h. Boxplot shows minimum, 1st quartile, median, 3rd quartile and maximum. Near outliers (o) and far outliers (*) are annotated with patient number. Significant differences between zones are indicated for each time block.

4.3.3.1.7 Summary of results by time

Overall, there was an initial ischaemic picture within the unburned skin and zone of stasis which lasted until 16-24 hours post burn. This was evidenced by low glucose and high LPR values. There was also a peak in glycerol seen at the start of the study within the zone of coagulation.

4.3.3.2 Comparison of Zones

4.3.3.2.1 Glucose *(Figure 33)*

Glucose in Zone C was initially low, being significantly lower than Zone A ($P<0.001$) and Zone B ($P<0.05$), for Block 1 and lower than Zones A and B in Block 2 ($P<0.001$), but being indistinguishable from the other zones by 18 hours post-burn.

4.3.3.2.2 Lactate *(Figure 34)*

Lactate in Zone C started high, and declined with time towards the levels in Zone A. Lactate in Block 1 was significantly higher in Zone C than in Zone A ($P<0.01$), and this persisted into Block 2, with a significant difference between Zones A and C ($P<0.05$).

Lactate in Zone B followed a similar pattern, being higher than Zone A ($P<0.001$) for Block 1.

4.3.3.2.3 Pyruvate (Figure 35)

Overall, pyruvate levels were constant for all zones. There were some slight differences for Blocks 1 and 3, but no overall trends. Pyruvate in Block 1 was significantly higher in Zone C than in Zone A ($P<0.05$).

Pyruvate in Block 3 was significantly higher in Zone C than in Zone A ($P<0.05$), and higher in Zone A than Zone B ($P<0.01$).

4.3.3.2.4 LPR (Figure 36)

There was an overall trend in higher LPR in Zones B and C at the beginning of the study, returning to the baseline level of Zone A by Block 3. LPR in Block 1 was significantly higher in Zone C than in Zone A ($P<0.05$) and in Zone B than Zone A ($P<0.01$).

LPR in Block 2 was significantly higher in Zone B than in Zone A ($P<0.05$).

4.3.3.2.5 Glycerol (Figure 37)

Glycerol was highest in Zone A for the first three time blocks. In Block 1, this reached significance when compared to both Zones B and C ($P<0.05$).

Glycerol in Block 2 remained significantly higher in Zone A than in Zone B ($P<0.01$).

Glycerol in Block 3 was also significantly higher in Zone A than in Zone B ($P<0.05$).

4.3.3.2.6 Urea (Figure 38)

Urea was overall constant throughout the time zones and between the zones. Urea in Block 1 was significantly lower in Zone A than in Zone C ($P<0.05$).

Urea in Block 5 was significantly higher in Zone B than in Zone C ($P<0.05$).

There was good concurrence in urea results between zones for each individual patient (

Figure 39, Figure 40, Figure 41,

Figure 42, Figure 43, Figure 44, Figure 45, Figure 46,

Figure 47 and Figure 48).

Figure 39: Urea results for Patient S01 showing concurrence between zones

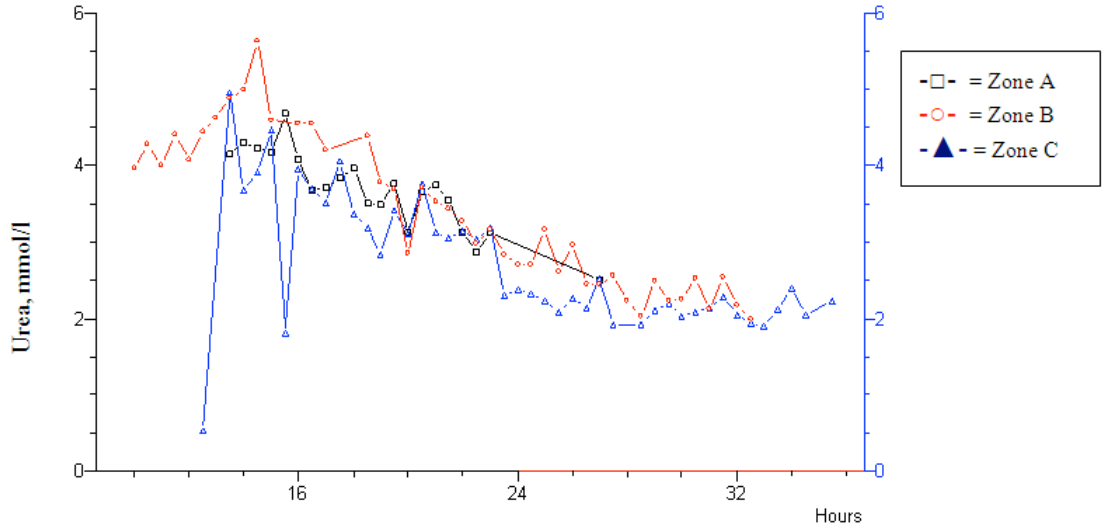


Figure 40: Urea results for Patient S02 showing concurrence between zones

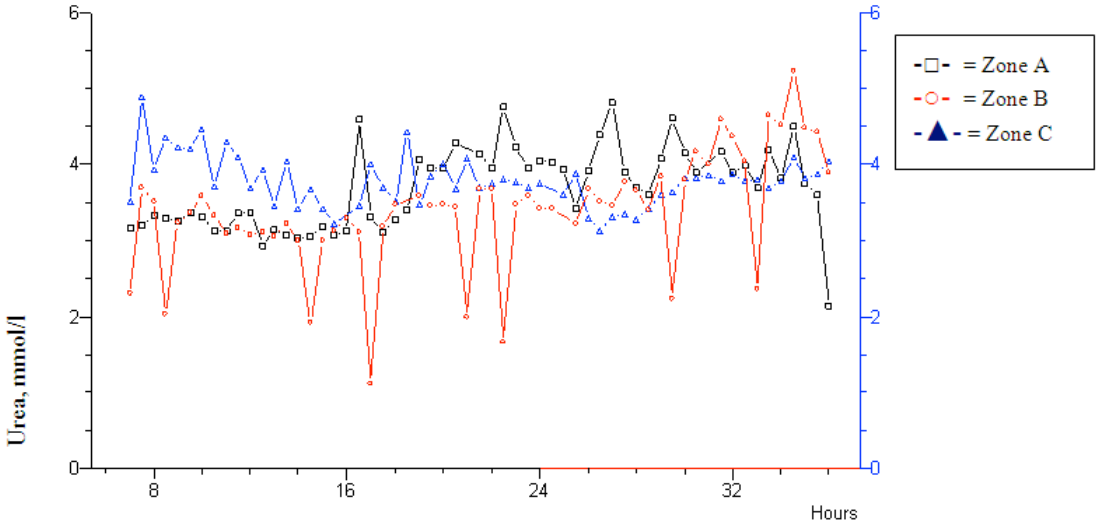


Figure 41: Urea results for Patient S03 showing concurrence between zones

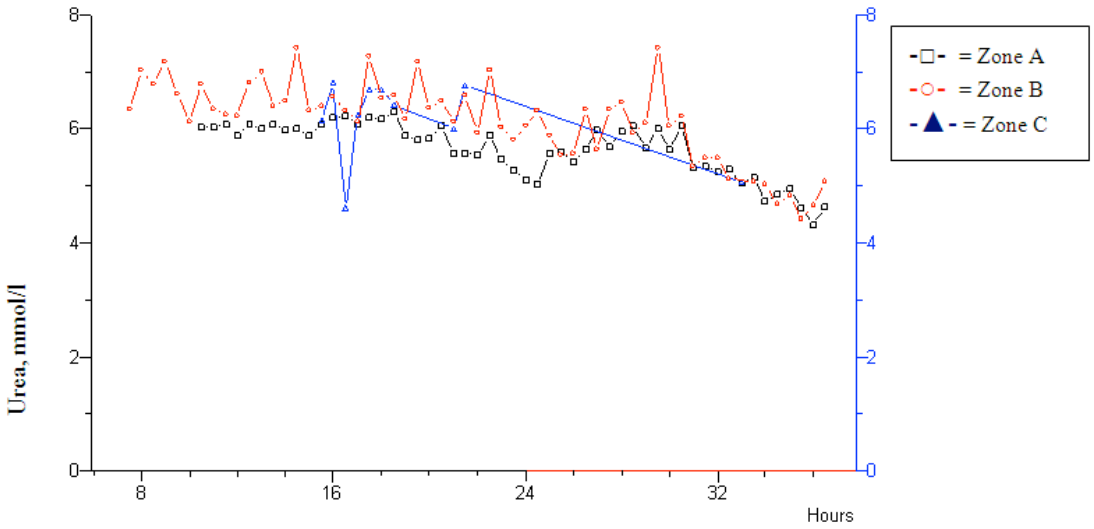


Figure 42: Urea results for Patient S04 showing concurrence between zones

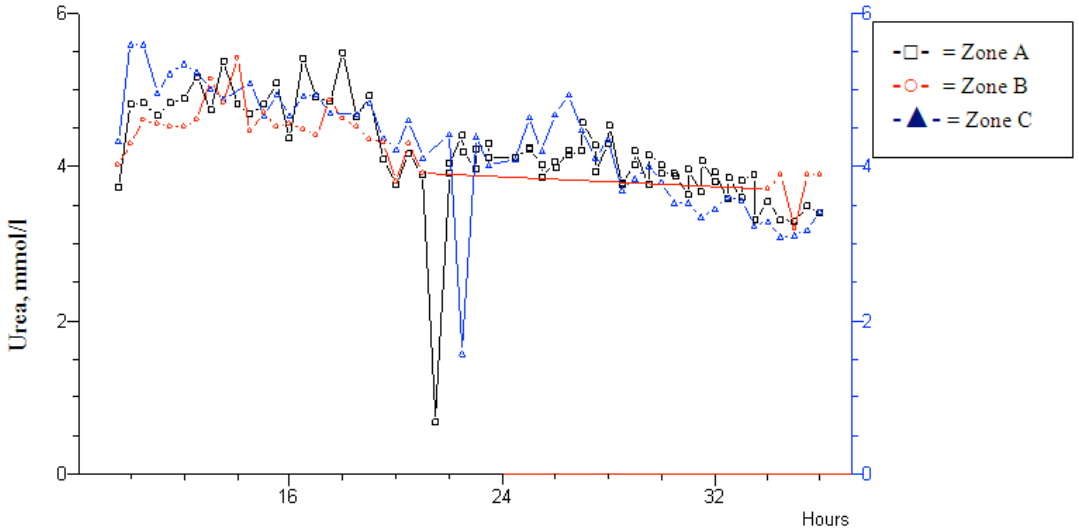


Figure 43: Urea results for Patient S05 showing concurrence between zones

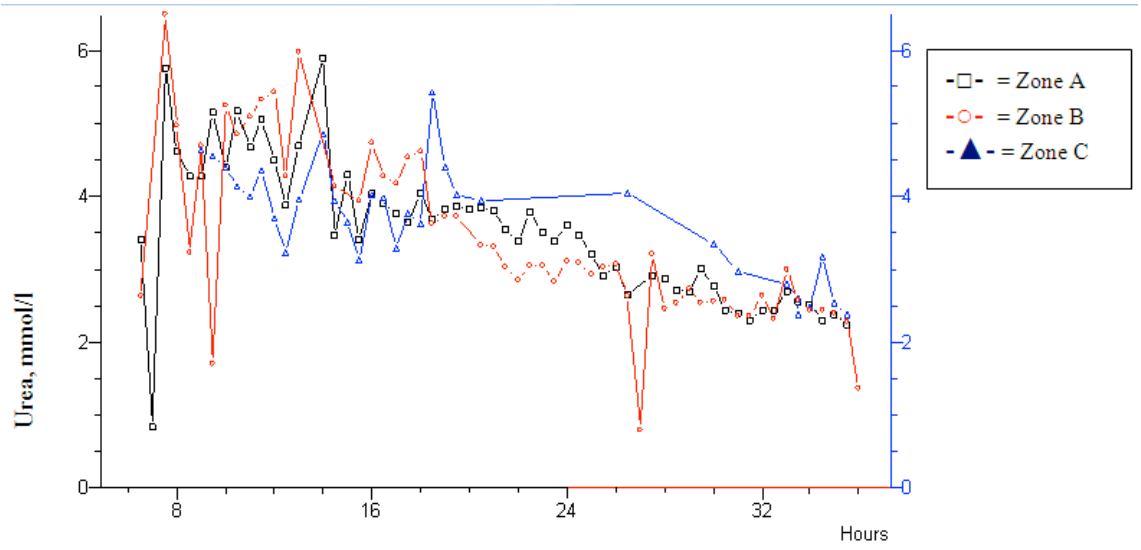


Figure 44: Urea results for Patient S06 showing concurrence between zones

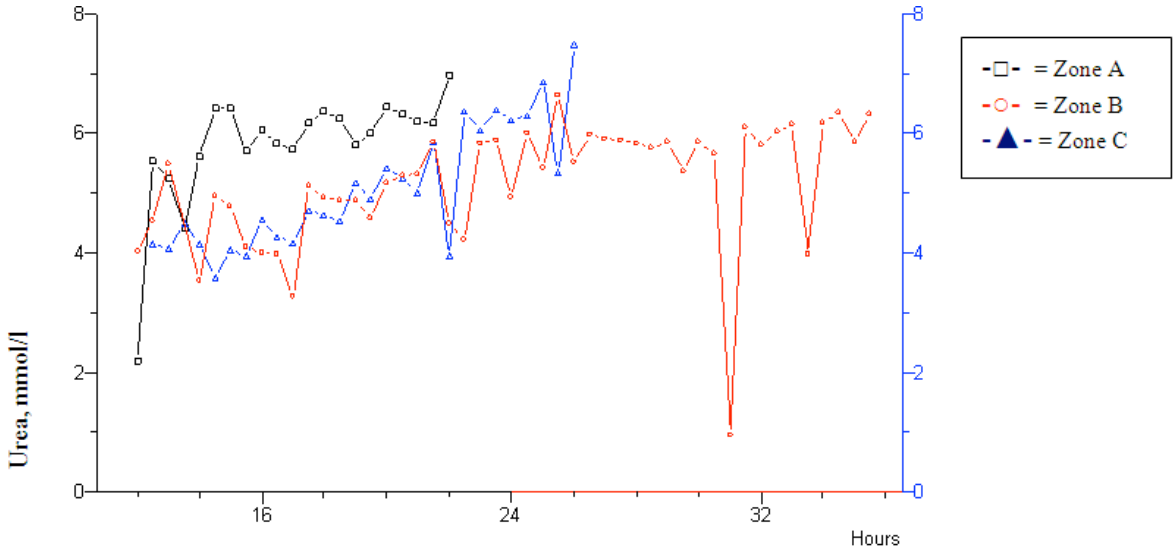


Figure 45: Urea results for Patient S07 showing concurrence between zones

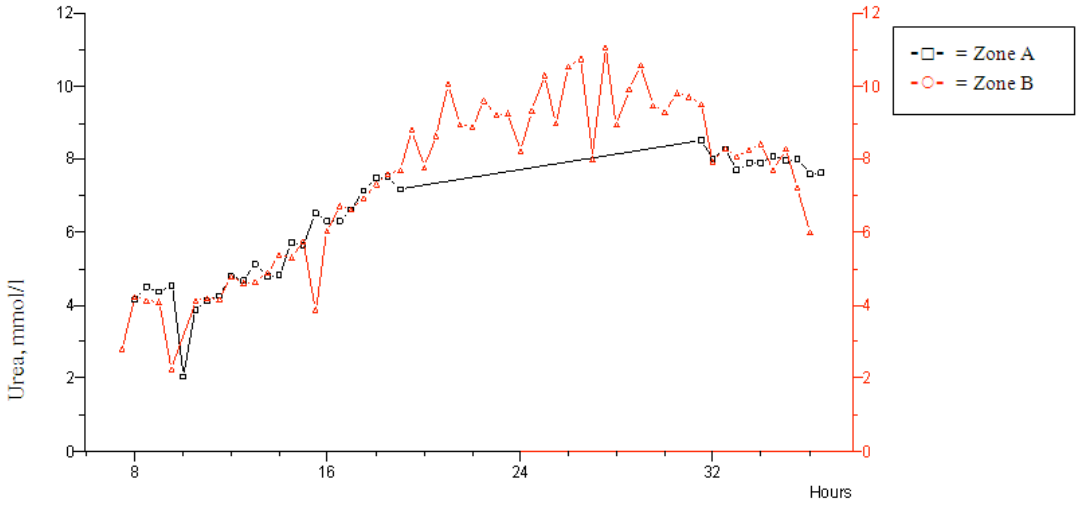


Figure 46: Urea results for Patient S08 showing concurrence between zones

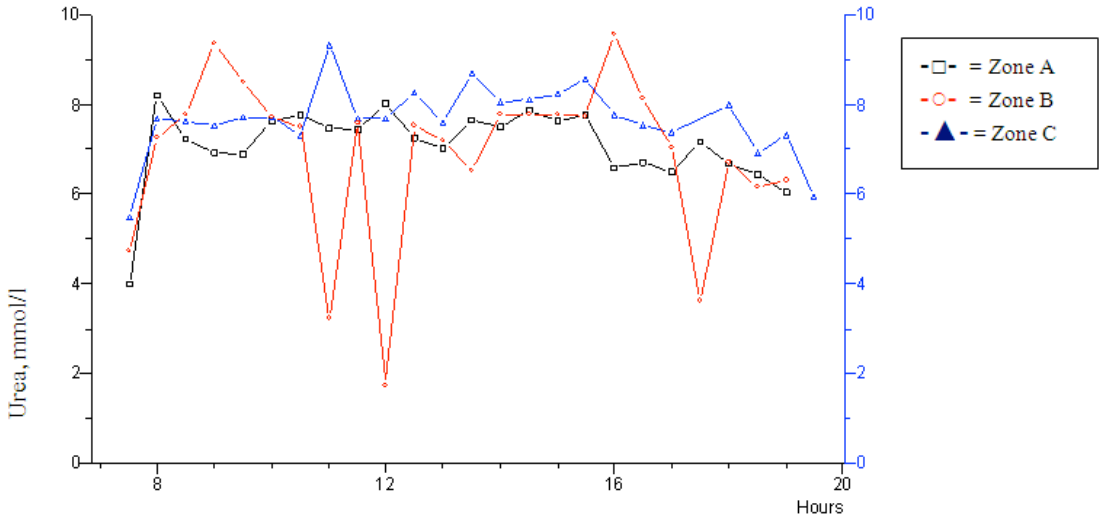


Figure 47: Urea results for Patient S09 showing concurrence between zones

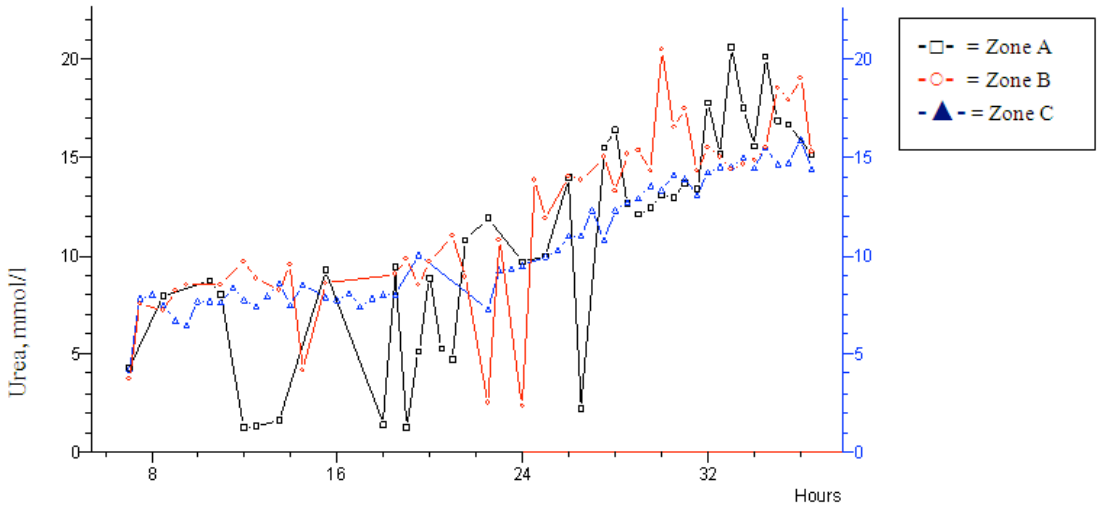
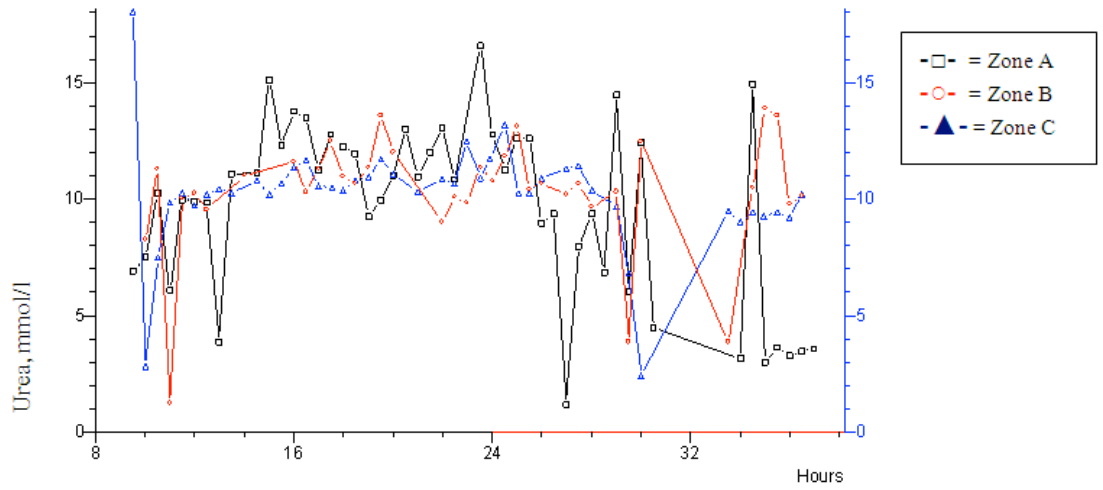


Figure 48: Urea results for Patient S10 showing concurrence between zones

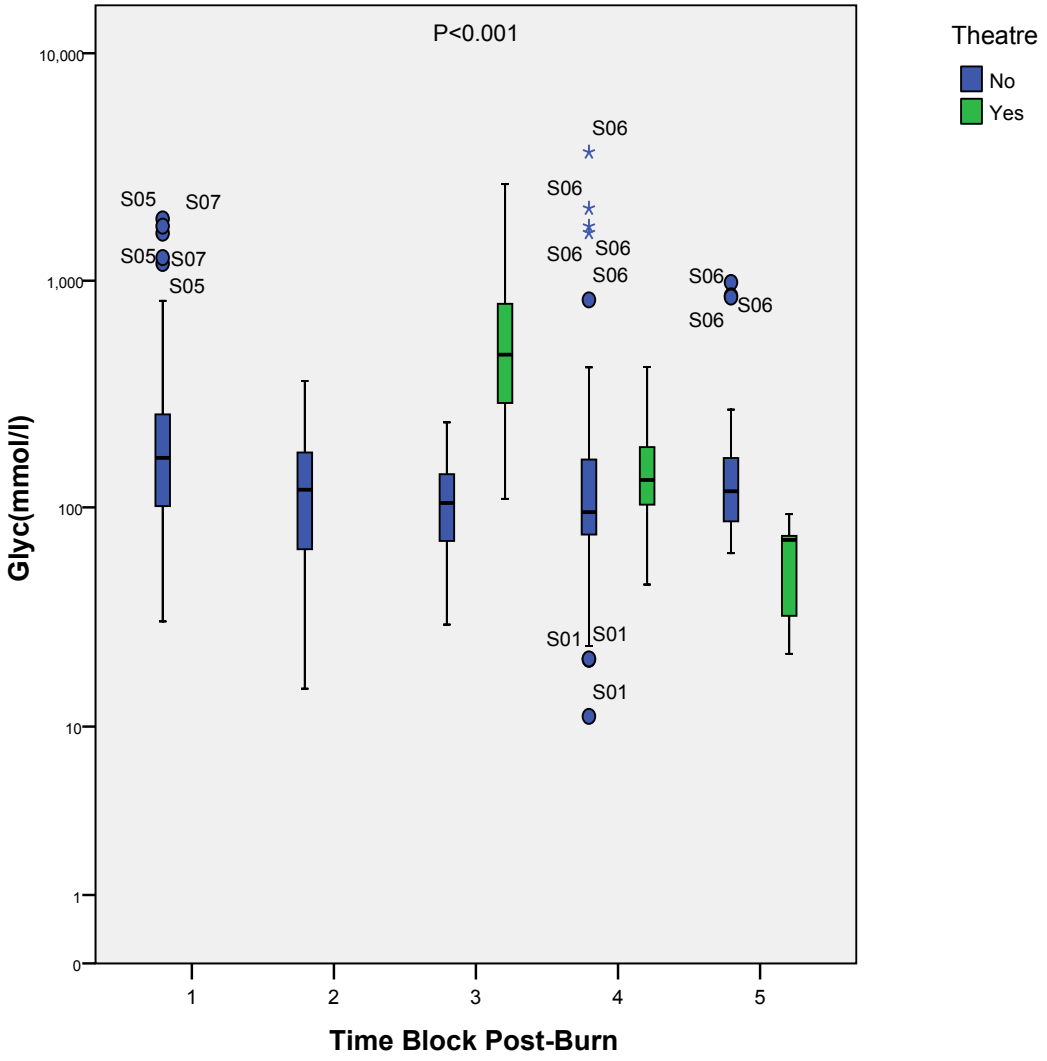


4.3.3.3 Trends with Theatre Episode

Two patients (S09 and S10) were taken to theatre within the study period. Both patients were taken to theatre during Block 3. Differences in the glycerol levels are illustrated in Figure 49,

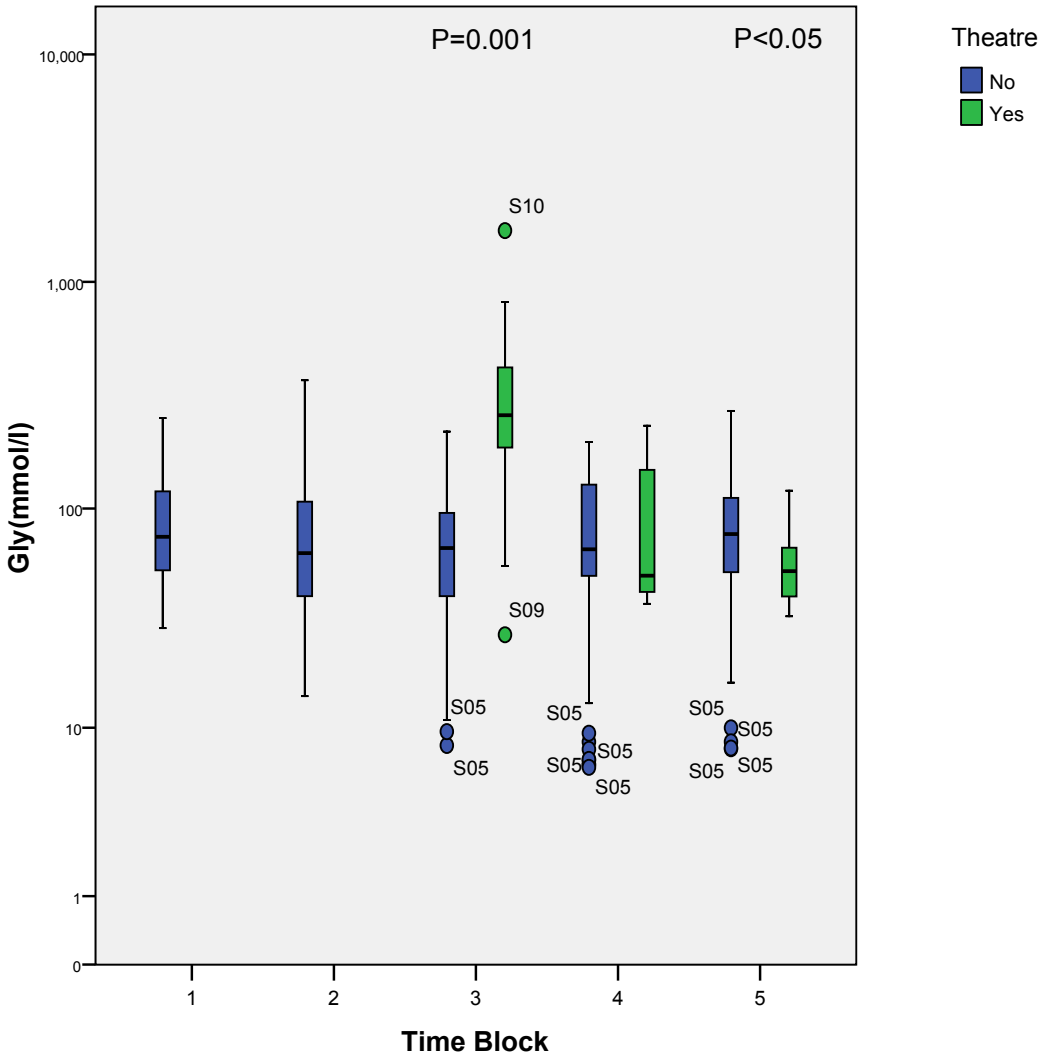
Figure 50 and Figure 51.

Figure 49: Boxplot to show the differences in glycerol levels between patients attending theatre and patients not attending theatre for Zone A



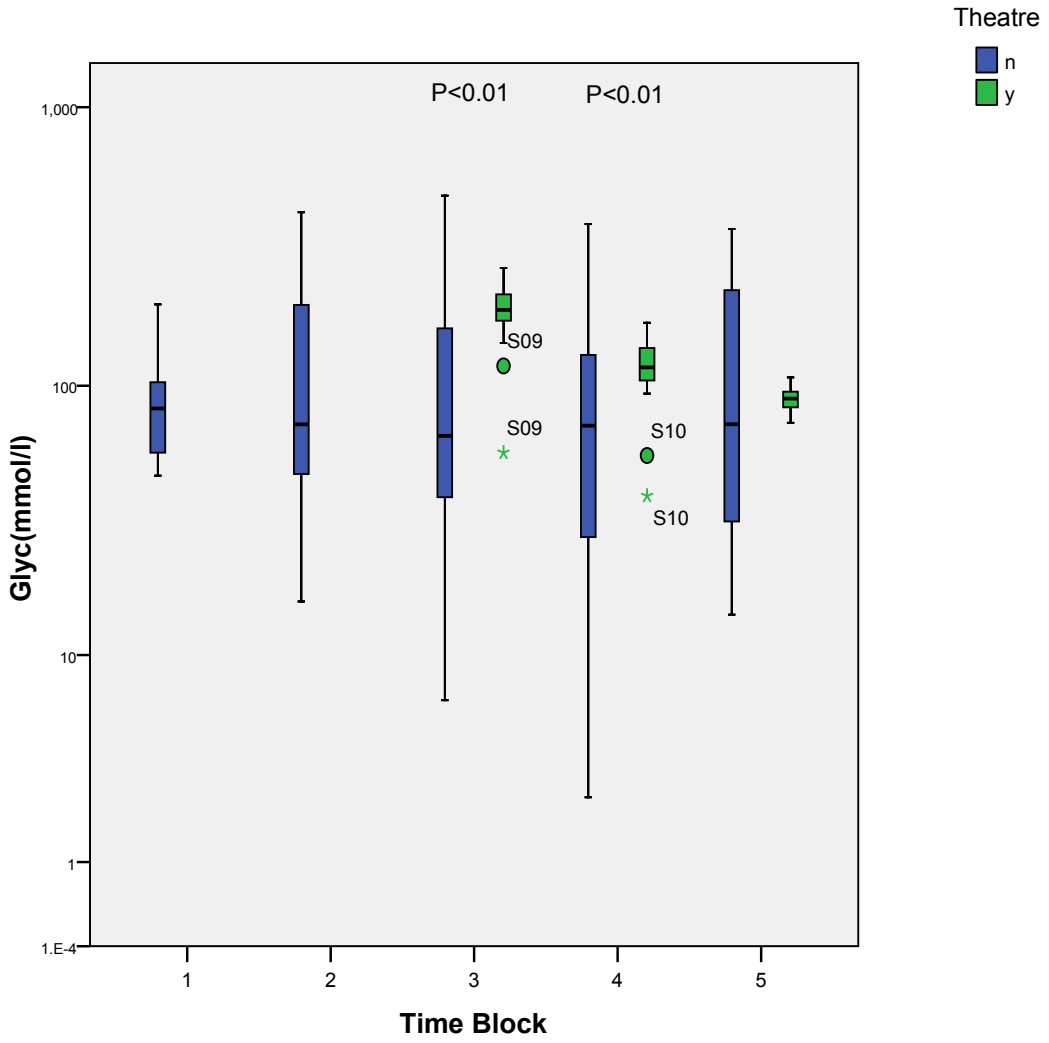
Time blocks refer to time post-burn as follows: 1=6.5-12h, 2=12.5-18h, 3=18.5-24h, 4= 24.5-30h, 5=30.5-36h. Boxplot shows minimum, 1st quartile, median, 3rd quartile and maximum. Near outliers (o) and far outliers (*) are annotated with patient number. Significant differences between zones are indicated for each time block.

Figure 50: Boxplot to show the differences in glycerol levels between patients attending theatre and patients not attending theatre for Zone B



Time blocks refer to time post-burn as follows: 1=6.5-12h, 2=12.5-18h, 3=18.5-24h, 4= 24.5-30h, 5=30.5-36h. Boxplot shows minimum, 1st quartile, median, 3rd quartile and maximum. Near outliers (o) and far outliers (*) are annotated with patient number. Significant differences between zones are indicated for each time block.

Figure 51: Boxplot to show the differences in glycerol levels between patients attending theatre and patients not attending theatre for Zone C



Patents S09 and S10 attended theatre within time block 3.

Time blocks refer to time post-burn as follows: 1=6.5-12h, 2=12.5-18h, 3=18.5-24h, 4= 24.5-30h, 5=30.5-36h. Boxplot shows minimum, 1st quartile, median, 3rd quartile and maximum. Near outliers (o) and far outliers (*) are annotated with patient number. Significant differences between zones are indicated for each time block.

In all three zones, there was a greater measured glycerol in the dialysate of the two patients attending theatre, during and for some time after their operative procedure.

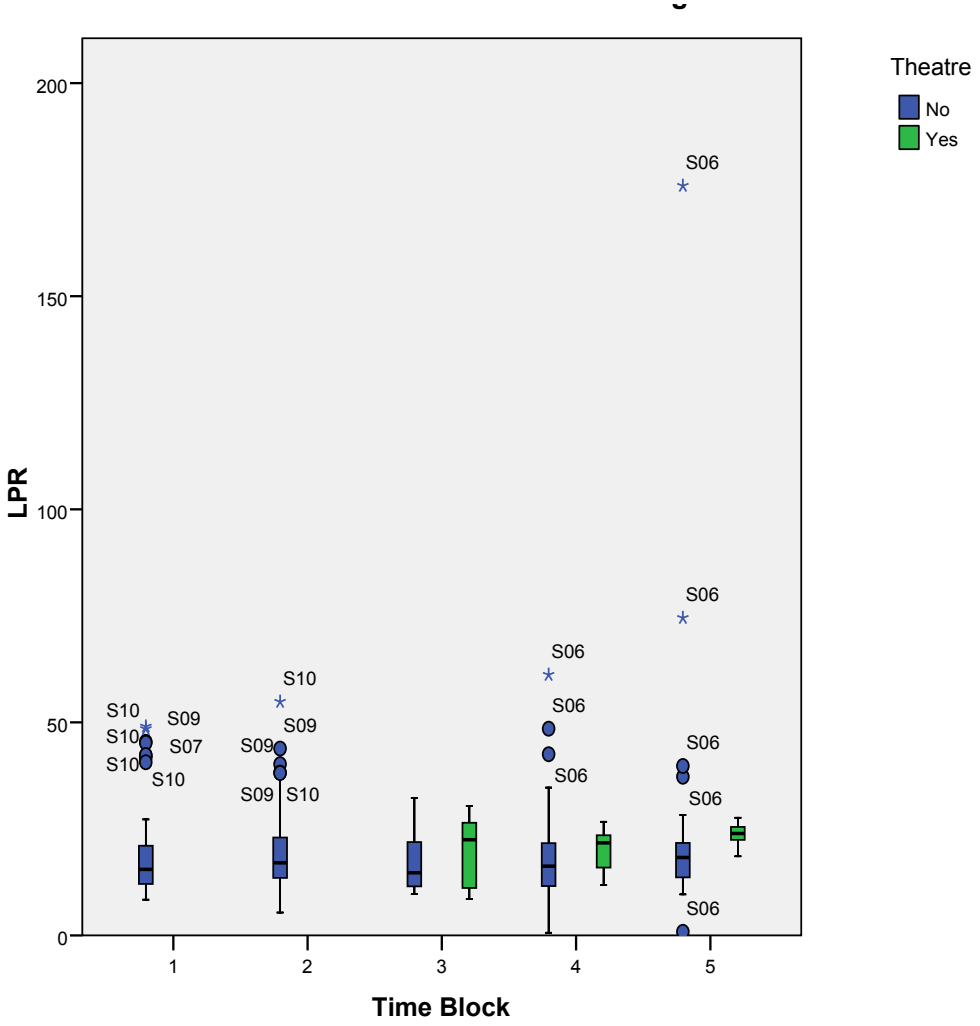
This difference was significant in Zone A during Block 3 only ($P<0.001$, Figure 49). In Zone B, glycerol was significantly higher in the theatre group during Block 3 ($P<0.001$), but significantly lower by Block 5 ($P<0.05$, Figure 50). Glycerol in Zone C was significantly higher during Block 3 in the group which went to theatre ($P<0.01$). This difference persisted into Zone 4 ($P<0.01$, Figure 51).

Differences in LPR were also noted (Figure 52, Figure 53, Figure 54).

Although there was a trend towards higher LPR results in Blocks 3, 4 and 5 in Zones A and B of the patients who went to theatre, this was not statistically significant (Figure 52, Figure 53).

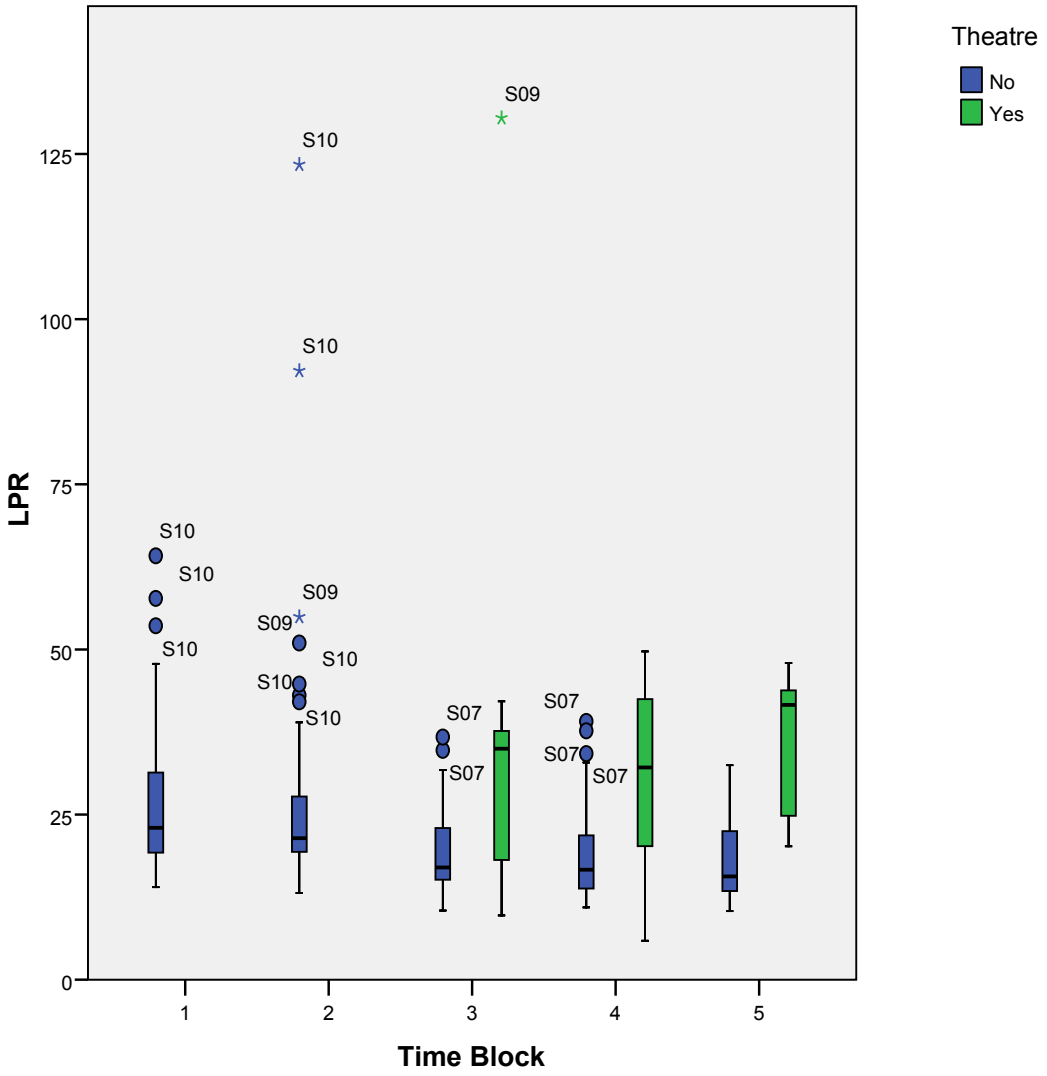
Interestingly, in Zone C, LPR was significantly lower in patients who had attended theatre for Block 3 ($P<0.001$), Block 4 ($P<0.05$), and Block 5 ($P=0.01$) (Figure 54).

Figure 52: Boxplot to show the differences in LPR in Zone A between patients attending theatre and patients not attending theatre



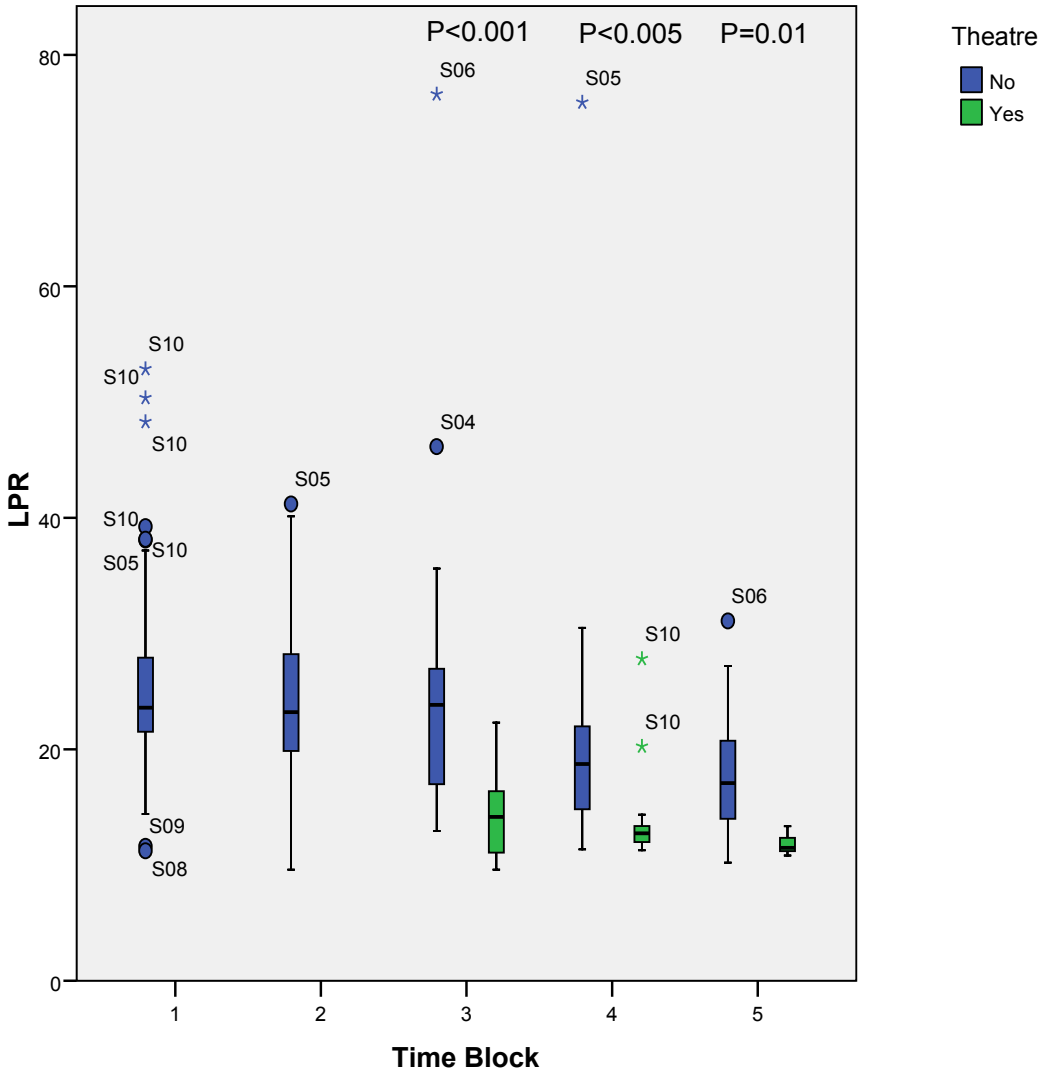
Time blocks refer to time post-burn as follows: 1=6.5-12h, 2=12.5-18h, 3=18.5-24h, 4= 24.5-30h, 5=30.5-36h. Boxplot shows minimum, 1st quartile, median, 3rd quartile and maximum. Near outliers (o) and far outliers (*) are annotated with patient number. Significant differences between zones are indicated for each time block.

