AN IMMUNE-ENDOCRINE APPROACH TO IDENTIFY BIOMARKERS OF OUTCOMES FOLLOWING MAJOR BURN INJURY IN ADULTS

by

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Abstract

Severe thermal injury induces a profound immune-inflammatory, endocrine and hypermetabolic response associated with poor outcomes including delayed wound healing, sepsis, multiorgan failure (MOF) and mortality. One urgent clinical need is to understand the mechanisms mediating the systemic response in order to identify prognostic biomarkers of outcome and develop new therapies.

This thesis analysed stored serum samples from a large observational study of severely burned patients (≥20% TBSA) at the Queen Elizabeth Hospital Birmingham. The HPA/HPG, Vitamin D, inflammatory, immune and adipokine responses were characterized from day of injury to 12 months post-injury and related to clinical outcomes including sepsis, MOF, wound healing, scarring and mortality.

Low DHEA, DHEAS, Testosterone and Vitamin D (25D3, Free 25D3, Bioavailable 25D3) status showed significant associations with poor outcomes including delayed wound healing, sepsis and mortality independent of age, gender and injury severity. Higher levels of DHEA, DHEAS, testosterone, Vitamin D and adiponectin were associated with improved scarring. Current burn treatments with potential influence on the endocrine system were also assessed. Corticosteroid use was associated with poor prognosis including sepsis, MOF and mortality, whereas Oxandrolone use was associated with improved outcomes. The data reveal several novel biomarkers of outcome that could also have therapeutic value.

This thesis is dedicated to the memory of my beloved grandmother

Efat Ahmad Taher

May your peaceful soul rest in the heavens above

Still miss you everyday

I hope this makes you proud

Acknowledgements

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“It has been said that something as small as the flutter of a butterfly's wing can ultimately cause a typhoon halfway around the world” - Chaos Theory

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CHAPTER 1: INTRODUCTION

1.1 The Burden of Thermal Injury

1.1.1 Aetiology and Epidemiology of Burn Injuries

Burn injury is a form of traumatic injury to skin or other tissues, incurred primarily through a heat-based external stimulus. In the UK the most common mechanisms of thermal injury and subsequent admission to burn centers are scald (39%), flame/flash (30%) and contact (19%)(1). In the US, the most prevalent causes of burn injury are flame, scald, contact, electrical and chemical accounting for 43%, 34%, 9%, 4% and 3% respectively (2). The incidence of the above causes behind burn injury have remained stable for many years (3). Other geographical regions have reported similar observations (4-6).

Thermal injury is a serious worldwide public health issue. In the UK, approximately 250,000 individuals sustain burn injuries with 112,000 attending emergency department annually (7). Almost 189,000 acute burn admissions were reported in England from 1991-2010, with the number of burn-related hospitalizations appearing to increase across these decades (8). Similarly, 40,000 burned patients were admitted in hospitals for treatment in the US , with an average of greater than 200 admissions to burn centers per year (2). Globally, the incidence of burns ranked 4th in all injuries that required medical assistance with 11 million people affected (9).

1.1.2 Outcomes of Thermal Injury

Although there is an increasing trend of burn hospitalizations in some countries, a global decreasing trend in burn incidence and severity has been reported (10). Furthermore, mortality rates have dramatically improved with time in the UK, US and

several other countries (10-13). This can be attributed to medical advancements in burn treatment, evolution of a multidisciplinary team approach in burn care and development of regional systems optimizing distribution and allocation of burned patients to the appropriate facilities and surveillance of medical care (11, 14-17). Despite this, a global annual estimate of 180,000 deaths is attributed to thermal injuries – with most occurring in low and middle-income countries (18). Therefore, more effective and efficient burn treatment and management is required to further improve survival rates following thermal injury.

With the number of burn survivors increasing, thermal injury is becoming a leading cause of morbidity (18). Morbidity following thermal injury can be classified into short-term and long-term consequences. Short-term causes of increased morbidity in burned patients include sepsis, multiorgan failure (MOF) and delayed wound healing. Long-term reasons for morbidity following thermal injury include scarring and development of chronic illness.

Burned patients are at increased risk of developing infections. Pneumonia, urinary tract infections, cellulitis and wound infections ranked in the top 5 of the ten most clinically relevant complications following thermal injury (19). Bacteremia and sepsis have been reported to affect approximately 26 - 69% of burned patients (20-23). Furthermore, the incidence of multiorgan dysfunction and failure following thermal injury is 63% and 19-37% respectively (20, 24) . Both sepsis and multiorgan dysfunction/failure are also the most reported causes of mortality in the burn population (25). Another recognized cause of mortality following injury is connected

with burn wounds. Survival rates following thermal injury has improved dramatically owing to early burn excision and wound closure through aseptic techniques, as well as advances in general wound care (26, 27). Therefore, clinical and research effort is currently focusing on addressing wound healing duration and prevention/minimization of sub-sequent scarring (28).

The incidence of scarring following thermal injury has been reported to be approximately 32-72% (29). Physical consequences of burn scars include contractures which potentially could affect activities of daily living. Additionally, burn scars can be symptomatic and have psychological consequences. Survivors of thermal injury may complain of scar pruritis and pain, as well as feeling shame and social anxiety (30). Anxiety in burn patients with visible scarring is probably attributed to experiencing stigmatizing behavior on a regular basis such as staring, startled reaction, whispering, teasing, rude comments, eye contact avoidance and manifestations of pity (29). Subsequently, it may lead to social isolation adversely affecting mental health (31). Burn patients have significantly higher mental health-related hospital admission, and are at increased risk of developing mood/anxiety disorders, psychotic disorders and alcohol/drug behavioral disturbances years following initial injury (32).

In addition to long-term mental health effects of burn injury, multiple reports demonstrated associations between thermal injury and various chronic illnesses. Epidemiological studies observed increased risk of various disease states in burned patients compared to un-injured patients including cardiovascular disease, diabetes

mellitus, gastrointestinal disease, infectious diseases, musculoskeletal diseases, neuro-pathologies and respiratory infections (33-39).

1.2 Pathophysiology of Thermal Injury

Cutaneous thermal injury can be caused by various sources of heat including flame, scalds with hot solutions and contact with hot objects. Other mechanisms include chemical, friction/trauma, frostbite, electrical and radiation. The haemodynamic consequences following burns is dependent on the mechanism of injury. Burns sustained through chemical and electrical means, due to their different pathological nature and the fact that they were not studied in this thesis, are not discussed further.

Thermal injury results in immediate, multiple pathophysiological responses, both at local and systemic levels. Such responses differ significantly from other forms of trauma. Therefore, a thorough understanding of the physiological implications of severe thermal injury is required to improve clinical burn care.

1.2.1 Pathology of Burn Wound and its Clinical Relevance

Burn wounds were described into three pathological zones by Mr. Douglas Jackson in 1953 (40). These include the central zone of coagulation or necrosis, intermediate zone of stasis and peripheral zone of hyperemia. The zone of coagulation is the zone of maximal direct damage and contains irreversible necrosis. The zone of stasis is a compromised zone associated with tissue ischemia. This zone is potentially salvageable with adequate resuscitation and treatment (41). The zone of hyperaemia is characterized by increased tissue perfusion and usually recovers without complications. Burn injury is a dynamic process with the overall depth and surface area

of the injury open to influence by addressing the zone of stasis and halting cellular apoptosis (42). By limiting the depth and surface area of burn wounds, the prognosis of burned patients can be improved (43, 44). The zones of burn wounds are summarised in Figure 1.1.

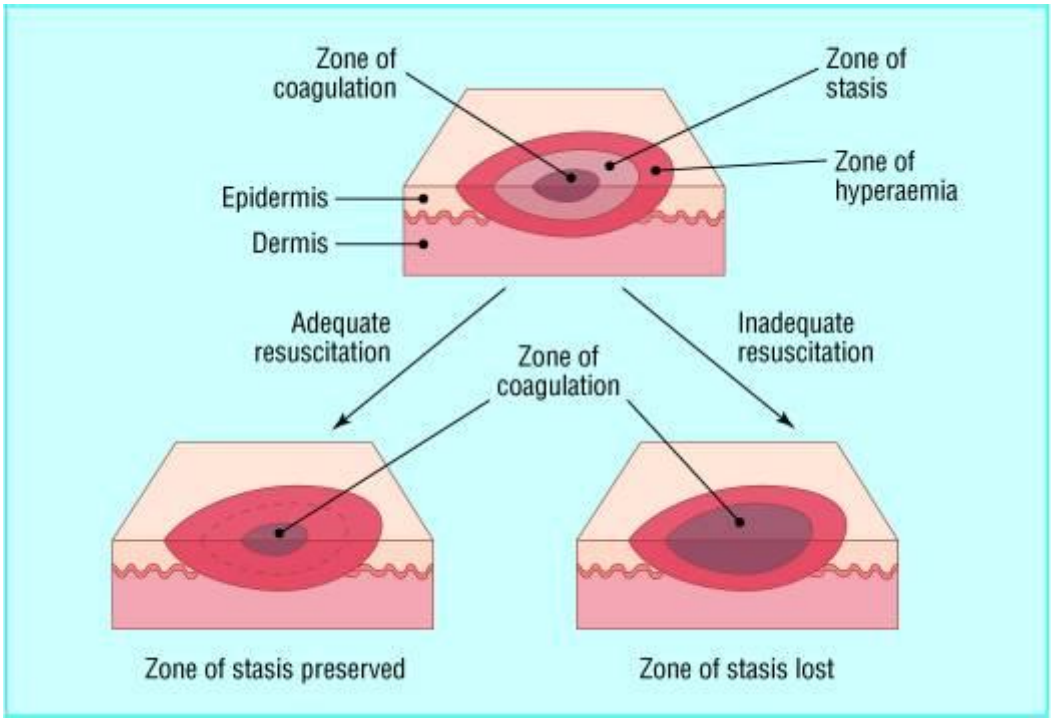


Figure 1.1. Overview of the three concentric zones of burn wounds.

The three zones of burn wounds form the local response following thermal injury. Burn exhibits a dynamic pathology. Inadequate treatment following thermal injury leads to progressive necrosis of burn wounds and loss of zone of stasis. Figure taken from "ABC of burns: pathophysiology and types of burns" by Hettiaratchy S et al (41).

Burn wounds are clinically classified by depth and extent of surface area involved. Depth of thermal injury is sub-divided into 4 categories: superficial, superficial partial thickness, deep partial thickness and full thickness. Superficial burns are confined to the epidermis and typically exhibit erythema and pain that resolves within a few days

with minimal medical intervention, usually without complications. Hence, the extent of epidermal burns is not usually calculated. Superficial partial thickness burns encompass both epidermis and papillary dermis. Superficial partial thickness wounds are characterized by blisters, erythema and edema, as well as blanching on pressure with brisk capillary refill on release. Deep partial thickness burns extend into the reticular dermis. Manifestations of deep partial thickness injuries include fixed red staining, marked oedema, moist wounds, slow capillary refill and reduced sensation. Partial thickness burn wounds usually requires medical and/or surgical intervention and is associated with patient outcomes including hypertrophic scarring(45). Full thickness burn involves all layers of skin and may extend into subcutaneous tissues, fascia and other sub-dermal structures. Full thickness injuries invariably require excision of necrotic tissues and resurfacing surgery including skin grafts/skin substitutes (46). Correct diagnosis of burn depth is thus clinically important. Re-epithelization of burn wounds occurs by a complex set of actions involving somatic and stem cells within different skin layers, with keratinocyte migration from skin appendages and release of various growths factors from cells within reticular dermis (46, 47). The anatomy of skin in relation to burn depth is illustrated in Figure 1.2.

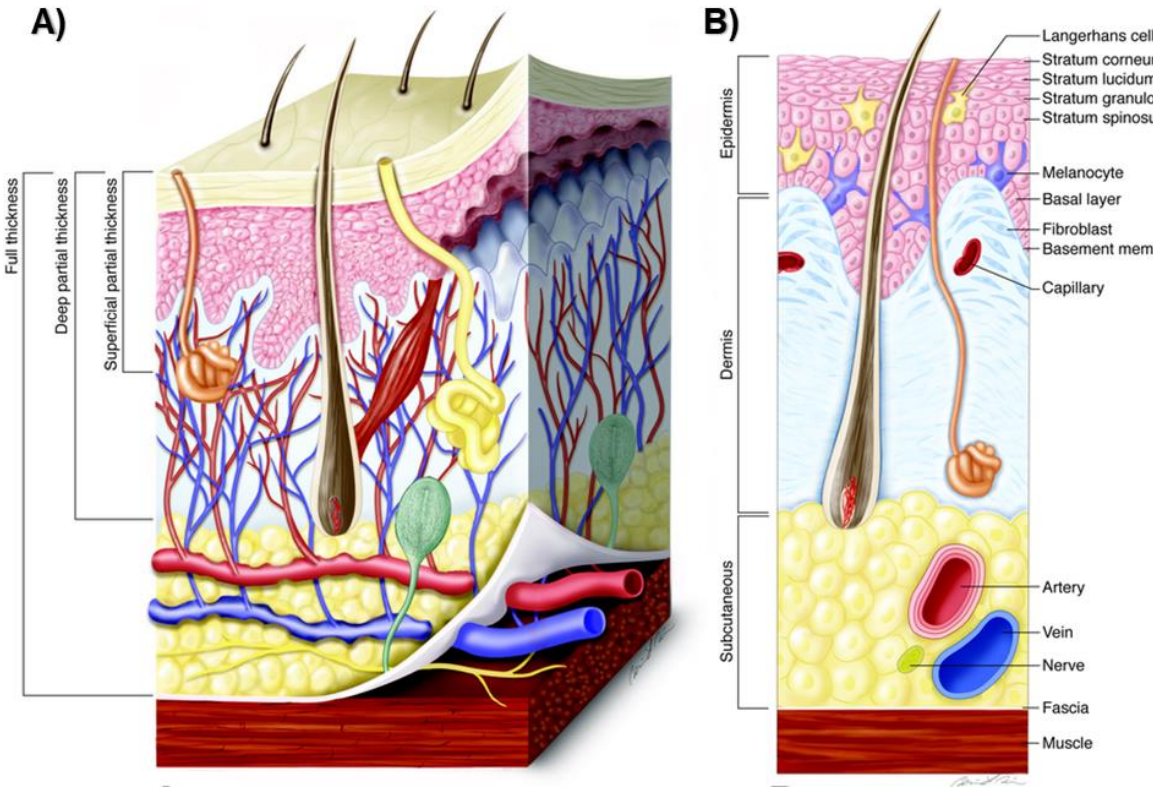


Figure 1.2. Overview of burn depth in relation to skin anatomy.
A) Three-dimensional illustration of skin anatomy and burn depth. B) Skin histological layers with dermal and sub-dermal contents. Figure taken from "A Review of the Local Pathophysiologic Bases of Burn Wound Progression" by Shupp JW et al (48).

The extent of burn wound surface area can be assessed by at least, 3 methods. These include the Wallace rules of 9s, the Lund and Browder chart and the 1% patient's palmar surface (49-51). These burn size estimation techniques are illustrated in Figure 1.3. Among these methods estimation of burn surface area using the Lund and Browder chart is reported to be the most accurate (52). However, burn estimation using these methods is subjective with significant wide discrepancy between assessors (53, 54), which could be attributed in part to clinical experience. Nevertheless, estimating the injury size is clinically important. Burned patients with total burn

surface area (TBSA) ≥10% should be referred to a burn unit or center as they may require specialized care including systemic medical interventions (55-57).

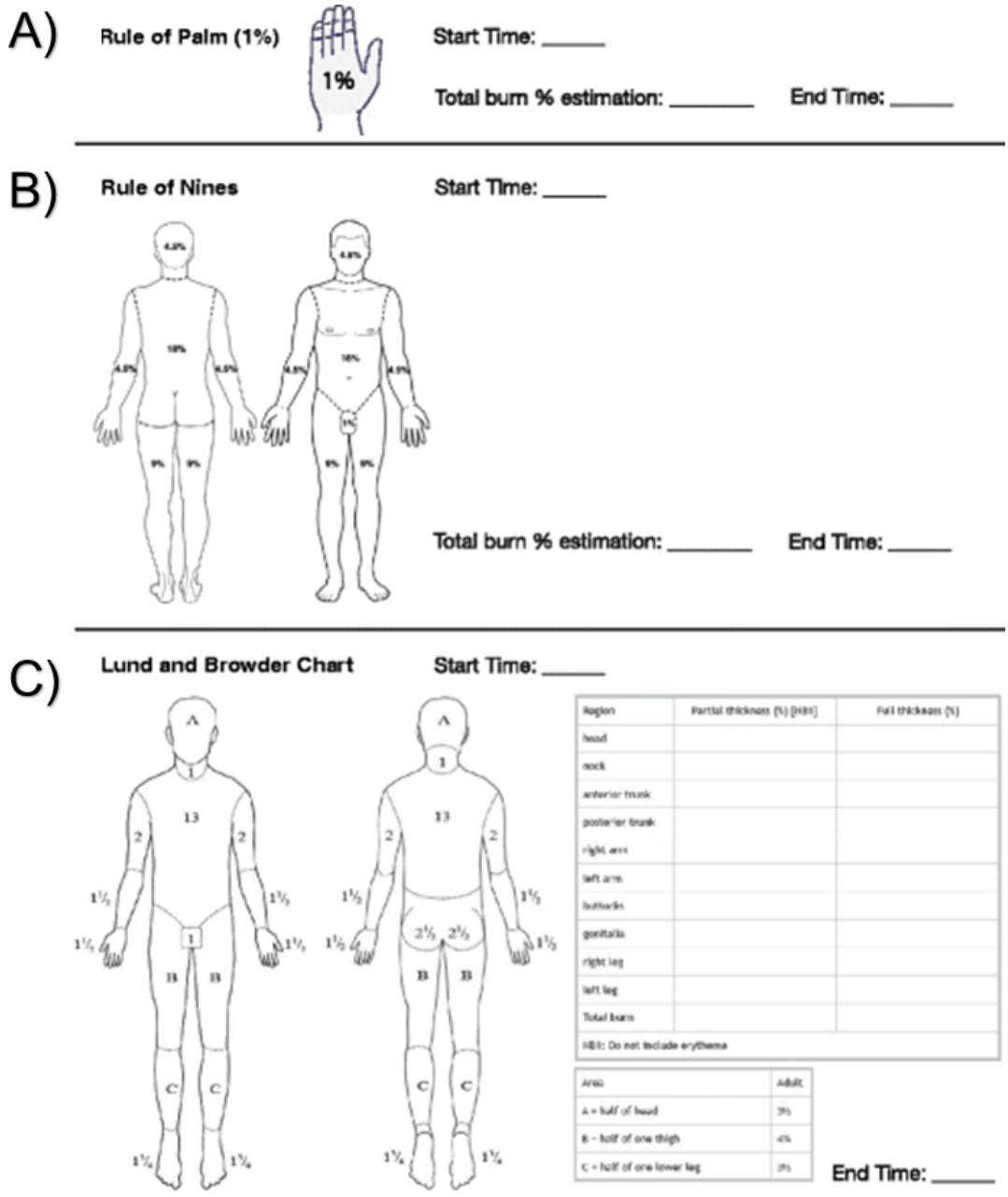


Figure 1.3. Methods of estimating burn wound surface area.

A) Patient's palmar surface equates to approximately 1%. B) Wallace's rule of nines. C) Lund and Browder Chart. Figure acquired from "The validation study on a three-dimensional burn estimation smart-phone application: accurate, free and fast?" by Cheah AKW et al (58).

1.2.2 Systemic Response Following Thermal Injury

The primary aim of establishing referral criteria is to capture patients with severe-enough burns at risk of profound systemic pathophysiological response (59). Furthermore, prompt clinical treatment of patients following severe thermal injury is essential to improve prognosis (60). However, the definition of severe burns in the literature has been variable. Recently, adult patients with ≥20% TBSA have demonstrated robust and complex pathophysiological responses comparable to those with larger thermal injury (61). Response following severe thermal injury is characterized by burn shock and global disruption of immune-inflammatory, metabolic and endocrine processes and these will now be discussed in more detail as they form a central aspect of this thesis.

1.2.3 Hypovolemia and Shock Following Severe Thermal Injury

Severe thermal injury results in significant systemic and microcirculatory alterations associated with shock. Shock is clinical phenotype of circulatory failure with subsequent tissue hypoperfusion, inadequate cellular oxygenation and metabolism (62). Burned patients exhibit substantial distributive shock and significant tissue trauma. Severe thermal injury leads to increased vascular permeability with subsequent fluid shifts and intravascular volume depletion, as well as oedema formation (63). This process results in hypovolemia and causes tissue injury secondary to translocation of fluids and protein into burned and non-burned tissues (64, 65). Subsequently, an imbalance between hydrostatic and oncotic pressures develops. The new interstitial contents create an osmotic gradient that draws in additional fluid from the vasculature, with subsequent and further loss of protein into the oedema fluid

(66). This cascade results in marked hypoproteinemia and massive oedema formation. Subsequently, intravascular hypovolemia and haemoconcentration occurs within the first 12-24 hours of severe thermal injury (67). This first period following severe burns is called the 'ebb' phase and typically lasts 24-72 hours (65). Approximately 58% of burn-related deaths occur within the first 72 hours (68). This indicates that burn shock is still a major cause of mortality. Corticosteroids are sometimes used to address burn shock refractory to resuscitation following severe thermal injury. The use of corticosteroids in burned patients will be discussed further in Chapter 3.

Following the 'ebb phase', the second period of burn resuscitation begins and is called the 'hyperdynamic and hypermetabolic' or 'flow' phase. At 24-48 hours post-thermal injury, microvasculature integrity and systemic vascular resistance, as well as cardiac output improves leading to enhanced blood flow to burn wounds (69). The pathophysiological response following thermal injury is summarized in Figure 1.4. Severe burn affects multiple organs and tissues with inflammation, immune dysfunction, hypermetabolism and endocrine disturbances being the hallmarks of the pathophysiological response following injury (63, 70, 71).

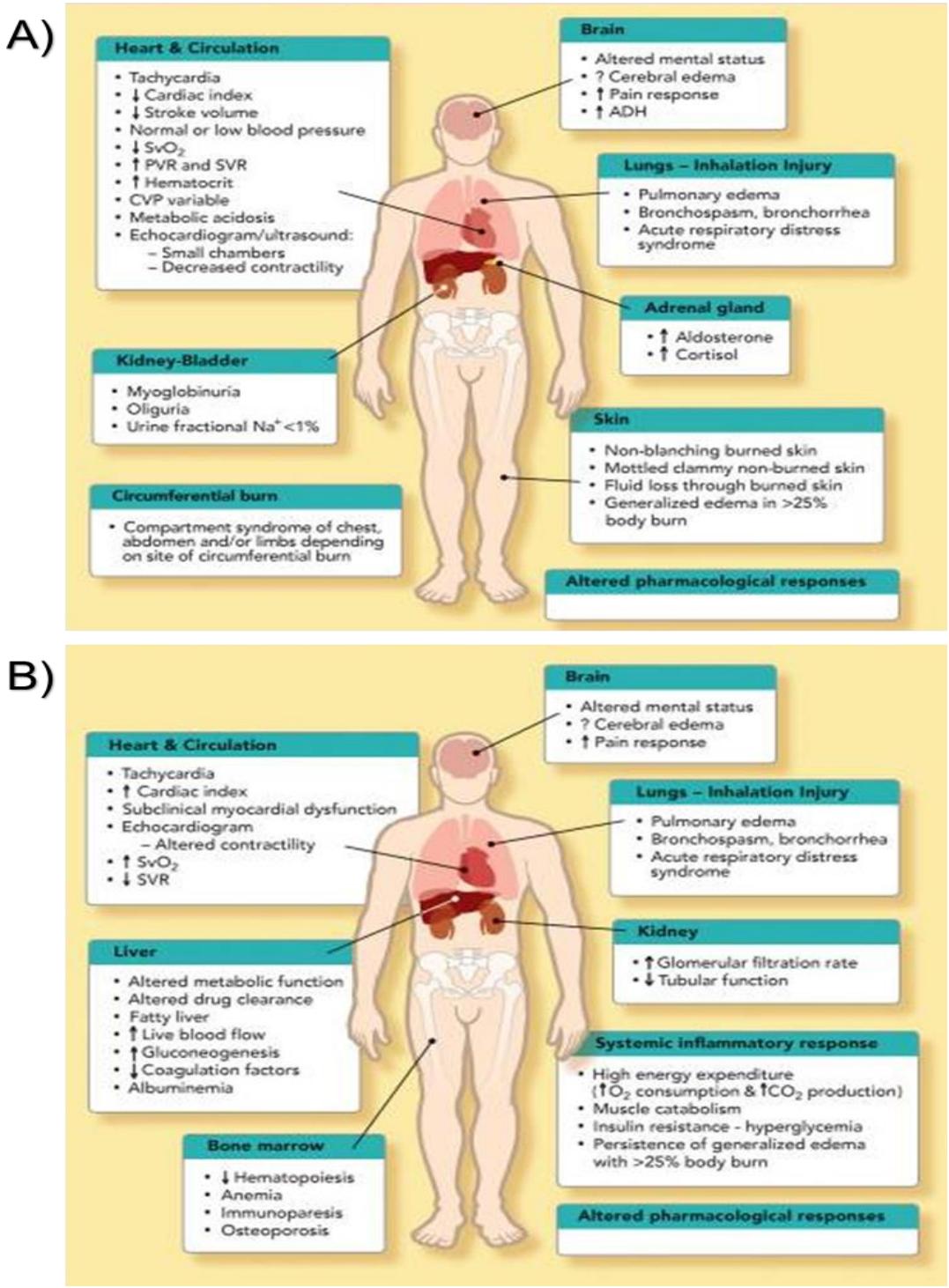


Figure 1.4. The pathophysiological responses following severe thermal injury. Severe thermal injury induces multiple reactions with consequences on various organs and tissues. A) The pathophysiological response during the 'ebb' phase in burned patients. B) The pathophysiological response during the 'flow' phase in burned patients. Figure acquired from "Acute and Perioperative Care of the Burn-Injured Patient" by Bittner EA et al (69)

1.2.4 Systemic Inflammatory Response following Severe Thermal Injury

Burns and other traumatic injuries are associated with the release of damage-associated molecular patterns (DAMPs) (72-74). DAMPs originate from the extracellular matrix, such as decorin and biglycan, or intracellular compartments(75). DAMPS deriving from intracellular compartments can be cytosolic (such as F-actin and S100 proteins), nuclear (e.g. histones and DNA), mitochondrial (such as mtDNA and Formyl peptide), endoplasmic reticulum-based (e.g. Calreticulin), Granular (such as Defensins and Cathelicidin) and Plasma membrane-derived (such as Syndecans and Glypicans) (75). Following tissue injury or cellular apoptosis, discharged DAMPs initiate an immune response and sterile inflammation (72, 76). Inflammation is an acute host reaction to tissue injury and pathogens. It is a defense mechanism that aims to remove injurious stimuli, restore tissue homeostasis and initiate the healing process (77). However, in extreme circumstances like burn injury, severe tissue trauma results in an overwhelming release of inflammatory mediators into the circulation resulting in complex multi-system effects. Clinically, this is recognized as systemic inflammatory response syndrome (SIRS). The definition of SIRS was set up in a consensus conference involving the American College of Chest Physicians and Society of Critical Care Medicine in 1991(78). Clinically SIRS is diagnosed when 2 of following criteria are met: Temperature >38^{o}C or <36^{o}C, Heart Rate >90 beats/min, Respiratory Rate >20 breaths/min or $PaCO_2$ <4.3kPa and White Cell Count >12,000 cells/mm^3, <4000 cells/mm^3 or immature bands >10%. Additionally, the same consensus conference defined sepsis, septic shock and multiple organ dysfunction syndrome (MODS)(78). These definitions are as outlined: Sepsis – SIRS with documented microbial source,

Septic Shock – SIRS with hypotension refractory to fluid resuscitation, and MODS – SIRS with organ dysfunction.

Originally, severe forms of trauma were thought to induce an initial excessive SIRS response followed sequentially by a compensatory anti-inflammatory response syndrome (CARS)(79). Furthermore, multiple inflammatory events or 'second hits' were thought to complicate patient outcomes by inducing a secondary exaggerated response (80). This classical consecutive SIRS/CARS paradigm was questioned when septic murine models exhibited concurrent pro- and anti-inflammatory responses (81, 82). Recently, a clinical study examined circulating leukocytes in trauma patients observing similar findings and as a result a new paradigm of concomitant SIRS and CARS has been proposed (83). Burned patients demonstrated simultaneous and rapid significant elevations of systemic anti- and pro-inflammatory gene expressions. Furthermore, outcomes following injury were associated with the magnitude and duration of the acute inflammatory response. No evidence of 'second hit' phenomenon was observed. The SIRS/CARS paradigms of burns and major trauma are illustrated in Figure 1.5.

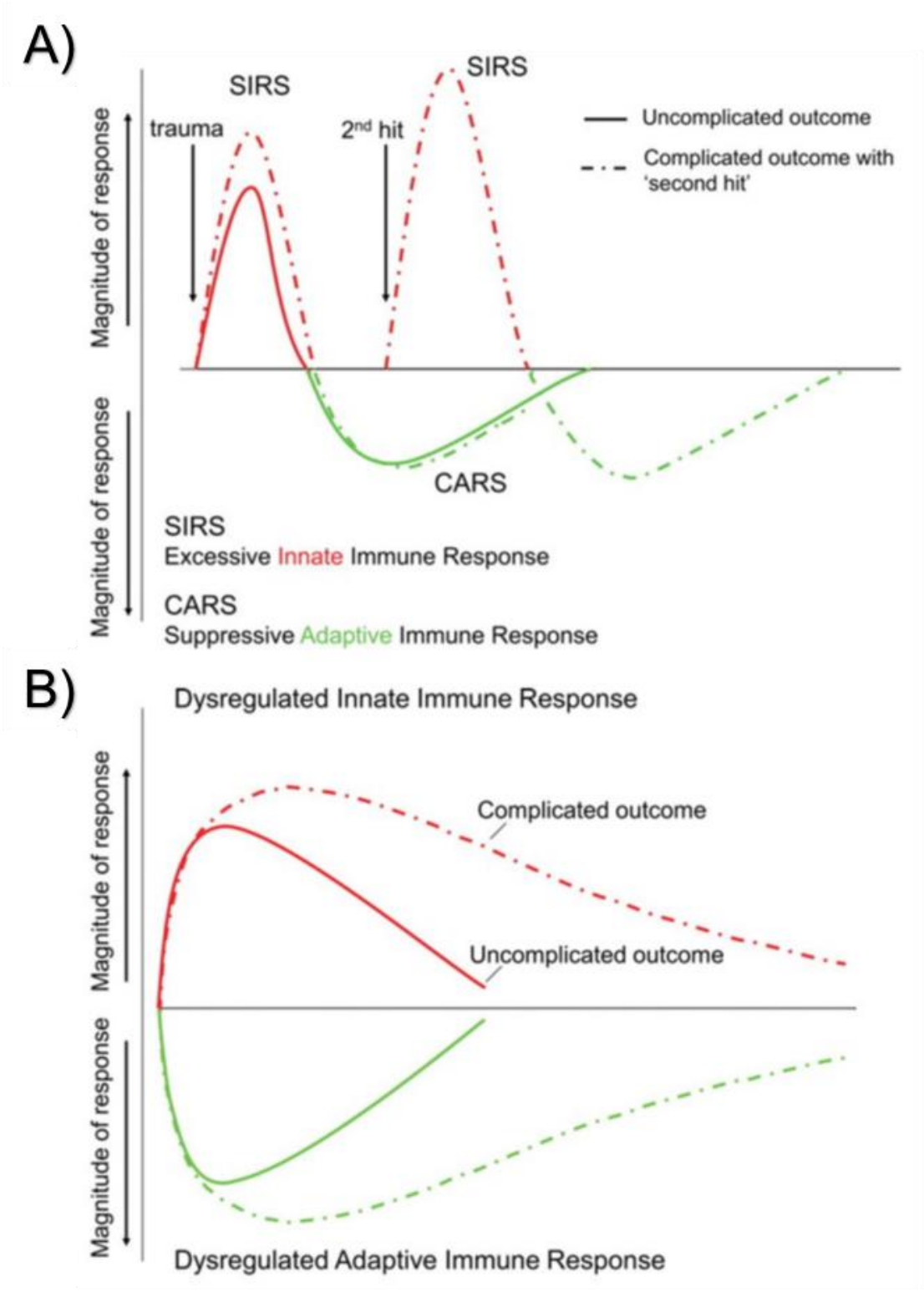


Figure 1.5. Inflammatory paradigms following injury.
A) Classical trauma paradigm demonstrating SIRS followed temporally by CARS with a 'second hit' phenomenon. B) New proposed trauma paradigm of simultaneous SIRS and CARS. Figure taken from "A genomic storm in critically injured humans" by Xiao W et al (83).

1.2.5 Cytokine Response following Severe Thermal Injury

The severe inflammatory response exhibited following burn injury is primarily mediated by cytokines released from innate immune cells as a result of activation by DAMPs (84, 85). Cytokines are proteins with autocrine/endocrine properties that modulate cellular activities and interactions including angiogenesis, cellular apoptosis, proliferation and repair, as well as the immune response itself (86). Similar to the SIRS/CARS paradigm, the understanding of cytokine responses following injury has been evolving. Initially, outcomes were proposed to be driven by the sequential release of cytokines, referred to as the "cytokine cascade"(87). More recently the term "cytokine storm" has emerged (88) from large scale studies such as the "Glue Grant" study in the US. The cytokine storm concept suggests outcomes are associated with an exaggerated release of primarily pro-inflammatory cytokines. The current understanding involves an interplay between various cytokines of different roles which aim to dampen the inflammatory response and restore body homeostasis and immune equilibrium (89). Cytokines can be broadly classified into pro and anti-inflammatory roles and their numerous functions have been extensively reviewed (90) and are summarized in Table 1.1.

Severe thermal injury induces a hyper-inflammatory response with multiple cytokines demonstrating up-to 200 fold elevations compared to un-injured individuals (91). Similar perturbations of cytokine kinetics in burned patients were observed in multiple studies (61, 92-97). Furthermore, alterations in circulating cytokine levels post-burn are significantly associated with outcomes following thermal injury. Elevated serum levels of IL-1Ra, IL-6, IL-8 and MCP-1 at day 1 post-burn injury were significantly

associated with mortality (95, 97). Potentially, these cytokines may be used as prognostic biomarkers of survival once clinically validated. Interestingly, these perturbations in cytokine profiles can be persistent and may last for up to 3 years following severe thermal injury (94)

Studies profiling the cytokine response in burned patients have demonstrated that not all pro-inflammatory cytokines are elevated (91, 93, 94, 97). This observation could be due to various influencing variables including age, gender, burn size, injury severity, time of sample from injury, patient co-morbidities and treatments given prior to sampling. Additionally, such an observation may suggest the presence of a possible regulatory mechanism attempting to maintain cytokine equilibrium and its associated functions including the immune response.

Table 1.1. Overview of cytokines and other signaling proteins analyzed in this thesis

Cytokine/Protein	Source	Role
Interleukin-1 Receptor Antagonist (IL-1Ra)	Immune cells including monocytes and neutrophils Epithelial cells Adipocytes	Anti-Inflammatory effects(98)
Granulocyte-Colony Stimulating Factor (GCSF)	Immune cells including macrophages Endothelial cells	Anti-inflammatory effects(99)
Interleukin 6 (IL6)	Immune cells including macrophages Myocytes Adipocytes	Anti-inflammatory and Pro-inflammatory effects(100, 101)
Interleukin 8 (IL8)	Immune cells including macrophages Epithelial cells Endothelial cells Myocytes	Pro-inflammatory effects(102)
Interleukin 10 (IL10)	Immune cells including monocytes and lymphocytes	Anti-inflammatory effects(103)
Interleukin 12-p70 (IL12p70)	Immune cells including dendritic cells, macrophages and neutrophils	Pro-inflammatory effects(104)
Interleukin 17 (IL17)	Immune cells including natural killer cells and T cells	Pro-inflammatory effects(105)
Monocyte Chemoattractant Protein-1 (MCP-1)	Immune cells including Epithelial cells Endothelial cells Fibroblasts Myocytes	Pro-inflammatory effects(106)
Tumour Necrosis Factor alpha (TNFα)	Immune cells including macrophages, and lymphocytes	Pro-inflammatory effects(107)
Insulin-like Growth Factor-1 (IGF-1)	Liver	Anti-inflammatory effects(108)
Interleukin-1 beta (IL-1β)	Macrophages	Proinflammatory effects(109)
Transforming Growth Factor beta 1 (TGF-β1)	Immune cells	Pro-inflammatory effects(110)

1.2.6 Immune Dysfunction following Severe Thermal Injury

Dysfunction of the immune system has been demonstrated in burned patients and is mediated by a 'genomic storm' that dysregulates both the innate and acquired immune system (83). The innate immune system responds instantaneously without programming or differentiation and consists of cells like neutrophils and monocytes. The acquired immune system needs activation and programming by cell-to-cell contact by antigen presenting cells such as dendritic cells and consists of T- and B-lymphocytes. The various roles and components of the innate and acquired immune system have been summarized in several comprehensive reviews (111).

Severely burned patients exhibit significant dysfunction of neutrophils with simultaneous release of immature granulocytes, these immature cells may in part explain reduced neutrophil function after burn injury (112). Thermal injury results in immediate and persistent impairment in chemotaxis, phagocytosis and reactive oxygen species production (112, 113). Furthermore, burn injury induces significant changes in surface expression of adhesion molecules and chemokine receptors of neutrophils facilitating their recruitment to the burn site (114) and also to other organs such as the lungs, contributing to outcomes such as Acute Respiratory Distress Syndrome (ARDS). Moreover, cell-free DNA, a marker of neutrophil extracellular traps, are elevated during the acute phase of burn injury (112). Interestingly, neutrophil phagocytic capacity, immature granulocyte count and cell-free DNA levels in blood at day one post-thermal injury were predictive of subsequent sepsis development (112). This indicates that burns induced significant alterations to neutrophil function resulting in increased susceptibility to infection and poorer outcomes. Alterations in the innate

immune system following severe thermal injury may also have long-term implications. Burned patients exhibited a subpopulation of peripheral blood mononuclear cells that potentially may modulate connective tissue cells, such as fibroblasts, by producing pro-fibrotic cytokines and mediators (115).

Severe thermal injury significantly reduces levels of circulating lymphocytes during the acute phase (116). This could be secondary to burn-induced dysregulation of lymphocyte apoptosis (117). Abnormal circulating lymphocyte levels in burned patients is associated with poor prognosis including increased risk of infections (118).

1.2.7 Metabolic Response following Severe Thermal Injury

Burn injury induces an exaggerated metabolic response that differs from other forms of trauma and critical illness in magnitude and persistence (119, 120). Significant metabolic disturbances following severe thermal injury are characterized by significant elevated metabolic rates, hyperdynamic circulation, disrupted temperature regulation and several biochemical/physiological alterations resulting in a severe catabolic state. This state is termed the hypermetabolic response. This hypermetabolic response is characterized by significant cardiac dysfunction, protein catabolism and lean body mass loss, hyperglycemia and substantial insulin resistance, as well disruption of lipid metabolism and fat composition (91, 121). This hypercatabolic state results in significantly increased metabolic rates with resting energy expenditure (REE) reaching 180% of predicted values (122). Furthermore, hypermetabolic responses following injury correlate with patient age, burn mechanism and burn size (92, 123, 124). Metabolic rates of burned patients increases in a curvilinear fashion from being

approximately normal for TBSA <10% to double that of predicted values at ≥40% TBSA (125). Additionally, severely burned patients demonstrate significantly increased resting metabolic rates at 6, 12 and 24 months following injury (91, 94, 120). Such significant increases in catabolism can lead to poor outcomes including loss of lean body mass, decreased immune defenses, slower wound healing and mortality (120, 126). The hypermetabolic response and associated outcomes are summarized in Figure 1.6.

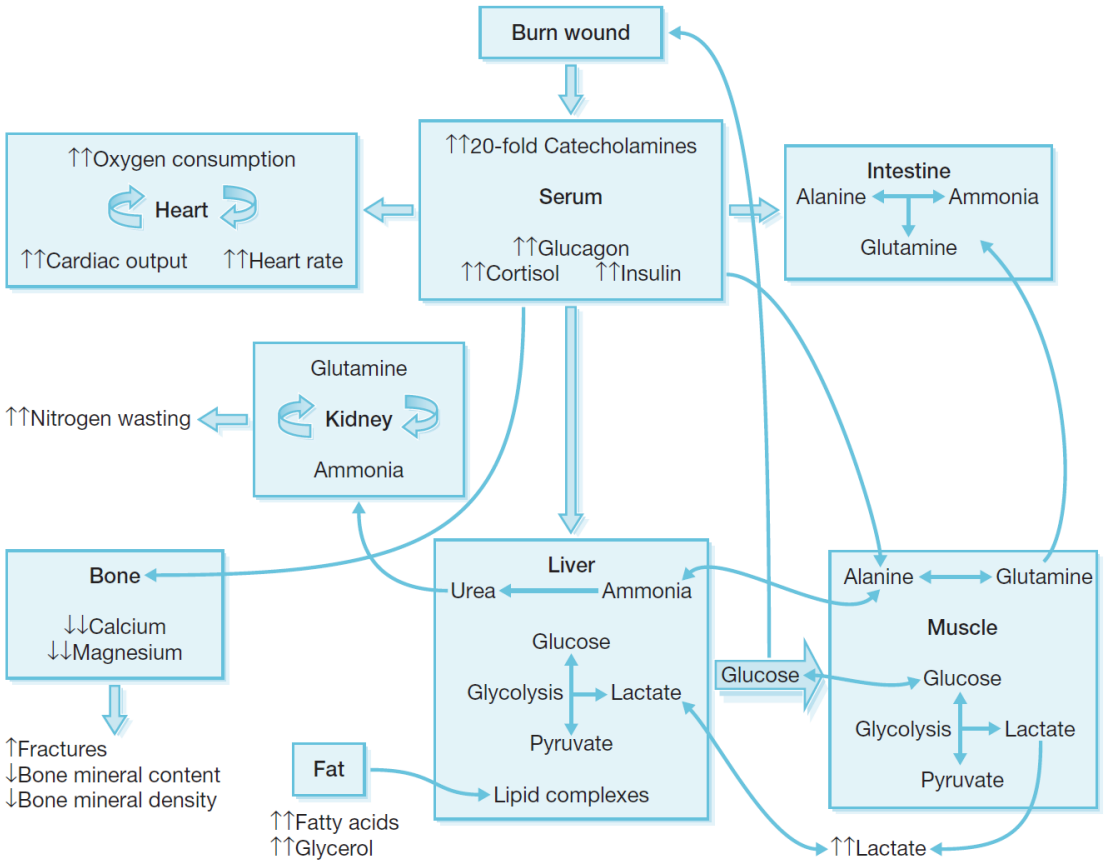


Figure 1.6. The effects of metabolic dysfunction following severe thermal injury. Figure taken from Total Burn Care (5th Edition) (121).

Due to poor outcomes associated with the hypermetabolic response following severe thermal injury, research has been focused on dampening the response and addressing responsible mediators. One of which is the endocrine and stress response following thermal injury (94, 121). Current pharmacological agents used to dampen the hypermetabolic response following severe thermal injury is oxandrolone. Oxandrolone use in burned patients will be discussed in Chapter 3.

1.2.8 Endocrine Response following Severe Thermal Injury

The endocrine system is crucial in initiating and managing systemic responses following trauma (127). Major thermal injury induces an exaggerated sympathetic activation eliciting significant 'flight or fight' stress responses. In turn, this leads to major disturbances in multiple endocrine systems including the hypothalamic-pituitary-adrenal (HPA) axis and gonadal hormones (128). Severely burned patients thus exhibit elevated serum levels of catecholamines and cortisol (91). Additionally, urinary catecholamines and cortisol levels in burned patients are increased, 5-8 fold, following injury (91). This surge in systemic levels of catecholamines and cortisol reflects the stress levels exhibited in burned patients, a phenotype that can persist for years following injury (94).

Other hormones profiled following thermal injury include dehydroepiandrosterone (DHEA), dehydroepiandrosterone-sulfate (DHEAS), estrogen, progesterone, testosterone (91, 94, 129). Although the status of these hormones is affected in burned patients, the responses these hormones displayed are diverse. The status of other hormones have been investigated but not characterized longitudinally following

thermal injury including vitamin D and adipokines (130, 131). Furthermore, no studies have explored the effects and status of upstream metabolites of vitamin D, HPA and gonadal axes on outcomes following thermal injury. Moreover, no studies evaluated the kinetics of HPA and gonadal hormones, vitamin D hormones and adipokines immediately following thermal trauma and in the long term. By profiling the post-thermal injury responses of various hormones longitudinally and analyzing associations with outcomes, potential clinical and therapeutic benefits may be identified and explored further to improve outcomes of burned patients.

1.3 Current Knowledge Gaps

The systemic response following thermal injury is similar to those observed in critical illness and trauma; however, the magnitude, persistence and severity of the post-burn response is significantly greater(132). This potentially also includes the endocrine response following severe thermal injury (71), though this is less well researched. Observations and reports investigating such responses in critically ill and trauma populations, burned or otherwise, could be of value. Such data may act as a guide for setting up studies or optimize analytical and experimental methodologies. Therefore, reviewing the literature reporting on endocrine responses following critical illness and trauma is valuable. This includes the HPA and gonadal axes, vitamin D metabolism pathway and adipokines. The endocrine responses of these hormones and their associations with outcomes in critically ill and trauma populations have been summarized and published (133-136) and are described in brief here.

1.4 Sex Steroid Hormones in Critical Illness and Trauma

1.4.1 Gender Disparities on Outcomes Following Trauma

Sepsis and subsequent MOF continue to be a major cause of morbidity and mortality in trauma patients (137). Gender differences for sepsis, MOF and mortality have been reported in the literature. In a study of 681,000 trauma patients, females demonstrated significantly lower complications and mortality rates compared to males (138). A recent meta-analysis of 100,566 male and 39,762 female trauma patients found male gender was associated with higher incidence of complications, lengthier hospital stay and increased mortality (139). In addition, male gender has been identified as a risk factor in the development of infection and MOF (140-142) and males suffer from significantly lower survival rates following sepsis when compared to females, 31% vs. 74% (143). This suggests that sex steroid hormones (SSH) may play a role in the maintenance of immune-inflammatory function in the trauma setting. This is further supported by the work of Haider *et al* who concluded that females aged 13-64 exhibited significantly lower mortality outcomes following trauma-associated shock when compared to males, and that this difference was abolished in the extremes of age when the effects of sex hormones were either absent or diminished (144). Trentzsch *et al*, who performed a matched-pair analysis of 29,353 prospectively recorded trauma cases, concluded that males were more susceptible to MOF, sepsis and mortality (145).

Female patients appear to benefit from better physiological reserves and thereby are more protected against the consequences of trauma and shock. A prospective clinical study reported that female trauma patients required less fluid resuscitation volumes

(12L vs 8L, $p < 0.05$), less Starling curve intervention (42% vs 15%, $p < 0.05$) to maintain oxygen delivery index and less inotrope and/or vasopressor support (36% vs 10%, $p < 0.05$) compared with similarly injured male patients and a standardized management protocol(146). Another prospective clinical study involving more than 4,000 patients reported that premenopausal women exhibited lower serum lactate levels and required less blood transfusion despite having more severe injuries(147).

However, the role of gender in modifying the response to trauma is still not clear cut, with multiple conflicting clinical reports in the literature. Rappold *et al* concluded that the female gender offered no protection from the development of acute respiratory distress syndrome, pneumonia or sepsis nor was it associated with decreased mortality rates post trauma (148). This finding was replicated by other studies which have demonstrated equivalent mortality rates in both genders following traumatic injury (149-151). Other studies have suggested that female gender is a risk factor in trauma patients and is associated with increased complication and mortality rates (152-154). These conflicting findings may be attributed to many factors such as study sample size, triage, treatment speed, management protocol etc.

This apparent lack of agreement in the literature highlights the need for further studies in better controlled environments, comparing similar types of injury and taking age and gender into account in order to obtain more conclusive data. In addition, there remains a paucity of data on the mechanisms that may underlie gender differences in humans, with the majority of such research done in animal models of trauma. In this section, the potential impact of gender and SSH on different aspects of the response to

trauma are discussed, and it has been made clear where the data rely almost entirely on animal studies.

1.4.2 The Effects of Sex Steroid Hormones on the Immune-Inflammatory Response following Trauma

Various clinical and experimental studies have demonstrated that gender influences both humoral and cell-mediated immune responses and SSH receptors have been identified in multiple lymphoid tissues such as the bone marrow, spleen and thymus, as well as in different immune cells including lymphocytes, mast cells, granulocytes and macrophages (155). Trauma has been shown to lead to immune dysfunction which, in turn, is associated with increased susceptibility to sepsis, MOF and mortality (156-159). The processes driving immuneparesis after trauma are complex and include the cytokine storm elicited by tissue damage, which includes concomitant release of pro- and anti-inflammatory cytokines and the suppression of a variety of cell-mediated immune responses, which we have reviewed previously (160). This immune suppression is mediated largely by the effects of cortisol released as a result of activation of the hypothalamic-pituitary-adrenal axis, but there is evidence that sex hormones represent an additional influence.

Wichmann *et al* reported significant gender differences in B-lymphocyte, T-lymphocyte and natural killer (NK) cell counts following surgery despite comparable preoperative cell counts (161), with men showing reductions in cell numbers for up to 5 days. In addition, women exhibited a more pronounced pro-inflammatory response, with elevated circulating IL-6 levels, postoperatively (161). Conversely, other studies have

observed increased levels of IL-6, TNF-α and procalcitonin in male trauma patients compared to females (162, 163). What may be pertinent is the ratio of pro- to anti-inflammatory cytokines and the chronicity of the response: a profound initial inflammatory response may favour prevention of infection, but if inflammation is not resolved promptly this can prevent wound healing and lead to organ damage.

Experimental studies in animal models of trauma have shown the modulation of immune responses by sex hormones. Overall testosterone appears to have anti-inflammatory and immune suppressive effects, promoting synthesis of anti-inflammatory cytokines such as IL-10 by murine macrophages (164), reducing NK cell activity and the synthesis of pro-inflammatory cytokines, such as TNF-α, via the inhibition of nuclear factor kappa B (NFκB) (165, 166). Testosterone has also been associated with decreased expression on macrophages and monocytes of toll-like receptor 4 (TLR4) which is involved in the activation of the innate immune system and production of inflammatory cytokines (167) by DAMPs.

Progesterone also exerts an immunosuppressive effect by inhibiting the activation of NFκB and increasing the expression of suppressor of cytokine signalling protein 1 (SOCS1)(168). In addition, progesterone reduces the activity of macrophages and NK cells, as well as the synthesis of antibodies by B cells (169-172). Elevated levels of progesterone during pregnancy have been associated with decreased development of pro-inflammatory helper T-cell type 1 (Th1) immune responses while promoting the immune responses of Th2 including the synthesis of anti-inflammatory cytokines such as IL-4, IL-5 and IL-10 (173).

In contrast, estradiol has typically been shown to enhance cell-mediated and humoral immune responses. It augments NK cell cytotoxicity, as well as stimulating the production of pro-inflammatory cytokines including IL-1β, IL-6 and TNFα (171, 174) and inhibits the synthesis of anti-inflammatory cytokines such as IL-10 (175). In addition, oestrogens have been shown to increase survival and prevent apoptosis of immune cells (176, 177). The balance of sex hormones in the circulation may thus be a key modulator of immune responses to trauma and tissue injury in humans.

Several murine studies have shown depressed immune responses in males as well as oophorectomized and aged females following trauma, haemorrhage and sepsis (178, 179). Interestingly, pre-treatment of female mice with 5-dihydrotestosterone (DHT) prior to trauma haemorrhage resulted in depressed macrophage function and reduced levels of cytokines comparable to that seen in males (180, 181). Moreover, castration and depletion of male sex hormones prior to trauma haemorrhage resulted in enhanced immune responses (182-184). In contrast, female sex hormones are associated with enhanced cell-mediated immune responses to trauma. Elevated systemic levels of estradiol in proestrus female mice played a pivotal role in post trauma and haemorrhage immunocompetence (185). Furthermore, administration of 17β-estradiol (E2) was associated with improved survival rates in animal models of sepsis (186). A single dose of estradiol following trauma-haemorrhage and resuscitation was shown to restore depressed immune responses (187).

In animal studies, the effect of SSH on splenic immune response has been evaluated with studies demonstrating that E2 played a critical role in restoring splenic

macrophage and immune functions post injury by depressing pro-inflammatory cytokine production (185, 188). Furthermore, Knoferl *et al* reported that splenocyte proliferation and the release of IL-2, IL-3 and IFN-γ were suppressed in oophorectomised females following trauma-haemorrhage to levels comparable to those observed in males(185). Moreover, castration prior to injury attenuated the depression of major histocompatibility complex (MHC) II (Ia) expression in mice, thereby improving cell-mediated immunity (189). Oestrogen enhances splenic macrophage (TNF-α and IL-6) and T-lymphocyte (IL-2 and IL-6) cytokine secretion following trauma (190-192). In addition, E2 and ER-α agonist, prevented the apoptosis of splenic dendritic cells and attenuated the depression of splenic dendritic cell cytokine production, co-stimulating factors and MHC II expression as well as antigen presentation capacities (193). These effects of E2 on splenic function appear to be predominantly mediated via ER-α (192, 193). This protective role of female sex hormones is associated with significantly increased survival rates in animal models (185).

Clinical studies investigating the effect of SSH on the immune-inflammatory cascade following trauma are more limited. Male patients of virtually all age groups have been reported to have higher incidence of sepsis following trauma and haemorrhagic shock suggesting the immune-suppressive effect of testosterone (194). In addition, Zolin *et al* reported that early elevations and increasing levels of testosterone over the initial 24 hour period after injury were associated with an exaggerated inflammatory response and significantly increased risk of nosocomial infections and MOF. Interestingly, high circulating levels of estradiol at 24 hours were associated with a four-fold greater risk

of developing MOF (195). Another study observed negative correlations between estradiol levels and TNFα on day 1 and day 2 following trauma. However no significant relationships were identified between SSH levels and IL-6, IL-8 or leukocyte counts (196). Moreover, Lopez *et al* concluded that while there is sexual dimorphism in the leukocyte genomic response following severe injury that are associated with more severe and prolonged organ failure, these differences were not in sex-linked genes or linked to differences in systemic levels of cytokines and therefore do not translate into sex-specific organ dysfunction or 28 day in hospital mortality (197).

The overall picture in relation to the impact of gender onthe immune-inflammatory response to trauma and potential impact on outcomes such as sepsis, is one of a protective immune enhancing role of oestrogens and a contrasting immune suppressive effect of androgens. However, most data are derived from animal studies with very few studies in humans and there is thus a need for clinical research and RCTs to determine benefits of SSH in maintaining immune competence after trauma.

1.4.3 Potential Clinical and Therapeutic Value of Sex Steroid Hormones

1.4.3.1 Anabolic Androgenic Steroids

Oxandrolone is an anabolic androgenic steroid (AAS) that is derived from testosterone and has a high anabolic:androgenic ratio (10:1)(198). Oxandrolone has been shown to improve prognosis of various catabolic conditions including severe burns and trauma(199). It is the only AAS approved by the FDA for weight restitution following extensive surgery and severe trauma.

To date, there has been one multicenter prospective randomized double-blind trial investigating the effects of oxandrolone in adult patients with severe burns. The authors reported significantly shorter lengths of inhospital stay in the oxandrolone group compared to placebo and this difference was strengthened when deaths were excluded and hospital stay indexed to burn size(200). A recent meta-analysis of 15 randomized controlled trials reported that oxandrolone use was associated with shorter inhospital length of stay by 3 days, donor site healing time reduced by 4.4 days, time between surgical procedures reduced by 0.7 days, as well as reduced weight loss by 5kg and nitrogen loss 8.19g/day. Moreover oxandrolone use in the rehabilitation phase was associated with reduced weight loss by 0.86kg/week and lean body mass by 5% as well as gaining 3.99% and 10.78% lean body mass following severe thermal injury by 6 and 12 months respectively(201). Interestingly, oxandrolone and propranolol (β-blocker used in burns for its anti-catabolic effects) attenuated burn-induced growth arrest in pediatric patients following thermal injury by shortening its duration by 84 days and increased growth rate by 1.7 cm per year(202). The use of oxandrolone in pediatric burn patients up to 2 years is associated with greater improvements in bone mineral content, bone mineral density and height velocity(203).

1.4.3.2 DHEA/DHEAS

DHEA, a major steroid hormone circulating in plasma, is produced in response to stress and is an intermediate that can be metabolised to both testosterone and oestrogen. It has been reported to exhibit pre-dominantly oestrogenic effects in the male androgenic milieu (204). In view of the immuno-enhancing properties of oestrogen, studies have investigated the effect of DHEA in animal models of trauma-haemorrhage

and sepsis. Angele *et al* demonstrated that administration of DHEA attenuated depression of splenic and peritoneal macrophage function post-injury and improved mortality rates from subsequent sepsis in a rodent model (205). Furthermore, DHEA, post trauma-haemorrhage, restored splenocyte functions by directly stimulating T-cell functions and preventing increases in serum corticosterone (206). Interestingly, DHEA has been shown to antagonize the immunosuppressive effects of glucocorticoids such as dexamethasone on lymphocyte proliferation (207) and the sulphated form of DHEA, DHEAS, has been shown to potentiate neutrophil function via direct activation of neutrophil nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and reactive oxygen species (ROS) generation (208).

There are no human trials of DHEA intervention in trauma and this androgenic hormone has mainly been used in trials for Addisons Disease and some chronic inflammatory conditions including Rheumatoid Arthritis. As the HPA axis is disrupted after trauma we suggest that supplementation with DHEA may offer a novel, safe and inexpensive route in improving a range of outcomes after injury.

1.4.3.3 Androgen Receptor Antagonists

Several animal studies have indicated that testosterone depletion exerts numerous beneficial effects prior to any systemic insult. Administration of flutamide following trauma-haemorrhage and resuscitation normalised depressed splenic and peritoneal macrophage cytokine release (209). Angele *et al* showed that flutamide administration for three consecutive days not only restored diminished immuno-inflammatory responses but also decreased mortality rates associated with subsequent septic

challenge (210). Lin *et al* evaluated the use of flutamide in animal models of heatstroke, reporting that flutamide attenuated hypothermia, decreased the number of apoptotic cells within the hypothalamus, spleen, liver and kidney, diminished the plasma index of toxic oxidized radicals such as nitric oxide metabolites, attenuated systemic inflammatory responses including TNF-α and IL-6 release and reduced the infiltration of neutrophils into the lungs. All of which contributed to significantly improved mortality rates (211). Furthermore, flutamide is frequently used in the clinical management of testicular cancer over prolonged periods without major adverse effects. Therefore, short-term use can be considered safe and feasible. Again, there are currently no human studies investigating administration of androgen antagonists following trauma or burn injury.

1.5 Vitamin D in Critical Illness and Trauma

1.5.1 Vitamin D and Acute Clinical Care

Vitamin D insufficiency and deficiency is common in the general population (212), and can be present at up to 76% in critically ill patients (213). This is concerning as vitamin D is increasingly recognized for its wide ranging biological effects, including modulation of bone metabolism and muscle mass, enhancing immune function and cardiovascular effects (214, 215). Despite these roles, the clinical implications of hypovitaminosis D remains partially understood and therefore often overlooked in acute clinical contexts including burns and trauma. The literature investigating vitamin D deficiency and its consequences in adult burn patients is limited. Following thermal injury, patients are at prone to develop low vitamin D levels, the impact on short and long-term outcomes of which are relatively unknown.

1.5.2 Biological Effects of Vitamin D

Classically, Vitamin D is associated with musculoskeletal health by maintaining calcium homeostasis and bone mineralization, decreasing the risk of muscle weakness, osteopenia, osteoporosis and fractures (215, 216). Vitamin D exerts most of its physiological effects via $1,25(OH)_2D$ which when bound to its cognate nuclear Vitamin D receptor (VDR) is able to act as a transcription factor in concert with its retinoid x receptor heterodimer partner (217, 218). Gene expression analysis of 53 different tissues from over 500 human donors has shown VDR gene expression in more than half of samples including adipose tissue, adrenal glands, bladder, colon, fibroblasts, kidney, liver, lung, lymphocytes, pituitary glands, skin (219, 220). Accordingly, vitamin D actions are not limited to the skeletal system. The effects of vitamin D on various cell types and tissues are summarised in Table 1.2.

Of relevance to this thesis, vitamin D has a broad range of beneficial effects on the immune system (221). An association between the adaptive immune system and vitamin D status was initially observed when VDR levels were shown to be enhanced in activated T- and B-cells (222). In VDR-expressing T-cells, $1,25(OH)_2D$ promotes a tolerogenic immune response by favoring Th2 and Treg cell differentiation over the more inflammatory Th1 and Th17 cells, thereby limiting deleterious inflammatory activity (223-227). Other immune-modulatory effects of vitamin D include differential modulation of the response of the innate immune system (monocytes, macrophages and dendritic cells)(228) with upregulation of anti-microbial peptides such as cathelicidin and β-Defensin 2 from various cells including human keratinocytes and intestinal epithelial cells (229, 230); enhancing autophagy of intracellular

microbes(231) and regulation of antigen-presentation in dendritic cells, monocytes and macrophages to facilitate a non-exaggerated immune response(232). Crucially, antigen-presenting cells from the innate immune system express the vitamin D-activating enzyme CYP27B1, and are therefore able to metabolise 25(OH)D in a tissue-specific fashion (233). This 'intracrine' mode of 25(OH)D metabolism appears to be the principal mechanism by which vitamin D is able to regulate T-cell function (234), and provides a mechanism by which vitamin D-deficiency (low serum 25(OH)D) can influence immune function. The various effects of vitamin D upon the immune response are summarised in Table 1.2.

Vitamin D Binding Protein (VDBP) and albumin are the main transporters of vitamin D. However, sterol-binding capacity is not the only attribute of VDBP and albumin. Multiple roles of VDBP have been described including actin scavenging, binding of fatty acids and endotoxins, modulation of immune and innate immune responses, as well as the influence on bone metabolism via VDBP-macrophage activating factor(235). Albumin has been reported to exert antioxidant, immune-modulatory and anti-inflammatory effects, as well as antibiotic transportation and endothelial stabilization(236, 237).

Table 1.2. Effects of Vitamin D on various human cell types and tissues

Target Cells / Tissues	Effects of Vitamin D	Reference
Adipocytes	• Inhibits intracellular fat accumulation • Enhances basal lipolysis without cell toxicity • Upregulation of β-oxidation-related genes, lipolytic enzymes and vitamin D responsive genes • Increased levels of nicotinamide adenine dinucleotide and sirtulin 1 expression	(238)
Cardiomyocytes	• Inhibition of cell proliferation without apoptosis • Downregulation of expression of genes associated with cell cycle regulation • Promotes cardiomyotube formation • Induces cardiac differentiation	(239, 240)
Hepatocytes	• Protects against insulin resistance • Downregulates fibrogenic TGF-β signaling • Anti-inflammatory effects by inhibiting monocyte activation, TNF-α and IL-1 expression	(241-243)
Myocytes	• Modulation of calcium homeostasis and influx • Induces cellular proliferation and differentiation • Protects against insulin resistance • Stimulation of arachidonic acid mobilization	(244, 245)
Nephrocytes	• Upregulation of cellular metabolic activity, IL-6 and reactive oxygen species • Restoration of transepithelial barrier function	(246)
Neurons	• Neuroactive steroid modulating spontaneous regular firing, actin potential duration and intrinsic excitability • Enhances sensitivity to neurotransmitters and neurotransmitter receptors • Upregulation of neuronal growth factors, nerutrophin 3 and glial cell line-derived neurotrophic factor	(247, 248)
T Cells	• Inhibits Th1/Th17 chemokine/cytokine secretion (CXCL-10, IFN-γ, TNF-α and IL-17) • Enhances Th2 cytokine release (IL-4 and IL-5)	(249),(226)
B Cells	• Downregulates the proliferation of memory B cells • Inhibits plasma cell differentiation • Reduces Ig production	(250)
Antigen Presenting Cells	• Inhibits the expression of class II MHC molecules (HLA-DR) • Inhibition of co-stimulating molecules expression (CD80, CD83 and CD86) • Augments chemotaxis and phagocytosis of monocytes • Downregulates the maturation of dendritic cells • Induces tolerogenic dendritic cells capable of inducing Treg cells / Inhibits IL-12 p70 release • Decreases macrophage-stimulated pro-inflammatory cytokine production (IL-1, IL-1β, IL-6, IL-8, MCP-1 and RANTES)	(251), (252),(253), (254)
Macrophages	• Attenuates M1 Macrophages pro-inflammatory response • Promotes M2 Macrophage response	(256, 257)
NK cells	• Inhibition NK cell development and differentiation • Reduced INF-γ and cytotoxicity	(255)

1.5.3 Vitamin D Status in Critically Ill and Trauma Patients

Considering the pleotropic effects of vitamin D, its role in the severely ill has been a subject of growing interest. Thousands of patients are admitted to intensive care units (ICU) each year (258), and up to 77% of critically ill patients have vitamin D deficiency (213, 259-262). Alizadeh *et al* reported that 74% of critically ill surgical patients exhibited vitamin D deficiency (263). Similarly, Dickerson *et al* reported that 76% of critically ill patients following traumatic injury were vitamin D deficient or severely deficient(213). In such contexts, it is important to recognize patient demographic factors that may be associated with vitamin D deficiency including age, ethnic group (skin pigmentation), obesity, medical history (such as malabsorption/chronic pathologies and liver/renal disease), season, latitude and time of day(215). However, it is also vital to comprehend that vitamin D-deficiency may itself be a consequence of illness.

Low serum 25(OH)D levels, has shown a significant association with the magnitude of the critical illness and SIRS (262, 264, 265). The well-documented immunomodulatory effects of $1,25(OH)_2D$ suggest that vitamin D deficiency may be a causative factor for critical illness and resulting morbidity and mortality. The observed vitamin D deficiency in critically ill and trauma maybe due to diminished epidermal vitamin D production secondary to limited sunlight exposure and malnutrition, as well as enhanced conversion of 25(OH)D to active $1,25(OH)_2D$ to meet increased tissue demand, notably to promote 1,25(OH)2D-mediated immunoregulatory effects (266). Finally, critical illness, notably in the setting of inflammation, may promote enhanced catabolism of 25(OH)D and $1,25(OH)_2D$ to downstream metabolites via the enzyme 24-

hydroxylase (CYP24A1) (267). Interpretation of circulating vitamin D levels in critical illness is further complicated by the fact that critically ill patients usually require major fluid resuscitation resulting in low levels of 25(OH)D and 1,25$(OH)_2$D secondary to acute fluid shifts and haemodilution(268). Vitamin D concentration in critically ill patients post-resuscitation may take, at least, a few days to recover(268). Secondly, VDBP and albumin levels fall, as part of the systemic inflammatory response, reducing plasma levels of 25(OH)D significantly (269-271). This appears to be the case in the acute phase of the response to injury. Furthermore, disruption of the vitamin D axis in ICU patients can be attributed to hepatic, parathyroid and renal dysfunction, as well as reduced end organ resistance (272).

Clinical studies have associated low levels of circulating vitamin D with various poor outcomes in critically ill patients including sepsis (273, 274), organ failure (275, 276), short and long term mortality (259, 273, 275, 277, 278). Similar findings have also been reported among both critically ill surgical or trauma patients. For example, low vitamin D levels correlated with higher infection rates, length of stay, duration of organ dysfunction, ICU readmission, surgical intensive care treatment costs and mortality (279-282). However such associations are not universal, other observational studies have reported no association between vitamin D deficiency, sepsis and mortality (283, 284), as well as other ICU outcomes such as duration of ventilation and length of stay (285). Despite this, several meta-analysis studies have concluded that vitamin D deficiency is associated with significantly increased susceptibility to infections and sepsis, as well as greater incidence of mortality in critically ill patients (286-288).

