MD Thesis

MICROVESICLES AS MEDIATORS OF INFLAMMATION IN SEVERE BURN INJURY

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Declaration of originality

I hereby declare that this work is my own and that by others is fully acknowledged and referenced herein.

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Abstract

The host response to a severe burn injury is characterised by exaggerated systemic inflammation. Early clinical manifestations include shock, respiratory failure, renal failure and immunosuppression [1]. The signalling pathways that propagate the inflammatory response are unclear but have traditionally been thought to involve overspill of proinflammatory cytokines [2]. The importance of microvesicles, sub-cellular membrane-bound particles, is increasingly being recognised in the context of intercellular communication. Although circulating microvesicles are elevated in proinflammatory states such as sepsis [3], their relevance to the post-burn inflammatory response has not previously been evaluated.

We hypothesised that circulating microvesicles play a crucial role in propagating the post-burn inflammatory response. Our overall aims were to 1) optimise protocols for the processing and analysis of plasma samples for microvesicle content; 2) characterise the circulating microvesicle profile associated with severe burn injury; 3) develop *in vitro* techniques to assess microvesicle production and function.

The major findings of this work were that microvesicles derived from leukocytes, neutrophils, monocytes and endothelial cells were significantly elevated within 24 hours of burn injury. Microvesicle levels fell rapidly and were significantly decreased by day two post-injury. Total leukocyte- and neutrophil-derived microvesicles were significantly higher in non-survivors of burn injury as compared to survivors. *In vitro* studies demonstrated that neutrophil microvesicle release could be elicited by incubation with opsonised heat-killed cells. Pilot analysis of burn patient samples, using an endothelial co-culture assay, suggested that microvesicles may regulate the innate immune response to burn injury.

These findings indicated that circulating microvesicles are an important component of the post-burn inflammatory response. Their precise activity is likely to be subtype-specific but the association of neutrophil-derived microvesicles with patient outcome alludes to a key role in burn pathophysiology.

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Abbreviations

ABSI	Abbreviated burn severity index
ANOVA	Analysis of variance
ARDS	Acute respiratory distress syndrome
ARF	Acute renal failure
BOBI	Belgian outcomes in burn injury
DAD	Diffuse alveolar damage
DAMP	Damage-associated molecular pattern
EDTA	Ethylenediaminetetraacetic acid
GM-CSF	Granulocyte macrophage colony stimulating factor
HBSS	Hank's balanced salt solution
HMGB1	High mobility group protein B1
HPMEC	Human pulmonary microvascular endothelial cell
ICAM-1	Intercellular adhesion molecule-1
ICU	Intensive care unit
iNOS	Inducible nitric oxide synthase
IL	Interleukin
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein-1
MODS	Multiple organ dysfunction syndrome
MV	Microvesicle
NADPH	Nicotinamide adenine dinucleotide phosphate
NTA	Nanoparticle tracking analysis
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PFP	Platelet-free plasma
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PRR	Pattern-recognition receptor
ROS	Reactive oxygen species
SIRS	Systemic inflammatory response syndrome
SOFA	Sequential organ failure assessment
TBSA	Total body surface area
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-alpha
VCAM-1	Vascular cell adhesion molecule-1

Chapter 1

Introduction

1.1 Burns as a clinical problem

Burn injuries are a major global public health issue, resulting in over 300,000 deaths per year, 95% of which occur in low income countries [4]. In the United States, an estimated 450,000 patients per year require medical treatment for burns. 40,000 are admitted to hospital and 3,400 will die from their injuries [5,6]. In Europe, the annual incidence of severe burns is 0.2-2.9 per 10,000 inhabitants, where a severe burn is classified as an injury requiring specialised inpatient care [7]. In 2014, over 3,000 UK patients required hospital admission following burn injury [8]. Although the incidence of thermal injury has been reduced in middle and high-income countries, by the use of safety features such as smoke detectors [4], the care of patients with burn injuries requires considerable resources. This is due to prolonged hospitalisation and rehabilitation phases of care, as well as long-term wound management. For example, in elderly burn patients, the median length of hospital admission is 26 days [7], as compared to 9.4 days for other medical conditions [9].

A severe burn injury is a unique example of critical illness, where an exaggerated inflammatory response characterizes the early phase of the condition. This is exacerbated by loss of the protective cutaneous covering, necessitating repeated surgical procedures. Clinical manifestations include shock, respiratory failure, renal failure and immunosuppression [1]. Burn shock is a unique combination of distributive shock and hypovolaemia [10]. The distributive component results from widespread tissue oedema, distant from the site of injury. It is secondary to the systemic inflammatory response but far exceeds any possible beneficial effect [11]. Immunosuppression is linked to the higher incidence of sepsis in burn patients, in comparison to that seen following other injuries. Burn sepsis is also associated with a higher mortality [12].

This project investigated the role of sub-cellular particles known as “microvesicles” in the context of post-burn inflammation. The characterisation of microvesicles in burned patients formed the first part of the study, followed by *in vitro* analyses of microvesicle production and function. In this introductory chapter I will detail what is currently known about the response to a severe burn injury. I will then explain the possible relevance of microvesicles to this process. This will lead onto our overall hypothesis and aims.

1.2 Aetiology and management of burn injuries

Mechanisms of thermal injury

Burn injuries can be subdivided into thermal, electrical, chemical or radiation injuries [13,14]. Thermal injuries are further divided into flame, scald, contact and flash burns [13] and constitute the largest group of injuries, for patients managed both in and out of out of hospital [15]. The precise aetiology varies according to age, with scalds being more prevalent in paediatric populations and flame burns more common in adults [1,7,16].

Scalds typically result from spilling hot water or being immersed in hot bathing water. They tend to cause superficial dermal injuries [17]. Flame burns tend to be more extensive in depth [17] and area [18]. They are associated with a greater inflammatory response, increased hypermetabolism and worse outcomes [18]. Contact burns are less common and usually affect a small area, although they can occasionally cause severe damage to deep structures [19,20]. Flash burns result from sudden intense heat, for example following explosion of volatile compounds [21].

The likelihood of electrical burns is primarily determined by the voltage, with deep burns being common at higher voltages (>1000 volts) [22]. However, due to differing tissue resistances, there can be extensive muscle and bone necrosis despite relatively minor external burns [23]. Consequently, rates of limb or digit amputation are much higher in victims of electrical burns than those with thermal or chemical injuries [24]. Chemical burns constitute a small fraction of total burn cases and the number is falling, primarily due to improved industrial safety regulations [25]. There are specific implications for injuries caused by certain chemicals; for example, hydrofluoric acid exposure can result in systemic toxicity that is disproportionate to the area of skin involved [26,27]. Significant radiation burns are also relatively uncommon but may occur following therapeutic radiotherapy for cancer [14,28].

Classification of burns

Burn wounds are assessed according to their location, depth and the total body surface area (TBSA) affected. This guides initial management, particularly regarding fluid resuscitation [29] and determines if a patient should be referred to specialist burn centre. Burns were

historically judged by their external appearance alone but by the 1950s, it was realised that the burn depth was of crucial importance [30]. Deeper burns result in fewer viable ectodermal appendages and a prolonged healing time [31]. Clinical examination is the most commonly used method for depth assessment, although it is inaccurate in 20-40% of cases, with a tendency towards over-estimation [31–33]. The current depth classification stratifies injuries as follows [33–37]:

- Superficial
- Partial thickness
    - Superficial dermal partial thickness
    - Deep dermal partial thickness
- Full thickness

Superficial burns involve only the epidermis. Although painful, they respond to simple wound care and heal without scarring within seven days. They are not included when assessing the percentage of body surface area that is injured. Most superficial dermal burns will also heal spontaneously but this process takes slightly longer – up to 14 days. They are associated with some blistering and are very painful. Deep dermal burns preserve only the deeper skin structures. Blisters will develop early and skin sensation is decreased. There is widespread destruction of blood vessels and the capillary refill time is prolonged. Full thickness burns affect both layers of skin and may extend to deeper tissues. Injured skin sometimes has a leathery or waxy appearance. Due to destruction of nerve endings, these burns are classically painless.

Initial management of burn injuries

The priorities following a burn injury are to stop the burning process; assess the patient's airway, breathing and circulation; determine the severity of the burn and commence early fluid resuscitation [38]. Most severely burned patients are intubated early to secure the airway and provide respiratory support. Direct airway and pulmonary inhalation injury can occur and is associated with significant mortality [39]. Secondary tissue damage is also a risk due to generalised oedema, a feature of the burn inflammatory response [40]. Ventilatory strategies typically follow the lung-protective approaches that have been adopted for other ICU

patients. Specific interventions such as high-frequency oscillatory ventilation (HFOV) have not been shown to be beneficial in the burn population [41].

The cardiovascular instability evident after a major burn is the result of hypovolaemic and distributive shock [40,41]. Hypovolaemia results from intravascular fluid loss into both burned and unburned tissue and this process can continue for up to 48 hours [42]. Myocardial depression may also arise, as a consequence of increased circulating pro-inflammatory mediators and abnormal calcium homeostasis [10,43]. Fluid resuscitation is required to mitigate these factors and is a crucial aspect of early burn care [44]. Although a number of formulas exist to guide fluid administration, the Parkland formula is the most well-known [1]. It uses the patient's weight and burn area to calculate a total volume of fluid to be infused over the first 24 hours post-injury. Although under-resuscitation is now uncommon, an increasing concern is the administration of excessive intravenous fluid [45]. This places the burn patient at increased risk of pulmonary oedema, limb compartment syndrome and abdominal compartment syndrome [46]. One retrospective study in Australia confirmed that fluid volumes significantly exceeded Parkland targets [47] and a multi-centre investigation in the US associated excess fluid with increased morbidity and mortality [48]. Other research has demonstrated increased rates of pneumonia [49] and orbital oedema [50]. Novel therapies designed to modulate the post-burn inflammatory response may lessen the requirements for such aggressive fluid therapy.

The final aspect of initial burn management is the early surgical excision of dead tissue. The benefits of primary eschar excision were recognised as early as the 1950s [30]. This approach was not adopted initially because it was associated with physiological instability and increased infective complications [51]. It was not until the 1970s that perioperative care of the severely burned patient had reached a level where aggressive burn excision was possible. This progress had been accompanied by improvements in surgical techniques [52]. A retrospective paediatric study was published in 1973, comparing primary surgical excision and grafting with topical dressings only [53]. In the second group grafting was performed only after spontaneous separation of dead tissue. The authors found that mortality and wound healing time was lower in children undergoing early surgery. A further retrospective analysis from the same centre, published in 1986, argued that prompt excision in all age groups

contributed to improved outcome [54]. The early excision of necrotic tissue has been supported by a number of other studies [55–58] and it is now regarded as a standard of care [59]. Although patients with inhalational injuries historically fared less well following early excision [60], it is possible that modern ventilation strategies would contribute towards better outcomes, if such studies were repeated.

The rationale for early surgery is that it removes the key driver for ongoing release of inflammatory mediators, i.e. necrotic burnt tissue. This is thought to attenuate the systemic inflammatory response, reducing the rate and duration of secondary infective and metabolic complications [59]. There is certainly some experimental evidence to support this position. A study of paediatric patients found lower levels of inflammatory mediators, including IL-6 and TNF-α, in patients undergoing early burn excision [61]. Other paediatric investigations identified less muscle breakdown [62] and infection [62,63] in the prompt surgery cohort. A possible criticism of these studies is that because early surgery was locally established as best practice, it was not possible to conduct randomised trials. The investigators therefore relied on recruiting the control cohort from other centres, raising the possibility that non-surgical aspects of care, such as wound dressing, might have affected outcomes. However, animal models provided further evidence, demonstrating reduced levels of macrophage hyperactivity [64] and inflammatory cytokines [65] following early burn excision. If it is genuinely the case that attenuation of the post-burn inflammatory response leads to improved patient outcomes, then augmentation of this apparent surgical benefit may be possible if novel mediators can be targeted.

Burn scoring systems

As well as being crucial to the initial management of the patient, classification of burns also allows prognostic scoring. Accurate scoring systems facilitate decision-making, clinical research and quality control [66]. They also provide a context for measurements of clinical outcomes, the focus of increasing attention in the UK [67]. It has long been established that mortality increases with a greater area of injury and that age is also a key factor [68]. The Baux score, described in the 1960s, used a combination of age and burn surface area to generate a value that approximated to the mortality rate [69]. Comprehensive improvements in burn

care over the intervening years has rendered this particular scoring method pessimistic [70] and a number of alternative systems have been devised.

In 1982, Tobiesen *et al* published the abbreviated burn severity index (ABSI) [71]. The authors used logistic regression analysis of 1352 patients to identify three additional variables that could be included to improve prognostication: gender, the presence of inhalation injury and the presence of full-thickness burns. It proved to be accurate in both the derivation and validation cohorts [67,72] and remains applicable today [73,74]. A retrospective analysis of 1665 patients, published by Ryan *et al* in 1998 [75], confirmed that increasing age, larger burn area and inhalation injury were all risk factors for death.

More recently, McGwin *et al* analysed a much larger data set of 54,219 burn patients, collected from multiple centres, to tackle some of the perceived weaknesses of earlier systems [76]. They developed a model that incorporated pneumonia and co-existing trauma as predictive variables. It was validated in a separate group of 14,442 patients and found to be accurate. Others have argued that the inclusion of these additional variables does not substantially improve mortality prediction over earlier systems and is not warranted [77]. A further complication is that it is difficult to reliably diagnose pneumonia in ventilated patients [78]. A simpler model was published in 2009 by the Belgian Outcomes in Burn Injury (BOBI) group [66]. The authors modified the dichotomous approach used by Ryan *et al*, splitting age and percentage burn area into multiple categories, as with the ABSI. Although the 5246 patients analysed were from a single centre, the model has been externally validated in several cohorts [79,80], including a higher-risk ICU population [81]. This scoring system is user-friendly and has been adopted in our centre, in conjunction with the ABSI.

The scoring systems described above are relatively simple and easy to calculate. In contrast, the risk prediction models published for general ICU patients tend to employ larger numbers of physiological variables. Historically there has been some success at simplification. APACHE II (acute physiology and chronic health evaluation II), published in 1985, reduced the number of variables from the 34 used in the original APACHE to 12 [82]. However, comparison with models that use fewer variables suggests that it is the complexity of these systems that gives them their prognostic power [83]. Increasing recognition of the

heterogeneity of syndromes such as sepsis is driving the use of large data sets to characterise disease phenotypes [84], although this approach can be problematic [85]. These developments are likely to prove relevant to the care of patients with severe burns, due to the high prevalence of "ICU" conditions such as sepsis and the acute respiratory distress syndrome (ARDS) [86–88].

1.3 Local response to injury

Three zones of burn intensity were proposed by Jackson in 1953: the central zone of coagulation, the intermediate zone of stasis and the outer zone of hyperaemia [30]. The zone of coagulation is characterised by complete blood vessel obliteration and skin necrosis; it is non-viable. The hyperaemic zone comprises structurally normal skin, which is metabolically active. Vasodilation is present, blood flow is increased and full recovery is usual [17]. The zone of stasis, adjacent to the area of necrosis, is viable but at risk of progression. Inflammatory cell infiltration, compromised perfusion and oxidative stress increase this risk [89,90]. Consequently, a crucial part of burn care is to prevent conditions that promote progression of this zone over the first 48-72 hours, such as vasoconstriction and desiccation [31]. Both early apoptosis and delayed necrosis are present in the zone of ischaemia [91].

A number of inflammatory mediators are released into the surrounding tissues following a burn injury, including adenosine triphosphate (ATP) [92]. ATP is an example of a damage-associated molecular pattern molecule (DAMP) and as discussed below, these molecules are key to the innate response to tissue injury. Although inflammation promotes wound healing, by clearing cellular debris and protecting against pathogens, excessive inflammation can result in collagen degradation and keratinocyte apoptosis [90]. This contributes to burn wound progression.

The inflammatory phase of wound healing, which occurs acutely, overlaps with the proliferation phase, which begins after several hours but can continue for weeks [93]. Burn depth is the major factor affecting the healing of burn wounds. Because the epidermis is derived from ectoderm, it is capable of regenerative healing [1]. Deep dermal injuries result in fewer ectodermal appendages surviving and therefore slow tissue regeneration [31]. Healing of deeper wounds depends on migration of keratinocytes from surrounding uninjured tissue and/or from meshed skin grafts [1]. Full thickness burns result in destruction of all appendages and require skin grafts as they will not regenerate [42]. The maturation phase of healing persists for months post-injury and encompasses maturation of the regenerated epidermis, as well as contraction of the burn wound, a myofibroblast-mediated process [93].

Inhalation injury

The lungs may be injured directly by inhalation of smoke. This is predominantly a chemical rather than thermal effect, due to efficient heat dispersion in the upper airway [39,94]. The initial result is an increase in bronchial blood flow, contributing to pulmonary oedema [95]. This may play a significant role in the extension of the injury from the airways to the lung parenchyma and is mediated by nitric oxide [96]. Reactive oxygen and nitrogen species contribute to tissue injury and increased pulmonary vascular permeability, leading to small airways obstruction. Overventilation of the non-obstructed respiratory units promotes further inflammation and tissue injury [96].

1.4 Systemic response to burns

The stress response to severe burn injury is characterised by a marked inflammatory component [97]. The acute phase starts immediately after trauma, persists for several weeks and its magnitude is related to the burn size [98]. The inflammatory response progresses with time [99], contributing to remote organ damage and in severe cases leading to multiple organ dysfunction syndrome (MODS) [100]. The long-term, chronic consequence is a persistent metabolic disturbance that causes a profound catabolic state [101,102]. Inflammatory mediators, such as TNF-α, IL-1β and IL-6 are elevated in the early phase but do not necessarily correlate with clinical progress [103]. Moreover, the pathway that leads from local release of mediators at the burn injury site to the development of early systemic inflammation remains undefined.

The systemic inflammatory response syndrome

In 1992, an American College of Chest Physicians/Society of Critical Care Medicine (ACCP/SCCM) consensus conference was held, with the principle aim of standardising the definitions of sepsis, shock and organ failure [104]. Although the primary goal was to define the host response to infection, it was emphasised that a very similar response can result from a variety of insults, including trauma, pancreatitis and burns. The term "systemic inflammatory response syndrome" (SIRS) was proposed to describe this process. It was defined as the presence of two or more of the following parameters:

- Core temperature > 38°C or <36°C
- Heart rate > 90 beats/minute
- Respiratory rate > 20 breaths/minute or $PaCO_2$ 32 mmHg
- White cell count > 12 x 10^9/litre or < 4 x 10^9/litre

The term "sepsis" was reserved for those cases where SIRS was a result of documented infection. Patients with SIRS were recognised to suffer from organ dysfunction to a highly variable degree and the term "multiple organ dysfunction syndrome" (MODS) was proposed to describe this disorder. It was defined as the presence of altered organ function, requiring

intervention to maintain homeostasis. A requirement of these definitions was that the organ dysfunction was a result of the systemic reaction to the initial insult [105], an important concept of great relevance to burns.

Although widely adopted [106], the limitations of this approach to inflammation were recognised early on. The definitions were considered sensitive but they lacked specificity for any particular insult [105]. More than two thirds of critically ill patients may meet the SIRS criteria and some argued that it was effectively useless [107]. The authors themselves acknowledged that the criteria were likely to evolve over time, as knowledge improved [108]. A further conference was held in 2001, with the hope that novel biomarkers would allow better prognostication and diagnosis [106]. Unfortunately, this did not prove to be the case and the definitions were left unchanged. Those involved commented that the biochemical features of inflammation may be fairly consistent, regardless of the initial insult.

The limitations of using clinical criteria to identify inflammatory states echoes the difficulties experienced with ARDS, as described below. This was underscored by a recent large trial which demonstrated that not only are the SIRS criteria non-specific, they are also insufficiently sensitive for identifying serious infection [109]. The most recent 2012 Surviving Sepsis guidelines do not actually mention SIRS in their diagnostic criteria [110] and this reflects an increasing recognition of the heterogeneity of sepsis [111]. In burn patients, awareness of the ubiquity of the SIRS criteria may even distract physicians from occult infections [112]. Elucidation of inflammatory pathways in burn patients is therefore important for two reasons. Firstly, it may allow identification of those patients who would benefit from modulation of post-burn inflammation [113]. Secondly, it may facilitate recognition of serious infections, something that is known to be difficult in this population [87]. Clinical criteria alone are insufficient to perform either of these roles.

Cardiovascular dysfunction

The initial clinical manifestations of the inflammatory response are generalised oedema and hypovolaemia [113]. This necessitates aggressive fluid resuscitation as described above. Early experimental physiology partly explained this by identifying increased vascular permeability, although it was not sufficient to account for such extensive acute oedema. Instead,

increased extravascular osmotic pressure was found to draw water from the intravascular space [114]. Fluid resuscitation using intravenous colloid was historically contraindicated, as it was thought to worsen oedema via this osmotic "pull" [115]. More recent evidence suggests these fears are unfounded [41]. The time to peak oedema formation increases with burn size; animal studies suggest that after a 40% burn the peak does not arise until 12 hours [116]. The oedema plateau is thought to occur primarily due to improved clearance, as the fluid and protein flux persists for several days [117]. In the acute phase, burn oedema can cause life-threatening compromise of the airway, particularly in the presence of facial or airways burns [37]. The associated hypovolaemia can be profound, causing severe cardiovascular instability if not treated promptly [1].

Respiratory dysfunction

Patients with severe burn injuries commonly develop respiratory dysfunction and this is the organ system most frequently affected, in both the early and late stages of post-burn critical illness [118]. Burn patients are at risk from respiratory compromise due to a range insults [119]. In addition to direct inhalational injury, as described above, the lungs can be exposed to large quantities of circulating inflammatory mediators, as they receive 100% of the cardiac output [120]. However, the precise mechanisms linking thermal injury with this remote organ damage are unclear [121]. Neutrophil invasion is known to be a key step [122] and this process is mediated by CXCR2 [120]. Our research group has previously demonstrated that intravascular lung-marginated monocytes play an important role in the development of lung injury, following both direct pulmonary and indirect extra-pulmonary insults [123–126], although burn injuries were not investigated. Gut-derived factors may also be important to the pathogenesis [121].

One manifestation of respiratory dysfunction is ARDS and this has been associated with burn injuries [127]. The pathological hallmark of ARDS is diffuse alveolar damage (DAD) but identification of this requires lung biopsy or examination at autopsy [128]. Attempts have therefore been made to diagnose ARDS using clinical criteria. In 1994, the American-European Consensus Conference defined ARDS as the presence of impaired oxygenation and bilateral pulmonary radiograph infiltrates, in the absence of left heart failure [129]. Unfortunately, the diagnostic accuracy of this model is low and up to half of the patients

meeting the criteria do not have ARDS [130]. The 2012 Berlin definition attempted to address some of the limitations of previous systems and graded ARDS into different severities [131]. Despite these revisions, diagnostic accuracy remains poor and a direct measure of lung injury is lacking [132]. In burn patients, the reported incidence of ARDS varies from 2-17% [88,127], although in those requiring mechanical ventilation, the rate may be as high as 40-50% [119,133]. The influence on post-burn outcome is unclear. A paper based on the 1994 criteria did not find a significantly greater mortality in burn patients with ARDS [88] but a study using the Berlin definitions found that more severe ARDS was associated with an increased risk of death [127]. These discrepancies in incidence and outcome may reflect the poor diagnostic ability of the clinical criteria.

Severe burns usually necessitate repeated surgical procedures and these may require transfusion of considerable volumes of blood products. This places patients at risk of transfusion-related acute lung injury (TRALI) [134]. Thought to be initiated by donated anti-leukocyte antibodies, this leads to increased microvascular permeability and pulmonary oedema [135]. A further concern following severe burns is the predisposition to respiratory infections [133]. Inhalation injuries increase the risk of subsequent pneumonia and infection is also an independent risk factor for death [136]. Multidrug-resistant organisms are isolated in a significant proportion of cases [137] and this is likely to be an increasing problem in the future. Because systemic inflammation is so prevalent, it can be difficult to identify the onset of new respiratory infections [138]. Elucidation of inflammatory pathways could have clinical utility in this context, as measurement of specific mediators could potentially be used to exclude infection [86,139,140].

Renal dysfunction

The kidneys are also commonly affected following severe burn injuries, particularly in the second week post-injury [118]. The risk of renal dysfunction increases with age [141] and burn size [142,143]. The incidence of renal dysfunction reported in the literature varies from 1% to 36%, according to the burn population studied and the criteria used [144]. There is also variability in the published outcomes but historically, the mortality rate was over 80% in those patients requiring renal replacement therapy (RRT) [142,145,146]. A consensus definition of

acute renal failure (ARF) in critically ill patients was published in 2004 [147]. This quantified renal dysfunction into five categories using the "RIFLE" criteria:

- Risk
- Injury
- Failure
- Loss of function
- End-stage renal disease

Several studies have subsequently applied these criteria to ascertain the incidence of acute kidney injury (AKI) in patients with burn injuries [141,144,148–150]. The incidence found by these investigators varied from 24% to 53% but the rate of "failure", requiring RRT, ranged from 3% to 28%. Further refinements of the RIFLE criteria have been proposed by the "AKIN" and "KDIGO" groups [151]; it is unclear what impact these systems would have on the variability of renal dysfunction incidence in burn patients. Importantly, the outcome of patients who are affected seems to be improving [149].

A number of factors are thought to contribute to the pathophysiology of AKI post-burn injury, including pro-inflammatory mediators, sepsis and nephrotoxic drugs [144,146,150,152]. Inadequate fluid resuscitation was considered an important cause of renal dysfunction and early fluid therapy has been shown to reduce mortality from ARF [153]. Other studies found, however, that most patients with AKI received appropriate quantities of fluid and argued that the inflammatory response was a more important factor [154].

The incidence of AKI is highest in the most unwell patients, as determined by scores such as ABSI [150,155]. In patients with severe burns, renal failure rarely occurs in isolation and frequently progresses to multi-organ failure [118,156]. In addition to the circulatory, respiratory and renal dysfunction described above, severely burned patients are also at risk of liver, haematological and gastrointestinal failure [157]. A concept of organ "crosstalk" is emerging, which suggests that injured systems, in particular the kidneys, can exacerbate inflammatory damage occurring elsewhere [158].

Hypermetabolism

The metabolic response to trauma was first discovered by Cuthbertson in the 1930s [159]. He found that urinary losses of nitrogen, potassium, phosphorus and sulphur were all increased in patients with long bone fractures, to a greater extent than would be expected by local tissue injury. He concluded that this represented catabolism of lean tissue and subsequent investigations revealed a co-existing rise in oxygen consumption. In burn patients, the acute systemic response to injury starts immediately after injury [10] and the clinical features are described above. From a metabolic viewpoint, this phase is characterised by low oxygen consumption, low glucose tolerance and low catabolism [101]. The chronic response is characterised by hypermetabolism and increased catabolism. These phases have been described as the "ebb" and "flow" of the burn response [160].

The magnitude of the hypermetabolic response is related to the size of burn injury [98]. In the early part of this phase, the metabolic rate increases by 120-180%. This steadily decreases with time but hypermetabolism remains detectable at 12 months post-injury [161,162]. The severity and duration of the hypermetabolic response is significantly longer than that seen following other insults [101,163]. Distinctive hormonal changes occur, with increased catecholamine and cortisol levels, thyroid axis suppression, inappropriately low growth hormone secretion and reduced IGF-1 production [164,165]. This drives increased proteolysis, lipolysis and glycogenolysis, with net nitrogen loss [98]. The consequence is a prolonged reduction in lean body mass, with a profound loss of function that necessitates a considerable period of rehabilitation [102]. The liver and skeletal muscle are the major sites of increased oxygen consumption post-burn injury [101,166]. At the cellular level, mitochondrial dysfunction has been demonstrated in muscle tissue and may be an important factor [162].

Current treatments can ameliorate some of the consequences of hypermetabolism but do not suspend it. Propranolol opposes the action of elevated catecholamines, decreases energy expenditure and attenuates muscle loss [167]. Oxandrolone, an anabolic steroid, has been shown to improve lean body mass and reduce length of stay [168]. These agents have been studied in patients with more severe injuries (greater than 30-40% TBSA) and are used in our centre for patients with a TBSA of over 40%. Non-pharmacological approaches include early surgery and adequate nutrition; patients with burn injuries develop specific

deficiencies of micronutrients such as copper and zinc [163]. Some studies have suggested that early, aggressive nutritional support may attenuate the hypermetabolic response but the evidence is inconclusive [169].

The mechanisms that underlie prolonged hypermetabolism are not fully understood but are presumed to involve complex mediator cascades [170]. Cytokines (such as TNF-α, IL-1 and IL-6), reactive oxygen species and neutrophil-adhesion molecules are all capable of regulating the hypermetabolic response [163,170]. This incomplete understanding may explain why attempts to modulate post-burn hypermetabolism have had mixed results.

1.5 Pathogenesis of the inflammatory response to burn injury

Patients with a heterogeneous group of traumatic injuries, including burns, develop a state of systemic inflammation. This has been labelled SIRS, although this definition has certain limitations, as described above. In severe cases, this can lead to multi-organ failure and a number of mechanisms have been proposed to explain this process, which are thought to overlap [171]. They include increased production of cytokines, microcirculatory dysfunction with impaired global oxygen delivery, dysregulated endothelial cell-leukocyte interactions and translocation of intestinal microflora. Before consideration can be given to the possible roles of microvesicles in propagating the inflammatory response to burn injury, it is necessary to review the current paradigm.

Innate immune response

In vertebrates, two systems constitute immunological defence: the innate immune system and the adaptive immune system [172,173]. The innate immune system can be considered the first line of defence and evolved before the divergence of vertebrates and invertebrates [172]. The cellular component of the innate system includes phagocytes (such as neutrophils, monocytes and macrophages) which are responsible for mediating the innate response [174]. The complement system is the effector of the humoral innate system [175]. Both the cell-mediated and humoral components also have essential roles in regulating the adaptive immune response [176].

The innate immune system is not completely non-specific but is able to discriminate between “self” and various pathogens [174]. These pathogens are recognised by pattern-recognition receptors (PRR), which are germline-encoded and limited in number. In the context of infection, these PRRs recognise microbial components, termed pathogen-associated molecular patterns (PAMPs) [177]. One of the best studied PAMPs is lipopolysaccharide (LPS), which is a vital structural component of the outer membrane of Gram-negative bacteria and is a potent immunostimulant [178]. An important sensor for LPS is Toll-like receptor 4. The Toll-like receptors (TLRs), of which there are 11 in humans, are a major group of PRRs and are highly conserved in evolution [174]. Via two signal transduction pathways, TLR activation leads ultimately to upregulation of nuclear factor-kappa B (NF-κB),

a key transcription factor. NF-κB transcribes genes that encode for inflammatory cytokines and chemokines, regulate expression of adhesion molecules and promote survival of neutrophils [179].

DAMPs

Damage-associated molecular patterns (DAMPS), also known as alarmins, are analogous to PAMPs but are of endogenous origin [180]. They are released following cell necrosis caused by insults such as trauma, radiation, ischaemia and burns. They are therefore important in the formation of "sterile" inflammation. Apoptotic cells do not release DAMPs [181] but it is becoming evident that cells can secrete them under conditions of high stress [182]. DAMPs differ from other inflammatory mediators, such as classical cytokines, because they usually serve essential intra-cellular functions [183]. This represents a further analogy with PAMPs. Examples of DAMPs include galectins, annexins, heat-shock proteins and the "dual function" cytokines IL-1α and IL-33 [181,184,185].

The role of high mobility group protein B1 (HMGB1) as a DAMP has received much attention and it is thought to be a key mediator of inflammation [180]. HMGB1 was originally discovered as a chromatin-binding factor that bends DNA, allowing assembly of specific protein complexes [186,187]. It contributes to nucleosome stability and regulates a number of key nuclear events, including DNA repair and gene transcription [188]. Extracellular HMGB1 promotes inflammation in the context of both infectious and sterile tissue damage, acting synergistically with endogenous and exogenous inflammatory mediators [186]. Further evidence of its importance was provided by the finding that the post-trauma inflammatory response is diminished by blocking HMGB1 activity [189]. More recently, elevated HMGB1 levels have been found in patients with burn injuries [190]. This was noted early after burn injury and the levels in non-survivors were significantly higher than in survivors. HMGB1 binds multiple receptors, including TLR4 but its ability to recruit leukocytes is dependent on formation of a heterocomplex with the chemokine CXCL12, which binds to the CXCR4 receptor [182].

Cellular recruitment

Neutrophils, monocytes and macrophages are activated by DAMPs in response to tissue damage [191,192]. Macrophages act as sentinel cells that initiate neutrophil recruitment, by inducing processes such as cytokine release and increased vascular permeability [193]. Macrophages isolated from sites of tissue damage release TNF-α, interleukin-6 (IL-6) and inducible nitric oxide synthase (iNOS), when exposed to hypoxia. This may be an important factor in macrophage activation at the local level [194]. Most tissue macrophages are derived from circulating monocytes, although under steady-state conditions considerable local proliferation occurs. Following inflammatory insults there is increased dependence on recruitment of circulating precursors [194,195]. This is dependent on chemokines such as monocyte chemoattractant protein-1 (MCP-1), which is elevated following thermal injury and may in turn be influenced by TNF-α [196,197].

Neutrophils have long been considered the primary effector cell of the sterile inflammatory response [198]. They are rapidly activated following tissue injury and their lifespan is also lengthened, which results in the increased presence of primed neutrophils at the site of inflammation [193,199]. Priming of neutrophils, which has been demonstrated to occur following major tissue damage, results in augmented chemotaxis, adhesion, release of reactive oxygen species and secretion of proteases [200–202]. Priming agents, such as granulocyte macrophage colony stimulating factor (GM-CSF) and tumour necrosis factor-alpha (TNF-α), are increased following trauma [191] and severe burn injuries [98].

Although neutrophils contribute to wound healing, they have the potential to cause significant collateral tissue damage and their recruitment is tightly controlled [203,204]. Following recognition of tissue damage by sentinel cells, such as macrophages and mast cells, neutrophils are recruited rapidly. The recruitment cascade involves tethering, rolling, adhesion and transmigration [193]. A key initial step is the expression of endothelial adhesion molecules, including E-selectin and P-selectin, close to the area of injury [203,205]. These mediate tethering, along with L-selectin expressed on the neutrophil surface. Rolling of the neutrophil along the endothelium allows it to bind with chemokines, triggering expression and conformational activation of integrins on the neutrophil. Interestingly, inhibition of neutrophil rolling in animal models of burn injury reduces subsequent oedema and tissue

necrosis [206]. The integrins interact with endothelial ICAM-1, which arrests the neutrophil and permits firm adhesion. Finally, transmigration across the endothelial barrier occurs, a process mediated by the proteins PECAM-1 and VE-cadherin, amongst others [205]. Following the initial neutrophil extravasation to the site of injury, there is a secondary migration of monocytes. This is promoted by extravasated neutrophils and is assisted to some extent by platelets [207,208].

Cellular activity

Once in the site of injury, primed neutrophils undergo their primary function of phagocytosing damaged cells and matrix proteins, along with any bacteria that may be present [93]. Phagocytosis triggers an increase in oxygen consumption, through the activity of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, resulting in production of reactive oxygen species (ROS). This is known as the neutrophil respiratory burst [209]. Examples of ROS include the superoxide anion (O_2^-), the hydroxyl radical ($^{\bullet}HO$) and hydrogen peroxide (H_2O_2). Intracellular granules also release an array of biologically active proteases and peroxidases, the latter requiring H_2O_2 as a co-substrate [210,211]. During this phase of activity, the neutrophils undergo a transcriptional burst that underpins their roles in early inflammation, resolution of inflammation and wound healing [212].

The potency of the ROS and enzymes released by neutrophils means there is great potential for host tissue damage in inflammatory syndromes. This may occur via several mechanisms, including premature neutrophil activation, extracellular release of cytotoxins and failure to adequately terminate acute inflammation [213]. Neutrophil elastase has been implicated in the pathogenesis of lung disorders such as emphysema and acute lung injury [214]. The accumulation of extravascular fluid following burn injury is a particular problem and neutrophil-derived heparin binding protein may be a mediator of this process [215].

In addition to phagocytosis, monocytes and their progeny have important roles in immunomodulation and antigen presentation [209]. A distinction is made between “classical” and “non-classical” monocytes subsets. Classical or “inflammatory” monocytes differentiate into tissue macrophages and release inflammatory mediators. Their accumulation is slower than non-classical or “resident” monocytes, which perform a patrolling function [195]. A

number of studies have demonstrated monocyte hyporesponsiveness following burn injury. Development of monocytes in bone marrow is disturbed in animal models of burn [216] and their differentiation into dendritic cells is impaired [217]. Low human leukocyte antigen-DR (HLA-DR) expression has been found in patients with burn injuries and this correlates with both mortality and the risk of sepsis [218,219]. In addition, there is increased appearance of immunosuppressive M2 monocytes post-burn injury, which increases susceptibility to infection. This is reversed by administration of propranolol, which suggests monocyte function is strongly influenced by catecholamines [220,221].

Oxidative stress and nitric oxide

ROS form continuously as a by-product of aerobic respiration and are also necessary for cell signalling [222,223]. In health, a balance exists between the formation of ROS and their removal by scavenging compounds. When this reduction-oxidation (redox) balance is disturbed, by either excess ROS production or impaired scavenging, a state of oxidative stress occurs [224]. Oxidative tissue damage is elicited by the uncontrolled levels of ROS, as well as by reactive nitrogen species (RNS) and reactive lipid species. It has been known for some time that oxidant-induced lipid peroxidation occurs early following burn injury, reflecting widespread cell membrane disruption [225]. An end product of lipid peroxidation is the formation of malondialdehyde (MDA). Levels of MDA rise within the first 24 hours of injury, in accordance with the severity of injury, with the oxidative stress persisting for weeks after injury [226]. Increased circulating concentrations of lipid peroxides have been detected following burn injury [227].

Numerous pathways result in ROS production but there are four main sites of ROS generation: mitochondrial electron transport, peroxisomal fatty acid metabolism, cytochrome P450 reactions and NADPH oxidase activity. The latter occurs particularly in the phagocytic respiratory burst, as described above [223]. As well as being initiated by components of the innate immune response such as neutrophils, oxidative stress in itself amplifies the systemic inflammatory response. This is thought to be mediated by increased cytokine secretion, via ROS-dependent signalling pathways that activate NF-κB [227,228].

RNS also play a key role in post-burn oxidative stress and are produced by the reaction between nitric oxide (NO) and ROS [223]. Increased NO production occurs following burn injury and this may contribute to vascular leak, as well as mediating vascular tone [229]. Some studies demonstrate a correlation between burn size and NO levels, although this finding is not universal [230]. Activity of the inducible form of nitric oxide synthase (iNOS) underlies the higher NO levels and iNOS has been detected in burned human skin. NO reacts with superoxide ion to form peroxynitrite. This may have an important direct role in inducing remote organ damage, such as pulmonary dysfunction [231]. When protonated, it also produces hydroxyl and nitrogen dioxide radicals [223]. The net result is further amplification of oxidative stress, leading to direct cellular damage and upregulation of the inflammatory process. Antioxidant therapy with agents such N-acetylcysteine (NAC) results in biochemical and clinical evidence of oxidative stress attenuation [227,232,233].

Cytokine and chemokine response

Soluble cytokines are established as important mediators of the post-burn inflammatory process and have been investigated in many studies. Following initiation of the inflammatory cascade, a number of pro-inflammatory cytokines are rapidly upregulated [234]. TNF-α is a potent mediator of inflammation. It activates neutrophils and macrophages, induces endothelial adhesion molecule expression, enhances vascular leak and is pro-coagulant [235]. It also induces secretion of secondary cytokines such as IL-1β and IL-6. IL-1β has potent pro-inflammatory effects and elevated levels have been detected in animal tissue following burn injury and non-survivors of thermal trauma [103,236–238]. IL-6 induces production of acute phase proteins and regulates B-lymphocyte function. Early studies found the greatest IL-6 response in patients with larger injuries [103] and higher levels are also associated with the presence of post-burn sepsis [239]. It is the cytokine linked most consistently with post-injury complications and injured skin itself seems to be a significant source of IL-6 [240]. The growth factors granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF) are also upregulated following burn injury, with levels partially influenced by burn size [98,234]. G-CSF is released mainly from monocytes and macrophages and acts to increase neutrophil proliferation and function. Although it is found at significantly higher levels in non-survivors of burn injury [238], its action its actually thought to be protective, reducing the risk of post-burn infection [235,241].