Figure 53: Boxplot to show the differences in LPR in Zone B between patients attending theatre and patients not attending theatre



Time blocks refer to time post-burn as follows: 1=6.5-12h, 2=12.5-18h, 3=18.5-24h, 4= 24.5-30h, 5=30.5-36h. Boxplot shows minimum, 1st quartile, median, 3rd quartile and maximum. Near outliers (o) and far outliers (*) are annotated with patient number. Significant differences between zones are indicated for each time block.

Figure 54: Boxplot to show the differences in LPR in Zone C between patients attending theatre and patients not attending theatre



Time blocks refer to time post-burn as follows: 1=6.5-12h, 2=12.5-18h, 3=18.5-24h, 4= 24.5-30h, 5=30.5-36h. Boxplot shows minimum, 1st quartile, median, 3rd quartile and maximum. Near outliers (o) and far outliers (*) are annotated with patient number. Significant differences between zones are indicated for each time block.

4.3.4 Laser Doppler Images

All ten patients had LDI taken at the beginning of the study. Time between reported burn and first LDI ranged from 6 – 11.5 hours. Nine patients had LDI taken at the end of the study. One patient (S08) did not have an end-point LDI, as the burn was excised at 24 hours, and he was withdrawn from the study. Time from burn to final LDI ranged from 36 – 49 hours. Plates 1-10 show the laser Doppler images for patients S01-S10. Position of Probe B is indicated on the images with a black dotted line. Table 21 summarises the results.

A technical error occurred with the laser Doppler imager, resulting in suboptimal images for Patients S01 and S03. In both cases, the site of Probe B was not included within the visible part of the image, so progression at the site of the probe could not be assessed. However in both cases, a comment on overall progression of the wound could be made as the majority of the wound was imaged.

For patients S06 and S09, the initial images showed lower than expected blood flow globally. In S06, the first image was so poor that no comment on progression could be made about the wound globally. However, since there was moderate to good flow (yellow/pink colour) surrounding Probe B in the final image, it was assumed that there could not have been any wound progression here. In wound S09, enough of the wound was recognisable in

the initial LDI image to allow an interpretation on wound progression both globally, and around Probe B.

Five patients showed evidence of overall wound progression; only one of these showed evidence of progression at the site of the probe. Three patients showed no evidence of wound progression in the wound as a whole. Table 21 details these results.

Table 21 to show results of LDI for each patient

Patient	LDI Time post-burn	Area blue (cm^2)	Evidence of wound progression	Colour over stasis probe	Evidence of progression over probe
S01	9	0.6		n/a	
	36	0	No	n/a	n/a
S02	6	0		Yellow	
	48.5	8.12	Yes	Green/blue	Yes
S03	6.5	25.87		Yellow/green	
	38.5	52.9	Yes	n/a	n/a
S04	8	13.7		Yellow	
	43.5	35.91	Yes	Yellow/red	No
S05	6	54.67		Yellow/green	
	45	78.2	Yes	Yellow/green	No
S06	11.5	n/a		Unrecordable	
	36.5		n/a	Yellow/Pink	No
S07	6.5	13.37		Yellow	
	49	26.11	Yes	Yellow/Red	No
S08	6.5				
	n/a	n/a	n/a	n/a	n/a
S09	7	17.86		Blue	
	48.5	5.3	No	Yellow/Green	No
S10	9	18.44*		Blue	
	42	*	No	Yellow/green	No

* Full extent of wound not visible on either scan, so exact measurement would have been inaccurate. There does not appear to be any significant progression.

4.3.5 Biochemical correlation to LDI findings

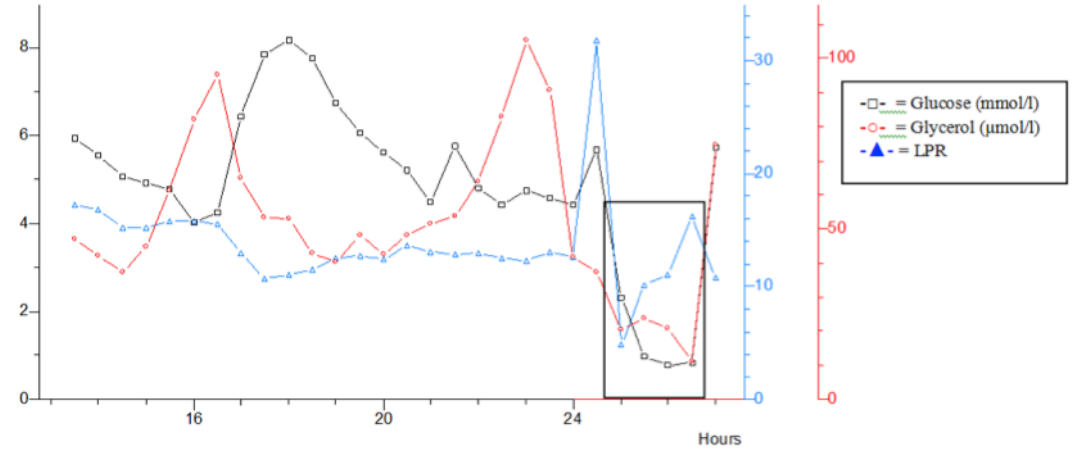
Table 22 shows a summary of the correlation between biochemistry (LPR and glycerol), LDI and wound outcome. LPR levels of above 25 are universally accepted as signifying anaerobic conditions. Glycerol of 450µmol/l was taken as the upper limit of normal as per results of Study 1 (Section 4.2.6). Results of individual patients are correlated to their LDI images in the paragraphs below:

Table 22: Wound outcomes and possible predictive factors

Patient	LPR>25 for more than 2h			Glycerol >450			LDI Progression		Healing	
Zone:	A	B	C	A	B	C	General	Probe B	Excision	Graft take/Healing
S01	N	N	N	N	N	N	N	n/a	Day 2	Good
S02	N	Y	Y	N	N	N	Y	Y	N	>2 weeks
S03	N	N	n/a	N	N	n/a	Y	n/a	Day 5	Good
S04	N	N	Y	N	N	Y	Y	N	N	>2 weeks
S05	N	Y	Y	Y	N	N	Y	N	Day 2	Burn progression. Regrafted
S06	Y	N	N	Y	N	N	n/a	N	Day 5	Good
S07	Y	Y	n/a	Y	N	n/a	Y	N	Day 2	Graft failure at distant site
S08	N	Y	N	N	N	N	n/a	n/a	Day 1	Good but with overgranulation. Healing >6 weeks
S09	Y	Y	N	Y	Y	N	N	N	Day 1	Graft loss neck (distant site). Regrafted
S10	Y	Y	Y	Y	Y	N	+/-	N	Day 1	Graft loss

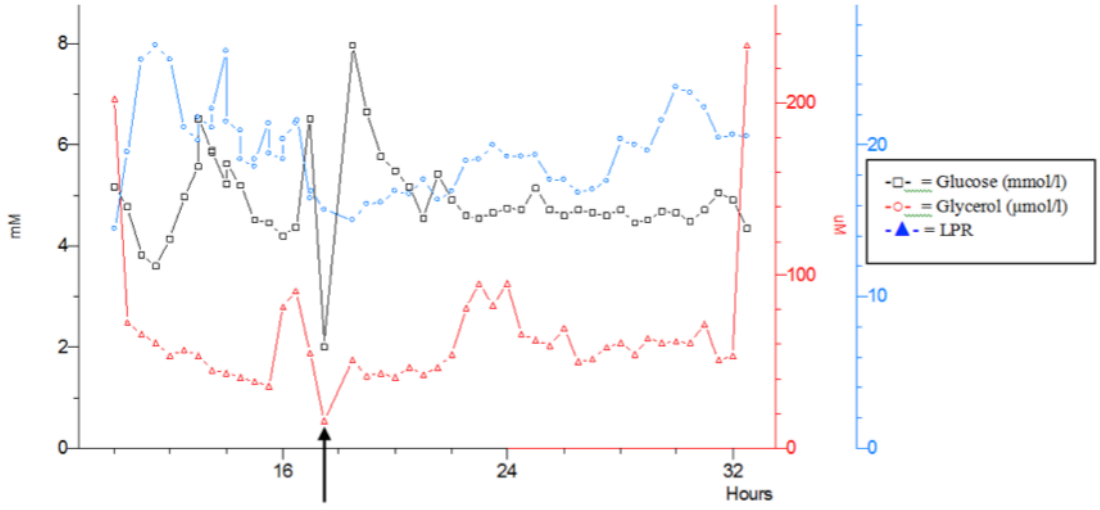
Patient S01

Figure 55: Graph to compare glucose with LPR and glycerol, in the Zone of Coagulation, for patient S01. Results within the boxed area were noted to be low across all analytes, and from a low volume sample.



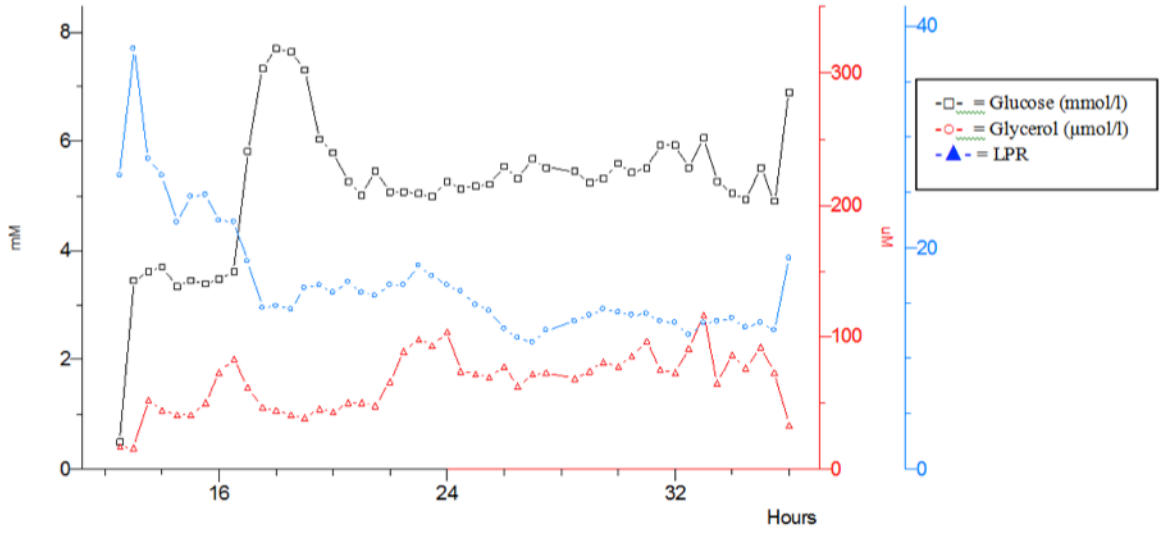
In Figure 55, there was a gentle decline in glucose levels to 16 hours, followed by a peak and another decline. The highest glucose reading was marginally outside the normal range, the rest being with the normal range. LPR was stable throughout, and well below ischaemic levels, apart from a single high reading at +24.5h. Glycerol was well within the normal range, and shows a cyclical pattern.

Figure 56: Graph to compare glucose with LPR and glycerol, in the Zone of Stasis, for patient S01. Arrow indicates a low volume sample.



Glucose levels in Figure 56 varied with time in the same manner as seen in the zone of coagulation (above) and unburned skin (below). Glucose results were within the normal range at all times, as were LPR values and glycerol. A single result (arrowed) showed low readings across all analytes and is likely to be anomalous. There was no evidence of an ischaemic environment.

Figure 57: Graph to compare glucose with LPR and glycerol, in the Unburned skin, for patient S01



Glucose in Figure 57 showed a similar course to Figure 55, but without the early peak. The initial low-normal level was followed by a peak at +18-20h, but was still within normal limits. LPR in this area was initially borderline ischaemic, falling rapidly as glucose levels increased. Glycerol was within normal range throughout, and there was evidence of the cyclical change seen in the other zones, with peaks at 17h and 23h, and an additional one at 32h.

Summary for S01

Biochemical parameters show little evidence of ischaemia in any of the zones. LDI (Plate 1), although suboptimal, gives an overall impression of improvement in wound perfusion over the study period, and there is no clear evidence of wound progression.

Patient S02

Figure 58: Graph to compare glucose with LPR and glycerol, in the Zone of Coagulation, for patient S02

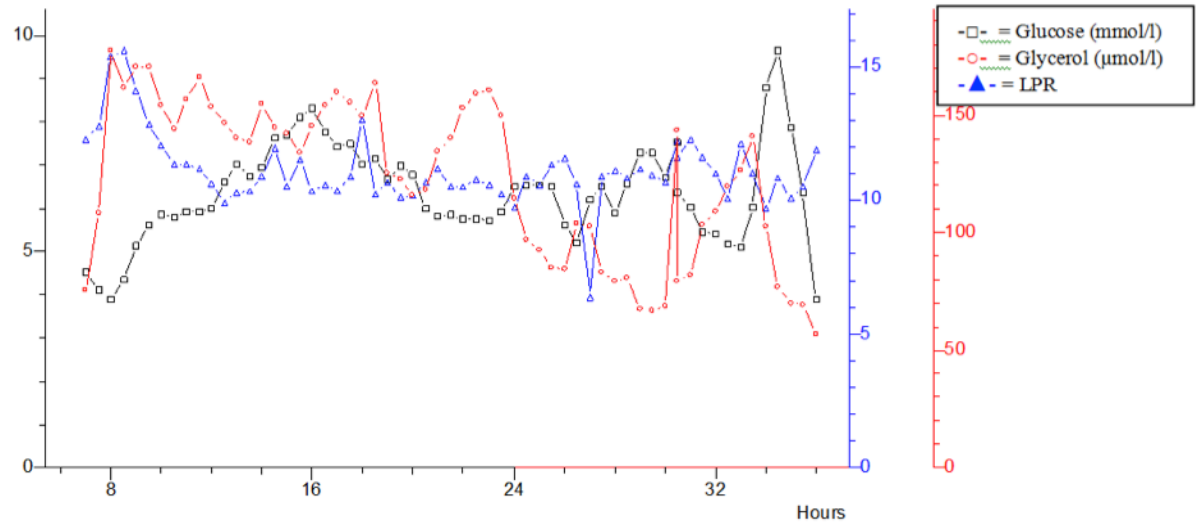


Figure 58 shows glucose levels within the normal range throughout the study period. This patient was recruited at 7.5 hours post injury and there was no initial peak of glycerol evident. LPR and glycerol show a stable course, with no ischaemia.

Figure 59: Graph to compare glucose with LPR and glycerol, in the Zone of Stasis, for patient S02

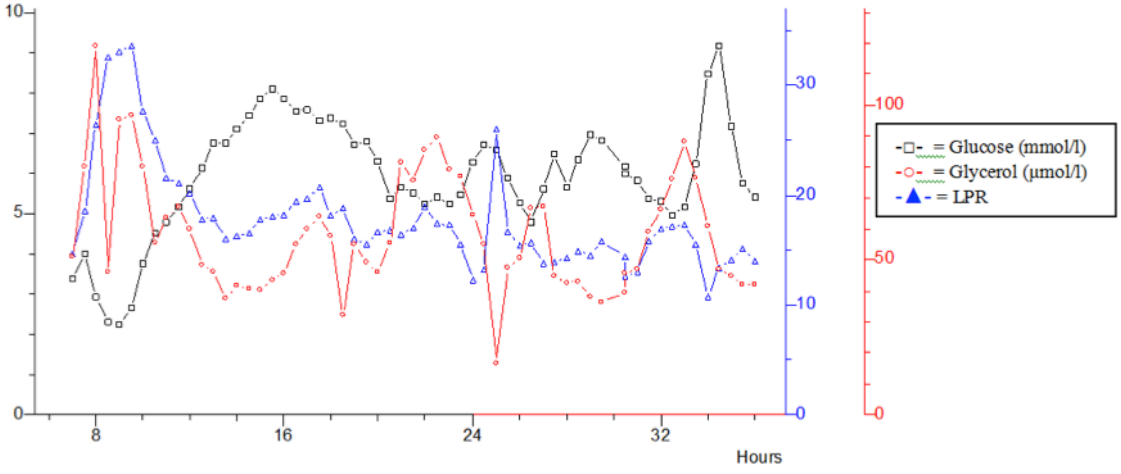
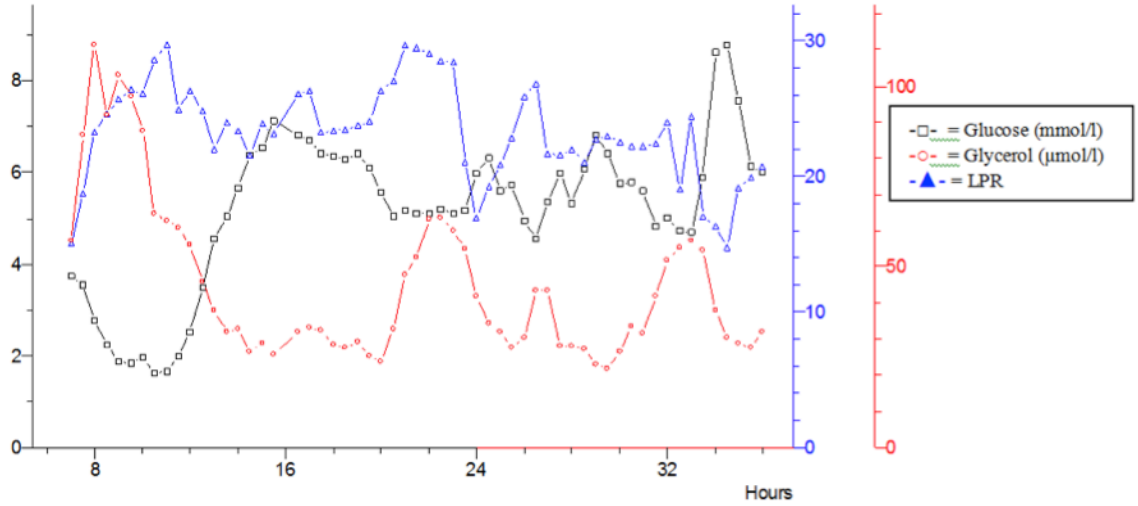


Figure 59 shows a low glucose for the first few hours with a corresponding elevation in LPR which entered the ischaemic range for 3 hours. Throughout the remainder of the study there

was no other notable event, and levels were non-ischaemic. There was no elevation in glycerol.

Figure 60: Graph to compare glucose with LPR and glycerol, in the Unburned skin, for patient S02



In Figure 60, there is a more profound reduction in glucose and elevation in LPR indicating an ischaemic environment lasting 4 hours for glucose and persisting for 12 hours for LPR. There was no corresponding rise in glycerol. A background cyclical trend in glycerol was seen.

Summary for S02

Biochemical parameters indicated some evidence of ischaemia for a short period in the zone of stasis (Figure 59), and for a lengthier period in the unburned skin (Figure 60). No corresponding rise in glycerol was seen. LDI showed some patchy areas of reduced perfusion of the burn, which were less well perfused at the end of the study, but had not progressed to full thickness injury (Plate 2).

Patient S03

Figure 61: Graph to compare glucose with LPR and glycerol, in the Zone of Coagulation, for patient S03

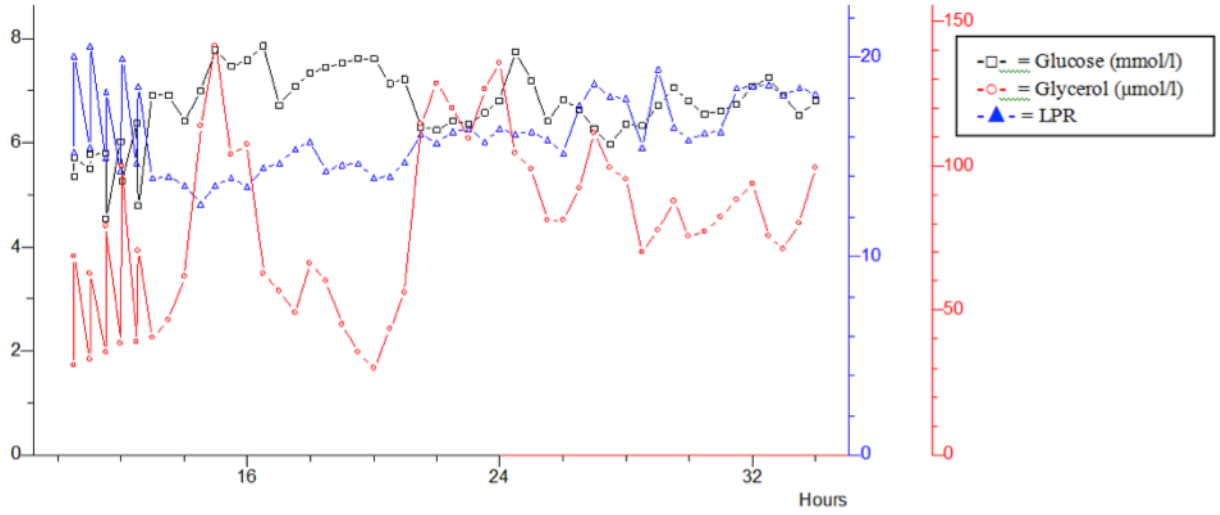


Figure 61 showed constant levels of glucose lactate and glycerol within their normal ranges throughout the study period. The saw-toothed appearance over the first few hours was seen in each of the analytes, which is suspicious of an artifactual cause.

Figure 62: Graph to compare glucose with LPR and glycerol, in the Zone of Stasis, for patient S03

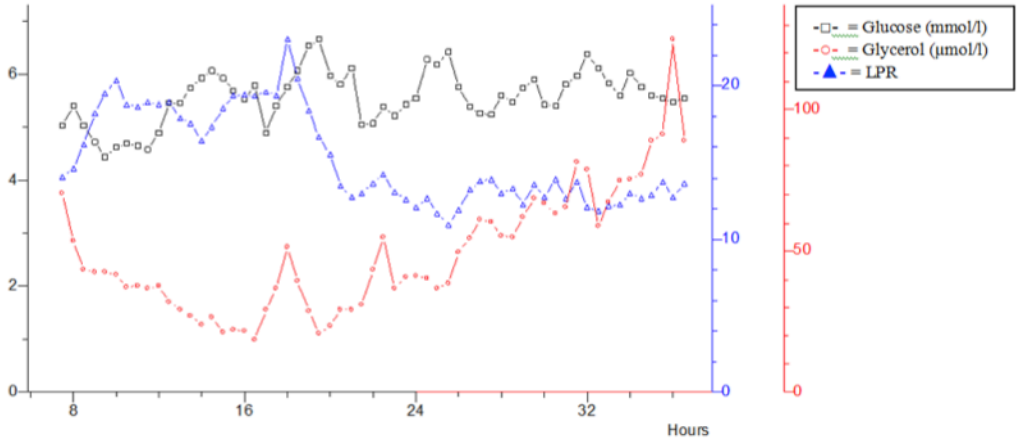


Figure 62 showed a steady state of glucose throughout the study period. LPR was within normal range for the entire time, as was glycerol. There is no evidence of ischaemia.

Figure 63: Graph to compare glucose with LPR and glycerol, in the Unburned skin, for patient S03

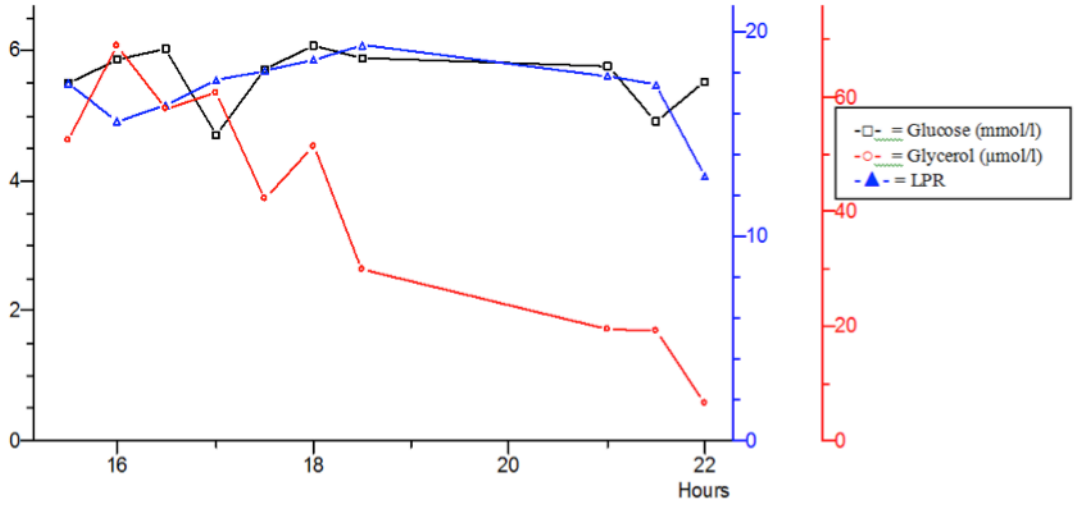


Figure 63 had incomplete readings due to a faulty probe. Readings which were present were all within the normal ranges.

Summary of S03.

All readings for all zones were within the normal range, although there was a significant amount of missing data for the unburned skin (Figure 63). The 48h LDI image was suboptimal as an incomplete image was captured, and the position of the hand was different. Nevertheless, there is a suggestion of wound progression over the dorsum of the hand (Plate 3).

Patient S04

Figure 64: Graph to compare glucose with LPR and glycerol, in the Zone of Coagulation, for patient S04

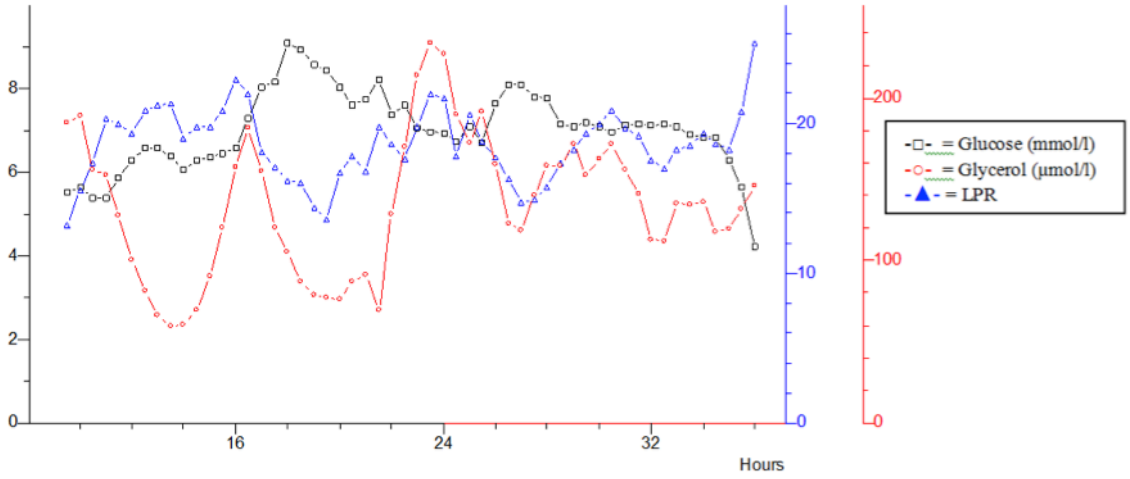


Figure 64 showed a steady state of parameters within the normal range. There was a baseline sinusoidal pattern to the glycerol results noted in several patients over all zones.

Figure 65: Graph to compare glucose with LPR and glycerol, in the Zone of Stasis, for patient S04

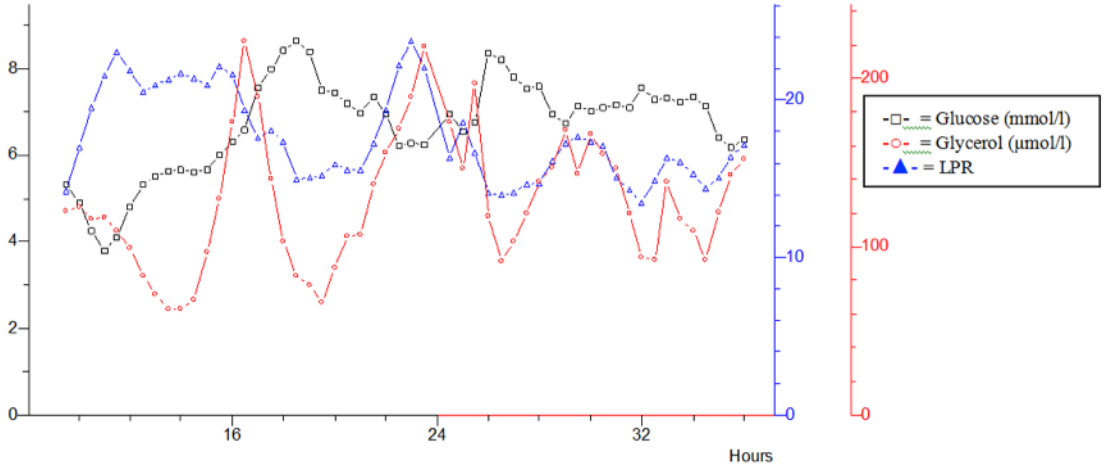


Figure 65 showed no evidence of ischemia, all parameters being within the normal range. An interaction between glucose and LPR could be seen here with a pattern of opposing peaks and

troughs. Each trough in glucose was accompanied by a peak in LPR and vice versa. The sinusoidal pattern in glycerol can be seen here.

Figure 66: Graph to compare glucose with LPR and glycerol, in the Unburned skin, for patient S04

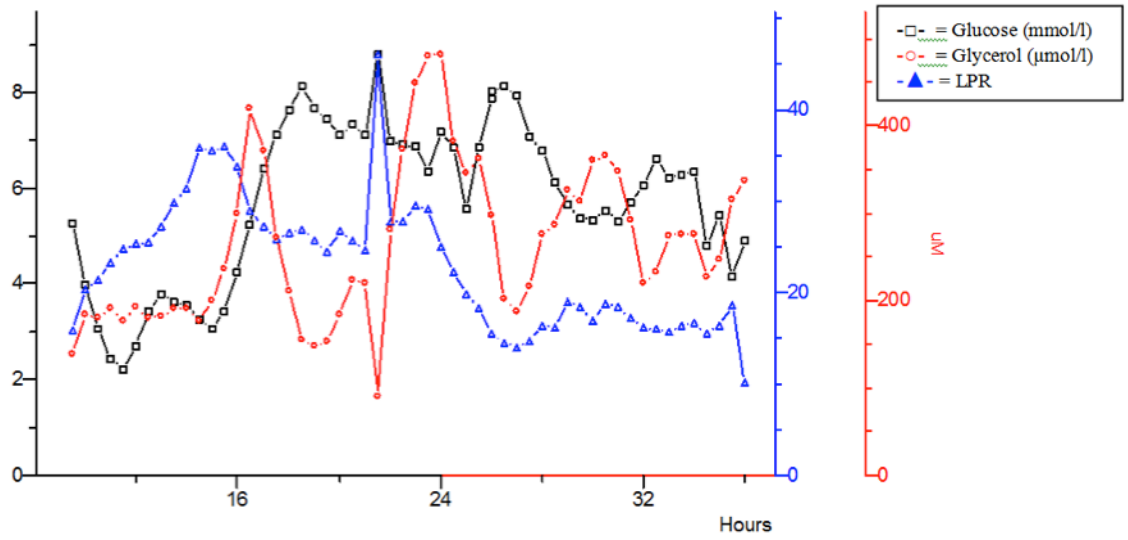


Figure 66 showed an initial ischaemic period lasting 8 hours for glucose and 14 hours for LPR. Glycerol levels were a little higher than in some of the other patients, but were not outside of the normal range. There was a cyclical pattern to the glycerol.

Summary of Patient S04

Ischaemia was longer lasting in the unburned skin of this patient (Figure 66), but not present in the zone of stasis (Figure 65). LDI showed evidence of wound progression (Plate 4).

Patient S05

Figure 67: Graph to compare glucose with LPR and glycerol, in the Zone of Coagulation, for patient S05. The scale for glycerol has been reduced, leaving the first three points off the graph, in order to make the baseline fluctuations visible

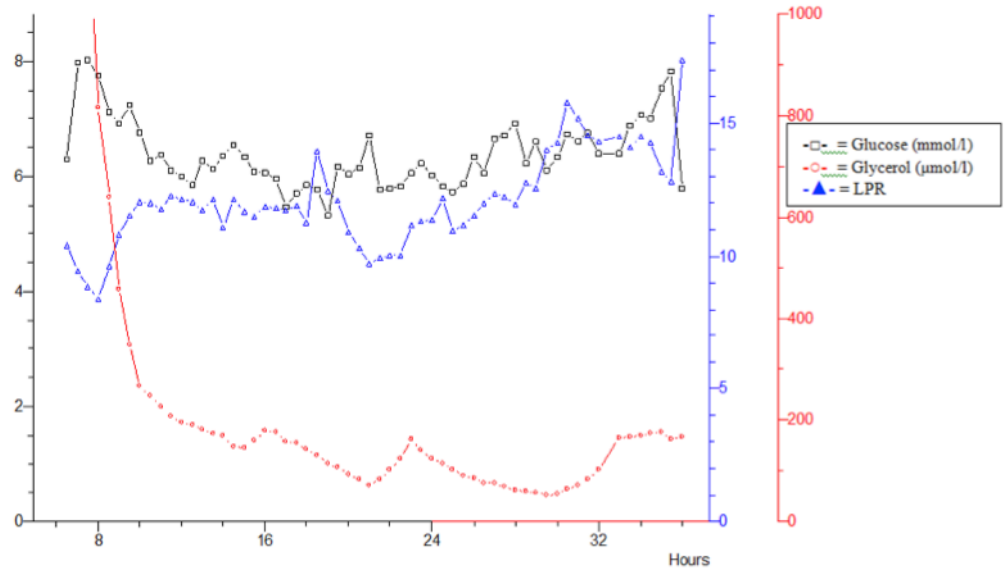


Figure 67 showed a high initial peak of glycerol, reaching above 1800µmol/l at 7h post-burn, and remaining above the normal range until 9 hours post-burn. Glucose and LPR did not show any evidence of ischaemia.

Figure 68: Graph to compare glucose with LPR and glycerol, in the Zone of Stasis, for patient S05

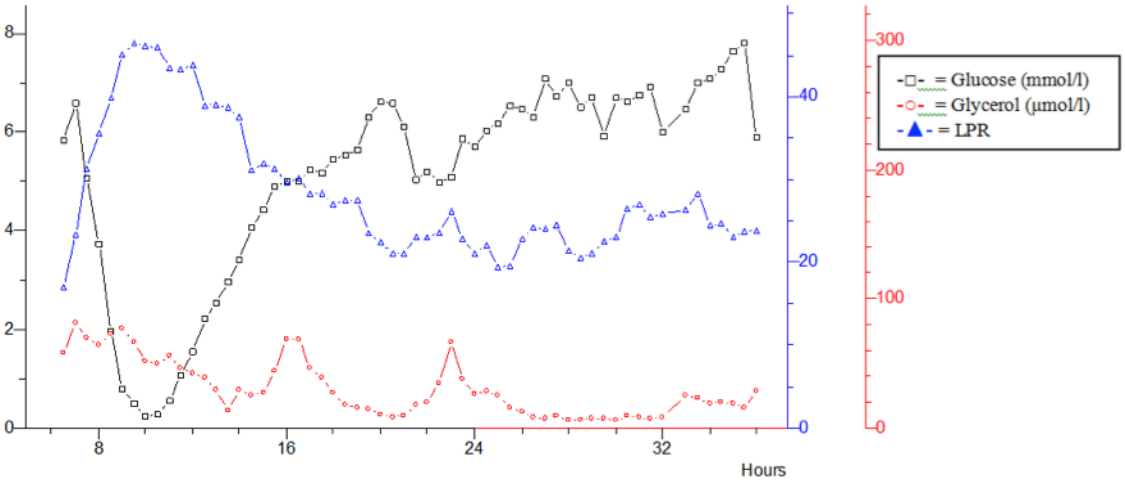


Figure 68 showed a profound trough in glucose which lasted for 6 hours and was accompanied by a rise in LPR which remained in the ischaemic range for 12 hours, and remained borderline ischaemic for the remainder of the study period. Glycerol changed cyclically, but remained within the normal range.

Figure 69: Graph to compare glucose with LPR and glycerol, in the Unburned skin, for patient S05. Arrowed results were under the detection limit for glycerol

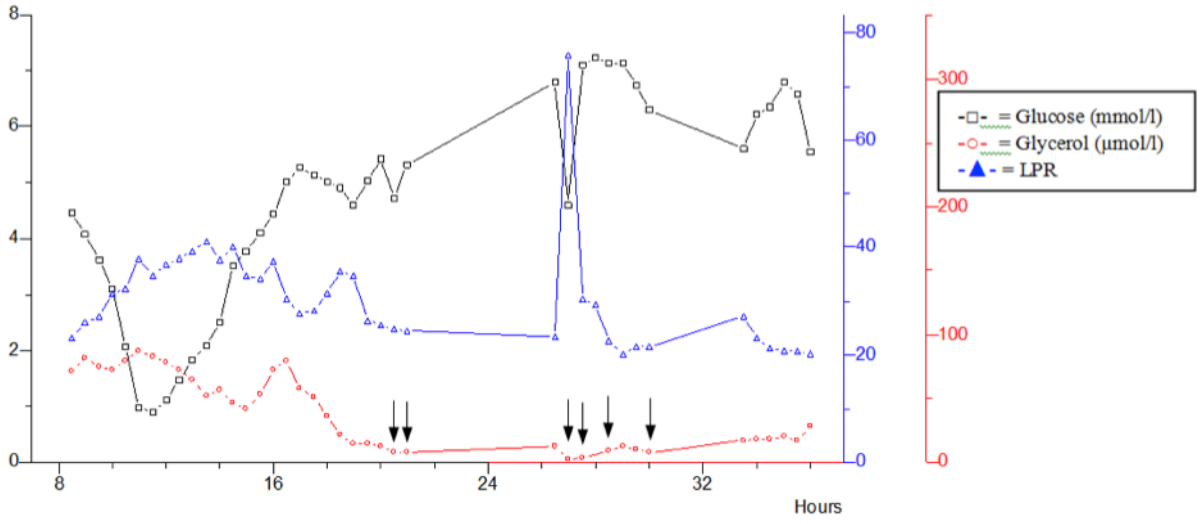


Figure 69 showed a trough in glucose and a peak in LPR which corresponded to that seen in the zone of stasis (Figure 68) but this normalised by 18 hours post-burn, lasting for 8 hours and 10 hours for glucose and LPR respectively. Glycerol remained unaffected.

Summary for S05

There was evidence of a significant period of ischaemia affecting the zone of stasis (Figure 68) and the unburned skin (Figure 69). LDI showed progression of the burn (Plate 5).

Patient S06

Figure 70: Graph to compare glucose with LPR and glycerol, in the Zone of Coagulation, for patient S06. Arrowed results were from a low volume sample and were consistently low across all analytes

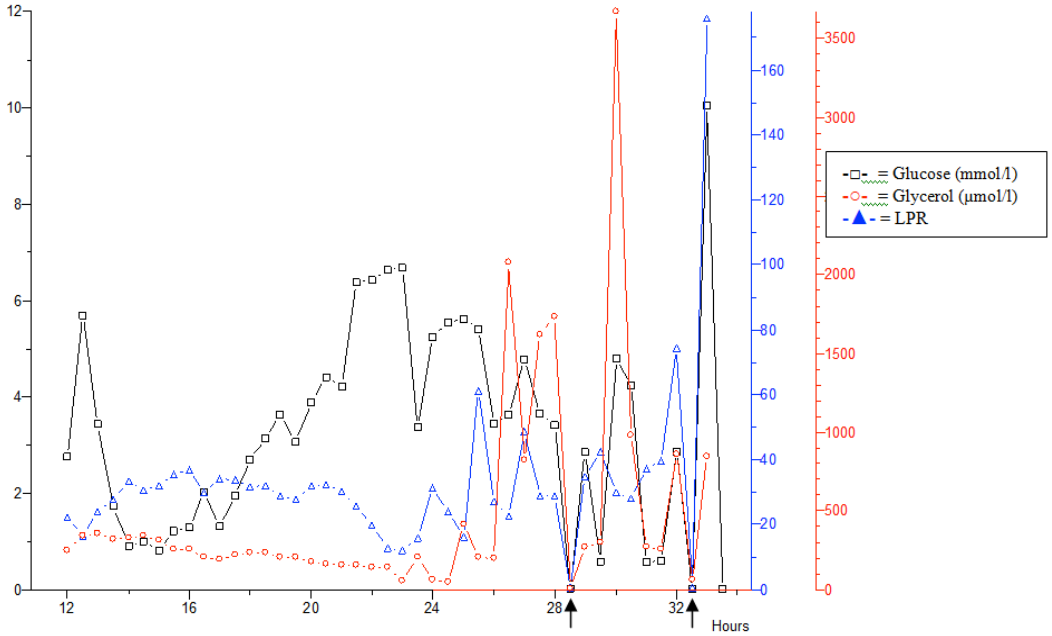


Figure 70 showed an initial fall in glucose with an elevation in LPR, which returned to non-ischaemic values by 22 hours post burn. Towards the end of the study there was a second ischaemic period with a fall in glucose, a rise in LPR and some peaks of glycerol outside of the normal range. This period was unusual as many of the high results were isolated with lower results interspersed, however the general trend seemed to be that of ischaemia.

Figure 71: Graph to compare glucose with LPR and glycerol, in the Zone of Stasis, for patient S06

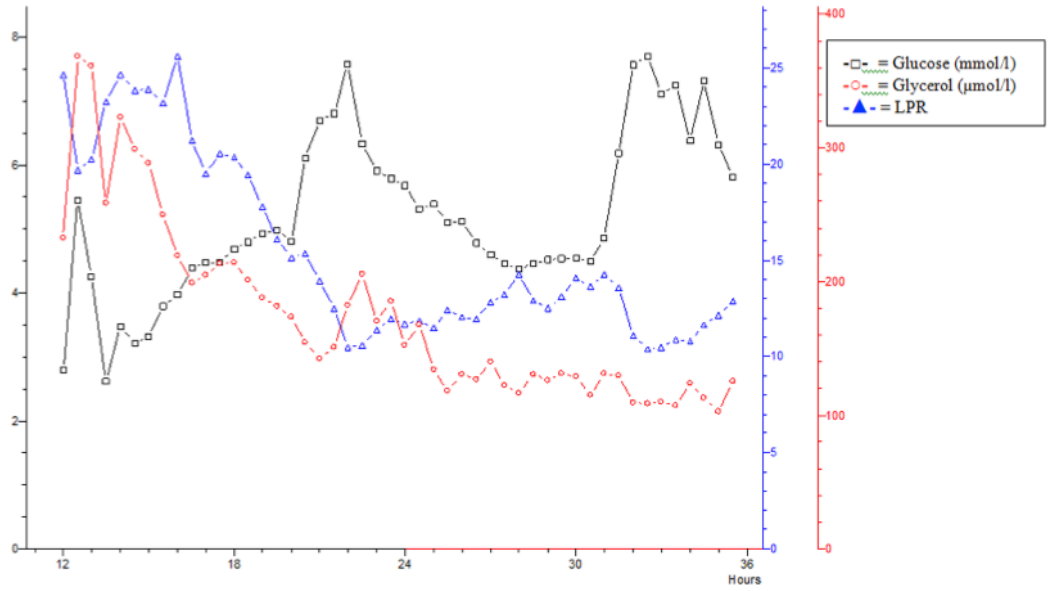


Figure 71 showed a short ischaemic period for glucose and a corresponding borderline-high peak in LPR. Glycerol remained in the normal range.