1.5.4 Therapeutic Value of Vitamin D in Critically Ill Patients

Vitamin D_3 supplementation maybe associated with decreased mortality in the general population (289). In addition, vitamin D status has been associated with adverse outcomes in the critically ill. Despite this, there are only a few clinical studies that have evaluated vitamin D supplementation in critically ill patients. In 2014, Amrein *et al* conducted the largest randomized controlled trial (RCT) to date investigating the influence of a high dose bolus enteral vitamin D3 supplementation on outcomes of 475 critically ill medical and surgical adult patients with vitamin D deficiency (≤20 ng/mL), the VITdAL-ICU trial (290). The authors concluded that high-dose vitamin D3 did not reduce hospital length of stay, hospital mortality or 6-month mortality (290). However, they observed lower hospital mortality following subgroup analysis of patients with severe vitamin D deficiency (≤12 ng/mL) at baseline (290). A systematic review and meta-analysis of 7 RCTs (716 patients) concluded that vitamin D administration was associated with decreased mortality in critically ill patients without serious adverse events (291). Interestingly, another recent meta-analysis of 6 RCTs (695 patients) reported no improvement on outcomes in critically ill patients supplemented with vitamin D (292). The difference between these two studies is related to inclusion and exclusion of various trials in the analysis. Other potentially important confounders in both studies are the inclusion of trials investigating cholecalciferol (vitamin D) and calcitriol ($1,25(OH)_2D$) using various dosing regimens that were administered through different routes (enteral and intravenous). Furthermore, the VITdAL-ICU trial has a larger cohort than all the other RCTs combined and therefore has major influence in the statistical analysis. These trials are summarized in Table 1.3.

Table 1.3. Summary of clinical trials investigating vitamin D supplementation in critically ill patients

Trial	Settings & Cohort Size	Intervention Regimen	Outcome	Adverse Events
Amrein 2011 (293)	Single Center ICU 25	Oral - Single dose cholecalciferol 540,000 IU	No difference in clinical outcome or mortailty	None
Amrein 2014 (290)	Single Center ICU 475	Oral - Single dose cholecaciferol 540,000 IU followed by monthly 90,000 IU for 5 months	Significant lower hospital mortality in severe vitamin D deficient patients only.	At month 6, 11% of vit D group developed total calcium levels of >10.6mg/dL
Leaf 2014 (294)	Multicenter ICU patients with severe sepsis and septic shock 67	IV - Single dose calcitriol 2µg	No influence on clinical outcome, mixed effects on inflammatory markers	None
Quraishi 2015 (295)	Single center ICU patients with severe sepsis and septic shock 30	Oral - Single dose cholecalciferol 200,000 IU or 400,000 IU	Associated with increased cathelicidin levels, no effect on CRP	None
Nair 2015 (296)	Single center ICU patients with SIRS 50	Oral - Single dose cholecalciferol 150.000 IU or 300.000 IU	Increased levels of cathelicidin and reduction in interleukin 6	None
Han 2016 (297)	Multicenter ventilated ICU patients 30	Oral - Daily doses cholecalciferol 50,000 IU or 100,000 IU for 5 days	Hospital length of stay significantly decreased	None
Alizadeh 2016 (298)	Single center Surgical ICU patients 59	IM - Single dose cholecalciferol 600,000 IU	Adiponectin significantly elevated.	Not reported
Han 2017 (299)	Multi center ventilated ICU patients 30	Oral - Daily doses cholecalciferol 50,000 IU or 100,000 IU for 5 days	Increased systemic mRNA expression of human cationic antimicrobial protein. No effect on circulating cathelicidin and human β-defensin.	None

1.6 Adipokines in Critical Illness and Trauma

1.6.1 Obesity and Adipose Tissue in Critical Illness

Obesity is a complex multifactorial condition that affects over a third of the world's population (300). With increasing prevalence of overweight and obese individuals (301, 302), obesity is being described as a global pandemic (303) as obesity greatly impacts the individual's health status and quality of life(304, 305) being a major risk factor for various pathologies including cancer, cardiovascular disease, diabetes and osteoarthritis (306).

In this context a recent and intriguing observation is that all-cause mortality is reported to be significantly lower in overweight and some obese patients (307). This phenomenon, where outcomes are paradoxically better in overweight and obese patients compared to normal weight individuals, is described as the 'obesity paradox' and is the subject of increasing interest in scientific and medical communities (308-311). The underlying mechanisms behind this phenomenon remain poorly understood and this is particularly the case in critically ill populations where the data on the obesity paradox are limited.

Adipose tissue is one of the largest organs in the human body. Importantly, it is no longer deemed an inert tissue that serves the roles of thermal/mechanical insulation protecting internal organs from external stimuli (such as cold and shock) or as an energy storage modality. Since the discovery in 1994 of leptin, an adipokine or adipose-derived hormone capable of controlling body energy balance (312), adipose tissue is now recognised as endocrine organ able to influence metabolism and

inflammatory status. As a result extensive research has been carried out investigating potential roles of adipokines in various clinical conditions including autoimmune and inflammatory disorders and connective tissue diseases (313), metabolic disorders (314, 315), cardiovascular and neurovascular diseases (316), and cancer (317, 318).

Despite increasing interest in adipose tissues' role in clinical pathologies, its role in the context of critical illness including burns and trauma remains to be fully elucidated. With thousands of critically ill patients admitted to intensive care units every year (258) some interesting observations have been made. Patients requiring prolonged critical care were reported to lose lean body mass while adipose tissue mass remained preserved or even increased (319, 320). Furthermore, although morbid obesity (BMI ≥ 40 kg/m^2) is an independent risk factor for mortality in critically ill patients (321), improved survival rates were observed among overweight (BMI 25-30 kg/m^2) and obese (BMI 30-40 kg/m^2) patients compared to normal BMI patients during critical illness (321-324). These paradoxical findings have stimulated research in to the interplay between critical illness and adipose tissue and their influence on patient outcomes. Moreover, the profound inflammatory and metabolic response to burn and trauma related critical illness suggest a potential involvement for adipose tissue and adipokines.

1.6.2 Biological Effects of Adipokines

There are approximately 600 identified hormones secreted by adipose tissue (325), providing a rich source of potential novel biomarkers and therapeutic targets for the management of various pathologies. In this thesis, the focus will be on Adiponectin, Ghrelin, Leptin, Resistin and Visfatin as the best characterised adipokines.

Adiponectin is released exclusively from white adipose tissue(326), and is the most abundant adipose-specific adipokine, with expression in subcutaneous fat being greater than visceral fat (327). Adiponectin has anti-inflammatory effects (328). Ghrelin is an orexigenic hormone that is an endogenous ligand to growth hormone and was initially thought to be produced mainly by the stomach (329), but has subsequently been identified in other tissues including adipose tissue (330). Ghrelin signaling is associated with adiposity, changes in fat distribution and mobilisation, independent of growth hormone and dietary intake(331, 332). Leptin is primarily secreted by subcutaneous white adipose tissue, the amount of leptin secreted into the circulation is proportional to adipose tissue mass and nutritional status(328). Leptin exhibits structural similarities to cytokines (333) and is pro-inflammatory (334). Resistin is also a pro-inflammatory adipokine expressed by adipocytes and other tissues including skeletal muscle (335, 336). Visfatin, also called pre-B-cell colony enhancing factor, is primarily secreted by adipocytes in visceral white adipose tissue and exhibits pro-inflammatory effects (337).

Relevant to this thesis, adipokines have been reported to influence skin and adipose tissue. Adiponectin and ghrelin have been observed to exert anti-inflammatory and

anti-fibrotic effects on skin (338-340) and were reported to enhance wound healing rates (341, 342). Similarly, leptin has been observed to enhance human epidermal keratinocyte and epithelial cell proliferation, differentiation and migration, as well as promote angiogenesis within dermal connective tissues (343). However, leptin was also found to be overexpressed in hypertrophic and keloid scars (344). This could be due to increased pro-inflammatory cytokine release associated with leptin, as seen in inflammatory skin conditions (345). Visfatin has been reported to enhance chemokine and antimicrobial peptide production in human keratinocytes (346, 347), as well as exhibit anti-fibrotic properties(348).

Adiponectin and leptin have been reported to induce browning of adipose tissue (349, 350) and adiponectin promotes adipogenesis as well as increasing lipid accumulation and insulin responsiveness of adipocytes (351). In contrast, leptin inhibits insulin-dependent glucose uptake and lipogenesis and reverses insulin-induced lipolysis (352). Ghrelin stimulates adipogenesis and glucose uptake, as well as inhibiting lipolysis, apoptosis and autophagy of adipocytes (353, 354). Resistin and visfatin enhance pro-inflammatory cytokine expression in adipose tissue including TNF-α and IL-6 (355, 356). Similarly, resistin and visfatin induce insulin resistance in adipocytes (356, 357). The influence of these adipokines is not limited to skin and adipose tissue. The beneficial and detrimental effects of these adipokines on various cell types and tissues are summarized in Figure 1.7. and in recent detailed reviews (316, 358-365).

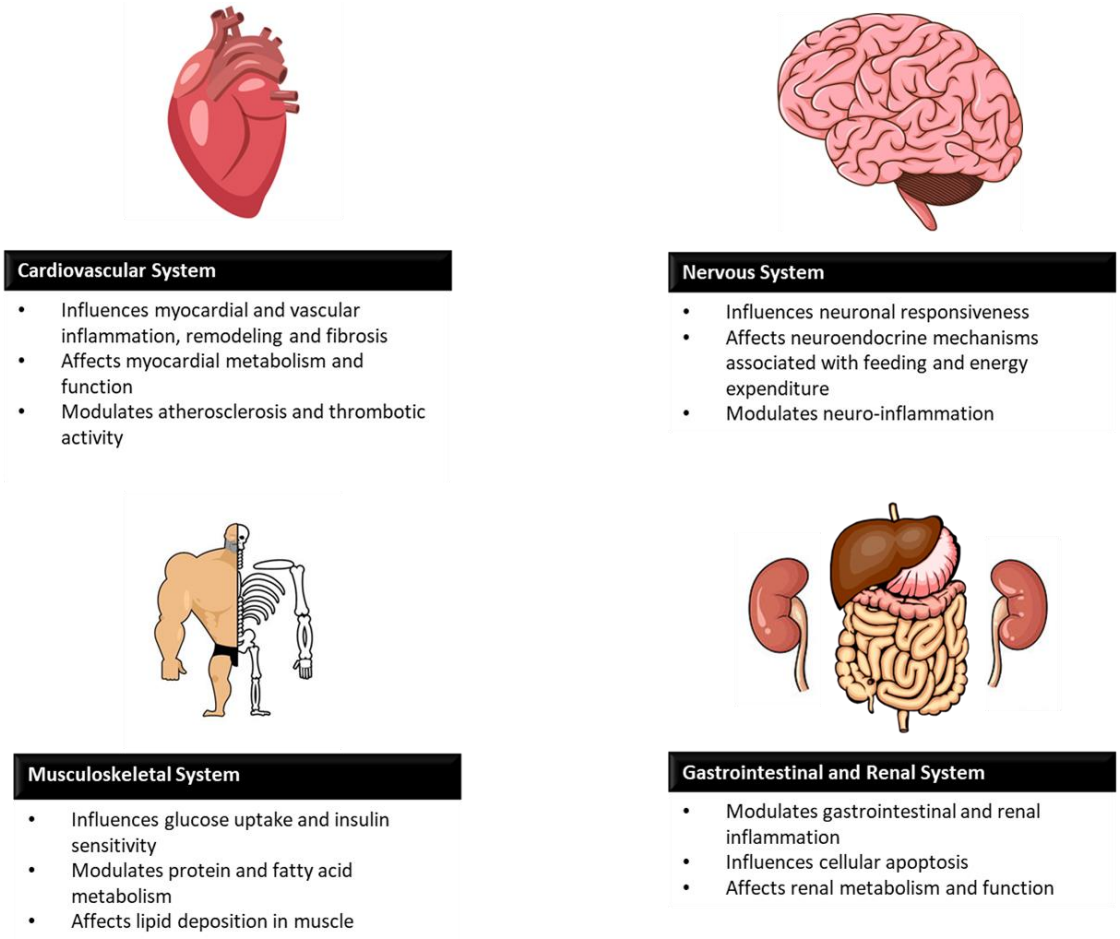


Figure 1.7. Effects of adipokines on various tissues and organs

1.6.3 Adipokines in Critical Illness

Several studies have demonstrated acute reductions in circulating adiponectin levels in critical illness (131, 366-371). In addition, an inverse association was reported between serum adiponectin levels and severity of illness as measured by C-Reactive Protein (CRP), Simplified Acute Physiology Score (SAPS) II and Sequential Organ Failure Assessment (SOFA) scores (367, 368, 371). Similar findings were observed in patients with acute pancreatitis, where adiponectin levels in the blood were negatively associated with severity of disease and incidence of tissue necrosis (369). Furthermore,

adiponectin levels progressively increase with patient recovery (367, 370). Although the above findings indicate that decreased serum adiponectin levels may lead to poor outcomes, other research has reported different findings. Circulating adiponectin levels in severely ill patients did not correlate with inflammatory markers including IL -6, IL-10 and TNF-α (131, 372, 373) and clinical scores including Acute Physiologic Assessment and Chronic Health Evaluation (APACHE) II score and SOFA (372, 374, 375). Furthermore, higher blood adiponectin levels were associated with increased risk of mortality during critical illness(376-379).

Only two studies have investigated circulating ghrelin levels in critical illness. Wade *et al* reported significantly reduced ghrelin levels in severely burned patients correlating with metabolic/caloric needs. No other associations with other parameters such as injury severity and inflammatory status were observed (131). Santacruz *et al* also observed significantly reduced plasma ghrelin levels in critically ill patients but saw no correlations with feeding status (380).

Leptin levels in the blood have been reported to be elevated in critical illness (381-384). Furthermore, leptin was positively associated with pro-inflammatory status of severely ill patients, as measured by CRP, IL-6, soluble tumour necrosis factor receptor-1 and TNF-α (382, 384-387). Additionally, other studies have reported that serum-soluble leptin receptor (SLR) in patients correlated with inflammatory response and illness severity as measured by IL-6, lactate, procalcitonin and APACHE II score (388, 389). Interestingly, elevated levels of leptin were observed in survivors of acute sepsis (381), while increased soluble leptin receptor (SLR) levels in critically ill patients were

associated with increased mortality (388). However, other studies have reported different findings. Blood leptin levels in severely ill patients were similar or reduced compared to healthy volunteers (131, 370, 371, 385, 388, 390) and no associations were found between circulating leptin levels and inflammatory status, illness severity, or mortality in critical illness (370, 371, 383, 385, 387, 388, 390). These contradictory reports could be owing the complex condition mediated properties of leptin, as well as difference in patient demographic, pathologies of critical illness and management given.

In contrast to the heterogeneity of results reported on the impact of adiponectin, ghrelin and leptin on critical illness outcomes, the influence of resistin and visfatin on outcomes of severely ill patients is consistent in the literature. Critically ill patients exhibit significantly elevated circulating levels of resistin (131, 371, 374, 375, 387, 391-396) and visfatin (387, 397-403). Additionally, both resistin and visfatin significantly correlated with pro-inflammatory responses (including CRP, IL-6, IL-8 and TNF-α), and worse clinical severity scores (including APACHE II, Glasgow Coma score, multiple organ dysfunction score, SAPS II and SOFA)(131, 371, 374, 375, 387, 391-395, 397-403). Furthermore, high resistin and visfatin levels in blood were associated with poor outcomes including mortality (392, 393, 398-402).

A systematic review examining the evidence for adipokines having an influence on critical care patients has been published recently (404). It concludes that although strong observations were reported indicating the influence of adipokines on the prognosis of critical illness, additional larger studies that incorporate more diverse

cohorts (such as age, gender, BMI, ethnic groups and different pathologies) is required to better understand the relationship between adipokines and critical illness. This is essential in order to validate the potential clinical value and utility of adipokines as diagnostic and/or prognostic biomarkers, as well their potential as therapeutic targets in critical illness including burn and trauma. Furthermore, studies to date have investigated the association of adipokines with critical illness in the acute setting only. This focus on the acute setting has further limited the translation of adipokines in clinical settings. Importantly, since medical care advancements have improved survival rates after critical trauma (405-407), greater emphasis is now placed on the prevention and treatment of potentially debilitating long-term sequelae experienced by survivors of severe illness including chronic critical illness (408-410), prolonged pathophysiological responses(94) and scarring (411).

1.7 Conclusions

The endocrine response following critical illness have demonstrated promising clinical value. Data investigating the HPA, HPG, vitamin D and adipokine status in critically ill populations are limited. This is especially true in burns patients where published studies investigating the endocrine response and influence on outcomes following severe thermal injury are scarce. Exploring the endocrine response following in severely burned patients may lead to the identification of novel biomarkers and treatments. Ultimately, this can significantly improve the short-term and long-term prognosis of burns patients.

1.8 Thesis Hypothesis

We hypothesize that major burn injury results in significant endocrine disturbances affecting HPA, HPG, vitamin D and adipokine responses and that the status of these hormones post-injury is associated with outcomes including sepsis, MOF, mortality, wound healing and scarring. We hypothesize that supplementation of adiponectin, DHEA, testosterone and vitamin D may improve outcomes.

1.9 Thesis Aims

The aims of this thesis were to:

- Determine the status of the adrenal and gonadal axes, vitamin D axis and adipokine levels in severely burned patients;
- Characterize these endocrine responses longitudinally from day 1 following severe thermal injury till month 12 post-injury;
- Explore associations between the longitudinal endocrine response and clinical outcomes in burned patients including mortality, MOF, sepsis, wound healing and scarring;
- Identify any potential clinical or systemic biomarker that may improve prognosis of burned patients or identify novel therapeutic targets via statistical modeling;
- Investigate the effects of pharmacological agents, corticosteroids and oxandrolone, on the endocrine response and outcomes following severe thermal injury.

CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1 Scientific Investigation of Biological Pathways following Thermal Injury Study (SIFTI)

The Scientific Investigation of biological pathways Following Thermal Injury in Adults and Children (SIFTI) is a UK multi-centre prospective observational cohort study (UKCRN ID: 13654 / IRAS ID: 2003666). The aim of the study is to characterise and profile the immune, inflammatory, coagulation, endocrine and metabolic response in burned adult, elderly and paediatric patients. Participating burn centres include the Queen Elizabeth Hospital Birmingham (QEHB), Birmingham Children's Hospital, Saint Andrews Hospital, Chelmsford, UK and Queen Victoria Hospital, East Grinstead, UK. This is the first observational study of 150 patients following thermal injury involving multiple burn centres to be conducted in Europe. The reports in this thesis investigates only a subset of patients with ≥20% TBSA recruited in this study. This sub-study is called endocrine-SIFTI (e-SIFTI).

Ethical Approval for the SIFTI study was granted by the National Research Ethics Service Committee East Midlands, UK (Reference 12/EM/0432).

2.1.1 Study Cohort

Fifty-two burn patients admitted in the QEHB burn centre from January 2013 till October 2015 were recruited into the study. The inclusion and exclusion criteria of the e-SIFTI study are summarised in Table 2.1. Eligible patients were recruited into the study following written informed consent. When patients initially lacked capacity to consent secondary to injury severity, enrolment into the study was achieved through a

legal consultee, either personal or nominated, until they regained capacity to consent themselves.

Inclusion Criteria	Exclusion Criteria
Arrival to burn centre within 24 hours of injury	Deep electrical or chemical burn injury
Adult/Elderly (16-99 years) burn patients (≥20% TBSA)	Associated multiple injuries with injury severity score (ISS) >25
Adult/Elderly patients with full thickness burns (>1% TBSA)	Decision not to treat
	Congestive cardiac failure (ejection fraction <20%)
	Active malignancy
	History of prolonged glucocorticoid therapy
	Multiple limb amputations (>1)

Table 2.1. Inclusion and exclusion criteria for e-SIFTI study.

All recruited burn patients were managed according standardised QEHB burn care guidelines on review. This includes fluid resuscitation using intravenous crystalloid infusion, Hartmann's solution, as guided by Parkland's formula (4ml/kg/%TBSA burn) for the first 24 hours, invasive monitoring of arterial blood pressure and central venous

pressure. Resuscitation endpoints include urine output of 0.3-0.5mLs/kg/hr and mean arterial pressure of >60mmHg. In the presence of persistent hypotension and/or oliguria despite adequate intravenous crystalloid infusion, 5% human albumin solution was given as boluses. Further cardiovascular support is achieved through pharmacological means involving inotropes, such as noradrenaline, when required. Other organ support measures used when clinically needed include mechanical ventilation and haemodialysis. Early enteral nutrition was started in all patients according to a standard formula, as well as glutamine and trace elements supplementations through enteral and intravenous means respectively. Other pharmacological interventions include the use of oxandrolone by burn surgeons and intensivists for its anabolic effects (200, 412), as well as systemic steroids by intensivists for shock control as per surviving sepsis campaign guidelines (413, 414).

All burn wounds were debrided as per local guidelines with 24 hours of admission. Patients requiring burn excision underwent surgery within 72 hours of admission. Coverage of resulting wounds was decided by burns consultant performing the surgical procedure and typically involves the use of split thickness autografts, cadaveric allografts, porcine xenografts and skin substitutes either alone or in-combination. Patients with full thickness burns who were medically unfit for surgical intervention were initially treated with daily application of topical silver sulfadiazine/cerium nitrate. This is to achieve a dry adherent eschar till the patient is optimised for excisional surgery.

Ten healthy volunteers were recruited as the control cohort to allow comparison. At the time of enrolment, all control participants were in good health and without significant co-morbidity. In addition, all participants were not on any medication known to influence their immune or endocrine status and had not had an acute infection in the preceding 2 weeks.

2.1.2 Blood Sampling

Blood samples of burned patients were acquired at specific timepoints; day 1 (within 24h post-injury), day 3 (±1 day), day 7 (±1 days), day 14 (±3 days), day 21 (±3 days), month 2 (±3 days), month 3 (±7 days), month 6 (±7 days) and month 12 (±7 days) as illustrated in Figure 2.1. Blood samples were collected into BD vacutainers® (Becton Dickinson, Oxford, UK) containing 1/10 volume of 3.2% trisodium citrate, EDTA, lithium heparin or z-serum clotting activator. Patients who died or were lost to follow-up were included in the analysis.

Figure 2.1. Timeline of blood sampling in e-SIFTI study.

Abbreviations: D (Day), M (Month).

One-off blood samples were collected from healthy study participants. Samples from healthy volunteers and burns patients (excluding day 1) were taken in the mornings between 0800 – 1000 hours.

2.1.3 Preparation of Serum

Blood samples collected in BD vacutainers® containing z-serum clotting activator were allowed to clot for 30 minutes at room temperature prior to processing. The samples were then centrifuged at 1500xg for 10 mins at room temperature. The top two-thirds of the serum was carefully removed and stored at -80°C in aliquots prior to analysis.

2.1.4 Measurement of Immune Function, Cytokines and Hormones

Quantification of immune function was performed by Dr. Peter Hampson, Dr. Jon Hazeldine and Dr. Robert Dinsdale. Immune functions assessed in this study include neutrophil extracellular trap generation (NETs), phagocytosis and production of reactive oxygen species (ROS) by neutrophils and monocytes. Quantification of immune function in healthy volunteers and burned patients was performed using commercially available kits for ROS generation in response to *E.coli* (Phagoburst, BD Pharmingen, UK) and phagocytosis of FITC-conjugated *E.coli* (Phagotest, BD Pharmingen, UK) with quantification by flow cytometry. This methodology has been published in detail previously (112).

Methodologies used to quantify steroid hormones, vitamin D and adipokines are described in chapters 3, 4 and 5 respectively. Quantification of cytokines and other hormones was performed Dr. Jon Hazeldine, Dr. Robert Dinsdale and Khaled Altarrah. This panel included: IL-1Ra, GCSF, IL-6, IL-8, IL-10, IL-12p70, IL-17, MCP-1, TNF-α, IGF-1,

IL-1β and TGF-β1. These cytokines in healthy volunteers and burned patients were measured using commercially available kits (Bio-Rad, Hertfordshire, UK and RnD Systems™, Oxfordshire, UK) using enzyme-linked immunosorbent assay ELISA or multiplex technology. Processing and quantifications of cytokines and other analytes were done as per manufacturer's instructions.

2.1.5 Data Collection

Data including patient demographics, burn injury characteristics, daily physiological status and clinical outcomes were prospectively recorded using case-report forms (CRF). Recorded outcomes used in this thesis include sepsis, MOF, wound healing, 28-day mortality and in-hospital mortality.

2.1.6 Definitions of Clinical Outcomes

Sepsis in burn patients was defined using the American Burn Association criteria agreed in 2007(415). Sepsis was diagnosed when at least 3 trigger criteria were met along with positive microbiological culture or when clinical response to antimicrobials was observed. The trigger criteria include: Temperature >39°C or <36.5°C, progressive tachycardia >110 beats per minute, progressive tachypnoea >25 breaths per minute or minute ventilation >12 L/min, presence of thrombocytopenia (platelet count <100,000/mcl), hyperglycaemia (Untreated plasma glucose >200 mg/dl or intravenous insulin >7 units/hr IV or significant resistance to insulin - >25% increase in insulin requirements over 24 h) and poor tolerance to enteral feedings >24 h (abdominal distension, enteral feeding intolerance [2x feeding rate], uncontrollable diarrhoea >2500 ml/day) (112).

MOF was defined using the Denver2 score as published in 1996 (416). A diagnosis of MOF following injury was made when a score >3 was established on two consecutive days involving at least two organ systems. This scoring system is summarised in Table 2.2. Denver2 scoring has demonstrated better specificity for outcomes associated with post-traumatic MOF compared to other scoring systems (417-419). This method has been validated for use following trauma (420), and its use in burn patients has been published (112, 421).

Organ dysfunction was quantified using components of the SOFA scoring system as described (422, 423). SOFA score evaluates the extent and rate of failure of 6 different organs and systems. These include respiratory, cardiovascular, hepatic, coagulation, renal and neurological systems. The SOFA scoring system is summarised in Table 2.3. Global SOFA scores have demonstrated reduced specificity, compared to Denver2 scores, in predicting outcomes following trauma (417, 419). Despite this, its individual components were reliable in describing organ dysfunction and predicting outcomes in critically ill/trauma patients (424-426).

Wound healing is the total duration of time taken in days for wounds to be considered 95% healed as per daily clinical review by a consultant burn surgeon. Mortality is analysed as two entities and is defined as non-survivors following thermal injury within 28 days of injury or during hospital admission accordingly. Patient characteristics and study process are summarised in Supplementary Table 1 and Supplementary Figure 1 respectively.

Organ Dysfunction Score	Respiratory Dysfunction (ARDS Score)	Renal Dysfunction (Creatinine - mg/dL)	Liver Dysfunction (Bilirubin – mg/dL)	Cardiac Dysfunction (Inotrope Use)
1	>5	>1.8	>2	Minimal*
2	>9	>2.5	>4	Moderate**
3	>13	>5	>8	High***

Table 2.2. Components of Denver2 Scoring System for MOF

Global score of all components of Denver2 is used to diagnose MOF (dysfunction should not related to chronic disease). ARDS score is a global score based on chest x-ray findings, PaO2:FiO2 ratio, minute ventilation, positive end-expiratory pressure and static compliance(416). Liver dysfunction should not be secondary to biliary obstruction or resolving hematoma. Cardiac dysfunction is defined as cardiac index <3L/min/M^2 requiring dopamine or dobutamine support. * <5µg/kg/min, ** 5-15 µg/kg/min, ***>15 µg/kg/min.

System Dysfunction	0	1	2	3	4
PaO2:FiO2 ratio (mmHg)	≥400	<400	<300	<200 with mechanical ventilation	<100 with mechanical ventilation
Glasgow Coma Scale	15	13-14	10-12	6-9	<6
Vascular Pressure/Support	MAP ≥70mmHg	MAP <70mmHg	Minimal Inotropes*	Moderate Inotropes**	High inotropes***
Bilirubin (mg/dL)	<1.2	1.2-1.9	2.0-5.9	6.0-11.9	>12.0
Platelets (x10^3/µL)	≥150	<150	<100	<50	<20
Creatinine (mg/dL) or Urine output (mL/24hrs)	<1.2	1.2-1.9	2.0-3.4	3.5-4.9 or <500	>5.0 or <200

Table 2.3. Components of SOFA scoring system for organ dysfunction

SOFA scores each organ and body systems. This is done on daily basis using the worst physiological values over 24 hours. Higher scores indicate poorer prognosis. * dopamine ≤ 5 µg/kg/min or dobutamine, ** dopamine > 5 µg/kg/min OR epinephrine ≤ 0.1 µg/kg/min OR norepinephrine ≤ 0.1 µg/kg/min, *** dopamine > 15 µg/kg/min OR epinephrine > 0.1 µg/kg/min OR norepinephrine > 0.1 µg/kg/min.

2.2 Birmingham Objective Scar Scale Study

The Birmingham Objective Scare Scale (BOSS) is a UK single centre prospective, non-blinded single-arm observational study (IRAS ID: 181543). The study utilises 3 independent assessors and aims to measure the intra and inter-rater reliability of different objective scar measurement tools in terms of reproducibility. Furthermore, this study aimed to create a global objective scar scale to be used in burn patients by combining the scores of various scar measurement tools used. This study was conducted in the Wellcome Trust Clinical Research Facility at QEHB.

Ethical Approval for the BOSS study was granted by the South Birmingham National Research Ethics Service Committee West Midlands, UK (Reference 15/WM/0378).

Details regarding recruitment, procedures and ethics were provided by Dr. Kwang Chear Lee as he was an investigator in this study and was a member of the assessment team that evaluated the scars (K C Lee, PhD thesis, University of Birmingham 2019). Only scar measurements performed in BOSS were analysed in this thesis.

2.2.1 Study Cohort

A total of 23 e-SIFTI participants were invited to undergo scar assessments. A summary of inclusion and exclusion criteria is listed in Table 2.4. Eligible participants were recruited into this study following written informed consent. Eleven e-SIFTI participants were enrolled and had their scars assessed at an average of 18 months following the date of 95% wound healing.

Inclusion Criteria	Exclusion Criteria
Age >18 years	History of pathological skin conditions
Presence of hypertrophic scar	Chronic steroid use
Scar age ≥6 months (calculated from date of 95% wound healed)	Presence of scars only in facial and/or perineal regions
History of skin grafting during burn treatment	
Burn wound with documented delayed healing (>2 weeks)	
Scar size ≥10cm^2	

Table 2.4. Inclusion and exclusion criteria for scarring measurements in e-SIFTI cohort.

2.2.2 Scar Assessment

A snapshot scar assessment was done to each participant. A single site with the worse scarring as regarded by both the burn patient and assessors was chosen as the area for investigation. Furthermore, one site of normal skin with similar anatomical qualities (contralateral or adjacent anatomical site) was also chosen as controls to allow for comparison and analysis of objective scar measures (Figure 2.2). Within the chosen sites, a 3x3 cm area was demarcated. A 1cm circle within this region in scar and normal skin sites was then selected and marked using a stencil and marker in each participant for evaluation using a variety of scar measures. Evaluation of chosen sites was

conducted during the same day by three appropriately trained individuals of different clinical backgrounds including a clinician, nurse and therapist. Assessment of these chosen sites involved the use of subjective and objective scar measurement tools.

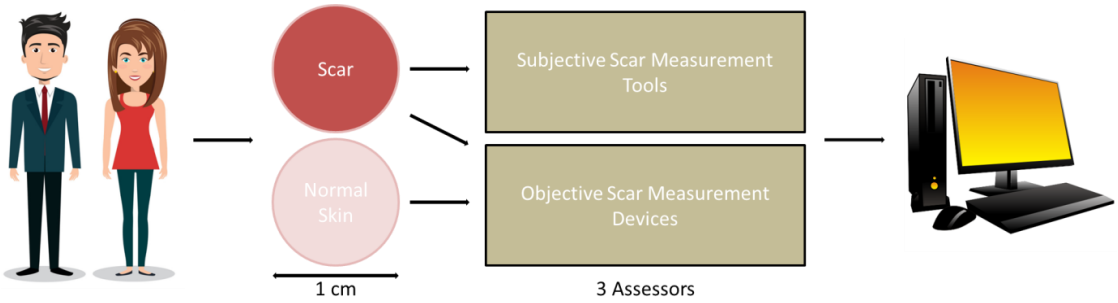


Figure 2.2. Overview of scar assessment pathway.

Burn patients whose scars were actively treated with pressure garments or topical medications, such as silicone gels or moisturisers, were asked to remove them prior to their appointments by at least 20 minutes. Both scar and normal sites were examined in the same temperature-controlled room (22±1°C) with the patient lying in the same position for each consecutive assessment. Furthermore, the temperature and humidity of the room was measured and monitored throughout the process.

2.2.3 Subjective Scar Measurement Tools

Two published subjective scar measurement tools were used in this study to assess the scar site, the modified Vancouver scar scale (mVSS) (427) and the patient and observer scar assessment scale (POSAS) (428). The mVSS utilises a numerical score of four scar features including height, pliability, vascularity and pigmentation as shown in Table

2.5. The assessors assign a numerical score for each of these characteristics upon evaluation of the chosen scar site.

Score	Pliability	Height	Vascularity	Pigmentation
0	Normal	Flat	Normal	Normal
1	Supple	<2mm	Pink	Hypopigmentation
2	Yielding	2-5mm	Red	Mixed
3	Firm	>5mm	Purple	Hyperpigmentation
4	Ropes			
5	Contracture/Adherent			

Table 2.5. Parameters of mVSS.

The POSAS tool comprises of two subjective scar scales as evaluated by both the patient and assessor. Both scales consist of 6 scar characteristics that are scored numerically on a scale from 1-10 as compared to normal skin. The patient segment of the POSAS examines the following scar features; pain, itch, colour, stiffness, thickness and irregularity. While the observer portion of the POSAS assesses scar vascularity, pigmentation, thickness, relief, pliability and surface area. Furthermore, an overall score in each scale is given to the scar site as judged by the patient (OPSS) and assessor/observer (OOSS) respectively.

POSAS Patient scale

The Patient and Observer Scar Assessment Scale v2.0 / EN

Date of examination:
Name of patient:
Observer:
Location:
Date of birth:
Research / study:
Identification number:

	1 = no, not at all									10 = yes, very much = so
HAS THE SCAR BEEN PAINFUL THE PAST FEW WEEKS?	○	○	○	○	○	○	○	○	○	○
HAS THE SCAR BEEN ITCHING THE PAST FEW WEEKS?	○	○	○	○	○	○	○	○	○	○
	1 = no, as normal skin									10 = yes, very different = so
IS THE SCAR COLOR DIFFERENT FROM THE COLOR OF YOUR NORMAL SKIN AT PRESENT?	○	○	○	○	○	○	○	○	○	○
IS THE STIFFNESS OF THE SCAR DIFFERENT FROM YOUR NORMAL SKIN AT PRESENT?	○	○	○	○	○	○	○	○	○	○
IS THE THICKNESS OF THE SCAR DIFFERENT FROM YOUR NORMAL SKIN AT PRESENT?	○	○	○	○	○	○	○	○	○	○
IS THE SCAR MORE IRREGULAR THAN YOUR NORMAL SKIN AT PRESENT?	○	○	○	○	○	○	○	○	○	○
	1 = as normal skin									10 = very different = so
WHAT IS YOUR OVERALL OPINION OF THE SCAR COMPARED TO NORMAL SKIN?	○	○	○	○	○	○	○	○	○	○

POSAS Observer scale

The Patient and Observer Scar Assessment Scale v2.0 / EN

Date of examination:
Name of patient:
Observer:
Location:
Date of birth:
Research / study:
Identification number:

PARAMETER	1 = normal skin	2	3	4	5	6	7	8	9	10 = worst scar imaginable = so	CATEGORY
VASCULARITY	○	○	○	○	○	○	○	○	○	○	PALE | PINK | RED | PURPLE | MIX
PIGMENTATION	○	○	○	○	○	○	○	○	○	○	HYPO | HYPER | MIX
THICKNESS	○	○	○	○	○	○	○	○	○	○	THICKER | THINNER
RELIEF	○	○	○	○	○	○	○	○	○	○	MORE | LESS | MIX
PLIABILITY	○	○	○	○	○	○	○	○	○	○	SUPPLE | STIFF | MIX
SURFACE AREA	○	○	○	○	○	○	○	○	○	○	EXPANSION | CONTRACTION | MIX
OVERALL OPINION	○	○	○	○	○	○	○	○	○	○	

Explanation

The observer scale of the POSAS consists of six items (vascularity, pigmentation, thickness, relief, pliability, surface area). All items are scored on a scale ranging from 1 (like normal skin) to 10 (worst scar imaginable).
The sum of the six items results in a total score of the POSAS observer scale. Categories boxes are added for each item. Furthermore, an overall opinion is scored on a scale ranging from 1 to 10.
All items are scored by comparing the scar (as compared to normal skin on a comparable anatomic location.

Explanatory notes on the items:

- **VASCULARITY** Presence of vessels in scar tissue assessed by the amount of redness. Scored by the amount of blood retain after blanching with a piece of Plexiglas.
- **PIGMENTATION** Blownish colouration of the scar by pigment (melanin); apply Plexiglas to the skin with moderate pressure to eliminate the effect of vascularity.
- **THICKNESS** Average distance between the subcutical-dermal border and the epidermal surface of the scar.
- **RELIEF** The extent to which surface irregularities are present (preferably compared with adjacent normal skin).
- **PLIABILITY** Suppleness of the scar tested by wrinkling the scar between the thumb and index finger.
- **SURFACE AREA** Surface area of the scar in relation to the original wound area

Figure 2.3. Parameters of POSAS patient and observer scales.

Figure taken from www.posas.org**(391)**.

2.2.4 Objective Scar Measurement Devices

The different features of scars were objectively measured by an appropriate corresponding device. Measurement of scar and normal skin thickness was done using Dermascan® C USB (Cortex Technology ApS, Denmark). Scar and normal skin pliability were assessed using Cutometer® MPA 580 (Courage and Khazaka GmbH, Germany). Scar and normal skin pigmentation were examined using DSM II Colorimeter® (Cortex Technology ApS, Denmark). The use of such devices in the assessment of burn scars has been published in a recent systematic review by Lee KC *et al* (429).

The Dermascan® C USB is a high frequency (20MHz) ultrasound scanner that comes with a dedicated software (Advance Control 6 Analysis SW Package, Cortex) and provides high resolution images of soft tissue with automated skin thickness measurement. A thin layer of conducting ultrasound gel was applied to the probe which was held perpendicular to the examined site. This provides and records an echographic image of the assessed site. All measurements were carried out with the ultrasound frequency set at 1580m/s, using a medium focus transducer with a 12mm wide viewing field and 15mm depth penetration. All scans were set at Mode 4 and a gain profile of 13. All images were analysed in B-Mode. This mode provides a two-dimensional pixelated image of varying intensities representing the amplitude of reflected signal and its use in burn scar assessment has been published (430). Normal skin appears highly echogenic as it is composed of dense connective tissue and collagen, while scar tissue is hypoechoic due to increased water content possibly secondary to aberrant proteoglycan metabolism (431). The recorded thickness of scar

and normal skin is the distance (mm) measured between the stratum corneum and the inner surface of the dermis.

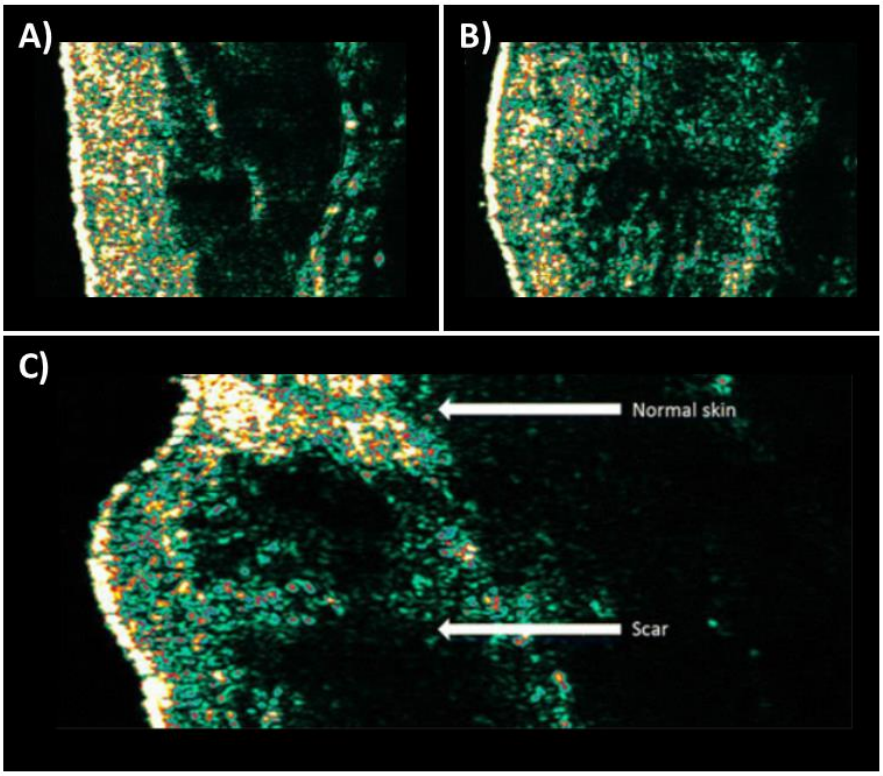


Figure 2.4. Dermascan® C USB images of normal skin and scar tissue.

A) Ultrasound image of normal skin. B) Ultrasound image of scar tissue. C) Ultrasound image of normal skin and adjacent scar tissue. Figure taken from "A systematic review of objective burn scar measurements" by Lee KC et al **(429)**.

The Cutometer® MPA 580 is an instrument that assesses skin elasticity by measuring the amount of skin displacement within the probe's hollow aperture via an optical system. This is achieved by placing the probe over the examined area and creating an air-tight seal. Negative pressure is then generated pulling the skin or scar into the aperture. All measurements were carried out using mode 1 sequence of "on/off"

pressure cycle. This involves the delivery of 3 cycles of negative pressure (500mbar) for 2 seconds followed by no pressure for a further 2 seconds. In addition, a 6mm diameter hollow aperture probe was used for all assessments in this study as this probe size was reported to be most efficient in measuring the dermis' viscoelastic properties (427, 432). This method of scar evaluation in burn patients has been published (427). Reported parameters in this study are R0 and R2. R0 is defined as the maximum deformation (extension) of skin or scar. R2 is defined as the final retraction to maximum deformation ratio of examined area.

The DSM II Colorimeter® is a handheld device that examines skin and scar's level of erythema and pigmentation. The device utilises 2 colour technologies, narrow-band spectrophotometry and tristimulus reflectance colorimetry, in a single measurement. This instrument consists of a probe with a transparent dome housing 2 white LED lights and colour sensor. All measurements were taken by gently placing the probe perpendicular to the examined site, to avoid blanching of skin or scar, to ensure accuracy of readings. The use of this device in the assessment of burn scars has been published (433). The following parameters were then recorded: Erythema, Melanin (pigmentation), L* (paleness/lightness/brightness), a* (redness/erythema) and b* (blueness/pigmentation).

2.2.5 Data Collection

Patient demographics, location normal skin and scar sites, subjective and objective scar measures and patient satisfaction were documented in CRFs. Recorded subjective

and objective scar measures of participants previously enrolled in the SIFTI study only are used in this thesis.

2.3 Statistical Analysis

As the number of patients with severe burn injury (≥20% TBSA burn) recruited was small, the timepoints investigated were combined to reflect post-injury stages clinically and statistically. This process is illustrated in Supplementary Figure 2. The clustered timepoints were allocated to the following groups: D01, D03-D07, D14-D28 and M02-M12. This potentially increases the generalisability and eases clinical interpretation of the data, as well as minimises attrition effects over time. Furthermore, grouping of timepoints allows more robust modelling and analysis due to increased numbers in groups (434).

All statistical analyses were done using Prism® version 7 (GraphPad Software Inc., California, USA) and IBM SPSS® Statistics version 25 (IBM Corp, New York, USA). All data were checked for normality using the Shapiro-Wilk test. Normally distributed data are reported as mean and standard deviation (SD), while non-normal data are presented as median and inter-quartile range (IQR). When comparing with healthy controls, one-way ANOVA followed by Bonferroni's post hoc test was performed when data were normally distributed. When the data distribution was non-normal, Kruskal-Wallis test followed by Dunn's multiple comparison test was performed. Continuous variables were compared using independent t or Mann-Whitney tests depending on normality of data. Categorical variables were assessed using Chi-Square test. Spearman's rank or Pearson's correlation co-efficient was used to determine

correlations between continuous variables such as analyte levels and clinical outcome dependant on data normality. Linear and binary logistic regression were utilised to allow multivariate analysis depending on outcome (categorical or continuous). Multivariate regression analysis was performed to account for variables that may influence outcomes and estimate treatment effects. Both uni-analyte and multi-analyte regression models were performed. Multi-variate cox regression analysis was performed to assess survival or hazard distributions of outcomes and estimate treatment effects in burns patients accounting for confounding variables. Area under the Curve (AUC)/Receiver Operating Characteristics (ROC) curve analysis was performed to assess predictive strength of studied biomarkers and/or statistical regression models found significant in categorical clinical outcomes including sepsis, MOF and mortality.

Heatmap data visualization and qualitative analysis was performed by Dr. Animesh Acharjee. The mean of each feature (including cytokines, immune function, injury severity and hormones) across different timepoints is shown in figures. Each feature was standardised by subtracting the mean of each feature and dividing by their respective standard deviation. This was done to allow comparison of all features included in this qualitative analysis. The distance between each feature was measured using a hierarchical clustering method. Data visualisation was performed using ggplot2 library in R version 3.6.0 (The R Foundation for Statistical Computing, Vienna, Austria).

In instances where burn patients received treatments of interest such as hydrocortisone, a propensity score matching (PSM) analysis was performed using

above software packages and extension bundle Propensity Sore Matching version 3.0.4 for IBM SPSS® Statistics version 25 (IBM Corp, New York, USA), Python version 3.4 for IBM SPSS® Statistics version 25 (IBM Corp, New York, USA) and R version 3.3.1 (The R Foundation for Statistical Computing, Vienna, Austria). Propensity scores were calculated via logistic regression analysis of timepoints and variables of interest including patient demographics, injury characteristics and haemodynamic status. This was done to balance the differences in co-variates between the treated and non-treated groups. The matching ratio was set at 1:1 using nearest neighbour matching algorithm with replacement. The maximum caliper distance was at 0.2. As the model allows replacement, multiple controls may match with cases if the propensity scores were within the caliper distance. This matching method was used to minimise bias (435-437). Analysis following PSM, was performed as described earlier.

Data are represented as bar charts, box-and-whisker plots or line graphs unless otherwise stated. Box and whisker plots utilised the Tukey method with the whiskers calculated as 1.5 times the inter-quartile range added or subtracted from 75^{th} or 25^{th} percentile respectively. Any value outside this calculated range is designated as an outlier and charted accordingly. Bar charts and line graphs are plotted as mean values with error bars representing the standard error of the mean. Statistical significance is set at $p < 0.05$ unless otherwise stated.

CHAPTER 3: STEROID STATUS AND ITS INFLUENCE ON OUTCOMES FOLLOWING SEVERE BURN INJURY

3.1 Introduction

The HPA and HPG axes are a cascade of processes resulting in steroidogenesis from cholesterol. The processes and pathways involved in steroidogenesis are outlined in Figure 3.1. Burn injury and other forms of physical trauma have been reported to affect HPA and HPG axes (129, 438). Despite this, clinical studies investigating the outcomes associated with disturbances in steroidogenic processes following trauma and thermal injury remain limited. The current understanding of the influence of steroid hormones on outcomes following injury and potential mechanisms behind such observations was previously summarised in chapter 1 and in a recent review(133).

There is growing evidence that steroids, including sex steroid hormones, are major determinants of prognosis following burn injury. An 11-year review of data in the UK Greater Manchester region reported that the largest proportion of burn-related deaths (24.8%) was among older individuals (≥75 years in age) and that the relative risk of mortality was approximately 1.5x higher in males (439). An analysis of the international burn injury database for England and Wales (2003-2011) concurred that patients aged 65 years or over suffered longer in-hospital length of stay, as well as the highest mortality rates among all other age groups, 19.24%. Interestingly in this analysis mortality was generally higher in females than males over the eight-year period (1.86 % vs. 1.32%) and in each individual year examined (440). This was further supported by Moore *et al* who showed that risk of death in women admitted to intensive care post-thermal injury was double when compared with males, OR 2.35 (441). Gender dimorphism in burn injury thus appears to be the opposite to other forms of injury. This is further supported by Summers *et al* who concluded female

gender is associated with poorer outcomes following severe thermal injury(442). A systematic review of literature published from 1965 till 2012 identified the female gender as a risk factor for hypertrophic scarring in patients who survived their burn injury(443).

Due to the limited number of clinical studies investigating gender dimorphism on outcomes following thermal injury, animal studies offer insight into potential mechanisms that may explain these epidemiological findings. Anathakrishnan *et al* described similar responses in rats following burn injury (40% TBSA) and trauma-haemorrhage, in which both acute lung and intestinal injury were potentiated by oophorectomy and prevented by castration (178). Wigginton *et al* stated that a single intravenous dose of E2 reduced burn injury severity through regulation of the immuno-inflammatory cascade, as well as its anti-oxidant and anti-apoptotic properties (444) and other studies reported estradiol administration, following severe thermal injury, attenuated body mass loss associated with the hypermetabolic response (445). Gregory *et al* suggested that gender dimorphism relating to immune function following severe thermal injury was mediated by oestrogen and its impact on IL-6 production. This study reported that while intact females, at day 10 post-burn, exhibited three times higher levels of plasma IL-6, they also demonstrated suppression of splenocyte proliferation and delayed type hypersensitivity reactions (446). In contrast, Gatson *et al* found administration of E2 after thermal injury attenuated both brain inflammation and apoptotic signalling by down-regulating TNF-α, IL-1β and IL-6 levels within brain tissue (447). Increasing concentrations of estradiol, through castration or treatments with E2 or anti-androgens, post-burn was also associated with reduced remote organ

inflammation (448). The data concerning the involvement of oestrogens in regulating the response to burn injury remains mixed and is dominated by studies in animal models, with few studies involving human patients. Comparisons of burn injury outcomes in pre- and post-menopausal women, or those on HRT would be beneficial, albeit difficult, in this respect.

Steroids exert various physiological effects on different organ systems. While the use of anabolic steroids in major burn jury is well-established (201), the therapeutic use of other forms of steroids such as corticosteroids remains controversial and the benefits are unclear (449, 450). In such circumstances, the literature relating trauma and non-trauma critical illness is referred to by clinicians to guide their clinical practice. This, potentially, may not be beneficial to all patients due to unique and extensive differential pathological responses involved following major thermal injury influenced by factors such as patient sex and age(133).

In order to address gaps in our understanding of the steroid response to burn injury and associations with clinical outcomes, an observational cohort study, SIFTI, was set up to characterise the steroid response in the serum of burned patients from admission to 12 months post-injury (see chapter 2).

In addition to this extensive kinetic profiling of the endocrine response to burn injury, the study also addressed the lack of a detailed analysis of steroid metabolism after burn injury. Technological advances, notably the use of liquid chromatography/tandem mass-spectrometry (LC-MS/MS) analysis has allowed detailed analysis of systemic steroid status in a variety of conditions(451-453).

Therefore, high performance LC-MS/MS was applied to the serum samples. Additionally, this study investigated any potential associations with short-term and long outcomes, as well as identifying any potential clinical value relating to steroid status post-burn injury. Finally, the use of current steroid-based therapies and their influence on outcomes in patients admitted to a tertiary burn centre was also evaluated.

We hypothesize that major burn injury results in significantly affects the HPA/G axis. HPA/G hormonal disturbances post-injury are associated with outcomes including sepsis, MOF, mortality, wound healing and scarring. We hypothesize that supplementation of DHEA and testosterone may improve outcomes.

The aims of this thesis include determining the status of the adrenal and gonadal axes in severely burned patients; characterising these HPA/G axis longitudinally from day 1 following severe thermal injury till month 12 post-injury; exploring associations between the longitudinal HPA/G response and clinical outcomes in burned patients including mortality, MOF, sepsis, wound healing and scarring and identify any potential biomarkers that may improve prognosis of burned patients or identify novel therapeutic targets. Furthermore, we investigate the effects of corticosteroids and oxandrolone on the HPA/G response and outcomes following severe thermal injury.

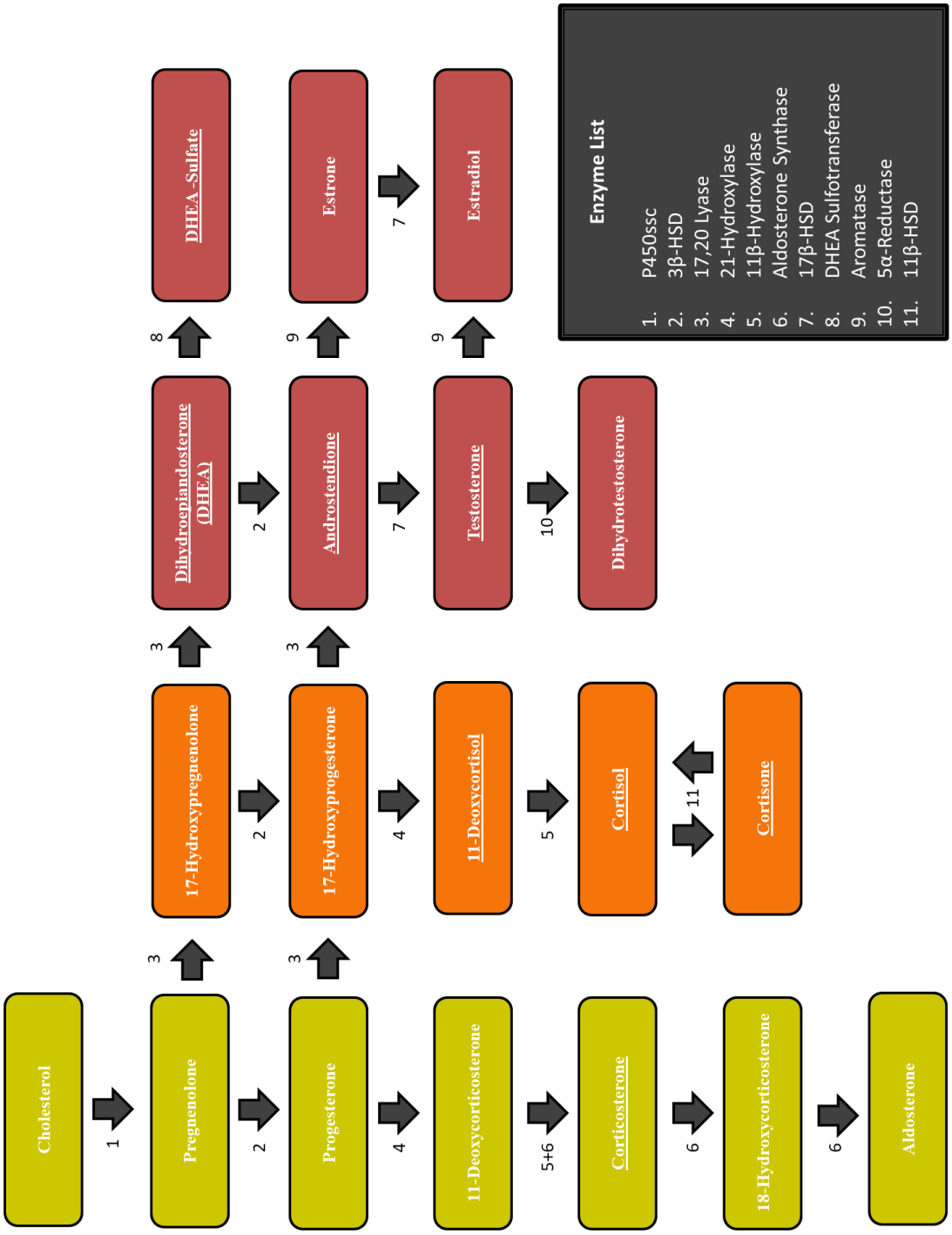


Figure 3.1. Overview of the steroidogenesis process.
Underlined analytes were analysed in this thesis.

3.2 Methods

The general methodology of the studies described here, including ethical approval and statistical analysis, are described in Chapter 2. In this chapter, quantification of steroid hormones and DHEAS was performed by Khaled Altarrah in a GCP validated lab in the IMSR, Birmingham University Medical School. Raw data acquired from LC-MS/MS analysis was quantified using Waters TargetLynx™ Application Manager (Waters Ltd, Elstree, UK) quantitative software by Dr. Angela Taylor. Overall data analysis was performed by Khaled Altarrah.

3.2.1 Measurement of Serum Steroid Hormone and DHEAS

All serum steroids were assessed using liquid chromatography/tandem mass-spectrometry (LC/MS-MS). Cortisol, cortisone, 11-deoxycortisol and corticosterone were extracted from 200-400µL of serum using a double liquid-liquid extraction consisting of 2x1mL of tert-butyl-methyl-ether (MTBE). Following evaporation of the MTBE layer to dryness, the samples were then reconstituted in methanol/water (50/50%) and analysed as previously described (454). Samples were then re-dried and derivatised to form oximes by employing 100 µL derivatization mixture (0.16 g hydroxylamine in 8 mL pyridine). Following this, testosterone, androstenedione and DHEA were measured as previously described (455, 456). DHEAS were measured from 20µL serum following protein precipitation using 20µL ZnSO4, 0.1mM and 100µL acetonitrile. The acetonitrile fraction was removed and evaporated to dryness before reconstitution in methanol/water 50/50 and analysis by LC-MS/MS using a Waters Xevo TQ-S mass spectrometer (Waters, Manchester, UK)(457, 458).

All samples were analysed using a Waters Xevo mass spectrometer with Acquity uPLC (Waters Ltd, Elstree, UK). DHEAS analysis was carried out in negative mode, whereas other steroids and steroid-oxime analysis was performed in positive mode. All steroids were separated using an optimised gradient system consisting of H_20 with 0.1% formic acid (FA) and methanol with 0.1% FA. All analytes were quantified relative to a linear calibration series with appropriate internal standards. Each steroid was measured relative to its deuterated analogue with the exception of androstenedione and cortisone which were quantified to testosterone-d3 and cortisol-d4 respectively. Each steroid was identified by matching retention times and two mass transitions in comparison to a reference compound. Precision data for each steroid assay has been previously described (415, 459-461).

3.3 Results

3.3.1 Patient Demographics

Fifty-two burn patients admitted to QEHB and 14 healthy volunteers were enrolled into this study. Owing to limited samples, only 46 burn patients had their serum analysed for HPA/G hormones. The process is summarised in *Supplementary Figure 3*. The baseline characteristics including age and gender were similar between healthy volunteers and burn patients. Median burn size was 42% TBSA and median revised BAUX score was 98. Burn patients underwent fluid resuscitation for the first 24 hours post-injury receiving a median of 18.6L of intravenous fluids (IVF) equating to a median of 5.53 mls/kg/%TBSA. Haemodilution effects of fluid resuscitation were measured using haematocrit (HCT). The median time for the first sample taken from burn

patients following injury was 7 hours (±6 hours SEM). HCT levels were similar between burn patients at day 1 post-injury and healthy volunteers. Twenty-two patients were given intravenous glucocorticoids to address treatment refractory shock, starting at median day 3 post-injury for median total duration of 5 days. Thirty-four burn patients were given oxandrolone to manage the post-burn hypermetabolic response, starting at median day 5 following injury for a median total duration of 19 days.

Burn patients at day 1 following injury had significantly elevated levels of cortisol (p=0.011), 11-deoxycortisol (p=0.003), corticosterone (p=0.007), androstenedione (p=0.001) and DHEA (p=0.033) compared to healthy volunteers. Cortisone levels were significantly lower in burn patients (p=0.01). Testosterone and DHEAS levels did not differ between burn patients and healthy volunteers. These findings are summarised in Table 3.1.

3.3.2 Patient outcomes

All burn patients recruited into the e-SIFTI study are included in this analysis, including non-survivors and participants lost to follow-up. From a total of 46 burn patients who had their serum analysed, 19% died at or before 28 days following injury and 35% died during their admission episode. Thirty-five percent of burn patients were diagnosed with MOF at median day 3 (min-max 2-11) post-injury. Seventy percent of burn patients developed sepsis at median day 5 (min-max 3-14) following injury. Other parameters including Time to 95% heal, as well as subjective and objective scar measures are summarised in Table 3.2. Scar measures were performed at an average of 18 months following the date of 95% wound healing.

	Healthy Controls	n	Burns Patients	n	p-value
Age	38 (31 - 70)	14	41 (33 – 55)	46	0.8
Gender (M/F)	8/6	14	30/16	46	0.753
% Total Burn Surface Area	-	-	42 (25 – 53.5)	46	-
Revised Baux Score	-	-	98 (79-111)	46	-
Hematocrit	0.399 (0.371-0.415)	12	0.429 (0.374-0.468)	31	0.127
Cortisol(nmol/L)	**201.7 (166.7 – 223.9)**	**14**	**548.5 (172 – 673)**	**46**	**0.011**
Cortisone (nmol/L)	**52.8 (47.9 – 63.6)**	**14**	**36.4 (28.4 – 51.5)**	**46**	**0.01**
11-Deoxycortisol (nmol/L)	**0.9 (0.9 - 1.4)**	**14**	**2.85 (1.4 – 6.0)**	**46**	**0.003**
Corticosterone (nmol/L)	**0.4 (0.4 – 3.7)**	**14**	**13.55 (1.8 – 34.7)**	**46**	**0.007**
Androstenedione (nmol/L)	**1.65 (1.4 – 2.2)**	**14**	**3.9 (2.4 – 6.4)**	**46**	**0.001**
Testosterone (nmol/L)	3.2 (1.3 – 9.1)	14	2.45 (1.4 – 4.5)	46	0.506
DHEA (nmol/L)	**1.9 (1.9 – 16.2)**	**14**	**15.6 (3.7 – 34.5)**	**46**	**0.033**
DHEAS (µmol/L)	3.2 (1.14 – 5.61)	14	2.1 (1.5 – 4.35)	46	0.507

Table 3.1.Demographics and serum analyte levels in healthy volunteers and burns patients at day 1 post injury.

Continuous variables are shown as median values with inter-quartile range. Burn patients and healthy volunteers were compared using Mann-Whitney test for continuous variables and Chi-squared test for categorical variables. Significant relationships are highlighted in bold.

Outcomes	Measure	n	Details
28D Survivor (Y/N)	9/37	46	Mortality at 28 days
Survivor (Y/N)	16/30	46	Mortality in hospital
MOF (Y/N)	16/30	46	DENVER score >3 for 48 hrs
Sepsis (Y/N)	32/14	46	ABA Sepsis Trigger Criteria ≥3
Time to 95% Heal (Days)	35 (18-59)	23	Higher number worse outcome
mVSS Total Score	6.67 (6-7)	11	Higher number worse outcome
Overall Observer Scar Score (POSAS)	4.67 (4-6)	11	Higher number worse outcome
Overall Patient Scar Score (POSAS)	8 (3-10)	11	Higher number worse outcome
Ultrasound Scar Thickness	1.7 (1.35-2.48)	11	Higher number worse outcome
Ultrasound Scar Intensity	0.45 (0.24-0.54)	11	Higher number worse outcome
Cutometer Scar Pliability R2	0.97 (0.93-1.17)	11	Lower number worse outcome
Cutometer Scar Pliability R0	0.56 (0.4-0.69)	11	Higher number worse outcome
DSM Colorimeter - Erythema Scale	1.27 (1.07-1.77)	11	Higher number worse outcome
DSM Colorimeter - Melanin Scale	1.21 (1.04-1.32)	11	Higher number worse outcome
DSM Colorimeter - L Scale	0.81 (0.72-0.86)	11	Subjective – scar paleness
DSM Colorimeter - a Scale	1.18 (1.02-1.43)	11	Higher number worse outcome
DSM Colorimeter - b Scale	0.79 (0.48-0.97)	11	Higher number worse outcome

Table 3.2. Summary of patient outcomes following injury.

Continuous variables are shown as median values with inter-quartile range.

3.3.3 Longitudinal Steroid Response Following Thermal injury

Cortisol, corticosterone, androstenedione and DHEA were all significantly elevated in burn patients at day 1 following injury (Table 3.1). Cortisol levels remained significantly elevated for the first month following thermal injury compared to healthy volunteers, median 353.7 nmol/L vs. 201.7 nmol/L ($p = 0.001$). In contrast cortisone levels in burn patients remained significantly reduced till day 28 post-injury compared to healthy controls, median 35.0 nmol/L vs. 52.8 nmol/L ($p < 0.001$), this suggests an activation of the 11βHSD1 pathway generating cortisol from cortisone. Interestingly, cortisone levels remained low despite a 5-day course of intravenous glucocorticoids being started for 63% of patients at timepoint D03-D07. The significant elevation of systemic cortisol and simultaneous reduction of cortisone demonstrate the prolonged pro-inflammatory state of patients following severe thermal injury, which may be the cause of any elevation as this enzyme is activated by inflammatory cytokines(462).

Testosterone levels in burn patients were significantly reduced at D03-D07 compared to healthy volunteers, median 1.3 nmol/L vs 3.2 nmol/L ($p=0.027$). This timepoint matches the median start day of oxandrolone supplementation at day 5. Testosterone began to increase and return to healthy volunteer levels thereafter. DHEAS levels in blood started to decrease following severe thermal injury becoming significant at week 2-4 post-injury compared to healthy controls, median 1.5 µmol/L vs. 3.2 µmol/L ($p = 0.026$). The steroid levels in serum of burn patients are summarised longitudinally in Figure 3.2.

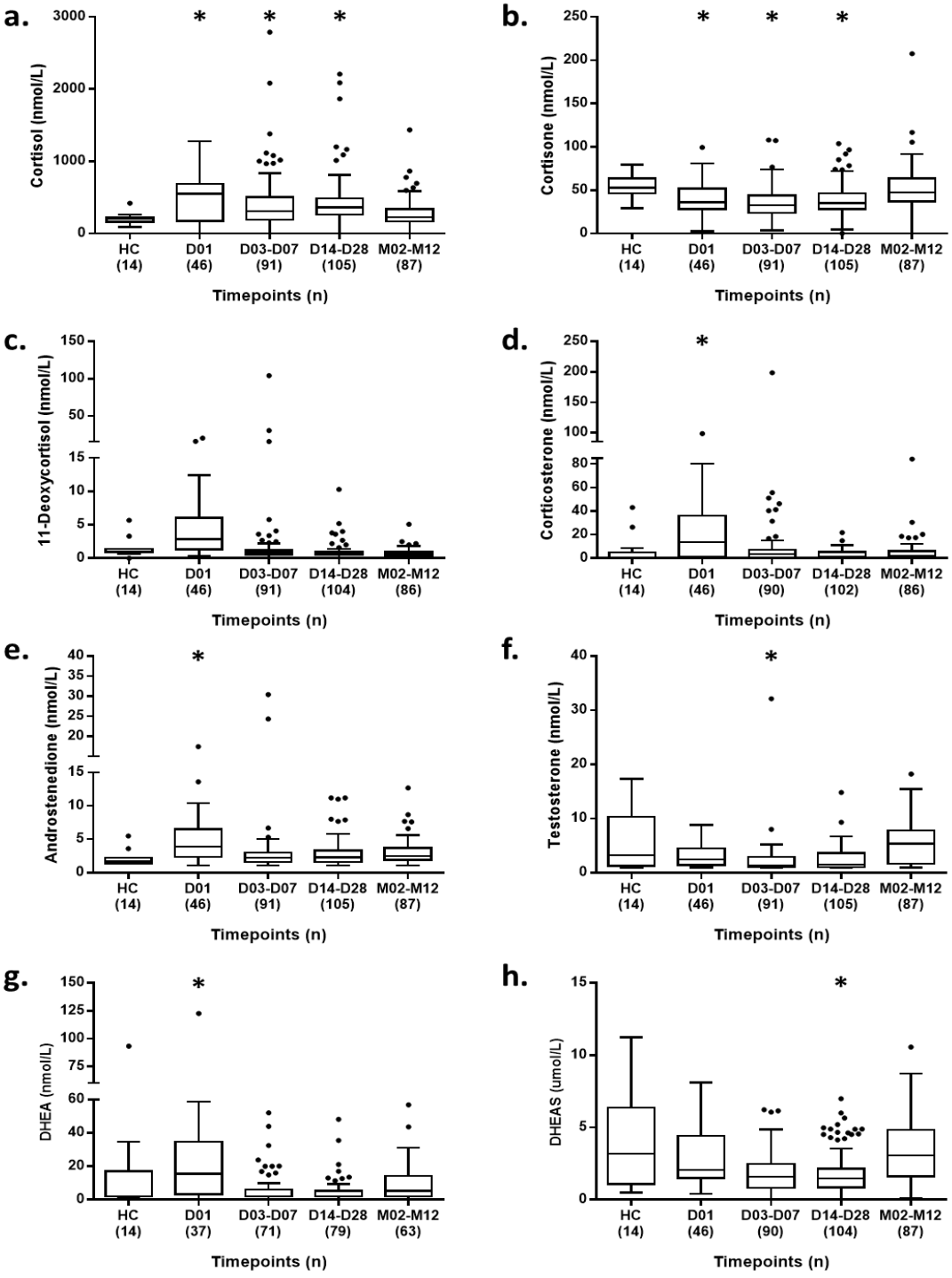


Figure 3.2. **Burn injury significantly disrupts the HPA/G endocrine response.** Systemic steroids were analysed longitudinally over a 12-month period. a. Cortisol; b. Cortisone; c. 11-Deoxycortisol; d. Corticosterone; e. Androstenedione; f. Testosterone; g. DHEA; h. DHEAS. Analyte levels at timepoints were compared to healthy controls (HC) using Kruskal-Wallis test with Dunn's multiple corrections; * $p < 0.05$.

