UNIVERSITY OF BIRMINGHAM

Metabolic Pathways, Immune Regulation and Clinically Relevant Biomarkers Impacted by Landiolol Therapy in Sepsis

by

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Abstract

Background: Sepsis affects 245,000 people annually in the UK with up to 48,000 deaths. Globally sepsis is involved with the death of 11 million people. It has been hypothesised that β-blockade provides protection to patients during septic shock through reducing inflammation, regulating metabolism, reducing vasopressor requirements and subsequently reducing cardiac dysfunction. A previous clinical trial conducted in Rome by Morelli and colleagues in 2013, showed remarkable improvements in mortality but were criticised for the high mortality in the group who did not receive beta-blockade. STRESS-L, a multi-centre randomised trial of patients with established and severe septic shock (as measured by treatment with high dose noradrenaline >24 hours at recruitment) and a tachycardia with heart rate >95 bpm used Landiolol and aimed to perform the largest study of beta-blockade in septic shock so far performed, and to provide mechanistic insight to β-blockade in septic shock. STRESS-L stopped recruitment early following recommendation from the Data Monitoring Committee due to concerns surrounding mortality.

Methods: 100 patients (419 plasma samples) taken at time points: Day 0, 1, 2, 4, 6 and end of noradrenaline therapy following randomisation into STRESS-L were analysed by flow infusion electrospray ionisation high-resolution mass spectrometry (FIE-HRMS)-based metabolomics. Luminex multiplex analysis was further used to identify metabolic and immune changes respectively. Commercially-sourced ELISAs were used to measure CK-MB and Pro-BNP concentrations as indicators of cardiac damage. Metabolomic data were analysed by several chemometric statistical tests, including PCA, PLS-DA and data-visualisation cluster analysis. Enrichment and AUC/ROC analysis was run using MetaboAnalyst. Other statistics analysed used IBM® SPSS version 28 software package.

Results: STRESS-L enrolled 126 patients, 63 of whom received Landiolol. 58% of patients were male in both treatment arms, with the control arm seeing a 28-day mortality of 25% and the interventional arm saw 36%. Landiolol did not influence the trial primary outcome, SOFA score, sub-analysis did show improvement in survivor category. Noradrenaline requirement was minimally greater in the interventional arm (+0.05 mcg/kg/min) compared to control; no clinically relevant difference of mean arterial pressure was observed. Landiolol provided excellent heart rate management. Cytokine analysis showed IFNγ and IL-2 were both higher in standard care. IL-1Ra was higher in Landiolol treated patients. Analysis by mortality status showed non-survivors more hyper-inflamed with increases in TNFα, IL-6 and IL-8. In survivors, Landiolol treated patients had reductions in cardiac troponins and CK-MB not observed in standard care. AUC analysis showed cTnI was a good predictor of mortality along with IL-10 in early timepoints. Landiolol treated survivors also had a consistently lower cTn level despite higher of noradrenaline dosage (at the time point) not replicated in standard care. Metabolomic analysis showed poor separation by treatment allocation and generally patient mortality.

Conclusion: STRESS-L was stopped before achieving its full patient recruitment. Mortality was not significantly higher in the clinical study. Landiolol offered cardiac protection not observed to the same degree in standard care, particularly regarding noradrenaline dosage and tachycardia effects on cardiac stress (as a measure of cTnI and CK-MB). The study also showed Landiolol feasibly acting as an anti-inflammatory. Metabolomic analysis provided inconclusive outcomes but presented insight into SOFA score separations between survivors and non-survivors.

Dedication(s)

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To my grandmother, Virginia, who passed peacefully on
18th April 2022

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Contents

1. Introduction

1.1: General Overview

Sepsis in the UK affects 245,000 people annually, 10% of these are children. According to the UK's leading sepsis charity, *The UK Sepsis Trust*, at least 48,000 people die yearly in the UK from sepsis. Global numbers are even more striking with 11 million lives lost (Sepsis Trust, 2021). Financially, sepsis is a burden and costly to both the National Health Service (NHS) and the economy. Estimates by HM Government placed the ballpark cost to NHS at around £2.0 Billion in 2015, this is now expected to be drastically higher as not only people begin to live longer increasing one's risk of sepsis, but also with rises in antimicrobial resistance and community-acquired infections (DHSC, 2015).

The definition has been made consensus in three iterations. This thesis uses the '*Sepsis-3*' description provided by Singer *et al.* (2016). Sepsis is defined as '*a life-threatening organ dysfunction caused by a dysregulated host response to infection'*, with organ dysfunction identified by a change in Sequential Organ Failure Assessment (SOFA) score of ≥2 points resulting from known or suspected infection. The SOFA score (Vincent *et al.,* 1996) is a measure of the degree of organ dysfunction in patients with sepsis. The score was formulated by consensus and consists of 6 domains: central nervous, cardiovascular and respiratory systems, coagulation, liver, and renal function (Vincent *et al.*, 1996). Patients are scored 0-4, where 4 is the most severe for a single category. Over the 6 domains, the total score is between 0 to 24. Whilst the SOFA score is an important tool in the categorisation of organ dysfunction, such scoring is not without limitation. For example, central nervous system assessment (using Glasgow Coma Scale, GCS), is often poorly scored or irrelevant in patients

who are sedated (personal communication). As a result, some recent clinical trials use a modified SOFA scoring system, excluding the CNS as a measure.

The symptomatology of systemic infection and early-onset sepsis is vague, often with unspecific clinical manifestations such as occasionally slurred communication, urine output reduction and skin with a blotchy meningitis-like rash. These occur in addition to generic signs of infection, e.g., elevation in heart rate and (systemic) inflammation. As the disease progresses to critical/end stages more symptoms become apparent such as reduced platelets, increased bilirubin levels, reduced GCS, increased creatinine, and a requirement for ventilation. If left untreated sepsis will almost certainly be fatal (The Sepsis Manual, 5e, Sepsis Trust, 2021).

The clinical manifestations of sepsis are explored in greater depth in section 1.2, which include changes in not only the immune and coagulation systems (1.2.1), but also metabolism (1.2.2) and the cardiovascular system (1.2.3), among others. All of which are investigated within this project in order to fulfil the mechanistic outcomes of the clinical trial STRESS-L (Study into the Reversal of Septic Shock with Landiolol (β-blockade)), upon which this project is based.

Epidemiologically, the causation of sepsis, whether community-acquired or nosocomial is a result of infection. Bacterial infections are predominately responsible though viral and fungal also occur (Figure 1). Infections can arise at any site though the respiratory tract is the most frequent tissue affected (Figure 2).

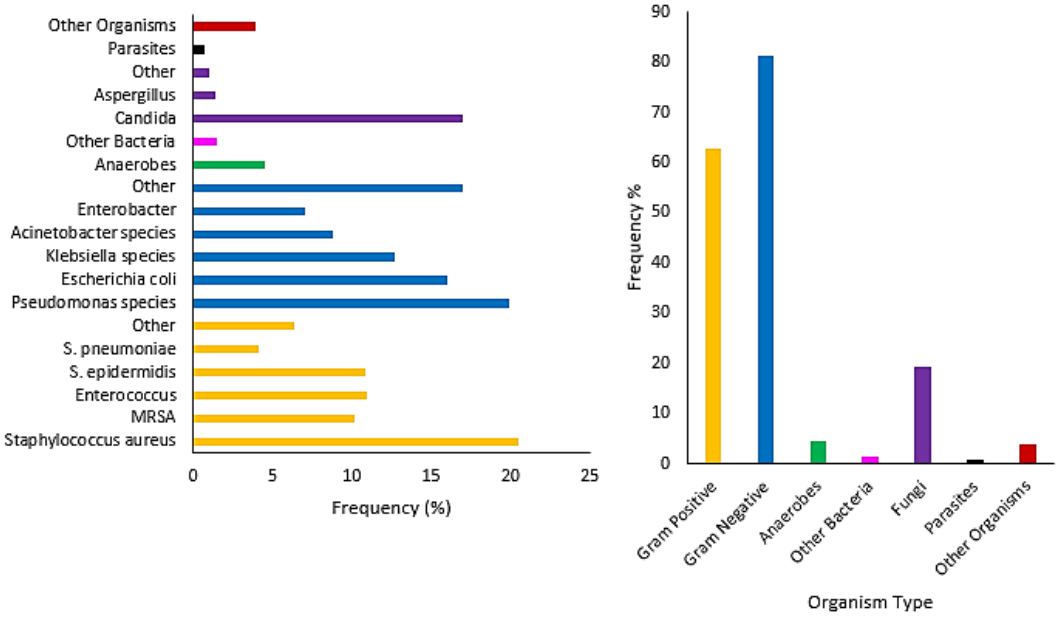


Figure 1: Organism type and frequency responsible for causing sepsis and sepsis-like presentations in patients. Figure made from data from Mayr et al. (2014).

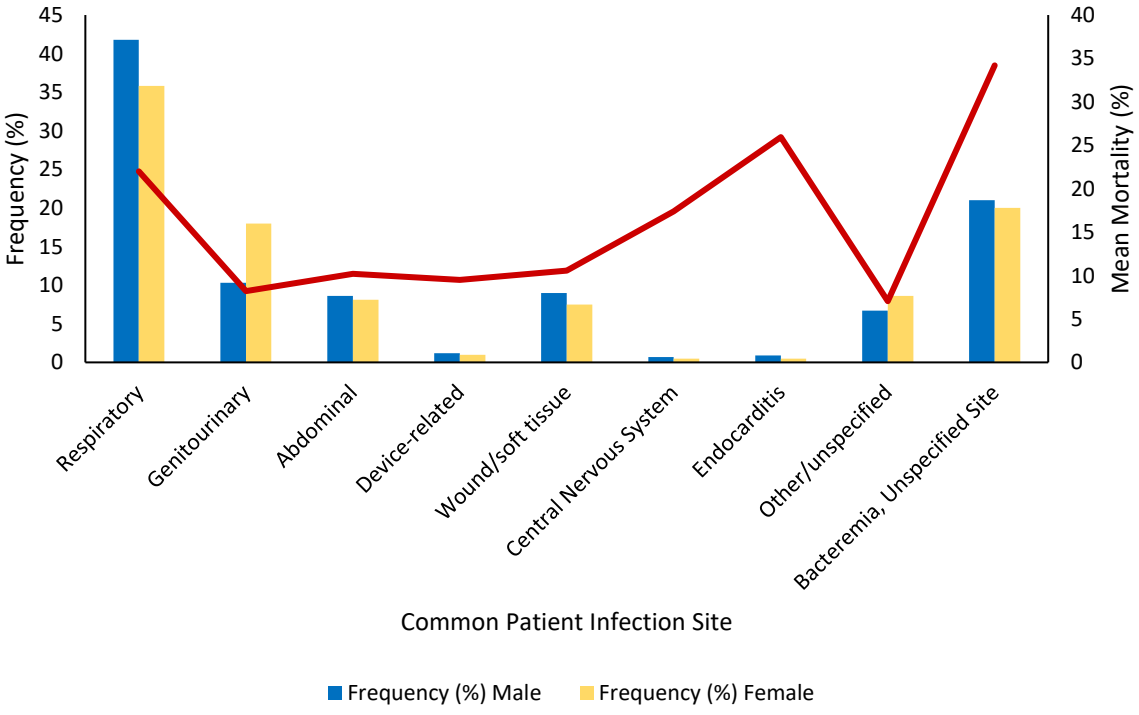


Figure 2: Common patient infection site by frequency (%) on left against mean mortality (%) right for severe sepsis. Sex: Male (Blue) and Yellow (Female). Red line denoted mortality for respected site. Figure made from data obtained from Mayr et al. (2014).

The greatest cause of mortality is bacteraemia (unspecified location). Whilst both sexes have diagnoses of sepsis in all infection locations, there are some notable incidences where a single sex appears to develop sepsis more commonly. For example, there is a notable increase in female diagnosis of sepsis from genitourinary sites – this is expected due to anatomical configuration and the established greater UTI infection and cystitis rate (Foxman, 2002), whilst Figure 2 highlights males are more predisposed to respiratory-based infection. This is likely correlated to higher-risk occupational exposures, such as those in construction, high pollution-based works, and potential increased cigarette smoking (Schachter *et al.*, 2009; Peters *et al.*, 2014). There are less apparent frequency differences among the other sites of infection.

Whilst pathophysiology and clinical presentations are discussed later in this thesis (Section 1.2); it is a common presentation in many septic patients to develop some degree of cardiovascular/cardiopulmonary dysfunction, including often reversible septic cardiomyopathy and onsets of supraventricular tachyarrhythmia and physiological derangement such as hypotension for example. These clinical presentations often result in reduced cardiac output (CO) and stroke volume (SV). The relationship between the various cardiac parameters are mathematically demonstrated in Equations 1 – 5. As functions are connected physiologically, disruption in one variable has consequences on other parameters.

Equation 1: Relationship between Cardiac Output (CO) and Stroke Volume (SV) and Heart Rate (HR)

$$CO = SV \times HR$$

Equation 2: Relationship between Stroke Volume (SV) and End Diastolic Volume (EDV) and End Systolic Volume (ESV)

$$SV = EDV - ESV$$

Equation 3: Relationship between Left Ventricular Ejection Fraction (%) and Stroke Volume (SV) with End Diastolic Volume (EDV)

$$LVEF\% = \frac{SV}{EDV} \times 100$$

Equation 4: Relationship between Mean Arterial Pressure (MAP) and Cardiac Output (CO) with Systemic Vascular Resistance (SVR)

$$MAP = CO \times SVR$$

Equation 5: Relationship between Systemic Vascular Resistance (SVR), Mean Arterial Pressure (MAP), Central Venous Pressure (CVP) and Cardiac Output (CO).

$$SVR = \left(\frac{MAP - CVP}{CO}\right)$$

In some patients, β-blockers have a negative inotropic effect on the heart. Further, they can manage inappropriate arrhythmia leading to better haemodynamic compliance. Equation 4 illustrates that cardiac output (Equation 1) multiplied by vascular resistance (Equation 5) gives light to MAP. Therefore, as MAP is the product of CO and SVR, theoretically any decrease in HR from β-blockade may drive a greater stroke volume, which preserves cardiac output.

Whilst explored in greater depth in section 1.4.2.1, this concept was supported by a single-site study in Rome by Morelli *et al.* (2013) which looked to increase the efficiency of the heart. Although the study also reported marked improvements in mortality, ICU length of stay, and haemodynamically more compliant interventional patients in comparison to the control group. Morelli did not mechanistically explore the outcomes.

This thesis will explore the pathophysiology and dysfunction caused by sepsis generally, and the impact of β-blockade in sepsis patients, investigating the mechanistic outcomes of STRESS-L. As part of the metabolomic analysis, β-blockade-induced metabolic changes will be identified. Additionally, studies will include cytokine analysis to determine effects on immune and inflammatory responses. Finally, measurement plasma creatine kinase myocardial-band (CK-MB), troponin-I (cTnI), *NT* Pro-B-type natriuretic peptides (Pro-BNP) levels will aim to identify cardiomyocyte damage, cardiac-remodelling or cardiac protection in providing an overview of the cardiovascular system response to those treated with β-blockade.

1.2: General Pathophysiology in Sepsis

This section provides an overview of the physiological effects of sepsis and severe sepsis, with a focus on sepsis-induced system and organ dysfunction. The systems explored are the immune system and the cardiovascular system, along with metabolism and bioenergetics. Of particular interest will be those who can be potentially influenced with Landiolol.

1.2.1: Immune and Coagulation Dysfunction

The dysfunction of the immune system caused by sepsis is extensive, complex, and dynamic. Sepsis affects, whether directly or indirectly, the entirety of the immune system. Activation of the immune system occurs through the interaction and recognition of a pathogen. In humans, the immune system is divided into two discrete yet connected defence systems, innate and acquired (adaptive) immune systems. The former is responsible for the immediate response to infection whilst the latter mediates the longer term more specific response.

Initiation of the innate primary immune response differs by the type of microorganism involved in the infection, i.e., whether viral, bacterial or fungal and is propagated following interaction between pattern recognition receptors (PRR) in immune cells and pathogen-associated molecular patterns (PAMPs). Examples of common PAMPs are lipopolysaccharides (LPS) in bacterial pathogens, β-glucans in fungi and nucleic acids of viruses (Athman and Philpot, 2004; Soltanian *et al.*, 2009). PRRs are an umbrella category formed of several classes, some of the most notable Toll-like Receptors (TLRs) (Takeuchi and Akira, 2010). TLRs are expressed on innate immune cells such as macrophage and neutrophils and have been implicated in initialising the inflammatory response and are of particular interest in sepsis and infectious disease medicine. TLRs initiate the inflammatory reaction by activating macrophages (Mϕ) to produce cytokines such as IL-6, TNF and IL-1β (Seki and Brenner, 2008),

which can then lead to systemic inflammatory responses and recruitment of other immune cells to site of infection. TLRs also cause a range of other responses such as activating transcriptional factors (e.g., TLR1 and 2 with MAL and MyD88 [Myeloid differentiation protein 88] leading to activation of TRAF6 to IKK-α/-β/-γ, inducing IκBa, p50 and p65 finally 'activating' inflammatory cytokines such as TNF-α, IL-1, IL-6) to induce other potent mediators in host defence – Figure 3 (Kaisho and Akira, 2006; Fajar, 2016).

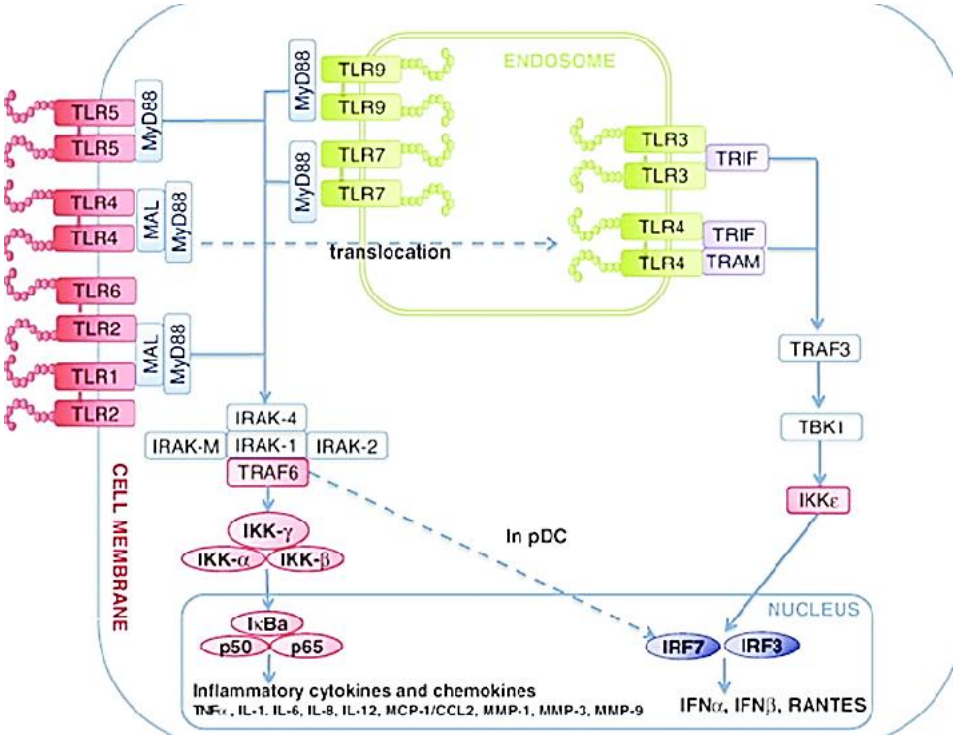


Figure 3: Schematic of TLR signalling pathways highlighting TLR and transcription factors regulation, releasing inflammatory cytokines and chemokines. Figure taken from Fajar (2016).

The acquired/adaptive immune system is based on B and T lymphocytes which express B-cell and T-cell receptors specific for single antigens (Chaplin, 2010). The innate immune system plays a key role in the activation of the adaptive system, presenting antigen via macrophages and dendritic cells, and cytokines that influence the differentiation of these cells into specific effector lineages (Warrington *et al.,* 2011). As the focus of the thesis is the inflammatory

response, the adaptive system will not be discussed further here. The longer-term response to sepsis is explored in literature elsewhere, e.g., Brady *et al.* (2020).

As infection progresses towards sepsis, the immune response has two parallel responses. These are the inflammatory and immunosuppressive elements. The inflammatory phase is dominated by pro-inflammatory mediators such as IL-1β, TNF-α and IL-17 for example. The overwhelming and acute release of these cytokines drives a process known as the "*Cytokine Storm*". This cytokine storm (Figure 4) leads to several pathological presentations in key systems, such as causing pneumonitis in the lungs, elevated enzymes and hepatomegaly in the liver, to acute renal injury and failure. Nervous system dysfunctions include aphasia, delirium and seizures and dysregulations in the cardiovascular and lymphatic systems, e.g., endothelial damage, increased vascular permeability, vasodilatory shock, hypotension and cardiomyopathy. A full account is given by Fajgenbaum and June (2020), where they highlight also constitutional, rheumatological, gastrointestinal and skin-related pathologies. Due to the extensive volume of endothelium within the body, the vascular dysfunction has drastic consequences on capillary leakage, issues with perfusion and blood clotting. Dysfunction and damage to these cells can result in release of von Willebrand factor (vWF) driving blood clotting leading to embolus or diseases such as *thrombotic thrombocytopenic purpura* when vWF is at a greater concentration than circulating ADAMTS13 (enzyme cleaving vWF preventing fatal clotting) as observed by Lin *et al.* (2016). Notably in sepsis, disseminated intravascular coagulation (DIC) and platelet aggregation is a critical problem requiring therapeutic intervention. Furthermore, and discussed later in the cardiovascular section, endothelial cells discharge microparticles partially responsible for the hypotensive presentations seen in severe sepsis (Helbing *et al.,* 2014; Delano and Ward, 2016).

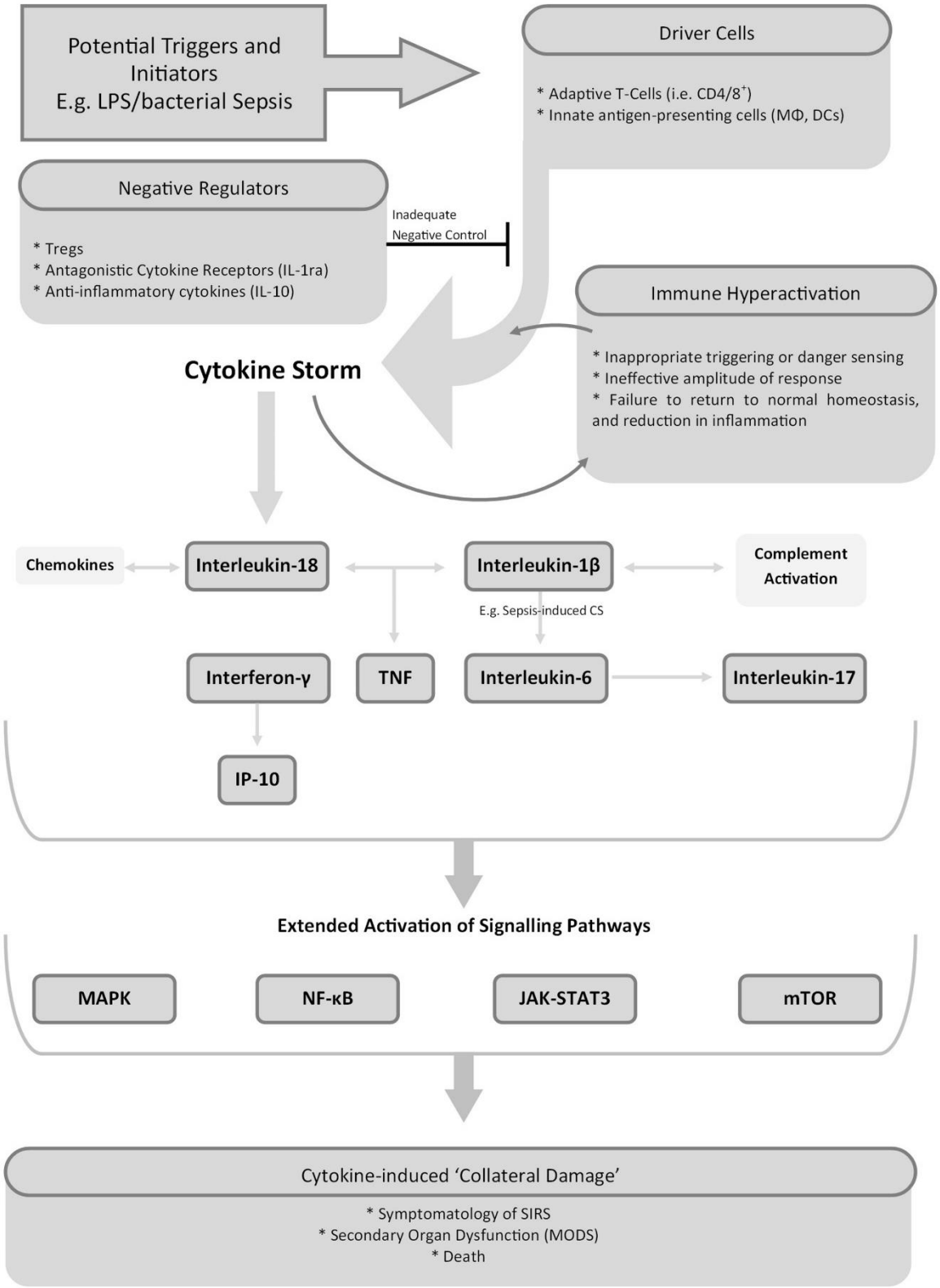


Figure 4: Schematic of Cytokine Storm which occurs from an inappropriate response to potential triggers, and later the failure to resolve the hyperactive immune response, driving severe inflammatory response syndrome, organ dysfunction and eventual death. The figure adapted from Fajgenbaum and June (2020). IL: Interleukin, Treg: Regulatory T-Cells, IP-10: Interferon-inducible Protein 10, TNF: tumour necrosis factor, MΦ: macrophage, DCs: Dendritic Cells, CS: Cytokine Storm, MAPK: mitogen-activated protein kinase, NF-κB: nuclear factor kappa B. JAK-STAT3: Janus kinase-signal transducer and activator of transcription 3, mTOR: mammalian target of rapamycin.

Delano and Ward (2016) highlighted that some patients recover from the cytokine storm, older patients with multi-morbidity often remain in a hyperinflammatory state leading to continued immunological dysregulation and immune exhaustion further reducing the ability to clear infection. This hyper-inflamed state also has impact on metabolic pathways – skewing towards catabolism with significant loss of tissue such as skeletal muscle (Lang *et al.,* 2007; Costamagna *et al.,* 2015).

Whilst the hyperinflammatory phase causes extensive systemic damage, it is often the hypoinflammatory or immunosuppressive element which can cause the greater morbidity and mortality rates. This is because the hypo-inflammatory element characterised by an increased concentration of circulating anti-inflammatory cytokines (such as IL-10), further increases in lipid mediators – such as resolvins, and increased action of regulatory T-cells (Tregs). Resolvins are synthesised from ω-3-polyunsaturated fatty acids and inhibit synthesis of pro-inflammatory mediators in addition to regulating the migration and clearance of leukocytes, particularly endothelium (Sommer and Birklein, 2011; Moro *et al.*, 2016). Whilst Tregs cause immunosuppression or immune tolerance by inhibiting the proliferation of T-cell and cytokines. Normal regulated function is to maintain homeostatic and self-tolerant states, in the cases of dysregulation, Tregs prolong immunotolerance, and contribute to mortality (Kondelkova *et al.*, 2010). Subsequently, due to the combination of these increased anti-inflammatory mediators, the inability to mount an effective/sufficient response to the primary infection or the failure to clear secondary infections lead to poor outcomes. Frequent readmissions to hospital are often seen following sepsis and is likely related to the persistent immune dysfunction (Delano and Ward, 2016; Fry *et al.,* 2021). Goldhill *et al.* (2004) reported that with each additional day admitted to a clinical environment, patient life expectancy decreases.

Therefore, it is imperative to establish what drives this immune dysregulation and dysfunction to establish therapeutic options. Research by Delano and Ward (2016) identified from post-mortem splenocytes a notable decline in $CD4^+$ and $CD8^+$ T-cells which would impact adaptive immune responses. Four days or more of lymphopenia is correlated with increased long-term mortality (Heffernan *et al.*, 2012). A longitudinal study by Pachot *et al.* (2005) showed a significant reduction of *T-Bet* and *GATA3* expression in lymphocytes in septic patients. T-Box transcription factor TBX21 (*T-Bet*) is a transcription factor responsible for differentiation of $CD4^+$ T-cells to the pro-inflammatory Th_1 subtype (Wan and Flavell, 2007). *GATA3*, is another key transcription factor whose primary function is the differentiation of Th_2 cells, the anti-inflammatory subtype, and it also influences Treg cellular function, metabolism and immunological homeostasis (Wan, 2014). The combination of reduction in both Th_1 and Th_2 differentiating factors is a significant contributor to the reported immune paralysis and dysfunction seen in septic patients, with the dominance of the inflammatory response mediated by macrophages.

Another potential contributor to morbidity during infection and sepsis is one of the methods neutrophils employ to eradicate pathogens, namely the formation of neutrophil extracellular traps (NETs). NETs are formed by the release chromatin to form web-like structures that can trap bacteria. NETs are released through a process of depolarisation and actin arrest by neutrophils, and disassembling of the nuclear envelope (Papayannopoulos, 2018). As NETs are associated with potential pathogenesis in sepsis, but also the clearance of the causative pathogen of sepsis, it is imperative to understand their function. NETs are important in infection control but also can cause tissue damage, circulatory obstruction, cell degradation (Tan *et al.,* 2021). Further investigations are needed to understand the full extent of NET mediated pathogenesis.

How normal functionality is returned in surviving patients is poorly understood, however the balance of cytokines that influence the inflammatory and anti-inflammatory functions of immune cells are clearly important. In addition, there are other soluble factors that are also key in resolving the immune response to infection, notably the lipid based resolvins, protectins and lipoxins. These lipid-based mediators are part of the *'specialised pro-resolving mediators'* (SPMs) superfamily as reviewed by Serhan and Levy (2018). Each of these families are structurally different but synthesised from either eicosapentaenoic acid, docosapentaenoic acid or docosahexaenoic acid – all of which are fatty acids. A notable, and potent lipoxin, is LXA_4 (Lipoxin A_4), notable resolvins include species from both D- and E-series (e.g. RvD1) and PD1 (protectin D1) (Serhan and Levy, 2018). As mentioned previously, these are synthesised from fatty acids, and reported deficiencies in genes responsible for fatty acids biosynthesis show reduced production of these key mediators. Thus, this suggests, as both the pro-inflammatory and anti-inflammatory elements of the immune system work simultaneously to support a homeostatic balance, when septic dysregulation occurs – for example the heart switching to favour fatty acids as an energy source, this may increase the hyperinflammatory/pro-inflammatory element and lose homeostatic balance due to less FFA availability to continue synthesis of inflammation resolvers. And, hence when the energy currency of the heart reverts to other mediums, we see homeostatic balance resolve. The metabolomics analysis within this thesis could potentially provide additional evidence on lipid profiles in septic patients, and indeed, changes associated with β-blockade. There is mixed clinical opinion as to the extent of β-blocker's impact on the lipid profile, particularly from the older literature. Nevertheless, most new studies argue little clinical significance. One interesting study by Iaccarino *et al.* (2005) however did note patients with β_2-AR gene

polymorphism(s) had experienced increased dyslipidaemia events whilst in-receipt of β-blockers, particularly those with β_2AR-^{27}Glu mutation, compared to the ^{27}Gln-allele.

The immune system being interconnected to many essential systems, it is without surprise that the immune system also modulates and impacts other essential systems like coagulation, particularly during sepsis. Giustozzi *et al.* (2021) note the essential role both coagulation and immune systems have in the fight against infection during sepsis. Inflammation impacts coagulation function and coagulation subsequently impacts the inflammatory response. As such, there is a homeostatic feedback cycle where one change affects the entire equilibrium. Research by Walborn *et al.* (2018) has established that IL-1β, IL-2, IL-6, IFN-γ and TNF-α cause a reduction in and exhaustion of platelets and clotting factors inducing (micro)vascular thrombosis, leading to DIC. Giustozzi *et al.* (2021) highlighted thrombocytopenia (i.e. <150/µL platelets) in ICU patients as associated with higher mortality, thrombocytopenia is now included within the Sequential Organ Failure Assessment panel (SOFA). Whilst not fully understood, the causation for thrombocytopenia could be dysfunction in platelet synthesis, and haemophagocytosis lymphohistiocytosis (Castillo and Carcillo, 2009).

Whilst several cytokines have been associated as drivers of DIC, in 2001 the committee on DIC, proposed DIC be divided into two categories: non-overt and overt. The difference between overt and non-overt primarily is whether homeostatic systems are compensated for or not. In the cases of overt, homeostatic mechanisms are decompensated with progressive severity, whilst in non-overt the system is maintained but under pressure (Giustozzi *et al.,* 2021). Pathology of DIC is initiated through the acute and excessive release of tissue factor [CD142 or platelet tissue factor], which drives the release of thrombin in superfluous amounts which activates VIII, IX and XI factors responsible for normal coagulation (Giustozzi *et al.*

2021). As a result of coagulation system stimulation, inflammation is further increased through the upregulated expression of the protein bradykinin which also causes vasodilation and increases in the complement pathway, namely protein C3a. This protein, formed through the cleavage of component 3 is regarded as an anaphylatoxin (Mamane *et al.,* 2009). Anaphylatoxins are known to increase chemotaxis activity of immune cells such as macrophages and increase both smooth muscle contraction and vascular permeability, i.e., the indirect effect of coagulation disorders through sepsis leads to greater pathophysiology within the complement system and promote further immune activation, particularly tissue infiltration by macrophages. Furthermore, C3a has suspected roles in insulin resistance which is an established presentation of many sepsis patients admitted to intensive care (Shim *et al.*, 2020; Rivas and Nugent, 2021).

Finally, in addition to the reported points above, consequences of complement system activation and/or dysfunction can lead to multiple-organ dysfunction syndrome (MODS). The process is typically enacted through an excess of the anaphylatoxin C5a driving neutrophil dysfunction leading to MODS. Some of these dysfunctions include reduced membrane potentials, oxidative burst, and glycolytic fluxing (Mollnes and Huber-Lang, 2020). The activation and potential pathophysiological role of the complement system is shown in Figure 5 (Mollnes and Huber-Lang, 2020; Oncul and Afshar-Kharghan, 2020).

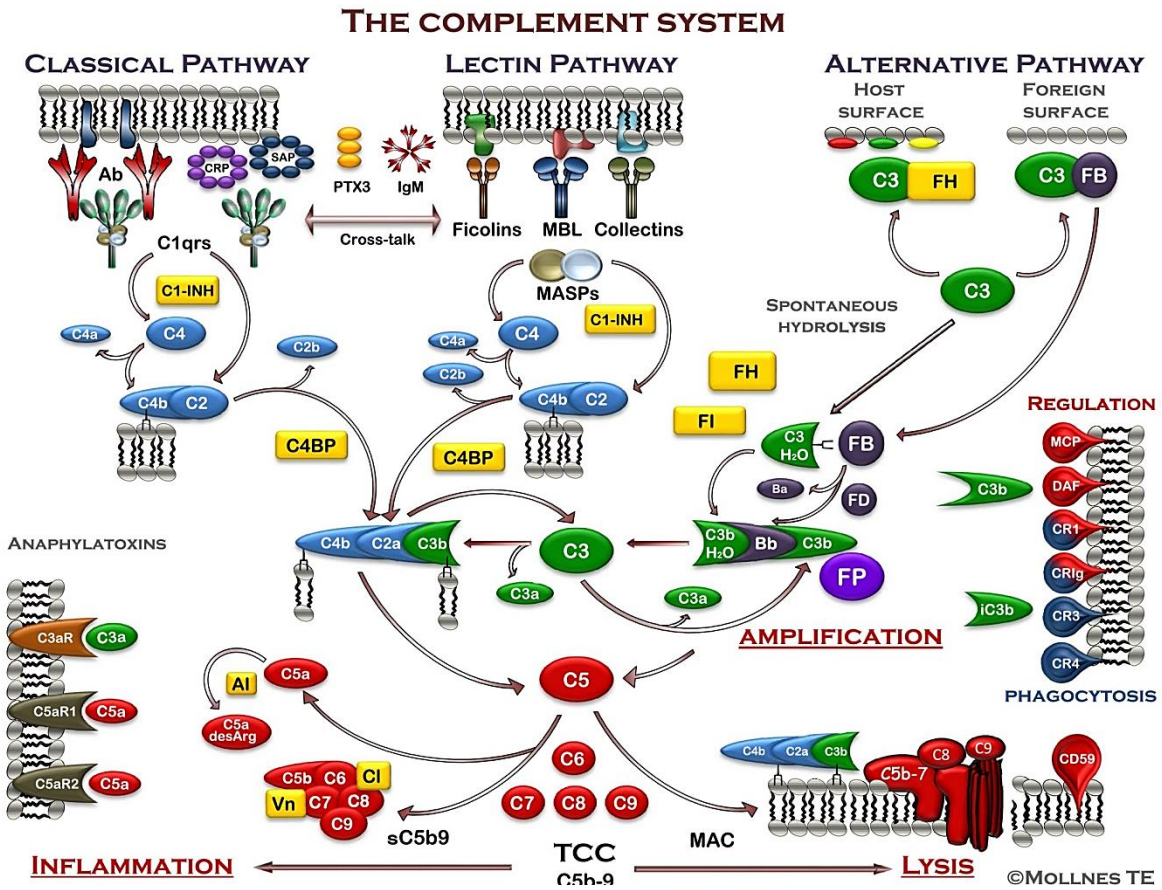


Figure 5: The 3 pathways (classical, lectin and alternative) of complement activation and resultant impacts, i.e. inflammation, cell lysis and immune cell regulation. The diagram is obtained from the works of Mollnes and Huber-Lang (2020).

Considering the brief overview of the immune system in sepsis progression, it is apparent that sepsis causes widespread malfunction and that this is a driver of morbidity and mortality. Sepsis and severe sepsis are not simple conditions, rather a disease caused by several interconnected processes, pathways and homeostatic mechanisms becoming dysregulated, some failing.

1.2.2: Metabolic Response to Sepsis

Metabolism is a set of essential chemical reactions responsible for sustaining life, supplying energy to ensure physiological conditions are maintained and elimination of chemical waste. Human physiology is remarkably complex, and as such metabolic pathways are extensive. Romero *et al.* (2005) estimated there are around 135 metabolic pathways in humans, however additional research indicates the true number is closer to 317 (Gómez-Romero *et al.,* 2020). Networks often are interconnected, and it therefore comes as no surprise that sepsis-induced metabolic dysregulation can cause extensive changes to these life sustaining reactions, and drive dysfunction of individual reactions, organs or indeed whole systems.

1.2.2.1: Glycolysis and Warburg Effect

Whilst much literature is available surrounding metabolism and metabolic-related diseases in patients, surprisingly little is explicitly known about pathway changes seen in septic patients. Some metabolite biomarkers have translational value and are currently exploited within the clinic to provide prognosis indicators. A notable metabolite in sepsis that is a strong indicator of a patient in crisis is *L*-lactate. Lactate [2-hydroxypropanoic acid] is a terminal by-product of glycolysis, produced from pyruvate reaction with lactate dehydrogenase, the shift to glycolysis is known as the Warburg effect. Van Wyngene *et al.* (2018) reported elevated lactate levels to be associated with poorer outcomes in patients, particularly when combined with alterations in the metabolism of amino acids and fatty acids. The wider consequences of lactic acidosis (plasma pH <7.35) may be in decreased utilisation (or uptake) of oxygen by the brain which leads to a reduced GCS score, uncontrolled arrhythmias and cardiopulmonary failures. According to Kimmoun *et al.* (2016) septic patients with a plasma pH <7.00 had a mortality of 100%.

The Warburg effect is a phenomenon seen in several other disease, including cancer, where glucose is readily converted to lactate as an 'energy requirement' (Warburg, 1925). When lactate rises rapidly lactatemia with acidosis is a common presentation in sepsis. Hyperlactatemia and lactic acidosis [lactatemia with acidosis] may arise as a result of tissue hypoxia; in sepsis oxygen supply to tissues is usually maintained or even raised which suggests other mechanisms are at work. Instead, problems with glycolytic flux and cytopathic hypoxia – cells not consuming the oxygen available to them are thought to be involved (Gutierrez and Wulf, 2005; Van Wyngene *et al.* 2018). Additionally, it is likely a combination of immune cells producing lactate, production within the lungs as a result of acute lung injury (established in sepsis), and the hypermetabolic state (Suetrong and Walley, 2016) are other contributors.

Severe metabolic acidosis causes a reduction in the adrenoceptors on the cell membrane by the process of internalisation, nitrosylation and subsequent downregulation because of catecholamines (endogenous and exogenous) supporting cardiovascular function and paradoxically has the potential to make the patient less responsive to both endo- and exogenous adrenergic hormones. As there are few therapeutic options to correct severe acidosis this dysfunction continues to be a key indicator of mortality (Velissaris *et al.*, 2015).

Immunological cells contribute to increased lactate levels as naïve immune cells predominantly obtain energy and expenditure from glycolysis and oxidative phosphorylation. The full extent of metabolism interaction in immune cell function has been reviewed by several authors (Cheng *et al.,* 2016; Park and Zimjewski, 2017; Preau *et al.*, 2021). A resting immune cell uses glucose (transported into cells by Glut1), glycolysis reactions occur (glucose -> glucose-6-P -> pyruvate), and pyruvate is converted to acetyl-CoA (via pyruvate decarboxylation) which can enter the TCA cycle and later oxidative phosphorylation.