Figure 72: Graph to compare glucose with LPR and glycerol, in the Unburned skin, for patient S06

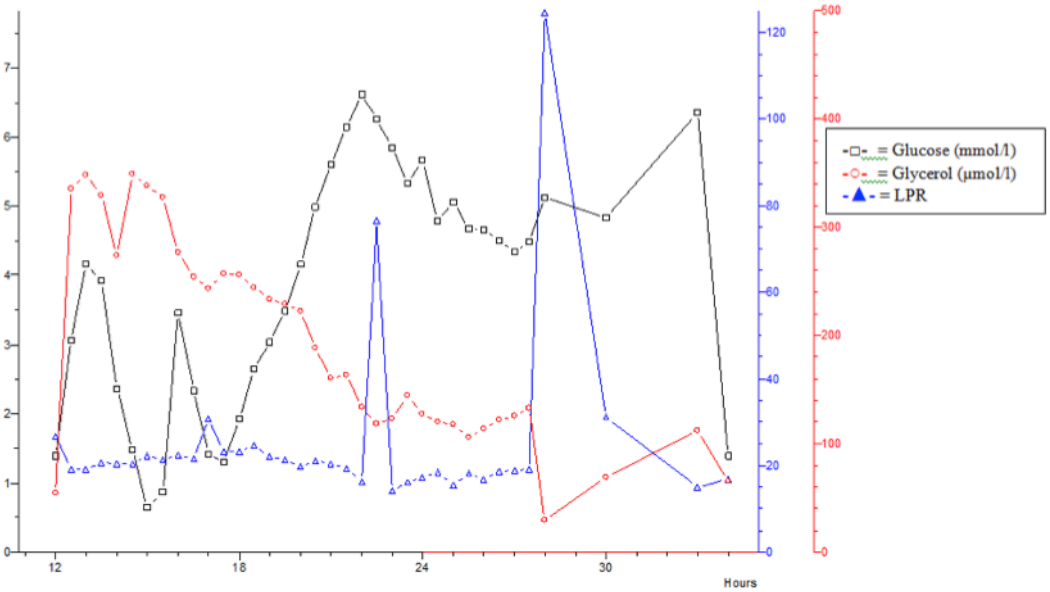


Figure 72 showed swinging glucose levels, but without the mirroring of LPR, which suggested a non-ischaemic cause. Two single high readings of LPR were unrelated to the other readings. Glycerol remained within the normal range.

Summary of S06

Zone of coagulation (Figure 70) showed evidence of ischaemic metabolism and an unusual event towards the end of the study period resulting in fitful production of glycerol. There was a short early ischaemic period in zone of stasis (Figure 71) which was not evident in the unburned skin (Figure 72). LDI evidence of wound progression in this patient was difficult to interpret because the initial scan was not informative. The final image showed a well perfused zone of stasis, and a poorly perfused zone of coagulation (Plate 6).

Patient S07

Figure 73: Graph to compare glucose with LPR and glycerol, in the Zone of Coagulation, for patient S07

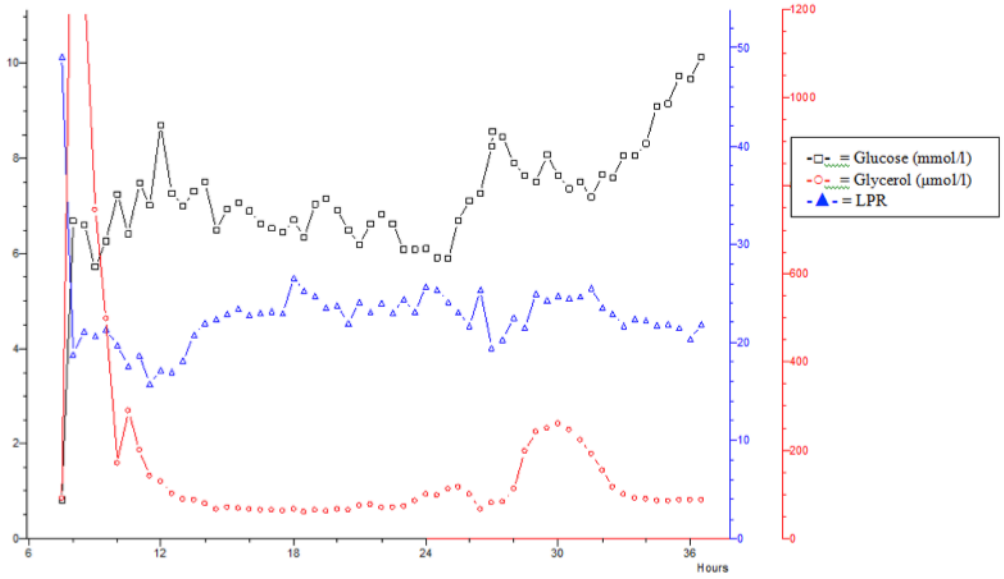


Figure 73 showed non-ischaemic levels of glucose but borderline-ischaemic levels of LPR throughout. There was an initial high peak of glycerol of over 1700 µmol/l at 8 hours post burn, but for the remainder of the time glycerol remained within normal limits.

Figure 74: Graph to compare glucose with LPR and glycerol, in the Zone of Stasis, for patient S07

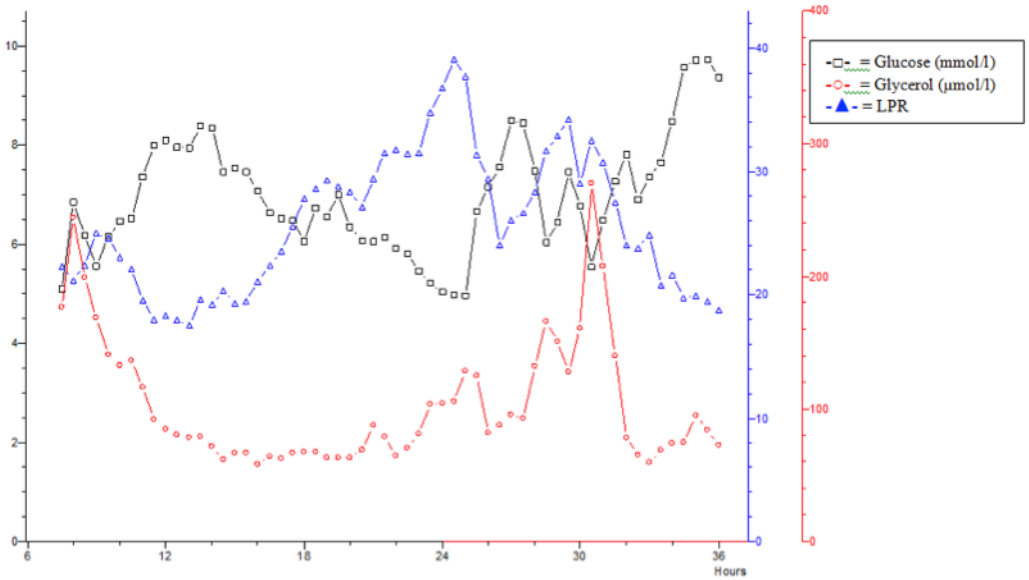


Figure 74 showed normal levels of glucose, but there was evidence of ischaemia from LPR readings between 16 and 30 hours post burn. There was no elevation in glycerol outside of the normal range.

There were no results for unburned skin in S07 as the patient had pulled out the probe.

Summary for S07

There was evidence of prolonged ischaemia in the zone of stasis, but no significant rise in glycerol (Figure 74). There was evidence of wound progression on LDI (Plate 7).

Patient S08

Figure 75: Graph to compare glucose with LPR and glycerol, in the Zone of Coagulation, for patient S08

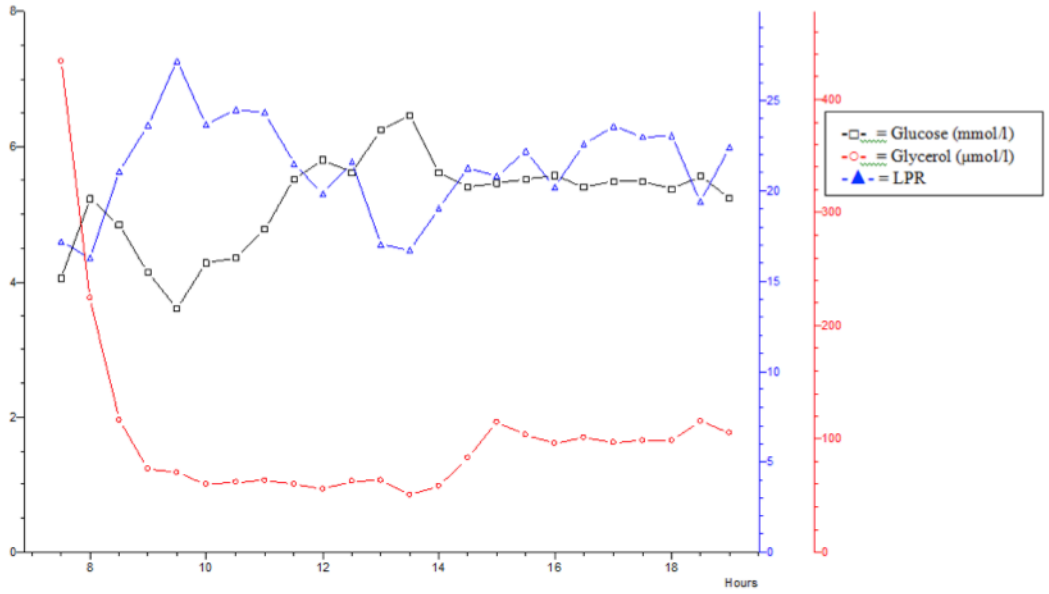


Figure 75 showed glucose within the normal range throughout. An interaction between LPR and glucose was evident but neither remained in the ischaemic range for more than half an hour. Glycerol showed an early high peak of over 400 µmol/l at 7.5 hours post injury, but was within the normal range for the remainder of the time.

Figure 76: Graph to compare glucose with LPR and glycerol, in the Zone of Stasis, for patient S08

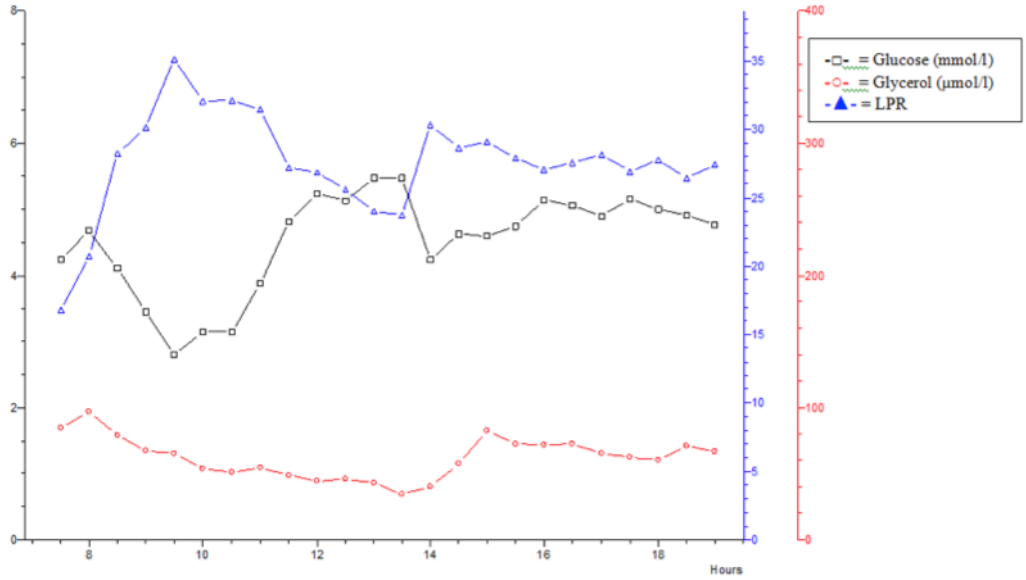


Figure 76 showed a slight decline in glucose in the first few hours after recruitment, and this was accompanied by a rise in LPR which remained within the ischaemic range for 4 hours and was borderline ischaemic until the premature end of the study period.

Figure 77: Graph to compare glucose with LPR and glycerol, in the Unburned skin, for patient S08

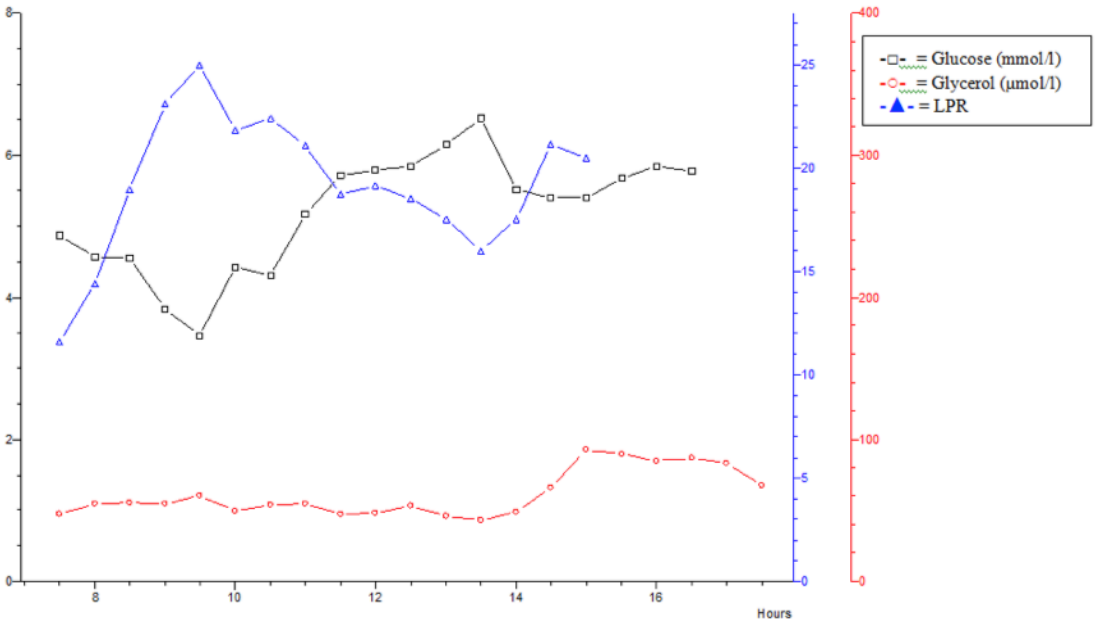


Figure 77 showed normal glucose, LPR and glycerol levels throughout the shortened study period.

Summary for S08

The study period was curtailed by the patient being taken to theatre for burn excision. There was evidence of an ischaemic environment in the zone of stasis (Figure 76). LDI results could not be correlated as there was no LDI image for the end of the study period (Plate 8).

Patient S09

Figure 78: Graph to compare glucose with LPR and glycerol, in the Zone of Coagulation, for patient S09. Arrows indicate low volume samples with low results for all analytes (full arrow) or some results (dashed arrow)

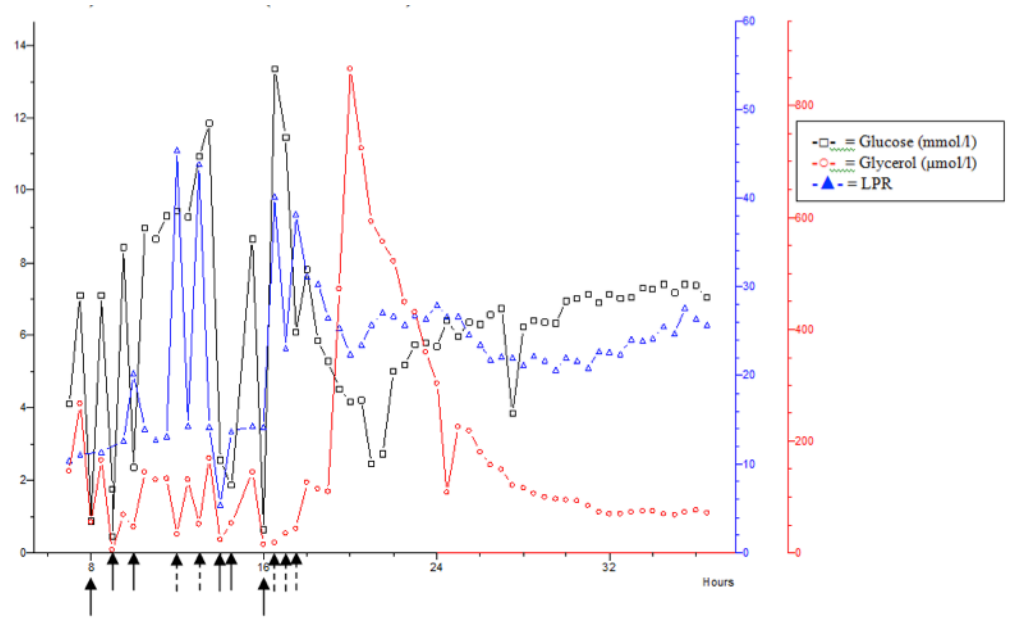


Figure 78 had many intermittent low readings for all analytes during the first few hours. These were all low volume samples (arrowed), indicating possible spurious results. On observing the other zones, it appeared that glucose remained in the normal or high range within the first 12 hours. LPR remained within the normal range within this time, but did become moderately elevated at and beyond 16 hours following injury. A peak of glycerol occured at 19h, the time at which this patient was taken to theatre.

Figure 79: Graph to compare glucose with LPR and glycerol, in the Zone of Stasis, for patient S09. Arrows indicate low volume samples with low results for all analytes (full arrow) or some results (dashed arrow)

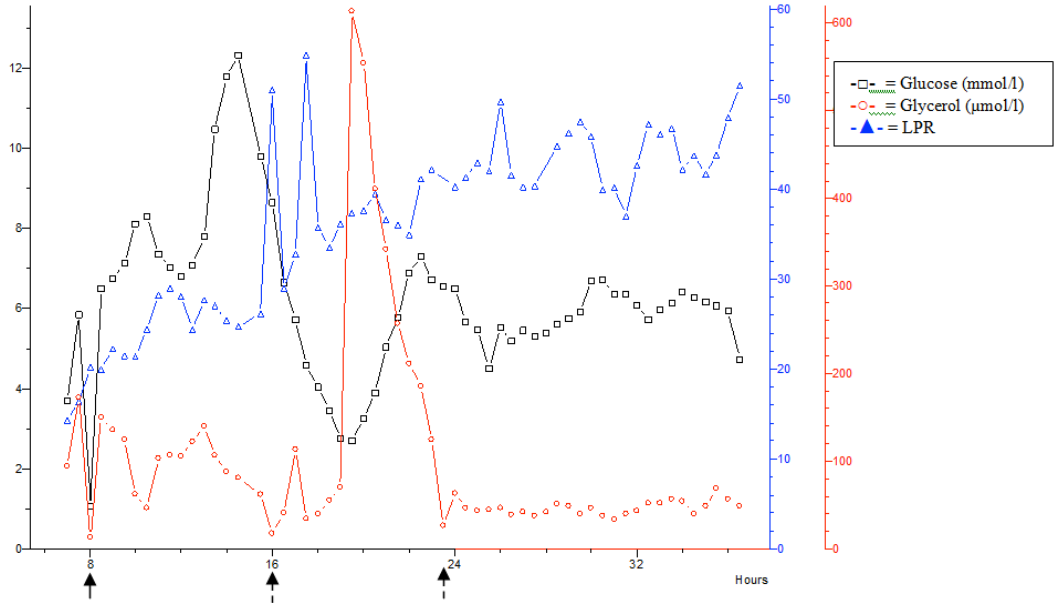


Figure 79 showed elevated glucose for four hours, with a peak at 14 hours post-burn. LPR showed a steadily increasing course which reached ischaemic levels at 16 hours and lasted until the end of the study period at 36 hours. There was a high peak of glycerol at 20h during the theatre episode.

Figure 80: Graph to compare glucose with LPR and glycerol, in the Unburned skin, for patient S09

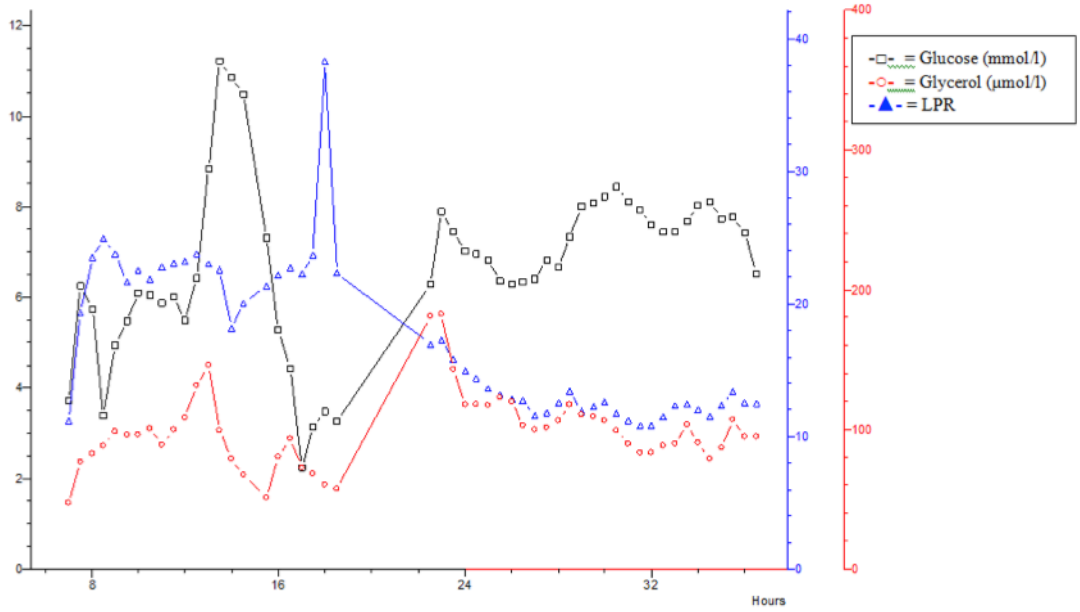


Figure 80 showed a similar pattern of glucose to the other two zones. LPR did not become ischaemic and showed a downward trend after 20h. Glycerol remained within normal limits throughout although data was missing from this probe during the theatre episode due to it being displaced when the patient was positioned on the table, and reinsertion was not possible until after the procedure had finished.

Summary of S09

This patient was known to be diabetic, which explains the high levels of glucose not seen in the other patients. There was an increase in LPR in the zone of stasis which commenced before, and continued after the theatre episode, indicating ischaemia in this zone only (Figure 79). Interestingly, the opposite trend occurred in the unburned skin, which became less ischaemic following theatre (Figure 80). A peak of glycerol occurred at the time of theatre in zones of coagulation (Figure 78) and stasis (Figure 79). Assessment of progression on LDI was inconclusive in this patient due to the poor initial image (Plate 9).

Patient S10

Figure 81: Graph to compare glucose with LPR and glycerol, in the Zone of Coagulation, for patient S10. Arrow indicates low volume samples with low results for all analytes. Diamond arrows indicate very high volume samples due to pump malfunction.

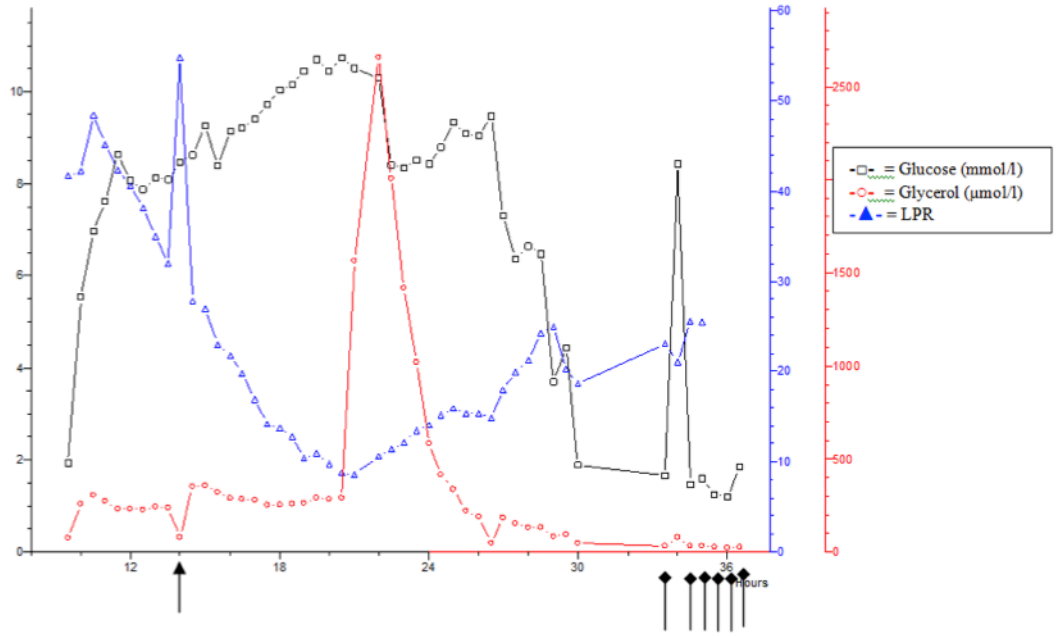


Figure 81 showed higher levels of glucose than other patients initially. An initially ischaemic LPR level dropped within the first few hours to reach non-ischaemic levels by 15 hours post-burn. From 20h it then steadily increased, reaching borderline ischaemic levels again by the end of the study period. There was a single peak of glycerol at 21h, which coincided with a theatre episode.

Figure 82: Graph to compare glucose with LPR and glycerol in the Zone of Stasis, for patient S10. Arrows indicate low volume samples with low results for all analytes (full arrows) or some analytes (dashed arrows). Diamond arrow indicates very high volume samples

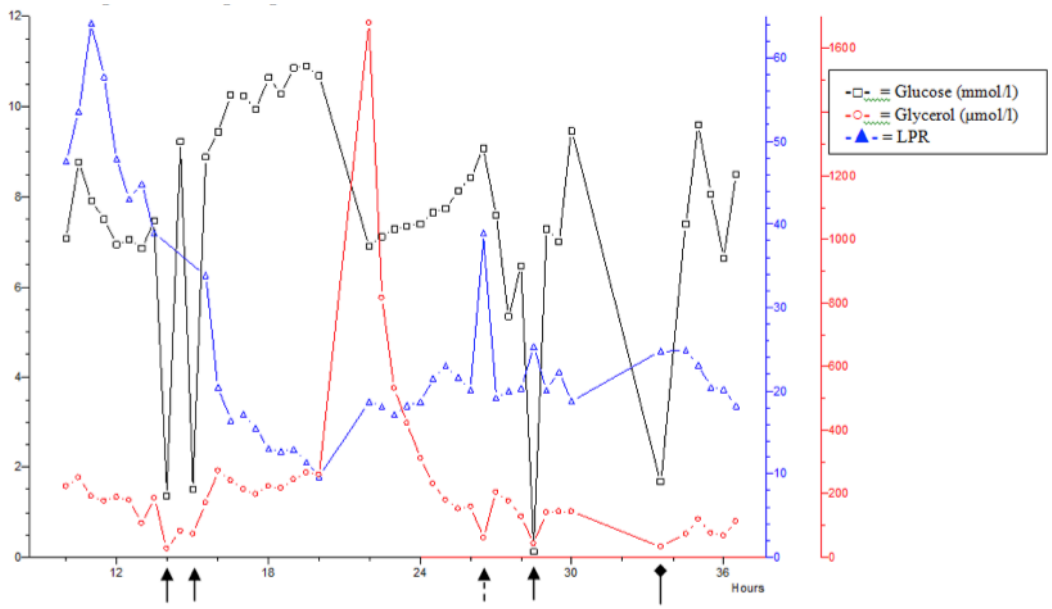


Figure 82 showed high glucose levels, similar to the zone of coagulation, and an initially high LPR which dropped into the normal range by 15h, and then steadily climbed again after 22h. A peak of glycerol was seen at 21h.

Figure 83: Graph to compare glucose with LPR and glycerol, in the Unburned skin, for patient S10. Arrows indicates low volume samples with low results for all analytes (full arrow), or some analytes (dashed arrow). Diamond arrow indicates very high volume sample

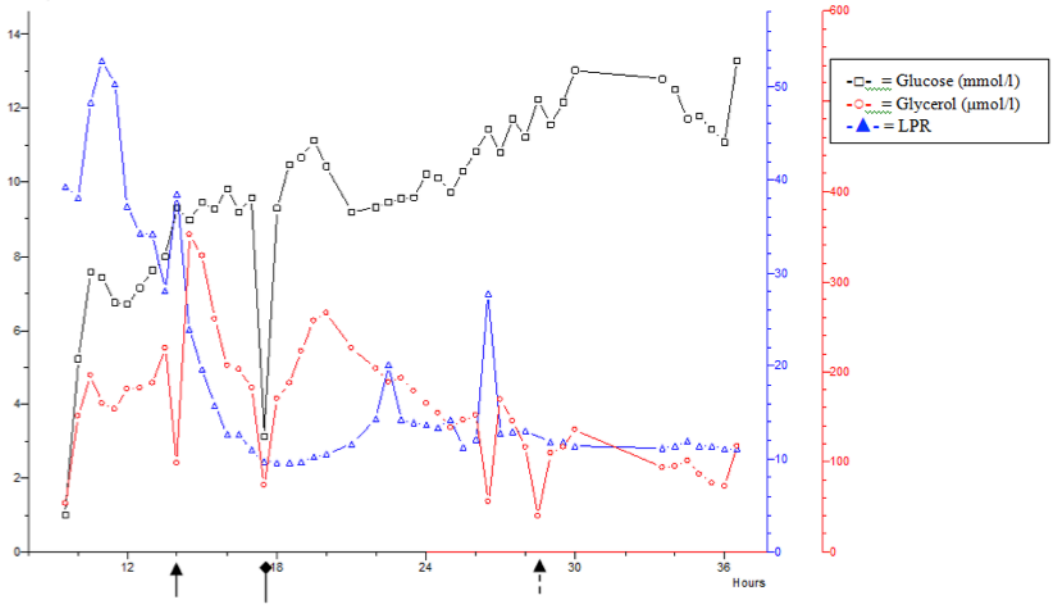


Figure 83 showed low glucose which climbed rapidly into the high range by 14 hours post burn. LPR was initially high, but rapidly fell to non-ischaemic levels by 15 hours, and remained there. There was no peak in glycerol in this zone which remained within the normal range throughout the study.

Summary of S10

Initially high glucose in zones of coagulation (Figure 81) and stasis (Figure 82) was unusual. Interestingly, the high glucose came together with high LPR values, which could indicate hypoxia, but not necessarily ischaemia. LPR and glycerol appeared to be affected, in both of these zones, by the visit to theatre at 20h, but the unburned skin is not (Figure 83). LDI evidence of progression was borderline, with no progression at the site of the Zone of stasis probe (Plate 10).

4.4 Results of Clinical Study 3 – Large Burns

Two patients were recruited into this group over the time period. This small number was mainly due to the fewer numbers of patients presenting with large burns. Two patients with large burns could not be recruited because a study on smaller burns was already underway at the time of their admission, so the microdialysis pumps were already in use. Large burn patients are analysed separately, and compared to the small burn group.

4.4.1 Patient Details

Patient L01 was aged 31 years and sustained a 35%TBSA flame burn to her face, arms, chest and back in a house fire. There was evidence of inhalational injury and suspicion of cyanide poisoning. Probes A and B were sited on the chest, and probe C in the left leg. Probe insertion was 15 hours after injury, and took place in theatre, whilst the patient was undergoing escharotomies to the arms. Serum lactate was >3 mmol/l post-operatively, and took some time to normalise. Hypovolaemia was noted post-operatively, requiring 2.5 litres of colloid in addition to calculated crystalloid requirement in the last 12 hours of the resuscitation period. She was on noradrenaline throughout the study period.

Patient L02 was aged 37 years and had a 50% flame burn to his arms, chest, legs, face and neck. He commenced the study 7 hours after injury. Probes were inserted whilst he was in theatre, during which he had an escharotomy to his right arm, and excision of the thigh and left arm burns. Propofol was used for induction of anaesthesia. Probes were sited on the right arm, as this was the only area of full-thickness burn which was not excised during the theatre visit. He was nursed on ITU throughout the study, and at 44 hours post-burn he had a further theatre visit

for change of dressings. During this theatre episode, no propofol was given as the patient was already sedated with morphine and midazolam. Although the study had been completed by this time, measurements were restarted for the theatre visit, at the end of which the probes were removed in order for burn excision to take place. There was no opportunity to take laser Doppler images of this patient.

4.4.2 Biochemical Characteristics of Zones

4.4.2.1 **Glucose** (Figure 84, Figure 85)

Glucose in three zones showed a slow upward trend in patient L01 from around 4 mmol/l at the start of the study to around 6 mmol/l by the end (Figure 84). This was consistent with the results from the small burns (Figure 33). Patient L02 (Figure 85) showed an initial peak, then trough in the glucose results for all zones, but most marked in Zone C. This was different from all of the small burn patients. From 20 hours onwards, there was a slow climb in Zones A and B, more in keeping with the patterns seen in the small burn patients. In Zone C, however there was a more pronounced trough at 20 hours, a second peak and trough by 28 hours, and then a rapid climb to join the levels of A and B by 32 hours.

Figure 84: Graph to show changes in glucose with time for each zone in patient L01

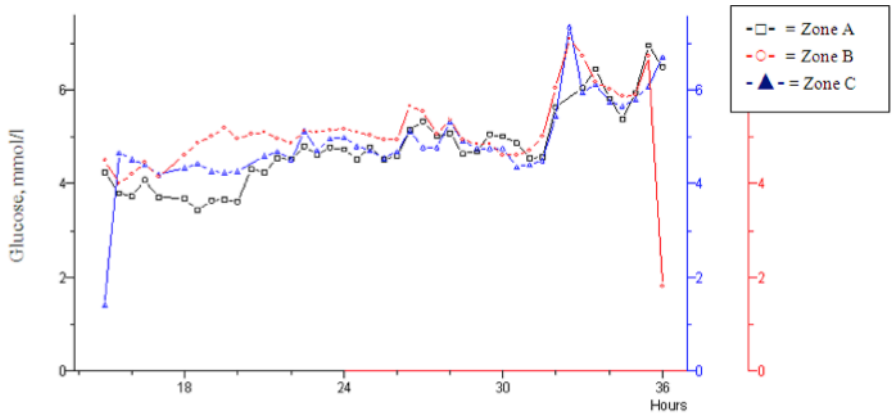
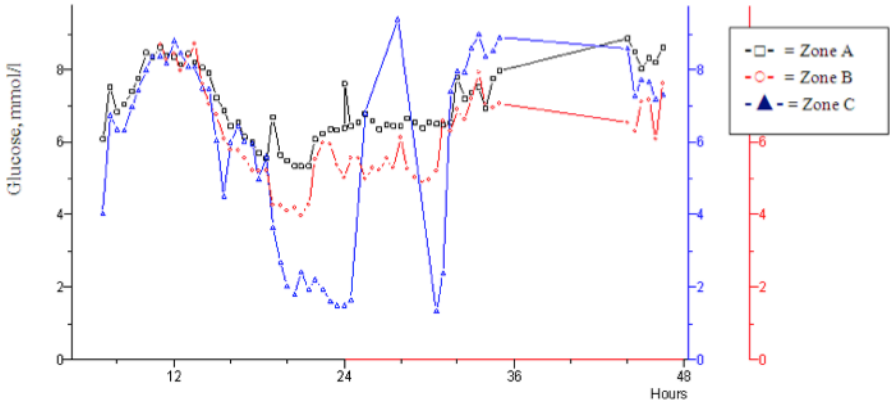


Figure 85: Graph to show changes in glucose with time for each zone in patient L02



4.4.2.2 **Lactate Pyruvate Ratio** (Figure 86, Figure 87)

For patient L01 (Figure 86), the LPR in Zone A is initially high, falling to non-ischaemic levels by 21 hours, but remaining higher than the other zones. LPR in zones B and C remains below 25 throughout, except for the initial reading for zone B. This was in contrast to the overall pattern for the small burns, where Zones B and C showed the higher LPR readings (Figure 36). Patient S10 was the only patient in the small burns group to show high LPR readings in Zone A at the beginning of the study period (Figure 81).

In patient L02 (Figure 87), all three zones had LPR readings which remained below 25 for the entire study period. There was a slow climb in LPR in zone B throughout the study, and this zone had the highest readings for the majority of the time. The results here were entirely consistent with results from patients with small burns.

Figure 86: Graph to show changes in LPR with time for each zone in patient L01

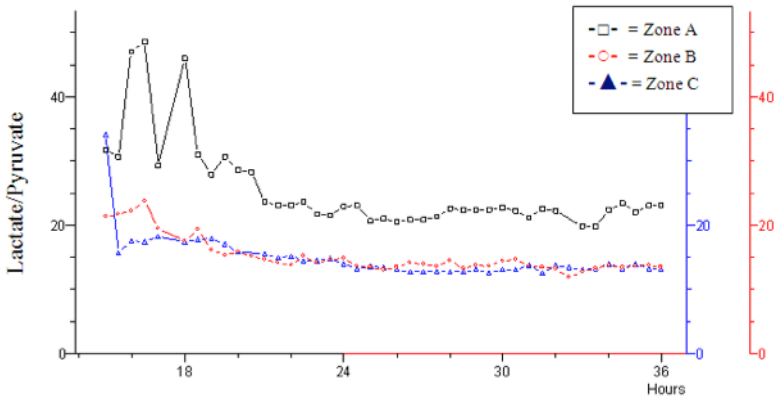
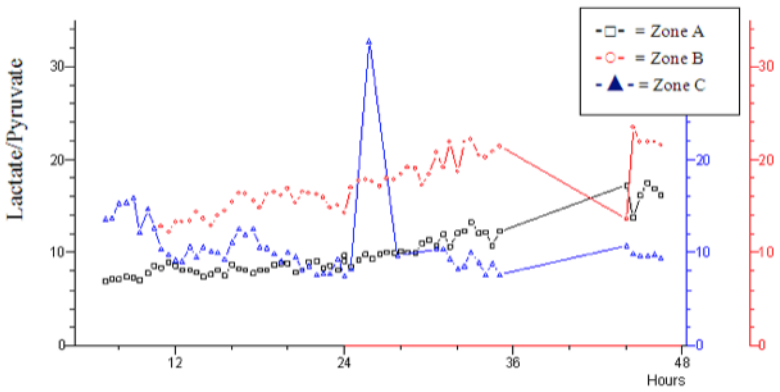


Figure 87: Graph to show changes in LPR with time for each zone in patient L02



4.4.2.3 **Glycerol** (Figure 88, Figure 89)

Patient L01 (Figure 88) had an initial peak of glycerol in Zone A at recruitment, followed by a fall, then a second, higher peak at 18 hours post-burn. This early peak could correspond to that seen in some of the small burn patients recruited early in the post-burn period (Figure 67, Figure 73, Figure 75), although as this patient was not recruited until 15 hours post-burn. It is more likely that the peak seen here is a reflection of the theatre episode during which the probes were inserted, and as such corresponds to the peak seen in S09 (Figure 78, Figure 79) and S10 (Figure 81, Figure 82). There is a subtle corresponding peak in Zone B. An additional peak in glycerol in Zone A, during her hypovolaemic episode in ITU, is not mirrored in Zone B as significantly as it was in patients S09 and S10 during their theatre visits. For Zones B and C there was a subtle mirroring of the two peaks and troughs, but with measurements not exceeding 400 μmol/l. There was then a gradual decline with time in these zones.

Patient L02 (Figure 89) showed an initial high peak in glycerol in Zone A, which declined with time. Since patient L02 was recruited within 7 hours of the burn, this is consistent with the peak seen in Zone A of those small burn patients who were recruited early (Figure 67, Figure 73, Figure 75). It may have been added to by the theatre visit. Unfortunately samples from Zone B contained blood in the initial stages, so had to be discarded, thus no comment can be made as to the presence or absence of a corresponding peak in Zone B during this time. The second theatre visit at 44h did not cause a glycerol peak in any zone.

Figure 88: Graph to show changes in glycerol with time for each zone in patient L01

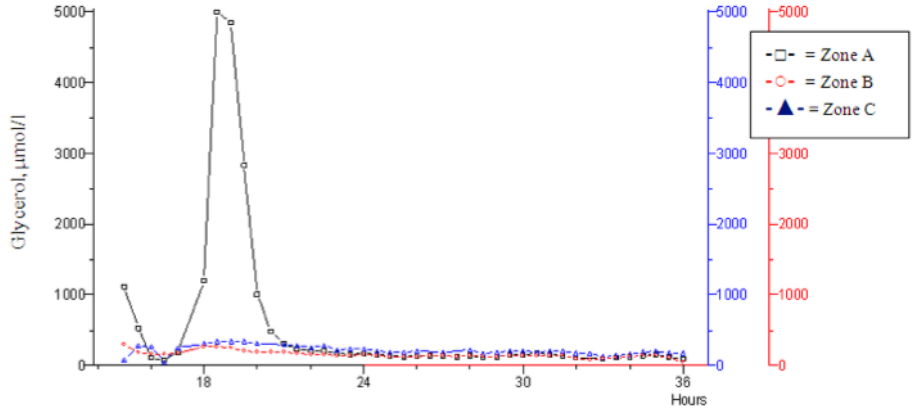
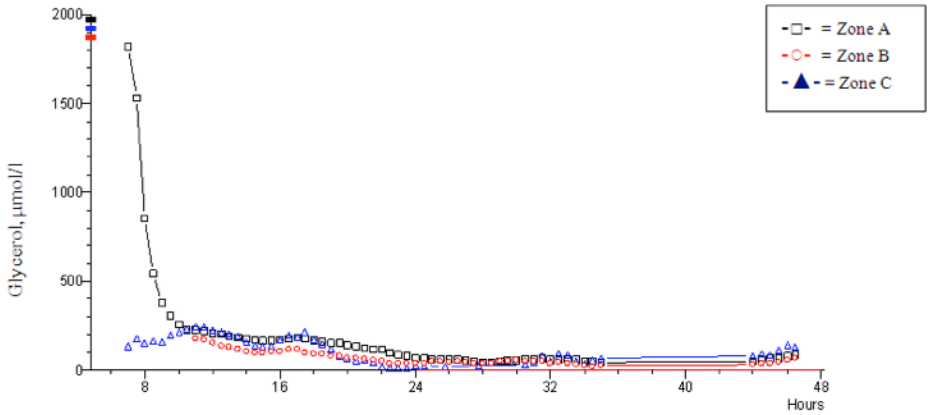


Figure 89: Graph to show changes in glycerol with time for each zone in patient L02



4.5 Amino Acid profiles with time and by zone for all burn patients

There was sufficient microdialysate to analyse the amino acids on eight patients. These were S02, S04, S05, S06, S08, S09, S10, L01. Age range of these patients was 19-68

years, with 3 male and 5 female patients. Percentage total body surface area (%TBSA) burn was 4 - 35%. Catheter insertion occurred at 6.5 – 12h post burn.

4.5.1 General Trends

The following amino acids and related amines exhibited no statistically significant changes in levels in the 28 hours post-burn injury, either with respect to time or to the location zone of the catheter: alanine, β-alanine, ethanolamine, γ-aminobutyric acid (GABA), glutamine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, taurine, threonine, tryptophan, tyrosine and valine (data not shown).

4.5.1.1 Excitatory amino acids

Two-way ANOVA revealed a significant effect of both time ($p<0.0001$) and catheter location ($p=0.0008$) on aspartate levels (Figure 90). Aspartate decreased over time at all three locations but the rate of decrease was slower in Zones A and B. In Zones B and C, the level of aspartate at 14h was significantly lower than that at 6h (Student's t-test, $p<0.05$) and remained so thereafter. In contrast, in Zone A, aspartate levels did not significantly differ from the 6h time point at any stage of the period of microdialysis.