The chemokine IL-8 elicits significant neutrophil accumulation at sites of inflammation and levels have been found to correlate with burn mortality [237,242], as is the case with monocyte chemoattractant protein-1 (MCP-1/CCL2) [238]. MCP-1 specifically attracts monocytes and macrophages and is produced constitutively but is also induced by oxidative stress, growth factors and cytokines such as TNF-α [197,243,244]. It is released by several cell types, including epithelial, endothelial and microglial cells. Of note, production of MCP-1 by non-inflammatory skin cells is upregulated following burn injury [245].

The large-scale post-burn upregulation of pro-inflammatory cytokines and chemokines is accompanied by increased production of anti-inflammatory cytokines [246]. Suppression of immune responses is important to avoid chronic inflammation and autoimmunity [247]. Interleukin-10 (IL-10) is the most widely studied anti-inflammatory cytokine in the context of burn injury. It exerts an inhibitory effect on the inflammatory response, by suppressing pro-inflammatory cytokine production [237,248]. Several studies have demonstrated increased levels of IL-10 following burn injury and higher levels are also associated with a worse prognosis [190,234,248,249]. Animal models suggest that neutrophils are the major source of IL-10 following burn injury [250].

Transforming growth factor-β (TGF-β) controls several aspects of the inflammatory response and accelerates wound healing [100,247,251]. Levels increase following burn injury and over-production has been linked to excessive suppression of the immune system [252,253]. TGF-β also exerts pro-inflammatory effects post-burn, for example by inducing differentiation of immature T cells into inflammatory Th-17 cells [254]. This dual function is not exclusive to TGF-β, with other cytokines such as IL-6 capable of exhibiting both pro- and anti-inflammatory effects [255]. Three further anti-inflammatory cytokines have been identified more recently: IL-27, IL-35 and IL-37. These have been proposed as potential therapeutic targets in the context of inflammation [247]. Cytokine inhibition has been investigated as a means of modulating the post-burn inflammatory response but immunotherapy has not yet demonstrated a clinically-relevant effect [113,248].

Complement

The complement system is a key component of the innate response to both foreign and altered host cells [191]. It is composed of a collection of plasma proteins, being produced mainly in the liver, and cell surface proteins [256]. The complement system has four major functions: target recognition, opsonisation to assist phagocytosis, stimulation of inflammation via anaphylatoxin generation and direct cell killing by the membrane attack complex (MAC) [257]. The MAC forms by self-association of C5b, cleaved from C5, with C6-C9. Any altered host cell, debris or microorganism that lacks specific identifying molecules will support complement deposition, via the "alternative pathway". This is permanently active and results in covalent binding of C3b, an activated form of component C3, which opsonises targets and recruits leukocytes [258]. The alternative pathway can be amplified by properdin, which recognises DAMPs and PAMPs [259,260]. Activation of the "lectin" and "classical" pathways occurs via pattern recognition molecules, such as C1q and ficolins. All three pathways converge at the point of C3 cleavage, which amplifies opsonisation by C3b, lyses cells via formation of the MAC and its insertion into target membranes, promotes inflammation, and activates the endothelium [256,258,261]. Although MAC formation is conventionally considered to be a C3-dependent process, more recent evidence demonstrated that thrombin can act as a C5 convertase, which links complement to coagulation [259,262].

Early complement activation has been clearly demonstrated following major injury, with the magnitude of activation correlating with injury severity [259,262]. The alternative pathway takes a central role but there is also an association between thrombin generation and complement activation following trauma. Activation of the complement pathway following burn injury has been acknowledged for some time, with the first report being published in 1963 [263]. Subsequent animal models of burn demonstrated that this activation occurred within minutes of injury and resulted in the deposition of C3 in burned tissue. Furthermore, this complement activation contributed to lung oedema [264,265]. *In vitro* studies showed that heat-killed cells could fix C3, which in turn led to neutrophil recruitment [266]. In burn patients, complement has been shown to influence neutrophil function post-burn [267,268] and circulating C3 levels inversely correlate with injury severity, consistent with its deposition in injured tissue [269].

Inflammation and coagulation

Systemic inflammatory states are frequently accompanied by simultaneous activation of the coagulation system. This close functional linkage can be traced back to the early stages of evolution and is preserved throughout vertebrates [270]. Interestingly, the pattern recognition molecules of the innate immune system, such as C3, function in a similar manner to contact factors of the coagulation cascade. Increasing recognition of the interface between inflammation and coagulation generated a great deal of interest, particularly in the context of sepsis, where physiologic anticoagulants were found to be depleted [271]. Unfortunately, attempts to modulate this hypercoagulable state have not proved successful, despite the early promise of activated protein C [272].

Given the close physiological relationship of inflammation and coagulation, as well as the activation of clotting associated with major trauma [273], it was assumed that severe burn injuries would also be associated with a hypercoagulable state [274]. Several investigations have supported this assumption, with both hypercoagulability and hyperfibrinolysis identified early after burn injury [274–276]. Furthermore, the magnitude of the clotting activation was found to be related to organ dysfunction and clinical outcome. In contrast, more recent evidence suggests that burn patients have normal coagulation parameters on admission, becoming hypercoagulable during recovery [277]. This discrepancy highlights the importance of delineating the specific pathways that are activated as part of the inflammatory response to burn injury.

Post-burn immunosuppression

Severe burn injury results in profound alterations to the host response to pathogens and infectious complications remain a major cause of death in this population [278]. This can partly be attributed to loss of skin, which acts as the primary barrier to microorganisms. However, burn patients also exhibit classical signs of immunosuppression, such as loss of delayed hypersensitivity reactions, prolonged allograft survival and reduced T-cell proliferation [279]. This has been termed the compensatory anti-inflammatory response syndrome (CARS) and is described as a systemic deactivation of the immune system, in an attempt to restore homeostasis from a severely inflamed state [280]. CARS was first described by Bone in 1996 and resulted from a reappraisal of sepsis pathophysiology. This was prompted by the failure

of a number of sepsis trials that attempted to modulate inflammatory mediators [281]. He hypothesised that some survivors of the initial inflammatory insult develop an excessive anti-inflammatory response, resulting in immunosuppression. A final stage of "immunologic dissonance" was posited, whereby the imbalance between pro- and anti-inflammatory mechanisms became persistent.

The concept of immunosuppression following an inflammatory insult was not new, having been reported for several decades in patients sustaining burn injuries, as well as major trauma [280]. Lymphocyte dysfunction was recognised early on to be a key factor in this immunosuppression and evidence in the 1980s suggested this was due to upregulation of "suppressor" T cells in the post-burn period [282,283]. T cells are critical to the adaptive immune response and a crucial subtype is T helper cells (T_h), which are classically generated by antigen-presenting cells (APCs), such as macrophages and dendritic cells [284]. A number of studies identified a characteristic switch, from T_h1 to T_h2 activity, following burn and major trauma. The resulting bias away from cell-mediated immunity and towards humoral immunity is thought to predispose burn patients to secondary sepsis [173,253].

A newly discovered T_h subset, known as T_h17 cells, has also been linked to post-burn immunosuppression. These are generated in animal models of burn injury and are antagonistic to T_h1 cells [285]. Furthermore, the T_h17 effector cytokines IL-17 and IL-22 have been detected at increased levels at the site of burn injury [284]. Some discrepancies exist, however, with an investigation of burn patient samples failing to identify increased T_h17 production [286].

Defective CD3 signalling may underlie some of the T cell alterations present following burn injury [278,287] and increased levels of MCP-1 have also been implicated [288]. A number of alterations to effectors of the innate immune system are also likely to contribute to post-burn immunosuppression. Monocyte deactivation or hypo-responsiveness is thought to be an important factor, as discussed above [216]. Impaired dendritic cell differentiation has also been demonstrated [289]. Further investigation of the inflammatory pathways that mediate these responses may generate novel targets for modulation, with the ultimate aim of reducing infectious complications post-burn.

1.6 Microvesicles

Microvesicles (MVs) are small, functionally-active, membrane-bound structures that are of emerging importance in the pathogenesis of systemic inflammation [290,291]. They are derived directly from the plasma membrane of activated or dying parent cells [292]. Microvesiculation is a ubiquitous cellular mechanism but it increases under conditions of stress. Importantly, the released vesicles retain proteins and lipids characteristic of parent cells [293]. Following preliminary studies in the 1940s, the presence of cell-derived vesicles was first confirmed by Wolf in 1967, using electron microscopy [294]. Wolf termed them "platelet dust" and this "dust" was thought to account for the pro-coagulant activity of cell-free serum [295]. Although platelets are the main source in health, erythrocytes, leukocytes and endothelial cells also release microvesicles [296].

Definitions

The most widely used collective term for cell-derived, phospholipid bilayer-bound structures is "extracellular vesicles" [294,297,298]. Occupying the size range between molecules and cells, this broad population is thought to consist of a number of distinct subtypes, with varying physicochemical characteristics [299]. However, the nomenclature relating to these subtypes is ambiguous and inconsistent [292,294,300]. The three major types referred to in most publications are as follows [292,294,297,298,301]:

- Exosomes
- Microvesicles
- Apoptotic bodies

Multiple other terms have been used in the literature, including ectosomes, microparticles, membrane particles, exosome-like vesicles, shedding vesicles and apoptotic vesicles [292,299]. For the purposes of this thesis, we will refer to the three major types of vesicles listed above. Exosomes are the smallest subtype, with a diameter of 50-100nm – similar to viruses. They are released from all cells, with production increased by cell activation [298]. Classically, they are thought to form within multivesicular endosomes, which then fuse with

the plasma membrane, resulting in exosome release. An alternative pathway of release is thought to exist, with exosomes budding directly from the plasma membrane [294].

This latter method of formation and release is also thought to occur with microvesicles, the next major category of extracellular vesicles. These have a diameter of 100-1000nm, (comparable to bacteria) and are released only in response to cell activation or stress [297,298]. Many publications use the term "microparticles" instead of microvesicles and they are largely interchangeable, although some authors use "microvesicles" as a collective term for both exosomes and shedding vesicles [292,302]. We will refer to microvesicles in this thesis because the term "microparticle" can also apply to non-biological structures, such as those used for drug delivery. Apoptotic bodies are the largest type of extracellular vesicle, with a diameter of 1-5µm, which overlaps the size range of platelets. Unsurprisingly, they are released by cells undergoing apoptosis and a distinguishing feature is that they contain nuclear components. They are therefore capable of transferring genes to other cells [298,301].

It is important to note at this stage that there is considerable overlap of the characteristics of these three types of extracellular vesicles, including size. There is currently no consensus on the markers that can be used to distinguish the origin or biogenesis of extracellular vesicles once they have left the cell [303]. Different separation protocols may allow enrichment of one subtype over another but any preparation will be heterogeneous. The introduction of artefacts, by processes such as fixation for electron microscopy, will also influence the accuracy of vesicle classification [299]. Because microvesicles are thought to be produced primarily in response to cell activation or stress, this subtype will be the focus of our investigations. In practice, current technologies do not allow them to be studied in isolation.

Role of microvesicles in inflammation

It is only recently that the key function of MVs in intercellular communication has been fully appreciated [304]. The ability of MVs to package and deliver signals between cells may have important inflammatory and immuno-modulatory effects [305]. In patients with sepsis, elevated levels of functionally-active leukocyte-derived MVs have been described [306]. Major trauma has also been associated with increased MV release [307]. In contrast, despite the profound systemic inflammation in patients with burn injuries, there are no studies to date

that have evaluated microvesicles in this condition. An increasing body of evidence suggests that microvesicles have fundamental roles in mediating the different phases of systemic inflammation.

As discussed earlier, the release of DAMPs in response to tissue injury is crucial to initiation of the inflammatory response. Evidence suggests that the activity of DAMPs such as HMGB1 is dependent on the formation of complexes with other DAMPs or cytokines, with vesicular structures providing a vehicle for the assembly and delivery of these complexes to remote targets [183,308]. Other DAMPs associated with vesicular structures include heat-shock proteins and S100 proteins [297].

A number of investigations also support a key role for microvesicles in propagating the inflammatory response. Macrophages release MVs in response to activation of purinergic $P2X_7$ receptor ($P2X_7R$) and TLRs [309,310]. Phospholipids delivered by these MVs are capable of activating other macrophages, in a NF-κB-dependent process. A similar autocrine process may occur between monocytes [311] and in the context of non-sterile inflammation [177,312,313]. Another means by which microvesicles can mediate intercellular communication is by the packaging of cytokines, such as TNF-α [314]. IL-1β has significant pro-inflammatory effects and the release of IL-1β-enriched microvesicles is recognised to be a key pathway by which this molecule is secreted [315]. IL-18, which induces the synthesis of inflammatory cytokines and upregulates adhesion molecule expression, also associates to microvesicles [316]. $P2X_7R$ appears to be important to the release of both IL-1β- and IL-18-enriched microvesicles [317].

Microvesicle-mediated endothelial activation

We have seen that following recognition of tissue damage, the endothelial adhesion cascade is important for recruiting neutrophils and monocytes to the site of injury. *In vitro* investigations has demonstrated that microvesicles released by neutrophils are capable of potentiating this process, leading to inflammatory gene expression, IL-6 release and upregulation of adhesion molecules [318]. Procoagulant responses in the endothelium are also enhanced by this signalling pathway [319]. Further investigations have suggested that neutrophil-derived microvesicles contain myeloperoxidase and are capable of injuring endothelial cells [320]. Monocyte-derived MVs have also been shown to activate and injure

endothelial cells, in addition to mediating procoagulant effects [302,321,322]. Taken together, these studies support the idea that leukocyte-derived microvesicles make a key contribution to the widespread endothelial dysfunction that is associated with systemic inflammation.

MVs released by endothelial cells are also involved in leukocyte-endothelial crosstalk. They are capable of activating monocytes and modulating their procoagulant activity [323,324]. Maturation of dendritic cells and production of inflammatory cytokines is induced by endothelial MVs [325]. In addition, endothelial MVs exhibit autocrine effects, eliciting inflammatory responses and dysfunction in nearby endothelial cells and pericytes [326–328]. MVs derived from platelets may modulate the interaction between leukocytes and endothelial cells [329]. Clinical studies have demonstrated elevated levels of endothelial MVs in a number of disease states associated with dysregulated endothelial function. These include sepsis [330] acute coronary syndromes [331], atherosclerosis [332] and pulmonary hypertension [333]. They may therefore have value as prognostic markers [332,334].

Immunomodulation by microvesicles

The biological actions of MVs are diverse and include effects which are immunosuppressive and potentially protective [3,335]. Neutrophil-derived MVs, for example, have been found to downregulate inflammatory activity in macrophages, dendritic cells and natural killer cells [336–340]. *In vitro* analysis of endothelial cells demonstrated decreased survival when MV production was inhibited [341]. One possible mechanism for this protective MV effect is the delivery of functional microRNA to recipient cells [342]. In patients with sepsis, high levels of endothelial MVs have been associated with increased survival [343]. These investigations underscore the fact that microvesicles represent a heterogeneous population. Their precise role in mediating inflammation *in vivo* is likely to depend both on their composition and site of action [344].

1.7 Analysis of microvesicles

Detection of MVs is problematic, owing to their small size and low refractive index [345]. A number of techniques have been used in their detection but the most commonly used is flow cytometry, being used by 75% of departments processing clinical samples [346]. This is an optical method that is able to collect both light scatter and fluorescence information for each "event" detected. The benefits of flow cytometry include high sample turnover rate, enumeration of large number of events and the detection of two or more antigens simultaneously. Unfortunately, most cytometers are designed to study larger structures and have a sensitivity limit of around 300-500nm, excluding a significant proportion of extracellular vesicles. It is also somewhat subjective and difficult to automate [347,348]. Flow cytometric MV analysis will be discussed in more detail in chapter three, in conjunction with the pre-analytical aspects of investigating microvesicles.

It is difficult to visualise particles of less than 1µm using conventional optical microscopy, with the resolution limit of the best microscopes lying at around 200nm. Furthermore, enumeration of particles using this technique is laborious and impractical [347,348]. A more useful method is electron microscopy, of which there are two types: transmission electron microscopy (TEM) and scanning electron microscopy (SEM) [349]. TEM uses a similar principle as light microscopy, transmitting a beam of electrons (instead of photons) through a thin specimen, which is then focused to create an image. TEM has a higher resolution of around 1nm [345] - this is sufficient to resolve internal morphological features [347]. The fixation techniques required may alter this morphology, however. It is also only capable of providing semi-quantitative information. The resolution of SEM is less than that of TEM but it provides three-dimensional images [349].

Enzyme-linked immunosorbent assays (ELISAs) have also been used for MV analysis, albeit much less extensively than flow cytometry. ELISAs are purported to generate reproducible results [350,351] but they require the presence of a specific target that can be captured by the assay. A common approach is to use annexin V, which binds to phosphatidylserine on the MV surface. Unfortunately, a large proportion of MVs fail to bind annexin V [352]. Furthermore, ELISAs do not provide any information regarding vesicle size.

A more recent method of analysing MVs is nanoparticle tracking analysis (NTA). This uses a combination of light microscopy and video software to track the Brownian motion of individual vesicles [353]. The sensitivity limit of this technique varies according to the refractive index of the particle being analysed. For extracellular vesicles, which have a low refractive index, the limit is around 50nm, i.e. significantly lower than the limit of flow cytometers [354]. NTA may not be as accurate as the current generation of flow cytometers, does not allow multi-parameter fluorescence detection and is relatively slow. Consequently, flow cytometry is still considered superior for clinical research [345] and is the method we used in our investigations.

1.8 Hypothesis and aims

The principal aim of this project was to investigate the microvesicle profile associated with the post-burn inflammatory response. We hypothesised that due to widespread tissue injury and inflammatory cell activation, severe burn injury would produce an acute, significant and potentially specific pattern of MV release, generating targets for subsequent analysis. We also hypothesised that changes in the MV profile may reflect evolution of the inflammatory response.

In order to test these hypotheses, it was first necessary to optimise our sample processing and flow cytometry protocols. This work is described in chapter three. We applied these protocols in a clinical observational study, comparing blood sampled from burn patients, sepsis patients and healthy volunteers. The results of this analysis are described in chapter four. To conclude our project, we investigated the burn-related conditions necessary to generate MVs *in vitro* and developed an assay relevant to microvascular inflammation, to assess the role of microvesicles in the propagation of organ injury. This assay was then applied to samples taken from burn patients. These investigations constitute chapter five.

Chapter 2

Materials and Methods

2.1 Equipment and software

Equipment	Application	Supplier
Cyan ADP Analyser	Flow cytometry	*Beckman-Coulter, High Wycombe, UK*
Eppendorf 5417R microcentrifuge	Centrifugation	*Eppendorf, Hamburg, Germany*
Heraeus Multifuge 3 S-R	Centrifugation	*Thermo Fisher Scientific, Waltham, USA*
Leucosep Tube 163 290	Whole blood separation	*Hugo-Sachs Elektronik, March, Germany*
Nuaire DH autoflow incubator	Cell culture	*Nuaire Group, Caerphilly, UK*
Nylon cell strainer (40µm)	BAL fluid filtration	*BD Falcon, New Jersey, USA*

Table 2.1 *Equipment*

Equipment	Application	Supplier
FlowJo v10.0.7	Flow cytometry analysis	*Flowjo, LLC, Ashland, OR, USA*
Graphpad Prism 6 for Windows	Plotting / statistical analysis	*GraphPad, La Jolla, CA, USA*
Summit v4.3.04	Flow cytometry acquisition	*Beckman-Coulter, High Wycombe, UK*

Table 2.2 *Software*

2.2 Materials

Reagent	Application	Supplier
Adenosine triphosphate (ATP)	Cell stimulation	*Tocris Bioscience, Bristol, UK*
Bovine serum albumin (BSA)	Buffer/media constituent	*Sigma Aldrich, St Louis, USA*
Calcein AM	Cytoplasmic staining	*Invitrogen, Paisley, UK*
Calcium ionophore A23187	Cell stimulation	*Tocris Bioscience, Bristol, UK*
Cell dissociation solution	Cell harvesting	*Sigma Aldrich, St Louis, USA*
CFDA-SE	Cytoplasmic staining	*Thermo Fisher Scientific, Waltham, USA*
DMEM F12 media	Cell culture	*Invitrogen, Paisley, UK*
Eagle's MEM media	Cell culture	*Sigma Aldrich, St Louis, USA*
EGM-MV	Cell culture	*Lonza, Basel, Switzerland*
EDTA	Buffer constituent	*Sigma Aldrich, St Louis, USA*
Foetal calf serum (FCS)	Buffer constituent	*Sigma Aldrich, St Louis, USA*
Hanks Balanced Salt Solution (HBSS)	Buffer	*Sigma Aldrich, St Louis, USA*
Human albumin solution (HAS)	Media constituent	*Baxter, Newbury, UK*
K-SFM media	Cell culture	*Invitrogen, Paisley, UK*
Lipopolysaccharide (LPS)	Cell stimulation	*Autogen Bioclear, Calne, UK*
Phorbol myristate acetate (PMA)	Cell stimulation	*Tocris Bioscience, Bristol, UK*
Phosphate buffered saline (PBS)	Buffer	*Invitrogen, Paisley, UK*
Sodium azide	Buffer constituent	*Sigma Aldrich, St Louis, USA*
Staphylococcus aureus	Cell stimulation	*Invitrogen, Paisley, UK*
Triton	Detergent lysis	*Sigma Aldrich, St Louis, USA*
Trypsin-EDTA	Cell harvesting	*Lonza, Basel, Switzerland*
Trypsin Neutralizing Solution	Cell harvesting	*Lonza, Basel, Switzerland*
Zymosan	Cell stimulation	*Invitrogen, Paisley, UK*

Table 2.3 *Reagents and pharmacological agents*

Equipment	Application	Constituents
Flow wash buffer (FWB)	Flow cytometry	PBS, 2% FCS, 0.1% sodium azide, 5mM EDTA

Table 2.4 *"In house" buffers*

Flow cytometry

Antibodies were fluorophore conjugated and anti-human. Table 2.1 below contains details of the antigen, fluorophore and manufacturer for each antibody.

Antigen	Fluorophore	Clone	Manufacturer
CD45	AF 647	HI30	*BioLegend, San Diego, USA*
CD14	PE	HCD14	*BioLegend, San Diego, USA*
CD11b	AF 647	M1/70	*BD Horizon, Oxford, UK*
CD66b	PE	G10F5	*BioLegend, San Diego, USA*
CD105	PE	43/A3	*BioLegend, San Diego, USA*
	PE-Cy7	43/A3	*BioLegend, San Diego, USA*
CD62e	PE	HAE-1f	*BioLegend, San Diego, USA*
CD106	APC	STA	*BioLegend, San Diego, USA*
C3c	FITC	Polyclonal	*Thermo Fisher Scientific, Waltham, USA*

Table 2.5 ***Flow cytometry antibodies***
AF 647 – Alexa Fluor 647
PE – Phycoerythrin
PE-Cy7 – Phycoerythrin-Cyanine 7
APC – Allophycocyanin
FITC – Fluorescein isothiocyanate

2.3 Blood processing and cell isolation

Blood collection

Informed consent or assent was obtained for all participants. For microvesicle analysis, 18ml of venous or arterial blood was collected into lithium heparin Vacutainers and processed immediately. For leukocyte isolation, 18ml of venous blood was collected into EDTA Vacutainers and processed immediately. For serum preparation, 18ml of venous blood was collected into clot activator Vacutainers.

Preparation of plasma and serum

To prepare platelet-rich plasma (PRP), whole blood was centrifuged at 200g for 10 minutes. The supernatant (PRP) was collected immediately. To prepare platelet-poor plasma (PPP), PRP was centrifuged for a further 20 minutes at 1500g. For serum preparation, clotted whole blood was centrifuged at 1400g for 5 minutes. The supernatant was collected and centrifuged at 20,000g for 10 minutes to yield serum (supernatant).

Peripheral blood mononuclear cell (PBMC) isolation

PBMCs were isolated from healthy volunteers by density gradient centrifugation of whole blood. This was performed using Leucosep tubes (Greiner Bio-One) and Histopaque-1077 (Sigma), according to the manufacturers' instructions. After separation, PBMC were washed in 10ml sterile PBS and centrifuged for 10 minutes at 250g. This was repeated twice, before the cells were resuspended in HBSS and counted using flow cytometry. Cells were kept on ice until used.

Neutrophil isolation

Following PBMC separation, the neutrophil- and erythrocyte-rich pellet was recovered aseptically, mixed gently with ice-cold sterile 6% dextran solution, and allowed to sediment for 30 minutes. The neutrophil-rich, erythrocyte-depleted upper layer was aspirated and centrifuged to pellet the cells. Residual erythrocytes were lysed using ice-cold sterile water for 20 seconds, before isotonicity was restored by adding one volume of 10x PBS to 9 volumes of cell suspension. The neutrophils were then washed twice in 10ml of cold HBSS, centrifuged at 250g for 10 minutes and counted using flow cytometry.

2.4 Cell culture

Cells were cultured in 175cm^2 flasks, at 37^{o}C, in 95% air / 5% CO_2. Media were changed every 48 hours. Where Trypsin-EDTA was used for harvesting, cells were observed using a microscope until detached. Trypsin Neutralizing Solution (TNS) was then added immediately. Wash steps were performed using fresh medium and cells were centrifuged at 250g for 5 minutes unless otherwise stated.

RAW 264.7 mouse macrophage cell line

RAW 264.7 cells (Sigma) were cultured in DMEM F12 media. Once confluent, cells were harvested with cell dissociation solution (CDS), washed in PBS and centrifuged. Cells were resuspended in HBSS and counted using the flow cytometer. For subculturing, one quarter of the cells were transferred to fresh culture flasks. For seeding into plates, 2 x 10^6 cells were added to wells (growth area 9.5cm^2) then incubated in fresh media at 37^{o}C until confluent.

HK-2 human kidney epithelial cell line

HK-2 cells (ATCC) were cultured in K-SFM media. For subculturing, 80% confluent cells were harvested using Trypsin-EDTA. One quarter of the washed cells were transferred to fresh culture flasks. For experiments, cells were detached using CDS, washed in PBS and centrifuged. Cells were resuspended in HBSS and counted using the flow cytometer.

BJ Human fibroblast cell line

Fibroblasts (BJ, ATCC) were cultured in Eagle's MEM / 10% foetal calf serum. For subculturing, confluent cells were harvested using Trypsin EDTA. One quarter of the washed cells were transferred to fresh culture flasks. For experiments, cells were detached using CDS, washed in PBS and centrifuged. Cells were resuspended in HBSS and counted using the flow cytometer.

Primary human endothelial cells

Primary human pulmonary microvascular endothelial cells (HPMECs, Promocell) were cultured in EGM-MV medium. For cell passaging and plate seeding, cells were harvested using Trypsin-EDTA. Cells were washed in fresh medium and centrifuged at 250g for three minutes, then seeded at confluence ($2x10^5$ / well) into 24 well plates.

PBMC-endothelial cell co-culture experiments

Following stimulation (see below), endothelial cells were detached using cell dissociation solution, then diluted in FACS wash buffer (FWB), before centrifugation at 200g for 5 minutes. The cell pellet was resuspended in 120µl of FWB and stained with fluorescent antibodies raised against identification marker and relevant activation markers. After staining and washing, the samples were analysed by flow cytometry. Cell adhesion molecules levels were expressed as MFI of stained cells with background values subtracted.

2.5 Microvesicle preparation

Neutrophil and PBMC preparations were resuspended in Eppendorf tubes in HBSS / 0.5% Human Albumin Solution (HAS), at 1 x 10^7 cells per ml. Cells were rested for one hour at 37^{o}C, before incubating with the relevant stimulant for a further hour at 37^{o}C. Incubations were performed under sedimenting (static) or suspension (rotating) conditions. The microvesicles were isolated by centrifuging the cells at 200g for 10 minutes, before collecting the supernatant. Microvesicles were analysed as described below.

For incubation of PBMC-endothelial co-culture with microvesicles, the cell-free supernatant was centrifuged for 30 minutes at 20,000g to pellet microvesicles, which were then resuspended in 200µl 0.5% HAS/EBM. The microvesicles were added to the endothelial cell-PBMC co-culture and incubated for 4 or 16 hours. Endothelial cells were retrieved and analysed as above.

Opsonisation of particulate stimulants

Cells or microbial particles were opsonised by suspension in 500µl of serum and incubation in the rotator for one hour at 37^{o}C. The stimulant was then washed to remove residual serum, before being added to the leukocyte preparation.

2.6 Flow cytometry

Flow cytometry acquisition was performed using a Cyan ADP flow cytometer, with Summit software (version 4.3.04). Acquired microvesicle and cell data was analysed using FlowJo, version 10.0.7.

Microvesicle analysis

Microvesicle samples (10µl) were incubated with 10µl of antibodies (see Table 2.1) for 30 minutes at 23^{o}C, and then diluted with 1ml of filtered PBS. 500µl was removed from each sample and added to 5µl of Triton X-100 10%. 10µl of counting beads were added to each sample before data acquisition.

Cell analysis

Endothelial cells (50µl) were incubated with 50µl of antibodies for 30 minutes at 4^{o}C, and then washed with 4ml of wash buffer (WB), before centrifugation at 200g for 5 minutes. Cell pellets were resuspended in 400 µl of WB before flow cytometry data acquisition.

Cytometric bead array

Soluble cytokines, chemokines and growth factors were measured using a cytometric bead array kit (BD CBA Soluble Flex Set System) as per manufacturer's instructions. Standards, capture beads and phycoerythrin (PE) detection reagents were prepared prior to performing the assay. Human platelet-poor plasma was prepared as below. 50µl of 10 serially-diluted standards were added to 10 tubes. 50µl of each sample was added to the appropriate assay tubes. 50µl of capture beads were added to each tube, before incubation at room temperature for one hour. 50µl of detection reagent was then added to each tube before incubation for a further two hours. Samples were washed with 1ml of wash buffer, and centrifuged at 200g for 5 minutes. The supernatant was discarded before the sample was resuspended in a further 300µl of wash buffer. Data was acquired using a Cyan ADP and analysis was performed using FCAP Array software.

2.7 Statistical analysis

Means and standard deviations are provided for parametric data. Means are shown where all data points are displayed. A t-test was used to compare two groups of data. For three or more groups, a one-way analysis of variance (ANOVA) was used with Dunnett's multiple comparison test (if comparing groups to a control) and Tukey's multiple comparison test (if comparing each group to all others). Paired values were compared using a paired t-test. Nominal variables were compared with Fisher's exact test and Pearson's correlation coefficient was used to measure correlations.

For non-parametric data, medians and interquartile ranges are provided. Medians are shown where all data points are displayed. The Mann-Whitney test was used to compare two groups of data. For three or more groups, the Kruskal-Wallis test was used with Dunn's multiple comparison test. Paired values were compared using the Wilcoxon signed rank test and Spearman's correlation coefficient was used to measure correlations. Normality was assessed using the D'Agostino-Pearson omnibus test.

Chapter 3

Optimisation and validation of flow cytometry protocols for analysing microvesicles

Summary

The most commonly used method for quantifying and characterising microvesicles is flow cytometry. However, these small particles lie at the detection limits of conventional flow cytometers, in the range where optical and electronic noise become problematic. A number of attempts have been made to standardise microvesicle analysis, but there is no universally-approved approach. Individual cytometers also vary in their size detection threshold and resolution. Before analysing samples from burn patients, we sought to establish the capabilities and limitations of our cytometer and to optimise methods for processing and storing samples.

Calibrated sizing beads were used to determine the physical characteristics of the cytometer when investigating small particles. After optimising machine setup, including troubleshooting signal distortions produced by mismatched sheath and sample fluid composition, we evaluated different approaches to the detection and analysis of microvesicles generated *in vitro*. Our initial findings suggested that it was better to discriminate microvesicles from background noise on the basis of fluorescence, rather than size. Fluorescence triggering for acquisition of heterogeneous microvesicle populations requires generic stains to label microvesicles according to common biochemical properties. We evaluated two cytoplasmic dyes that have previously been used in cell proliferation studies. The second of these, calcein-AM, showed promise with isolated microvesicles, but when used to study plasma samples, this dye increased background noise without increasing microvesicle detection. We eventually concluded that for microvesicles in plasma samples, side scatter was the best discriminator. Further steps were then taken to exclude artefact, including detergent lysis of microvesicles and reference to a biological size standard from ionophore-stimulated platelets.

The optimised cytometry protocol was then used to study sample processing. We found that the centrifugation steps described in many publications led to an unacceptable loss of microvesicles. We also confirmed that freezing of platelet-rich plasma had a minimal effect on non-platelet microvesicles. By this stage we were satisfied that we had evaluated and optimised all the components necessary for a patient sample protocol.

3.1 Background

Flow cytometers are designed to measure laser light scattered by a cell at two distinct angles, whilst simultaneously measuring fluorescence emission signals [355]. Cells are drawn from a sample tube and are constrained as they pass the sensing laser by a cylinder of sheath fluid. This process is known as hydrodynamic focussing [356]. Light is reflected in all directions. The forward scatter light, correlating to cell size, is collected at narrow angles. The side scatter light, reflecting structural complexity, is collected at wide angles in conjunction with fluorescence signals. The information then undergoes computer analysis. The process is summarised in figure 3.1 below.

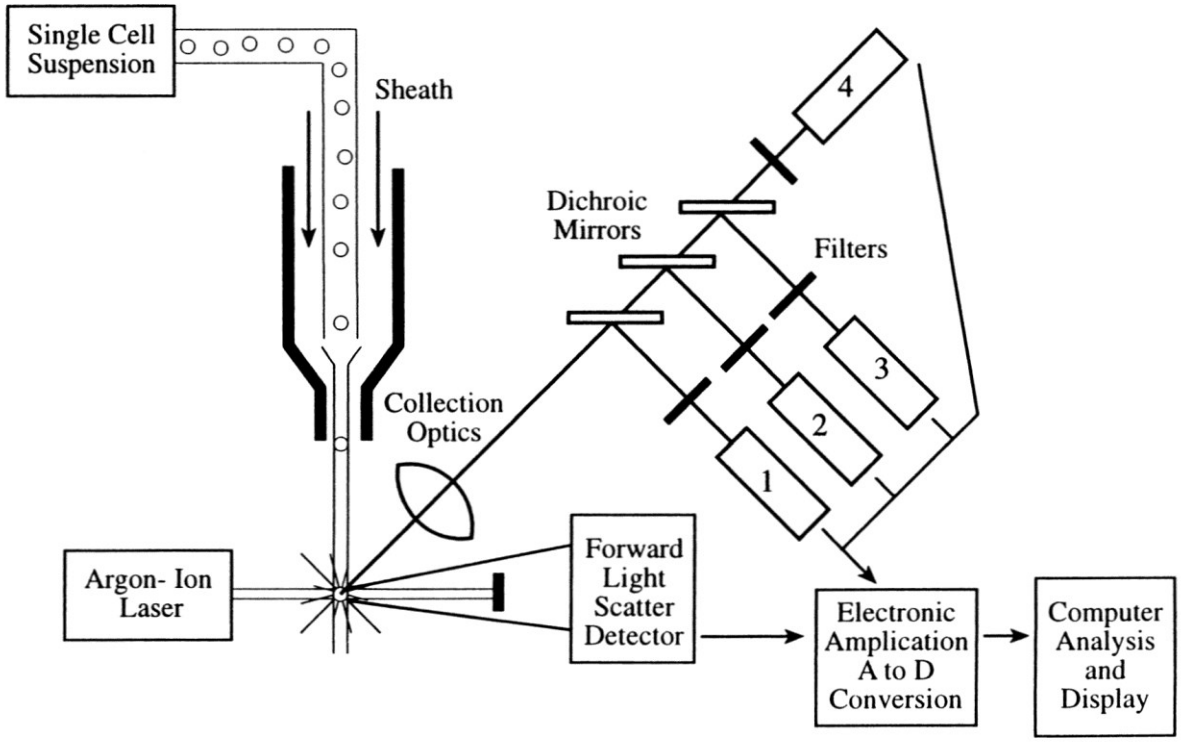


Figure 3.1 Schematic of typical flow cytometer

1: Side scatter detector 2-4: Fluorescence emission detectors [357]

(reproduced with permission)

Flow cytometry is the most widely-used technique for analysing microvesicles because it allows simultaneous multi-parametric analysis of thousands of particles per second [358]. However, its use can be problematic and lacks standardisation [347–349,359,360]. For example, the

relationship between forward scatter and size breaks down as the particle size approaches the laser wavelength of 400-500nm [361]. Before we could begin investigating microvesicles from patients with burn injuries, we sought to develop an optimised flow cytometry protocol. There were two separate issues to consider: firstly, the intrinsic analytical capabilities and limitations of our particular cytometer, a Beckman Coulter Cyan ADP. Secondly, the pre-analytical factors relevant to human samples.

Flow cytometry-based microvesicle analysis

The diameter range of microvesicles is often reported as between 100nm and 1µm [348], although this range does vary, with some authors including particles up to 1.5µm [362]. Others argue that the lower size limit is arbitrary and advocate inclusion of all particles below a certain size [360]. Regardless of the precise values chosen, most cytometers have a threshold for detection of around 400-500nm; detection is possible below this value but unreliable [360,363,364]. The absolute limit of sensitivity is around 300nm, or 150nm where the cytometer has been designed specifically for microvesicles [345,353]. Therefore, when using flow cytometry for microvesicle analysis, a certain proportion of the population of interest will be missed.

When assessing the capabilities of a flow cytometer, consideration should be given to the standard used for calibration. Fluorescent beads of specific sizes are often utilised and have been proposed as a means for comparing results from different centres, using different machines [365]. The location of these beads on a cytometry scatter plot can be used to define a size-restricted group of events commensurate with microvesicles. The assumption in this process is that all particles of a given size will scatter laser light to the same degree. Unfortunately, polystyrene beads have a higher refractive index than particles of a cellular origin [366] and will scatter more light than similarly-sized microvesicles. Silica calibration beads may mitigate this problem to some degree, as their refractive index more closely matches that of microvesicles [364], although these were not available commercially to us. Therefore the most appropriate use of calibration beads would be to ensure consistency between samples, with a precise cut-off ideally referenced to a biological standard.

The next issue to consider is the choice of a "threshold" or trigger. When analysing cells, usual practice is to select a forward scatter value that lies between the population of interest and the noise floor. The cytometer will then record every event above this value. Noise can represent small particles in the sample solution and sheath fluid. It also originates from low level signals in the cytometer electronics and stray light detected by the optics [367] – this will be present regardless of actual particle content. Microvesicles lie very close to or within the noise floor, meaning the smallest microvesicles may be unresolvable from noise on physical characteristics alone [368]. Raising the threshold to exclude noise excludes particles of interest. Because of this dilemma, a number of authors have advocated the use of alternative triggers, such as fluorescence, to improve discrimination of microvesicles from noise [349,368–370].

A further potential source of error is the co-detection of immune complexes. These are protein aggregates, consisting of antibodies and bound antigen, that have physiologic but also pathophysiologic roles [371] and pose a particular problem when derived from fluorescence-conjugated antibody probes. The biophysical properties of microvesicles overlap with those of immune complexes and they cannot be distinguished by centrifugation or light scattering. This contrasts with particles such as lipoproteins, which are significantly smaller [372]. For this reason, several papers have suggested the use of detergent to lyse microvesicles, leaving insoluble protein complexes intact [372–374]. These events can then be excluded from the final microvesicle count.

Sample processing

A number of pre-analytical factors are also thought to affect microvesicle counts. The overall aim of sample processing is to minimise *ex-vivo* or post-harvest production of microvesicles, whilst retaining the greatest possible yield from the system being analysed. An important part of minimising post-sampling microvesicle release is to remove cells as rapidly as possible. One approach used for blood samples is to perform a two-stage centrifugation, at ~200g and ~1500g [375–377]. The lower speed centrifugation removes leukocytes and erythrocytes, whilst the medium speed centrifugation reduces platelet contamination. However, this is another area with a lack of standardisation. Some authors argue a further centrifugation, at a medium speed, is needed to produce platelet-free

plasma (PFP) [377]. Others suggest a high speed of 13000g can be used for shorter periods [378], which conflicts with data indicating that larger microvesicles are lost at speeds as low as 1200g [379].