Whereas, during sepsis, this process is altered as activated immune cells utilise aerobic glycolysis (Kornberg, 2020) and essentially prevent translation to acetyl-CoA leading to lactate production (Figure 6). Aerobic glycolysis is upregulated by the mTOR pathway (mechanistic target of rapamycin) transcriptional factor HIF-1α (Van Wyngene *et al.,* 2018). Despite being energetically unfavourable to remain in the glycolytic state over oxidative phosphorylation because the kinetic turnover and synthesis rate of ATP is quicker, this dominates in activated immune cells. The consequences of the Warburg effect during sepsis include causing changes to the redox potential in the mitochondria, increasing reactive oxygen species (ROS) generation and diversion of resources into different pathways (Liberti and Locasale, 2016) – the metabolic changes are discussed later. We understand that contrary to the initial belief by Otto Warburg that mitochondrial inactivation drives the Warburg effect, literature has since demonstrated that indeed the Warburg effect is under complex control biochemically by tissue-related factors, and that practically mitochondrial inactivation is a rarity (Bar-Or *et al.*, 2018). There are several biochemical processes that regulate the energy utilisation in sepsis. These are reviewed by Bar-Or *et al.* (2018), but include the inactivation of the pyruvate dehydrogenase complex (PDC). This complex has regulatory control and influence over the initial (catalytic) reaction of phosphoenolpyruvate to pyruvate. *PKM2*, a pyruvate dehydrogenase kinase, part of the PDC can inhibit the creation of acetyl-CoA, and hence prevent initial entry to the TCA and subsequently oxidative phosphorylation. This ensuring, whilst reduced amounts of ATP (2 compared to ~36) synthesis per mole of glucose, quicker utilisation of such substrate. They further reported another control mechanism – the accumulation of succinate. Succinate, when at significant concentrations, indirectly (through prolyl hydroxylase inhabitation) stabilises hypoxia-induced transcription factor-alpha; thus,

this produces hypoxic cell properties established in sepsis despite there being no hypoxic environment through upregulation of hypoxia-related genes (Van Wyngene *et al.* 2018).

Due to the inability to adequately enter the TCA cycle, the increased glycolysis (anaerobic) significantly increases the creation of lactate. The accumulation of lactate in the bloodstream gives rise to hyperlactatemia and eventually lactate acidosis. Significant lactate concentration elevations are associated with increased mortality in septic patients, and indeed remains one of the most important disease severity biomarkers clinically.

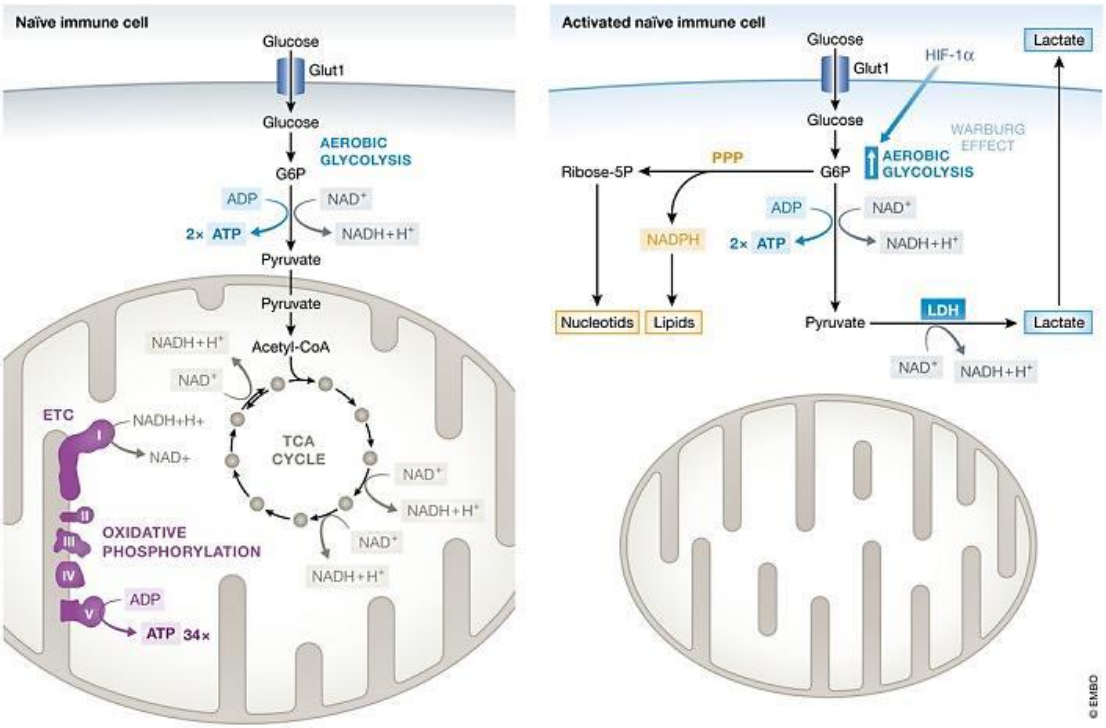


Figure 6: The left diagram highlights the usual metabolic pathway seen in a resting immune cell whereas the right shows an activated immune cell influenced by HIF-1α (hypoxia-inducible factor-1 alpha) despite no tissue hypoxia evident but cytopathic hypoxia seems present. The activated cell experiences the Warburg effect and pyruvate is converted to lactate via LDH (lactate dehydrogenase). Subsequently, lactate concentration increases both inter and extra-cellular leading to pathology. It is notable on the right diagram that no TCA (tri-carboxylic acid) cycle is present, neither is the ETC (electron transport chain) during oxidative phosphorylation – unlike in the left inactivated state. In the inactive immune cell there is little nucleotide and lipid synthesis through PPP (pentose phosphate pathway). ATP: Adenosine Triphosphate; ADP: Adenosine diphosphate; NAD (Nicotinamide adenine dinucleotide); G6P: Glucose-6-Phsophate; GLUT1: Glucose Transporter 1. This diagram is taken from Van Wyngene, Vandewalle and Libert, (2018).

Evidently, lactate is an important indicator of sepsis progression and severity. Not only is it an important indicator, but lactate has also been linked to upregulating toll-like receptors, NF-κB signalling and promoting the release of HMGB1.

As there are quite extensive changes in glucose metabolism, it is foreseeable patients will suffer from either hyper- and hypoglycaemia (dysglycemia). At the start of sepsis there is usually hyperglycaemia, whilst towards the later stages hypoglycaemia can occur due to changes in insulin sensitivity (Rivas and Nugent, 2021). The high energy requirements of the hyperimmune response and the energy required by other systems is provided in part from the oxidation of lipids. The release of (free) fatty acids (FFA) is paramount in the initial stages of providing energy. FFA undergo β-oxidation in the required organ, however FFAs in too great a concentration are also toxic and "fatty-deposits" can develop in organs (e.g. steatosis), which would drive organ dysfunction. The increase in blood concentration of FFAs from lipolysis is controlled by the transcription factor, peroxisome proliferator-activated receptor-alpha (PPARα), which influences ketogenesis and beta-oxidation (Van Wyngene *et al.,* 2020). The rapidly rising levels of FFAs fail to be metabolised by β-oxidation due to the associated downregulation of PPARα in sepsis patients. Further analysis by metabolomics investigating the metabolic disturbance identified the process is impaired by acylcarnitine-dependent fatty acid transport into the mitochondrion from the cytosol (Preau *et al.,* 2021).

Figure 7 illustrates the key metabolic responses to sepsis, in comparison to starvation which also results from energy shortages. Illustrated by the figure is the accumulation of free fatty acids, and the shortage of ketone bodies, in addition to mitochondrial damage. Van Wyngene *et al.* (2018) reports downregulations of PPARα, β-oxidation and ketone body production in

sepsis across the heart, liver and kidney. The opposite is true in the "starvation" response, where indeed upregulations of PPARα, β-oxidation and ketone body production are observed.

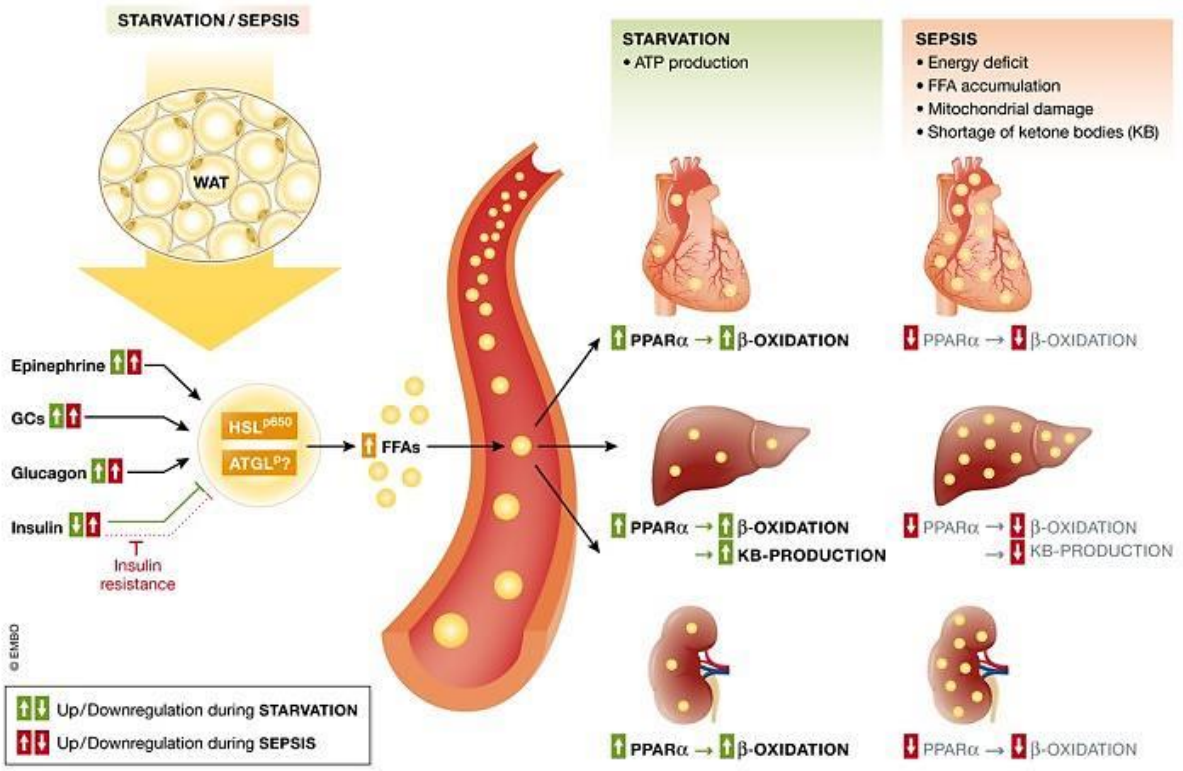


Figure 7: The effects sepsis has on energy use in comparison to starvation. Epinephrine (adrenaline), glucocorticoids (GCs), glucagon are all upregulated in both responses, whereas insulin resistance is upregulated in sepsis but downregulated in starvation. These changes influence Hormone Sensitive Lipase (HSL^{p650}) and potentially Adipose Triglyceride Lipase ($ATGL^{p}$) increasing free fatty acids (FFAs) which in the starvation response upregulates PPARα in the heart increasing the incidence of β-oxidation, whereas the upregulation in the liver increases β-oxidation and ketone bodies (KB)-production. Increases of β-oxidation is established in the kidney also. This in contrast to the established pathophysiology in sepsis which sees an energy deficit, highlights the dysregulation in metabolism (particularly in the mitochondria), increase FFA concentration and there is a shortage of KB. All the upregulated factors seen in starvation are downregulated in sepsis. Figure from Van Wyngene et al. (2018).

The change in muscle metabolism is important to note as a study published by Preau *et al.* (2021) identified little change in mitochondrial morphology however noted that in septic patients, citrate synthesis in monocytes were markedly lower in comparison to control patients, and that indeed, complexes I, III and IV were reduced in muscles. This is unsurprising considering the extent of catabolism and muscle wastage seen in these patients. The decline in citrate synthesis is also not unsurprising due to the extent of metabolic reprogramming and the re-routing of pyruvate to lactate rather than pyruvate to acetyl-CoA entering the cycle by

oxaloacetate. Considering the re-routing, it would be expected a complete stop or near complete decline in TCA metabolites stemming from pyruvate or glycolysis; however, another glycolytic intermediate, phosphoenolpyruvate, can also undergo reaction (by phosphoenolpyruvate carboxylase) converting to oxaloacetate directly entering the cycle. The literature has already recognised the damage caused by ROS within the mitochondrion, including damage to mtDNA, proteins and membranes and therefore hindering ATP synthesis capabilities. The acute presence or rapid increase in ROS species can cause Complex I-III's iron-sulphur centres to inactivate, essentially stopping the ETC functioning and a complete shutdown of ATP synthesis (Guo *et al.,* 2013). Septic patients' platelets showed leaky respiration (ATP synthesis interruption) and increased respiratory capacity which proposes a defective glycolytic and mitochondrial relationship (Preau *et al.,* 2021).

A recent review by Patil *et al.* (2019) examines the regulation of immune cell function by TCA metabolites and reverse electron transition. They propose that succinate moves into the cytoplasm and acts as a stabilising agent for HIF-1α to support its function of increasing glycolysis and pro-inflammatory polarisation. mROS also seems to have a stabilising effect on HIF-1α, and succinate interacts with SUCNR1 (succinate receptor 1) and increases IL-1β, TNF-α and other pro-inflammatory cytokines. This suggests in order to modulate the hyperinflammatory state in some patients to preserve tissue and prevent extensive organ necrosis, the TCA cycle will also need to be controlled. Interestingly, the literature shows that catecholamines induce metabolic stress and at high-doses are associated with system-wide dysfunction and increased mortality (Bruning *et al.*, 2021). β-blockade, by Landiolol for example, would be expected to result in a decline in metabolic-related stress caused by catecholamines (i.e. increases in succinate's pro-inflammatory role and mROS production), with beneficial effects on the initial hyperimmune response. Whether this is an effect seen in

sepsis is not fully established, however, Grisanti *et al.* (2019) noted that patients already prescribed β-blockade (in primary care) have decreased leukocyte responsiveness to injury and infection. This could explain why older patients in-receipt of β-blockers fail to yield a significant inflammatory response initially or remain in a hypo-immune or immune tolerant state. This has not been shown comprehensively in the literature.

1.2.2.2: Mitochondrial Changes

Published literature has reported dysfunction with mitochondria including morphological, impacts on genetic information, membrane potential, dynamics, biogenesis and naturally metabolism to name a few. Whilst literature is available, little consequences is known about the mitochondria during sepsis. A summary of mitochondrial changes seen in sepsis are shown in Figure 8.

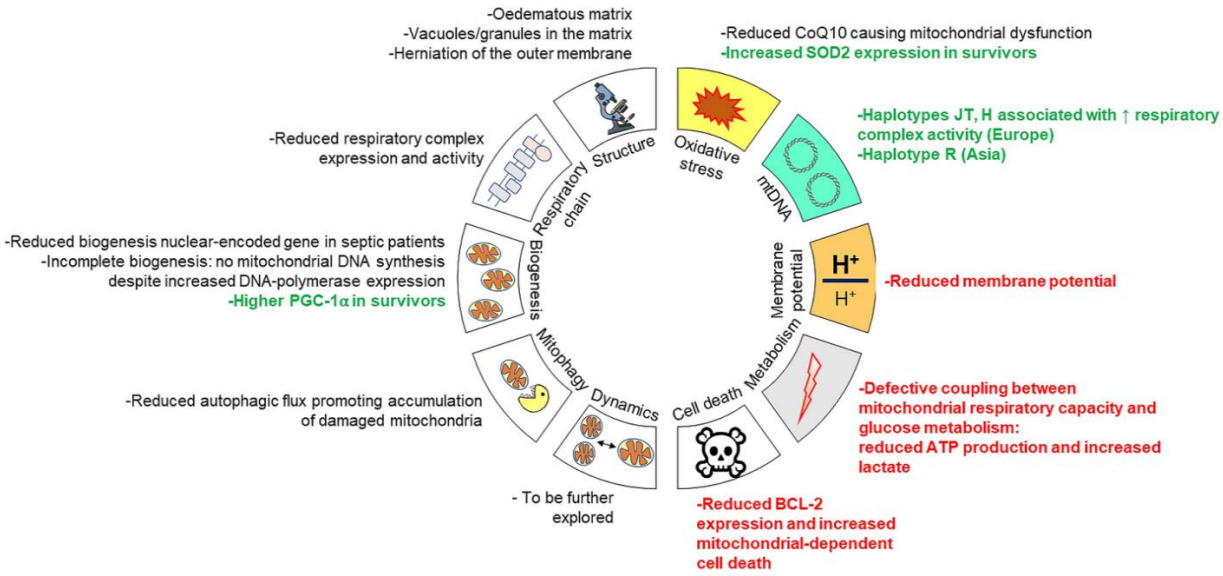


Figure 8: Obtained from Preau et al. (2021), a summary of mitochondrial changes from established septic shock patients. Text coloured red is correlated with poorer outcomes in patients, whilst green correlated with better outcomes.

Preau *et al.* (2021) explored the loss of function experienced in many of the key domains in the mitochondrion. The collective loss of functionality across these domains result in

significant impairment to metabolism and energy generation. In patients with severe sepsis there is a notable decline in nuclear-encoded genes, many of which are responsible for respiration and generation of new components. Literature previously established that mitochondria are prone to damage from oxidative damage (e.g. ROS), and with a reduction in the ability to regenerate damaged parts, recovery is likely hindered. Preau and colleagues noticed that PGC-1α were elevated >2-fold in sepsis survivors following *vastus lateralis* biopsy, but in non-survivors, expression did not change. As PGC-1α (peroxisome proliferator-activated receptor-gamma co-activator-1-alpha) is a key regulator of carbohydrate and lipid metabolism, and believed to be closely related to cardiomyopathy (Liang and Ward, 2006), it is suggestive elevations of PGC-1α may contribute to recovery. Though this is not confirmed. As a result of downregulation of respiratory related genes, there is reduced respiratory activity of Complexes I, III and IV which could account for the lack of energy performance, mitochondrial myopathy, and thus inducing muscle atrophy – often more severe in those mechanically ventilated (Levine *et al.,* 2008).

Among other dysfunction observed is the accumulation of dysfunctional and damaged mitochondria. Increases in oxidative stress, leading to damage and mitochondria failure, along with the inactivation of mitophagy pathways leads to this accumulation which ultimately results in the cell death. Large amounts of cell death, including with immune infiltration, can drive organ dysregulation and dysfunction (Singer, 2014; Kubli and Gustafsson, 2012). Other causes of mitochondrial death include reduced mitochondrial membrane potential (ψm), this decrease in ψm is observed across several cells including immune cells (monocytes for example). A depolarisation-type event is considered the first and irreversible step towards cell death, linking to either necrosis or apoptosis depending on

the severity. Whilst not reviewed here, Δψm is intimately connected to regulating proteins of cell death (Fas, Hsp70 and Bcl-2) (Adrie *et al.,* 2001).

1.2.2.3: Amino Acid Metabolism

Finally, with other key pathways affected, amino acids and protein degradation are also common in septic patients. Hasselgren and Fischer (1998) highlighted that those with sepsis often undergo catabolic reactions, for example in muscles, which results in extensive loss of muscle mass. This catabolic loss, combined with ICU-acquired muscle weakness and loss, is a driver of extensive wastage. Several pathways have been implicated in this muscle breakdown for example the ubiquitin-dependent proteolytic pathway (Lecker *et al.,* 1999).

The constituent building blocks of proteins are amino acids (AA). Several studied have clearly illustrated dysfunction associated with amino acid metabolism in septic patients (Druml *et al.,* 2001; Biolo *et al.*, 2007; Kao *et al.,* 2009; Su *et al.,* 2015). A small clinical trial in 2001 consisting of 9 septic patients and 8 healthy controls found that there were significant differences in the plasma concentrations of 12 amino acids: isoleucine, leucine, phenylalanine, threonine, valine, arginine, histidine, proline, glycine, serine, branched-chain AA, and glucogenic AA (Druml *et al.,* 2001). Interestingly, of these amino acids, 6 (leucine, threonine, arginine, histidine, glycine, and serine) showed reduced levels due to increased clearance rates. Su *et al.* (2015) run a similar trial with 35 septic patients which also found several amino acids to be significantly different (*p<.001)* between SIRS, sepsis, and controls on admission to the ICU. These amino acids were: arginine, aspartic acid, ethanolamine, glutamine, phenylalanine, branch-chained amino acids, carnosine, citrulline, cystathionine, ornithine, phosphoethanolamine, sarcosine, tryptophan, valine, δ-hydroxylysine and phosphoserine. Some of the former amino acids being significantly lower in sepsis patients than controls, such

as branched-chain amino acids, carnosine, ornithine, histidine, valine, among others. Many had a stable tendency between controls and septic patients (e.g. methionine, cystine, serine i.a.) Whereas others had increased concentrations such as Arginine, glutamine, phenylalanine and others. A complete comparison of the study metabolites is obtainable from Su *et al.* (2015). They also stated neutrophil effectiveness and function is linked with lower asparagine levels. Muscle catabolism is promoted by the reduced levels of valine, isoleucine and leucine (Holeček, 2018). Whilst not reported within Druml's original study; Kao *et al.* (2013) noted glutamine had a lower plasma concentration in septic patients compared to control patients, and this mechanistically could be due to increased glutamine clearance. It appears many of the higher secreted amino acids are essential. Therefore, administrating essential amino acids to patients would expect to improve outcomes. However, Su *et al.* (2015) showed patients given supplemental amino acids declined quicker and with more severe SOFA scores. This suggests one of two relationships: 1) administrating supplemental amino acids to patients causes greater dysregulation by maintaining inflammation and preventing the immunosuppressive element; or 2) that the immunosuppressive phase with reduced neutrophil functionality is indeed more beneficial to septic patients due to the increased opportunity to repair damaged tissue and reduce bystander tissue damage by neutrophils. But on the contrary, many reports support immunosuppression to be more damaging as reactivation of latent viruses causes increased concern, and failure to clear the initial infection (Hotchkiss *et al.*, 2013a; 2013b). Further evidence to support the need for more trials with amino acids follows a report by Kao *et al.* (2009) of a discrepancy referred to as *L*-arginine paradox, here plasma concentrations of arginine limit synthesis of nitric oxide, however decreased nitric oxide synthesis correlates with worse outcomes seen in patients demonstrating reduced arginine plasma levels. That noted, one of the largest clinical trials

investigating nitric oxide had to be stopped prematurely following an increased mortality trend in the group where a nitric oxide inhibitor was administered (Bailey *et al.,* 2007). They hypothesised the failure of the trial could possibly be due to the decision to use a non-selective NO inhibitor, rather than selective, and subsequently it is possible that the non-selective inhibitor reduced all isoforms driving extensive microvasculature dysfunction.

Much the same applies to the increased clearance of histidine. Histidine is the precursor to histamine, which has an essential role in inflammation (Branco *et al.,* 2018; Akdis and Blaser, 2003). Increases in histidine and arginine clearance in sepsis could suggest progression into the immunosuppressive phase. It was noted in a heart failure study that β-blocker use was associated with increases in arginine bioavailability (Tang *et al.*, 2013). This suggests that β-blocker use in septic patients in the inflammatory stage may even result in greater mortality, especially if hyper-inflammation is a key driver of mortality in the patient population.

1.2.3: Cardiovascular Dysfunction

The cardiovascular system is subject to dysfunction in sepsis as with any other critical system. The pathophysiology seen within sepsis is extensive, and the term 'sepsis-induced myocardial dysfunction' (SIMD) was coined to describe the unique dysregulation in sepsis (Lv and Wang, 2016). This dysfunction occurs across both right and left sides of the heart, though this is often reversible in patients within 7-10 days (Li *et al.,* 2013b). In others where key inflammatory mediators remain such as chemokines and cytokines, ROS and NO, these lead to cardiac remodelling (hypertrophy and cell death) and poor cardiovascular recovery (Zhao *et al.*, 2020). As mentioned in previous sections, the immune system interaction and influence on the cardiac system is significant. Septic shock and sepsis impair myocardial compliance and affects the contractility of the cardiac myocytes and impairs their function. It is unclear how

this occurs; particularly as cardiac dysfunction could be driven by several complex and interconnected pathways.

Firstly, the clinical presentation or haemodynamic status of septic patients can be grouped into two almost phase like categories: warm and cold shock (or hyperdynamic and hypodynamic, respectfully). Warm shock is associated with low systemic resistance, a normal or elevated cardiac output, and typically flushed warm skin; thus, in contrast to cold shock which sees patients with clammy and sweaty-type skin, hypotension and reduced cardiac output. The latter phase becomes more evident in severe sepsis and shock, or towards later disease progression (Li *et al.,* 2013b). Figure 9 published from Kakihana *et al.* (2016) illustrates the direct warm and cold shock phases, also the mechanisms by which these are proposed to occur. The release of cytokines and nitric oxide drives both cardiomyocyte hyperresponsiveness and vasodilatation. The latter considered 'warm shock', which leads to damaged cells, themselves subsequently releasing DAMPs leading to cardiomyocyte injury through further immune cell infiltration. Taken together leads to endothelial injury which is characterised by 'cold shock'. Cold shock patients ultimately present with multi-organ failure. There is another pathway leading to cold shock presentation. This is through PAMPs, PRRs and the generation of NETs. These NETs (reviewed elsewhere in this thesis) directly contribute to endothelial injury – driving cold shock and MOF. But further, NETs cause the release of myocardial depressant factors (these include TNFα and IL-1β and others explored in Table 1.) (Kakihana *et al.*, 2016).

Briefly, some of these clinical manifestations include both diminishing diastolic and systolic ventricular functions, the latter commonly referred to as 'cardiac depression' – complications with cardiac output and return. Further features include reduction in left ventricle ejection

fraction (LVEF), altered coronary blood flow with a decline in oxygen usage by the myocardium. Other possible dysfunctions experienced include irregular tissue motion and thickening of walls, and particularly common in septic presentations are sepsis-induced arrhythmias (Aneman and Vieillard-Baron, 2016; Suzuki *et al.,* 2017).

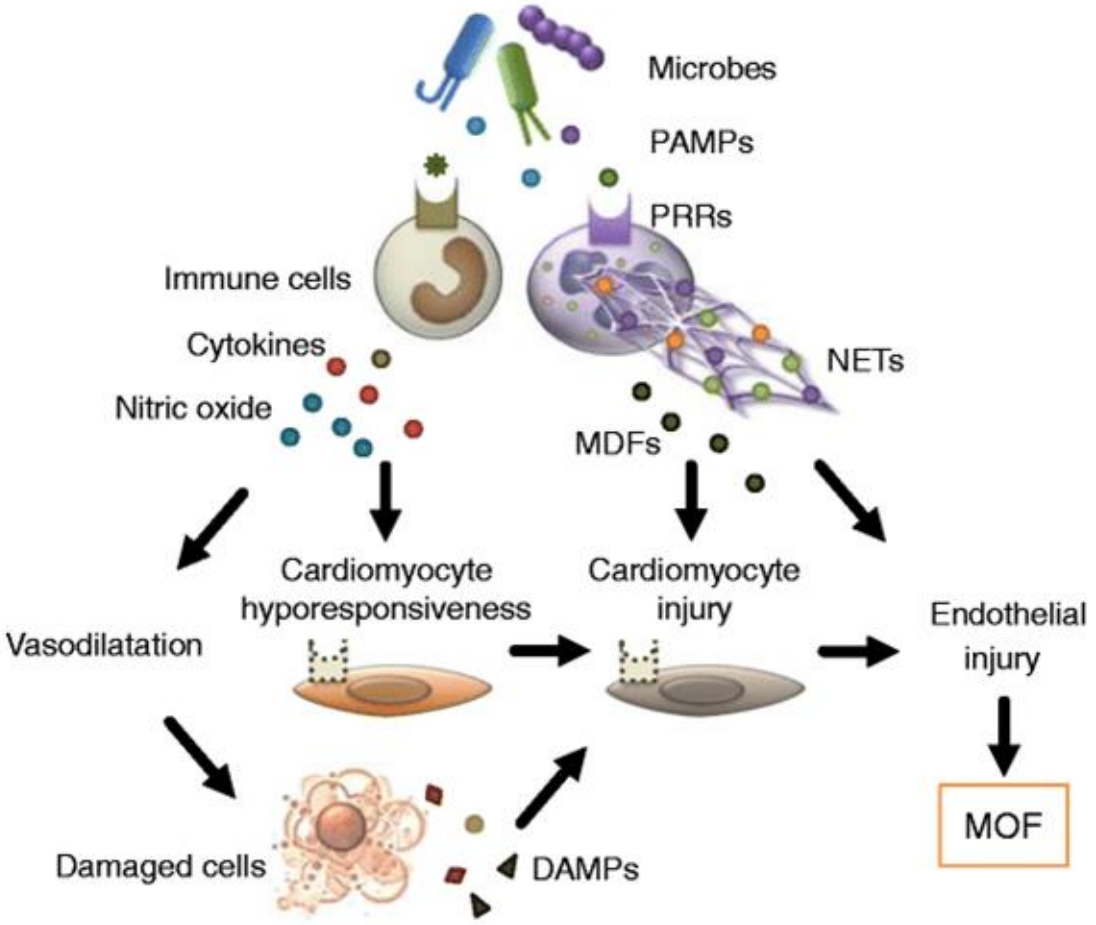


Figure 9: Warm and Cold Shock pathways, left pathway leads to warm shock presentations, whereas the right (with MOF) is typically characterised as cold shock and is notable with multi-organ failure. MOF: multiorgan failure, DAMPs: Damage Associated Molecular Patterns, MDFs: myocardial depressant factors; NETs: neutrophil extracellular traps, PRRs: pattern recognition receptors. Figure adapted from Kakihana et al. (2016)

1.2.3.1: Inflammation and Cardiovascular Function

Notable inflammatory influencers yielding significant effects are summarised in Table 1.

Table 1: The functional impact of key mediators has on the cardiovascular system. Immune identifiers initially obtained from Li, Ge, Peng and Chen (2013). Effect examination and references are provided for each mediator. IL: interleukin; TNF: Tumour Necrosis Factor; IFN: Interferon; HMGB: High Mobility Group Box; CLP: Cecal Ligation Puncture; SR: Sarcoplasmic Reticulum

Immune Mediators	Effect	Reference
IL-1β	Displays negative inotropic effect – effecting contractibility; myocyte depression; increase sarcoplasmic reticulum Ca^{++}leakiness; induce mitochondrial injury	Li, Ge, Peng and Chen (2013); Kumar *et al.* (1996); Fernandes Jr, Akamine and Knobel (2008)
TNF-α	Displays negative inotropic effect – effecting contractibility; myocyte depression; increase sarcoplasmic reticulum Ca^{++}leakiness; induce mitochondrial injury	Li, Ge, Peng and Chen (2013); Kumar et al. (1996); Fernandes Jr, Akamine and Knobel (2008)
IL-2	Not well understood, likely contributes to release of TNF-α	Fernandes Jr, Akamine and Knobel (2008)
IL-4	Contributes to myocardial depression	Fernandes Jr, Akamine and Knobel (2008)
IL-6	Contributes to myocardial/cardiomyocyte depression; induce mitochondrial injury	Li, Ge, Peng and Chen (2013); Fernandes Jr, Akamine and Knobel (2008)
IL-7, IL-17A, IL-22, IL-33	Potential role, although not established	Li, Ge, Peng and Chen (2013)
IL-8, IL-10	Depressor action	Li, Ge, Peng and Chen (2013)
IL-18	Stimulation of cardiomyocyte hypertrophy; potentially proapoptotic	Li, Ge, Peng and Chen (2013)
IFN-γ	Mild depressor action	Fernandes Jr, Akamine and Knobel (2008)
HMGB1	Mediated dysfunction through TLR 4 pathways; increases SR leakage	Liu, Yu, Shou and Chai (2017)

From Table 1 it is apparent that IL-1β and TNF-α are potent causes of myocardial dysfunction with large ranging effects including impact on calcium trafficking. Also impacting sarcoplasmic reticulum (SR) is High Mobility Group Box 1 (HMGB1) increasing SR leakiness of Ca^{2+} (Liu *et al.,* 2017). The β-blocker Landiolol, is thought to reduce circulating levels of HMGB1 thus reducing both systemic inflammation and potentially protecting cardiomyocyte calcium overload. It further has the potential to mitigate some inflammatory-based dysregulation of cardiomyocytes (e.g. from extended presence of cytokines and NO and ROS) from the cardio-protective properties established with β-blockage, such as cardiac remodelling (Messerli *et al.,* 2009; Oliver *et al.,* 2019). One of the other potential benefits of continued stimulation of β_2-AR is the inhibitor and blocking effect of apoptosis and myocyte death. That noted however, whilst myocyte death is not impossible as injury does occur, the event is considered fairly rare in sepsis (Takasu *et al.*, 2013).

Protease activated receptors 1 and 2 have also been associated with initiating hypertrophy in cardiomyocytes which drives disease, suggesting a potential therapeutic target; however, Flaumenhaft and De Ceunynck (2017) highlighted that inhibiting these detrimentally blocks cytoprotective properties seen in several cells including fibroblasts within several preclinical models. Thereby, seeming counter indicated.

1.3: Adrenergic Responses

Adrenergic responses arise from autonomic nervous system (ANS), specifically the sympathetic nervous system, which is responsible for the "*fight-or-flight*" response (Shan *et al.*, 2010). The adrenergic response occurs through either an α or β receptor, of which 9 have been described (α: α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B}, α_{2C}; β: β_1, β_2, β_3) (Ciccarelli *et al.*, 2017). These receptors bind catecholamines, which are the first line treatments for unresponsive hypotension following attempts at initial fluid resuscitation in septic shock. There are several catecholamines, those commonly used clinically include adrenaline and noradrenaline. The function of catecholamines is to increase blood pressure by vasoconstriction (subsequently also vascular resistance and tone), increase cardiac output, heart rate and respiratory rate. Although the evidence base is not strong, noradrenaline is recommended as first-line treatment following fluid resuscitation (Evans *et al.*, 2021). Further support by Scanzano and Cosentino (2015) who reviewed adrenergic immune regulation, highlighting that immune cells have adrenergic receptors on their surface (adrenoreceptors, AR). These responses include altered migration and chemotaxis potential in neutrophils as demonstrated by Nicholls *et al.* (2018), changes in immune metabolism and altered cluster of differentiation expressions (such as CD18 for example) (Stolk *et al.*, 2020). Other than affecting neutrophils, cells including natural killer (NK), dendritic cells (DC) and monocytes are further impacted as they all express β-AR receptors (Scanzano and Cosentino, 2015). It would therefore not be unexpected the greater the concentration of catecholamines, greater the impact on these cells (dose-response), in addition to increasing incidence rates of global cardiac ischemia. With the administration of β-blockers (i.e., Landiolol in the case of STRESS-L), the antagonistic effect should reduce some of the dysregulations exerted by catecholamines. In addition to the advantages of β-blockade in routine clinical practice that could be applied to septic shock

management – such as managing supraventricular tachyarrhythmias (e.g., ventricular tachycardia and atrial fibrillation), reviewed by Rehberg *et al.* (2018) and Pemberton *et al.* (2015) in patients and Matsuishi *et al.* (2016) in a rat model. The addition of β-blockade, as discussed later in section 1.4 and 1.5, aims to alleviate and manage some of these presentations.

As the concentration of catecholamines increases, there are several other non-hemodynamic effects seen in patients, including: bronchodilation, metabolic dysfunction, insulin resistance, reduced motility and hypercoagulability for example (Hartmann *et al.*, 2017) – these are discussed elsewhere in the thesis.

As β-blockers occupy receptors through a competitive process impacting key systems, several models will be constructed to assess the difference between trial participants randomised to the control arm and experimental arm. An introduction to modelling in mechanistic studies and their importance are discussed in section 1.8. With the potential for β-blockade to alleviate some dysfunction and dysregulation in severe sepsis and septic shock – blockade has been proposed as a new treatment (section 1.4.2 and 1.5).

1.4: Treatment for Sepsis

The treatment decisions are driven by guidelines from the National Institute for Health and Care Excellence (NICE) and the Surviving Sepsis Campaign guidelines, NICE-'NG51' (2016) and Evans *et al.* (2021). As the clinical guidelines lay out the treatment options for sepsis, this thesis will not explore them further. However, this section will briefly explore catecholamines and their role in sepsis management.

1.4.1 Current Treatment

Catecholamines are drugs whose primary action leads to contraction of blood vessels and therefore raises or aims to increase blood pressure. Noradrenaline [norepinephrine] remains the first-choice vasopressor by national and international guidelines. Vasopressors and catecholamines have side effects and increased doses are associated with increased mortality (Martin *et al.,* 2015). These side effects include ischemia-related damage, onset of arrhythmias, and modulation of the patient's immune system in addition to changes in metabolism. These extensive effects raise the question as to whether their indicated use is appropriate particularly as Dhalla *et al.* (2010) indicated catecholamine use was associated with lethal ventricular arrhythmias (ischemia and coronary spasm) and sudden cardiac arrests in ICU patients. Conflict exists between potential risk of side effects (including mortality) or the potential to sustain life. The mechanism by which noradrenaline works is shown in Figure 10. Due to the nature of these catecholamines-based drugs, some of these (including norepinephrine) are affected by tachyphylaxis. Dosage tolerance adjustments are required for the same therapeutic effect to be observed in patients, which not only further increases the intensity and likelihood of side effects but also the mortality probability. During clinical presentations of vasoplegia, increasing the dosage of norepinephrine >20 mcg/min (with or without second and third vasopressor) places the patient in the high mortality category with greater than 80% mortality (Brand *et al.*, 2017). Vasopressor dosage (combined drugs) >40 mcg/min is positively associated with expected fatality. This is likely the result of refractory hypotension with poor organ perfusion leading multiorgan damage and eventually failure, subsequently being incompatible with life. For enrolment to STRESS-L, as inclusion criteria, patients required noradrenaline support >0.1 mcg/kg/min (Lall *et al.,* 2021).

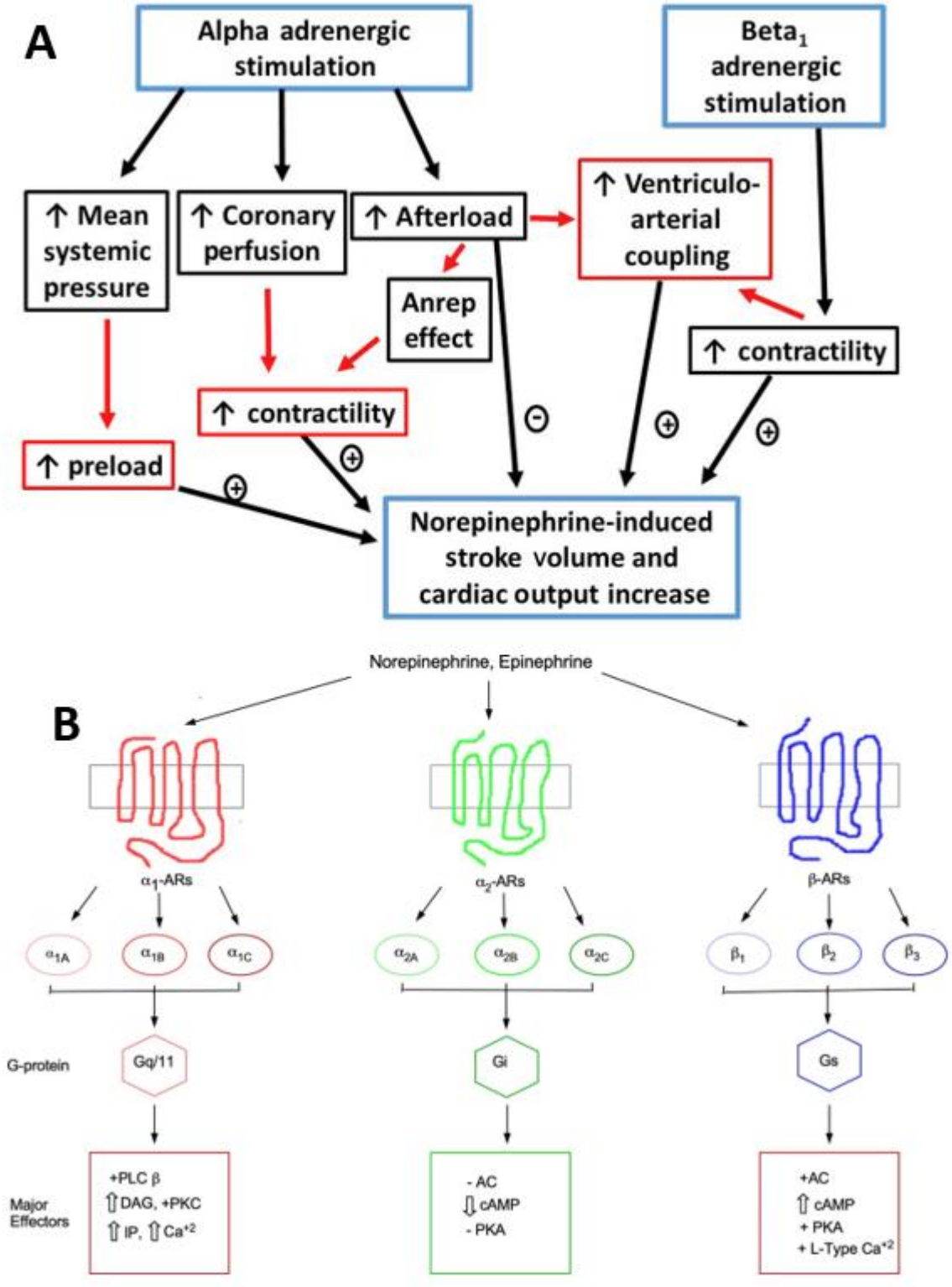


Figure 10: Figures highlight norepinephrine effects on administration. A) Obtained from the works of De Backer and Pinsky (2018). The red boxes show the impact functionally by norepinephrine, i.e., increases in preload and contractility for example whilst the black boxes illustrate the immediate effect seen based on administration. Figure B) obtained from Perez (2020) shows the signalling effects of norepinephrine's effect including upregulation of diacylglycerol (DAG), protein kinase A (PKA), intracellular calcium and inositol phosphate (IP) in the cases of α1-AR binding and through G_q/11 G-protein. Whilst not shown in the figure upregulations of Gs-GTP leads to positive chronotropic and inotropy affects. AC: adenylate cyclase; cAMP: cyclic adenosine triphosphate; AR: adrenergic receptor.