With respect to glutamate, a tendency towards a gradual decrease over the 28-hour monitoring period was also observed at all three sites, most notably in Zones B and C (Figure 91). However, this decrease did not achieve statistical significance at any time point. Probe location had no effect on the glutamate levels measured.

Figure 90: Barchart to show changes in aspartate levels with time post-injury for each zone. Mean and Standard Error bars are shown. * p<0.05 in comparison to 6-8h level for zone of stasis. # p<0.05 in comparison to 6-8h level for normal skin. Coagulation zone = Zone A; Stasis zone = Zone B; Normal skin = Zone C

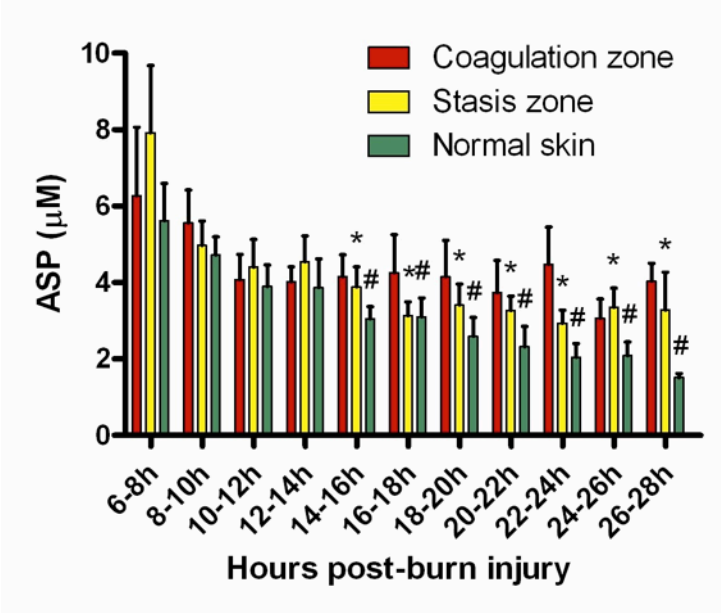
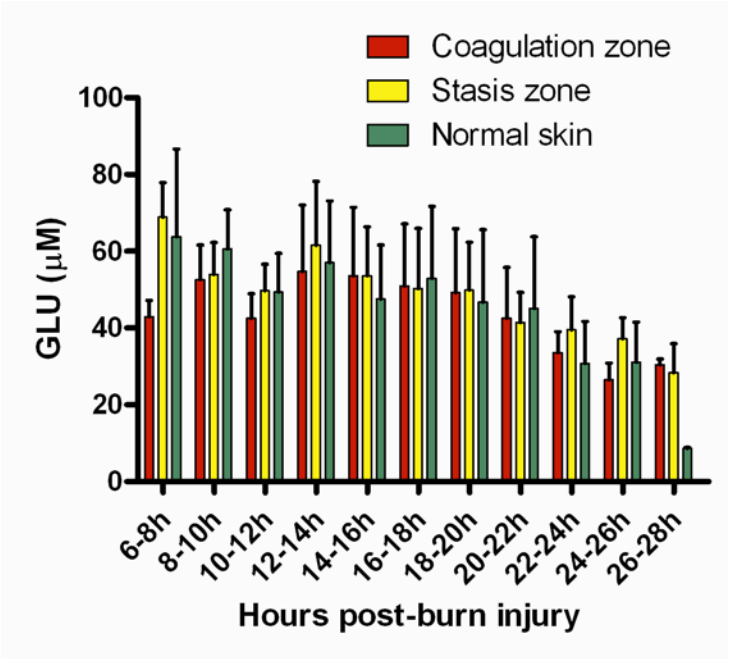


Figure 91: Barchart to show changes in glutamate levels with time post-injury for each zone. Mean and Standard Error bars are shown. Coagulation zone = Zone A; Stasis zone = Zone B; Normal skin = Zone C



4.5.1.2 Arginine and metabolites

Two-way ANOVA demonstrated no association between time after burn injury and levels of arginine, or of its amino acid metabolites, citrulline and ornithine. However, probe location had a highly significant effect on arginine levels ($p=0.0004$) and ornithine ($p=0.0068$) levels, with arginine being highest in Zone A, and lowest in Zone C, and the reverse being true for ornithine. Citrulline also tended to show lower levels in the Zone A, although this did not reach statistical significance. Figure 92 shows the time course of the ratio of ornithine to arginine levels as an indicator of arginase activity whilst Figure 93 illustrates the ratio of citrulline to arginine as a measure of nitric oxide synthase activity. In both cases, examination of the Zone C data reveals that the ratios increased over the first 18-20h, reflecting utilization of arginine, before then declining to basal levels. In Zone B, both ratios remain fairly constant throughout, whilst in Zone A, there is a tendency for the ratios to fall, although this did not reach statistical significance. Two-way ANOVA revealed a highly significant effect of probe location on ornithine to arginine ratio ($p=0.0003$) and a lesser effect of probe location on citrulline to arginine ratio, approaching significance ($p=0.06$). Post-hoc Bonferroni tests suggested that the ratio of ornithine to arginine in Zone C was higher than in Zone A at 10h, 12h and 14h post-implantation, although this difference did not quite reach statistical significance ($p>0.05$ but < 0.10).

Figure 92: Barchart to show changes in ornithine/arginine ratio with time post-injury for each zone. Mean and Standard Error bars are shown. Coagulation zone = Zone A; Stasis zone = Zone B; Normal skin = Zone C

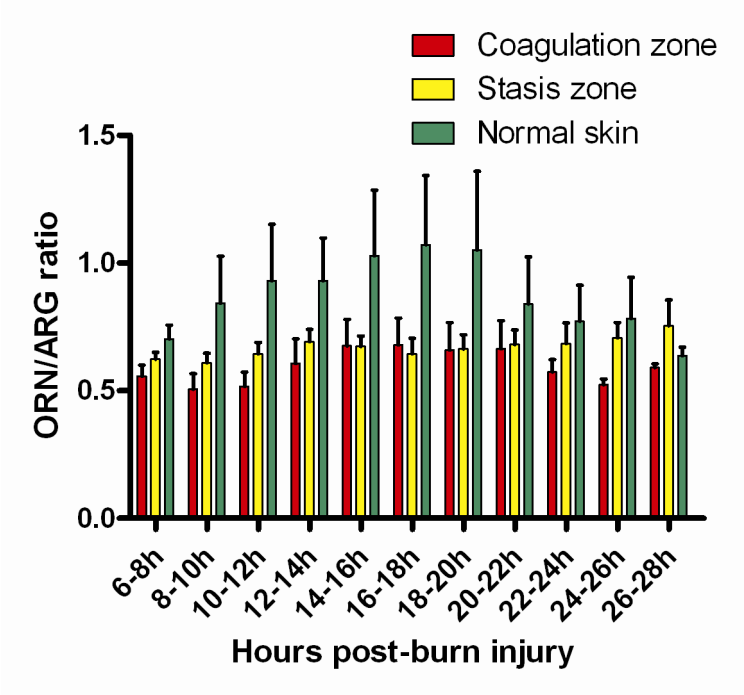
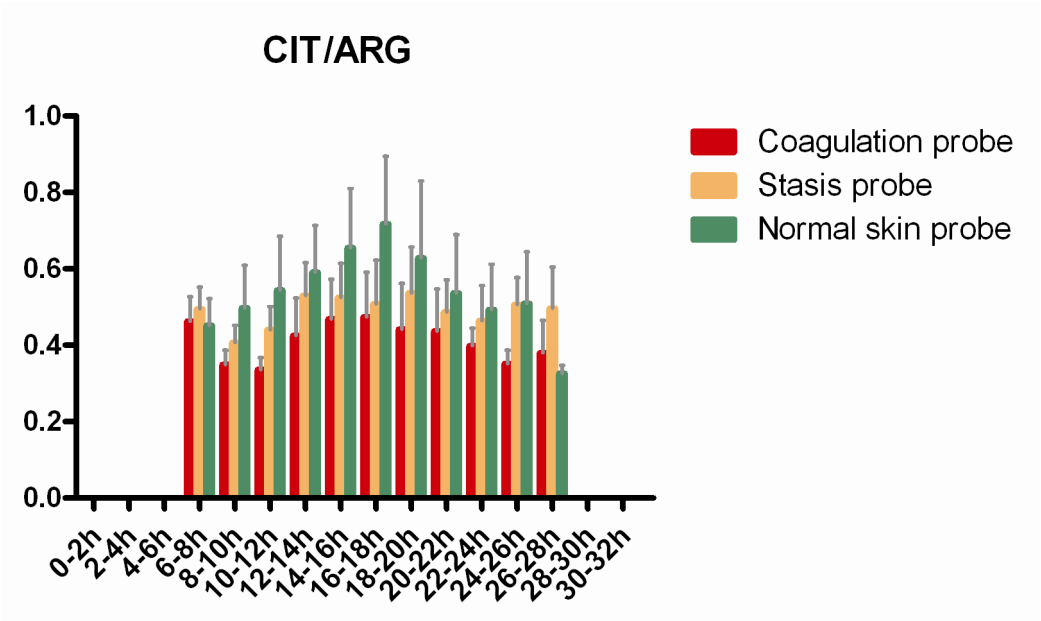


Figure 93: Barchart to show changes in citrulline/arginine ratio with time post-injury for each zone. Mean and Standard Error bars are shown. Coagulation probe = Zone A; Stasis probe = Zone B; Normal skin probe = Zone C



4.5.1.3 Other amino acids

Two-way ANOVA indicated a gradual increase in glycine with time post-burn ($p<0.0001$), but there was no difference related to catheter location. Comparison with the respective levels at 6h using Student's t-test did not reveal a significant difference at any time point.

4.5.2 Amino Acid Trends in Individual Patients

Since patients had different outcomes with regard to wound progression and graft take, the individual amino acid profiles were reviewed. Statistical analysis was not possible due to the small numbers involved

4.5.2.1 **Taurine** (Figure 94, Figure 95, Figure 96, Figure 97, Figure 98, Figure 99, Figure 100, Figure 101)

Four patients (Figure 96, Figure 98, Figure 99, Figure 100) showed levels of Taurine >100 μM in the zone of stasis, during the study period. In Figure 99 and Figure 100, these occurred in the time following being taken to theatre. In Figure 99, the results peaked at 120 μM within 2 hours of the start of the operation and declined with time. In Figure 100, the levels continued to rise postoperatively, and at the end of the study period measured 161 μM. In Figure 96, the first two measurements following probe insertion (6-8h, and 8-10h post burn) were 114 μM and 107 μM respectively, and levels

declined following this. In Figure 98, only the first result was elevated at 106 μM.

In all patients where serum measurements were available, Taurine levels in serum were higher than in skin at the corresponding time.

Figure 94: Barchart to show Taurine levels by zone over time post-burn for S02.

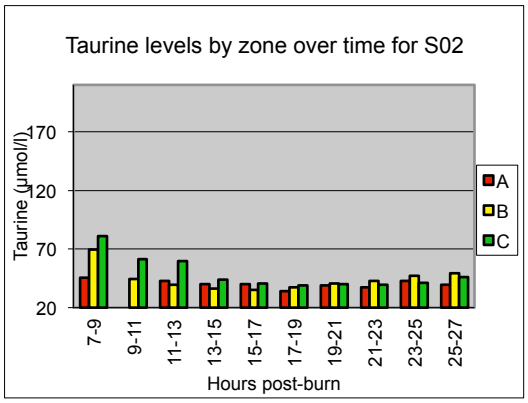


Figure 95: Barchart to show Taurine levels by zone over time post-burn for S04.

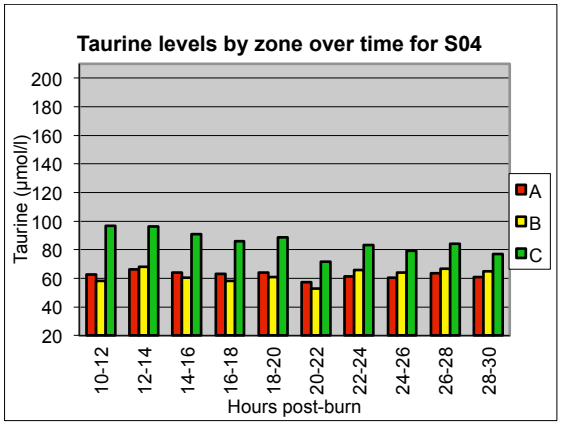


Figure 96: Barchart to show Taurine levels by zone over time post-burn for S05. Blue bars indicate serum levels

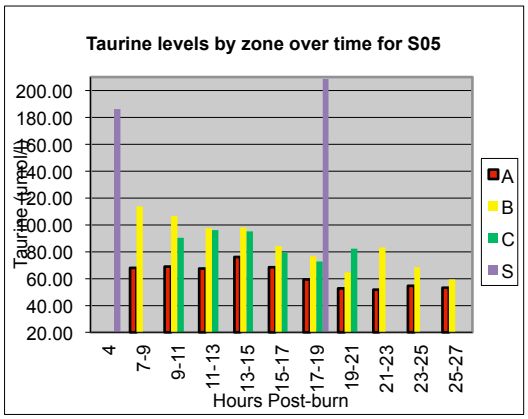


Figure 97: Barchart to show Taurine levels by zone over time post-burn for S06.

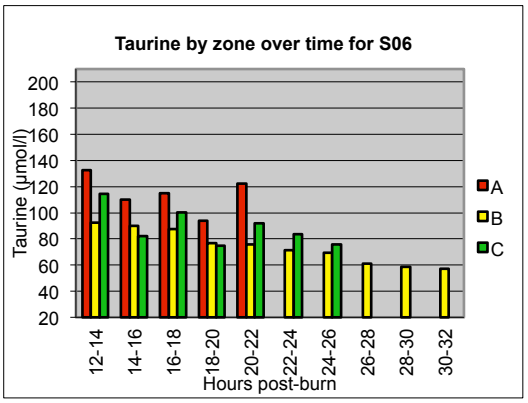


Figure 98: Barchart to show Taurine levels by zone over time post-burn for S08.

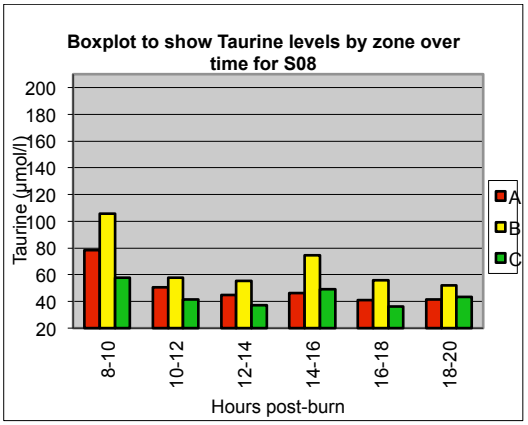


Figure 99: Barchart to show Taurine levels by zone over time post-burn for S09.

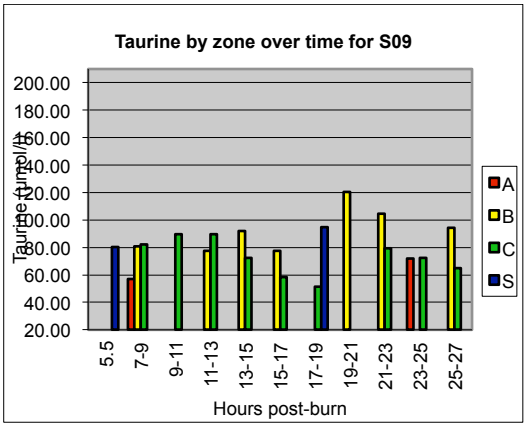


Figure 100: Barchart to show Taurine levels by zone over time post-burn for S10. Blue bars indicate serum levels

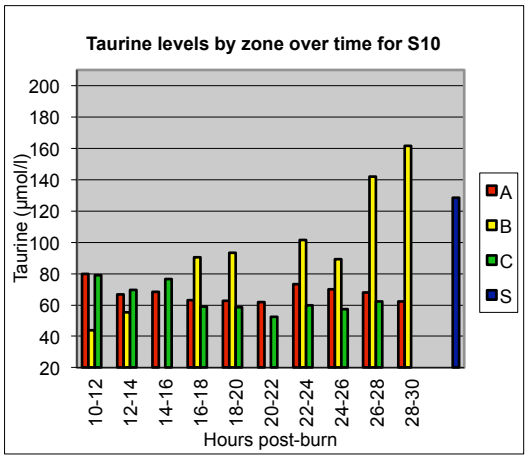
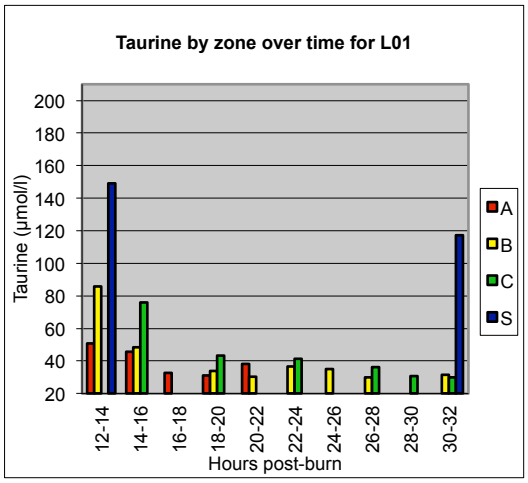


Figure 101: Barchart to show Taurine levels by zone over time post-burn for L01.



4.5.2.2 Citrulline/Arginine ratio and Ornithine/Arginine ratio

Citrulline/Arginine ratios and Ornithine/Arginine ratios are shown for patients S02 (Figure 102, Figure 103), S04 (Figure 104, Figure 105), S05 (Figure 106, Figure 107), S06 (Figure 108, Figure 109), S08 (Figure 110, Figure 111), S09 (Figure 112, Figure 113), and S10 (Figure 114, Figure 115).

Five patients had higher Citrulline/Arginine (Cit/Arg) ratios during the study period, S05, S06, S08, S09 and S10 (Figure 106, Figure 108, Figure 110, Figure 112, Figure 114). In most, the three zones did not show much variation, but in S05 (Figure 106), Zone C was markedly higher, and in S10 (Figure 114), Zone B showed a noticeable upward trend towards the end of the study period, not seen elsewhere.

In patient S02 (Figure 102), Cit/Arg was stable at a low level across all zones. Orn/Arg (Figure 103) showed an increase at 19h which then declined again in zones of stasis but persisted in unburned skin. This change occurred at around the same time as the increase in ischaemic conditions noted in Figure 60.

In Patient S04 (Figure 104), Cit/Arg remained low throughout. Figure 105 showed Orn/Arg to be elevated in the unburned skin,

which was concurrent with the ischaemic environment seen in this zone in Figure 66.

In patient S05 (Figure 106), Cit/Arg ratio in Zone A was 0.2-0.4, it was moderately raised in the Zone B (0.4-0.6), and much higher in Zone C (>1) (Figure 106). This was associated with very low levels of Arginine in Zone C (not shown), a high Orn/Arg ratio 1.5 – 2.7 (Figure 107), and higher Ornithine in Zone C than in Zone A (not shown). Raised ratios of Orn/Arg and Cit/Arg were evident at the same time as the ischaemic periods in the unburned skin (Figure 69). In the zone of stasis, there was also evidence of ischaemia (Figure 68).

Patient S06 had high Cit/Arg ratios (>0.7) in all zones throughout the study (Figure 108), associated with low Arginine levels. Orn/Arg ratio for this patient was also high throughout the study period, with Zone C ratios being consistently highest (Figure 109). Citrulline levels were constant with no large differences between zones. Ornithine levels were highest in the Zone C with a peak in the middle of the study period. There was no related ischaemic period in any of the zones for this patient.

Figure 110 showed a moderate, constant ratio of Cit/Arg in all zones for patient S08. This was stable throughout the study period, and there was no difference between zones. Orn/Arg ratio was slightly elevated, but all zones showed similar results (Figure 111).

Citrulline, Arginine and Ornithine individually did not show any marked trends. There was evidence of ischaemia in zone of stasis and unburned skin over this period (Figure 76, Figure 77).

Figure 112 showed incomplete data for patient S09, but with a tendency towards elevated Cit/Arg ratios in Zones B and C, associated with elevation of Citrulline. There was insufficient data to assess Zone A. A more marked pattern was seen in Orn/Arg ratios with Zone C reaching ratios of up to 1 (Figure 113). This was associated with high levels of Ornithine. These findings do not correlate with patterns of ischaemia seen in Figure 78, Figure 79 and Figure 80.

Figure 114 showed moderate levels of Cit/Arg initially, for patient S10, which increased in Zones A and B following the theatre episode. Zone C levels remained low. A similar pattern was seen for Orn/Arg in Figure 115, with highest ratios in Zones A and B.

The changes for both Figure 112 and Figure 114, were associated with a decreasing Arginine level and increasing Citrulline levels.

Figure 102: Histogram to show Cit/Arg ratio by zone with time for patient S02

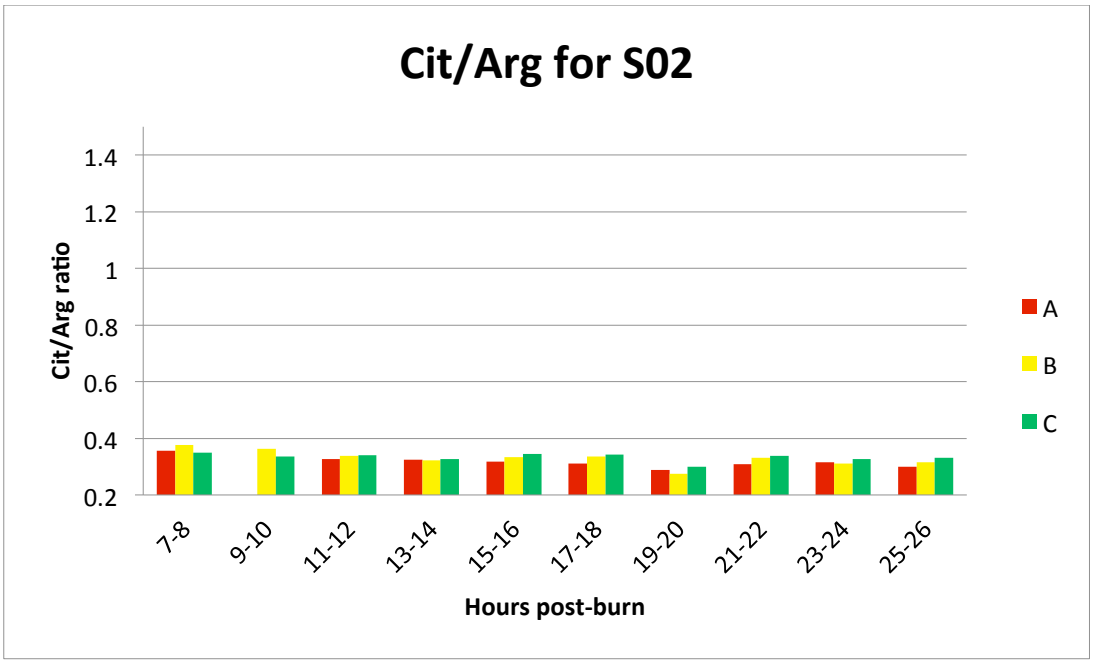


Figure 103: Histogram to show Orn/Arg ratio by zone with time for patient S02

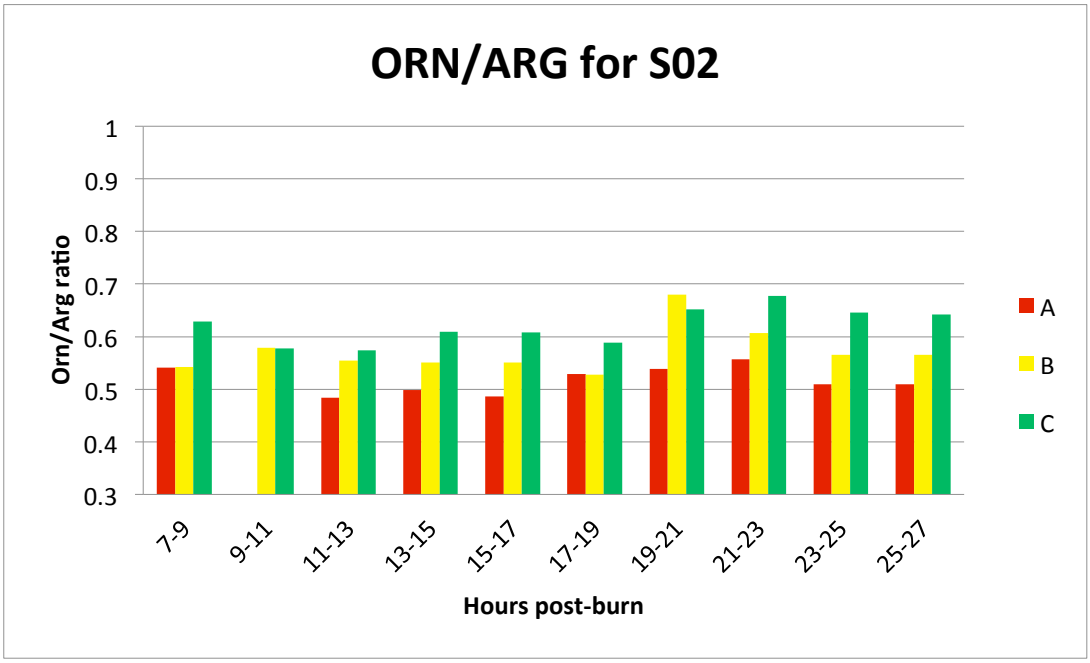


Figure 104: Histogram to show Cit/Arg ratio by zone with time for patient S04

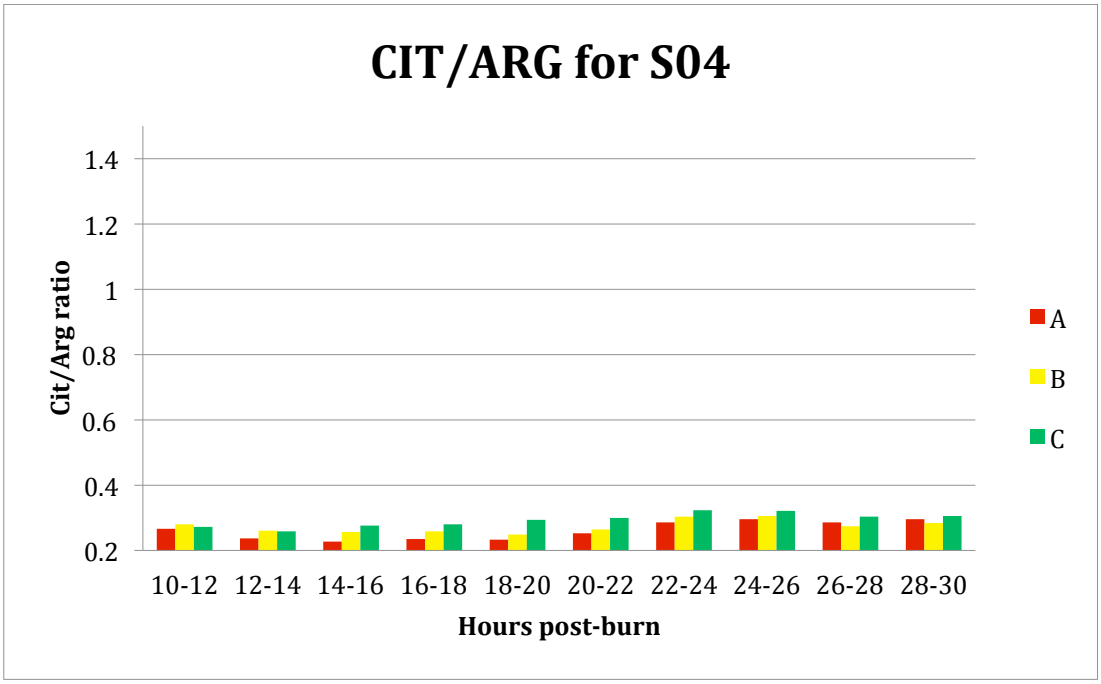


Figure 105: Histogram to show Orn/Arg ratio by zone with time for patient S04

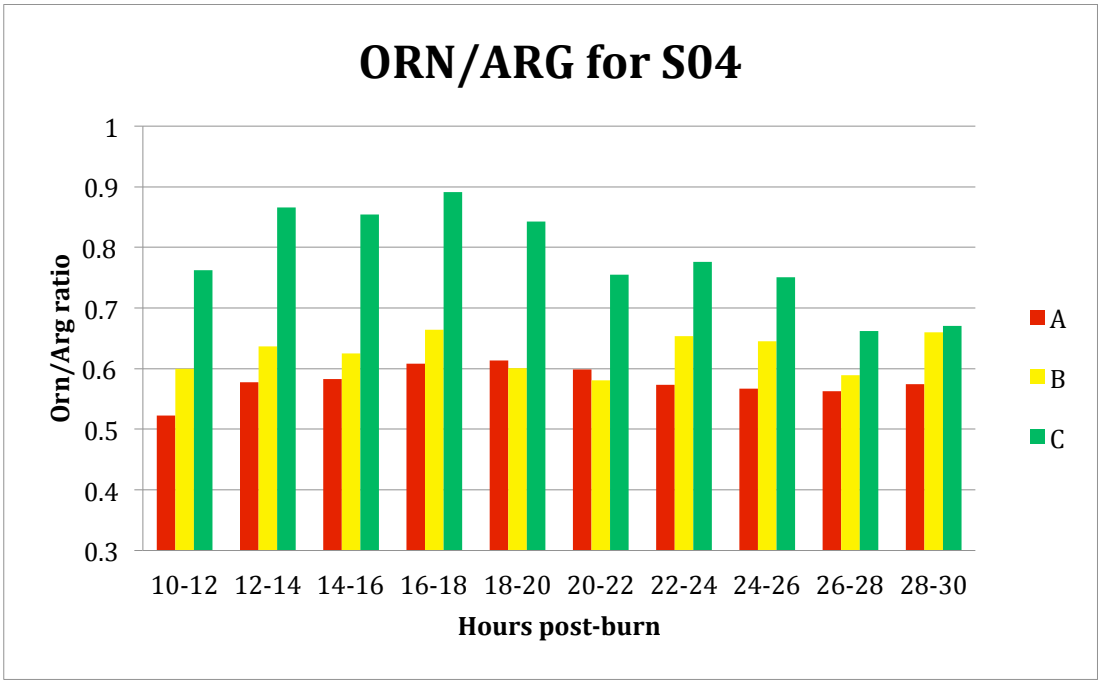


Figure 106: Histogram to show Cit/Arg ratio by zone with time for patient S05. Blue bars represent serum levels.

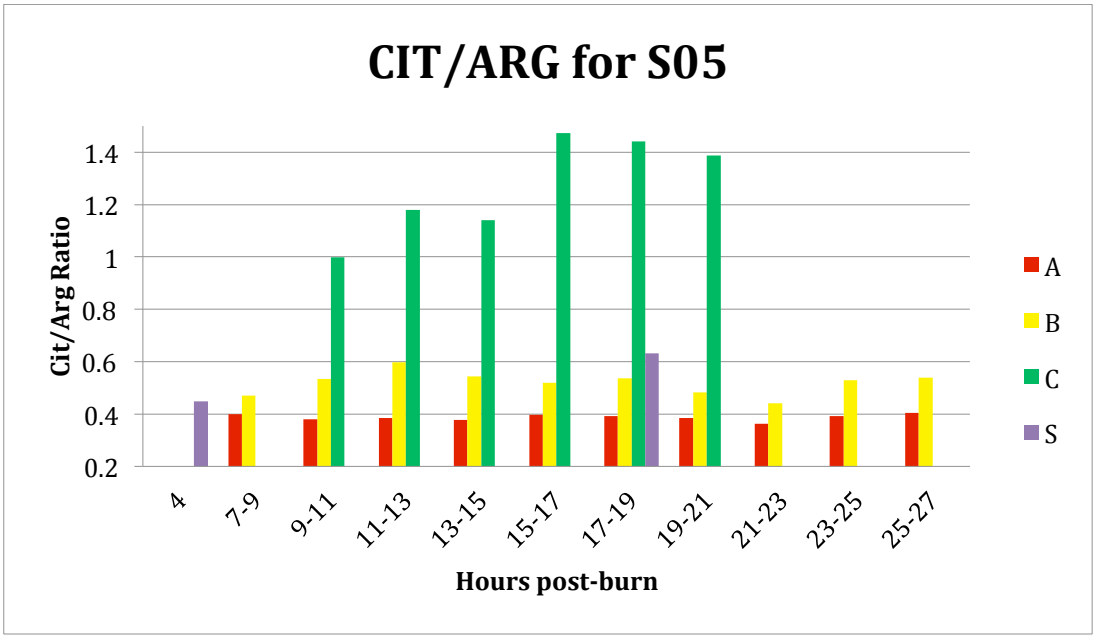


Figure 107: Histogram to show Orn/Arg ratio by zone with time for patient S05. Blue bars represent serum levels.

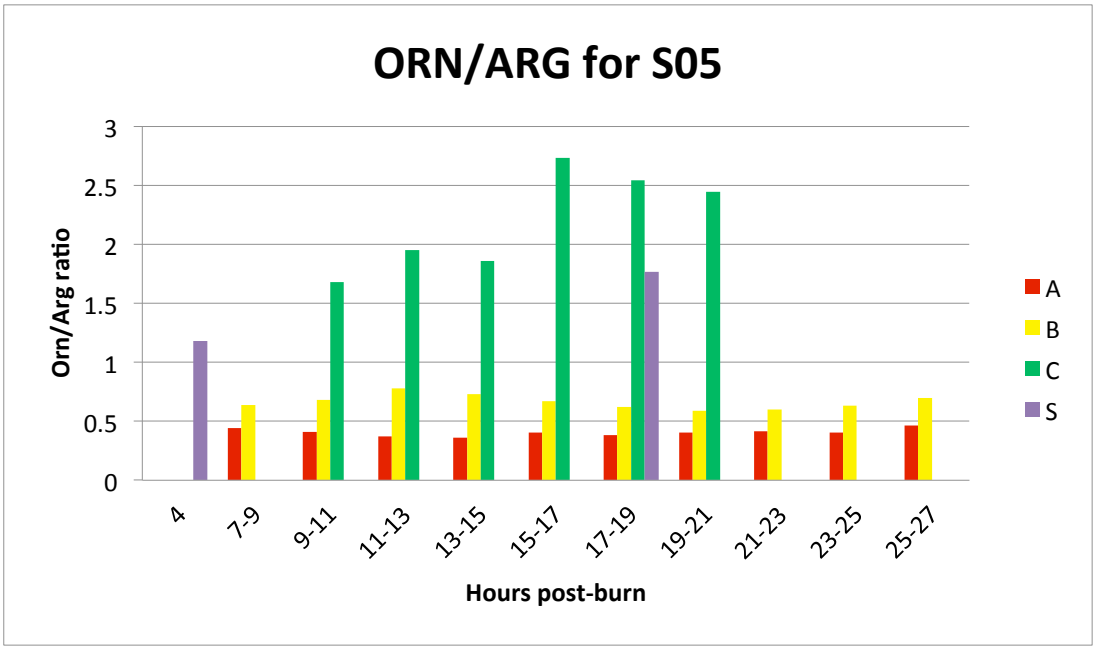


Figure 108: Histogram to show Cit/Arg ratio by zone with time for patient S06

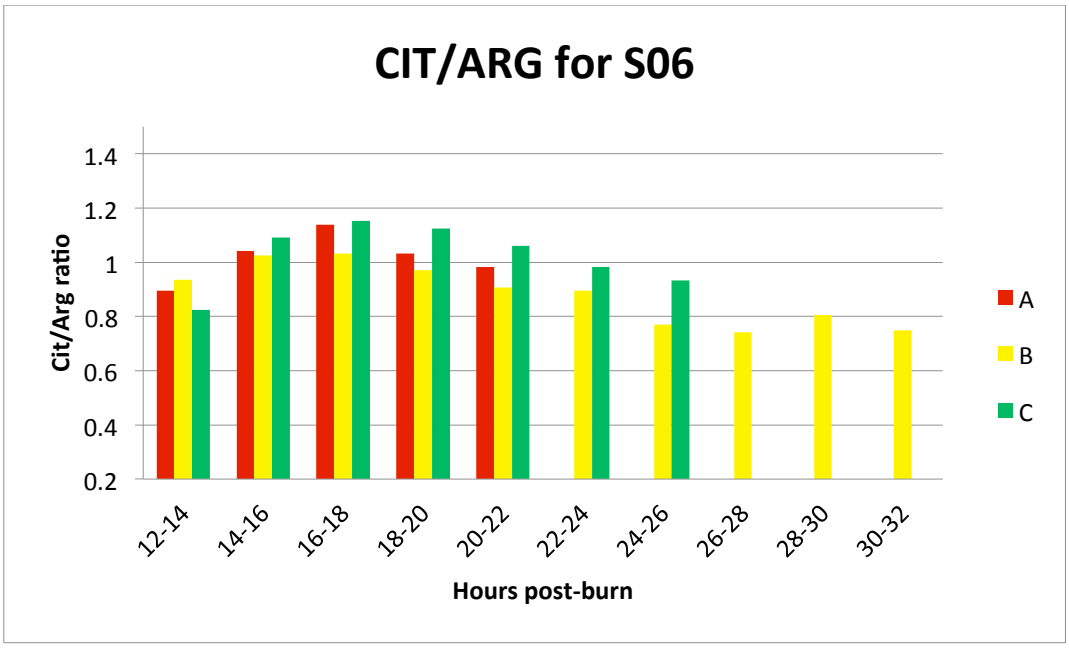


Figure 109: Histogram to show Orn/Arg ratio by zone with time for patient S06

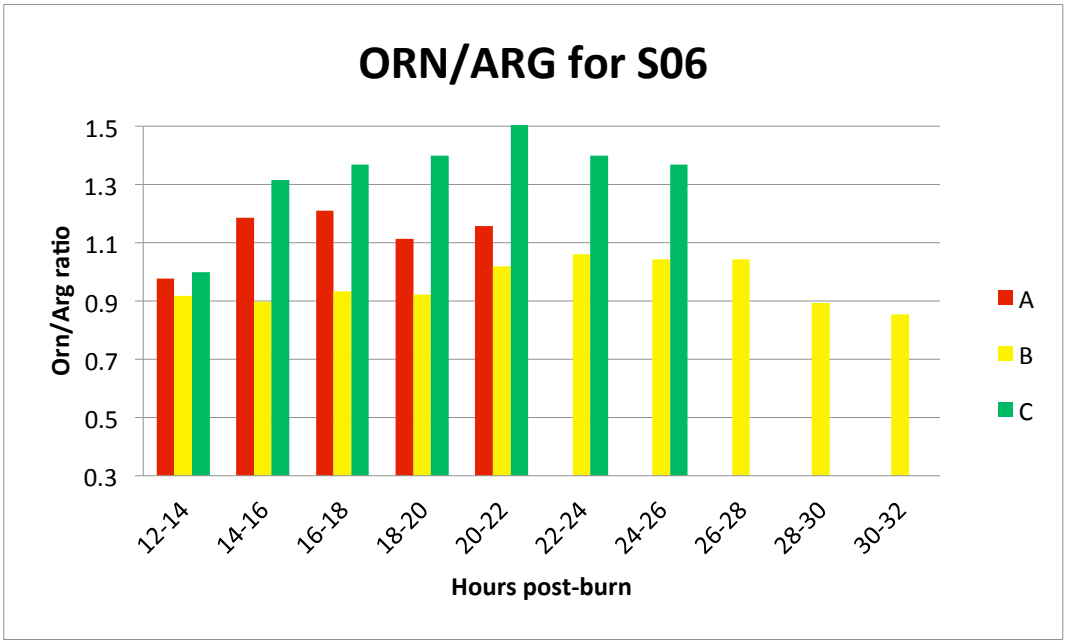


Figure 110: Histogram to show Cit/Arg ratio by zone with time for patient S08

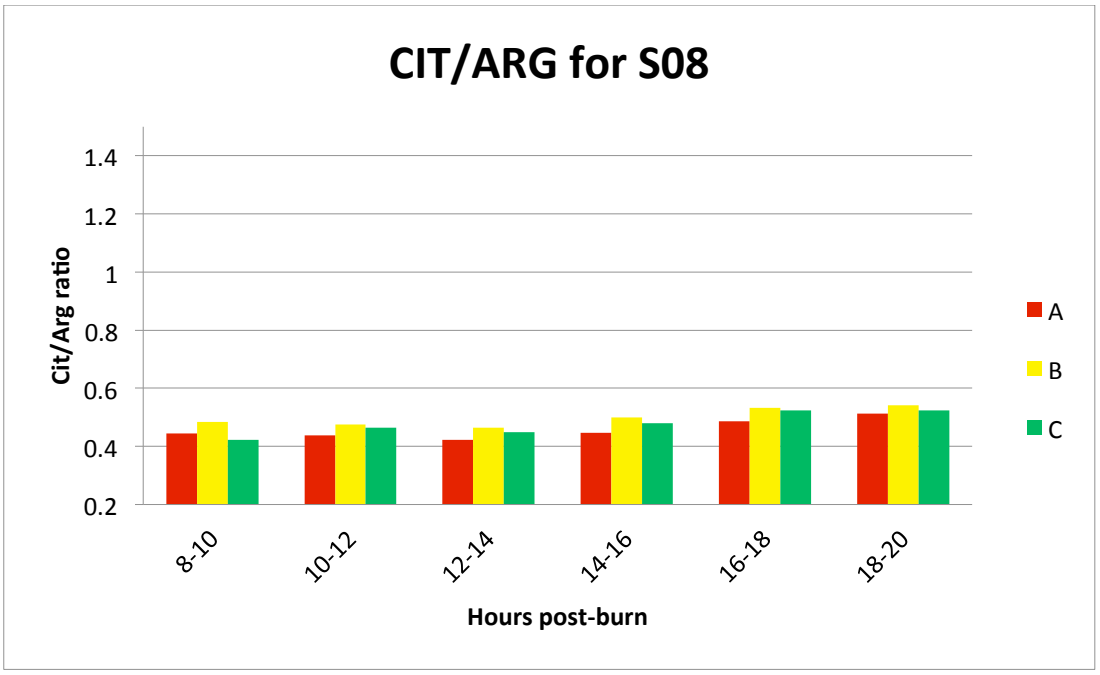


Figure 111: Histogram to show Orn/Arg ratio by zone with time for patient S08

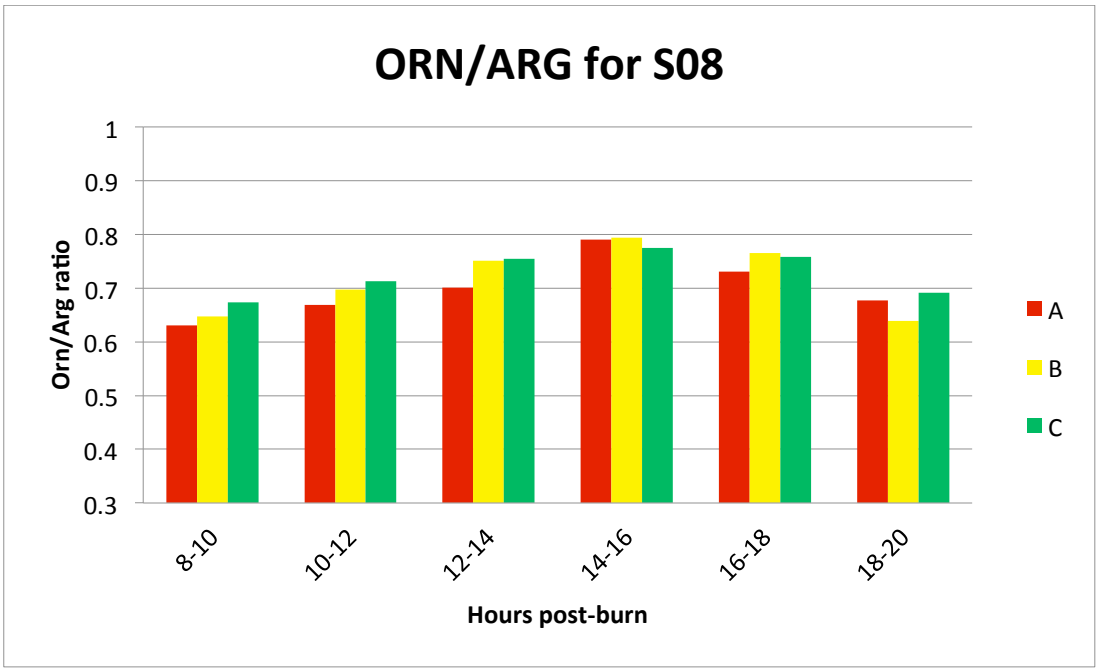


Figure 112: Histogram to show Cit/Arg ratio by zone with time for patient S09. Blue bars indicate serum levels.