3.3.4 Longitudinal Steroid Response and Mortality Following Thermal Injury

Systemic levels of cortisol were generally elevated among non-survivors of burn injury compared to survivors. Burns patients who died in-hospital had significantly elevated levels of cortisol in serum compared to survivors, median 640.1 nmol/L vs. 320.1 nmol/L ($p = 0.014$). Furthermore, cortisol levels were significantly elevated among burns in-hospital non-survivors compared to survivors at month 2 till month 12 post-injury, median 546.2 nmol/L vs. 219.4 nmol/L ($p = 0.008$). Cortisone levels following thermal injury were similar among in-hospital non-survivors and survivors at most timepoints, though non-survivors had significantly reduced levels of cortisone from month 2 onwards post-injury, median 32.3 nmol/L vs. 47.8 nmol/L ($p = 0.021$).

Interestingly, day 1 systemic 11-deoxycortisol levels were significantly higher in burns patients who died at/before 28 days and during their admission episode compared to survivors, median 6.0 nmol/L vs 2.5 nmol/L (p 0.034) and 5.8 nmol/L vs. 2.1 nmol/L ($p = 0.001$). Furthermore, corticosterone levels in serum were significantly increased in non-survivors compared to survivors of thermal injury, median 31.2 nmol/L vs. 8.7 nmol/L ($p = 0.029$). The longitudinal steroid response among survivors and non-survivors of severe thermal injury is summarised in Figure 3.3 and Figure 3.4.

3.3.5 Longitudinal Steroid Response and MOF Following Severe Thermal Injury

Burns patients diagnosed with MOF exhibited significantly different systemic steroid responses compared to patients who did not develop MOF. At Day 1 post-burn injury, patients with MOF had significantly increased serum levels of DHEA, median 32.8 nmol/L vs. 9.1 nmol/L ($p = 0.038$). Subsequently at D14-D28, burns patients with MOF exhibited significantly elevated systemic levels of cortisol, androstenedione and DHEA compared to patients who did not develop MOF, median 468.0 nmol/L vs. 347.4 nmol/L ($p = 0.001$), 2.9 nmol/L vs. 2.2 nmol/L ($p = 0.008$) and 3.25 vs. 1.9 nmol/L ($p = 0.026$) respectively. The longitudinal steroid response in burns patients who developed and did not develop MOF is summarised in Figure 3.5.

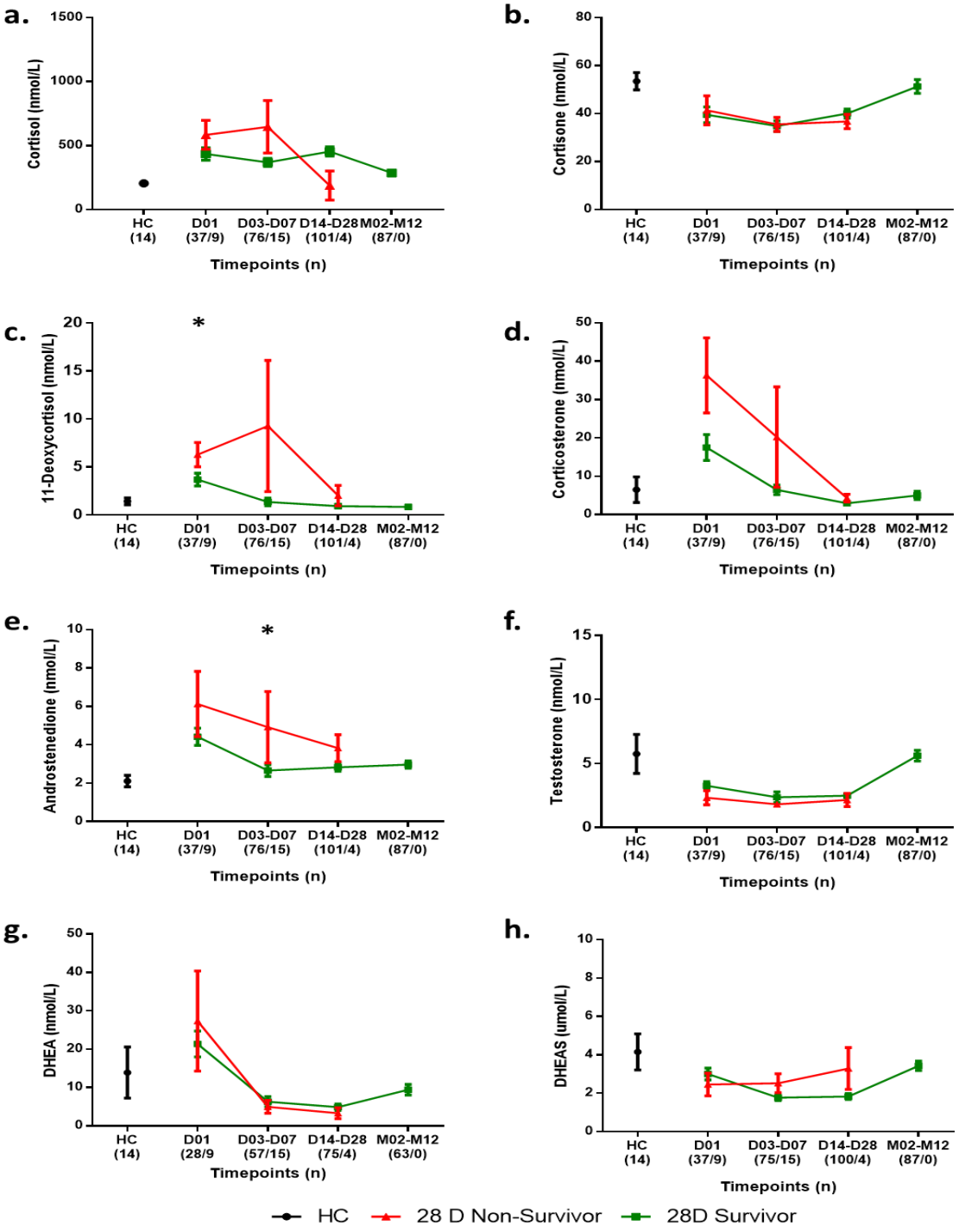


Figure 3.3. 11-Deoxycortisol and Androstenedione levels were significantly elevated in 28-day non-survivors following burn injury.

Serum steroid levels in 28-day burn survivors and non-survivors were analysed across all timepoints. a. Cortisol; b. Cortisone; c. 11-Deoxycortisol; d. Corticosterone; e. Androstenedione; f. Testosterone; g. DHEA; h. DHEAS. Analyte levels at each timepoint was compared between both cohorts (28D survivors vs. 28D non-survivors) using Mann-Whitney Test; * $p < 0.05$.

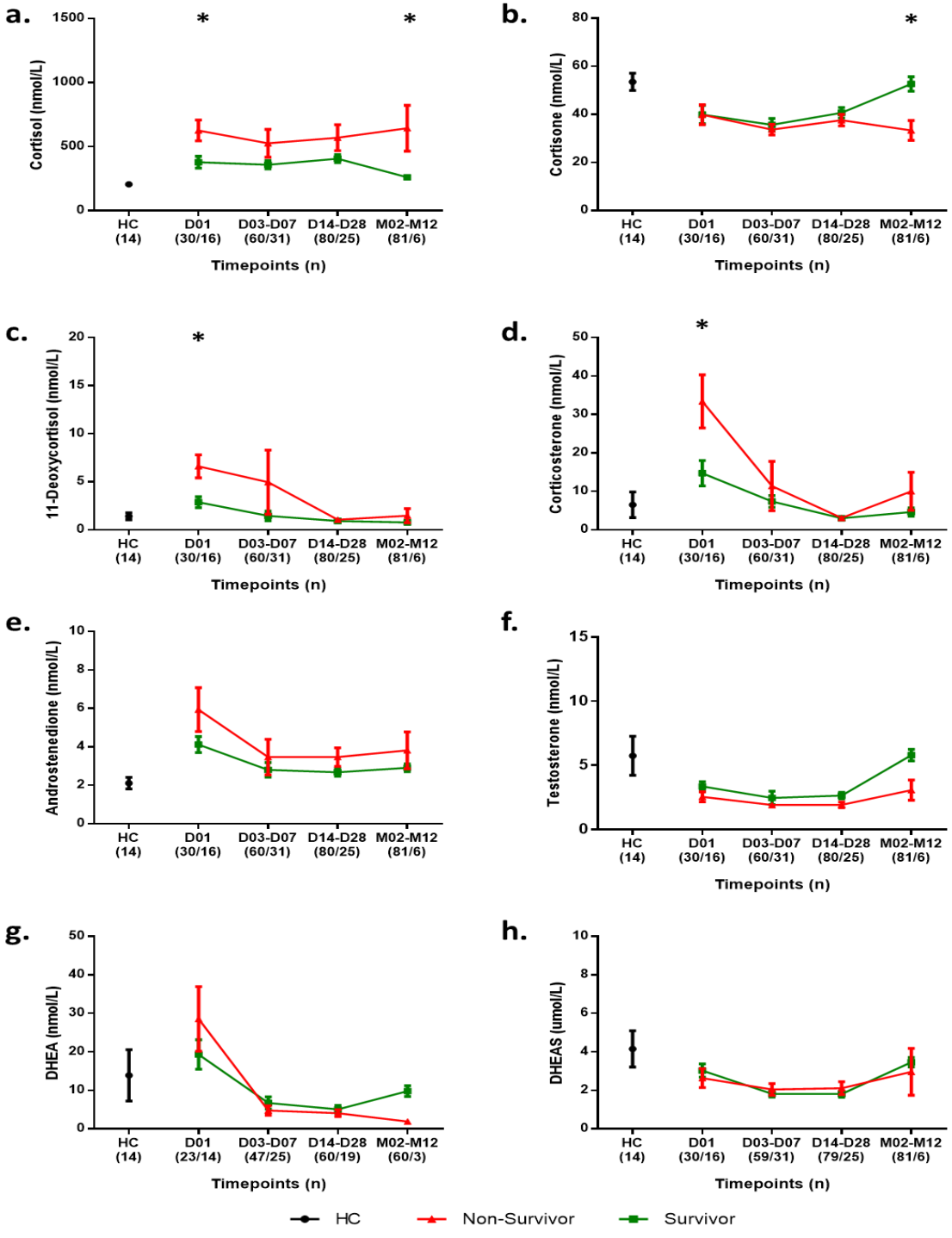


Figure 3.4. Cortisol, 11-Deoxycortisol and Corticosterone were significantly elevated among in-hospital non-survivors at Day 1 following burn injury.
Serum steroid levels in burn survivors and non-survivors were analysed across all timepoints. a. Cortisol; b. Cortisone; c. 11-Deoxycortisol; d. Corticosterone; e. Androstenedione; f. Testosterone; g. DHEA; h. DHEAS. Analyte levels at each timepoint were compared between both cohorts (survivors vs. non-survivors) using Mann-Whitney Test; * $p < 0.05$.

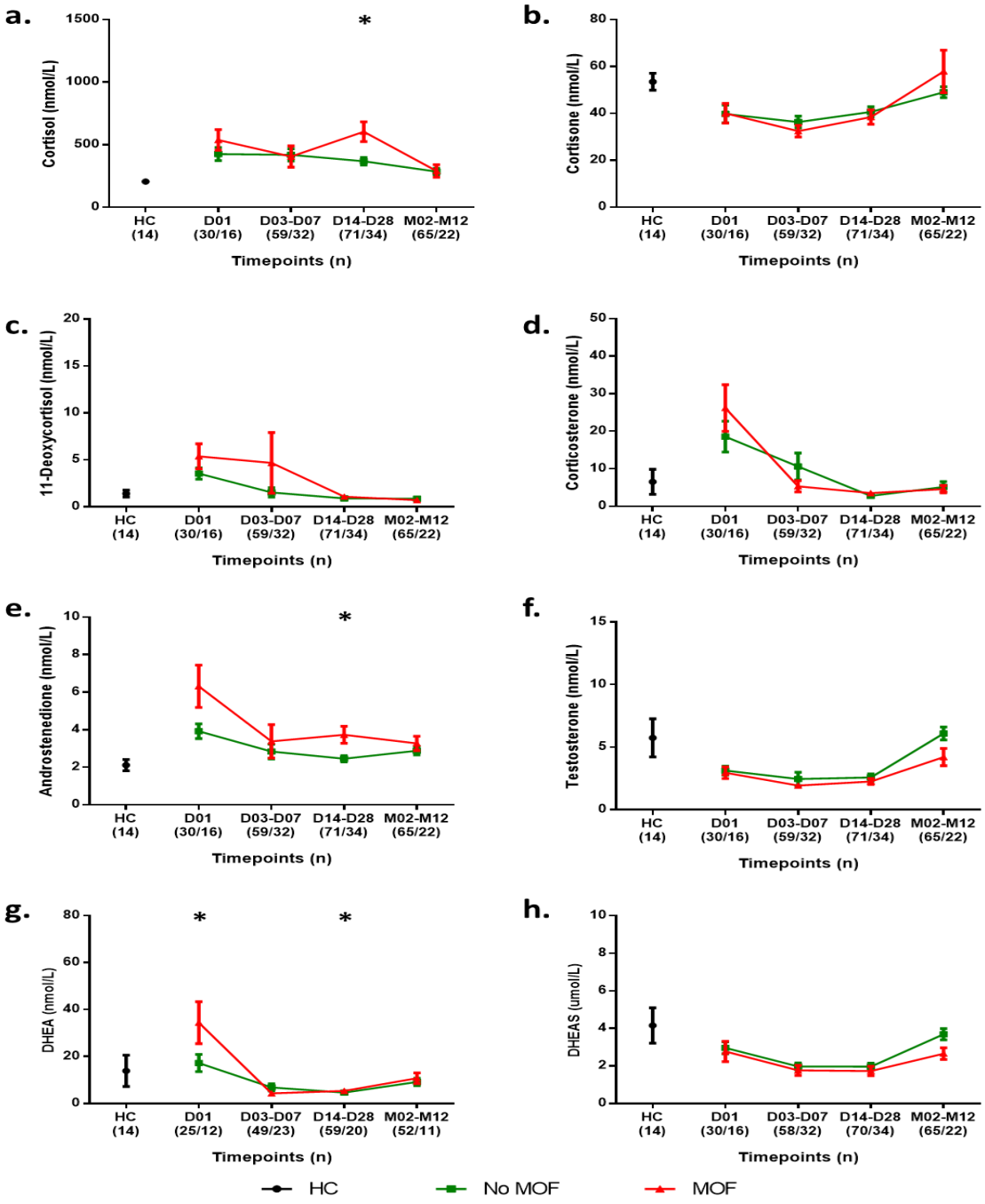


Figure 3.5. Cortisol, Androstenedione and DHEA were significantly elevated in burn patients diagnosed with MOF.

Serum steroid levels in burn patients who did and did not develop MOF were analysed across all timepoints. a. Cortisol; b. Cortisone; c. 11-Deoxycortisol; d. Corticosterone; e. Androstenedione; f. Testosterone; g. DHEA; h. DHEAS. Analyte levels at each timepoint were compared between both cohorts (MOF vs. No MOF) using Mann-Whitney Test; * $p < 0.05$.

3.3.6 Longitudinal Steroid Response in Septic and Non-Septic Patients Following Severe Thermal Injury

Septic burns patients demonstrated an altered and prolonged steroid response compared to non-septic patients. Androstenedione, DHEA and DHEAS levels in serum were significantly reduced in septic patients compared to non-septic patients at D03-D07 following thermal injury, median 2.0 nmol/L vs. 2.8 nmol/L ($p = 0.046$), 1.9 nmol/L vs. 4.6 nmol/L ($p = 0.017$) and 1.5 µmol/L vs. 1.9 µmol/L ($p = 0.017$) respectively. Thereafter, circulating levels of DHEA and DHEAS remained significantly lower in septic burn patients compared to non-septic patients at M02-M12, median 1.9 nmol/L vs. 10.3 nmol/L ($p = 0.004$) and 2.8 µmol/L vs. 4.0 µmol/L ($p = 0.007$) respectively.

Systemic cortisol levels were significantly elevated in septic patients compared to non-septic patients following thermal injury at D14-D28, median 411.5 nmol/L vs. 277.6 nmol/L ($p <0.001$). While testosterone levels in serum of septic burns patients were significantly depressed compared to non-septic patients at D14-D28, median 1.4 nmol/L vs. 2.2 nmol/L ($p = 0.047$). The longitudinal steroid response in septic and non-septic burns patients is summarised in Figure 3.6.

3.3.7 Longitudinal Steroid Response and Wound Healing following Severe Thermal Injury

Serum steroid levels were significantly associated with time taken to achieve wound healing at various timepoints throughout the study period. At D01 post-burn injury, lower levels of circulating testosterone and DHEAS were significantly associated with longer wound healing times, rho -0.421 ($p = 0.045$) and rho -0.602 ($p = 0.002$)

respectively. Furthermore, depressed testosterone levels in the serum of burns patients at D14-D28 post-injury were significantly associated with increased days taken to achieve wound healing, rho -0.389 (p = 0.001). Interestingly, longer wound healing times were significantly associated with prolonged reduction of circulating levels of various steroids including testosterone, DHEA and DHEAS. Correlations between longitudinal serum steroids and duration of wound healing are summarised in Figure 3.7.

3.3.8 Longitudinal Steroid Response and Scarring Following Severe Thermal Injury

3.3.8.1 Subjective Scar Measures

Systemic levels of steroids in burns patients were significantly associated with scarring outcomes, as measured subjectively by total mVSS and overall POSAS scores. Lower levels of serum 11-deoxycortisol at D03-D07 was significantly associated with worse scarring outcomes in both scar scoring systems. Thereafter, elevated systemic levels of cortisol were significantly associated with higher mVSS scores at D14-D28, rho 0.534 (p = 0.002). Interestingly, higher levels of DHEAS in blood at D14-M12 was significantly associated with higher OOSS scores, rho 0.375 (p = 0.002). Significant correlations between serum steroids measured longitudinally and subjective scar scoring systems are summarised in Figures Figure 3.8 and Figure 3.9.

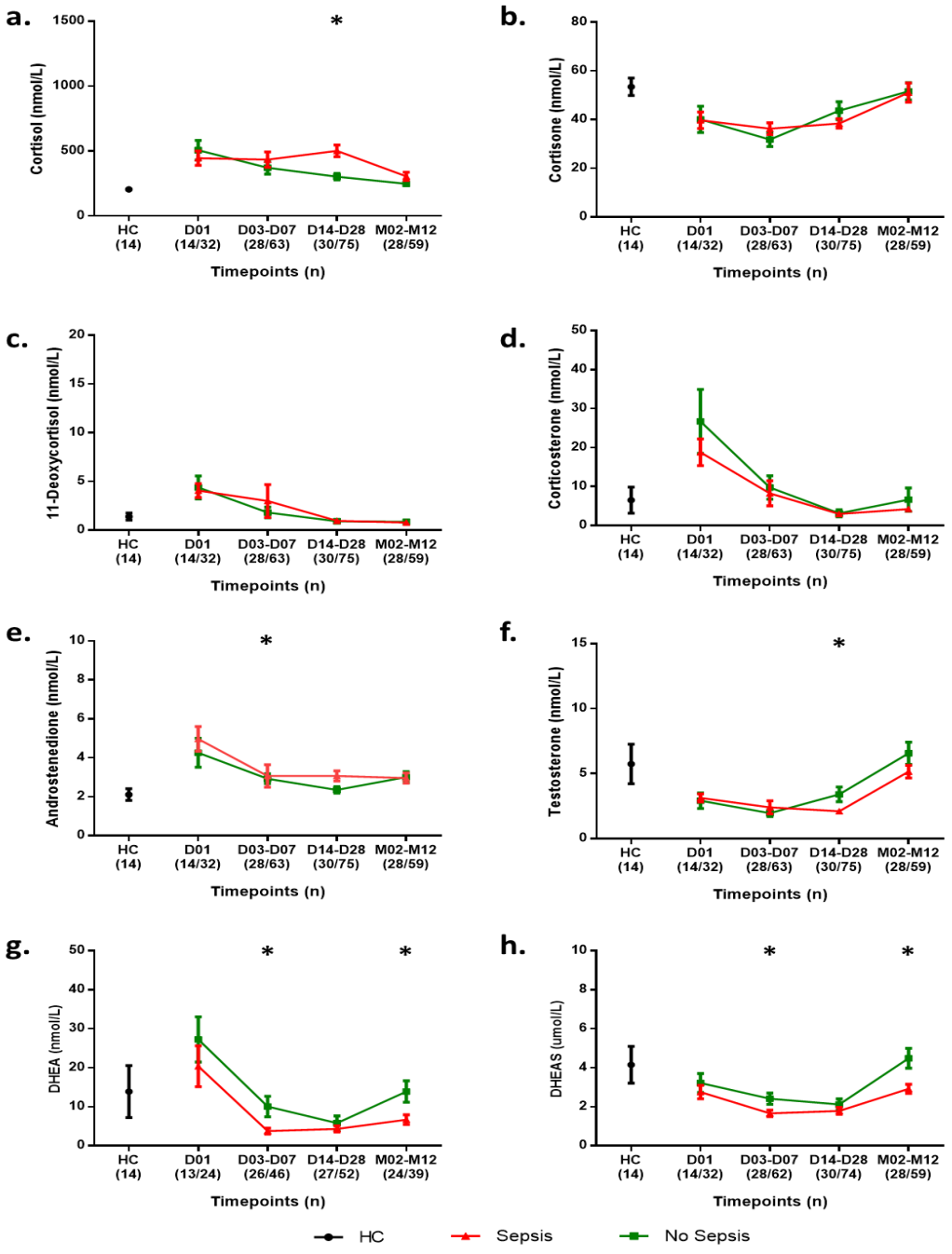


Figure 3.6. DHEA, DHEAS and Testosterone were significantly reduced in septic burn patients.

Serum steroid levels in septic and non-septic burns patients were analysed across all timepoints. a. Cortisol; b. Cortisone; c. 11-Deoxycortisol; d. Corticosterone; e. Androstenedione; f. Testosterone; g. DHEA; h. DHEAS. Analyte levels at each timepoint were compared between both cohorts (Sepsis vs. No Sepsis) using Mann-Whitney Test; * $p < 0.05$.

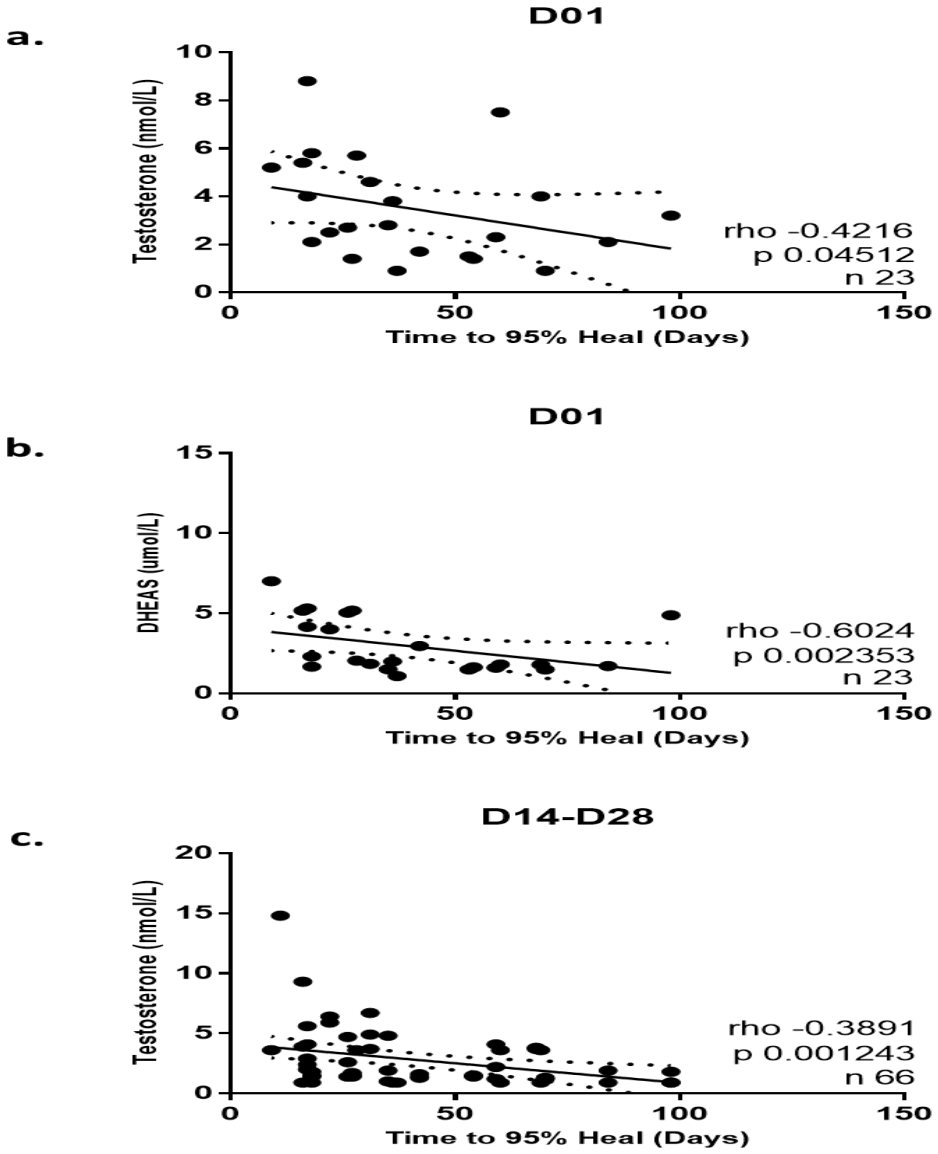


Figure 3.7. Testosterone and DHEAS significantly correlated with time to 95% would healing following severe thermal injury.
Serum steroid levels and days taken to achieve 95% wounds healed in burns patients were analysed across all timepoints. a. Testosterone at D01; b. DHEAS at D01; c. Testosterone at D14-D28. Correlations were performed between analyte levels at timepoints and time to 95% heal using Spearman's rank correlation co-efficient; * p <0.05.

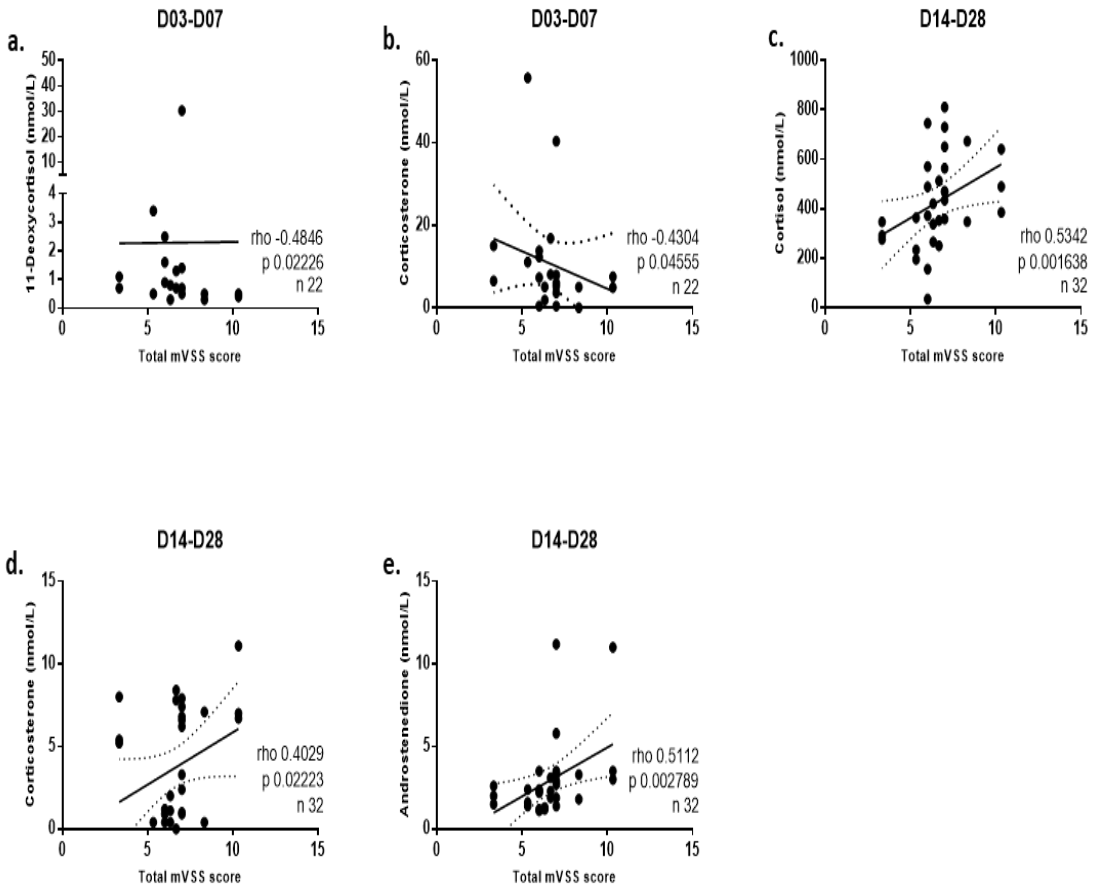


Figure 3.8. Steroid response and mVSS score correlations following severe thermal injury.

Serum steroid levels and total mVSS scores in burns patients were analysed across all timepoints. a. 11-Deoxycortisol at D03-D07; b. Corticosterone at D03-D07; c. Cortisol at D14-D28; d. Corticosterone at D14-D28; e. Androstenedione at D14-D28. Correlations were performed between analyte levels at timepoints and total mVSS scores using Spearman's rank correlation co-efficient; * $p < 0.05$.

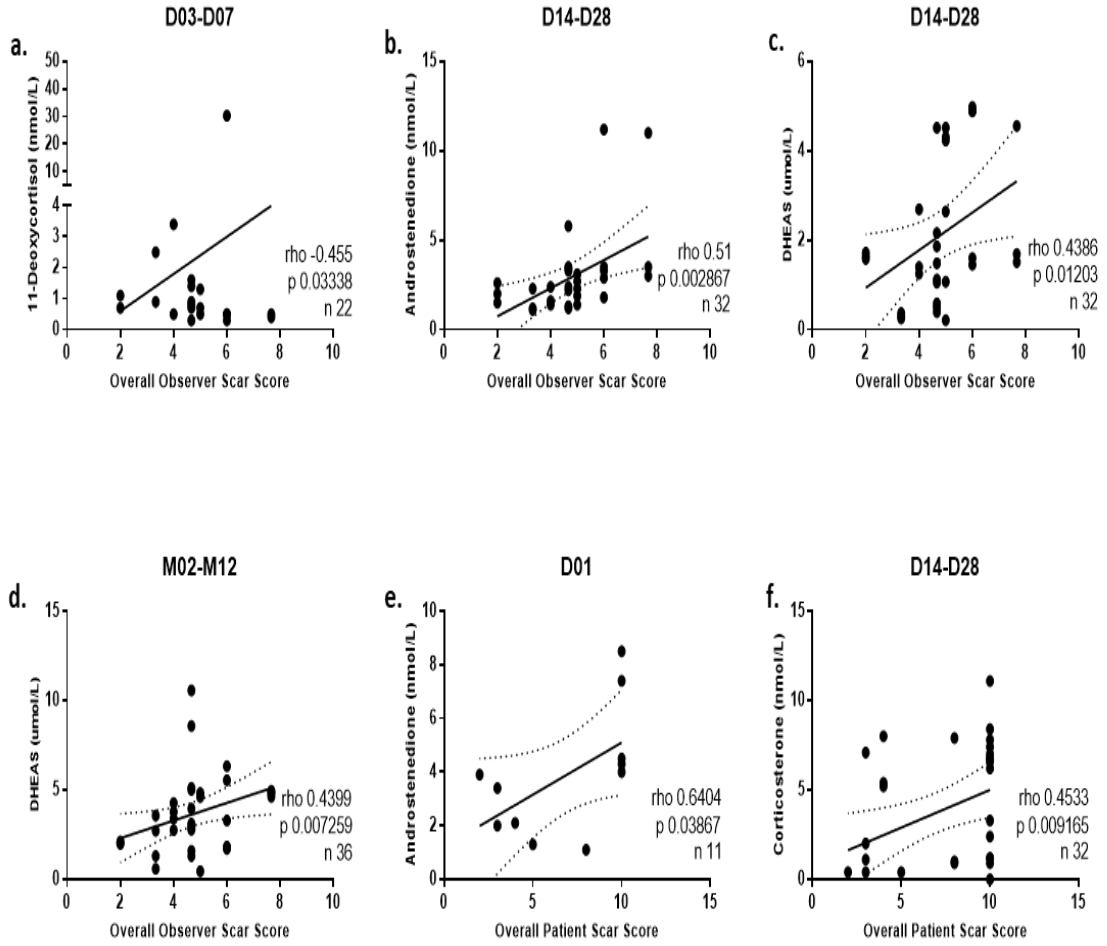


Figure 3.9. Steroid response and POSAS score correlations following severe thermal injury.

Serum steroid levels and POSAS (Patient and Observer) overall scores in burns patients were analysed across all timepoints. a. 11-Deoxycortisol vs. OOSS at D03-D07; b. Androstenedione vs. OOSS at D14-D28; c. DHEAS vs. OOSS at D14-D28; d. DHEAS vs. OOSS at M02-M12; e. Androstenedione vs. OPSS at D01; f. Corticosterone vs. OPSS at D14-D28. Correlations were performed between analyte levels at timepoints and overall POSAS scores using Spearman's rank correlation co-efficient; * $p < 0.05$.

3.3.8.2 Objective Scar Measures

Throughout the study period, serum steroid levels in burns patients were significantly associated with multiple objective scar measures. Elevated cortisol levels at D14-D28 were significantly associated with thicker scarring in burns patients, rho 0.571 (p <0.001). Furthermore, increased cortisone levels at D03-D07 post-thermal injury were significantly associated with more dense scarring, rho 0.47 (p 0.027). Interestingly, testosterone and DHEAS levels in serum had a significant inverse association with dense scars at M02-M12 post-burn injury, rho -0.363 (p 0.029) and rho -0.448 (p 0.006) respectively. Significant correlations between systemic steroids and ultrasound scar measures are summarised in Figure 3.10.

Scar pliability and firmness as measured by cutometer demonstrated significant relationships with systemic steroid levels of burns patients. Immediately following thermal injury, lower androstenedione levels were significantly associated with increased scar pliability, rho -0.755 (p = 0.010). Furthermore, increased DHEAS levels in serum at D03-D28 post-burn injury were significantly associated with better scar pliability, rho 0.434 (p = 0.001). Additionally, elevated testosterone levels in blood of burns patients at M02-M12 were significantly associated with improved scar pliability, rho 0.515 (p = 0.001). Similarly, reduced systemic testosterone levels at D14-D28 post-burn injury were associated with increased scar firmness, rho -0.368 (p = 0.038). Additionally, decreased levels of DHEA in burns patients were significantly associated with firmer scars, rho -0.491 (p = 0.020). Significant correlations between serum steroid levels in burns patients and cutometer scar measures are summarised in Figure 3.11.

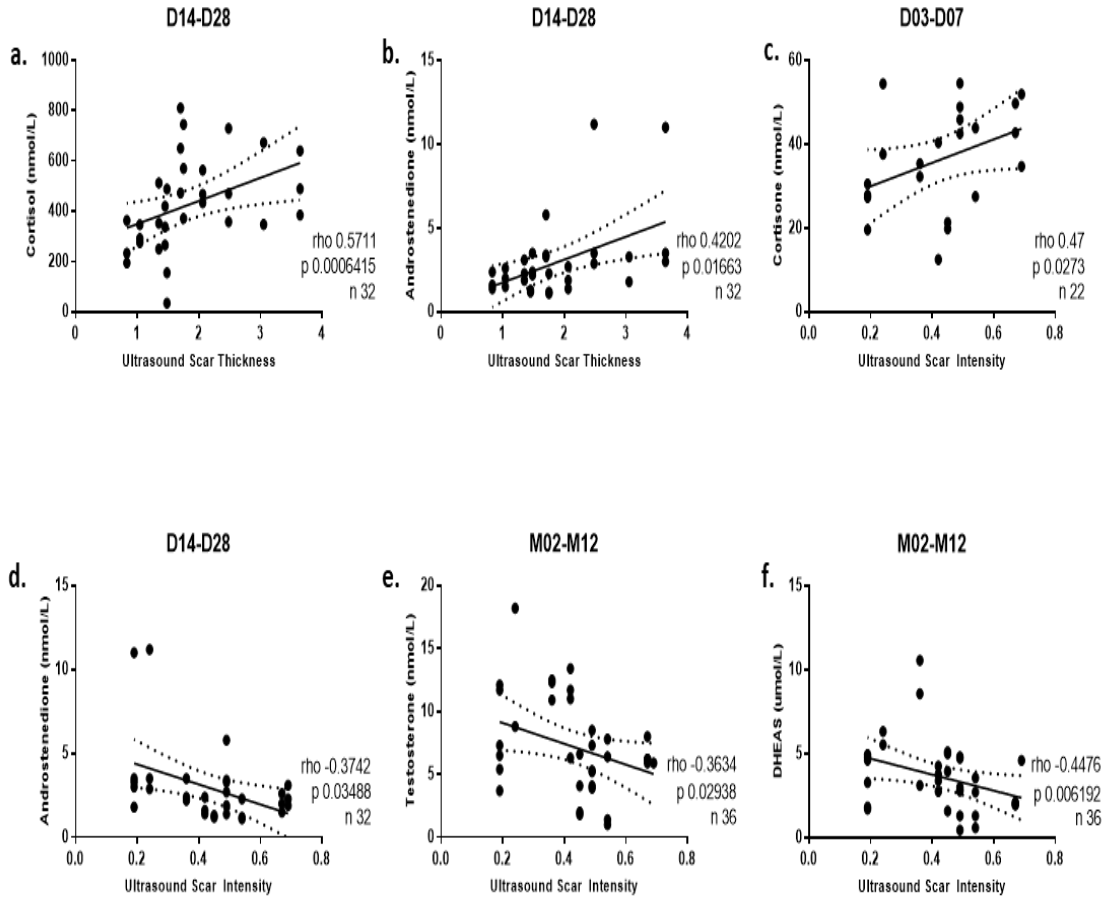


Figure 3.10. Steroid response and ultrasound scar measure correlations following severe thermal injury.

Serum steroid levels and scar thickness/intensity in burns patients as measured by ultrasound were analysed across all timepoints. a. Cortisol vs. Scar Thickness at D14-D28; b. Androstenedione vs. Scar Thickness at D14-D28; c. Cortisone vs. Scar Intensity at D03-D07; d. Androstenedione vs. Scar Intensity at D14-D28; e. Testosterone vs. Scar Intensity at M02-M12; f. DHEAS vs. Scar Intensity at M02-M12. Correlations were performed between analyte levels at timepoints and ultrasound scar measures using Spearman's rank correlation co-efficient; * $p < 0.05$.

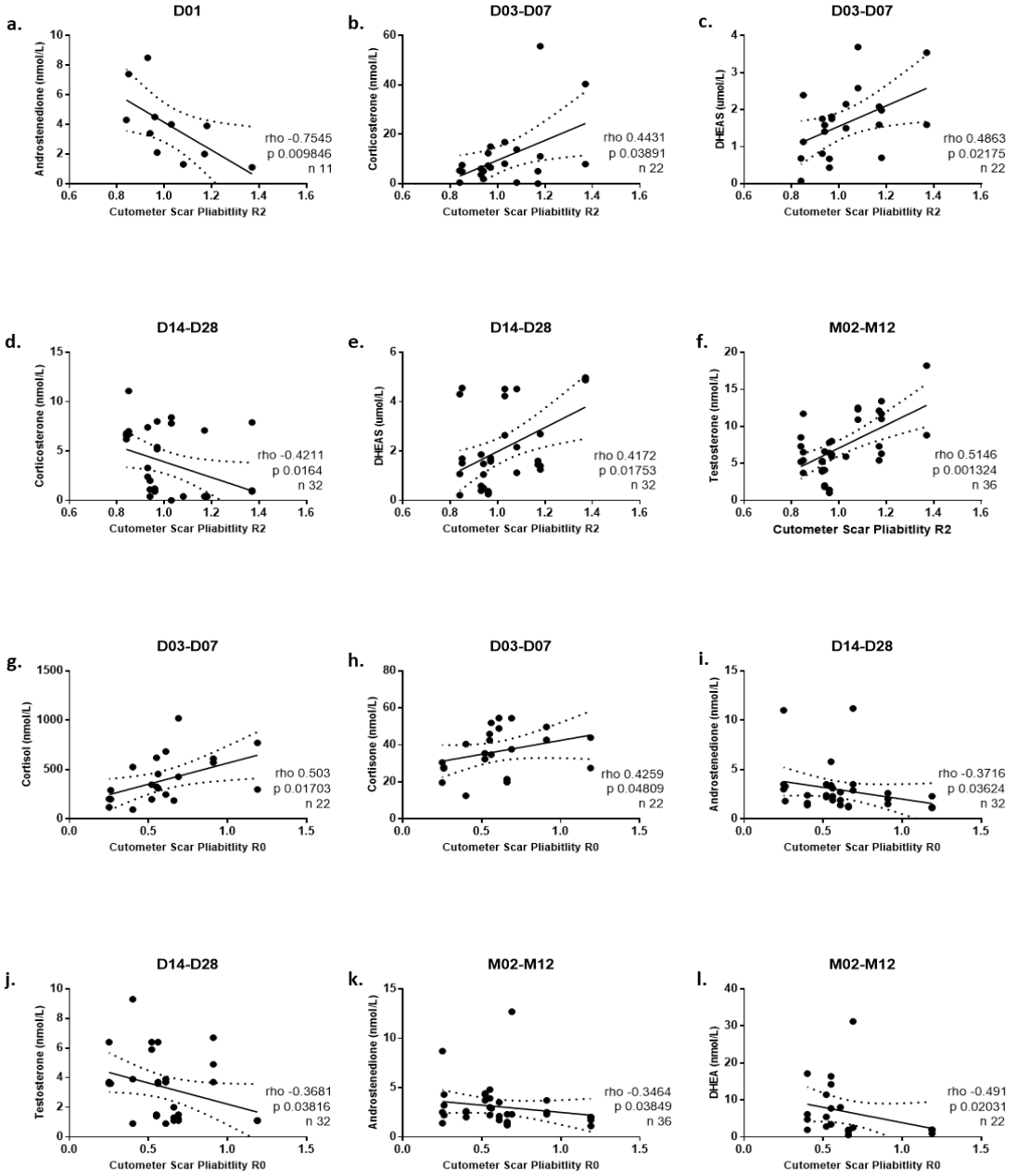


Figure 3.11. Steroid response and cutometer scar measure correlations following severe thermal injury.

Serum steroid levels and scar pliability (R2)/firmness (R0) in burns patients as measured by cutometer were analysed across all timepoints. a. Androstenedione vs R2 at D01; b. Corticosterone vs. R2 at D03-D07; c. DHEAS vs. R2 at D03-D07; d. Corticosterone vs. R2 at D14-D28; e. DHEAS vs. R2 at D14-D28; f. Testosterone vs. R2 at M02-M12; g. Cortisol vs. R0 at D03-D07; h. Cortisone vs. R0 at D03-D07; i. Androstenedione vs. R0 at D14-D28; j. Testosterone vs. R0 at D14-D28; k. Androstenedione vs. R0 at M02-M12; l. DHEA vs. R0 at M02-M12. Correlations were performed between analyte levels at timepoints and cutometer scar measures using Spearman's rank correlation co-efficient; * $p < 0.05$.

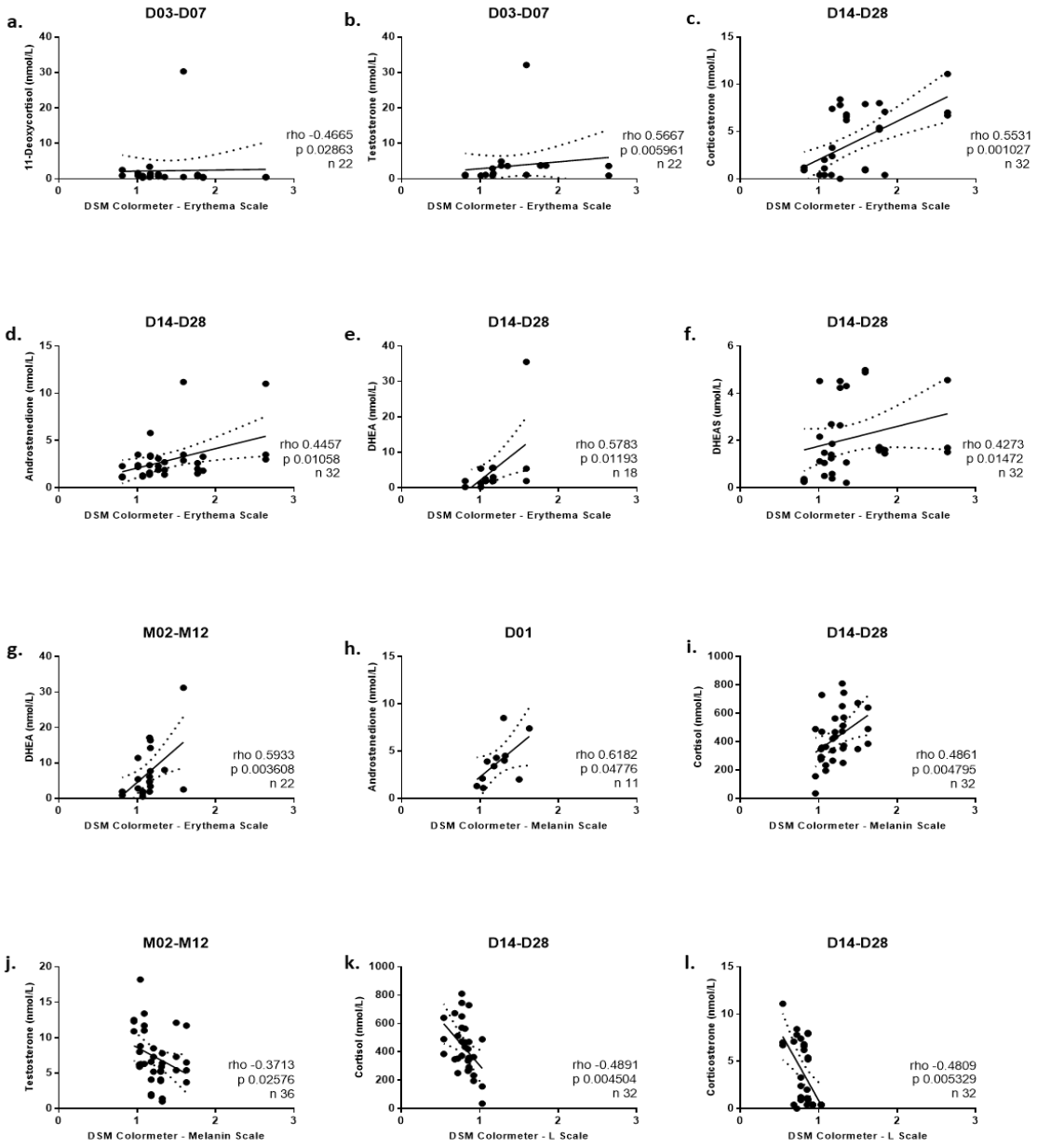


Figure 3.12. Steroid response and colorimeter scar measure correlations following severe thermal injury (Part 1).

Serum steroid levels and scar erythema/pigmentation in burns patients as measured by colorimeter were analysed across all timepoints. a. 11-Deoxycortisol vs. Erythema at D03-D07; b. Testosterone vs. Erythema at D03-D07; c. Corticosterone vs. Erythema at D14-D28; d. Androstenedione vs. Erythema at D14-D28; e. DHEA vs. Erythema at D14-D28; f. DHEAS vs. Erythema at D14-D28; g. DHEA vs. Erythema at M02-M12; h. Androstenedione vs. Melanin at D01; i. Cortisol vs. Melanin at D14-D28; j. Testosterone vs. Melanin at M02-M12; k. Cortisol vs. L at D14-D28; l. Corticosterone vs. L at D14-D28. Correlations were performed between analyte levels at timepoints and colorimeter scar measures using Spearman's rank correlation co-efficient; * $p < 0.05$.

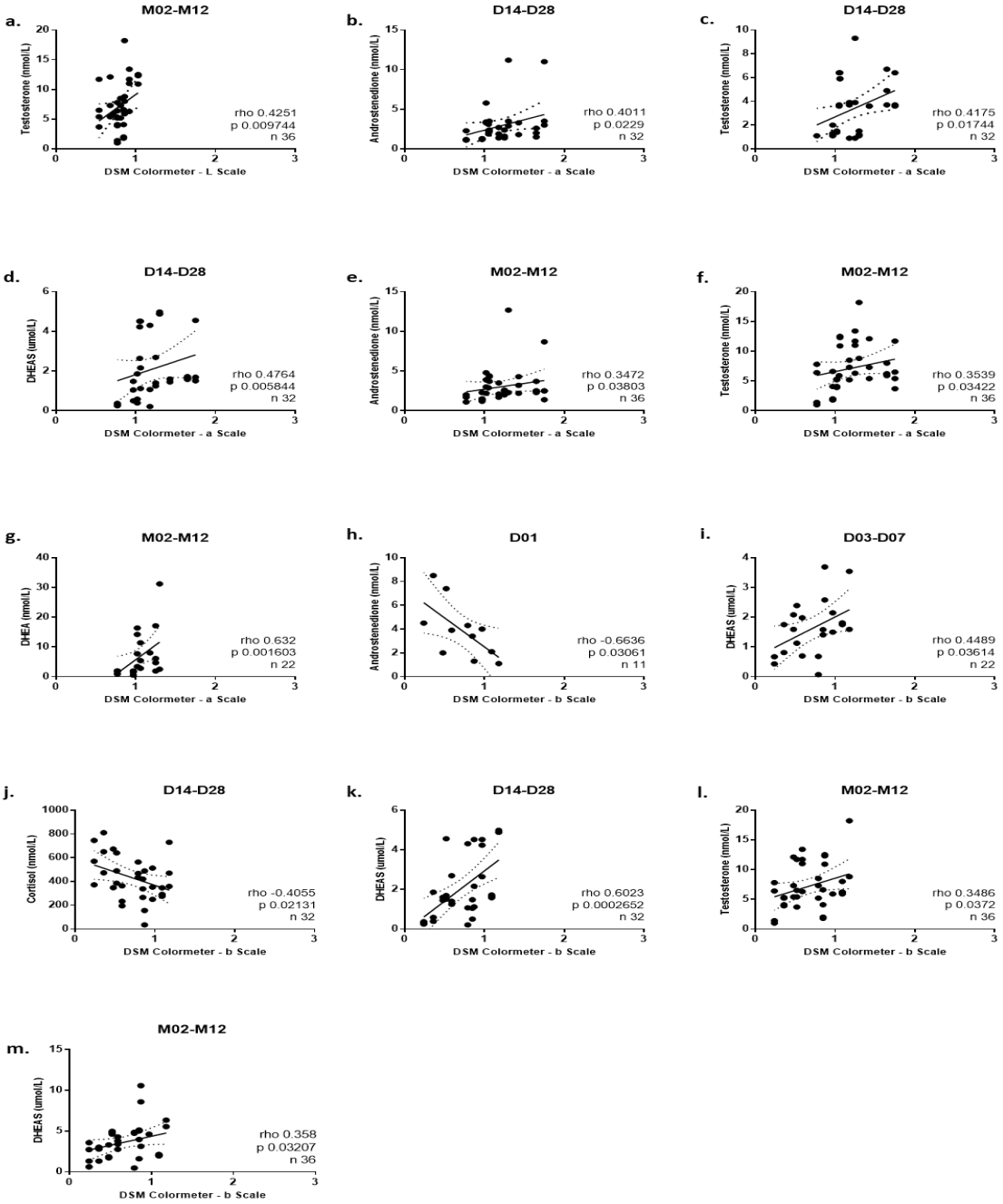


Figure 3.13. Steroid response and colorimeter scar measure correlations following severe thermal injury (Part 2).

Continuation from Figure 3.12. a. Testosterone vs. L at M02-M12; b. Androstenedione vs. a at D14-28; c. Testosterone vs. a at D14-D28; d. DHEAS vs. a at D14-D28; e. Androstenedione vs. a at M02-M12; f. Testosterone vs. a at M02-M12; g. DHEA vs. a at M02-M12; h. Androstenedione vs. b at D01; i. DHEAS vs. b at D03-D07; j. Cortisol vs. b at D14-D28; k. DHEAS vs. b at D14-D28; l. Testosterone vs. b at M02-M12; m. DHEAS vs. b at M02-M12.

Throughout the study period, serum steroid levels demonstrated significant associated with subsequent scar erythema and pigmentation in burns patients. Significant correlations between systemic steroids levels and colorimeter scar measures are summarised in Figures Figure 3.12 and Figure 3.13.

3.3.9 Therapeutic Potential of Steroids following Severe Thermal Injury

The data so far suggest that supplementing burns patients with testosterone, DHEA and DHEAS may reduce the risk of developing sepsis and mortality following injury. At D03-D07, DHEA and DHEAS demonstrated significant association with sepsis development in burns patients independent of age, gender and injury severity, $p = 0.023$ and $p = 0.027$ respectively. Supplementing patients with DHEA or DHEAS at D03-D07 post-thermal injury may increase the odds of not developing sepsis by 13% and 65% for 1ng/mL increase of serum DHEA and DHEAS respectively.

Circulating levels of testosterone at D03-D07 was significantly associated with and predictive of 28-day mortality in burns patients, $p = 0.028$. This was independent of age, gender, injury severity and physiological state on admission. Supplementing patients with testosterone at D03-D07 post-burn injury may increase the odds of 28-day survival by 104% for each 1 nmol/L increase in serum testosterone levels. Furthermore, serum DHEA levels at D14-D28 post-thermal injury was significantly associated with and predictive of in-hospital mortality independent of patient and injury characteristics, $p = 0.036$. A 1 nmol/L increase in serum DHEA in burns patients may improve the odds of survival following injury by 34%. The multi-variate analysis

and significant therapeutic potential of steroids following severe thermal injury are summarised in Table 3.3.

					Measure of Benefit (No Adverse Outcome)		
Timepoint	Outcome	Analyte	Co-Variates	p- value	Odds Ratio	Effect on OR for 1 unit (%)	Required units to increase OR by 1
D03-D07	Sepsis	DHEA	Age, TBSA, Inhalation Injury, Gender, APACHE II	0.019	1.15	13.8	7.25
D03-D07	Sepsis	DHEAS		0.042	1.58	46.7	2.14
D03-D07	Sepsis	Cortisone		0.044	0.96	-4.3	-23.26
D03-D07	Sepsis	DHEAS	Age, TBSA, Inhalation Injury, Gender, APACHE II, All Steroids	0.014	2.15	76.6	1.31
D03-D07	Sepsis	Cortisone		0.047	0.93	-7.1	-14.1
D03-D07	28D Mortality	Testosterone		0.028	2.83	104.2	1.0
D14-D28	Mortality	Corticosterone		0.047	0.68	-38.4	-2.6
D14-D28	Mortality	DHEA		0.036	1.40	33.6	3.0
D14-28	MOF	Cortisol		0.022	0.99	-1	-100

Table 3.3. Supplementing DHEA, DHEAS and Testosterone may improve outcomes following severe thermal injury.
Outcomes and Steroids at all timepoints were assessed using uni-analyte and multi-analyte regression models to estimate treatment effects. Multi-variate binary logistic regression was performed accounting for patient demographics, injury severity and/or physiological state on admission. Significant associations $p < 0.05$.

Timepoint	Outcome	Analyte	Co-Variates	p-value	1% analyte increase reduces healing days by (%)
D01	95%heal	Testosterone	Age Gender TBSA Inhalation Injury Excision No. Grafting No.	0.024	0.666
D03-D07	95%heal	Testosterone		0.032	0.287
D14-D28	95%heal	Testosterone		0.007	0.274
D01	95%heal	DHEAS		0.034	0.555
D03-D07	95%heal	Corticosterone		0.039	0.101

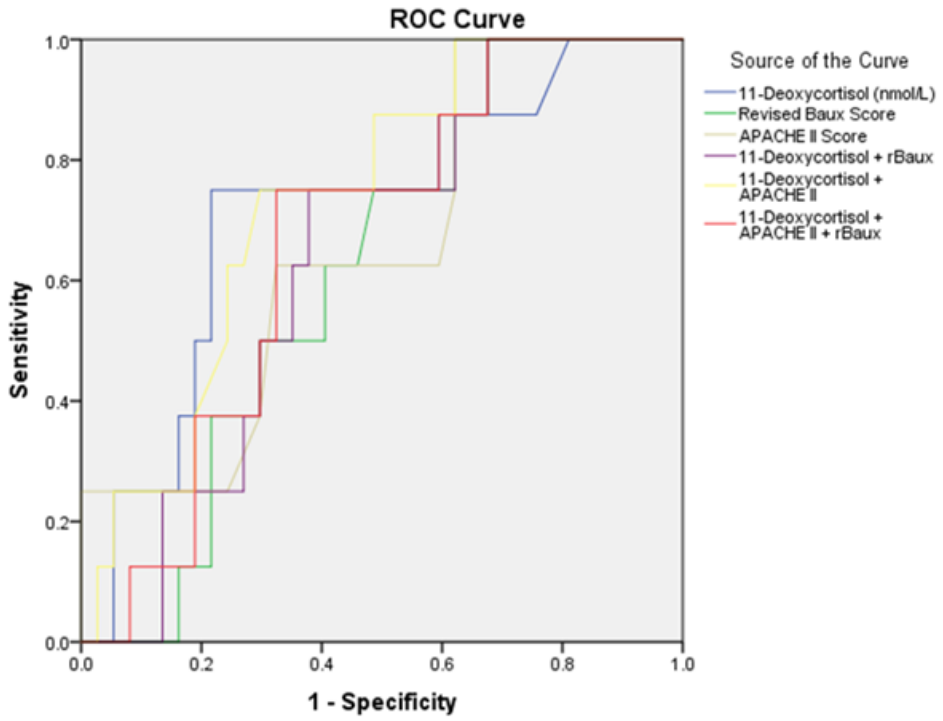
Table 3.4. Supplementing DHEAS and Testosterone may improve wound healing times following severe thermal injury.
Wound healing and steroids at all timepoints were assessed using uni-analyte and multi-analyte regression models to estimate treatment effects. Multi-variate linear regression was performed using categorical variables and natural logarithm of continuous variables accounting for patient demographics, injury severity and surgical procedures performed. Significant associations $p < 0.05$.

On admission, testosterone and DHEAS were significantly associated with wound healing times in burns patients independent of baseline patient and injury demographics, as well as surgical interventions. Increasing serum testosterone and DHEAS by 100% on admission may decrease wound healing duration by 66% and 55% respectively. Multi-variate analysis and significant therapeutic potential of steroids on wound healing following thermal injury are summarised in Table 3.4. Multi-analyte multi-variate regression models of steroids and wound healing in burns patients demonstrated minimal therapeutic value. Therefore, multi-analyte regression data on wound healing are not reported. Multi-variate regression analysis could not be performed using scarring data due to low patient numbers.

3.3.10 Steroids Potential as Diagnostic/Prognostic Biomarkers Following Severe Thermal Injury.

At D01 post-burn injury, serum levels of 11-Deoxycortisol in patients was significantly different between survivors and non-survivors (Figure 3.3 and Figure 3.4). 11-Deoxycortisol levels in serum may predict 28-day mortality and in-hospital mortality with 72% and 79% accuracy. When combining circulating levels of 11-Deoxycortisol with APACHE II score on admission, the predictive power is further increased to 74% for 28-day mortality and 84% for in-hospital mortality. AUC/ROC curve analysis for 11-Deoxycortisol and mortality outcomes are summarised in Figure 3.14 and Figure 3.15.

a.



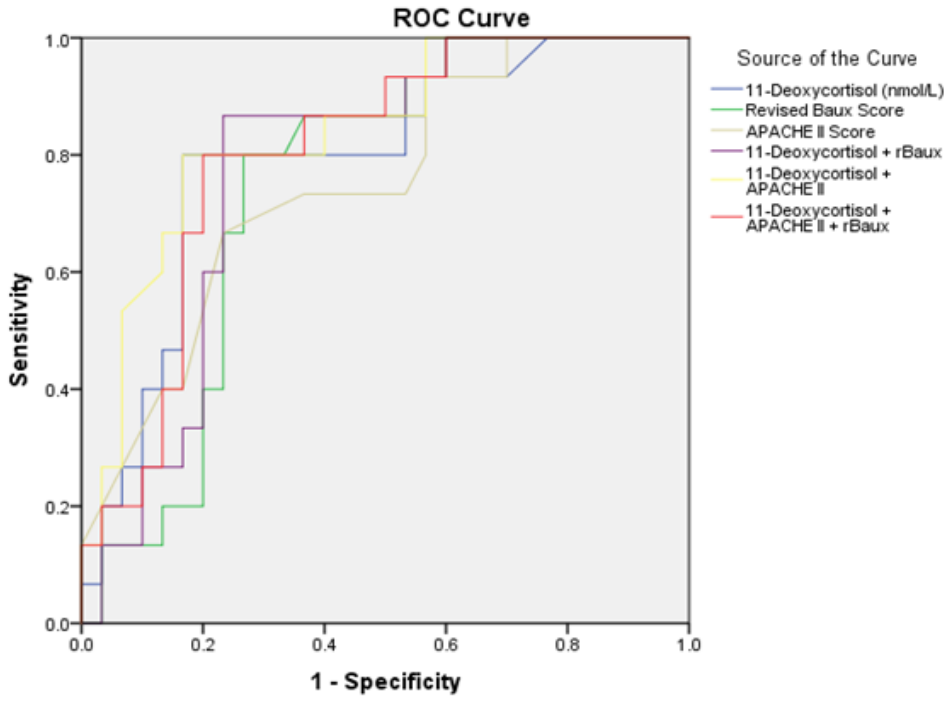
b.

Test Result Variable(s)	AUC
11-Deoxycortisol (nmol/L)	0.716
rBaux Score	0.617
APACHE II Score	0.657
11-Deoxycortisol + rBaux	0.642
11-Deoxycortisol + APACHE II	0.735
11-Deoxycortisol + APACHE II + rBaux	0.666

Figure 3.14. Serum 11-Deoxycortisol levels at D01 post-injury and 28-day mortality in burns patients.

Predictive strength of serum 11-Deoxycortisol levels at D01 post-injury on 28-day mortality were assessed using AUC/ROC curve analysis. a. ROC curve analysis of 11-Deoxycortisol levels and clinical scores both alone and in-combination; b. AUC analysis of 11-Deoxycortisol levels and clinical scores both alone and in-combination.

a.



b.

Test Result Variable(s)	AUC
11-Deoxycortisol (nmol/L)	0.791
Revised Baux Score	0.750
APACHE II Score	0.750
11-Deoxycortisol + rBaux	0.780
11-Deoxycortisol + APACHE II	0.838
11-Deoxycortisol + APACHE II + rBaux	0.804

Figure 3.15. Serum 11-Deoxycortisol levels at D01 post-injury and in-hospital mortality in burns patients.

Predictive strength of serum 11-Deoxycortisol levels at D01 post-injury on in-hospital mortality were assessed using AUC/ROC curve analysis. a. ROC curve analysis of 11-Deoxycortisol levels and clinical scores both alone and in-combination; b. AUC analysis of 11-Deoxycortisol levels and clinical scores both alone and in-combination.

3.3.11 Longitudinal Steroid and Immune-Endocrine Response following Severe Thermal Injury

The immune and endocrine systems are closely integrated. Acute severe burn injury induces major simultaneous and persistent immune-endocrine responses. Following injury, patients exhibited increased expression of both upstream and final metabolites of the HPA axis. Furthermore, elevated levels of both pro-inflammatory and anti-inflammatory cytokines and hormonal responses were observed following thermal injury. In addition, increased expression of multiple immune functions was exhibit in acutely burned patients(112).

Burn patients exhibited a prolonged differential immune-endocrine response when compared to healthy volunteers. The behaviour and expressions of immune and hormonal status between burned patients and healthy volunteers remained altered for at least one year following injury. This was particularly in the case of cortisol, cortisone, testosterone, DHEAS, neutrophil responses (ROS generation and phagocytosis), IGF-1, IL-β1 and TGF-β1.

At D01-D28, burns patients demonstrated a higher systemic expression of cortisol and cortisone. Interestingly, the expression of circulating cortisol was higher when compared to cortisone during the acute phase of burn injury followed by reversal in levels of circulating cortisol and cortisone levels at M02-M12. Additionally, higher systemic expression of pro-inflammatory cytokines, including IL-1β, TNF-α, IL-17 and IL12p70, was observed for the first month following thermal injury. This further

suggests the conversion of cortisone to cortisol secondary to induction of 11βHSD1 by the overwhelming systemic pro-inflammatory milieu following severe thermal injury.

Immediately following major burn injury, patients had elevated levels of circulating DHEAS and testosterone that coincided with increased levels of their respective upstream metabolites, DHEA and androstenedione. Throughout the course of treatment and management of acutely injured patients, the systemic concentrations of all four analytes declined progressively. Interestingly, DHEAS and testosterone levels in blood of burned patients increased dramatically at M02-M12 post-injury while their upstream metabolites remained low. This suggests increased production of DHEAS and testosterone from their precursors secondary to increased demand and utility by various cells and tissue during the prolonged hypermetabolic response following severe thermal injury.

The longitudinal immune-endocrine status in burned patients and healthy volunteers are summarized in Figure 3.16.