The use of norepinephrine in patients with sepsis, caused by microbial infection, appears to be counterproductive as Evans *et al.* (1948) highlighted that it stimulates the growth of microbial organisms such as *Escherichia coli*. This is further supported by research by Sandrini *et al.* (2014) and Freestone *et al.* (2000) which links norepinephrine with growth stimulation of *pneumococcal* species, increases in the virulence genes of organisms and direct formation of biofilms by several prokaryotes. The mechanism of increasing microbial growth could be related to the role of transferrin and iron-acquisition by bacterial species (O'Donnell *et al.,* 2006) as norepinephrine increases availability of transferrin-bound iron (Freestone *et al.*, 2000). Subsequently, this suggests norepinephrine use in microbial-positive septic patients could increase mortality by maintaining bacterial populations, especially those with antimicrobial resistance (AMR), and prolong the dysregulated inflammatory response. In addition, Stolk *et al.* (2016; 2020) reported the immunomodulation caused by norepinephrine leading to immune paralysis (Figure 11). Importantly, any options to reduce the dosage of norepinephrine should be taken where clinically appropriate.

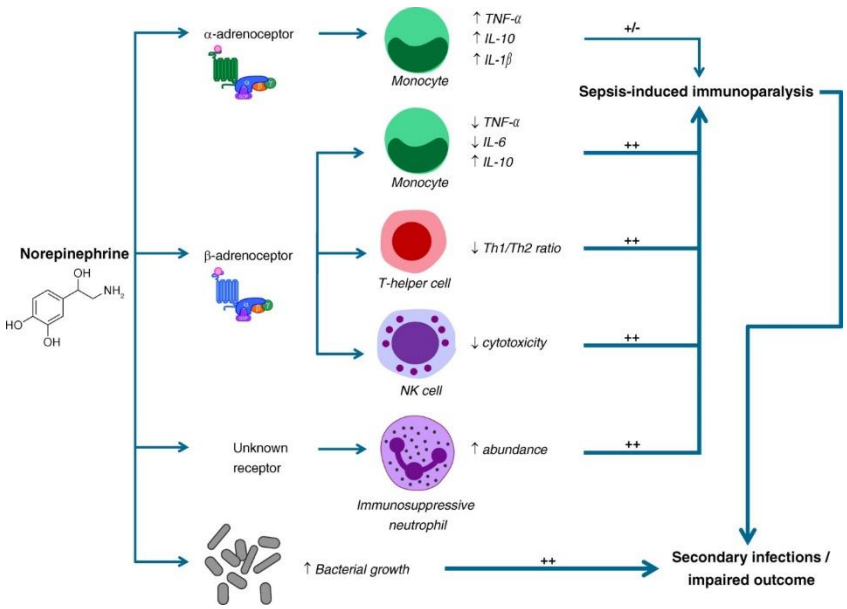
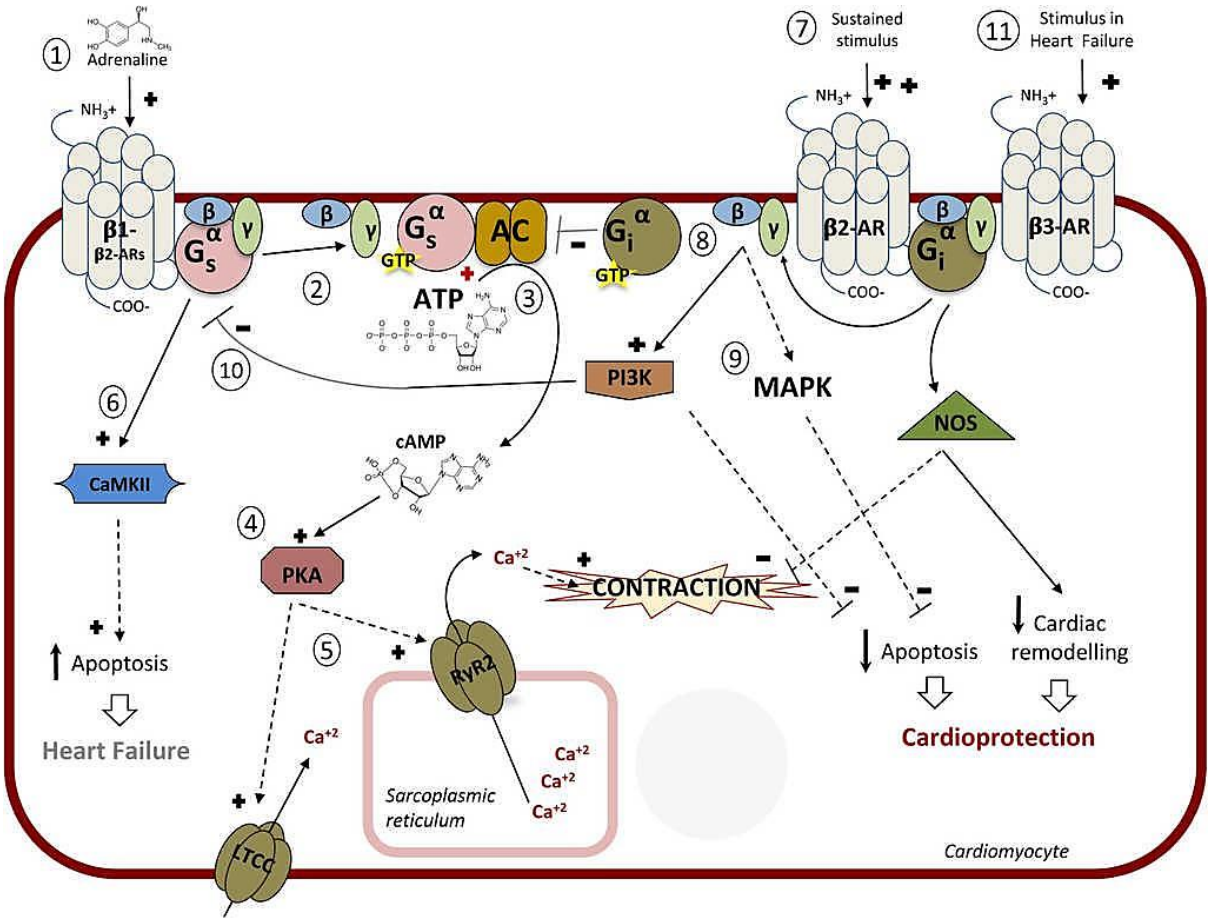


Figure 11: Potential mechanisms whereby norepinephrine induces and contributes to sepsis-induced immune paralysis. Taken from Stolk et al. (2016).

1.4.2 New Treatment

A proposed a new treatment option is the use of β-blockade. β-blockers, are indicated to treat several conditions ranging from angina, heart failure, high blood pressure, anxiety and hyperthyroidism depending on particular drug prescribed. There are two main classes of β-blockers: *non-selective* and *selective* – also termed *cardiac-selective.* They differ typically by pharmaceutical receptor interaction. For instance, *non-selective* β-blockers antagonise both β_1- and β_2-adrenergic receptors, whereas *selective* β-blockers typically interact more with β_1-receptors. Hence, newer generation drugs (i.e. *cardiac-selective*) are more suitable for patients who are asthmatics due to the lack of smooth muscle counter-interaction.

Beta-blockers interact directly with adrenoreceptors, antagonising their signalling. Landiolol is a cardiac-selective β-blocker, thereby favouring the ligand interaction with β_1-receptors. Adrenergic receptors (section 1.3) are part of a larger family of G-protein coupled-receptors (GPCRs), reviewed by Wallukat (2002). Adrenergic receptors are the predominant receptors found within the myocardium, and the focus of β-blocker interaction. Cyclic AMP (cAMP) is the hallmark of adrenergic receptor signalling, upregulating protein kinase A (PKA) which acts on L-type calcium channels and ryanodine receptors to increase force of contraction, and whilst also controlling relaxation properties (Oliver *et al.*, 2019). Other signalling pathways and kinases, e.g. phosphatidylinositol-3-kinase has roles in cardiac protection for example. Full signalling is illustrated in Figure 12.



Rev Esp Cardiol. 2019;72:853–62

Figure 12: Signalling pathways initiated by β-adrenoreceptors. 1) Initial binding of adrenaline or noradrenaline (catecholamines) to GCPR β-adrenoreceptors which induces coupling to G_s (heterotrimeric) protein. This causes the dissociation of β and γ subunits from the $G_{\alpha s}$-Guanosine Triphosphate (GTP) unit – activating adenylate cyclase (denoted AC on diagram) (2). Thus, causing the transition from adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP) (3). cAMP activates/upregulates protein kinase A (PKA) (4) which is principally responsible for the increase to contractual force and rate of cardiac relaxation through upregulation/activation by calcium at the L-type calcium channel (denoted LTCC) and ryanodine receptor (denoted RyR2) on the sarcoplasmic reticulum (5). Point 5 in turn increases contraction. At point 6, prolonged stimulation by catecholamines (i.e., A/NA) induces cell-death type response, apoptosis, through upregulated CaMKII (protein kinase - calcium/calmodulin complex) leading to heart failure. Binding to $β_2$-AR with continued stimulus causes $G_{i(α)}$-coupling (7) subsequently inhibiting adenylate cyclase conversion of ATP to cAMP. The $G_iβγ$-subunit causes cardiac protection by preventing apoptosis (by inhibiting) through phosphatidylinositol-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways (9). The former, PI3K, also inhibits progression to CaMKII (6). Finally, the activation of nitric oxide synthase (NOS) decreases cardiac remodelling and inhibits repeated (inappropriate) contraction, aiding cardiac protection (11). Taken from Oliver, Mayor and D'Ocon (2019).

1.4.2.1: β-Blockade Potential in Sepsis and Shock

Research into β-blocker use in sepsis has produced varying outcomes. Of the many clinical trials, few exist without limitations. Wang *et al.* (2015) looked at the effect of combining Esmolol with Milrinone in 60 patients with sepsis reporting a decline in 28-day mortality and improvement in cardiac function. Other studies, such as those by Kakihana *et al.* (2020) in a multicentre trial (J-Land 3S) of Landiolol in 151 patients showed positive outcomes with greater heart rate compliance in the study group than control. The authors also reported a reduction of new arrhythmias once Landiolol therapy had commenced, however serious adverse events had been recorded in 6% of patients including cardiac arrest. Whether the cardiac arrest had a causative link to Landiolol or was a sepsis-induced dysfunction is unclear. For comparison, adverse effects occurred in 59% of control group patients. One of the more substantive trials was that led by Morelli in 2013, who enrolled patients with septic shock with tachycardia who were in receipt of high-dose noradrenaline therapy (Morelli *et al.,* 2013). For this group, high drug dosage is correlated with mortality rate >60%. This study found that both mortality rate and ICU length of stay in the interventional arm was reduced, as well as β-blockers being safe in septic care and with greater hemodynamical compliance. Some of the issues associated with this study were that the trial occurred at a single site with a small number of trial participants (154 patients, 77 in each arm). This study also had a high mortality rate (80.5%) in control participants which may unintentionally distort the result in the experimental arm, in addition to the investigators not exploring any mechanisms.

In the J-Land 3S study (Kakihana *et al.,* 2020) there were also suggested differences between the sexes in 28-day mortality with the benefit established more in males (Matsuda *et al.*, 2020). In studies using a rat model of sepsis, Mathieu *et al.* (2018), showed sex differences

for several outcomes, including stroke and diastolic volume(s) differences in males compared to female rats. With much of Landiolol-induced effects benefitting male participants – such as resolving cardiac dysfunction, whereas in female Wistar rats producing more deleterious effects such as reducing left ventricle ejection fraction. Also, intriguingly, the same J-Land 3S study showed that if the infection site responsible for sepsis is elsewhere than a respiratory-based organ, the 28-day mortality rate favours control arm, but if infection is within a respiratory-based organ there is favour towards the interventional Landiolol group. This suggests that Landiolol possibly has a location sensitive response, and thus a different β-blocker may need to be selected depending on location of infection. Further studies will be required to provide additional evidence.

Whilst this brief overview of previous trials is far from extensive, one common underlying outcome is that Landiolol is generally well-tolerated in adult patients with sepsis. The main limitation common among all these trials is the lack of mechanistic and explorative investigation, with very few (if any substantial) observed in human participants longitudinally.

1.4.2.2: Landiolol (β-blocker) – Structure, Properties and Mechanism of Action

Landiolol hydrochloride (Figure 13) is a new ultra-fast acting β-adrenergic agent with an extremely high cardioselectivity ratio $\beta_1/\beta_2 = 255$ and low toxicity (Atarashi *et al.*, 2000; Shiga, 2022). This is compared to the closest rapidly acting β-blocking drug Esmolol with selectivity of $\beta_1/\beta_2 = 33$. Landiolol bears resemblance to Esmolol despite the extensive cardio-selectivity ratio differences. Developed in Japan to originally treat intraoperative tachyarrhythmia, this has expanded to the treatment of left ventricular dysfunction and atrial fibrillation (Matsuishi *et al.*, 2020). One benefit of Landiolol is that it is not renal and hepatic metabolism dependent

– greatly expanding its use among patients with both reduced eGFR (estimated glomerular filtration rate) and hepatic failure (Atarashi *et al.*, 2000).

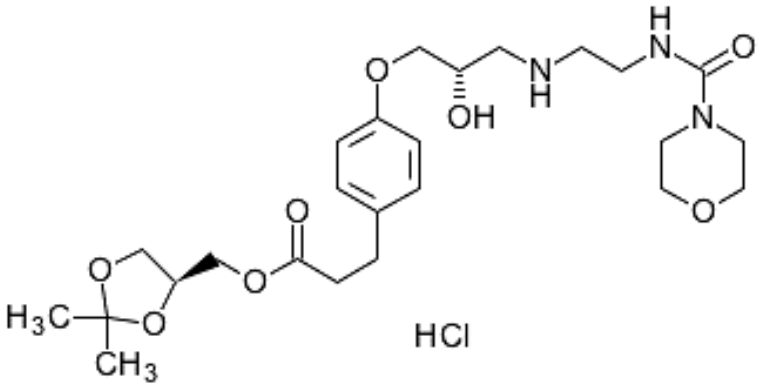


Figure 13: Chemical structure of Landiolol Hydrochloride from KEGG Entry: D01847

The pharmacological properties of a drug are vitally important, Landiolol shows a linear PK-PD relationship across its suggested dose regime meaning drug efficacy should increase linearly with drug dose. The drug reaches steady-state condition approximately 15 minutes by continuous infusion without loading dose, however when initiated with a loading dose, this can be achieved at 2 minutes, hence it is an "ultra-quick acting" β-blocker ideal for us in emergency and acute settings (Atarashi *et al.*, 2000). This is likely as the drug binds <10% to protein allowing rapid diffusion into tissue. The metabolism of the drug occurs both by hydrolysis and (β-)oxidation depending on the stage of biotransformation. Principally, metabolism occurs by pseudocholinesterases in the serum, and hepatic carboxylesterases. With regards to drug elimination from the system, this occurs readily with up to 75% of bio-transformed metabolite eliminated in urine within 4 hours, almost 100% within 24 hours Atarashi *et al.*, 2000; STRESS-L Drug Characteristics, 2020).

Some properties of Landiolol include an anti-inflammation or immune modulation type-response. The exact mechanisms by which this occurs are not widely understood but includes

interaction with adrenergic receptors on immune cells (Scanzano and Cosentino, 2015). The interaction of adrenergic signalling and immune cells are reviewed in Section 1.3. Several studies have reported a reduction in circulatory cytokines such as high-mobility group box-1 (HMGB1), TNF-α and interleukins (Hagiwara *et al.,* 2009). Evidence from the PASCAL trial ("Prevention of Atrial Fibrillation in Patients having Cardiac Surgery with Landiolol Hydrochloride for Coronary Artery Bypass Grafting") has also shown the reduction of serum IL-6 among other cytokines with Landiolol (Sezai *et al.*, 2011). IL-6 is an important mediator of inflammation including induction of C-reactive protein (Tanaka *et al.,* 2014) and contributor to pathology such as increasing vascular permeability. Elevations of IL-6 have been associated with greater levels of mortality in sepsis and septic shock (Remick *et al.,* 2005). However other studies have reported conflicting data with Matsuishi *et al.* (2016) showing Landiolol had no effect on IL-6 in a rat model of early sepsis. Further studies are required to provide insight, particularly in humans.

As with IL-6, Landiolol's impact on HMGB-1 is not explicitly clear. It is understood that HMGB-1 contributes to acute lung injury and endotoxin lethality at least in mice, with the former expected in humans also. Other consequences can also include necrosis-type injuries (Park *et al.*, 2006). There is little to no substantial research of the mechanistic effect of Landiolol, on HMGB-1, other than the results reported by Hagiwara *et al.* (2009) previously. Ackland *et al.* (2010) also observed reductions in plasma IL-6, IL-18 and chemoattractant protein-1 on commence of beta-blockade. A mortality reduction was also seen in these Wistar rats if beta-blocker therapy was initiated before sepsis become established. Peripheral blockade agent in this trial however was not Landiolol.

Although Hagiwara and colleagues reported reductions in TNF-α with Landiolol (Hagiwara *et al.,* 2009), Matsuishi *et al.* (2016) did not confirm these findings. The role of TNF-α in sepsis pathophysiology includes increasing the production of macrophages and prolonging their action, affecting coagulation and inducing fever (Schulte *et al.,* 2013). Despite the conflicting views on whether β-blockade impacts TNF-α directly or indirectly, previous β-blockers such as Esmolol have reduced circulating cytokines, and therefore, its presumed Landiolol should also reduce to their levels (Wei *et al.,* 2016).

Interestingly, it is not clear the mechanisms of action of Landiolol, and indeed how it acts as an indirect anti-inflammatory. One hypothesis could be because of its biotransformation to benzoic acid. Benzoic acid has several anti-inflammatory effects including reduced neutrophil elastase release and NET generation (Chen *et al.,* 2008). Neutrophil elastase is a serine proteinase responsible for immune cell mediated tissue damage (Wang, 2018). Sandhaus and Turino (2013) have implicated neutrophil elastase in emphysema and other lung-based damage. It would be intriguing to identify if Landiolol reduces neutrophil elastase and elastase mediated damage in sepsis patients, the latter can be measured indirectly by assessing levels of an elastase breakdown produce in serum (Sapey *et al.,* 2019).

From the few pre-clinical studies already conducted, a reduction in heart rate has been reported, whilst little to no effect on systolic and diastolic pressures and few adverse events such as bradycardia, hypotension or bronchial spasm (Atarashi *et al*., 2000). As such Landiolol appears to be a drug of considerable promise. This is particularly true in patients with refractory hypotension (unresponsive to adequate supportive measures, including fluid resuscitation) presenting with ventricular dysfunction and tachyarrhythmia. Krumpl *et al.* (2020) showed that Landiolol had successfully reduced heart rate within 1 minute (1-6

minutes). Noteworthy, heart rate reduction has been reported to occur in separate discrete phases varying by reduction effect and intensity. Whether this is directly related to the actions of Landiolol or to desensitisation of a catecholamine ligand is unclear, as the latter reduction was considerably less than initial reported reduction on infusion. Furthermore, Krumpl and colleagues did report the bradycardic effect of the drug which reduced rapidly with termination of infusion, though recovery from heart rate was incomplete. Considering the complex physiology experienced in septic shock with multi-factorial systems failing, Landiolol lends itself to rhythm control in cardiac failure and dysregulation such as atrial fibrillation. B-blockade, using Landiolol, may therefore be a useful tool for clinicians trying to address arrythmias without simultaneously impacting already established reductions in blood pressure, particularly helping the decision as to whether to increase or maintain current dosage of vasopressors. It would thus be interesting to identify the impact Landiolol has on key markers of cardiac insult and myocardial injury such as Pro-β-natriuretic peptide, troponin-I and creatine kinase myocardial band (CK-MB).

There are clear metabolic changes occurring in patients with sepsis and septic shock – reviewed earlier, but little is known with regards to the effect Landiolol would have on metabolism in sepsis when administered to the septic patient. The few studies already published are of beta-blockers are mainly in animal models and have not been on a large scale. Few studies have conducted longitudinal analysis, very little if any in human participants to the extent of STRESS-L.

1.5: Study into the Reversal of Septic Shock with Landiolol (β-blockade) (STRESS-L)

Study into the Reversal of Septic Shock with Landiolol (β-blockade), abbreviated STRESS-L, was a clinical trial based within the United Kingdom, having run at approximately 41 intensive care units (ICUs) led by Professor Tony Whitehouse at Queen Elizabeth Hospital Birmingham. This trial aims to build upon and independently verify the results of a previous trial with Esmolol by Morelli and co-workers (Lall *et al.,* 2021; Morelli *et al.,* 2013). To manage the clinical manifestations of sepsis, current guidelines include use of catecholamines, commonly noradrenaline (discussed in section 1.4.1). However, extended use of noradrenaline adversely impacts immune function, metabolic pathways, and the cardiac system (Stolk *et al.*, 2016). The research conducted by Morelli and colleagues in 2013 showed that patients treated with high dose noradrenaline in addition to a β-blocker improved not only their haemodynamic parameters, but importantly reduced mortality (Morelli *et al.*, 2013). The study did not examine the mechanism of how β-blockers impacted the septic patient to improve outcome. Furthermore, the study was small (having 77 patients in each arm) and based at a single site, as a result, insufficient evidence exists to support recommendation of β-blockade in septic patients mainstream. STRESS-L aims to fill these gaps in research and to provide greater mechanistic information. In addition to drastically expanding the number of study sites (41) to ensure a multi-site effect and increased participant number (target 340, recruited 126 before pre-mature closure), STRESS-L will also examine the effects β-blockade has on cardiac function, metabolism, and the inflammatory response. These data may also help to identify biomarkers to indicate patients predisposed to greater severity of sepsis and those most likely to benefit from β-blockers.

The trial's hypothesis is: "that a reduction in heart rate using Landiolol infusion in patients with septic shock and tachycardia improves organ failure as a measure by the Sequential Organ Failure Assessment (SOFA) score during the 14 days after study drug administration through a reduction in cardiac and immune dysfunction" (STRESS-L protocol Warwick CTU, 2020). The primary objective of the trial is to confirm the effectiveness and safety of β-blockers as a treatment for septic shock and seek the mechanisms of protection in patients with tachycardia treated with prolonged vasopressor drugs (>24-hours). The secondary outcomes of STRESS-L are improvement in mortality, length of hospital stay in ICU, individual organ failure and use of vasopressors. The mechanistic aspect of the study includes the exploration of: 1) cardiac protection, i.e., analysis of plasma creatine kinase (CK-MB), Pro-B-Type natriuretic peptide and troponin-I; 2) metabolic pathways changes, identifying impaired pathways, shifts fuel usage, fatty acid profiling and other bio-energetic considerations by mass spectrometry-based metabolomic techniques; 3) immune modulation via analysis of pro- and anti-inflammatory cytokines. These form the basis of this thesis.

The inclusion criteria are shown in Methods (section 2.2) Figure 14 and the trial consort flow diagram in Figure 15.

1.6: Metabolomics

Metabolomics is the mass study of metabolites (i.e. small molecules, usually <1500 Da) simultaneously within a sample, such as: urine, saliva or blood, in order to deduce and explore the biochemical processes occurring within the body. Importantly, this technology provides insight into how complex real-time physiology is altered by internal and external stresses or disease (such as sepsis) or indeed the effects caused by medicinal agents (such as β-blockers) in the treatment of a disease (Clish, 2015). Metabolomic-techniques provide a powerful approach for the detection of new clinically relevant biomarkers. For example, in the cases of phenylacetic acid and acetic acid have sufficient discrimination ability to determine non-small cell lung cancer to small cell lung cancer (O'Shea *et al.*, 2016). This in addition to providing the foundations to real-time tracking of metabolic pathway disruption, to be used in risk prediction and severity of disease diagnosis (Zhang *et al.,* 2015). Metabolomics has the potential to drive personalised medicine approaches including the management of severe sepsis. It is widely known that sepsis is a very heterogeneous condition, with standard treatment impacting patients differently (Molnár *et al.,* 2016).

This project employs metabolomic technology which allows the simultaneous analysis of thousands of metabolites giving a high level of information and combined with real-time data analytics using both open source and closed source applications will allow biomarker detection. The use of metabolomics in sepsis is discussed by Su *et al.* (2014) and Eckerle *et al.* (2017).

The development pace of metabolomics continues to grow rapidly, with greater functionality being added to platforms. Metabolomic techniques can be combined with other platform-based applications such as MetaboAnalyst which increases the functional use of the data to

incorporate research-relevant entities, such as area under the curve (AUC) and receiver operating characteristic (ROC) which are used as discriminatory powers for biomarker discovery. Other data uses from MetaboAnalyst include network exploration to discover metabolite interaction with biological systems, metabolic pathway enrichment processes and analysis, cluster visualisation and statistical meta-analysis for example (Pang *et al.,* 2021).

Whilst metabolomics research in sepsis is limited, recent paper published by Pandey *et al.* (2021) used NMR-based metabolomics to identify changes in patient metabolism and metabolic profiles in septic patients with additional co-morbidities. The study which consisted of 50 septic shock patients and 20 'healthy' controls reported dysregulations in several pathways including lipid and carbohydrate metabolism and amino acids. They found several biomarkers, both physiologically and statistically significant, which included elevations in lactate, 3-hydroxybutyrate, amino acids (glycine and proline), creatinine, *n*-acetyl glycoprotein and bile among others in sera of septic patients; where on the contrary, citrate, phospholipids, choline, and lipoproteins (e.g. LDL/HDL) had significantly declined in patients presenting with septic shock when compared with health controls. With regards to distinguishing patients with established multimorbidity, in these there were notable further concentration increases in amino acids such as serine and valine, 1,2-propandiol and greater elevations in lactate. Where lactate is an already established gold-standard indicator in likely disease progression or severity. Pandey *et al.* (2021) also shared other pathologies that could manifest in patients with co-morbidities, including cholestasis. This results in the accumulation or secretion impairment of bile, a metabolite previously noted as elevated in septic shock patients. This project will employ mass spectrometry-based metabolomics rather than NMR-based as mass spectrometry (MS) has both a greater resolution and coverage and is better for performing lipidomic analysis (Holčapek *et al.,* 2018*;* Wang *et al.,* 2020). The

complementary nature of Pandey's paper will aid biomarker discovery because of the analytics used, though their patients were not treated with β-blockers experimentally. They used Orthogonal Projections Latent Structure-Discriminant Analysis (OPLS-DA) with unsupervised PCA. Whereas this project looks to expand on these data using Partial-Least Squares-Linear Discriminant Analysis (PLS-LDA) with PCA.

1.7: Predictive Modelling of Outcome Data

Whilst the aim of this thesis is not to construct a predictive and prescriptive model to stratify patients following recent literature showing some sepsis patients have significant adverse reactions in the ITU when given β-blockers, whilst others benefit considerably with marked reductions in mortality and length of stay. This thesis will however use the rich data generated from metabolomic data, cytokine analysis and blood chemistry combined with clinical data to determine mechanistic changes in patients in receipt of standard care and those with standard care and Landiolol. This would be to determine and illustrate if any clear relationships exist between data variables. To ensure models can facilitate meaningful clinical discussion and effective patient management (if later translational potential exist), models should comply with the statement of *TRIPOD* (Transparent Reporting of Multivariable Prediction Model for Diagnosis or Prognosis) and comply with the guidelines of *PROBAST* (Prediction Model Risk of Bias and Assessment Tool) (Collins *et al*., 2015; Wolff *et al*., 2019). With the increasing use of data mining, artificial intelligence and machine learning, the number of predictive models has been increasing rapidly over the last several years, however many fail to become an integrated part of clinical medicine decision making with the

exception of few, APACHE and APACHE II, and SOFA for example (Bonnett *et al.,* 2019). This thesis will construct several statistical models, including:

- SOFA Score and treatment arm (i.e., SOFA with Standard Care vs. SOFA with Standard Care + Landiolol)
- Age and treatment (β-blocker)
- Sex and treatment (β-blocker)
- β-blocker and cytokine balance
- Cardiac markers and treatment

1.8: Project Aims and Objectives

This project aims to identify the mechanisms by which Landiolol hydrochloride, a new β-blocker, impacts upon septic shock, specifically effects on metabolism, inflammation and markers of cardiac damage. Additionally, using data analytics the project will attempt to identify biomarkers of disease severity and progression.

The study will use samples and clinical data from a multi-centre clinical trial, STRESS-L, which is assessing the efficacy of the β-blocker Landiolol in patients with sepsis. The project has the following objectives:

1. To access the impact of Landiolol on metabolism in septic patients by MS-based metabolomics analysis;
2. To determine effects on Landiolol on systemic inflammation and markers of cardiac damage in patients with sepsis;
3. To identify biomarkers of sepsis severity and progression using clinical data combined the data generated in objectives 1 and 2;

2. Methods

2.1 Ethics

Patient samples were collected, stored and processed in line with the protocol of the STRESS-L study which was approved by the East of England – Essex Research Committee *17/EE/0368*, and authorised by the MHRA.

2.2 Patient Screening and Clinical Trial Enrolment

Patients were selected to join the clinical trial by the local principal investigator (PI) at each designated clinical site following the criteria outlined in the study protocol (Lall *et al.,* 2021). Patient randomisation for enrolment occurred which was run by Warwick CTU. The inclusion criteria and study consort diagram are shown in Figure 14 and 15 respectively.

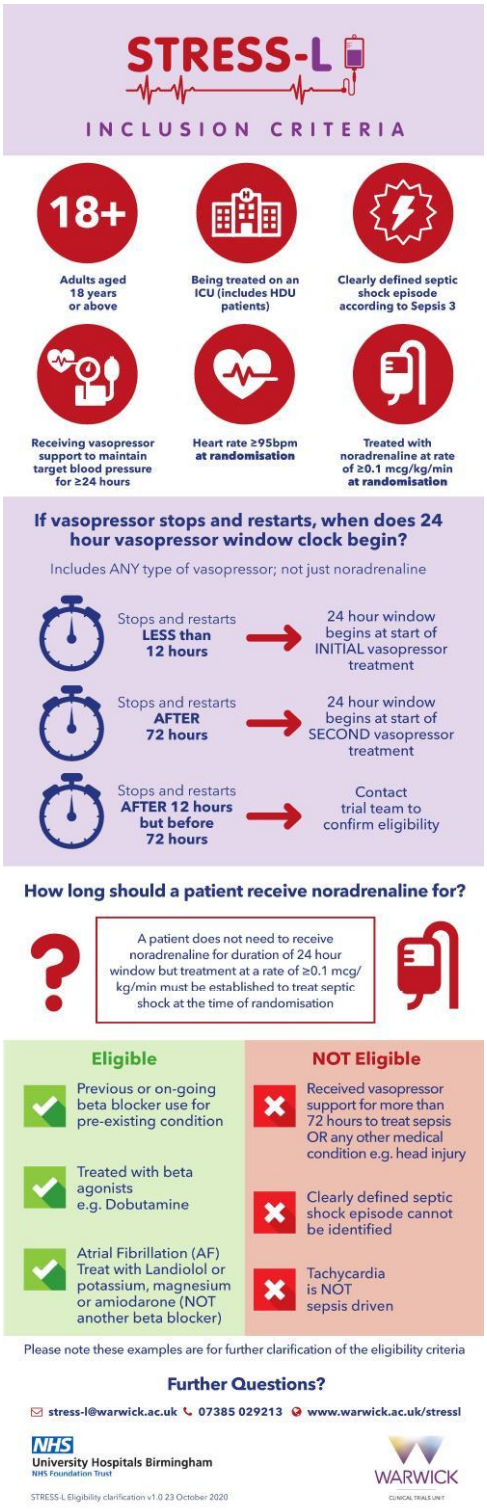


Figure 14: Inclusion Criteria from Warwick Clinical Trials Unit - STRESS-L

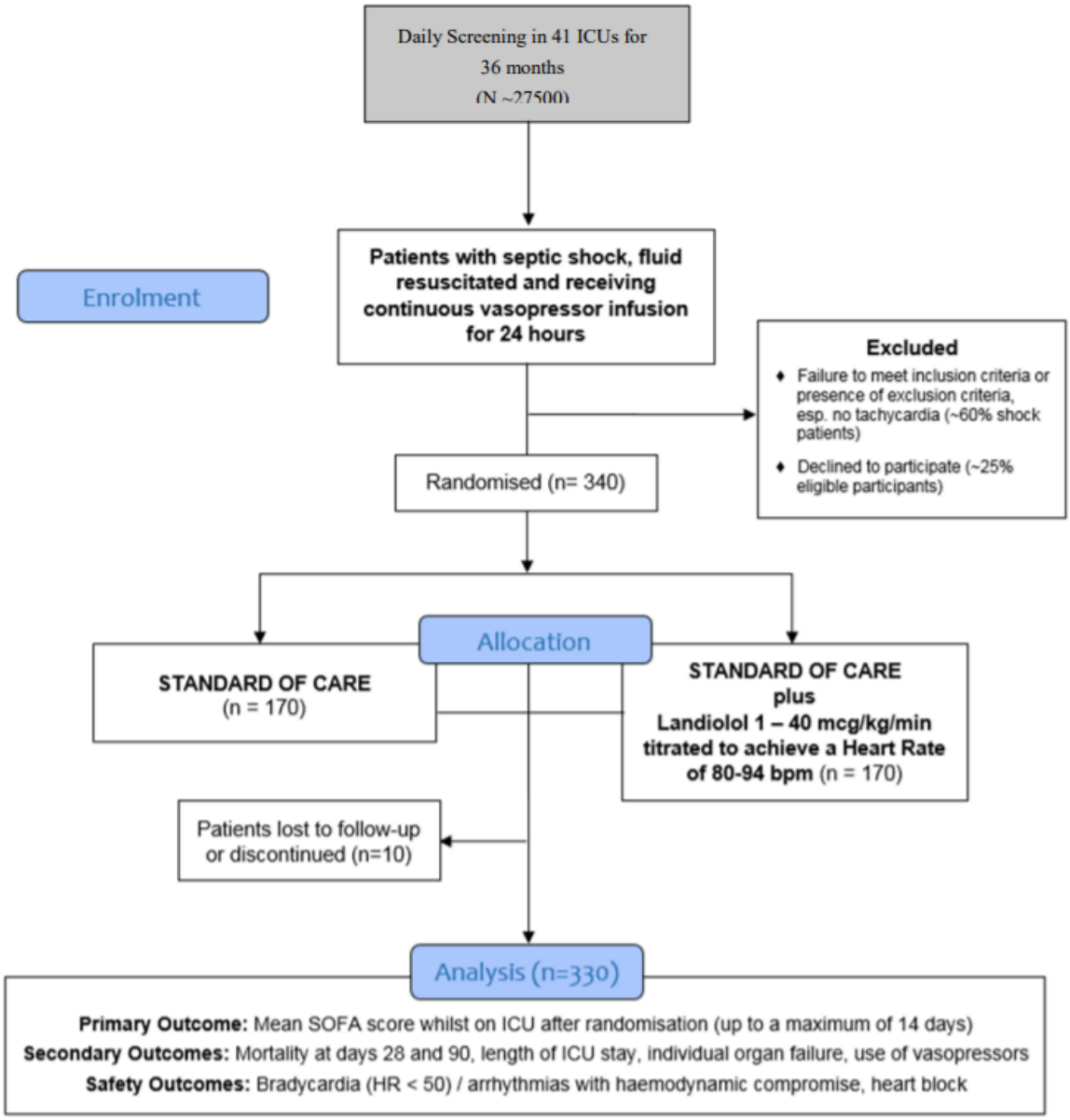


Figure 15: Study Consort Flow from Lall et al. (2021).

2.3 Sample Collection and Storage

Patient samples were collected on days 0, 1, 2, 4, 6 and at the end of noradrenaline therapy (EoNT). Samples obtained on day 0 were collected preceding administration of Landiolol.

Whole blood samples were collected in Lithium Heparin Vacutainers (BD Biosciences, UK) and plasma isolated as per the laboratory manual protocol version 3 (July 2020) and subsequently stored at -80 °C in labelled Cryovials.

Plasma Isolation: Lithium heparinised whole blood was subjected to centrifugation at 1500 x *g* for 10 minutes at room temperature before the plasma layer was isolated and 500 µL aliquots transferred to 8 cryovials and stored at -80 °C.

2.4 Metabolomic and Data Analysis

Sample Preparation and Processing: Plasma (200 µL) was added to high performance liquid chromatography (HPLC)-grade MeOH:$CHCl_3$ (400 µL, 4:1 *v/v*), vortexed and subjected to centrifugation (6000 x *g*, 15 minutes, 4 °C). To perform flow infusion electrospray ionisation high-resolution mass spectrometry (FIE-HRMS), 70 µL of the sample was aliquoted to a 0.2 mL flat-bottom micro-insert HPLC vial for analysis. Metabolites extracted were analysed by FIE-HRMS at Aberystwyth University's specialist high-resolution metabolomic facility.

Using both negative and positive polarities, metabolite fingerprints were generated, with ion intensities obtained using resolution setting of 280,000 for 3.5 minutes between mass/charge ratio 55-120 Da. Of the metabolite extractions, 20 µL were injected by an autosampler in a flow of 100 $\mu L.min^{-1}$ MeOH:H_2O (70:30 *v/v*) before the data was aligned using MatLab (The MathWorks, in-house package) to ensure single positive and negative ionisation mean intensity matrix (runs x mass/charge) around spectra apex.

Data and Statistical Analysis: Raw data obtained from the mass spectrometer was managed using in-house statistical package on MatLab for data alignment. The data was subjected to $\log_{10}$ transformation in readiness for processing by MetaboAnalyst 5.0 (Pang *et al.* 2021). Utilising the platform packages, MS peaks to pathways identifies metabolites and substantially affected metabolic pathways (*H. sapiens* as model organism, tolerance = 3 *ppm*), later exploiting the *mummichog* algorithm as part of the enrichment analysis of data to detect possible metabolic pathway matches, in addition to examining local enrichment ultimately recording true activity and excluding false matches. To ensure the accuracy of these plots, the data was subjected to this algorithm to avoid *a priori* identification of metabolites.

In addition to enrichments, several statistical tests were applied, including chemometric analytics (principal component analysis, partial least squares linear discriminant analysis); as well as data-visualisation cluster analysis packages generating heatmaps and dendrograms to examine relationships built on the foundation of random decision forest statistical analysis. Other standard statistical tests were applied including box plots and over representation analysis.

Furthermore, analytics such as area under the curve (AUC) analytics will be applied to assess the discrimination potential to evaluate potential clinically-relevant biomarkers.

2.5 Cytokine and Statistical Analysis

Luminex multiplex technology follows the basic principle of a standard sandwich-based enzyme-linked immunosorbent assay (ELISA), except that it permits the quantification of multiple cytokines simultaneously, from a single patient sample as small as 50 µl.

Sample Analysis: Isolated plasma samples were assayed using commercially available kits and following manufacturer's guidelines. Cytokine analysis (IFN-γ, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-

8/CXCL8, IL-10, IL-12 p70, IL-17, TNF-α, Trop-I) were completed using multiplex luminometry/ Luminex Discovery Assay (Bio-techne, UK), Kit Number: LXSAHM-12. Using Red (λ635 nm) and Green (λ532 nm) lasers fitted on the platform Luminex^{200} (Bio-Plex), the samples were analysed in conjunction with Bio-Plex Manager (software package). Samples were run neat.

Data and Statistical Analysis: All statistical analysis were performed using SPSS version 28 (IBM). Dependent on data output, either parametric or non-parametric testing was applied. In all cases $p<0.05$ was regarded as being statistically different. All data are reported as mean values ± SD unless otherwise stated. Where appropriate independent t-tests were conducted in order to determine and explore the statistical differences in patients in receipt of β-blocker (Landiolol) with standard therapy or those in the control group, being in receipt of standard NICE (The National Institute for Health and Care Excellence) treatment as per best practice guidance. This includes analysis of co-variance. Where necessary in order to reduce type-I data error due to several analytical tests, *Bonferroni correction* was applied.

2.6 Cardiac Biomarker and Statistical Analysis

Sample Analysis: Circulating cardiac biomarkers were quantified using commercially available ELISA kits, Human NT Pro-BNP DuoSet ELISA (Bio-techne, UK), Number: DY3604-05, following manufacturers guidelines. Samples were run without dilution. CK-MB concentrations were also measured using commercially available ELISA kits, Human Creatinine Kinase MB ELISA kit (colorimetric) (Novus Biologicals, Bio-techne, UK) kit: NBP2-75309, samples processed without dilution following manufacturer's guidelines.

Results

To evaluate the influence β-blockade has on clinical outcome (3.1), metabolism and system energetics (3.2), immune response (3.3) and cardiac performance (3.4), application of several techniques including mass spectrometry metabolomics, multiplex analysis and ELISAs were employed. Table 2 presents a summary of participants key parameters.

*Table 2: **Summary Clinical Demographics of Trial Participants;** * Non-Parametric Tests (Significance Level is 0.05 / 5%)*

N = 126	Standard Care Only (control) N= 63	Landiolol and Standard Care (experimental) N= 63	p-value*
Sex - male, female, unknown (% male)	37, 25, 1 (58.7%)	37, 26, 0 (58.7%)	ns
Mean Age (range)	55.35 (65.40) SD = 17.27	55.90 (63.30) SD = 16.21	.847
Mean 14 Day SOFA Score (range)	8.12 (13.03) SD = 3.28	8.82 (13.70) SD = 3.94	.516
Mean Weight/ kg (range)	78.06 (86.80) SD = 18.97	81.24 (119.00) SD = 24.22	.565
Mean Heart Rate /bpm (range)	97.95 (164.00) SD = 19.62	93.08 (205.00) SD = 17.47	<.001
Mean MAP (range)	75.83 (122.00) SD = 11.01	74.42 (124.00) SD = 12.02	<.001
Mean Noradrenaline Infusion Rate ug/kg/min (range)	0.25 (2.66) SD = .25	0.30 (2.77) SD = .28	<.001
Mean Highest Creatinine Level µmol/L (range)	136.00	128.00	ns
Confirmed Atrial Fibrillation (n%)	21 (33%)	17 (26%)	ns
28-day Mortality (alive, dead, unknown)	47, 16, 0	39, 23, 1	.002

3.1: Clinical Analysis and Parametrics

Using the clinical data obtained over a 14-day period, plots were generated to demonstrate relationships between trial arm allocations (i.e. either 'standard treatment only' or 'Landiolol with standard treatment') and patient outcome with varying clinical parameters.