The storage of samples is another key question, of particular relevance to studies involving patient samples. Complete processing and analysis of samples from patients recruited outside normal working hours is not always possible. This is particularly relevant with conditions that require emergency treatment, such as burn injuries. Therefore it is advantageous if samples are amenable to basic processing and storage, with analysis being performed at a later date. Adequate sample storage would also facilitate multi-centre studies, by avoiding the need for multiple analysers, with each requiring independent calibration [365]. The light scatter parameters of microvesicles are not thought to be altered by freezing [333] but the optimal storage protocol is not clear. Freezing platelet-rich plasma (PRP) is certainly a problem when analysing platelet microvesicles, because the cellular source is by definition still present in the fluid. A considerable increase in platelet 'microvesicle' counts is therefore seen in freeze-thawed platelet-rich plasma [378]. This effect has also been described with platelet poor plasma (PPP) [378,380], although some studies found PPP to be unaffected [359]. In contrast, leukocyte and endothelial microvesicle counts have not been shown to change after a freeze-thaw cycle with plasma depleted of cells [380].

3.2 Aims

The aims for this chapter were as follows:

1. Determine the optimal flow cytometry set up and protocol for analysing microvesicles on a Cyan ADP cytometer
2. Develop a sample processing and storage protocol suitable for studying microvesicles from patient samples

3.3 Protocols

Cell culture and stimulation

RAW 264.7 cells (murine monocyte-macrophage cell line) were cultured in flasks and wells as described in chapter two. For stimulation, cells were washed with PBS, then stimulated for 30 minutes with either ATP (3mM) or ATP + A23187 (3mM + 10µM). The fluid from each well was aspirated and centrifuged in microfuge tubes at 200g for 10 minutes, to pellet cells. Microvesicles were analysed in the supernatant.

Blood collection

For analysis of circulating microvesicles, 20ml of blood was obtained from volunteers who had given informed consent, or assent in the case of patients unable to give consent. The blood was collected into lithium heparin Vacutainers and processed immediately.

Microvesicle staining

A 10µl volume of the microvesicle preparation was incubated with PE or APC-conjugated monoclonal antibodies, specific to the cell surface marker to be analysed, for 30 minutes at room temperature. Antibody volumes were titrated to maximise separation between negative and positive events (logarithmic scale). The total staining volume was 20µl and filtered PBS was added where necessary to ensure constant volume. Staining was terminated by diluting samples with 1ml of filtered PBS.

Sensitivity of Cyan ADP Flow Cytometer as determined by fluorescent beads is improved with fluorescence trigger but discriminatory power is lost in the sub-500nm size range

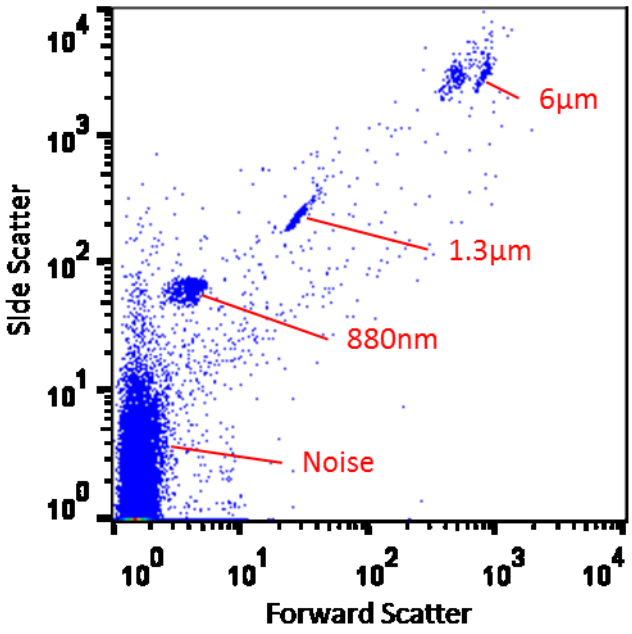
Our first task was to establish the intrinsic characteristics and capabilities of our flow cytometer, in the size range at which we would expect to find microvesicles. A commonly described method is to use calibrated fluorescent beads to define the forward scatter resolution limit, which is the most appropriate parameter to use when defining microvesicle size [365].

Spherotech polystyrene fluorescent beads (20µl), ranging in size from 220nm to 1.3µm, were suspended in 400µl of distilled water. Accucheck counting beads (20µl), with an approximate size of 6.36 and 6.4µm, were also included for comparison. Cytometry data was acquired for a fixed period of 60 seconds, at a speed calibrated to ensure constant flow. Forward and side scatter voltages were based on normal settings for cell acquisition and the threshold set initially to the lowest value (0.01%).

Size calibration and counting beads were acquired with a forward scatter trigger and evaluated on forward scatter vs side scatter plots (figure 3.2A). 6µm, 1.3µm and 880nm beads were successfully detected and could be discriminated from each other. However, it was not possible to detect a distinct population of 490nm or 220nm beads. In contrast, using a fluorescence trigger enabled the detection of discrete populations of 490nm and 220nm beads (figure 3.2B). Their positions on the scatter plot suggest that these beads are obscured by noise when a forward scatter trigger is used.

The ability of the Cyan flow cytometer to discriminate on forward scatter alone was then evaluated (figure 3.3). This was important because we could not reliably predict the extent to which genuine microvesicles would vary in their side scatter. When using a forward scatter trigger, only the 6µm and 1.3µm beads were distinct, with the 880nm beads blending into the noise signal (figure 3.3A). When using a fluorescence trigger, it was also possible to discern the 880nm beads, although the 490nm beads and 220nm beads did not have discrete forward scatter signals (figure 3.3B).

A



B

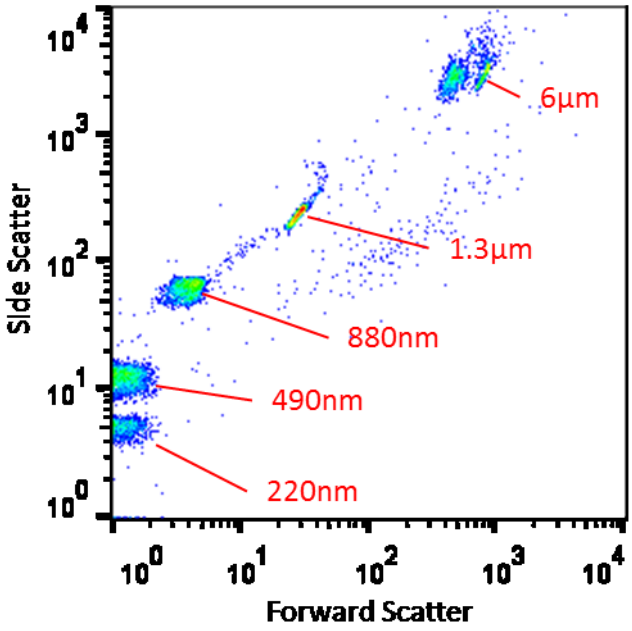
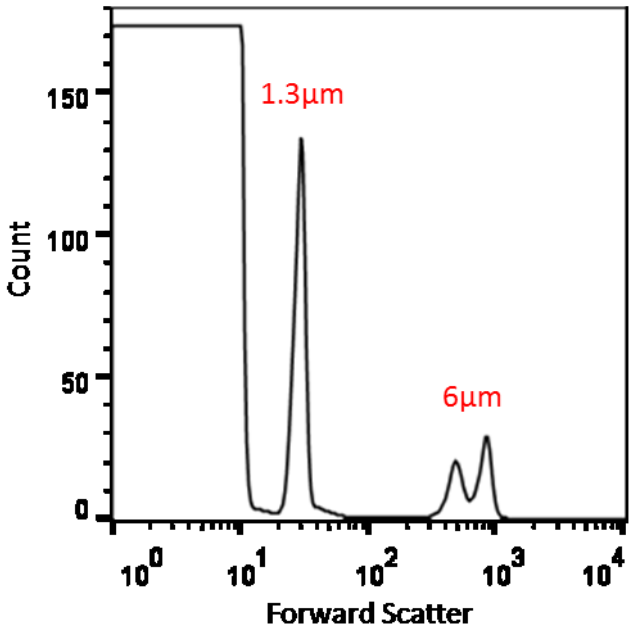


Figure 3.2 ***Analysis of forward scatter and side scatter of sizing beads using a Cyan ADP cytometer***

Fluorescent beads of size 490nm and 220nm were obscured by noise when using a forward scatter trigger (A). Fluorescence trigger (channel 1) allows detection and discrimination of all bead populations (B)

A



B

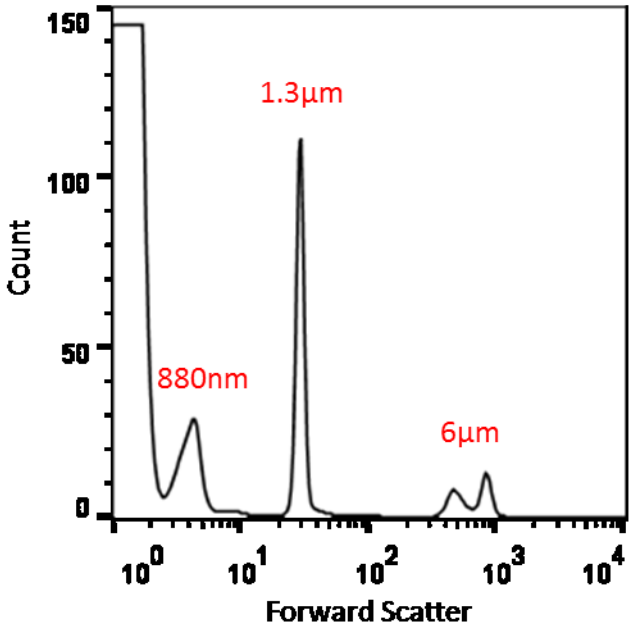


Figure 3.3 ***Analysis of forward scatter of sizing beads using a Cyan ADP cytometer***

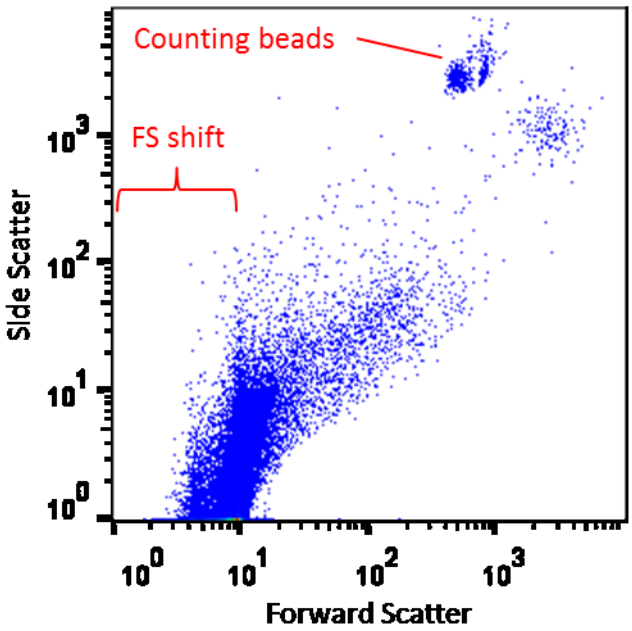
Cyan can only detect discrete forward scatter signals for 1.3µm and 6µm beads when forward scatter trigger is used (A). Fluorescence trigger also allows discrimination of forward scatter signal for 880nm beads (B). Y axis is adjusted to demonstrate bead peaks. Noise is therefore off-scale but distinct bead peaks not visible when scale increased

Forward scatter events in microvesicle size range are affected by sheath fluid with mismatched refractive index

It became apparent in our early experiments that the forward scatter signal of small particles was often distorted and shifted away from zero. An example of the scatter plots obtained is shown in figure 3.4A. Because the affected area of forward scatter would typically be excluded when analysing cells, this phenomenon would normally be overlooked. However, we did not detect the distortion when the sizing beads were studied (figure 3.4A), which were resuspended in distilled water, whereas our *in vitro* microvesicle samples were resuspended in a physiological saline (PBS). Because the sheath fluid being used at this point was also distilled water, we suspected that an interaction between the sheath fluid and sample fluid was occurring. Investigations of small particles have previously demonstrated that a mismatch in refractive indices, between sheath and sample fluids, distorts forward scatter [381]. The effect was maximal with the smallest particles but affected cells up to 5µm in diameter, well in excess of the size range we were focusing on. We concluded that using sheath solutions with matched refractive indices was critical for all further microvesicle analyses. All subsequent work was performed with a commercial isotonic sheath solution (Isoflow, Beckman Coulter).

Before analysing a batch of samples, we acquired scatter data using filtered PBS for at least 10 minutes. By plotting time against forward scatter, it was possible to confirm that the forward scatter signal was stable. If water had previously been used for sheath fluid, we found stabilisation to occur after approximately 9 minutes after switching to Isoflow. A plot demonstrating this effect is shown in figure 3.4B.

A



B

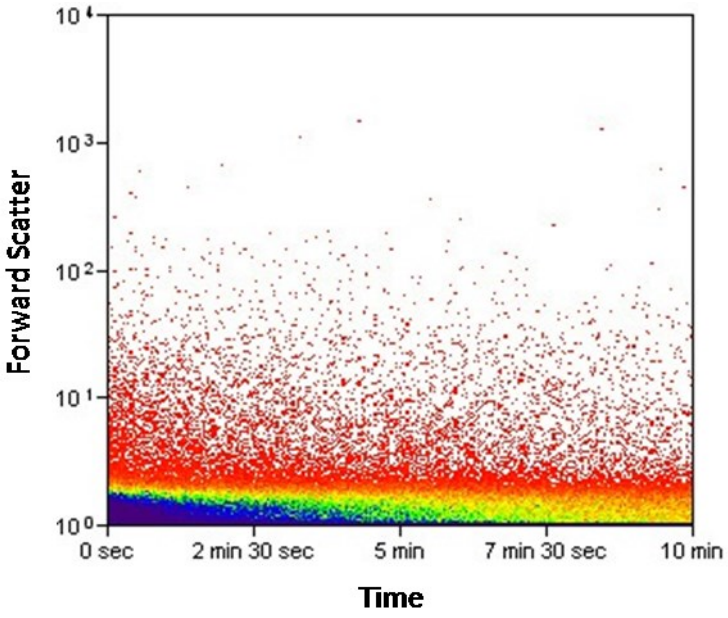


Figure 3.4 ***Effect of mismatched sheath fluid on forward scatter***

Forward scatter noise is shifted away from baseline when refractive index of sheath fluid does not match that of sample (A). Substituting unmatched sheath fluid (water) for matched Isoflow results in forward scatter noise returning to baseline. This process takes approximately 9 minutes (B)

A combination of surface labelling and fluorescence trigger allows detection of RAW 264.7 cell microvesicle response to stimulation

Our initial bead data had suggested that a fluorescence trigger could be useful for reducing background noise. We next sought to establish if a fluorescence trigger would enable our cytometer to detect microvesicles generated *in vitro*, from the RAW 264.7 murine monocyte-macrophage cell line. Local experience showed these cells produce significant quantities of microvesicles when stimulated with ATP and calcium ionophore.

RAW 264.7 cells were cultured and stimulated with ATP and ionophore A23187. Released microvesicles were stained with allophycocyanin (APC)-conjugated anti-CD11b antibodies then diluted with PBS. Analysis was performed using a fluorescence trigger (channel 8, APC) set to the minimum value (0.01%). Microvesicles were defined as all events with a forward scatter value smaller than that of the 1.3µm beads described earlier. Unstained and stained samples were compared.

The absolute numbers of microvesicles detected were relatively low, when unstained samples were analysed (figure 3.5). There was an increase in counts seen following stimulation with ATP + A23187 (924 ± 1291 to 1674 ± 2237 MV μl^{-1}) but this was not significant. In contrast, the stained samples yielded a significant increase in microvesicle counts following stimulation with ATP alone (8599 ± 265 to 18794 ± 6466 MV μl^{-1}) and ATP + A23187 (38149 ± 8102 MV μl^{-1}). These results demonstrated that by using a fluorescence trigger, it was possible to clearly identify microvesicles with only a relatively low background event counts. However, this technique was dependent on fluorescent labelling and detection could not be based upon an intrinsic auto-fluorescence signal alone.

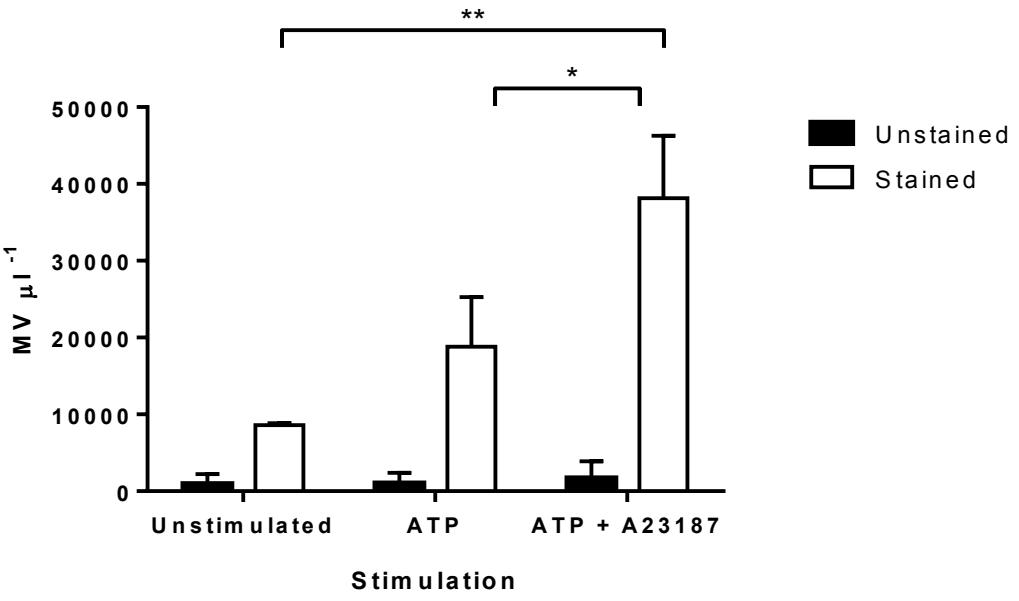


Figure 3.5 ***Comparison of auto-fluorescence and surface marker labelling when using a fluorescence trigger to detect RAW cell microvesicles***

Use of fluorescent CD11b antibody in conjunction with fluorescence trigger allows detection of increased RAW cell microvesicle levels following stimulation with ATP + A23187. Absolute numbers are lower and significant post-stimulation increase is not detected by use of auto-fluorescence only (unstained samples)

**p<0.05*

***p<0.01*

2-way ANOVA with Tukey's multiple comparisons test

(n=2)

Cytoplasmic staining of RAW 264.7 microvesicles with calcein-AM allows detection of greater numbers of microvesicles than staining with CFDA-SE

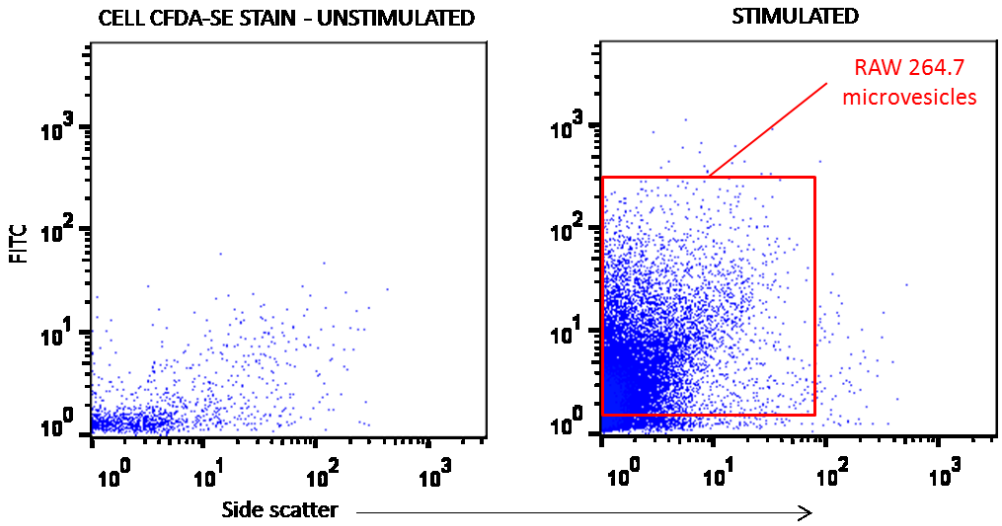
Although the combination of surface marker labelling and a fluorescence trigger could be useful for analysing microvesicles in single-cell preparations, it would not be valid for fluids with multiple cell types, such as human plasma. Such an approach could be affected by weak or variable surface marker expression on cells and their subsequent packaging into microvesicles. Identification of microvesicles with generic markers based on their physical and/or biochemical properties could potentially overcome this limitation. Our first candidate was a cytoplasmic dye: carboxyfluorescein diacetate succinimidyl ester (CFDA-SE). This is converted by intracellular esterases to carboxyfluorescein succinimidyl ester (CFSE), which is fluorescent [382]. CFDA-SE is conventionally used for cell proliferation assays [382–384] but has also been used for tracking the uptake of microvesicles [385,386].

When parent RAW cells were stained pre-stimulation (ATP + A23187) with CFDA-SE, a marked increase in microvesicle response was detectable (1425 ± 988 to 30078 ± 2423 MV μl^{-1}) (figure 3.6A). However, when the microvesicles were stained directly, the number of post-stimulation microvesicles detected was much smaller (6962 ± 5004 MV μl^{-1}) (figure 3.6B).

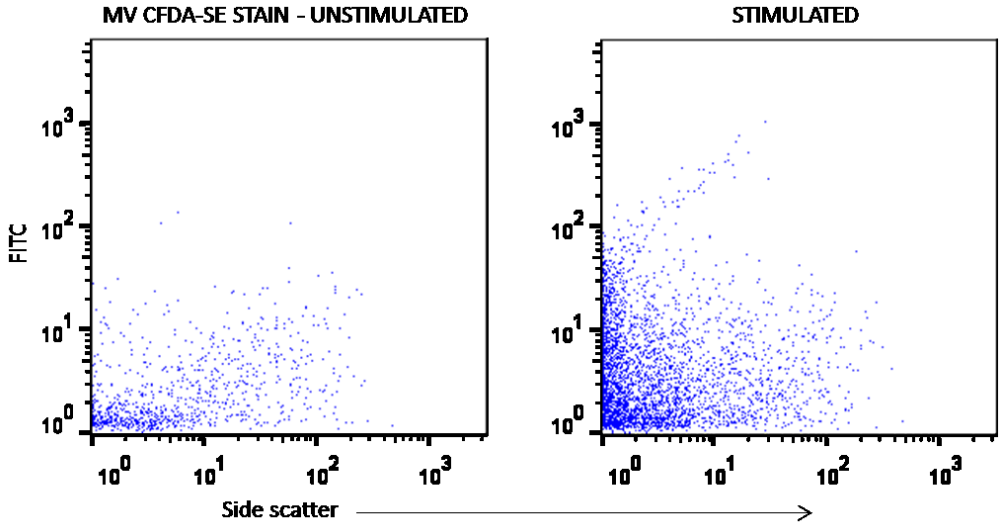
Staining of parent cells would not be an appropriate technique for staining circulating microvesicles that had already been released *in vivo*. As direct MV staining with CFDA-SE was sub-optimal, we evaluated an alternative cytoplasmic dye, calcein acetoxymethyl ester (calcein-AM). Calcein-AM is similar to CFDA-SE, in that it is converted to a fluorescent form by intracellular esterases. However, it does not covalently link to intracellular molecules [382,387] and also exhibits less dye leakage than CFSE [388,389]. Before testing calcein-AM with human samples, we investigated its behaviour with RAW microvesicles.

RAW cells were stimulated as above. The resultant microvesicles were stained directly with calcein-AM and fluorescent anti-CD11b antibodies. When using calcein-AM (20nM) and a fluorescence trigger, a clear CD11b+ microvesicle population was detectable. The number of microvesicles detected using 200nM calcein-AM was markedly increased (figure 3.6C).

A


CELL CFDA-SE STAIN - UNSTIMULATED
STIMULATED
RAW 264.7
microvesicles
FITC
Side scatter

B


MV CFDA-SE STAIN - UNSTIMULATED
STIMULATED
FITC
Side scatter

C

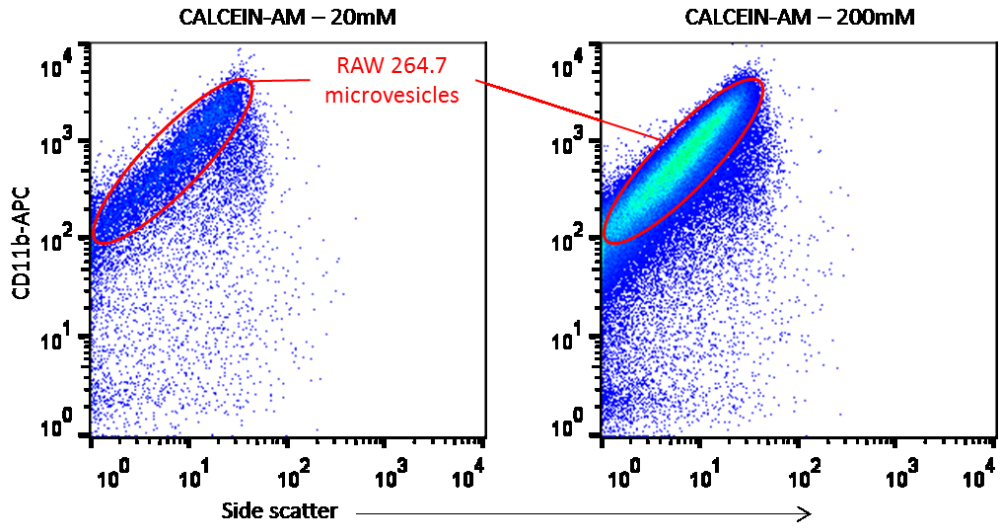

CALCEIN-AM – 20mM
CALCEIN-AM – 200mM
RAW 264.7
microvesicles
CD11b-APC
Side scatter

Figure 3.6 ***Effect of cytoplasmic staining on detection of RAW microvesicles when using fluorescence trigger***

CFDA-SE (5µm) staining of parent RAW 264.7 cells, prior to stimulation, leads to greater microvesicle detection (A) than is the case when RAW microvesicles are stained directly (B)

Clear population of CD11b+ RAW microvesicles is detectable using direct calcein-AM stain (20nM). Microvesicle detection is enhanced by higher concentration of calcein-AM (200nM) (C)

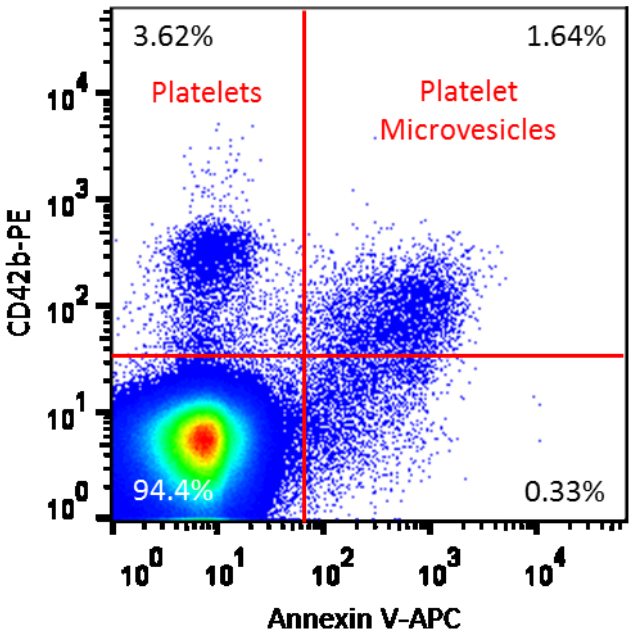
Investigation of calcein-AM as a generic marker for plasma microvesicles

We next investigated calcein-AM as a generic staining method in platelet rich plasma. Although not previously described, we considered that this method might be an ideal approach for identifying microvesicles in burn patients. We speculated that plasma would be a significant source of small non-vesicular particles, such as immune complexes and other protein aggregates. By preferentially staining vesicles of cellular origin with a relatively inert, non-protein-binding cytoplasmic dye such as calcein-AM, we hoped a fluorescence discriminator could be used to maximise the microvesicle count, whilst minimising noise.

Blood was sampled as above and centrifuged to pellet cells. Plasma was collected and analysed immediately. Samples were stained with a combination of calcein-AM (200nM), anti-CD42b and annexin V-APC. Annexin V binds to phosphatidylserine, which is externalised during microvesicle formation [377,390]. CD42b (glycoprotein 1b alpha), is widely used as a platelet surface marker [343,391–395]. As a comparator, we also assessed side scatter as the trigger parameter. This has lower levels of background light detection than forward scatter [368], with the potential for improved resolution, as evidenced by our earlier sizing bead analysis. Although the exact side scatter value will not reflect the size of microvesicles [396], using this parameter as the trigger had the potential for reducing noise [367]. Repeat sample acquisitions were performed, using fluorescence (FITC) and side scatter triggers separately. Microvesicles were defined as events positive for annexin V and CD42b, with a forward scatter less than 1.3µm sizing beads.

When using the combination of calcein-AM staining and a fluorescence trigger, it was possible to identify separate platelet and platelet microvesicle populations (figure 3.7A). However, by using a side scatter trigger, it was possible to detect a higher number of platelet microvesicles, despite a lower overall number of events (figure 3.7B). We attributed this to relatively weak calcein-AM staining of microvesicles, as well as staining of non-vesicular plasma constituents, i.e. the side scatter trigger had greater sensitivity and specificity for microvesicles. We therefore concluded that side scatter was a more suitable trigger parameter to use for analysing microvesicles in plasma.

A



B

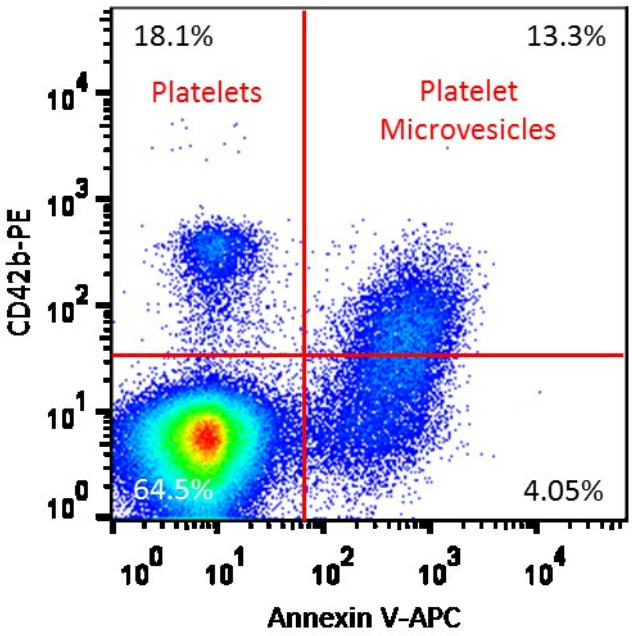


Figure 3.7 ***Comparison of fluorescence trigger and side scatter trigger for detecting platelet microvesicles stained with cytoplasmic and surface dyes***

Flow cytometry plots of platelet microvesicles (AV+/CD42b+) in human plasma. Microvesicle detection is lower when using direct calcein-AM stain and fluorescence trigger (A) than when using side scatter trigger (B), despite more events being detected overall

Platelet microvesicles are sensitive to lysis with Triton X-100

Next we investigated methods to verify identification of events as microvesicles. The presence of immune complexes in the fluid being analysed may lead to overestimation of microvesicle numbers [372]. Self-aggregation of fluorescent antibodies may also form protein complexes that can mimic vesicular events [298]. Some authors therefore advocate the use of a detergent lysis step, to differentiate insoluble protein aggregates from genuine microvesicles [300,373,397].

We tested this procedure by adding Triton X-100 (TX-100), a non-ionic surfactant, to the microvesicle samples after they had been analysed. Addition of TX-100 (final concentration 0.1%) to plasma samples resulted in the disappearance of virtually all annexin V+/CD42b+ events (figure 3.9 A and B). We concluded these represented genuine platelet microvesicles. This was also the case when TX-100 was added to samples containing RAW cell-derived microvesicles. The small number of detergent-insoluble events remaining can be subtracted from the total gated event count, to give a final microvesicle count.

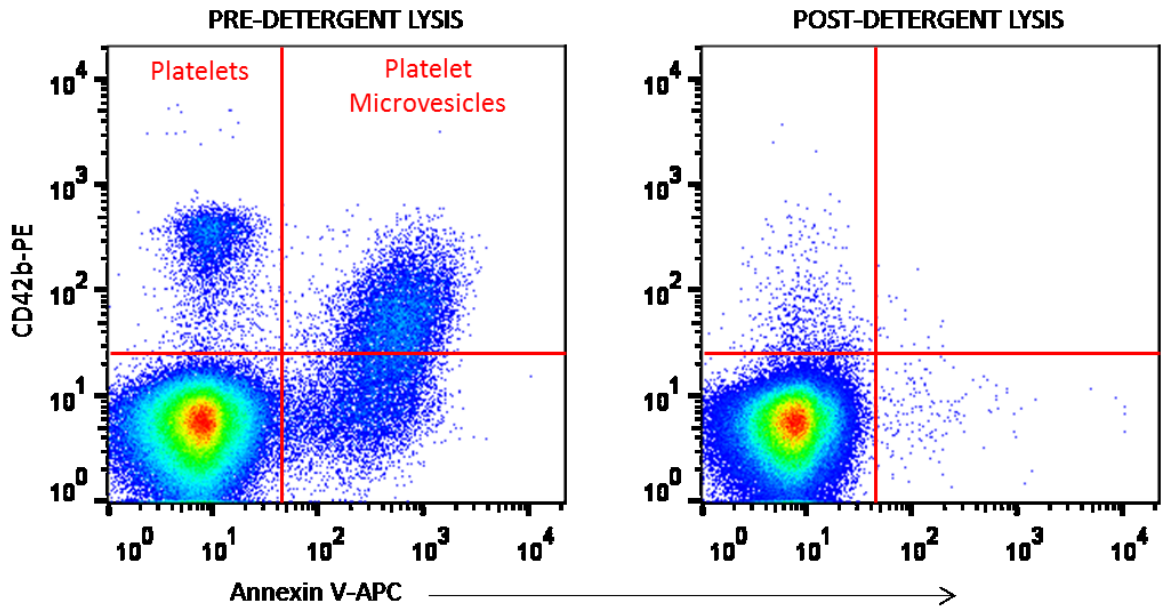


Figure 3.8 ***Effect of Triton X-100 detergent on platelet microvesicles***

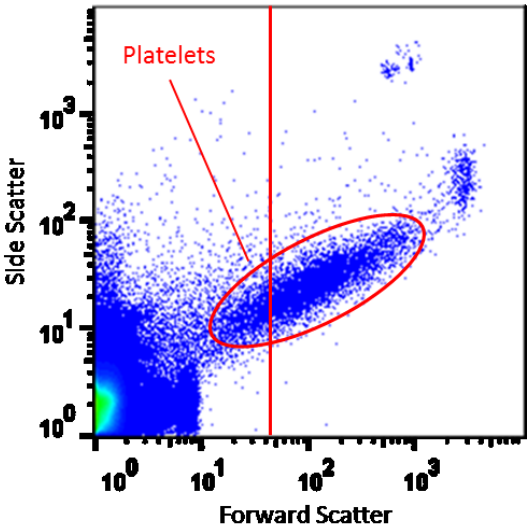
Majority of Annexin V+ / CD42b+ events (A) are lysed by Triton X-100 detergent (B), indicating they are genuine platelet microvesicles

Calibration of the forward scatter size gate using platelet microvesicles

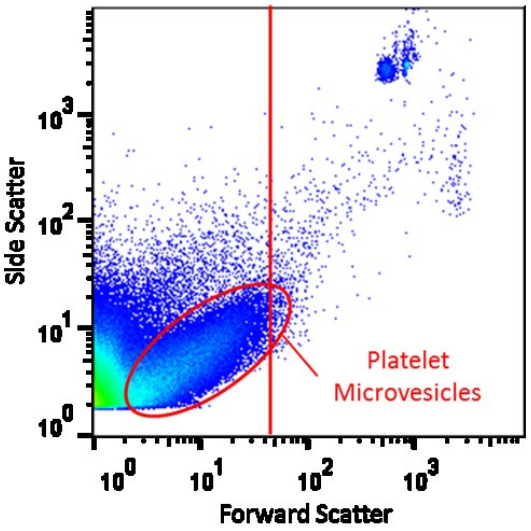
We next sought to establish a biological reference point so that a forward scatter value could be set to define microvesicles accurately. Sizing beads have conventionally been used for this purpose, however as explained above, some beads scatter more light than similarly-sized membrane-bound particles [366]. One approach is to create artificial phospholid vesicles of known diameter [364]. A more relevant method is to stimulate plasma to generate a population of microvesicles, which can then be compared to sizing beads. This technique has been used previously, with calcium ionophore A23187 as the stimulant [380].

Platelet-rich plasma was stimulated with A23187 (10µM) for 30 minutes. The plasma was then stained as above to analyse platelet microvesicle content. In unstimulated plasma, the smallest platelets coincided with the forward scatter value of 1.3µm sizing beads (figures 3.9A and C respectively). Following stimulation of plasma, a microvesicle population became evident, the upper forward scatter limit of which also corresponded to 1.3µm sizing beads (figure 3.9B and C). The microvesicle population was both annexin V+ / CD42b+ and TX-100-sensitive. We were therefore confident that these were the expected platelet microvesicles. We concluded that the 1.3µm beads were an appropriate means to define a microvesicle scatter gate for future experiments.

A


Platelets
Side Scatter
Forward Scatter

B


Platelet
Microvesicles
Side Scatter
Forward Scatter

C

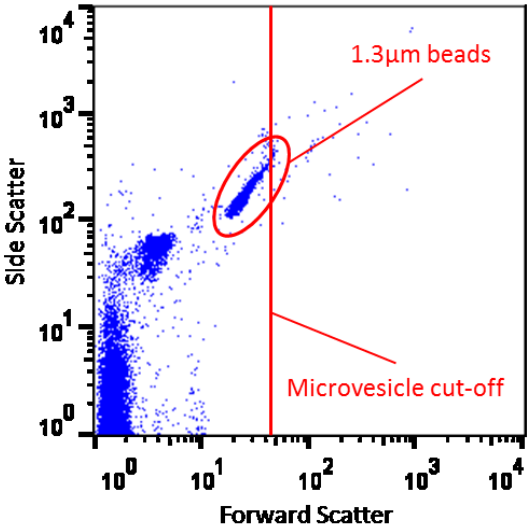

1.3µm beads
Microvesicle cut-off
Side Scatter
Forward Scatter

Figure 3.9 ***Flow cytometry plots demonstrating calibration of microvesicle gate***

(A) Human platelet-rich plasma, with platelet population indicated

(B) Following stimulation of plasma with A23187, a platelet microvesicle population is visible

(C) Upper forward scatter limit of the microvesicle population corresponds to 1.3µm sizing beads

Microvesicle background fluorescence as determined by matched isotype controls

Antibody isotype controls have traditionally been used in flow cytometry to distinguish "fluorescent-positive" from "fluorescent-negative" populations [398]. Staining with isotype controls of irrelevant specificity indicates the component of the fluorescence signal emitted by cells due to Fc receptor or non-specific antibody binding [399], in addition to the intrinsic auto-fluorescence of the cell. However, isotypes controls exhibit limitations and drawbacks. They are secreted by abnormal "hybridoma" cells and subtle differences in immunoglobulin structure can exist, despite being matched for subclass. Errors can also be introduced by differences in manufacturing protocols, fluorophore-antibody conjugate ratios and antibody concentrations. The appropriateness of isotype controls for cellular analysis has consequently been questioned [398–400].