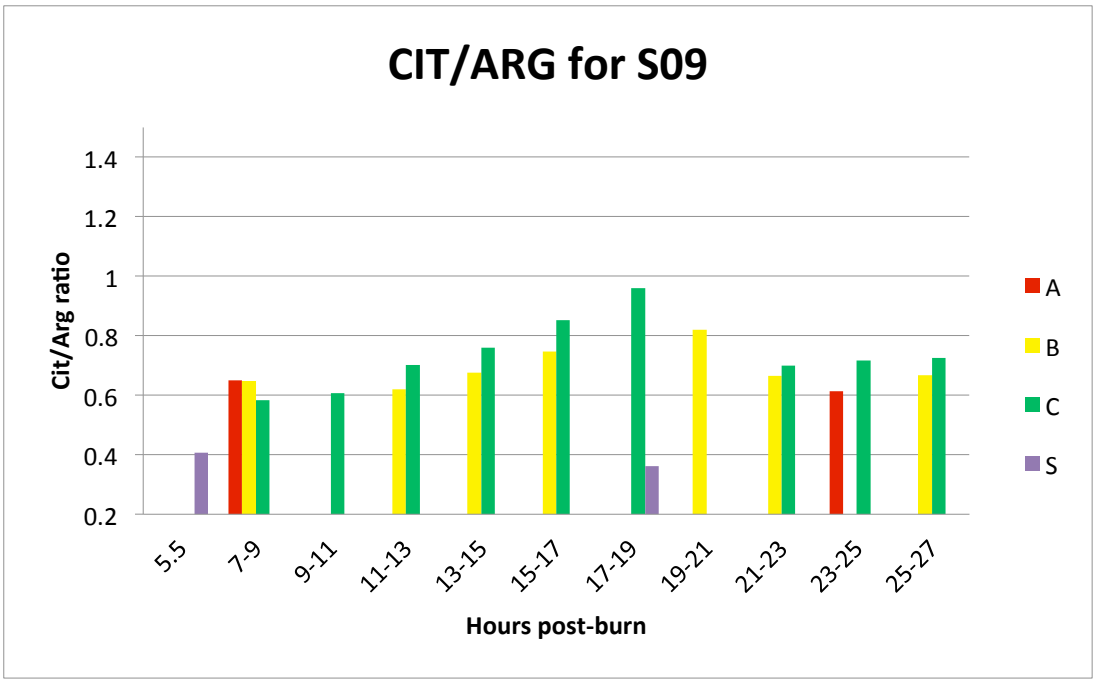


Figure 113: Histogram to show Orn/Arg ratio by zone with time for patient S09. Blue bars indicate serum levels.

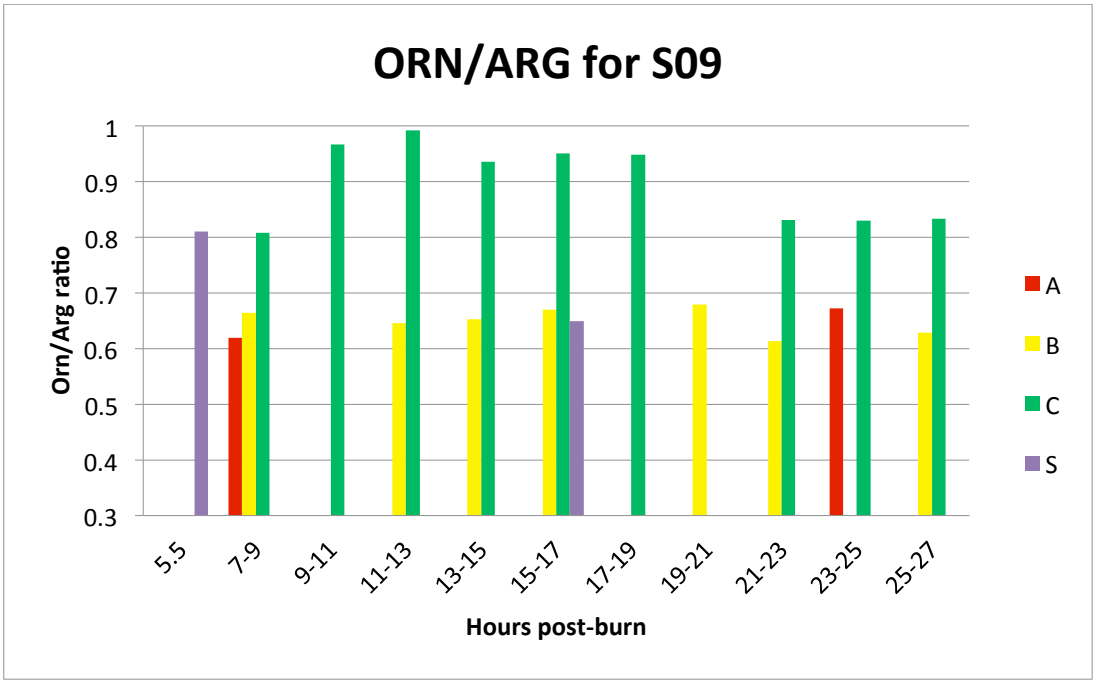


Figure 114: Histogram to show Cit/Arg ratio by zone with time for patient S10. Blue bar indicates levels from the combined dialysate of two serum samples taken at the beginning and end of the study period

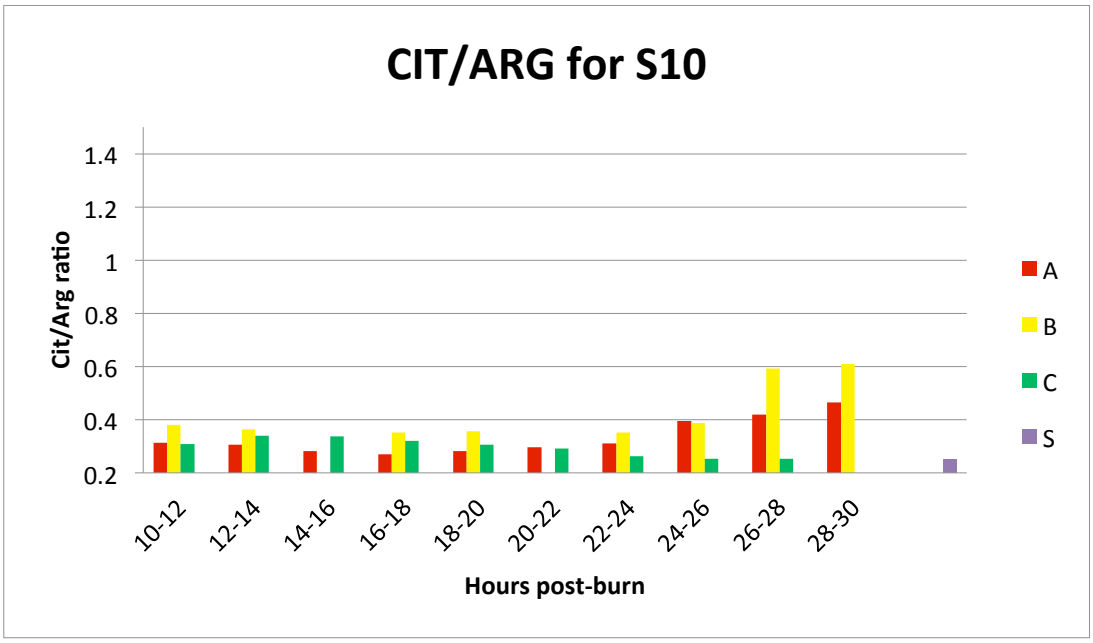
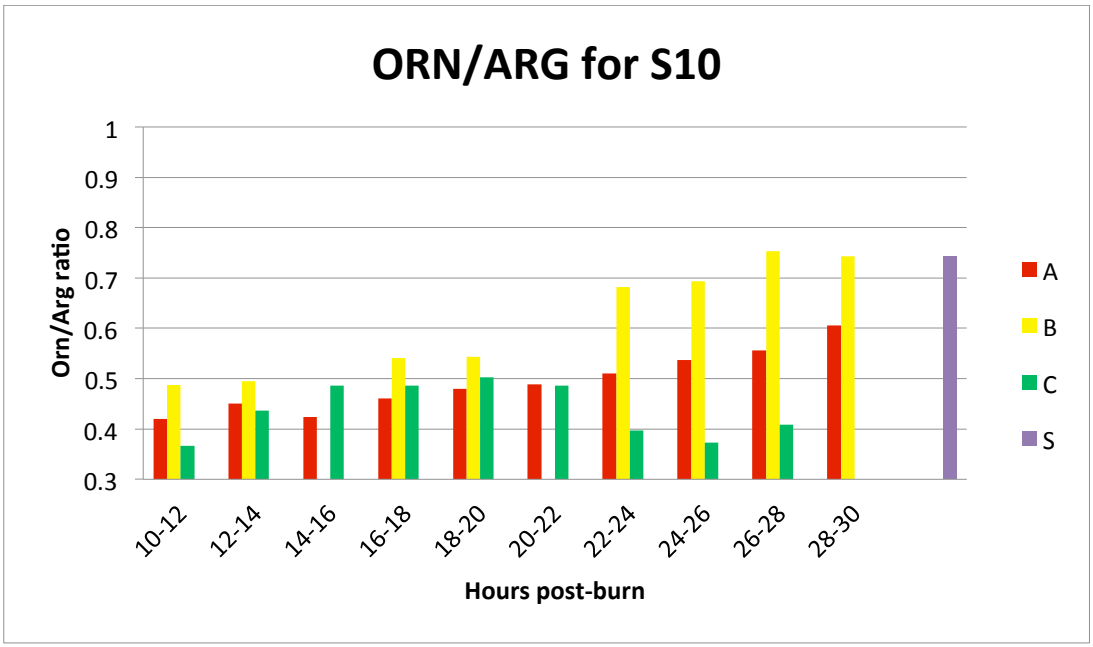


Figure 115: Histogram to show Orn/Arg ratio by zone with time for patient S10. Blue bar indicates levels from the combined dialysate of two serum samples taken at the beginning and end of the study period



5 DISCUSSION

Burn injury is a traumatic insult, which triggers a cascade of events within the human body. These include both systemic and local responses. Much work has been done on the systemic component of the burn injury, and the majority of treatments for burns are monitored using systemic parameters. Local response to injury has an effect on the duration of healing and the presence and quality of scarring, and yet little work has been done on monitoring the burn wound. Douglas Jackson's model of the zones of burn injury, together with the concept of wound progression, remains the hypothetical model for managing the burn wound. Clinical examination remains the mainstay of wound monitoring, and traditionally this is undertaken at presentation, and repeated at 48 hours at which point progression of the wound has already occurred. Laser Doppler Imaging has been an attempt at objectively measuring depth of wound, and has been validated for use at 48hours post injury (45), but is not suitable as a method of continuous monitoring.

Microdialysis has been used within other specialties where monitoring of the local environment provides vital information. Monitoring of the brain following neurological injury has allowed investigation of secondary injury in the field of neurosurgery (83). Measurements in transplanted livers warn of vascular insufficiency and rejection ahead of systemic parameters and could potentially lead to an improved graft survival rate (84) (85).

The aim of this thesis was to investigate the possibility of using microdialysis to monitor the burn wound, using it to understand and ultimately prevent wound progression.

Since the Jackson model indicates capillary stasis, and therefore ischaemia as a mechanism by which wounds progress, glucose metabolites were chosen as a marker for wound ischaemia, and glycerol for cell death. These metabolites have been shown to signal ischaemia and cell death in other tissues (55) (85) (57).

Although these metabolites have been previously studied in other tissues, no normal range could be found for them in the dermis. A few studies in normal volunteers had been performed of lactate and glucose in human dermis using the microdialysis technique (Table 23). Pyruvate had also been studied in some of these experiments, but results were expressed as LPR only with no pyruvate values specified. No studies investigating glycerol had been undertaken. The normal volunteer results in this thesis have allowed a normal range of glucose, lactate, pyruvate, LPR and glycerol to be calculated.

Lactate and glucose levels in this thesis were consistent with the higher levels reported in the literature, in particular those taken after a glucose load, during oral glucose tolerance test conditions. There was a wider range in the patients in this thesis than that published in the literature.

There is some difficulty in direct comparison, since many of the results in the literature had not been published as raw data. Jansson and Krogstad both used the 'no net flux' method to calibrate the probes in vivo over 3 hours, and then performed a linear regression to calculate the percentage recovery of the probes (52) (76). This percentage was then used to calculate the published result. Glucose was calibrated by the 'hot loss' technique, adding labelled glucose to the perfusion fluid and calculating the amount of glucose lost through the membrane during dialysis. Petersen used the 'no net flux' method for both lactate and glucose quantification (77) (78). All

authors used relatively high flow rates. Also, microdialysis probes were of different specification (See Table 23)

The studies cited in the literature have used fasting subjects, with or without a subsequent glucose tolerance test. For reasons of matching the conditions of the volunteers in this study as closely as possible with the burns patients in the proceeding studies, the subjects in thesis were not starved. Therefore the values obtained for both glucose and lactate would not be expected to be as extreme, or with such a narrow range as those obtained in the fasting state or following the glucose challenge.

Table 23: table to compare lactate and glucose results from dermal microdialysis in normal volunteer experiments in the literature

Author	n	Lactate Range (mmol/l)	Glucose Range (mmol/l)	Comments
Petersen, 1992[76]	7		5.3	Flow rate 3µl/min. Single dialysis fibre, 216µm d, 2kDa pore, 20mm length. Recovery 20.5+/- 0.7%.
Jansson, 1996[86]	7	1.001+/- 0.024	5 10 after OGTT (data from graph)	Flow rate 3 µl/min. Probe 0.3mm d, 35mm length, 50kDa pore size. Recovery 48%. Fasted subjects.

Petersen, 1999[87]	8 10	2.48 +/- 0.17 2.52 +/- 0.32 raised by 18% after OGTT	5.49 +/-0.18 6.31 +/- 0.18 raised by 41% after OGTT	Flow rate 3 µl/min. Recovery 20.1 +/- 0.9% for lactate, 14.3 +/- 0.5% for glucose. Results calculated using no net flux method. Subjects fasted initially, followed by oral glucose tolerance test (OGTT).
Krogstad, 1996[51]	15	1.171 +/- 0.228	5.2 +/- 0.8	Flow rate 2.5µl/min. Probe 0.3mm d, 35mm length, 50kDa pore size. Recovery calculated at 36 +/- 9%
Breuning	10	0.72-5.76	3.81-7.75	Flow rate 0.3µl/min. Probe 0.6mm d, 30mm length, 20kDa pore size. Raw data. Patients not starved

5.1 Discussion of Results of Burns

Clinical identification of burn wound zones

The sites chosen for the monitoring of progression of the burn wounds in this study were based on the wound model described by Douglas Jackson in 1953. (7). His method of clinically determining the zones involved a tourniquet test, and described the blood flow and presence of de-oxygenated blood to indicate patency of the dermal

plexus and the metabolic state of the tissue in order to identify the three different zones (See Section 1.2.2). In order to follow this method, it would have been necessary to recruit only patients with a burn on a limb (i.e. amenable to the use of a proximal tourniquet), thus vastly reducing the number of patients meeting the inclusion criteria, and rendering the study non-viable in the two year time frame.

The zone of coagulation was easy to identify without a tourniquet as an area of full thickness burn. Unburned skin was chosen rather than zone of hyperaemia as this was also easy to select, and provided a good control. A different method of identification of the zone of stasis was employed: an area within the same burn wound as the full thickness burn, and immediately adjacent to it, which showed epithelial loss, but showed blanching to pressure at the time of recruitment. This method was used to define the zone of stasis throughout the study. It was felt that this method identified the zone which, in clinical practice, is most often observed to convert into full thickness burn in adverse conditions. The placement of the probe in this area was supported by the distinct differences in the results between this zone and the other two probe sites in the study.

Consideration was given to the statistical methods used in this study. A power calculation was performed at the study design stage, and based on the only other burn study to attempt to measure differences between zones of a burn wound using the microdialysis technique (58). Repeated measures analysis was not possible due to missing values. Analysis of time-related trends was therefore undertaken using Generalised Estimating Equations. It was felt that an overview of the trends with time was what was required of this study, and a decision was made to group the data into six-hour blocks (12 samples in each time block in most instances) for statistical

analysis. A post-hoc Tukey analysis was used to identify differences between time blocks and between zones.

Within Zone A, equivalent to Jackson's zone of coagulation, Glycerol showed significant changes with time, the first two 6-hour time blocks being higher than the final one. On observing the data more closely, it was seen that those patients recruited within 7 hours of burn injury showed high peaks of glycerol in this zone only. This can be interpreted as evidence of the primary injury. The cells in this zone have recently been damaged, and cell wall breakdown products (including glycerol) are abundant in the interstitial spaces. This early peak resolves within a few hours to a quiescent background level similar to that in the surrounding zones. The second significant elevation at 12-18h is accounted for by two patients who were taken to theatre within the study period. This is discussed further below. One patient showed glycerol levels which did not fit in to any pattern displayed by the other patients. High peaks and troughs were seen in zone A for patient S06, which are preceded by spikes in LPR. There is no clear explanation for this, but differences from the other patients included co-existing soft tissue trauma, and alcohol/drug withdrawal symptoms.

There was no significant change in glucose, lactate, pyruvate or LPR with time in Zone A. This is consistent with the injury to the zone of coagulation. Heat injury causes obliteration of most of the normal tissue architecture in a full thickness burn, and denaturation of most cellular elements. Without these functioning elements, there is no direct nutritional supply from the vascular structures, and little, if any, metabolic activity. It would be expected that glucose, LPR and glycerol would all remain constant in this zone.

Though no statistically significant trend was observed for the group, two individuals showed ischaemic LPR levels, one of whom also had the unusual glycerol course.

In summary for zone A, the zone of coagulation, there was evidence of a response to the burn injury within 7 hours of the injury, and a further peak of glycerol in those attending theatre.

Glucose in Zone B showed a statistically significant upward trend with time. This may indicate return of blood flow to the area. In only 1 patient, S05, was the glucose level in this zone below 2mmol/l, a level comparable to that of a non-vascularised free flap (56). In this patient the low glucose was accompanied by a rise in LPR to >25, indicating ischaemia, but there was no rise in glycerol to indicate cell death. There was evidence of wound progression in this patient, although not over the area containing the probe.

According to Jackson, the zone of stasis has a static circulation with vessels which are in spasm, or filled with microthrombi. This would be expected to interfere with glucose delivery to Zone B. Since the cells remain viable initially, it might be expected that glucose within the zone of stasis would start to fall, as it is used for cellular metabolism, but not replenished by the circulation, leading eventually to cell death and wound progression if the blood flow is not restored. The findings of this study are contrary to this, the indications being a return to circulation and a restoration of normal glucose levels.

LPR in Zone B did not show any statistically significant variation with time and therefore no evidence of ischaemia. However when studying the raw data, it was clear that there were some trends for individual patients. These were not picked up with the statistical methods used, due to the size of the time blocks chosen for comparison.

The majority of patients showed an initial rise in LPR within the first few hours after probe insertion, followed by a steady decline from hour 10 onwards. This would indicate an intitially ischaemic environment, probably due to vasoconstriction in response to the burn, which improves with time, indicating restored blood flow to this zone, rather than wound progression. This is also consistent with the LDI results showing most patients not to have progression around the zone B probe. Patients S07 and S09, were an exception and continued to show an upward trend. Patient S07 showed evidence of wound progression on LDI (although not over the probe site), but for S09, LDI comparison was inconclusive.

Zone B did not show any significant change in glycerol during the study period, except for the two patients attending theatre. This would indicate that for the majority of patients within this study, conditions were not so severe as to cause any further cellular damage in this zone. Interestingly, some patients did show evidence of wound progression on LDI in the wider zone B area through this study period. It may be possible that the presence of the microdialysis catheter had some protective effect on the zone immediately surrounding it, preventing cell death despite unfavourable conditions causing progression of the wound in other parts. A possible mechanism for this could be the provision of hydration to this area from the perfusate of the catheter in an area under threat from desiccation. The movement of water from the probe into the surrounding tissues has been previously noted (86). Another possibility is that the mechanism for wound progression is not related to ischaemia, although any mechanism causing cell death would be expected to produce a rise in glycerol, even if LPR was unaffected. Other work distinguishing between necrosis and apoptosis has favoured the former in burn progression (87)

In summary for zone B, the zone of stasis or peripheral partial thickness area, there was no evidence of any ischaemic trends for the group as a whole, though a few patients showed individual ischaemic levels of glucose and LPR. The only rise in glycerol was seen in the two patients who attended theatre. There was no corresponding evidence of wound progression from LDI.

An unburned area, Zone C, was chosen on a contralateral limb, or an area >10cm distant from the burn to reflect the systemic effect of the burn and its initial treatment. All patients showed an initially low glucose, more than half of them below the normal range, and most showing a fall within the early hours. Glucose remained significantly low for the first 12 hours, when compared to the final time block, before increasing gradually in all patients. This trend is consistent with the stress response following injury, with vasoconstriction of an intact cutaneous vascular bed. This was corrected in the hours following admission, allowing return of cutaneous perfusion. Glucose increase with time may also reflect another facet of the stress response, the release of glucocorticoids leading to gluconeogenesis and anti-insulin effects. Falling lactate levels, a constant pyruvate level, a significantly declining LPR and a stable glycerol level all support this explanation. The combination of a low glucose and high LPR indicates an ischaemic environment. The lack of a corresponding rise in glycerol confirms that this vasoconstriction is not harmful to the uninjured tissue.

Glycerol was significantly higher in Block 3 due to peaks in patients S04, S06 and S10. These peaks were all within the normal range, except for patient S04 which briefly exceeded the upper limit by a small margin. Glycerol was otherwise stable, and within the normal range, which is consistent with the clinical knowledge that

distant unburned skin is not in danger of necrosis following a burn wound elsewhere. A subtle, cyclical fluctuation of glycerol could be seen in all three zones, when inspecting the individual results. This may reflect a systemic release of glucocorticoids, and the resulting breakdown of fat, releasing glycerol in the nearby subcutaneous layer, or the normal production of glycerol form the pilosebaceous units (88).

In summary for Zone C, the unburned tissue distant to the wound, there were statistically significant changes including a low glucose and a high LPR when compared to the other zones. Glucose increased, and LPR decreased with time, indicating a normalizing physiology. There was no raised glycerol at any point.

Samuelsson et al was the only other paper to investigate the burn wound in humans using microdialysis. Patients had more severe injuries (>25% body surface area burn). The zone chosen for their 'burned skin' catheter was that of a deep dermal burn area, and probably correlates somewhere between Zone A and Zone B of this study. Their 'uninjured' catheter corresponds to Zone C. Measurements were taken 3 hourly from Day 1-5 post-burn, with complete data only available for Day 2-4. There were no results from earlier in the post-burn period. Data was analysed by grouping into 1 day time blocks. Statistical comparison was undertaken between injured patients and controls only. There was no statistical comparison between days of injury. A rise in glucose was seen with time in the injured area, consistent with our own results, and absolute values for their Day 1-2 were consistent with the data in this thesis. LPR results were significantly higher than controls for both injured and uninjured patients, and were highest on Day 2 (47 for burned skin and 33 for uninjured skin). These

results were higher than in our patients. Glycerol was significantly higher than controls on Days 3 and 4 (136.6umol/l in burned and 123.3umol/l in uninjured skin). These values were consistent with those in this thesis. There was no comment about an early peak of glycerol, and it is assumed that the measurements had not been started within 7 hours of injury. There was no mention of high glycerol during surgery, however it was noted that sampling was interrupted during theatre.

Biochemical Trends with Theatre Episodes

Elevated glycerol levels were observed in both of the small burn patients who were taken to theatre within the study period, and this occurred in Zones A and B. Patient S09 did not have results for Zone C in this time period, but patient S10 did not show any simultaneous rise in glycerol in Zone C. Peaks of glycerol also occurred in Zone A of the large burn patient L01 at the time of theatre visit (15h), and in patient L02 at time of probe insertion during first theatre visit (7h), but not on a subsequent theatre visit at 44h. One interpretation of these finding is that both Zones A and B contain some viable cells which are susceptible to secondary injury, and that a secondary injury may be caused during the anaesthetic or operative process, leading to cell death and glycerol release. Another interpretation is that there is a confounding factor associated with the theatre visit. Propofol, an anaesthetic agent, contains 2.25% glycerol in its preparation. It is possible that the rise in glycerol seen during each theatre episode is related to the use of this drug. It may be seen in Zones A and B only, due to the damaged endothelium allowing the drug to leak into the extracellular fluid. It is not seen in Zone C as this area contains intact endothelium. The observation in patient L02 that a peak in glycerol is seen on a first theatre visit (where

propofol was administered), but not on a second visit (where propofol was not given) adds circumstantial evidence to this argument. This reasoning does not explain the peak in glycerol in Zone A in patient L01 at 18h, since no theatre visit occurred at this time, and there was no documentation of propofol administration at this time. One other paper investigated the influence of propofol on metabolism (89). This study showed no difference in subcutaneous fat metabolism when fat harvest under general anaesthetic with propofol was compared to local anaesthetic. No studies investigated the effect of propofol on dermis or in a wound.

In summary, the three patients in this study who attended theatre all showed some striking findings not seen in other patients. A high level of glycerol in the burned areas (Zones A and B) could be evidence of a secondary injury, although a confounding factor such as leakage of glycerol is also a possible explanation.

LDI

LDI assessment at the beginning and end of the study was undertaken to assess for the presence of wound progression, in particular over the area where Probe B was sited. There was LDI evidence of progression of the wound in the area of the probe located in Zone B in only one patient.

Interestingly, there was LDI evidence of progression of part of the wound, but not in the area adjacent to the probe, in a further four patients. One explanation for this is could be that the presence of the microdialysis probe has a protective effect, possibly due to hydration. This has not been previously documented.

There was evidence of no progression in the wound in 2 patients. There were problems with one of the scan images in 5 patients, and this affected the diagnosis of progression in 3 patients. One patient did not complete the study, so did not have a

48h LDI scan. In two patients, there was a malfunction in the scanner, meaning a portion of the scan was missing. For both of these patients it was possible to make a comment on global progression from the appearance of the scan picture, but it was not possible to identify progression over the location of the probe in zone B. In another two patients, there was a problem with low flow for the initial images, most likely due to reduced skin temperature from exposure of the wound. No comment on global progression could be made. However, since the probe in Zone B was within a well perfused area in the second scan, it can be assumed that there was no progression in this zone, and so a result has been ventured on this in the table.

The presence of Glycerol >450μmol/l (outside the normal range) or LPR >25 (universally accepted ischemic level) for at least 2hours in each zone was compared to LDI evidence of global wound progression. Some correlation could be drawn when comparing LPR>25 for >2h in Zone B or Zone C with evidence of wound progression. Of the five patients who showed clear evidence of wound progression in part of the wound, three had LPR>25 in at least one of these zones. Of two patient who clearly showed no progression, one did not have LPR>25 for a sustained length of time. No correlation could be made between glycerol and LDI progression. There were too many incomplete results in this study to enable a valid conclusion to be drawn on this matter.

Comparisons to wound healing could not be made due to the wide variation in the management, and in the timing of surgical procedures.

In summary, although the dataset was incomplete due to technical issues, there was evidence of wound progression in five patients, but in only one of them was this evident over the site of probe B. A possible protective effect of the microdialysis method must be considered.

In order to be able to draw a conclusion on the relationship between LPR or glycerol monitoring and wound progression, more patients would need to be studied, or a standardized animal model of wound progression used. If this study were to be repeated, it would be vital to ensure that the patients were normothermic before the initial scan, and that the LDI machine was fully functional, with a backup machine where possible.

Amino Acids

Local and systemic responses to burn injury include vasodilation and increased permeability, both of which are mediated by nitric oxide (NO) (90). Nitric oxide is formed from Arginine by the enzyme Nitric Oxide Synthase (Figure 116). Nitric oxide itself is a very volatile substance, and its presence is usually measured by proxy markers, the most frequently used being either nitrite/nitrate ratio, or Citrulline/Arginine ratio.

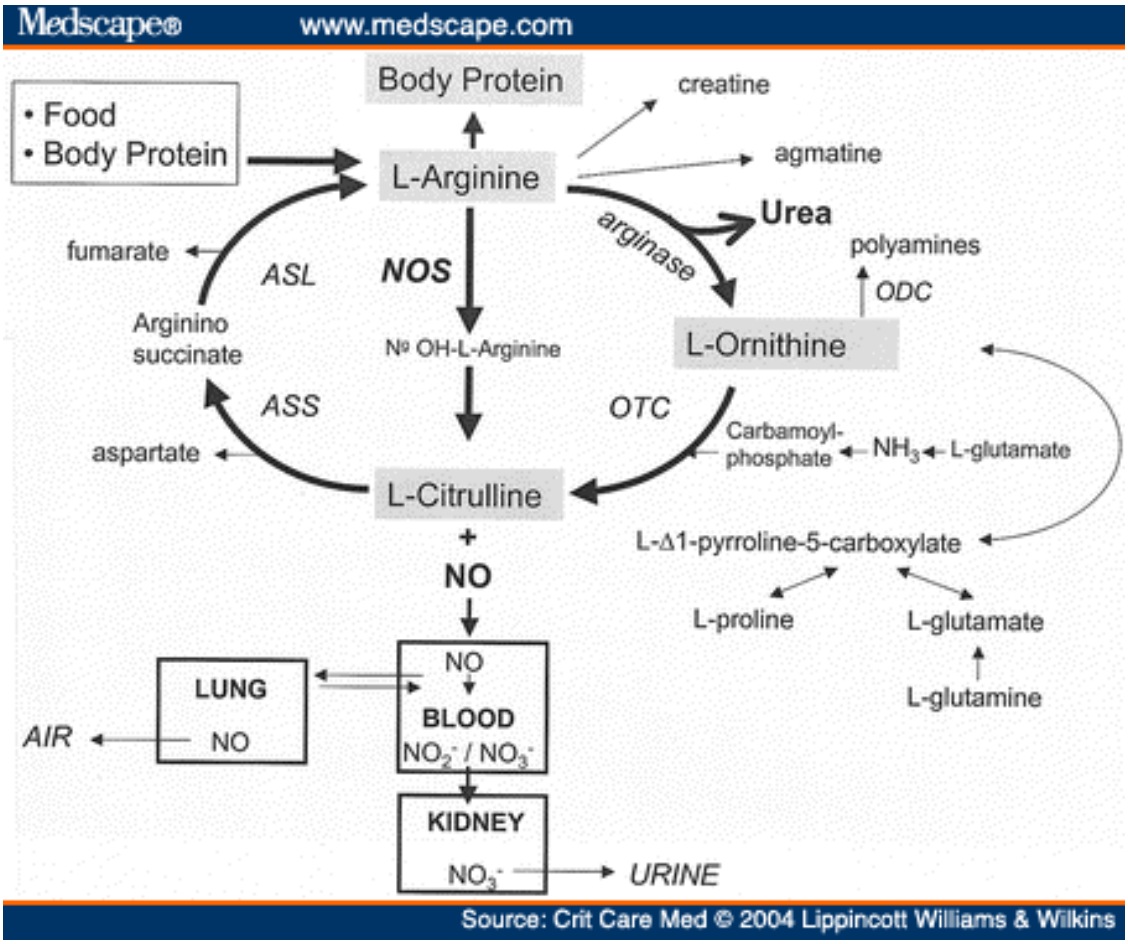


Figure 116: Metabolism of Arginine bia the nitric oxide synthase adn arginase routes. Copied from YC Luiking et al. Sepsis: An Arginine Deficency State? Crit Care Med 2004 32(10): 2135, with permission from Lipincott Williams and Wikins

Much of the work in this area examines NO production from markers in the systemic circulation, with very little information on the burn wound itself. Two animal studies measured higher levels of NO (represented by nitrite/nitrate) in the wound, one using a mouse ear burn model which was then homogenized (91) and the other a mouse foot model with perfusion of the subcutaneous space (92). Inoue showed generally increased levels of NO which were inhibited by the administration of the NO inhibitor L-NAME. Neither investigated more specifically the site of NO production within the injured area. In a burn wound animal model, NO induction by giving intravenous L-

arginine, was found to significantly reduce plasma extravasation in the burn wound, but to increase diuresis and proteinuria (93). Infusion of L-arginine prior to the ischaemic event in an ischaemia-reperfusion model increased the amount of NO available and prevented necrosis (38). The limited time window for success suggests that the either the endothelial uptake of L-arginine, or the nitric oxide manufacturing system are damaged during the reperfusion injury (37).

Blood flow in the wound, related to nitric oxide has been investigated by Lindblom et al (93) They found no difference between those treated with a NO synthase inhibitor NG-nitro-L-arginine (L-NNA), or precursor, L-Arginine, in blood flow in the full thickness burn area, but found that L-NNA reduced blood flow in the surrounding partial thickness area, indicating that NO has a role in blood flow in this area.

Inducible Nitric Oxide Synthase (iNOS) upregulation has been measured from histological specimens of human burn wounded skin. Paulsen et al showed that there was increased iNOS expression in keratinocytes at the healing borders of the burn wound, none in the eschar, and baseline levels in the adjacent unburned skin. Expression within the endothelium of capillaries, and arteriolar smooth muscle was noted, but not formally compared between zones. They conclude that NO has a role in both the acute burn wound and the ongoing healing process (94).

No studies have attempted to measure NO production continuously within the wound. As mentioned above, NO is volatile, must be measured by proxy markers. The amino acids citrulline and arginine are small enough to diffuse into the microdialysis catheter so could be collected using the microdialysis method. Since the method of analysis involved the use of HPLC, results for all amino acids were available and were therefore analysed in this study for any interesting findings.

The majority of amino acids in this study showed no significant change with regard to catheter location or time post-burn injury. This confirms the validity of the experimental method, and strengthens the argument that those amino acids showing changes are reflecting true alterations in physiology within the wound.

Arginine metabolism

Amino acids involved in nitric oxide and urea cycle metabolism showed significant differences between zones in this study. Extracellular arginine levels were consistently higher in the wounded areas than in the uninjured skin, with the zone of coagulation showing the highest levels at each time point.

Arginine is the only substrate for nitric oxide (Figure 116). Arginine can also be converted to urea and ornithine by arginase. This pathway is involved in collagen synthesis and cell proliferation. Both pathways co-exist in the healing wound (95) (96)

Arginine is transported into the cell by specific transporters in response to inflammatory cytokines (97) (98) (99), and this process is co-induced with iNOS (100) (101) (102) (103) (104) (105) (106), increasing the capacity to form NO. Thus the high extracellular arginine found in the burned areas may indicate a loss of cellular uptake, and a decreased capacity for intracellular NO synthesis.

Arginine levels measured in an incised surgical wound model have been previously shown to be undetectable in the wound fluid (95). The disparity between this study and the findings in this thesis may be explained by the thermal mechanism of injury, which causes a wide zone of injury, and involves denaturation of proteins.

Decreased endothelial cell nitric oxide synthesis has been linked to reperfusion injury (37) Thus the higher level of arginine in the injured zones in our study may indicate a sensitivity to secondary injury, and thus wound progression.

Nitric oxide synthase activity utilizes arginine and produces citrulline, and thus nitric oxide synthesis can be measured indirectly by the ratio of citrulline/arginine in the extracellular fluid. Citrulline/Arginine ratio showed the highest levels in unburned skin, with levels reaching a peak at 18 hours, then declining. Levels were relatively constant in Zone B.

High citrulline/arginine ratios, and low arginine levels in the unburned skin perhaps reflect the intact mechanism of cellular uptake and nitric oxide synthesis. This is consistent with findings of increased NO production in the peripheral tissues causing vasodilatation and systemic capillary leak, summarized by Rawlingson (90) However it differs from the work by Paulsen et al which suggests decreased iNOS expression in the surrounding unburned skin, though this work concentrated on keratinocyte expression, and mostly referred to timepoints beyond Day 1 (94).

The reduced levels within the burned tissue have little to compare to within the literature, and may indicate an interesting finding that the wound itself shows an incomplete response or ability to respond to the insult.

Arginine can also be converted to urea and ornithine by arginase. This pathway is involved in collagen synthesis and cell proliferation. Both the nitric oxide and

the arginase pathways co-exist in the healing wound (96) (95). Ornithine/Arginine ratio is therefore an indirect marker of arginase activity. Arginase has been found to be upregulated in wound-derived fibroblasts as compared to normal dermal fibroblasts in surgical wounds in the rat (107). Schaffer et al looked at the time course of nitric oxide synthesis in incised wounds and wound cells using radiolabelled arginine in a murine model (95). They found that arginine itself was virtually undetectable in the wound and postulated that this was due to its immediate use. Labeled citrulline peaked at 24 hours then declined slowly, indicating that this was the peak of nitric oxide synthesis. Ornithine continued to rise until day 10 when it plateaued, indicating that arginase activity followed this pattern.

Ornithine/arginine ratio in this thesis showed significant differences between zones, being highest in the unburned skin. In Zones A and B, the ratio remained relatively constant with time. In Zone C, unburned skin, it appeared to increase until 22 hours post-burn, and then began to decline, though this difference with time did not attain statistical significance. The differences between zones indicated a loss of arginase activity within the burned areas and this may indicate damage to the cellular uptake mechanisms or the enzymatic pathways.

An animal study by Schwacha et al did not show a significant difference between excisional wounds and excisional plus burn wounds in the amount of

arginase activity in wound homogenates at Day 3 (108). This study did not perform a comparison to uninjured skin.

A human study by Debats et al showed increased levels of citrulline, ornithine and nitric oxide in skin graft donor site wounds compared to plasma (109). Arginine was slightly lower in the wound compared to plasma (but not significantly different). This would indicate an increase in arginase activity in the wound. Arginase was measured directly; Arginase-1 was not present in control skin, but present in PMNs in the wounded skin. Arginase-2 was present in keratinocytes, smooth muscle and endothelial cells in both wounded and control skin, but the wounded area showed additional staining in macrophages, fibroblasts and PMNs, with significantly more quantities in the wounded skin. There was no difference between measurements at Day 2 and 5. As discussed above, the burn wound has different features from a surgical wound, and is not directly comparable, but it is interesting to see similar changes in the incised wound in this model, to those seen in the unburned skin in the burn patients in this thesis, adding strength to the argument that the normal healing processes are attenuated in the burn wound itself, but are present in the apparently uninjured skin distant from the wound.

Five patients showed particularly high Citrulline/Arginine ratios. This was accompanied by a similar rise in Ornithine/Arginine in most of these patients, indicating changes in both the nitric oxide synthase and arginase pathways.

Further study of individual patterns in Citrulline, Ornithine and Arginine supported evidence of a rise in Nitric Oxide production in two patients in Zone

B: S09 and S10. These patients both exhibited a decline in Citrulline/Arginine ratio in Zone C after their theatre episodes (at 19h for S09 and 20h for S10). Citrulline/Arginine and Ornithine/Arginine ratios showed similar trends across the study period and between zones, in all patients except for S04, which showed a high Ornithine level in Zone C. Two patients, S10 and to a lesser extent S02, showed evidence of increase in Ornithine/Arginine ratio in Zone B. Four patients, S04, S05, S06, S09, showed evidence of markedly elevated Ornithine/Arginine ratio in Zone C, particularly patients S05 and S09. There was no consistent correlation of either Citrulline/Arginine or Ornithine/Arginine ratios with ischaemia as shown by raised LPR.

It might be expected that the 'normal skin' would be the most stable physiologically, and yet it is here that the greatest changes appear to be occurring. One explanation is that the 'normal skin' has a physiological response to the catheter implantation, whereas the traumatized tissue has a lesser (or different) reaction to the new insult.

Examination of the individual results did not reveal any patterns with regard to changes in Citrulline/Arginine or Ornithine/Arginine in relation to episodes of ischaemia as measured by sustained periods of LPR>25. It was noted that the two patients who attended theatre showed raised Citrulline/Arginine ratios consistent with increased NO production in Zone B, following the theatre episode. This may be due to a secondary insult, or possibly a direct effect of glycerol in the tissues. However care must be taken with interpretation as this is a very small subgroup.

Localised changes in amino acid metabolism can be affected by systemic disease. Holowatz showed that arginase was upregulated in patients with hypertension (110). The decrease in arginine available for nitric oxide production was evidenced by attenuated dermal reflex vasodilatation. This patient group was a heterogenous one in terms of age and comorbidity and some variation in individual results may be attributable to other factors such as these.

Although the data in this thesis is observational, it has shown a difference in the response of the three zones to injury with both the nitric oxide synthase and arginase pathways affected to varying degrees, with the most profound effect being on the arginase pathway. These results could be interpreted as a loss of the ability of cells in the burn wound to uptake or to process Arginine, possibly due to the thermal damage to endothelial cells beyond the immediate borders of the wound. Further study would be necessary to differentiate the effect of probe insertion from that of the burn injury, and to further distinguish the effect of the changes seen on wound healing or wound progression.

Excitatory amino acids and skin: Aspartate and Glutamate

Aspartate and glutamate have been proposed as chemical transmitters of mechanoreceptor responses in the skin, and there is evidence for the involvement of both ionotropic and metabotropic glutamate receptors in mediating this (111-113). Supporting evidence has shown aspartate and glutamate levels in skin biopsies were lower in motor neuron disease patients

with the levels associated with the duration of illness. (114) Glutamate has been implicated in keratinocyte signaling pathways and is linked to wound healing (115).

Aspartate levels in this thesis were initially high, and decreased with time in all zones, but the decrease was more blunted in the zones of stasis and coagulation. Glutamate levels showed a similar trend, although this did not attain significance either for time or probe location.

The data presented here are consistent with similar data on these transmitter amino acids in liver (62). A proposed mechanism for the findings is that initial implantation injury results in the release of aspartate and glutamate into the extracellular space. The injury quickly stabilizes and uptake mechanisms begin to remove the transmitter amino acids from the extracellular space to avoid over-stimulation of receptors, and toxicity. Our results showed that the downward trend with time was much reduced for both the zones of coagulation and stasis. This suggests that the uptake mechanisms have been impaired by the burn injury locally, and this could in turn lead to over-stimulation of receptors, or toxicity, and a delay or prevention of normal wound healing mechanisms. Thus, although normal skin is damaged by probe implantation, endogenous uptake mechanisms quickly restore the 'status quo'. In contrast, these uptake mechanisms have been rendered less efficient by the burn, more so in the zone of coagulation than in the zone of stasis, so these tissue sites take longer to recover from probe implantation and may not recover fully at all.

Taurine

Taurine has osmoregulatory and neuromodulatory roles which have been studied in the central nervous system (116). Its neuromodulatory role protects the cell from overstimulation of excitatory amino acids in the context of ischaemic injury, and has been linked to glutamate receptor stimulation, and to nitric oxide activity (117). It is known to protect the cell from free radicals, and improves in vitro polymorphonuclear cell function in normal and burned patients (118). Its osmoregulatory role has been explored in the brain. Taurine levels were found to increase when neuronal and glial cells were placed in a hypotonic environment, and prevented further swelling of the cells in vitro (117).

Two patients within this thesis showed rising levels of extracellular taurine within the zone of stasis during the study period.These are the two patients with the strongest suspicion of a secondary insult, as shown by a rise in LPR and glycerol after a theatre episode. Of note was a concomitant rise in Citrulline/Arginine ratio in S10 following the theatre episode, indicating increase in nitric oxide production. The two patients with raised levels of taurine within the first 4 hours of recruitment were both within the group, recruited early, in which significant peaks of glycerol were found in the zone of coagulation This supports the theory that this early glycerol peak is due to the primary injury.

This observational study has revealed that there are significant differences in levels of excitatory amino acids, and amino acids involved in the urea pathway between different zones of burn when compared to unburned skin in acute burn

patients. The response of excitatory amino acids appears to be dampened in burned skin. Arginine levels were significantly higher, and Citrulline/Arginine ratio significantly lower in the zones of stasis and coagulation, than in the unburned skin, indicating that there is a reduction of arginine uptake, and synthesis of nitric oxide in the areas of injury. It appears that the thermal injury shows different trends from an incisional wound. The protective amino acid, Taurine, showed increased levels in the zone of stasis following a possible secondary ischaemic event.