A clinical variable of particular interest in STRESS-L, and specifically the primary outcome, is improvement in SOFA score. As a generalised measure of disease severity, separation of SOFA score by treatment type (Figure 16), sex (Figure 17), age and weight (Figure 18) and mortality (Figure 19) are shown.

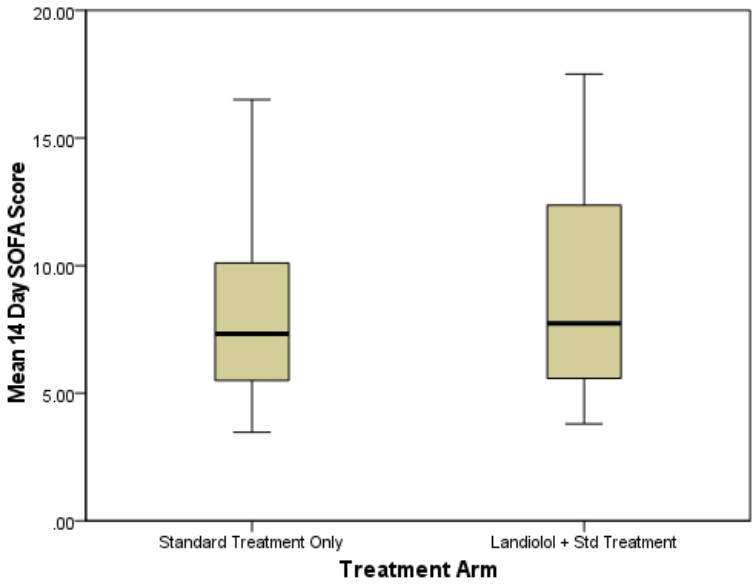


Figure 16: ***Mean 14 Day SOFA Score by Treatment Arm Allocation.*** *Data are median ± 95%CI, p=0.516.*

Figure 16 illustrates the 14-day SOFA score within the experimental (mean 8.82 ± 3.94) and standard of care (mean 8.12 ± 3.28) arms, the difference was no significant (p=0.516). To further breakdown possible variances in SOFA score, the influence of participant sex was explored, separated by treatment arm (Figure 17), however there was no difference by sex (p=0.28).

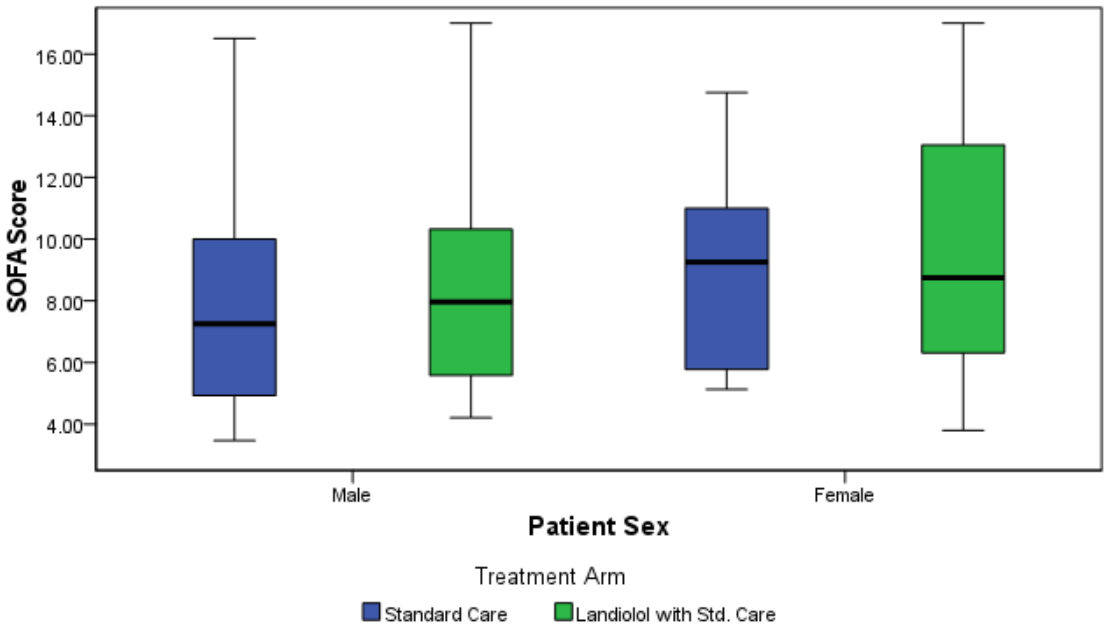


Figure 17: ***Mean 14 Day SOFA Score by Sex, separated by Treatment Arm****. Data are median ± 95%CI.*

The analysis of whether age influences SOFA score and whether there are any treatment-dependent outcomes was explored (Figure 18), illustrates a positive (increasing) SOFA score trend in the Landiolol group with age but not in the control group.

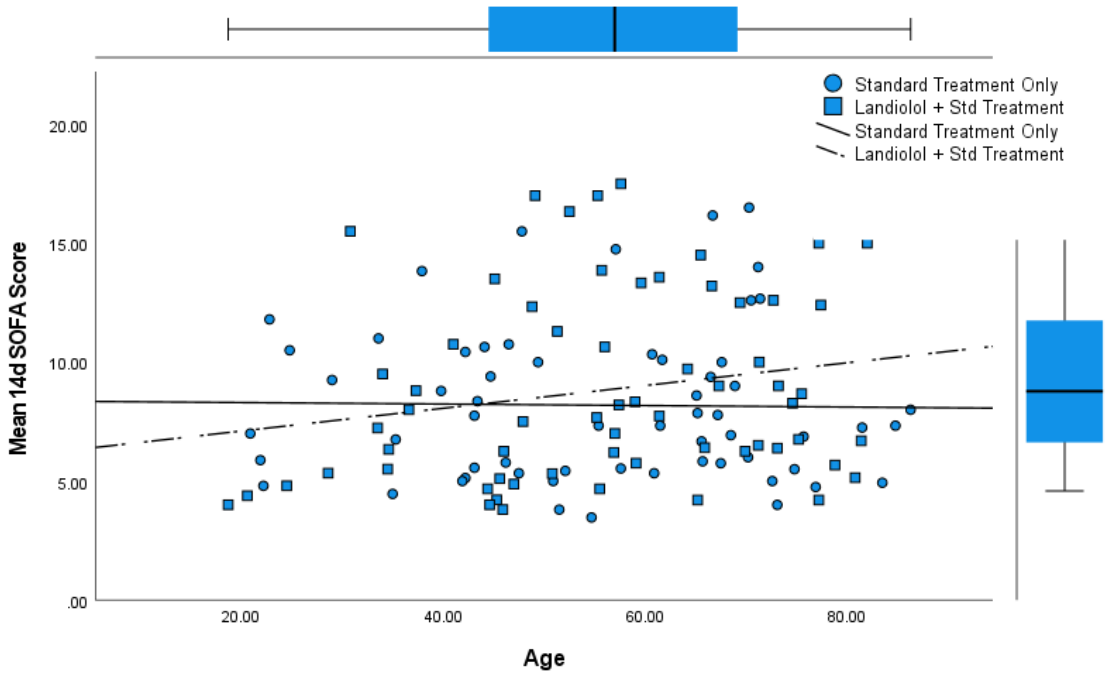


Figure 18: ***Regression Model for Influence of Age on Mean 14 Day SOFA Score****, p=0.001*

To conclude the overall SOFA score analysis, it is important to establish that a higher SOFA score correlates to increased patient mortality at 28-days (Figure 19). 28-day patient mortality showed a deceased rate of 27.03% for males on standard treatment, and 38.89% for Landiolol treatment. In females 28-day mortality was 24% and 34.62% for standard treatment and Landiolol respectively (*p=0.002)*. 90-day mortality for females did not change, for males on standard treatment this increased to 32.43% and Landiolol increased to 50% (*p=<0.001)*. Results suggest either a delay in male mortality or a greater compensatory period, as SOFA score did not statistically differ between treatment groups or sex. This suggests some degree of sexual dimorphism in response to septic shock or treatment.

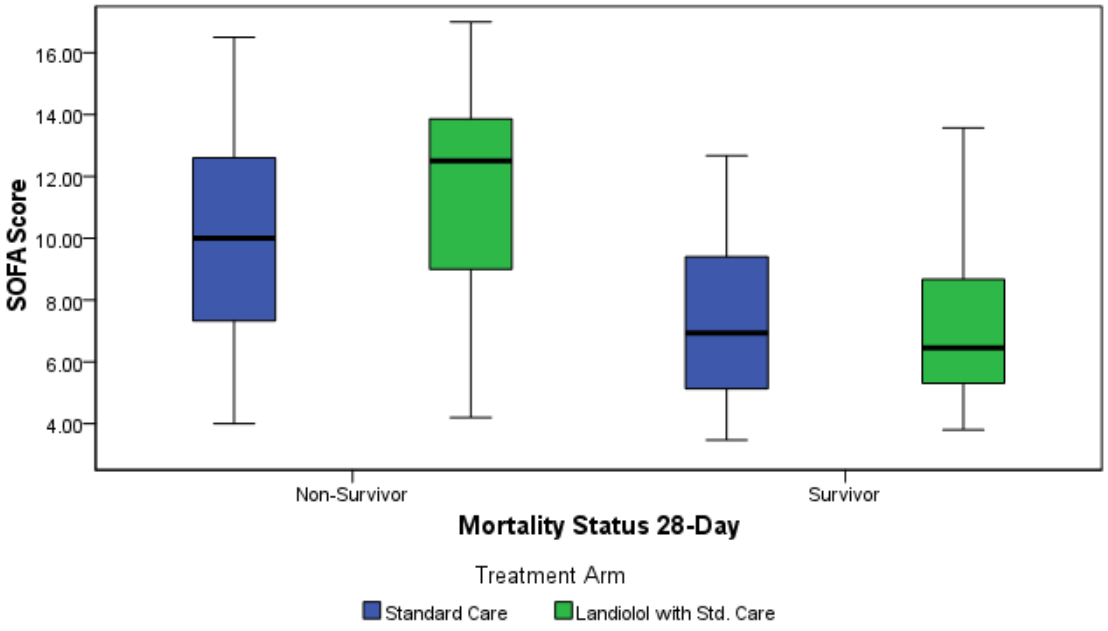


Figure 19: ***28-Day Mortality Status by 14-day SOFA Score.*** *Data are median ± 95%CI, p=0.002*

To establish any treatment-dependency increases in SOFA scores relative to mortality status, a relationship tree was conducted to visualise and provide statistical insight.

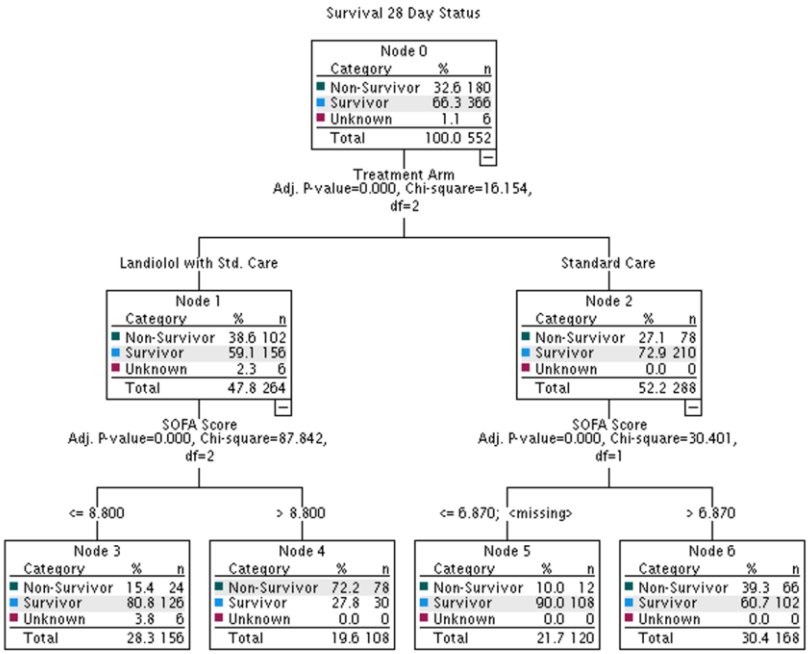


Figure 20: ***Relationship Tree Exploring Survival Status by Treatment Allocation and SOFA Score***

As sepsis progresses into septic shock, it is common for patients to experience a degree of organ dysfunction, either from a single organ system or multiple organ systems (MODS). A measurable indicator of renal function is creatinine levels (Figure 21).

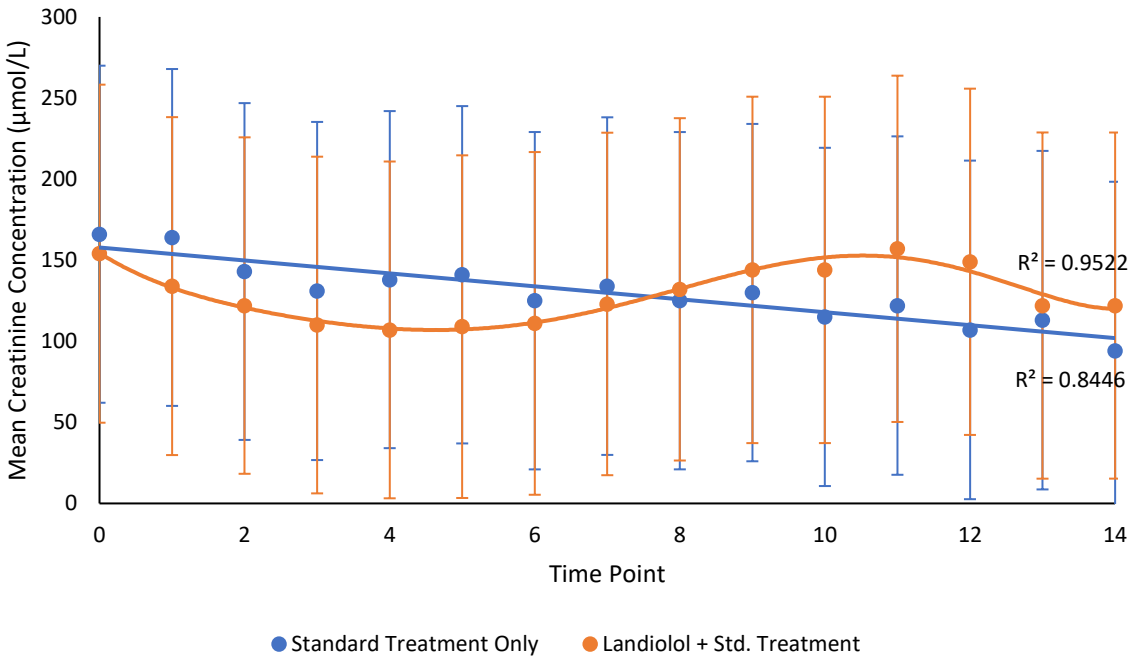


Figure 21: ***Mean Concentration of Creatinine by Trial Allocation.*** *Sampling occurred longtindinally over 14-day period.*

The mean concentration of creatinine was measured over 14-days, separated by treatment allocation. The data suggests slight differences in kinetics as the Landiolol treatment arm was best fitted with a polynomial trendline, compared to a linear fitting with standard treatment only. Both trendlines have good co-efficient of determination value (R^2=0.9522 and R^2=0.8446, respectfully). In the standard treatment only, there is a clear linear trend of decreasing creatinine over time, whilst a more pronounced decrease is noted in the Landiolol group initially.

An important consideration for STRESS-L is how haemodynamically compliant or stable the patients are when administered the β-blocker compared to standard treatment only. When administered Landiolol, physiologically there is an expectation to decrease heart rate, stabilising mean arterial pressure, and subsequently show a decreased requirement for noradrenaline therapy over those receiving standard septic shock care only. Figure 22 summarises the haemodynamic changes of several key parameters (e.g. MAP and HR), observed across the treatment allocations.

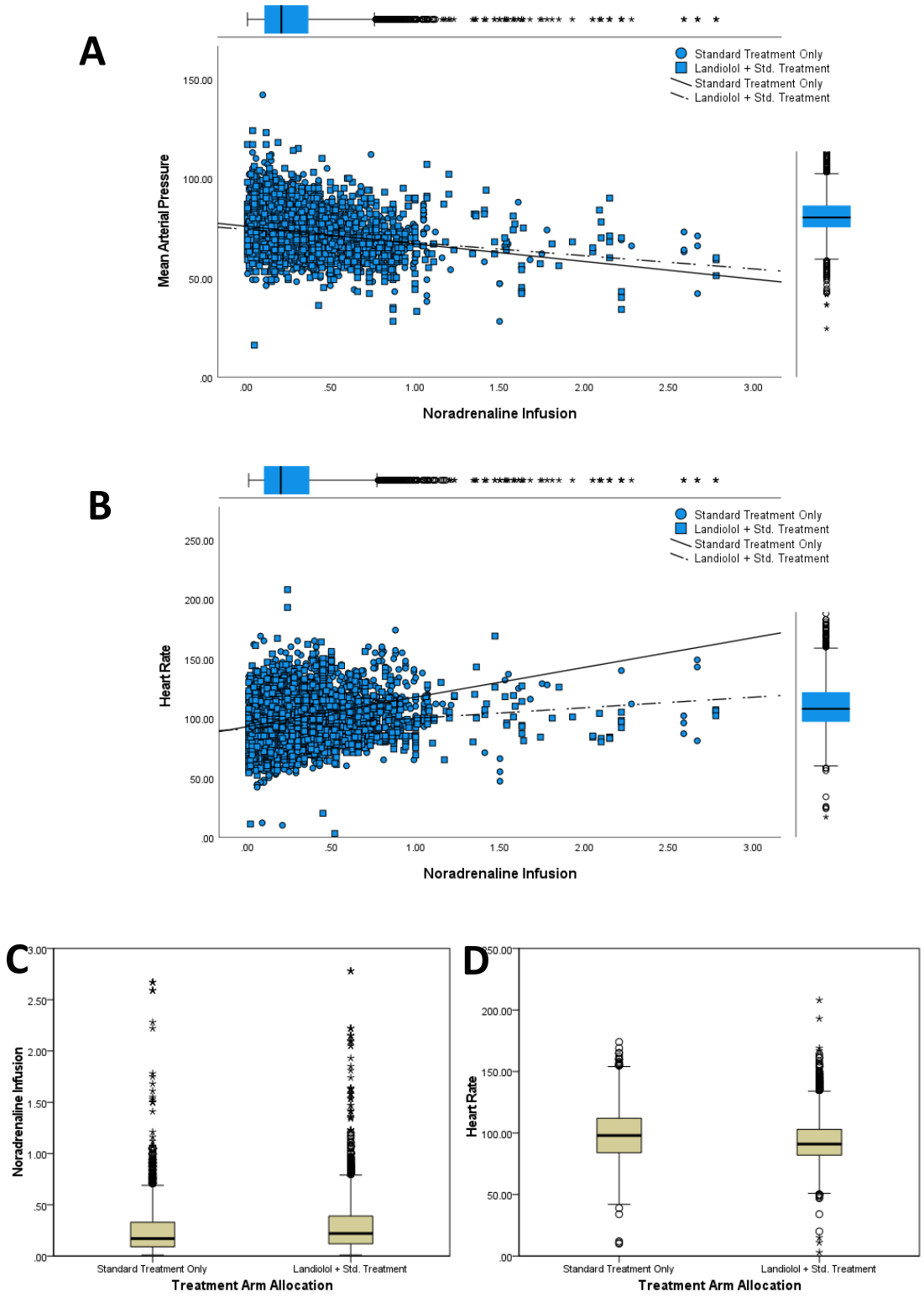


*Figure 22: **Haemodynamic Status of Participants**; A) regression variable plot for arterial pressures by noradrenaline infusion (mcg/kg/min), B) regression variable plot for heart rate by catecholamine infusion dosage (mcg/kg/min), C) noradrenaline requirement by treatment arm and D) heart rate by treatment arm. C and D plot median ± 95% CI.*

Non-parametric analysis and a Mann-Whitney U Test was conducted on heart rate (p<0.001), mean arterial pressure (p<0.001) and noradrenaline infusion rate (p<0.001), demonstrating key haemodynamic variables differed by treatment allocation. Data show that patients allocated to receive Landiolol had a lower mean HR compared to standard treatment alone. However, the standard treatment group required on average 0.05 mcg/kg/min less noradrenaline infusion compared to those administrated Landiolol, taken together with standard treatment group having on average approximately 1 mmHg higher MAP than Landiolol. However, the difference of 1 mmHg is clinically insignificant. The regression variable plot constructed in Figure 22A illustrates at dosage extremes of noradrenaline in both treatment groups MAP continues to decline despite titration of catecholamine, highlighting an almost un-rescuable and moribund state in these patients. Figure 22B shows the relationship between HR and noradrenaline infusion; the trend for standard treatment only illustrates as greater infusion of noradrenaline occurs, an increase in heart rate is observed, causing a reduction in meaningful cardiac output. Whilst this increase is also established in the Landiolol group, it is less pronounced, suggesting some degree of cardiac output protection.

With atrial fibrillation (AFib) usually occurring in around 30% ICU-admitted septic shock patients, incidences of AFib were reported during the course of STRESS-L, with onset either already established at baseline (Day 0) or new incidences through the study time course. Confirmed AFib was present in 21 standard care patients and 17 on Landiolol. Within the standard care group, 57% of these AFib presentations were established at baseline, whilst 47% were noted at baseline for Landiolol. Incidences of AFib occurred more frequently in males (68.4%) than females (31.6%) (p=0.001). The exact mechanism by which patients go on to present with atrial fibrillation is still unclear. Literature suggests possible energy and

mitochondrial dysfunctions and the involvement of inflammasome signalling. To provide some further insight into possible involvement, the immune system and metabolic pathways were analysed. Metabolomic analysis was conducted on 419 plasma samples from 100 patients collected longitudinally over days 0, 1, 2, 4, 6 and when the patient stopped the need for noradrenaline (EONT).

3.2: Metabolomics Analysis

NOTE: Metabolite annotation and PCA/PLS-DA plots were generated in collaboration with Professor Luis A. J. Mur of Aberystwyth University. Further metabolomic analysis in the appendix is my non-collaborative work.

Metabolic derangement is an established dysfunction observed in septic shock, with varying degrees of dysregulation seen across patients, a barrier to effective standardisation of treatment. Basic changes in glycolytic pathways, to meet the increased demand by immune cells to produce a sufficient immune response, together with dysregulation in lipids, fatty acids, amino acids, oxidative phosphorylation, and tri-critic acid cycle can all occur in sepsis. The primary outcome of STRESS-L is the improvement of SOFA score in patients administered Landiolol compared to standard care. However, the clinical data (section 3.1) did not show improvements in the interventional arm, contradicting previous reports (Morelli *et al.,* 2013). To establish possible explanations for this finding, both unsupervised and supervised analysis of mass spectrometry-based metabolomics was conducted on 100 patients (419 samples). Firstly, data was separated by treatment allocation using principal component analysis for both negative and positive ionisations (Figure 23a and 23b, respectively).

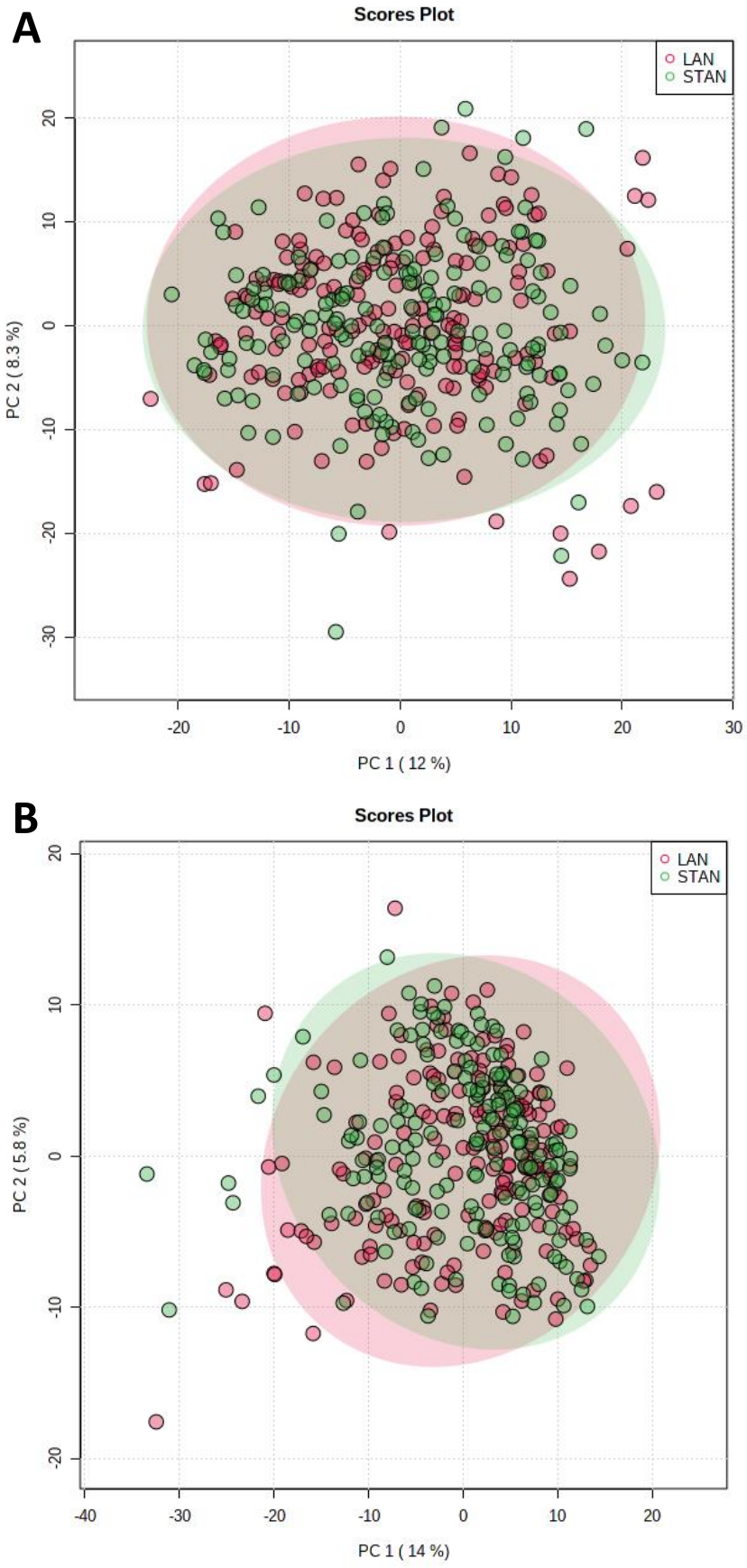


Figure 23: ***Principal Component Analysis for Separation of Patients Assigned by Treatment Allocation****. Plot A uses negative ionisation mode and Plot B using Positive Ionisation Model. LAN = Landiolol; STAN: Standard Care.*

Both ionisation modes showed no meaningful separation by treatment arm using unsupervised PCA. Analysis was repeated using supervised partial-least squares linear discriminant analysis (PLS-DA) for both ionisations (24a and 24b).

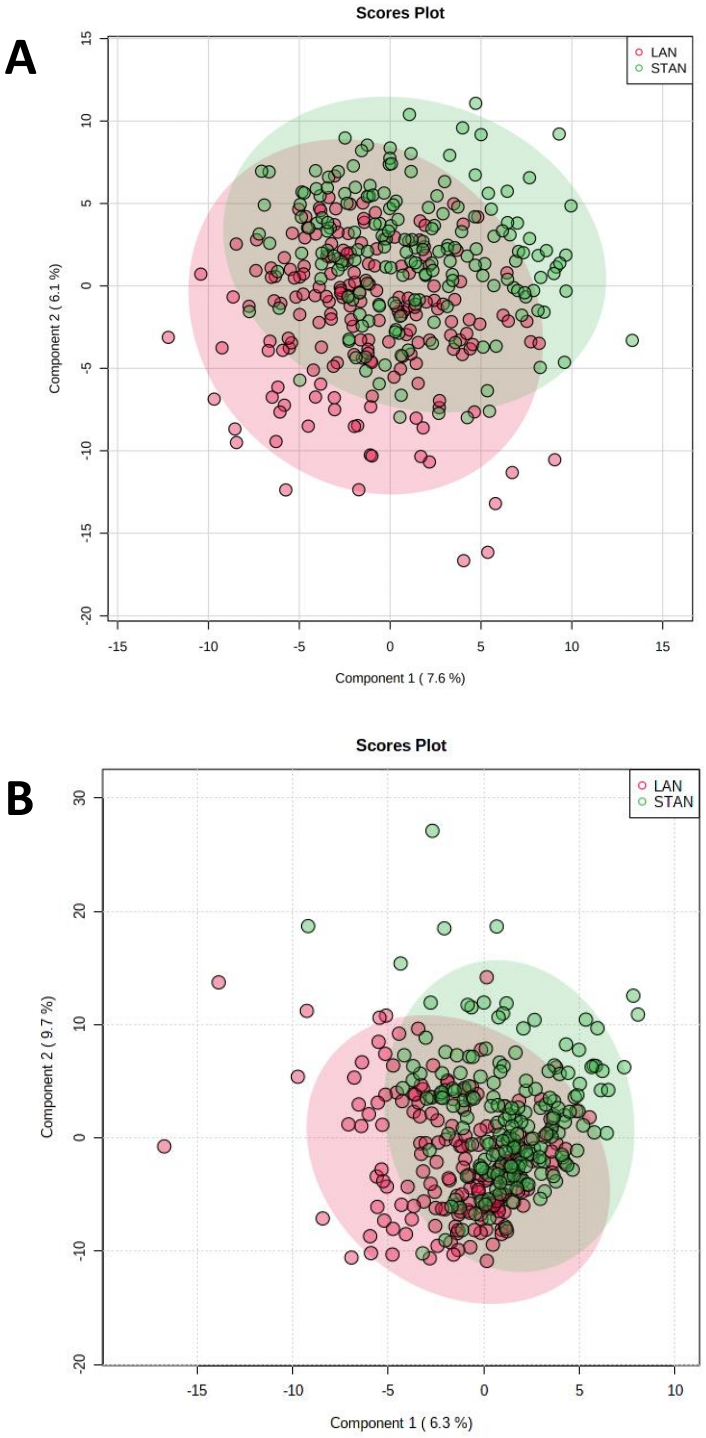


Figure 24: ***Partial Least Squares Linear Discriminant Analysis for Separation of Patients Assigned by Treatment Allocation.*** *Plot A uses negative ionisation mode and Plot B using Positive Ionisation Model. LAN = Landiolol; STAN: Standard Care.*

Some degree of separation was observed using the supervised method, although this was minimal. To establish potential treatment differences through sampling time points (days: 0, 1, 2, 4, 6 and End of NorAd), individual models were generated (Appendix 1). Whilst these models were constructed, it is important to recognise these are possibly overfitted. The models constructed include PCA, PLS-DA and OPLS-DA, the latter certainly an overfitted model. Both supervised and unsupervised analytics were also applied to survival status. As positive ionisation showed no separation (data not shown), only the negative ionisation mode are shown from now on. The PCA and PLS-DA plot showed modest separations between alive and deceased patients (Figure 25a and 25b).

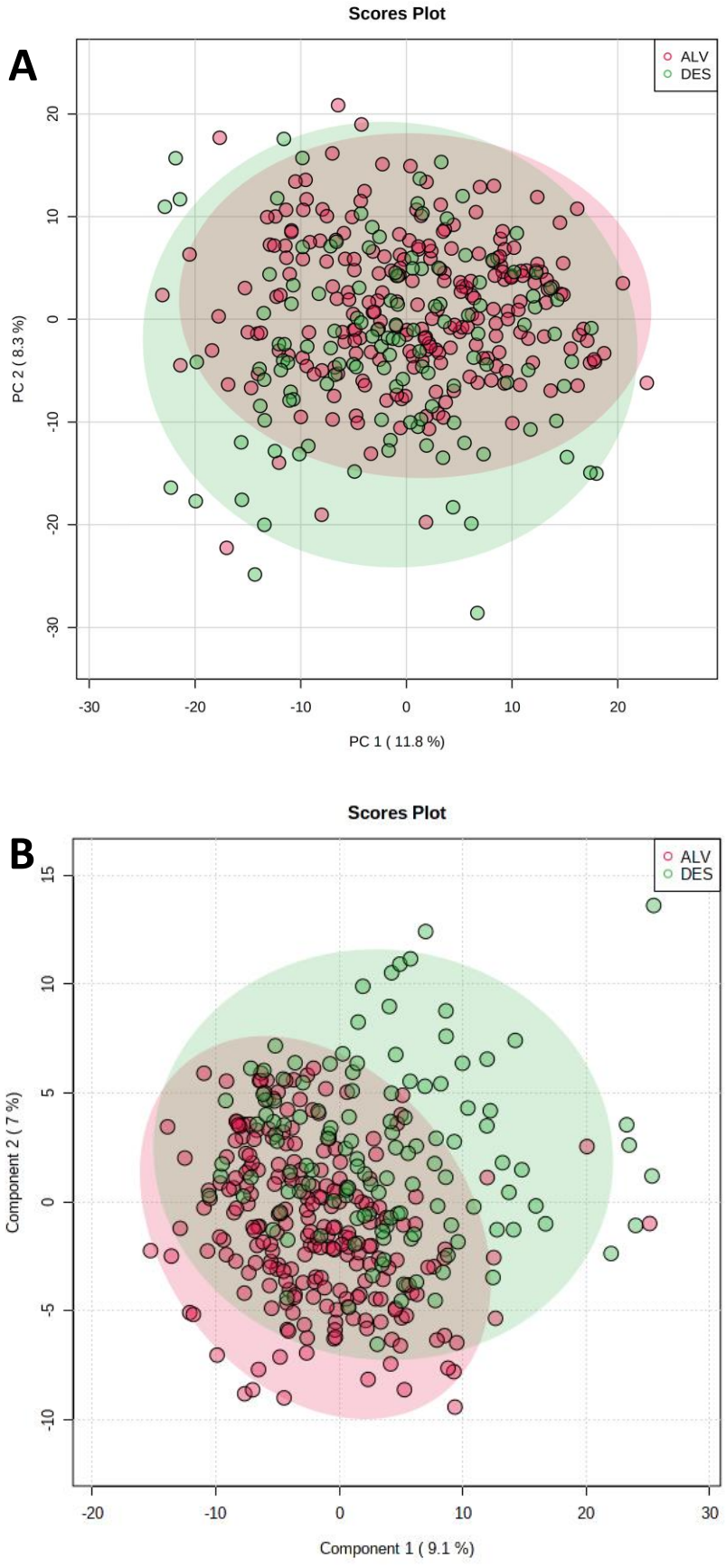


Figure 25: ***Principal Component Analysis (A) and Partial Least Squares (B) for Separation of Patients by 28-day Mortality Status****. Both plots use negative ionisation mode. ALV = Alive; DES: Decreased*

As there is a degree of separation by 28-day survival, whilst more apparent in supervised modelling (PLS-DA) than unsupervised, 28-day survival has also been separated by sampling timepoints in Appendix 1. Using the methods described previously (PCA, PLS-DA and OPLS-DA) there are similar model overfitting concerns.

As SOFA scores are associated with increased mortality, models were constructed for SOFA score against combined treatment allocations. Research by University Hospitals Birmingham NHS Foundation Trust (UHB) Critical Care Services (data not published) showed patients with a SOFA score ≥10 are associated with significantly higher mortality rates and therefore considered as 'severe', those with SOFA scores between 1-4 have been assigned as 'mild' and 5-9 as 'moderate'. These groupings were then used in the models here. Separation by PCA for mild, moderate, and severe sepsis is shown in Figure 26A, and separations by PLS-DA in Figure 26B.

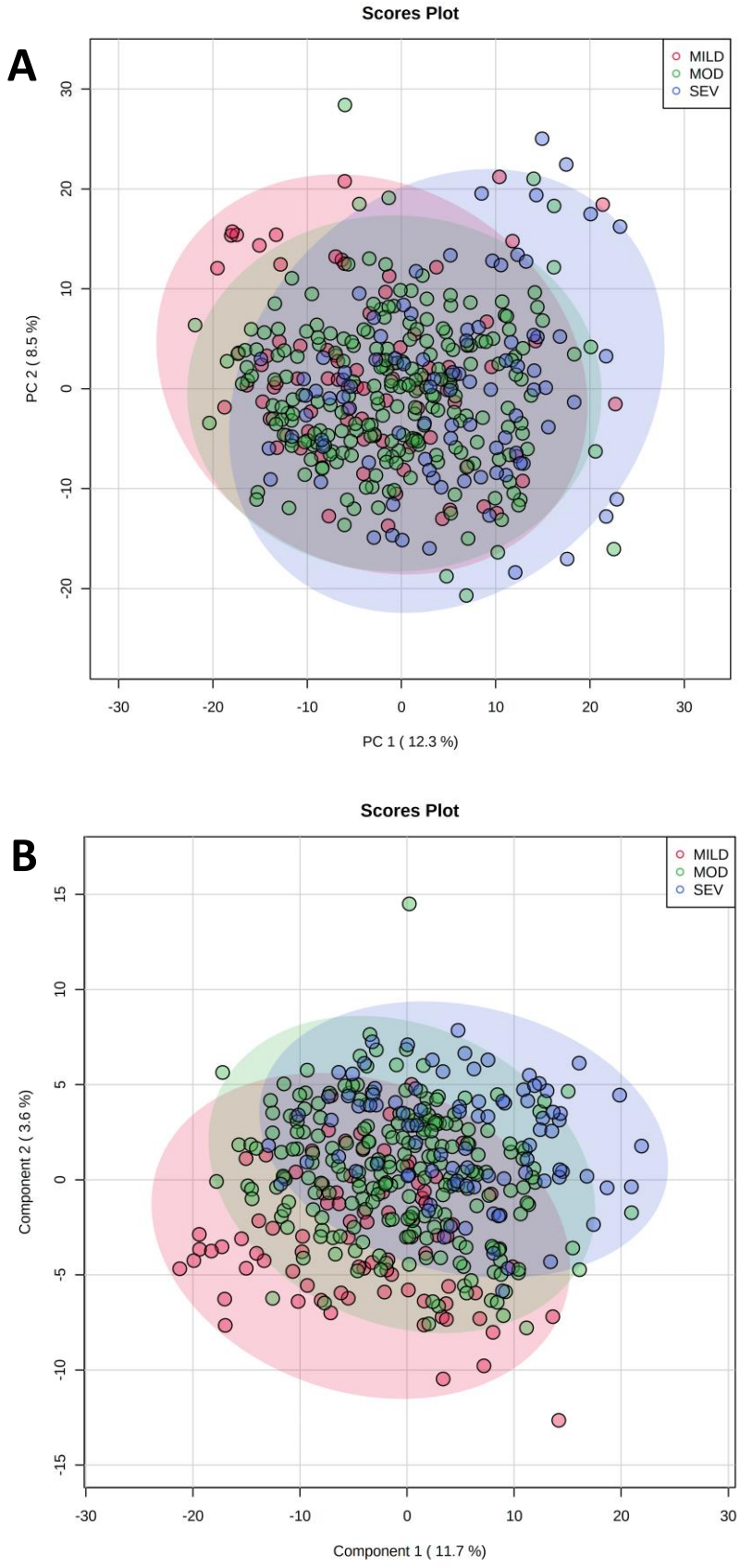


Figure 26: ***Principal Component Analysis (A) and Partial Least Squares (B) of SOFA Score****. Both plots use negative ionisation mode.*

In PLS-DA models there was some degree of separation between mild and the moderate and severe sepsis groups. SOFA score categories separated further for mortality were analysed by PCA (Figure 27) and showed separation in the mild group for survivors and non-survivors.

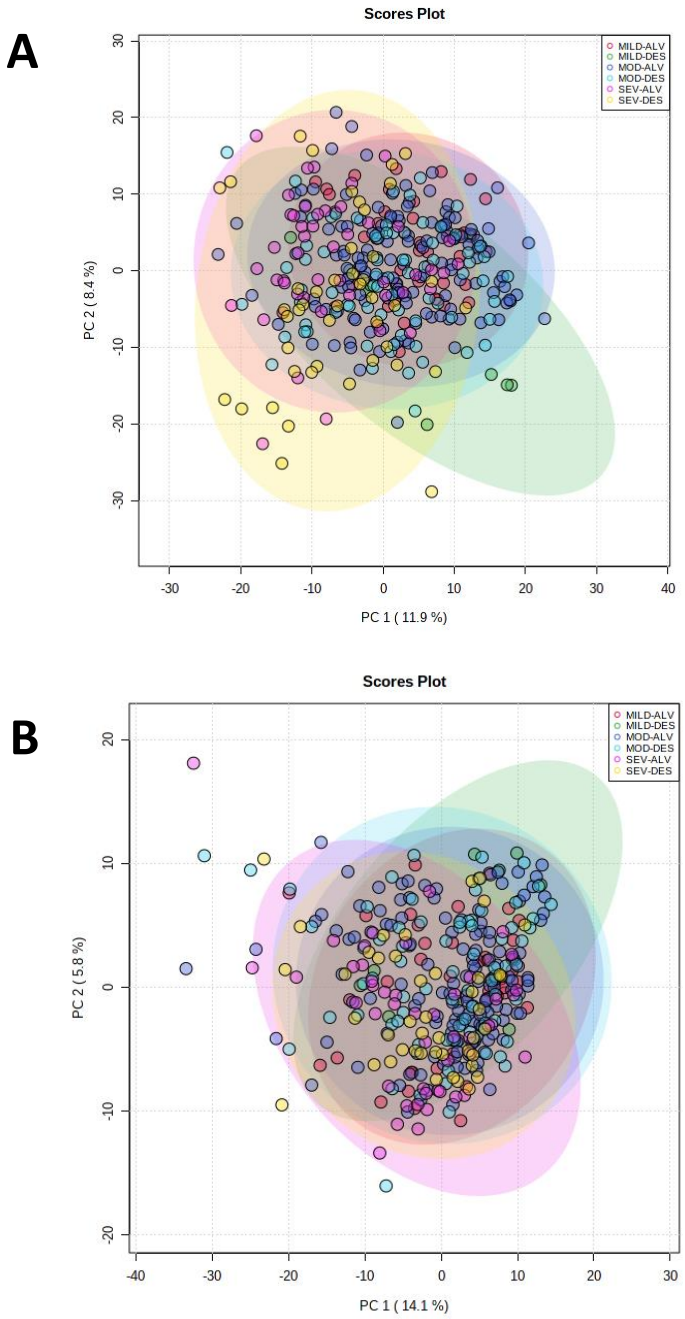


Figure 27: ***Principal Component Analysis of SOFA Score Classification with 28-Day Survival Status****. Plot A uses negative ionisation mode and Plot B using Positive Ionisation Model*

To determine the extent of separation, using the negative ionisation mode, PCA and PLS-DA plots were generated (Figure 28 and 29, respectively).