When analysing microvesicles, these issues become more significant. Because of a smaller surface area, antibody binding to microvesicles is much less than that to cells. Considerable variations in the binding of isotype controls have been demonstrated and this would have a disproportionate effect on microvesicle quantification [400]. These problems may be exacerbated by the issues of their broad size range and overlap with machine noise. We therefore did not routinely use isotype controls in our quantification of microvesicles. However, it was important to demonstrate the absence of significant non-specific antibody binding.

Plasma samples were analysed for CD45+ (protein tyrosine phosphatase receptor type C), CD66b+ (CEACAM8) and CD105+ (endoglin) microvesicles. Triton X-100 detergent was then added to each sample, to identify non-vesicular events. The results were compared to plasma samples stained with matched isotype controls. Because of low numbers of microvesicles in healthy volunteers, this was necessarily performed on samples from burn patients. The number of fluorescent-positive events identified by isotype controls was low in each case (figure 3.10). We concluded that non-specific antibody binding was not a significant contribution to microvesicle fluorescence in our samples.

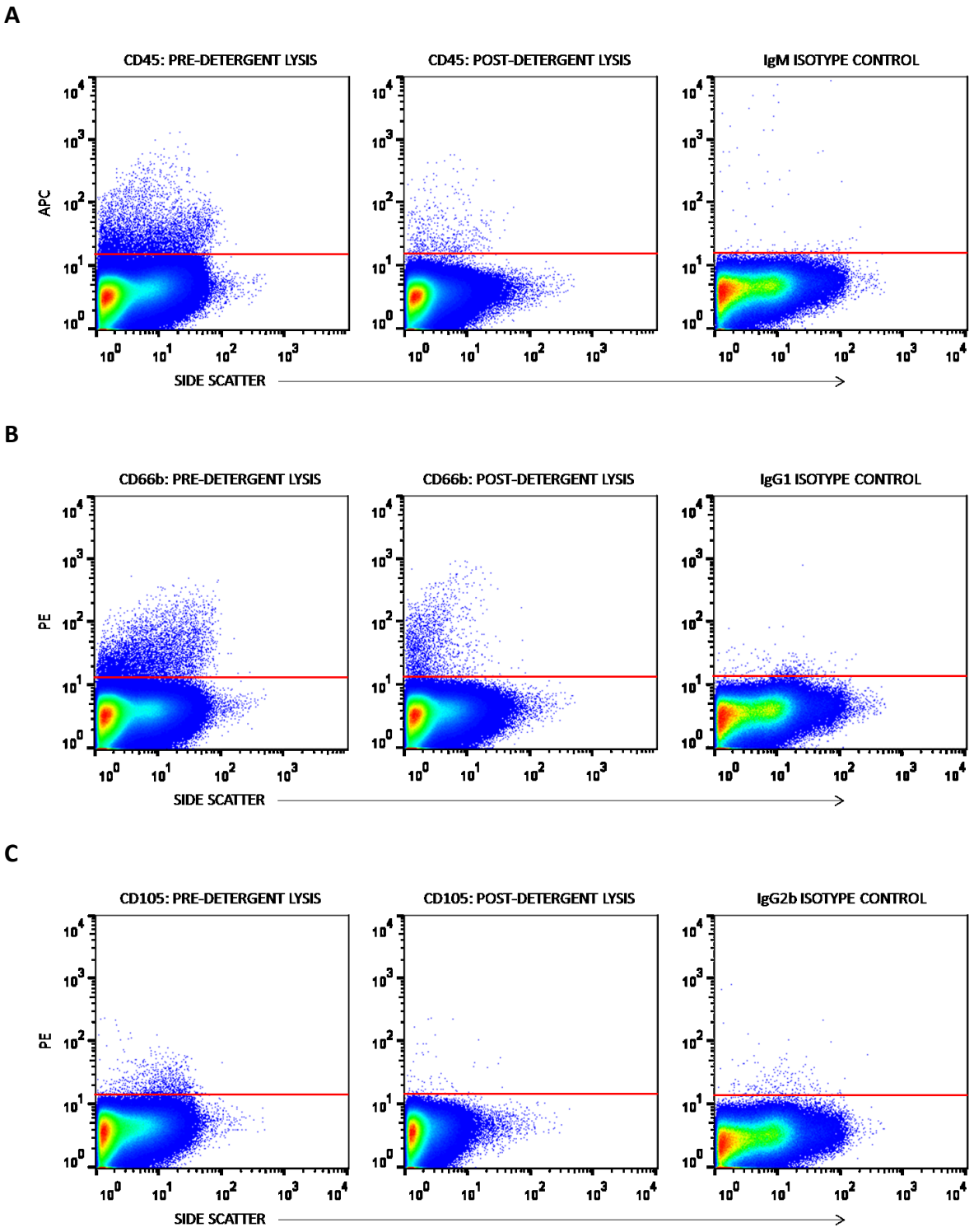


Figure 3.10 ***Cytometry plots of burn patient plasma samples labelled with antibodies to cell-surface markers and matched isotype controls***

Detergent-sensitive CD45+ (A), CD66+ (B) and CD105+ (C) microvesicle populations are demonstrated above red cut-off line. Staining with isotype controls does not demonstrate significant non-specific antibody binding

3.5 Results – Effect of sample processing and storage

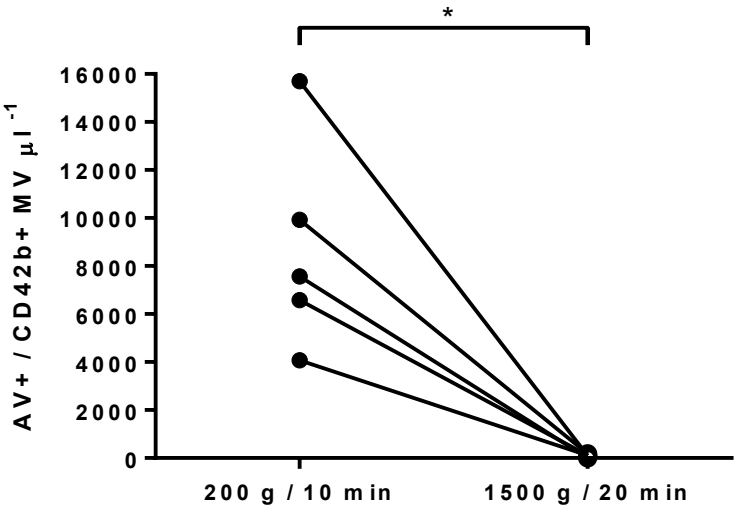
Centrifugation protocols that deplete platelets also result in significant loss of microvesicles

Several sources advocate multiple plasma centrifugation steps, of increasing speed, to remove as many platelets as possible [359,377]. One argument for this is to minimise generation of microvesicles from residual platelets when a sample is freeze-thawed [380,401]. However, other investigations have found that even a moderate speed centrifugation will result in some microvesicle loss [378]. We therefore investigated the effect of different centrifugation speeds on the microvesicle levels detected by our flow cytometer.

Healthy volunteer blood was centrifuged at 200g for 10 minutes to generate platelet-rich plasma (PRP). This was the minimum speed we could use to obtain a sufficient yield of plasma from whole blood. A further centrifugation at 1500g for 20 minutes was performed to generate platelet-poor plasma (PPP). PRP and PPP were analysed for platelet microvesicle content. Samples were used immediately after venepuncture to minimise *ex vivo* microvesicle production from platelets and events were size-gated to minimise the inclusion of platelets in the microvesicle counts. A second analysis was performed using neutrophil microvesicles generated according to the protocols developed in chapter 5. 400µl of neutrophil microvesicles (cell-free, mean 27,000 MV μl^{-1}) were added to 600µl of ultracentrifuged fresh human serum. This was centrifuged at 200g for 10 minutes, with half of the supernatant undergoing a further centrifugation at 1500g for 20 minutes.

The second centrifugation step resulted in a significant reduction of both platelet (figure 3.11A) and neutrophil (figure 3.11B) microvesicle counts. We therefore concluded that microvesicle levels and composition in PPP were unlikely to reflect those circulating *in vivo* and that PRP was a more suitable plasma preparation to investigate.

A



B

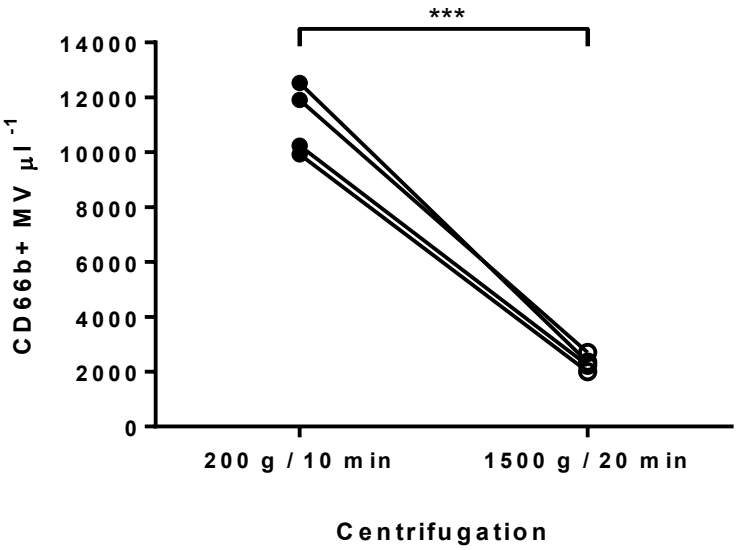


Figure 3.11 Effect of serial centrifugations on microvesicle counts

Platelet-derived (A) (n=5) and neutrophil-derived (B) (n=4) microvesicle counts are significantly reduced when centrifugation at 200g for 10 minutes is followed by further centrifugation at 1500g for 20 minutes

**p<0.05*

****p<0.001*

Paired t test

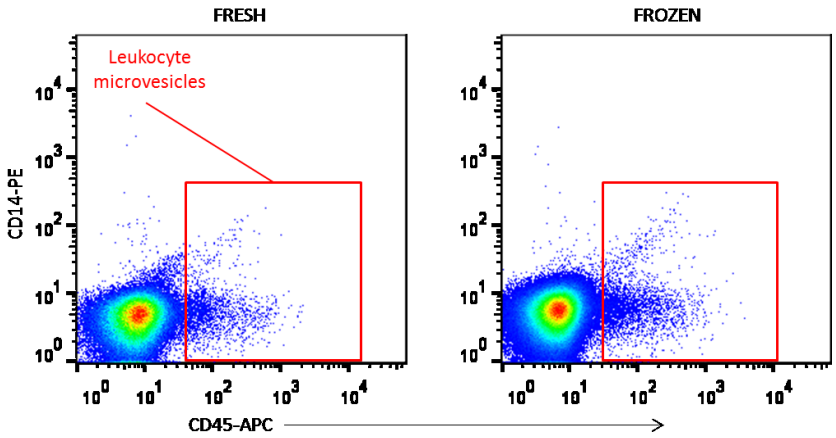
Leukocyte microvesicle detection levels in platelet plasma are minimally changed by one freeze-thaw cycle

Storage of microvesicles would have considerable advantages for studying patients with burn injuries. Such patients are admitted on an emergent basis and samples cannot always be collected during daylight hours. Presentation to non-specialist hospitals is also relatively rare. A reliable storage method would also facilitate multi-centre studies [365]. Several publications have shown that freezing of PRP and PPP increases platelet microvesicle counts [333,378,380]. Leukocyte and endothelial cell microvesicle counts have been shown to be stable after a freeze-thaw cycle [380], although this analysis was performed using PPP. Given the deleterious effects of higher centrifugation speeds as highlighted above, it was important to confirm that storage of PRP had no effect on detection of non-platelet microvesicles. Because of the very low circulating levels in healthy volunteers, this analysis necessarily included samples from patients.

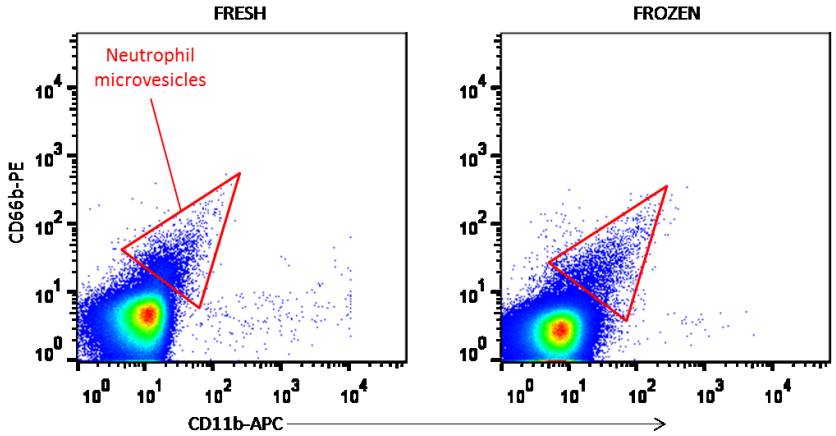
PRP was prepared as before and aliquots were frozen at -80°C. Fresh samples were analysed for leukocyte microvesicle content. Leukocyte microvesicles were defined using a similar method for platelet microvesicles, but CD45 was used as the surface marker. Neutrophil microvesicles were defined as CD11b+ / CD66b+. Frozen samples were thawed at room temperature before analysis using the same procedure; these results were then compared to the matched fresh sample.

The appearance of leukocyte (CD45+), monocyte-macrophage (CD45+ / CD14+) and neutrophil (CD11b+ / CD66b+) microvesicle populations were similar, when fresh and frozen-thawed samples were compared (figure 3.12, A and B). In contrast, platelet microvesicles are clearly altered by the freezing process, with a disappearance of AV-platelets and consequent loss of distinction between platelets and microvesicle populations. It appeared that freeze-thawing results in most platelet (CD42b+) events binding AV, presumably due to loss of membrane integrity and access of AV to the phosphatidylserine-rich inner face of the plasma membrane.

A



B



C

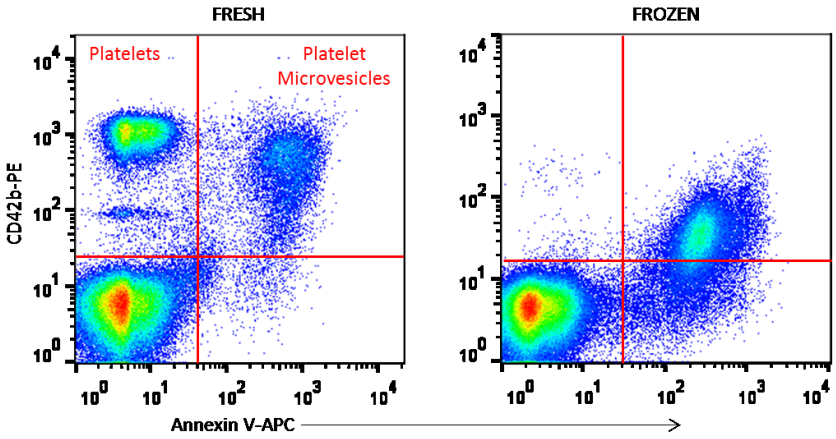
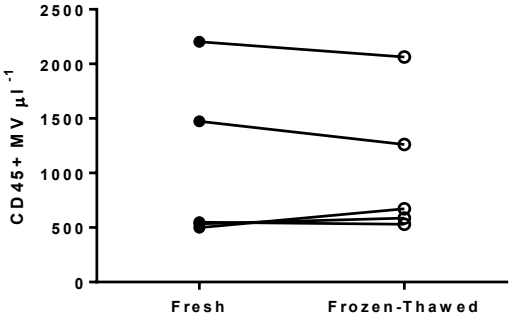


Figure 3.12 ***Effect of freeze-thaw cycle on leukocyte and platelet microvesicle plots***

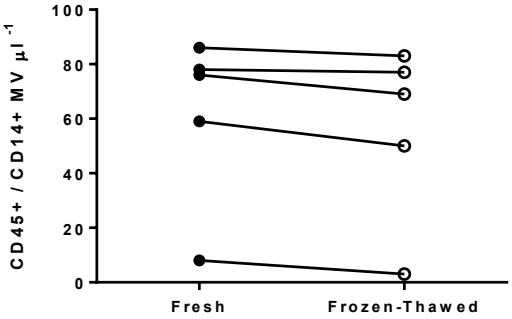
Plasma CD45+ / CD14+ (A) and CD11b+ / CD66b+ (B) microvesicle flow cytometry plots are similar when fresh and frozen-thawed samples are compared. AV+ / CD42b+ microvesicle plots are different when fresh and frozen-thaw samples are compared, with complete loss of platelets (C)

The total leukocyte microvesicle counts were minimally affected by the freeze-thaw cycle, with only minor increase or decreases in numbers observed (figure 3.13A). This was also the case for monocyte-macrophage microvesicles (figure 3.13B) and neutrophil microvesicles (figure 3.12C).

A



B



C

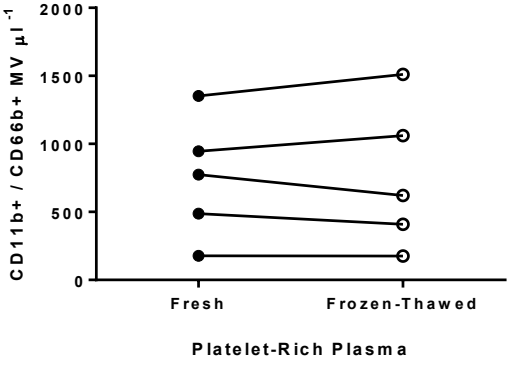


Figure 3.13 Effect of freeze-thaw cycle on leukocyte microvesicle counts

Total leukocyte (CD45+) (A), monocyte-macrophage (CD45+/CD14+) (B) and neutrophil (CD11b+/CD66b+) (C) microvesicle counts in platelet-rich plasma were minimally affected by one freeze-thaw cycle (n=5)

3.6 Discussion

In order to analyse microvesicles from patient samples, we undertook a series of flow cytometry-oriented optimisation and validation studies. Despite its versatility and precision for cell analysis, there is no universal standardised approach to using flow cytometry for microvesicle analysis, although this is an active and advancing aspect of research in this field [303,346,365,402]. We therefore sought to apply some of the techniques used by other researchers to our flow cytometer set up (BC Cyan ADP) to generate a robust, reliable method of analysing microvesicles, whilst identifying potential limitations.

First, we used calibrated sizing beads to establish some baseline characteristics of our cytometer. We found that when using a forward scatter trigger, we could detect and discriminate beads of 1.3µm. However, the forward scatter signal of smaller beads merged with background noise. When utilising a fluorescence trigger, this noise was reduced and it was possible to discern the beads of size 880nm. By incorporating the side scatter signal, the 490nm and 220nm beads could also be discriminated from each other. The cytometer behaviour observed here was largely to be expected. It is known that as particle size decreases, light scatter decreases in a non-linear fashion [347,368]. This explains why it was not possible to detect separate forward scatter signals for the smallest beads, even when noise was reduced by using a fluorescence trigger.

The performance of newer cytometers, for example the Gallios (Beckman Coulter) is improved by analysing forward scatter light over a relatively wide angle [346,358]. Cytometers that are designed specifically to analyse small particles, such as the A50 (Apogee), can distinguish beads of 100nm diameter [358,392]. However, our preliminary results did show that our cytometer was capable of detecting particles in the size range of microvesicles. They also demonstrated that background noise could be reduced considerably by avoiding use of forward scatter as an event acquisition trigger.

Having established that sheath-sample fluid refractive index matching was essential to avoid distortion of forward scatter, our next set of experiments assessed the feasibility of using a fluorescence trigger to detect microvesicles. This was an approach suggested by several

sources [349,367–370]. We began by investigating microvesicles released by RAW 264.7 cells, a murine leukaemic monocyte macrophage cell line. When fluorescent antibodies to CD11b were combined with a fluorescent trigger, the detection of microvesicles increased dramatically. This demonstrated the principle of using a dye to amplify an intrinsic property of the microvesicles. However, for it to be applicable to human samples, we needed a generic label that could be used for heterogeneous microvesicle populations. CFDA-SE was evaluated in the first instance, a cytoplasmic dye converted to a fluorescent form (CFSE) by intracellular esterases. We found that if the parent cells were stained prior to stimulation, a large increase in RAW microvesicle count could be detected following stimulation. However, this effect was markedly reduced when the microvesicles were stained directly. The reason for this was unclear. It is known that CFSE has a cytotoxic effect [403,404] but if this induced microvesicle release it might be expected in the unstimulated as well as stimulated cells. Another possible cause is that only a protein-conjugated form of CFSE (CFR2) is fully retained in the cytoplasm; this conjugation may not occur to the same extent in vesicles, due to selective packaging of contents.

We next trialled calcein-AM (C-AM), which had theoretical advantages over CFDA-SE in that it does not rely on protein binding and is retained in the cytoplasm to a greater extent [387–389]. Unfortunately, we could not demonstrate a benefit of using a C-AM/fluorescence trigger combination, when compared to a forward scatter trigger. In fact, the use of both parameters as a discriminator proved problematic, due to the sheer number of events detected. This could only be avoided by raising the threshold value, which is in itself an issue, as genuine particles of interest can be excluded [368]. In contrast, the use of a side scatter trigger enabled the consistent acquisition of data from plasma samples, without compromising the microvesicle count. Furthermore, the side scatter trigger resulted in a greater signal to noise ratio. A major advantage of the side scatter trigger is that it provides an intrinsic detection signal, minimally affected by the type of microvesicle or biological fluid. It also removes the need for a dedicated labelling step. We therefore concluded that side scatter was the best initial discriminator for analysing plasma samples with our Cyan. The principle remains promising however, and colleagues in our research group have had success using PKH26, a lipophilic dye [382], for *in vitro* investigations.

In order to distinguish microvesicles from non-vesicular particles, such as immune complexes, we investigated a detergent lysis method using TX-100. This procedure had previously been described [372]. The vast majority of the population we had identified as microvesicles proved sensitive to lysis, increasing our confidence in our results. A further advantage of this step was that it provided a suitable background fluorescence level for each antibody combination, as well as allowing subtraction of false positive events from the microvesicle total. A common approach when performing conventional flow cytometry is to use isotype control antibodies, to establish the non-specific background signal. However, this technique can represent a major source of error if used with microvesicles. Because the frequency of microvesicle antigens is low and varies randomly, variations in isotype binding can significantly affect microvesicle quantification [400]. Our analysis of burn patient samples did not reveal significant background fluorescence following labelling with isotype controls.

Because microvesicles, unlike cells, do not form a discrete population of similar dimensions on a flow cytometry plot, it is necessary to use a cut-off value of some sort. This is commonly performed by using polystyrene sizing beads [365]. However, these refract light to a greater extent than membrane-bound particles [366]. We therefore created a biological standard by stimulating platelets to generate microvesicles, as described by Ayers *et al* [380], the upper limit of which matched the forward scatter of 1.3µm beads. This was advantageous as it would not be practical to generate the microvesicle population for every experiment.

The only way to reliably differentiate microvesicles from small platelets on the basis of size would be to reduce the forward scatter value we were using as a cut-off, sacrificing sensitivity for specificity. As an additional discriminator, platelet-specific surface markers are commonly used in combination with annexin V [359,365,378,392] and this was the approach we used. Annexin V binds to phosphatidylserine, externalised during the vesiculation process [390]. However, some studies have suggested that only a minority of platelet microvesicles bind annexin V [352]. Therefore, the numbers of platelet microvesicles are likely to be underestimated if small platelets are excluded with any degree of confidence. The problems faced when quantifying platelet microvesicles continue when the effect of centrifugation is considered. As discussed above, depletion of platelets from plasma is advocated to

minimise *ex vivo* microvesicle production. However, we found that even the production of PPP, which requires less centrifugation than PFP, removed the majority of microvesicles. This was consistent with other published data [378]. We concluded that none of the plasma preparations described was likely to yield representative microvesicle counts. One caveat is that platelet microvesicles from inflamed patients might be smaller and sediment at higher centrifugal forces, which could allow greater separation using centrifugation. Freeze-thaw analysis of ionophore stimulated-plasma could be used to test this idea. In contrast to platelets, leukocytes are separated from plasma by low-speed centrifugation. *Ex vivo* leukocyte microvesicle production should therefore be minimised, whilst preserving microvesicle yield.

The last issue we addressed in this chapter was the storage of microvesicles. Leukocyte microvesicles have previously been shown to tolerate a freeze-thaw cycle when stored in PPP [380] but we wanted to confirm that this would be the case when PRP was stored. Endothelial cell microvesicle counts have also been shown to be minimally changed by freezing [380]. Endothelial cells circulate at very low levels, with a mean level of 0.4 per millilitre in health, vs 7 x 10^6 leukocytes per millilitre [405]. There is therefore minimal potential for *ex vivo* endothelial microvesicle generation.

Chapter 4

Circulating microvesicles in patients with burn injuries

Summary

The period following a severe burn is characterised by marked systemic inflammation. Microvesicles are thought to play a key role in propagating the inflammatory response associated with other conditions such as sepsis but they have not been investigated in patients with burn injuries. We sought to establish whether microvesicle release by leukocytes and endothelial cells, as part of the inflammatory response to burn injury, would be altered in comparison to that seen in healthy volunteers. We also sought to identify any changes in microvesicle profile over the days following burn injury.

Blood samples were taken from patients admitted to the Burn Intensive Care Unit, within 24 hours of injury. Samples were also taken from healthy volunteers (negative controls) and patients with severe sepsis (positive controls). Plasma was obtained by centrifugation and stored for subsequent analysis. An optimised flow cytometry protocol was used to quantify microvesicles derived from leukocytes, neutrophils, monocytes/macrophages and endothelial cells. Clinical information including laboratory blood results, severity of illness scores and outcome data was also recorded.

On day zero, elevated circulating levels of all microvesicle populations were evident in burn patients, in comparison to healthy volunteers. These counts then decreased rapidly over the subsequent 48 hours. Leukocyte counts did not correlate with microvesicle levels and decreased at a slower rate following injury, implying that increased microvesicle numbers are not a simple reflection of leukocytosis. A strong correlation was found between total leukocyte microvesicle levels and the ABSI and BOBI injury severity scores. Leukocyte-derived microvesicles were also significantly elevated in non-survivors of burn injury.

The results suggested that leukocyte and endothelial cell-derived microvesicles may be important to the systemic inflammatory response that occurs following a severe burn. They provide targets for investigation in mechanistic studies and show potential for use as prognostic markers in burn patients.

4.1 Background

The systemic inflammatory response precipitated by a severe burn leads to widespread vascular leak, hypermetabolism, hypercatabolism and a propensity towards multi-organ failure [113,406]. This inflammatory response is thought to be mediated at least in part by circulating cytokines [103,234,239,248]. An accumulating body of evidence supports the idea that in other inflammatory conditions, such as sepsis, microvesicles play a key role in the immuno-vascular response [3,318,319,329]. However, the production of microvesicles following severe burns or contribution to post-burn inflammation has not previously been investigated.

Following tissue damage such as that caused by burn, endogenous DAMPs are released, which activate resident macrophages [193]. This initiates recruitment of neutrophils to the site of damage, followed by secondary recruitment of circulating monocytes [191,192,207,208]. Endothelial activation, which upregulates surface adhesion molecules, is key to the recruitment of inflammatory leukocytes [203,205]. These processes are mediated by cytokines and chemokines such as TNF-α, IL-6 and MCP-1, which are elevated following burn injury [194,196,197]. Microvesicles are elicited by multiple inflammatory stimuli, including DAMPS and cytokines, and therefore their release from myeloid leukocytes and endothelial cells should be expected in the acute post-burn phase. The potential sources of circulating leukocyte and endothelial microvesicles in burn patients can be categorised into two groups. Firstly, the areas of ischaemic and inflamed tissue that surround coagulated necrotic tissue – Jackson's zones of stasis and hyperaemia [17]. Secondly, remote organ systems injured by (and perpetuating) the systemic inflammatory response – in particular, the cardiovascular system, lungs and kidneys [118].

Leukocyte-derived microvesicles, circulating at low levels in health, have been found at elevated levels in patients with other inflammatory conditions such as sepsis and major trauma [313,330,385,407]. *In vitro* studies have suggested these microvesicles can act as inflammatory mediators; neutrophil-derived microvesicles for instance activate endothelial cells [318,319]. Monocyte-derived microvesicles have been shown to exhibit autocrine activation of monocyte/macrophages [311] and increase nitrosative stress in endothelial cells

[408]. It was therefore relevant to the post-burn inflammatory response and organ dysfunction to establish if these populations could be detected in patients with burn injuries.

Endothelial microvesicles have also been associated with inflammation, both as mediators and markers [334,409]. In the context of end-stage renal failure, elevated microvesicle levels were associated with clinical and experimental evidence of endothelial dysfunction [410]. Endothelial microvesicles are also capable of inducing pulmonary inflammation by initiating a cytokine cascade [411]. *In* vitro studies have suggested that they can act as paracrine mediators, inducing an inflammatory response in "naïve" endothelium [326]. This was found to be a p38-dependent process. It is not clear, however, that elevated levels of endothelial microvesicles are associated with a poorer outcome in patients. In severe sepsis, increased counts correlated with improved survival [343] and some argue endothelial microvesicles may have protective effects [412].

As part of this observational study, we also recorded clinical data, including outcome and severity of illness scores. A number of clinical scoring systems have been devised for patients with burn injuries. The abbreviated burn severity index (ABSI) is in widespread use and is based on a combination of five variables [72]. Although published in 1982, recent reviews have established that it remains methodologically sound and robust as a predictive tool [67,73,80]. A more recent system was developed by the Belgian Outcome in Burn Injury (BOBI) group. This has been validated externally [79] and is also robust [67]. Both of these systems use hospital mortality as their outcome.

We included in our analysis samples from healthy volunteers and patients with severe sepsis. We expected to find low levels of circulating leukocyte and endothelial microvesicles in healthy participants [3,334,413] and this was our negative control group. In contrast, elevated levels of both populations were expected to be found in sepsis patients [330,343] and this group served as a positive control.

4.2 Aims

The specific aims in this chapter were as follows:

1. To characterise leukocyte and endothelial-derived microvesicle populations in patients with severe burn injuries
2. To relate the microvesicle analysis to clinical data including severity of illness scores and patient outcomes

4.3 Protocols

Patient selection

Our study was approved by a local research ethics committee (NRES Committee London – Camden & Islington, reference 12/LO/1543) and our Department of Research & Development (reference C&W12/101). All participants were over 18 years of age and were excluded if pharmacologically immunosuppressed pre-injury, or known to be infected with blood-borne viruses. A prospective power calculation was not possible because the "effect size" was unknown and previous research led us to suspect microvesicle counts would not be normally-distributed [414]. However, based on our admission rates, we expected that it would be possible to recruit 15-20 burn patients during the study. All patients admitted to the Burns Intensive Care Unit at Chelsea and Westminster Hospital were screened for eligibility. Patients were excluded if they had injuries judged too extensive for active treatment.

Healthy volunteers were recruited from laboratory staff at Imperial College London and from clinical staff at Chelsea and Westminster Hospital Intensive Care Unit. Volunteers were excluded if they were suffering from intercurrent illness or if they had undergone venesection within seven days. Patients with severe sepsis, as defined by the American College of Chest Physicians/Society of Critical Care Medicine (ACCP/SCCM) Consensus Conference Committee, were recruited from the General Intensive Care Unit at Chelsea and Westminster Intensive Care Unit.

Sampling

Initial sampling of burn patients occurred within 24 hours of injury. Further samples were taken on days two and five. Blood (18mls) was obtained from arterial cannulas, or central venous catheters if arterial blood was unavailable. A single sample was taken from the healthy volunteers and severe sepsis patients, who were sampled within 48 hours of identification of sepsis. The sepsis group included both community and hospital-acquired infections, with variable prodrome duration. Blood was transferred into lithium heparin Vacutainers and processed immediately for storage at -80^{o}C. Samples were subsequently analysis.

Analysis

Thawed platelet-rich plasma (PRP) was stained with fluorescent antibodies, using the protocol explained in chapter 3, in the following combinations:

1. CD45-APC / CD14-PE
2. CD11b-APC / CD66b-PE
3. CD105-PE
4. CD106-APC / CD62e-PE

Fluorophores were limited to PE and APC to standardise sensitivity and minimise spectral overlap.

4.4 Results

Patient demographics and baseline characteristics

Details of the burn injuries group are shown in table 4.1. The parameters included are those required to calculate the Abbreviated Burn Severity Index (ABSI) and Belgian Outcomes in Burn Injury (BOBI) scores. Burn patients were recruited to the study as soon after admission as possible and all participants were sampled within 24 hours of injury. However, as the majority of patients were transferred from other hospitals, a short delay was unavoidable. The mean time from injury to sampling was 11 ± 4.9 hours.

A total of 16 burn patients were recruited during the study, with a median burn size of 28% of Total Body Surface Area (TBSA). The median ABSI value was 8, corresponding to a predicted mortality of 30-50% [73]. Actual hospital mortality for the cohort was 31%, with all five deaths occurring in ICU following a planned withdrawal of care. Significant differences between survivors and non-survivors were observed for age and the presence of inhalation injury.

Table 4.2 demonstrates the clinical data for patients with severe sepsis. A total of 15 participants were recruited. 53% had a pulmonary source of infection, with most of the remainder having an abdominal source. The median Acute Physiology and Chronic Health Evaluation (APACHE) II score was 21, equivalent to a predicted mortality of 30-40% [415]. Actual hospital mortality was 33%. There were no statistically significant differences between the burn and sepsis patients in terms of age, gender, ICU length of stay, hospital length of stay, or mortality rates.

Burn Patients	**All (*n* = 16)**	**Survivors (*n* = 11)**	**Non-survivors (*n* = 5)**	***p***
Male sex, n (%)	11 (69)	7 (64)	4 (80)	1
Age, mean ± SD (years)	47 ± 23	38 ± 19	66 ± 20	0.02
Inhalation injury present, n (%)	8 (50)	3 (27)	5 (100)	0.03
Full thickness burn present, n (%)	11 (69)	6 (55)	5 (100)	0.12
Burn TBSA %, median (IQR)	28 (18-36)	30 (18-35)	25 (16-39)	0.98
ABSI, median (IQR)	8 (7-8)	7 (6-8)	10 (8-12)	0.03
BOBI, median (IQR)	3 (1-5)	2 (1-3)	6 (5-7)	0.001
Days in ICU, median (IQR)	15 (7-37)	15 (4-32)	12 (5-79)	0.98
Days in hospital, median (IQR)	42 (18-77)	54 (27-96)	12 (5-79)	0.22

Table 4.1 ***Demographics data for burn patients***

SD – standard deviation; TBSA – Total Body Surface Area; IQR – interquartile range; ABSI – Abbreviated Burn Severity Index; BOBI – Belgian Outcome in Burn Injury

Sex, presence of inhalation injury and presence of full thickness burn were compared with Fisher's exact test. Ages were compared using unpaired t test. TBSA, ABSI, BOBI and lengths of stay were compared using Mann-Whitney test

Sepsis Patients	**All (n = 15)**
Male sex (%)	9 (60)
Age, mean ± SD (years)	58 ± 16
Source of sepsis, n (%)	
Pulmonary	8 (53)
Abdominal	6 (40)
Soft tissue	1 (7)
APACHE II, mean ± SD	20 ± 9
Days in ICU, median (IQR)	11 (7-22)
ICU mortality, n (%)	4 (27)
Days in hospital, median (IQR)	34 (23-145)
Hospital mortality, n (%)	5 (33)

Table 4.2 ***Demographics data for sepsis patients***

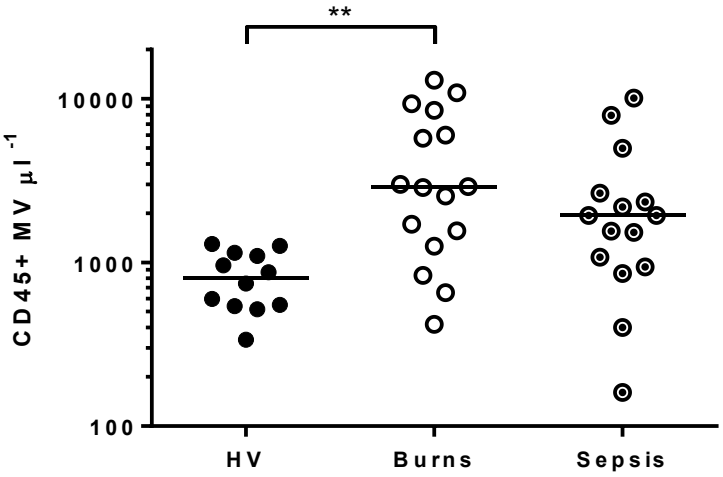
SD – standard deviation; APACHE II - Acute Physiology and Chronic Health Evaluation II; IQR – interquartile range

Circulating leukocyte-derived microvesicle populations are elevated in patients with severe burn injuries

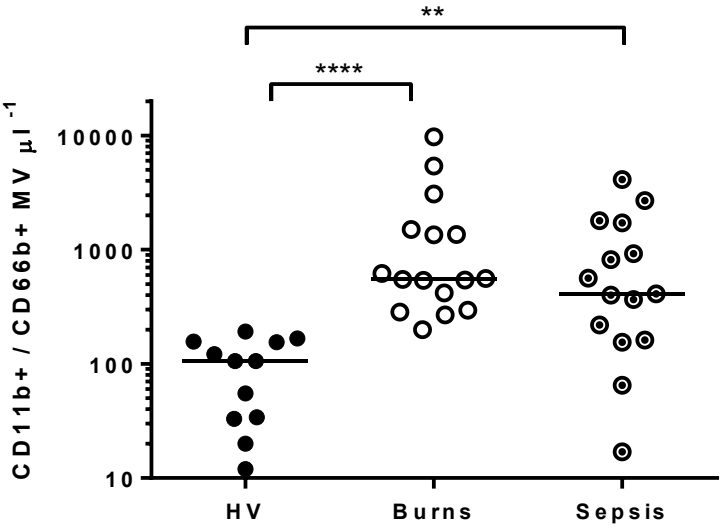
Given the onset of the post-burn inflammatory response is known to be rapid [406,416] and our interest in the role of MV in propagation of systemic inflammation, our initial focus was the acute post-burn phase. Leukocyte-derived microvesicles were quantified within 24 hours of burn injury. Sampling, processing, storage and analysis were performed using the protocols described in chapter 3. The results were compared to those from healthy volunteers and patients with severe sepsis.

Patients with burn injuries had significantly elevated levels of total leukocyte-derived (CD45+) microvesicles within 24 hours of admission compared to healthy volunteers (Fig 4.1A). However, levels of CD45+ microvesicles in sepsis patients were not significantly different from those in burns patients. Neutrophil-derived (CD11b+/CD66b+) microvesicles were elevated in burn patients and sepsis patients compared to healthy volunteers (figure 4.1B), with no significant difference between the burn and sepsis groups. By contrast, monocyte/macrophage-derived (CD45+/CD14+) microvesicles in burn patients were significantly higher than in both the healthy volunteer and the sepsis groups (figure 4.1C).