5.2 Discussion of Microdialysis Technique

The microdialysis system used was easy to handle, and straightforward to use. The probe insertion technique was mastered quickly, and insertion into the dermis was shown to be reproducible on ultrasound examination. Both volunteers and patients tolerated the insertion procedure well. Retention of the probes in the skin for the study period was generally very good, although a few probes were lost due to displacement. There were no adverse events. For the majority of the time, the probe and pump system produced consistent and reproducible results.

A few abnormal sample volumes were noted (See Table A20 in Appendix C).

There appeared to be a tendency of the analyzing equipment to give falsely low results if the sample volume was insufficient. Once this was recognized, the approximate volumes of the sample vials were noted in patients S09 and S10 to

allow identification of possible anomalous results. Since the calibration study assessing the effect of volume on analysis was inconclusive, these low volume results were included in the statistical analysis.

On several occasions it was noted that the pump output was inappropriately high, possibly due to a temporary power failure initiating the pump flush sequence. In addition to any effect on the analytical process, a higher flow rate will have decreased the relative recovery, leading to a falsely low result for all substances within that sample. Episodes where this occurred have been noted in Appendix B Table A20.

Microdialysis samples from a number of probes contained blood, and therefore had to be discarded. The maximum pore size of the microdialysis probes was 20kDa, which is less than the size of haemoglobin (68kDa). This finding may have been due to damage of the probe on insertion into the skin. This explanation is consistent with information from the manufacturer's website (119). An alternative insertion method, which puts less pressure on the probe, may need to be sought to prevent this occurrence.

Since the microdialysis probe must be placed invasively into the tissue, there is potential for a tissue reaction to the probe, which could confound results. This is of particular importance in the burn wound, since many of the processes to be measured are those relating to inflammation. Additionally, any time for equilibration of the probes must be minimal to allow the earliest possible reliable sampling in this acutely presenting study group.

Local anaesthetic was given in the normal volunteers and the unburned and zone B areas of the burn patients to make probe insertion less painful. EMLA

was used in normal volunteers as it was thought to be more acceptable for this group. 1% lignocaine injection was given to the burn patients in the interest of a timely probe insertion, and because the occlusive dressing required with EMLA would be difficult to use on an area with epidermal loss. The effects of local anaesthesia are discussed below, and were not thought to have an effect on results. However, a potential confounding factor cannot be ruled out and therefore ideally a more detailed study on the comparative effects on EMLA vs 1% lignocaine would be required.

Groth (120) studied the effects of insertion trauma on human skin. She found significant increases in skin blood flow, colour and erythema which had normalised by 120 minutes. This was confirmed by Anderson (121) and Petersen (77), who found increases in perfusion lasting 60 minutes and 90-135 minutes respectively. The former and the latter of these three studies used linear probes, with may represent a greater degree of trauma than with the double lumen probe. Probe insertion without prior anaesthesia was found to have a change in blood flow of a greater magnitude, and lasting for a longer period than those inserted with anaesthesia (122). Skin thickness was increased as a result of probe insertion by up to 38% as measured by ultrasound, and did not return to normal within 2 hours. The finding of a low echogenic area around the probe would suggest oedema formation (122). However Ault et al found no oedema or tissue disruption (123). After 6 hours a lymphocyte infiltrate has been noted in skin (124) and after eight days a fibrosis was apparent at the insertion site in liver (125).

Results in this thesis showed that an equilibration period is necessary for some but not all substances. Glucose, Lactate and Pyruvate individually were stable from the time of probe insertion. Glycerol required 30 minutes equilibration time, and LPR calculation required one hour. This knowledge is important when interpreting results from probes inserted into burn patients. There is no need to completely disregard all results for 90 minutes as was implied from previous studies. However, results need to be interpreted with caution with respect to LPR (for the first 60 minutes) and Glycerol (for the first 30 minutes). It is likely that the small rise in Glycerol is caused by insertion trauma. Lactate, Pyruvate and Glycerol molecules are all smaller than Glucose, and if the difference were due to diffusion characteristics, it would be more likely that glucose would show the longest equilibration time.

The hypothesis asserts that the changes observed within the burn wound are due to the initial burn injury and to the natural progression of the burn proceeding. The assumption is that the insertion of the probes had no lasting effect on the natural progression. This assumption was based on the findings of Study 1 in normal volunteers and on the supporting literature. However such an assumption, based on findings in uninjured skin, may not hold true for extrapolation to burned skin. The constraints of a clinical study meant that recruitment and probe insertion could not be commenced at a uniform time following the burn. Based on the findings of Study 1, a decision was made to analyse the data based on time post burn rather than time post probe insertion. The striking findings of glycerol in the zone of coagulation appear to support the validity of this assumption. There were marked peaks only in patients

recruited early in the post-burn period which were not present in those recruited later. This supports the interpretation that observed changes were attributable to the burn and not to the secondary trauma of probe insertion.

For consistency, the same method was used to interpret the amino acid data. However no data were available for amino acids from probe insertion into normal volunteers, and the same assumptions that probe insertion causes only temporary changes may not be valid. Whereas lactate, pyruvate, glucose and glycerol are markers of basic glucose metabolism in the tissues, some of the amino acids are a reflection of the neuro-humoral responses in the wound. Certain amino acids are much more likely to show changes related to probe insertion, and so should be interpreted with caution in this study. This effects of the microdialysis probe on different substances and physiological processes is highlighted in a study by Stenken & Church (126). A further study would be necessary to ascertain the effect of probe insertion on amino acids in the burn wound.

To allow a calculation of the actual tissue concentration of a substance measured within the microdialysate, a recovery coefficient is required. Recovery of substances from the tissues using microdialysis can only reach 100% at a theoretical flow rate of zero.

In-vivo and in-vitro systems are different and require different methods of calibration. An in-vitro system is static. There is no replenishment of substances by a capillary supply, therefore dilution occurs as substances are removed in the dialysate, but there is no other change in the environment. Direct comparison to

a known concentration of a prepared solution, or time-based methods, such as no-net-flux can be used to calculate recovery.

An in vivo system is dynamic. Concentration of substances is constantly changing, in response to the local and systemic environment. The microdialysis system competes with capillaries for uptake of substances, and changes in blood flow affect rates of recovery. Levels of substances are in constant flux and are very difficult to control for. True values are almost impossible to attain. Most methods of calculating recovery are performed in series, and are based on the premise that there is no change in the environment.

Flow-rate variation (127), one of the first methods of calibration to be published, uses the principle that relative recovery varies with flow rate. At high flow rates, recovery is low and it increases with lower flow rates. 100% recovery occurs at a flow rate of zero. Therefore a series of flow rates are utilised, and the results plotted on a graph. Extrapolation to zero flow gives the true value in the tissue. This is described by the equation $C_{dial} = C_0(1-e^{-rA/F})$, where C_{dial} is the concentration of the compound in the dialysate collected, C_0 is the concentration in the fluid surrounding the probe, r is the mass transport coefficient, A the surface area of the membrane and F is the flow rate. Disadvantages of this method include the length of time needed to perform these serial tests, particularly when using lower flow rates where collection of adequate sample volumes for analysis can be lengthy. Also, extrapolation contains an exponential function which may be difficult to determine. It is suitable for in-vitro studies only.

Method of no-net-flux (128) utilises the premise that there is only a change in the concentration of the perfusate if there is a concentration gradient by which the substance in question can diffuse across the membrane. If the concentration in the perfusate equals that in the dialysate, then there will be no movement across the membrane (i.e. "no net flux"). Varying concentrations of the substance in question are added to the perfusate, both higher and lower than the assumed concentration in the tissue. Where the concentration in the perfusate is lower than that of the tissue, the substance will move into the microdialysis system, and the dialysate concentration will increase. Conversely, where the concentration in the perfusate is higher than that of the tissue, the substance will move out into the surrounding tissue, leaving a lower concentration in the dialysate. Concentration of the perfusate (C_{perf}) is plotted against the difference between the concentration of the dialysate and the concentration of the perfusate ($C_{dial} - C_{perf}$). On the graph plot, the points are joined with a straight line, and where this line crosses zero, the point of no net flux, can be assumed to be the actual tissue concentration of the substance. This method is more accurate than the flow rate variation method since interpolation rather than extrapolation is used. However this method is also time-consuming, and assumes a steady state throughout the experimental period. This is most suitable for an in-vitro, or steady state in-vivo environment.

Dynamic no-net-flux (129) (130) takes advantage of parallel study design in perfusing each individual in the study with a different concentration for one unit of time, and then pooling the results. While this reduces the time needed for calibration, it also assumes all probes, and all individuals will behave in the same way, and gives an average calibration for the whole group. Higher

numbers of participants are therefore required to overcome this inter-subject or inter-probe variability. For certain studies, particularly those where all other conditions can be controlled, this method is a useful alternative. However in the clinical setting, there is too much inter-patient variability both in individual physiological response, and in the disease severity, for this method to be feasible.

Retrodialysis (131) uses the principle that the passage of a substance across the membrane is equal in both directions. A substance of known concentration, and which does not already exist in the peri-probe tissue, is added to the perfusion solution. Measurement of the remainder in the dialysate allows calculation of recovery, as loss of this substance should be equal to recovery of the other substances in question. Recovery can thus be calculated using the following formula: Recovery (%) = 100 – (C_{dial}/C_{perf} x100). A variation of this method is the use of an internal standard. This consists of the addition of a standard amount of a substance to the perfusion fluid throughout the study period. The concentrations both of the remaining calibration standard, and of the substance in question can be expressed as a ratio to give recovery for any one sample during the entire study period. This method is therefore of particular use in the clinical setting as a continuous measure of recovery is then available. The proviso for this method is that the substance added to the perfusate does not interfere with the biochemical environment being monitored. In the zone of stasis, an additional problem may be that due to the postulated impairment of capillary function in this zone, the clearance of this substance may be impaired, allowing it to build up within this zone. While this would indeed provide useful information on the alterations in recovery as a result of this ‘closed’ system, it is

impossible to predict the effect of this substance on the very metabolism being monitored.

The Endogenous reference substance method (69) (132) has the advantage of being less time-consuming, and not requiring steady-state conditions. It relies upon the existence of a substance within the tissue to be studied which diffuses freely between compartments, and is small enough to enter the microdialysis system. This substance should not be affected by the environment which is being studied. Several substances have been investigated, but urea has been acknowledged to be an ideal candidate, due to its properties of freely diffusing within tissue. It has been proposed that its recovery in vivo should be similar to its recovery in vitro for this reason (132). However, this takes into account only its properties of equal diffusion through tissues, and not the difference in tortuosity between living tissue and liquid media. An experimental study in humans by Strindberg supported this argument, showing an in-vivo to in-vitro recovery ratio of 0.4, comparable to that of the other substances tested. In this study, comparison of plasma and interstitial concentrations was performed, and urea was found to be equal in the muscle compartment and the plasma. These authors have therefore suggested that true interstitial values can be calculated for all substances, based on a plasma reading of urea, and the dialysate concentrations of urea and of the other substances in question (69). In contrast, Brunner performed a similar study to assess the validity of the urea endogenous reference technique, and found that results for glucose were significantly different when calculated by the urea endogenous reference technique and the no-net-flux method. He explained this was most likely due to a difference in the

ratio between in vivo and in vitro recovery of the two substances, a basic supposition of the urea endogenous reference method.

For the in-vitro component of this thesis, direct comparison with a solution of known concentration was used. The solution itself was tested before and after microdialysis had taken place, and the microdialysate was compared to it. Probe recovery overall was high (Appendix B, Tables A6-A9), as would be expected with a slow perfusion rate of 0.3µl/min. A single probe measured consistently low during the calibration experiments. The remainder of the probes showed recovery rates of around 100%. This showed that the results obtained in these experiments, for glucose, lactate, pyruvate and glycerol, could be reasonably assumed to reflect the actual values in interstitial fluid throughout these experiments. Interestingly, the majority showed recovery rates above 100%. Reasons for this are not clear, and there is no discussion of this in the literature.

Dilution of the environment being sampled (in this case the calibration solution) can occur during microdialysis sampling in a closed system. This in theory could lead to a lower denominator, giving a falsely high % recovery. In this study, the calibration solution was measured immediately prior to the start of microdialysis, to prevent this error.

Contamination of a sample from residue in the probe could lead to a falsely high result (if the previous probe environment was more concentrated). This would give a falsely high % recovery. There is no evidence to suggest contamination of samples from residue in the probe had any effect in this study. In the pre-study calibration (Appendix B, Tables A6-A9, Column C1, 01, 02), a new probe

was placed into the calibration solution and microdialysis took place for two continuous 30 minute periods. In all cases except NV11, the first result (01) was lower than the second (02), showing that any contaminant, from probe manufacture or initial flush sequence, was serving to dilute rather than concentrate the samples. Indeed at the end of study calibration (13), where in some cases the probe had been in the more concentrated environment of the volunteer's skin, results show % recovery values which are almost identical to those from the first calibration.

A sampling error of the analyser overestimating the numerator, and/or underestimating the denominator could explain this, however it would be expected that this would occur in a random fashion. In this series, not only was there a significant majority of the probes reading >100%, but the probes themselves showed consistent readings for pre- and post- microdialysis calibration, and for the different substances. This points towards a sampling equipment related reason.

Evaporation of water from the calibration fluid during the microdialysis process could be another cause. Since the sample of calibration fluid measured in the analyser, was taken prior to microdialysis taking place, the measured sample may be more dilute than the actual fluid sampled during the microdialysis process. If this was the case, it would be expected that the values at the end of the experiment (columns C2; % recovery 2) would show a higher value than those at the beginning (columns C1; % recovery 1), however this was not the case for the majority of results.

The probe used in NV02 showed a lower % recovery than the other probes. This was consistent at the beginning (% recovery 1) and the end of the study (% recovery 2), indicating a malfunction with the probe. It is possible that part of the membrane was blocked, giving the effect of a shorter probe, and thus a reduced % recovery.

In summary, all of the results except one are close to 100%, showing that recovery with this method is consistently high. It is unclear why the % recovery calculated was often >100%. It must be kept in mind that there may be an occasional underperforming probe.

The endogenous reference technique seemed to be most appropriate for the in-vivo studies undertaken in this thesis. At the planning stage of this study, the endogenous reference technique using urea was not known to the author. A late addition was made to the protocol after the commencement of the study, to attempt to gain the information needed to perform these calculations. There were a number of limitations to the data thus collected. Instead of protocol-based collection of timed blood samples and simultaneous microdialysis, blood samples drawn during the course of routine care, at varying times, had to be used retrospectively, after permission for their use was obtained from the Ethics Committee. Accurate data on time of blood collection was not available and had to be estimated from the biochemistry laboratory booking system. Length of storage before microdialysis occurred was variable. It is therefore impossible to comment on the accuracy of this reference technique in this study.

In this thesis, urea showed good concordance between zones in the burns patients, and therefore shows promise for use as an endogenous substance. Any further study should ideally include microdialysis of serum samples synchronous with the dermal microdialysis, to allow the endogenous reference substance method to be further evaluated. Alternatively, the retrodialysis method could be used, which would require further investigation into a suitable substance, which would not interfere with local metabolism.

Best attempts were made to maximise recovery in this study to ensure that results are as close as possible to real values. It is known that recovery is proportional to length of membrane, and to flow rate. The longest membrane commercially available was used, together with the lowest practical flow rate, 0.3µl/min. This is commonly accepted as the optimum flow rate in order to balance sampling volumes (and time intervals) with maximum recovery. The in-vitro methods of calculating recovery in this thesis showed results around 100% recovery. With a recovery ratio close to 1.0, the requirement for an accurate coefficient decreases.

Thus the endogenous reference technique for calibration using urea in these experiments was not possible. This means that absolute values cannot be calculated with accuracy. However this does not affect the validity of the analysis of patterns of changes with time and across zones.

In order to assess the effects of sample volume and storage conditions on the results, a set of supporting studies was undertaken in this thesis. Some statistically significant differences were identified amongst the results of these experiments, although the size of the differences was very small. There was no clear trend in samples becoming either more or less concentrated over the study period with each particular storage condition or sample volume, nor was there any storage condition or sample volume which consistently produced more concentrated samples.

Since Day 1 samples for storage analysis were identical, any differences observed could not be attributed to the variability in storage conditions. In the case of pyruvate and glycerol there were significant differences at the $P<0.05$ level between Day 1 samples. This may have been due to the error range of the analytical equipment, or due to a Type 1 statistical error. Each sample took 7 minutes for full analysis, so the maximum length of time between analyses was 63 minutes, leaving room for the possibility of machine drift or evaporation of samples.

Differences between days, and thus between batches, introduces the possibility of variation due to recalibration of the analyser. If the differences were attributable to storage conditions or sample volume alone, it would be expected that a trend in the direction of the difference (either becoming more or less concentrated) would be observed. This was not the case: the differences appeared to be random, supporting the likelihood that the significant differences can be attributed to slight variations in the analytical process.

A total of eight results lay outside of two standard deviations from the mean. For each of lactate, pyruvate, glucose and glycerol, there was a single outlying result. Reviewing the results revealed that the outliers were all from different sample vials, and different variables. These are likely to be random occurrences. Four outliers were found within the urea results. The three below 2 standard deviations from the mean were all located within the High Volume samples. The one abnormally high result was from the Low Volume samples. On initial observation there appears to be a trend here, however the High Volume samples, as well as containing the three lowest results, also contained some of the highest results, so it does not appear that the low results are entirely due to the high sample volume. The Low Volume samples were all within the high range but there was no trend with time, so any concentrating effect caused by the low volume of the samples is not affected by repeated freeze-thaw cycles.

Although statistically significant, the size of these differences is very small, and the clinical significance of this is doubtful. Further clarity would be gained by repeating this study with a larger sample size.

11 samples would be required for a two-way ANOVA to have at least an 80% chance of detecting a difference in means at the 5% level of significance for glucose, lactate, pyruvate and urea. Glycerol showed a non-normal distribution and would therefore need to by transformed before analysis.

In summary, this thesis has shown that microdialysis of the burn wound environment is both possible, and promising. The procedure was well tolerated by patients and volunteers, and there were no adverse effects. A normal range

for glucose, lactate, pyruvate, glycerol and urea within the dermis was calculated from volunteers, a useful baseline for future studies. Significant findings within the burn wounds included an initial peak of glycerol in Zone A early following burn injury. Low glucose together with high LPR gave evidence of peripheral ischaemia in the unburned skin early on, with a normalization over time. Significant alterations in physiology from a theatre episode raised the possibility of either capillary leak in the injured zones, or cell breakdown in response to the surgical or anaesthetic manipulation. High arginine and low citrulline/arginine ratio in the burned areas may indicate a failure of nitric oxide synthesis in zone A with a reversal of this trend in the unburned skin. This has not been previously described in relation to the zones of the burn. Arginase activity, which relates to wound healing processes, was also found to be greatest in the unburned skin.

The microdialysis technique itself, and supporting studies have been critiqued, and a series of recommendations follows for future work.

6 CONCLUSIONS AND FURTHER WORK

The microdialysis method is a safe and valid technique to monitor metabolism within the skin, in normal subjects, and following a burn injury. Probe insertion was consistently achieved within the dermal layer of the skin, and was well tolerated by patients. It was shown that changes in ambient temperature do not affect microdialysis results when using the probes and flow rates selected for this study. The microdialysis method is the first time in-vivo measurements of the biochemistry of a burn wound have been possible, giving a window into the workings of the wound environment. This series of studies represent the first steps in gaining the ability to monitor progression of a burn in-vivo. Results have shown differences between the zones of the burn wound, and changes in the environment with time following burn. Some consistent patterns have been identified, including evidence of primary injury, with a high peak of glycerol in patients recruited in the early hours post-burn. Some prolonged periods of low glucose and high LPR were observed, consistent with hypoperfusion and ischaemia, but in no patient did this lead to a rise in glycerol (an indicator of cell death) in any zone. Theatre visits had an effect on the biochemistry of these zones, with release of glycerol in zones of stasis and coagulation, however this was not preceded by low glucose or high LPR, and may have been due to anaesthetic agents. Burn progression is multifactorial, and burns patients are complex. There was insufficient evidence from these studies to conclusively link microdialysis changes with wound progression, as only one of the patients showed progression over the area of the probes. There was no evidence to suggest that any rises in glycerol were caused by an ischaemic mechanism.

Significant results in the amino assays were found to support the previous theories on loss of endothelial cell nitric oxide synthesis in the burned areas. The burn wound showed behaviour distinctive from that of a surgical wound in that high levels of arginine were measured, in contrast to other studies of incised wounds. Low Cit/Arg in the burned areas as compared to unburned areas, indicated low nitric oxide synthase activity in the injured zones. This is consistent with the burn wound model showing endothelial damage beyond the immediate cellular damage, and thus the wound being susceptible to further injury, though this further injury was not observed within our patient cohort.

These studies have given a valuable insight into the exciting opportunities which the microdialysis technique brings to the field of burns. As a series of pilot studies, this thesis has identified patterns in the course of the burn wound which would be worthy of further research. From the experience of these studies, the following recommendations can be made for further research;

1) The normal volunteer studies give valuable baseline data for comparison with diseased states in future studies.
2) Identification of zones of burn, as described by Jackson, was found to be difficult in this study, since the method used in the original paper required tourniquet control of the area. This was not possible to apply in wounds of the trunk. While it is of academic interest to define these areas accurately, it is perhaps unnecessary for evaluating burn wound progression. Since in clinical practice the burn wound can be seen to progress in depth from a superficial to a deep burn, monitoring of an area of burn of superficial or mid-dermal depth

on initial examination should be adequate to detect progression. This theory was supported by the differences in the biochemistry seen in the three distinct areas monitored in the studies in this thesis. However caution should be used since there was little evidence of any wound progression in any of the 'zone of stasis' areas. A better way of defining this zone may be sought, for example using confocal microscopy (See Section 1.2.3.4).

3) Monitoring of progression of a burn using the microdialysis technique may have some intrinsic difficulty. One potential confounding factor is the introduction of hydration, via the microdialysis cannula, directly to the area in which the mechanism of progression may be linked to profound dehydration. An animal study would be ideal to study the question of whether the microdialysis technique has a protective effect on the wound, preventing progression. This question would need to be answered before a larger study, to look at whether burn progression can be predicted, could be commenced.

4) Further investigation could be made into the reason for some of the volume variations of samples seen in this study. A simple mechanical fault could be ruled out by running the pump in an in vitro environment and measuring volumes produced each half an hour. The effect of a dehydrated environment, on volumes produced, could also be evaluated experimentally.

5) Analysis calibration studies in this thesis were performed on a small sample number (n=3). The resulting statistically significant differences, although clinically small, led to some uncertainty in their interpretation. These experiments could be repeated with a larger sample number to clarify the effects of volume and storage conditions on the analysis.

6) The endogenous reference method for in-situ calibration was not addressed adequately in these studies, as the concept was introduced late into the study design. The urea endogenous reference method did not produce promising results, because the method (comparisons with urea from blood samples drawn at varying times and analysed retrospectively) was flawed. We would recommend blood samples to be taken as part of the study protocol at pre-determined times, and the serum microdialysed contemporaneously. This would require an additional microdialysis pump and probe. Alternatively a different reference method could be used, for example the retrodialysis technique (see Introduction 1.3.2.2).

7) Interpretation of results in this study was based on the assumption that the insertion of the probe had no effect on the tissue being studied. Therefore, although probes were inserted at different times post-injury, all results were analysed with reference to time post-burn. It is worth noting that if the probe insertion itself causes a response in the tissues (i.e. a second injury), the method of analysis in this study may have hidden interesting data trends. A further study design could address this issue by standardising the time of probe insertion in relation to the time of burn. This would certainly be possible in an animal model.

8) LDI is a widely accepted, validated method for measuring burn wound depth, however results were somewhat disappointing in this study. More familiarity with the LDI may improve the quality of images, and attention should be paid to ensuring appropriate skin temperature of the wound being imaged to avoid a globally hypoperfused image seen in at least 2 patients in this study.

9) The interesting finding of raised glycerol in zones of coagulation and stasis during theatre episodes warrants further investigation. Microdialysis of a series of animal or human burn wounds in a standardised scenario, with or without the administration of propofol, or of a systemically administered, labeled substance would clarify the source of the additional glycerol seen in the wound, and ascertain whether further investigation on a secondary insult during the theatre episode is warranted.

10) Significant differences in Citrulline/Arginine ratios, and in Arginine values were seen in burned areas of these patients, but without evidence of wound progression. Whilst loss of nitric oxide production itself may not cause reperfusion injury, loss of its protective effect, in combination with a large secondary insult, may have a permissive effect in allowing wound progression. This could be further assessed using a standardized animal model for burn wound progression.

11) The microdialysis technique could be used to monitor other substances in the burn wound such as cytokines and Damage Associated Molecular Pattern Molecules (DAMPs). This could give insight into cell signaling pathways, and the ongoing immune response seen particularly in patients with major burns. DAMPs have a broad spectrum of molecular weight from around 6kDa to 100kDa. Pore size of the probes needs to be 4x the size of the molecules to be investigated to allow good recovery, and commercialy available probes have a maximum pore size of 100kDa. A custom probe may need to be made in order to investigate some of the larger molecules.

Appendix A: Ethics Approval and Patient Consent

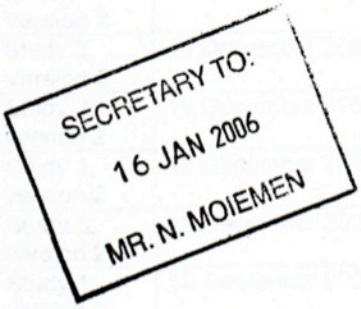
Ethics committee approval was given for the study by the Black Country Ethics Committee. Ref 05/Q2702/76. See below for copy of approval form, and samples of the consent forms and patient information sheets prepared for the study.

Dudley Local Research Ethics Committee
Dudley Beacon and Castle PCT
Public Health
Second Floor
St Johns House
Union Street
Dudley
DY2 8PP

Telephone: 01384 366871
Facsimile: 01384 366485

Elly

13 January 2006



Mr Naiem Moiemen
Honorary Senior Lecturer University of Birmingham
University Hospital Birmingham NHS Foundation Trust
Burns Unit, Selly Oak Hospital
Raddlebarn Road, Selly Oak
Birmingham
B29 6JD

Dear Mr Moiemen

Full title of study: **Investigation of dermal metabolism in burned and unburned human skin using the microdialysis method to quantify peripheral resuscitation following burn injury**

REC reference number: **05/Q2702/76**

Thank you for your letter of 21 December 2005, responding to the Committee's request for further information on the above research and submitting revised documentation.

The chairman considered the further information on the 11th January 2006.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The favourable opinion applies to the research sites listed on the attached form.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Trial R&D number: RRK 2975

Patient Identification Number for this trial:_____

CONSENT FORM

Investigation of dermal metabolism in burned and unburned skin

Study 1 – Normal skin

Name of Researchers: Miss E Breuning, Mr N Moiemen, Dr P Gosling, Dr D Richards

1. I confirm that I have read and understand the information sheet dated _____

(version _____) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

2. I understand that my participation is voluntary and that I am free to withdraw at any time

without giving any reason, without my medical care or legal rights being affected.

3 I agree to have a sensor inserted into the skin of my forearm for 4 hours. I agree that

samples of fluid taken from my skin may be used for analysis and stored for further study.

4. I agree that any photographs or other images taken during this period may be

used in future scientific publications. I understand that I will not be identifiable from these images.

5. I understand that relevant sections of any of my medical notes and data collected during

the study, may be looked at by responsible individuals from the research team, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.

6. I agree to my GP being informed of my participation in the study.

7. I agree to take part in the above study.

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature

_____	_____	_____
Researcher	Date	Signature

Participant Information Sheet – Study 2

Study Title: Investigation of dermal metabolism in burned and unburned human skin

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

- Part 1 tells you the purpose of this study and what will happen to you if you take part.
- Part 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Part 1

What is the purpose of the study?

Background

Burn injuries present the patient and the medical team with several problems.

Small burn injuries cause localised problems in the injured area. A burn is an open wound which is prone to infection before it heals. Infection can cause the patient to become unwell. It also delays healing and makes scars worse.

Extensive burns behave differently to small burns. In addition to the above problems, extensive burns can lead to swelling of the normal tissues as well as the burned skin itself. This process can be life-threatening, but in surviving patients leads to further delays in healing, and can increase the area of the scar.

We know that patients whose wounds heal quickly are much less likely to suffer complications and will leave hospital sooner than those who take longer to heal.

Research into the processes that occur in burn wounds is vital to our understanding of how these wounds heal. This will help us to identify ways in which we can improve treatment in order to improve healing, leading to lower infection and improved survival rates in large burns, and improvement in the long-term appearance of scars.

Zones of injury

A burn wound can be divided into three areas which behave differently. The central zone is permanently damaged, but there is a zone surrounding this which can heal or can turn into a permanently damaged area depending on various factors. The normal skin in small burns is assumed to be unaffected, but in larger burns the normal skin changes too. It is important that we study each of these zones of the burn to gain a better understanding of what factors improve the chances of healing.

Microdialysis

Microdialysis is the name of the process by which we can obtain a sample of fluid from the skin. The equipment is composed of a thin probe which contains a type of filter, similar to that found in a kidney dialysis machine. This is the part which is inserted into your skin. It is put in place using a needle which is then removed. The probe is connected by plastic tubing to a small pump which pumps very small amounts of sterile fluid through the probe. The fluid is collected in tiny plastic bottles (a small droplet every 10 minutes), which are then removed for analysis.

Why have I been chosen?

We need to study twelve patients who have had a burn injury which is less than 15% of their body surface area. You have been chosen because you fit into this category, and because your burn was sustained within the last 5 hours.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason.

What will happen to me if I take part?

If you agree to take part, you will have exactly the same treatment for your burn as if you did not take part. The only difference is that we will insert three probes into different areas of your burn wound to allow us to measure the changes in the skin from normal.

The following sets out what will happen during this period:

1**. Local Anaesthetic Injection**: A small injection will be given to you in two places around your burn. This is local anaesthetic to numb the area. This is the only part of the study which is uncomfortable. It means that you won't feel the probes being put in. The centre of your burn doesn't have any nerve fibres, so it is not necessary to have an injection here.

2. **Cuff Inflation**: A blood pressure cuff will be put on your arm or leg above the site of your burn. It will be inflated so it is tight for no longer than 3 minutes. This will help us to identify the correct areas of the burn in which the probes will be placed.

3. **Insertion of microdialysis probe**: A microdialysis probe will be inserted into the numbed skin of your burn in three areas. A needle (similar to those used for drips in hospital) will be used to put the probe in place. The needle will be removed, but the probe remains in the skin. This is connected to a small pump (about the size of a mobile phone) which will be started immediately. Fluid is collected in a small plastic tube. These tubes will be changed every 30 minutes throughout the study and will be labelled for analysis.

4. **Your stay in hospital**: Your treatment will continue as normal while you are in hospital, except that every 30 minutes we will disconnect the sample from the machine and connect a new collecting tube. This should not disturb you. You will receive standard dressings on your burn wound. These are the same as you would have received if you were not in the study. The equipment will not restrict you from moving around.

5. **Removal of probe**: After 36 hours, the study will be complete and the probe will be removed. No further input will be required from you. If for any reason it becomes necessary for you to leave hospital before 36 hours is complete, we will remove the probe and stop the study early.

N.B. If you feel uncomfortable at any point, please inform one of the nursing staff. The study can be stopped at any time. If you change your mind at any point, you are free to do so.

If you have any concerns following the procedure you are most welcome to contact us using the details below.

Expenses and payments

No payment will be made to you for your participation.

What do I have to do?

You will be admitted to the ward as usual. The Research Doctor will explain the procedures to you before doing anything. We would ask you not to interfere with the probes or equipment while they are attached to you, since some parts of the equipment are quite delicate. If they cause you any discomfort, please speak to a member of staff who will deal with the problem. You may walk around with the equipment, but please speak to a member of staff if you wish to leave the ward, or if you wish to take a shower.

What is the drug, device or procedure that is being tested?

Microdialysis is the name of the process by which we can obtain a sample of fluid from the skin. The equipment is composed of a thin probe which contains a type of

filter, similar to that found in a kidney dialysis machine. This is the part which is inserted into your skin. It is put in place using a needle which is then removed. The probe is connected by plastic tubing to a small pump which pumps very low volumes of sterile fluid through the probe. The fluid is collected in tiny plastic bottles (a small droplet every 30 minutes), which are then removed for analysis.

What are the side effects of any treatment received when taking part?

You are not being given any medication or treatment during this study. The needle placed in your arm is taking samples from your skin using a sterile solution to collect it. There is a small potential risk of having an allergy to the materials present in the needle, probe or tubing.

What are the other possible disadvantages and risks of taking part?

The needle, probe, tube and fluid are all sterile, and the skin is cleaned before the needle is inserted, however there is a very small risk of getting an infection from where the needle is inserted, as would be possible with any scratch.

The local anaesthetic injection is in routine use in hospitals. It is usually used to numb the skin before a minor operation. There is a theoretical risk of allergy.

The fluid in the pump is a sterile, neutral solution. It does not contain any drugs. There are no long-term risks from this study.

There is no reason to suspect that any part of this study could harm an unborn child. However the results we obtain from your skin could be different if you are pregnant. If you know you are pregnant, we will not perform the study on you at this time. If you find out later that you were pregnant at the time of the study, there is no need for concern, however please contact us so that we can adjust our results if necessary.

What are the possible benefits of taking part?

There are no direct benefits to you in taking part. We hope that as a result of this study, there may be benefits to burns patients in the future.

What happens when the research study stops?

You will not be required to have any more participation after the day you attend. However, if you wish to receive information on how the study is progressing or details of any scientific publications, then please inform us and we will send you a summary sheet at the end of the study period.

What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

Will my taking part in the study be kept confidential?

Yes. All the information about your participation in this study will be kept confidential. The details are included in Part 2.

Contact Details:

Elly Breuning

Burns Research Fellow

Tissue Services

Burns Unit

Selly Oak Hospital

Raddlebarn Road

Birmingham

B29 6JD

Tel: 0121 627 8718 (office hours)

Tel: 0121 6271627 ask for Elly Breuning (out-of-hours, emergency only)

This completes Part 1 of the Information Sheet.

If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

Part 2

What will happen if I don't want to carry on with the study?

You can withdraw from the study at any time. Information and samples we have already collected may still be used unless you specifically request that they are not.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.

Harm

In the event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation against University Hospital Birmingham NHS Foundation Trust NHS Trust, but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you.

Will my taking part in this study be kept confidential?

When you are enrolled into the trial, you will be given a unique code number. Your data will be stored in a booklet identified with this code. Details of your name, address and contact details will be kept separately in case we need to contact you for any reason. The contact details will be kept securely and only the researchers and the hospital Research and Development office will have access.

All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it. Procedures for handling, processing, storage and destruction of their data are compliant with the Data Protection Act 1998.

Involvement of the General Practitioner/Family doctor (GP)

A letter will be sent to your GP to inform them that you are taking part in this study.

What will happen to any samples I give?

The microdialysis samples taken from you during this study will be stored and tested in the laboratories at University Hospital Birmingham and at the University of Birmingham. All samples will be stored with an identification code with no direct reference to your details. Samples will be kept for up to 10 years following completion of the study to allow results to be checked and re-analysed if necessary. Samples will then be destroyed.

Will any genetic tests be done?

No genetic tests will be done in this study.

What will happen to the results of the research study?

Results of the study will be published in a scientific journal. You will not be identifiable from these results.

Who is organising and funding the research?

The study is sponsored by University Hospital Birmingham Foundation NHS Trust. Funding is being sought from burns charities and medical research charities.

Who has reviewed the study?

This study has been reviewed by the Dudley Research Ethics Committee and has been given a favourable ethical opinion for conduct in the NHS.

You will be given a copy of this information sheet and a copy of the signed consent form to keep.

Thank you for taking the time to read this sheet and considering taking part in the study.

Appendix B: Raw data for calibration experiments

Table A1: Raw data for glucose in calibration experiments (mmol/l)

	Group	A	B	C	D	E
	Storage temperature	Freezer, -80°C	Fridge, 2-5°C	Room, 22°C	Freezer, -80°C	Freezer, -80°C
	Volume	Medium	Medium	Medium	High	Low
Day	Sample					
1	a	6.10	6.67	5.99	6.00	5.79
	b	5.83	5.80	5.97	6.14	5.97
	c	6.03	5.77	5.84	5.77	6.07
2	a	6.25	5.69	5.53	5.54	5.72
	b	5.85	5.93	5.57	5.65	5.93
	c	5.77	5.35	5.59	5.42	5.74
3	a	5.66	5.54	5.54	5.74	5.82
	b	5.64	5.52	5.42	6.01	6.23
	c	5.53	5.44	5.40	5.93	6.22
1	Ave (a,b,c)	5.99	6.08	5.93	5.97	5.94
2	Ave (a,b,c)	5.96	5.66	5.56	5.54	5.80
3	Ave (a,b,c)	5.61	5.50	5.45	5.89	6.09

Table A2: Raw data for lactate in calibration experiments (mmol/l)

	Group	A	B	C	D	E
	Storage temperature	Freezer, -80^{o}C	Fridge, 2-5^{o}C	Room, 22^{o}C	Freezer, -80^{o}C	Freezer, -80^{o}C
	Volume	Medium	Medium	Medium	High	Low
Day	Sample					
1	a	2.79	2.88	2.84	2.80	3.89
	b	2.64	2.61	2.70	2.73	2.78
	c	2.73	2.68	2.66	2.73	2.79
2	a	2.59	2.52	2.50	2.76	2.72
	b	2.52	2.70	2.55	2.63	2.56
	c	2.56	2.59	2.59	2.63	2.65
3	a	2.70	2.55	2.52	2.63	2.60
	b	2.75	2.54	2.57	2.67	2.72
	c	2.70	2.51	2.50	2.64	2.81
1	Ave (a,b,c)	2.72	2.72	2.73	2.75	3.15
2	Ave (a,b,c)	2.56	2.60	2.55	2.67	2.64
3	Ave (a,b,c)	2.72	2.53	2.53	2.65	2.71

Table A3: Raw data for pyruvate in calibration experiments (µmol/l)

	Group	A	B	C	D	E
	Storage temperature	Freezer, -80°C	Fridge, 2-5°C	Room, 22°C	Freezer, -80°C	Freezer, -80°C
	Volume	Medium	Medium	Medium	High	Low
Day	Sample					
1	a	240	235	248	235	244
	b	235	228	250	232	252
	c	225	235	240	232	249
2	a	238	239	244	259	248
	b	252	256	237	247	248
	c	250	236	245	244	253
3	a	230	233	234	246	238
	b	222	222	213	241	247
	c	223	222	216	235	249
1	Ave (a,b,c)	233	233	246	233	248
2	Ave (a,b,c)	247	244	242	250	250
3	Ave (a,b,c)	225	226	221	241	245

Table A4: Raw data for glycerol in calibration experiments (µmol/l)

	Group	A	B	C	D	E
	Storage temperature	Freezer, -80°C	Fridge, 2-5°C	Room, 22°C	Freezer, -80°C	Freezer, -80°C
	Volume	Medium	Medium	Medium	High	Low
Day	Sample					
1	a	475	471	483	486	493
	b	452	463	478	489	496
	c	466	468	480	468	485
2	a	483	469	469	497	509
	b	480	462	463	478	484
	c	495	458	459	474	475
3	a	458	454	451	480	508
	b	457	442	442	473	506
	c	463	458	441	468	517
1	Ave (a,b,c)	464	467	480	481	491
2	Ave (a,b,c)	486	463	464	483	489
3	Ave (a,b,c)	459	451	445	474	510

Table A5: Raw data for urea in calibration experiments (mmol/l)

	Group	A	B	C	D	E
	Storage temperature	Freezer, -80°C	Fridge, 2-5°C	Room, 22°C	Freezer, -80°C	Freezer, -80°C
	Volume	Medium	Medium	Medium	High	Low
Day	Sample					
1	a	14.0	13.9	13.8	12.8	14.1
	b	13.8	14.1	14.2	13.8	14.5
	c	13.9	14.3	14.0	13.6	14.4
2	a	13.9	13.8	13.7	14.1	14.4
	b	13.9	13.6	13.6	14.0	14.7
	c	14.1	13.7	13.8	14.2	14.9
3	a	14.2	13.8	13.8	13.0	14.2
	b	14.7	13.8	13.7	12.5	14.4
	c	14.3	13.9	13.6	13.8	14.6
1	Ave (a,b,c)	13.9	14.1	14.0	13.4	14.3
2	Ave (a,b,c)	14.0	13.7	13.7	14.1	14.7
3	Ave (a,b,c)	14.4	13.8	13.7	13.1	14.4

Appendix B: Raw Data for Study 1

Table A6: Glucose results (mmol/l) for calibration fluid (C1, C2) and microdialysis of calibration fluid at the start (01,02) and end (12,13) of the study

Volunteer number	C1	01	02	%recovery 1 (02/C01)	12	13	C2	% recovery 2 (13/C02)
NV02	5.48	1.83	3.42	62%	5.97	3.22	5.82	55%
NV03	5.83	3.87	6.12	105%	7.30	6.00	5.63	107%
NV04	5.91	4.08	6.37	108%	6.64	6.26	6.07	103%
NV05	6	0.90	7.15	119%	6.40	6.76	5.70	119%
NV06	5.59	2.12			4.87	5.22	5.59	93%
NV07	5.76	3.54	5.40	94%			5.24	
NV08	5.44	3.35	5.52	101%	4.83	5.69	5.50	104%
NV09	5.66	4.78	6.51	115%	5.49	6.02	5.86	103%
NV10	5.32	4.12	5.55	104%	6.17	5.50	5.49	100%
NV11	6.00	6.02	5.96	99%			5.67	

Table A7: Lactate results (mmol/l) for calibration fluid (C1, C2) and microdialysis of calibration fluid at the start (01,02) and end (12,13) of the study

Volunteer number	C1	01	02	%recovery 1 (02/C01)	12	13	C2	%recovery 2 (13/C02)
NV02	2.41	1.02	1.78	74%	2.67	2.48	2.61	95%
NV03	2.76	2.01	2.80	102%	3.20	3.04	2.58	118%
NV04	2.73	2.13	3.07	113%	3.15	2.90	2.84	102%
NV05	2.75	0.49	3.44	125%	3.64	3.01	2.76	109%
NV06	2.77	1.10			4.43	3.37	2.62	128%
NV07	2.23	1.55	2.22	100%			2.10	
NV08	2.19	1.45	2.21	101%	2.63	2.16	2.14	101%
NV09	2.09	2.20	2.90	139%	2.60	2.58	2.48	104%
NV10	2.35	2.09	2.43	103%	3.02	2.88	2.33	124%
NV11	2.32	2.39	2.35	101%			2.25	

Table A8: Pyruvate results (µmol/l) for calibration fluid (C1, C2) and microdialysis of calibration fluid at the start (01,02) and end (12,13) of the study

Volunteer number	C1	01	02	%recovery 1 (02/C01)	12	13	C2	%recovery 2 (13/C02)
NV02	226	102	167	74%	202	234	245	96%
NV03	258	178	268	104%	250	275	246	119%
NV04	259	198	288	112%	234	286	260	110%
NV05	259	42	333	129%	258	289	258	112%
NV06	257	104			272	304	242	126%
NV07	245	141	222	91%			225	
NV08	232	154	242	105%	230	239	227	105%
NV09	228	199	274	120%	222	249	232	108%
NV10	230	183	240	104%	222	243	228	106%
NV11	238	252	245	103%			230	

Table A9: Glycerol results (µmol/l) for calibration fluid (C1, C2) and microdialysis of calibration fluid at the start (01,02) and end (12,13) of the study

Volunteer number	C1	01	02	%recovery 1 (02/C01)	12	13	C2	%recovery 2 (13/C02)
NV02	481	200	319	66%	252	495	524	94%
NV03	511	328	511	100%	318	534	460	116%
NV04	499	376	541	109%	382	736	513	144%
NV05	540	93	662	123%	354	599	548	109%
NV06	497	225			330	609	480	127%
NV07	485	333	449	93%			457	
NV08	465	300	485	104%	280	484	456	106%
NV09	452	388	538	119%	700	750	462	163%
NV10	459	366	464	101%	408	706	456	155%
NV11	499	532	517	103%			490	

Table A10: Glucose results (mmol/l) for the equilibration period (03, 04, 05) and study period 1 (06,07)

Volunteer number	03	04	05	06	07
NV02	5.97	5.96	6.30	7.33	6.41
NV03	6.17	6.43	6.18	5.98	6.42
NV04	*	*	1.27	2.77	6.07
NV05	*0.01	7.19	6.20	6.52	5.71
NV06	6.12	4.26	3.33	3.16	4.35
NV07	5.37	*0.02	1.79	2.59	5.40
NV08	4.77	4.41	5.52	5.97	5.02
NV09	8.33	8.23	7.43	7.47	6.04
NV10	5.81	7.25	6.15	5.53	5.70
NV11	*	*	2.41	5.14	5.82

* Anomalous result, and below detection limit of analyser. Due to an air bubble in the microdialysis system, the collecting vial is underfilled, and there is insufficient sample for accurate analysis.