Figure 28: ***Principal Component Analysis of Mild SOFA Score Classification with 28-Day Survival Status****. Plot generated using negative ionisation*

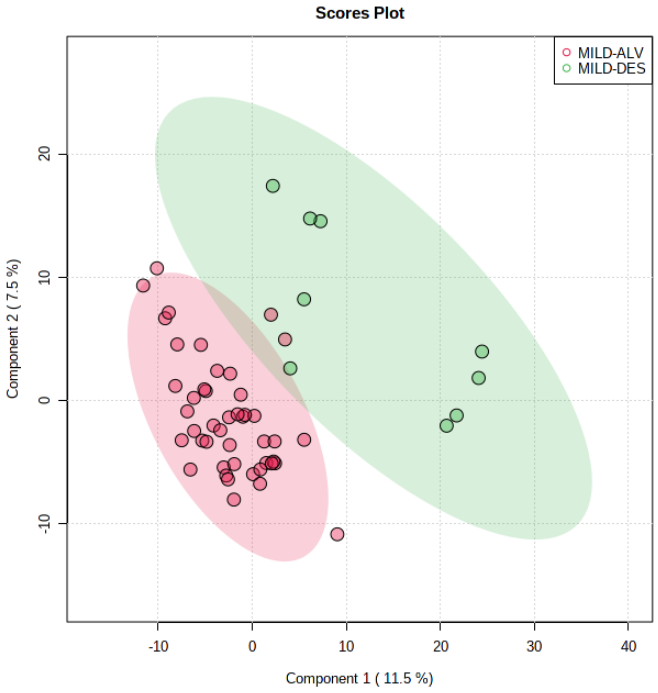


Figure 29: ***PLS-DA of Mild SOFA Score Classification with 28-Day Survival Status.*** *Generated by negative ionisation mode*

There are clear separations by PLS-DA for ‘mild’ SOFA score with 28-day survival status. To confirm a good statistical fitting and an accurate model, component and performance evaluation was conducted. Results indicate a good model. The appropriateness of model fitting (R^2, referred to variations) was high, whilst the predictive ability of the model (Q^2) provides clarity of appropriateness of prediction ability. the model had a Q^2 >0.4.

As separations were observed, a dendrogram-heatmap was generated based on the PLS-DA negative ionisation data (Figure 30).

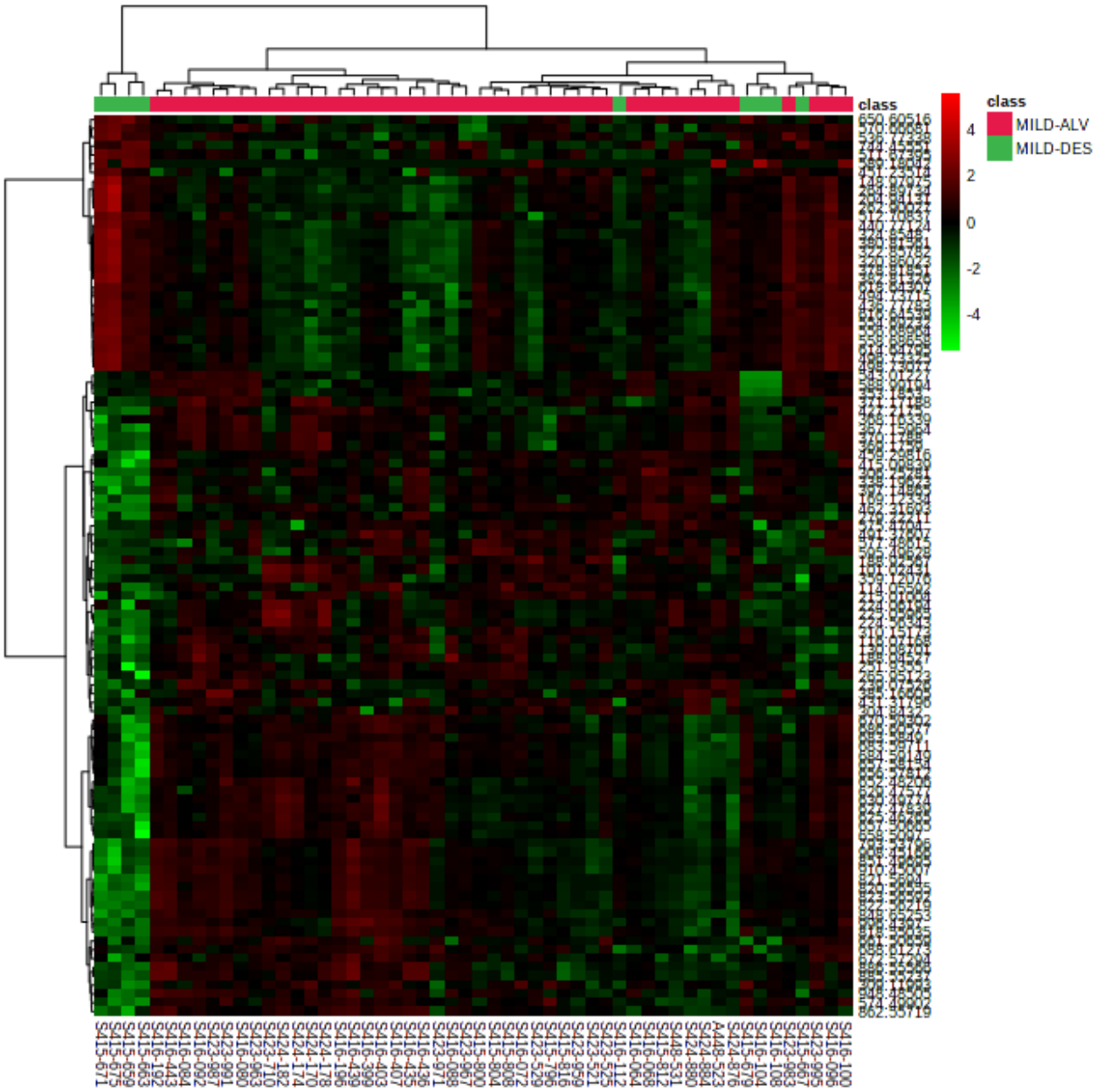


Figure 30: ***Heatmap-Dendrogram of Survivor and Non-Survivor Separations*** *(Negative Ionisation) for mild SOFA score with alive and deceased status.*

As clear variances were observed, putative annotation (by mummichog algorithm) to determine which metabolites were different was carried out to provide insight into possible reasons for unexpected results observed in the trial. The established benefit of using the mummichog algorithm for metabolite identification is the added resilience to random errors in metabolite identification, as annotation occurs at the group level through the concept of

collective behaviour. Figure 31 shows an annotated dendrogram-heatmap for the mild SOFA score with survival outcome.

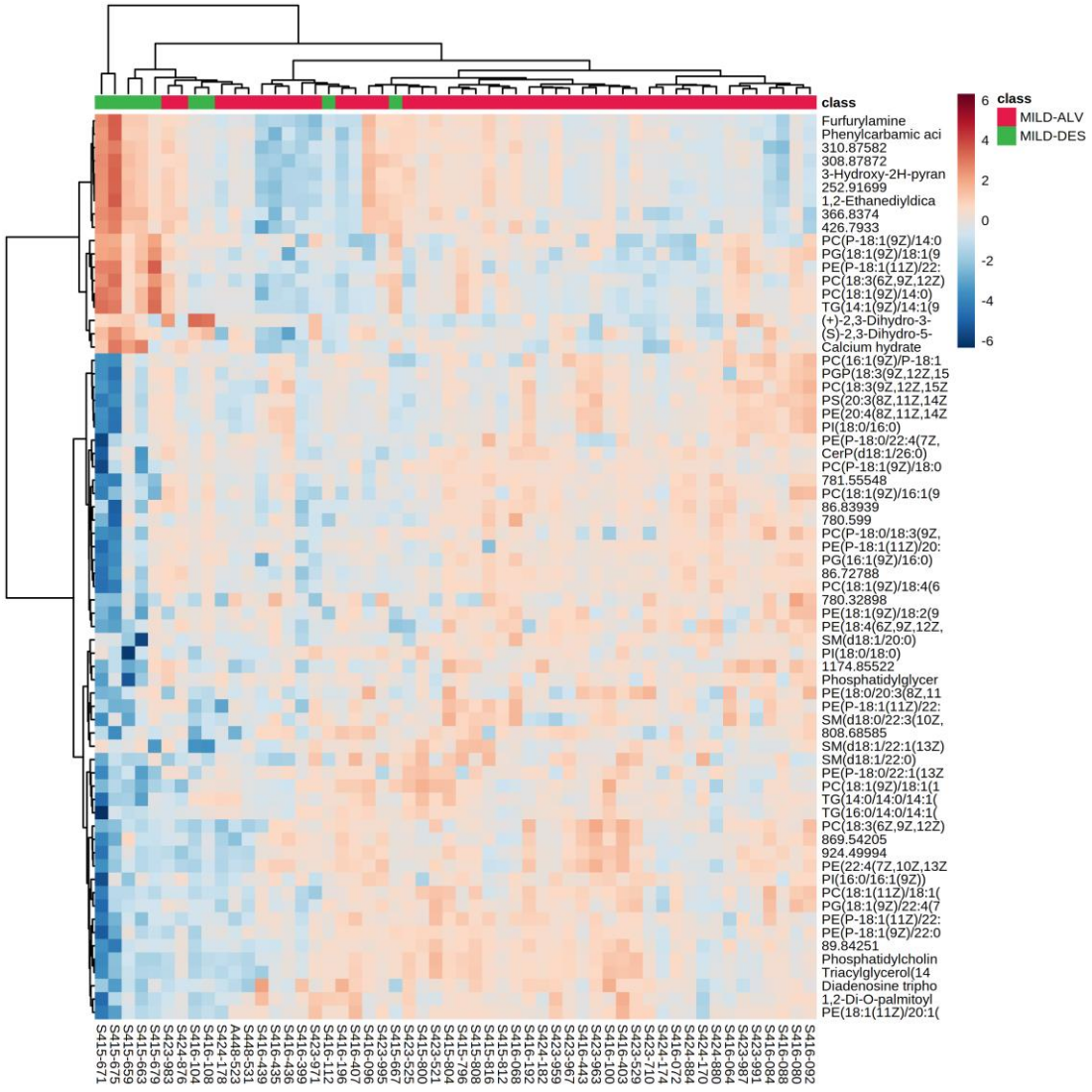


Figure 31: ***Annotated Dendro-heatmap for Mild SOFA Score (score points 1-4) and survival outcome. ALV = Alive, DES = deceased. Negative Ionisation Mode.***

The results indicate differences are predominantly observed in lipid metabolism. The full annotated metabolites and lipids reported in Figure 31 are displayed in Appendix 2. The significantly different metabolites and lipids that were used in AUC/ROC analysis, include:

PE(18:1(11Z)/20:1(11Z));

PC(P-18:1(9Z)/18:0);

PE(P-18:1(11Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z));

Phosphatidylcholine(18:3(9Z,12Z,15Z)/20:2(11Z,14Z));

Triacylglycerol(14:0/18:3(9Z,12Z,15Z)/14:1(9Z));

PI(16:0/16:1(9Z));

Furfurylamine;

Phosphatidylglycerol(16:1(9Z)/18:3(9Z,12Z,15Z));

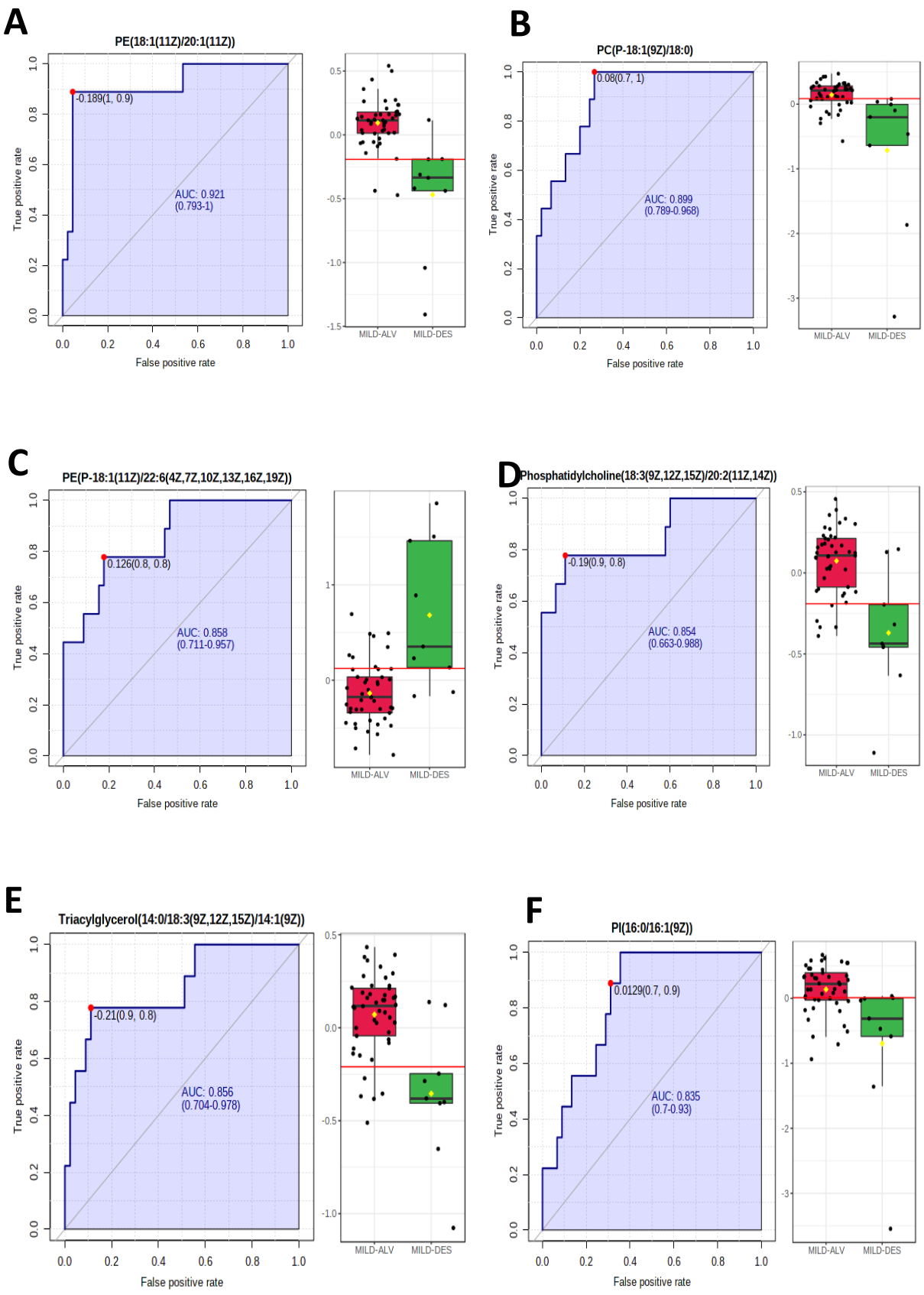
Phenylcarbamic acid;

3-Hydroxy-2H-pyran-2-one;

1,2-Ethanediyldicarbamodithioic Acid;

(+)-2,3-Dihydro-3-methyl-1H-pyrrole;

For these identified metabolites, AUC/ROC and difference plots, are shown in Figure 32 A-L.


A
PE(18:1(11Z)/20:1(11Z))
-0.189(1, 0.9)
AUC: 0.921
(0.793-1)
True positive rate
False positive rate
MILD-ALV
MILD-DES
B
PC(P-18:1(9Z)/18:0)
0.08(0.7, 1)
AUC: 0.899
(0.789-0.968)
C
PE(P-18:1(11Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))
0.126(0.8, 0.8)
AUC: 0.858
(0.711-0.957)
D
Phosphatidylcholine(18:3(9Z,12Z,15Z)/20:2(11Z,14Z))
-0.19(0.9, 0.8)
AUC: 0.854
(0.663-0.988)
E
Triacylglycerol(14:0/18:3(9Z,12Z,15Z)/14:1(9Z))
-0.21(0.9, 0.8)
AUC: 0.856
(0.704-0.978)
F
PI(16:0/16:1(9Z))
0.0129(0.7, 0.9)
AUC: 0.835
(0.7-0.93)

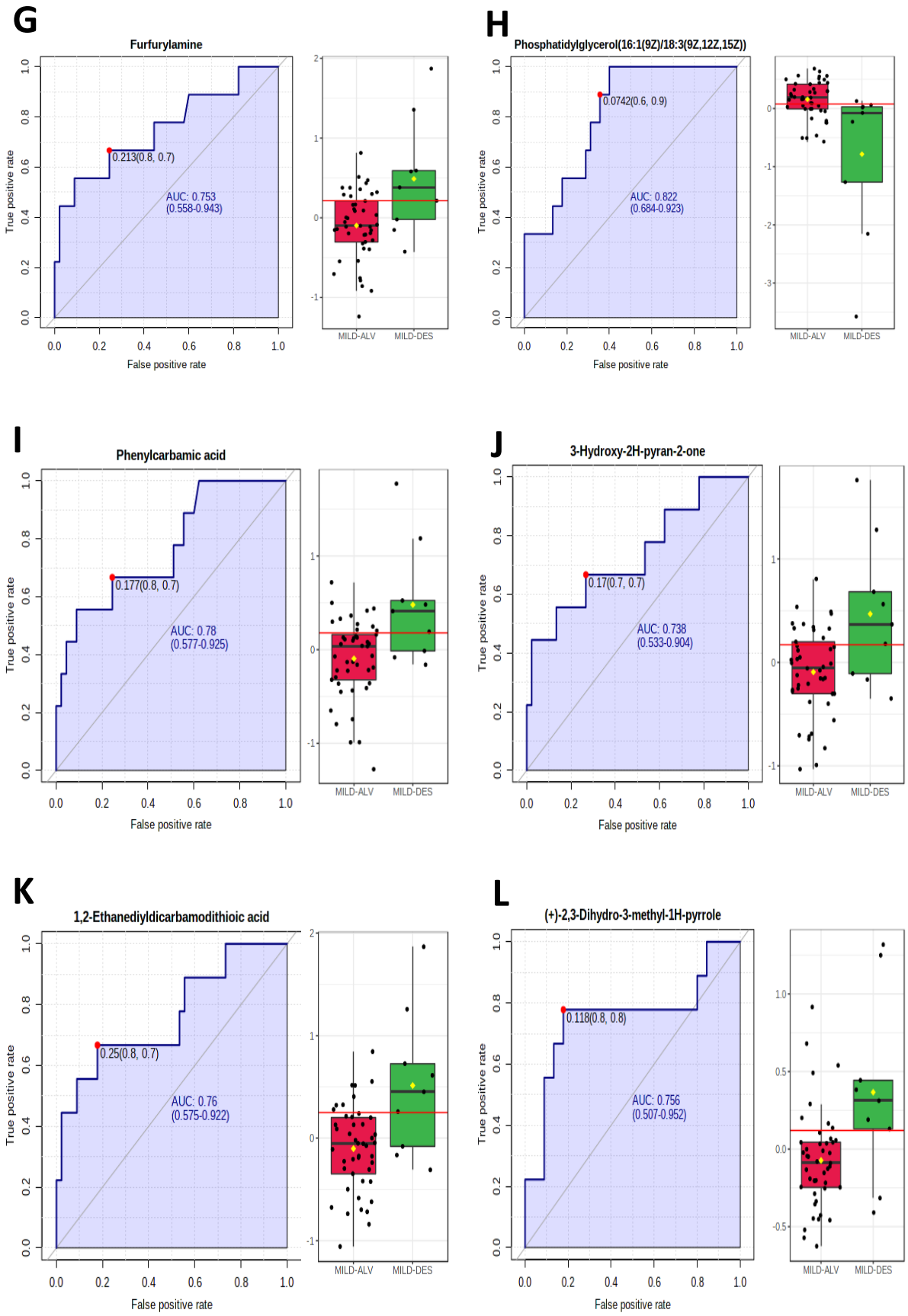


Figure 32: ***Significant Metabolites Identified in Heatmap with AUC/ROC plot and Box Plot – Analytes A to L named on Plot.***

Using the annotated metabolites/lipids, enrichment and pathway analysis was conducted, with Holm-Bonferroni method applied to account for multiple tests. Analysis indicates significant pathways for the Mild-(x)Survivor includes: glycerophospholipid metabolism, linoleic acid metabolism, α-Linolenic acid metabolism, glycosylphosphatidylinositol (GPI)-anchor biosynthesis, sphingolipid metabolism, arachidonic acid metabolism and purine metabolism. Although the total number of hits per pathway were low, the maximum pathway receiving 2, with the maximum impact recorded of 0.20 for the glycerophospholipid metabolism.

Analysis of SOFA score with mortality yielded for the moderate group little significance and poor separation (data not shown). For the severe group PCA, PLS-DA plots and a heatmap for positive ionisation are shown in Figure 33 A-C. Of note, the model performance was poor with unsatisfactory performance scoring for accuracy, R^2 and lower Q^2 values. Annotation of the 5 metabolites: *m/z*=780.17004, *m/z*=148.00385, *m/z*=169.98569, *m/z*=58.06542 and *m/z*=154.08368 subsequently did not occur.

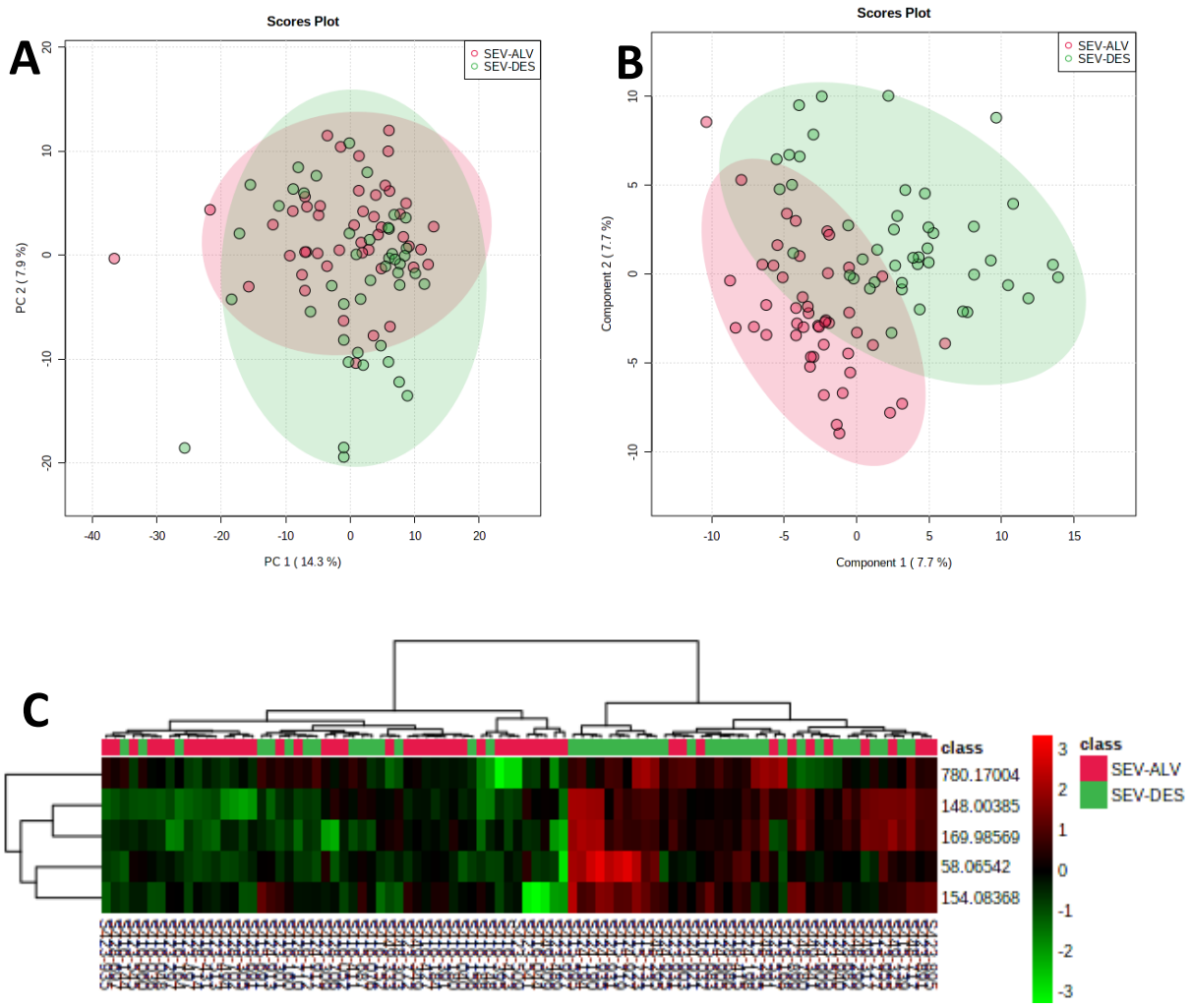


Figure 33: ***Analysis of (A) PCA, (B) PLS-DA and (C) Heatmaps for the Positive Ionisation for the most Severe SOFA group associated with mortality status.***

3.3: Cytokine Profile Assessment

With little clear separation observed in the metabolomic studies when explored by treatment, the changes in cytokine profiles between treatment groups and survival status were then examined. Any statistically significant cytokines were then analysed with SOFA score.

Separation by Treatment: Non-parametric tests, namely Mann-Whitney U Test, were applied to the whole trial population, followed by direct analysis of differences observed between survivors and non-survivors to establish if Landiolol had an effect. The separation by survival status is necessary due to the high mortality rate associated with this critical care population,

where loss of life may occur, irrespective of treatment. This is to prevent sampling bias. Table 3 compares the cytokines by treatment allocation.

Table 3: ***Non-Parametric Analysis Conducted for Separation of Treatment Allocation by Whole Population, Survivors Only and Non-Survivors. Statistically significant results are indicated with asterisk (*). Non-parametric test for general distribution of concentration over sampling points/longitudinally.***

Cytokine	Whole Population *p* – value	Survivors Only *p* – value	Non-Survivors Only *p* – value
IFN-γ	0.500	0.006*	0.847
IL-2	0.301	0.030*	0.667
IL-5	0.671	0.073	0.428
IL-8	0.755	0.308	0.791
IL-12	0.604	0.283	0.468
TNF-α	0.640	0.051	0.618
IL-1Ra	0.349	0.035*	0.441
IL-4	0.876	0.188	0.917
IL-6	0.603	0.473	0.655
IL-10	0.876	0.139	0.246
IL-17	0.969	0.099	0.040*

Statistical significance was not achieved for any cytokines across the whole population. Although sub-analysis between survivors and non-survivors showed 4 statistically significant results. In non-survivors, IL-17 was significantly elevated in Landiolol patients only, whereas, the concentration of IFN-γ and IL-2 were both greater in standard care patients compared to Landiolol. The opposite was true for IL-1Ra, a greater concentration was observed in Landiolol compared to standard care.

The raw data and percentage change from baseline plots for all patients are shown in Appendix 3 and 4, respectively. Reduced concentrations of IFNγ, TNFα and IL-2 were observed in Landiolol patients compared to standard treatment, suggesting a causal relationship may exist between Landiolol and the reduction of pro-inflammatory cytokines in trial survivors. Figure 34 shows %Δ relative to baseline for significant survivor cytokines.

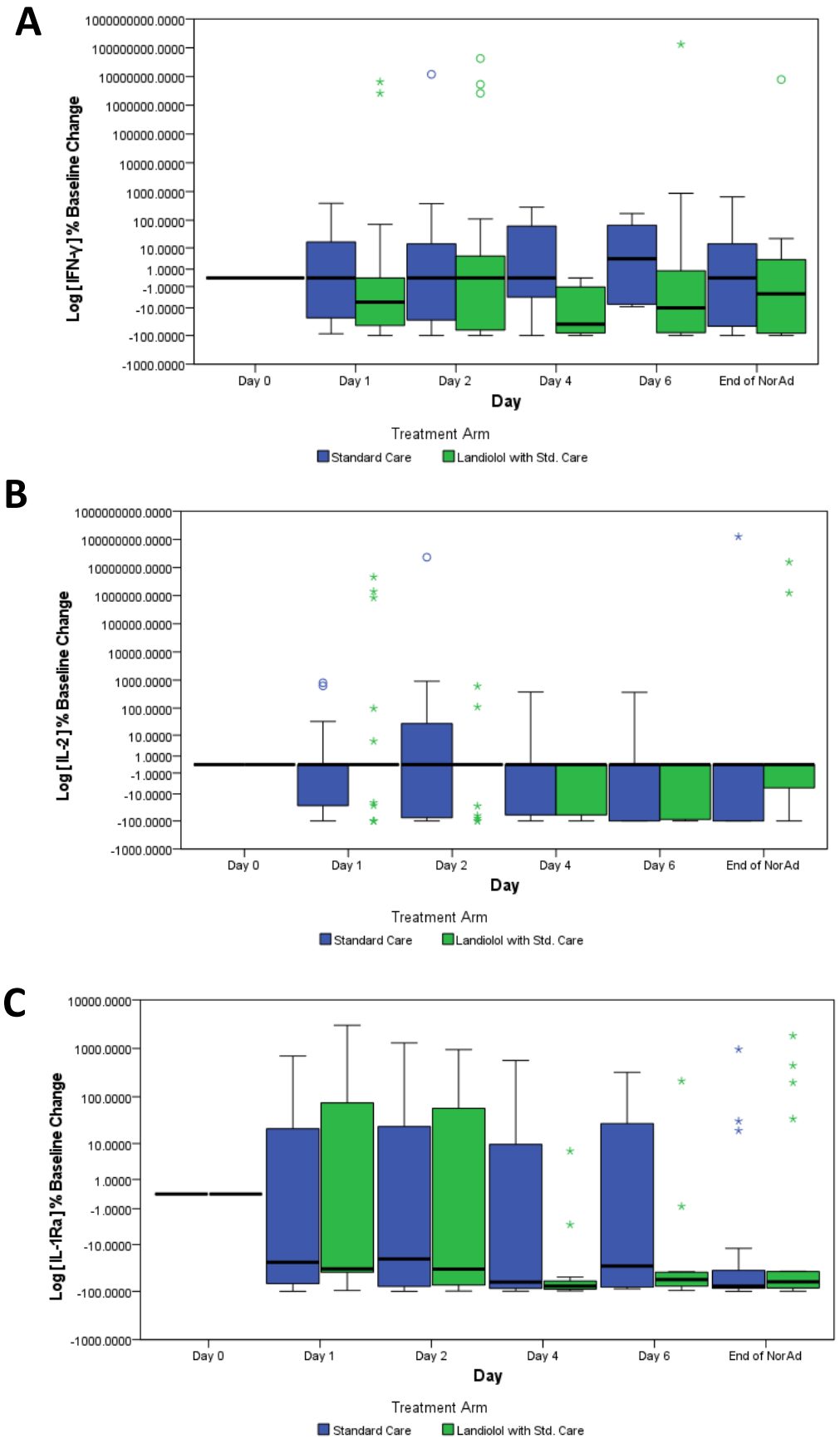


Figure 34: ***Percentage Baseline Change Relative to Day 0 in Trial Survivors Separated by Treatment Arm.*** *A) IFNγ, B) IL-2 and C) IL-1Ra. Data shown longitudinally from non-parametric distribution over sampling timepoints.*

Figure 34 demonstrates, particularly in the case of IFNγ, that relative to baseline patients administered Landiolol had a more pronounced decline compared to standard care only. Equally, IL-1Ra shows a similar relationship particularly with increasing timepoint. Whilst less pronounced in IL-2, the plot illustrates the range is reduced in Landiolol, and unlike that observed in control patients Landiolol patients did not show percentage increases longitudinally. The data for IL-17 is shown in Figure 35 showing %Δ to baseline by treatment allocation. These data illustrate how dynamic %Δ to baseline over time occurs in non-survivors, a classic hallmark of septic shock, dominated by hyperdynamic and dysregulated changes. In most instances, IL-17 is more elevated in Landiolol non-survivors, with a greater range observed immediately by day 1.

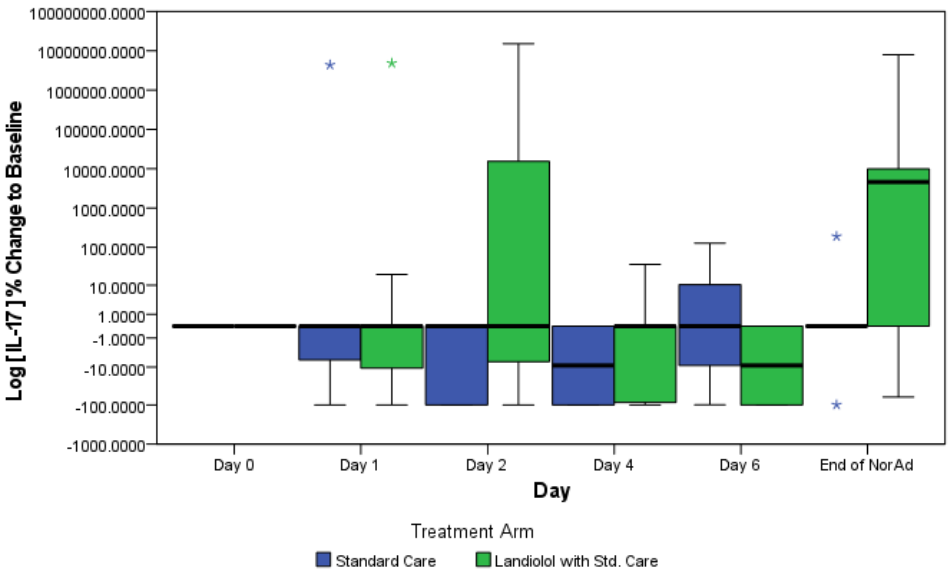


Figure 35: ***Percentage Change Relative to Baseline for IL-17 as seen in Trial Non-Survivors, Separated by Treatment***

Overall, results suggest Landiolol has a degree of influence on cytokine concentrations when compared to standard care alone.

Survival Status: Using the same 11 cytokines run in the previous analysis, non-parametric testing was further applied to identify differences in survival independent of treatment allocation, and then individually by Landiolol patients and patients continuing with standard care only (Table 4).

Table 4: ***Non-Parametric Analysis of Cytokine Concentrations by Survival Status. Analyses run by combined treatment type, Landiolol only and Standard Care only.*** *Asterisk (*) indicates significant cytokines*

Cytokine	Combined *p* – value	Landiolol *p* – value	Standard Care *p* – value
IFN-γ	0.205	0.023*	0.809
IL-2	0.017*	0.006*	0.642
IL-5	0.183	0.022*	0.922
IL-8	<0.001*	<0.001*	<0.001*
IL-12	0.193	0.186	0.169
TNF-α	0.205	0.036*	0.147
IL-1Ra	0.078	0.050*	0.023*
IL-4	0.001*	0.003*	0.025*
IL-6	<0.001*	<0.001*	<0.001*
IL-10	<0.001*	<0.001*	0.016*
IL-17	0.873	0.110	0.070

Analysis reported 5 global (combined treatment) statistically significant cytokines between survivors and non-survivors, compared to 9 out of the 11 for Landiolol, and 5 for standard care. Whilst these data provide insight into the differences between survivors and non-survivors who were administered Landiolol, it is important to recognise this insight comes with a sampling bias, particularly the inability to sample deceased patients. Subsequently, non-survivors could be subject to more extreme data fluctuations not necessarily experienced by survivors. Results consequently are interpreted with caution.

In-depth analysis of cytokine data suggests Landiolol-administered non-survivors were generally more hyper-inflamed than survivors, with lower percentage reductions in cytokines IFNγ, IL-4, IL-6 and IL-8 compared to baseline. Interestingly in the earlier time points, non-survivors showed a reduced %Δ of anti-inflammatory IL-1Ra. Intriguingly however, IL-10

reduced sharply on infusion of Landiolol, and continued reducing over time in survivors. This is on the contrary to non-survivors which saw pronounced percentage increases over days 1 and 2 before a reduction is eventually seen by day 6. Although non-survivor patients sampling by day 6 were reduced. Some cytokines percentage change relative to baseline for Landiolol survivors and non-survivors are presented in Figure 36.

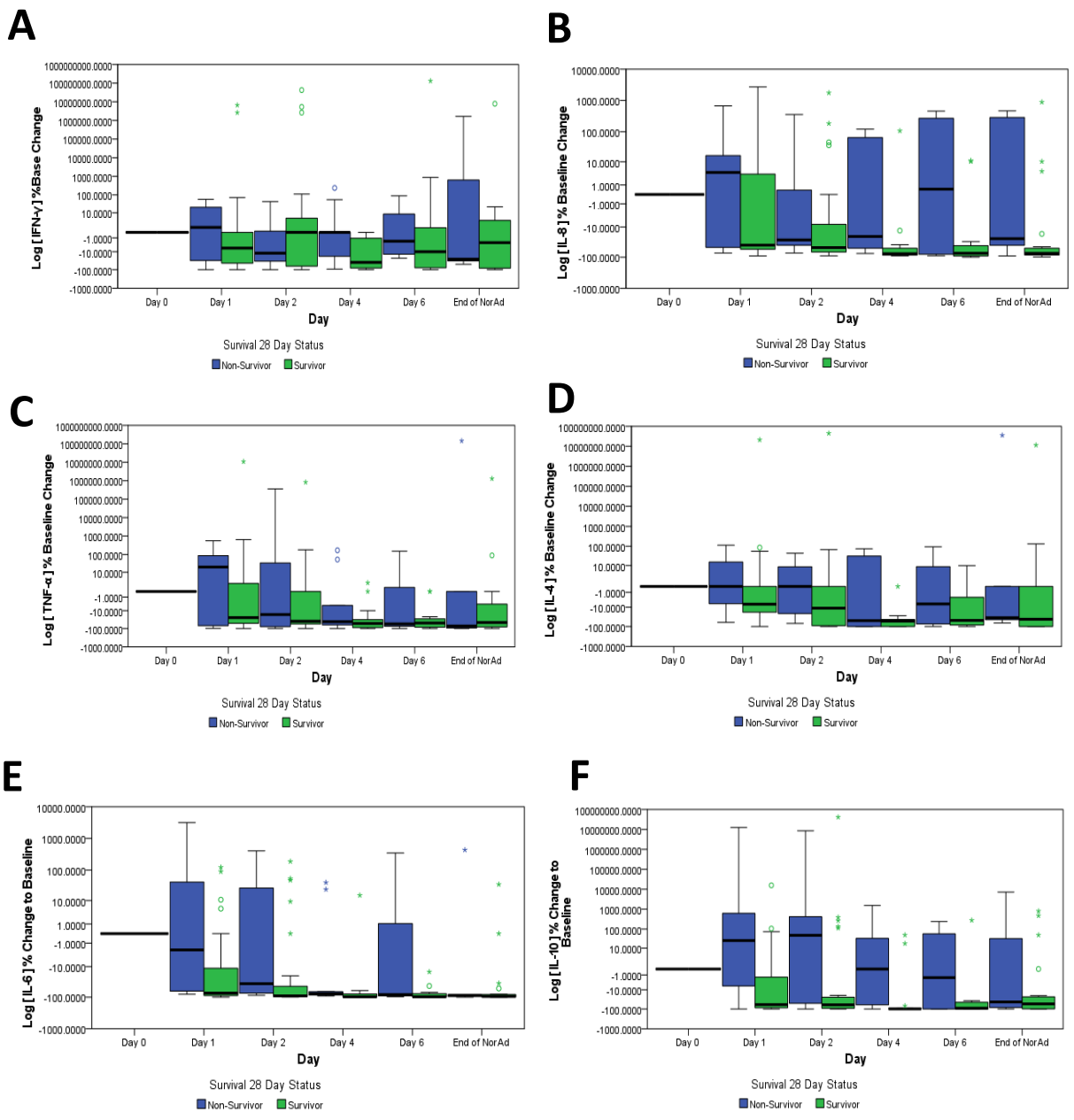


Figure 36: ***Survivor and Non-Survivor for Landiolol Recipients. Plots Representing Percentage Change Relative to Baseline Over Time.*** *A) IFNγ, B) IL-8, C) TNFα, D) IL-4, E) IL-6 and F) IL-10*

Analysis of the cytokines showed the percentage change of IL-6 concentration is less initially in non-survivors compared to survivors in Landiolol, with non-survivors displaying a large range of %Δ to baseline. In survivors, a decline was observed immediately at day 1 and remained lower than at baseline. TNFα had a pronounced percentage decline relative to day 0 sampling point and overtime this remained decreased in survivors, whilst in non-survivors a decline to the same degree was not initially seen and a larger range was observed. Evaluation of IL-10 showed % increases in concentration relative to baseline for days 0-4 in non-survivors which suggests Landiolol non-survivors may be more immunosuppressed when taken together with no cytokine reduction in Th_2-mediated IL-4. Increasing concentrations of IL-10 in non-survivors were not observed in Landiolol survivors. For both IL-10 and IL-4, concentration reductions of these were seen immediately from day 1. Most pronounced survivor reductions were seen in IL-10.