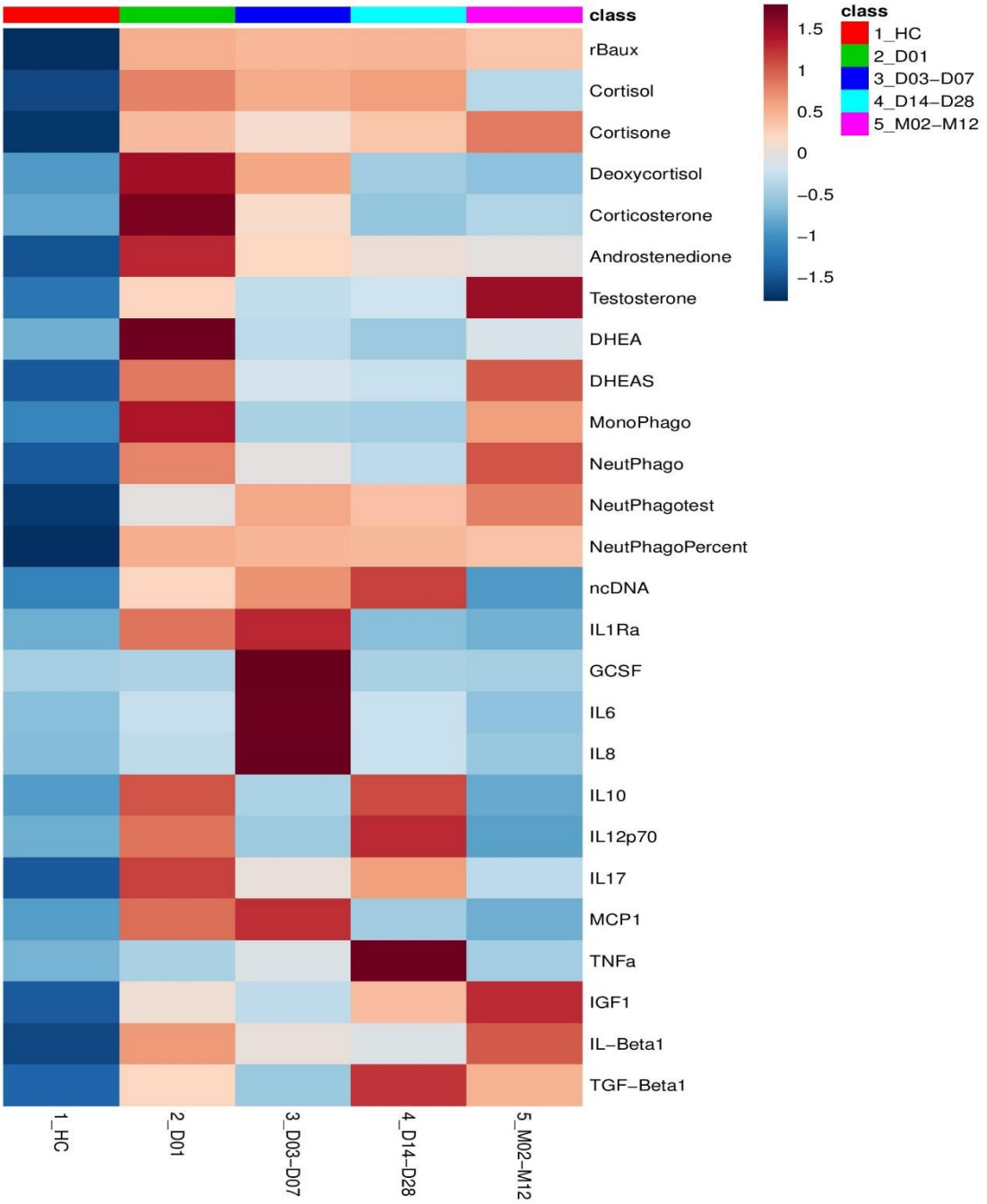


Figure 3.16. The longitudinal steroid and immune-endocrine response following major burn injury.

Circulating status of cytokines, immune function and steroid hormones were analysed across all timepoints accounting for the revised Baux score. Immune-endocrine status of healthy volunteers was performed to allow comparison. This heatmap represent expressions of molecules in serum by colour with high expressions being red and low expressions being blue. Immune function tests include Monocyte and neutrophil ROS generation (Phagoburst MFI), neutrophil phagocytosis MFI (% positive) and nuclear cfDNA (marker of NETs). Cytokines and other hormones analysed include IL-1Ra, GCSF, IL-6, IL-8, IL-10, IL-12p70, IL-17, MCP-1, TNF-α, IGF-1, IL-β1 and TGF-β1.

3.3.12 Subgroup Analysis

3.3.12.1 Gender Influence on Longitudinal Steroid Response following Severe Thermal Injury

Male and female burns patients exhibited significant differences in the steroid response following injury, despite the injury severity (rBaux Score) being similar between both cohorts, p 0.565.

Male burns patients had significantly reduced levels of systemic cortisone at D01- D28 post-injury compared to male healthy controls, median 34.3 nmol/L vs 52.8 nmol/L (p <0.001). Systemic levels of cortisone were similar between female healthy controls and burns patients at all timepoints. Furthermore, male patients had significantly higher levels testosterone compared to female patients at D01 and D14-M14 post-burn injury, median 3.5 nmol/L vs. 1.6 nmol/L (p 0.001) and 4.0 vs. 1.3 nmol/L (p <0.001) respectively.

Male burns patients exhibited generally lower levels of DHEA compared to female patients with D01 post-injury being significant, median 8.8 nmol/L vs. 21.6 nmol/L (p = 0.036) respectively. Conversely, male patients demonstrated generally increased levels of DHEAS compared to female patients with D03-D07 and M02-M12 post-thermal injury being significantly different, median 1.8 µmol/L vs 1.3 µmol/L (p = 0.005) and 3.4 µmol/L vs. 2.0 µmol/L (p = 0.008). Systemic steroid levels in male and female burns patients are summarised longitudinally in Figure 3.17.

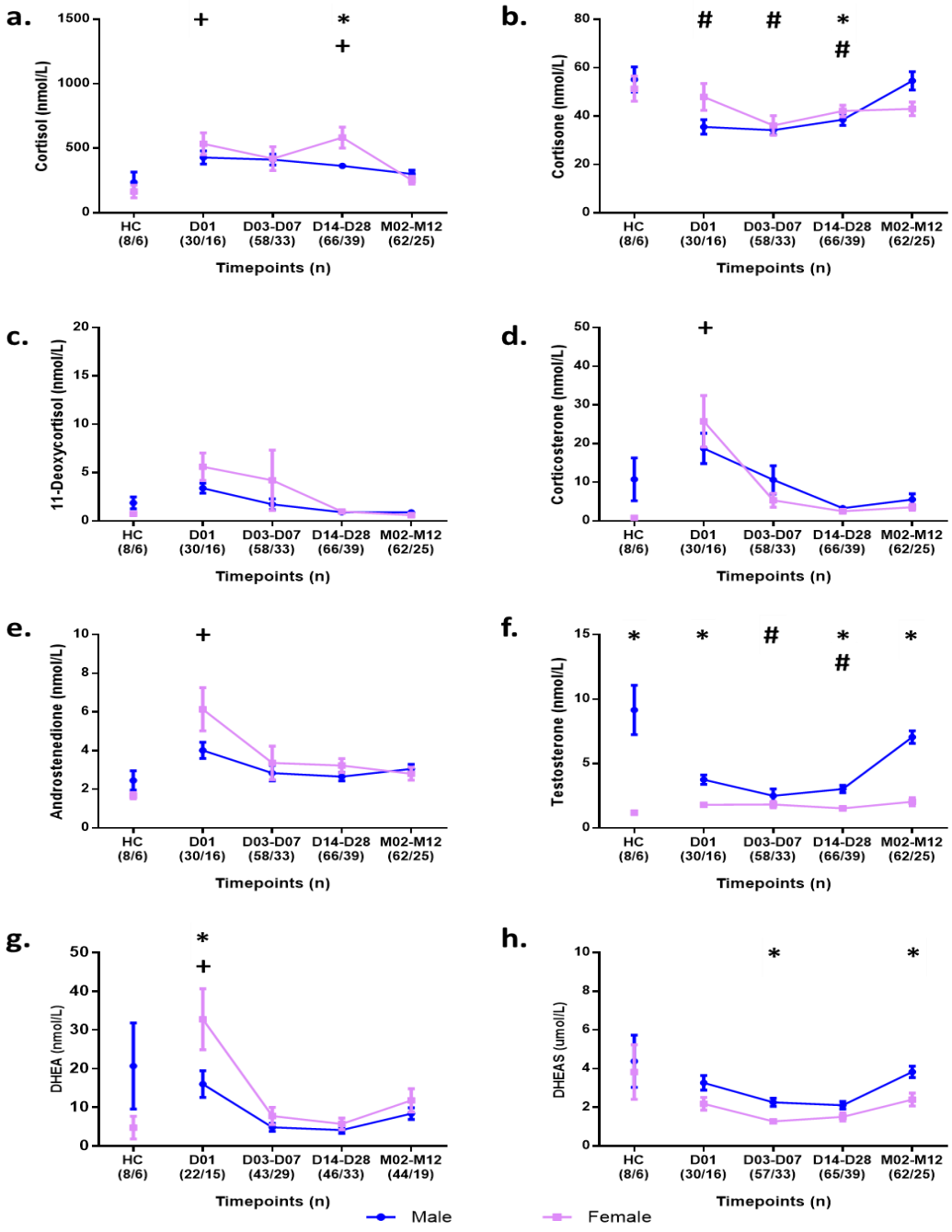


Figure 3.17. Longitudinal steroid response in females and males following burn injury.

Serum steroid levels in female and male burns patients were analysed across all timepoints. a. Cortisol; b. Cortisone; c. 11-Deoxycortisol; d. Corticosterone; e. Androstenedione; f. Testosterone; g. DHEA; h. DHEAS. Analyte levels at each timepoint was compared between both cohorts (Female vs. Male) using Mann-Whitney Test; * $p < 0.05$. Analyte levels of female and male burns patients were compared to female and male healthy controls (HC) using Kruskal-Wallis test with Dunn's multiple corrections; + Female vs. HC Female, $p < 0.05$; # Male vs. HC Male, $p < 0.05$.

3.3.12.2 Influence of age on Longitudinal Steroid Response following Severe Thermal Injury

Age significantly affected the steroid response following burn injury. Age is defined as young (< 65 years) and old (≥65 years). Injury severity and gender were similar between both cohorts, p = 0.328 and p = 0.618 respectively.

Older patients had significantly elevated circulating levels of cortisol and 11-deoxycortisol following severe thermal injury compared with younger patients, median 643.8 nmol/L vs. 263 nmol/L (p = 0.001) at D01-D07 and 6.8 nmol/L vs. 2.1 nmol/L (p = 0.012). Subsequently at D14-D28 post-thermal injury, older patients exhibited significantly decreased serum cortisol levels compared to younger patients, median 233.9 nmol/L vs. 372.2 nmol/L (p = 0.012). Circulating steroid levels in young and old burns patients are summarised longitudinally in Figure 3.18.

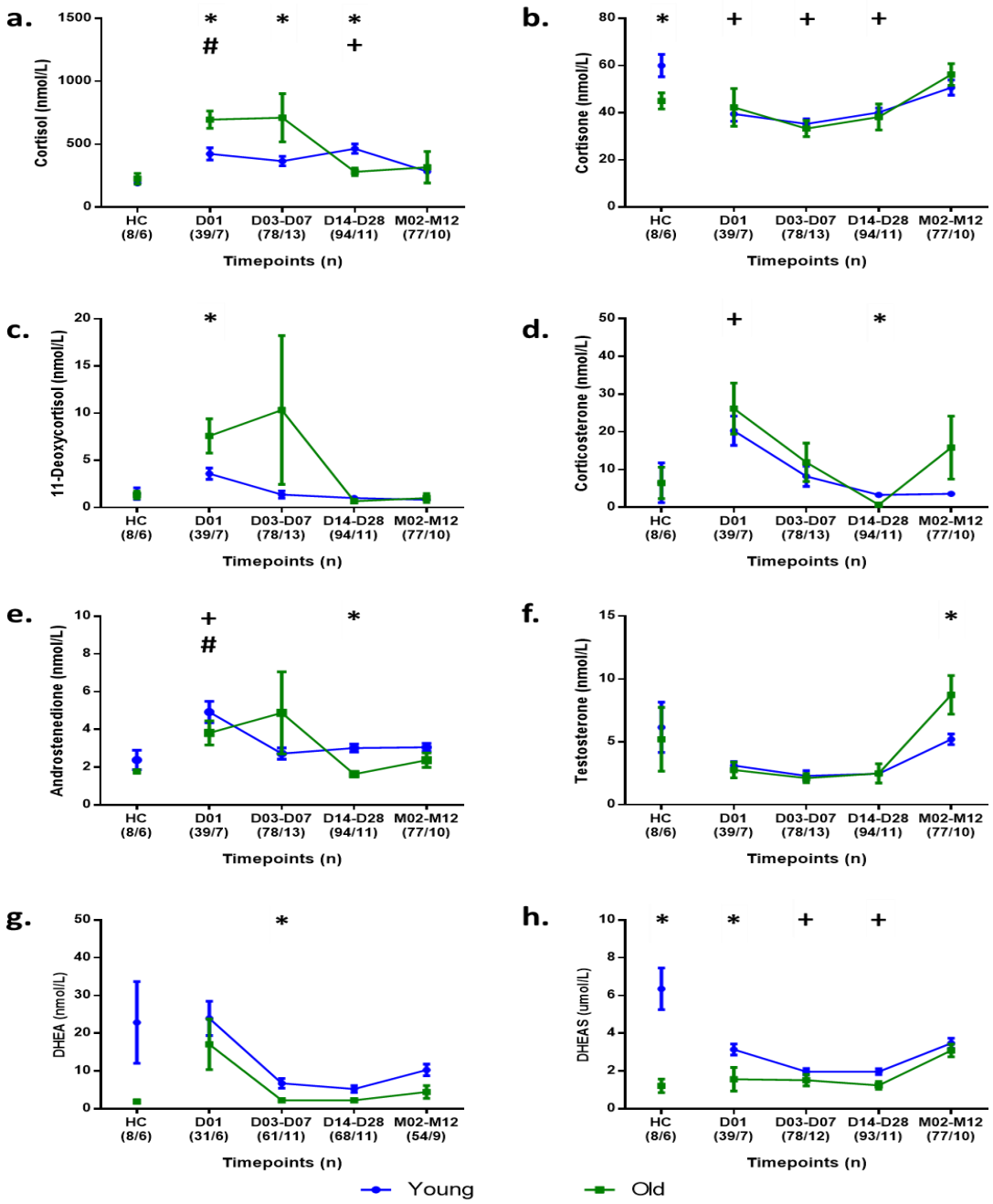


Figure 3.18. Longitudinal steroid response in young (<65 years) and old (≥65 years) patients following burn injury.

Serum steroid levels in young and elderly burns patients were analysed across all timepoints. a. Cortisol; b. Cortisone; c. 11-Deoxycortisol; d. Corticosterone; e. Androstenedione; f. Testosterone; g. DHEA; h. DHEAS. Analyte levels at each timepoint was compared between both cohorts (Young vs. Old) using Mann-Whitney Test; * p <0.05. Analyte levels of young and old burns patients were compared to young and old healthy controls (HC) using Kruskal-Wallis test with Dunn's multiple corrections; + Young vs. HC Young, p <0.05; # Old vs. HC Old, p <0.05.

3.3.12.3 Corticosteroid Influence on Longitudinal Steroid Response and Outcomes following Severe Thermal Injury

Patient demographics were similar between controls and corticosteroids groups. Corticosteroid-treated patients had significantly elevated revised Baux score compared to controls, median 106 vs. 84 (p 0.006). Patient demographics and outcomes are summarised in Supplementary Table 2. Corticosteroid treatment did not appear to influence the longitudinal steroid response in burns patients. Serum levels of steroid hormones in control and corticosteroid treated groups following severe thermal injury are summarised in Figure 3.19.

PSM analysis was performed to explore indications of corticosteroid administration and investigate the differences in outcomes of treated and non-treated burns patients. In this sub-group analysis of outcomes, 52 burns patients with ≥20% TBSA were assessed. Twenty-two patients received corticosteroids. The analysis accounts for sequential organ dysfunction over 4 timepoints spanning two weeks following severe thermal injury.

Corticosteroid-treatment and control burns patients were matched according to age, gender, TBSA, presence of inhalation injury, revised Baux score and timepoints. Out of 110 controls and 83 corticosteroid-treated burns patients, 98 and 41 were matched respectively. Matching was done within the designated caliper distances. Standardised mean differences (SMD) of co-variates pre- and post-matching were assessed for co-variate balance. Median SMD of pre-matched covariates was -0.278 with some co-variates exceeding 1.0. Median SMD of co-variates post-matching was 0.041 with all

co-variates within 0.130 range with exception of one count being 0.375. Furthermore, propensity score comparison between control and corticosteroids groups was not significant post-matching (p 0.793). This indicates robust matching. Propensity scores before and after matching are summarised in Figure 3.20.

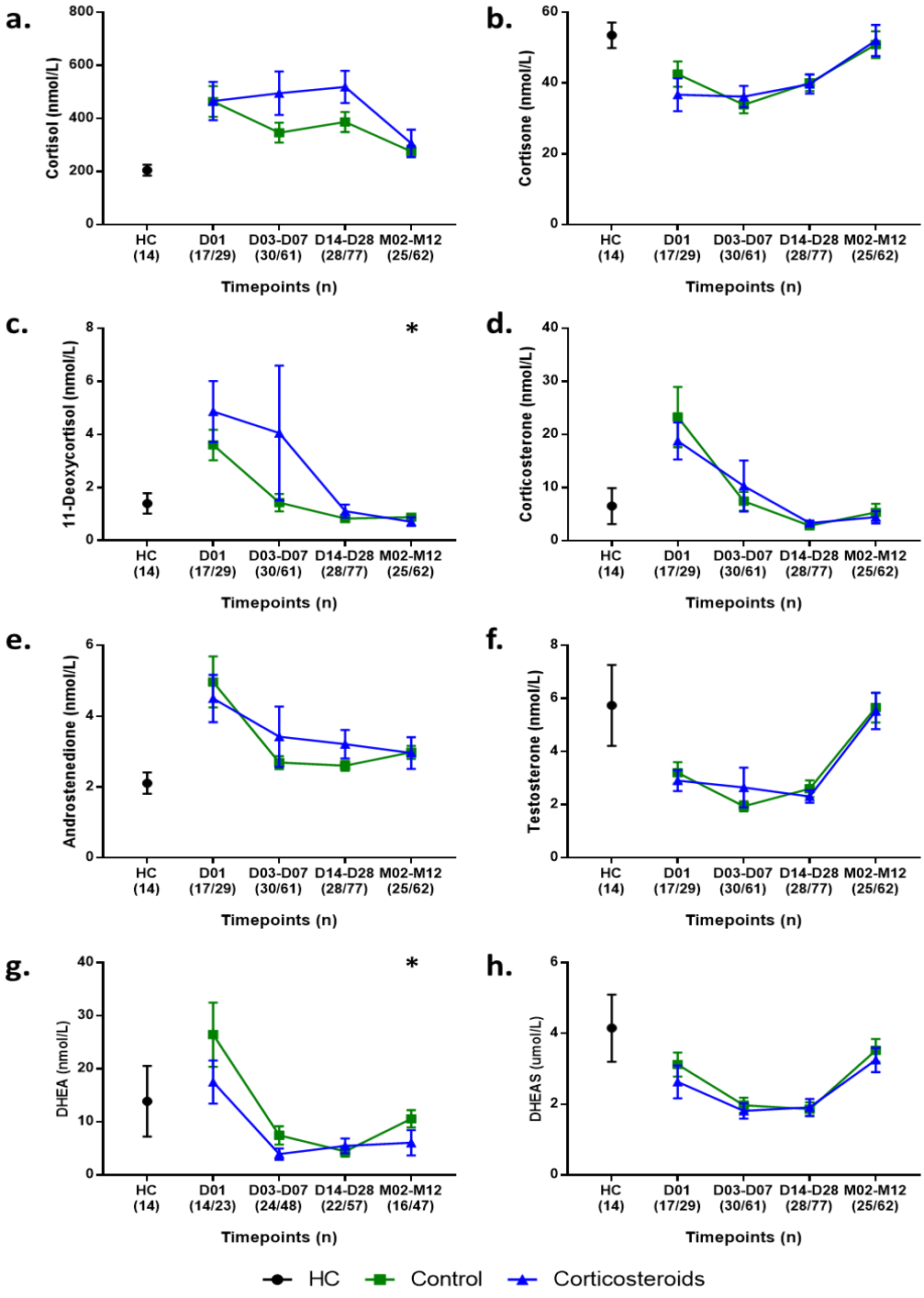


Figure 3.19. Longitudinal steroid response in burns patients who received and did not receive corticosteroids.

Serum steroid levels in burns patients who received corticosteroids and those who did not (control) were analysed across all timepoints. a. Cortisol; b. Cortisone; c. 11-Deoxycortisol; d. Corticosterone; e. Androstenedione; f. Testosterone; g. DHEA; h. DHEAS. Analyte levels at each timepoint was compared between both cohorts (Corticosteroid vs. Control) using Mann-Whitney Test; * $p < 0.05$.

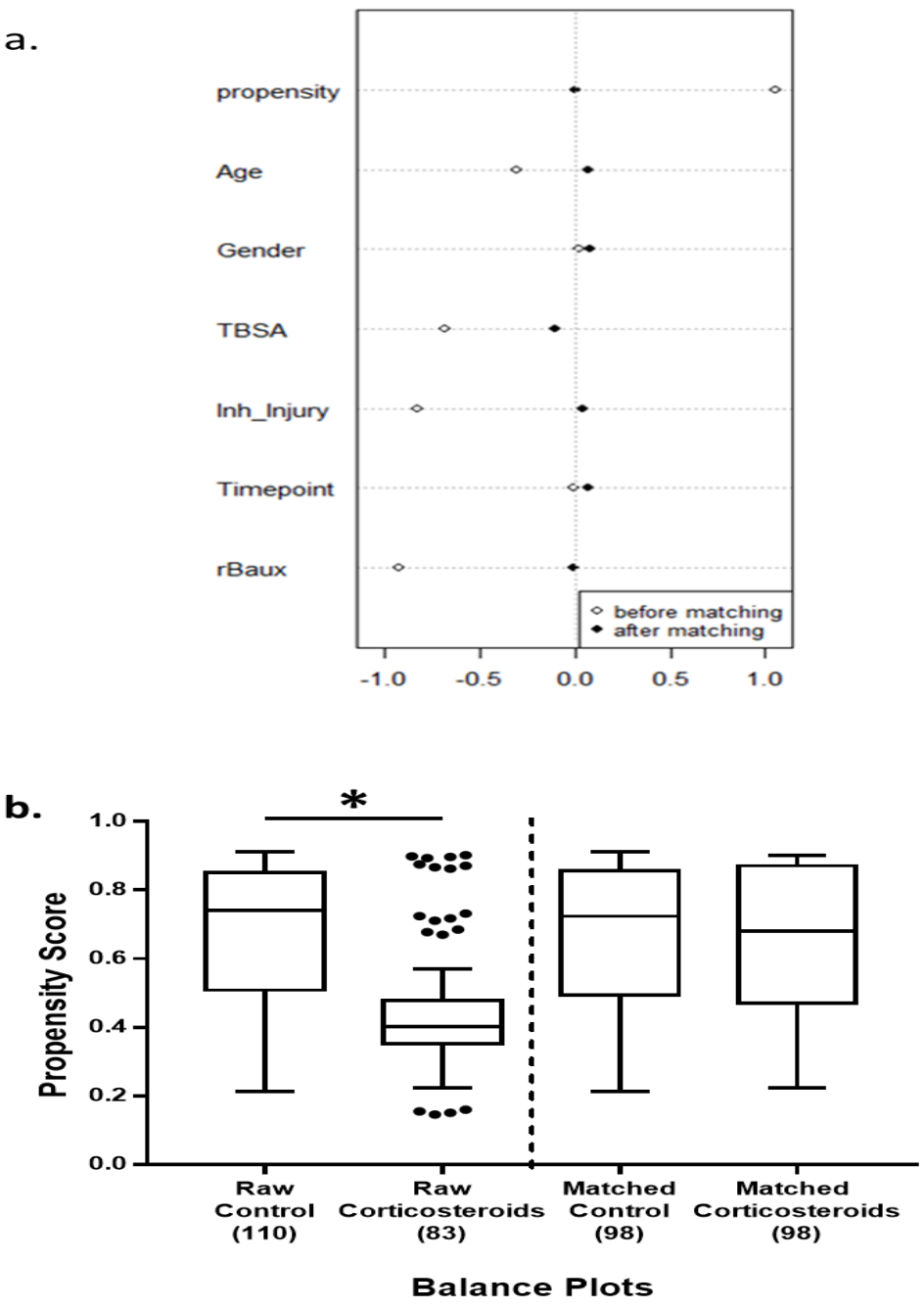


Figure 3.20. Propensity score balance plots matching control and corticosteroids burns patients.

Patient demographics, injury characteristics and timepoints were matched using PSM analysis. a. Propensity scores dot plot of baseline demographics and timepoints before and after matching. b. Propensity scores boxplot of control and corticosteroids burns patients before and after matching. Propensity scores of control and corticosteroid cohorts were compared using Mann-Whitney Test; * $p < 0.05$.

Following severe thermal injury, sequential organ failure assessment (SOFA) cardiac scores were utilised as an indicator of haemodynamic instability and vasoplegia during the acute stage. Corticosteroid therapy was started at median day 3 following burn injury for a median total duration of 5 days. At D03 post-injury, burns patients who were started on corticosteroid therapy have significantly higher SOFA cardiac score compared to control group, median 4 vs. 1 ($p = 0.027$). Furthermore, SOFA cardiac score was significantly higher in treated group compared to controls at D14 post-injury, median 1 vs. 0 ($p = 0.037$). This may indicate that the corticosteroid cohort were generally more critically ill than controls. SOFA cardiac scores of corticosteroid and control cohorts are summarised longitudinally in Figure 3.21.

Corticosteroid therapy had significant effects on outcomes following severe thermal injury. Burns patients treated with corticosteroids had significantly increased odds of developing sub-sequent sepsis, OR 6.0($p <0.001$). Furthermore, corticosteroid therapy was associated with significantly increased odds of mortality, OR 2.86 ($p = 0.016$). In addition, MOF is independently associated with corticosteroid use. These effects are independent of baseline characteristics and SOFA cardiac scores with their associated timepoints. The influence of corticosteroid therapy in burns patients is summarised in

Figure 3.22. Furthermore, survival distributions of significant outcomes associated with corticosteroid therapy in severely burned patients are summarised in Figure 3.23. This could be secondary to a corticosteroid induced alternations in immune-endocrine response as outlined in Figure 3.24.

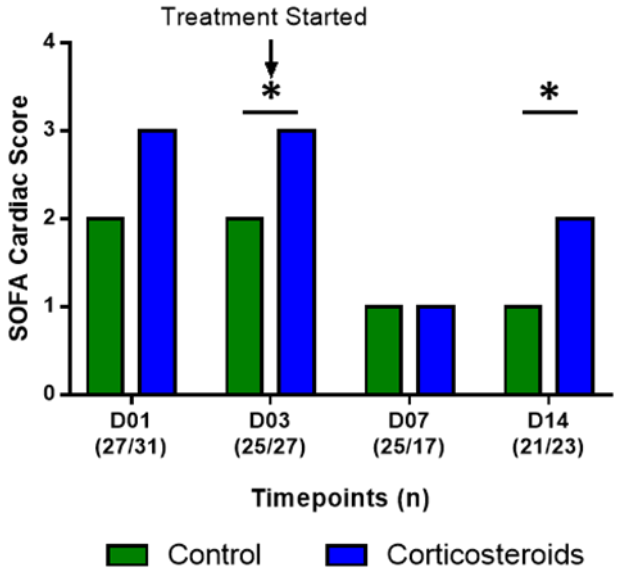


Figure 3.21. SOFA cardiac scores of PSM-matched control and corticosteroid burns patients.
Extent cardiac dysfunction of burns patients was examined at D01, D03, D07 and D14 post-injury. SOFA cardiac scores of control and corticosteroid cohorts were compared at each timepoint using Mann-Whitney Test; *p <0.05.

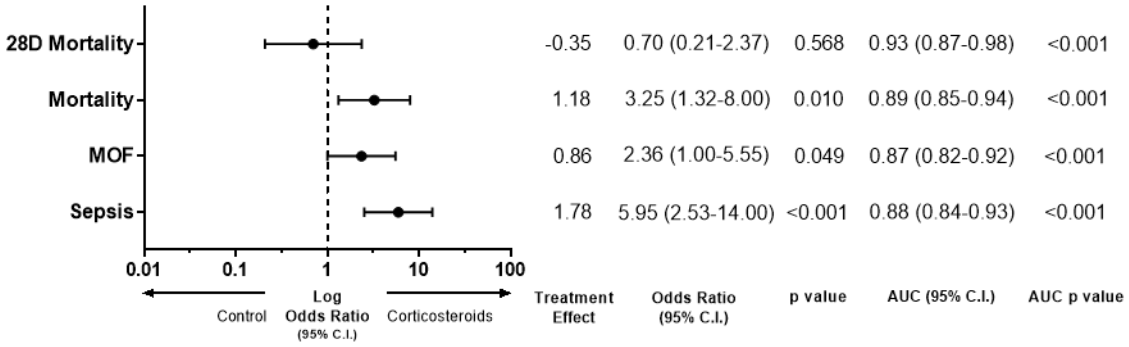


Figure 3.22. Corticosteroid use independently associated with mortality, MOF and sepsis following severe thermal injury.
Outcomes in PSM-matched control and corticosteroid groups were compared using logistic regression model accounting for age, gender, TBSA, inhalation injury, timepoints, SOFA cardiac score and corticosteroid use. Forest plots are odds ratios of outcomes with horizontal lines as 95% confidence intervals; *p <0.05. AUC analysis performed for each significant outcome regression model; * AUC p value <0.005.

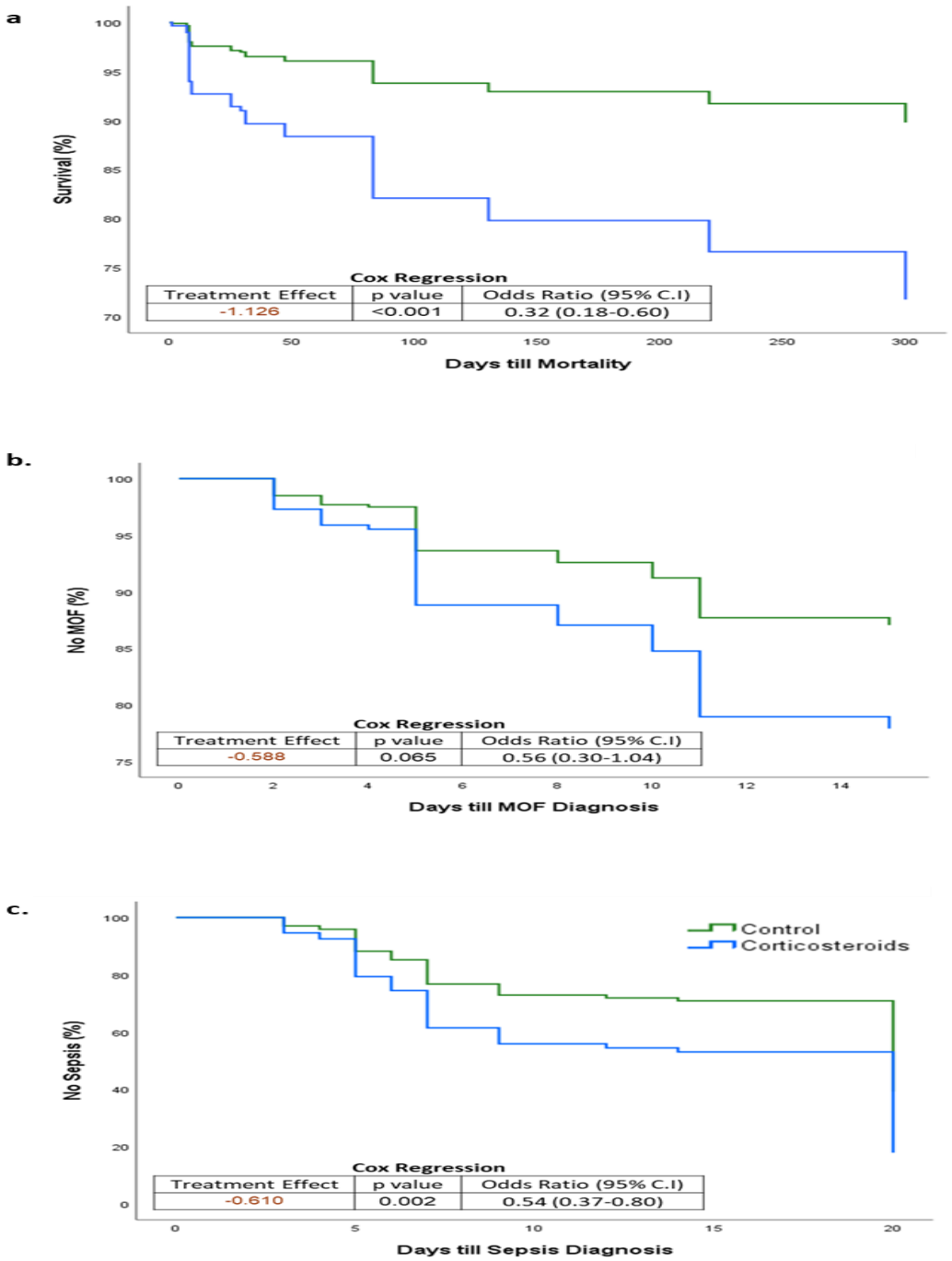


Figure 3.23. Kaplan-Meier survival graphs demonstrating significant distribution differences for in-hospital mortality and sepsis among control and corticosteroid treated burns patients.

Survival distribution analysis in PSM-matched control and corticosteroid groups was performed using Cox Regression accounting for age, gender, TBSA, inhalation injury, timepoints, SOFA cardiac score and corticosteroid use. a. Survival curve distributions for in-hospital mortality in control and corticosteroid groups. b. Survival (No MOF) curve distributions for MOF diagnosis in control and corticosteroid groups. c. Survival (No Sepsis) curve distributions for Sepsis diagnosis in control and corticosteroid groups; *p <0.05

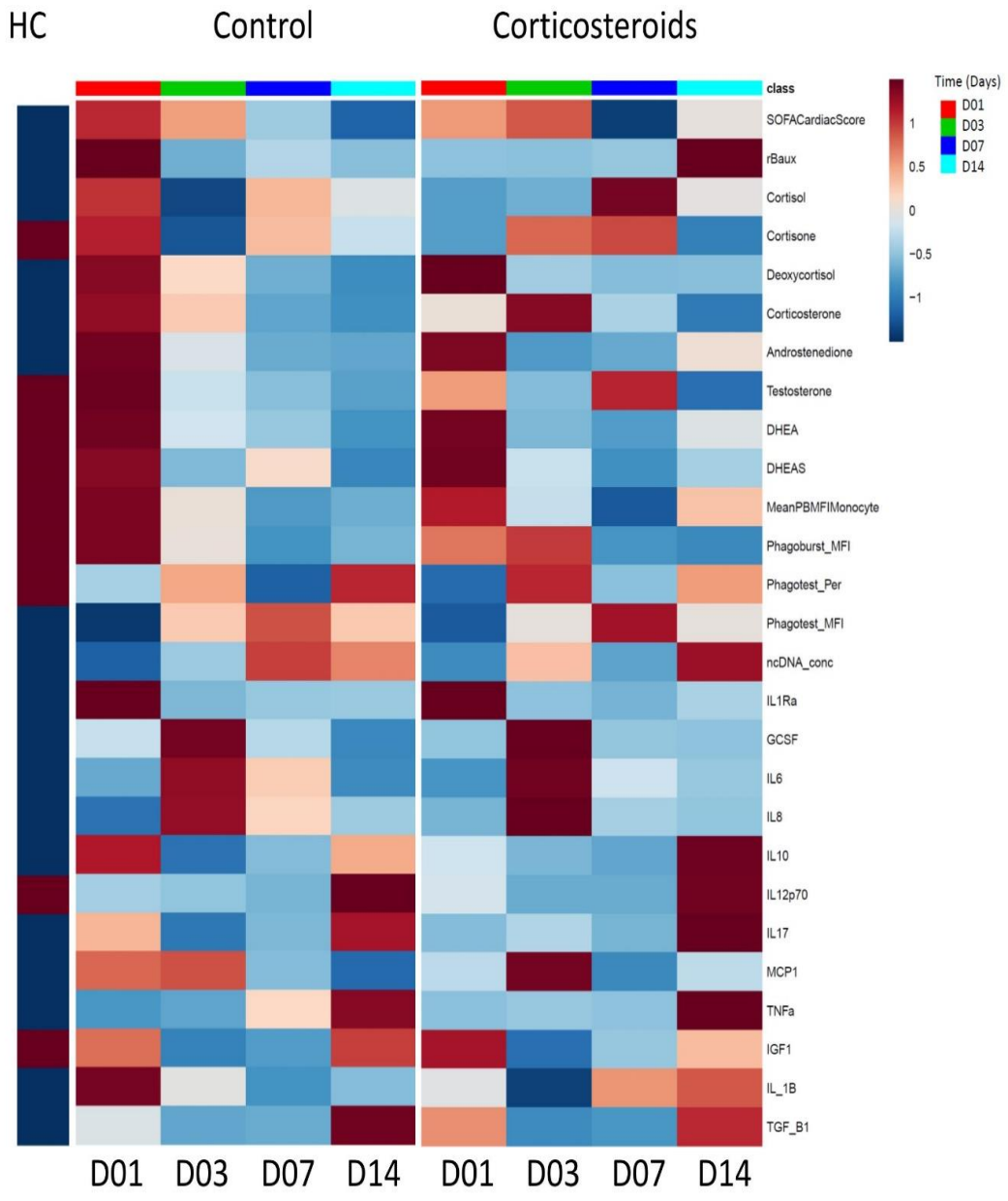


Figure 3.24. The longitudinal steroid and immune-endocrine response of PSM-matched burns patients treated and not treated (control) with corticosteroids. Circulating status of cytokines, immune function and steroid hormones were analysed at day 1, day 3, day 7 and day 14 post-thermal injury accounting for the revised Baux score and SOFA cardiac score. This heatmap represent expressions of molecules in serum by colour with high expressions being red and low expressions being blue. Immune-endocrine status of healthy volunteers was assessed for reference. Immune function tests include Monocyte and neutrophil ROS generation (Phagoburst MFI), neutrophil phagocytosis (Phagotest, % positive) and Nuclear cfDNA concentration (marker of NETs). Cytokines and other hormones analysed include IL-1Ra, GCSF, IL-6, IL-8, IL-10, IL-12p70, IL-17, MCP-1, TNF-α, IGF-1, IL-β1 and TGF-β1.

3.3.12.4 Influence of oxandrolone on the Longitudinal Steroid Response and Outcomes following Severe Thermal Injury

Patient demographics were similar between controls and corticosteroids groups. Oxandrolone-treated patients had significantly higher % TBSA compared to controls, median 45 vs. 27 (p 0.027). Patient demographics and outcomes are summarised in Supplementary Table 3.

Oxandrolone treatment may influence specific steroid hormones in burns patients. On treatment, systemic cortisone and 11-deoxycortisol levels were significantly reduced in the oxandrolone therapy group compared to the patients who did not receive oxandrolone (controls) at D03-D07, median 36.3 nmol/L vs. 42.3 nmol/L (p = 0.02) and 0.8 vs. 0.9 nmol/L (p = 0.010) respectively. Serum cortisol levels in burns patients treated with oxandrolone were generally reduced compared to the control group at D03-D07 post-injury, median 295.0 vs. 309.0 nmol/L (p = 0.088). Furthermore, serum DHEA levels in burns patients were significantly higher in the oxandrolone group compared to controls, median 2.9 nmol/L vs 1.9 nmol/L (p = 0.013) This may indicate that oxandrolone-treated burns patients were more stable systemically than the controls. The longitudinal steroid response in control and corticosteroid groups following severe burn injury is summarised in Figure 3.25.

PSM analysis was performed to explore the systemic status of burns patients who received and did not receive oxandrolone administration, as well as investigating the differences in outcomes of treated and non-treated burns patients. In this sub-group analysis of outcomes, all burns patients within e-SIFTI were assessed. Thirty-four burns

patients received oxandrolone. The analysis accounts for sequential organ dysfunction over 4 timepoints spanning two weeks following severe thermal injury. Oxandrolone-treated and control burns patients were matched according to age, gender, TBSA, presence of inhalation injury, revised Baux score and timepoints. Out of 51 controls and 115 oxandrolone-treated burns patients, 32 and 93 were matched respectively. Matching was done within the designated caliper distances. Standardised mean differences (SMD) of co-variates pre- and post-matching were assessed for co-variate balance. Median SMD of pre-matched covariates was 0.124with some co-variates exceeding 1.0. Median SMD of co-variates post-matching was 0.02 with all co-variates within 0.2 range. Furthermore, propensity score comparison between control and groups was not significantly different post-matching ($p = 0.570$). This indicates robust matching. Propensity scores before and after matching are summarised Figure 3.26.

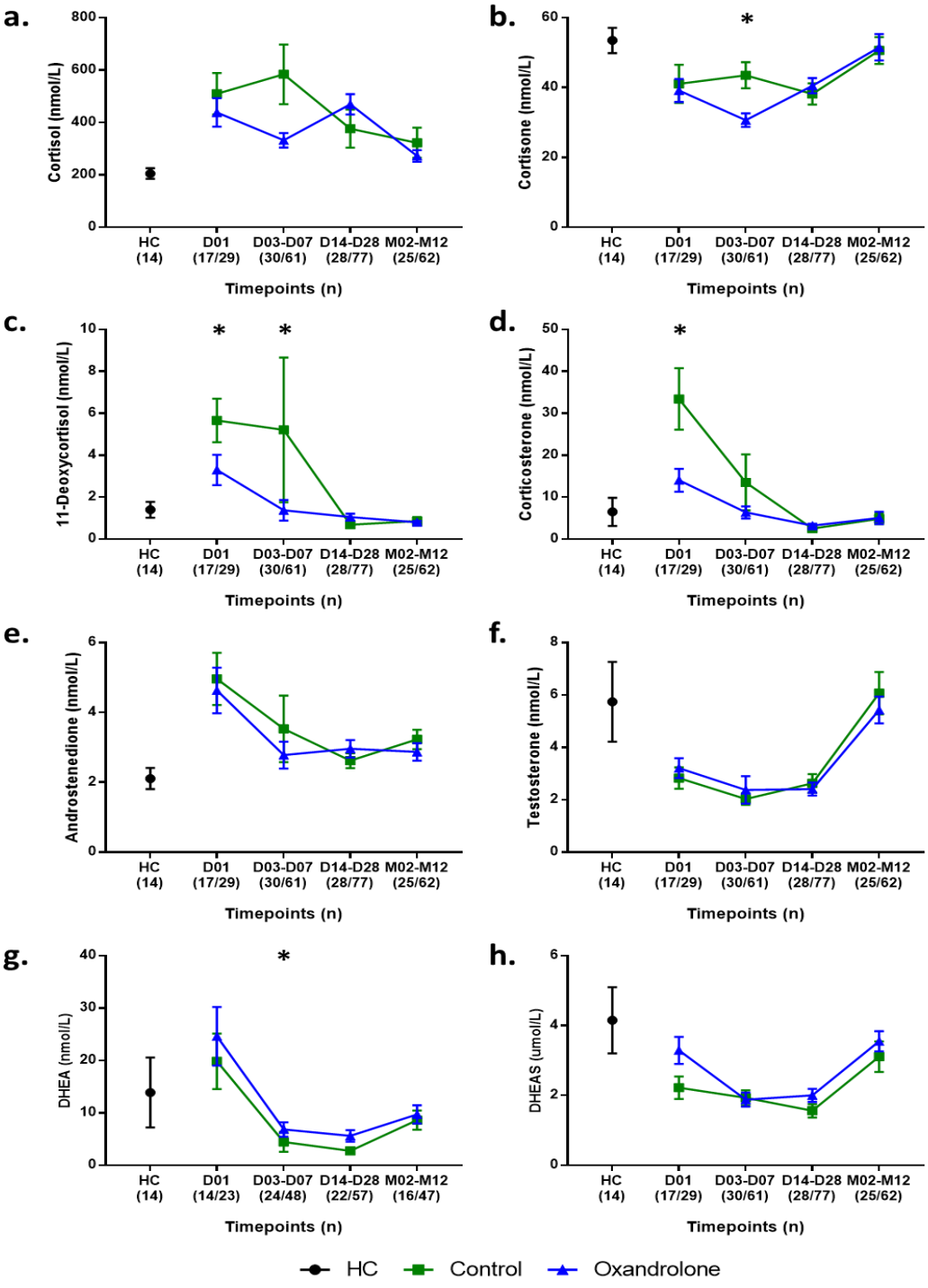


Figure 3.25. Longitudinal steroid response in burns patients who received and did not receive oxandrolone.

Serum steroid levels in burns patients who received and did not receive oxandrolone were analysed across all timepoints. a. Cortisol; b. Cortisone; c. 11-Deoxycortisol; d. Corticosterone; e. Androstenedione; f. Testosterone; g. DHEA; h. DHEAS. Analyte levels at each timepoint was compared between both cohorts (Oxandrolone vs. Control) using Mann-Whitney Test; * $p < 0.05$.

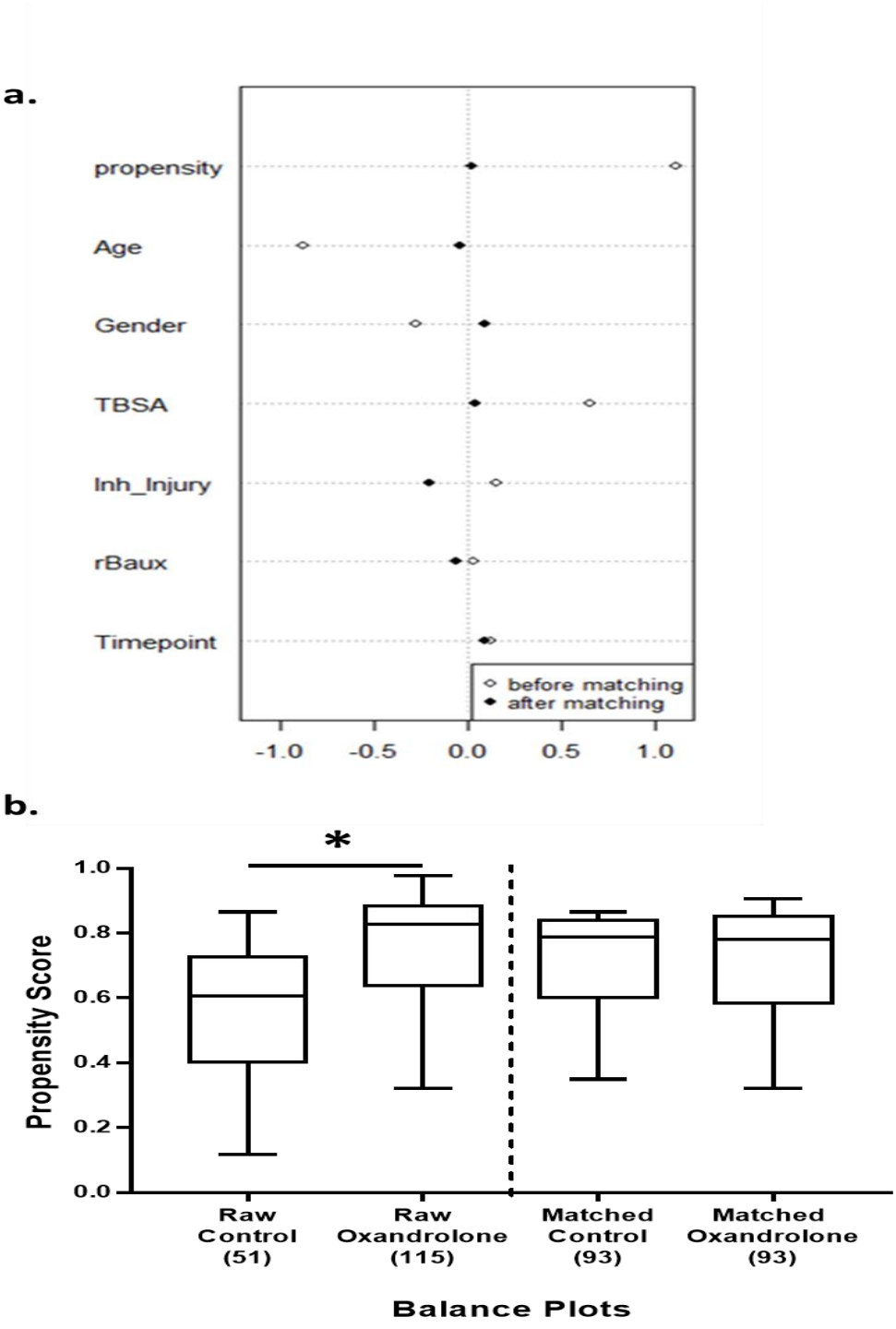


Figure 3.26. Propensity score balance plots matching control and oxandrolone burns patients.

Patient demographics, injury characteristics and timepoints were matched using PSM analysis. a. Propensity scores dot plot of baseline demographics and timepoints before and after matching. b. Propensity scores boxplot of control and oxandrolone burns patients before and after matching. Propensity scores of control and oxandrolone cohorts were compared using Mann-Whitney Test; * $p < 0.05$.

Following severe thermal injury, sequential organ failure assessment (SOFA) liver scores were utilised as liver function dictates the initiation/delay of oxandrolone therapy in burns patients following the establishment of nutrition. Oxandrolone treatment was started at median day 5 following burn injury for a median total duration of 19 days. At D03 post-injury, burns patients who were subsequently started on oxandrolone therapy had similar SOFA liver scores compared to the control group, median 3 vs. 3 ($p = 0.744$). Following administration of oxandrolone, treated burns patients exhibited significantly lower SOFA liver scores compared to controls at D07 post-injury, median 2 vs. 3 ($p < 0.001$). This may indicate that the oxandrolone may have been omitted in burns patients due to liver dysfunction. SOFA liver scores of oxandrolone and control cohorts are summarised longitudinally in Figure 3.27.

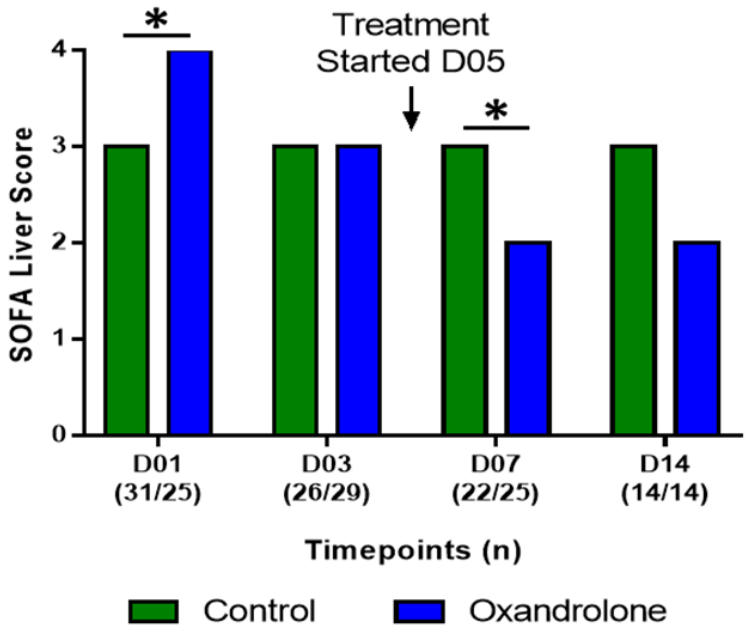


Figure 3.27. SOFA Liver scores of PSM-matched control and oxandrolone burns patients.
Extent of liver dysfunction of burns patients was examined at D01, D03, D07 and D14 post-injury. SOFA liver scores of control and corticosteroid cohorts were compared at each timepoint using Mann-Whitney Test; *$p < 0.05$.

Oxandrolone therapy had significant effects on outcomes following severe thermal injury. Oxandrolone treatment reduced the risk 28-day mortality ($p < 0.001$), in-hospital mortality ($p < 0.001$) and sepsis ($p = 0.008$). Conversely, oxandrolone use was associated with significantly increased risk of MOF, OR 7.90 ($p < 0.001$). These effects are independent of baseline characteristics and SOFA liver scores with their associated timepoints. The influence of oxandrolone therapy in burns patients is summarised in Figure 3.28. Furthermore, survival distributions of significant outcomes associated with oxandrolone treatment in severely burned patients are outlined in Figure 3.29. These effects could be due to alternations in immune-endocrine response mediated by oxandrolone as illustrated in Figure 3.30.

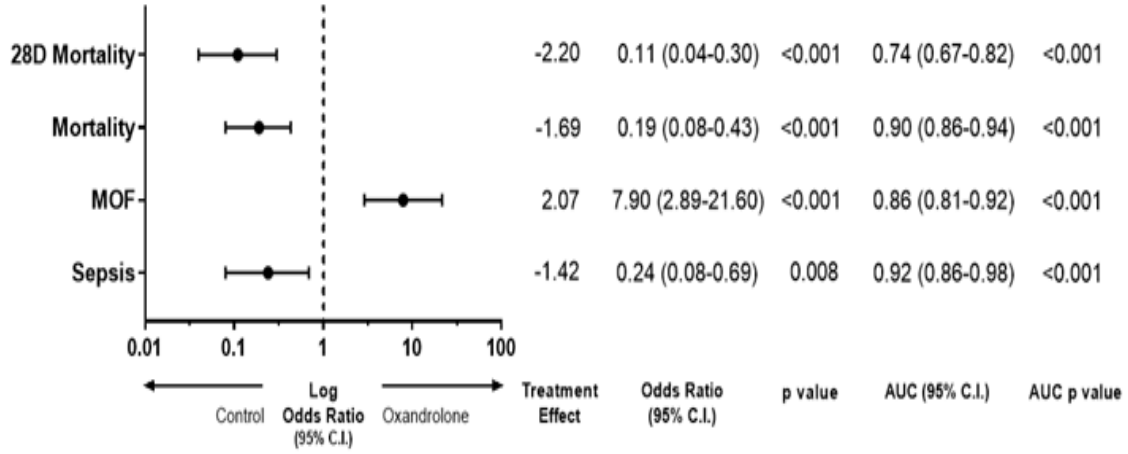


Figure 3.28. Oxandrolone use was independently associated with improved sepsis and survival rates following severe thermal injury.
Outcomes in PSM-matched control and oxandrolone groups were compared using logistic regression model accounting for age, gender, TBSA, inhalation injury, timepoints, SOFA liver scores and oxandrolone use. Forest plots are odds ratios of outcomes with horizontal lines as 95% confidence intervals; *$p < 0.05$. AUC analysis performed for each significant outcome regression model; * AUC p value <0.005.

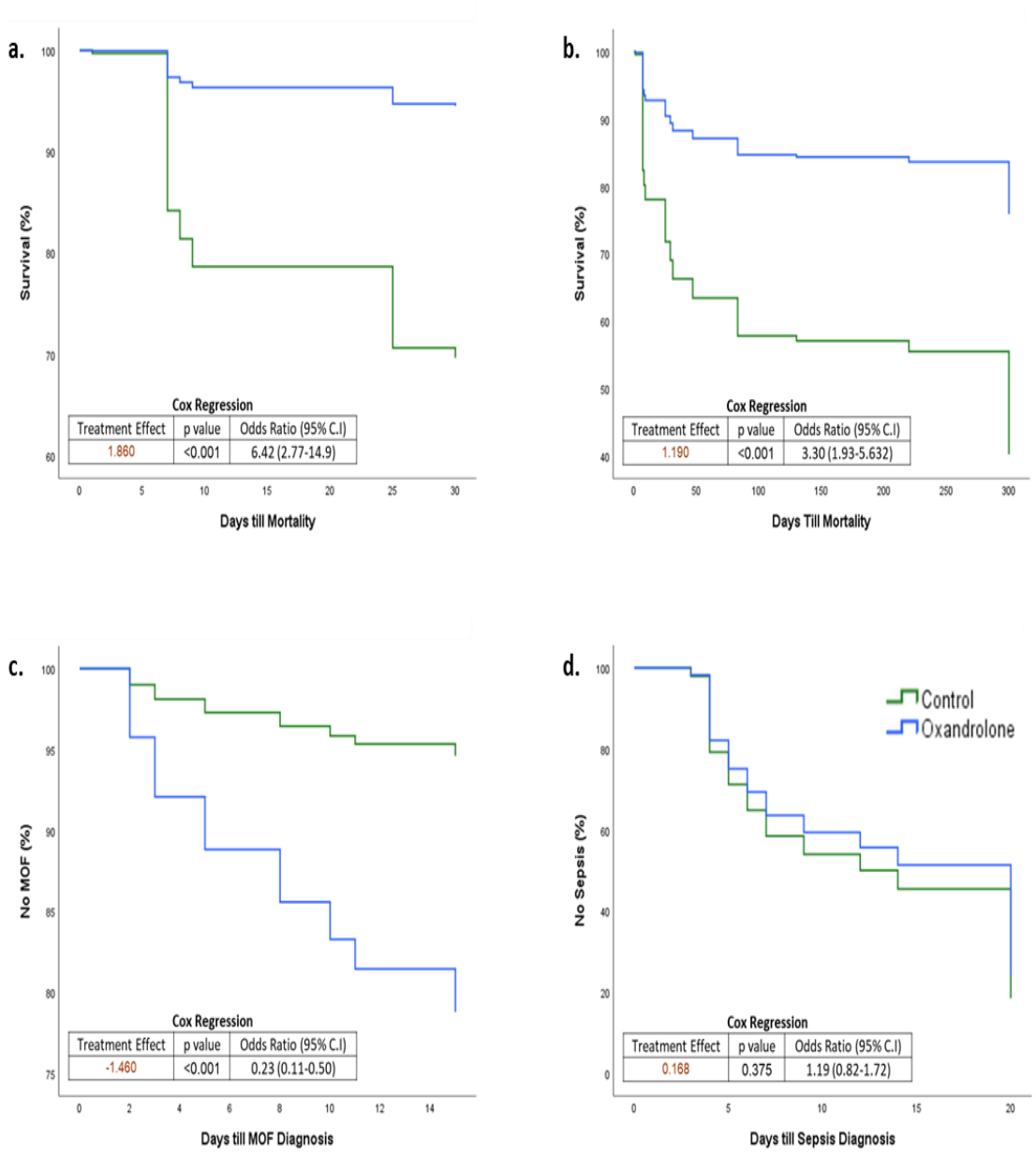


Figure 3.29. Kaplan-Meier survival graphs demonstrating significant differences in 28-mortality, in-hospital mortality and MOF distributions in control and oxandrolone treated burns patients.

Survival distribution analysis in PSM-matched control and corticosteroid groups was performed using Cox Regression accounting for age, gender, TBSA, inhalation injury, timepoints, SOFA liver score and oxandrolone use. a. Survival curve distributions for 28-day mortality in control and oxandrolone groups. b. Survival curve distributions for in-hospital mortality in control and oxandrolone groups c. Survival (No MOF) curve distributions for MOF diagnosis in control and corticosteroid groups. d. Survival (No Sepsis) curve distributions for Sepsis diagnosis in control and corticosteroid groups; *p <0.05

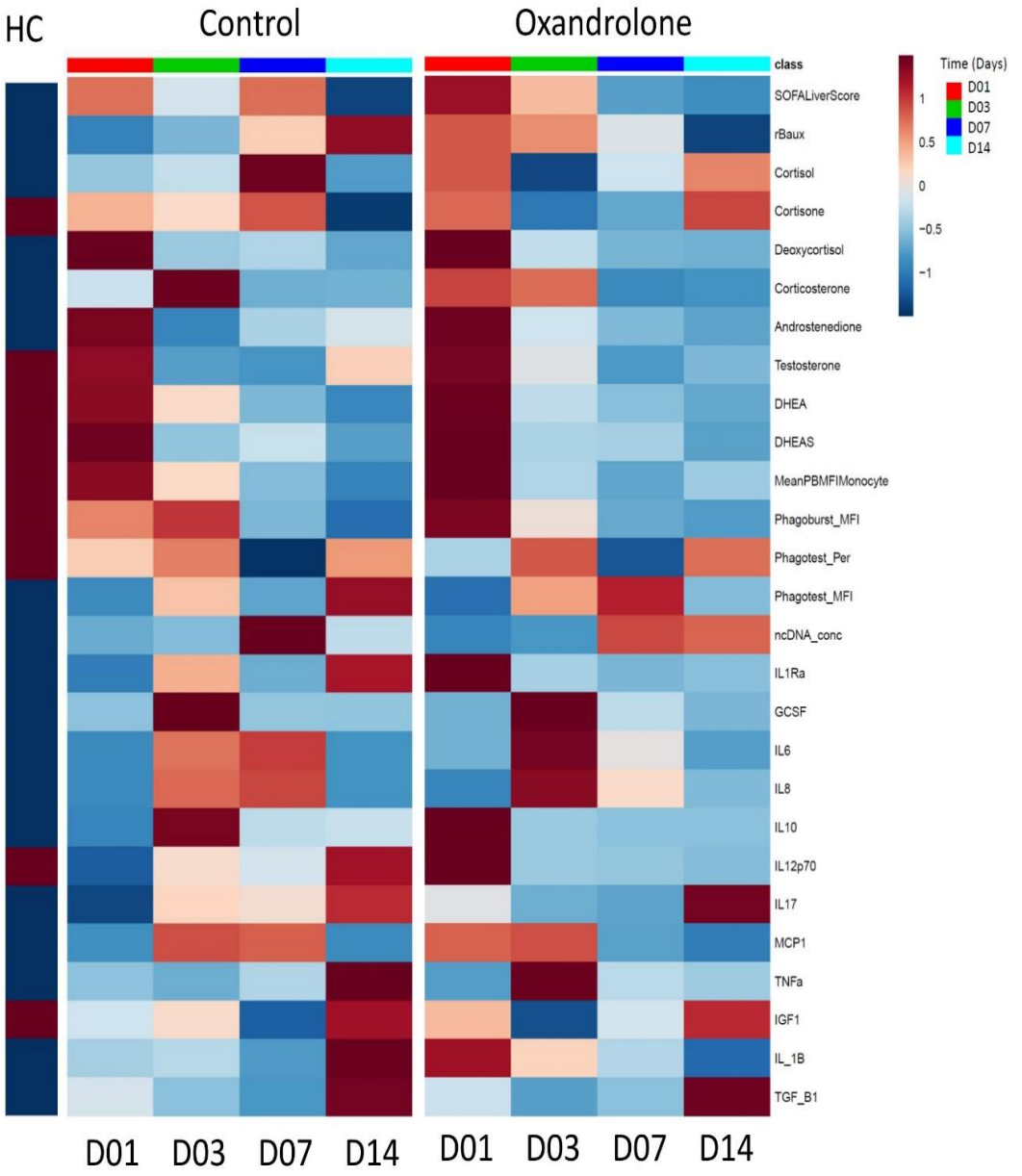


Figure 3.30. The longitudinal steroid and immune-endocrine response of PSM-matched burns patients treated and not treated (control) with oxandrolone.
Circulating status of cytokines, immune function and steroid hormones were analysed at day 1, day 3, day 7 and day 14 post-thermal injury accounting for the revised Baux score and SOFA Liver score. This heatmap represent expressions of molecules in serum by colour with high expressions being red and low expressions being blue. Immune-endocrine status of healthy volunteers was assessed for reference. Immune function tests include Monocyte and neutrophil ROS generation (Phagoburst MFI), neutrophil phagocytosis (Phagotest, % positive) and Nuclear cfDNA concentration (marker of NETs). Cytokines and other hormones analysed include IL-1Ra, GCSF, IL-6, IL-8, IL-10, IL-12p70, IL-17, MCP-1, TNF-α, IGF-1, IL-β1 and TGF-β1.

3.4 Discussion

On admission, severely burned patients are assessed using multiple clinical scoring systems aiming to predict outcomes (463-465). This is primarily done to aid clinical judgement and treatment, as well as resource allocation. Even when the decision for active treatment is made, identification of burned patients at risk of developing adverse outcomes remains a challenge. This could be attributed to overlap of symptoms and signs of sepsis and MOF/organ dysfunction with SIRS following major burn injury (466). Furthermore, any delay in identification or treatment of sepsis or organ dysfunction was shown to significantly increase mortality (467, 468). This has led to multiple studies identifying potential biomarkers to predict outcomes (112, 469-471).

This study was carried out to profile the steroid response for up to 12 months following severe burn injury using high sensitivity LC-MS/MS to gain an in depth understanding of steroid species changing after injury. It also investigated the associations between the altered steroid axis and outcomes in burn patients at various timepoints throughout the first year following thermal injury. Furthermore, this study explored the diagnostic and therapeutic potential of steroid changes and examined the efficacy of current steroid-based therapeutics, specifically oxandrolone, used as standard of care in a tertiary burn center in the West Midlands region, UK. This is the first report exploring these areas simultaneously.

Several reports have observed age and gender differences in short and long-term outcomes following major burn injury, with females and older patients associated with

poorer prognosis (406, 440-443). Although the mechanisms behind such observations are complex, it may partly be explained by differences in adrenal and gonadal steroid hormones. Although some studies have investigated the influence of steroids following burn injury, these studies typically involved testosterone and/or estradiol use in experimental animal models (133). These animal burn models suggest the beneficial role of estradiol in ameliorating the immune-inflammatory and hypermetabolic responses following injury(444, 445). Additionally, anti-androgens and castration treatments were reported to reduce remote end-organ inflammation and damage in animal models following thermal injury(178, 448). However, such studies should be interpreted with caution due to poor correlation between animal models and human counterparts of similar pathologies (472). This may, in part, explain the significant differences in outcomes following major burn injury between experimental animal models and clinical epidemiological studies.

Bergquist et al explored the adrenal/gonadal steroid axis in 16 adult burn patients prospectively and profiled the adrenal/gonadal response longitudinally till day 21 post-thermal injury using LC-MS/MS(129). The findings reported by Bergquist et al include: serum cortisol, cortisone and DHEA remained within reference range throughout the study period; reduced circulating levels of testosterone throughout the study period; estrone was mostly elevated throughout the study period while estradiol was within or below the reference range.

There were therefore significant differences and similarities in the observations made in Bergquist et al report and this study. Firstly, serum cortisol in burned patients were

significantly increased compared to healthy volunteers in this study and other published literature (473, 474). Simultaneously, serum cortisone levels in patients were significantly reduced compared to healthy individuals for the first month post-burn injury. This observation is novel as no other published reports describing cortisone levels following thermal injury were identified. Severe thermal injury is a stressor that is associated with release inflammatory cytokines and subsequent systemic elevation of cortisol (474, 475). Inflammatory environments were reported to induce the enzyme 11β-HSD1 converting cortisone to cortisol at both local and systemic levels (462, 476, 477). Subsequently, this systemic increase in cortisol levels can lead to significant immune-inflammatory modulation and metabolic effects in burned patients including immunosuppression and catabolism (475, 478-480). In this study, acute thermal injury resulted in immediate increased immune function (including ROS production, phagocytosis and NETosis), inflammatory cytokines (including IL-1β, TNF-α, IL-10, IL12p70, IL-17 and MCP1), cortisol and cortisone. Low circulating cortisone in burned patients compared to healthy volunteers suggests increased metabolism and conversion to cortisol through induction of 11β-HSD1 pathway. Subsequently, at D03-D28 following injury, burned patients maintained elevated systemic cortisol with reduced immune function, indicated by lower ROS production. These data are novel as we could not find critical care or trauma studies exploring cortisol associations with various immune function in the literature. However, studies have reported similar findings following severe psychological stress in bereaved individuals(481, 482). Furthermore, similar results were reported in equine models(483). Interestingly, this inverse relationship between neutrophil oxidative

burst and cortisol remains maintained at M02-M12 post-burn injury when systemic cortisol was low while ROS production expression of circulating monocytes and neutrophils was high. This highlights the need for robust quantitative statistical analysis of the data to conclude these findings.

Secondly, severe thermal injury significantly elevated systemic levels of DHEA but did not affect circulating levels of testosterone in patients at D01 in this study. Apart from Bergquist et al's study (129), no other studies explored the gonadal axis in adult burned patients using LC-MS/MS techniques. Jeschke et al has reported similar findings with testosterone levels in serum being maintained immediately following thermal injury in the paediatric population (474). Due to scarcity of data, definitive conclusions may prove difficult to make on the status of sex steroids following thermal injury. However, the status of DHEA/DHEAS and testosterone proved important in this study due to their association with mortality, sepsis and wound healing following multi-variate analysis (discussed below). This highlights the need for further exploration and profiling of sex steroid hormones status in critically ill populations including burns and trauma.

Berquist et al's report on the influence of steroids on outcomes has been limited to mortality (129). Elevated serum levels of androstenedione, cortisone, DHEA, pregnenolone, 17-OH pregnenolone and 17OH-progesterone at D01 post-injury was associated with mortality following thermal injury (129). Sepsis data was collected but no associations were reported (129). This could be attributed to the low number of burned patients recruited during the study period and the high cost of sample

processing using high sensitivity LC-MS/MS. Other limitations include the male-only cohort and the analysis being confined to the acute phase of injury. Thus, generalisability and clinical applicability of the results cannot be made from this study.

In this study, the longitudinal HPA and HPG responses in female and male burned patients, as well as young and elderly patients were assessed from day one up to 1-year post-injury. Significant differences in circulating steroid hormone levels and their up-stream metabolites were observed between the cohorts of both groups. Similarly, significant differences in the HPA and HPG status on burn patients who developed adverse outcomes and those who did not. Such steroid hormones influencing outcomes included cortisol, DHEA, DHEAS and testosterone, as well as their respective up-stream metabolites. However, results should be adjusted to confounding variables including age, gender and injury severity, to generalise interpretation and permit clinical translation.