A


**
10000
1000
100
CD45+ MV µl-1
HV
Burns
Sepsis

B


**
****
10000
1000
100
10
CD11b+ / CD66b+ MV µl-1
HV
Burns
Sepsis

C

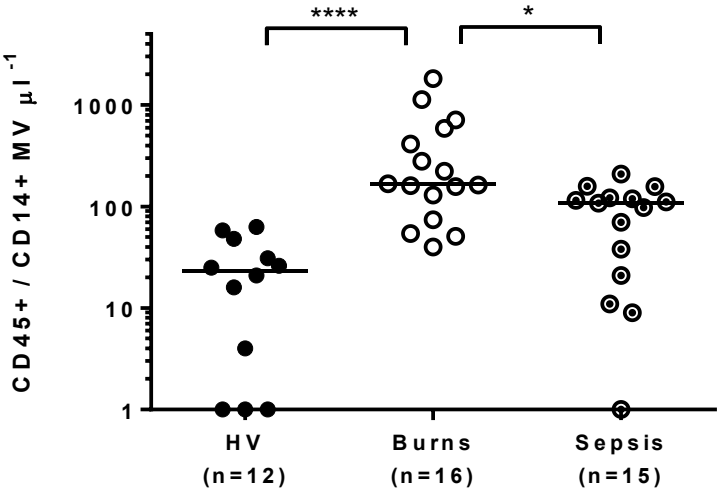

****
*
1000
100
10
1
CD45+ / CD14+ MV µl-1
HV
(n=12)
Burns
(n=16)
Sepsis
(n=15)

Figure 4.1 ***Comparison of circulating leukocyte microvesicle levels in healthy volunteers, patients with burn injuries and patients with severe sepsis***

Blood was sampled from burn patients within 24 hours of injury and from sepsis patients within 48 hours of identification of sepsis

(A) Circulating leukocyte (CD45+) microvesicle levels are significantly elevated in patients with burn injuries, in comparison to healthy volunteers (HV). Levels in patients with severe sepsis are not significantly different from those found in burn patients

(B) Circulating neutrophil (CD11b+/CD66b+) microvesicle levels are significantly elevated in patients with burn injuries and severe sepsis, in comparison to healthy volunteers (HV). Levels found in burn patients are not significantly different from those found in sepsis patients

(C) Circulating monocyte/macrophage (CD45+/CD14+) microvesicle levels are significantly elevated in patients with burn injuries, in comparison to both healthy volunteers (HV) and patients with severe sepsis

Kruskal-Wallis test with Dunn's multiple comparisons test

**p<0.05*

***p<0.01*

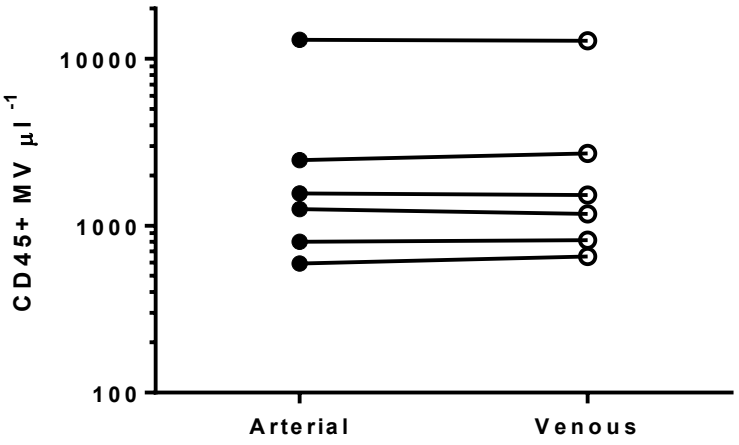
*****p<0.0001*

Leukocyte-derived microvesicle levels in burn patients are similar in arterial and venous circulations

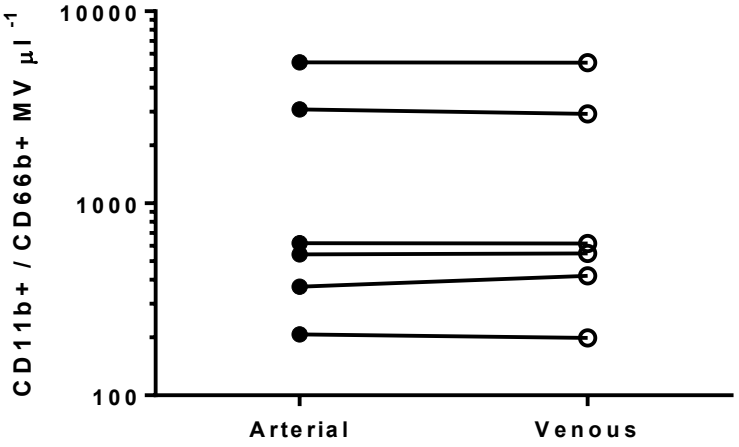
Animal and human studies suggest that clearance of microvesicles occurs rapidly, which implies that microvesicles are continuously produced [393,417,418]. Uptake is primarily performed in the liver and spleen by macrophages [419,420]. Production of microvesicles, in the context of burns, may occur at sites of injury but also in remote organs such as the lungs. Microvesicle levels may therefore vary according to sampling site. Paired samples of arterial and venous blood were taken simultaneously from burn patients, within 24 hours of injury. Leukocyte-derived microvesicles were subsequently quantified.

No significant differences were found between arterial and venous samples, in terms of total leukocyte-derived, neutrophil-derived or monocyte/macrophage-derived microvesicles (figure 4.2A-C). This suggested that the circulating microvesicle counts reflected a steady-state between production and uptake that was relatively consistent throughout the circulation.

A


10000
1000
100
CD45+ MV µl-1
Arterial
Venous

B


10000
1000
100
CD11b+ / CD66b+ MV µl-1
Arterial
Venous

C

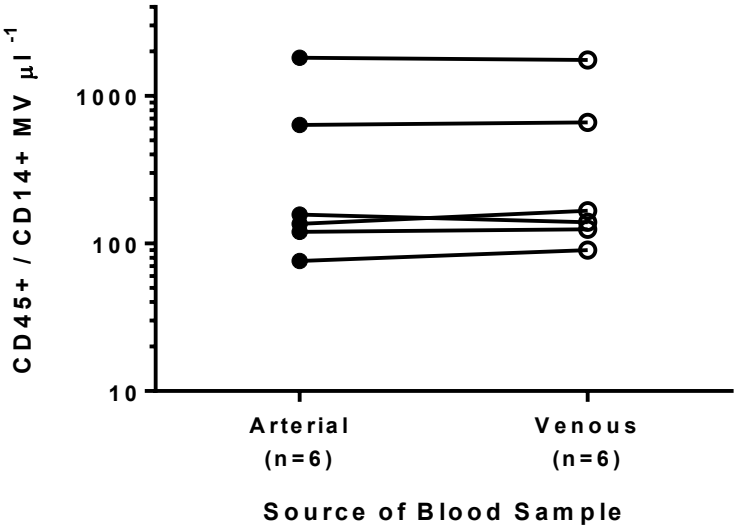

1000
100
10
CD45+ / CD14+ MV µl-1
Arterial
(n=6)
Venous
(n=6)
Source of Blood Sample

Figure 4.2 ***Comparison of post-burn leukocyte microvesicle counts in paired arterial and venous samples***

Blood was sampled simultaneously from indwelling arterial and central venous catheters, within 24 hours of burn injury

No significant difference in leukocyte (A), neutrophil (B) or monocyte/macrophage (C) microvesicle levels, between arterial and venous samples

Wilcoxon matched-pairs signed rank test
(n=6)

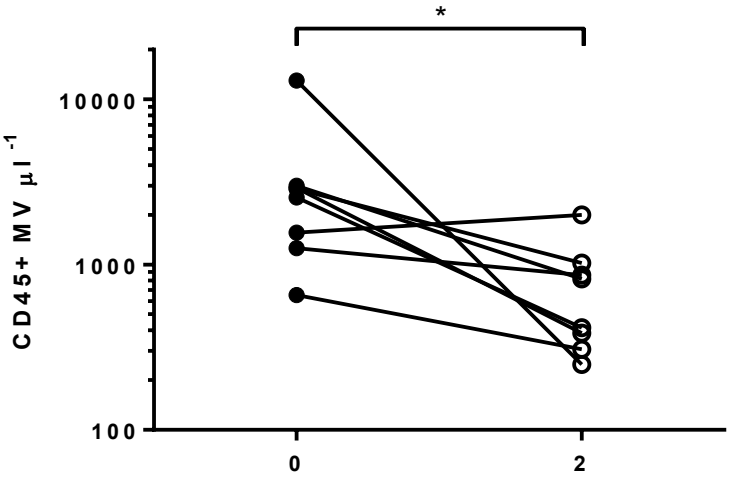
Circulating leukocyte-derived microvesicle levels decrease by day two post-injury

Having identified increased quantities of circulating leukocyte-derived microvesicles in the first 24 hours following burn injury, we next sought to characterise changes in this profile over time. Studies investigating temporal changes in cytokine levels tend to show an early peak after burn injury with a decrease over time [103,421], although this is not universal [406]. In contrast, some clinical parameters such as temperature and leukocyte count remain persistently elevated for at least 20 days post-injury [422].

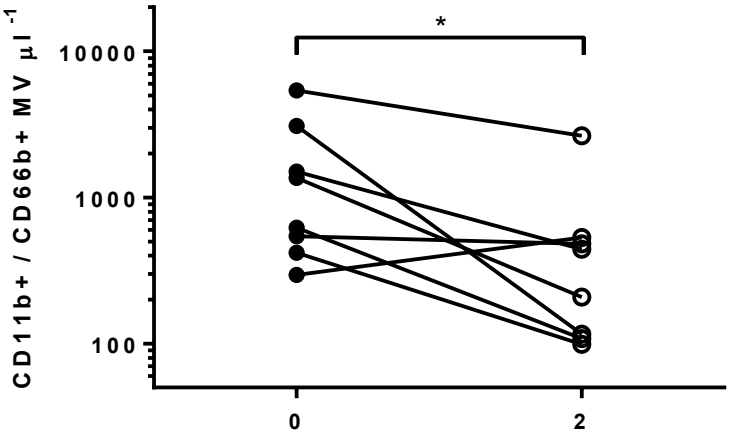
To evaluate early post-burn microvesicle kinetics, repeat blood samples were taken where possible. Paired analyses of samples from day zero and day two post-burn (*n*=8) indicated that the numbers of leukocyte-derived microvesicles declined significantly in all subpopulations. The median total leukocyte-derived MV count reduced from 2711 (1335-2977) MV μl^{-1} to 617 (327-983) MV μl^{-1} (figure 4.3A). Median neutrophil-derived MV levels reduced from 989 (450-2686) MV μl^{-1} to 325 (110-520) MV μl^{-1} (figure 4.3B). The median monocyte/macrophage MV count fell from 223 (158-996) MV μl^{-1} to 60 (3-185) MV μl^{-1} (figure 4.3C). Total leukocyte and neutrophil cell counts were also significantly lower at day two post-burn. However, the magnitude of reduction was much smaller in each case; mean leukocyte cell counts fell from 18.8 ± 8.5 $x10^6$ ml^{-1} to 14 ± 6.2 $x10^6$ ml^{-1} (figure 4.4A) and neutrophil counts decreased from 14.6 ± 6.9 $x10^6$ ml^{-1} to 10.8 ± 5.4 $x10^6$ ml^{-1} (figure 4.4B) Circulating monocyte counts were not significantly lower at day two (Figure 4.4C).

Samples were also available from day five post-injury but due to the relatively low *n* number of 5, these were not included in the statistical analysis. Median levels of CD45+ and CD45+/CD14+ microvesicles were the same at day five as day two, whereas median CD11b+/CD66b+ microvesicle counts were further reduced at day five (data not shown).

A


*
10000
1000
100
CD45+ MV µl-1
0
2

B


*
10000
1000
100
CD11b+ / CD66b+ MV µl-1
0
2

C

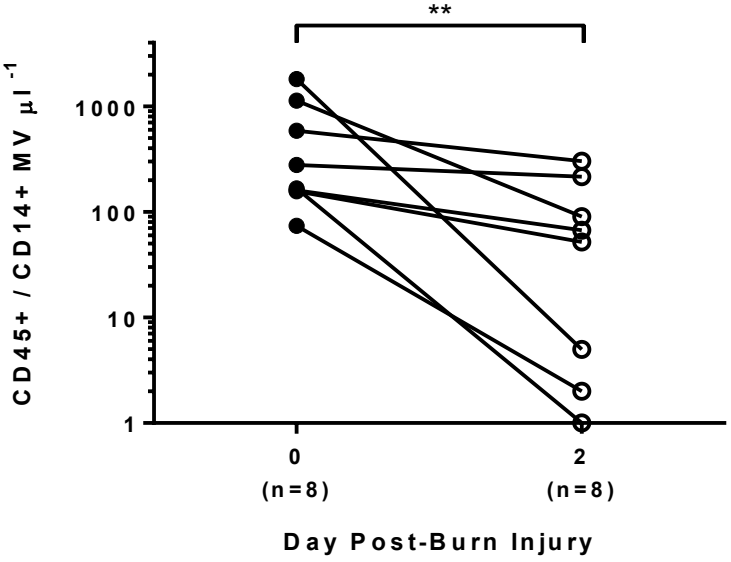

**
1000
100
10
1
CD45+ / CD14+ MV µl-1
0
(n=8)
2
(n=8)
Day Post-Burn Injury

Figure 4.3 ***Change in circulating leukocyte microvesicle levels over time following burn injury***

Blood was sampled on day zero (n=8) and day two (n=8)

(A) Significant decrease in leukocyte (CD45+) microvesicle levels by day two post-burn injury

(B) Significant reduction in neutrophil (CD11b+/CD66b+) microvesicles by day two post-burn injury

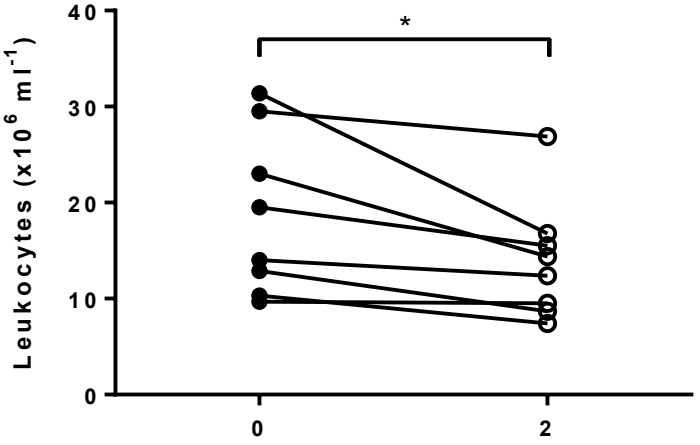
(C) Significant decrease in monocyte/macrophage (CD45+/CD14+) microvesicles by day two post-burn injury

Wilcoxon matched-pairs signed rank test

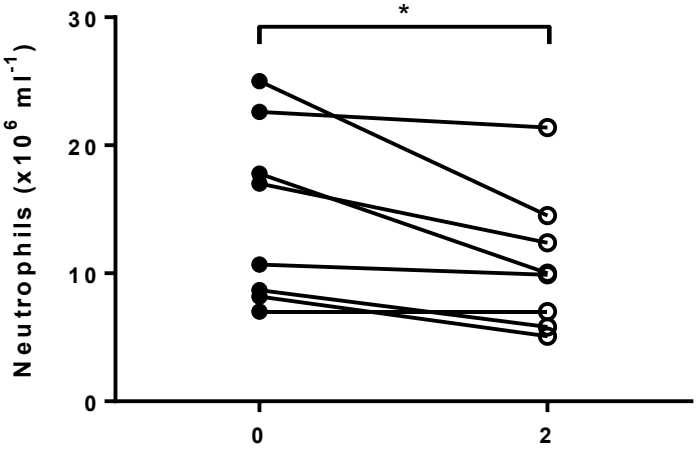
**p<0.05*

***p<0.01*

A


Leukocytes (x10^6 ml^-1)
40
30
20
10
0
*
0
2

B


Neutrophils (x10^6 ml^-1)
30
20
10
0
*
0
2

C

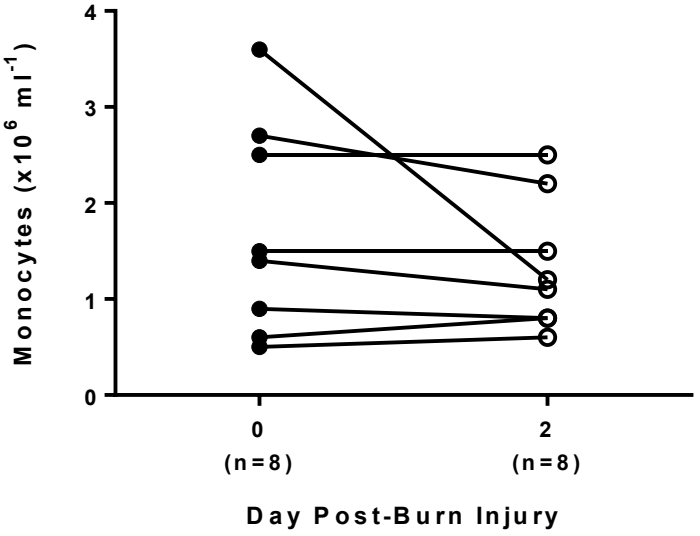

Monocytes (x10^6 ml^-1)
4
3
2
1
0
0
(n=8)
2
(n=8)
Day Post-Burn Injury

Figure 4.4 ***Change in leukocyte cell counts over time following burn injury***

Cell counts were recorded from clinical laboratory analysis of blood sampled on day zero (n=8) and day two (n=8) post-injury

(A) Significant reduction in total leukocytes by day two post-burn

(B) Significant reduction in neutrophils by day two post-burn

(C) Similar levels of monocytes at day zero and day two

Paired t test

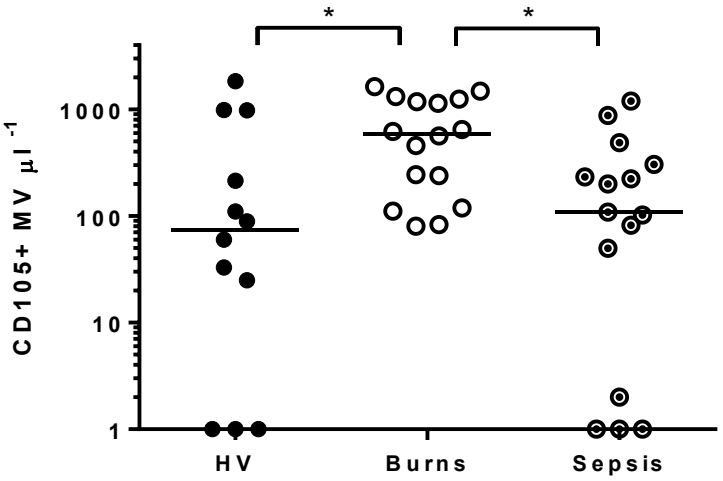
Circulating endothelial cell-derived microvesicle populations are elevated in patients with severe burn injuries

We next investigated endothelial cell-derived microvesicles because of evidence linking them to endothelial dysfunction in sepsis [343], trauma [423] and related organ dysfunction [410,411]. In addition to mediating pro-inflammatory states, they may also have clinical utility as biomarkers [332,334,409].

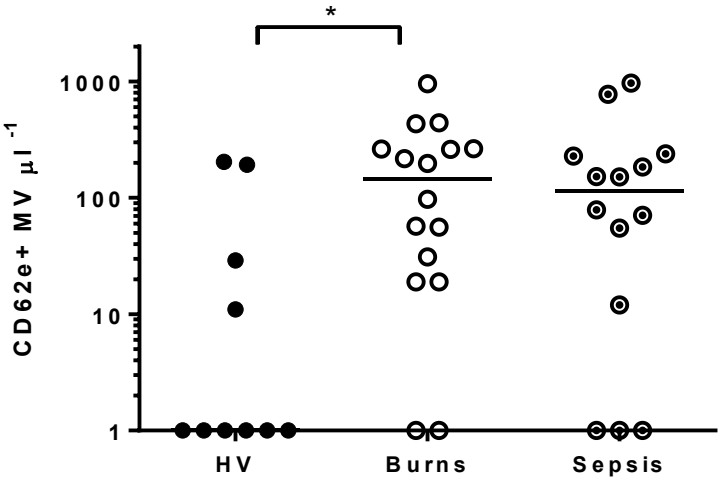
Because of the known heterogeneity in endothelial microvesicle surface molecule expression [331,334,424], antibodies to three separate markers were selected: CD105 (endoglin), CD62e (E-selectin) and CD106 (VCAM-1). CD105 is constitutively expressed by endothelial cells [425], whereas CD62e (E-selectin) and CD106 (VCAM-1) are upregulated following endothelial activation [418,426].

CD105+ microvesicles were found to be significantly elevated in burns patients, in comparison to both healthy volunteers and patients with severe sepsis (figure 4.5A). In contrast to the uniformly low leukocyte microvesicle levels in healthy volunteers, levels of CD105+ in some individuals were higher than the patient group mean values. Levels of CD62e+ and CD106+ microvesicles were significantly higher in burn patients, as compared to healthy volunteers but were not significantly different from sepsis patients (figure 4.5B and 4.5C respectively).

A


*
*
1000
100
10
1
CD105+ MV µl-1
HV
Burns
Sepsis

B


*
1000
100
10
1
CD62e+ MV µl-1
HV
Burns
Sepsis

C

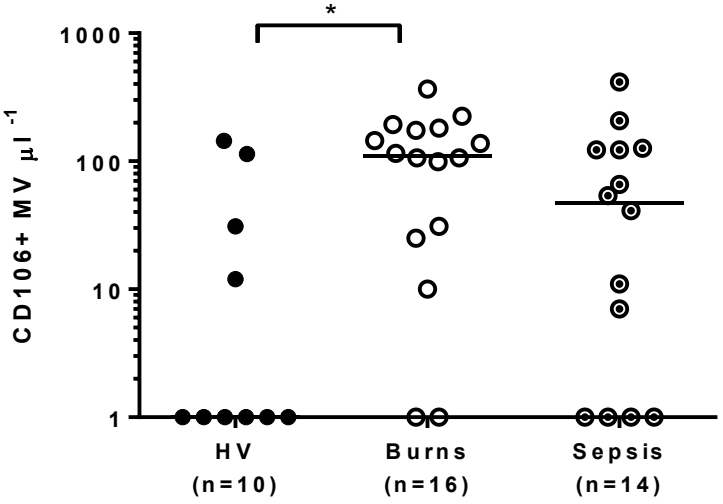

*
1000
100
10
1
CD106+ MV µl-1
HV
(n=10)
Burns
(n=16)
Sepsis
(n=14)

Figure 4.5 ***Comparison of circulating endothelial levels in healthy volunteers, patients with burn injuries and patients with severe sepsis***

Blood was sampled from burn patients within 24 hours of injury and from sepsis patients within 48 hours of identification of sepsis

(A) Circulating CD105+ microvesicle levels are significantly elevated in patients with burn injury, in comparison to both healthy volunteers and patients with severe sepsis

(B) Circulating CD62e+ microvesicle levels are significantly elevated in patients with burn injury, in comparison to healthy volunteers. Levels found in patients with severe sepsis are not significantly different from those found in burn patients

(C) Circulating CD106+ microvesicle levels are significantly elevated in patients with burn injury, in comparison to healthy volunteers. Those found in patients with severe sepsis are not significantly different from those found in burn patients

Kruskal-Wallis test with Dunn's multiple comparisons test

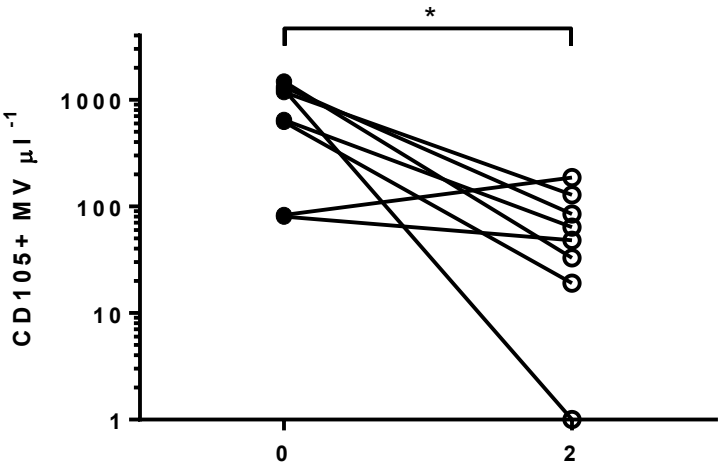
**p<0.05*

Circulating endothelial cell-derived microvesicles rapidly decrease post-burn injury

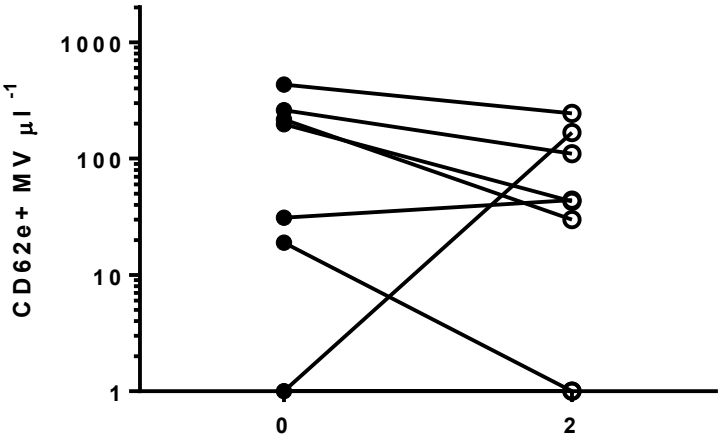
As was the case with circulating leukocyte microvesicles, endothelial microvesicles also decreased by day two post-burn. This reduction was statistically significant in the case of CD105+ microvesicles, (figure 4.6A). The reduction in CD62e+ and CD106 microvesicle counts was less consistent and not statistically significant (figure 4.6B and C respectively).

Levels of the three endothelial microvesicle populations were similar at day five (*n*=5) to those found at day two (data not shown). Circulating endothelial cells were not quantified.

A


*
CD105+ MV µl-1
1000
100
10
1
0
2

B


CD62e+ MV µl-1
1000
100
10
1
0
2

C

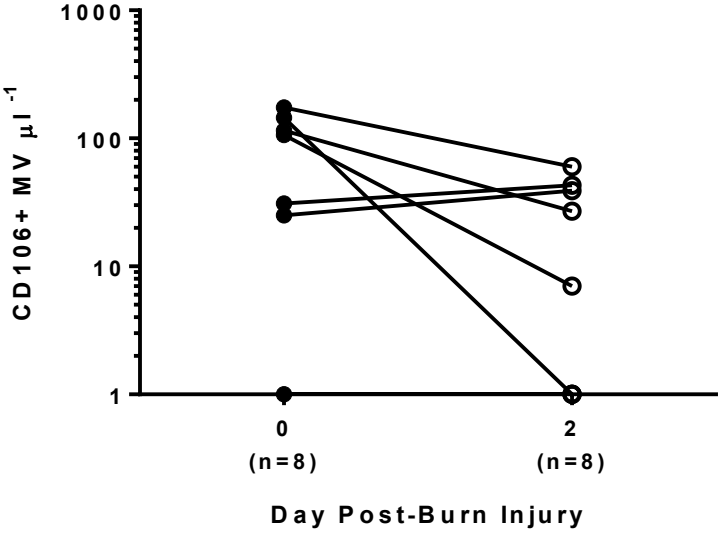

CD106+ MV µl-1
1000
100
10
1
0
(n=8)
2
(n=8)
Day Post-Burn Injury

Figure 4.6 ***Change in circulating endothelial microvesicle levels over time following burn injury***

Blood was sampled on day zero (n=8) and day two (n=8) post-burn

(A) Significant decrease in CD105+ microvesicle levels by day two

(B) Statistically non-significant reduction in CD62e+ microvesicles by day two

(C) Statistically non-significant reduction in CD106+ microvesicles by day two

Wilcoxon matched-pairs signed rank test

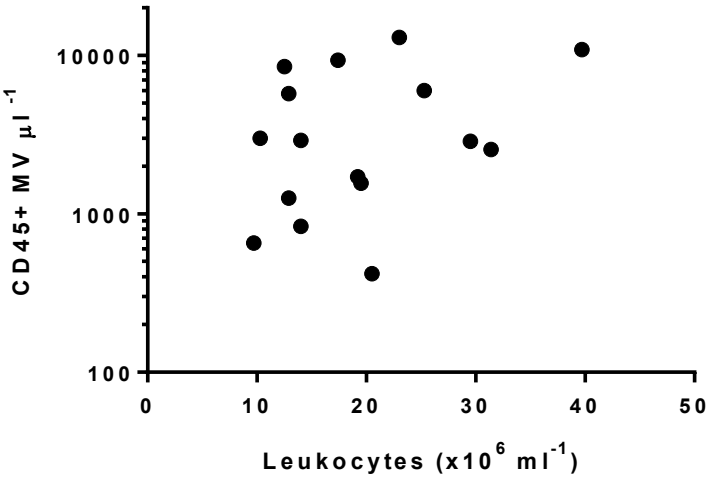
**p<0.05*

Levels of circulating leukocyte-derived microvesicles do not correlate with circulating leukocytes

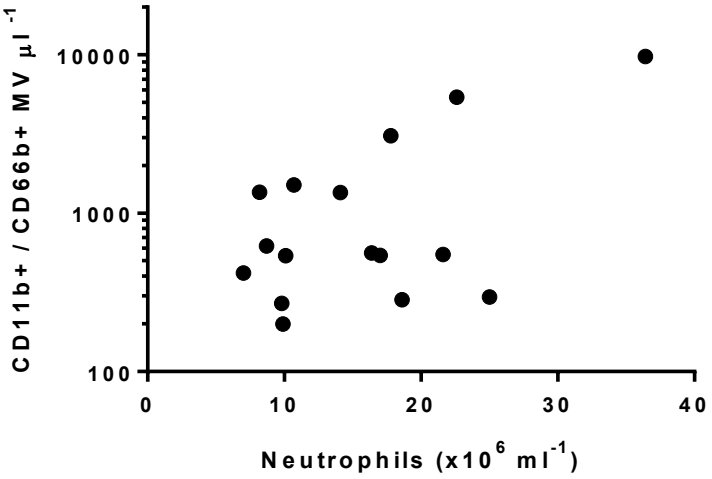
The elevation of leukocyte-derived circulating microvesicle levels may simply be a reflection of the increased numbers of circulating leukocytes in severe burns injury, as found here and in previous studies [198,217,219,422,427,428]. We therefore compared leukocyte and microvesicle counts to establish if there was a direct relationship. Total leukocyte numbers were compared to CD45+ microvesicles, neutrophils to CD11b+/CD66b+ microvesicles and monocytes to CD45+/CD14+ microvesicles, in burns and sepsis patients. Correlation was determined using Pearson's or Spearman's correlation coefficient, as appropriate.

In the burns group no significant correlation was found between leukocytes and CD45+ microvesicles (figure 4.7A), neutrophils and CD11b+/CD66b+ microvesicles (figure 4.7B), or monocytes and CD45+/CD14+ microvesicles (figure 4.7C). Analysis of the severe sepsis group generated the similar findings (figure 4.8A-C). Therefore, the increased microvesicle levels in both disease states were not merely results of leukocytosis and instead were likely to indicate enhanced microvesicle production by cells in response to the burn injury.

A


CD45+ MV µl-1
10000
1000
100
0
10
20
30
40
50
Leukocytes (x10^6 ml^-1)

B


CD11b+ / CD66b+ MV µl-1
10000
1000
100
0
10
20
30
40
Neutrophils (x10^6 ml^-1)

C

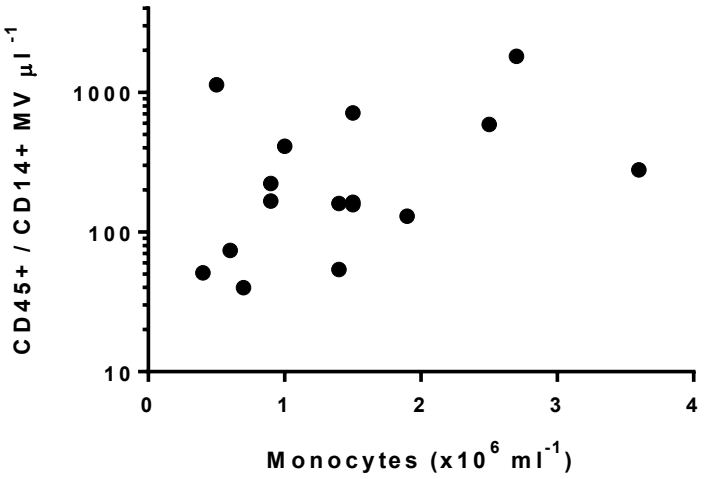

CD45+ / CD14+ MV µl-1
1000
100
10
0
1
2
3
4
Monocytes (x10^6 ml^-1)

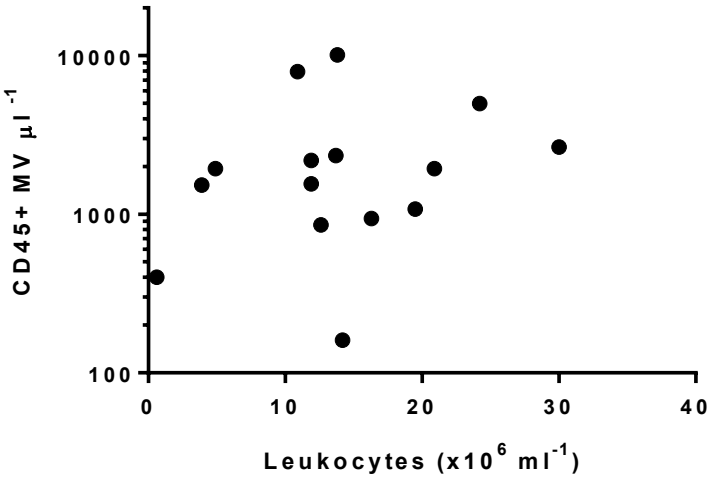
Figure 4.7 ***Comparison of cell and microvesicle counts on day zero post-burn injury***

No significant correlation found between leukocyte and CD45+ microvesicle counts (A), neutrophil and CD11b+/CD66b+ microvesicle counts (B), or monocyte and CD45+/CD14+ microvesicle counts (C)

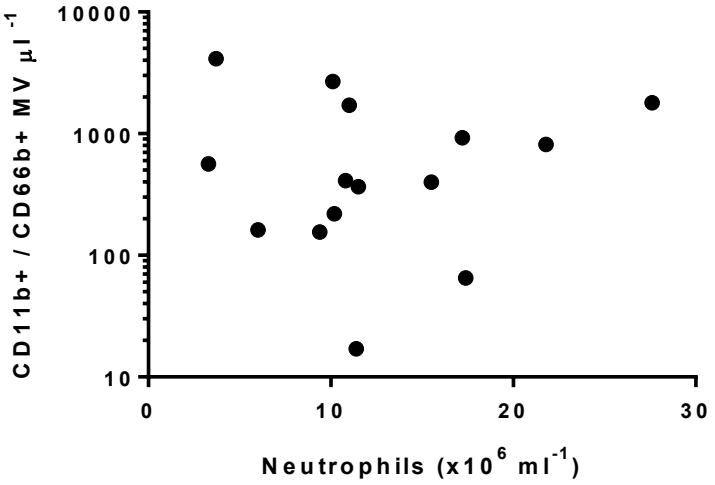
Correlation determined using Pearson's correlation coefficient (A) and Spearman's correlation coefficient (B and C)

(n=16)

A


CD45+ MV µl-1
10000
1000
100
0
10
20
30
40
Leukocytes (x10^6 ml^-1)

B


CD11b+ / CD66b+ MV µl-1
10000
1000
100
10
0
10
20
30
Neutrophils (x10^6 ml^-1)

C

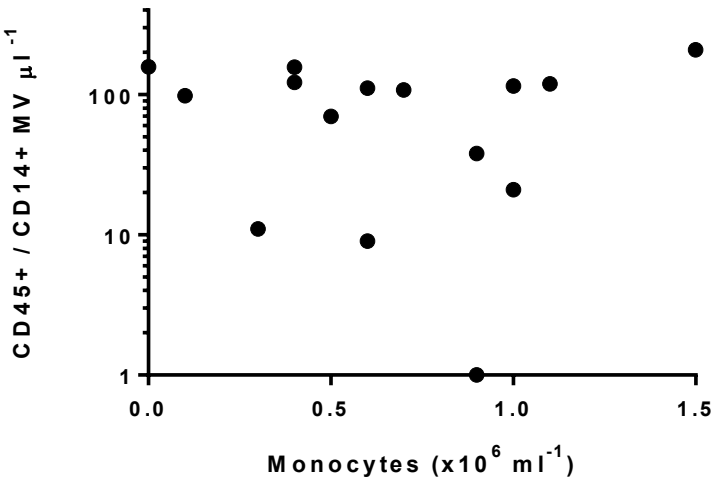

CD45+ / CD14+ MV µl-1
100
10
1
0.0
0.5
1.0
1.5
Monocytes (x10^6 ml^-1)

Figure 4.8 ***Comparison of cell and microvesicle counts in patients with severe sepsis***

No significant correlation found between leukocyte and CD45+ microvesicle counts (A), neutrophil and CD11b+/CD66b+ microvesicle counts (B), or monocyte and CD45+/CD14+ microvesicle counts (C)

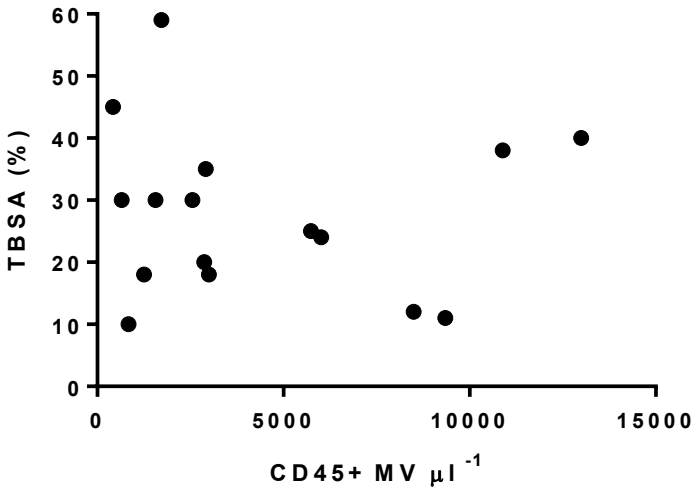
Correlation measured using Spearman's correlation coefficient (A and B) and Pearson's correlation coefficient (C)
(n=15)

Circulating leukocyte-derived microvesicle levels correlate with the ABSI and BOBI burn severity scores

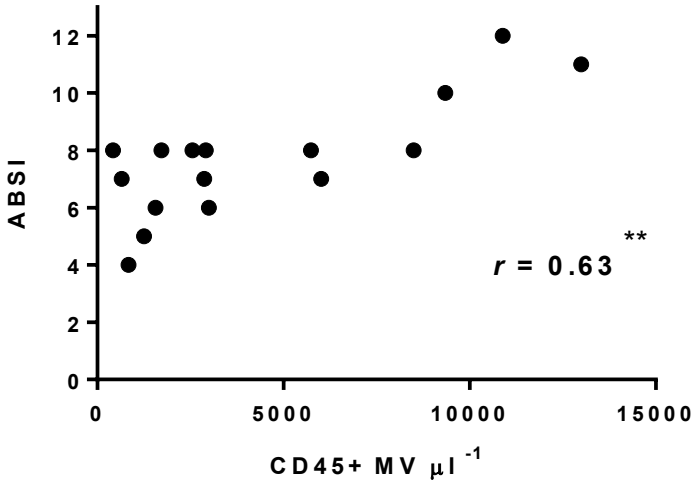
Microvesicles have been proposed for use as biomarkers across a range of diseases [303,353,429–431] but they have not previously been investigated in burn patients. Our objective was to test whether there was any relationship between circulating microvesicle levels and conventional means of quantifying burn severity. We therefore compared the %TBSA, ABSI and BOBI with each patient's initial microvesicle counts.

No significant correlation was identified between any microvesicle population and %TBSA. An example of the correlation analysis is shown for total leukocyte (CD45+) microvesicles vs %TBSA in figure 4.9A. A significant, strong correlation was found between CD45+ microvesicles and the ABSI score (r=0.63, $p<0.01$) (figure 4.9B). A significant strong correlation was also identified between CD45+ microvesicles and the BOBI score (r=0.67, $p<0.01$) (figure 4.9C). No significant correlations were found between these scores and other leukocyte or endothelial microvesicle populations (data not shown).