Analogous analysis was conducted for cytokines in the standard arm (Figure 37). Some similarities were identified between standard care only and those administered Landiolol. These cytokines in particular may provide the greatest insight. Cytokine differences in standard care saw IL-4, IL-6, IL-8, IL-10 and IL-1Ra all generally had higher concentrations in non-survivors, although Figure 37 demonstrates such great variations in the survivors group that the non-surviving group changes appear not as pronounced as observed in the Landiolol patients.

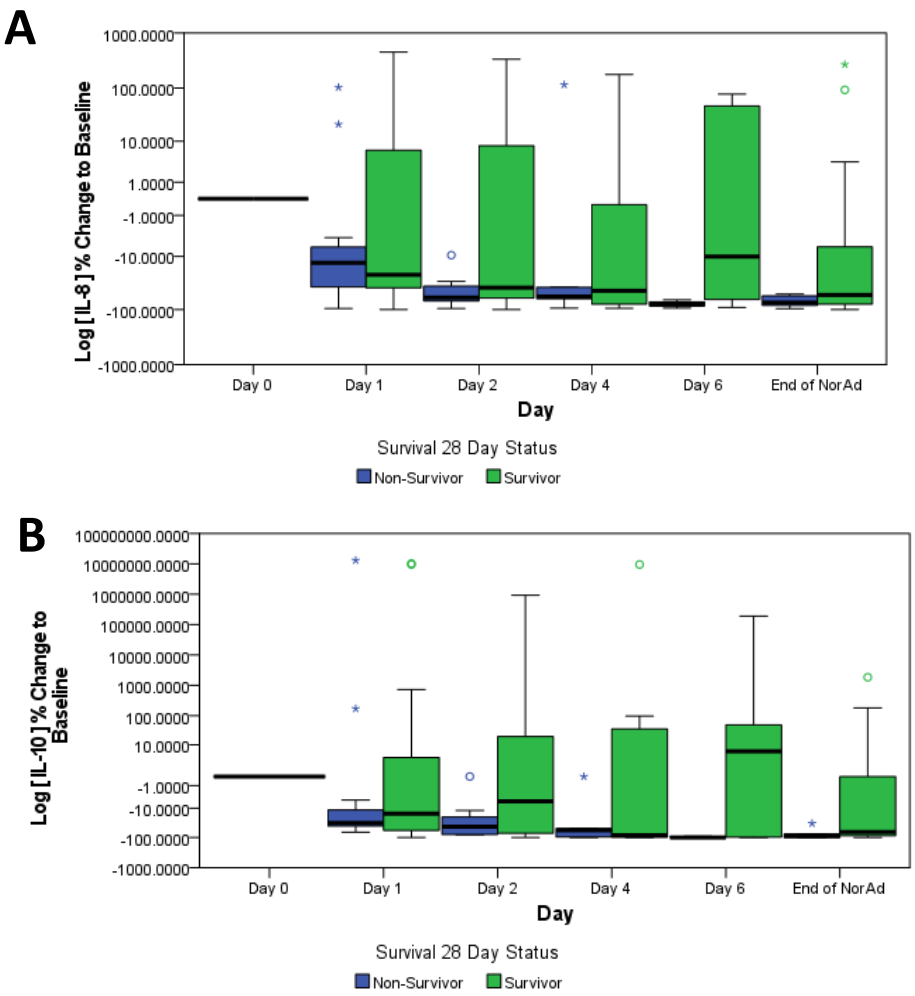
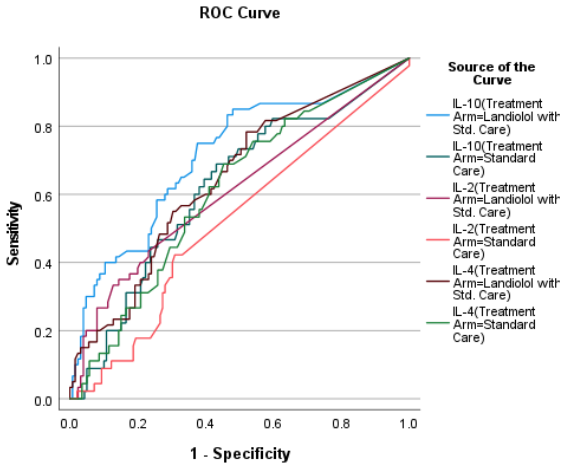


Figure 37: ***Survivor and Non-Survivor for Standard Care Recipients. Plots Representing Percentage Change Relative to Baseline Over Time.*** *A) IL-8 and B) IL-10*

Although, Figure 37 presenting IL-8 and IL-10 show interestingly other than Day 1, IL-8 shows gradual (though small) % median increase in survivors compared to non-survivors. In all cases the survivor % concentration change range is substantially greater. By day 6, both the range and median of IL-8 is notably less decreased relative to baseline in standard care survivors compared to non-survivors. Particularly interesting, IL-10 shows generally increasing concentration in survivors, despite the initial reduction at day 1, with box plot data range always greater than baseline which is not observed in non-survivors. With greater concentrations of both pro-inflammatory IL-8 and anti-inflammatory IL-10 observed in survivors, this suggests more stable immune homeostasis compared to non-survivors.

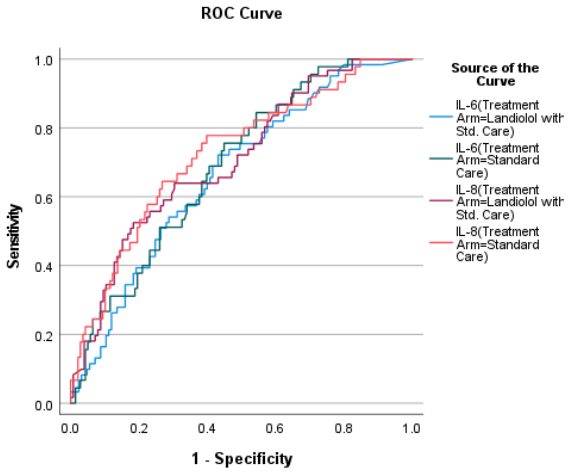
As there appears to be a relationship between several cytokines, particularly IL-10 showing clear separation in both treatment allocations, the statistically significant treatment-independent cytokines (IL-2, IL-4, IL-6, IL-8 and IL-10) were analysed further using area under the ROC for mortality prediction in Figure 38. The AUROC value for IL-10, notably with Landiolol, indicates a good prediction potential with a value >0.7. However, only IL-10 in Landiolol patients was a 'good' predictor, the remaining anti-inflammatory cytokines gave values <0.7. IL-8 shows up as a good predictor of mortality across both treatment arms with AUROC values >0.7. This cytokine combined with IL-10 may provide a good overall survival indicator in Landiolol patients, whereas IL-8 alone may provide sufficient stratification potential in standard care. For comparation, in this thesis SOFA score obtained a mortality area under the ROC ~0.74.



Area Under the ROC Curve

Test Result Variable(s)	Treatment Arm	Area
IL-2	= Landiolol with Std. Care	.611
	= Standard Care	.518
IL-10	= Landiolol with Std. Care	.714
	= Standard Care	.620
IL-4	= Landiolol with Std. Care	.645
	= Standard Care	.610

The test result variable(s): IL-2, IL-10, IL-4 has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.



Area Under the ROC Curve

Test Result Variable(s)	Treatment Arm	Area
IL-8	= Landiolol with Std. Care	.706
	= Standard Care	.724
IL-6	= Landiolol with Std. Care	.665
	= Standard Care	.688

The test result variable(s): IL-8, IL-6 has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.

Figure 38: ***Area Under the ROC Curve for Anti-inflammatory Cytokines and Inflammatory Cytokines.*** *Area separated by treatment arm*

Atrial Fibrillation: Analysis of Afib incidence against cytokine concentration was carried out. A Mann-Whitney U test was run to compare the distribution of cytokine concentration by confirmed Afib status, and 3 analytes showed significance (Table 5). The analysis indicates TNFα was statistically different in those with confirmed Afib, as was IL-4 at the global level. Interestingly, whilst not significant at the 5% level, IL-8 levels trended towards significance between those with and without Afib.

Table 5: ***Statistical Analysis (Non-Parametric Test, Mann-Whitney U) of Cytokine Concentrations by incidence of confirmed Afib status.*** *Statistically significant results denoted with asterisk (*)*

Cytokine	Plasma Concentration *p* – value	Landiolol Only *p* – value	Standard Care Only *p* – value
IFN-γ	0.402	0.624	0.515
IL-2	0.307	0.220	0.819
IL-5	0.941	0.777	0.812
IL-8	0.069	0.017*	0.935
IL-12	0.464	0.698	0.534
TNF-α	0.034*	0.101	0.203
IL-1Ra	0.187	0.134	0.694
IL-4	0.026*	0.082	0.188
IL-6	0.424	0.791	0.340
IL-10	0.360	0.087	0.635
IL-17	0.936	0.241	0.219

At the individual treatment level, IL-8 concentration was statistically different in those with and without Afib who were administered Landiolol, this was not significant in standard care. AUROC analysis showed TNF-α in the Landiolol arm a value of 0.607 and standard care 0.580; IL-4 gave 0.600 and 0.581, respectively. AUC/ROC for IL-8 in Landiolol patients gave a value of 0.649 thus none of these cytokines were good predictors for Afib.

Finally, as SOFA score was the primary outcome of the trial, cytokine data was superficially used to determine increases in SOFA score by treatment arm through a relationship tree (Figure 39).

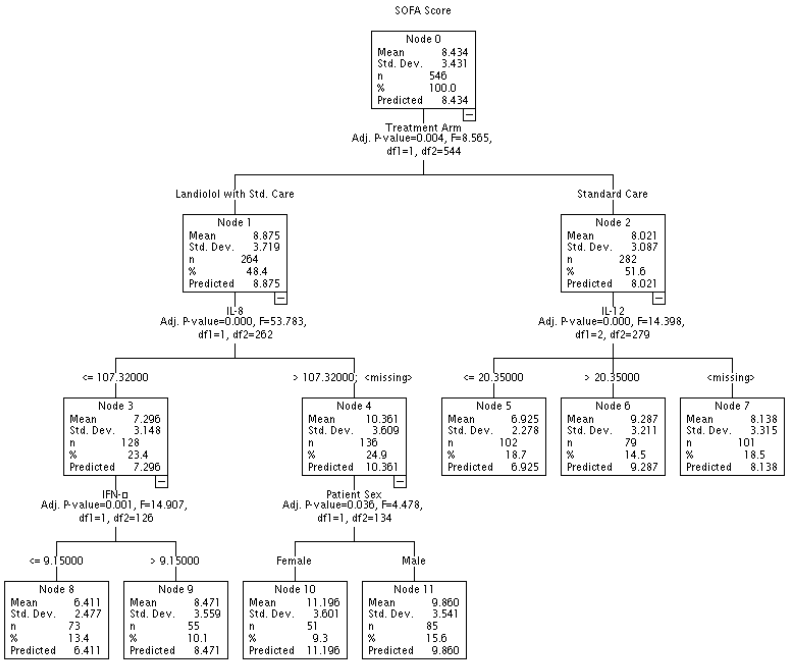


Figure 39: ***Relationship Tree Exploring Influential Factors on Primary Outcome***

The tree indicates, as confirmed in clinical analysis, Landiolol had a higher mean SOFA score compared to standard care. The largest driver of SOFA score increase in the Landiolol group was increases in IL-8. Concentrations >107 pg/mL led to a mean increase of 3 points in SOFA score (p<0.001). With IL-8 appearing significant in several categories, namely: survival and Afib onset, in addition to a driver of organ failure, results indicate a significant role for this cytokine within our study cohort. The relationship tree also highlights an increase >107.3 pg/mL is associated with crossing the inflection from mild or moderate to severe sepsis.

Within the standard care cohort, the driver of elevated SOFA scores appears to be related to IL-12. Surprisingly this cytokine was not initially identified or correlated with increases in mortality in Table 2, despite seeing an increase in organ failure score with elevating concentrations. A concentration increase >20.350 pg/mL was associated with an increase of more than 2 points, and indirectly an expected increase in mortality. Unlike that observed with IL-8, the highest concentration seen on the tree (node 6) did not pass the critical inflective point for sepsis severity of =>10 points.

3.4: Cardiac Biomarkers

As a β-blocker is routinely used to manage a variety of conditions, predominantly associated with the cardiac system or sympathetic nervous system, it is expected a degree of change will be observed in patients administered Landiolol over those in receipt of standard therapy only. With the primary outcome of STRESS-L being an improvement in overall mean SOFA score, it is important to ascertain the key relationships between essential cardiac markers and change in SOFA score. Further, as observed in section 3.1, Landiolol patients required greater infusion of noradrenaline to maintain MAP, the analysis will subsequently also investigation if Landiolol provided any protection against cardiac damage, stress and global ischemia-related injury associated with dosage extremes of catecholamine-induced vasoconstriction.

Firstly, analysis of the treatment differences was performed, using Mann-Whitney U Test to identify if cTnI, CK-MB and Pro-BNP were statistically different by treatment arm. Results presented in Table 6.

Table 6: Cardiac Biomarkers Separated by Survivors and Non-Survivors to Evaluate Differences by Treatment Allocation. Non-Parametric Test (Mann-Whitney U). Statistical significance denoted by asterisk (*).

Biomarker	Combined *p* – value	Survivors Only *p* – value	Non-Survivors Only *p* – value
cTn-I	0.377	0.012*	0.130
CK-MB	0.085	0.020*	0.865
Pro-BNP	0.718	0.703	0.701

The results demonstrate in survivors only, a statistical difference between standard care and Landiolol patients, with cTnI noticeably greater in the standard care arm (Figure 40). Further a more extensive CK-MB range, an earlier generation/less specific test to cTnI, was observed for standard care which suggests Landiolol protects to a degree from cardiac stress despite increases in pressor dosage which contributes to cardiac stress. Females had greater cTnI levels in standard care compared to males, and with no median difference observed in males

independent of treatment (data not shown). Female Landiolol patients have a reduced median concentration compared to standard care, although concentration range is comparable to standard care in male patients, which is still not reduced to the degree observed in male Landiolol patients.

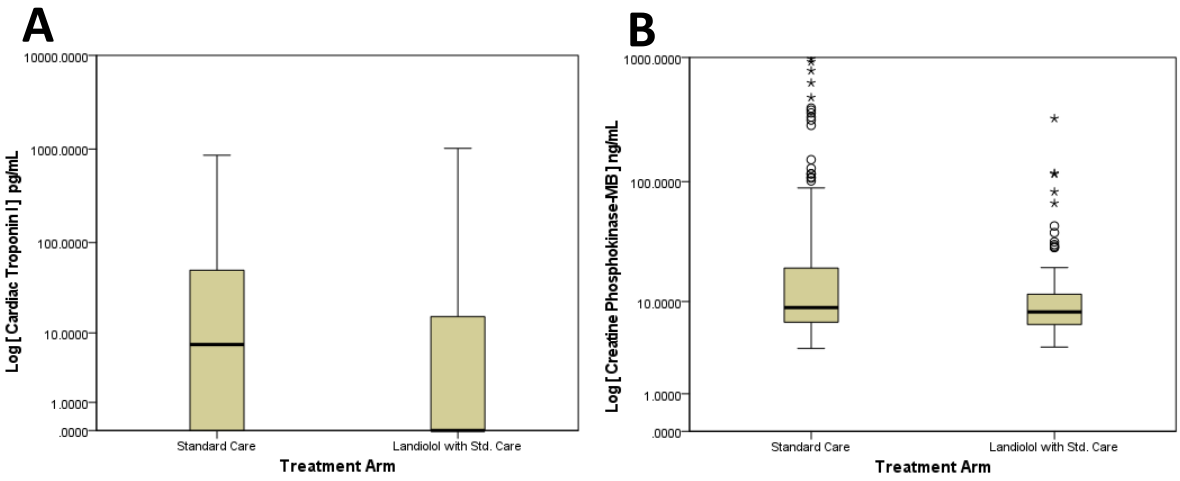


Figure 40: ***Statistically Significant Cardiac Biomarkers in Survivors. A) cTnI and B) CK-MB.*** *Data are median ± 95%CI*

Whilst a difference was observed in survivors for treatment allocation, as noted in other analyses within this thesis, a difference was usually reflected by mortality status. Non-parametric analysis was run to determine difference between survivors and non-survivors within the intervention and control arms (Table 7).

Table 7: ***Analysis of Mortality Status within Landiolol and Standard Care Only Arms.*** *Significant results denoted with asterisk (*). Statistical analysis performed with Mann Whitney U Test, significance regarded at 5%.*

Biomarker	Combined *p* – value	Landiolol *p* – value	Standard Care *p* – value
cTn-I	0.001*	<0.001*	0.866
CK-MB	0.293	0.028*	0.776
Pro-BNP	0.210	0.239	0.635

The results indicate stress markers (cTnI and CK-MB) were statistically different in the Landiolol group, with the significance for cTnI is shared in the treatment-independent category. No results achieved significance in standard care between survivors and non-survivors. Specific analysis examining the differences observed in cTnI and CK-MB found that in non-survivors both were higher than survivors, cTnI data shown in Figure 41. Whilst CK-MB concentration differences between non-survivors and survivors were significant, the change was not as pronounced as observed with cTnI (p=*0.028*). cTnI was elevated in non-survivors independent of treatment *p*=0.001.

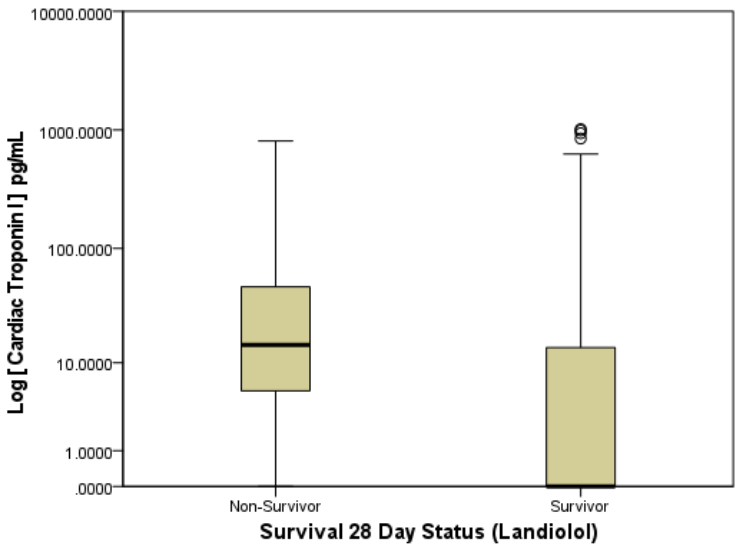
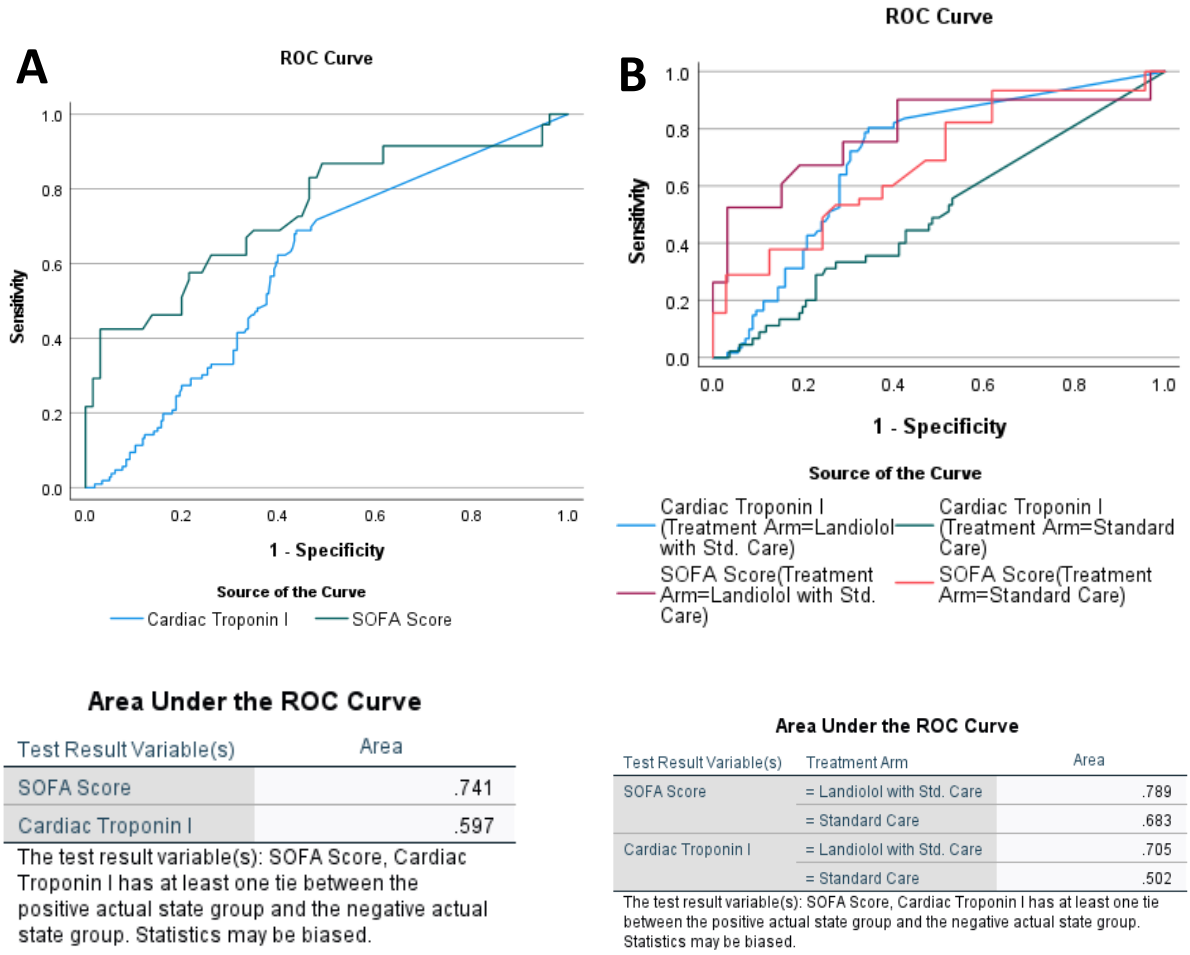


*Figure 41: Log(**cTnI) Concentration by Mortality Status for Landiolol.** Data are median ± 95%CI*

As a statistical relationship exists between cardiac health biomarker increase and mortality, to ascertain its predictive potential within our cohort, AUC/ROC analysis was conducted (Figure 42).



Area Under the ROC Curve

Test Result Variable(s)	Area
SOFA Score	.741
Cardiac Troponin I	.597

The test result variable(s): SOFA Score, Cardiac Troponin I has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.

Area Under the ROC Curve

Test Result Variable(s)	Treatment Arm	Area
SOFA Score	= Landiolol with Std. Care	.789
	= Standard Care	.683
Cardiac Troponin I	= Landiolol with Std. Care	.705
	= Standard Care	.502

The test result variable(s): SOFA Score, Cardiac Troponin I has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.

*Figure 42: **Area Under the ROC Curve Predictors of Mortality**. A) Combined Treatment Arm; B) Separation by Treatment Allocation indicate differing predictive values.*

When treatments are combined an unsatisfactory AUROC of 0.597 was obtained compared against the established indicator, SOFA Score at 0.741. When cTnI is separated by treatment allocation, Landiolol gave an AUROC of >0.7, whilst control arm was 0.502. Thus, cTnI is a good indicator of mortality outcome for those in-receipt of Landiolol only. An AUROC analysis performed for CK-MB reported an overall value of 0.532 for both treatments, Landiolol = 0.598 and control = 0.486. Pro-BNP also performed poorly, combined = 0.547, Landiolol = 0.565 and control = 0.524.

Regression analysis was performed to identify any influence varying dosage of noradrenaline infusion had on cardiac stress indicators (Figure 43). Clinical analysis in 3.1 demonstrated noradrenaline requirement was greater in Landiolol than controls, it is imperative as a result to identify what degree of protection, if any, does Landiolol provide compared to controls.

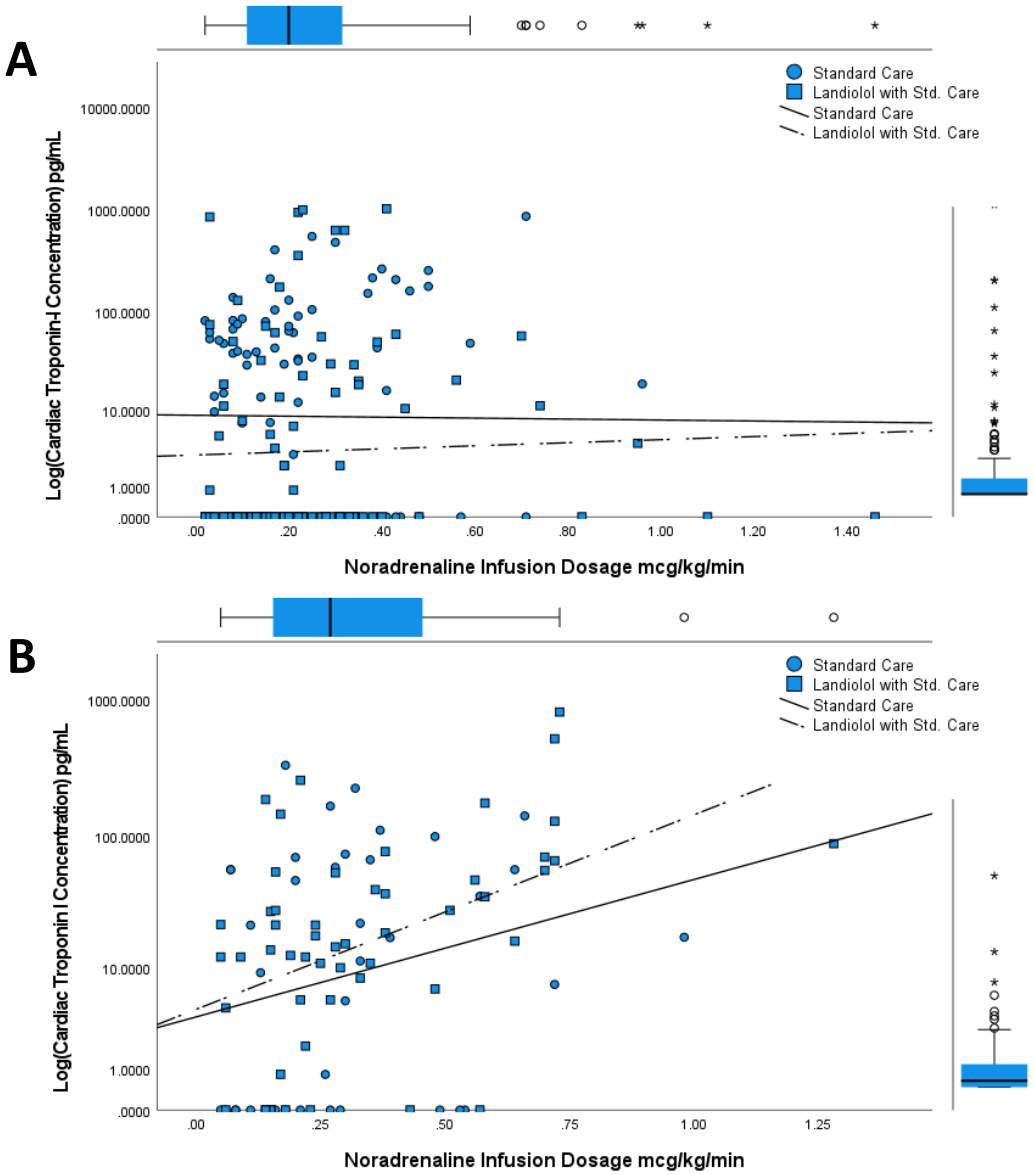


Figure 43: ***Effect of Increasing Noradrenaline Infusion on Cardiac Troponin-I Plasma Levels****. Effects separated by Treatment in A) Survivors B) Non-Survivors*

For purposes of complete analysis, separation has further been conducted by sex (Figure 44)

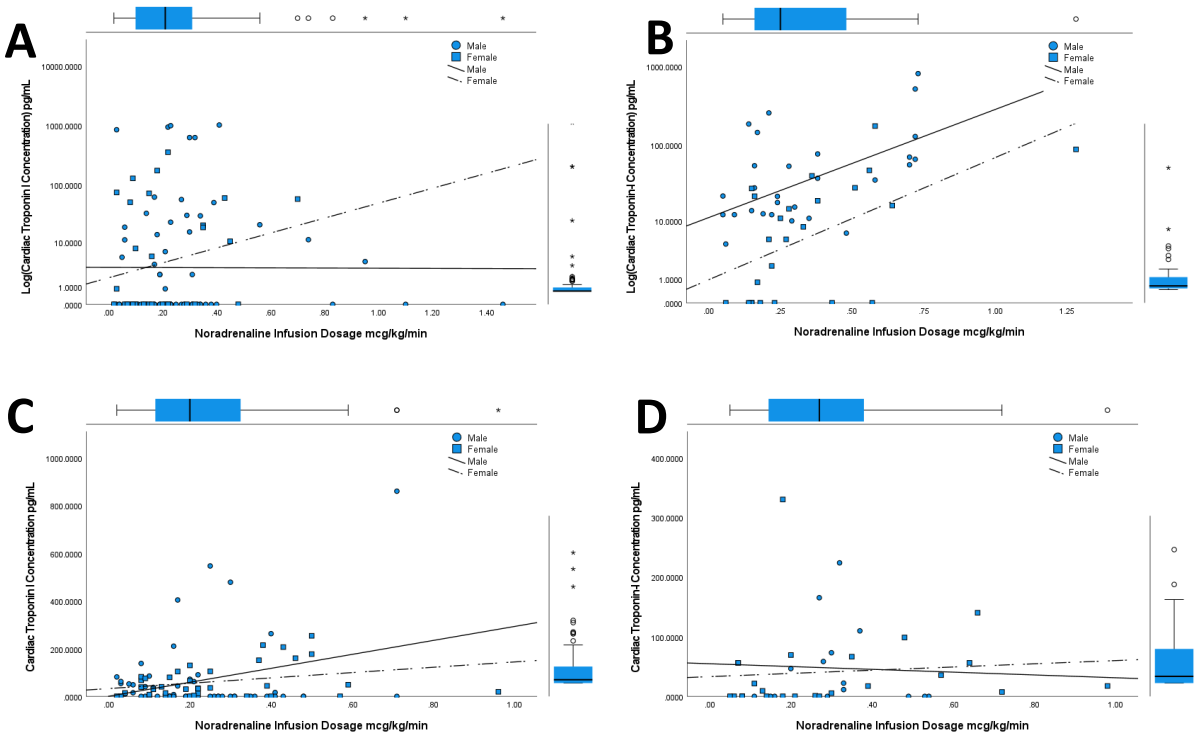


Figure 44: ***Sex-Mediated Responses to Increasing Noradrenaline Dosage in Survivors and Non-Survivors.***
Plot A) Survivor –Landiolol B) Non-Survivor – Landiolol C) Survivor – Standard Care D) Non-Survivor – Standard Care

Figure 43 illustrates Landiolol survivors had lower cTnI compared to standard care survivors, most pronounced at lower infusion rates, although still remaining lower even at the highest infusion rate. cTnI levels were higher at extremes of pressor dosage independent of treatment allocation in non-survivors compared to that of survivors. Figure 43 shows in these non-survivors, those administered Landiolol had greater cTnI plasma level compared to standard care non-survivors. Sub-analysis of these groups exploring sex-mediated responses found males had lower cTnI compared to females in the Landiolol survivor group whereas the opposite was true for non-survivors (Figure 44). Non-survivor males had prominent elevations compared to females. In standard care survivors, independent of sex cTnI elevations were seen as pressor infusion increased generally, notably males again had higher concentrations compared to females. Little change was observed at varying dosage in non-surviving standard care patients. Non-parametric analyses using Mann-Whitney U Test reported p=0.021 for cTnI

levels across treatment arms for survivors, and *p*=0.130 for difference across treatment groups for non-survivors. CK-MB were not investigated as cTnI has superseded this test in routine clinical practice, and no statistical differences were seen with Pro-BNP.

The concentration of Pro-BNP which has a plasma $t_{1/2}$ of 2 hours is elevated with cardiac events, including the onset of Afib. The cohort participants with confirmed AFib were analysed to establish any relationship. Patients with a higher NT Pro-BNP concentration, particularly >2650 pg/mL had increased occurrence of AFib compared to those with Pro-BNP <2650 pg/mL (*p*=0.002). In those with increased concentrations, male patients were affected more than females (*p*=0.007). The area under the ROC was analysed and reported combined group value of 0.592, Landiolol = 0.597 and control = 0.586. Analysis for the other markers cTn-I and CK-MB were also conducted. cTn-I had an AUROC of combined = 0.657, Landiolol = 0.719 and control = 0.596; CK-MB had combined = 0.575, Landiolol = 0.642 and control = 0.512 (Figure 45).

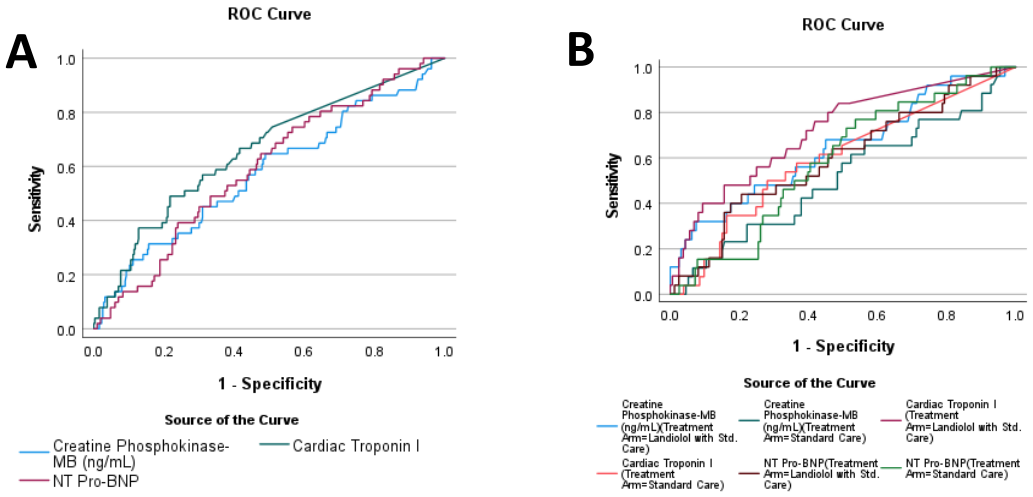


Figure 45: ***Receiver Operating Characteristic Curves for Cardiac Biomarker as a Predictor of Atrial Fibrillation Onset*** *a) Combined treatments B) Treatments separated*

Further analysis which included separating the cohort by survival status first, revealed a treatment-independent AUROC for: cTnI = 0.641 [95% CI: 0.5-0.782], CK-MB = 0.603 [95% CI: 0.467-0.738] and Pro-BNP = 0.477 [95% CI: 0.347-0.606] for non-survivors. Equivalent analysis for survivors showed: cTnI = 0.657 [0.549-0.764], Pro-BNP = 0.649 [0.550-0.749] and CK-MB = 0.548 [0.427-0.669] (Table 8).

*Table 8: **Area Under the ROC for Onset of AFib.** Data separated by survival status, and later treatment allocation. CI: Confidence Interval; [a]=Null hypothesis assumes true area = 0.5*

Mortality Indicator	Test variable	Allocation	Area Under ROC	95% CI [Lower-Upper]	Asymptotic Significance p-value[a]
Survivors	cTnI	Landiolol	0.670	0.506-0.835	0.042
		Std Care	0.633	0.495-0.772	0.059
	Pro-BNP	Landiolol	0.733	0.577-0.890	0.004
		Std Care	0.592	0.463-0.720	0.169
	CK-MB	Landiolol	0.560	0.365-0.755	0.547
		Std Care	0.529	0.377-0.682	0.708
Non-Survivors	cTnI	Landiolol	0.764	0.622-0.906	<0.001
		Std Care	0.522	0.295-0.749	0.852
	Pro-BNP	Landiolol	0.422	0.246-0.597	0.382
		Std Care	0.577	0.382-0.772	0.438
	CK-MB	Landiolol	0.739	0.610-0.868	<0.001
		Std Care	0.455	0.234-0.676	0.692

Table 8 results suggests the onset of Afib is heavily influenced by cardiac failure markers in Landiolol survivors, whilst onset is observed by both cTnI and Pro-BNP in non-survivors (area >0.7). Both cardiac failure and stress markers are poor predictors for Afib onset in control patients. This possibly suggests a casual relationship between Landiolol and vulnerability to new onsets of Afib not seen in standard care.

As described in section 3.1, Landiolol prevented (in both survivors and non-survivors) increases in heart rate from increasing dosage of pressor. This was not replicated to the same extent in standard care, demonstrating a degree of support not seen without β-blockade. The primary use of the pressor was to maintain MAP, subsequently, regression models were

created to explore any relationships between increasing MAP and cTnI. Results indicated in survivors a higher cTnI was observed at the lower blood pressures presumably related to increased pressor dosage, as pressures reached the desired range, a decline in cTn was seen again possibly related to reduced pressor support. Interestingly, in Landiolol the initial cTnI concentration at the lower MAP values were considerably lower than standard care, and as pressures increased cTnI concentration remained unchanged. This suggests possibly a degree of protection from increased noradrenaline infusion not seen in standard care. In non-survivors, a similar relationship was observed between the trial arms, although cTnI concentrations were considerably greater in both treatment allocations (data not shown). As blood pressure is in part controlled by the renin-angiotensin-aldosterone axis, which is strongly related to renal function, brief analysis was conducted to evaluate consequence of increasing renal creatinine levels and MAP (Figure 46).

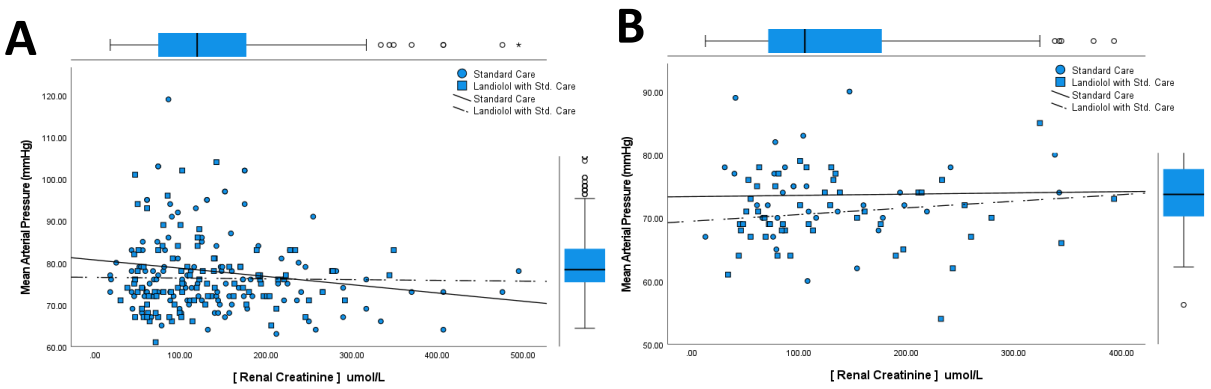


Figure 46: ***Relationship between Renal Function (estimated by clearance of creatinine) and Mean Arterial Pressures in Survivors (A) and Non-Survivors (B).***

Figure 46 shows little change is observed between trends for Landiolol in survivors and non-survivors, other than the increased pressure in survivors, with regards to renal creatinine levels and MAP. Although in standard care, as renal creatinine increases, a decline is observed in MAP. Which suggests despite possible kidney function deterioration, blood pressure is to a degree maintained in Landiolol not observed to the effect in standard care.

Finally, to conclude the cardiac analysis and relationship between cardiac performance and renal function, data was analysed to ascertain any association between cardiac stress, failure markers and renal function. This analysis provides insight into whether Landiolol could provide a degree of renal protection and possibly the degree of influence increasing renal creatinine concentration (as a measure of decreasing renal performance) has on cTnI and BNP levels (Figure 47).

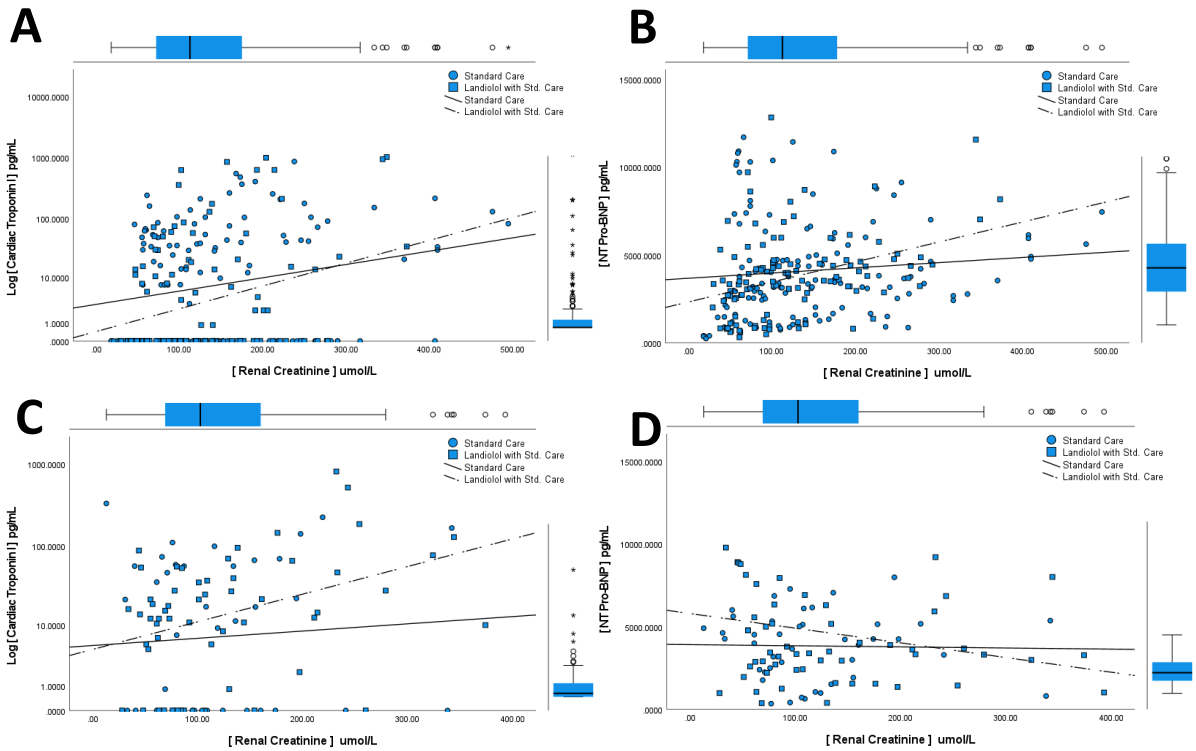


Figure 47: ***Associations between Renal Creatinine Concentration and Markers of Cardiac Stress (cTn) and Cardiac Failure (Pro-BNP) by Treatment Allocation for Survivors (A and B) and Non-Survivors (C and D).***

At lower renal creatinine, both cTnI and Pro-BNP levels were lower in Landiolol compared to standard care only. As kidney function continues to decline (based on increase in creatinine), an increase in cTnI was observed for both interventional and control participants. At 300 µmol/L, the increase appears more pronounced in Landiolol than controls. Pro-BNP showed little increase with standard care across increasing renal concentration, whilst initially at a lower concentration, as renal concentration reaches 150 µmol/L or pro-BNP 4800 pg/mL, a considerable increase in cardiac failure biomarker is observed for Landiolol participants.