In this study, 11-deoxycortisol, an upstream metabolite to cortisol, was found to be significantly elevated at D01 among non-survivors of major burn injury. Circulating levels of 11-Deoxycortisol at D01 post-injury had a moderate predictive value for 28-day and in-hospital mortality, AUROC 0.72 and 0.79 respectively. The predictive value of serum 11-Deoxycortisol levels increased when accounting for APACHE II score of severely burned patients, AUROC 0.74 (28-day mortality) and 0.84 (in-hospital mortality). When compared to other published biomarkers, serum 11-Doxycortisol levels perform similar to, or better in predicting mortality at D01 post-injury. For example, Cato et al reported platelets as a predictor of survival following major burn

injury with AUROC of 0.53 (stand-alone) and 0.85 when combined with rBaux (471). Furthermore, Yang et al reported circulating neutrophil gelatinase-associated lipocalin levels may predict mortality in burn patients admitted to intensive care, AUROC 0.80 at 6 hours post-admission (484).

Additionally, lower levels of circulating DHEA, DHEAS and testosterone during the acute phase were independently and significantly associated with sepsis, 28-day mortality and mortality in this study. This suggests that supplementation of these steroids may improve outcome for the following reasons. DHEAS and testosterone were reported to exert important immune-inflammatory and metabolic effects. DHEAS directly increases superoxide generation by human neutrophils and therefore enhances immune system response to pathogens (208). While testosterone was reported to regulate neutrophil function by increasing phagocytic capacity and supressing oxidative stress in neutrophils (485). Both DHEAS and testosterone modulated the immune-inflammatory responses in a dose-dependent fashion (208, 485). Furthermore, serum DHEAS and testosterone levels were reported to be directly associated with greater muscle mass in healthy individuals (486). This could be attributed to anabolic effects secondary to improve local synthesis of dihydrotestosterone in skeletal muscle from DHEA and testosterone (487). Furthermore, DHEAS demonstrates to protective effects of skeletal muscle and maintaining muscle mass (488). Oxandrolone is an anabolic androgenic steroid derived from testosterone. Oxandrolone is currently approved the food and drug administration in the US as adjunctive therapy for weight restitution following

extensive surgery, chronic infections or severe trauma(489). The effects of oxandrolone therapy following severe thermal injury will be discussed further below.

The importance of addressing the acute challenges of severe thermal injury is paramount. However, with improved medical advancements and treatment, survival rates have significantly improved with time (405, 407). This, in turn, has led to further interest in addressing longer-term sequelae of major burns including wound healing rates and scarring (28, 490, 491). In this study, the steroid responses following thermal injury significantly correlated with time to achieve 95% wound healing and subsequent scarring. In agreement with the published literature (492) low levels of circulating testosterone following acute burn injury was significantly associated with longer durations to achieve wound closure. Similarly, lower serum levels of DHEAS at D01 post-thermal injury was associated with longer wound healing times. Furthermore, circulating levels of testosterone and DHEAS in burn patients at D01 post-injury were independently and significantly associated with lower wound healing times. This suggest that supplementation of DHEAS and testosterone at D01 following major burn injury may significantly reduce wound healing times for the following reasons. DHEAS stimulated keratinocyte and fibroblast migration through genomic and non-genomic signalling in human dermis and epidermis (493). Furthermore, DHEA was reported to promote pro-collagen synthesis and inhibit collagen degradation (494). Testosterone had little effect on keratinocyte and dermal fibroblast migration in a human epidermal study (493). However, oxandrolone treatment was reported to significantly accelerated wound healing in murine full thickness burn models (495). Potential mechanisms behind oxandrolone improving wound healing rates remains to be

established. By addressing wound healing duration, post-burn scarring outcomes may be improved (28).

Significant correlations were observed between DHEA, DHEAS, testosterone and scarring outcomes at various timepoints following major burn injury. Lower levels of the above steroids were associated with worse scarring as assessed subjectively and objectively. This indicates that potential long-term modulation of these responses during the acute phase of burn injury maybe of value. However, no clinical studies investigating the influences of these steroids on scarring outcomes following burn or trauma have been reported in the literature. A larger prospective study designed to allow multi-variate analysis accounting for surgical intervention and wound healing times would be ideal prior to suggesting potential therapeutic value in these steroids in the context of scarring.

Steroid use in burn care and management is a subject for discussion involving various specialities including scientists, burn surgeons and intensive care physicians. Steroid therapies involving DHEA and DHEAS following burn and trauma are yet to be established. Anabolic androgenic steroids, such as oxandrolone, became gold standard in burn care due to its multiple positive effects (496, 497). While the use of corticosteroids, such as hydrocortisone, remain controversial in burn (449, 450, 498-501). In this study, both oxandrolone and corticosteroids were used in burned patients when clinically required as dictated by treatment protocols at the tertiary burn centre. In both groups, no major differences in steroid levels during the first year following burn injury in patients who received treatment and those who did not were seen.

Corticosteroid therapy in critically ill septic patients has been fiercely debated (502, 503). In 2016, the Surviving Sepsis Campaign (SSC) adopted the use of intravenous hydrocortisone therapy in critically ill patients with treatment-refractory septic shock (504). Since then, two major RCTs reported the benefits of corticosteroid use in critical illness (505, 506). This was the rationale behind corticosteroid use in burns patients recruited into this study. Hence, corticosteroids were given to address persistent vasoplegia despite adequate resuscitation as indicated by increased cardiac SOFA scores. Despite this, corticosteroid therapy was associated with poorer outcomes independent of injury severity and hemodynamic status. Corticosteroid use in burned patients significantly increased the odds ratio (OR) and hazard ratio (HR) of subsequent mortality, MOF and sepsis.

Ever since initial reports of the successful use in burn injury of oxandrolone this became standard-of-care (496, 497). Since then, multiple studies reported the beneficial effects of oxandrolone during the acute and rehabilitative phases of burn injury (201, 507). Observations made in this study agree with the published literature. Oxandrolone use following burn injury was associated with reduced risk of sepsis and improved 28-day and in-hospital survival rates respectively. Interestingly, oxandrolone use was significantly associated with increased risk of MOF. This association between oxandrolone and MOF should be interpreted with caution for the following reasons. Firstly, the 95% confidence interval of the odds ratio is 18.7 which is large. Secondly, the median start day of oxandrolone therapy was at D05 post-burn injury while the median day of MOF diagnosis in this study was D03 post-burn injury. Thirdly, no

significant associations between oxandrolone and MOF were previously reported (507, 508).

This study has limitations. The status of oestrogen, progesterone and their respective upstream metabolites in burned patients were not quantified. This was due to mass spectrometer specifications. Furthermore, steroid-binding globulins levels following thermal injury were not measured due to limited amounts of serum and extensive nature of the SIFTI study. Quantification of female sex steroid hormones and steroid-binding globulins would have been important assessing their relative influence on outcomes following thermal injury. Therefore, observational studies investigating the longitudinal response of these molecules are encouraged.

3.5 Conclusions

Severe burn injury influences the HPA and HPG axis. The status of steroid hormones in burned patients appears to affect outcomes following injury including mortality, sepsis, wound healing and scarring. The data reported here suggest that steroid use following thermal injury is a double-edged sword. The use of corticosteroids in severely burned patients was associated with significantly increased odds of sepsis, MOF and in-hospital mortality independent of injury severity and haemodynamic status. While oxandrolone therapy following major thermal injury was significantly associated with improved prognosis. Scarring data suggests continued oxandrolone use or testosterone supplementation following discharge may improve post-burn scarring. Similarly, our data suggest that DHEA and/or DHEAS may be of potential therapeutic value and immediate long-term supplementation may improve outcomes. Further

clinical studies are required to validate these observations. Pilot studies assessing the effects of immediate and long-term use of oxandrolone/DHEA/DHEAS on short and long-term outcomes following major burn injury would prove useful prior to designing RCTs.

CHAPTER 4: VITAMIN D STATUS AND ITS INFLUENCE ON OUTCOMES FOLLOWING SEVERE BURN INJURY

4.1 Introduction

The Vitamin D axis involves conversion of inert vitamin D, cholecalciferol, from sun and diet into 25D3 and 1,25D3 by various organs. The pathways and metabolism of vitamin D are summarized in Figure 4.1. Vitamin D insufficiency/deficiency is common among the general population, with 20% of adults and up to 24% of children affected (212). Moreover, low vitamin D status has been reported in up to 76% of critically ill patients (213). Similarly, low vitamin D levels were observed among burned patients (130, 509). Despite this, mechanisms affecting vitamin D status following critical illness remain unclear and its influence over patient outcomes is poorly understood. The literature addressing these issues was previously summarised(134).

Critically ill populations are clearly identified to be at-risk of vitamin D deficiency, but there are sub-populations where there is insufficient literature on the status of vitamin D and its influence on outcomes, including patients with thermal injuries. Although studies investigating vitamin D levels in burns patients are scarce, vitamin D levels have been shown to decrease following thermal injury (130, 510). This may be both as a primary effect of the injury or secondary response to the injury itself and/or the clinical management initiated such as fluid resuscitation and use of pressure garments.

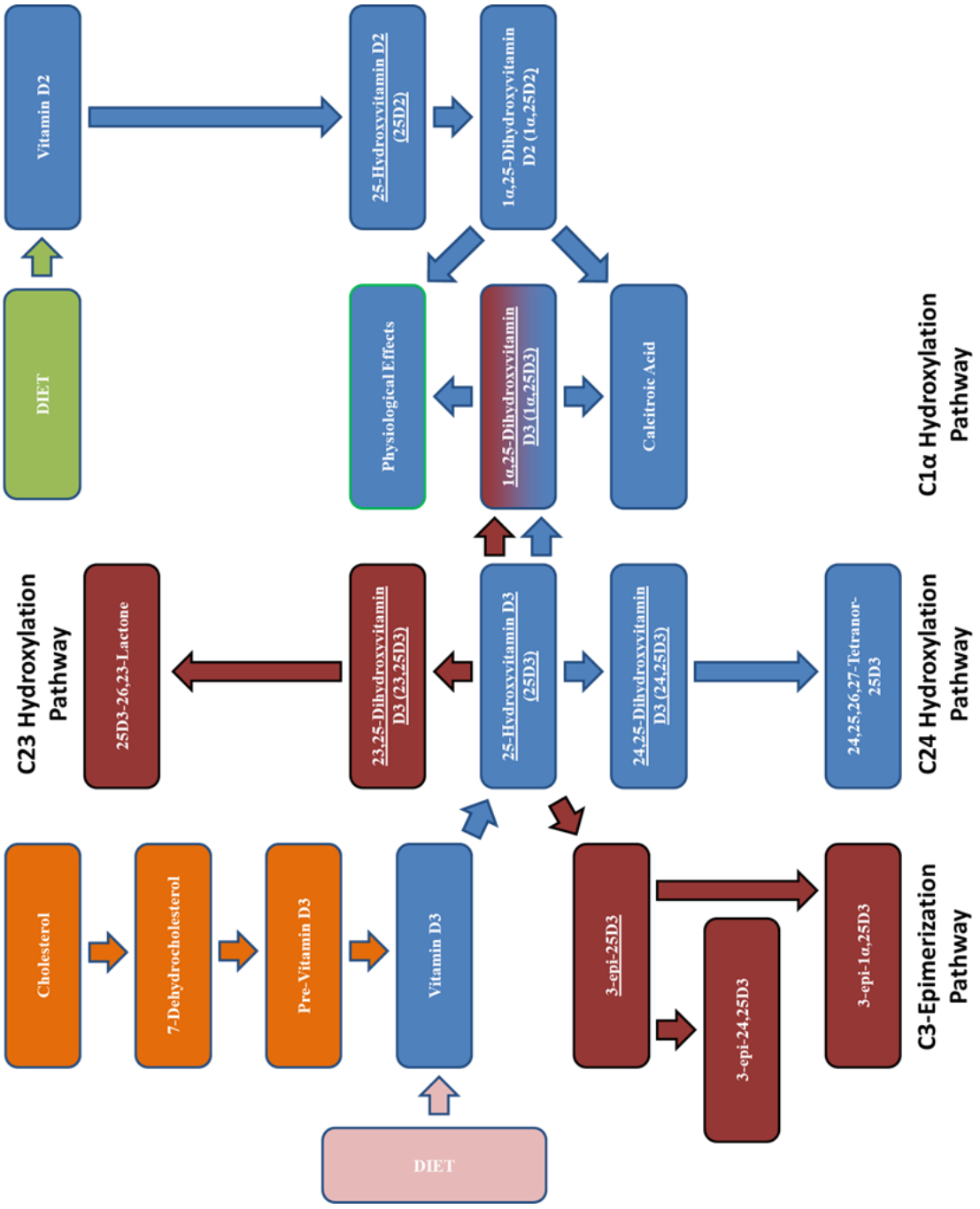


Figure 4.1. Overview of vitamin D biosynthesis and metabolism.

The underlined vitamin D metabolites were examined in this chapter.

Severe burn injury thus induces persistent disturbances of multiple immuno-inflammatory and physiological responses simultaneously, befitting the designation: persistent inflammation, immunosuppression, and catabolism syndrome, PICS (511). This includes reduced circulating levels of vitamin D and its carrier proteins, VDBP and albumin (130). Based on the literature concerning non-burn and burn trauma, we propose that this can be explained through a variety of potential mechanisms: First, as previously postulated (266), there may be attempts to maintain immune homeostasis via increased conversion of 25D to active 1,25D, thereby reducing circulating 25D levels. In individuals who are vitamin D-sufficient prior to injury, this effect may have a negligible impact on serum 25D status, but for those who are vitamin D-deficient at injury, this effect may lead to exacerbation of low serum 25D concentrations. Second, fluid resuscitation and a compromised vascular integrity results in decreased serum vitamin D levels and its metabolites secondary to haemodilution and fluid shifts (268). VDBP and albumin are also affected in the acute response, thereby reducing bound vitamin D levels and impacting its delivery to target tissues (269-271). This phenomenon of binding protein extravasation would be temporary as microvascular integrity is re-established 6 hours following thermal injury (512). Third, VDBP levels are reduced in the acute stage buffering actin's deleterious effects as part of the actin scavenging system (513, 514).

Although not firmly established, it appears that VDBP levels recover during the acute phase of thermal injury (509), while albumin levels may recover as early as 6 months (515). Due to this multiplicity of factors, interpretation of 25D levels and diagnosing vitamin D deficiency in burn patients remains challenging (516) .

Vitamin D levels following severe thermal injury can also be reduced secondary to extrinsic causes including prolonged in-hospital stay (including ICU), prolonged immobilization and lack of supplementation. Although critically ill burn patients receive oral or enteral feed supplements, current regimens have proved ineffective in replenishing vitamin D levels in the acute phase (510). Furthermore, current long-term burn management regimens involve scar management comprising mainly of sun avoidance and protection, as well as the use of pressure garments. These factors minimize sun exposure, hence reducing 25D levels. In addition, both burn scar and adjacent normal skin in burn patients exhibit subnormal conversion levels of 7-DHC to pre-D_3 compared to healthy individuals (517). This further potentiates vitamin D deficiency, resulting in low levels of 25D and 1,25D for many years, at least seven, following burn injury (518). The potential causes of hypovitaminosis D following injury is summarized in Figure 4.2.

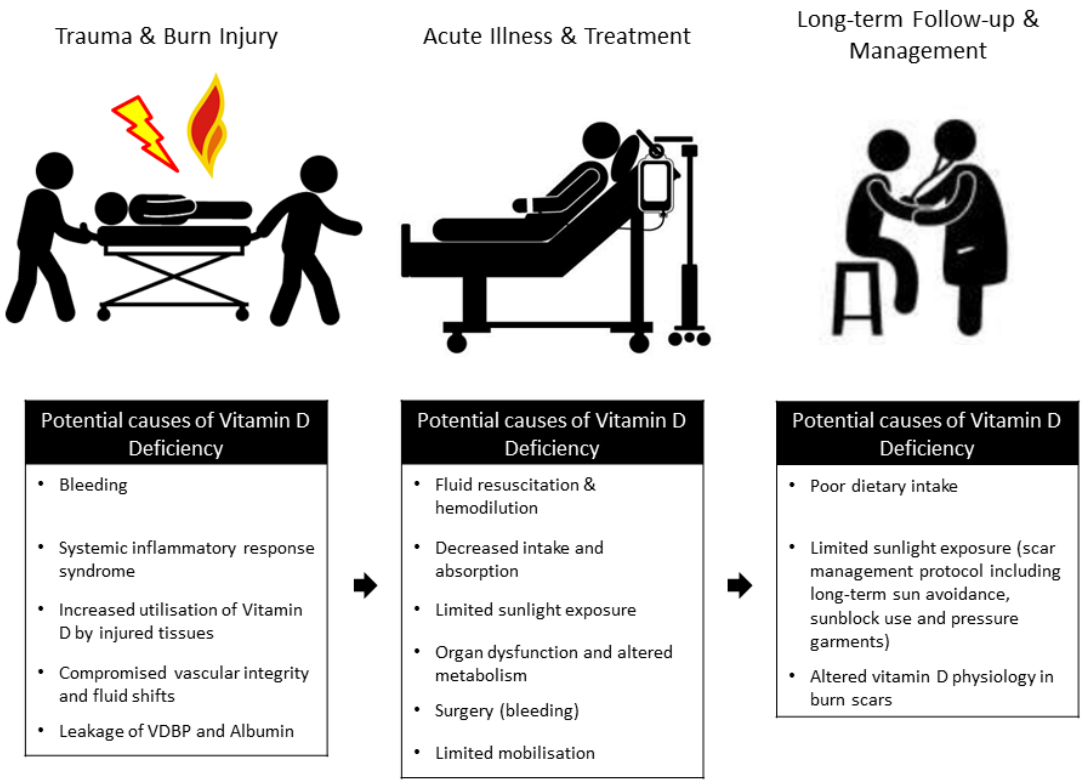


Figure 4.2. Potential causes of low vitamin D status in burned patients

Low vitamin D levels in patients with minor burns, median TBSA of 5%, has been associated with increased length of hospital stay (519). Although not statistically significant, the authors also observed higher complication rates in burn patients with low vitamin D including sepsis, pneumonia, cardiovascular complications and graft loss (519). It is important to note that low vitamin D status in this study cohort most likely represents the population's pre-injury 25D levels rather than a consequence of the burn. Furthermore, this study has some limitations. Thirty percent of the cohort was admitted to ICU with relatively minor injuries, which is unusual. No description of patient pre-morbid state or other pathologies were reported which may potentially

affect vitamin D status or outcomes in general. In addition, the cohort is comprised mostly of minor partial thickness thermal injuries limiting its application in severe burns. There are no reports investigating the influence vitamin D levels on short-term outcomes of major burn patients.

Low serum levels of vitamin D in major burn patients have shown to persist for at least one year (509). Long term outcomes assessed include bone mineral density and leg muscle strength. Although not statistically significant, quadricep muscle strength was lower at month 12 than the month of injury in burn patients with low vitamin D levels (509). Another long-term consequence of major thermal injury is scarring. A cross-sectional study has reported a strong negative correlation between circulating 25D levels at year 1 post-injury and subjective scar measures (mVSS) (520). Both studies were limited by small sample sizes. There are no other studies investigating the influence of vitamin D on long-term outcomes of burned adult patients. While studies have reported increased incidence of long bone fractures among children with major burns following discharge(521). This is most likely attributed to reduced bone mineral density and vitamin D levels (518, 522). Vitamin D supplementation in paediatric burn patients may be beneficial in reducing fracture risk(522).

Due to current gaps in our understanding of the impact of vitamin D metabolism changes in trauma patients, vitamin D supplementation in critical illness and burned patients is often overlooked. We hypothesize that major burn injury results in significantly affects the vitamin D metabolism axis and that Vitamin D status post-injury are associated with outcomes including sepsis, MOF, mortality, wound healing

and scarring. We hypothesize that supplementation of vitamin D may improve outcomes.

The aims of this thesis include determining the status of the vitamin D axis in severely burned patients; characterising these vitamin D axis longitudinally from day 1 following severe thermal injury till month 12 post-injury; exploring associations between the longitudinal vitamin D response and clinical outcomes in burned patients including mortality, MOF, sepsis, wound healing and scarring and identify any potential clinical biomarkers that may improve prognosis of burned patients or identify novel therapeutic targets.

4.2 Methods

The general methodology used including ethical approval and statistical analysis are described in Chapter 2.

Only this chapter incorporates healthy volunteer data and serum vitamin D levels from the healthy ageing study published by Dr. Zaki Hassan-Smith (523, 524), conducted in Wellcome Trust Clinical Research Facility at QEHB. Details regarding volunteer recruitment, procedures and ethics were provided Dr. Zaki Hassan-Smith and Dr. Carl Jenkinson as they were investigators in this study. In addition to the published data, this information is outlined in Dr. Zaki Hassan-Smith's doctorate thesis. This is data is added to the healthy volunteer data recruited in e-SIFTI. The healthy ageing study did not perform vitamin D derivatisation and hence not all vitamin D metabolites were quantified as compared to the e-SIFTI cohort. HAS participants with full demography data were used in statistical analysis used in this chapter.

In this chapter, quantification of vitamin D was performed by Khaled Altarrah in a GCP validated lab in the IMSR, Birmingham University Medical School. Raw data acquired from LC-MS/MS analysis was quantified using Waters TargetLynx™ Application Manager (Waters Ltd, Elstree, UK) by Dr. Carl Jenkinson. Overall data analysis was performed by Khaled Altarrah.

4.2.1 Healthy Ageing Study (HAS)

4.2.1.1 Study Cohort

A total of 98 healthy adult volunteers (60 female/38 male; age 20-75 years) from the local population were invited to participate. A summary of inclusion and exclusion

criteria are summarised in Table 4.1. Eligible participants were recruited into this study after obtaining written consent. Results presented in this chapter only include HAS participants who had their vitamin D metabolites analysed. Ethical Approval for the study was obtained from the Coventry and Warwickshire Research Ethics Committee, UK (Reference 07/H1211/168).

Inclusion Criteria	**Exclusion Criteria**
Adult (≥16 years)	Pregnancy
BMI 20-30kg/m^2	Significant past medical history including diabetes mellitus, ischemic heart disease, cerebrovascular disease, severe respiratory disease and epilepsy
Females in follicular phase of menstrual cycle	Glucocorticoid therapy in past 12 months
	Use of medication/drugs affecting growth hormone release
	Oral anti-coagulant use

Table 4.1. Inclusion and exclusion criteria for the Healthy Ageing study.

4.2.1.2 Blood sampling

One-off blood samples were collected from fasted study participants and were taken in the mornings between 0900 – 1100 hours.

4.2.1.3 Preparation of Serum

Blood samples collected in BD vacutainers® containing z-serum clotting activator were allowed to clot for 30 minutes at room temperature prior to processing. The serum was carefully extracted thereafter and stored at -80°C in aliquots prior to analysis.

4.2.1.4 Data Collection

Data including patient demographics and observations were prospectively recorded using CRFs. Participants with vitamin D metabolite levels only are used in this chapter.

4.2.2 Measurement of Vitamin D Metabolites

All serum vitamin D metabolites were assessed using LC-MS/MS as previously described (523, 525). Vitamin D metabolites including 25OHD3, 3-epi-25OHD3, $24,25(OH)_2D3$ and 25OHD2 were extracted from 50-220µl of serum using supportive liquid-liquid extraction. Vitamin D metabolites were eluted from the SLE plate using two 800µL volumes of MTBE/ethyl acetate (90/10%) following protein precipitation using 80µL of methanol, 50µL isopropanol and 80µL of water. Samples were eluted from the SLE plate under gravity and a vacuum (5Hg) was applied. The solvent was then evaporated using nitrogen at 50°C after each 800µL addition. Following this, samples were reconstituted in 125µL of water/methanol (50/50%) and analysed. Samples were then re-dried and processed via Vitamin D derivatisation using 200µL derivatisation mixture (0.5mg of 4-Phenyl-1,2,4-triazoline-3,5-dione (PTAD) per 1mL acetyl nitrile). Following this, 1a25D3, 23,25D3 and 24,25D3 were measured previously published (525).

All samples were processed using an ACQUITY ultra performance liquid chromatography (uPLC) coupled to a Waters Xevo TQ-S mass spectrometer (Waters, Manchester, UK). Vitamin D metabolite analysis was performed in positive ion node and ionisation was done using electrospray ionisation (ESI) mode. Chromotography separation of all analytes was carried out using a Lux Cellulose-3 chiral column (100mm,2mm,3µm) maintained at 60°C. All metabolites were separated using an optimised gradient system consisting of methanol, water and 0.1% formic acid and a flow rate of 330µL/min. All vitamin D metabolites were quantified using a linear calibration series with appropriate internal standards. Each metabolite was recognised by matching retention times and two mass transitions in comparison to a reference compound. Each metabolite was quantified against a vitamin D internal standard, using the internal standards; 25OHD3-d3, 3-epi-25OHD3-d3, 24,25$(OH)_2$D3-d6 and 1α$(OH)_2$D3-d3. Precision data for each vitamin D metabolite assay has been previously published (525).

4.2.3 Measurement of VDBP

VDBP levels were measured from serum samples using ELISA Kits (K2314, ImmunoDiagnostik, Germany). Plate preparation and reconstitution of assay reagents were done accordingly as per manufacturer's protocol. Samples were prepared and diluted in sample buffer solution (SAMPLEBUF) to a factor of 40,000 utilising a three-step procedure. Following this, 100µL of the final diluted samples were added to the plates and left to incubate for 1 hour at room temperature with shaking at 550rpm.

Following incubation, the well contents were discarded and washed 5 times using 250µL of wash buffer solution to remove unbound material. Next, 100µL of peroxidase-labelled antibody was added to each well and left to incubate for 1 hour at room temperature with shaking. The contents of the wells were then discarded and washed 5 times using wash buffer to remove unbound antibody. One hundred microliters of TMB solution was then added to each well, followed by incubation at room temperature for 10 min. Following this, 100µL of stop solution was added to each well.

Following the assay procedure, absorption of each plate was determined using BioTek ELx808™ Absorbance Microplate Reader and BioTek Gen5™ Data Analysis Software (BioTek®, Swindon, UK) at 450nm against 630nm as reference. VDBP levels were extrapolated from standard curves using Graphpad PRISM® (GraphPad Software Inc., California, USA). Interplate and intraplate variability are 14.9% and 4.5% respectively. The standard curves are illustrated in Supplementary Figure 4.

4.2.4 Free and Bioavailable 25vitD3 Calculations

The concentration of free 25vitD3 in serum was evaluated using the formula described by Bikle et al (526). The concentration of the bioavailable (free and albumin-bound) fraction of 25vitD3 in blood was calculated using the formula published by Vermeulen et al (527). These formulae are illustrated in Supplementary Figure 5. The use of these formulae in clinical studies investigating vitamin D, including burn and critically ill patients has been previously published (130, 299, 528, 529).

4.3 Results

4.3.1 Patient Demographics

Forty-six patients admitted to the burn centre in QEHB and 110 healthy volunteers participated in this study. The process is illustrated in Supplementary Figure 6. Both burns patients and healthy volunteers had similar demographics with exception of gender. Injury and burn resuscitation details were previously described in Chapter 3. HCT between both burn and healthy cohorts were similar.

Both healthy volunteers and burn patients were vitamin D deficient with the median 25D3 levels of both cohorts being <20ng/mL. Additionally, burn patients at day 1 exhibited decreased levels in almost all vitamin D metabolites and VDBP compared to the healthy volunteers. Vitamin D metabolites significantly reduced in burn patients included 25D3 ($p<0.001$), 1α25D3 ($p<0.001$), 3-epi-25D3 ($p<0.001$), 23,25D3 ($p = 0.018$) and 25D2 ($p<0.001$) levels. Furthermore, the median levels of free and bioavailable 25D3 in burn patients were 8.9 fg/mL and 0.3 ng/mL respectively. These findings are summarized in Table 4.2. The outcomes for burn patients assessed in this chapter are described in Chapter 3, Table 3.2.

4.3.2 Longitudinal Vitamin D Response Following Thermal Injury

Burn patients exhibited a global decrease in vitamin D metabolites. Levels of 25D3 in serum of patients was significantly reduced up until one-month post-injury compared to healthy volunteers, median 8.6 ng/mL vs 16.2 ng/mL ($p <0.001$). Although 25D3 returned to healthy control levels, on further analysis of the timepoints 25D3 levels in

burn patients became decreased at month 12, median 9.5 ng/mL vs. 16.2ng/mL ($p = 0.017$).

Likewise, 1α25D3 remained significantly decreased in burn patients throughout the study period compared to healthy individuals, median 36.2 pg/mL vs 82.5 pg/mL ($p <0.001$). Similarly, levels of 25D2 in serum were significantly lower in patients compared to controls throughout the first-year post-injury, median levels were undetectable vs. 0.18 ng/mL ($p <0.001$). VDBP was significantly reduced at day 1 in burn patients ($p<0.001$) but returned to healthy control levels thereafter. Vitamin D metabolites including biomarkers of alternative vitamin D metabolism pathways are summarised in Figure 4.3.

	Healthy Controls	n	Burns Patients	n	p-value
Age	44 (28-61)	110	41 (33 – 55)	46	0.901
Gender (M/F)	**45/65**	**110**	**30/16**	**46**	**0.006**
% Total Burn Surface Area	-	-	42 (25 – 53.5)	46	-
Revised Baux Score	-	-	98 (79.0-111.3)	46	-
Hematocrit	0.399 (0.371-0.415)	12	0.429 (0.374-0.468)	31	0.127
25D3 (ng/mL)	**16.2 (9.5-22.9)**	**103**	**3.8 (2.0-12.6)**	**38**	**<0.001**
1α25D3 (pg/mL)	**82.5 (67.4-119.7)**	**8**	**28.6(15.9-47.3)**	**38**	**<0.001**
3-epi-25D3 (ng/mL)	**1.8 (1.1-2.3)**	**104**	**0.4 (0.1-0.8)**	**38**	**<0.001**
24,25D3 (ng/mL)	2.1 (0.8-4.0)	104	1.4 (0.8-2.5)	38	0.093
23,25D3 (pg/mL)	**126.5(43.8-181.4)**	**8**	**35.1 (19.8-61.6)**	**38**	**0.018**
25D2 (ng/mL)	**0.2 (UD-0.8)**	**104**	**UD (UD-UD)**	**38**	**0.001**
VDBP (µg/mL)	**696.1(475.0-756.6)**	**14**	**303.6(211.4-441.3)**	**45**	**<0.001**
Free 25D3 (pg/mL)	-	-	1.1 (0.5-3.2)	36	-
Bioavailable 25D3 (ng/mL)	-	-	0.3 (0.2-0.8)	36	-
Albumin (g/L)	-	-	34.0 (26.0-41.5)	44	-
Calcium (mmol/L)	-	-	2.1 (2.0-2.3)	40	-
Phosphate (mmol/L)	-	-	1.26 (1.1-1.5)	27	-

Table 4.2. Demographics and serum analyte levels in healthy volunteers and burns patients at day 1 post injury.
Continuous variables are shown as median values with inter-quartile range. Burn patients and healthy volunteers were compared using Mann-Whitney test for continuous variables and Chi-squared test for categorical variables. Significant relationships are highlighted in bold.

4.3.3 Longitudinal Vitamin D Response among Survivors and Non-survivors Following Thermal Injury

Circulating levels of vitamin D and associated metabolites in blood were generally increased in survivors of severe burn injury compared to non-survivors. At D03-D07, burns survivors before/at 28 days post-injury had significantly elevated levels of 25D3, 24,25D3 and 23,25D3 compared non-survivors, median 6.5 ng/mL vs. 3.2 ng/mL (p 0.039), 1.4 ng/mL vs. 0.7 ng/mL (p 0.006) and 35.2 pg/mL vs. 20.7 pg/mL (p 0.028) respectively.

Patients who survived their admission episode following thermal injury had significantly elevated levels of 25D3, 24,25D3 and 23,25D3 at D03 till D28 post-injury, median 10.6 ng/mL vs. 5.5 ng/mL (p <0.001), 2.1 ng/mL vs 1.0 ng/mL (p <0.001) and 59.0 pg/mL vs. 29.0 pg/mL (p <0.001) respectively. Furthermore, serum levels of free and bioavailable 25D3 were significantly elevated in burns patients who survived their in-hospital admission compared to non-survivors at D03-D28 post-injury, median 1.3 pg/mL vs. 0.86 pg/mL (p = 0.003) and 0.2 pg/mL vs. 0.1 pg/mL (p = 0.001) respectively. Additionally, VDBP levels in blood were significantly increased in survivors of thermal injury compared to non-survivors at D14-D28, median 764.4 µg/mL vs. 590.9 µg/mL (p = 0.002). The longitudinal vitamin D response in burns survivors and non-survivors are summarised in Figure 4.4 and Figure 4.5.

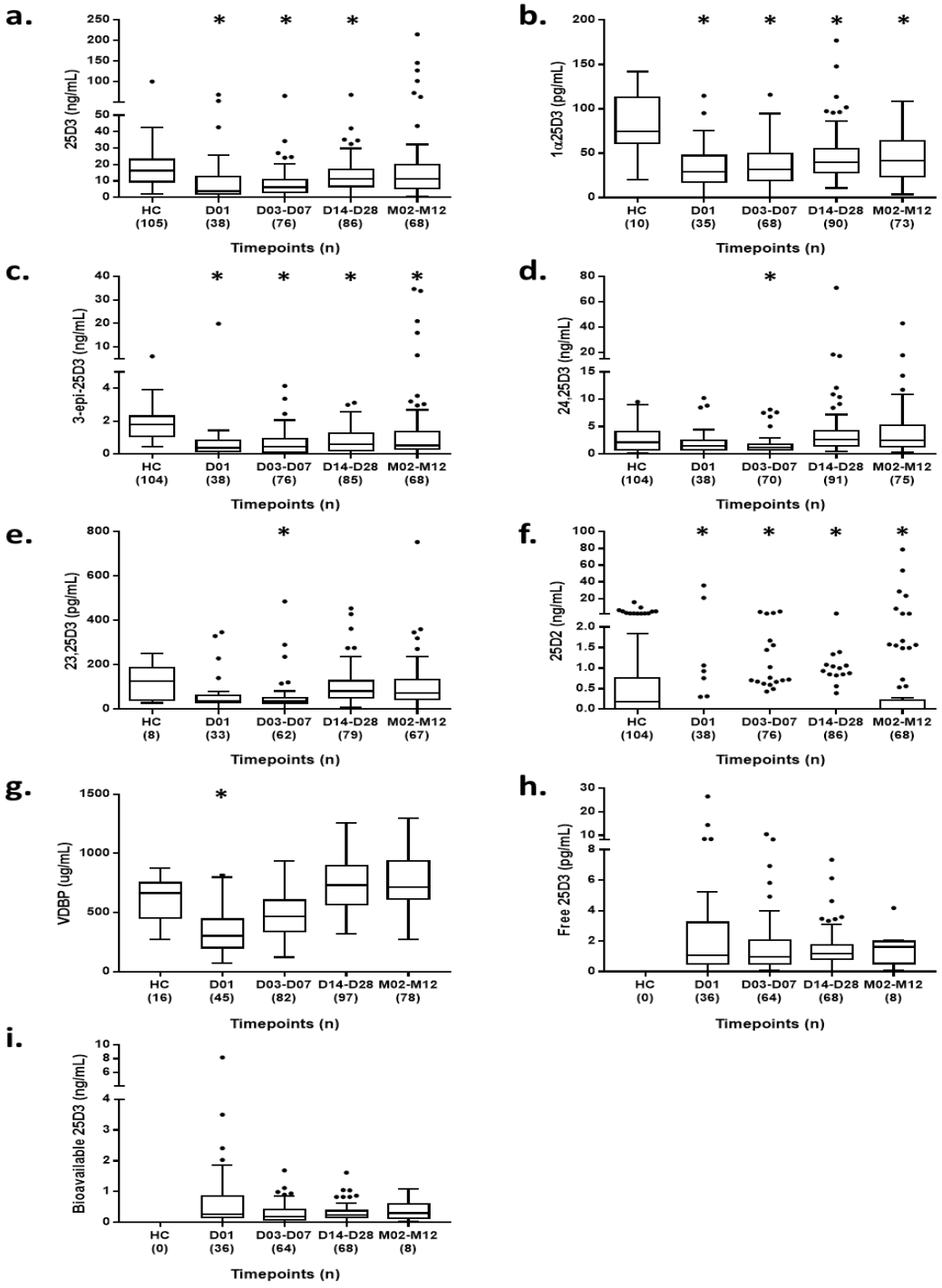


Figure 4.3. Severe burn injury causes significant reductions multiple vitamin D metabolites.

Systemic vitamin D metabolites were analysed over a 12-month period. a. 25D3; b. 1α25D3; c. 3-epi-25D3; d. 24,25D3; e. 23,25D3; f. 25D2; g. VDBP; h. Free 25D3; i. Bioavailable 25D3. Analyte levels at timepoints compared to healthy controls (HC) using Kruskal-Wallis test with Dunn's multiple corrections; * $p < 0.05$. Analyte levels at timepoints compared to healthy controls (HC) using Kruskal-Wallis test with Dunn's multiple corrections; * $p < 0.05$.

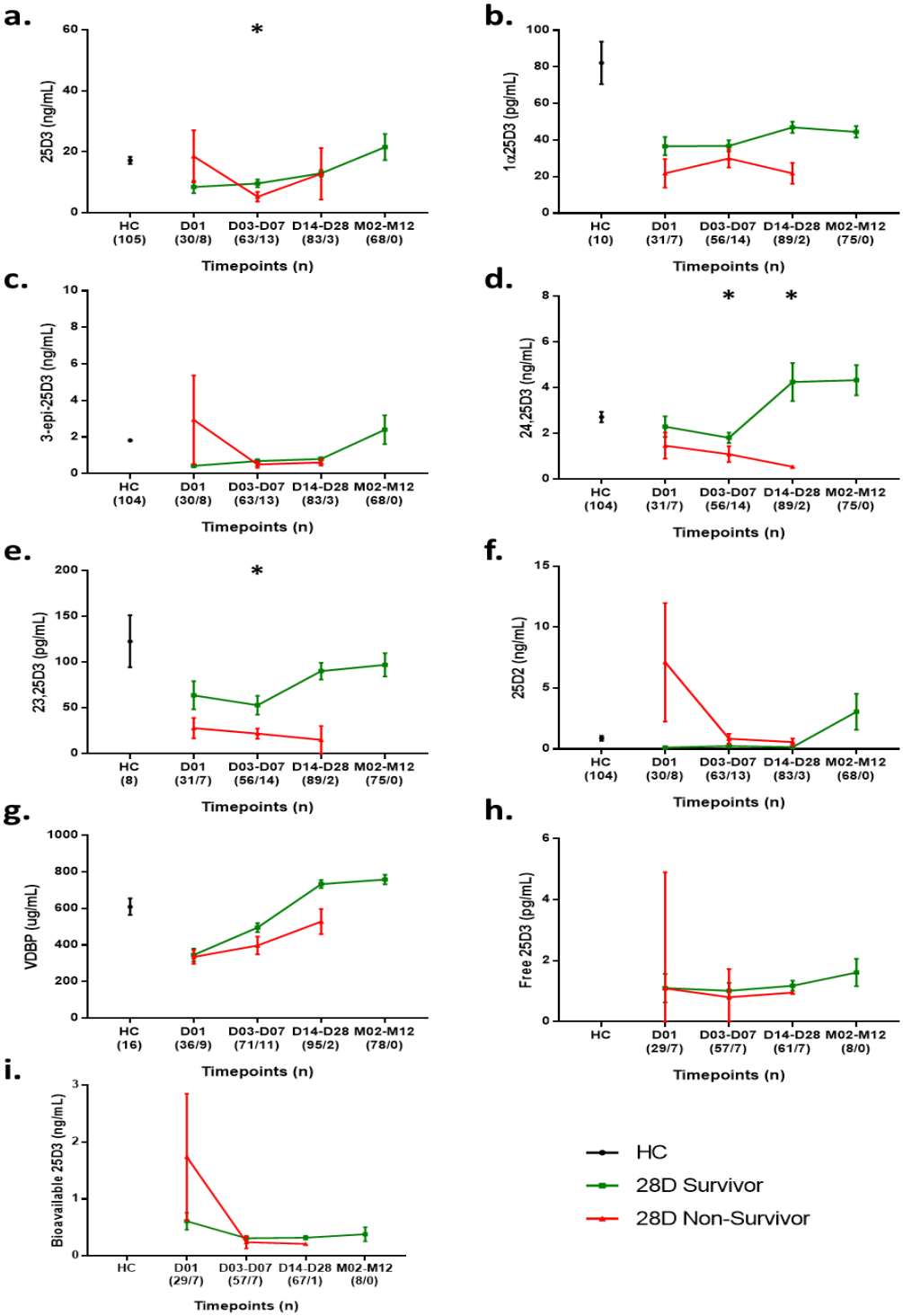


Figure 4.4. 28D burn survivors had significantly elevated levels of 25D3, 24,25D3 and 23,25D3 compared to non-survivors.

Serum vitamin D levels in 28-day burn survivors and non-survivors were analysed at all timepoints. a. 25D3; b. 1α25D3; c. 3-epi-25D3; d. 24,25D3; e. 23,25D3; f. 25D2; g. VDBP; h. Free 25D3; i. Bioavailable 25D3. Analyte levels at each timepoint was compared between both cohorts (28D survivors vs. 28D non-survivors) using Mann-Whitney Test; * $p < 0.05$.

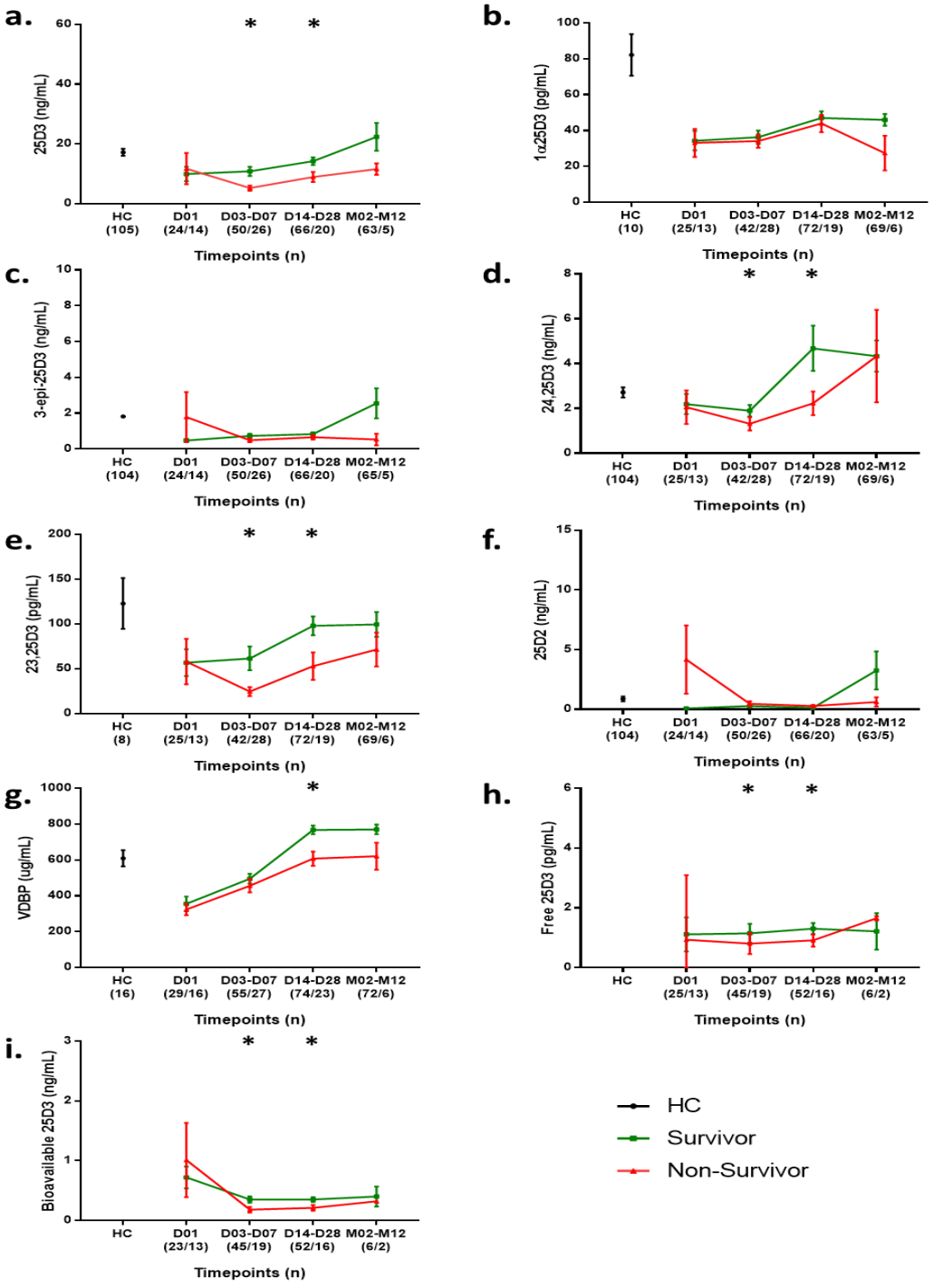


Figure 4.5. Burns survivors had significantly elevated levels of 25D3, free 25D3, bioavailable 25D3, 24,25D3, 23,25D3 and VDBP.

Serum vitamin D levels in burn survivors and non-survivors were analysed at all timepoints. a. 25D3; b. 1α25D3; c. 3-epi-25D3; d. 24,25D3; e. 23,25D3; f. 25D2; g. VDBP; h. Free 25D3; i. Bioavailable 25D3. Analyte levels at each timepoint was compared between both cohorts (Survivors vs. Non-survivors) using Mann-Whitney Test; * $p < 0.05$.

4.3.4 Longitudinal Vitamin D Response and MOF following Severe Thermal Injury

Following burn injury, patients who subsequently developed MOF demonstrated a different vitamin D response compared to patients without MOF. At D03-D07, patients with MOF had significantly lower levels of 25D3 and free 25D3 compared to those without MOF, median 3.0 ng/mL vs. 4.2 ng/mL ($p = 0.014$), 0.6 pg/mL vs. 1.2 pg/mL ($p = 0.013$) respectively. Furthermore, patients with MOF had significantly lower levels of 23,25D3 and bioavailable 25D3 compared to those without MOF at D03-D28 post-thermal injury, median 34.3 pg/mL vs. 57.6 pg/mL ($p <0.001$) and 0.2 ng/mL vs. 0.3ng/mL ($p = 0.012$) respectively. Additionally, burns patients diagnosed with MOF has significantly reduced levels of 24,25D3 compared to those without MOF at D14-D28 post-injury, median 1.8 ng/mL vs. 2.8 ng/mL ($p = 0.024$). The longitudinal steroid response in burns patients who developed and did not develop MOF is summarised in Figure 4.6.

4.3.5 Longitudinal Vitamin D Response in Septic and Non-Septic Patients Following Severe Thermal Injury

Immediately following burn injury, non-septic burns patients exhibited generally higher levels of vitamin D metabolites in serum compared to septic patients for the duration of the study. Circulating levels of 25D3, free and bioavailable 25D3 were significantly elevated in non-septic patients compared to septic patients at D01 following thermal injury, median 13.1 ng/mL vs. 2.6 ng/mL ($p = 0.006$), 3.4 pg/mL vs. 0.9 pg/mL ($p = 0.017$) and 1.2 ng/mL vs. 0.2 ng/mL ($p = 0.031$) respectively. Additionally, 25D3 and 3-epi-25D3 levels in serum were significantly increased in non-septic burns patients

compared to septic patients at M02-M12, median 18.0 ng/mL vs. 9.4 ng/mL (p= 0.005) and 0.8 ng/mL vs. 0.4 ng/mL (p = 0.025) respectively.

Furthermore, systemic levels of 1α25D3, 24,25D3 and 23,25D3 were significantly higher in non-septic patients compared to septic patients at D14-28 following thermal injury, median 48.9 pg/mL vs. 35.8 pg/mL (p = 0.013), 3.2 ng/mL vs. 2.2 ng/mL (p = 0.016) and 96.4 pg/mL vs. 56.8 pg/mL (p <0.001). The longitudinal vitamin D response in septic and non-septic burns patients is summarised in Figure 4.7.

4.3.6 Longitudinal Vitamin D Response and Wound Healing following Severe Thermal Injury

Systemic levels of vitamin D metabolites were significantly associated with wound healing times in severely burned patients throughout the study. Burn patients with lower levels of 25D3 in serum at D01 post-injury had significantly longer times of wound healing, rho -0.495 (p = 0.037). Furthermore, lower circulating levels of 24,25D3 at D03-D07 post-burn injury were significantly associated with longer wound healing times in patients, rho -0.427 (p = 0.012). Additionally, 23,25D3 levels in serum of burns patients at D14-D280 post injury demonstrated a significant inverse relationship with duration of wound healing, rho -0.423 (p <0.001). Conversely, circulating levels of 3-epi-25D3 and 25D2 in patients at D14-28 post injury had significant positive correlation with time taken to achieve wound healing. Significant correlations between vitamin D metabolites at various timepoints and duration of wound healing are summarised in Figure 4.8.

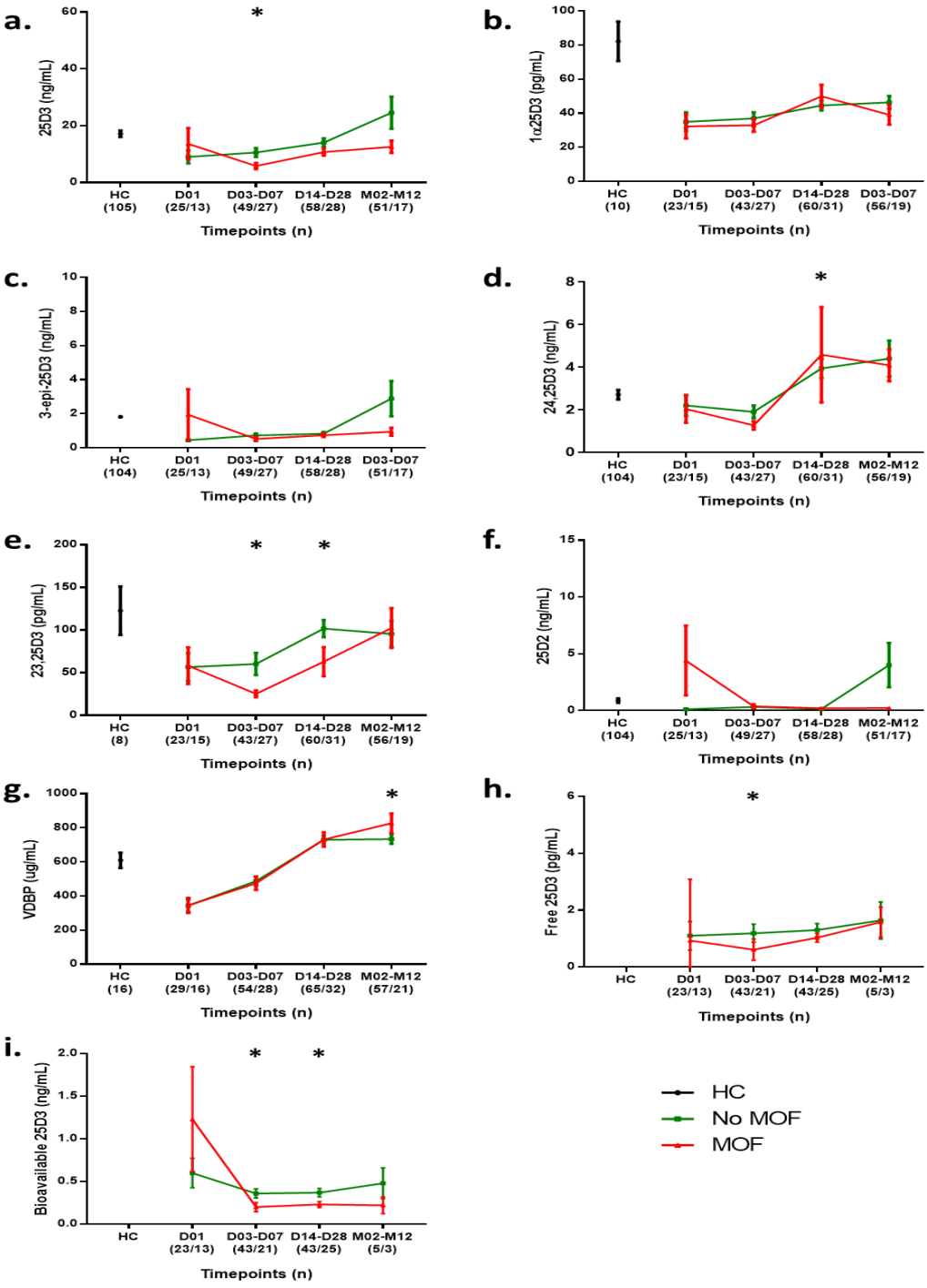


Figure 4.6. Burn patients without MOF exhibited significantly elevated levels of 25D3, free 25D3, bioavailable 25D3 and 23.25D3 compared to burn patients with MOF. Serum vitamin D levels in burn patients who developed and did not develop MOF were analysed across all timepoints. a. 25D3; b. 1α25D3; c. 3-epi-25D3; d. 24,25D3; e. 23,25D3; f. 25D2; g. VDBP; h. Free 25D3; i. Bioavailable 25D3. Analyte levels at each timepoint was compared between both cohorts (MOF vs. No MOF) using Mann-Whitney Test; * $p < 0.05$.

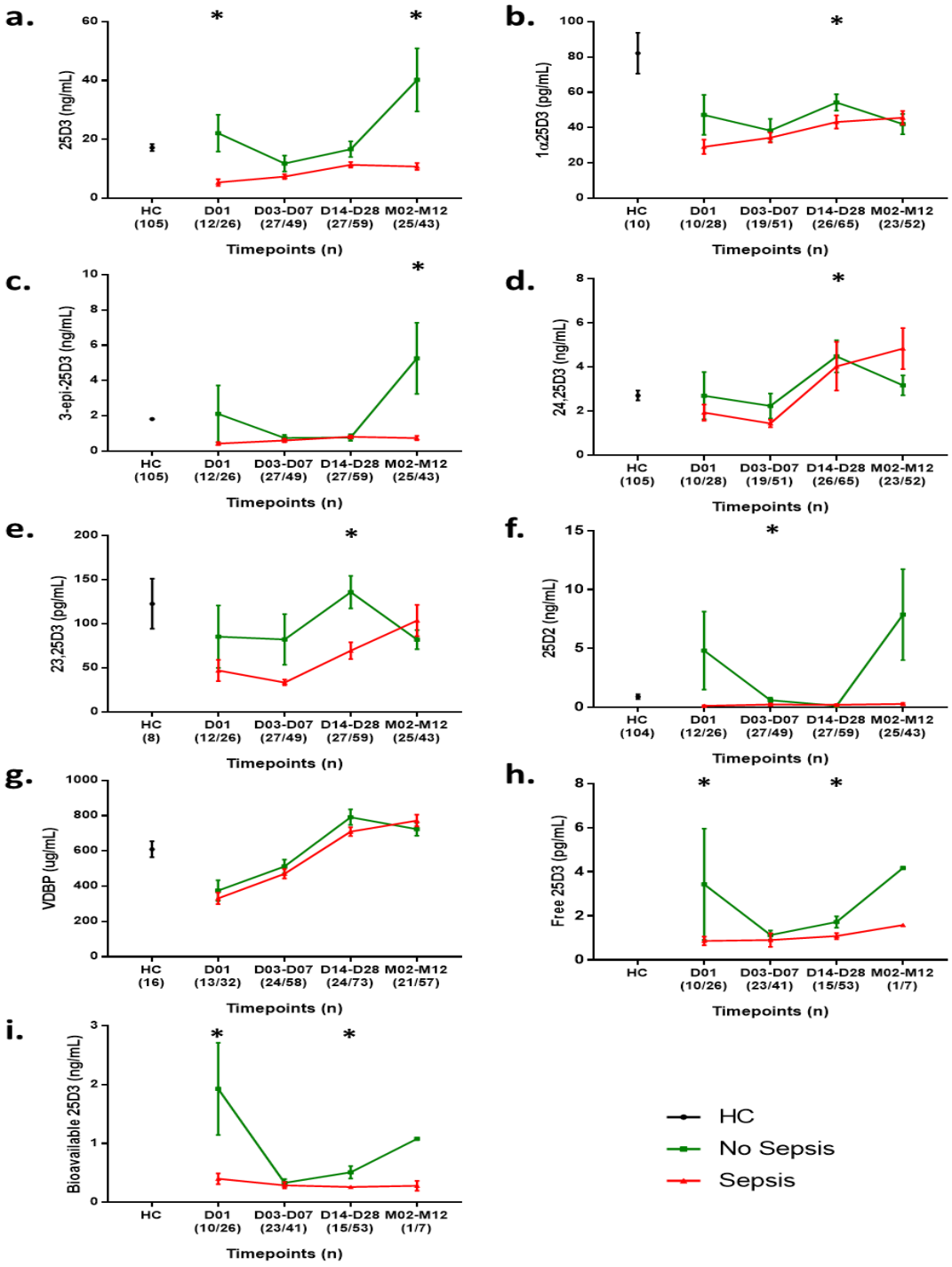


Figure 4.7. Septic burn patients had significant reductions of all vitamin D metabolites compared to non-septic patients.

Serum vitamin D levels in septic and non-septic burns patients were analysed across all timepoints. a. 25D3; b. 1α25D3; c. 3-epi-25D3; d. 24,25D3; e. 23,25D3; f. 25D2; g. VDBP; h. Free 25D3; i. Bioavailable 25D3. Analyte levels at each timepoint was compared between both cohorts (Sepsis vs. No Sepsis) using Mann-Whitney Test; * $p < 0.05$.

4.3.7 Longitudinal Vitamin D Response and Scarring following Severe Thermal Injury

4.3.7.1 Subjective Scar Measures

Circulating levels of vitamin D metabolites were significantly associated with scarring outcomes in burns patients throughout the study period. At D03-D07 post-burn injury, decreased serum 25D2 levels were significantly associated with worse scarring as assessed by clinicians, mVSS rho -0.570 ($p = 0.015$) and OOSS rho -0.567 ($p = 0.015$). Furthermore, reduced systemic levels of bioavailable 25D3 significantly correlated with poor scar formation as assessed by both clinicians and patients. At D14-D28 post-thermal injury, bioavailable 25D3 had negative correlations with mVSS and OOSS, rho -0.583 ($p = 0.009$) and rho -0.485 ($p = 0.036$) respectively. At D03-D07, a negative correlation was observed between bioavailable 25D3and OPSS in burns patients, rho -0.589 ($p= 0.015$). A significant negative correlation between free 25D3 and OPSS was observed at D03-D07 post-thermal injury, rho -0.509 ($p = 0.039$). Interestingly, higher serum levels of VDBP at M02-M12 was associated with worse scarring outcomes, mVSS rho 0.364 ($p = 0.035$). Significant correlations between serum vitamin D metabolites measured longitudinally and subjective scar scoring systems are summarised in Figure 4.9.

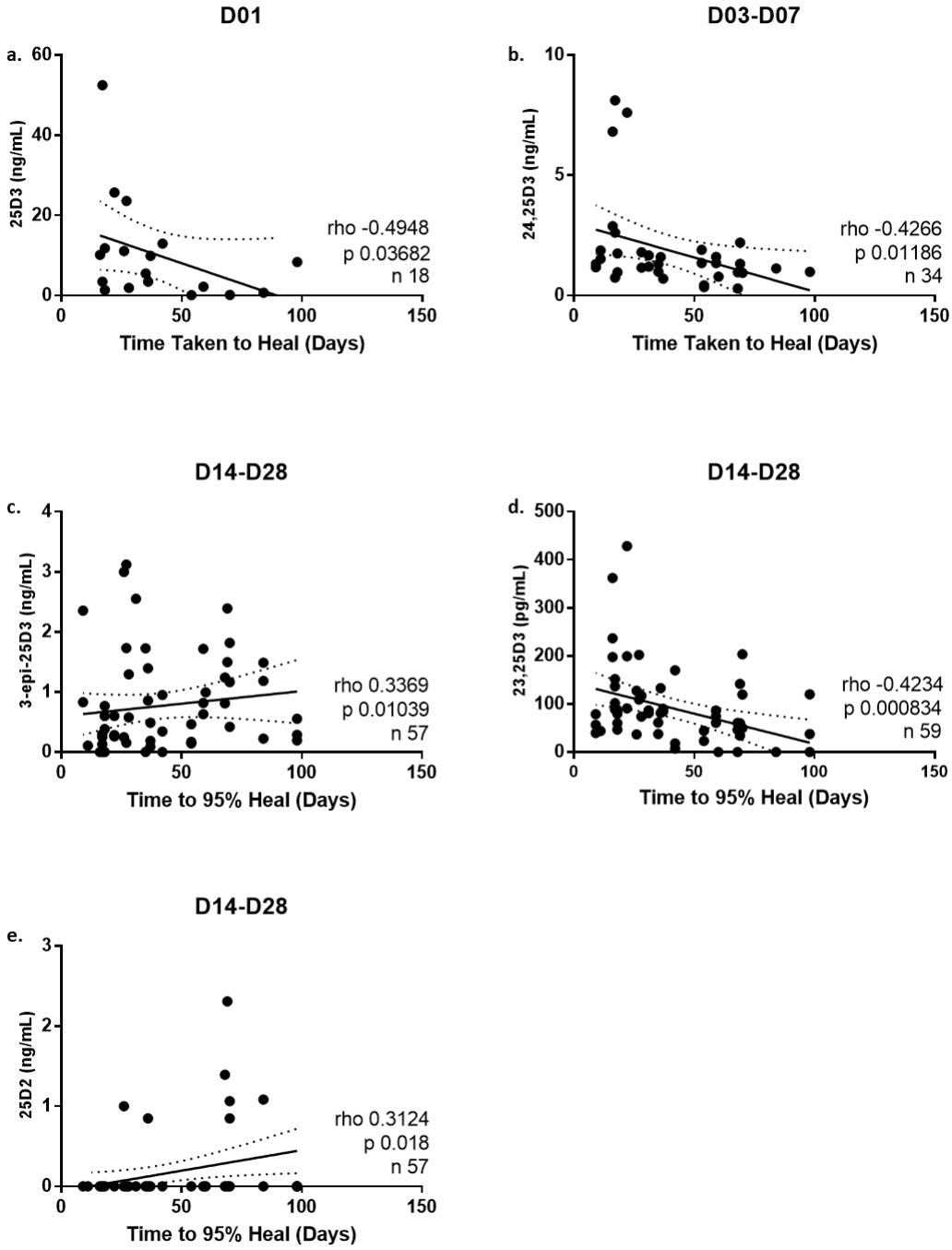


Figure 4.8. Reduced serum 25D3 at day 1 post-burn injury was significantly associated with longer wound healing times.

Serum vitamin D levels and days taken to achieve 95% wounds healed in burns patients were analysed across all timepoints. a. 25D3 at D01; b.24,25D3 at D03-D07; c. 3-epi-25D3 at D14-D28; d. 23,25D3 at D14-D28; e. 25D2 at D14-D28. Correlations were performed between analyte levels at timepoints and time to 95% heal using Spearman's rank correlation co-efficient; *p <0.05.

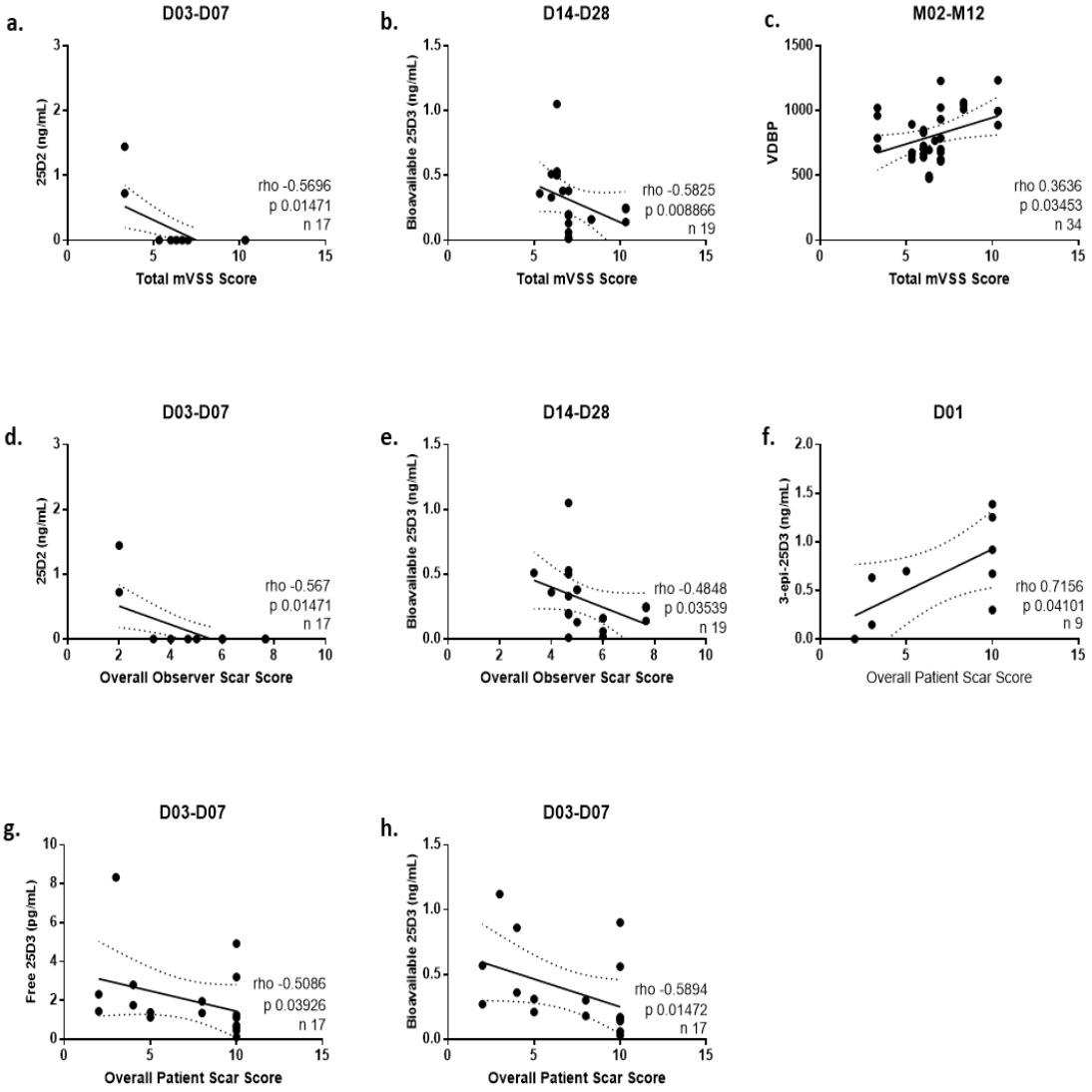


Figure 4.9. Vitamin D response and correlations with subjective scar measures following severe thermal injury.

Serum vitamin D levels and scar scores as measured using subjective scar assessments (mVSS and POSAS) in burns patients were analysed across all timepoints. a. 25D2 vs. mVSS at D03-D07; b. Bioavailable 25D3 vs. mVSS at D14-D28; c. VDBP vs. mVSS at M02-M12; d. 25D2 vs. OOSS at D03-D07; e. Bioavailable 25D3 vs. OOSS at D14-D28; f. 3-epi-25D3 vs. OPSS at D01; g. Free 25D3 vs. OPSS at D03-D07; h. Bioavailable 25D3 vs. OPSS at D03-D07. Correlations were performed between analyte levels at timepoints and subjective scar scores using Spearman's rank correlation co-efficient; * p <0.05.

4.3.7.2 Objective Scar Measures

Systemic levels of vitamin D metabolites were significantly associated with scarring outcomes in burns patients as per objective measures via various devices. Reduced levels of bioavailable 25D3 in burns patients was significantly associated with increased scar thickness, rho -0.549 ($p = 0.015$). Furthermore, increased serum levels of VDBP at M02-M12 following thermal injury significantly correlated with increased scar thickness, rho 0.466 ($p = 0.006$). Additionally, reduced levels of 1α25D3, 24,25D3 and 23,25D3 in blood were significantly associated with firmer scars, rho -0.369 ($p = 0.041$) at D14-D28, rho -0.561 ($p = 0.021$) at D03-D07 and rho -0.358 ($p = 0.012$) at D03-D28 respectively. Significant correlations between serum vitamin D metabolites measured longitudinally and objective scar measures via ultrasound and cutometer are summarised in Figure 4.10.