A


60
50
40
30
20
10
0
TBSA (%)
0
5000
10000
15000
CD45+ MV µl -1

B


12
10
8
6
4
2
0
ABSI
r = 0.63
**
0
5000
10000
15000
CD45+ MV µl -1

C

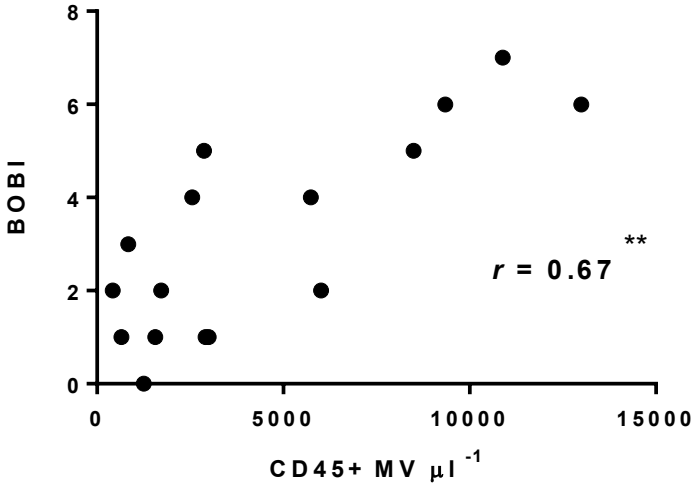

8
6
4
2
0
BOBI
r = 0.67
**
0
5000
10000
15000
CD45+ MV µl -1

Figure 4.9 Correlation between microvesicle counts and indices of burn injury

(A) No correlation was found between circulating CD45+ microvesicle levels and percent of Total Body Surface Area (TBSA) burned

(B) Significant, strong correlation between circulating CD45+ microvesicle levels and Abbreviated Burn Severity Index (ABSI) score

(C) Significant, strong correlation between circulating CD45+ microvesicle levels and Belgian Outcomes in Burn Injury (BOBI) score

Correlation measured using Pearson's correlation coefficient (A) and Spearman's correlation coefficient (B and C)

***p<0.01*

(n=16)

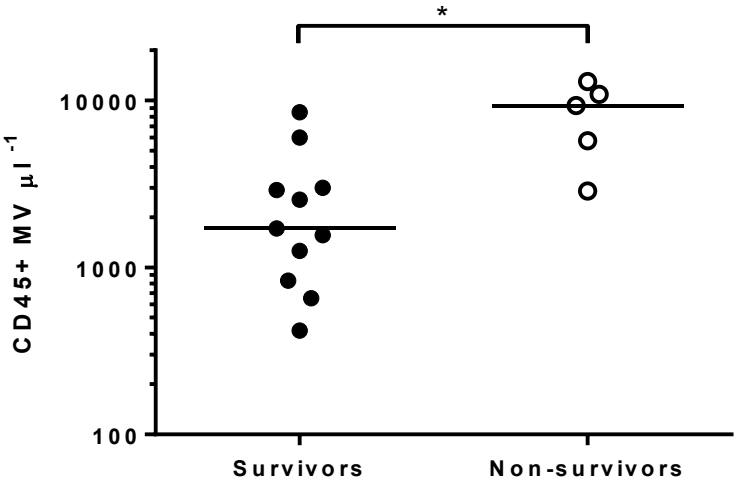
Circulating leukocyte microvesicle levels are reduced in survivors of severe burn injuries

To complete our analysis we compared microvesicle levels with actual mortality. This is an approach that has been taken with numerous biomarkers in burn patients [237,416,432–434]. The positive correlation between leukocyte microvesicles and ABSI/BOBI scores suggested these microvesicles may be increased in non-survivors of burn injury. All burn patients were followed to hospital discharge or death. Patients were classified as survivors or non-survivors accordingly. Levels of each microvesicle population, as measured in samples taken on day zero, were compared between each group.

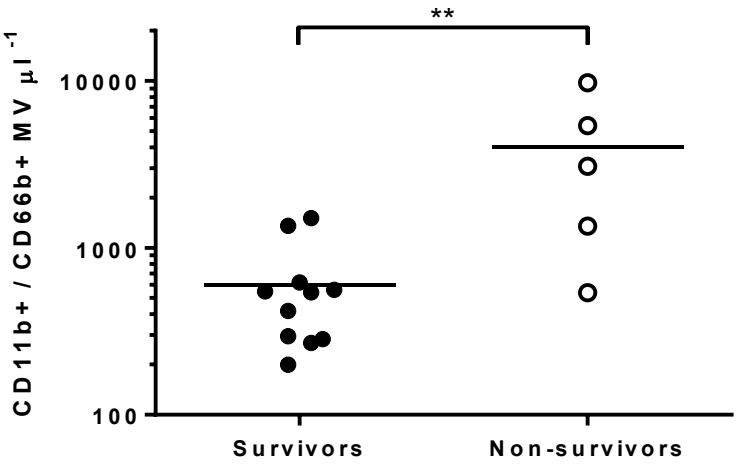
Total leukocyte (CD45+) microvesicles were significantly elevated in non-survivors as compared to survivors (figure 4.10A). Neutrophil (CD11b+/CD66b+) microvesicles were also significantly increased in the non-survivors group (figure 4.10B). A statistically non-significant increase in monocyte/macrophage (CD45+/CD14+) microvesicles was identified (figure 4.10C). No differences were found in CD105+ or CD62e+ endothelial microvesicle levels (figure 4.11A and B). In non-survivors, there was a non-statistically significant increase in CD106+ endothelial microvesicle levels (figure 4.11C).

We performed a similar analysis using the data obtained from patients with severe sepsis. In non-survivors, we identified statistically non-significant increases in total leukocyte and neutrophil microvesicles (data not shown).

A


*
10000
1000
100
CD45+ MV µl-1
Survivors
Non-survivors

B


**
10000
1000
100
CD11b+ / CD66b+ MV µl-1
Survivors
Non-survivors

C

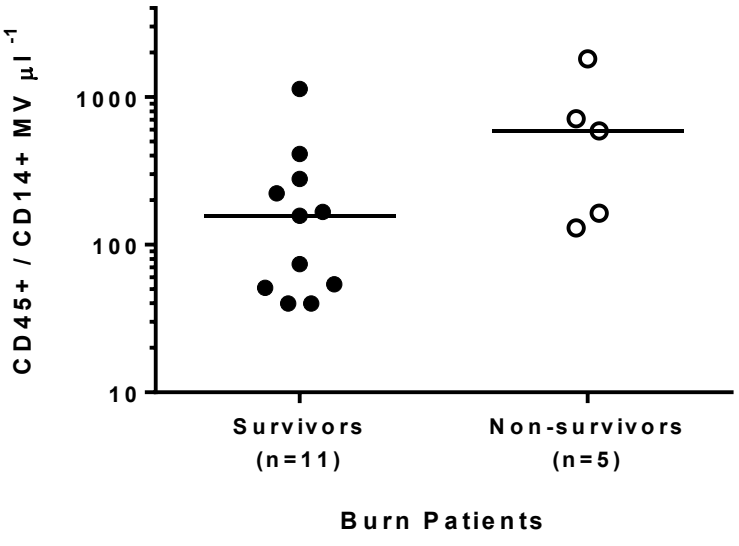

1000
100
10
CD45+ / CD14+ MV µl-1
Survivors
(n=11)
Non-survivors
(n=5)
Burn Patients

Figure 4.10 Comparison of leukocyte microvesicle counts in survivors and non-survivors of burn injury

(A) Circulating CD45+ microvesicles are significantly elevated in non-survivors

(B) Circulating CD11b+/CD66b+ microvesicles are significantly elevated in non-survivors

(C) Non-significant increase in CD45+/CD14+ microvesicles in non-survivors

Mann-Whitney test (A and C)

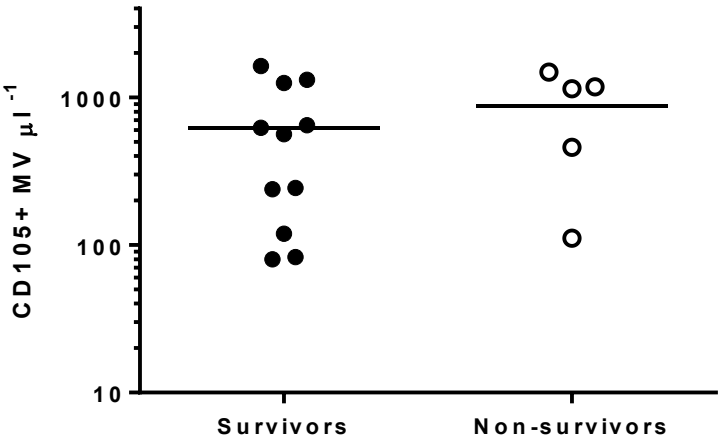
Unpaired t test (B)

**p<0.05*

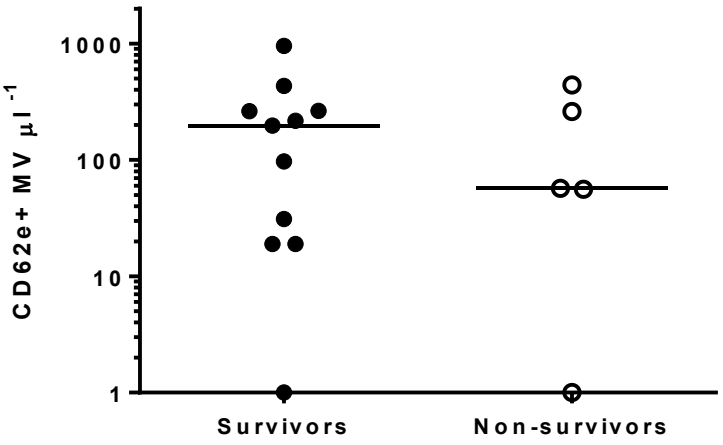
***p<0.01*

(n=11 (survivors), n=5 (non-survivors))

A


CD105+ MV µl-1
1000
100
10
Survivors
Non-survivors

B


CD62e+ MV µl-1
1000
100
10
1
Survivors
Non-survivors

C

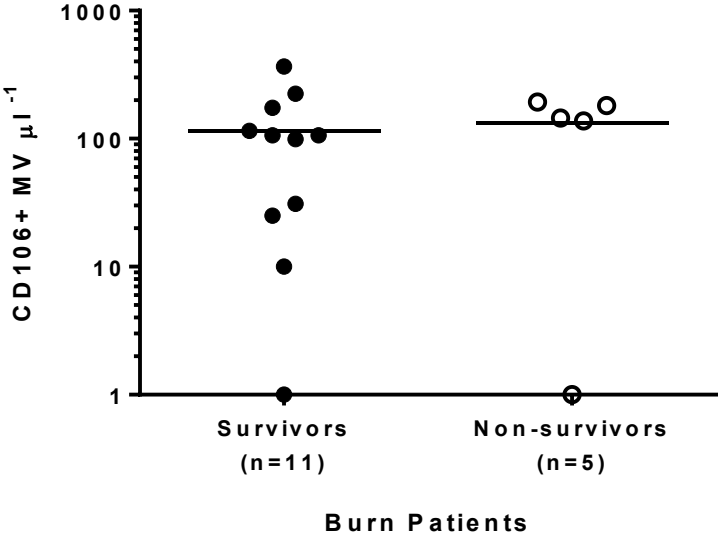

CD106+ MV µl-1
1000
100
10
1
Survivors
(n=11)
Non-survivors
(n=5)
Burn Patients

Figure 4.11 Comparison of endothelial microvesicle counts in survivors and non-survivors of burn injury

No statistically significant difference in CD105+ (A), CD62e+ (B) or CD106+ (C) endothelial microvesicle counts, between survivors and non-survivors.

Unpaired t test (A and C)
Mann-Whitney test (B)
(n=11 (survivors), n=5 (non-survivors))

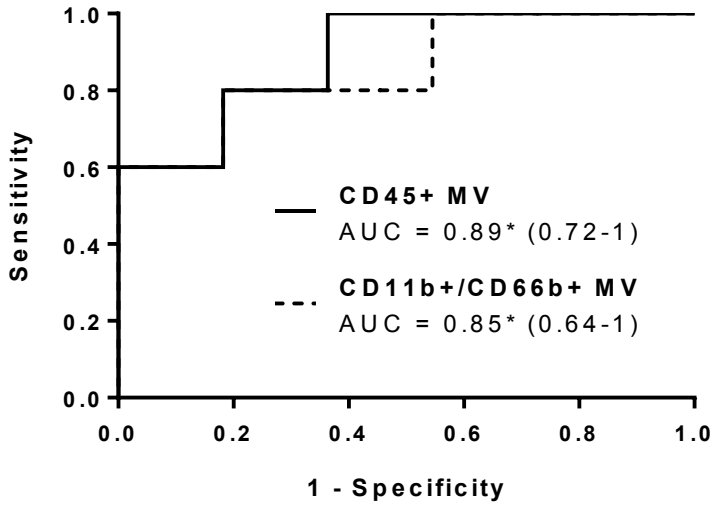
Circulating leukocyte microvesicle levels are predictive of mortality of patients with burn injuries

Accurate prediction of the risk of death associated with burn injuries facilitates clinical decision-making and stratification for research purposes [66]. The best performing prediction models at present are the ABSI and BOBI score, which are composites of clinical data such as burn area and age [66,67,73,79,80]. Given that numbers of total leukocyte microvesicles were markedly elevated in patients who did not survive their injuries, we reasoned that specific microvesicle values might be used to predict death.

Burn patients were classified as before as survivors or non-survivors. A receiver operating characteristic (ROC) curve was constructed, using the day zero CD45+ and CD11b+/CD66b+ microvesicle counts as a discriminator. For comparison, ROC curves were also constructed using the same patients' ABSI and BOBI values. Accuracy of each test for predicting death was measured by the area under the ROC curve, with 1 representing a perfect test and 0.5 a test that performs no better than a random classifier [435].

In our population, the initial CD45+ microvesicle count performed well as a predictor of death, with an area under the curve (AUC) of 0.89 (95% confidence interval 0.72-1, p=0.01). A cut-off value of 7253 CD45+ MV μl^{-1} yielded a specificity of 0.9 and sensitivity of 0.6 (likelihood ratio 6.6). The CD11b+/CD66b+ count also performed well, with an AUC of 0.85 (95% confidence interval 0.64-1, p=0.02) (figure 4.12A). The ABSI score demonstrated comparable performance, with an AUC of 0.85 (95% CI 0.62-1, p=0.03). The BOBI score displayed excellent predictive ability, with an AUC of 0.96 (95% CI 0.88-1, p=0.004) (figure 4.12B).

A



B

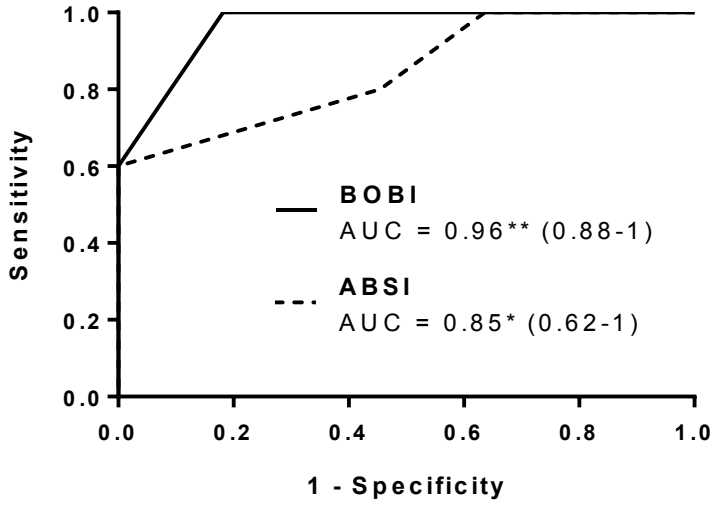


Figure 4.12 ***Receiver-operator characteristic (ROC) curves for mortality prediction in patients with burn injuries***

(A) Good prediction of mortality by day zero circulating CD45+ and CD11b+/CD66b+ microvesicle levels

(B) Good prediction of mortality using ABSI score; excellent prediction of mortality using BOBI score

AUC = Area Under the Curve. 95% confidence intervals in parentheses

**p<0.05, **p<0.01*

4.5 Discussion

Quantification of circulating microvesicles

The primary aim of this study was to characterise the leukocyte and endothelial microvesicle profile associated with severe burn injuries. These findings were then compared to clinical data to assess the potential significance of microvesicles as mediators of burns pathophysiology. For the first time, we have demonstrated that levels of circulating leukocyte and endothelial microvesicles are increased significantly in patients with severe burns. The preliminary findings indicate that microvesicles could be an important component of the inflammatory response to acute severe burn injury and have potential value as prognostic markers.

It is important to first consider whether our burn population was representative of Burn ICU patients in general. By comparing our patients to larger cohorts and published epidemiology, the applicability of any findings can be judged. A preponderance of male patients was noted, which was consistent with data from both the UK [15] and Europe [7]. The age distribution was also consistent, with the majority of patients falling within the 0-49 years category [436]. The median burn injury area in our cohort was 28% TBSA (range 11 to 59%). Although the precise definition of a "severe" burn injury varies [7], this is above the threshold of 15-20% TBSA at which shock can develop [47]. The mortality predicted by the burn severity scores showed good agreement with actual mortality. We concluded that the patients we had recruited were reasonably representative of "severely burned" patients in general.

Patients with severe sepsis were included as a positive control and reference group because previous investigations have associated this syndrome with increased levels of leukocyte and endothelial microvesicles [3,330,343]. Recruitment was opportunistic and was not designed to capture a group that was representative of all severe sepsis patients. However, comparison of our patients with those of other sepsis studies allows our microvesicle analysis to be placed in context and some judgement of wider applicability to be made. The age, male preponderance, APACHE II score and mortality rate of participants was similar to recent data from UK ICUs [437]. The mortality rate was also similar to international data [438],

although it was higher than recent figures from Australia [109,439]. ICU and hospital lengths of stay were longer in our cohort than in the UK and Australian studies [109,437,439]. Lengths of stay are affected by illness severity but can also be influenced by institutional factors [440]. Overall, the demographic data did not suggest our severe sepsis group was markedly different from those investigated in larger studies.

The elevation in levels of circulating leukocyte microvesicles in patients with severe burns is consistent with the upregulation demonstrated in other systemic inflammatory states [330]. In the sepsis group, total leukocyte microvesicle counts were not significantly different from burn patients and a similar range of values was evident. Total leukocyte (CD45+) microvesicle numbers were of a similar magnitude to those of neutrophil microvesicles, suggesting that this was the predominant cellular origin of CD45+ microvesicles. CD45 is also expressed on lymphocytes [441] and lymphocyte activation has been demonstrated following burn injury [442], although the response is relatively slow [443]. This is a microvesicle population which could be evaluated in future studies.

Increased neutrophil mobilisation from bone marrow and recruitment to sites of inflammation occurs rapidly following tissue injury [193,199]. Neutrophils have been shown to release microvesicles *in vitro* in response to multiple inflammatory stimuli [444]. The neutrophil-derived circulating microvesicles in burn patients may therefore originate directly from burned tissue, specifically the viable zones of stasis and hyperaemia [17] . They could also originate from areas of remote organ damage, such as the lungs and kidneys. Multiple organ dysfunction is often evident following burn injuries and this has been linked to neutrophil sequestration [118,445]. However, the acute increases seen within 24 hours of admission, before significant organ injury has developed, argues in favour of a primary (burn) injury site of origin. In the sepsis patient group, where the course and stage of inflammation is less defined, the elevated circulating neutrophil levels may reflect generation at the site of infection [385] but also vascular sites of neutrophil sequestration and infiltration in remote organs [446].

The potential function of these neutrophil-derived microvesicles in the context of burn injuries is less clear. There is certainly some *in vitro* evidence that neutrophil microvesicles

can mediate an inflammatory effect, either through a direct action on endothelial cells [318–320,447] or by adhering to other mediators such as monocytes [448]. Furthermore, analysis of burn-activated neutrophils showed them to be capable of increasing endothelial permeability [449]. However, other studies have suggested neutrophil microvesicles have immunomodulatory effects [338,450,451] and may in fact help to preserve vascular integrity [452].

The increased levels of CD14-expressing microvesicles observed in burn patients was a potentially significant finding. This population is likely to be derived mainly from classical subset CD14+ monocytes and tissue macrophages, although CD14 is also expressed at low levels by non-myeloid cells [453]. There is evidence to suggest that monocyte-derived microvesicles are capable of mediating endothelial dysfunction [302,321,454]. Vesicles released by macrophages have also been shown to stimulate an inflammatory response [177]. The higher levels in burn patients as compared to sepsis patients should be interpreted with caution. In burn patients, the time of injury is usually established to within a few minutes. In contrast, sepsis is known to be a heterogeneous syndrome where the time of onset, prior to identification, is unknown [455]. It is important to note that previous investigations identified a rapid reduction of neutrophil microvesicle counts in sepsis patients but found monocyte microvesicle levels to be sustained for longer [330]. This suggests that the timing of sampling, in relation to the initial insult, cannot fully explain the differences between burn and sepsis patients. The next step would be to investigate a more stringently selected sepsis cohort, perhaps focusing on a single source and narrower time window. If our provisional result was replicated it would support the notion that the roles of microvesicles in mediating these inflammatory states are distinct.

The rapid decline in leukocyte-derived microvesicles after burn injury was also a noteworthy result, particularly when compared to the corresponding leukocyte counts, as it cannot be explained solely by changing numbers of circulating leukocytes. One possible explanation for this finding is that the early excision of burned tissue is removing the stimulus for microvesicle production. Another possibility is that reduced microvesicle production by leukocytes reflects a developing state of “immunoparalysis”. In this model, a pro-inflammatory state is followed by an exaggerated anti-inflammatory response, resulting in deactivation of monocytes and neutrophils [313]. This phenomenon has been described in the

context of major trauma [456], sepsis [457] and ARDS [458]. Monocyte hyporesponsiveness in particular has been demonstrated following burn injuries [216,219]. The net result is that the patient is more susceptible to subsequent infections [191,219,456]. The decreases we have shown in this study will need to be confirmed in a larger patient group, as serially-sampled patients were limited in number. If the frequency and duration of sampling can also be increased, it may ultimately prove possible to associate specific microvesicle profiles with major clinical events. For example, a lull in microvesicle release at 24-48 hours, followed by a subsequent large peak in production, may reflect secondary sepsis.

The lack of correlation between circulating leukocyte counts and microvesicle levels could be explained by our hypothesis that a major site of microvesicle production in burn patients is injured tissue. Neutrophil recruitment and monocyte influx is known to occur in burned tissue [197,445]. An additional factor is that there may be varying rates of microvesicle clearance. Microvesicles have been shown to have a short half-life in the circulation [418], which implies that the results we obtained reflect a balance of continuous production and removal [417]. If the clearance capacity of individual patients varies or was temporarily overloaded, for example due to altered macrophage function [459], this would also affect circulating microvesicle counts. From a methodological point of view, the fact that microvesicle and circulating leukocyte counts do not correlate argues against post-sampling *ex vivo* microvesicle release being a significant factor.

Our investigations also revealed significantly elevated levels of endothelial cell microvesicles in burn patients. The difference in comparison to healthy volunteers was less stark than that seen with leukocyte-derived microvesicles but was significant for all three surface markers analysed. The results support the idea that endothelial microvesicles could be used as markers of endothelial dysfunction in burns, as has been proposed for other diseases [331,333]. The fact that we detected a reduction in endothelial microvesicles by day two, suggests they could have sufficient sensitivity for this purpose. It would be interesting to directly compare temporal changes in endothelial microvesicles following burn injury, with the profiles of soluble inflammatory mediators. There is also scope for expanding the panel of surface markers used to quantify endothelial markers, to include PECAM-1 (CD31) [413], ICAM-1

(CD54) [461] and VE-cadherin (CD144) [391]. *In vitro* studies suggest these could be used to identify phenotypically distinct endothelial microvesicles [424].

We did not set out to analyse platelet microvesicles in our study because we hypothesised that leukocyte and endothelial sources would be most likely to reflect the intravascular inflammatory response, local or systemic. Furthermore, because of the methodological issues described in the previous chapter, we did not consider their quantification to be sufficiently accurate. Nevertheless, there is some evidence platelet microvesicles contribute to inflammatory processes, in addition to their pro-coagulant activity [462,463]. Further refinements in cytometry technique might allow more reliable analysis in the future. Although erythrocyte microvesicles may be an important to blood transfusion [464–466], they have not been shown to mediate inflammatory processes and were not analysed here.

Comparison with severity of illness and clinical outcomes

Previously published work has correlated microvesicle levels with severity of illness in a wide variety of disease states, including sepsis [343], trauma [467], ARDS [414] and pulmonary hypertension [333]. In burn patients, a number of clinical scoring systems have been devised. The simplest is the percentage of the patient's skin affected by burn (TBSA). It has long been recognised as a key factor in patient mortality [67] and it forms a major part of burn scoring systems [66,72]. The ABSI is in widespread use and it remains methodologically sound and robust as a predictive tool [67,73,80]. A simpler and more recent scoring method, adopted by our burns unit BOBI system. This has been validated externally [79] and is also robust [67]. Both of these systems use hospital mortality as their outcome.

A number of correlations between microvesicle levels and clinical parameters were found. A strong correlation was found between total leukocyte (CD45+) microvesicle levels and the validated burn severity scores, which predict the risk of death for each patient. In contrast, microvesicle levels did not correlate with burn area alone. There is a degree of biological plausibility to this finding, as the factors that constitute the burn severity scores could theoretically also influence circulating microvesicle levels. The burn area, burn depth and presence of inhalational injury all reflect the extent of injured tissue. Alternatively, advanced age may reduce the ability to clear microvesicles, which would increase their

numbers as discussed above. It is unclear how gender would affect microvesicle levels but this factor is not included in the BOBI score and is allocated a low weighting in the ASBI value [80]. However, the same arguments could be applied to the other microvesicle populations and they did not correlate strongly with the burn severity scores. The reason for this is unclear and is worthy of further investigation.

The next step of our analysis was to compare microvesicle levels with actual mortality. This is an approach that has been taken with numerous biomarkers in burn patients [237,416,432–434]. When the burn patients were subdivided into survivors and non-survivors, a clear pattern emerged, with leukocyte microvesicles being significantly elevated in those patients who died before hospital discharge. The greatest difference was seen with neutrophil microvesicles but there was also a significant difference when total leukocyte microvesicle counts were analysed. Monocyte-macrophage microvesicles were also increased in non-survivors but this difference was not statistically significant. For the reasons outlined above, this is a plausible result, with the factors known to influence mortality, such as burn area and age [73], also having the potential to affect microvesicle levels.

It is not yet certain that microvesicles would contribute to the pathophysiology of SIRS in severe burns, and if so by what mechanism [412]. It is possible that they directly mediate remote organ inflammation and dysfunction. An alternative explanation is that higher initial microvesicle production increases the risk of subsequent immune dysfunction. Neutrophil microvesicles have antibacterial effects [468], so if microvesicle production or availability is diminished post-burn, the risk of secondary infection may be increased. For comparison, no significant differences in leukocyte, neutrophil or monocyte counts were found between survivors and non-survivors.

We used the strong association between higher leukocyte microvesicle counts and mortality to guide our final analysis. Construction of receiver operating characteristic (ROC) curves suggested that leukocyte- and neutrophil-derived microvesicles might be useful as discriminators to predict mortality. Their performance was comparable to the validated ABSI and BOBI prediction systems and superior to other novel biomarkers described in the literature, such as neutrophil extracellular traps (NETs) [434]. An obvious caveat is that the

groups we analysed (survivors / non-survivors) contained low numbers of patients. However, if this finding can be validated in a larger cohort, leukocyte-derived microvesicles may represent a clinically useful prognostic marker for burn patients.

Chapter 5

Investigation of microvesicle production and pro-inflammatory activity in relation to heat injury *in vitro*

Summary

The analysis of samples from burn patients in the previous chapter had demonstrated significantly elevated levels of circulating microvesicles. These were derived predominantly from neutrophils but we also detected monocyte/macrophage-derived microvesicles, as well as a population from endothelial cells. We speculated that a potential trigger for leukocyte microvesicle release may be their direct interaction with burned tissue, following their recruitment to the site of injury. We also hypothesised that the microvesicles produced locally by recruited leukocytes might contribute to systemic inflammation by mediating endothelial dysfunction.

To address these questions in human cells in vitro, we first developed an optimised system for microvesicle production. We chose to focus on neutrophil MV production because of the relative abundance of neutrophils during the early stages of local inflammation and our observation of high levels of neutrophil-derived MV in burn patient plasma. Isolated neutrophils were stimulated with defined agonists previously shown to induce MV, including phorbol myrisate acetate (PMA), zymosan and a calcium ionophore (A23187). We initially saw a negligible MV response, however following assessment of different stimulation conditions, we found that significant quantities of microvesicles could be elicited by maintaining neutrophils in suspension throughout the 37^{o}C incubation period.

Demonstration that neutrophil microvesicle production was elicited by opsonised particulate stimulants led us to consider opsonisation of heat-injured cells as a model to investigate neutrophil microvesicle release at the burn injury site. We performed pilot studies in which serum-opsonised heat-treated epithelial cells were co-incubated with neutrophils in suspension culture. This method produced a significant release of microvesicles.

The second aim of our *in vitro* experiments was to test the activity of burn-relevant microvesicles *in vitro*. We used PBMC-endothelial cell co-culture as *in vitro* model to assess MV-induced microvascular inflammation. Incubation with microvesicles from A23187-stimulated neutrophils led to a significant upregulation of E-selectin on endothelial cells. We

then assessed the activity of MV derived from a human skin explant model of burn injury, and demonstrated a clear upregulation E-selectin expression. In contrast, incubation with a mixed population of microvesicles, isolated from burn patient plasma, led to downregulation of both E-selectin and VCAM-1 expression.

We concluded that heat-injured cells could elicit a microvesicle response from neutrophils and this may be one pathway responsible for the microvesicle populations seen in burn patients. We also concluded that microvesicles from sources relevant to burn injury are capable of influencing endothelial activation, potentially contributing to the development of systemic inflammation and organ injury after severe burn injury.

5.1 Background

Our investigations to this point had shown that circulating leukocyte- and endothelial cell-derived microvesicles are significantly elevated following severe burn injury. The most abundant microvesicles were those derived from neutrophils. We also identified a significantly elevated population of monocyte/macrophage-derived microvesicles, in the same range or higher than that seen in patients with severe sepsis. Leukocyte microvesicles were therefore prime candidates for further evaluation in the context of systemic propagation of burn inflammation.

In vitro interaction between thermal injury and leukocyte microvesicle production

Our initial priority was to explore the experimental conditions that would reproducibly generate leukocyte microvesicles *in vitro*. The importance of this was twofold. Firstly, to investigate the mechanisms linking thermal injury to leukocyte activation and microvesicle release. Secondly, to provide a reliable source of microvesicles, to develop assays for measuring microvesicle inflammatory activity, including those derived from patient plasma. Our initial focus was neutrophil MV production because of the relative abundance of neutrophils during the early stages of local inflammation and our finding of high levels of neutrophil-derived MV in burn patient plasma.

A range of defined stimuli have been shown to promote neutrophil microvesicle release *in vitro*, such as formyl-methionyl-leucyl-phenylalanine (fMLP) [336,444], phorbol myristate acetate (PMA) [469] and calcium ionophore (A23187) [320]. However, such stimuli may not be relevant to burn wound microenvironment, an important consideration as the type of stimulus may be key to determining the phenotype of elicited MV. A large neutrophil microvesicle response can be generated by *Staphylococcus aureus* particles, if opsonised by incubating with normal serum [468]. This finding is relevant to burns because previous studies of the burn wound microenvironment have suggested that activation and chemotaxis of neutrophils by heat-killed fibroblasts is a complement-dependent process [266]. A number of other studies have highlighted the role of complement in the response to burn injuries [6,94,470–473] and separately, complement activation products have been demonstrated to play a role in microvesicle induction [474]. We therefore reasoned that opsonisation of heat-killed

cells may be necessary to generate a significant microvesicle response in neutrophils. To our knowledge, the effect of heat-damaged cells on leukocyte microvesicle production has not previously been investigated.

Endothelial dysfunction following severe burn injury

Endothelial dysfunction is known to be a key component of the innate immune response to severe infection and major trauma. Although the initial insult is very different, endothelial dysfunction is also evident following severe burns. This leads to widespread leak of fluid and proteins into the interstitium [475], a procoagulant state [275] and vasoplegia [476]. This is exacerbated by leukocyte adhesion and activation in the microvasculature [477]. The potential interaction between leukocyte microvesicles and endothelial cells was therefore of considerable interest. The concept of leukocyte-derived microvesicles contributing to an inflammatory state, by way of endothelial dysfunction, is not new. Neutrophil-derived microvesicles have previously been shown to activate [318,319] and injure [320] endothelial cells *in vitro.* Monocyte-derived microvesicles have been found to induce loss of endothelial cell integrity and apoptosis [302]. Microvesicles released into the circulation following burns may therefore play an important role in the propagation and/or the amplification of systemic inflammation and organ injury. A basic pathway consistent with burn pathogenesis can be envisaged, whereby inflammatory leukocytes are recruited to heat-injured tissue, leading to their activation and release of microvesicles. These microvesicles in turn activate remote endothelial cells, contributing to organ inflammation and injury. To address these concepts we aimed to develop protocols that would lead ultimately to an *in vitro* bioassay system for assessing the pro-inflammatory of circulating patient-derived MV in burn and other pro-inflammatory conditions.

5.2 Aims

1. Investigate methods for *in vitro* microvesicle generation, relevant to burn injury
2. Optimise assays using *in vitro*-generated microvesicles to measure their effect on microvascular inflammation in the context of systemic inflammation
3. Apply the vascular inflammation assay to evaluate burn patient microvesicles

5.3 Protocols

Leukocyte isolation

Density gradient centrifugation of whole blood was used to isolate peripheral blood mononuclear cells (PBMCs). Neutrophils were isolated from the resultant cell pellet by dextran sedimentation. For experiments involving total leukocytes, dextran-sedimentation of whole blood was performed. Further details of these methods are given in chapter two.

Microvesicle generation

The leukocytes were resuspended in 0.5% Human Albumin Solution (HAS) to a volume of 450µl, such that the neutrophil concentration was $1x10^7$ ml^{-1}. Cells were rested for one hour at 37^{o}C, before incubating with 50µl of stimulant for a further hour at 37^{o}C. The microvesicles were then isolated by centrifugation and analysed as described above. Stimulants (bacteria or cells) requiring opsonisation were resuspended in serum and incubated on a rotator as described earlier.

Leukocyte-endothelial co-culture

Primary human pulmonary microvascular endothelial cells (HPMECs) were seeded as described in the methods chapter. They were co-cultured with PBMCs for one hour at 37°C, the PBMCs having been resuspended in 200µl of 0.5% human albumin solution/endothelial basal medium (HAS/EBM). The co-culture was then stimulated with microvesicles for 16 hours. Following stimulation, endothelial cells were recovered and stained with antibodies specific for E-selectin (CD62e) and VCAM-1 (CD106).

Initial evaluation of microvesicle release from *in vitro* stimulated neutrophils

We performed pilot experiments, consisting of stimulation of primary human neutrophils, to ascertain if our optimised flow cytometry protocol was able to replicate results published by other authors. PMA was chosen in the first instance, as this has previously been shown to generate a significant microvesicle response from neutrophils [468]. Neutrophils were isolated from whole blood and stimulated with PMA at a final concentration of 1µM. Microvesicles were collected from cell supernatants and stained with PE-conjugated anti-CD66b monoclonal antibody. Baseline levels of CD66b+ microvesicles were low and there was only a small increase in count following PMA stimulation (figure 5.1).

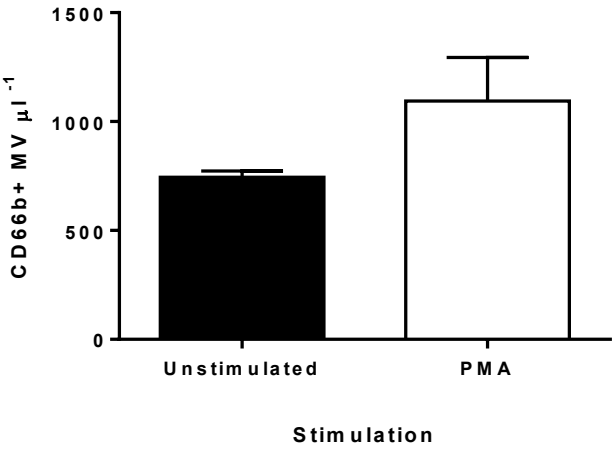


Figure 5.1 ***Minimal increase in neutrophil-derived microvesicle release following PMA stimulation of neutrophils in static environment*** *Unpaired t test (n=2)*

Given the minimal increase in neutrophil microvesicle release elicited by PMA stimulation, we next investigated the effect of zymosan particles, to see if a greater response could be elicited. Zymosan (yeast wall extract) is an established tool for generating sterile inflammation and has been shown to increase microvesicle production from neutrophils [340]. However, the results were unclear, with low CD66b+ microvesicle counts despite clear evidence of cell activation (upregulated CD11b expression). We concluded that this stimulation environment, despite prior optimisation of our flow cytometry protocol, was not sufficient to replicate the microvesicle response demonstrated in other studies [340,468].

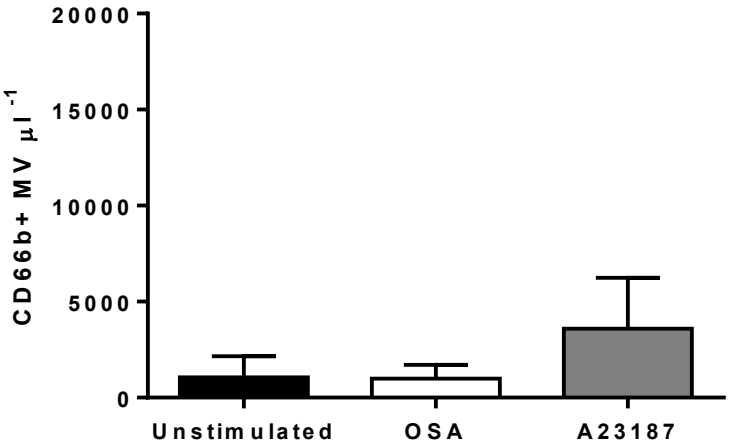
Optimal microvesicle release from neutrophils requires maintenance of cells in suspension

We next investigated experimental conditions that might influence neutrophil activation and MV production. Neutrophils are known for the ease by which they can become activated as a result of *in vitro* isolation methods [478,479]. Early activation could theoretically affect the subsequent microvesicle response to stimulation. To eliminate the prolonged isolation procedure as a factor contributing to low microvesicle production, we elected to truncate the cell separation and stimulate neutrophils in a mixed leukocyte preparation. A further consideration was that during the rest and stimulation incubations, a significant amount of cell sedimentation was observed. A potential consequence was cell-cell contact-dependent activation, which may have adversely affected MV release. We therefore investigated stimulation of cells in suspension, to ensure thorough mixing of cells throughout the stimulation phase.

Mixed leukocytes were prepared as above, before stimulation with opsonised *S. aureus* (OSA) particles (5×10^7 in 50µl HBSS) or calcium ionophore A23187 (10µM). These stimuli have previously been shown to be effective inducers of neutrophil microvesicle release [320,468]. The stimulation was performed in either static or rotated Eppendorfs. As before, static stimulation did not lead to significant increases in neutrophil microvesicle counts above baseline (1063 ± 1099 MV μl^{-1}) (Figure 5.2A). However, after rotated stimulation, there was a significant increase in microvesicle levels with both opsonised *S. aureus* (2239 ± 1352 to 8954 ± 286 MV μl^{-1}, $p<0.001$) and A23187 (19160 ± 774 MV μl^{-1}, $p<0.0001$) (Figure 5.2B).

In order to confirm that opsonisation was an important factor in the rotated stimulation environment, we also evaluated the effect of non-opsonised *S. aureus* (NOSA) on the mixed leukocyte preparation. The neutrophil microvesicle counts generated by NOSA stimulation were not significantly higher than baseline levels (data not shown). This was consistent with the literature [468].