For non-survivors, Landiolol treatment gave a higher concentration of pro-BNP in early timepoints, although little change is observed overall. With regards to cTnI, Landiolol was seen to have a notable increase as renal function deteriorates, not seen to the same degree in control non-survivors. Taken together, these data suggest that Landiolol provides a degree of protection in early elevating creatinine levels from exacerbating kidney dysfunction, although cannot maintain lower cardiac stress above 300 µmol/L similar to lesser concentrations. At the extremes of renal creatinine concentration (indicative of worsening kidney function), cardiac failure may eventually be more severe in Landiolol recipients.

Discussion

The initial 2013 trial which underpinned STRESS-L led by Professor Andrea Morelli reported marked improvements in mortality (49.4% intervention to 80.5% control arm), reductions in noradrenaline requirements, greater kidney function in addition to reduced markers of myocardial injury in the β-blockade (Esmolol) group compared to those in-receipt of standard therapy alone. Study clinicians noted the possibility of a chance finding, particularly with high mortality in the group not treated with beta-blockade. However, a second trial (also using Esmolol) reporting 7 years later by Gadallah *et al.* (2020) also found a significant reduction in heart rate and mortality, further coupled with reduction in ITU length of stay and reduced durational need for mechanical ventilation. In addition to a reduced SOFA score in the interventional patients. Notably however, this trial is small and not without limitation, including uncommon study procedures and statistical analysis, subsequently was analysed with caution. A further pilot study conducted a year later by Levy *et al.* (2021) found mixed support for the two other studies. Despite the trial closing early due to low enrolment, Levy observed a significant reduction in heart rate in addition to reductions in pro-inflammatory proteins. However, Esmolol infusion was ceased in 33% of trial participants due to a marked increase for noradrenaline by almost 0.30 μg/kg/min. In these 33%, even at the lowest doses of Esmolol, refractory cardiac failure was established. Interestingly, diuresis was significantly decreased during Esmolol infusion, but a non-significant increase in clearance rate 0.2%/min was observed.

Results obtained in STRESS-L were not as sharply defined, with some ambiguity. The trial observed statistically significant reductions in heart rate similarly found in the former trials. Haemodynamic analysis saw mean arterial pressures greater in the control arm, finding statistical significance, although clinically an insignificant change of 1 mmHg overall. As

observed in Levy's trial, STRESS-L also saw an increased need for noradrenaline infusion in the intervention arm. Notably however, this requirement was less pronounced with an average 0.05 μg/kg/min increase. Increases in noradrenaline saw a pronounced increase in heart rate, particularly at extremes of dose. Landiolol however demonstrated excellent compliance and resilience in maintaining heart rate to a degree considerably lower, not replicable or observed in standard therapy alone (Figure 22F). Gadallah *et al.* (2020) saw a SOFA score reduction in patients administered Esmolol, despite treatment not being administrated continually. This coupled with the study not reporting the frequency of initiation and termination of the β-blocker infusion (or length of time) making it difficult to accurately determine a reduction in SOFA score was associated with drug use. In STRESS-L, those assigned to continue standard therapy reported a mean SOFA score of 8.12 (SD: 3.28) whilst those in-receipt of Landiolol reported 8.82 (SD: 3.94), the difference is not statistically significant (p=0.516). No significant differences between SOFA score and sex were observed in the study cohort. Unexpectedly, whilst bearing no difference in control patients, increases in patient age negatively impacted SOFA score with higher results seen in Landiolol treated patients. This suggests, older patients may inevitably have increased SOFA scores (i.e., increased dysfunction and severity of disease) if in-receipt of β-blockade.

STRESS-L confirms that higher SOFA scores are correlated with increased mortality rates, with statistical significance at p<0.001 reported between survivors and non-survivors (Lopes Ferreria *et al.,* 2001). Interestingly, non-survivors administered Landiolol had a much greater SOFA score compared to non-survivors in standard care. Both Morelli *et al.* (2013) and Gadallah *et al.* (2020) reported reductions in mortality in interventional arms at 28-days. These findings were not observed in STRESS-L, on the contrary, mortality was numerically but not statistically greater at both 28-days and 90-days follow-up periods in the β-blockade

group. Overall, 28-day patient mortality rates show a male deceased rate of 27.0% for standard treatment, and 38.9% for Landiolol. In females, these are 24% and 34.6% respectfully. Over a 90-day follow-up period, mortality status for females remained unchanged, although in males control participants saw an increase to 32.4% and Landiolol allocation saw an overall mortality rate reach 50%. These data suggest sexual dimorphism in the effects of Landiolol. This demonstrates improvement with critical care over time but may demonstrate that STRESS-L was studying a group with different characteristics from Morelli.

Both Morelli's and Levy's trials demonstrated to some degree either a direct improvement in renal function (eGFR) or an increased clearance rate (suggesting greater renal function), respective. Whilst Levy did report incidence of diuresis (p=0.016), on average function was greater than standard care. STRESS-L found an increased renal clearance of mean creatinine concentration in the β-blocker arm, particularly a pronounced increase in days 1-6. Although, after day 6, β-blocker patients had lower clearance of creatinine than control patients (Figure 21), notably this could be a selection bias. Whilst a decrease of creatinine concentration was observed in the Landiolol patients, physicians were free to initiate and terminate renal replacement therapy as clinically necessary. Subsequently, it is difficult to accurately determine or indeed, deduce a cause-and-effect relationship between the drug and renal function or renal protection from these data. That noted, interestingly Hasegawa *et al.* (2021) reported at 72 hours, ultra-short acting β_1-blockers improved clearance of lactate. Whilst clearance of lactate is more complicated than creatinine, this suggests a relationship (though still unclear) between improved metabolite clearance and β_1-blockade, and therefore better patient outcome as lactate is currently used to determine adequacy of intervention. As a result, the effects of Landiolol on renal function should be investigated further, particularly as Landiolol's metabolism and biotransformation is not hepatic or renally-dependent, rather

relying on plasma pseudocholinesterase. Analysis conducted in the later sections concerned with renal-dependent influences on mean arterial pressure (MAP) suggests Landiolol may offer a degree of protection at earlier stages of renal dysfunction as an indirect correlation with less cardiac stress (measured through cTnI concentrations) and the maintenance of MAP in Landiolol patients not observed to the same degree in control participants despite significantly elevating renal creatinine (Figure 50A). Results indicated as creatinine concentration increased, arterial pressure in control participants declined ~12 mmHg, possibly observing a decline below 70 mmHg MAP, this on the contrary to Landiolol patients who saw no to little fluctuation in MAP despite reduced kidney function. This presuming intimate connection with renin-angiotensin-aldosterone axis and the regulation of vascular resistance and tone. Using the same regression models, results indicate as kidney function deteriorates continually, an increase in cTnI levels is observed in both treatment arms, initially this increase is much less pronounced in Landiolol, although at renal [creatinine] >300 μmol/L, a more pronounced trajectory is seen. Research by Hall *et al.* (2016) and Bakris *et al.* (2006) report that β-blockers use, particularly β_1-selective (associated with renin release and cardiac output), may reduce the progression of renal dysfunction, or even prevent this acute onset of dysfunction; this could partially occur through reduced renal oxygen consumption and concurrent management of the unusual 'cytopathic hypoxia' observed in severe septic shock. Interestingly, in an abstract presented by Kiyonaga *et al.* (2018), they too report Landiolol was found to have organ-protecting effects. With these taken together, it is possible Landiolol may offer a degree of renal protection not observed in standard care patients.

Atrial fibrillation (Afib) and supraventricular arrhythmias are common presentations established in septic shock with prevalence rates nearing 50% in the most severe patients (Seemann *et al.*, 2015). STRESS-L data show control patients had a greater Afib incidence rate

than intervention arm. The J-Land 3S study (Kakihana *et al.*, 2020) which explored the efficacy and indeed safety of Landiolol for the management of septic-related tachyarrhythmias found adverse and serious adverse events (including cardiac arrest) was associated with Landiolol use in this cohort, although serious adverse reactions/events were reported in the control cohort without administration of Landiolol. The STRESS-L result supported the earlier RCT *PASCAL* by Sezai *et al.* (2011) which reported Afib prevalence was reduced by Landiolol in post-operative patients, with reduction of ischemia related presentations, reduction in inflammation (supporting the concept Landiolol operates in part as an anti-inflammatory), and inhibition of sympathetic overdrive. Unexpectedly, whilst noted standard therapy patients had greater Afib prevalence overall, most of these confirmed cases were already established at baseline. Some of the confirmed Afib cases in the interventional arm were initiated after the infusion of Landiolol, which suggests Landiolol in our cohort did not entirely prevent new onsets. Nevertheless, it is also not possible to determine the extent of the onsets that Landiolol had prevented.

Levy et al. (2021) reported pro-inflammatory proteins were reduced in all patients of their study, including those administered Esmolol, and the PASCAL trial hypothesised Landiolol also had anti-inflammatory properties. As Esmolol is considered the precursor to Landiolol, this is not entirely unexpected. One major limitation to several studies conducted before, both in humans and animal models, is the lack of exploration of mechanisms by which β-blockers may act as anti-inflammatories. Subsequently, STRESS-L longitudinally sampled patients in both trial arms to explore any changes. Due to the high probability of mortality in this critical patient population, irrespective of treatment allocation, participants were first separated by survival status and assessed for differences. Within the survivors, 3 cytokines were regarded as statistically different. IFNγ and IL-2 both had greater concentrations in the control arm,

whereas IL-1Ra had a greater concentration in the intervention arm. Further, TNFα strongly trended towards significance in the survivors' group with a *p*=0.051, with the concentration also being reduced in Landiolol patients. With decreases in IFNγ, IL-2 and TNFα concentrations all observed in Landiolol, and an increase in IL-1Ra, results indicate Landiolol indeed reduces inflammation. This result supports earlier research by Ohtsuka *et al.* (2001) which found β-blockade reduced circulating levels of TNFα, in addition to IL-10 in cardiomyopathy patients; further literature by Hajighasemi and Mirshafiey (2016) which reported using an earlier generation blockade agent (Propranolol) also reduced TNFα expression. Results obtained from STRESS-L, previous literature and Levy *et al.* (2021) provide good evidence β-blockers have a role as anti-inflammatories. Although with the trial closing prematurely and the relatively small participant and sample number, STRESS-L would have benefitted from increased sampling opportunities. Further explicit studies should be carried out to confirm anti-inflammatory effects of Landiolol. Expression of IL-17 was significantly elevated in Landiolol non-survivors compared to control participants, and Simundic *et al.* (2017) noted prolonged arterial hypertension (or possibly prolonged vasoconstriction) is correlated with an increase in serum IL-17A levels, particularly more pronounced if further in-receipt of any diuretics. A study examining the use of diuretics in septic shock observed an elevated mortality rate in patients who at any point received a diuretic drug within their stay (Bandak *et al.,* 2020). Contradictory to our results, a 2019 study conducted by Razazi *et al.* (2019) observed that elevations in IL-17 were correlated with early sepsis resolution and increased survival. Cytokine differences were examined by treatment allocation to determine differences between survivors and non-survivors in respect to their own treatment. Interestingly, IL-17 was not significant in between survivors and non-survivors in the Landiolol arm. This suggests for IL-17 Landiolol non-survivors compared to standard care were likely

moving to resolution rather than mortality. This implies drivers of mortality are multifactorial. Rather interestingly, differences between Landiolol survivors and non-survivors, involve a variety of pro- and anti-inflammatory cytokines and notably Landiolol non-survivors were more inflamed than survivors, demonstrated by less percentage change to baseline in IFNγ, IL-4, IL-6 and IL-8. In an ovine hyperdynamic model of sepsis, Calzavacca *et al.* (2014) noted that β-blockade (by Atenolol) did not affect IL-6 or TNFα levels in surviving participants, but instead drove an increase in IL-10 secretion. Results in this thesis agree with the increase in IL-10 secretion on administration of β-blockade (likely through increased binding of β_2AR due to blockade of β_1), but a significant increase was only observed in non-survivors rather than survivors. Survivors in STRESS-L's trial instead demonstrated reductions in IL-10 immediately and longitudinally. Simultaneous analysis of standard care participants saw some cytokine similarities compared to Landiolol. All expressed higher concentrations in non-survivors, including IL-4, IL-6, IL-8, IL-10 and IL-1Ra. Collectively, it appears many non-survivors within standard care are immunosuppressed (or have a lower immune response), which is on the contrary to observations in Landiolol. Other studies and literature support results observed in the general non-survivors cohort, including increased IL-6 and IL-10 (Wu *et al.*, 2009); in addition to IL-4 and IL-8 by (Bozza *et al.*, 2007). Regardless of treatment arm: IL-2, IL-4, IL-6, IL-8 and IL-10 were collectively observed to be different. These cytokines were taken forward for AUROC analysis to predict mortality and stratify patients. Overall predictive ability was not high, IL-10 in the intervention arm was a good predictor (area >0.7) and IL-8 (area >0.7) for both treatment allocations. It appears cytokines are not well suited for stratification and prognostic indication, highlighted not only by the infrequent use in clinical environments, but further supported by Lvovschi *et al.* (2011) in an observational study which reported other studies may have reached inappropriate conclusions and clinical conditions not translating to

pre-defined cytokine profiles. Particularly not in acute settings (e.g. emergency departments) and, indeed, unless serial sampling occurs – profiling offers little clinical relevance. Many drugs used in the intensive care setting (analgesics, sedatives, and vasopressors) can all drastically alter cytokine concentrations.

The primary outcome of STRESS-L was improvement in SOFA Score, this was not demonstrated overall. Sub-analysis excluding the non-survivors saw a lower SOFA score for Landiolol compared to control patients. Analysis of the non-survivors only saw Landiolol patients have the higher score when compared to controls. Analysis of prominent cytokines concluded that IL-8 was the leading driver in Landiolol patients' SOFA score increase. A concentration >107 pg/mL gave an increase of 3 points. Bozza *et al.* (2007) also noted IL-8 was the largest driver of SOFA score in their cohort and a good predictor of 28-day mortality. The results from STRESS-L also appeared to show to some degree a difference in response from males to females; statistical significance was reached for both IL-10 and IL-17 across treatment arms, which suggests possibly a degree of sexual dimorphism within our cohort. Both IL-10 and IL-17 were elevated in females when compared to males.

Overall, insight from cytokine analysis, showed that survivors were generally less inflamed in Landiolol patients compared to standard care, which indirectly suggests less reduced bystander injury to tissue. In the non-survivor cohort, IL-17 appeared elevated in Landiolol patients more than that observed in standard care. IL-17 has been associated with neutrophil activation (i.e., promotor of neutrophilic inflammation) but is further expressed on a variety of other immune cells (including CD8, NK-cells and lymphoid cells), and has the potential to induce further inflammatory-related chemokines expression (Zenobia et al., 2015). Survival analysis originally saw Landiolol non-survivors to be hyper-inflammatory compared to

survivors. Although simultaneously, on administration of Landiolol a pronounced increase in IL-10 is observed in non-survivors not seen in survivors; thus, coupled with no decline in Th_2-mediated IL-4, when taken together suggests Landiolol patients are becoming progressively more immunosuppressed as time progresses. The increase in IL-10 concentration, combined with increasing pressor requirement, tentatively hints towards findings observed in a former trial, BALTI-2.

Literature for several years has demonstrated β-blockers exert their effect through interaction with a range of β-adrenergic receptors localised on the plasma membrane of not only cardiac cells, but a range of other cells including renal and immune cells. The myocardium expresses all 3 βARs (β_1, β_2 and β_3), in addition to immune cells expressing βAR. In the case of monocytes for example, activation of βARs lead to anti-inflammatory properties, where catecholamine binding to βARs on neutrophils prohibit effective migration and inhibit oxidative metabolism (Scanzano and Cosentino, 2015). Unlike the specificity observed in other β-blockers, Landiolol is ultra-selective with a β_1:β_2 of 255, whereas the closest competitor is Esmolol with β_1:β_2 of 33 (Atarashi *et al.,* 2000). As a β_1-antagonist with such great specificity, there is likely increased binding (and interaction) with β_2AR by catecholamines much above and beyond normal interaction should β_1 not be occupied. Published research has indicated continuous interaction with receptors can result in receptor desensitisation, and this could account for increased noradrenaline requirement seen in Landiolol not observed in standard care only (catecholamine dose tolerance), although protection by β-blockade should upregulate the receptor (Haeusler, 1990) rather than the contrary. This suggests that possibly β_1 is not necessarily involved to a similar extent as β_2AR. Ağaç *et al.* (2018) demonstrated in an animal model that stimulation of β_2AR lead to rapid increases in IL-10 secretion. This could account for the increases in concentration of IL-10

observed in non-surviving Landiolol patients. BALTI-2 was an RCT which terminated early due to increases in mortality and investigated β_2 agonism (using the agent Salbutamol). The premise behind BALTI-2 was hypothesised patients with acute respiratory distress syndrome (ARDS) would benefit from *i.v.* Salbutamol by reducing airway plateau pressure and extravascular pulmonary fluid, although study results indicated those assigned Salbutamol had higher mortality rate, in addition to much greater risk of new onset tachyarrhythmias and lactatemia with acidosis (Smith *et al.*, 2012). With Landiolol being an antagonist, selectively binding to β_1AR, this increases the substate availability (until saturation) for β_2AR and β_3AR. Increased availability (agonistic behaviour) downregulates subsequent receptors.

A recently published article by Ajoolabady *et al.* (2022) explored the mechanisms of Afib onset. They report the growing evidence of the inflammasome and inflammasomal signalling attributed to its pathogenesis. The activation of NLRP3 inflammasome is recognised as a significant driver of AFib pathogenesis. The process of AFib onset or initiation is believed to occur through a 2-step mechanism of *priming* and *triggering*. Inflammation, a key driver of Afib, namely the increasing presence of cytokines (such as IL-1β, IL-18, TNF-α, IL-6 and IL-8) and reactive oxygen species can cause apoptosis (though this is considered rare in septic shock), hypertrophy and atrial fibrosis. Cytokine infiltration to the left atrium was correlated with rhythm change. With increased cardiac damage affecting blood flow (such as hypertrophic cardiomyopathy, fibrosis and ischemia), more cytokines migrate into the atrial furthering the cycle, and worsening the pathology. Analysis was carried out to establish statistically significant cytokines associated with Afib in our cohort, and results indicate TNF-α and IL-4 were both elevated in patients with confirmed atrial fibrillation. Our results to some degree show a relationship or support comments made by Ajoolabady and colleagues. Additionally, the results obtained from STRESS-L further support the concept that immune

cell infiltration is aided considerably by β_2AR, as a secondary mice model which lacks β_2AR showed considerably less immune infiltration to the myocardium, with the authors also reporting less cardiomyocyte death, and decreases in hypertrophy and fibrosis associated with persistent stimulation (Tanner *et al.,* 2021).

As a measure of cardiac stress, troponin-I and the older generation CK-MB were analysed, and measurement of failure was conducted by Pro-BNP levels. Results obtained demonstrated in survivors, Landiolol patients had significantly lower concentrations of both cTnI and CK-MB compared to patients receiving standard care only. No differences were observed for Pro-BNP. Suggesting cardiac protection from ischaemia but that cardiac function remained the same. Further, no statistically different results were observed in non-survivors. Particularly, on analysis of survival status by treatment allocation saw cTnI and CK-MB were statistically different between Landiolol survivors and non-survivors (p<0.001 and p=0.028, respectively). Both cardiac markers were higher in non-survivors. No statistical significance difference was observed in standard care between survivors and non-survivors. Area under the ROC was conducted for cTnI as a predictor of mortality in Landiolol patients and compared to SOFA score, cTnI observed good prediction potential, cTnI as a predictor in standard care only was remarkably less good. Cheng *et al.* (2015) also confirmed cTnI was a good predictor of mortality in severe sepsis and septic shock.

As seen here and in Levy *et al.* (2021) trial, those assigned a β-blocker required on average a greater dose of vasopressor. Using regression models, analysis was conducted to establish if Landiolol offered any greater protection against noradrenaline dosage compared to control patients. Results demonstrated that cTnI was lower in the Landiolol arm than standard care, even at infusion rates >1.4 mcg/kg/min. This shows that intervention patients had less cardiac

stress from catecholamine infusion than control patients. This result is unexpected, increases in noradrenaline is usually associated with increases in cardiac damage. Landiolol in several other un-related incidences including percutaneous coronary intervention saw myocardial protection (Park *et al.*, 2013) as did the attenuation of post-ischemic cardiac dysfunction in rats (Yasuda *et al.*, 2001).

Chiefly, cardiac analysis shows in survivors, Landiolol not only had overall lower incidences of cardiac stress, but also had fewer increases in cTnI at extremes of pressor dosage not observed in standard care, but further maintained arterial pressures also not observed in standard care.

The abstract by Kiyonaga and colleagues, discussed possible metabolic protections offered by Landiolol in system energetics and pathway regulation. They suggested Landiolol protected against hypoxic conditions created by increases in glycolysis and unsustainable oxygen consumption, including increased ROS and lactic acid seen in non-Landiolol treated patients. Although Landiolol is thought not to extend this protection to the mitochondria from TNFα-induced damage, which can cause left ventricular dysfunction or neurotoxicity (Mariappan *et al.*, 2009; Doll *et al.*, 2015). As β-blockers reduce the oxygen demand and workload, it is possible β-blockade could alleviate some dysfunction established in septic shock. Consequently, metabolomic analysis was employed to identify if any changes or improvements were seen. The data did not show good separation by treatment allocation using both unsupervised (PCA) and supervised (PLS-DA) analyses. On conducting analysis, whilst the use of both positive and negative ionisations modes provides the greatest discovery and analysis of the metabolome (Lei *et al.*, 2011), it become apparent that the negative ionisation mode proved most informative in our study, in agreement with Liggand *et al.*

(2017). Analysis by treatment allocation showed little separation between those administered Landiolol and those continuing with standard therapy. We did not perform analysis by separation of survivors from non-survivors by treatment. However, analysis was conducted to identify separations based on survival status. Here too, little separation was observed by PLS-DA, and even less by PCA. STRESS-L's primary outcome of SOFA score improvement saw a degree of separation, particularly in the mild category (SOFA Score 1-4), although minimally. Finally, to ensure comprehensiveness in analysis, noting the relationship between SOFA score and mortality in septic patients reported here and elsewhere, unsupervised and supervised models were constructed for SOFA score with survival status. Results demonstrated separation between survivors and non-survivors who had a SOFA score of 1-4. Whilst a separation was shown with PCA, significantly better separation was observed with the supervised model; to ensure no overfitting was present, a fitting performance analysis saw good accuracy, good predictive potential (Q^2) and variations (R^2) was good. Based on this ideal model, an unannotated preliminary heatmap with dendrogram was constructed, and clear variances were observed. Putative annotation through mummichog algorithm identified most the important metabolites. Of these, 12 were carried forward for AUROC analysis. Areas obtained ranged from 0.921 [95% CI: 0.793-1] to 0.739 [95% CI: 0.533-0.904]. Before considering in-depth reasons for observed elevations and declines in survivors and non-survivors, enrichment analysis was conducted to identify any over-represented pathways. Results concluded glycerophospholipid metabolism, linoleic acid metabolism, glycosylphosphatidylinositol (GPI)-anchor biosynthesis, sphingolipid metabolism, arachidonic acid metabolism and also purine metabolism were enriched. This suggests many of the changes between survivors and non-survivors are related to cellular signalling, membrane-related recognition and regulatory roles. From the 12 compounds, 6 were elevated in

survivors and 6 were elevated in non-survivors. The compounds elevated in non-survivors were: PE(P-18:3(11Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)), Furfurylamine, Phenylcarbamic acid, 3-Hydroxy-2H-pyran-2-one, 1,2-Ethanediyldicarbamodithioic acid, and (+)-2,3-Dihydro-3-methyl-1H-pyrrole. Interestingly, many of the elevated metabolites in survivors not observed in non-survivors are lipids. Elevations observed include: PE(18:1(11Z)/20:1(11Z)), PC(P-18:1(9Z)/18:0), phosphatidylcholine(18:3(9Z,12Z,15Z)/20:2 (11Z,14Z)), PI(16:0/16:1(9Z)), Phosphatidylglycerol(16:1(9Z)/18:3(9Z,12Z,15Z)).

In survivors, both identified phosphatidylcholine (PC) metabolites were greater than observed in non-survivors. Synthesis of PCs occurs by two pathways, either CDP-choline pathway or alternatively the phosphatidylethanolamine N-methyltransferase pathway (Henneberry *et al.*, 2002). Predominantly biosynthesised in the liver (by hepatocytes), a downregulation or reduced synthesis of PC can affect transport processes in the Golgi complex for example (Testerink *et al.*, 2009) or even induce cellular death. With a lower level of PCs observed in non-survivors could suggest increased trafficking dysfunction and apoptotic death. Phosphatidylcholines are bound (usually non-covalently) to cytochrome C oxidase (CCO, complex IV) and is further considered a precursor to platelet activating factor (O'Flaherty, 1987). The latter could imply a reason for DIC observed in critically unwell shock patients whilst the former accounts for mitochondrial and respiratory failure. Although DIC is uncommon and was not measured in STRESS-L. PC binds to CCO to facilitate (catalyse) the reduction of O_2 to H_2O, thereby maintaining the electrochemical proton gradient across the mitochondrial membrane to enable adequate synthesis of ATP (Hugentobler *et al.*, 2020). This suggests with lower PC concentrations available in non-survivors, the proton motive force (and possibly in turn membrane potential) is reduced. Therefore seeing increased oxidative phosphorylation dysfunction in non-survivors due to the insufficient quantity to bind at

complex 4 reported similarly by Khaliq *et al.* (2020). Literature by Gil-de-Gómez *et al.* (2020), showed (in mouse model) that immune cells can transform PC to (lyso)phosphatidylethanolamine through arachidonic acid remodelling in macrophages. Interestingly, results obtained in STRESS-L show elevations in both non-survivors and survivors for phosphatidylethanolamine (PE) and related compounds. As with PCs, PEs are biosynthesised through CDP-pathway, alternatively can be synthesised in the mitochondria and ER through decarboxylation of phosphatidylserine as evidenced by Shiao *et al.* (1995). Indicated by Vance and Tasseva (2013), PE is essential for glycosylphosphatidylinositol (GPI)-anchor cell-surface signalling and Tasseva *et al.* (2013) also noted deficiency in phosphatidylethanolamine modifies Mt morphology and OxPhos functionality – particularly impairing the membrane integrity and integration of respiratory complexes, predominantly I and IV. They report studies have demonstrated even minor decreases in PE has profound consequences on Mt functionality. Further, and important in this cohort, PE has important roles in response to cardiac ischemia, with Musters *et al.* (1993), showing after extended periods of ischemia had led to destabilisation of lipid bilayer and irreversible damaged observed. Whilst the data obtained here cannot confirm a casual association between decreased PC and an increase in PE in non-survivors, a potential relationship is suggested. Nonetheless, with increased respiratory leakiness and dysfunction associated more with non-survivors, it suggests that the lower concentrations of PE(18:1(11Z)/20:1(11Z)) is an important indicator. This metabolite had the greatest AUROC value of 0.921 as predictor of mortality. Intriguingly, Phosphatidylglycerol(16:1(9Z)/18:3(9Z,12Z,15Z)), a metabolite elevated in survivors is considered essential for the functionality of respiratory chain, plasticity and signal transduction (PubChem: 52927236) and is a major influencer of the cardiolipin biosynthesis

pathway, further supports the concept that in non-survivors the metabolic dysregulations are key drivers in mortality and lack of energy production.

Among the other observed metabolites, elevations in Furfurylamine and Phenylcarbamic acid were both seen in non-survivors. The former metabolite is a known hepatotoxin and has roles associated with cholinergic signalling. Research by Giebelen *et al.* (2009) noted cholinergic agents (notably agonists) were found to increase TNFα and IFNγ and PubChem CID: 3438 has indicated furfurylamine with the onset of toxic pneumonitis. With increases in TNFα and IFNγ noted in non-survivors from immune analysis also, this suggests this metabolite in higher concentrations drives hepatic damage and systemic inflammation. Similarly, Phenylcarbamic acid is associated with neurological and cholinergic messaging, and higher concentrations of its parent compound (carbamic acid) is associated with neurotoxicity and onset of ataxia in hens (Fisher and Metcalf, 1983). Carbamic acid has been associated with (and possibly conjugated with) a secondary amine of sertraline (Tremaine *et al.*, 1989) and Shaffer *et al.* (2005) noted it's further involvement in γ-aminobutyric acid type-A receptor (GABAa) receptor. We did not investigate the association of these compounds with neurological complication of critical illness such as delirium or Critical Illness Weakness. Another metabolite, also higher in non-survivors, 3-Hydroxy-2H-pyran-2-one, has roles in controlling blood brain barrier and tight junction permeability of primary choroid plexus cells (Aerts *et al.*, 2015), as an inhibitor of this metabolite attenuated this increased permeability.

1,2-Ethanediyldicarbamodithioic acid – considered by nature a fungicide, which was found elevated in non-survivors has been demonstrated by Chung *et al.* (2019) using its main metabolite breakdown (ethylenethiourea) induces renal functional and structural damage in mice, combined with modulating neuronal properties – including neurotoxicity and

neurodegeneration through voltage-gated K+ channels (Li *et al.*, 2013a). Fungal overgrowth is a feature of septic shock in some patients which may represent the relative immunosuppression induced to resolve the pro-inflammatory state. Our findings may represent a response to this in non-survivors.

In summary, metabolomic analysis has indicated non-survivors are particularly vulnerable to disorder in the respiratory chain and general signalling, are sensitive to neurotoxicity and possibly onset of delirium before the disease is incompatible with life. Due to time limitation in the thesis, metabolomics could not be separated by survival status first and then treatment similarity to the analysis conducted in immune and cardiac biomarkers. Overall, metabolomic data suggests markers associated with mitochondrial metabolism will be good prognostic discriminators in the "mild" SOFA score category. Due the possible challenges of incorporating metabolic function into the SOFA score, a retrospective single-centre study run by Goutay *et al.* (2021) looked to combine SOFA score with metabolic failure, age and comorbidity assessment (MACA) to produce a SOFA-MACA score, which saw better mortality prediction than SOFA scoring alone. The results presented here, highlighting the metabolic potential in mild-SOFA patients may show better stratification with the SOFA-MACA model; and subsequently should be investigated further.

Conclusion

The trial reporting of increased mortality in the interventional arm could be a chance-finding and the study would have greatly benefitted from increased sampling opportunities. Landiolol appears to offer a degree of cardiac protection not observed in standard care, particularly against pressor dosage, refractory tachycardia and varying arterial pressors. Landiolol also appeared to act as an anti-inflammatory, though a rapid increase in IL-10 on initiation of treatment, and in particular early timepoints, is associated with greater mortality and possibly could act as an extremely early indicator to terminate β-blocker treatment. Cardiac troponin-I is was strongly associated with mortality in the Landiolol cohort. Finally, whilst metabolomic analysis in this thesis did not demonstrate separation by treatment initially, results did provide novel lines of enquiry in the mild-SOFA group, highlighting fatality in this group is possibly driven by changes in mitochondrial and metabolic pathways.

Limitations of Current Study and Future Investigations

Study Limitations

Following advice from the Data Monitoring Committee/Trial Steering Group, STRESS-L closed early to recruitment, recruiting 126 patients out of 340, i.e., 37% of target. Whilst 126 patients is still substantial with ~550 patient samples longitudinally, results may be considered underpowered for clinical implementation and clinically-relevant findings presented here will need to be revalidated. Further limitations of this project, due to the time constraints, scope and size of a Masters degree, limited analysis was conducted, and this work should be taken further to explore full outcomes of such rich clinical datasets, immune profiles, cardiac markers and metabolomics.

Further Investigations

Results showed in all cases, phosphatidylcholines were elevated in survivors compared to non-survivors, and further investigations should establish the relationship between Landiolol and PC concentrations. Metabolomic results, whilst not possible in this thesis due to time constraints, should be separated by survival status first (as saw with immune and cardiac results) to establish changes in the cohort. Finally, another line of exploration includes working with a researcher in Cardiovascular Sciences, College of Medical and Dental Sciences, who has developed an organoid model. Plasma samples from STRESS-L could be used to bath organoids and investigate whether Afib for example can be induced in this model.

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Appendix 1 – Further Metabolomic Modelling

(Day Separation by Treatment – Principal Component Analysis)

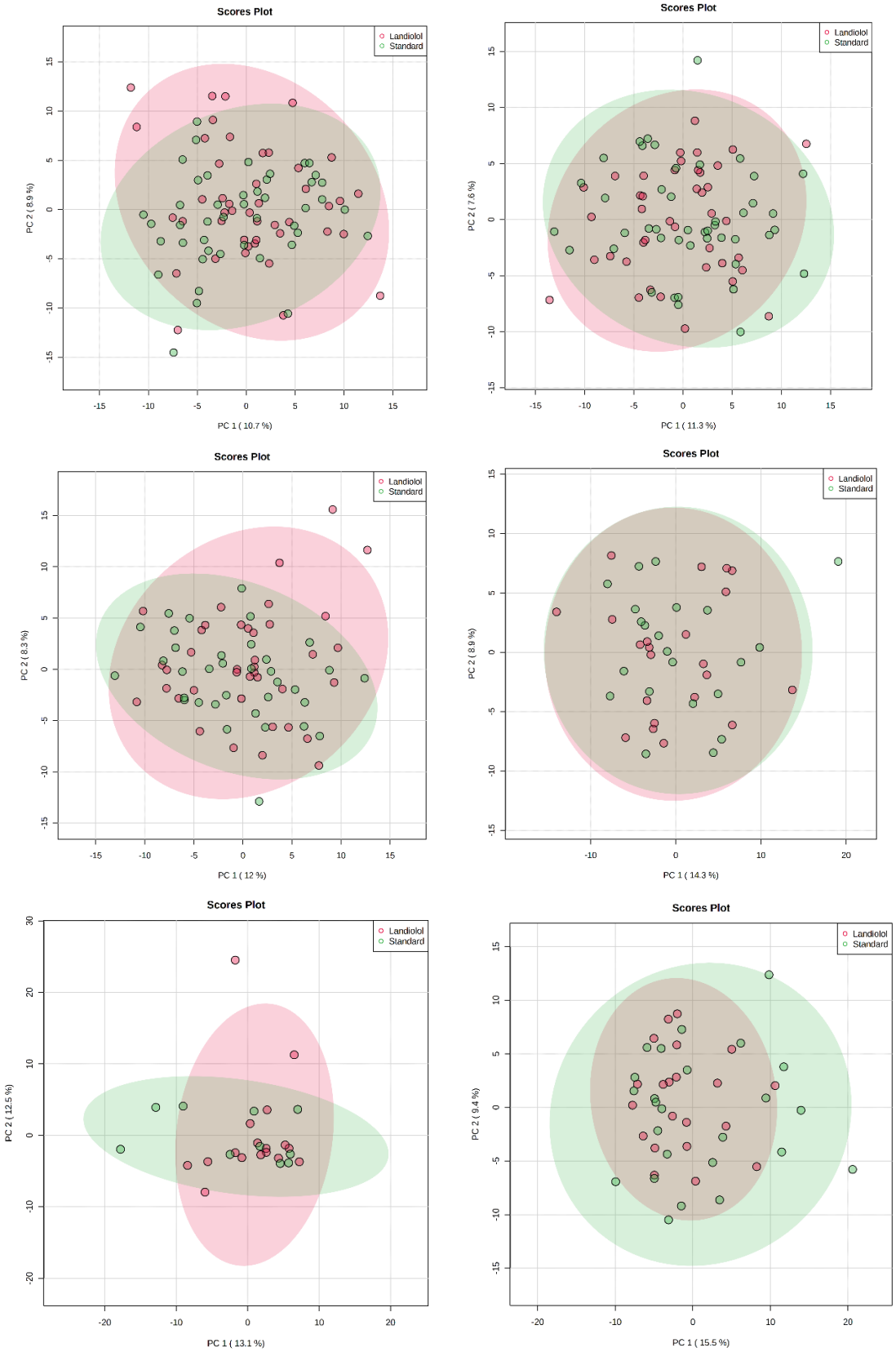


Figure 48: ***Negative Ion Day PCA Plots for Separation by Treatment*** *A) Day 0, B) Day 1, C) Day 2, D) Day 4, E) Day 6 and F) EONT*

(Day Separation by Treatment – Partial Least Squares Linear Discriminant Analysis)

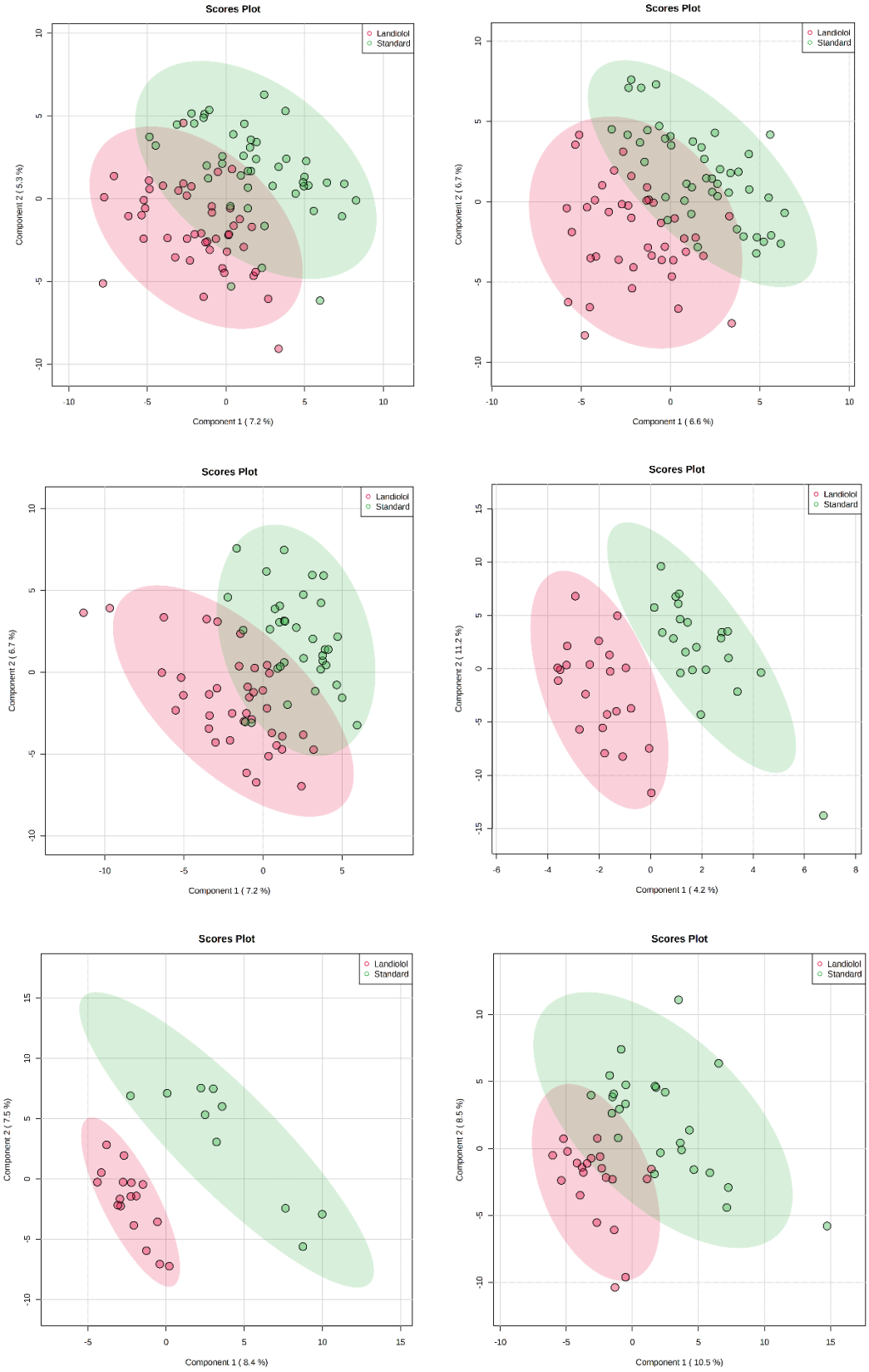


Figure 49: ***Negative Ion Day PLS-DA Plots for Separation by Treatment*** *A) Day 0, B) Day 1, C) Day 2, D) Day 4, E) Day 6 and F) EONT*

(Day Separation by Treatment – Orthogonal Partial Least Squares)