Throughout the study period, serum levels of vitamin D metabolites significantly correlated with subsequent scar erythema and pigmentation in burns patients. Significant correlations between systemic vitamin D analyte levels and colorimeter scar measures are summarised Figure 4.11 and Figure 4.12.

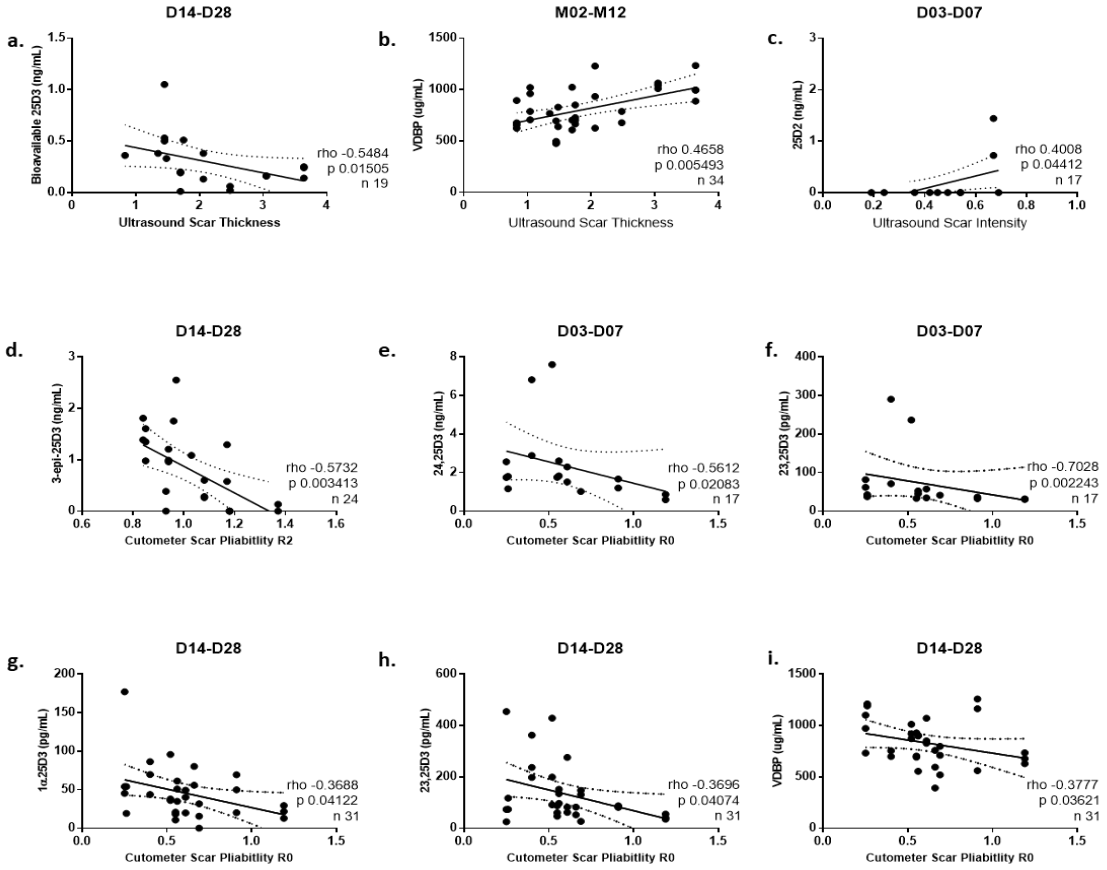


Figure 4.10. Vitamin D response and correlations with objective scar measures via ultrasound and cutometer following severe thermal injury.

Serum vitamin D levels and objective scar measures (ultrasound and cutometer) in burns patients were analysed across all timepoints. a. Bioavailable 25D3 vs. Scar Thickness at D14-D28; b. VDBP vs. Scar Thickness at M02-M12; c. 25D2 vs. Scar Intensity at D03-D07; d. 3-epi-25D3 vs. R2 at D14-D28; e. 24,25D3 vs. R0 at D03-D07; f. 23,24D3 vs. R0 at D03-D07; g. 1α25D3 vs. R0 at D14-D28; h. 23,25D3 vs. R0 at D14-D28; i. VDBP vs. R0 at D14-D28. Correlations were performed between analyte levels at timepoints and objective scar measures using Spearman's rank correlation co-efficient; * $p < 0.05$.

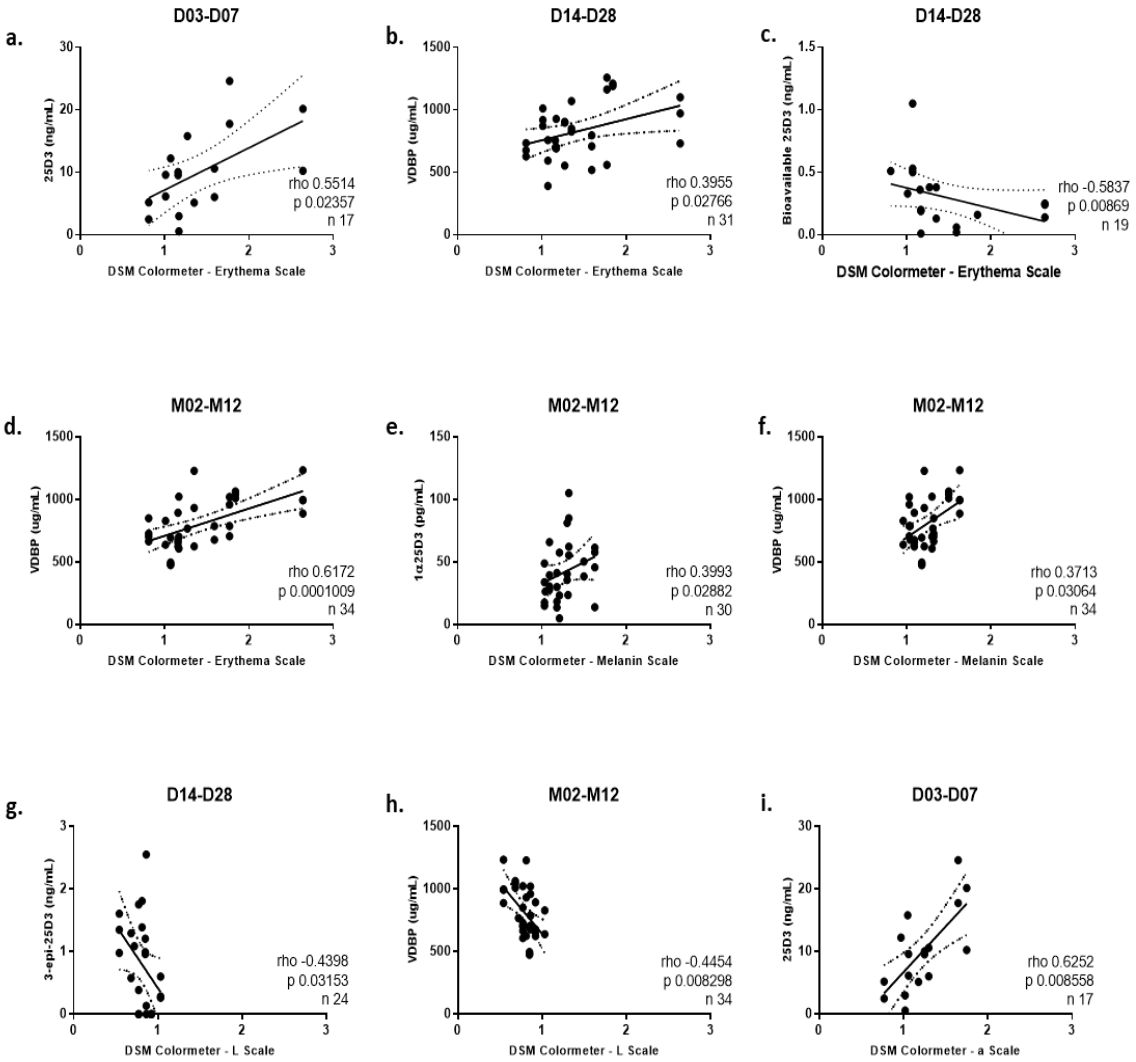


Figure 4.11. Vitamin D response and colormeter scar measure correlations following severe thermal injury (Part 1).

Serum levels of vitamin D metabolites and scar erythema/pigmentation in burns patients as measured by colorimeter were analysed across all timepoints. a.25D3 vs. Erythema at D03-D07; b. VDBP vs. Erythema at D14-D28; c. Bioavailable 25D3 vs. Erythema at D14-28; d. VDBP vs. Erythema at M02-M12; e. 1α25D3 vs. Melanin at M02-M12; f. VDBP vs. Melanin at M02-M12; g. 3-epi-25D3 vs. L at D14-D28; h. VDBP vs. L at M02-M12; i. 25D3 vs. a at D03-D07. Correlations were performed between analyte levels at timepoints and colormeter scar measures using Spearman's rank correlation co-efficient; * $p < 0.05$.

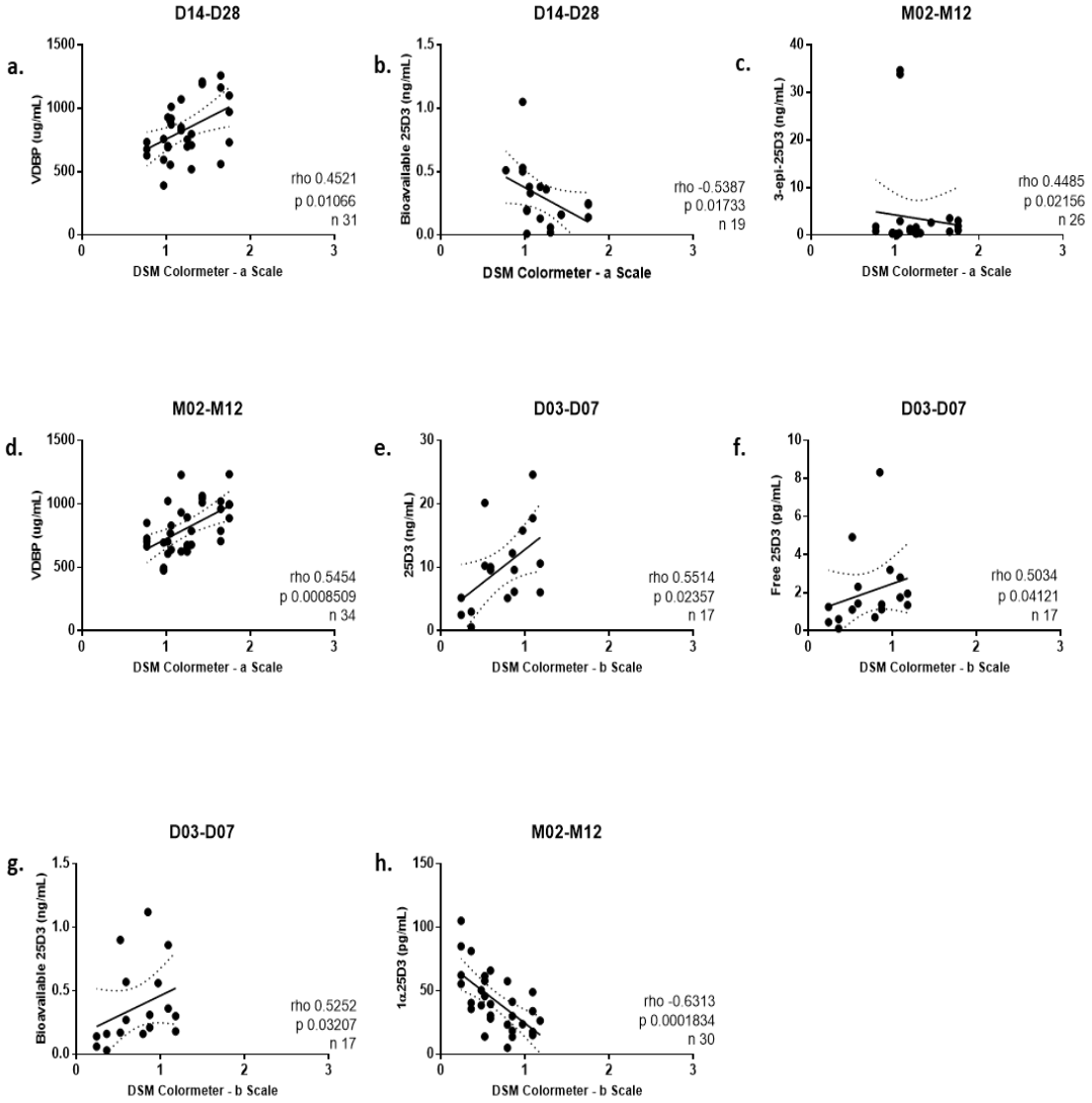


Figure 4.12. Vitamin D response and colormeter scar measure correlations following severe thermal injury (Part 2).

Continuation from Figure 4.11. a. VDBP vs. a at D14-D28; b. Bioavailable 25D3 vs. a at D14-D28; c. 3-epi-25D3 vs. a at M02-M12; d. VDBP vs. a at M02-M12; e. 25D3 vs. b at D03-D07; f. Free 25D3 vs. b at D03-D07; g. Bioavailable 25D3 vs. b at D03-D07; h. 1α25D3 vs. b at M02-M12.

4.3.8 Therapeutic Potential of Vitamin D following Severe Thermal Injury

Supplementing burns patients with vitamin D may reduce the odds of developing sepsis and mortality. Immediately following injury, serum levels of 25D3, Free 25D3 and Bioavailable 25D3 were associated with and predictive of sub-sequent sepsis development in burns patients, $p = 0.016$, $p = 0.022$ and $p = 0.025$ respectively. This effect was independent of patient demographics and injury severity. Furthermore, increasing circulating levels of 25D3, Free 25D3 and Bioavailable 25D3 by 1ng/mL may increase the odds of avoiding sepsis development by 18%, 64% and 199% respectively. Furthermore, systemic levels of 23,25D3 at D03-D07 post-thermal injury were associated with and predictive of 28-day mortality. Increasing serum levels of 23,25D3 in burns patients by 1 pg/mL may increase the odds of 28-day survival by 4.2%. At D14-D28 post-injury, serum levels of 25D3 were significantly associated with and predictive of mortality during admission episode. Increasing circulating 25D3 levels by 1 ng/mL may increase odds of survival following severe thermal injury by 32%. The multi-variate analysis and significant therapeutic potential of vitamin D metabolites following severe thermal injury are summarised in Table 4.3.

Supplementing vitamin D immediately following severe thermal injury may significantly reduce wound healing duration. On admission, systemic levels of 25D3 and bioavailable 25D3 in burns patients were significantly associated with and predictive of time taken to achieve 95% wound healing, p 0.042 and p 0.031 respectively. This effect was independent of patient demographics, injury severity and number of surgical interventions. Increasing serum levels of 25D3 and bioavailable 25D3 immediately following burn injury by 100% may reduce days taken to achieve

wound healing by 14% and 13% respectively. Multi-variate analysis and significant therapeutic potential of vitamin D metabolites on wound healing following thermal injury are summarised in Table 4.4. Multi-analyte multi-variate regression models of vitamin D metabolites and wound healing in burns patients demonstrated non-significant therapeutic value. Therefore, multi-analyte regression data on wound healing are not reported. Multi-variate regression analysis could not be performed using scarring data due to low patient numbers.

4.3.9 Vitamin D Metabolites Potential as Diagnostic/Prognostic Biomarkers Following Severe Thermal Injury

Serum 25D3 levels between septic and non-septic burns patients were significantly different at D01 post-injury (Figure 4.7). Circulating 25D3 levels at D01 post-thermal injury may predict patients not developing sepsis by 76% accuracy. The accuracy is further increased when adjusting 25D3 levels to injury severity, 80% with rBaux score and 81% with APACHE II score. AUC/ROC curve analysis for 25D3 and sepsis outcomes are summarised in Figure **4.13**.

Serum levels of 25D3, 24,25D3 and 23,25D3 in burns patients were significantly different between survivors and non-survivors at D03-D07 post-injury (Figure 4.4 and Figure 4.5). Levels of 24,25D3 in serum may predict 28-day survival with 76% accuracy. Furthermore, serum 24,25D3 levels may predict in-hospital mortality with 76% accuracy. This is further enhanced when accounting for 23,25D3 and clinical scoring systems, 82% with rBaux and 84% with APACHE II. AUC/ROC curve analysis for vitamin D metabolites and survival outcomes are summarised in Figure **4.14** and Figure **4.15**.

					Measure of Benefit (No Adverse Outcome)		
Timepoint	Outcome	Analyte	Co-Variates	p-value	Odds Ratio	Effect on OR for 1 unit (%)	Required units to increase OR by 1
D01	Sepsis	25D3	Age, TBSA, Inhalation Injury, Gender, APACHE II	0.014	1.25	22.2	4.50
D01	Sepsis	Free 25D3		0.018	1.88	63.3	1.58
D01	Sepsis	Bioavailable 25D3		0.026	7.30	199.1	0.50
D01	Sepsis	25D2		0.043	3.77	132.7	0.75
D03-D07	28D Mortality	23,25D3		0.049	1.042	4.2	23.81
D14-D28	Mortality	VDBP		0.022	1.004	0.4	250.00
D14-D28	MOF	VDBP		0.041	0.997	-0.3	-333.33
M02-M12	MOF	VDBP		0.035	0.997	-0.3	-333.33
D14-D28	Mortality	25D3	Age, TBSA, Inhalation Injury, Gender, APACHE II, All Analytes	0.030	1.38	32.1	3.12
D14-D28	Mortality	VDBP		0.026	1.02	1.5	66.67

Table 4.3. Potential Therapeutic Effects of Vitamin D on Binary Outcomes following Severe Thermal Injury.

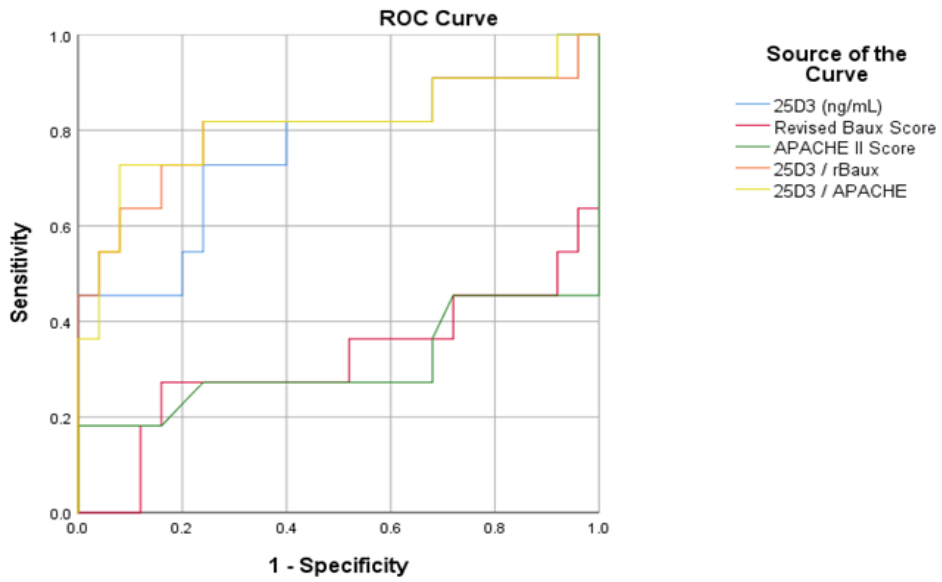
Outcomes and Vitamin D metabolites at all timepoints were assessed using uni-analyte and multi-analyte regression models. Multi-variate binary logistic regression was performed accounting for patient demographics, injury severity and/or physiological state on admission. Significant associations $p < 0.05$.

Timepoint	Outcome	Analyte	Co-Variates	p-value	1% analyte increase reduces healing days by (%)
D01	95%heal	25D3	Age Gender TBSA Inhalation Injury Excision No. Grafting No.	0.042	0.14
D01	95%heal	Bioavailable 25D3		0.031	0.13

Table 4.4. Potential Therapeutic Effects of Steroids on Wound Healing following Severe Thermal Injury.

Wound healing and Vitamin D metabolites at all timepoints were assessed using uni-analyte and multi-analyte regression models. Multi-variate linear regression was performed using categorical variables and natural logarithm of continuous variables accounting for patient demographics, injury severity and surgical procedures performed. Significant associations $p < 0.05$.

a.



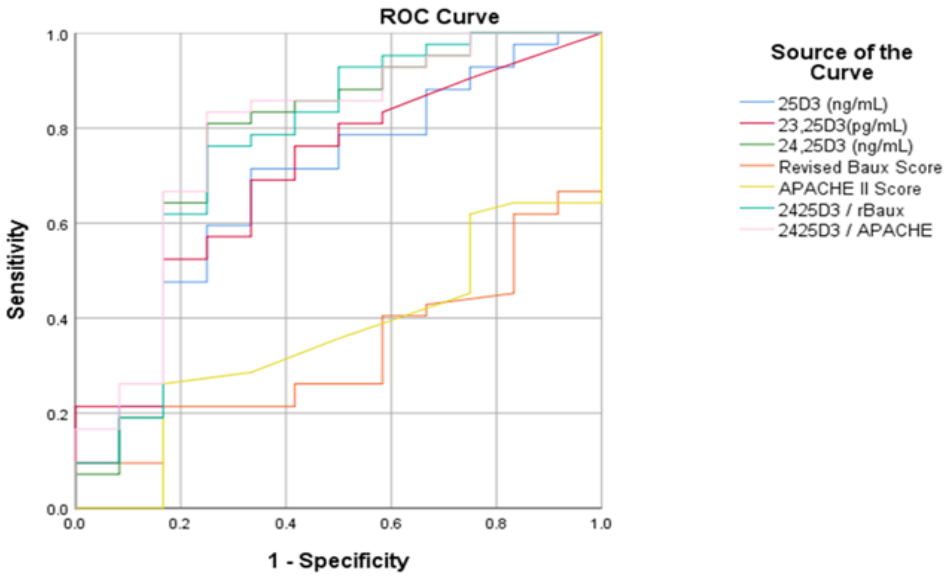
b.

Test Result Variable(s)	Area
25D3 (ng/mL)	0.756
rBaux Score	0.316
APACHE II Score	0.311
25D3 / rBaux	0.804
25D3 / APACHE II	0.811

Figure 4.13. Serum 25D3 levels at D01 post-injury and not developing sepsis in burns patients.

Predictive strength of serum 25D3 levels at D01 post-injury on burns patients not developing sepsis were assessed using AUC/ROC curve analysis. a. ROC curve analysis of 25D3 levels and clinical scores both alone and in-combination; b. AUC analysis of 25D3 levels and clinical scores both alone and in-combination.

a.



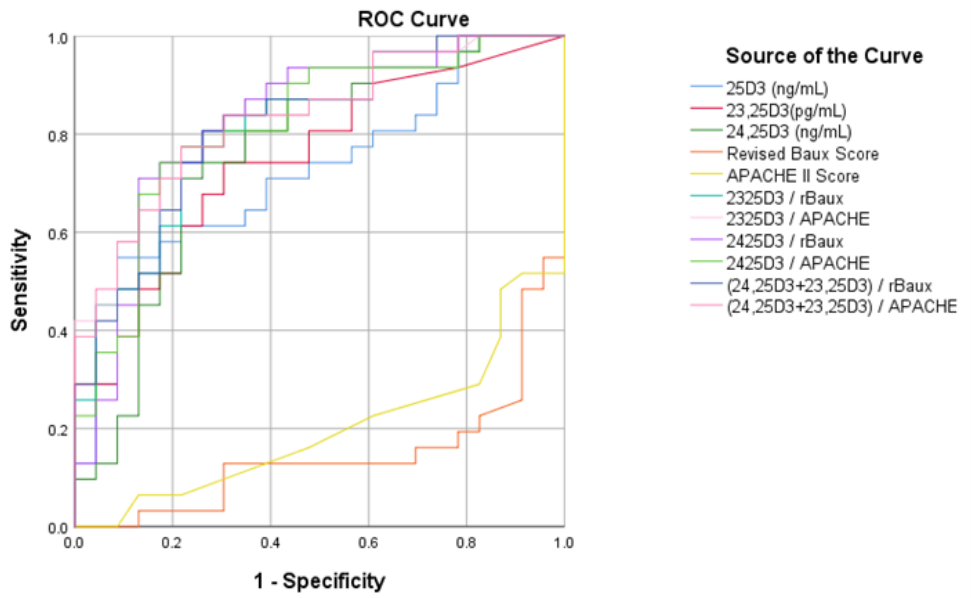
b.

Test Result Variable(s)	AUC
25D3 (ng/mL)	0.681
23,25D3(pg/mL)	0.698
24,25D3 (ng/mL)	0.764
rBaux Score	0.327
APACHE II Score	0.360
2425D3 / rBaux	0.762
2425D3 / APACHE II	0.782

Figure 4.14. Serum vitamin D metabolites levels at D03-D07 post-injury and 28-day survival in burns patients.

Predictive strength of serum levels of vitamin D metabolites at D03-D07 post-injury on 28-day survival were assessed using AUC/ROC curve analysis. a. ROC curve analysis of levels of vitamin D metabolites and clinical scores both alone and in-combination; b. AUC analysis of levels of vitamin D metabolites and clinical scores both alone and in-combination.

a.



b.

Test Result Variable(s)	AUC
25D3 (ng/mL)	0.721
23,25D3(pg/mL)	0.749
24,25D3 (ng/mL)	0.759
rBaux Score	0.144
APACHE II Score	0.199
2325D3 / rBaux	0.809
2325D3 / APACHE II	0.828
2425D3 / rBaux	0.825
2425D3 / APACHE II	0.816
(24,25D3+23,25D3) / rBaux	0.819
(24,25D3+23,25D3) / APACHE II	0.835

Figure 4.15. Serum vitamin D metabolites levels at D03-D07 post-injury and in-hospital survival in burns patients.

Predictive strength of serum levels of vitamin D metabolites at D03-D07 post-injury on in-hospital survival were assessed using AUC/ROC curve analysis. a. ROC curve analysis of levels of vitamin D metabolites and clinical scores both alone and in-combination; b. AUC analysis of levels of vitamin D metabolites and clinical scores both alone and in-combination.

4.3.10 Longitudinal Vitamin D and Immune-Endocrine Response following Thermal Injury

Acute severe burn injury induces major simultaneous immune-endocrine responses. Following injury, patients exhibited increased expression of both upstream and final metabolites of the Vitamin D axis.

Severe thermal injury induced an immediate reduction of circulating 25D3 and VDBP while elevating free and bioavailable 25D3 in serum. This suggests increased utilisation of 25D3 in its free and bioavailable forms following acute thermal injury through autocrine means. Interestingly, there was also simultaneous increase in albumin, calcium, phosphate, monocyte ROS production, IL-1Ra and IL10 at D01 post-injury. This may indicate enhanced systemic activity and interaction of free and bioavailable 25D3 with various body systems including monocytes/immune cells, bone and gastrointestinal tract.

Major burn injury appears to activate alternative pathways of vitamin D metabolism. This is evident by the increased expression of circulating levels of 23,25D3 in burned patients at D01 post-injury compared to healthy volunteers. Furthermore, the C23-hydroxylation pathways remain active in patients for, at least, 12 months post-thermal injury. The impact of this vitamin D metabolism pathway remains to be established.

Burn patients exhibited a prolonged differential immune-endocrine response when compared to healthy volunteers. The immune and hormonal status between burned patients and healthy volunteers remain altered for, at least, one year following injury. This was particularly in the case in all vitamin D metabolites, immune responses (ROS

generation and phagocytosis), IL-β1 and TGF-β1 levels. Interestingly, the longitudinal response of IGF-1 was similar to 1,25D3 and 23,25D3 responses following severe thermal injury. This indicates possible interaction and feedback mechanism between the IGF and vitamin D axis in burned patients. Correlations between IGF-1 and vitamin D has been reported in other pathologies such as metabolic syndrome and cardiovascular disease (530-532). The longitudinal vitamin D and immune-endocrine status and interactions in burned patients and healthy volunteers are summarized in Figure 4.16.

4.3.11 Subgroup Analysis

4.3.11.1 Gender Influence on Longitudinal Vitamin D Response following Severe Thermal Injury

Male and female burns patients exhibited significant differences in the vitamin D response immediately following injury. Injury severity between both cohorts were similar, p = 0.972. At D01 post-thermal injury, male patients had significantly increased levels of 25D3, 3-epi-25D3, VDBP, free 25D3 and bioavailable 25D3 compared to female patients, median 8.4 ng/mL vs. 2.2ng/mL (p = 0.008), 0.46 ng/mL vs. 0.20 ng/mL (p = 0.016), 346.7 µg/mL vs. 225.4 µg/mL (p = 0.023), 1.3 pg/mL vs. 0.7 pg/mL (p = 0.028) and 0.34 ng/mL vs. 0.19 ng/mL (p = 0.034) respectively. Systemic vitamin D metabolite levels in male and female burns patients are summarised longitudinally in Figure 4.17.

4.3.11.2 Age Influence on Longitudinal Vitamin D Response following Severe Thermal Injury

No major differences were observed in the vitamin D response in young and older burns patients. Injury severity and gender were similar between both cohorts, $p = 0.076$ and $p = 0.708$. Circulating vitamin D metabolite levels in young and elderly patients following thermal injury are summarised longitudinally in Figure 4.18.

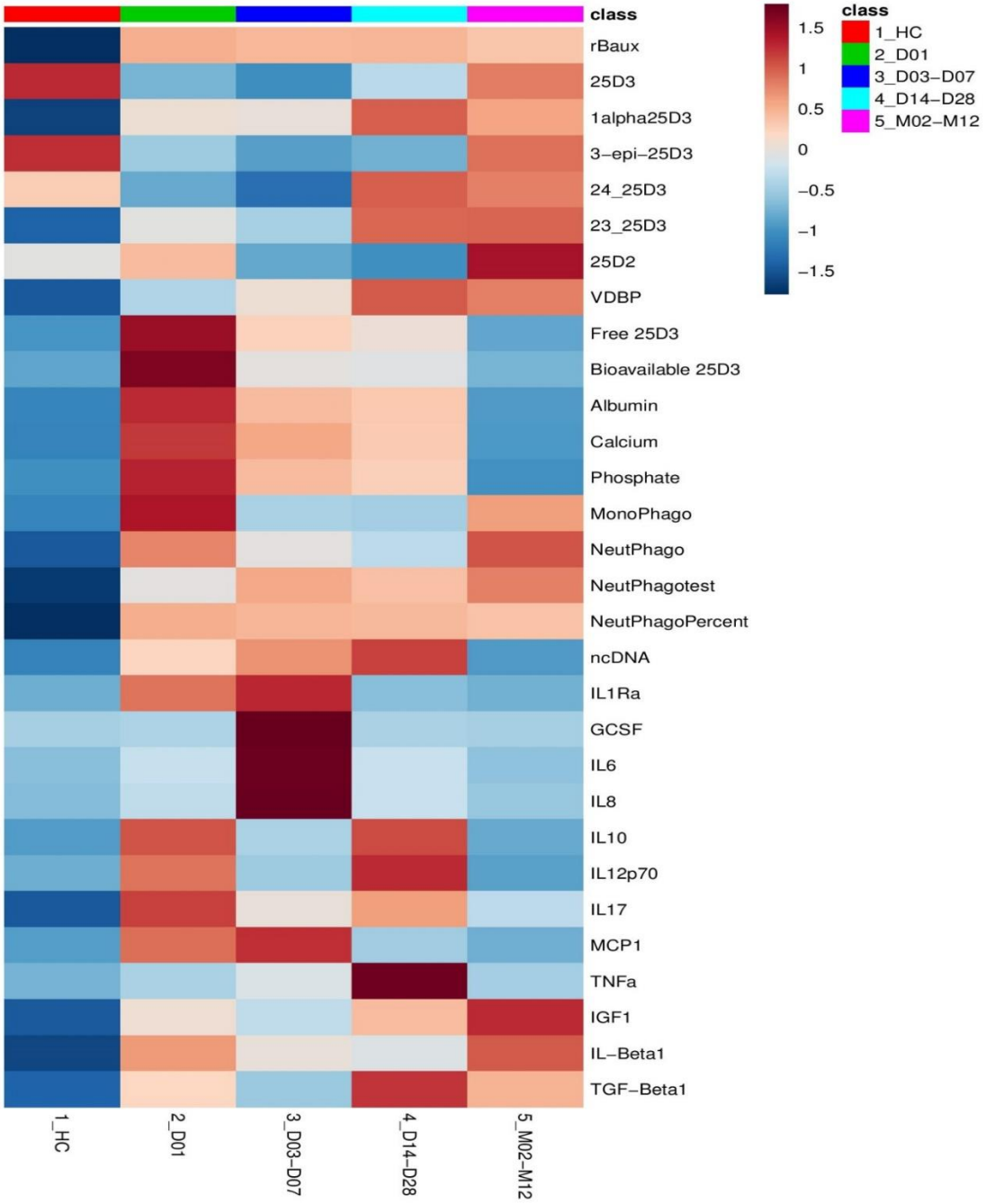


Figure 4.16. The longitudinal vitamin D and immune-endocrine response following major burn injury.

Circulating status of cytokines, immune function and vitamin D metabolites were analysed across all timepoints accounting for the revised Baux score. Immune-endocrine status of healthy volunteers was performed to allow comparison. This heatmaps represent expressions of molecules in serum by colour with high expressions being red and low expressions being blue. Immune function tests include Monocyte and Neutrophil ROS generation (Phagoburst MFI), monocyte and Neutrophil Phagocytosis (Phagotest, % positive) and Nuclear cfDNA concentration (marker of NETs). Cytokines and other hormones analysed include IL-1Ra, GCSF, IL-6, IL-8, IL-10, IL-12p70, IL-17, MCP-1, TNF-α, IGF-1, IL-β1 and TGF-β1.

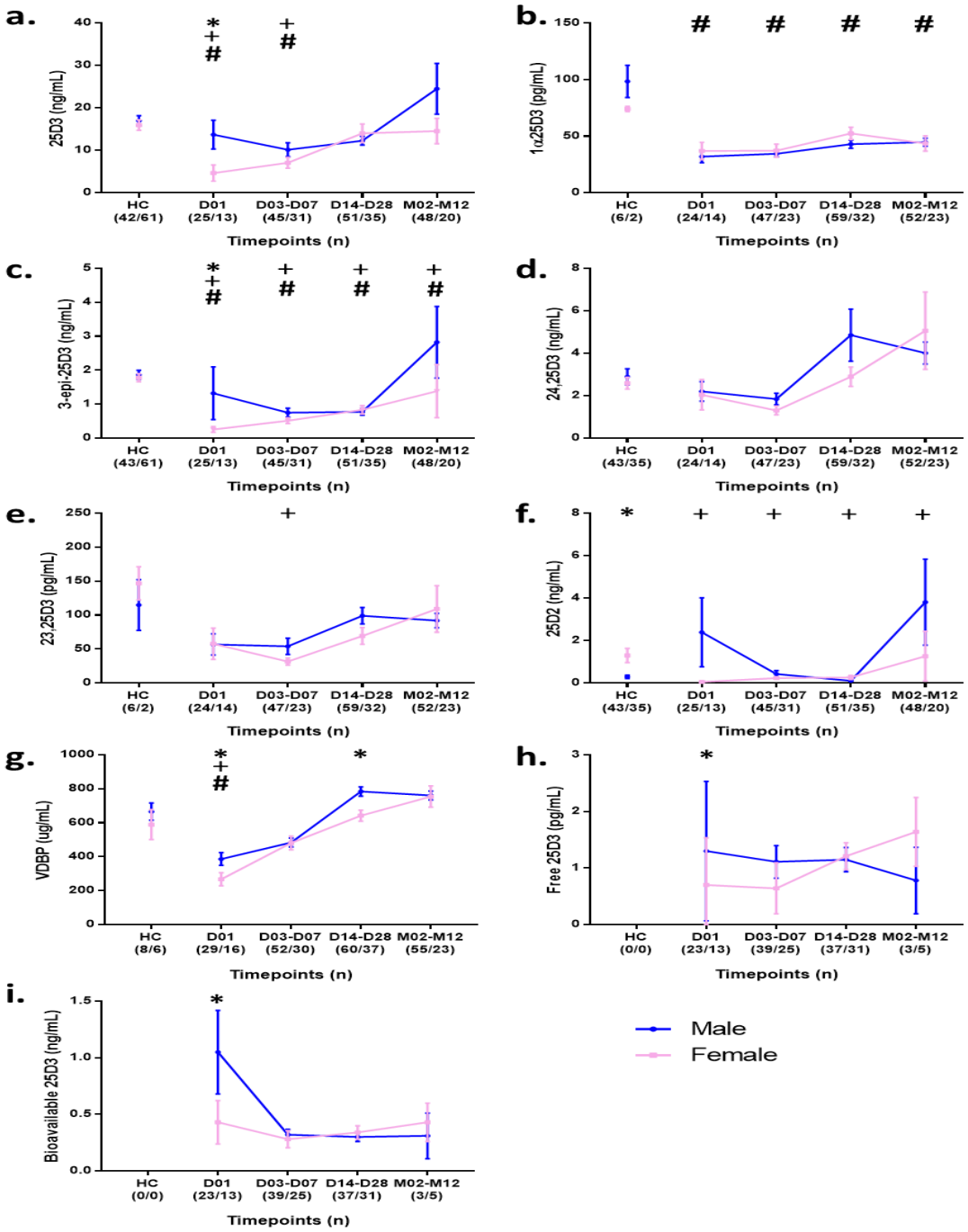


Figure 4.17. Longitudinal vitamin D response in females and males following burn injury.

Serum vitamin D metabolite levels in female and male burns patients were analysed across all timepoints. a. 25D3; b. 1α25D3; c. 3-epi-25D3; d. 24,25D3; e. 23,25D3; f. 25D2; g. VDBP; h. Free 25D3; i. Bioavailable 25D3. Analyte levels at each timepoint was compared between both cohorts (Female vs. Male) using Mann-Whitney Test; * $p < 0.05$. Analyte levels of female and male burns patients were compared to female and male healthy controls (HC) using Kruskal-Wallis test with Dunn's multiple corrections; + Female vs. HC Female, $p < 0.05$; # Male vs. HC Male, $p < 0.05$.

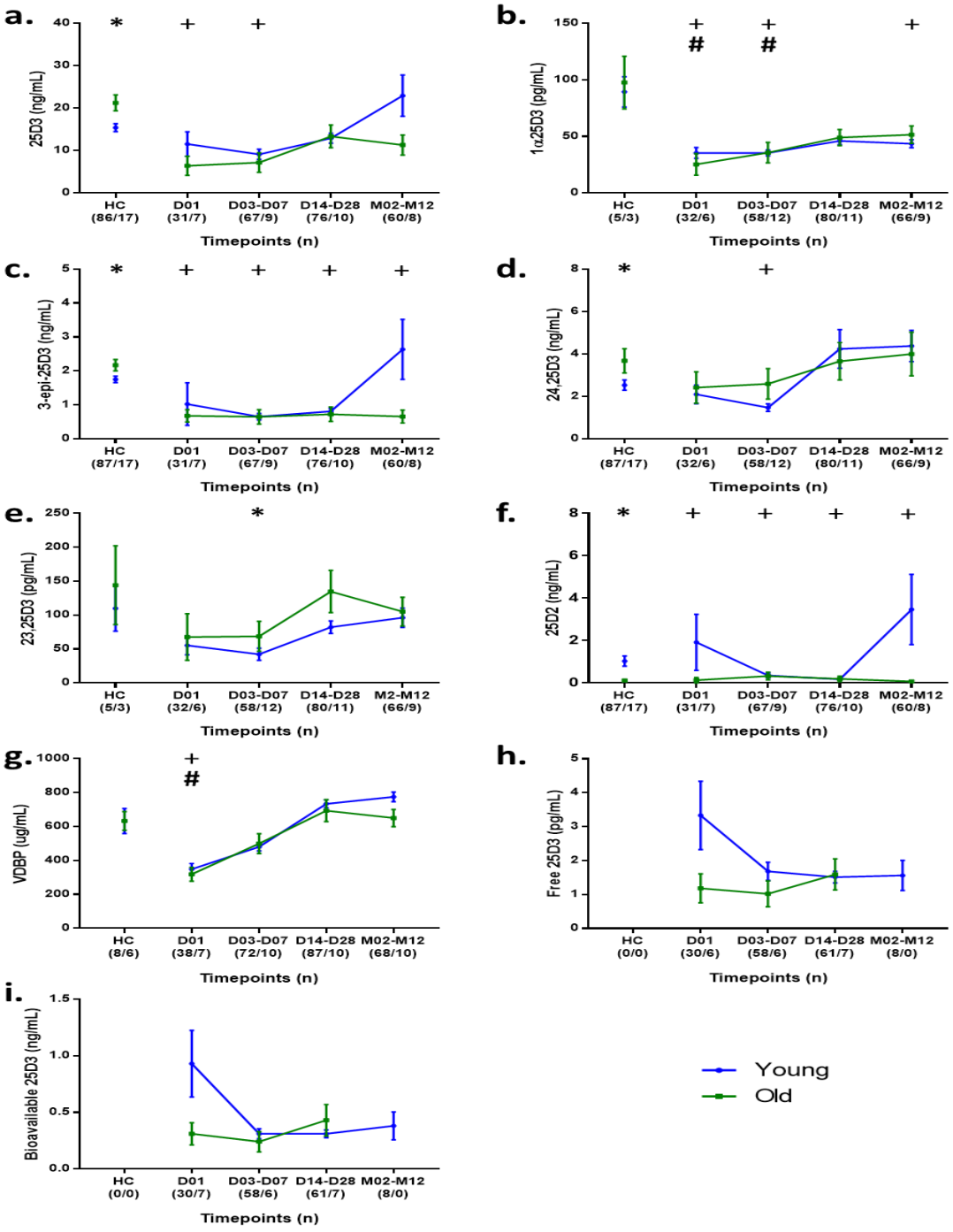


Figure 4.18. Longitudinal vitamin D response in females young and old following burn injury.

Serum vitamin D metabolite levels in young and elderly burns patients were analysed across all timepoints. a. 25D3; b. 1α25D3; c. 3-epi-25D3; d. 24,25D3; e. 23,25D3; f. 25D2; g. VDBP; h. Free 25D3; i. Bioavailable 25D3. Analyte levels at each timepoint was compared between both cohorts (Young vs. Old) using Mann-Whitney Test; * $p < 0.05$. Analyte levels of young and old burns patients were compared to young and old healthy controls (HC) using Kruskal-Wallis test with Dunn's multiple corrections; + Young vs. HC Young, $p < 0.05$; # Old vs. HC Old, $p < 0.05$.

4.4 Discussion

This study was carried out to profile the vitamin D response for up to 12 months following severe burn injury using high sensitivity LC-MS/MS. This study also investigated the associations of altered vitamin D metabolism and status with outcomes in burn patients throughout the first year following thermal injury. Furthermore, this study explored the diagnostic and therapeutic potential of vitamin D metabolites. This is the first report exploring these areas simultaneously and is warranted for the following reasons.

Rousseau et al investigated vitamin D status following major burn injury using LC-MS/MS and reported significantly low circulating 25D3, 1,25D3 in patients compared to healthy volunteers (130). Similar to their findings, here various vitamin D metabolites were significantly reduced immediately following thermal injury including 25D3 and 1,25D3. Additionally, Rousseau et al demonstrated that burned patients exhibited significantly reduced levels of systemic free 25D3 compared to un-injured individuals, 3.8 pg/ml vs. 6.2pg/ml ($p<0.05$)(130). In this study, median free 25D3 levels in serum of burned patients were 1.1 pg/ml at D01 and 1.6 pg/ml at M02-M12. This indicates that altered vitamin D status and metabolism observed in burned patients persisted for at least 12 months following injury. Similar findings were previously reported (509). Interestingly, Klein et al reported significantly lower levels of 25D in serum in burned patients 7 years post-injury (518). This could be attributed to a persistent hypercatabolic state observed following thermal injury and sub-optimal production of vitamin D by skin in burned patients (517). Despite exhibiting significantly decreased levels of serum 25D3, circulating levels of 1,25D3 in burned

patients remained within reference range (15-75 pg/mL). This may indicate that 25D3 is being utilised in an autocrine manner (non-classical vitamin D metabolism) following severe burn injury. Such significant reductions in vitamin D levels following major thermal injury may predispose patients to poorer outcomes.

During the acute phase following thermal injury, the status of various vitamin D metabolites was associated with sepsis, survival and wound healing. Burned patients with higher levels of vitamin D metabolites exhibited better prognosis. Higher circulating levels of 25D3, 23,25D3 and 24,25D3 during the first 21 days was observed among survivors following thermal injury. Furthermore, these metabolites demonstrated good discriminatory power between survivors and non-survivors post-thermal injury with AUROC >0.8 when adjusted with clinical scoring systems (Figure 4.14 and Figure 4.15). Furthermore, the status of 25D3, 24,25D3 and VDBP were independently associated with survival of burned patients. This indicates that vitamin D supplementation may help in further reducing mortality rates following major thermal injury. Similar observations were reported in critically patients (259, 277, 278). However, no other reports investigating the influence of vitamin D on survival post-major burn injury are currently reported.

Associations between low vitamin D status and sepsis were reported in critically ill populations (273, 274). Similarly, patients with higher serum levels of 25D3 immediately following major thermal injury did not progress to develop sepsis. This observation had good predictive value with AUROC >0.8 when adjusted to clinical scoring systems. Furthermore, circulating levels of 25D3, free 25D3 and bioavailable

25D3 demonstrated to be independently associated and predictive of subsequent development of sepsis in severely burned patients. Interestingly, high expressions of free and bioavailable 25D3 coincided with elevated expressions of monocyte ROS production at D01 post-injury. This is in keeping with reported immunomodulatory roles of vitamin D (533). Potential mechanisms revolve around the 'free hormone hypothesis' where free and bioavailable 25D access target cells via passive diffusion of lipid soluble 25D through cell membranes (534, 535). Furthermore, this suggests that immediate vitamin D supplementation following burn injury and increasing serum free and bioavailable 25D3 levels may prove to be immense potential therapeutic value in decreasing the risk of subsequent sepsis for the following reasons. Firstly, burned patients exhibit significant fluid shifts and protein translocation immediately following injury. Therefore, increasing circulating levels of free 25D3 may prove beneficial and improve monocytes and other immune cell functions through autocrine utilisation. Major thermal injury is known to significantly disrupt the immune system and response predisposing patients to sepsis (112). Secondly, burned patients exhibit significant systemic inflammatory response making the diagnosis of sepsis difficult and risks treatment delay (466). Lastly, delay of appropriate treatment is significantly associated with increased mortality and poorer prognosis (467). Hence, measurement of free and bioavailable 25D3 can be valuable in the management of burn patients.

A recent review highlighted the importance of free vitamin D measurements as free 25D levels are independent of confounding factors (such as liver function, kidney disease and pregnancy) and correlated well with pathological conditions including liver, kidney, tumour and allergic diseases (536). Interestingly, a recently published

case report described a 58-year-old with severe vitamin D deficiency was diagnosed with congenital VDBP deficiency associated with homozygous deletion of the GC gene (537). Secondary to VDBP absence, serum 25D levels were persistently undetectable. On further analysis, free and bioavailable 25D were quantified with free 25D being directly measured. Despite lifelong VDBP deficiency with undetectable 25D, the patient did not develop rickets or osteomalacia and only presented to the medical services in her fifth decade due to osteopenia and fragility fractures. This case report presents the strongest clinical evidence thus far, in support of the 'free hormone hypothesis' and intracrine vitamin D metabolism (534, 535, 538). No other reports studying vitamin D status and sepsis in severely burned patients are in the literature.

Vitamin D status and metabolism demonstrated significant correlations with wound healing and subsequent scarring. During the acute phase following thermal injury, circulating 25D3 levels and metabolism via both hydroxylation pathways had significant association with wound healing durations. Moreover, serum 25D3 and bioavailable 25D3 levels at D01 post-burn injury were associated and predictive of time taken to achieve wound closure independent of injury severity and subsequent surgical intervention. This suggests that immediate supplementation of 25D3 in severely burned patients may probably improve wound healing rates. Although, no studies examining wound healing and acute response of vitamin D following major thermal injury could be identified in the literature. Similar observations were made in other pathologies such as diabetic foot ulcers (539, 540).

In this study, various vitamin D metabolites throughout the first-year post-major thermal injury have shown significant correlations with subsequent objective and subjective scar measures. A relationship between lower vitamin D status in burned patients and poor scarring outcomes was previously reported (520). Furthermore, post-burn vitamin D deficiency was associated with worse biomechanical properties of subsequent hypertrophic scarring, including increased pigmentation, reduced skin barrier function, decreased scar pliability, and slower movement of interstitial fluid (541, 542). These observations could be secondary to amelioration of dermal immune-inflammatory responses by vitamin D(543). This suggests that long term vitamin D supplementation following thermal injury may be beneficial.

23,25D3 is one of the by-products of C23 hydroxylation pathway in vitamin D metabolism. In this study, higher serum levels of 23,25D3 were observed among survivors following thermal injury, as well as in burned patients who did not develop multi-organ failure and/or sepsis. Additionally, lower circulating levels of 23,25D3 following thermal injury was significantly associated with delayed wound healing and poor scarring outcomes. This is a novel finding as no literature describing 23,25D3 and the C23 hydroxylation metabolism in the context of clinical care and outcomes exists. The biological activity of 23,25D3 remains to be established. Furthermore, Prof. Martin Hewison's team and Prof. Ann Logan's team explored potential effects of this molecule on monocyte function and mRNA levels of α-SMA, fibronectin and collagen I respectively. These experiments did not reveal any biological activity/effects of this metabolite (unpublished data). This suggests that 23,25D3 may be an intermediary by-

product and biomarker of the c23 hydroxylation pathway of vitamin D metabolism. No published literature describing the biological activity of 23,25D3 were identified.

This study has limitations. The addition of participants of the HAS study as healthy controls with inclusion criteria different from the e-SIFTI healthy controls. The stringent exclusion criteria in the HAS study may not reflect the co-morbid status of the burnd cohort. Furthermore, fibroblast growth factor-23 (FGF-23) levels in serum were not quantified owing to limited serum quantity. FGF-23 plays a complex and essential role in vitamin D metabolism. FGF-23 inhibits renal 1α-hydroxylase and subsequent production of 1,25D3 (544, 545). Additionally, FGF-23 stimulates the catabolism of 1,25D3 by activating 24-hydroxylase(546). Burned patients exhibited significantly elevated systemic levels of FGF-23 and lower vitamin D status compared to healthy volunteers (130, 510). This indicates that elevated FGF-23 levels following thermal injury is a potential mechanism associated with significant reduction in vitamin D levels observed in severely burned patients in this study. Therefore, elevated circulating FGF-23 levels may have adverse effect outcomes following thermal injury. This is concerning, as increased systemic levels of FGF-23 was associated with poor outcomes, including mortality, in critically ill patients (547-549).

4.5 Conclusions

Major thermal injury significantly influences vitamin D status and metabolism in patients for at least 12 months post-injury. Lower levels of various vitamin D metabolites in burned patients were significantly associated with poorer prognosis including sepsis, mortality, wound healing and subsequent scarring. Data indicate that

immediate and long-term vitamin D supplementation follow severe burn injury may greatly improve outcomes. Further studies are encouraged to validate the effects of post-burn vitamin D status and outcomes. RCTs investigating the influence of vitamin D supplementation, 25D3, on the prognosis of burned patients may be required for clinical implementation.

CHAPTER 5: ADIPOKINE STATUS AND ITS INFLUENCE ON OUTCOMES FOLLOWING SEVERE BURN INJURY

5.1 Introduction

Adipose tissue is increasingly becoming recognised as a functional endocrine organ with potential roles in various pathologies including type 2 diabetes, cancer and dementia, through its pro-inflammatory actions(550-554). Hence, the relationship between adipose tissue and critical illness has been gaining interest.

Critical illness following injury is a multifactorial heterogeneous disorder characterised by an overwhelming pro-inflammatory response accompanied by a compensatory anti-inflammatory reaction and subsequent immunosuppression (555, 556). This classical paradigm also applies to severe forms of critical illness such as burns, the pathology of which we have described previously (133, 134). The human response to burn injury includes a so-called 'genomic storm'(557), consistent with simultaneous increased systemic inflammation, innate immune activation and anti-inflammatory response (94, 112), as well as suppression of adaptive immunity (557). In addition, burn patients and others with severe critical illness suffer from a prolonged hypermetabolic, hypercatabolic response (94, 558).

The metabolic response following thermal injury is characterised as a two phase response: the 'ebb' phase within 48 hours where metabolism, cardiac output and oxygen consumption are reduced, followed by the 'flow' phase at approximately 120 hours post-injury where these parameters increase and plateau (559). This metabolic response includes: peripheral lipolysis and free fatty acid (FFA) (513) oxidation leading to an acute, global and complex increase in FFA levels (560); systemic induction of endoplasmic reticulum stress and unfolded protein response (561); up to 6-fold

increase in breakdown rates of skeletal muscle protein (562); elevation in REE up to 140%(474) that can be prolonged (94).

Burns and other severe critical illnesses have been reported to influence adipose tissue morphologically and functionally. Saraf *et al* reported the impact of severe burn injury on subcutaneous white adipose tissue in children and observed significantly reduced adipocyte size, increased collagen deposition and cell mitochondria content, increased immune cells such macrophages, as well as increased inflammatory cytokine production (563). These morphological changes suggest "browning" of subcutaneous adipose tissue following thermal injury, a finding which was confirmed biochemically and functionally. Sidossis *et al* reported significantly increased mitochondrial density and mitochondrial respiratory capacity, as well as an 80-fold increase in the expression of uncoupling protein 1 (UCP1), a molecule abundantly observed in brown adipose tissue depots (564), in burn patients compared to healthy controls (565). In addition, Patsouris *et al* reported similar findings including significantly increased mitochondrial mass and adipose tissue browning markers in burn patients (566). This could be a compensatory mechanism since brown adipose tissue is known to induce thermogenesis, modulate energy expenditure and exert local tissue effects such as stimulating angiogenesis and influencing macrophage polarization (567). Similar morphological and metabolic activity alterations of adipose tissue have been reported in critically ill patients (568, 569). A functional aspect of adipose tissue is its endocrine role through the production of adipokines, adipocyte derived cytokines, and these may mediate many of the effects seen in burns and critical illness.

There are many adipokines now described and they exert both anti-inflammatory and pro-inflammatory effects on various body systems. These include adiponectin, ghrelin, leptin, resistin and visfatin Adiponectin is released exclusively from white adipose tissue (326), and is the most abundant adipose-specific adipokine, with greater expression in subcutaneous fat compared to visceral fat (327). Adiponectin exerts anti-inflammatory effects (328). Ghrelin is an orexigenic hormone that is an endogenous ligand to growth hormone and was thought to be produced primarily by the stomach (329), but has subsequently been identified in other tissues including adipose tissue (330). Ghrelin signalling, independent of growth hormone and dietary intake, is associated with adiposity, changes in fat distribution and mobilisation (331, 332). Leptin is primarily secreted by subcutaneous white adipose tissue, the amount of leptin secreted into the circulation is proportional to adipose tissue mass and nutritional status (328). Leptin exhibits structural similarities to cytokines (333) and is pro-inflammatory (334). Resistin is also a pro-inflammatory adipokine expressed by adipocytes and other tissues including skeletal muscle (335, 336). Visfatin, also called pre-B-cell colony enhancing factor, is primarily secreted by adipocytes in visceral white adipose tissue and exhibits pro-inflammatory effects (337).

More recently it has been shown that adipokine levels in the circulation have significant associations with outcomes in critically ill patients. These effects of adipokines on critical illness have been summarised in Chapter 1 and previously published (135). Systemic levels of adiponectin, ghrelin and leptin demonstrated heterogenous results and correlations illness severity, as measured through biomarkers and clinical scoring systems, and outcomes including mortality. Critically ill

patients exhibit significantly raised circulating resistin (131, 371, 374, 375, 387, 391-396) and visfatin levels (387, 397-403). Additionally, both resistin and visfatin significantly correlated with pro-inflammatory responses as measured via systemic biomarkers (including CRP, IL-6, IL-8 and TNF-α), and worse clinical severity scores (such as APACHE II, Glasgow Coma score, multiple organ dysfunction score, SAPS II and SOFA)(131, 371, 374, 375, 387, 391-395, 397-403). Furthermore, high resistin and visfatin levels in blood were associated with poor prognosis in critically ill populations including mortality (392, 393, 398-402).

A systematic review examining the evidence for adipokines having an influence on critical care patients has been published recently (404). It concludes that although strong observations were reported indicating the influence of adipokines on the prognosis of critical illness, additional investigations with more diverse study participants (such as age, gender, BMI, ethnic groups and different pathologies) are required to improve current understanding of adipokines in critically ill populations. This is essential in order to validate the potential clinical value and utility of adipokines as diagnostic and/or prognostic biomarkers, as well their potential as therapeutic targets in critical illness including burn and trauma. Furthermore, studies to date have investigated the association of adipokines with critical illness in the acute setting only. This focus on the acute setting has further limited the translation of adipokines in clinical settings. Importantly, since medical advancements have improved survival rates after critical trauma (405-407), greater emphasis is now placed on the prevention and treatment of potentially debilitating long-term sequelae experienced

by survivors of severe illness including chronic critical illness (408-410), prolonged pathophysiological responses(94) and scarring (411).

Due to the scarcity of reported data, the adipokine response to burn injury and its possible effects on prognosis of patients remains unclear. We hypothesize that major burn injury results in significantly affects adipokines and that systemic levels of adipokines post-injury maybe associated outcomes including sepsis, MOF, mortality, wound healing and scarring. We hypothesize that adiponectin and ghrelin may have potential therapeutic value following major burn injury.

The aims of this thesis include determining the status of the adiponectin, leptin, ghrelin, resistin and visfatin in severely burned patients; characterising these adipokines longitudinally from day 1 following severe thermal injury till month 12 post-injury; exploring associations between the longitudinal adipokine response and clinical outcomes in burned patients including mortality, MOF, sepsis, wound healing and scarring and identify any potential clinical biomarkers that may improve prognosis of burned patients or identify novel therapeutic targets.

5.2 Methods

General methodology of the study, including ethical approval and statistical analysis, are described in Chapter 2. It is important to point out that patient weight and body mass index were not used in this chapter despite their importance for adipokine levels. This is because the weight and height recorded for burn patients were estimated

values rather than absolute measurements due to the critical state of burn patients and logistics of the intensive care unit in QEHB.

5.2.1 Measurement of Adiponectin

Adiponectin levels in serum samples were measured using Duoset® ELISA Kits (DY 1062 RnD Systems™, Oxfordshire, UK). Plate preparation and reconstitution of assay reagents were done according to the kit protocol. Samples had been frozen at -80C and were thawed and diluted in Reagent Diluent at 1:5000. 100 µl of the final dilution was added to the plates and left to incubate overnight at 4°C with shaking.

Following incubation, the well contents were discarded and washed 3 times with 400µl of wash buffer. One hundred microliters of diluted biotinylated human adiponectin detection antibody were added to each well thereafter, followed by incubation for 2 hours with shaking at room temperature. The contents of the well were then discarded and washed as previously mentioned. Following this, 100µl of Streptavidin-HRP was added to each well and left to incubate for 20 minutes at room temperature in the dark. The contents of the well were then discarded and washed 3 times to remove unbound antibody. One hundred microliters of substrate solution were then added to each well and left to incubate for 20 minutes at room temperature in the dark. Thereafter, fifty microliters of stop solution were added to each well.

The absorption of each plate was determined using a BioTek ELx808™ Absorbance Microplate Reader and BioTek Gen5™ Data Analysis Software (BioTek®, Swindon, UK) at 450nm against 550nm as reference. Adiponectin levels were extrapolated from standard curves using Graphpad PRISM® (GraphPad Software Inc., California, USA).

Interplate and intraplate variability are 14.9% and 30% respectively. The standard curves are illustrated in Supplementary Figure 7.

5.2.2Measurement of Ghrelin, Leptin, Resistin, Visfatin

Levels of Ghrelin, Leptin, Resistin and Visfatin were measured in serum samples using a multiplex method and the Bio-Plex Pro™ Human Assay Kit (#171-304070M Bio-Rad Laboratories Inc., Hertfordshire, UK). Flat bottom plates and reconstitution of assay reagents including magnetic beads were prepared as per kit protocol. Samples were prepared and diluted using Sample Diluent to 1 in 4. Samples were then vortexed and 50µl was added to each well. The plates were incubated for 1 hour at room temperature with shaking.

Following incubation, the well contents were washed 3 times with 100µl of wash buffer. Next, 25µl of 1x diluted detection antibody was added to each well and left to incubate for 30 minutes at room temperature with shaking. The contents of the well were then washed 3 times with wash buffer. Fifty microliters of diluted 1x Streptavidin-PE was then added to each well and left to incubate for a further 10 mins at room temperature with shaking. The wells were then washed 3 times with 100µl of wash buffer to remove unbound antibody. All wash procedures were carried out using Bio-Plex Pro™ Wash Station (Bio-Rad Laboratories Inc., Hertfordshire, UK).

Following the final wash, the beads were re-suspended in 125µl of assay buffer and incubated for 30 seconds at room temperature with shaking. The fluorescence of each plate was determined and converted into absolute concentrations using Bio-Rad Bio-

Plex 200™ Plate Reader and Bio-Plex Manager™ (Bio-Rad Laboratories Inc., Hertfordshire, UK) with settings adjusted as per kit protocol.

5.3 Results

5.3.1 Patient Demographics and serum adipokines

Forty-seven patients following burn injury admitted to QEHB and 10 healthy volunteers were enrolled in this study. This process is illustrated in Supplementary Figure 8. Patient demographics including age and gender were similar to healthy volunteers (Table 5.1). Median TBSA was 42% and median revised BAUX score was 101. Median fluid resuscitation of burn patients was 18.8L of intravenous fluids equating to a median of 5.51 mls/kg/%TBSA for the first 24 hours. HCT levels were similar between healthy volunteers and burn patients at day 1 post injury.

Burned patients at day 1 had significantly reduced levels of adiponectin and leptin in serum compared to healthy volunteers, 38.8 μg/mL vs. 69.2 μg/mL ($p = 0.001$) and 2.8 ng/mL vs. 7.0 ng/mL ($p = 0.005$) respectively. Resistin and visfatin were significantly elevated in patients at day 1 post injury compared to healthy volunteers, 26.0 ng/ml vs 7.6 ng/ml ($p<0.001$) and 14.3 pg/ml vs 0.8 pg/ml ($P<0.001$) respectively. Systemic levels of ghrelin were similar between burn patients and healthy volunteers. Findings are summarised in Table 5.1.

	Healthy Controls	n	Burns Patients	n	P value
Age	38 (31-72)	10	41 (34-61)	47	0.875
Gender (M/F)	5/5	10	30/17	47	0.485
% Total Burn Surface Area	-	-	45 (25-53)	47	-
Revised Baux Score			101 (80-116)	47	-
Hematocrit	0.380 (0.369-0.404)	9	0.429 (0.374-0.459)	31	0.055
Adiponectin (µg/mL)	**69.2 (56.5-103.4)**	**10**	**38.8 (24.5-55.7)**	**46**	**0.001**
Leptin (ng/mL)	**7.0 (5.9-10.4)**	**10**	**2.8 (1.0-5.9)**	**47**	**0.005**
Ghrelin (ng/mL)	0.9 (0.4-1.7)	10	0.5 (0.3-0.9)	47	0.190
Resistin (ng/mL)	**7.6 (6.6-8.9)**	**10**	**26.0 (15.3-53.7)**	**47**	**<0.001**
Visfatin (pg/mL)	**0.8 (0.4-1.2)**	**10**	**14.3 (7.3-18.7)**	**47**	**<0.001**

Table 5.1. Demographics and serum adipokine levels in healthy volunteers and burns patients at day 1 post injury.
Continuous variables are shown as median values with inter-quartile range. Burn patients and healthy volunteers were compared using Mann-Whitney test for continuous variables and Chi-squared test for categorical variables. Significant relationships are highlighted in bold.

5.3.2 Patient Outcomes

All burn patients recruited into the SIFTI study are included in this analysis, including non-survivors and participants lost to follow-up. From a total of 47 burn patients, 21% died at or before 28 days following injury and 38% died during their admission episode. Thirty-eight percent of burn patients were diagnosed with MOF at median day 3 post-injury. Seventy percent of burn patients developed sepsis at median day 5 following injury. Patient outcomes are summarised in Table 5.2. Scarring outcomes for burn patients assessed in this chapter are as described in Chapter 3, Table 3.2

Outcomes	Measure	n	Details
28D Survivor (Y/N)	37/10	47	Mortality at 28 days
Survivor (Y/N)	29/18	47	Mortality in hospital
MOF (Y/N)	18/29	47	DENVER score >3 for 48 hrs
Sepsis (Y/N)	33/14	47	ABA Sepsis Trigger Criteria ≥3
Time to 95% Heal (Days)	36 (22-60)	23	Higher number worse outcome

Table 5.2. Summary of patient outcomes following injury.
Continuous variables are quoted as median values with inter-quartile range.

5.3.3 Longitudinal Adipokine response following thermal injury

Levels of anti-inflammatory adiponectin were significantly reduced in burn patients throughout the 1-year study period compared to healthy volunteers, median 69.2 µg/mL vs. 31.26 µg/mL ($p < 0.001$). Additionally, levels of the pro-inflammatory adipokine leptin were significantly depressed in patients at day 1 post-burn injury but returned to healthy control levels thereafter. Other pro-inflammatory adipokines, including resistin and visfatin remained significantly elevated in patients for 1-month post-thermal injury compared to healthy individuals, median 36.7 ng/mL vs. 7.6 ng/mL ($p < 0.001$) and 5.2 ng/mL vs. 0.9 ng/mL ($p < 0.001$) respectively (Figure 5.1).

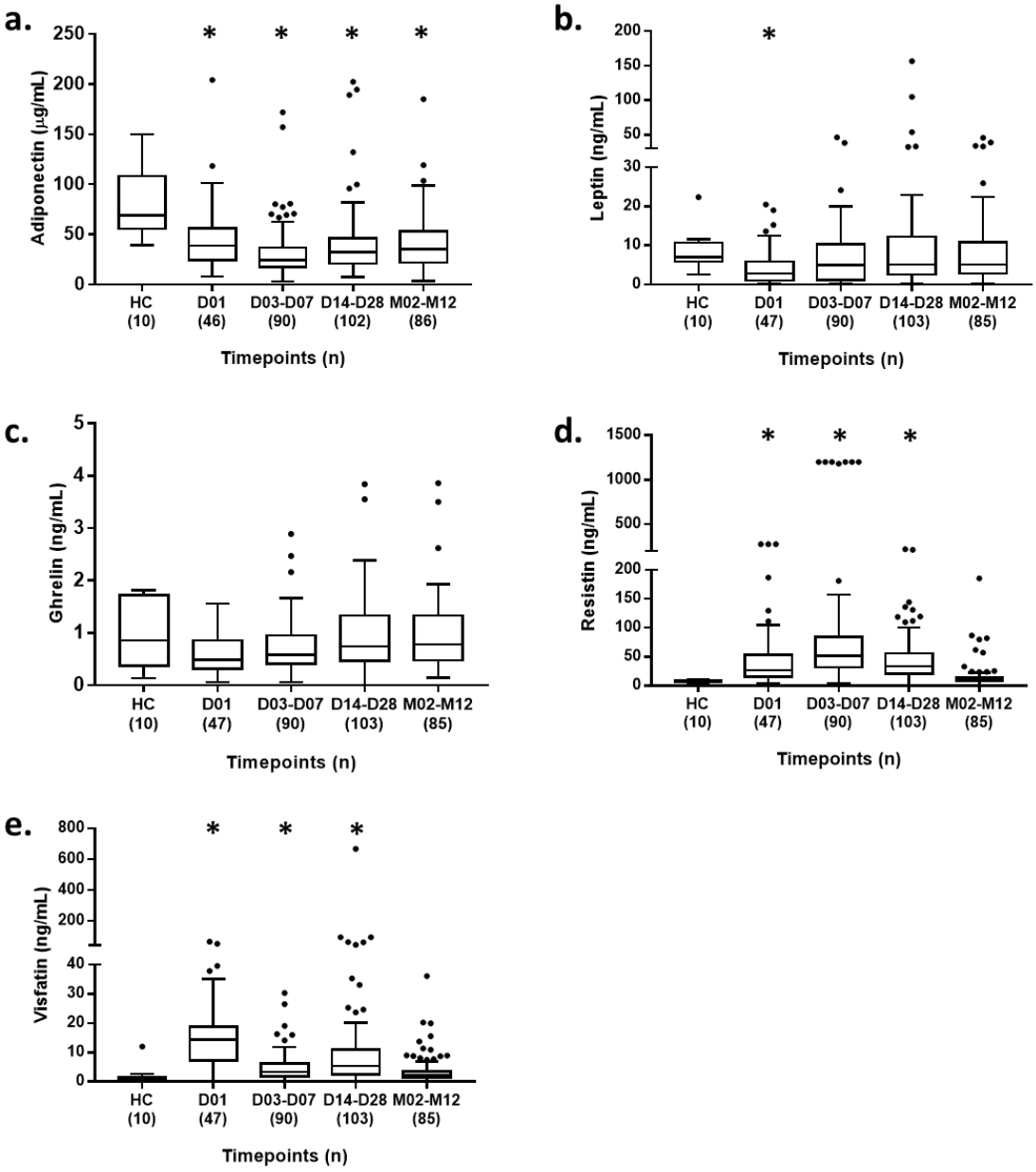


Figure 5.1. Major burn injury significantly reduces systemic adiponectin levels and simultaneously increases systemic resistin and visfatin.
Adipokines in serum were analysed over a 12-month period in burn injured patients. a. Adiponectin; b. Leptin; c. Ghrelin; d. Resistin; e. Visfatin. Analyte levels at timepoints compared to healthy controls (HC) using Kruskal-Wallis test with Dunn's multiple corrections; * $p < 0.05$.