A



B

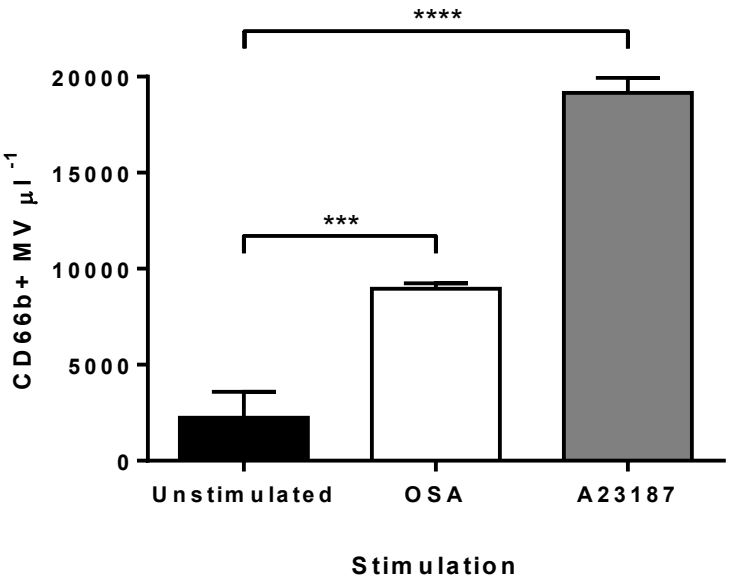


Figure 5.2 Effect of continuous rotation on neutrophil microvesicle release

(A) No significant increase in neutrophil microvesicle release, in response to stimulation by opsonised S. aureus (OSA) and A23187, in static environment

(B) Significant increase in microvesicle release, in response to OSA and A23187, in rotating environment

****p<0.001, ****p<0.0001*

One-way ANOVA with Dunnett's multiple comparisons test (n=3)

Isolation of neutrophils from other leukocyte sub-populations does not affect their production of MV

The next task was to investigate the effect of rotated stimulation on purified neutrophils, as we had suspected the isolation procedure may have affected their capacity to produce microvesicles. A pure neutrophil preparation facilitates elucidation of the direct neutrophil-specific pathways, while mixed leukocyte stimulation may be complicated by cross-regulation of microvesicle release.

Neutrophils were isolated as before and stimulated in the rotating incubator with opsonised *S. aureus* and A23187, in the same manner as that used for mixed leukocytes. Neutrophil numbers were the same in all treatments and experiments were run separately. No significant differences in neutrophil microvesicle levels were found between the mixed leukocyte and neutrophil preparations, as shown in figure 5.3. This applied to all three treatments, with reasonably low variability seen in both methods. We therefore concluded that modulation by other leukocytes was unlikely in this system and that provision of "pure" neutrophil-derived MV for activity assays was optimal using isolated neutrophils.

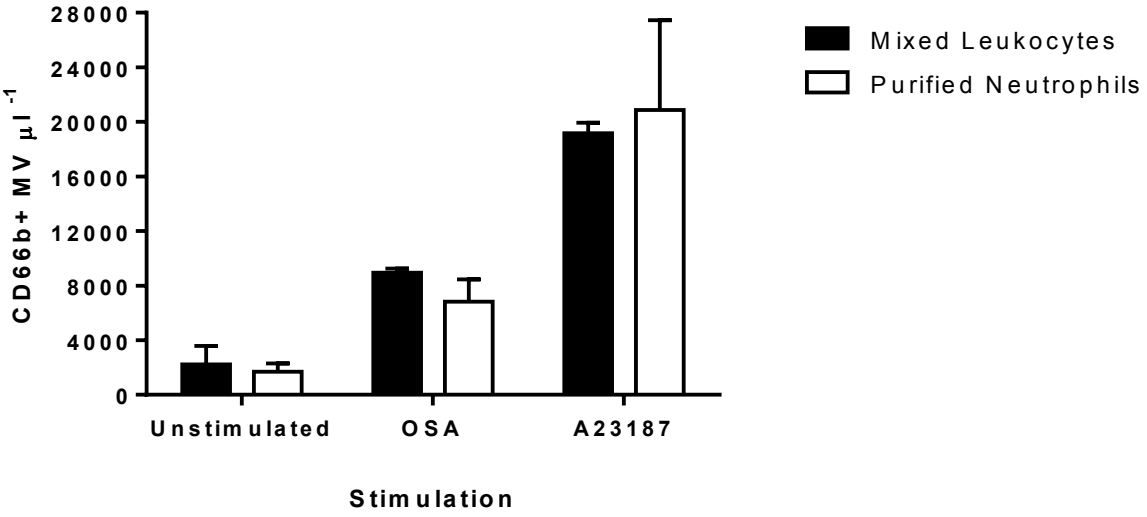


Figure 5.3 ***Comparison of neutrophil microvesicle release from mixed leukocytes and purified neutrophil preparations***

Rotated suspensions of mixed leukocytes and purified neutrophils yield similar numbers of neutrophil microvesicles when stimulated with opsonised S. aureus (OSA) and A23187

Unpaired t test (separate test for each treatment)
(n=3)

Development of a cell-based model of burn-injury and complement activation

Our experiments with purified neutrophils had demonstrated significant quantities of microvesicles, provided the stimulation was performed whilst the cells were mixed continuously. They also confirmed that opsonisation of *S. aureus* particles produced increased microvesicle generation, suggesting the importance of complement deposition as a co-stimulus. We hypothesised that heat injury of host cells, leading to fixation and activation of complement [266,470], may provide a stimulus for neutrophil microvesicle production post-burn injury.

We first evaluated the effect of heat treatment on complement activation, as one potential mechanism by which opsonisation may induce or enhance MV production. Heat injury and complement activation has previously been modelled using human kidney cells [470] and with the HK-2 human kidney epithelial cell line available, these cells were selected for pilot experiments. A severe burn will lead to widespread skin epithelial damage, so this choice was of relevance to burn injury.

HK-2 cells ($1.6x10^6$) were incubated in 100µl HBSS at 37 or 80^{o}C, for 15 minutes, based on previously published methods [266]. After centrifugation, the epithelial cells were resuspended in fresh human serum or HBSS and incubated at 37^{o}C, for one hour. To assess surface complement activation, the cells were washed and stained with anti-C3c. The geometric mean fluorescence intensity (gMFI) of viable non-opsonised HK-2 cells was 203, rising to 537 for viable opsonised cells. Non-opsonised heat-killed cells demonstrated a gMFI of 1634, rising to 8065 for heat-killed opsonised cells (see appendix). These results suggested increased complement activation on heat-killed cells and was consistent with work previously performed on kidney epithelial cells [470], as well as fibroblasts [266]. We also investigated shorter, more clinically-relevant heat exposure times and found that one minute at 80^{o}C was sufficient for a large increase in C3c deposition over baseline levels, with no further increase in complement activation with longer heating periods (data not shown).

Stimulation of neutrophils with heated-injured and opsonised HK-2 cells induces microvesicle release

To determine if heat-injured cells stimulate neutrophil microvesicle release and if this is enhanced by opsonisation, neutrophils were incubated with treated kidney cells in suspension. HK-2 cells ($1x10^6$) were heated for two minutes and serum-opsonised using the above method. After washing and resuspension, 50µl of heated-opsonised cells (approx. $5x10^5$) were added to the neutrophils and incubated for one hour.

Stimulation of neutrophils with the opsonised non-heated epithelial cells resulted in a significant increase in microvesicle release (1401 ± 811 to 5167 ± 1177 MV µl $^{-1}$, $p<0.01$) (Figure 5.4). However, the response seen after stimulation with the heated-opsonised cells was significantly greater again (8731 ± 1690 MV µl $^{-1}$, $p<0.05$). Without opsonisation, heat-injured cells did not induce significant MV release from neutrophils (2073 ± 811 MV µl $^{-1}$, $p<0.001$) suggesting that opsonisation and/or complement activation may be a key factor in neutrophil MV release following burn injury in vivo.

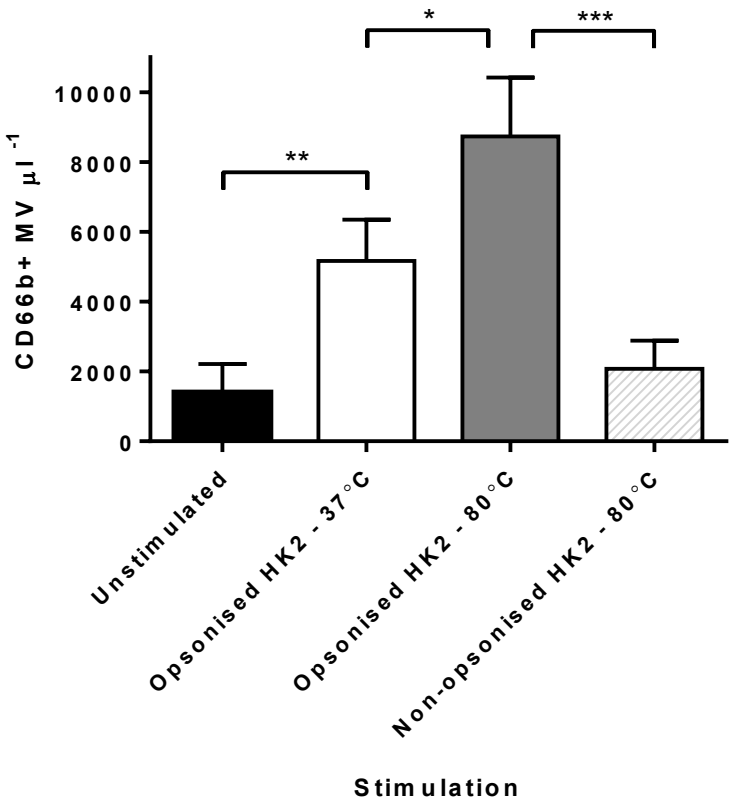


Figure 5.4 ***Neutrophil microvesicle release in response to stimulation with opsonised heat-killed HK-2 kidney epithelial cells***

Neutrophil microvesicle release is significantly increased over baseline by incubation with both opsonised heat-killed (80^{o}C) and opsonised viable (37^{o}C) kidney epithelial cells. Increase is significantly higher in response to opsonised heat-killed cells, than in response to both opsonised viable cells and non-opsonised heat-killed cells

**p<0.05*

***p<0.01*

****p<0.001*

One-way ANOVA with Tukey's multiple comparison test

(n=3)

Treatment of PBMC-endothelial cell co-cultures with neutrophil microvesicles induces upregulation of endothelial cell adhesion molecule expression

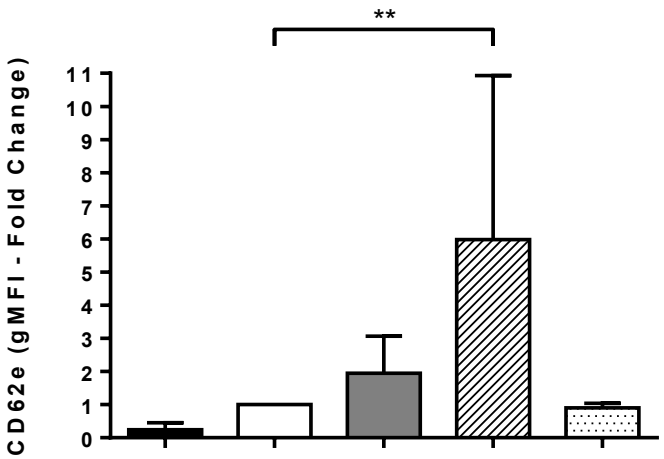
We used pulmonary microvascular endothelial cells as an *in vitro* indicator of vascular inflammation, with the addition of PBMCs to evaluate endothelial cell activation in the presence of capillary-sequestered leukocytes [126]. Our previous experiments had shown that the most productive and reliable microvesicle response was generated by stimulation with calcium ionophore (A23187) and therefore this was used for the initial experiments.

The neutrophils were stimulated with A23187 as above and microvesicle numbers were determined from the cell-free supernatant by flow cytometry. Supernatant from three Eppendorfs (13.5×10^7 neutrophils in total) was pooled and centrifuged at 20,000g for 30 minutes. Pelleted microvesicles were resuspended in 200µl of 0.5% HAS/EBM and added to HPMEC-PBMC co-cultures, that were pre-equilibrated for one hour in 200µl of 0.5% HAS-EBM. Controls included microvesicles from the same number of unstimulated neutrophils (13.5×10^7), as well as 10µl of supernatant obtained from pelleting the microvesicles. This was included as a control to assess the effect of any residual A23187 or soluble mediators associated with the microvesicle pellet. After incubation for 16 hours, media was aspirated from wells, centrifuged and frozen for future analysis. Endothelial cells were retrieved by non-enzymatic detachment, washed, stained and analysed for the presence of the activation markers E-selectin (CD62e) and VCAM-1 (CD106).

Due to variation in baseline expression of VCAM-1 and E-selectin, we normalised the data from different experiments, using values from unstimulated co-cultures as baseline values. Following incubation with microvesicles from stimulated neutrophils, there was a significant increase in E-selectin expression (fold-change in gMFI 6.0 ± 4.9, $p<0.01$) (figure 5.5A). In contrast, an increase was not seen following incubation with the A23187-treated neutrophil MV-free supernatants, which argued against the upregulation being triggered directly by the soluble stimuli (fold-change 0.90 ± 0.14). The neutrophil microvesicles released spontaneously were associated with only a modest increase in E-selectin expression (fold change 1.9 ± 1.1).

This overall pattern was replicated when CD106 expression was assessed (figure 5.5B). There was a non-statistically significant upregulation following incubation with microvesicles from stimulated neutrophils (fold-change 3.3 ± 3.1, p=0.05). Again, there was no increase in expression following incubation with the supernatant (fold change 0.83 ± 0.16) and only a modest upregulation with the spontaneously-released microvesicles (fold-change 1.3 ± 0.85).

A



B

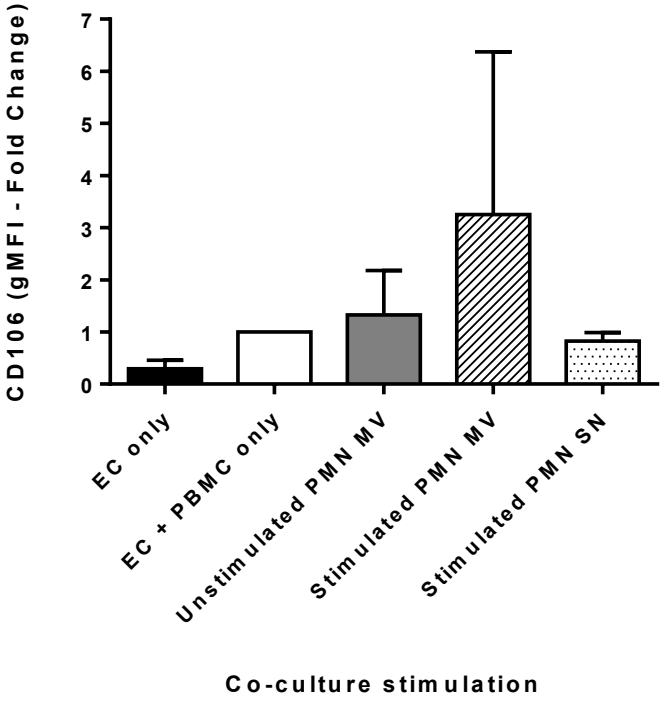


Figure 5.5 Stimulation of endothelial co-culture with neutrophil microvesicles

(A) Stimulation of endothelial cell (EC)-PBMC co-culture with neutrophil microvesicles (MV) leads to significant upregulation of E-selectin (CD62e) expression by endothelial cells

(B) Statistically non-significant VCAM-1 (CD106) upregulation

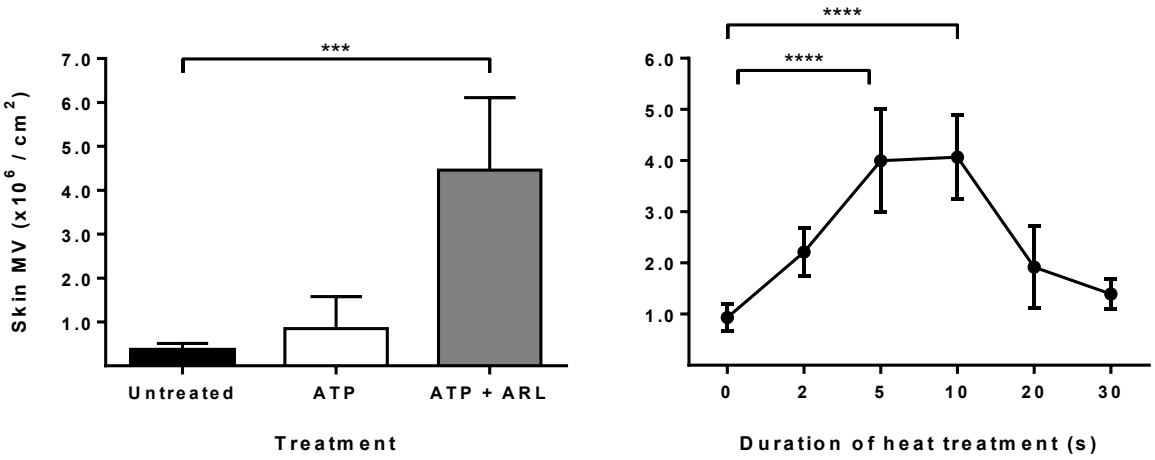
*SN=supernatant. **p<0.01. One-way ANOVA with Dunnett's multiple comparison test. (n=4-6)*

Detergent-sensitive microvesicle population can be generated by heat treatment of human skin explant

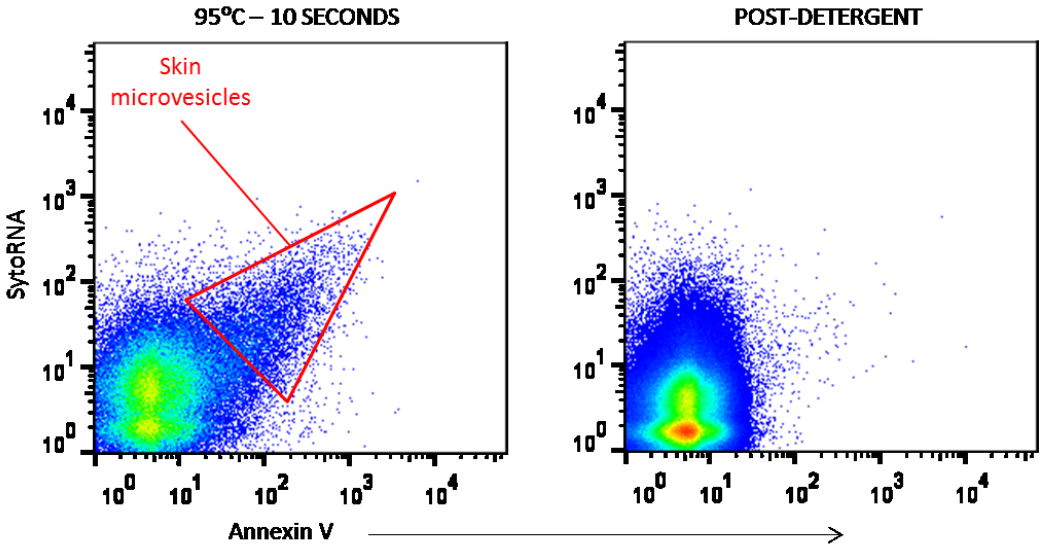
Having established the co-culture model of intravascular inflammation for assessing microvesicle function *in vitro*, we next investigated microvesicles generated using a human skin explant model of burn injury. These are highly relevant to burn injury and the microvesicles generated were an ideal candidate to test in the co-culture system. Optimisation of murine skin microvesicle generation was conducted within the group by Umar Katbeh, as part of a PhD thesis. Significant skin MV release could be induced by incubation with a combination of ATP (6mM) and ARL 67156 (1mM), a selective ecto-ATPase inhibitor. The optimal duration of skin heating, which generated a comparable level of microvesicles, was found to be 10 seconds, at 95^{o}C (figure 5.5A). These protocols were adapted for human skin explants and used for the work described here, which was conducted jointly.

Commercially-supplied skin samples, approximately 25cm^{2} in area, were taken from volunteers undergoing panniculectomy. These were delivered to the laboratory within 18 hours of excision. Subcutaneous fat was removed from the skin, in sterile conditions, before biopsies of a standardised diameter (8mm) were taken. Each piece of skin was then immersed in sterile PBS at 95^{o}C for 10 seconds to produce a thermal injury response that included oedema, as evidenced by substantial uptake of explant medium and weight increase (data not shown). After incubation at 37^{o}C for 2 hours, the fluid from each well was collected and centrifuged at 200g for 10 minutes, to pellet any cells or large debris. The supernatant contained a detergent-sensitive, annexin V+ / SytoRNA+ microvesicle population (figure 5.5B). The precise cellular source of these microvesicles was undetermined. Untreated control skin did not yield a similar microvesicle population (figure 5.5C).

A


***
Skin MV (x10^6 / cm^2)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
Untreated
ATP
ATP + ARL
Treatment
****
****
6.0
5.0
4.0
3.0
2.0
1.0
0
2
5
10
20
30
Duration of heat treatment (s)

B


95°C – 10 SECONDS
POST-DETERGENT
Skin microvesicles
SytoRNA
Annexin V

C

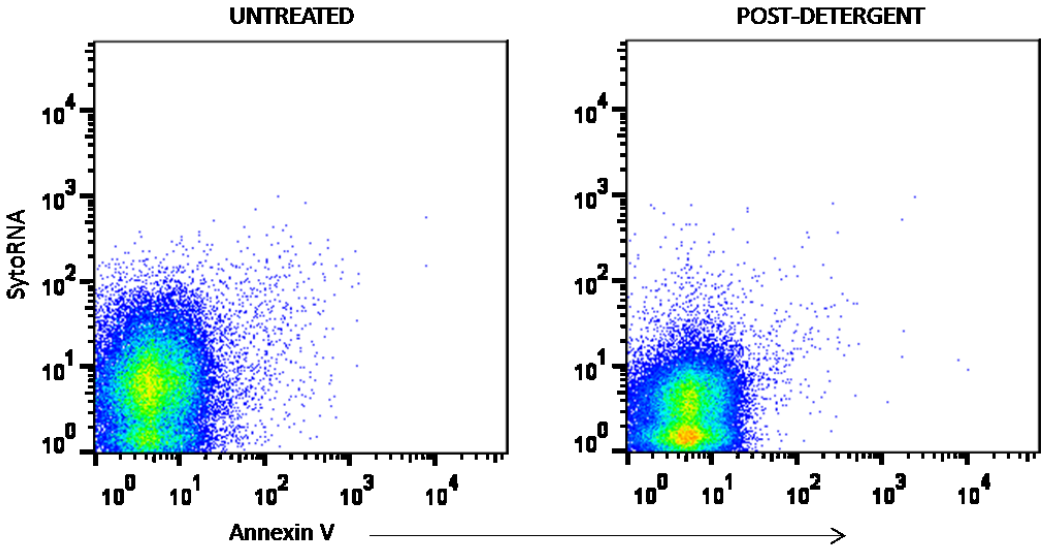

UNTREATED
POST-DETERGENT
SytoRNA
Annexin V

Figure 5.5 Generation of skin-derived microvesicles

(A) Significant microvesicle (MV) release elicited by incubation of murine skin with ATP/ARL. Similar levels of microvesicles are released by heat treatment of skin for 5 and 10 seconds, at 95^{o}C. MV levels are expressed per cm^{2} of skin sample. (n=4)

(B) Large numbers of detergent-sensitive microvesicles are released following 10 second 95^{o}C heat treatment of human skin samples

(C) Minimal levels of detergent-sensitive skin microvesicles are released following incubation of human skin samples at 37^{o}C

****p<0.001*

*****p<0.0001*

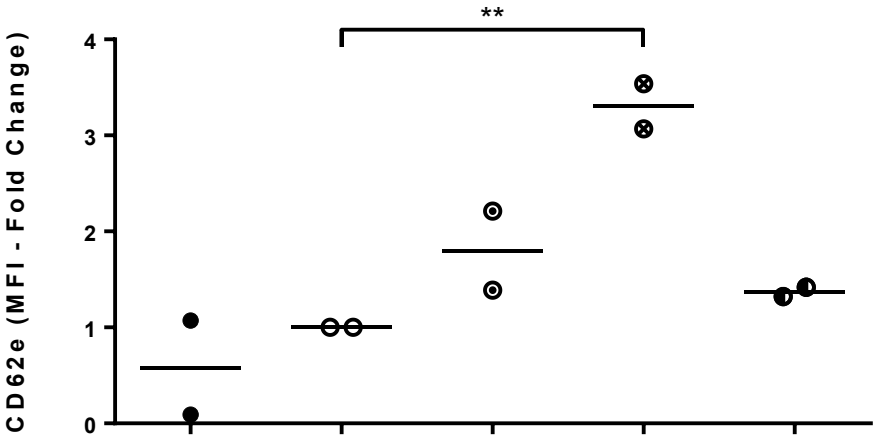
One-way ANOVA with Dunnett's multiple comparison's test

Stimulation of endothelial cell-PBMC co-culture with microvesicles from heat-injured human skin causes upregulation of endothelial cell adhesion molecules

To assess skin microvesicle activity, cell-free supernatant was collected as above. This was centrifuged (20,000g / 30 minutes) to pellet microvesicles, which were then resuspended in 200µl HAS/EBM. The microvesicles were added to the endothelial cell-PBMC co-culture and incubated for 16 hours. Endothelial cells were retrieved and analysed as above.

In response to stimulation with the heat-injured skin-derived microvesicles, there was a significant increase in E-selectin (CD62e) expression by endothelial cells (fold-change 3.3 ± 0.33, $p<0.01$) (figure 5.6A). However, significant changes were not seen following stimulation with either the microvesicles from untreated skin, or the heated skin microvesicle supernatant. A small increase in VCAM-1 (CD106) expression was also found following stimulation with the heated skin microvesicles (figure 5.6B). However, this did not reach statistical significance. Taken together with the neutrophil MV experiments, it appeared that E-selectin was a more sensitive marker of endothelial activation at the 16 hour time-point in this co-culture assay.

A



B

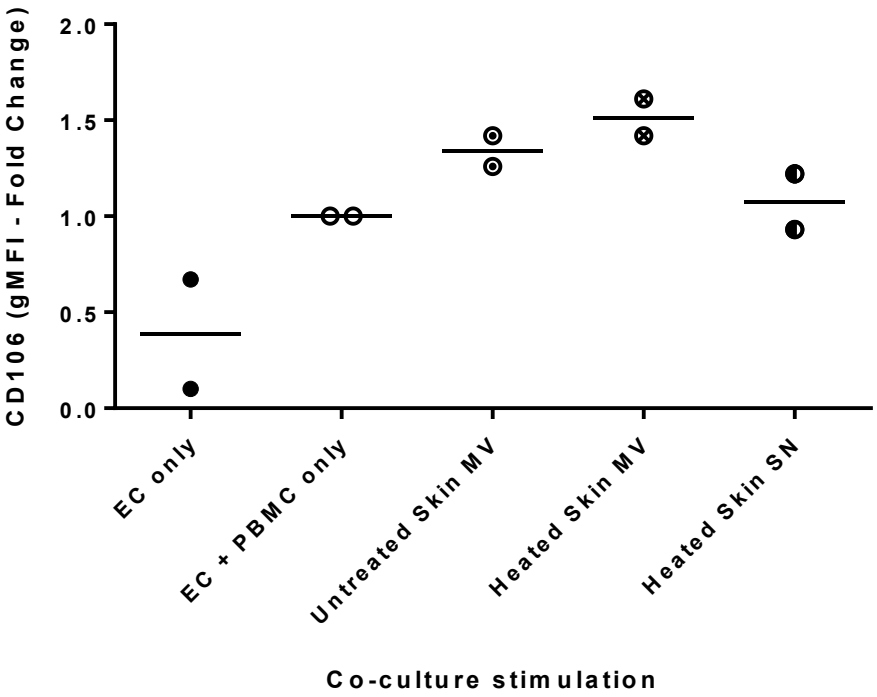


Fig 5.6 ***Stimulation of endothelial co-culture with skin-derived microvesicles***

(A) Stimulation of endothelial cell-PBMC co-culture with heated skin microvesicles leads to significant upregulation of E-selectin (CD62e)

(B) VCAM-1 (CD106) expression in response to same stimuli

***p<0.01*

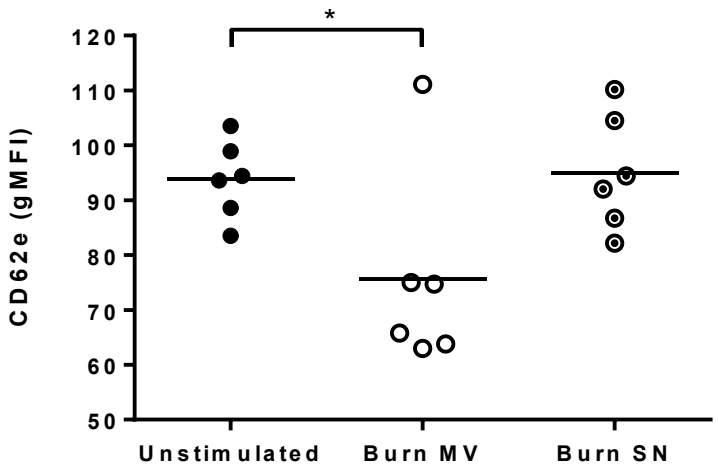
One-way ANOVA with Dunnett's multiple comparisons test (n=2)

Microvesicles isolated from burn patients downregulate endothelial cell adhesion molecule expression *in vitro*

Further optimisation of the co-culture system demonstrated that endothelial cell adhesion molecule expression was maximal at four hours. This was conducted within the group by Nikhil Tirlapur, as part of preliminary work towards a PhD thesis. We used this protocol to assess the activity of circulating microvesicles isolated from patients within 24 hours of burn injury. In this pilot study, samples from the patients with the six highest CD45+ microvesicle counts (in platelet-rich plasma) were analysed to maximise the likelihood of detecting microvesicle activity. Platelet-poor plasma (PPP) was used to minimise the effect of platelets on the co-culture. PPP was generated by an initial 200g / 10 minute centrifugation, followed by a 1500g / 20 minute centrifugation, prior to storage. After being thawed, the PPP was filtered, before centrifugation at 20000g to pellet the microvesicles, before resuspension in 400µl of 0.5% HAS/EBM. The PBMC-endothelial co-culture was then stimulated with the burn patient MV and MV-free supernatants, for four hours.

In contrast to the upregulation demonstrated in previous experiments, incubation with burn patient microvesicles resulted in a significant downregulation of endothelial adhesion molecules. The shorter incubation protocol was more consistent in terms of endothelial cell adhesion molecule expression, enabling data to be expressed as gMFI. E-selectin (CD62e) expression was reduced from a baseline gMFI of 93.7 ± 7.1 to 75.6 ± 18.2 ($p<0.05$) (figure 5.7A). VCAM-1 (CD106) expression also decreased, from a gMFI of 1385 ± 110, to 1154 ± 170 ($p<0.01$) (figure 5.7B). These findings are likely to represent attenuation of the endothelial adhesion molecule upregulation that results from the presence of PBMC alone. Significant downregulation was not found following incubation with the cell and MV-free supernatant, arguing against a significant contribution from soluble mediators.

A



B

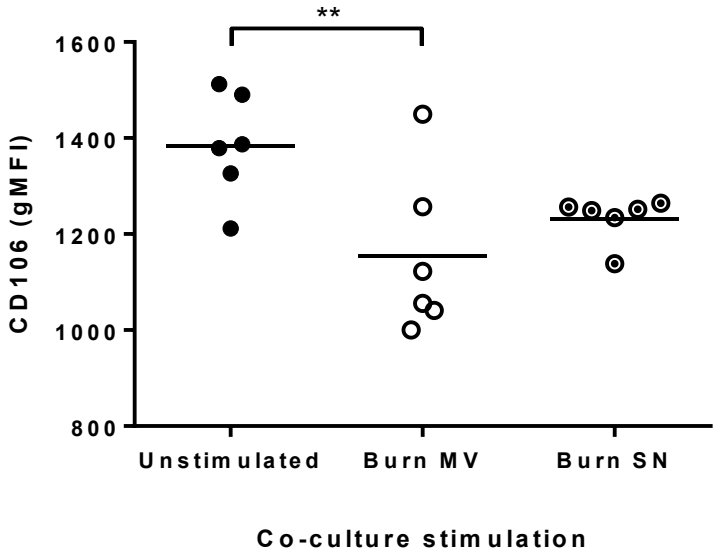


Figure 5.7 ***Incubation of endothelial co-culture with burn patient-derived microvesicles***

Incubation of PBMC-endothelial co-culture with microvesicles (MV) isolated from burn patients leads to significant downregulation of endothelial E-selectin (CD62e) (A) and V-CAM1 (CD106) (B) expression. MV-free supernatant (SN) does not cause significant downregulation.

**p<0.05*

***p<0.01*

One-way ANOVA with Dunnett's multiple comparisons test (n=6)

5.6 Discussion

In vitro generation of neutrophil-derived microvesicles

The overall objective in this chapter was to test our hypothesised role for microvesicles in burn-related inflammation, using *in vitro* models. In particular, we were interested in neutrophil microvesicle production as our *in vivo* studies had identified significantly elevated levels of this subpopulation, following burn injury. Our first specific aim was to investigate whether thermal damage would initiate microvesicle release from neutrophils *in vitro*. To do so, we had to be sure that we were stimulating the neutrophils in an optimised environment.

We conducted a pilot experiment, using PMA as the stimulant but we were unable to elicit a notable increase in microvesicle production. This was followed by further attempts at producing a microvesicle response, using zymosan, opsonised *S. aureus* and the calcium ionophore A23187. These stimuli have all previously been shown to stimulate microvesicle release from neutrophils [320,468,469]. Furthermore, the mechanisms by which these stimuli act are thought to be distinct: PMA is a protein kinase C activator [480], A23187 increases calpain activity via calcium influx [481–483], whilst zymosan and *S. aureus* can stimulate neutrophils once opsonised by complement and immunoglobulins [484,485]. Additionally, there is evidence that non-opsonised zymosan can stimulate neutrophils in a protein tyrosine kinase-dependent pathway [485]. Consequently, we felt there was sufficient diversity in the stimuli to suggest that a generic factor was preventing us from detecting a microvesicle response. A consistent phenomenon in these early experiments was a degree of cell settling, during the rest and stimulation phases. We therefore compared the effect of a rotated stimulation to a static stimulation, to achieve a homogeneous suspension. As seen above, we found that this dramatically increased the number of microvesicles detected, following both *S. aureus* and ionophore stimulation.

Although brief mention is made of linear agitation in the methods of some experiments [468], we were not able to find any research that had explicitly assessed the effect of rotation in this way. Previous investigations have shown that severe agitation may increase platelet microvesicle release, for example during transport of samples [402]. Certainly, there was a

small increase in “spontaneous” microvesicle levels following rotation but this was dwarfed by the numbers seen when stimulants were present, with a near 10-fold increase seen with ionophore stimulation. Clearly, this was not simply a case of increased background signal. The precise mechanism that underlies this finding is unclear and warrants further research. One possible explanation is that the microvesicles are being released when static but remain attached to their parent cells and are therefore lost during the initial centrifugation. A more interesting proposition is that accumulation of a local signalling factor is influencing microvesicle production when cells are static. A candidate molecule is nitric oxide, with research suggesting an inhibitory role in both neutrophils [486] and endothelial cells [487].

We next sought to confirm that opsonisation was an important part of provoking a microvesicle response from neutrophils. We found that although non-opsonised *S. aureus* could increase microvesicle counts, it did so at reduced levels in comparison to opsonised *S. aureus*. This was consistent with previous reports [5] and supported the idea that opsonisation was important to the microvesicle response to burn injury. Further experiments showed us that using LPS or ATP in the rotating environment did not generate a greater yield of microvesicles. We therefore continued using A23187 as our soluble stimulus.

We had hypothesised that the additional steps of neutrophil isolation may have contributed to the lack of microvesicle response seen in our initial experiments. However, the large increases seen following stimulation of a cell suspension suggested this was not the case. When we compared the two cell preparations directly, we found very similar levels of neutrophil microvesicles, following each stimulation. This led us to conclude that the neutrophil isolation method was in fact adequate for investigating microvesicles *in vitro*. It also suggested that the stimulants we were using were acting directly on the neutrophils, rather than via other leukocytes present in the culture. Additionally, this finding was of practical benefit as it meant the PBMCs, isolated simultaneously, could be used for our later co-culture experiments.

The final phase of this *in vitro* section investigated if a burn-relevant stimulus was capable of upregulating microvesicle production from primary neutrophils. We knew from previously published work that neutrophils would adhere to heat-killed fibroblasts and that this was serum-dependent [266]. Heat-killed kidney cells and fibroblast also activate complement [266,470]. Our preliminary research had confirmed that opsonised bacteria could increase microvesicle production significantly. We therefore hypothesised that heated cells, if opsonised, may have a similar effect on microvesicle production. The cells chosen were cultured from the HK-2 cell line, an immortalised kidney epithelial line.

Before incubating heat-injured HK-2 cells with neutrophils, we first confirmed that our heating and opsonisation protocol resulted in detectable complement activation. Although complement activation is not the only means by which pathogens can be opsonised [484], several studies have addressed the importance that complement plays in the response to burn injury [6,94,266,470–473]. We then introduced the opsonised heat-damaged epithelial cells into the neutrophil suspension discussed above. This resulted in a significant increase in the number of neutrophil microvesicles. Levels were also significantly higher than those seen after incubation with the opsonised non-heated HK-2 cells, whilst the non-opsonised heat-damaged cells did not increase microvesicle production at all. We concluded from this that heat-injured cells could indeed interact directly with neutrophils, to upregulate their microvesicle production, in a serum-dependent process. This was a key finding and one which has not been demonstrated elsewhere, to our knowledge.

It is important to highlight that the opsonised non-heated HK-2 cells also caused an increase in microvesicle production, whilst the difference between heated and non-heated counts was not as stark as that seen when examining complement activation alone. There are a number of possible explanations for this. The first is that the epithelial cells themselves may act as potent stimulus; although they are derived from a human cell line, the cells are not autologous and have been subjected to a number of different processing steps. Their viable state may also have increased their activating potential. Secondly, other factors present in serum, such as immunoglobulins, may be partly responsible for mediating the interaction with neutrophils, which we did not assess in the same way as complement activation. Thirdly, we may be witnessing a degree of saturation of the complement-mediated

pathway; the magnitude of response was very similar to that seen with opsonised *S. aureus*, although this may be coincidental. Finally, it may be the case that some microvesicles are bound with large epithelial cell fragments and therefore not accessible for analysis using the above protocol.

Optimisation of an *in vitro* assay to assess inflammatory activity of neutrophil-derived microvesicles

The second phase of our *in vitro* work was to evaluate the activity of the microvesicles we had generated. Our specific interest was their role in mediating endothelial dysfunction, an important feature of the post-burn response [475]. Although previous research had demonstrated that leukocyte-derived microvesicles may play a part in this process [302,318–320], we were interested in creating a more sensitive and physiologically-relevant model. Earlier research conducted in our department had shown that in murine models of sepsis, monocytes marginate to the pulmonary vasculature, where they produce direct activation of the endothelium [488]. In the context of burn injuries, monocyte recruitment is known to be an important part of wound healing and precedes infiltration of the damaged tissue [196,197,245]. We therefore hypothesised that microvesicles may exert a greater effect on endothelial cells in the presence of other leukocytes and that such a model may be more representative of the *in vivo* environment.

Following a simple pilot experiment, which suggested that endothelial cells in co-culture may indeed be more responsive to stimulation, we moved onto testing the microvesicles. We initially evaluated neutrophil microvesicles, generated by A23187 stimulation, as this had consistently yielded the highest counts. By using a soluble stimulant, we also minimised any potential ambiguities that might be caused by residual *S. aureus* or epithelial cell fragments. Having pre-equilibrated the endothelial cells and PBMCs for one hour, we then performed a 16 hour incubation with the neutrophil microvesicles.

The resultant endothelial E-selectin (CD62e) expression was significantly increased, with a 6-fold increase over baseline, a finding not previously reported. In contrast, microvesicles from unstimulated neutrophils did not lead to a significant upregulation of E-selectin. A similar pattern was seen with VCAM-1 (CD106), with a 3-fold increase in expression over

baseline following stimulation with the A23187-generated microvesicles. This change did not reach statistical significance but when taken in conjunction with the E-selectin data, it suggested the model had potential for assaying microvesicle-endothelial interactions.

The lower response seen with VCAM-1 was not the only limitation of our system. We focused our initial efforts on a relatively long stimulation period of 16 hours. Subsequent refinement of our protocols demonstrated that peak adhesion molecule expression occurred at 4-6 hours, consistent with information derived from cytokine stimulation [489]. We also observed considerable variation in the expression of each adhesion molecule between experiments, necessitating their analysis as fold-change. This issue was diminished in subsequent experiments using the shorter stimulation period.