Table A11: Lactate results (mmol/l) for the equilibration period (03, 04, 05) and study period 1 (06,07)

Volunteer number	03	04	05	06	07
NV02	2.19	1.48	1.82	3.00	3.14
NV03	2.33	1.65	1.71	2.08	2.84
NV04	*	*	0.38	0.92	1.91
NV05	*0.02	3.09	2.48	2.73	3.60
NV06	2.76	2.95	4.02	4.58	5.03
NV07	2.30	*	0.67	1.06	1.28
NV08	1.55	1.02	1.59	2.39	2.65
NV09	2.24	1.84	1.98	2.45	2.64
NV10	2.02	2.35	3.29	3.51	3.49
NV11	*	*	1.44	2.22	3.52

* Anomalous result, and below detection limit of analyser. Due to an air bubble in the microdialysis system, the collecting vial is underfilled, and there is insufficient sample for accurate analysis.

Table A12: Pyruvate results (µmol/l) for the equilibration period (03, 04, 05) and study period 1 (06,07)

Volunteer number	03	04	05	06	07
NV02	206	147	168	239	215
NV03	185	160	148	173	213
NV04	*	*	31	59	112
NV05	*	275	176	180	220
NV06	248	184	226	254	326
NV07	238	*	58	91	57
NV08	172	101	150	203	199
NV09	215	209	234	278	268
NV10	200	217	267	248	218
NV11	*	*	84	158	217

* Anomalous result, and below detection limit of analyser. Due to an air bubble in the microdialysis system, the collecting vial is underfilled, and there is insufficient sample for accurate analysis.

Table A13: Glycerol results (µmol/l) for the equilibration period (03, 04, 05) and study period 1 (06,07)

Volunteer number	03	04	05	06	07
NV02	275	36	25	22	27
NV03	191	66	87	113	138
NV04	*	*	86	219	397
NV05	*	299	48	54	57
NV06	409	93	146	156	124
NV07	197	*	23	11	4
NV08	240	44	62	67	70
NV09	194	73	74	84	94
NV10	278	39	39	38	39
NV11	*	*	48	30	37

* Anomalous result, and below detection limit of analyser. Due to an air bubble in the microdialysis system, the collecting vial is underfilled, and there is insufficient sample for accurate analysis.

Table A14: Lactate/Pyruvate ratio (LPR) calculations for the equilibration period (03, 04, 05) and study period 1 (06,07). Missing values where source data for either lactate or pyruvate was erroneous or missing

Volunteer number	03	04	05	06	07
NV02	12.6	10.3	11.5	12.0	13.4
NV03	*	*	12.2	15.5	17.0
NV04	*	11.2	14.1	15.2	16.4
NV05	11.1	16.1	17.8	18.0	15.4
NV06	9.7	*	11.5	11.7	22.5
NV07	9.0	10.1	10.6	11.7	13.3
NV08	10.4	8.8	8.5	8.8	9.9
NV09	10.1	10.8	12.3	14.2	16.0
NV10	*	*	17.1	14.1	16.2
NV11	12.6	10.3	11.5	12.0	13.4

* Anomalous results in lactate and pyruvate data, preventing calculation of LPR

Table A15: Glucose results (mmol/l) for the study period 1(06,07) and study period 2 (08,09) and study period 3 (10,11)

Volunteer number	06	07	08	09	10	11
NV02	7.33	6.41	5.34	5.80	5.67	6.09
NV03	5.98	6.42	7.02	*0.01	6.42	6.87
NV04	2.77	6.07	8.12	8.66	9.10	8.42
NV05	6.52	5.71	5.84	5.23	7.10	5.97
NV06	3.16	4.35	6.54	6.14	4.87	5.22
NV07	2.59	5.40	5.78	6.18	6.10	4.94
NV08	5.97	5.02	1.20	4.82	4.19	3.97
NV09	7.47	6.04	5.44	6.57	5.83	5.81
NV10	5.53	5.70	4.96	4.37	4.69	6.28
NV11	5.14	5.82	*0.42	5.84	4.86	5.25

* Anomalous result. Due to an air bubble in the microdialysis system, the collecting vial is underfilled, and there is insufficient sample for accurate analysis.

Table A16: Lactate results (mmol/l) for the study period 1(06,07) and study period 2 (08,09) and study period 3 (10,11)

Volunteer number	06	07	08	09	10	11
NV02	3.00	3.14	3.36	3.34	3.28	2.99
NV03	2.08	2.84	3.31	*	3.64	3.66
NV04	0.92	1.91	2.17	2.82	3.37	3.8
NV05	2.73	3.60	4.19	3.88	*0.79	4.45
NV06	4.58	5.03	6.12	5.77	5.53	6.11
NV07	1.06	1.28	2.17	2.29	1.77	1.33
NV08	2.39	2.65	*0.67	3.10	3.24	3.14
NV09	2.45	2.64	2.44	2.55	2.02	2.75
NV10	3.51	3.49	3.27	2.58	2.25	2.53
NV11	2.22	3.52	*0.32	3.95	3.45	2.87

* Anomalous result, and below detection limit of analyser. Due to an air bubble in the microdialysis system, the collecting vial is underfilled, and there is insufficient sample for accurate analysis.

Table A17: Pyruvate results (µmol/l) for the study period 1(06,07) and study period 2 (08,09) and study period 3 (10,11)

Volunteer number	06	07	08	09	10	11
NV02	239	215	207	191	163	146
NV03	173	213	243	*	246	236
NV04	59	112	122	165	178	166
NV05	180	220	229	204	*13	255
NV06	254	326	406	331	266	265
NV07	91	57	165	167	146	122
NV08	203	199	*2	211	225	203
NV09	278	268	239	236	197	206
NV10	248	218	187	136	120	168
NV11	158	217	*16	223	212	180

* Anomalous result, and below detection limit of analyser. Due to an air bubble in the microdialysis system, the collecting vial is underfilled, and there is insufficient sample for accurate analysis.

Table A18: Glycerol results (µmol/l) for the study period 1(06,07) and study period 2 (08,09) and study period 3 (10,11)

Volunteer number	06	07	08	09	10	11
NV02	22	27	38	29	30	31
NV03	113	138	140	*	95	87
NV04	219	397	299	160	118	139
NV05	54	57	*6	60	*5	62
NV06	156	124	136	126	110	120
NV07	11	4	14	14	13	17
NV08	67	70	*	80	74	95
NV09	84	94	95	101	102	113
NV10	38	39	45	39	41	45
NV11	30	37	*3	29	48	46

* Anomalous result, and below detection limit of analyser. Due to an air bubble in the microdialysis system, the collecting vial is underfilled, and there is insufficient sample for accurate analysis.

Table A19: Lactate/Pyruvate ratio (LPR) calculations for the study period 1 (06,07), study period 2 (08,09) and study period 3 (10,11). Missing values where source data for either lactate or pyruvate was erroneous or missing

Volunteer number	06	07	08	09	10	11
NV02	12.5	14.6	16.2	17.5	20.1	20.5
NV03	12.0	13.4	13.6	*	14.8	15.5
NV04	15.5	17.0	17.9	17.1	18.9	22.9
NV05	15.2	16.4	18.3	19.0	*63.1	17.5
NV06	18.0	15.4	15.1	17.4	20.8	23.1
NV07	11.7	22.5	13.2	13.7	12.2	10.9
NV08	11.7	13.3	*	14.7	14.4	15.5
NV09	8.8	9.9	10.2	10.8	10.3	13.4
NV10	14.2	16.0	17.5	19.0	18.7	15.0
NV11	14.1	16.2	*20.4	17.7	16.3	15.9

* Anomalous results in lactate and pyruvate data, preventing accurate calculation of LPR

Appendix C: Raw data for Study 2

TABLE A20: Missing Results, and labeling errors. Reasons for missing data are noted, together with the original value attributed to that data point, where applicable. 'Empty sample' refers to the finding of no microdialysate within the sample vial at the end of the sampling period. These samples were not put through the analyser. 'Blood in sample' refers to a sample contaminated with blood, presumed to be due to damage to the membrane during insertion. These samples were not put through the analyser to avoid potential damage to the analyzing equipment, and because the results were assumed to be invalidated by the presence of metabolically active cells within the sample.

Patient	Time post-burn (h)	Sample number	Substance not analysed	Original Value	Reason for missing result
S01	10	A01	Glu,L,P,Gly	n/a	Empty sample
S01	10.5	A02	Glu,L,P,Gly	n/a	Empty sample
S01	11	A03	Glu,L,P,Gly	n/a	Empty sample
S01	11.5	A04	Glu,L,P,Gly	n/a	Empty sample
S01	12	A05	Glu,L,P,Gly	n/a	Empty sample
S01	12.5	A06	Glu,L,P,Gly	n/a	Empty sample
S01	13	A07	Glu,L,P,Gly	n/a	Empty sample
S01	18	B17	Glu,L,P,Gly	n/a	Empty sample
S01	10	C01	Glu,L,P,Gly	n/a	Empty sample
S01	10.5	C02	L	0.008	Empty sample
			Glu,P,Gly	n/a	
S01	11	C03	L	0.009	Empty sample
			Glu,P,Gly	n/a	
S01	11.5	C04	Glu,L,P,Gly	n/a	Empty sample
S01	12.0	C05	Glu,L,P,Gly	n/a	Empty sample
S01	28.0	C37	Glu,L,P,Gly	n/a	Empty sample
S02		B47	Glu.L.P.Gly		Mislabeled as 10:15. Should be 09:45
S02	16.0	C19	Glu,L,P,Gly	n/a	Empty vial
S03	7.5	A01	Glu.L.P.Gly	n/a	Blood in sample
	8	A02	Glu.L.P.Gly	n/a	Blood in sample

	8.5	A03	Glu.L.P.Gly	n/a	Blood in sample
	9	A04	Glu.L.P.Gly	n/a	Blood in sample
	9.5	A05	Glu.L.P.Gly	n/a	Blood in sample
	10	A06	Glu.L.P.Gly	n/a	Blood in sample
		A55-59	Glu,L,P,Gly		Mislabelled as 27/1/7. Shd be 28/1/7
	7.5	C01	Glu,L,P,Gly	n/a	Blood in sample
	8	C02	Glu,L,P,Gly	n/a	Blood in sample
	8.5	C03	Glu,L,P,Gly	n/a	Blood in sample
	9	C04	Glu,L,P,Gly	n/a	Blood in sample
	9.5	C05	Glu,L,P,Gly	n/a	Blood in sample
	10	C06	Glu,L,P,Gly	n/a	Blood in sample
	10.5	C07	Glu,L,P,Gly	n/a	Blood in sample
	11	C08	Glu,L,P,Gly	n/a	Blood in sample
	11.5	C09	Glu,L,P,Gly	n/a	Blood in sample
	12	C10	Glu,L,P,Gly	n/a	Blood in sample
	12.5	C11	Glu,L,P,Gly	n/a	Blood in sample
	13	C12	Glu,L,P,Gly	n/a	Blood in sample
	13.5	C13	Glu,L,P,Gly	n/a	Blood in sample
	14	C14	Glu,L,P,Gly	n/a	Blood in sample
	14.5	C15	Glu,L,P,Gly	n/a	Blood in sample
	15	C16	Glu,L,P,Gly	n/a	Blood in sample
	19	C24	Glu,L,P,Gly	n/a	Empty sample
	19.5	C25	Glu,L,P,Gly	n/a	Empty sample
	20	C26	Glu,L,P,Gly	n/a	Empty sample
	20.5	C27	Glu,L,P,Gly	n/a	Empty sample
	22.5-36.5	C31-59	Glu,L,P,Gly	n/a	Probe pulled out and replaced. All further vials empty
S04	23.5	B30	Glu,L,P,Gly	n/a	missing sample point
S05	32.5	A53	Glu,L,P,Gly	n/a	Mislabeled as 14/4/7. Shd be 5/4/7
	32.5	B53	Glu,L,P,Gly	n/a	Mislabeled as13/4/7 Shd be 5/4/7
	6.5	C01	Glu,L,P,Gly	n/a	Blood in sample
	7	C02	Glu,L,P,Gly	n/a	Blood in sample
	7.5	C03	Glu,L,P,Gly	n/a	Blood in sample
	8	C04	Glu,L,P,Gly	n/a	Blood in sample
	21.5	C31	Glu,L,P,Gly	n/a	Blood in sample
	22	C32	Glu,L,P,Gly	n/a	Blood in sample
	22.5	C33	Glu,L,P,Gly	n/a	Blood in sample
	23	C34	Glu,L,P,Gly	n/a	Blood in sample
	23.5	C35	Glu,L,P,Gly	n/a	Blood in sample
	24	C36	Glu,L,P,Gly	n/a	Blood in sample
	24.5	C37	Glu,L,P,Gly	n/a	Blood in sample
	25	C38	Glu,L,P,Gly	n/a	Blood in sample
	25.5	C39	Glu,L,P,Gly	n/a	Blood in sample
	26	C40	Glu,L,P,Gly	n/a	Blood in sample

	28	C44	Gly	n/a	Insufficient sample for Gly analysis
	30.5	C49	Glu,L,P,Gly	n/a	Blood in sample
	31	C50	Glu,L,P,Gly	n/a	Mislabeled as 15/4/7. Shd be 5/4/7
	31.5	C51	Glu,L,P,Gly	n/a	Blood in sample
	32	C52	Glu,L,P,Gly	n/a	Blood in sample
	32.5	C53	Glu,L,P,Gly	n/a	Blood in sample
	33	C54	Glu,L,P,Gly	n/a	Blood in sample
S06	34	A44	Glu,L,P,Gly	n/a	Insufficient for analysis
	34.5	A45	Glu,L,P,Gly	n/a	Empty sample
	35	A46	Glu,L,P,Gly	n/a	Empty sample
	35.5	A47	Glu,L,P,Gly	n/a	Empty sample
	36	A48	Glu,L,P,Gly	n/a	Empty sample
	29	C34	Glu,L,P,Gly	n/a	Empty sample
	29.5	C35	Glu,L,P,Gly	n/a	Empty sample
	30	C36	Glu,L,P,Gly	n/a	Empty sample
		C37	Glu,L,P,Gly		Mislabelled as 08:00 Shd be 06:00
	31	C38	Glu,L,P,Gly	n/a	Empty sample
	31.5	C39	Glu,L,P,Gly	n/a	Empty sample
	32	C40	Glu,L,P,Gly	n/a	Empty sample
	32.5	C41	Glu,L,P,Gly	n/a	Empty sample
	33	C42	Glu,L,P,Gly	n/a	Empty sample
		C43	Glu,L,P,Gly		Mislabelled as 14:00 Shd be 09:00
	34	C44	Glu,L,P,Gly	n/a	Empty sample
		C45	Glu,L,P,Gly		Mislabelled as 16:00 Shd be 10:00
	35	C46	Glu,L,P,Gly	n/a	Empty sample
	35.5	C47	Glu,L,P,Gly	n/a	Empty sample
	36	C48	Glu,L,P,Gly	n/a	Empty sample
		B59	Glu,L,P,Gly	n/a	No sample
		C1-59	Glu,L,P,Gly	n/a	Blood in initial sample Then all remaining C's empty
	15.5	C17	L	n/a	?Insufficient for analysis
	16	C18	L	n/a	?Insufficient for analysis
	16.5	C19	L	n/a	?Insufficient for analysis
S07	17	C20	Glc,L	n/a	?Insufficient for analysis
	17.5	C21	Glu,L,P	n/a	?Insufficient for analysis
S08	18-36	C22-57	Glu,L,P,Gly	n/a	Study concluded early due to excision of burn in theatre
S09	15.0	A16a	Glu,L,P,Gly	-	Sample point missed, but numbering continues

		B16a	Glu,L,P,Gly	-	Sample point missed, but numbering continues
		C16a	Glu,L,P,Gly	-	Sample point missed, but numbering continues
		C24-30	Glu,L,P,Gly		Tubing cut whilst removing dressings in theatre. Probe replaced at C30
S10	21.5	A25	Glu,L,P,Gly	n/a	Empty sample
	29.5	A41	Glu	12.164534	Exchanged with C vial. Values reversed
			L	4	
			P	338.613	
			Gly	116.666	
		A43-48	Glu,L,P,Gly	n/a	Empty sample
		A53-55	L	n/a	No lactate result ?why
		B01	Glu,L,P,Gly	n/a	Empty sample
		B43-48	Glu,L,P,Gly		Empty samples
		B50	Glu,L,P,Gly	n/a	Empty sample
	20.5	C23	Glu,L,P,Gly	n/a	Empty sample
	21.5	C25	Glu,L,P,Gly	n/a	Empty sample
	29.5	C41	Glu	4.442825	C and A samples noted to be in wrong trays. Numbers exchanged
			L	3.909	
			P	193.059	
			Gly	93.713	
		C43-48	Glu,L,P,Gly	n/a	Empty samples

TABLE A21: Anomalous Results. Reasons for anomaly are noted, together with the original value.

Patient	Time post-burn (h)	Sample number	Analyte	Original Value	Changes made	Reason for inaccurate result
S01	25.0	A31	Glu L P Gly U	2.318 0.202 41.708 20.703 3.574	None	v minimal sample
S01	25.5	A32	Glu L P Gly U	0.997 0.344 34.056 23.855 n/a	None	v minimal sample
S01	26.0	A33	Glu L P Gly U	0.786 0.343 30.949 20.805 0.528	None	v minimal sample Under detection limit
S01	26.5	A34	Glu L P Gly U	0.850 0.321 19.784 11.185 0.272	None	v minimal sample Under detection limit
S01	17.5	B16	Glu L P Gly U	1.995 0.812 51.565 15.911 n/a	None	v minimal sample
S05	20.5	C29	Gly U	7.533 3.949		Under detection limit
	21	C30	Gly U	7.540 n/a		Under detection limit
	27	C42	Gly U	2.407 n/a		Under detection limit
	27.5	C43	Gly U	3.667 n/a		Under detection limit
	28.5	C45	Gly U	9.105 n/a		Under detection limit
	30	C48	Gly U	8.429 3.347		Under detection limit
S06	29.0	A34	Glu L P Gly	0.035 0.064 n/a 3.727		V minimal sample

			U	n/a		
	33	A42	Glu L P Gly U	0.034 .040 n/a 65.038 n/a	None	V minimal sample
	30	C37	Glu	4.830	30h	Wrongly attributed to 31h post burn
		C43	Glu	6.353	33h	Wrongly attributed to 37h
		C45	Glu	1.394	34h	Wrongly attributed to 39h
S09	8.0	A03	Glu L P Gly U	0.873 0.212 24.445 54.998 n/a		minimal sample
	9.0	A05	Glu L P Gly U	1.753 0.442 16.232 5.440 n/a		virtually empty Under detection limit
	10.0	A07	Gluco se L P Gly U	2.342 0.927 45.683 46.530 n/a		minimal sample
	12.0	A11	P Gly U	54.591 33.760 1.244		Minimal sample
	13.0	A13	P Gly U	76.352 51.302 n/a		Minimal sample
	14.0	A15	Glu L P Gly U	2.549 0.359 66.539 23.128 n/a		v minimal sample
	14.5	A16	Glu L P Gly U	1.861 1.113 81.118 53.428 n/a		minimal sample
	15.0	A16a	Glu,L, P,Gly, U	-		Sample point missed, but numbering continues
	16.0	A18	Glu L	0.632 0.652		V minimal sample

			P Gly U	45.913 15.140 n/a		
	16.5	A19	P Gly U	75.428 18.976 n/a		Minimal sample
	17.0	A20	L,P,Gly U	 n/a		Minimal samples, but results left
	17.5	A21	L,P,Gly U	 n/a		Minimal samples, but results left
	8.0	B03	Glu L P Gly U	1.065 0.319 15.827 13.671 n/a		V minimal sample
	15.0	B16a	Glu,L,P,Gly	-		Sample point missed, but numbering continues
	16.0	B18	L P Gly Urea	1.857 36.436 18.156 n/a		V minimal sample
	23.5	B33	P Gly U	47.131 27.155 n/a		Low sample
	28.0	B42	L	0.732		?Air bubble on lact analysis
	15.0	C16a	Glu,L,P,Gly	-		Sample point missed, but numbering continues
S10	14.0	A10	L P Gly U	1.704 31.071 79.256 1.636		Minimal sample
	29.5	A41	Glu L P Gly U	12.165 4.000 338.600 116.700 6.053	4.443 3.909 193.100 93.710 6.808	A&C Sample vials swapped (LPR 11.811 = 20.249)
	33.5->	A49, A51-55	Glu,L,P,Gly,U			Very full (no changes made to results)
	35.5->	A53-55	L	n/a		No lactate result ?why
	14.0	B10	Glu L	1.356 2.169		V minimal sample

			P Gly U	17.576 27.288 11.030		
	14.5	B11	Glu,L, P,Gly, U			Minimal sample (no changes made to results)
	15.0	B12	Glu U	1.503 n/a	unchanged	V minimal sample (no changes made to L,P,Gly)
	26.5	B35	L,Gly U	 n/a		Low vol sample (?gly and p affected)
	28.5	B39	Glu L P Gly U	0.136 2.703 106.697 42.010 n/a		v minimal sample
	33.5	B49	Glu L P Gly U	1.685 2.546 102.832 32.976 3.836		Very full therefore dilute ? pump malfunction
	14.0	C10	L P Gly U	2.664 69.297 98.976 n/a		Low volume sample
	17.5	C17	Glu L P Gly U	3.126 1.504 154.371 74.059 5.200	None	v full sample: likely pump malfunction
	28.5	C39	P Gly U	20.248 39.797 n/a		Minimal sample
	29.5	C41	Glu L P Gly U	4.443 3.909 193.059 93.713 6.808	12.165 4.000 338.600 116.700 6.053	C and A samples in wrong trays.

REFERENCES

1. History of treatment of burns. In: Herndon DN, editors. *Total Burn Care.* Galveston, USA: Saunders; 2006. p. 1-6.

2. BULL JP, FISHER AJ. A study of mortality in a burns unit: A revised estimate. Ann Surg 1954, Mar;139(3):269-74.

3. Bull JP. Revised analysis of mortality due to burns. The Lancet 1971;298(7734):1133-4.

4. Herndon DN, Gore D, Cole M, Desai MH, Linares H, Abston S, et al. Determinants of mortality in pediatric patients with greater than 70% full-thickness total body surface area thermal injury treated by early total excision and grafting. J Trauma 1987, Feb;27(2):208-12.

5. Bessey P. Wound care. In: Total Burn Care. Galveston, USA: Saunders.

6. Deitch EA, Wheelahan TM, Rose MP, Clothier J, Cotter J. Hypertrophic burn scars: Analysis of variables. J Trauma 1983, Oct;23(10):895-8.

7. JACKSON DM. [The diagnosis of the depth of burning]. Br J Surg 1953, May;40(164):588-96.

8. Singer AJ, Berruti L, Thode HC, McClain SA. Standardized burn model using a multiparametric histologic analysis of burn depth. Acad Emerg Med 2000, Jan;7(1):1-6.

9. deCamara DL, Raine TJ, London MD, Robson MC, Heggers JP. Progression of thermal injury: A morphologic study. Plast Reconstr Surg 1982, Mar;69(3):491-9.

10. Watts AM, Tyler MP, Perry ME, Roberts AH, McGrouther DA. Burn depth and its histological measurement. Burns 2001, Mar;27(2):154-60.

11. Nanney LB, Wenczak BA, Lynch JB. Progressive burn injury documented with vimentin immunostaining. J Burn Care Rehabil 1996;17(3):191-8.

12. Tyler MPH, Watts AMI, Perry ME, Roberts AHN, McGrouther DA. Dermal cellular inflammation in burns. An insight into the function of dermal microvascular anatomy. Burns 2001;27(5):433-8.

13. Boykin JV, Eriksson E, Pittman RN. In vivo microcirculation of a scald burn and the progression of postburn dermal ischemia. Plast Reconstr Surg 1980, Aug;66(2):191-8.

14. Ganong W. A review of medical physiology. 17th ed. Appleton & Lange; 1995.

15. Choi M, Rabb H, Arnaout MA, Ehrlich HP. Preventing the infiltration of leukocytes by monoclonal antibody blocks the development of progressive ischemia in rat burns. Plast Reconstr Surg 1995;96(5):1177.

16. Zawacki BE. Reversal of capillary stasis and prevention of necrosis in burns. Ann Surg 1974, Jul;180(1):98-102.

17. SEVITT S. Early and delayed oedema and increase in capillary permeability after burns of the skin. J Pathol Bacteriol 1958, Jan;75(1):27-37.

18. Gravante G, Palmieri MB, Esposito G, Delogu D, Santeusanio G, Filingeri V, Montone A. Apoptotic cells are present in ischemic zones of deep partial-thickness burns. J Burn Care Res 2006;27(5):688-93.

19. Knabl JS, Bauer W, Andel H, Schwendenwein I, Dado PF, Mittlböck M, et al. Progression of burn wound depth by systemical application of a vasoconstrictor: An experimental study with a new rabbit model. Burns 1999, Dec;25(8):715-21.

20. Horton JW. Free radicals and lipid peroxidation mediated injury in burn trauma: The role of antioxidant therapy. Toxicology 2003, Jul 15;189(1-2):75-88.

21. Horton JW. Free radicals and lipid peroxidation mediated injury in burn trauma: The role of antioxidant therapy. Toxicology 2003;189(1/2):75-88.

22. Latha B, Babu M. The involvement of free radicals in burn injury: A review. Burns 2001, Jun;27(4):309-17.

23. Kaufman T, Neuman RA, Weinberg A. Is postburn dermal ischaemia enhanced by oxygen free radicals? Burns 1989, Oct;15(5):291-4.

24. S Noble HG, Robson MC, Krizek TJ. Dermal ischemia in the burn wound. Journal of Surgical Research 1977;23(2):117-25.

25. Saliba MJ, Dempsey WC, Kruggel JL. Large burns in humans. Treatment with heparin. JAMA 1973, Jul 16;225(3):261-9.

26. Baskaran H, Toner M, Yarmush ML, Berthiaume F. Poloxamer-188 improves capillary blood flow and tissue viability in a cutaneous burn wound. J Surg Res 2001, Nov;101(1):56-61.

27. Robson MC, Kucan JO, Paik KI, Eriksson E. Prevention of dermal ischemia after thermal injury. Arch Surg 1978, May;113(5):621-5.

28. Mileski W, Borgstrom D, Lightfoot E, Rothlein R, Faanes R, Lipsky P, Baxter C. Inhibition of leukocyte-endothelial adherence following thermal injury. J Surg Res 1992, Apr;52(4):334-9.

29. Tarnow P, Jönsson A, Rimbäck G, Cassuto J. Increased dermal perfusion after skin burn injury by d-myo-inositol-1,2,6-trisphosphate. Burns 1996, Aug;22(5):363-8.

30. Lund T, Reed RK. Alpha-Trinositol inhibits edema generation and albumin extravasation in thermally injured skin. J Trauma 1994, Jun;36(6):761-5.

31. Giles N, Rea S, Beer T, Wood FM, Fear MW. A peptide inhibitor of c-jun promotes wound healing in a mouse full-thickness burn model. Wound Repair Regen 2008;16(1):58-64.

32. Morellini NM, Giles NL, Rea S, Adcroft KF, Falder S, King CE, et al. Exogenous metallothionein-iia promotes accelerated healing after a burn wound. Wound Repair Regen 2008;16(5):682-90.

33. Del Beccaro EJ, Heggers JP, Robson MC. Preventing the prostaglandin effect on dermal ischemia in the burn wound. Surg Forum 1978;29:603-5.

34. Heggers JP, Robson MC, Zachary LS. Thromboxane inhibitors for the prevention of progressive dermal ischemia due to the thermal injury. J Burn Care Rehabil 1985;6(6):466-8.

35. Kurose I, Wolf R, Grisham MB, Granger DN. Modulation of ischemia/reperfusion-induced microvascular dysfunction by nitric oxide. Circ Res 1994, Mar;74(3):376-82.

36. Kubes P, Suzuki M, Granger DN. Nitric oxide: An endogenous modulator of leukocyte adhesion. Proc Natl Acad Sci U S A 1991, Jun 1;88(11):4651-5.

37. Ma XL, Weyrich AS, Lefer DJ, Lefer AM. Diminished basal nitric oxide release after myocardial ischemia and reperfusion promotes neutrophil adherence to coronary endothelium. Circ Res 1993, Feb;72(2):403-12.

38. Lovett JE, Fink BF, Bernard A, Ochoa J. Analysis of nitric oxide activity in prevention of reperfusion injury. Ann Plast Surg 2001, Mar;46(3):269-73; discussion 273-4.

39. Luo G, Peng D, Zheng J, Chen X, Wu J, Elster E, Tadaki D. The role of NO in macrophage dysfunction at early stage after burn injury. Burns 2005, Mar;31(2):138-44.

40. Tokyay R, Zeigler ST, Kramer GC, Rogers CS, Heggers JP, Traber DL, Herndon DN. Effects of hypertonic saline dextran resuscitation on oxygen delivery, oxygen consumption, and lipid peroxidation after burn injury. J Trauma 1992, Jun;32(6):704-12; discussion 712-3.

41. Germonpré P, Reper P, Vanderkelen A. Hyperbaric oxygen therapy and piracetam decrease the early extension of deep partial-thickness burns. Burns 1996, Sep;22(6):468-73.

42. Kopal C, Deveci M, Oztürk S, Sengezer M. Effects of topical glutathione treatment in rat ischemic wound model. Ann Plast Surg 2007, Apr;58(4):449-55.

43. Kloppenberg FW, Beerthuizen GI, ten Duis HJ. Perfusion of burn wounds assessed by laser doppler imaging is related to burn depth and healing time. Burns 2001, Jun;27(4):359-63.

44. McGill DJ, Sørensen K, MacKay IR, Taggart I, Watson SB. Assessment of burn depth: A prospective, blinded comparison of laser doppler imaging and videomicroscopy. Burns 2007, Nov;33(7):833-42.

45. Riordan CL, McDonough M, Davidson JM, Corley R, Perlov C, Barton R, et al. Noncontact laser doppler imaging in burn depth analysis of the extremities. J Burn Care Rehabil 2003;24(4):177-86.

46. Hoeksema H, Van de Sijpe K, Tondu T, Hamdi M, Van Landuyt K, Blondeel P, Monstrey S. Accuracy of early burn depth assessment by laser doppler imaging on different days post burn. Burns 2009, Feb;35(1):36-45.

47. Vo LT, Papworth GD, Delaney PM, Barkla DH, King RG. A study of vascular response to thermal injury on hairless mice by fibre optic confocal imaging, laser doppler flowmetry and conventional histology. Burns 1998, Jun;24(4):319-24.

48. Ungerstedt U, Pycock C. Functional correlates of dopamine neurotransmission. Bull Schweiz Akad Med Wiss 1974, Jul;30(1-3):44-55.

49. Rooyackers O. Microdialysis to investigate tissue amino acid kinetics. Curr Opin Clin Nutr Metab Care 2005, Jan;8(1):77-82.

50. Brunner M, Müller M. Microdialysis: An in vivo approach for measuring drug delivery in oncology. European Journal of Clinical Pharmacology 2002;58(4):227-34.

51. Bradford HF. Glutamate, GABA and epilepsy. Prog Neurobiol 1995;47(6):477-511.

52. Krogstad AL, Jansson PA, Gisslén P, Lönnroth P. Microdialysis methodology for the measurement of dermal interstitial fluid in humans. Br J Dermatol 1996, Jun;134(6):1005-12.

53. Silva MA, Richards DA, Bramhall SR, Adams DH, Mirza DF, Murphy N. A study of the metabolites of ischemia-reperfusion injury and selected amino acids in the liver using microdialysis during transplantation. Transplantation 2005, Apr 15;79(7):828-35.

54. Dabrosin C. Microdialysis—an in vivo technique for studies of growth factors in breast cancer. Front Biosci 2005;10:1329-35.

55. Ungerstedt U, Rostami E. Microdialysis in neurointensive care. Curr Pharm Des 2004;10(18):2145-52.

56. Edsander-Nord A, Röjdmark J, Wickman M. Metabolism in pedicled and free TRAM flaps: A comparison using the microdialysis technique. Plast Reconstr Surg 2002, Feb;109(2):664-73.

57. Nielsen HT, Gutberg N, Birke-Sorensen H. Monitoring of intraoral free flaps with microdialysis. Br J Oral Maxillofac Surg 2011, Oct;49(7):521-6.

58. Papp A, Härmä M, Harvima R, Lahtinen T, Uusaro A, Alhava E. Microdialysis for detection of dynamic changes in tissue histamine levels in experimental thermal injury. Burns 2005, Jun;31(4):476-81.

59. Samuelsson A, Steinvall I, Sjöberg F. Microdialysis shows metabolic effects in skin during fluid resuscitation in burn-injured patients. Crit Care 2006;10(6):R172.

60. Plock N, Kloft C. Microdialysis--theoretical background and recent implementation in applied life-sciences. European Journal of Pharmaceutical Sciences: Official Journal of the European Federation for Pharmaceutical Sciences 2005;25(1):1.

61. Chaurasia CS. In vivo microdialysis sampling: Theory and applications. Biomed Chromatogr 1999, Aug;13(5):317-32.

62. Richards DA, Silva MA, Murphy N, Wigmore SJ, Mirza DF. Extracellular amino acid levels in the human liver during transplantation: A microdialysis study from donor to recipient. Amino Acids 2007, Sep;33(3):429-37.

63. Baumeister FA, Rolinski B, Busch R, Emmrich P. Glucose monitoring with long-term subcutaneous microdialysis in neonates. Pediatrics 2001, Nov;108(5):1187-92.

64. Khramov AN, Stenken JA. Enhanced microdialysis extraction efficiency of ibuprofen in vitro by facilitated transport with β-cyclodextrin. Anal Chem 1999, Apr 1;71(7):1257-64.

65. Sun L, Stenken JA. Improving microdialysis extraction efficiency of lipophilic eicosanoids. J Pharm Biomed Anal 2003, Dec 4;33(5):1059-71.

66. Plock N, Buerger C, Kloft C. Successful management of discovered ph dependence in vancomycin recovery studies: Novel HPLC method for microdialysis and plasma samples. Biomed Chromatogr 2005, Apr;19(3):237-44.

67. Höistad M, Chen KC, Nicholson C, Fuxe K, Kehr J. Quantitative dual-probe microdialysis: Evaluation of [3H]mannitol diffusion in agar and rat striatum. J Neurochem 2002, Apr;81(1):80-93.

68. Nicholson C, Syková E. Extracellular space structure revealed by diffusion analysis. Trends Neurosci 1998, May;21(5):207-15.

69. Strindberg L, Lönnroth P. Validation of an endogenous reference technique for the calibration of microdialysis catheters. Scand J Clin Lab Invest 2000, May;60(3):205-11.

70. Petersen LJ, Poulsen LK, Søndergaard J, Skov PS. The use of cutaneous microdialysis to measure substance p-induced histamine release in intact human skin in vivo. J Allergy Clin Immunol 1994, Oct;94(4):773-83.

71. Petersen LJ, Nielsen HJ, Skov PS. Codeine-induced histamine release in intact human skin monitored by skin microdialysis technique: Comparison of intradermal injections with an atraumatic intraprobe drug delivery system. Clin Exp Allergy 1995, Nov;25(11):1045-52.

72. Petersen LJ, Skov PS, Bindslev-Jensen C, Søndergaard J. Histamine release in immediate-type hypersensitivity reactions in intact human skin measured by microdialysis. A preliminary study. Allergy 1992, Dec;47(6):635-7.

73. Schmelz M, Luz O, Averbeck B, Bickel A. Plasma extravasation and neuropeptide release in human skin as measured by intradermal microdialysis. Neurosci Lett 1997, Jul 18;230(2):117-20.

74. Schmelz M, Petersen LJ. Neurogenic inflammation in human and rodent skin. News Physiol Sci 2001, Feb;16:33-7.

75. Clough GF, Church MK. Vascular responses in the skin: An accessible model of inflammation. News Physiol Sci 2002, Aug;17:170-4.

76. Jansson PA, Krogstad AL, Lönnroth P. Microdialysis measurements in skin: Evidence for significant lactate release in healthy humans. Am J Physiol 1996, Jul;271(1 Pt 1):E138-42.

77. Petersen LJ, Kristensen JK, Bülow J. Microdialysis of the interstitial water space in human skin in vivo: Quantitative measurement of cutaneous glucose concentrations. J Invest Dermatol 1992, Sep;99(3):357-60.

78. Petersen LJ. Interstitial lactate levels in human skin at rest and during an oral glucose load: A microdialysis study. Clin Physiol 1999, May;19(3):246-50.

79. de Boer J, Plijter-Groendijk H, Visser KR, Mook GA, Korf J. Continuous monitoring of lactate during exercise in humans using subcutaneous and transcutaneous microdialysis. Eur J Appl Physiol Occup Physiol 1994;69(4):281-6.

80. Lee YS, Yang JH, Choi JC, Eun HC. Age-dependent change of uric acid level in the dermis using cutaneous microdialysis. Gerontology 2005;51(4):231-3.

81. Papp A, Kiraly K, Härmä M, Lahtinen T, Uusaro A, Alhava E. The progression of burn depth in experimental burns: A histological and methodological study. Burns 2004, Nov;30(7):684-90.

82. Machens HG, Pabst A, Dreyer M, Gliemroth J, Görg S, Bahlmann L, et al. C3a levels and occurrence of subdermal vascular thrombosis are age-related in deep second-degree burn wounds. Surgery 2006, Apr;139(4):550-5.

83. Tisdall MM, Smith M. Cerebral microdialysis: Research technique or clinical tool. Br J Anaesth 2006, Jul;97(1):18-25.

84. Waelgaard L, Thorgersen EB, Line PD, Foss A, Mollnes TE, Tønnessen TI. Microdialysis monitoring of liver grafts by metabolic parameters, cytokine production, and complement activation. Transplantation 2008, Oct 27;86(8):1096-103.

85. Haugaa H, Thorgersen EB, Pharo A, Boberg KM, Foss A, Line PD, et al. Inflammatory markers sampled by microdialysis catheters distinguish rejection from ischemia in liver grafts. Liver Transpl 2012, Jul 5.

86. Hamrin K, Rosdahl H, Ungerstedt U, Henriksson J. Microdialysis in human skeletal muscle: Effects of adding a colloid to the perfusate. J Appl Physiol 2002, Jan;92(1):385-93.

87. Lanier ST, McClain SA, Lin F, Singer AJ, Clark RA. Spatiotemporal progression of cell death in the zone of ischemia surrounding burns. Wound Repair Regen 2011;19(5):622-32.

88. Fluhr JW, Darlenski R, Surber C. Glycerol and the skin: Holistic approach to its origin and functions. Br J Dermatol 2008, Jul;159(1):23-34.

89. Large V, Reynisdottir S, Eleborg L, van Harmelen V, Strömmer L, Arner P. Lipolysis in human fat cells obtained under local and general anesthesia. Int J Obes Relat Metab Disord 1997, Jan;21(1):78-82.

90. Rawlingson A. Nitric oxide, inflammation and acute burn injury. Burns 2003, Nov;29(7):631-40.

91. Inoue H, Ando K, Wakisaka N, Matsuzaki K, Aihara M, Kumagai N. Effects of nitric oxide synthase inhibitors on vascular hyperpermeability with thermal injury in mice. Nitric Oxide 2001, Aug;5(4):334-42.

92. Yonehara N, Yoshimura M. Interaction between nitric oxide and substance P on heat-induced inflammation in rat paw. Neurosci Res 2000, Jan;36(1):35-43.

93. Lindblom L, Cassuto J, Yregård L, Mattsson U, Tarnow P, Sinclair R. Importance of nitric oxide in the regulation of burn oedema, proteinuria and urine output. Burns 2000, Feb;26(1):13-7.

94. Paulsen SM, Wurster SH, Nanney LB. Expression of inducible nitric oxide synthase in human burn wounds. Wound Repair Regen 1998;6(2):142-8.

95. Schäffer MR, Tantry U, van Wesep RA, Barbul A. Nitric oxide metabolism in wounds. J Surg Res 1997, Jul 15;71(1):25-31.

96. Witte MB, Thornton FJ, Tantry U, Barbul A. L-Arginine supplementation enhances diabetic wound healing: Involvement of the nitric oxide synthase and arginase pathways. Metabolism 2002, Oct;51(10):1269-73.

97. Devés R, Boyd CA. Transporters for cationic amino acids in animal cells: Discovery, structure, and function. Physiol Rev 1998, Apr;78(2):487-545.

98. Van Winkle LJ. Endogenous amino acid transport systems and expression of mammalian amino acid transport proteins in xenopusoocytes. Biochimica Et Biophysica Acta (BBA)/Reviews on Biomembranes 1993;1154(2):157-72.

99. Kilberg MS, Stevens BR, Novak DA. Recent advances in mammalian amino acid transport. Annu Rev Nutr 1993;13:137-65.

100. Stevens BR, Kakuda DK, Yu K, Waters M, Vo CB, Raizada MK. Induced nitric oxide synthesis is dependent on induced alternatively spliced CAT-2 encoding l-arginine transport in brain astrocytes. J Biol Chem 1996, Sep 27;271(39):24017-22.

101. Gill DJ, Low BC, Grigor MR. Interleukin-1 beta and tumor necrosis factor-alpha stimulate the cat-2 gene of the l-arginine transporter in cultured vascular smooth muscle cells. J Biol Chem 1996, May 10;271(19):11280-3.

102. Cendan JC, Topping DL, Pruitt J, Snowdy S, Copeland III EM, Lind DS. Inflammatory mediators stimulate arginine transport and arginine-derived nitric oxide production in a murine breast cancer cell line. Journal of Surgical Research 1996;60(2):284-8.

103. Schmidlin A, Wiesinger H. Stimulation of arginine transport and nitric oxide production by lipopolysaccharide is mediated by different signaling pathways in astrocytes. J Neurochem 1995, Aug;65(2):590-4.

104. Durante W, Liao L, Schafer AI. Differential regulation of l-arginine transport and inducible NOS in cultured vascular smooth muscle cells. Am J Physiol 1995, Mar;268(3 Pt 2):H1158-64.

105. Bogle RG, Baydoun AR, Pearson JD, Moncada S, Mann GE. L-arginine transport is increased in macrophages generating nitric oxide. Biochem J 1992, May 15;284 (Pt 1):15-8.

106. Simmons WW, Closs EI, Cunningham JM, Smith TW, Kelly RA. Cytokines and insulin induce cationic amino acid transporter (CAT) expression in cardiac myocytes. Regulation of l-arginine transport and no production by CAT-1, CAT-2A, and CAT-2B. J Biol Chem 1996, May 17;271(20):11694-702.

107. Witte MB, Barbul A, Schick MA, Vogt N, Becker HD. Upregulation of arginase expression in wound-derived fibroblasts. J Surg Res 2002, Jun 1;105(1):35-42.

108. Schwacha MG, Nickel E, Daniel T. Burn injury-induced alterations in wound inflammation and healing are associated with suppressed hypoxia inducible factor-1alpha expression. Mol Med 2008;14(9-10):628-33.

109. Debats IB, Wolfs TG, Gotoh T, Cleutjens JP, Peutz-Kootstra CJ, van der Hulst RR. Role of arginine in superficial wound healing in man. Nitric Oxide 2009;21(3-4):175-83.

110. Holowatz LA, Kenney WL. Up-regulation of arginase activity contributes to attenuated reflex cutaneous vasodilatation in hypertensive humans. J Physiol 2007, Jun 1;581(Pt 2):863-72.

111. Cahusac P, Senok SS. Metabotropic glutamate receptor antagonists selectively enhance responses of slowly adapting type I mechanoreceptors. Synapse 2006;59(4):235-42.