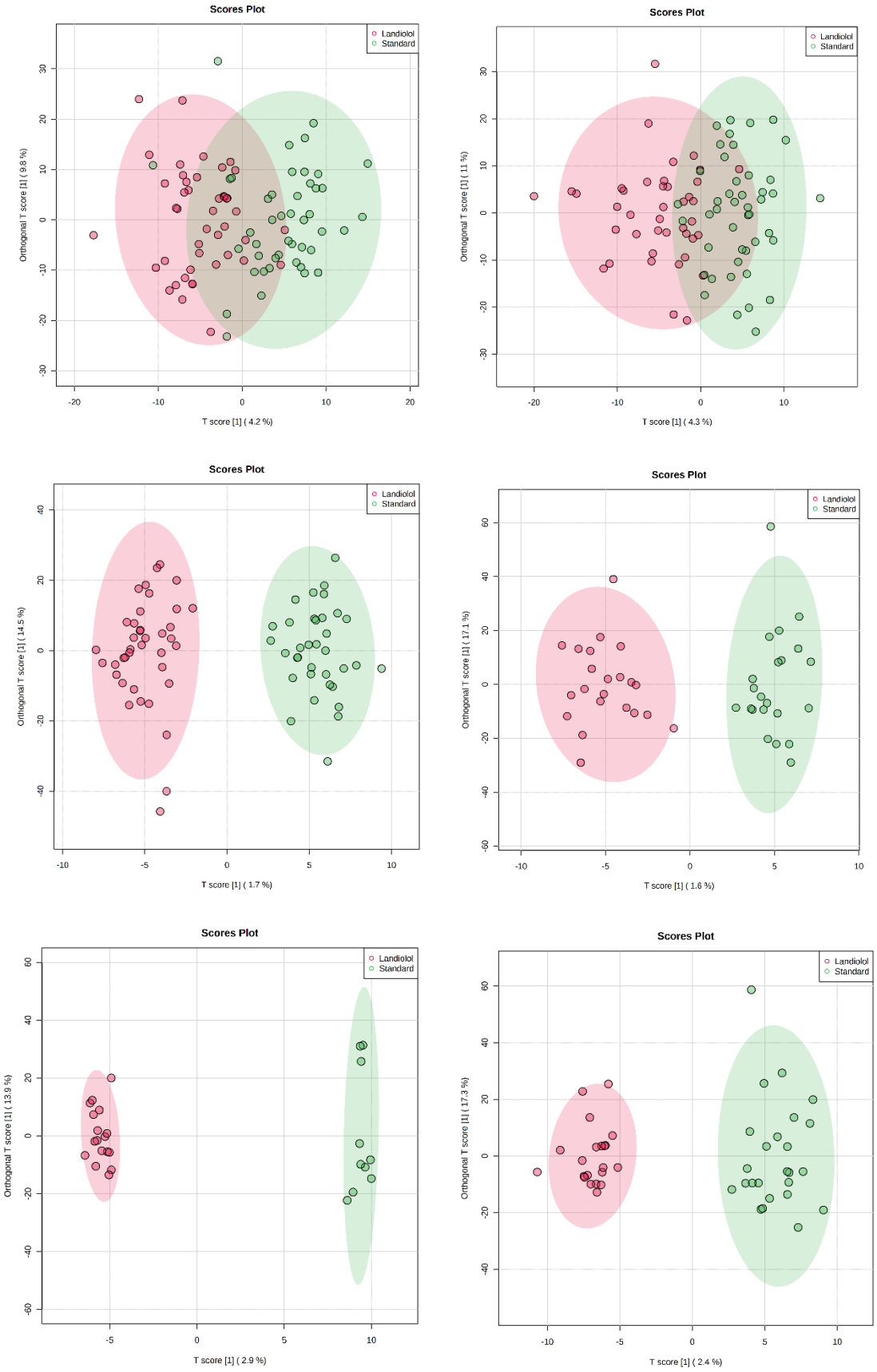


Figure 50: ***Negative Ion Day Orthogonal Partial Least Squares Plots for Separation by Treatment*** *A) Day 0, B) Day 1, C) Day 2, D) Day 4, E) Day 6 and F) EONT*

(Day Separation by Survival – Principal Component Analysis)

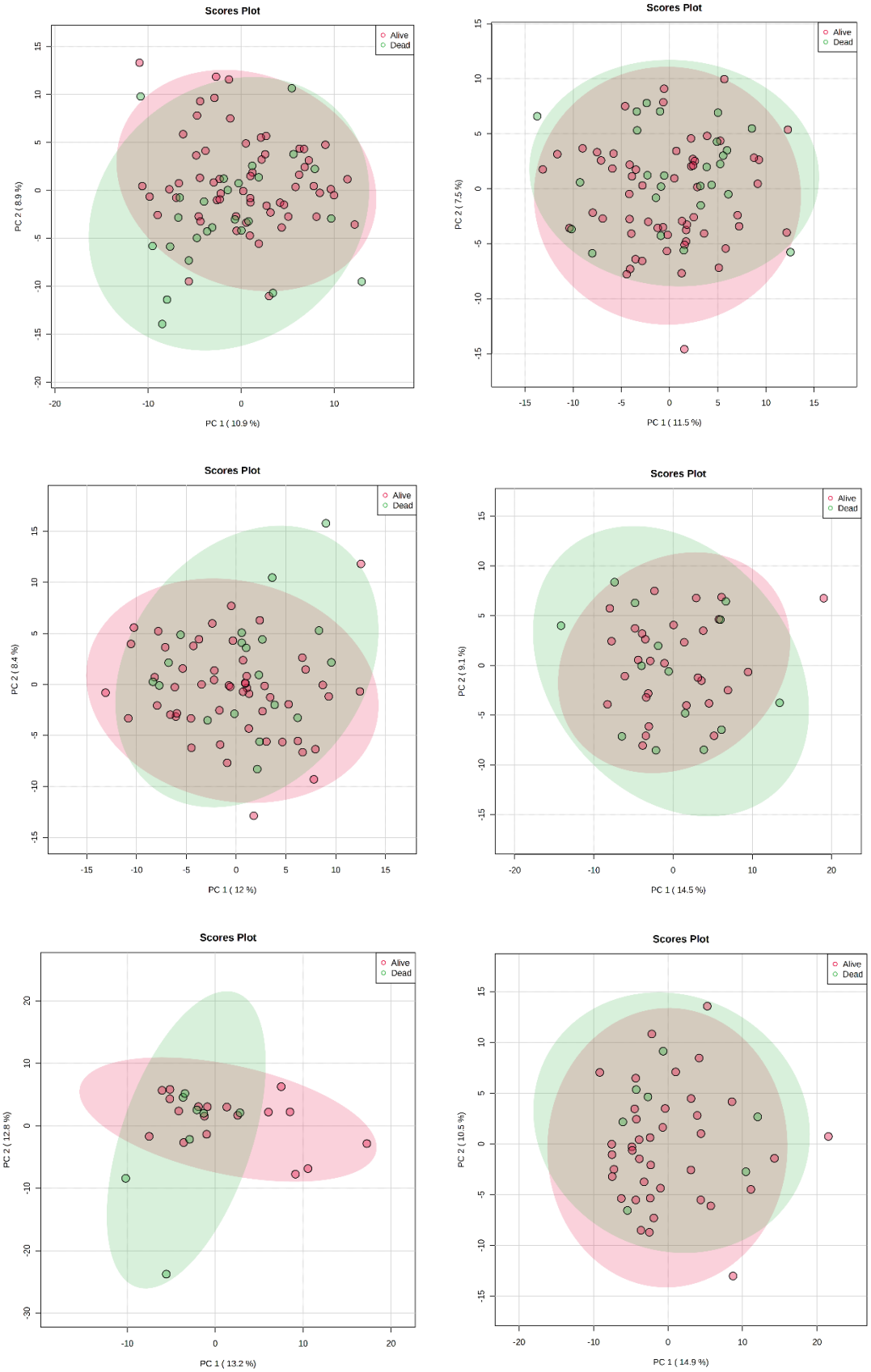


Figure 51: ***Negative Ion Day PCA Plots for Separation by Survival Status*** *A) Day 0, B) Day 1, C) Day 2, D) Day 4, E) Day 6 and F) EONT*

(Day Separation by Survival – Partial Least Squares Linear Discriminant Analysis)

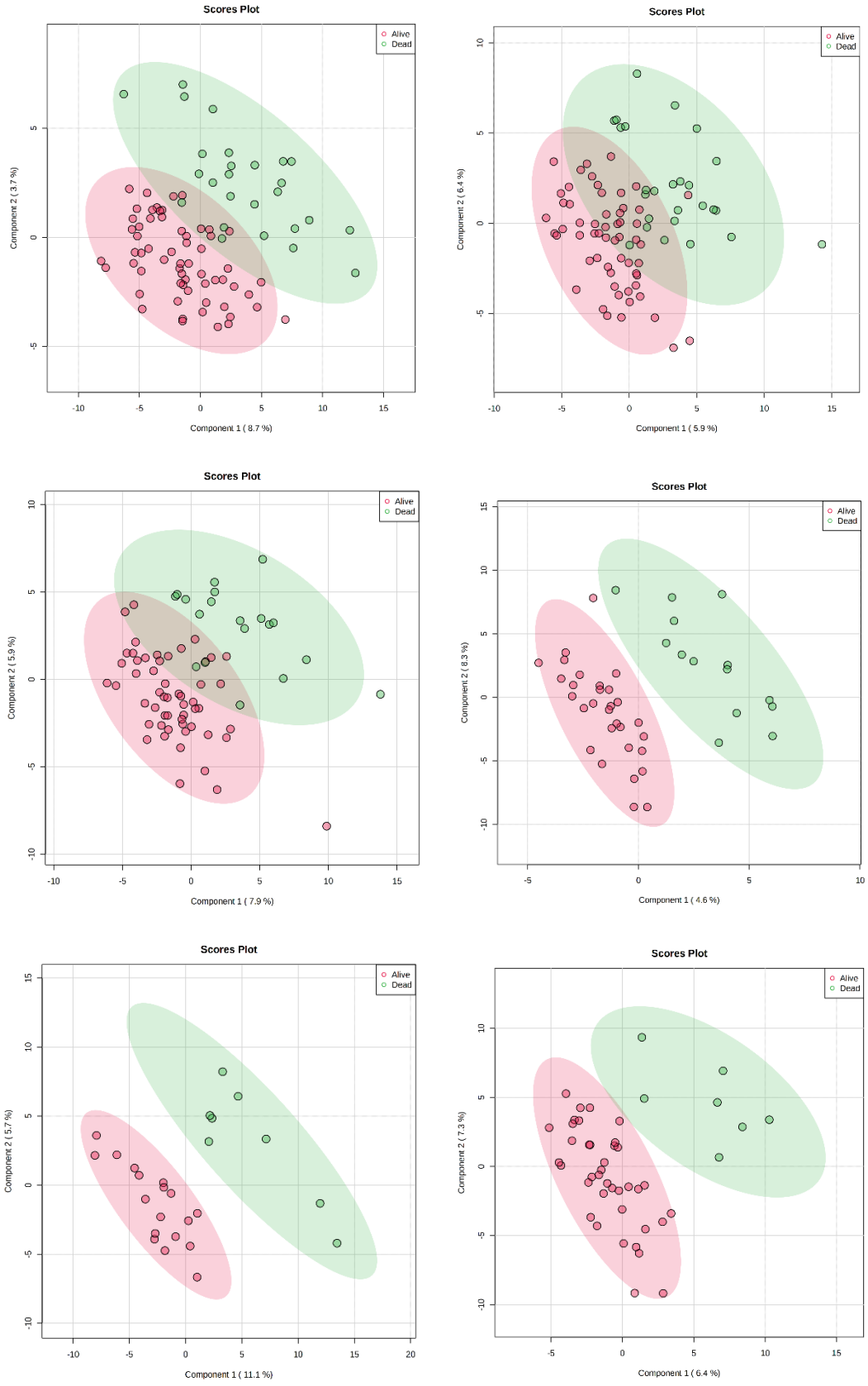


Figure 52: ***Negative Ion Day PLS-DA Plots for Separation by Survival Status*** *A) Day 0, B) Day 1, C) Day 2, D) Day 4, E) Day 6 and F) EONT*

(Day Separation by Survival – Orthogonal Partial Least Squares)

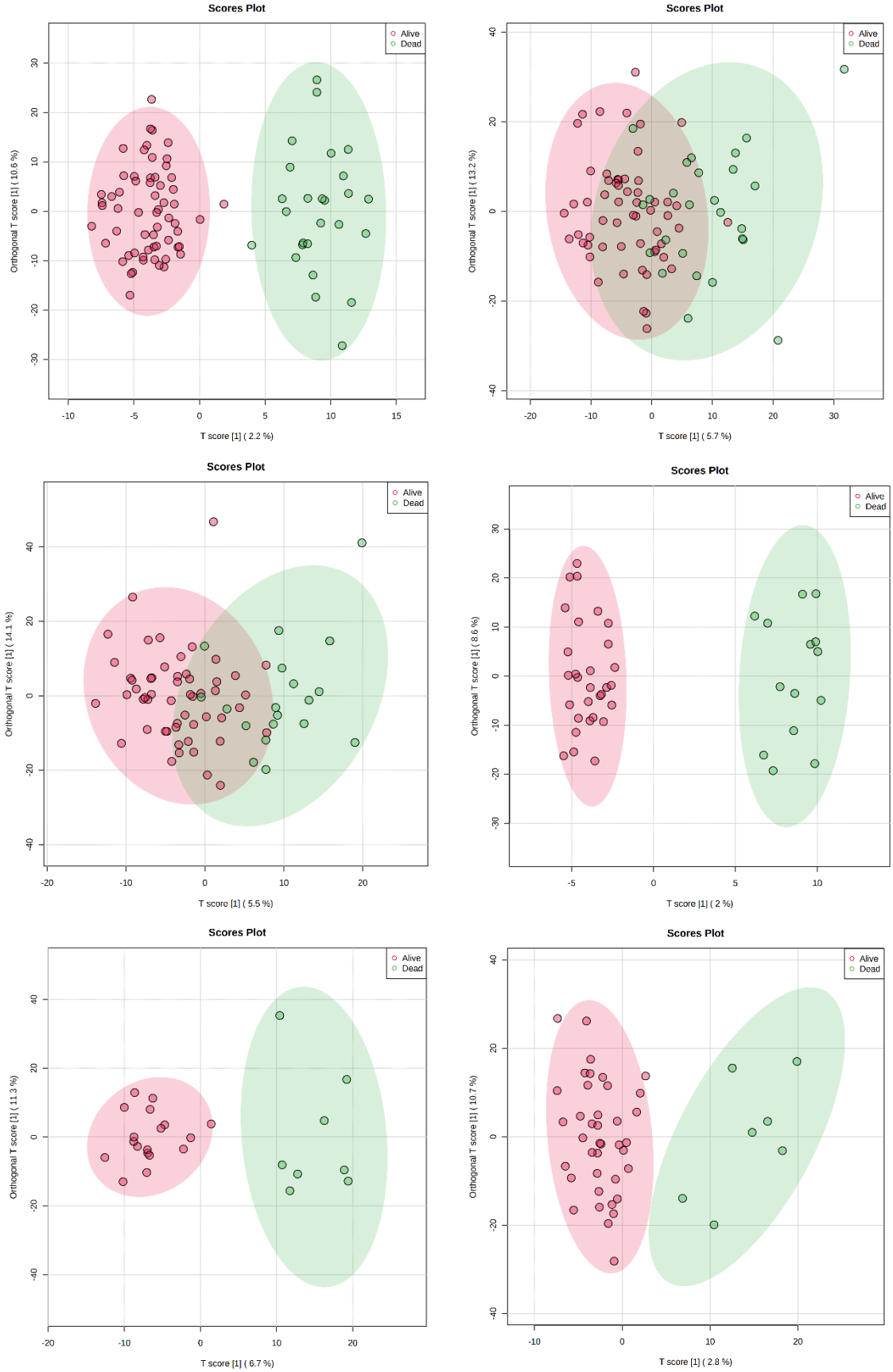


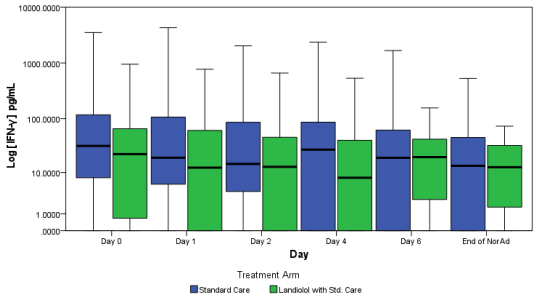
Figure 53: ***Negative Ion Day Orthogonal Partial Least Squares Plots for Separation by Survival*** *A) Day 0, B) Day 1, C) Day 2, D) Day 4, E) Day 6 and F) EONT*

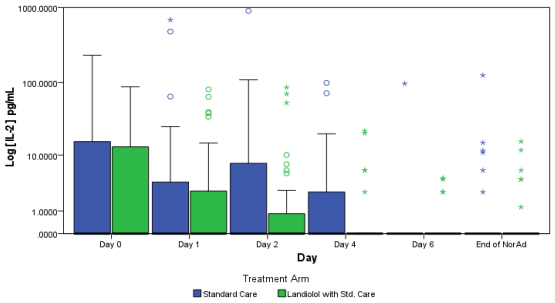
Appendix 2 – List of Annotated Metabolites from Figure 31

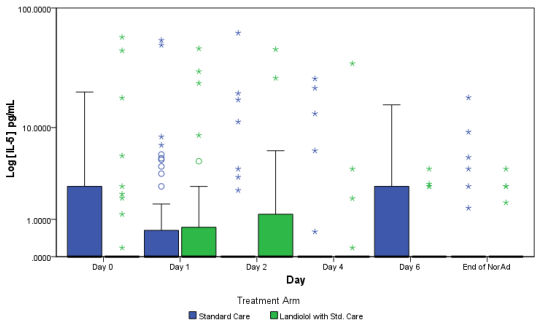
m/z: 86.72788
m/z: 86.83939
m/z: 89.84251
m/z: 252.91699
m/z: 308.87872
m/z: 310.87582
m/z: 366.8374
m/z: 426.7933
m/z: 780.32898
m/z: 780.599
m/z: 781.55548
m/z: 808.68585
m/z: 869.54205
m/z: 924.49994
m/z: 1174.85522
(+)-2,3-Dihydro-3-methyl-1H-pyrrole
(S)-2,3-Dihydro-5-hydroxy-2-methyl-1,4-naphthoquinone
1,2-Di-O-palmitoyl-3-O-(6-sulfoquinovopyranosyl)glycerol
1,2-Ethanediyldicarbamodithioic acid
3-Hydroxy-2H-pyran-2-one
Calcium hydrate
CerP(d18:1/26:0)
Diadenosine triphosphate
Furfurylamine
PC(16:1(9Z)/P-18:1(9Z))
PC(18:1(11Z)/18:1(11Z))
PC(18:1(9Z)/14:0)
PC(18:1(9Z)/16:1(9Z))
PC(18:1(9Z)/18:1(11Z))
PC(18:1(9Z)/18:4(6Z,9Z,12Z,15Z))
PC(18:3(6Z,9Z,12Z)/14:0)
PC(18:3(6Z,9Z,12Z)/22:5(7Z,10Z,13Z,16Z,19Z))
PC(18:3(9Z,12Z,15Z)/20:5(5Z,8Z,11Z,14Z,17Z))
PC(P-18:0/18:3(9Z,12Z,15Z))
PC(P-18:1(9Z)/14:0)
PC(P-18:1(9Z)/18:0)
PE(18:0/20:3(8Z,11Z,14Z))
PE(18:1(11Z)/20:1(11Z))
PE(18:1(9Z)/18:2(9Z,12Z))
PE(18:4(6Z,9Z,12Z,15Z)/20:5(5Z,8Z,11Z,14Z,17Z))
PE(20:4(8Z,11Z,14Z,17Z)/22:4(7Z,10Z,13Z,16Z))
PE(22:4(7Z,10Z,13Z,16Z)/22:4(7Z,10Z,13Z,16Z))
PE(P-18:0/22:1(13Z))
PE(P-18:0/22:4(7Z,10Z,13Z,16Z))
PE(P-18:1(11Z)/20:0)
PE(P-18:1(11Z)/22:0)
PE(P-18:1(11Z)/22:1(13Z))
PE(P-18:1(11Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))
PE(P-18:1(9Z)/22:0)
PG(16:1(9Z)/16:0)
PG(18:1(9Z)/18:1(9Z))
PG(18:1(9Z)/22:4(7Z,10Z,13Z,16Z))
PGP(18:3(9Z,12Z,15Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))
Phenylcarbamic acid
Phosphatidylcholine(18:3(9Z,12Z,15Z)/20:2(11Z,14Z))
Phosphatidylglycerol(16:1(9Z)/18:3(9Z,12Z,15Z))
PI(16:0/16:1(9Z))
PI(18:0/16:0)
PI(18:0/18:0)
PS(20:3(8Z,11Z,14Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))
SM(d18:0/22:3(10Z,13Z,16Z))
SM(d18:1/20:0)
SM(d18:1/22:0)
SM(d18:1/22:1(13Z))
TG(14:0/14:0/14:1(9Z))
TG(14:1(9Z)/14:1(9Z)/14:1(9Z))
TG(16:0/14:0/14:1(9Z))
Triacylglycerol(14:0/18:3(9Z,12Z,15Z)/14:1(9Z))

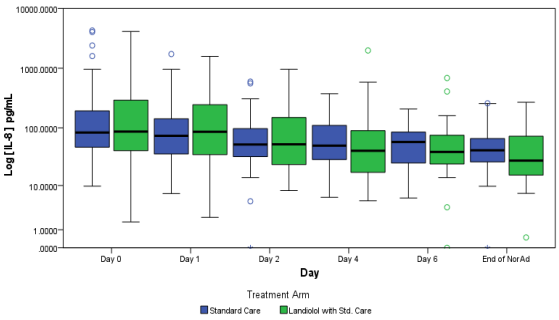
Appendix 3 – Raw Cytokine Concentration Plots, Longitudinal Sampling

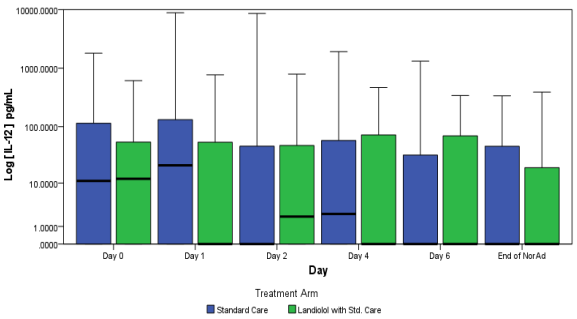
Separation by Treatment Allocation

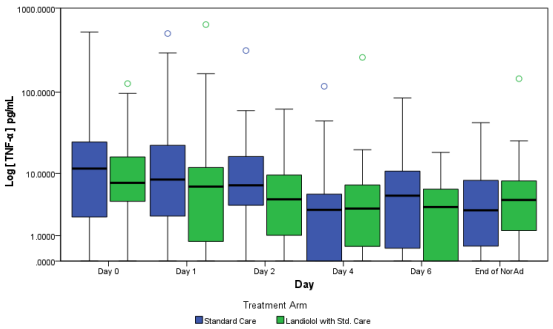


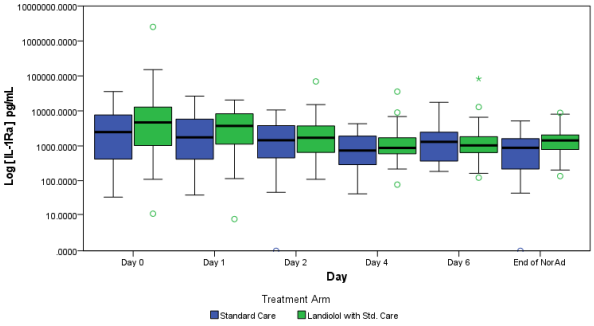


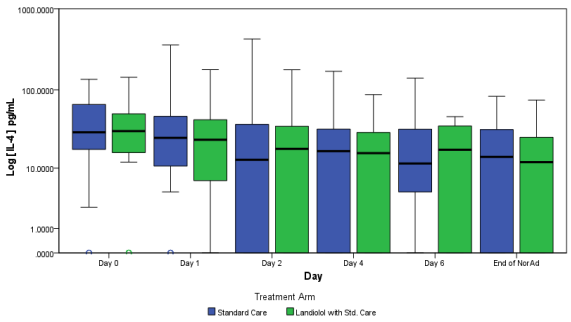


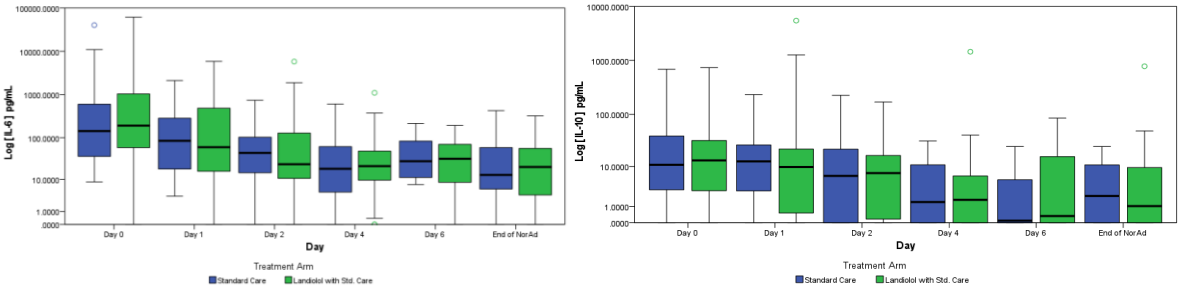


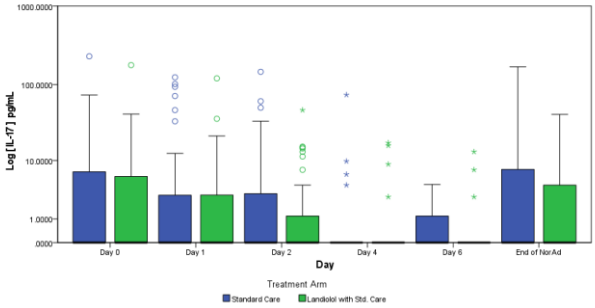




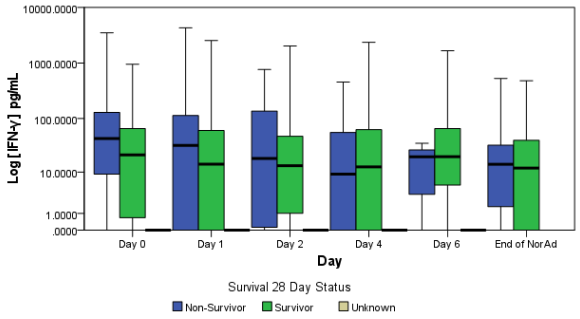


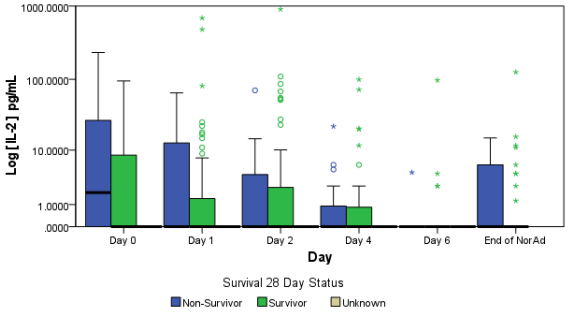


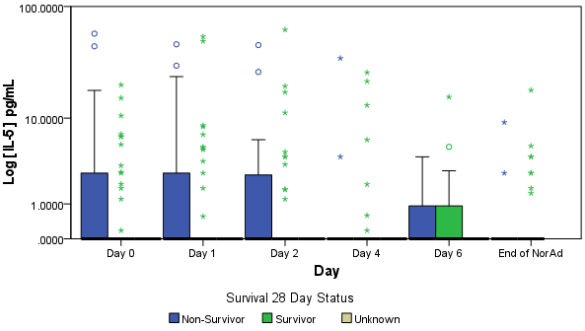


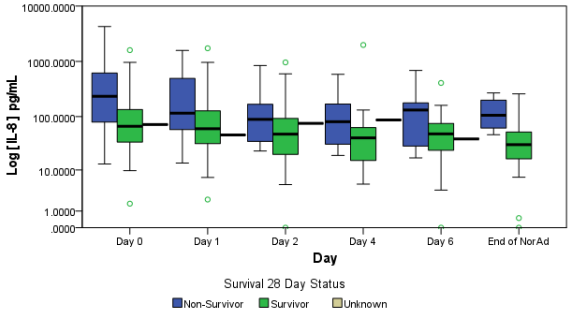


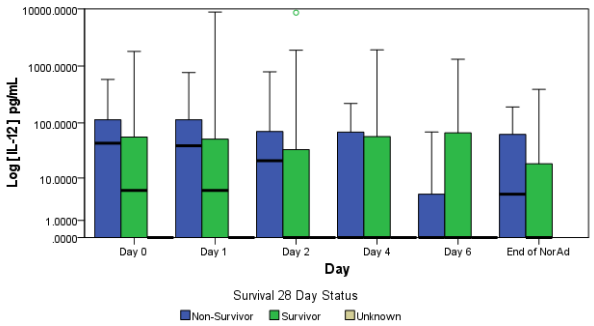
Separation by 28-Day Survival Status










Log [IL-12] pg/mL
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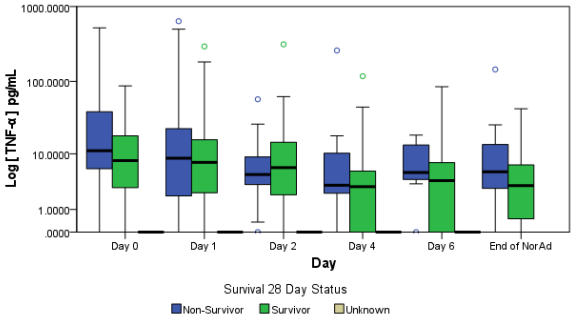
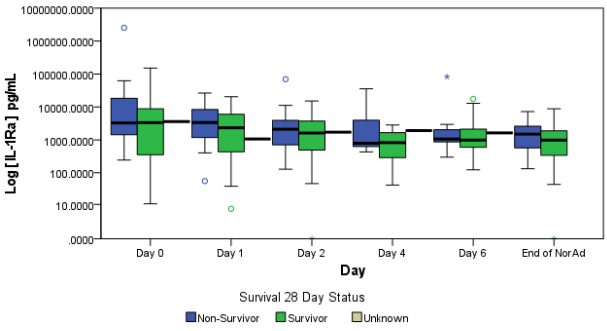
1000.0000
100.0000
10.0000
1.0000
.0000
Day 0
Day 1
Day 2
Day 4
Day 6
End of NorAd
Day
Survival 28 Day Status
Non-Survivor
Survivor
Unknown


Log [TNF-α] pg/mL
1000.0000
100.0000
10.0000
1.0000
.0000
Day 0
Day 1
Day 2
Day 4
Day 6
End of NorAd
Day
Survival 28 Day Status
Non-Survivor
Survivor
Unknown


Log [IL-1Ra] pg/mL
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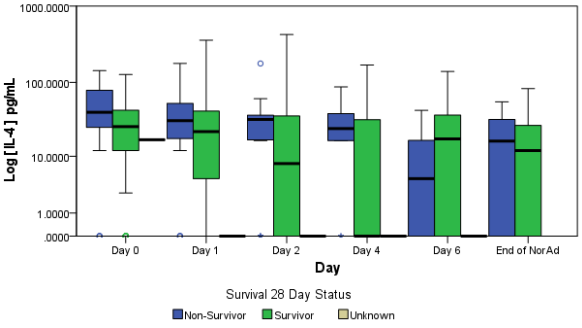
1000000.0000
100000.0000
10000.0000
1000.0000
100.0000
10.0000
.0000
Day 0
Day 1
Day 2
Day 4
Day 6
End of NorAd
Day
Survival 28 Day Status
Non-Survivor
Survivor
Unknown


Log [IL-4] pg/mL
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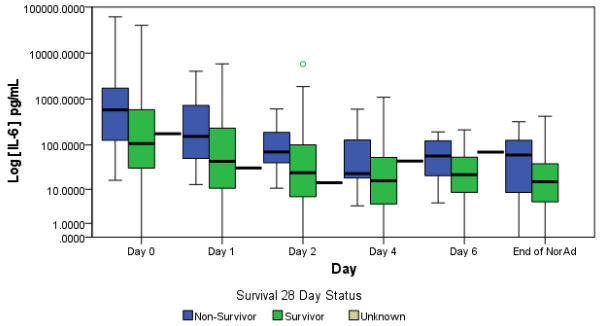
100.0000
10.0000
1.0000
.0000
Day 0
Day 1
Day 2
Day 4
Day 6
End of NorAd
Day
Survival 28 Day Status
Non-Survivor
Survivor
Unknown


Log [IL-6] pg/mL
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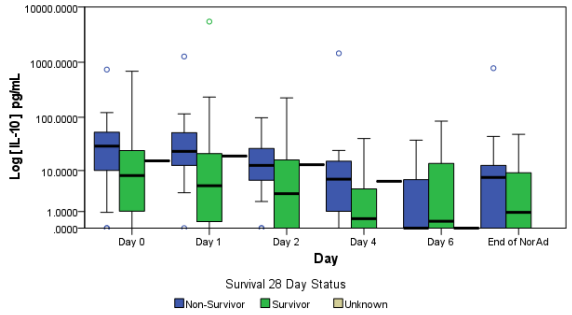
10000.0000
1000.0000
100.0000
10.0000
1.0000
.0000
Day 0
Day 1
Day 2
Day 4
Day 6
End of NorAd
Day
Survival 28 Day Status
Non-Survivor
Survivor
Unknown


Log [IL-10] pg/mL
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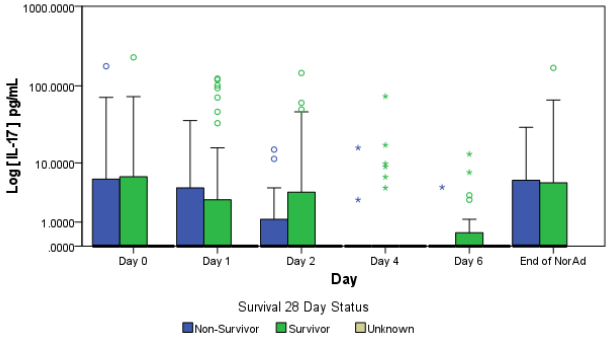
1000.0000
100.0000
10.0000
1.0000
.0000
Day 0
Day 1
Day 2
Day 4
Day 6
End of NorAd
Day
Survival 28 Day Status
Non-Survivor
Survivor
Unknown


Log [IL-17] pg/mL
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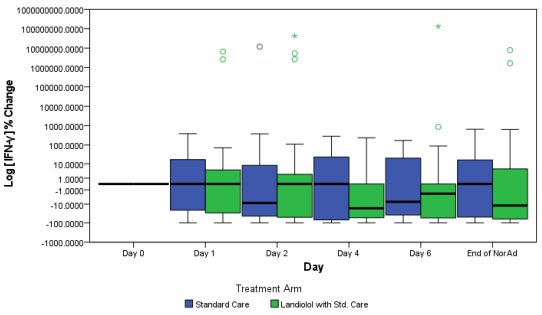
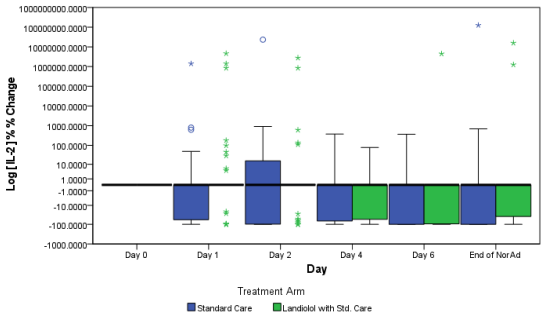
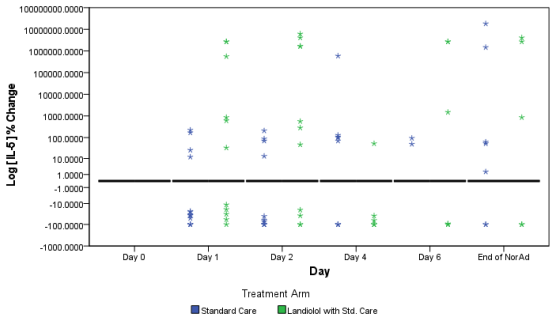
100.0000
10.0000
1.0000
.0000
Day 0
Day 1
Day 2
Day 4
Day 6
End of NorAd
Day
Survival 28 Day Status
Non-Survivor
Survivor
Unknown

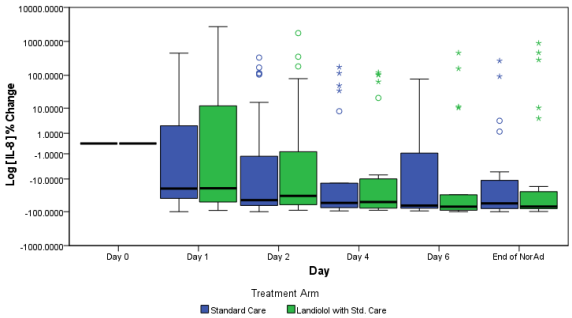
Appendix 4 – Percentage Change of Cytokine Concentration Relative to Baseline (Day 0) Plots, Longitudinal Sampling

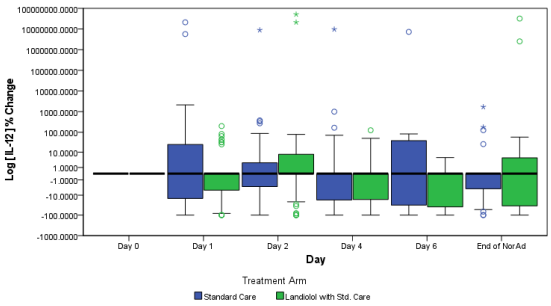
Separation by Treatment Allocation

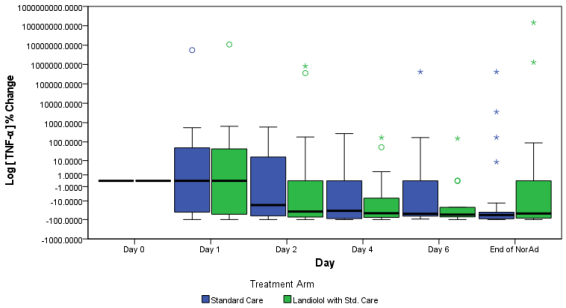


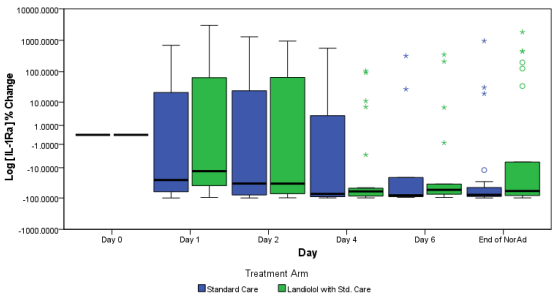


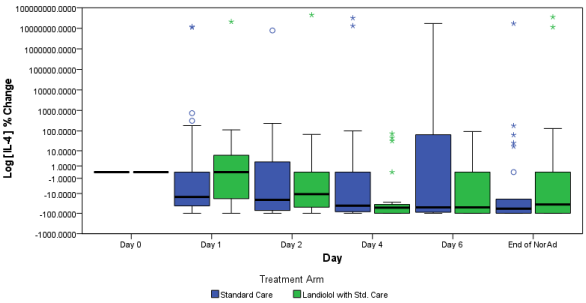


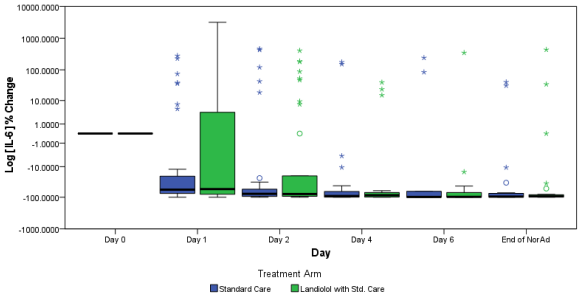


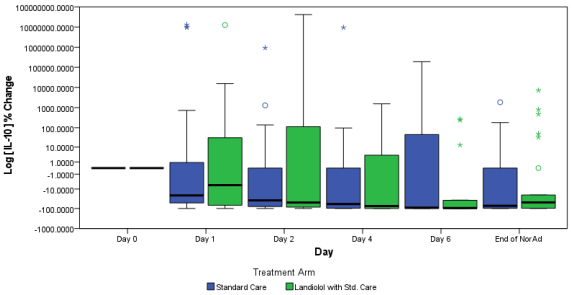









Log [IL-6] % Change
10000.0000
1000.0000
100.0000
10.0000
1.0000
-1.0000
-10.0000
-100.0000
-1000.0000
Day 0
Day 1
Day 2
Day 4
Day 6
End of NorAd
Day
Treatment Arm
Standard Care
Landiolol with Std. Care


Log [IL-10] % Change
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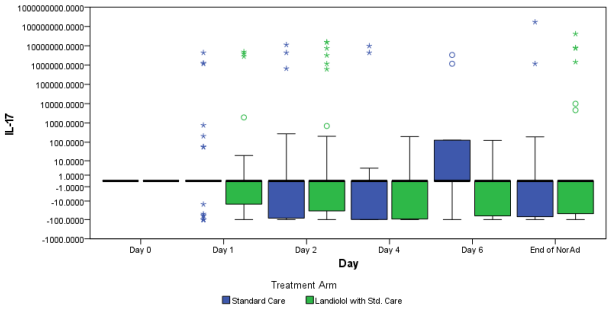
10000000.0000
1000000.0000
100000.0000
10000.0000
1000.0000
100.0000
10.0000
1.0000
-1.0000
-10.0000
-100.0000
-1000.0000
Day 0
Day 1
Day 2
Day 4
Day 6
End of NorAd
Day
Treatment Arm
Standard Care
Landiolol with Std. Care


IL-17
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100000000.0000
10000000.0000
1000000.0000
100000.0000
10000.0000
1000.0000
100.0000
10.0000
1.0000
-1.0000
-10.0000
-100.0000
-1000.0000
Day 0
Day 1
Day 2
Day 4
Day 6
End of NorAd
Day
Treatment Arm
Standard Care
Landiolol with Std. Care

~ ~ ~ ~