5.3.4 Longitudinal Adipokine response among survivors and non-survivors following thermal injury

Pro-inflammatory adipokines, visfatin and resistin, were significantly elevated in non-survivors compared to survivors following severe burn injury. Serum visfatin levels were significantly increased in burns patients who died before/at day 28 post-injury compared to survivors on admission till day 7, median 6.7 ng/mL vs. 4.8 ng/mL (p= 0.004). In addition, visfatin levels in blood were significantly higher in patients who died during their admission episode compared to survivors at day 1 post-burn injury, median 18.1 ng/mL vs. 10.5 ng/mL (p = 0.007).

Circulating levels of resistin were significantly elevated in patients who died during their admission episode following severe burn injury compared to survivors at D14 onwards, median 43.8 ng/mL vs 13.2 ng/mL (p <0.001). The longitudinal response of adipokines in burns survivors and non-survivors are summarised in Figures Figure 5.2 and Figure 5.3.

5.3.5 Longitudinal Adipokine response and MOF following severe thermal injury

Following burn injury, patients who developed MOF demonstrated a different adipokine response compared to patients without MOF. At D01 post-injury, burns patients with MOF had significantly elevated levels of visfatin in serum compared to patients without MOF, median 16.4ng/mL vs. 9.2 ng/mL (p = 0.034). Subsequently, patients with MOF had significantly increased levels of resistin and visfatin compared with patients without MOF at D14-D28 post-burn injury, median 54.6 ng/mL vs. 28.2 ng/mL (p <0.001) and 8.4 ng/mL vs. 4.0 ng/mL (p = 0.001) respectively. The longitudinal

adipokine response in burns patients who developed and did not develop MOF is summarised in Figure 5.4.

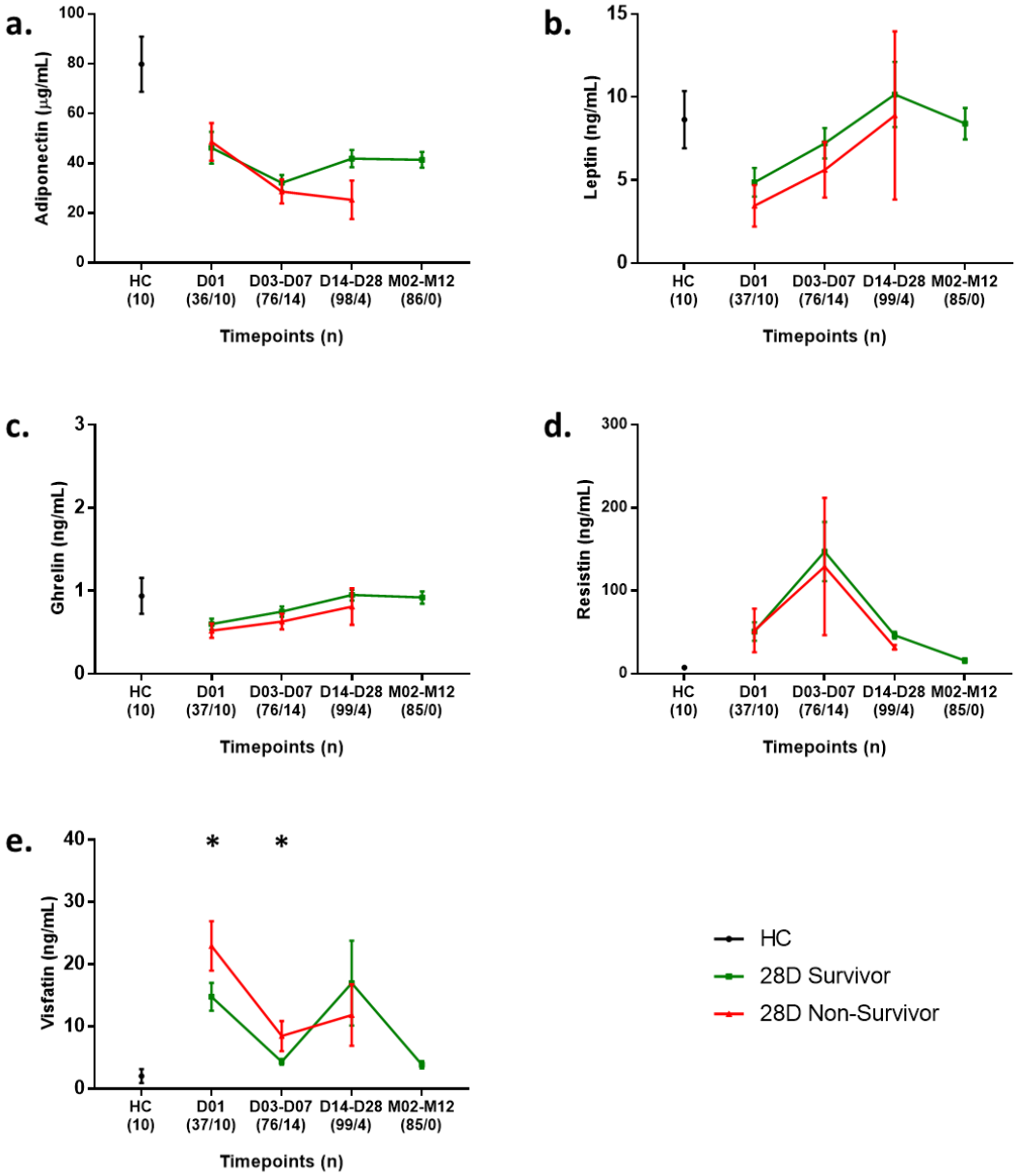


Figure 5.2. 28D non-survivors had significantly elevated levels of visfatin compared to 28D survivors following burn injury.
Serum adipokine levels in 28-day burn survivors and non-survivors were analysed across all timepoints. a. Adiponectin; b. Leptin; c. Ghrelin; d. Resistin; e. Visfatin. Analyte levels at each timepoint was compared between both cohorts (28D survivors vs. 28D non-survivors) using Mann-Whitney Test; * $p < 0.05$.

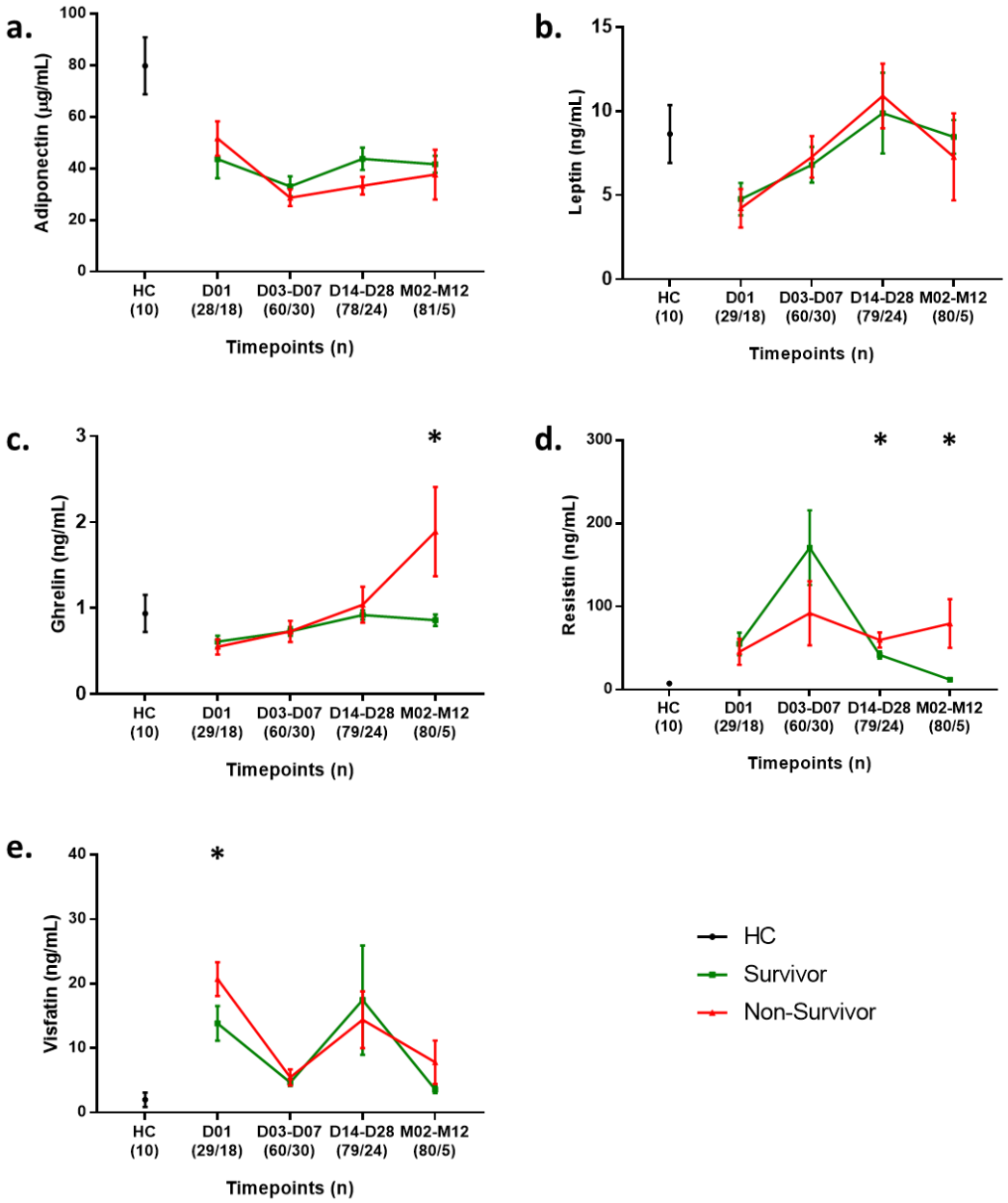


Figure 5.3. Burn non-survivors had significantly increased levels of resistin and visfatin compared to burn survivors.

Serum adipokine levels in burn survivors and non-survivors were analysed across all timepoints. a. Adiponectin; b. Leptin; c. Ghrelin; d. Resistin; e. Visfatin. Analyte levels at each timepoint was compared between both cohorts (survivors vs. non-survivors) using Mann-Whitney Test; * $p < 0.05$.

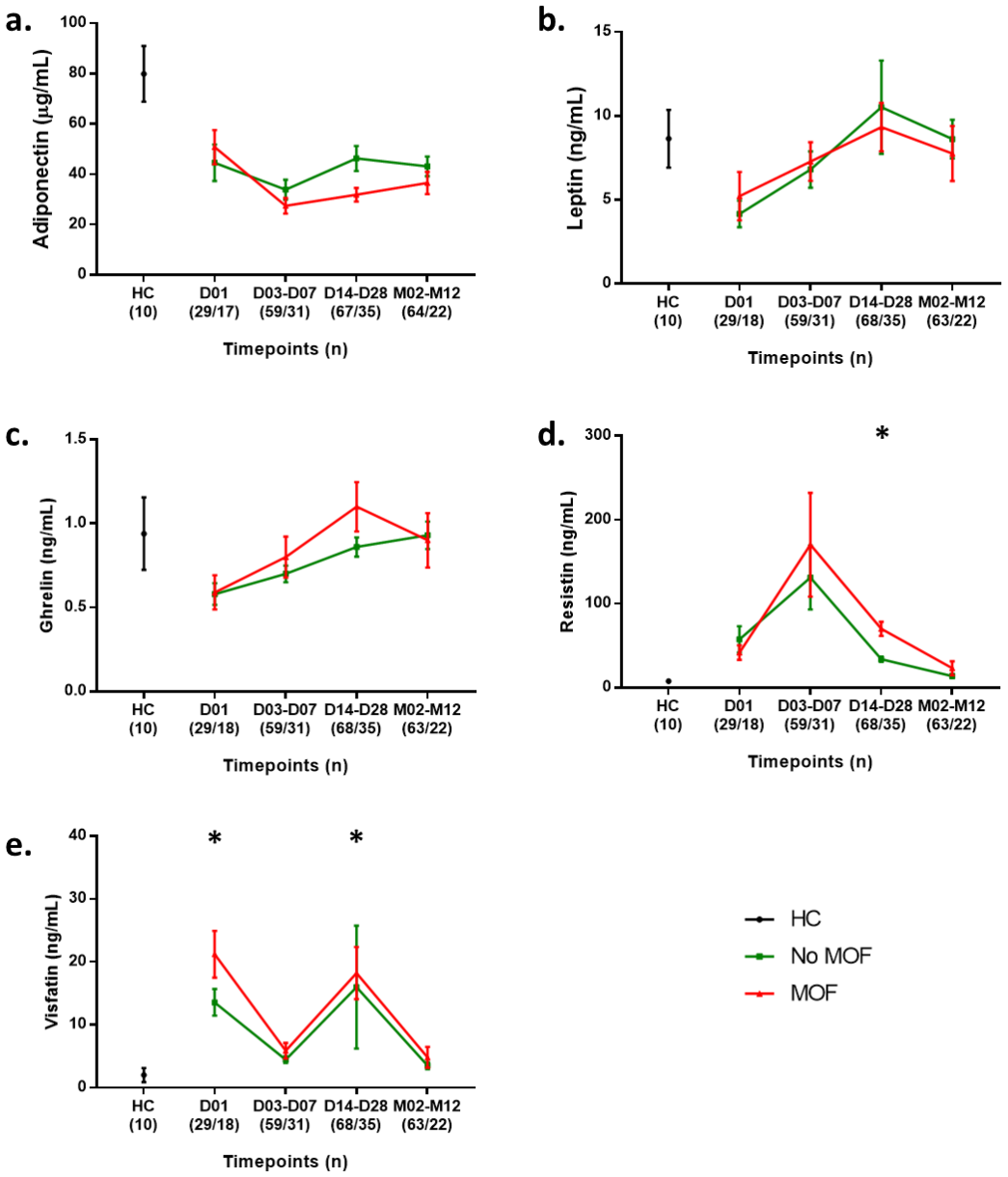


Figure 5.4. Burn Patients with MOF exhibited significantly increased levels of resistin and visfatin compared to burn patients without MOF.
Serum adipokine levels in burn patients who developed and did not develop MOF were analysed across all timepoints. a. Adiponectin; b. Leptin; c. Ghrelin; d. Resistin; e. Visfatin. Analyte levels at each timepoint was compared between both cohorts (MOF vs. No MOF) using Mann-Whitney Test; * $p < 0.05$.

5.3.6 Longitudinal Adipokine response in Septic and Non-Septic patients following severe thermal injury

Severely burned patients with sepsis demonstrated a significantly different inflammatory response compared to non-septic patients. Following thermal injury, septic patients exhibited a significant reduction of anti-inflammatory adipokine response and a simultaneous significant increase in pro-inflammatory adipokines. Both responses were prolonged.

Systemic adiponectin levels in septic burns patients remained significantly reduced from admission till M12 post-injury compared to healthy individuals, median 28.4 µg/mL vs. 69.2 µg/mL ($p < 0.001$). Furthermore, adiponectin levels in serum of septic burns patients were significantly reduced at D03-D28 post injury compared to non-septic patients, 25.8 µg/mL vs. 38.0 µg/mL ($p < 0.001$).

Systemic pro-inflammatory adipokine levels were significantly elevated in septic patients compared to non-septic patients at D14-28 post-burn injury. This included leptin (median 6.7 ng/mL vs. 3.7 ng/mL, $p = 0.018$), resistin (median 49.1 ng/mL vs. 16.9 ng/mL, $p < 0.001$) and visfatin (median 6.6ng/mL vs. 2.3ng/mL, $p < 0.001$). The longitudinal adipokine response in septic and non-septic burns patients is summarised in Figure 5.5.

5.3.7 Longitudinal Adipokine response and wound healing following severe thermal injury

Systemic levels of adipokines significantly correlated with wound healing in patients following severe thermal injury. At D03-D07 post-burn injury, circulating leptin levels in

patients had significant positive correlation with duration taken to achieve 95% wound healing, rho 0.377 (p 0.008). Furthermore, lower levels of leptin and resistin in serum of burns patients at D14-D28 significantly correlated with longer wound healing times, rho 0.333 (p 0.006) and rho 0.321 (p 0.009) respectively. Conversely, adiponectin levels in blood at D14-28 post-burn injury demonstrated a significant inverse relationship with duration of wound healing, rho -0.352 (p 0.006). Significant correlations between adipokines levels at various timepoints and duration of wound healing are summarised in Figure 5.6.

5.3.8 Longitudinal Adipokine response and scarring following severe thermal injury

5.3.8.1 Subjective Scar Measures

Serum adipokine levels were significantly associated with scarring outcomes following burn injury as measured using mVSS and POSAS. Serum pro-inflammatory adipokines, resistin and visfatin, at D14-D28 post-injury significantly correlated with higher mVSS and worse scarring, rho 0.566 ($p = 0.001$) and rho 0.619 ($p < 0.001$) respectively. Similarly, elevated circulating levels of resistin and visfatin in burns patients were significantly associated with poor scarring as per clinician assessment using POSAS, rho 0.391 ($p = 0.027$) at D14-D28 and rho 0.575 ($p < 0.001$) at D03-D28 respectively. Furthermore, elevated visfatin levels in patients were significantly associated with higher OPSS at D14-D28 post-burn injury, rho 0.386 ($p = 0.029$). Ghrelin levels in serum of burn patients during the acute and late stages post-injury demonstrated a significant inverse correlation with scarring outcomes. Significant correlations between serum adipokines measured longitudinally and subjective scar scoring systems are summarised in Figure 5.7.

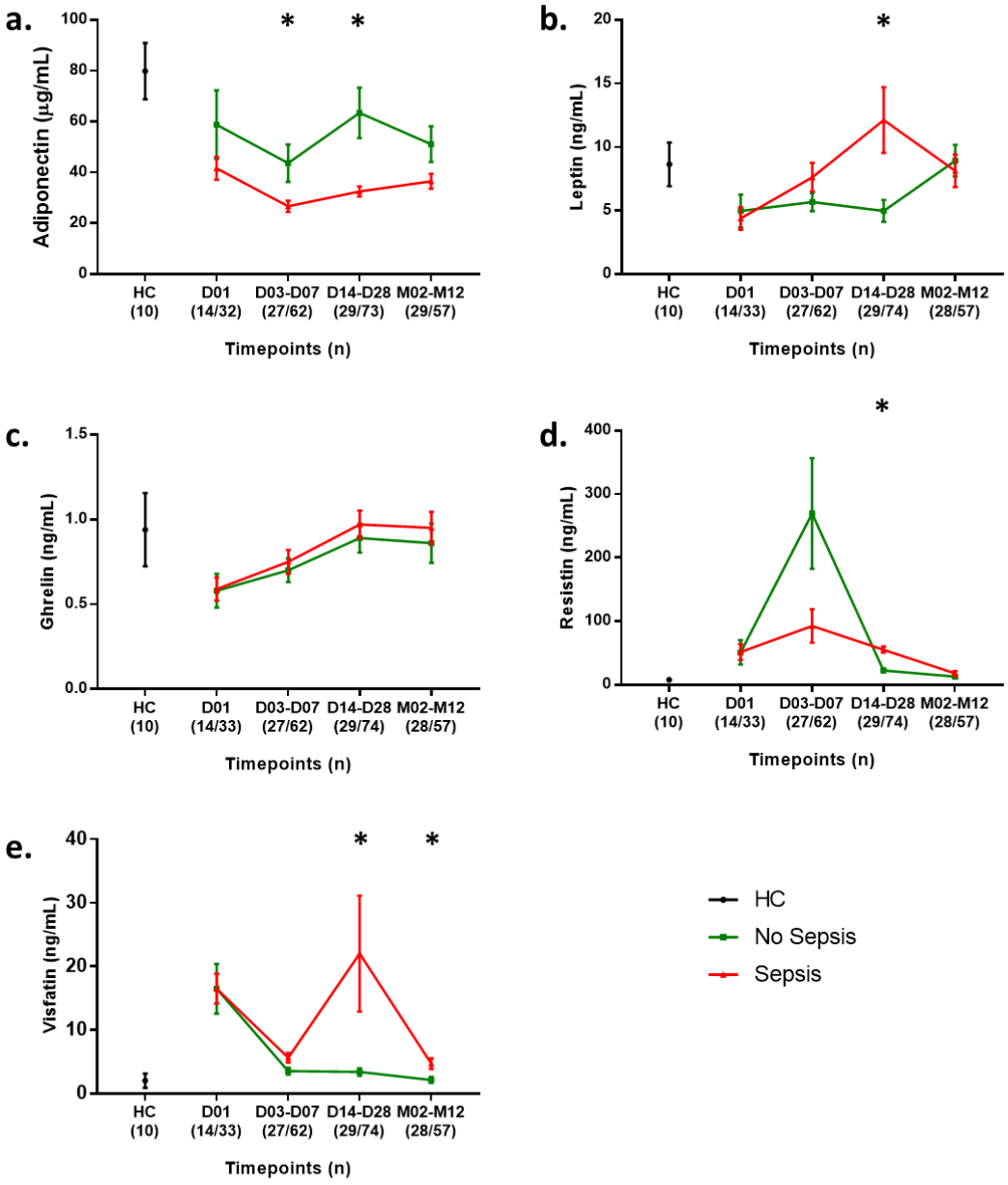


Figure 5.5. Septic burn patients exhibited significantly reduced levels of adiponectin with simultaneous elevated levels of leptin, resistin and visfatin compared to non-septic burn patients.

Serum adipokine levels in septic and non-septic burns patients were analysed across all timepoints. a. Adiponectin; b. Leptin; c. Ghrelin; d. Resistin; e. Visfatin. Analyte levels at each timepoint was compared between both cohorts (Sepsis vs. No Sepsis) using Mann-Whitney Test; * $p < 0.05$.

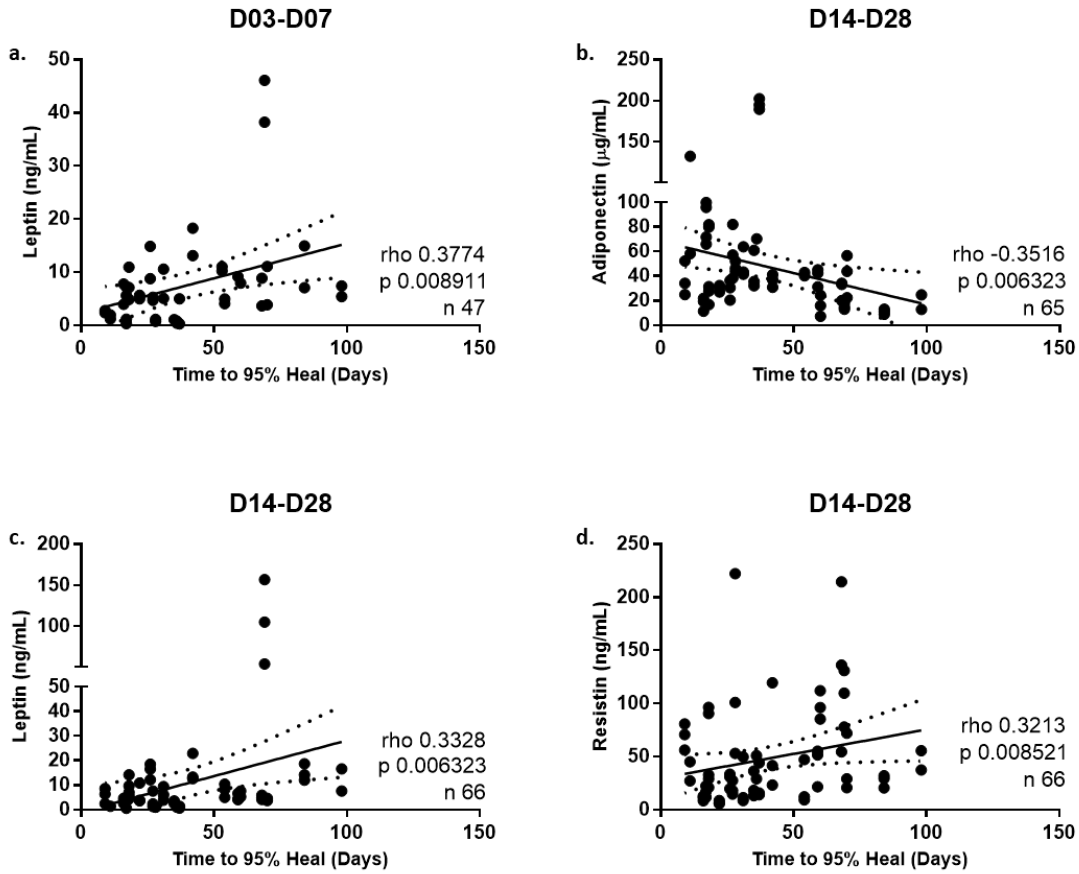


Figure 5.6. Reduced adiponectin levels were associated with longer wound healing times while leptin and resistin positively correlated with increased wound healing durations.

Serum adipokine levels and days taken to achieve 95% wounds healed in burns patients were analysed across all timepoints. a. Leptin at D03-D07; b. Adiponectin at D14-D28; c. Leptin at D14-D28; d. Resistin at D14-D28. Correlations were performed between analyte levels at timepoints and time to 95% heal using Spearman's rank correlation co-efficient; * $p < 0.05$

5.3.8.2 Objective Scar Measures

Adipokine response following severe thermal injury was significantly associated with subsequent scarring outcomes as per objective measures via various devices.

Circulating levels of resistin and visfatin were significantly associated with increased

scar thickness at D14-D28 post-thermal injury, rho 0.478 (p = 0.006) and rho 0.412 (p = 0.019).

At D03-D28, reduced leptin levels in serum of burns patients were significantly associated with increased scar thickness, rho -0.415 (p 0.002). Interestingly, elevated systemic levels of leptin significantly correlated with increased scar intensity at D03-M12 post-burn injury, rho -0.386 (p <0.001). Significant correlations between adipokine response and ultrasound scar measures are summarised in Figure 5.8.

Adipokine response following injury was significantly associated with subsequent scar pliability in burns patients. At D03-D28 post-injury, elevated adiponectin levels in serum were significantly associated with improved scar pliability, rho 0.393 (p = 0.003). Meanwhile, increased systemic levels of visfatin in burns patients at M02-M12 were significantly associated with poor scar pliability, rho -0.340 (p = 0.043). Interestingly, elevated ghrelin levels at D03-M12 post-thermal injury significantly correlated with increased scar firmness, rho 0.422 (p <0.001). Significant correlations between serum adipokine levels in burns patients and cutometer scar measures are summarised in Figure 5.9.

Throughout the study period, serum adipokine levels in burns patients demonstrated significant associated with subsequent scar erythema and pigmentation. Significant correlations between circulating adipokine levels and colorimeter scar measures are summarised in Figures 5.10 and 5.11.

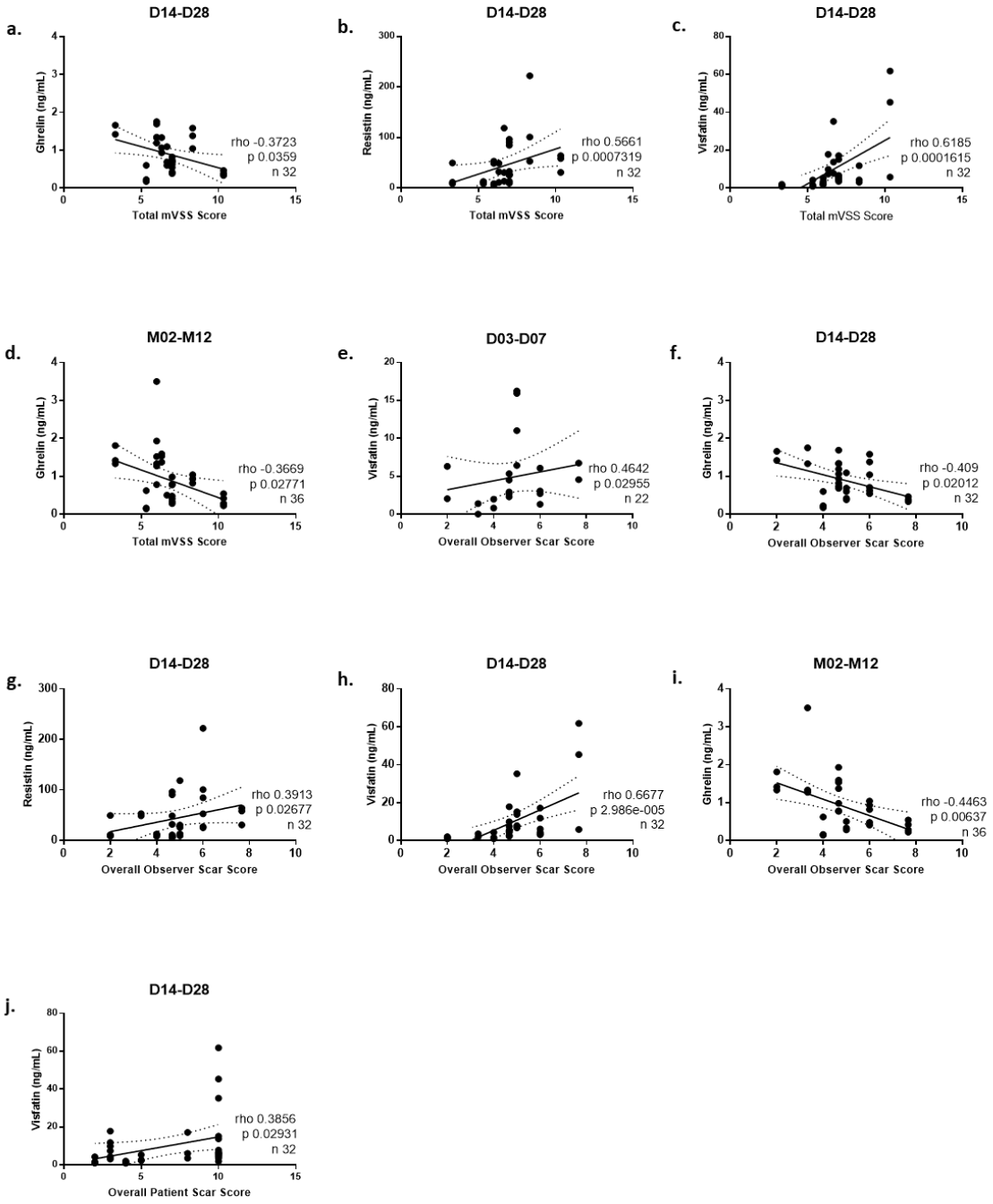


Figure 5.7. Adipokine response and correlations with subjective scar measures following severe thermal injury.

Serum adipokine levels and scar scores as measured subjectively using mVSS and POSAS in burns patients were analysed across all timepoints. a. Ghrelin vs. mVSS at D14-D28; b. Resistin vs. mVSS at D14-D28; c. Visfatin vs. mVSS at D14-D28; d. Ghrelin vs. mVSS at M02-M12; e. Visfatin vs. OOSS at D03-D07; f. Ghrelin vs. OOSS at D14-D28; g. Resistin vs. OOSS at D14-D28; h. Visfatin vs. OOSS at D14-D28; i. Ghrelin vs. OOSS M02-M12; j. Visfatin vs. OPSS at D14-D28. Correlations were performed between analyte levels at timepoints and subjective scar scores using Spearman's rank correlation co-efficient; * $p < 0.05$.

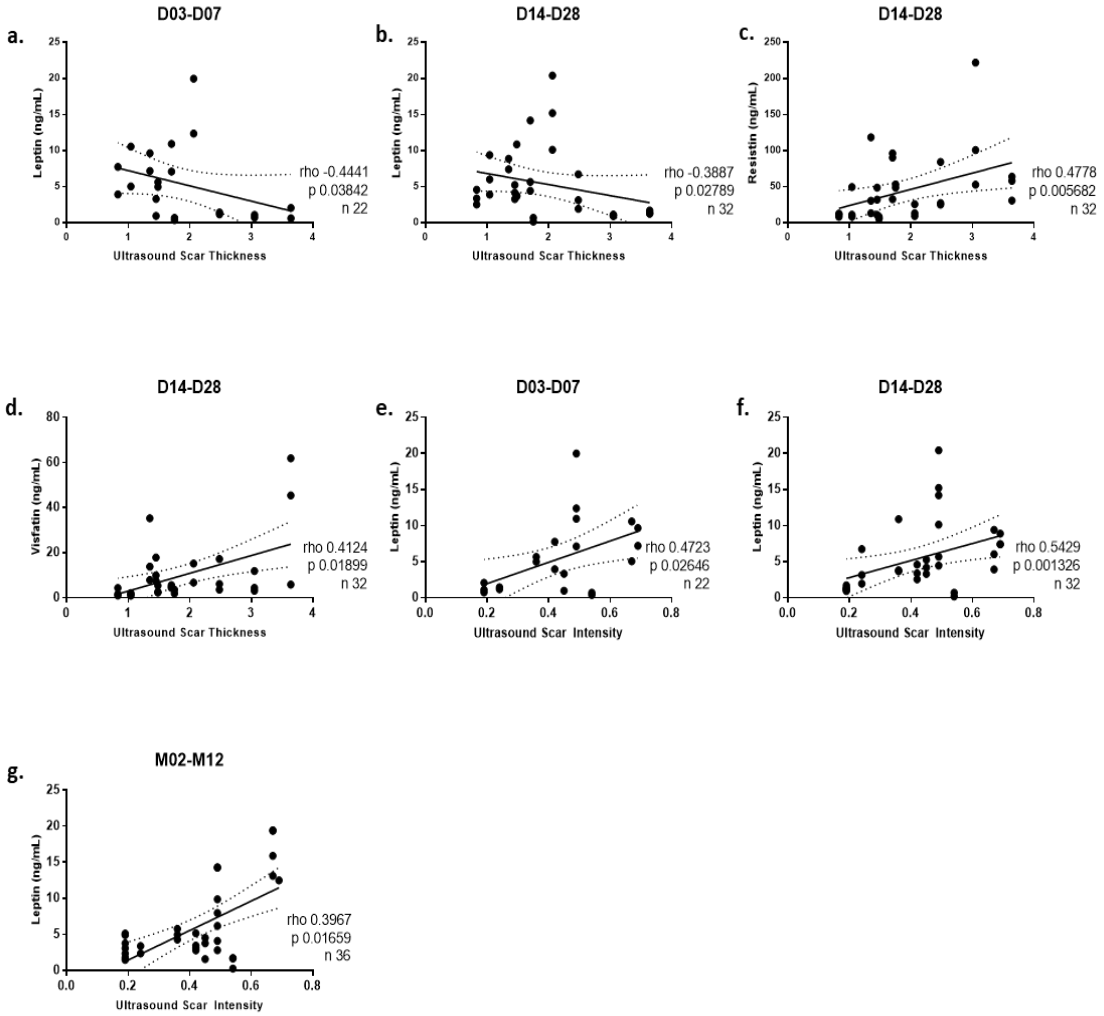


Figure 5.8. Adipokine response and ultrasound scar measure correlations following severe thermal injury.

Serum adipokine levels and scar thickness/intensity in burns patients as measured by ultrasound were analysed across all timepoints. a. Leptin vs. Scar Thickness at D03-D07; b. Leptin vs. Scar Thickness at D14-28; c. Resistin vs. Scar Thickness at D14-D28; d. Visfatin vs. Scar Thickness at D14-D28; e. Leptin vs. Scar Intensity at D03-D07; f. Leptin vs. Scar Intensity at D14-D28; g. Leptin vs. Scar Intensity at M02-M12. Correlations were performed between analyte levels at timepoints and ultrasound scar measures using Spearman's rank correlation co-efficient; * $p < 0.05$.

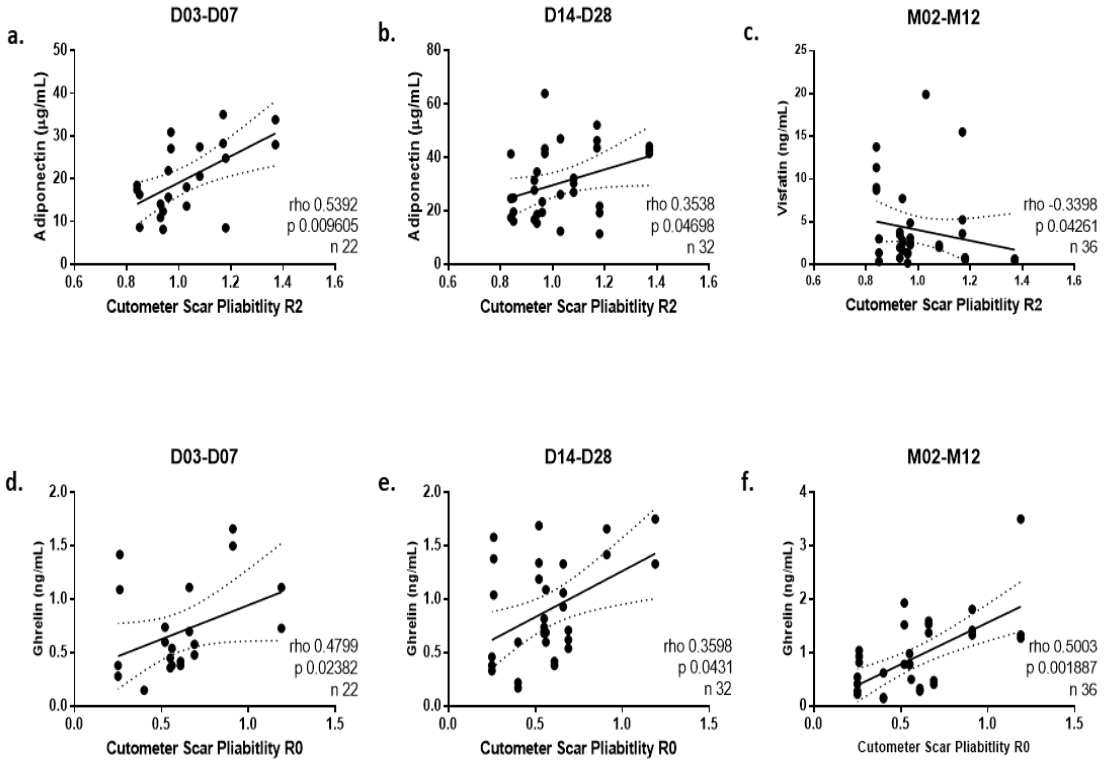


Figure 5.9. Adipokine response and cutometer scar measure correlations following severe thermal injury.

Serum adipokine levels and scar pliability (R2)/firmness (R0) in burns patients as measured by cutometer were analysed across all timepoints. a. Adiponectin vs. R2 at D03-D07; b. Adiponectin vs. R2 at D14-D28; c. Visfatin vs. R2 at M02-M12; d. Ghrelin vs. R0 at D03-D07; e. Ghrelin vs. R0 at D14-D28; f. Ghrelin vs. R0 at M02-M12. Correlations were performed between analyte levels at timepoints and cutometer scar measures using Spearman's rank correlation co-efficient; * $p < 0.05$.

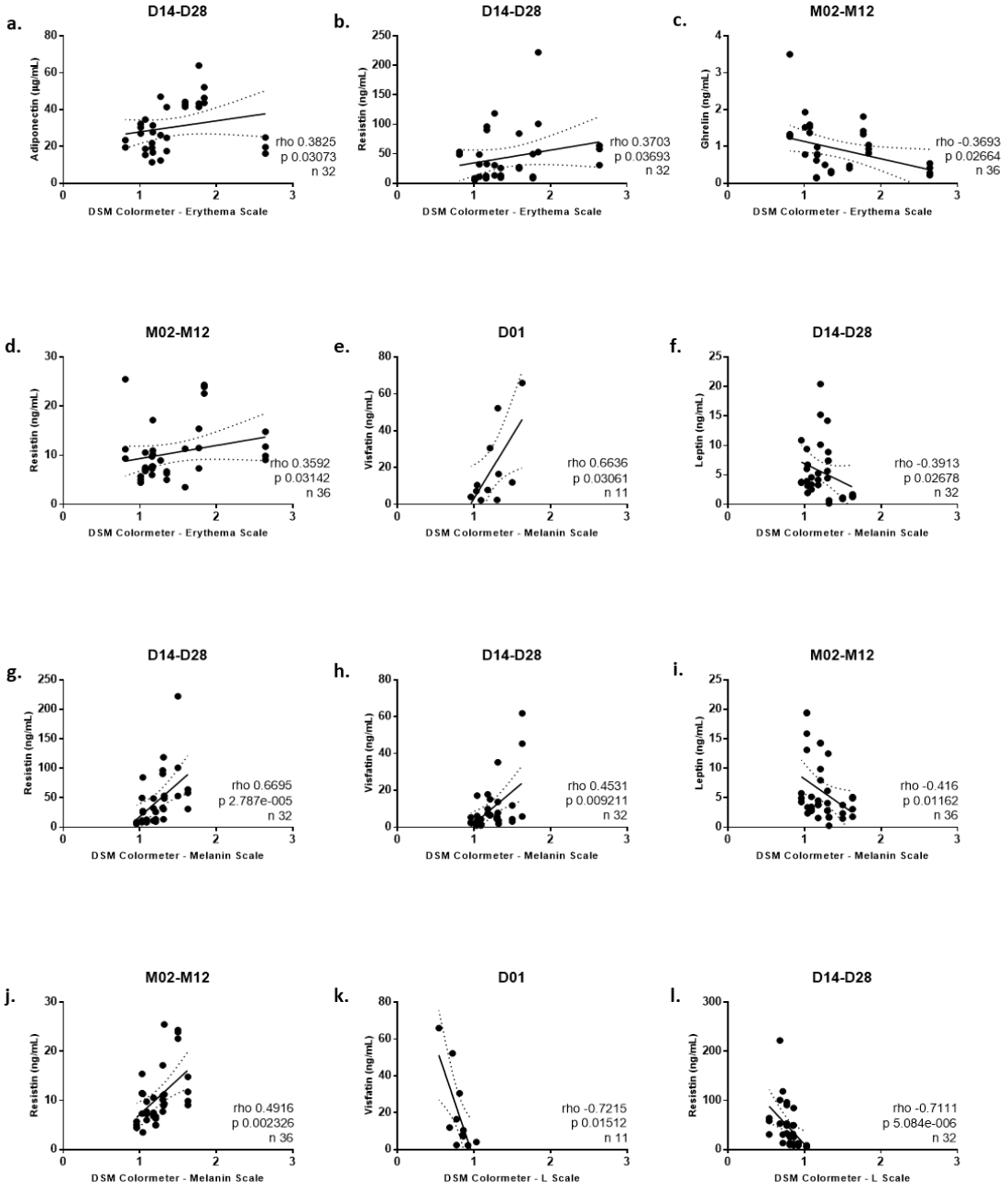


Figure 5.10. Adipokine response and colorimeter scar measure correlations following severe thermal injury (Part 1).

Serum levels of adipokines and scar erythema/pigmentation in burns patients as measured by colorimeter were analysed across all timepoints. a. Adiponectin vs. Erythema at D14-D28; b. Resistin vs. Erythema at D14-D28; c. Ghrelin vs. Erythema at M02-M12; d. Resistin vs. Erythema at M02-M12; e. Visfatin vs. Melanin at D01; f. Leptin vs. Melanin at D14-D28; g. Resistin vs. Melanin at D14-D28; h. Visfatin vs. Melanin at D14-D28; i. Leptin vs. Melanin at M02-M12; j. Resistin vs. Melanin at M02-M12; k. Visfatin vs. L at D01; l. Resistin vs. L at D14-D28. Correlations were performed between analyte levels at timepoints and colorimeter scar measures using Spearman's rank correlation co-efficient; * $p < 0.05$.

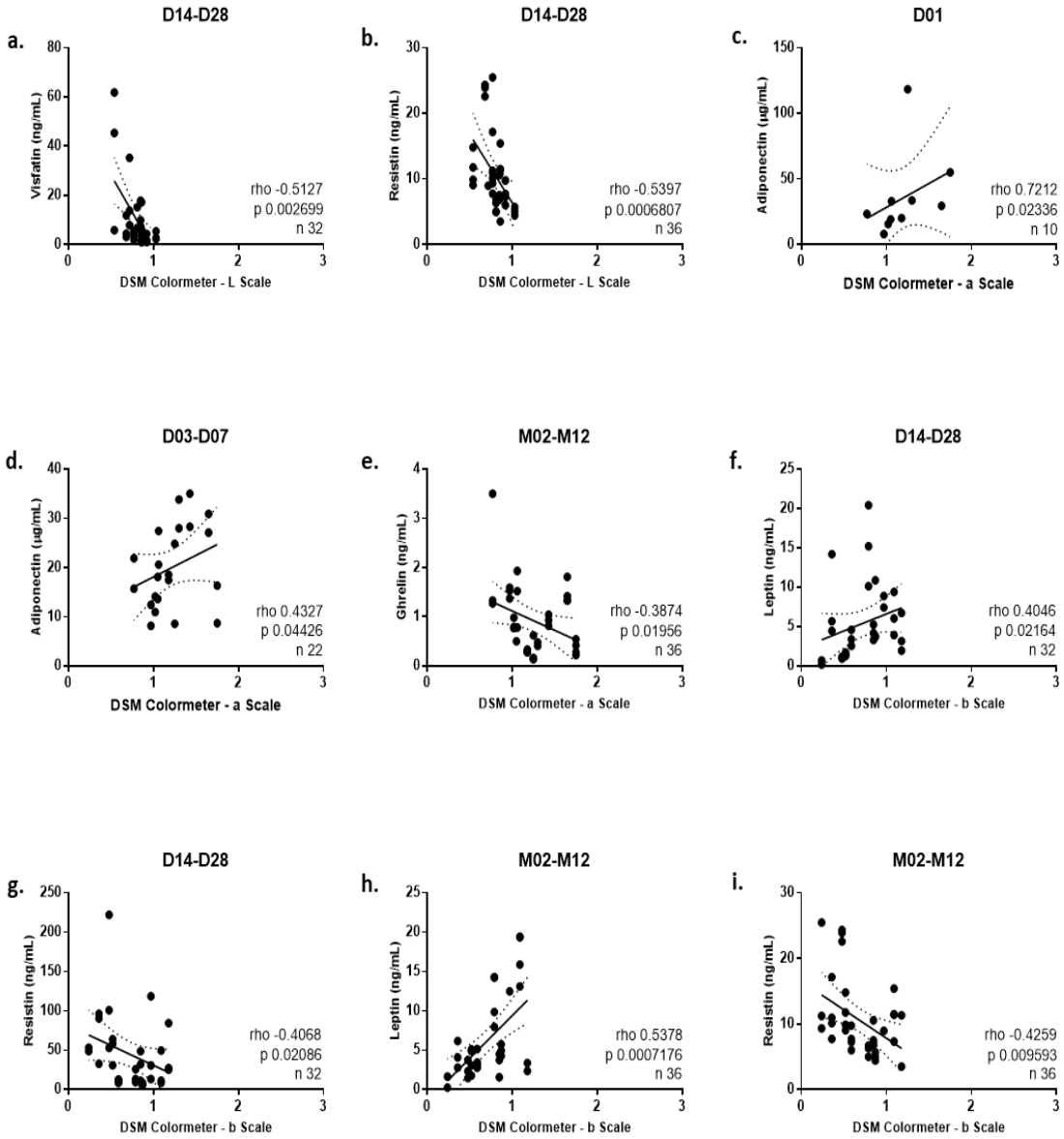


Figure 5.11. Adipokine response and colorimeter scar measure correlations following severe thermal injury (Part 2).

Continuation from Figure 5.10. a. Visfatin vs. L at D14-D28; b. Resistin vs. L at D14-D28; c. Adiponectin vs. a at D01; d. Adiponectin vs. a at D03-D07; e. Ghrelin vs. a at M02-M12; f. Leptin vs. b at D14-D28; g. Resistin vs. b at D14-D28; h. Leptin vs. b at M02-M12; i. Resistin vs. b at M02-M12.

5.3.9 Therapeutic potential of Adipokines following severe thermal injury

Reducing circulating levels of pro-inflammatory adipokines may improve survival of patients following severe burn injury. At D03-D07 post-burn injury, uni-analyte and multi-analyte regression models demonstrated that circulating levels of visfatin were significantly associated with and predictive of 28-day mortality, $p = 0.026$ and $p = 0.034$ respectively. Reducing serum levels of visfatin in burns patients by 1 ng/mL may increase the odds of 28-day survival by 16-17%. A significant association between resistin levels at D03-D07 post-thermal injury and sepsis has been observed. The multi-variate analysis and significant therapeutic potential of adipokines following severe thermal injury are summarised in Table 5.3.

Multi-variate regression models of adipokines and wound healing in burns patients demonstrated no significant therapeutic value. Multi-variate regression analysis could not be performed using scarring data due to low patient numbers.

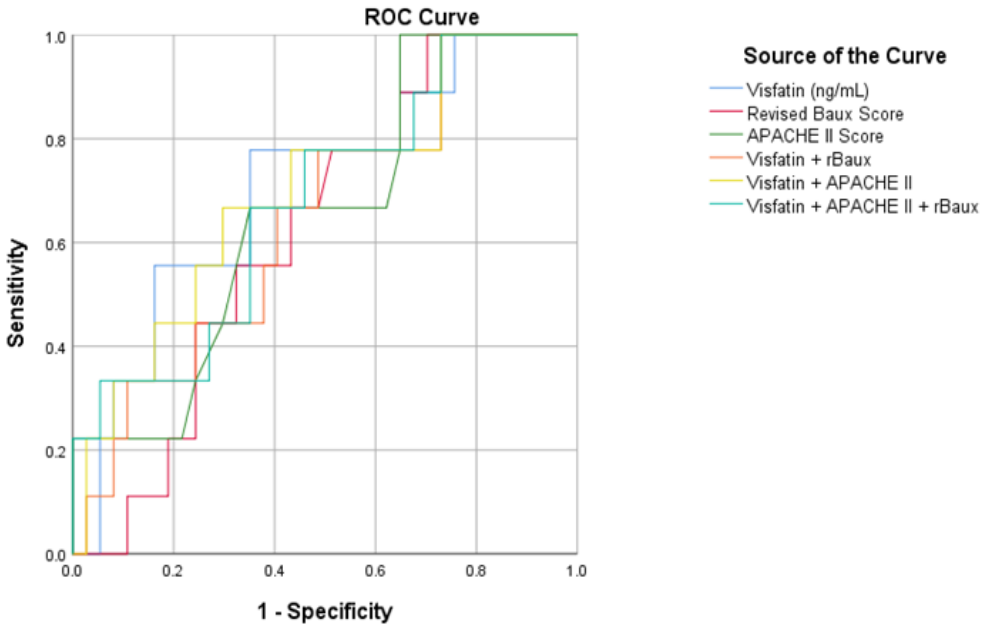
5.3.10 Adipokine potential as Diagnostic/Prognostic biomarkers following severe thermal injury

Serum visfatin levels in burns patients were significantly different between survivors and non-survivors at D01 post-injury (Figure 5.2 and Figure 5.3). The predictive power of visfatin, alone or in combination with clinical scores, did not exceed 80%. The AUC/ROC curve analysis for visfatin and survival outcomes are summarised in Figure 5.12 and Figure 5.13.

Timepoint	Outcome	Analyte	Co-Variates	p- value	Measure of Benefit (No Adverse Outcome)		
					Odds Ratio	Effect on OR for 1 unit (%)	Required units to increase OR by 1
D03-D07	Sepsis	Resistin	Age, TBSA, Inhalation Injury, Gender, APACHE II	0.034	1.002	0.2	500
D03-D07	28D Mortality	Visfatin		0.026	0.84	-16	-6.25
D14-D28	MOF	Ghrelin		0.038	0.377	-97.5	-1.03
D14-D28	MOF	Resistin		0.005	0.973	-2.7	-37.1
D03-D07	Sepsis	Resistin	Age, TBSA, Inhalation Injury, Gender, APACHE II, All Analytes	0.006	1.004	0.4	250
D03-D07	28D Mortality	Visfatin		0.034	0.83	-17	-5.88
D14-D28	MOF	Resistin		0.006	0.967	-3.3	-30.3

Table 5.3. Potential Therapeutic Effects of Adipokines on Binary Outcomes following Severe Thermal Injury.
Outcomes and Adipokines at all timepoints were assessed using uni-analyte and multi-analyte regression models. Multi-variate binary logistic regression was performed accounting for patient demographics, injury severity and/or physiological state on admission. Significant associations $p < 0.05$.

a.



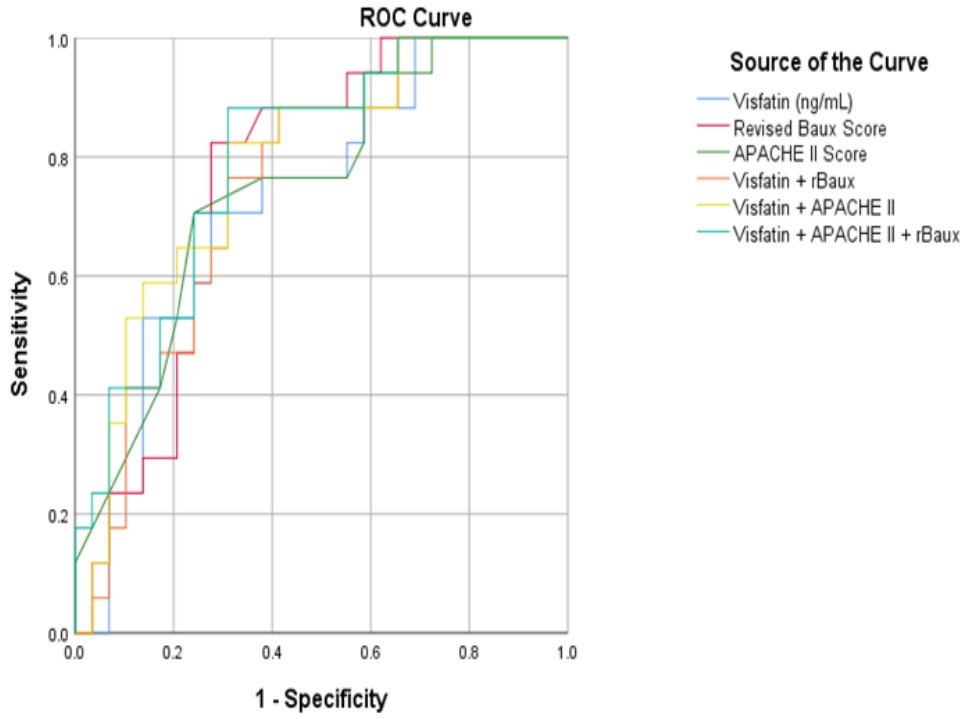
b.

Test Result Variable(s)	AUC
Visfatin (ng/mL)	0.709
rBaux Score	0.623
APACHE II Score	0.658
Visfatin + rBaux	0.646
Visfatin + APACHE II	0.697
Visfatin + APACHE II + rBaux	0.679

Figure 5.12. Serum visfatin levels at D01 post-injury and 28-day mortality in burns patients.

Predictive strength of serum levels of visfatin at D01 post-injury on 28-day mortality were assessed using AUC/ROC curve analysis. a. ROC curve analysis of levels of visfatin and clinical scores both alone and in-combination; b. AUC analysis of levels of visfatin and clinical scores both alone and in-combination.

a.



b.

Test Result Variable(s)	AUC
Visfatin (ng/mL)	0.726
rBaux Score	0.764
APACHE II Score	0.749
Visfatin + rBaux	0.751
Visfatin + APACHE II	0.785
Visfatin + APACHE II + rBaux	0.795

Figure 5.13. Serum visfatin levels at D01 post-injury and in-hospital mortality in burns patients.

Predictive strength of serum levels of visfatin at D01 post-injury on 28-day mortality were assessed using AUC/ROC curve analysis. a. ROC curve analysis of levels of visfatin and clinical scores both alone and in-combination; b. AUC analysis of levels of visfatin and clinical scores both alone and in-combination.

5.3.11 Longitudinal Adipokine and Immune-Endocrine response following severe thermal injury

Acute severe burn injury induces major simultaneous immune-endocrine responses. Following injury, patients exhibited increased expression of both pro-inflammatory and anti-inflammatory adipokines and cytokines. In addition, increased expression of multiple immune functions was seen in burned patients.

Systemic levels of adiponectin, ghrelin and leptin in burned patients were different compared to healthy volunteers. The changes in circulating adipokines occurred immediately following thermal injury and remained persistently high till, at least, M12 post-injury. This suggests prolonged utilisation of these adipokines during the hypermetabolic response following severe thermal injury. Furthermore, low neutrophil phagocytosis expression at D01 post-thermal injury coincided with elevated and reduced circulating expressions of adiponectin and leptin. This suggests possible immune-modulatory role of adiponectin and leptin in these patients (570). In addition, elevated serum adiponectin coincided with increased IL-1RA and IL10 at D01 post-thermal injury. While raised IL-6 in serum was present with reduced circulating levels of adiponectin at D03-D07 post-injury in burned patients. This relationship between IL-6 and adiponectin levels inverses thereafter. This indicates possible immune-inflammatory modulatory role of adiponectin following thermal injury(571, 572).

Pro-inflammatory visfatin level in serum was elevated at D01 and D14-28 following severe thermal injury indicating a time-dependent bi-phasic response (573). Interestingly, IL-10, IL-12p70 and IL-17 exhibited a similar bi-phasic response. This is

mostly likely attributed to the effects of visfatin on immune cells (348, 574, 575). This suggests the influence of visfatin on the immune system and its contribution to systemic inflammatory response following severe thermal injury.

Burn patients exhibited a prolonged differential immune-endocrine response when compared to healthy volunteers. The simultaneous anti-inflammatory and pro-inflammatory responses between burned patients and healthy volunteers remained altered for, at least, one year following injury. The longitudinal adipokine and immune-endocrine status and interactions in burned patients and healthy volunteers are summarized in Figure 5.14.

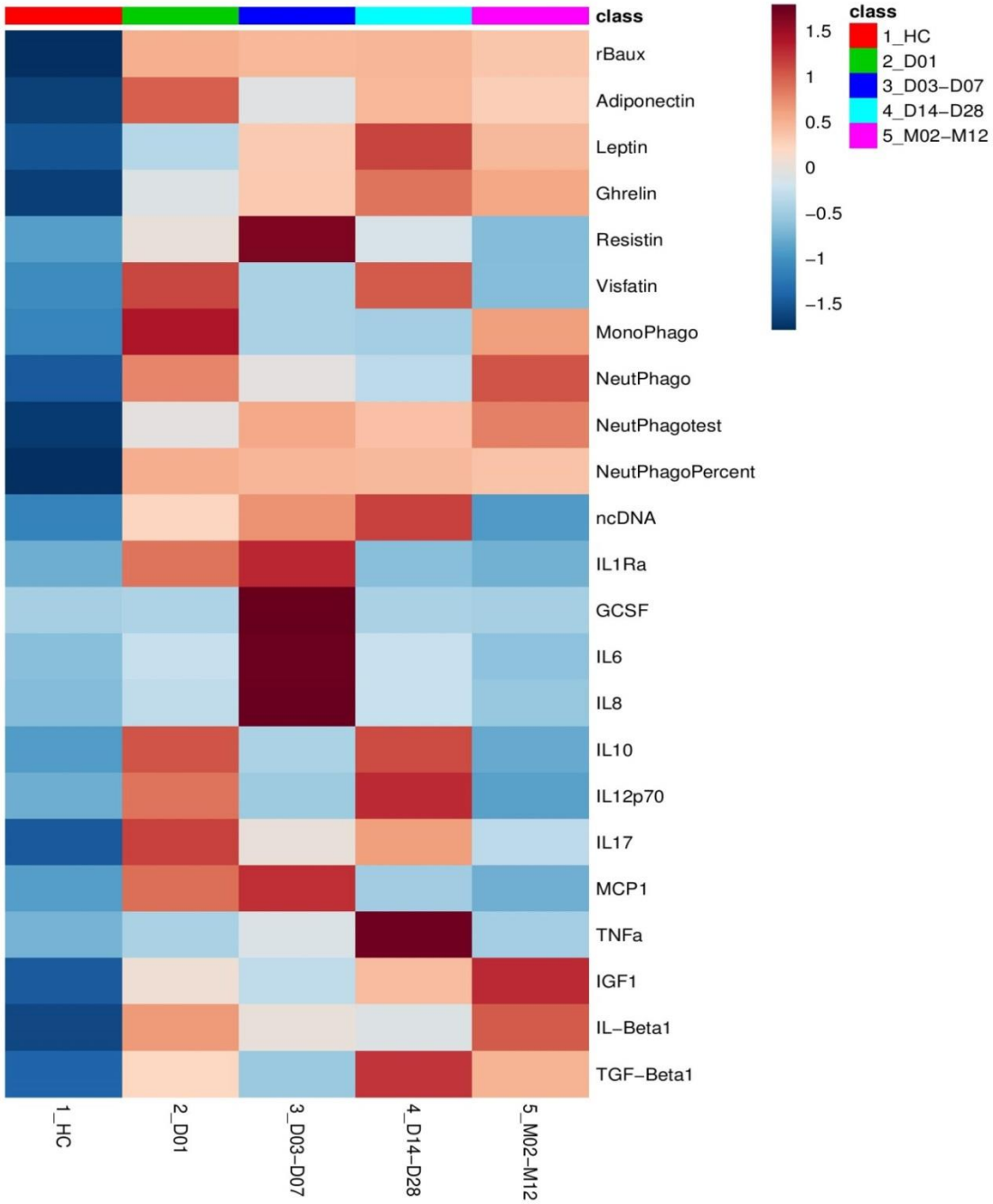


Figure 5.14. The longitudinal adipokine and immune-endocrine response following major burn injury.

Circulating status of cytokines, immune function and adipokines was analysed across all timepoints accounting for the revised Baux score. Immune-endocrine status of healthy volunteers was performed to allow comparison. This heatmaps represent expressions of molecules in serum by colour with high expressions being red and low expressions being blue. Immune function tests include Monocyte and Neutrophil ROS generation (Phagoburst MFI), Neutrophil and monocyte Phagocytosis (Phagotest, % positive) and Nuclear cfDNA concentration (marker of NETs). Cytokines and other hormones analysed include IL-1Ra, GCSF, IL-6, IL-8, IL-10, IL-12p70, IL-17, MCP-1, TNF-α, IGF-1, IL-β1 and TGF-β1.

5.3.12 Subgroup Analysis

5.3.12.1 Gender Influence on Longitudinal Adipokine Response following Severe Thermal Injury

Gender exerted significant effects on systemic levels of adipokines in major burns patients. Injury severity in both female and male burns patients was similar, p = 0.956. Male patients had a significantly reduced anti-inflammatory response at D03-M12 compared to non-injured males, median adiponectin 29.5 µg/mL vs. 77.8 µg/mL, p = 0.001. Furthermore, male patients demonstrated a significantly elevated pro-inflammatory state with increased levels of resistin and visfatin at D01-D28 following severe thermal injury compared male healthy volunteers, median 47.8 ng/mL vs. 7.3 ng/mL (p = 0.001) and 5.0 ng/mL vs. 1.0 ng/mL (p = 0.002) respectively.

Leptin levels in males and female burns patients were similar to their correspondent healthy controls at all timepoints following injury. Interestingly, female patients had significantly increased levels of leptin at D03-M12 following thermal injury compared male patients, median 8.2 ng/mL vs. 4.1 ng/mL (p <0.001). Circulating adipokine levels in male and female burns patients are summarised longitudinally in Figure 5.15.

5.3.12.2 Age influence on Longitudinal Adipokine response following severe thermal injury

Young and older burns patients exhibited significant differences in the adipokine response following injury. Injury severity was similar between both cohorts, p = 0.082 and p= 0.50 respectively. At D01-D07 post-thermal injury, elderly patients exhibited significantly increased serum adiponectin levels compared to younger patients, median

45.1 µg/mL vs. 25.9 µg/mL ($p < 0.001$). Interestingly, older burns patients had significantly reduced levels of systemic ghrelin at M02-M12 post-injury compared to younger burns patients, median 0.4 ng/mL vs. 0.8 ng/mL ($p = 0.009$). Serum adipokine levels in young and old burns patients are summarised longitudinally in Figure 5.16.

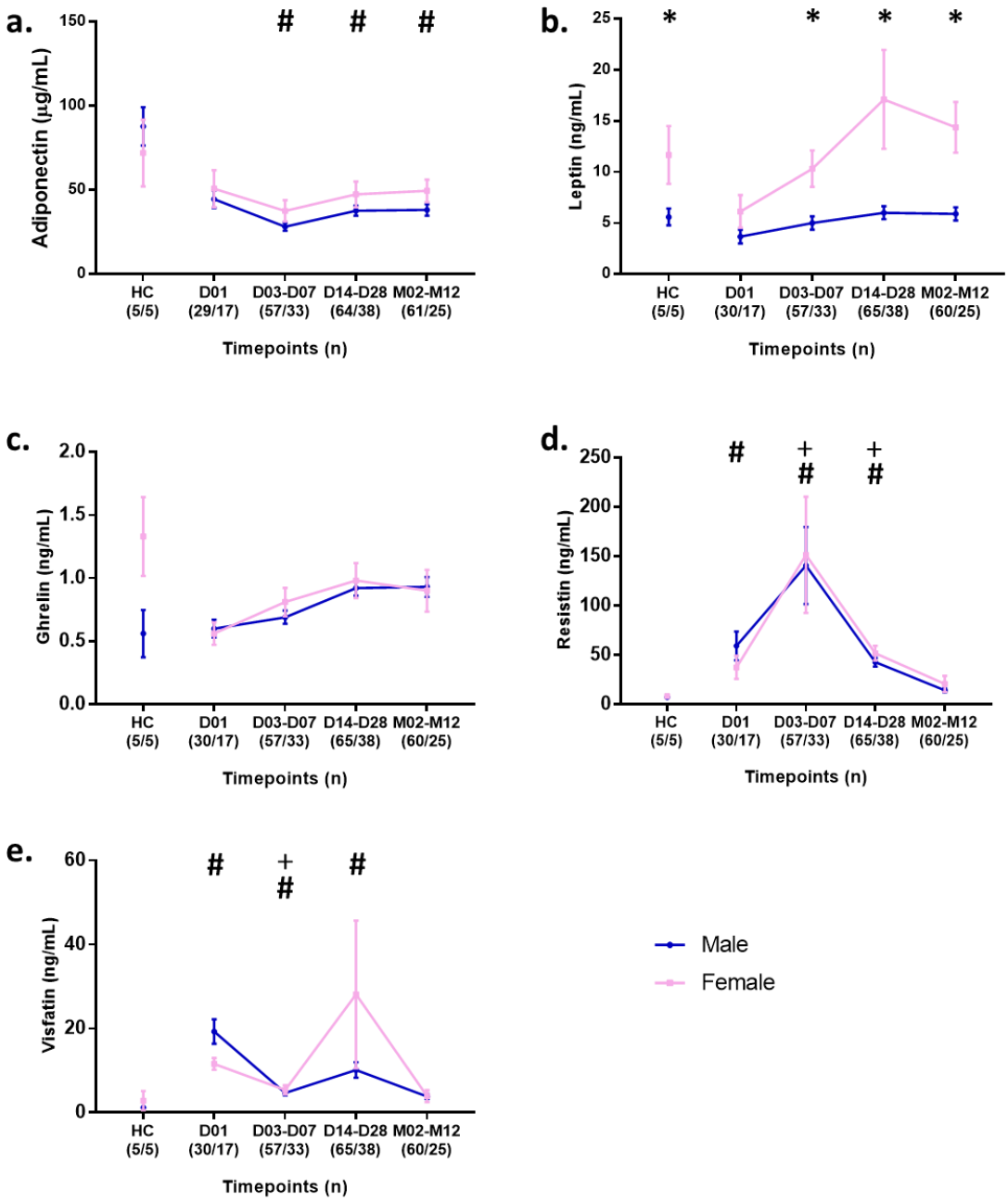


Figure 5.15. Longitudinal adipokine response in females and males following burn injury.