Limitations notwithstanding, we were interested in using this model to test heat-generated microvesicles from a different cellular origin, to see if they exhibited a similar response. *In vitro* studies in our department had demonstrated that samples of human skin, when subjected to thermal injury, produced increased levels of microvesicles (Katbeh U, unpublished work). We isolated these skin-derived microvesicles and incubated them with the co-culture system described above. Microvesicles from thermally-damaged skin elicited a significant upregulation of E-selectin. In contrast, microvesicles from untreated skin did not lead to E-selectin upregulation. The lack of response from heated skin supernatant, which was microvesicle-free, argues against the observed response being mediated by soluble mediators. As was the case with neutrophil-derived microvesicles, the effect on VCAM-1 expression was less marked and did not reach statistical significance.

The conclusions that can be drawn from this pilot study are limited. Further experiments using skin will be conducted but sample availability is unpredictable, as the donation is a sporadic process and relatively expensive. It should also be highlighted that due to a lack of specific surface marker, we were unable to quantify circulating skin microvesicles in our burn patient samples. However, a severe burn by definition involves widespread skin damage and our *in vitro* experiments have demonstrated a robust microvesicle increase after thermal skin damage. There is also growing evidence that microvesiculation is a universal feature of cellular life [490]. Given these points, it would be surprising if skin

microvesicles were not prevalent in burn patients and our studies suggest they may contribute to endothelial dysfunction.

In contrast to the upregulation of endothelial adhesion molecules demonstrated with neutrophil and skin microvesicles, the microvesicles isolated from burn patients exhibited a negative effect on endothelial activation in our co-culture assay. A number of factors need to be considered when interpreting this result. The first is that the microvesicles isolated from patients were a mixed population and would have contained platelet- and erythrocyte-derived MV, in addition to those derived from leukocytes and endothelial cells. Secondly, our earlier experiments had used PBMCs and neutrophils from autologous donors, whereas the burn patient microvesicles were necessarily from non-autologous donors. This may have modulated the co-culture response. It should also be noted that to minimise the effect of platelets, platelet-poor plasma was used as a source of microvesicles. From earlier experiments, we knew that PPP contains significantly less MV than platelet-rich plasma. It is possible that variable effects are seen at different concentrations of MV.

If the negative effect on endothelial activation is representative of *in vivo* activity, this finding would be consistent with a number of prior investigations. Neutrophil-derived MV have been shown to exhibit anti-inflammatory effects *in vitro* [336–338,340,450,452], although this finding is far from universal [318–320]. The available information regarding the function of MV derived from other cells also demonstrates variability, with platelet-derived MV apparently capable of both upregulating [461,462,491] and downregulating inflammatory pathways [329,492]. Separation of microvesicles by subtype from a complex medium such as plasma would be difficult but future experiments will aim to utilise immunomagnetic separation to delineate discrete phenotypes. The applicability of our results to all severely burned patients also needs to be assessed, as we focussed our pilot studies on those patients with the highest MV response. It may be the case that microvesicles from less severely injured patients are phenotypically, as well as quantitatively distinct.

Chapter 6

Final Discussion

As discussed in the introductory chapter, the systemic consequences of a severe burn injury are secondary to a distinct and overwhelming inflammatory response. Marked oedema and hypovolaemia in the acute phase are followed later by prolonged hypermetabolism and catabolism [233,460]. Despite decades of research into this phenomenon, the relationship between localised tissue injury and systemic inflammation remains poorly defined [493]. Although burn care and survival has improved markedly, our ability to directly modulate the inflammatory response and curb its deleterious effects is limited [113,221]. Identification of circulating mediators may facilitate novel therapeutic approaches, in addition to having diagnostic and prognostic utility.

The systemic component of the post-burn inflammatory response has conventionally been linked to dysregulated cytokine release. These cytokines, such as TNF-α and IL-6, are thought to spill over from sites of injury into the circulation [2,234]. However, clinical evolution of the inflammatory response correlates poorly with systemic cytokine profiles [238] and evidence of a causative relationship is lacking. Indeed, significant reduction in the activity of locally-released mediators would be expected as a result of dilution, clearance and specific neutralisation mechanisms. Therefore, soluble cytokines may not be the dominant propagating factor in post-burn inflammation.

Signalling by microvesicles represents an additional "third way" by which cells can communicate, bridging the classical dichotomy between soluble mediators and direct cell-cell contact [304]. They can function as unique biological ferries, capable of transporting a diverse range of cargo [494] and are elevated in disorders of the innate immune response, such as sepsis [3,330]. We hypothesised that due to widespread tissue injury and inflammation, significant microvesicle release would follow a severe burn injury. The overall objective of this project was to evaluate the microvesicle profile associated with burn injury, to examine its relevance to the propagation of inflammation and to establish the relationship with disease severity. To address this aim, we developed optimised protocols to quantify circulating microvesicles and applied them to samples obtained from burn patients. In an accompanying experimental arm, we investigated microvesicle production and function in assays relevant to burn injury and systemic inflammation.

6.1 Optimisation of flow cytometric analysis of microvesicles

Due to their small size, the analysis of microvesicles is challenging [349]. Flow cytometry is the most commonly used method and is the most suitable for clinical studies, particularly as the cellular source of microvesicles can be determined. Our initial experiments demonstrated that the performance of our cytometer was consistent with other "conventional" cytometers, i.e. those not specifically designed to investigate sub-cellular particles. It is important to note, however, that smaller microvesicles are undetectable by flow cytometers, such that underestimation of counts is a recognised limitation in most studies. Although purpose-built cytometers have improved sensitivity [392], emerging technologies such as nanoparticle tracking may be required to give a more comprehensive picture [353].

We sought to establish the optimal "threshold" parameter, used to trigger acquisition of a particle. This step was crucial to maximising sensitivity without overloading the cytometer, as complex biological fluids such as plasma contain a great number of small particles, not all of which are vesicles. We explored the use of cytoplasmic dyes, added directly to plasma, to increase sensitivity. Although our success was limited by a poor signal to noise ratio, presumably due to dye association with plasma lipoprotein complexes, this approach is worthy of further investigation. The adoption of differential detergent lysis allowed us to confirm the vesicular nature of particles detected by the cytometer. More recent research suggests alternative detergents might be used to differentiate between extracellular vesicle populations [495]. This would be highly advantageous, because the current classification of extracellular vesicles relies on arbitrary size ranges and cut-offs [294] which are only loosely related to phenotype and difficult if not impossible to apply practically [496,497].

Our demonstration of significant microvesicle loss at moderate centrifugation speeds, used to deplete platelets, was consistent with published research [376,378]. It supported our selection of platelet-rich plasma for studying leukocyte- and endothelial cell-derived microvesicles. Analysis of platelet-poor plasma, obtained by centrifuging samples at higher speed, would have resulted in significantly less microvesicles being detected. Furthermore, the microvesicle loss may not have been uniform, with larger particles being sedimented at lower speeds, potentially affecting the results qualitatively as well as quantitatively.

6.2 Circulating microvesicles in burn patients

Our clinical study was the first to demonstrate that leukocyte-derived and endothelial cell-derived microvesicles are significantly elevated following severe burn injury. Mortality from burn injury was associated with higher levels of leukocyte microvesicles. This observation may prove to be unique among conditions that trigger a systemic inflammatory response. In trauma patients for example, platelet-derived microvesicles were found at reduced levels in non-survivors [467] and in survivors of ARDS, circulating leukocyte microvesicles were significantly higher [414]. A rapid decline in circulating leukocyte and endothelial microvesicles was evident by day two post-injury, suggesting a decline in production and/or an increase in clearance. A similar transient elevation has been demonstrated in the context of meningococcal sepsis [330] and traumatic brain injury [498], indicating that microvesicles may have utility as markers of hyper-acute systemic inflammation.

Despite the relatively low patient numbers in this pilot study, it was interesting that leukocyte-derived microvesicles correlated with the ABSI and BOBI burn severity scores. In addition to burn size and age, a common component of these scores is the presence of inhalation injury. As predicted by the severity scores, inhalation injury was more common in non-survivors. This raises the possibility that injured lungs are contributing to the elevated circulating pool of microvesicles found post-burn. Both circulating [414] and intra-alveolar [499] microvesicle levels are elevated in patients with ARDS. Although our sample collection methods are not yet optimised, initial analysis of bronchoalveolar lavage fluid (BALF) from our burn patients revealed high levels of leukocyte-derived microvesicles (see appendix). Conversely, circulating microvesicles may also contribute to secondary lung damage, even in the absence of direct inhalation injury. Endothelial cell-derived microvesicles, for example, have been shown to induce pulmonary oedema and neutrophil recruitment in mice [500]. The lungs may therefore be an excellent candidate for studying the role of microvesicles in the development of remote organ inflammation and dysfunction post-burn injury.

6.3 In vitro assessment of microvesicle production and activity

We set out to develop assays to evaluate the activity of microvesicles derived from burn patients and from cell-based *in vitro* models of thermal injury. In our preliminary investigations of neutrophil microvesicles, we found that a static culture environment appeared to inhibit microvesicle release. In common with our other *in vitro* experiments, this was an artificial environment and may not reflect *in vivo* processes. However, a possible explanation is that a negative autocrine feedback mechanism exists, whereby local accumulation of mediators released from neutrophils under static conditions prevents microvesicle release. We do not yet know if this is a generic mechanism applicable to all cells. If a local signalling cascade exists that is specific to neutrophils, it might be exploitable pharmacologically. Our finding that neutrophil microvesicle release was enhanced by opsonised heat-killed cells was novel but in isolation, does not provide evidence that complement activation is the dominant stimulating factor *in vivo*. However, the results are noteworthy given that burn and trauma-induced complement activation is an established process [262–265]. The complement system may mediate the direct interaction of pathogens and neutrophil microvesicles, as ectosomes released by human neutrophils at sites of inflammation express clusters of complement receptor 1 [469].

The second aspect of our *in vitro* studies was to investigate the activity of the microvesicles we had identified in burn patients, to establish if a role in endothelial dysfunction was plausible. Having demonstrated pulmonary endothelial activation by neutrophil- and skin-derived microvesicles, we were surprised to discover a small but significant inhibitory effect of platelet-poor plasma derived from burn patients. The loss of a major fraction of plasma microvesicles by centrifugation is a concern and needs to be addressed in the future. However, if this inhibitory effect can be conclusively attributed to circulating microvesicles, it would be an important finding, considering the prevalence of immunosuppression in these patients. Although we have demonstrated an association between mortality and leukocyte-derived microvesicle levels, we have no evidence for causation as yet. These pilot investigations raise the possibility that microvesicles may contribute to the inappropriate dampening of the innate immune response , a feature of severe burn injury [279].

6.4 Concluding remarks

The classical paradigm of intercellular communication is based on direct cell-cell contact and the secretion of paracrine molecules. Over the past few years, the importance of sub-cellular vesicle exchange has become increasingly recognised [501]. It may even be the case that a significant proportion of "soluble" molecules measured in fluids are in fact vesicle-bound [334]. Ours is the first study to demonstrate acute increases in the level of circulating microvesicles following thermal injury. Moreover, leukocyte-derived microvesicles were found to be predictive of mortality, suggesting they may have prognostic utility.

The precise role of these microvesicles in mediating the post-burn inflammatory response remains to be elucidated. Our initial studies have provided conflicting evidence but it is possible that the net effect of neutrophil-derived microvesicles, the most numerous sub-population post-burn, is to modulate the inflammatory response [502]. It seems likely that significant heterogeneity exists even within microvesicle subtypes, with their activity also varying according to target cell phenotype.

Our priorities for future work are as follows:

1) Assess the activity of microvesicles derived from monocytes, platelets and endothelial cells, alone or in combination, on the endothelial-PBMC co-culture model
2) Compare these actions to those of specific microvesicle populations, isolated from burn patient samples
3) Increase our burn patient cohort and expand our investigations to include patients with inflammation secondary to major trauma
4) Investigate longitudinal microvesicle release in burn patients, particularly in the context of supervening sepsis, to assess potential diagnostic utility

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Appendix

7.1 Study Documentation

Consent Form for Volunteers

Title of Project:

Pilot study to evaluate gas and fluid-phase biomarkers of inflammation and oxidative stress in patients with burn injuries

Study Identification:

Initials:

Participant Declaration (please **INITIAL** all boxes which apply)

I have been given the chance to read and understand the information sheet relating to the above study	☐
I have been given the opportunity to ask questions and discuss the study	☐
I have been made aware of the risks/ benefits	☐
I understand that relevant sections of any data collected during the study may be looked at by individuals from regulatory authorities and/or Chelsea and Westminster Hospital NHS Foundation Trust/Queen Victoria Hospital NHS Foundation Trust/The Royal Marsden NHS Foundation Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	☐
I agree to the storage of my samples for future use in ethically approved research projects	☐
I understand that I am free to withdraw from this study at any time	☐

Participant

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

Name: Signature:

Date:

Person responsible for obtaining Informed Consent

"To the best of my knowledge I have provided the above individual with sufficient information to enable them to give informed consent".

Name: Signature:

Date: Position:

Witnessed by

Name: Signature:

Date: Position

Chelsea & Westminster Hospital NHS Foundation Trust
369 Fulham Road, London, SW10 9NH

Queen Victoria Hospital NHS Foundation Trust
Holtye Road, East Grinstead, RH19 3DZ

The Royal Marsden NHS Foundation Trust
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VOLUNTEER INFORMATION SHEET

08th October 2014
Version 3.0

STUDY TITLE

Pilot study to evaluate gas and fluid-phase biomarkers of inflammation and oxidative stress in patients with burn injuries

INVESTIGATORS

Dr Suveer Singh, Dr Nandor Marczin, Dr John Porter, Dr Julian Giles, Dr Vimal Grover.

WHAT IS THIS ALL ABOUT?

Introduction

This participant information sheet gives some information about a research study being carried out at Chelsea and Westminster Hospital, Queen Victoria Hospital and The Royal Marsden Hospital. It includes details on the type of information being collected and the aims of the study. If there is anything that you do not understand, or if you require information concerning this study, please ask the research staff for further information.

Invitation

You are being invited to take part in a research study. Before you decide whether or not to take part it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends and relatives. Ask us if there is anything that is not clear or if you would like more information.

Thank you for taking the time to read this form.

WHY ARE WE DOING THIS?

Purpose of the study

The main aim of this study is to measure specific gases, cells and proteins in the breath and biological fluids of patients with burn injuries. The information obtained will be compared with findings from other patient groups.

Explanation of project

Patients with burn injuries will develop inflammation at the site of their injury. If the burn injury is severe enough, this inflammation may spread to other sites in the body. In some patients this can affect how well organs work, for example the lungs or the kidneys. This is why some patients need to be looked after in the Burns Intensive Care Unit. Such inflammation affects the levels of cells and proteins in various body fluids, such as blood and lung fluid. It can also affect the production of certain gases, which can be detected in exhaled breath. Collectively, these are known as 'biomarkers'. Studies have identified that biomarkers may prove useful in the diagnosis and treatment certain conditions. To date, there has only been limited research to look at these biomarkers in patients with burn injuries. We aim to study a number of biomarkers and compare them to the overall condition of patients. In particular, we are interested in the gases that may be produced as very little is known about this aspect. In the future, the information we obtain might be useful for guiding patients' treatment.

Study Design

We plan to conduct this study over four years from 2012 to 2016. We wish to study seven groups of participants and we aim to recruit a total of 200 participants to our study.

The first group of patients will be patients with burn injuries, in whom we will measure lung, blood and urine levels of our biomarkers.

The second group will be patients who have injuries to their lungs, as a result of breathing in smoke.

The third group of will be patients who have developed a severe infection, such that they need to be cared for in the General Intensive Care Unit.

The fourth group will be patients in the Intensive Care Unit undergoing bronchoscopy for a suspected chest infection

The fifth group will be patients with low levels of white blood cells, who have developed a severe infection

The sixth group will be patients undergoing outpatient bronchoscopy.

The final group will be healthy volunteers.

WHY HAVE I BEEN CHOSEN?

You have been chosen as you are a healthy volunteer.

WHAT HAPPENS IF I TAKE PART?

Procedures undertaken, side effects and possible risks

1. Blood sampling

This is performed routinely in management of severe burns, smoke inhalation, and severe infection.

We will take blood samples from a vein in your hand or arm, up to a total of 30mls. To reduce the risk of infection or contamination of the samples, the skin will be thoroughly cleaned with antiseptic. To minimize any discomfort while performing this procedure, local anaesthetic can be applied to the skin before inserting a needle into the vein to draw blood. There is a slight risk of bruising which will subside soon.

2. Breath analysis

This is performed by one of two devices. The first is connected via plastic tubing to the breathing machine that supports you during surgery or while you are in intensive care. It is very similar to a device that we use routinely in these situations. The second uses small containers that store condensed fluid from the breathing machine. If you do not require support in intensive care or are not undergoing surgery, you will be required to blow into a simple mouthpiece. Your breath can then be analysed. Breath testing is very safe. We will perform breath testing twice but may repeat this if you become more unwell.

3. Urine collection

Most patients that we will be studying will require a urinary catheter at some point. This drains the bladder continuously and samples can be taken from this tube. If you do not require a urinary catheter as part of your standard treatment, we may ask you to provide a urine sample into a simple container. This non-invasive procedure is performed frequently as part of many medical tests.

Confidentiality

After obtaining the samples from you, they will be anonymised by giving them encoded reference number. This will be linked to your details only in a secured file which is kept separate from your medical notes. All other personal data that identifies you with the sample will be discarded.

Use of samples

We will take samples to laboratory and we will measure levels of our biomarkers in the sample. The results of the study will be submitted for publication in scientific journals, and they can be made available to you, if you wish to know at the end of the study.

If you decide to withdraw from the study after the procedure, and before submission of data for publication in scientific journals, we will discard those samples and data derived from them.

All samples will be stored at the end of the study for a period of three years.

Participation in the study

Finally, we would like to thank you for your interest in the study, even if you have decided not to take part. There is no pressure on you to participate and if you agree to participate you are free to withdraw at any stage without having to provide any reason. There will be no direct benefits to you for taking part in this study. It may allow us to establish the role of biomarkers in the early diagnosis of the source of infection in the future and help patients in similar circumstances. If you do decide to take part in the study all information relating to the study will be kept confidentially and you will not be identifiable in any reports of the study.

If you have any questions or concerns following your participation in the study please do not hesitate to contact the research team:

Dr John Porter (Tel: 020 8746 8518), Clinical Research Fellow, Chelsea & Westminster Hospital, 369 Fulham Road, London, SW10 9NH.

Dr Suveer Singh (Tel: 020 8746 8472), Consultant Physician in Respiratory and Intensive Care Medicine, Chelsea & Westminster Hospital, 369 Fulham Road, London, SW10 9NH.

Dr Julian Giles (Tel: 01342 414000), Consultant Anaesthetist, Queen Victoria Hospital, Holtye Road, East Grinstead, RH19 3DZ.

Dr Vimal Grover (Tel: 020 7352 8171), Consultant Anaesthetist and Intensivist, The Royal Marsden Hospital, Fulham Road, London, SW3 6JJ.

Thank you very much for helping with this project.

Consent Form for Patients

Title of Project:

Pilot study to evaluate gas and fluid-phase biomarkers of inflammation and oxidative stress in patients with burn injuries

Patient Hospital Number: Patient Study Identification:

Patient Initials:

Participant Declaration (please **INITIAL** all boxes which apply)

I have been given the chance to read and understand the information sheet relating to the above study	☐
I have been given the opportunity to ask questions and discuss the study	☐
I have been made aware of the risks/ benefits	☐
I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by individuals from regulatory authorities and/or Chelsea and Westminster Hospital NHS Foundation Trust/Queen Victoria Hospital NHS Foundation Trust/The Royal Marsden NHS Foundation Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	☐
I agree to the storage of my samples for future use in ethically approved research projects	☐
I understand that I am free to withdraw from this study at any time without prejudice to my future care/ treatment	☐

Participant

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

Name: Signature:

Date:

Person responsible for obtaining Informed Consent

"To the best of my knowledge I have provided the above individual with sufficient information to enable them to give informed consent".

Name: Signature:

Date: Position:

Witnessed by

Name: Signature:

Date: Position

Chelsea & Westminster Hospital NHS Foundation Trust
369 Fulham Road, London, SW10 9NH

Queen Victoria Hospital NHS Foundation Trust
Holtye Road, East Grinstead, RH19 3DZ

The Royal Marsden NHS Foundation Trust
Fulham Rd, London, SW3 6JJ

PATIENT INFORMATION SHEET

08th October 2014
Version 5.0

STUDY TITLE

Pilot study to evaluate gas and fluid-phase biomarkers of inflammation and oxidative stress in patients with burn injuries

INVESTIGATORS

Dr Suveer Singh, Dr Nandor Marczin, Dr John Porter, Dr Julian Giles, Dr Vimal Grover.

WHAT IS THIS ALL ABOUT?

Introduction

This participant information sheet gives some information about a research study being carried out at Chelsea and Westminster Hospital, Queen Victoria Hospital and The Royal Marsden Hospital. It includes details on the type of information being collected and the aims of the study. If there is anything that you do not understand, or if you require information concerning this study, please ask the research staff for further information.

Invitation

You are being invited to take part in a research study. Before you decide whether or not to take part it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends and relatives. Ask us if there is anything that is not clear or if you would like more information.

Thank you for taking the time to read this form.

WHY ARE WE DOING THIS?

Purpose of the study

The main aim of this study is to measure specific gases, cells and proteins in the breath and biological fluids of patients with burn injuries. The information obtained will be compared with findings from other patient groups.

Explanation of project

Patients with burn injuries will develop inflammation at the site of their injury. If the burn injury is severe enough, this inflammation may spread to other sites in the body. In some patients this can affect how well organs work, for example the lungs or the kidneys. This is why some patients need to be looked after in the Burns Intensive Care Unit. Such inflammation affects the levels of cells and proteins in various body fluids, such as blood and lung fluid. It can also affect the production of certain gases, which can be detected in exhaled breath. Collectively, these are known as 'biomarkers'. Studies have identified that biomarkers may prove useful in the diagnosis and treatment certain conditions. To date, there has only been limited research to look at these biomarkers in patients with burn injuries. We aim to study a number of biomarkers and compare them to the overall condition of patients. In particular, we are interested in the gases that may be produced as very little is known about this aspect. In the future, the information we obtain might be useful for guiding patients' treatment.

Study Design

We plan to conduct this study over four years from 2012 to 2016. We wish to study seven groups of participants and we aim to recruit a total of 200 participants to our study.

The first group of patients will be patients with burn injuries, in whom we will measure lung, blood and urine levels of our biomarkers.

The second group will be patients who have injuries to their lungs, as a result of breathing in smoke.

The third group of will be patients who have developed a severe infection, such that they need to be cared for in the General Intensive Care Unit.

The fourth group will be patients in the Intensive Care Unit undergoing bronchoscopy for a suspected chest infection

The fifth group will be patients with low levels of white blood cells, who have developed a severe infection

The sixth group will be patients undergoing outpatient bronchoscopy.

The final group will be healthy volunteers.

WHY HAVE I BEEN CHOSEN?

You have been chosen as you are in the category of participants who:

.....

WHAT HAPPENS IF I TAKE PART?

Procedures undertaken, side effects and possible risks

1. Blood sampling

This is performed routinely in the management of severe burns, smoke inhalation and severe infection.

We will take blood samples from a vein in your hand or arm. At the same time we will remove a further 10-20mls of blood (equivalent to a tablespoon) for the purposes of our study. To reduce the risk of infection or contamination of the samples, the skin will be thoroughly cleaned with antiseptic. To minimize any discomfort while performing this procedure, local anaesthetic can be applied to the skin before inserting a needle into the vein to draw blood. There is a slight risk of bruising which will subside soon. We will take 1 sample soon after you have been admitted and 1 further sample if you become more unwell.

2. Breath analysis

This is performed by one of two devices. The first is connected via plastic tubing to the breathing machine that supports you during surgery or while you are in intensive care. It is very similar to a device that we use routinely in these situations. The second uses small containers that store condensed fluid from the breathing machine. If you do not require support in intensive care or are not undergoing surgery, you will be required to blow into a simple mouthpiece. Your breath can then be analysed. Breath testing is very safe. We will perform breath testing twice but may repeat this if you become more unwell.

3. Urine collection

Most patients that we will be studying will require a urinary catheter at some point. This drains the bladder continuously and samples can be taken from this tube. If you do not require a urinary catheter as part of your standard treatment, we may ask you to provide a urine sample into a simple container. This non-invasive procedure is performed frequently as part of many medical tests. We will take 1 sample soon after you have been admitted and 1 further sample if you become more unwell.

4. Bronchoscopy

This is performed routinely for patients suffering from severe smoke inhalation, in some patients with infections and in the bronchoscopy clinic.

Patients who have inhaled smoke may need support from a breathing machine. Once the patient is asleep, a breathing tube is inserted via the mouth or nose. It is often necessary to look inside the lungs with a small camera (bronchoscope), inserted through the breathing tube. This is to look for signs of infection or objects blocking the lungs and if necessary to

wash the lungs out. Other patients undergo bronchoscopy for diagnosis or treatment in the outpatient clinic. We send some of the fluid collected for laboratory analysis. As part of our study, we would also send some fluid for biomarker analysis. It is not a painful procedure but it can cause coughing so we give drugs to minimise this. There is a risk of patients requiring more oxygen for a period after this procedure. For this reason, we will not perform it if you are requiring a very high amount of oxygen or high levels of support from the breathing machine. Bronchoscopy will only be performed if necessary for your treatment.

Confidentiality

After obtaining the samples from you, they will be anonymised by giving them encoded reference number. This will be linked to your details only in a secured file which is kept separate from your medical notes. All other personal data that identifies you with the sample will be discarded.

Use of samples

We will take samples to laboratory and we will measure levels of our biomarkers in the sample. The results obtained will be without significance for you personally; i.e. they will not guide your care in hospital. The medical team responsible for you will use existing proven tests and treatments to manage your condition in line with existing protocols. The results of the study will be submitted for publication in scientific journals, and they can be made available to you, if you wish to know at the end of the study. If you decide to withdraw from the study after the procedure, and before submission of data for publication in scientific journals, we will discard those samples and data derived from them. All samples will be stored at the end of the study for a period of three years.

Participation in the study

Finally, we would like to thank you for your interest in the study, even if you have decided not to take part. There is no pressure on you to participate and if you agree to participate you are free to withdraw at any stage without having to provide any reason. Whatever your decision it will not affect in any way the treatment you may be receiving at this hospital. There will be no direct benefits to you for taking part in this study. It may allow us to establish the role of biomarkers in the early diagnosis of the source of infection in the future and help patients in similar circumstances. If you do decide to take part in the study all information relating to the study will be kept confidentially and you will not be identifiable in any reports of the study.

If you have any questions or concerns following your participation in the study please do not hesitate to contact the research team:

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Dr Vimal Grover (Tel: 020 7352 8171), Consultant Anaesthetist and Intensivist, The Royal Marsden Hospital, Fulham Road, London, SW3 6JJ.

Thank you very much for helping with this project.

Consultee Declaration Form

Title of Project:

Pilot study to evaluate gas and fluid-phase biomarkers of inflammation and oxidative stress in patients with burn injuries

Patient Hospital Number: Patient Study Identification:

Consultee Declaration (please **INITIAL** all boxes which apply)

I have been consulted about my relative/patient I represent participating in this research and have been given the chance to read and understand the information sheet relating to the above study	☐
I have been given the opportunity to ask questions and understand what is involved	☐
In my opinion he/she would have no objection to taking part	☐
I understand that relevant sections of any of his/her medical notes and data collected during the study may be looked at by individuals from regulatory authorities and/or Chelsea and Westminster Hospital NHS Foundation Trust/Queen Victoria Hospital NHS Foundation Trust/The Royal Marsden NHS Foundation Trust, where it is relevant to him/her taking part in this research. I give permission for these individuals to have access to his/her records	☐
I understand that I can request that he/she is withdrawn from the study at any time without giving a reason without his/her legal rights or future care/treatment being affected	☐
I agree to the storage and future use of his/her samples for future use in ethically approved research projects	☐

Relative/representative

Name: Signature:

Date: Relationship:

Person responsible for obtaining assent

"To the best of my knowledge I have provided the above individual with sufficient information to enable them to give informed assent".

Name: Signature:

Date: Position:

Witnessed by

Name: Signature:

Date: Position:

Chelsea & Westminster Hospital NHS Foundation Trust
369 Fulham Road, London, SW10 9NH

Queen Victoria Hospital NHS Foundation Trust
Holtye Road, East Grinstead, RH19 3DZ

The Royal Marsden NHS Foundation Trust
Fulham Rd, London, SW3 6JJ

RELATIVE/REPRESENTATIVE INFORMATION SHEET

08th October 2014
Version 4.0

STUDY TITLE

Pilot study to evaluate gas and fluid-phase biomarkers of inflammation and oxidative stress in patients with burn injuries

INVESTIGATORS

Dr Suveer Singh, Dr Nandor Marczin, Dr John Porter, Dr Julian Giles, Dr Vimal Grover.

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Invitation

Your relative/patient you represent is being invited to take part in a research study. Before you decide whether or not you are happy for them to take part it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends and relatives. Ask us if there is anything that is not clear or if you would like more information.

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The third group of will be patients who have developed a severe infection, such that they need to be cared for in the General Intensive Care Unit.

The fourth group will be patients in the Intensive Care Unit undergoing bronchoscopy for a suspected chest infection

The fifth group will be patients with low levels of white blood cells, who have developed a severe infection

The sixth group will be patients undergoing outpatient bronchoscopy.

The final group will be healthy volunteers.

WHY HAS MY RELATIVE/PATIENT I REPRESENT BEEN CHOSEN?

They have been chosen as they are in the category of participants who:

.....

WHAT HAPPENS IF I AGREE FOR MY RELATIVE/PATIENT I REPRESENT TO TAKE PART?

Procedures undertaken, side effects and possible risks

1. Blood sampling

This is performed routinely in management of severe burns, smoke inhalation, severe infection and before weight-loss surgery.

We will take blood samples from a vein. At the same time we will remove a further 6-10mls of blood (equivalent to a tablespoon) for the purposes of our study. To reduce the risk of infection or contamination of the samples, the skin will be thoroughly cleaned with antiseptic. To minimize any discomfort while performing this procedure, local anaesthetic can be applied to the skin before inserting a needle into the vein to draw blood. There is a slight risk of bruising which will subside soon. We will take 1 sample soon after admission and 1 further sample if your relative/patient you represent becomes more unwell.

2. Breath analysis

This is performed by one of two devices. The first is connected via plastic tubing to the breathing machine that supports patients during surgery or while they are in intensive care. It is very similar to a device that we use routinely in these situations. The second uses small containers that store condensed fluid from the breathing machine. If your relative does not require support in intensive care or is not undergoing surgery, they may be asked to blow into a simple mouthpiece. The breath can then be analysed. Breath testing is very safe. We will perform breath testing twice but may repeat this if your relative/patient you represent becomes more unwell.

3. Urine collection

Most patients that we will be studying will require a urinary catheter at some point. This drains the bladder continuously and samples can be taken from this tube. If your relative does not require a urinary catheter as part of their standard treatment, we may ask them to provide a urine sample into a simple container. This non-invasive procedure is performed frequently as part of many medical tests. We will take 1 sample soon after admission and 1 further sample if your relative/patient you represent becomes more unwell.

4. Bronchoscopy

This is performed routinely for patients suffering from severe smoke inhalation and in some patients with infections.

Patients who have inhaled smoke may need support from a breathing machine. Once the patient is asleep, a breathing tube is inserted via the mouth or nose, or into the front of their neck. The breathing machine is connected to this tube. It is often necessary to look inside the lungs with a small camera (bronchoscope), inserted through the breathing tube.

This is to look for signs of infection or objects blocking the lungs and if necessary wash the lungs out. We send some of the fluid collected for laboratory analysis. As part of our study, we would also send some fluid for biomarker analysis. It is not a painful procedure but it can cause coughing so we give sedative drugs to minimise this. There is a risk of patients requiring more oxygen for a period after this procedure. For this reason, we will not perform it if your relative is requiring a very high amount of oxygen or high levels of support from the breathing machine. Bronchoscopy will only be performed if necessary for treatment.

Confidentiality

After obtaining the samples, they will be anonymised by giving them an encoded reference number. This will be linked to your relative's details only in a secured file which is kept separate from the medical notes. All names and personal data that identify your relative with the sample will be discarded.

Use of samples

We will take samples to laboratory and we will measure levels of our biomarkers in the sample. The results obtained will be without significance for your relative personally; i.e. they will not guide their care in hospital. The medical team responsible for your relative will use proven tests and treatments in line with existing protocols. The results of the study will be submitted for publication in scientific journals, and they can be made available to you or your relative, if you wish to know at the end of the study. If you decide to withdraw from the study after the procedure, and before submission of data for publication in scientific journals, we will discard those samples and data derived from them. All samples will be stored at the end of the study for a period of three years.

Participation in the study

Finally, we would like to thank you for your interest in the study, even if you have decided not to agree for your relative/patient to take part. There is no pressure on you to agree to participation and if you do agree you are free to change your mind at any stage without having to provide any reason. Whatever your decision it will not affect in any way the treatment your relative may be receiving at this hospital. There will be no direct benefits to your relative for taking part in this study. It may allow us to establish the role of biomarkers in the early diagnosis of infection in the future and help patients in similar circumstances. If you do agree for your relative to take part in the study all information relating to the study will be kept confidentially and you will not be identifiable in any reports of the study.

If you have any questions or concerns following your participation in the study please do not hesitate to contact the research team:

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Dr Suveer Singh (Tel: 020 8746 8472), Consultant Physician in Respiratory and Intensive Care Medicine, Chelsea & Westminster Hospital, 369 Fulham Road, London, SW10 9NH.

Dr Julian Giles (Tel: 01342 414000), Consultant Anaesthetist, Queen Victoria Hospital, Holtye Road, East Grinstead, RH19 3DZ.

Dr Vimal Grover (Tel: 020 7352 8171), Consultant Anaesthetist and Intensivist, The Royal Marsden Hospital, Fulham Road, London, SW3 6JJ.

Thank you very much for helping with this project.

7.2 Permissions

Figure 3.1 - "Schematic of typical flow cytometer" (chapter 3)

From: Alina Foo
Sent: 09 October 2015 22:16
To: Porter, John RS R S
Cc: Permissions
Subject: RE: Permission for image use

Dear Dr. Porter,

Thank you for requesting permission.

Please accept this email as your authorization to use the material as described in your correspondence below, and be sure to include a complete citation to the original source:

Michael Brown and Carl Wittwer. Flow Cytometry: Principles and Clinical Applications in Hematology. Clinical Chemistry 2000; v. 46, p.1221-1229. (Reproduced with permission from the American Association for Clinical Chemistry).

Kind regards,
Alina

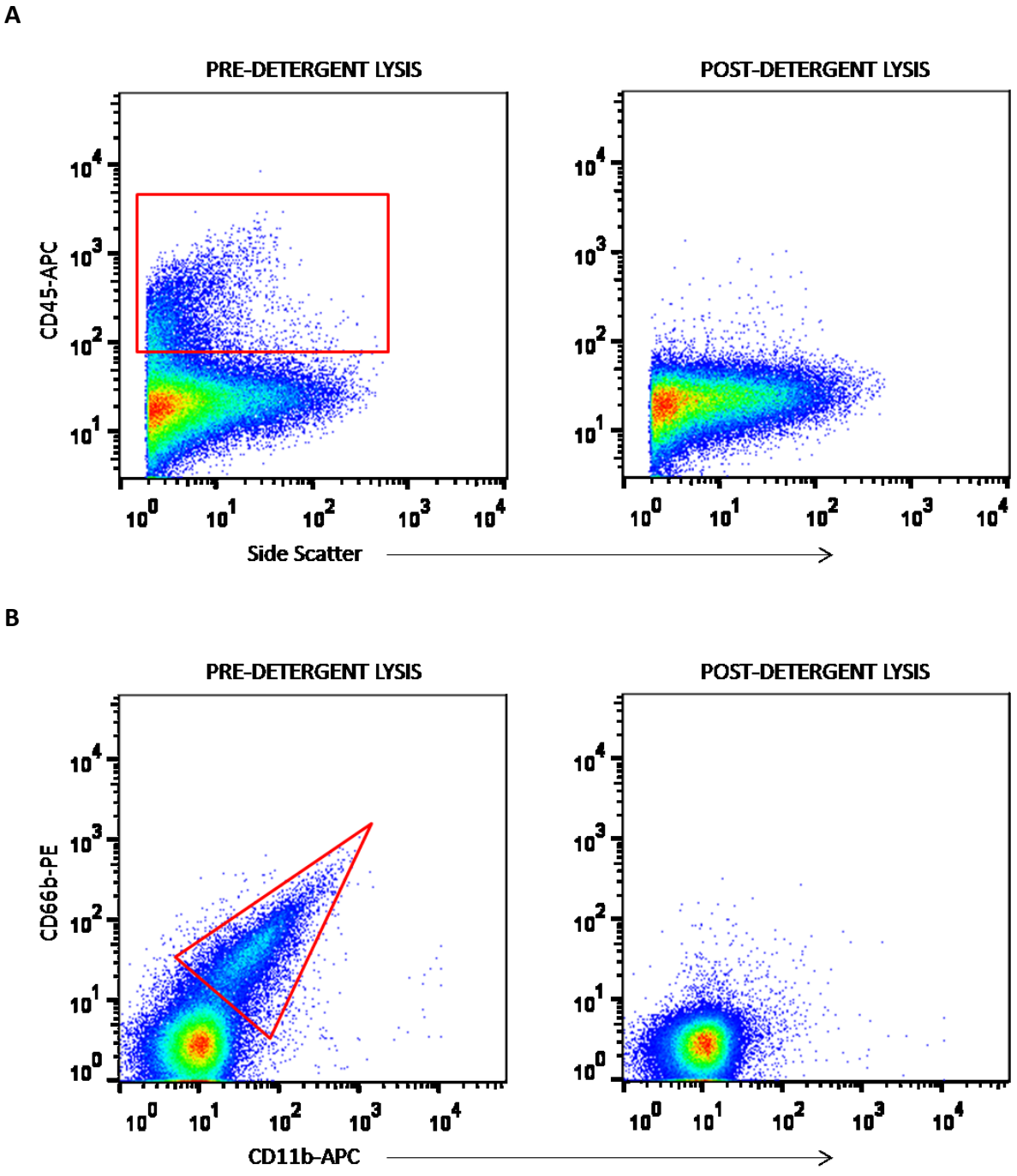
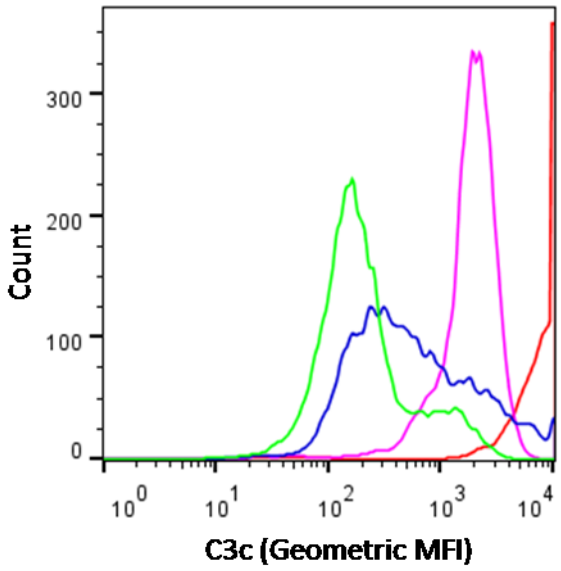


Figure 7.1 ***Pilot analysis of leukocyte-derived microvesicles in bronchoalveolar lavage fluid (BALF) sampled from patient invasively ventilated following inhalation injury***

Detergent-sensitive leukocyte-derived (A) and neutrophil-derived (B) microvesicle populations are indicated by red boxes

7.4 Complement activation in response to heat treatment of kidney epithelial cells



	HK2 Temp (^oC)	C3c (Geometric MFI)
	37 - HBSS	203
	37 - Serum	537
	80 - HBSS	1634
	80 - Serum	8065

Figure 7.2 ***Analysis of complement activation following heat treatment and opsonisation of HK-2 kidney epithelial cells***

Heating HK-2 epithelial cells to 80^oC prior to incubation with human serum leads to increased complement activation on cell surface, as demonstrated by increased levels of complement C3c, a stable C3-conversion product