112. Cahusac PM, Senok SS, Hitchcock IS, Genever PG, Baumann KI. Are unconventional NMDA receptors involved in slowly adapting type I mechanoreceptor responses? Neuroscience 2005;133(3):763-73.

113. Fagan BM, Cahusac PMB. Evidence for glutamate receptor mediated transmission at mechanoreceptors in the skin. Neuroreport 2001;12(2):341-7.

114. Ono S, Yamauchi M. Glutamate and aspartate are decreased in the skin in amyotrophic lateral sclerosis. Acta Neurol Scand 1992, Nov;86(5):481-4.

115. Genever PG, Maxfield SJ, Kennovin GD, Maltman J, Bowgen CJ, Raxworthy MJ, Skerry TM. Evidence for a novel glutamate-mediated signaling pathway in keratinocytes. J Invest Dermatol 1999, Mar;112(3):337-42.

116. Saransaari P, Oja SS. Taurine and neural cell damage. Amino Acids 2000;19(3-4):509-26.

117. Oja SS, Saransaari P. Modulation of taurine release by glutamate receptors and nitric oxide. Prog Neurobiol 2000, Nov;62(4):407-25.

118. Farriol M, Venereo Y, Rosselló J, Gomez P, Palao R, Orta X, Segovia-Silvestre T. Effects of taurine on polymorphonuclear phagocytosis activity in burned patients. Amino Acids 2002;23(4):441-5.

119. www.Mdialysis.com/faq_clinical

120. Groth L, Serup J. Cutaneous microdialysis in man: Effects of needle insertion trauma and anaesthesia on skin perfusion, erythema and skin thickness. Acta Derm Venereol 1998, Jan;78(1):5-9.

121. Anderson C, Andersson T, Wårdell K. Changes in skin circulation after insertion of a microdialysis probe visualized by laser doppler perfusion imaging. J Invest Dermatol 1994, May;102(5):807-11.

122. Groth L. Cutaneous microdialysis. Methodology and validation. Acta Derm Venereol Suppl (Stockh) 1996;197:1-61.

123. Ault JM, Riley CM, Meltzer NM, Lunte CE. Dermal microdialysis sampling in vivo. Pharm Res 1994, Nov;11(11):1631-9.

124. Mathy FX, Denet AR, Vroman B, Clarys P, Barel A, Verbeeck RK, Préat V. In vivo tolerance assessment of skin after insertion of subcutaneous and cutaneous microdialysis probes in the rat. Skin Pharmacol Appl Skin Physiol 2003;16(1):18-27.

125. Davies MI, Lunte CE. Microdialysis sampling for hepatic metabolism studies. Impact of microdialysis probe design and implantation technique on liver tissue. Drug Metab Dispos 1995, Oct;23(10):1072-9.

126. Stenken JA, Church MK, Gill CA, Clough GF. How minimally invasive is microdialysis sampling? A cautionary note for cytokine collection in human skin and other clinical studies. AAPS J 2010, Mar;12(1):73-8.

127. Jacobson I, Sandberg M, Hamberger A. Mass transfer in brain dialysis devices--a new method for the estimation of extracellular amino acids concentration. J Neurosci Methods 1985;15(3):263-8.

128. Lönnroth P, Jansson PA, Smith U. A microdialysis method allowing characterization of intercellular water space in humans. Am J Physiol 1987, Aug;253(2 Pt 1):E228-31.

129. Stahl M, Bouw R, Jackson A, Pay V. Human microdialysis. Curr Pharm Biotechnol 2002, Jun;3(2):165-78.

130. Olson RJ, Justice Jr JB. Quantitative microdialysis under transient conditions. Anal Chem 1993;65(8):1017-22.

131. Ståhle L, Arner P, Ungerstedt U. Drug distribution studies with microdialysis. III: Extracellular concentration of caffeine in adipose tissue in man. Life Sci 1991;49(24):1853-8.

132. Brunner M, Joukhadar C, Schmid R, Erovic B, Eichler HG, Müller M. Validation of urea as an endogenous reference compound for the in vivo calibration of microdialysis probes. Life Sci 2000, Jul 14;67(8):977-84.

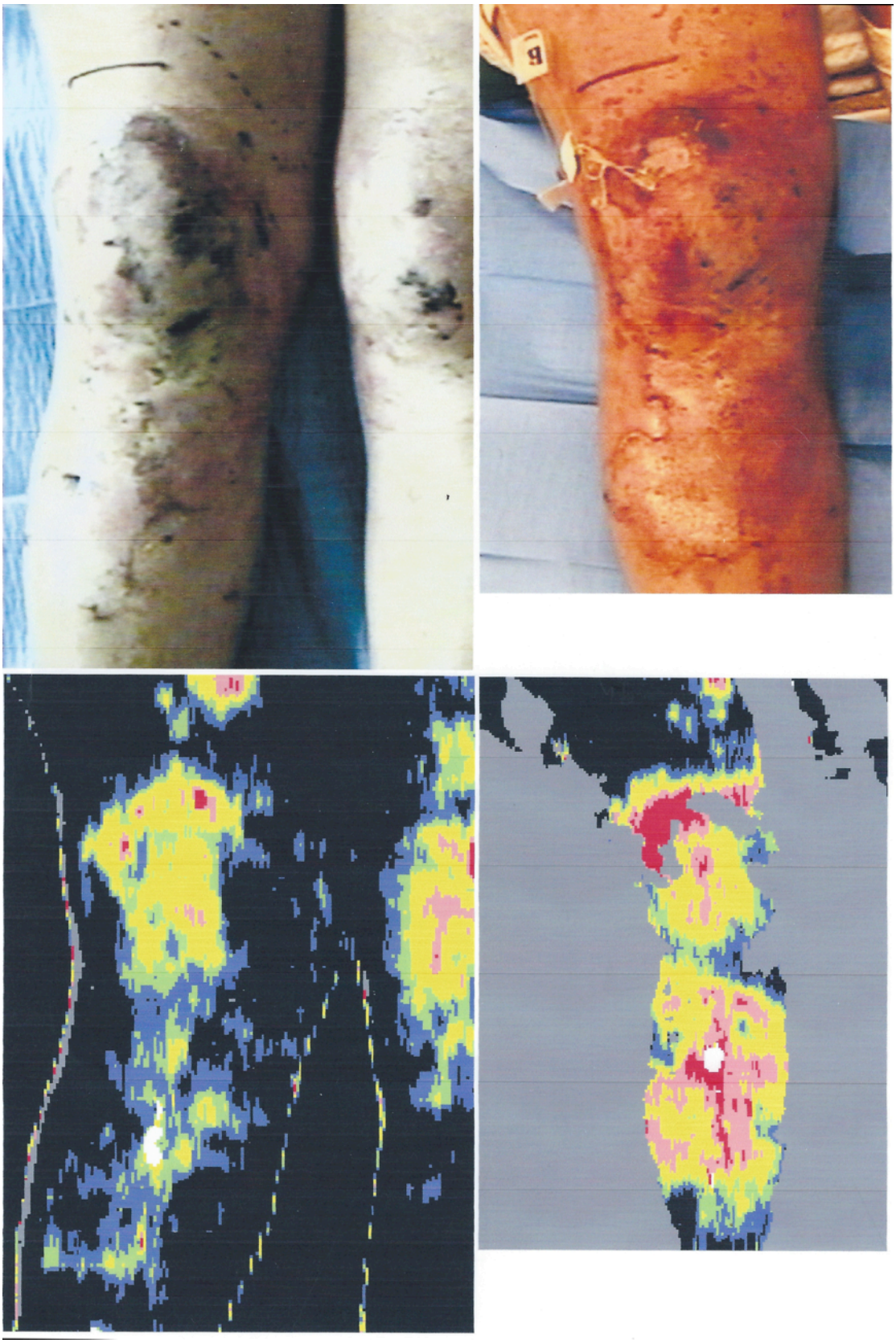


Plate 1: Photographs and LDI Images for Patient S01. Above left: Photograph of burn before probe insertion with 5cm standard line marked. Above right: Photograph of burn at end of study period with 5cm standard line marked, and probes A and B still in situ. Below left: LDI before probe insertion. Below right: LDI at end of study period.

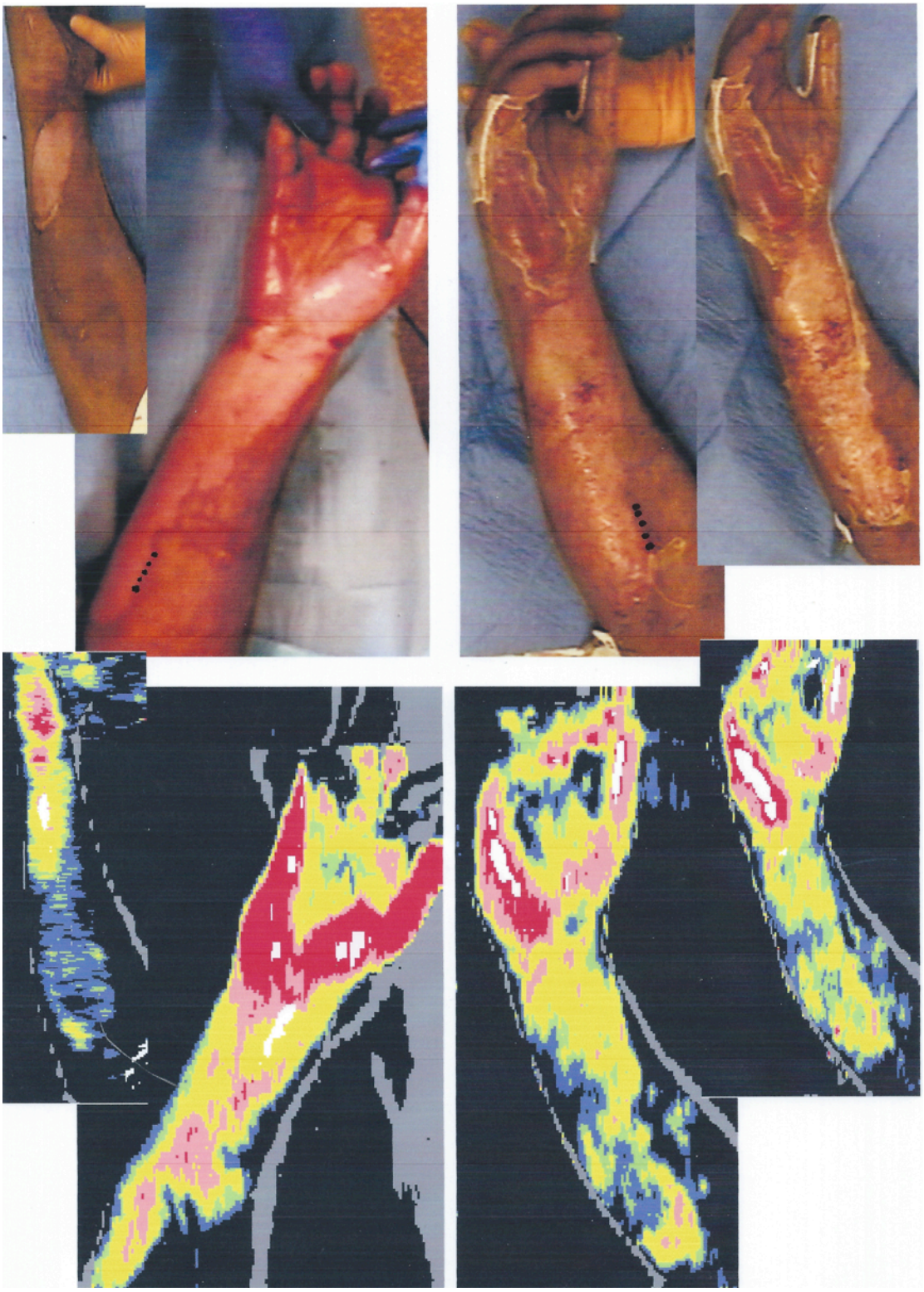


Plate 2: Photographs and LDI Images for Patient S02. Above left: Photograph of burn before probe insertion with site for probe B marked with a dotted line. Above right: Photograph of burn at end of study period with site of probe B marked with dotted line. Below left: LDI before probe insertion. Below right: LDI at end of study period.

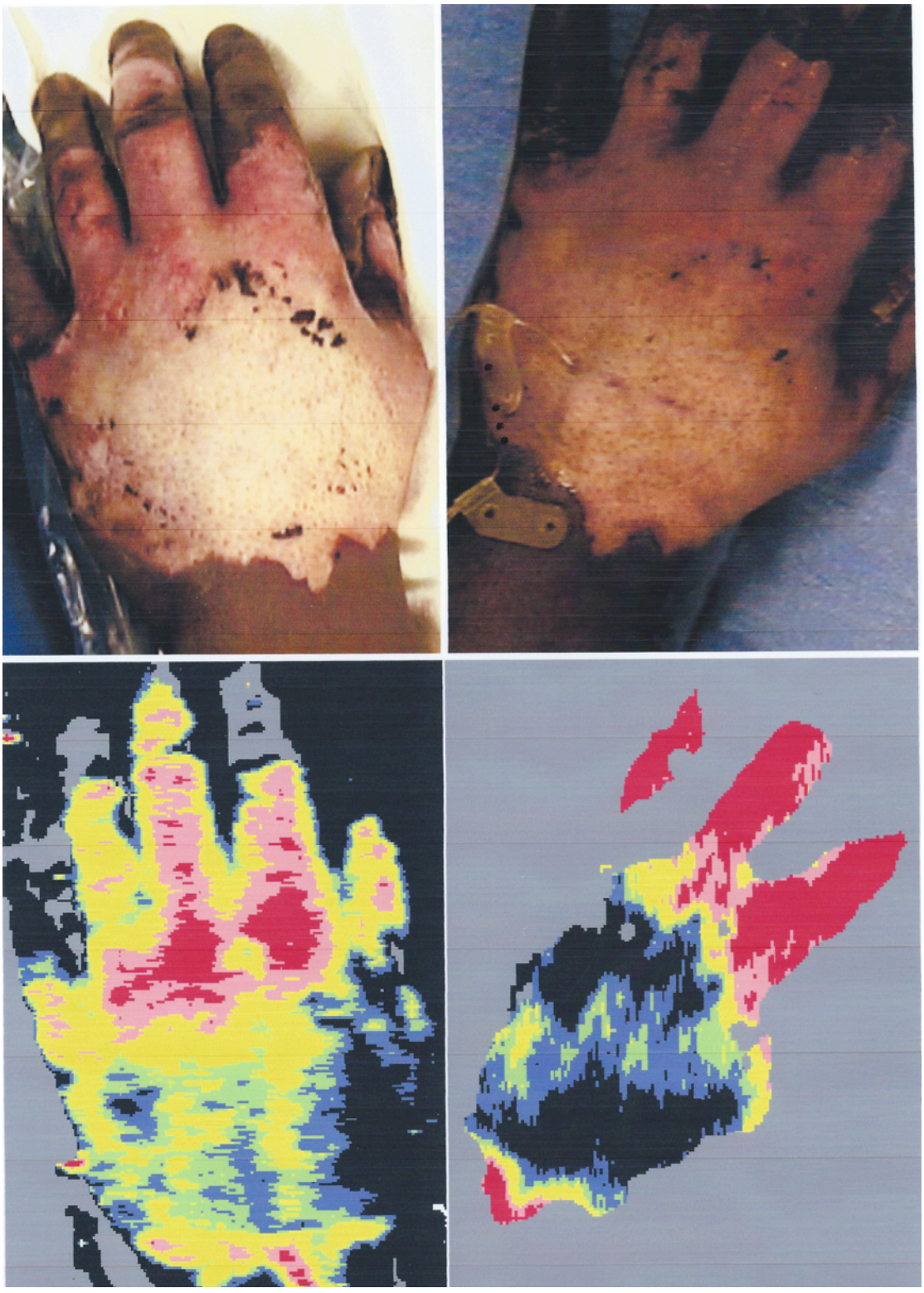


Plate 3: Photographs and LDI Images for Patient S03. Above left: Photograph of burn before probe insertion. Above right: Photograph of burn at end of study period with probe A (centre of wound) and probe B, loser left side of wound still In situ. Below left: LDI before probe insertion. Below right: LDI at end of study period.

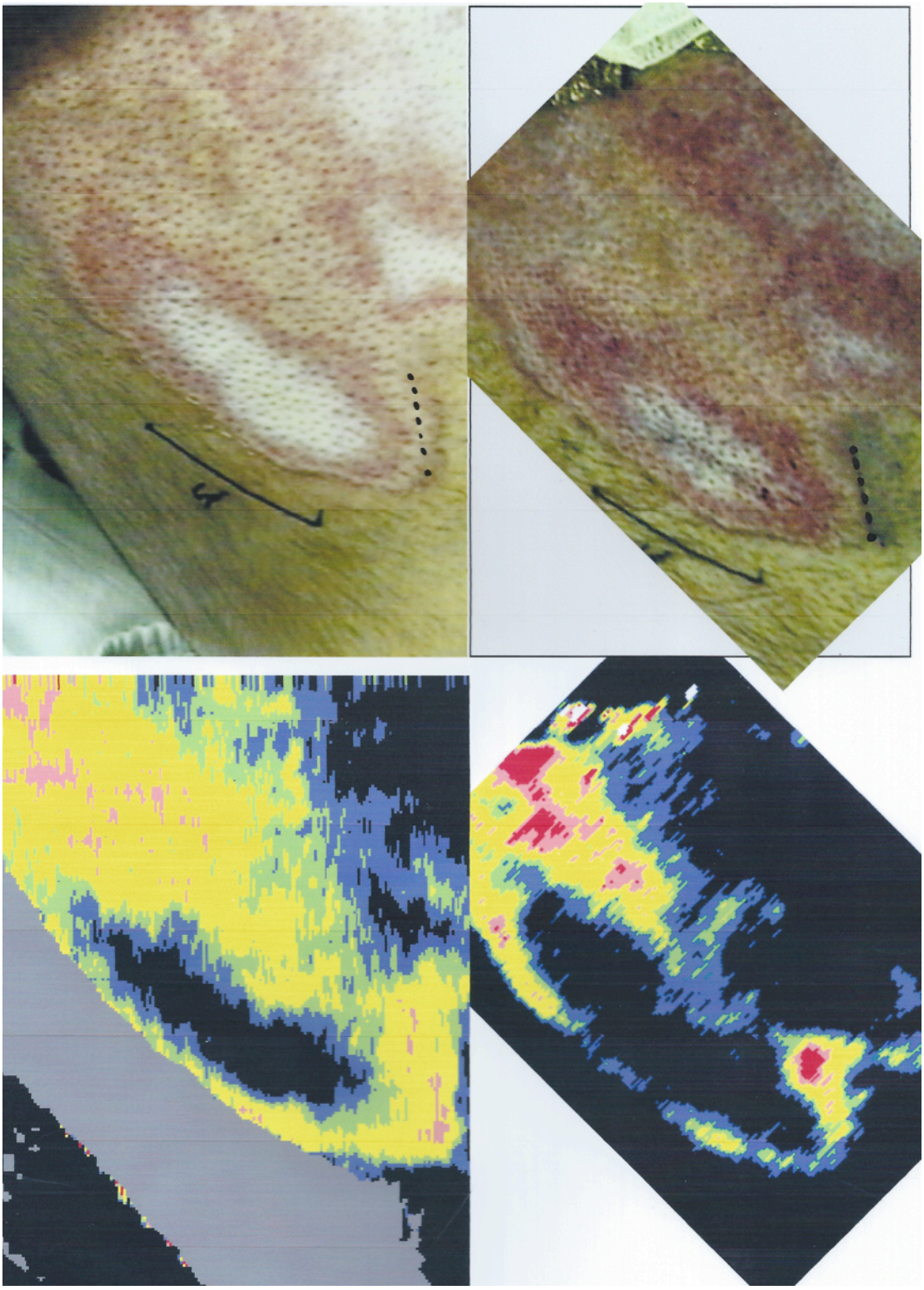


Plate 4: Photographs and LDI Images for Patient S04. Above left: Photograph of burn before probe insertion with standard 5cm line and site for probe B marked with a dotted line. Above right: Photograph of burn at end of study period with standard 5cm line, and site of probe B marked with dotted line. Below left: LDI before probe insertion. Below right: LDI at end of study period.

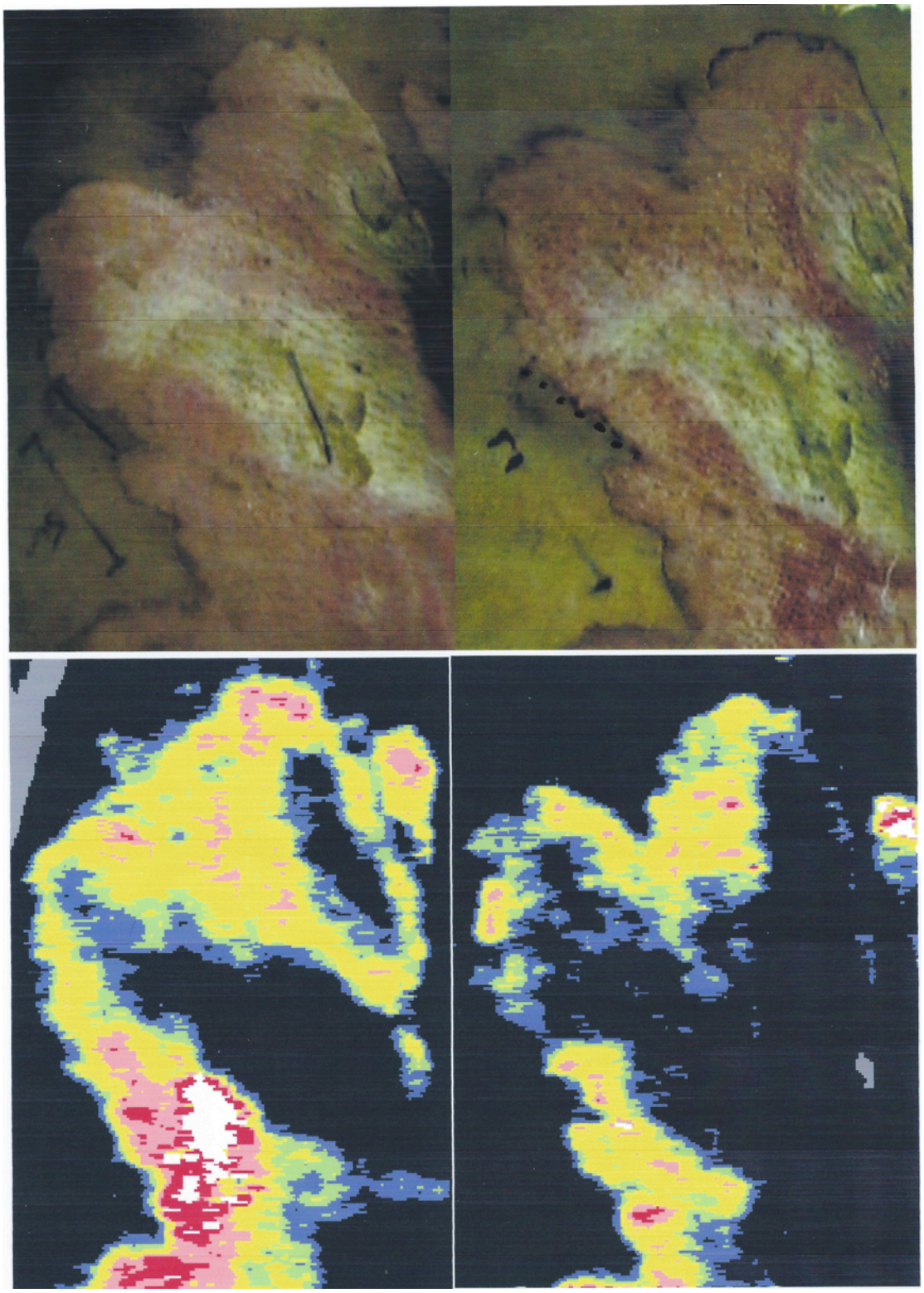


Plate 5: Photographs and LDI Images for Patient S05. Above left: Photograph of burn before probe insertion with standard 5cm line (far left), and site for probe B and A marked at edge and centre of wound respectively. Above right: Photograph of burn at end of study period with standard 5cm line, and site for probe B marked with dotted line. Below left: LDI before probe insertion. Below right: LDI at end of study period.

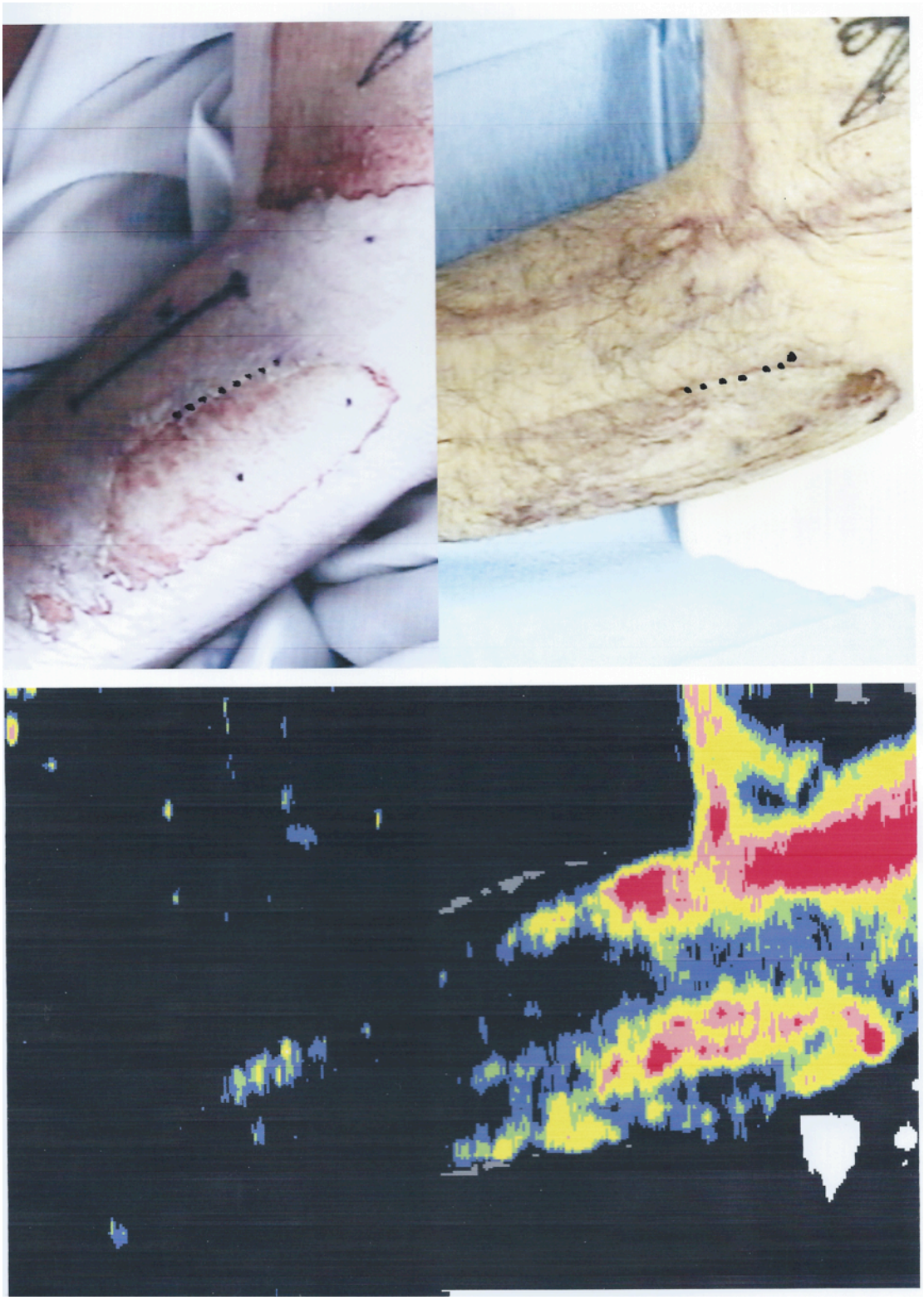


Plate 6: Photographs and LDI Images for Patient S06 Above left: Photograph of burn before probe insertion with standard 5cm line, site for probe B marked with dotted line, and probe A between two dots in centre of wound. Above right: Photograph of burn at end of study period with site of probe B marked with dotted line. Below left: LDI before probe insertion. Below right: LDI at end of study period.

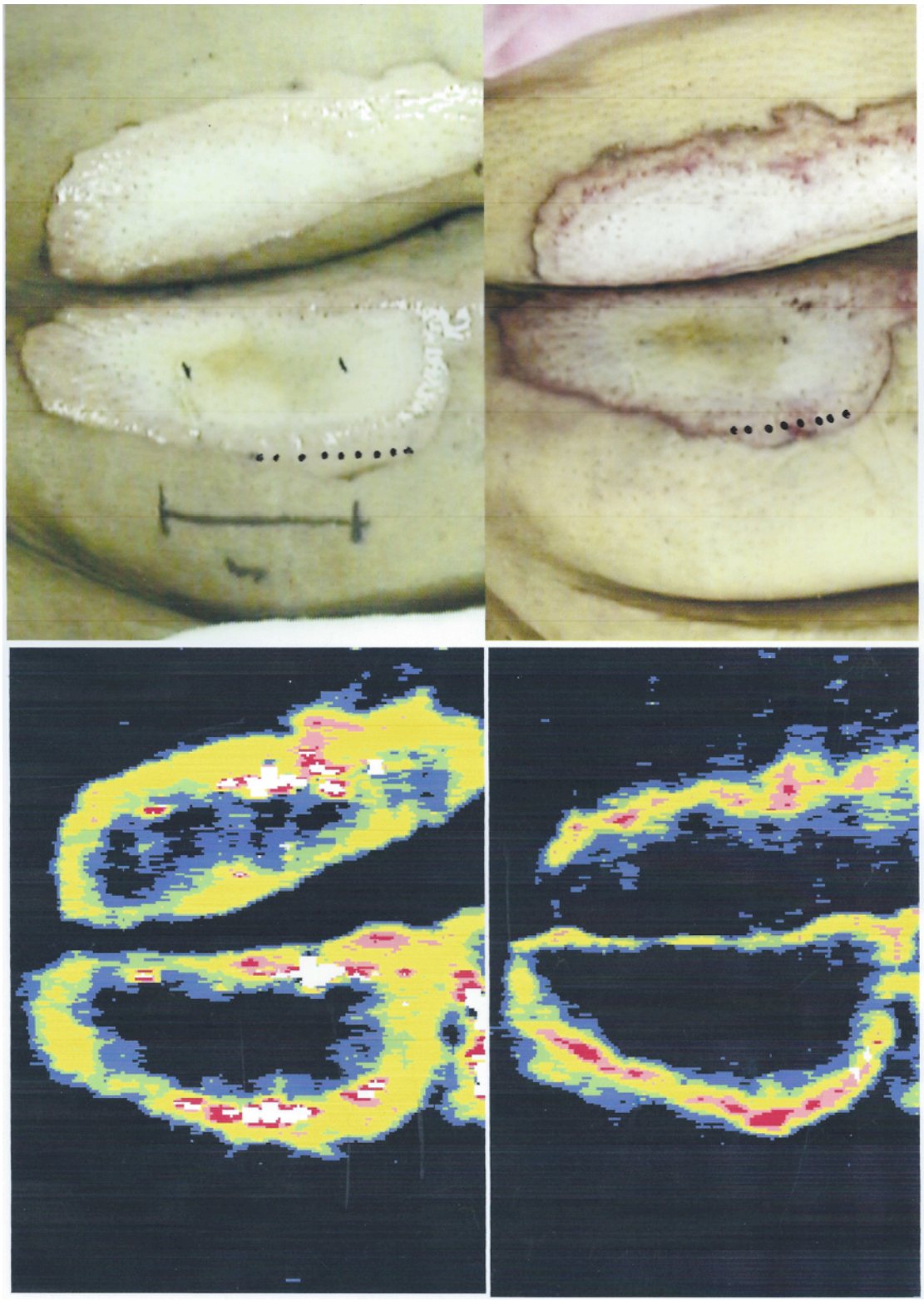


Plate 7: Photographs and LDI Images for Patient S07 Above left: Photograph of burn before probe insertion with standard 5cm line, site for probe B marked with dotted line, and probe A between two dots in centre of wound. Above right: Photograph of burn at end of study period with site of probe B marked with dotted line. Below left: LDI before probe insertion. Below right: LDI at end of study period.

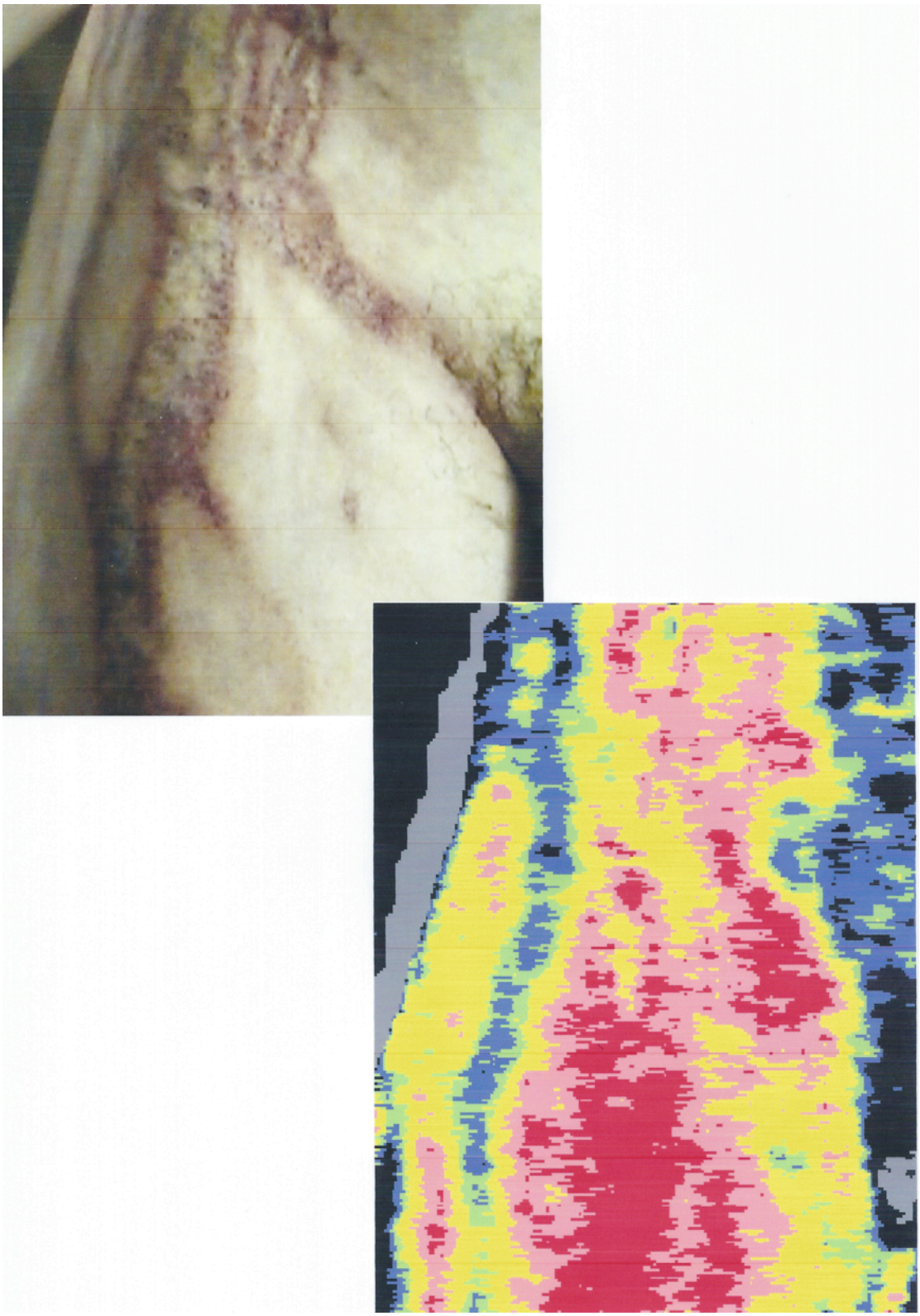


Plate 8: Photograph and LDI Image for Patient S08. Above: Photograph of burn before probe insertion. Below: LDI before probe insertion. No photograph/LDI image at end of study period as this patient did not complete the study.

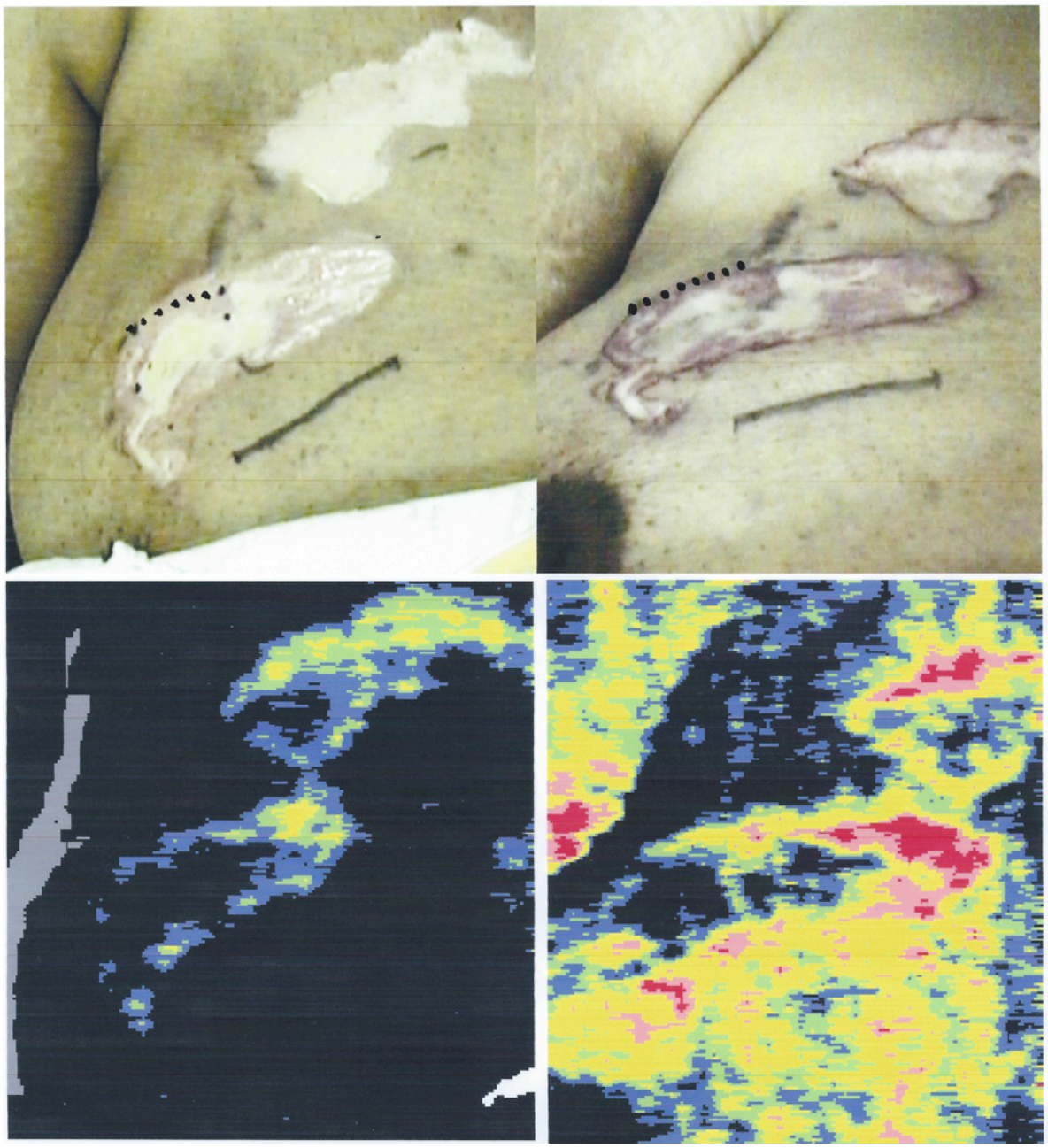


Plate 9: Photographs and LDI Images for Patient S09. Above left: Photograph of burn before probe insertion with standard 5cm line, and site for probe B marked with dotted line. Above right: Photograph of burn at end of study period with standard 5cm line, and site for probe B marked with dotted line. Below left: LDI before probe insertion. Below right: LDI at end of study period.

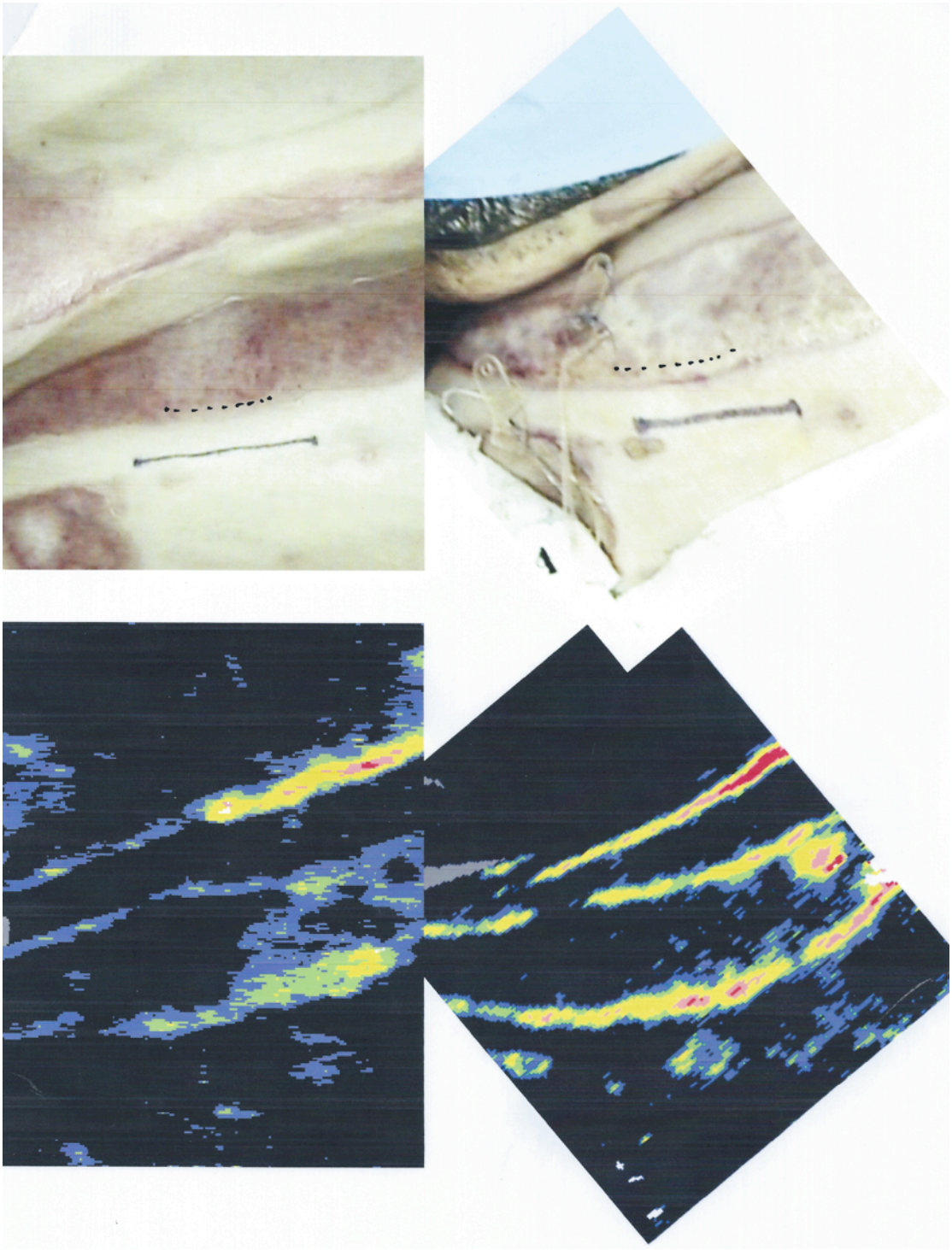


Plate 10: Photographs and LDI Images for Patient S10. Above left: Photograph of burn before probe insertion with standard 5cm line, and site for probe B marked with dotted line. Above right: Photograph of burn at end of study period with standard 5cm line, and site for probe B marked with dotted line. Probe A can be seen still in situ. Below left: LDI before probe insertion. Below right: LDI at end of study period.