Serum adipokine levels in female and male burns patients were analysed across all timepoints. a. Adiponectin; b. Leptin; c. Ghrelin; d. Resistin; e. Visfatin. Analyte levels at each timepoint was compared between both cohorts (Female vs. Male) using Mann-Whitney Test; * $p < 0.05$. Analyte levels of female and male burns patients were compared to female and male healthy controls (HC) using Kruskal-Wallis test with Dunn's multiple corrections; + Female vs. HC Female, $p < 0.05$; # Male vs. HC Male, $p < 0.05$.

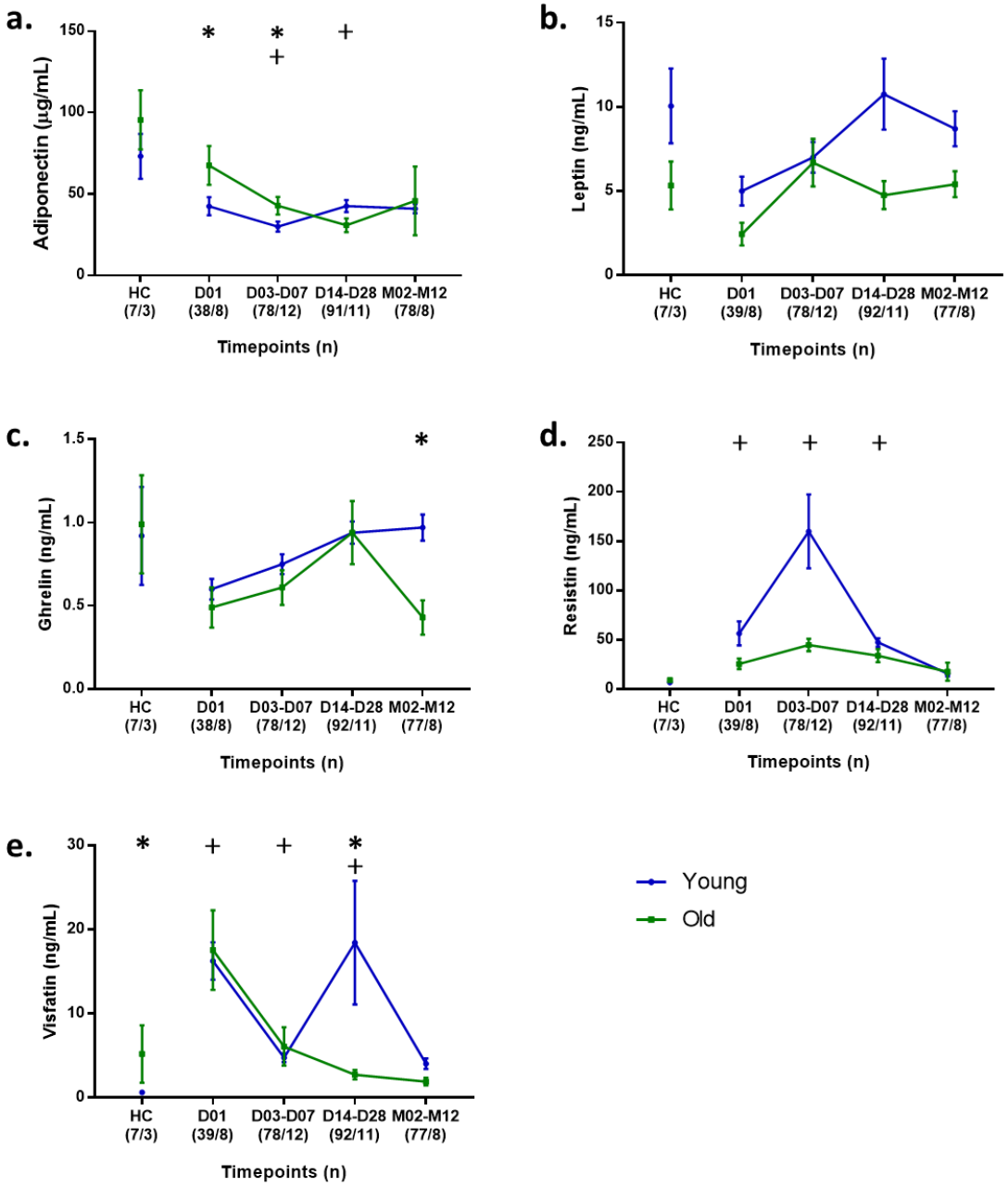


Figure 5.16. Longitudinal adipokine response in young and old patients following burn injury.

Serum adipokine levels in young and old burns patients were analysed across all timepoints. a. Adiponectin; b. Leptin; c. Ghrelin; d. Resistin; e. Visfatin. Analyte levels at each timepoint was compared between both cohorts (Young vs. Old) using Mann-Whitney Test; * $p < 0.05$. Analyte levels of young and old burns patients were compared to young and old healthy controls (HC) using Kruskal-Wallis test with Dunn's multiple corrections; + Young vs. HC Young, $p < 0.05$; # Old vs. HC Old, $p < 0.05$.

5.4 Discussion

This study was carried out to profile the adipokine response from day 1 till month 12 following severe burn injury using ELISA/Multiplex Technology. This study also investigated the associations of post-burn adipokine status with outcomes throughout the first year following injury. Furthermore, this study explored the diagnostic and therapeutic potential of adipokines. This is the first report exploring these areas simultaneously.

Major thermal injury has been reported to alter various properties of adipose tissue (135) and significant alterations in levels of serum adipokines were observed in burned patients (131, 576). Wade et al reported reductions in plasma concentrations of adiponectin, ghrelin and leptin while plasma levels of resistin were elevated following thermal injury (131). The Wade et al study had two major limitations. Firstly, the authors intended to collect samples three times per day for 7 days. Despite this, they presented direct statistical analysis between healthy volunteers and burned patients without profiling the response. Secondly, the authors only investigated the associations between adipokines and metabolic parameters. Another study profiled the response of adiponectin, ghrelin and leptin in burned children (576). The authors reported that adiponectin, ghrelin and leptin demonstrated similar trajectories following burn injury starting off low at week 1 with levels increasing thereafter mimicking the metabolic responses during the recovery process (576). Additionally, circulating resistin levels were elevated in burns population (396). In this study, adiponectin and leptin levels in serum were significantly reduced following sever thermal injury. Circulating levels of leptin in burned patients returned to healthy

volunteer levels by D03-D07 post-injury while systemic levels of adiponectin never recovered throughout the first-year post-injury. No difference in circulating levels of ghrelin between burned patients and healthy volunteers was observed in this study. In addition, serum levels of resistin and visfatin were significantly elevated immediately following thermal injury till day 28. Thereafter, circulating levels of resistin and visfatin in burned patient return to healthy volunteer levels. Similar observations were made in critically ill populations in terms systemic levels of most adipokines following pathologies such as sepsis and pancreatitis (371, 387, 394, 402). Interestingly, blood levels of leptin in acute critical illness were reported to be either increased or similar compared to reference range (371, 387) while burn patients exhibited significantly lower systemic leptin levels compared to un-injured individuals as illustrated in this study and others (131, 576). This indicates a potential unique response induced by major burn injury. Furthermore, this is the first study to characterise the adipokine response for 12 months following major thermal injury and in the context of critical illness. Interestingly, burned patients exhibited significant altered systemic adipokine concentrations and expressions compared to healthy volunteers throughout the study period.

Furthermore, age and gender had significant effects on adipokine response following severe thermal injury in this study. This may partly explain the differential outcomes between gender and age groups in burned patients (406, 440, 442). These are novel findings as no literature could be identified exploring longitudinal gender and age differential adipokine responses following burn injury or critical illness. The ageing process and sex hormones have been reported to influence adipose tissue affecting

adipokine levels outside the context of critical illness. These effects are discussed in detail in these reviews (577, 578).

Circulating levels of visfatin were significantly elevated among non-survivors immediately following severe thermal injury. Additionally, visfatin levels during the first week of burn injury were independently associated with predictions of 28-day mortality. However, the discriminatory power of this observation in burned patient was poor (AUROC <0.8). Furthermore, significantly increased levels of ghrelin and resistin at later timepoints were observed among non-survivors post-burn injury. Although no other reports describing similar observations in burned patients were identified, significant positive associations between resistin and visfatin with mortality were reported in critically ill populations (399, 579, 580). Interestingly, higher serum ghrelin concentration was reported as a positive predictor of survival in critically ill septic patients (581). However, observations of ghrelin response and mortality in this study should be interpreted with caution for the following reasons. This study was conducted in medical intensive care settings only and therefore limiting interpretation to this cohort only and therefore does not relate directly to critically ill surgical or trauma patients. Secondly, the median age of patients in this study was >60 years old while post-burn and trauma patients are usually younger. Further studies in the burns and trauma population are encouraged to validate this finding and scientific investigations are needed to explore potential mechanisms behind this observation.

Alterations of systemic adipokine concentrations were observed in burned patients who developed sepsis and/or multiorgan failure. Adiponectin levels in serum were

significantly reduced in septic patients post-thermal injury. While leptin, resistin and visfatin were significantly elevated in burned patients who subsequently developed MOF and/or sepsis. No other studies investigating the influence of adipokine status on short-term outcomes in burned patients have been published. However, similar observations were reported in critically ill patients who were admitted to intensive care with sepsis, septic shock and/or organ dysfunction (371, 402, 579, 582, 583). The mechanisms responsible for these observations in burns and critically illness remain to be fully elucidated. Further studies investigating the status of adipokines in critically ill populations at local tissue and systemic level are required to address this knowledge gap. Furthermore, quantification of associations and correlations between systemic adipokine levels and immune/inflammatory response using multi-variate statistical analysis may improve understanding of mechanisms of actions of adipokines in critical care contexts accounting for con-founding variables such as patient characteristics and health status, injury/illness severity and treatments.

Following severe thermal injury, low levels of anti-inflammatory adiponectin and/or high concentrations of pro-inflammatory leptin, resistin and visfatin significantly correlated with longer wound healing times and worse scarring outcomes. These associations remained significant at multiple timepoints. This suggests that adipokines may influence the process of wound healing and scarring. Interestingly, it was reported that adipokine concentrations may explain the wound healing and scar enhancing effects of fat grafting (584). Fat grafting is a surgical technique used to improve scar characteristics and forms an invaluable management option in the burns, plastic and reconstructive surgeons' repertoire. Despite being a validated surgical method, the

mechanisms of action associated with scar improvement outcomes remains to be established. Adipokines may offer a potential explanation due to their effects on procollagen synthesis, dermal matrix degradation, fibrosis, inflammation and vasculogenesis (585-587). In addition, involvement of adiponectin and leptin in scar pathogenesis and process has recently been reported(344, 588). Further studies Investigating the adipokine contents in the harvested fat graft and supernatant would prove useful. In addition, examining the adipokine and immune-inflammatory response using skin biopsies of treated and untreated scar longitudinally may prove invaluable in improving current understanding of mechanisms behind fat grafting and scarring outcomes measured objectively and subjectively.

A limitation in this study is the high intra-plate variability for Adiponectin ELISA. Hence, the quantification process was repeated twice. Despite this, the adiponectin intra-plate variability remained high. Further investigations quantifying adiponectin in burn patients are required to validate the observations reported in this study.

5.5 Conclusions

Major thermal injury significantly influences systemic adipokine concentrations in patients for a minimum of 12 months post-injury. Adipokines status post-burn injury had significant associations with prognosis of patients. Data suggest that adipokines may have potential therapeutic value in burn management. Furthermore, it potentially could explain the enhancing effects of current treatments utilising adipose tissue. Further studies are required to validate these observations, as well as improve understanding of mechanisms responsible for such observations.

CHAPTER 6: GENERAL DISCUSSION

6.1 Overview of the Thesis

The primary objective of this thesis was to determine the endocrine response to severe thermal injury using an -omics based approach. Omics-based studies involve the characterization and quantification of various biological molecules, including genomics, transcriptomics, proteomics and metabolomics. Such studies produce results that are holistic and highlight the dynamics and functionality of molecules in a targeted population. In this case, burned patients. This is referred to as a systems biology approach. Studies using systems biology frequently examine samples in a non-targeted, longitudinal and non-biased manner when the hypothesis remains unknown. Therefore, data acquired from such studies can then be used to define potential hypotheses for investigation (589). Such strategies are potentially useful in medicine.

Omics-based studies allow greater understanding of physiological and pathological processes following critical illness, burns and trauma. Furthermore, they allow clinical interpretation and identification of potential applications in screening, diagnosis and prognosis prediction. Furthermore, this approach may aide in the development of predictive, preventative and personalized patient care in hospitals and other clinical settings (590). Omics-based studies thus have potential clinical and scientific value in the health-care field. Such studies enable “precision medicine”, a concept where medical treatments account for individual variability to provide personalized management. Severe thermal injury can elicit major and prolonged pathological immune-metabolic responses that are associated with poor short and long-term outcomes (37, 71, 160, 591). Such severe post-burn responses can be, partly, explained by endocrine disturbances following injury (71, 127). Current knowledge relating to

burn care and pathology have been largely driven by traditional hypothesis-driven studies. Such strategies have led to significant advancements in the management of burned patients and subsequent clinical outcomes (405). Despite this, there is potential to improve overall prognosis of burned patients including scarring and re-integration to society by a systems biology and omics analysis approach (592).

This thesis has focused on the endocrine responses in a cohort of these patients following severe thermal injuries (TBSA ≥20%). These endocrine responses were quantified using state of the art LC-MS/MS and multiplex techniques. Characterization of the systemic endocrine response demonstrated the severe and persistence of hormonal alterations in burned patients. The data indicate that severe thermal injury may induce non-classical endocrine responses to maintain homeostasis. Furthermore, observations were made significant and independently associating circulating levels of various hormones to outcomes of severely burned patients. Additionally, and most importantly, immediate and long-term supplementation of hormones (such as 25D3, DHEA, DHEAS and testosterone) are suggested by these data in order to improve outcomes in all patients following thermal injury. Moreover, a sub-group analysis was performed to investigate the influence of current treatments, specifically oxandrolone and corticosteroids, demonstrating the overall effects of such treatments and potentially influencing their future use in burn care. The reports in this thesis are part of the comprehensive SIFTI study.

SIFTI was an ambitious observational study set up by the Scar Free Foundation Birmingham Burns Research Center and University of Birmingham in the UK. The

objective of SIFTI was to simultaneously characterize the immune, inflammatory, endocrine and metabolic responses in burned patients from admission to one year post injury in a tertiary burn center. A particular feature of SIFTI was the profiling of these responses over time, with frequent blood sampling in the first month after injury and then monthly out to one year. Furthermore, SIFTI enabled the exploration and identification of clinically valuable relationships of these responses simultaneously with outcomes of burned patients including survival, sepsis, MOF, wound healing and scarring.

6.2 Longitudinal Endocrine Response in Severely Burned Patients: An Omics-based Approach

Major thermal injury exerted significant effects on the endocrine system. Immediately following injury burned patients exhibited profound systemic disturbances of various hormones affecting anti-inflammatory, pro-inflammatory and stress hormones. Furthermore, significant differential endocrine responses were observed between age groups (young vs. elderly) and gender (female vs. male).

Severely burned patients demonstrated significant elevations in circulating levels of 11-deoxycortisol and cortisol, with simultaneous increases and decreases in systemic levels of anti-inflammatory corticosterone and cortisone respectively. The increase in cortisol with a decrease in cortisone is reflective of an increase in 11βHSD1 activity, the enzyme that converts cortisone to cortisol (462, 476, 477). Importantly this enzyme is activated by inflammatory cytokines such as TNFα (462), suggesting the inflammatory and endocrine responses are intimately linked both at local and systemic levels, in an

attempt to control the inflammatory response. Moreover, other circulating hormonal profiles post-thermal injury indicated significant reductions in anti-inflammatory hormones including vitamin D and adiponectin. Interestingly, profound changes in systemic pro-inflammatory hormones were observed simultaneously following major burn injury, including leptin, resistin and visfatin. Collectively, severe thermal injury induced a profound and prolonged pathophysiological systemic endocrine response characterized by simultaneous release of stress, pro-inflammatory and anti-inflammatory hormones. These observations support the paradigm, from a systemic endocrine response, of simultaneous SIRS/CARS responses following thermal injury as proposed Xiao et al (83). Collectively, the findings of the studies in the thesis are novel as no critical care reports including burns and trauma describe the systemic endocrine response in relation to the simultaneous SIRS/CARS response following injury/illness were identified.

Furthermore, severely burned patients exhibited a mixed endocrine response characterized by simultaneous significant and prolonged elevation and reduction of both anti-inflammatory and pro-inflammatory hormones. These findings indicate that the overwhelming pro-inflammatory and stress endocrine response following thermal injury is addressed by a simultaneous homeostatic anti-inflammatory response. Similar observations have been reported for circulating levels of pro and anti-inflammatory free fatty acids immediately following burn injury (61). This suggests the human response following burn injury potentially behaves similarly to inflammatory responses with counter-regulatory mechanisms described in sepsis (89). These findings highlight

the complex and severe nature of pathophysiological responses associated with burn injury.

Age had a significant influence on the hormonal response following thermal injury. Older burned patients exhibited significantly increased circulating levels of 11-deoxycortisol and cortisol compared to younger patients with similar injuries. The anti- and pro-inflammatory endocrine responses between young and older patients were also diverse post-burn injury. Older burned patients demonstrated lower systemic levels DHEA, DHEAS, leptin, resistin and visfatin, as well as most vitamin D metabolites, while exhibiting higher levels of adiponectin. The low level of DHEA and DHEAS likely reflect the age-related decline in these hormones, termed adrenopause (593, 594). The lower leptin is more surprising as the levels of this adipokine have been reported to increase with age (595, 596). This effect of ageing on leptin levels has been associated with changes in circulating sex steroid hormones, for example inverse relationship between testosterone and leptin, that is reported to be independent of body fat (596). However, other studies have demonstrated that adiposity and BMI are major determinants of systemic leptin levels with age and gender being secondary regulators (595, 597). Therefore, low serum leptin observed in this study could reflect a normal or lower BMI of our older patients. However, the lack of accurate BMI for our patients means this remains a hypothesis. Moreover, elderly patients demonstrated a more profound response at later time-points with significant elevation and reduction of circulating levels of testosterone and ghrelin at M02-M12 following thermal injury compared to younger patients.

These data in total highlight the extended dysregulation of the hormonal response exhibited by older patients following thermal injury which may, in part, explain the impaired immune response, delayed hypermetabolic responses and exhaustion phenotype reported in elderly burn patients predisposing them to poorer outcomes (598, 599). Elderly burns patients are reported to have higher mortality rates compared to younger adults (405, 406, 599). Interestingly, Jeschke et al observed no significant increased incidence of sepsis, bacteraemia, pneumonia, burn wound infection, renal failure, acute coronary syndrome, acute respiratory distress syndrome, pulmonary embolism and deep vein thrombosis in elderly compared to younger adult burns patients despite having increases APACHE II and Denver 2 scores (599). This could be secondary to the two-phase response exhibited by elderly burns patients. This two phase response is characterized by an early hypo-inflammatory and immune-senescent response followed by a late augmented reaction (598, 599). This could explain the substantial long-term mortality observed among elderly burn survivors (600).

Elderly burns patients had significantly elevated levels of circulating testosterone while exhibiting significantly lower systemic levels of ghrelin at M02-M12 post-injury compared to younger adults. Testosterone is known to exert anti-inflammatory and immune-suppressive effects. These are discussed in detail in the following studies and reviews (601-603). Furthermore, testosterone modulates glycolysis, glycogen synthesis and lipid and cholesterol metabolism at a molecular level and is discussed in detail in the following review (604). Therefore, elevated testosterone levels in elderly burns

patients at later timepoints, in this study, may be an attempt to dampen the augmented immune-inflammatory response discussed earlier.

Ghrelin is an appetite stimulating orexigenic hormone with multiple roles. Firstly, ghrelin enhances appetite and increases food intake in healthy individuals (605). Secondly, ghrelin plays important roles in energy homeostasis and metabolism affecting various tissues and organs with important consequences such as preventing muscle atrophy (606, 607). Additionally, Ghrelin exerts anti-inflammatory and immune-modulatory effects via multiple pathways and is comprehensively described in the following reviews (608, 609). Low levels of systemic ghrelin among elderly burns patients observed in this study exacerbates the late augmented immune-inflammatory response discussed earlier. Moreover, low ghrelin levels may lead to poor oral intake potentiating the consequences of the prolonged hypermetabolic response following thermal injury.

The findings of this thesis highlight the complexity of the multi-systemic endocrine responses observed in elderly burned patients in this study. The observations made in this thesis relating to the endocrine responses in elderly patients following severe thermal injury should be hypothesis-generating owing to low number of recruited patients over 65 years of age. This emphasizes the need for more studies to investigate immune-inflammatory, metabolic and endocrine responses in older patients following thermal injury. By understanding these responses, new age-specific treatments can be made to improve outcomes in this vulnerable population.

Gender also had significant effects on the endocrine response following thermal injury. Female burn patients demonstrated profound and prolonged stress and pro-inflammatory hormonal responses for the first-year post-injury compared to male burns patients. This response is characterized by increased levels of cortisol, leptin with simultaneous reduction in DHEAS and multiple vitamin D metabolites. These findings may potentially, in part, explain the poorer outcomes reported in female burn patients (439, 442).

Cortisol is a stress hormone known for its immune suppressive and anti-inflammatory effects(610). Therefore, increased levels of cortisol can lead to poor outcomes such as septic shock and mortality(611). DHEA/DHEAS antagonizes glucocorticoid effects on the immune systemic and enhances neutrophil functions while Vitamin D has influences on the immune system and exerts various immune-tolerogenic effects. The effects of DHEAS and Vitamin D on the immune-inflammatory responses are discussed in detail in the following reviews (533, 612, 613). Significantly reductions in serum DHEAS has been previously reported in septic shock and trauma (614). Additionally, high cortisol to DHEA ratios has been associated with mortality in critical illness (614). Furthermore, vitamin D deficiency is prevalent in critically ill populations predisposing them to poor outcomes including sepsis, organ failure and mortality (134).

Leptin is a complex molecule with multiple roles that appears to be condition-related. Leptin exerts protective anti-inflammatory effects during acute inflammation while being pro-inflammatory in various pathologies such as autoimmune disease (615, 616). The various effects of leptin on the immune system in the context of various disease

states, including systemic lupus erythematosus and arthritis, has been previously described (617). Leptin appears to positively correlate with pro-inflammatory status and illness/injury severity scores (135). In this study, significantly elevated serum levels of leptin were observed in septic patients and burn patients with delayed wound healing. These associations became non-significant during multi-variate analysis. Hence no conclusive statements can be made regarding the influence of leptin on outcomes following major burn injury. Furthermore, the function and effects of leptin following burn, critical illness and/or trauma remain poorly understood.

The observations made in this thesis in relation to gender differential endocrine-responses following thermal injury should be interpreted with caution owing to generally low number of male and female burn patients recruited in this study. Only one study investigating gender responses in critical illness was identified. Critically ill septic female and male demonstrated similar systemic responses and outcome rates (618). No literature exploring gender differential systemic responses following burns or trauma could be identified. Further studies are encouraged to investigate the hormonal mechanisms behind gender disparities and outcomes following injury to address this knowledge gap.

6.3 Potential Value of Endocrine Response as Clinical Biomarkers

Studies presented in this thesis demonstrated potential clinical utility of various hormones as outcome-predictive biomarkers. Immediately following severe thermal injury, 11-Deoxycortisol and vitamin D metabolites have demonstrated good discriminatory power for predicting subsequent poor outcomes. 11-Deoxycortisol is a

precursor to the stress glucocorticoid hormone cortisol and indicates a higher flux through the cortisol generating pathway, possibly influencing immune function and predisposing to infections and sepsis. Elevated circulating levels of 11-Deoxycortisol at day 1 post-burn injury were predictive for 28-day and in-hospital mortality. Meanwhile, increased systemic levels of 25D3, 23,25D3 and 24,25D3 following severe thermal injury were predictive of survival and being sepsis-free. This could be associated the immune-inflammatory modulation associated with vitamin D axis and its alternative metabolic pathways. The role of these alternative metabolic pathways of vitamin D remains to be understood. These are novel findings that highlight the urgent need to explore these 'non-classical' vitamin D responses as these alternative metabolic pathways were associated improved outcomes in patients following severe thermal injury.

Quantification of serum levels 11-deoxycortisol and vitamin D metabolites in patients during the first 24 hours of thermal injury may aid in patient management. This is very important for the following reasons. Injury severity in burns patients is assessed using various clinical scoring systems to determine probability of mortality and futility of treatment (465, 619, 620). Actively treated burns patients are at-risk of developing complications increasing their morbidity and mortality. This could be attributed to multiple reasons. Burns patients exhibit simultaneous overwhelming global inflammatory responses and an impaired multi-systemic response (61, 112, 417, 598). Subsequently, diagnosing subsequent adverse events, such as sepsis, can be challenging and may delay life-saving treatments. This can significantly affect their survival (467). Therefore, identification of high-risk burns patients through biomarkers

can help focus and guide necessary medical care minimizing the development of adverse events following severe thermal injuries.

6.4 Potential Value of the Endocrine Response as Therapeutics

The endocrine responses reported in this thesis demonstrate potential role of various hormones in the treatment of severe burn patients. During first 24 hours of injury, supplementation of testosterone, DHEAS and vitamin D (25D3) may reduce the risk of sepsis development and delayed wound healing in burns patients. Furthermore, administering testosterone, DHEA/DHEAS and vitamin D during the acute stage of admission may exert or maintain the same effects and improve survival rates following thermal injury. This indicates that regular daily supplementation of the above molecules may be more clinically valuable than a one off or once weekly dosing regimen. Furthermore, endocrine responses and expression remain significantly different in burns patients at later timepoints including months 2 till 12 post-injury. Moreover, significant associations were observed between various endocrine metabolites and scarring outcomes. This suggests that prolonged supplementation may be required to address longer-term outcomes.

Few medications that modulate endocrine systems are currently used in burn care. In this thesis, two medications used on burn patients were assessed which are part of treatment protocols for critically ill and/or burn patients, namely corticosteroids and oxandrolone. We found that administration of corticosteroids in burn patients was associated with poor outcomes including MOF, sepsis and mortality. While, treating burn patients with oxandrolone significantly reduced subsequent sepsis and improved

survival rates. The effects observed for corticosteroids and oxandrolone following thermal injury were independent of injury severity and relevant-organ status. Thus, the results could be interpreted for all flame and scald burn patients in general.

The use of corticosteroid therapy was reported to be beneficial in critical illness (505, 506), and is currently endorsed by international guidelines (504). The observations in this thesis have shown that corticosteroid use in burned patients can lead to adverse events despite amelioration of vasoplegia as indicated by cardiac SOFA scores. This highlights the severe nature of the injury and unique systemic responses associated with major burns. Therefore, treatments cannot be generalized to critically ill patients admitted to intensive care. Special care and consideration to the pharmacological effects of corticosteroids in relation to the biological consequences of burn injury compared to other critical illnesses. This is due to unique systemic responses observed in burned patients (557).

Oxandrolone is a testosterone analogue that accentuated the hypermetabolic response following severe thermal injury (621). Oxandrolone use demonstrated various beneficial effects in both the acute and recovery phases post-burn injury (201, 507). Furthermore, long-term oxandrolone supplementation resulted in greater improvements in lean body mass, bone mineral content/density and growth in children following severe burns (203, 622). Most of these reported beneficial effects relate to metabolic parameters and survival following thermal injury. Adding to current literature, oxandrolone use following major burn injury was significantly associated improved 28-day and in-hospital mortality, as well as lower sepsis rates. These

observations were independent of initial injury severity and daily liver function. This indicates that effects of oxandrolone may not be limited to metabolic response and can influence other body systems as per (Figure 3.30- Chapter 3). Jeschke et al investigated the endocrine and inflammatory responses in acutely burned children who received and did not receive oxandrolone reported no significant differences (412). However, oxandrolone use following discharge was associated with increased IGF-1, tri-iododothyronine uptake and free thyroxine index in the pediatric population following major burn injury (623, 624). This highlights the need to investigate the systemic responses of burned patients who received and did receive oxandrolone to identify, using robust analysis, potential mechanisms responsible for the observed improved outcomes.

Testosterone, as reported earlier and in chapter 3, demonstrated beneficial effects at day 1 following thermal injury. While, oxandrolone was given at median day 5 post-injury to burn patients in this study. This suggests that oxandrolone administration immediately post-burn injury may further improve outcomes.

6.5 Strengths of the Thesis

This research has multiple strengths. The e-SIFTI study was a prospective trial that combined extensive short and long-term clinical and outcome data collection at 10 different timepoints to investigate the endocrine response in the burn sub-populations. Furthermore, e-SIFTI contains the largest burns cohort to be examined in this manner. This sub-study forms a pillar of the larger SIFTI study. Furthermore, SIFTI was a pragmatic observational study that enables simultaneous bio-fluid analysis

immediately following severe thermal injury till month 12. Data and samples of burn patients were studied without affecting or influencing medical treatment given in a tertiary burn care center in the UK. This means the findings reported in this thesis are true representations of pathophysiological responses in burn patients treated using up-to-date gold standard burn care. Responses examined in SIFTI study include immune, inflammatory, endocrine and metabolic processes which were characterized and profiled in a parallel fashion. This ambitious project consisted of multiple large research groups and has not been conducted in UK or Europe previously.

e-SIFTI utilized optimal techniques to quantify endocrine responses following severe thermal injury. This was performed to allow accurate, reliable and reproducible data. Quantification of hormones post-burn injury was performed using an -omics based approach allowing for hypothesis generation and stimulation for further research, as well as enable the discovery of potential clinically relevant biomarkers and therapeutics. Statistical analysis performed allowed for generalizability and ease of clinical interpretation. Such techniques may potentially ease translation of the study findings to medical practice.

6.6 Limitation of the Thesis

The main limitations of the SIFTI study include healthy volunteer cohort, absence of important confounding factors relating to endocrine responses, statistical analyses utilised and the use of non-validated scar measurements.

The numbers of healthy controls studied in e-SIFTI are small. In order to address this, studies investigating the same hormones on healthy individuals utilizing the same

quantification techniques were incorporated into the e-SIFTI studies to increase numbers and obtain a more representative endocrine reference range of the local population for comparison. A major limitation is that these healthy volunteers were clinically healthy and, in the case of the HAS study, had stringent inclusion criteria. These individuals are probably at low risk of sustaining burn injury. Hence, the hormone status of the healthy controls may not be a true of representation of pre-injury endocrine status of burn patients when accounting to co-morbidities. The past medical history of enrolled burn patients is summarised in Supplementary Table 4 and Supplementary Table 5.

Another limitation in the studies presented in this thesis is the absence of confounding factors associated with endocrine status. These include nutrition, diet and exercise. Furthermore, burn patient weight was estimated and therefore, the measurements recorded were not reliable enough. This is probably due to the critical state of patients following thermal injury when controlling patient hemodynamic status is prioritized over accurate weight recording. The decision was made to exclude weight and BMI measurements from statistical analyses.

e-SIFTI studies in this thesis presents robust statistical analyses to demonstrate potential clinical utility of hormones as biomarkers and treatments. These treatment and biomarker effects of the quantified endocrine analytes are purely based on statistical modeling. Hence, the observations made hypothesis-generating and cannot be used to change medical burn management yet. Hence, pilot studies examining the effects of supplementation of hormones, such as DHEA, testosterone and vitamin D, in

burn patients are highly encouraged. These pilot studies are invaluable step to enable RCTs and subsequent medical practice change. Another statistical limitation is the difference of hormone levels between age groups and gender of healthy controls and burn patients. No validated statistical technique could be found to account the healthy control levels when analyzing age and gender endocrine responses following major injury. The statistical analysis in this thesis were overviewed by supporting statisticians, Dr. Animesh Acharjee.

e-SIFTI study correlated the endocrine response post-burn injury with various objective scar measure. Although the use of these objective measures in burn scar assessments were previously published in the literature, their use for scar evaluation remains to be clinically validated. Therefore, the observations made in this thesis are investigative and definitive conclusions cannot be made currently.

6.7 Conclusions

The observations reported in this thesis are novel with, hopefully, important clinical implications and may improve the outcomes of adult burned patients. However, the pathophysiological responses in the following severe thermal injury is indeed very complex. These responses include the endocrine, genomic, immune-inflammatory and metabolic reactions that occur in patients following major burns. Furthermore, exploring these responses at both systemic and local tissue levels in a longitudinal fashion holistically is key to further improve the short-term and long-term outcomes and debilitating consequences of severe thermal injury. To achieve this, burn research units must collaborate together and with research units of various specialties.

This thesis is the product of the efforts of clinical and scientific staff working in different clinical and research units within the University of Birmingham and University Hospitals Birmingham. These include: Burn center and Intensive Care in QEHB, Institute of Inflammation and Ageing (Trauma and Ageing Division and Neuroscience, Ophthalmology and Clinical Experimentation Division), Institute of Metabolism and Systems Research and Surgical Reconstruction and Microbiology Research Center. Only through collaboration and combined efforts and knowledge, advances in medical care can be made and patient outcomes improved.

6.8 Future Work and Directions

- Correlating endocrine responses following severe thermal injury with immune, inflammatory and metabolomic indices.
- Investigation of other endocrine process including growth hormone, insulin-like growth factors and associated binding proteins post-burn injury.
- Exploring systemic and local response of the extracellular matrix molecules including characterization of decorin, matrix metalloproteinases, tissue inhibitors of metalloproteinases and transforming growth beta in burn patients.
- Integrate and map the endocrine, immune, inflammatory responses following severe thermal injury.
- Identification of potential clinical biomarkers and therapeutics from above studies.
- Setting up clinical trials and pilot studies to assess the effects of DHEA, DHEAS and vitamin D supplementation following severe thermal injury

- Studying the effects of pre-injury statins and vitamin D supplementation on outcomes of burn patients via retrospective data collection and propensity matching statistical analyses

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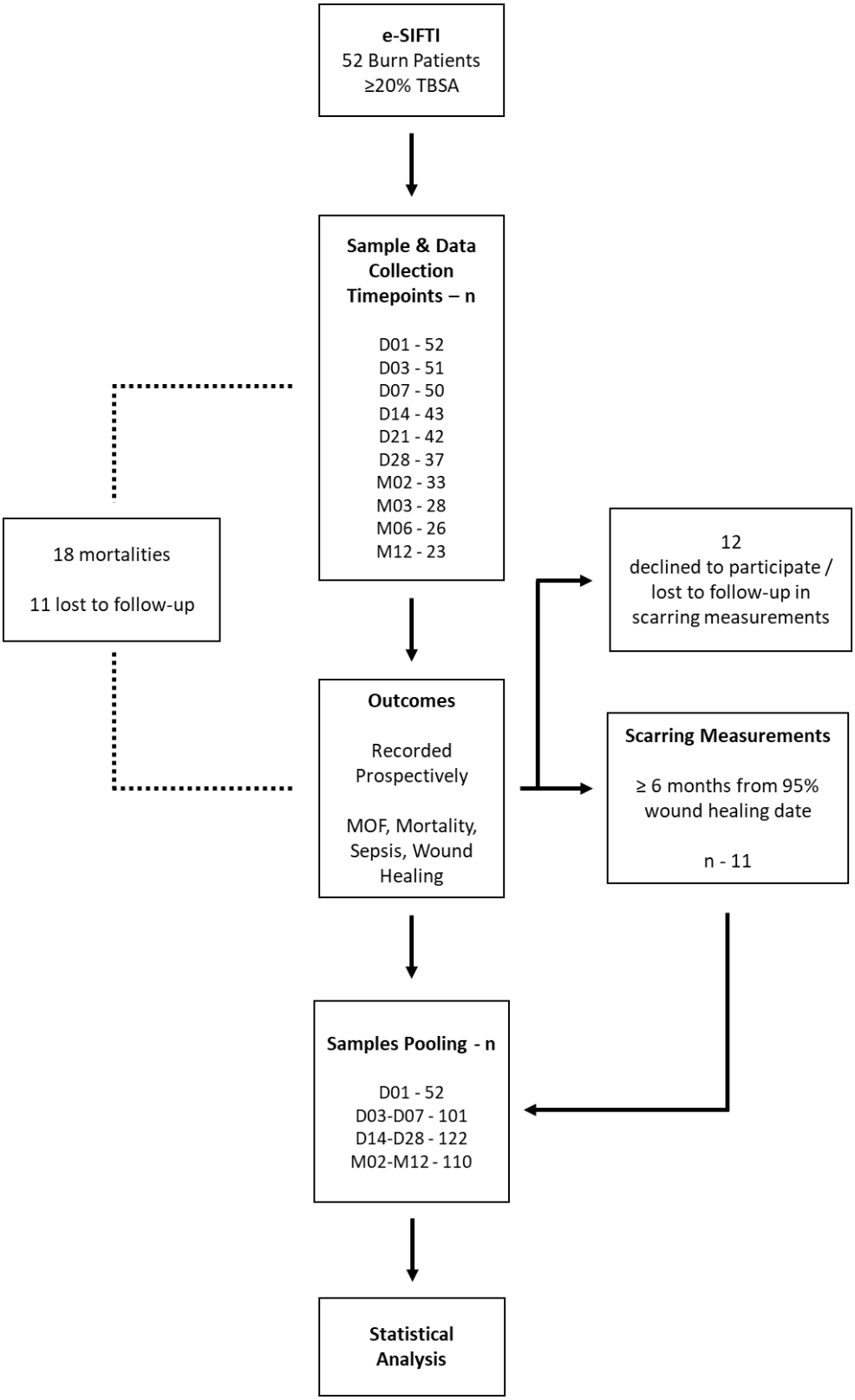
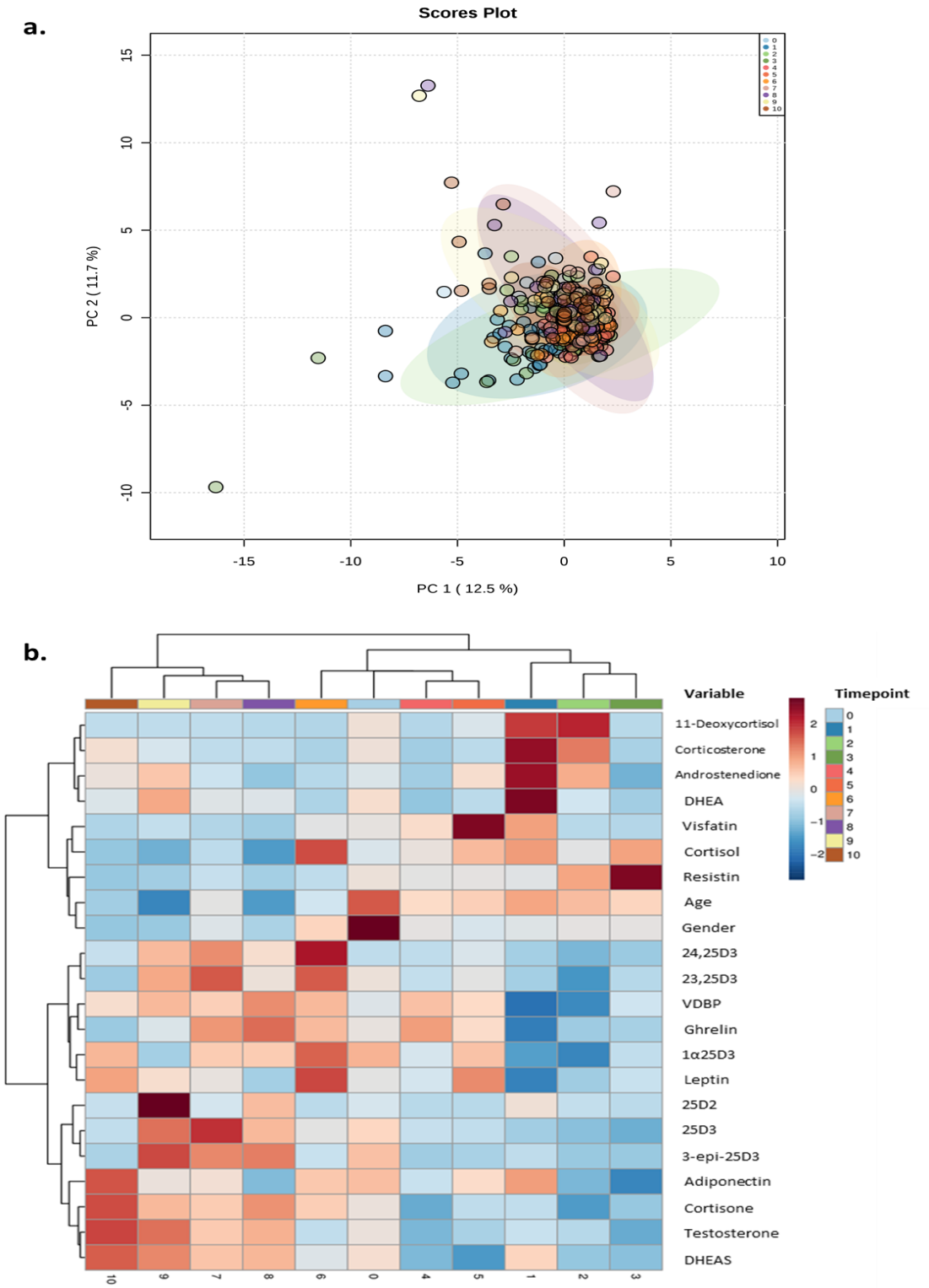
SUPPLEMENTARY DATA

Supplementary Table 1. Master table demonstrating demographics, injury severity, medications and outcomes of e-SIFTI participants.

	Healthy Controls	n	Burn Cohort	n
Age (Admission)	38 (31-70)	14	41 (33-55)	52
Gender (M/F)	8/6	14	33/19	52
TBSA (%)			42 (25-53.3)	52
Inhalation Injury (Y/N)			31/21	52
Revised BAUX Score			98 (74.975-113.26)	52
APACHE II Score			28 (17-31)	51
ITU Admission (Y/N)			40/12	52
Corticosteroid Given (Y/N)			22/30	52
Oxandrolone Given (Y/N)			34/18	52
Sepsis (ABA Criteria) (Y/N)			34/18	52
Multiorgan Failure (Y/N)			18/34	52
28D Patient Mortality (Y/N)			10/42	52
In-hospital Patient Mortality (Y/N)			18/34	52
Time Taken to Heal (Days)			33 (18-57)	28
mVSS Total Score			6.67 (6-7)	11
POSAS Overall Score (Clinician)			4.67 (4-6)	11
POSAS Overall Score (Patient)			8 (3-10)	11
Ultrasound Scar Thickness			1.7 (1.35-2.48)	11
Ultrasound Scar Intensity			0.45 (0.24-0.54)	11
Cutometer Scar Pliability R2			0.97 (0.93-1.17)	11
Cutometer Scar Pliability R0			0.56 (0.4-0.69)	11
DSM Colorimeter - Erythema Scale			1.27 (1.07-1.77)	11
DSM Colorimeter - Melanin Scale			1.21 (1.04-1.32)	11
DSM Colorimeter - L Scale			0.81 (0.72-0.86)	11
DSM Colorimeter - a Scale			1.18 (1.02-1.43)	11
DSM Colorimeter - b Scale			0.79 (0.48-0.97)	11

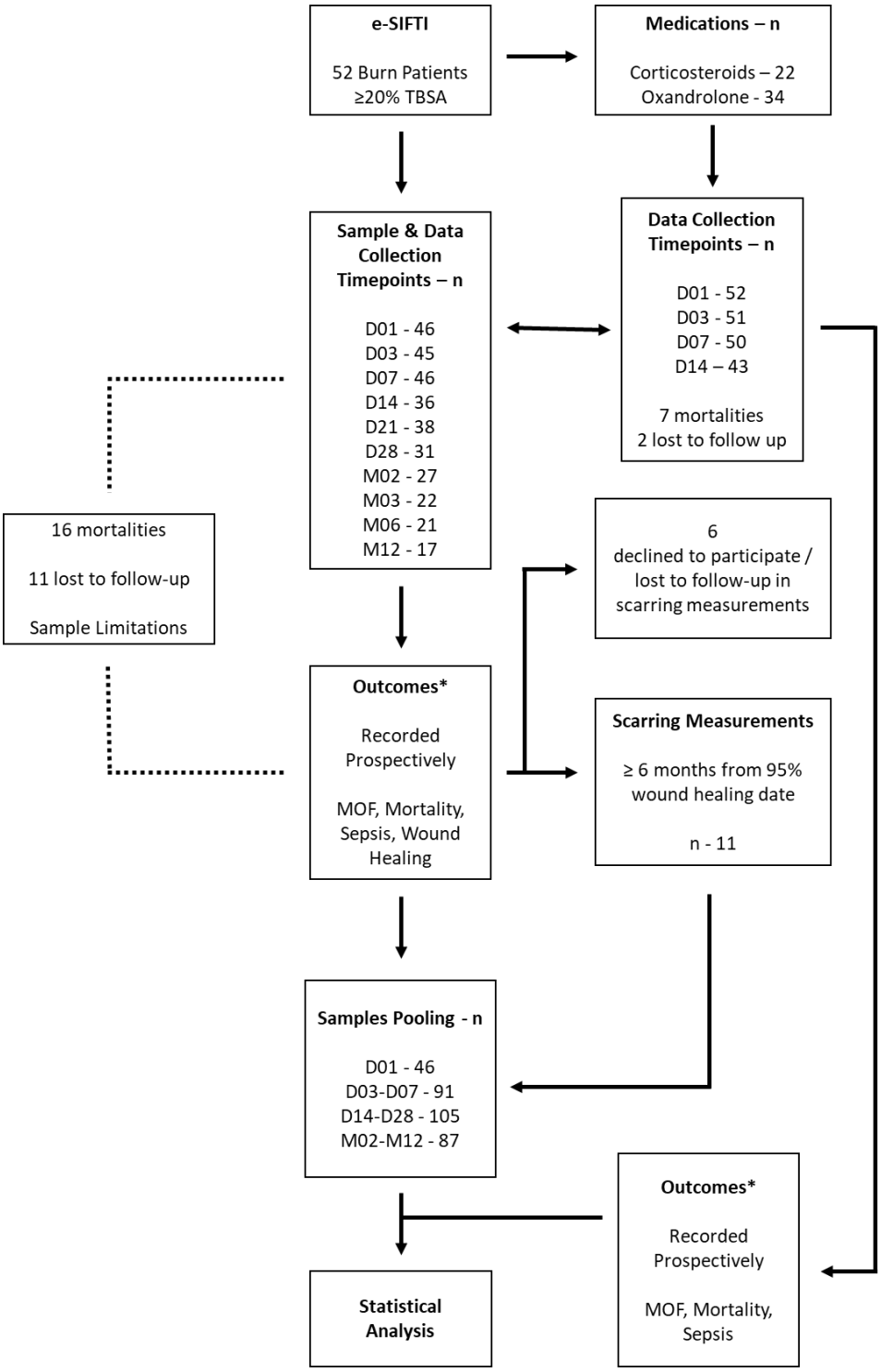


Supplementary Figure 1. Consort diagram of process of sample and data collection with subsequent data pooling of e-SIFTI cohort for statistical analysis.



Supplementary Figure 2. Statistical clustering of timepoints and hormones accounting for age and gender.

a. Principal component analysis demonstrating the structure and variance of data as per timepoints; b. Hierarchical clustering analysis with dendrograms of variables and timepoints. The dendrograms represent similarity between variables and timepoints. Timepoint legend: 0-HC; 1-D01; 2-D03; 3-D07; 4-D14; 5-D21; 6-D28; 7-M02; 8-M03; 9-M06; 10-M12.



Supplementary Figure 3. Consort diagram of process of sample and data collection with subsequent data pooling of e-SIFTI participants analyzed in CHAPTER 3: STEROID STATUS AND ITS INFLUENCE ON OUTCOMES FOLLOWING SEVERE BURN INJURY.

* Outcomes collected are the same.

	Total (n: 52)	Control (n:30)	Corticosteroids (n:22)	P value
Age	41 (33-55)	40 (29-55)	41 (35-63)	0.541
Gender (M/F)	33/19	19/11	14/8	0.982
% TBSA	42 (25-53.3)	30.5 (25-50)	48.5 (29-55)	0.078
Inhalation Injury (Y/N)	**31/21**	**13/17**	**18/4**	**0.005**
Revised Baux Score*	**98 (75.0-113.3)**	**83.6 (69.0-99.6)**	**105.5 (101.18-116)**	**0.006**
28D Survivor (Y/N)	10/42	5/25	5/17	0.585
Survivor (Y/N)	**18/34**	**7/23**	**11/11**	**0.046**
MOF (Y/N)	**18/34**	**6/24**	**12/10**	**0.010**
Sepsis (Y/N)	**34/18**	**15/15**	**19/3**	**0.006**

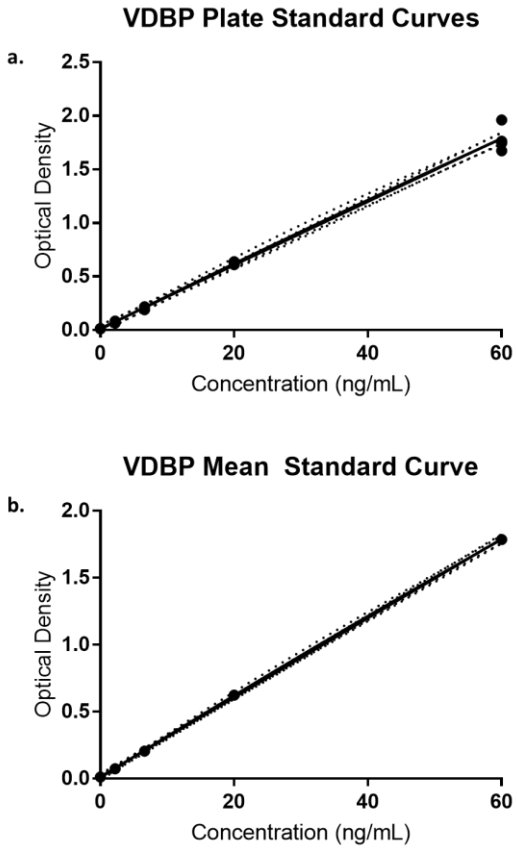
Supplementary Table 2. Demographics and outcomes of e-SIFTI participants treated and not treated with corticosteroids prior to PSM analysis.

Continuous variables are shown as median values with inter-quartile range. Controls and Corticosteroids cohorts were compared using Mann-Whitney test for continuous variables and Chi-squared test for categorical variables. Significant relationships are highlighted in bold. *Control and Corticosteroid cohorts were compared using independent t test as data distribution was normal.

	Total (n: 52)	Control (n:18)	Oxandrolone (n:34)	P value
Age	**41 (33-55)**	**52 (36-66)**	**39 (26-48)**	**0.011**
Gender (M/F)	33/19	11/7	22/12	0.798
% TBSA	**42 (25-53.3)**	**27.3 (23-50)**	**45 (30-55)**	**0.027**
Inhalation Injury (Y/N)	31/21	10/8	21/13	0.664
Revised Baux Score*	98 (75.0-113.3)	99.7 (83.46-116)	97.2 (72.7-110.1)	0.759
28D Survivor (Y/N)	10/42	6/12	4/30	0.06
Survivor (Y/N)	18/34	8/10	10/24	0.278
MOF (Y/N)	18/34	5/13	13/21	0.451
Sepsis (Y/N)	34/18	11/7	23/11	0.637

Supplementary Table 3. Demographics and outcomes of e-SIFTI participants treated and not treated with oxandrolone prior to PSM analysis.

Continuous variables are shown as median values with inter-quartile range. Controls and Corticosteroids cohorts were compared using Mann-Whitney test for continuous variables and Chi-squared test for categorical variables. Significant relationships are highlighted in bold. *Control and Corticosteroid cohorts were compared using independent t test as data distribution was normal.



Supplementary Figure 4. Standard Curves of the VDBP ELISA quantification.

a. Standard curves of all plates used for quantification; b. Mean standard curve for all plates quantified.

Bikle Formula

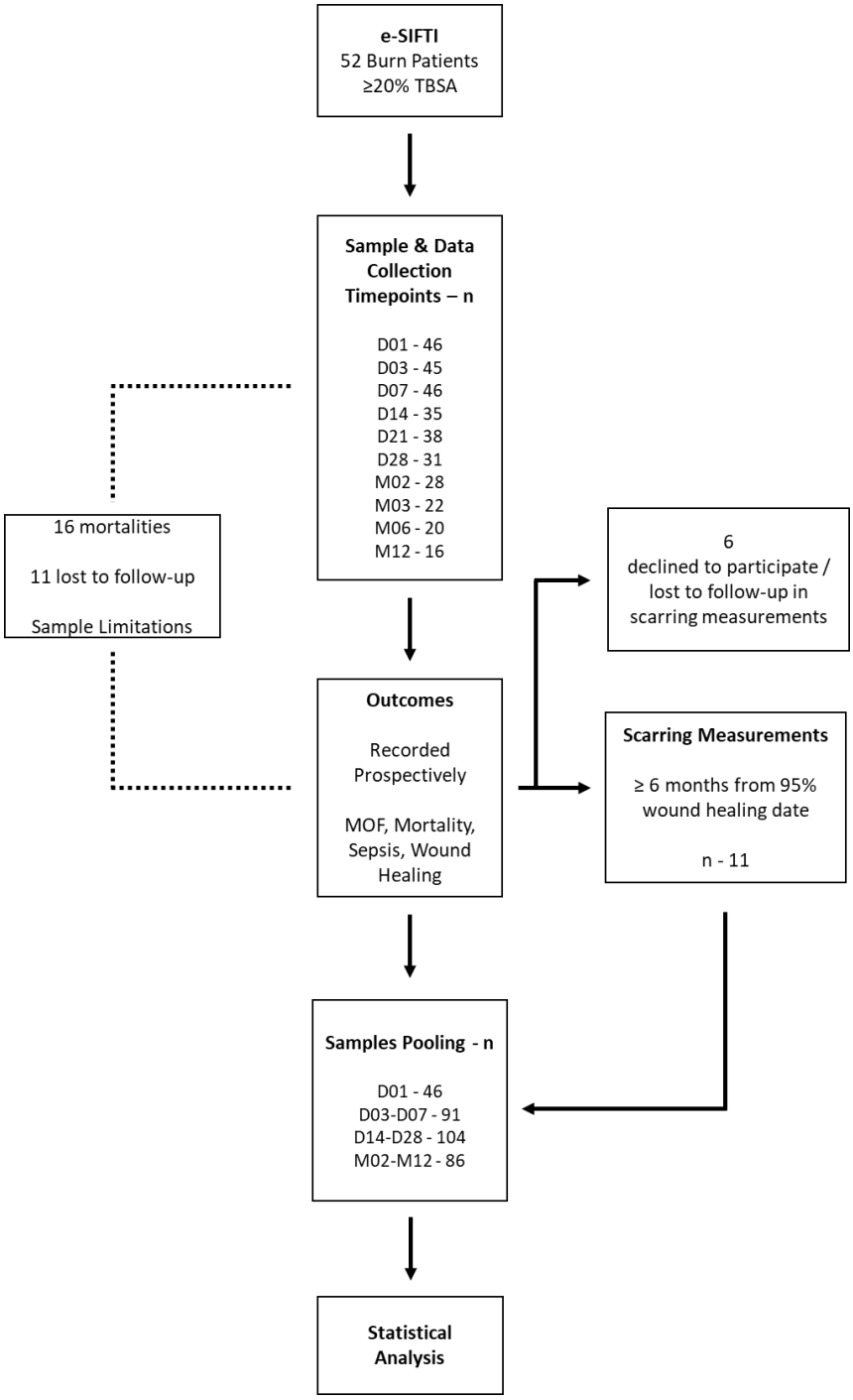
1. Free 25D3 = 25D3 ÷ (1+ (§ x Albumin) + (¥ x VDBP))

Vermeulen Bioavailable 25D3 Formula

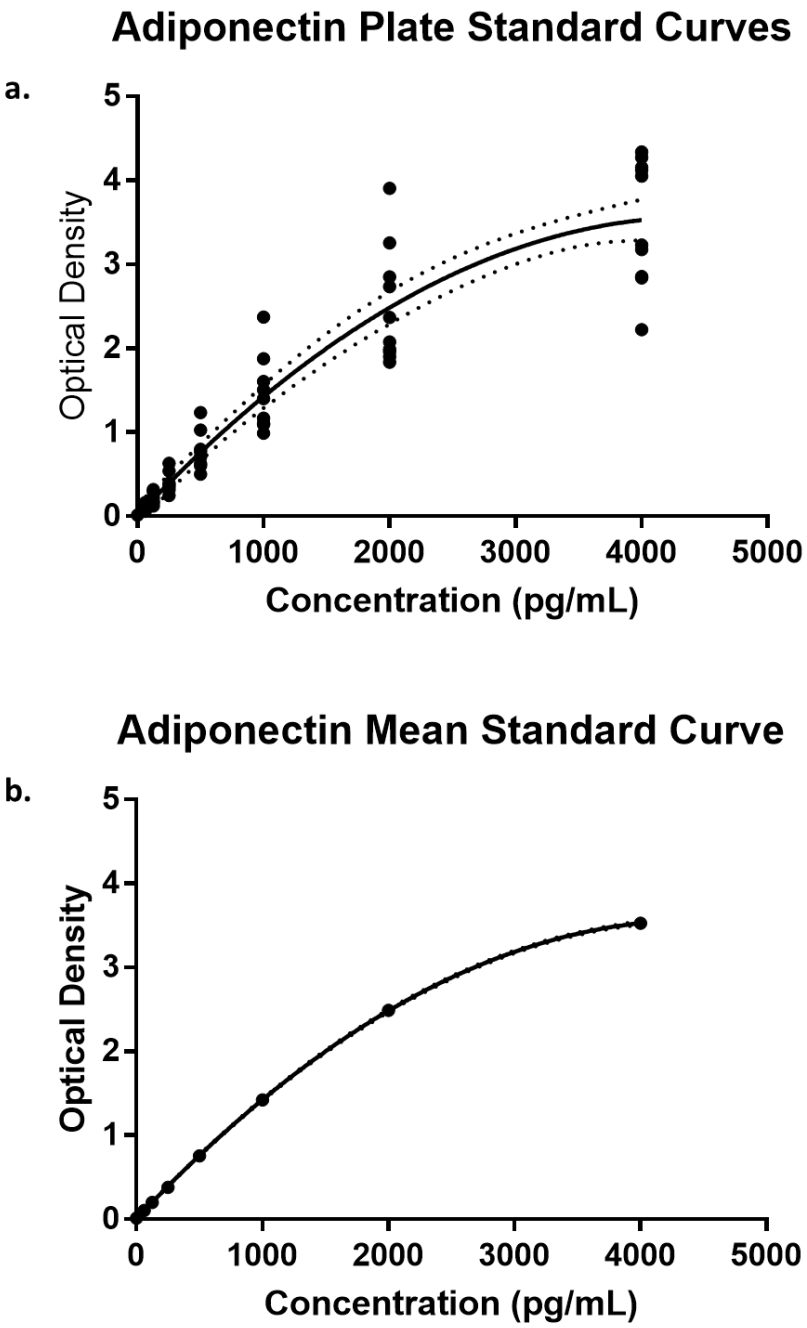
1. Free 25D3 = 25D3 ÷ (1+ (§ x Albumin)+(¥ x VDBP))
2. Albumin-bound 25D3 = Free 25D3 x § x Albumin
3. Bioavailable 25D3 = Free 25D3 + Albumin-bound 25D3

Supplementary Figure 5. Free 25D3 and Bioavailable 25D3 formulae.

§ Albumin Binding Co-efficient - $6x10^5$; ¥ VDBP Binding Co-efficient - $7x10^8$

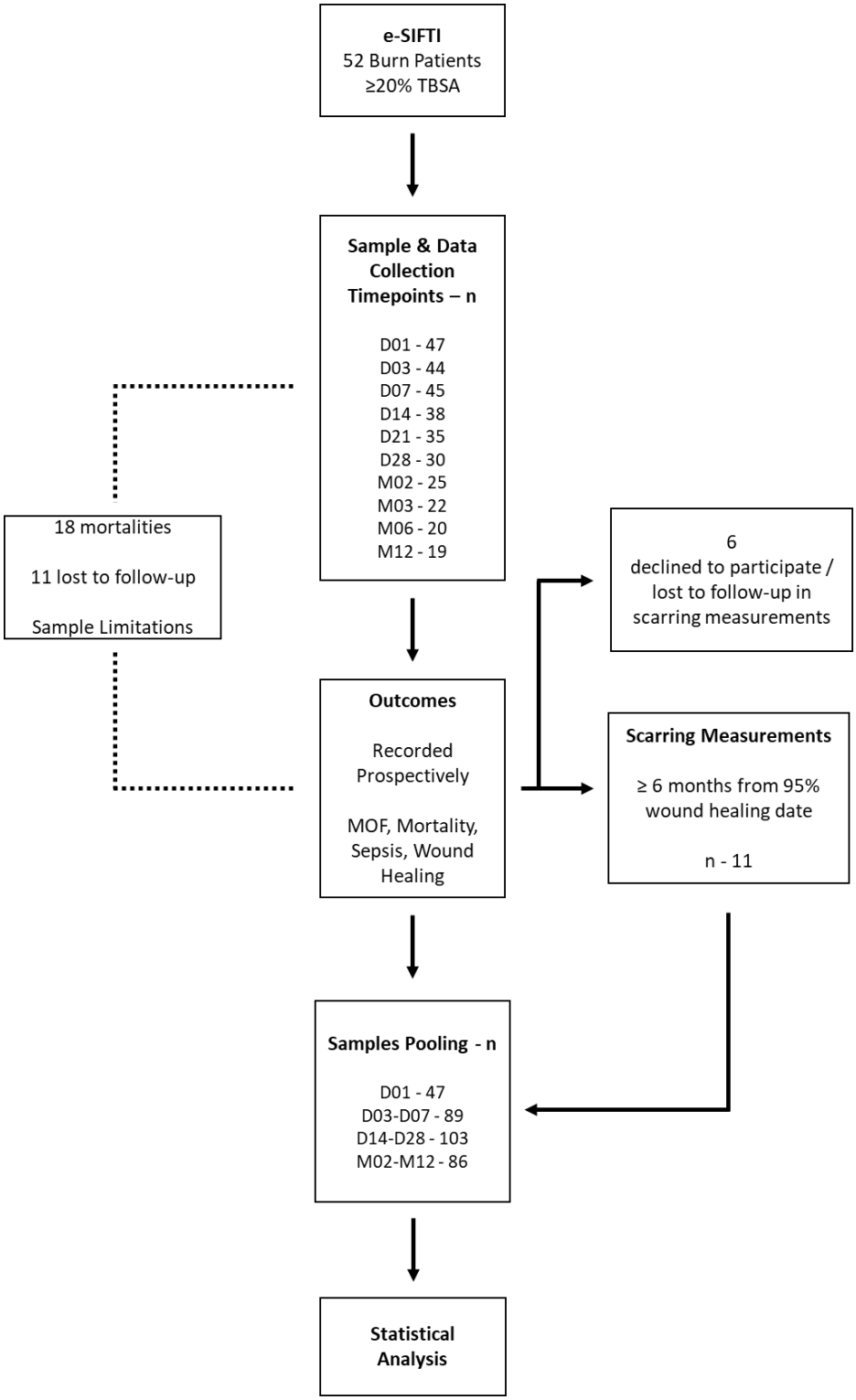


Supplementary Figure 6. Consort diagram of process of sample and data collection with subsequent data pooling of e-SIFTI participants analyzed in CHAPTER 4: VITAMIN D STATUS AND ITS INFLUENCE ON OUTCOMES FOLLOWING SEVERE BURN INJURY.



Supplementary Figure 7. Standard Curves of the Adiponectin ELISA quantification.

a. Standard curves of all plates used for quantification; b. Mean standard curve for all plates quantified.



Supplementary Figure 8. Consort diagram of process of sample and data collection with subsequent data pooling of e-SIFTI participants analyzed in CHAPTER 5: ADIPOKINE STATUS AND ITS INFLUENCE ON OUTCOMES FOLLOWING SEVERE BURN INJURY.

e-SIFTI ID.	Past Medical History
SIFT1-001	None
SIFT1-002	None
SIFT1-003	Congenital Hiatus Hernia
SIFT1-004	Bardet-Biedl Syndrome, Insulin Dependent Diabetes Mellitus, Hypertension, Chronic Kidney Disease 3 – Diabetic Nephropathy, Blindness, Hypogonadism, Obesity, Retinitis Pigmentosa, Situs Inversus
SIFT1-005	Excess Alcohol Intake
SIFT1-006	Hypertension; Rheumatoid Arthritis, Falls, Humerus Fracture
SIFT1-009	Mixed Anxiety/Depressive Disorder, Migraine, Chronic Fatigue Syndrome, Hypothyroidism, Previous Deliberate Self Harm
SIFT1-011	Depression
SIFT1-012	Hiatus Hernia, Hypertension
SIFT1-014	None
SIFT1-016	None
SIFT1-017	Depression
SIFT1-024	None
SIFT1-026	Asthma, Epilepsy
SIFT1-028	None
SIFT1-029	None
SIFT1-030	Schizophrenia, Chronic Obstructive Pulmonary Disease
SIFT1-031	None
SIFT1-032	Schizophrenia
SIFT1-035	None
SIFT1-039	None
SIFT1-040	Huntington's Disease
SIFT1-044	None
SIFT1-047	Schizophrenia, Cerebral Palsy
SIFT1-051	Huntington's Disease
SIFT1-052	Hypertension, Insulin Independent Diabetes Mellitus, Psychosis, Orbital Floor Fracture

Supplementary Table 4. Past medical history of patients with severe burn injury recruited in e-SIFTI (Part I).

e-SIFTI ID.	Past Medical History
SIFT1-054	Cerebellar Infarct, Depression with Psychosis, Previous Deliberate Self Harm, Epilepsy, Previous Excess Alcohol Intake
SIFT1-057	Hypertension, Obesity
SIFT1-058	Post-Traumatic Stress Disorder
SIFT1-059	Bipolar Affective Disorder
SIFT1-060	None
SIFT1-065	Depression, Excess of Alcohol Intake, Post-Traumatic Stress Disorder, Recreational Drug Use, Zygomatic Fracture
SIFT1-067	Cerebrovascular Accident-Bedbound, Chronic Obstructive Pulmonary Disorder, Osteoarthritis, Paroxysmal Atrial Fibrillation
SIFT1-068	None
SIFT1-070	None
SIFT1-071	None
SIFT1-072	Mixed Anxiety/Depressive Disorder, Caesarean Section
SIFT1-078	Excess Alcohol Intake
SIFT1-079	Colorectal Adenocarcinoma – Resected, Depression
SIFT1-080	None
SIFT1-081	Depression
SIFT1-083	Metastatic Prostate Cancer
SIFT1-085	Mood Affective Disorder
SIFT1-089	Cerebrovascular Accident, Chronic Obstructive Pulmonary Disease, Diabetes Mellitus, Excess Alcohol Intake, Gout, Hypertension
SIFT1-092	Excess Alcohol Intake
SIFT1-099	Alcoholic Cirrhosis of The Liver, Bipolar Affective Disorder, Excess Alcohol Intake
SIFT1-100	Hypertension, Cerebrovascular Disease, Depression, Excess Alcohol Intake
SIFT1-102	Head Injury
SIFT1-104	Obesity
SIFT1-105	Hyperlipidaemia, Hypothyroidism, Non-Insulin Dependent Diabetes Mellitus, Polyarthrosis, Schizophrenia
SIFT1-106	Cholecystectomy
SIFT1-114	Bipolar Affective Disorder
SIFT1-116	None

Supplementary Table 5. Past medical history of patients with severe burn injury recruited in e-SIFTI (Part II).