Peripheral mitochondrial function in multiple organ dysfunction syndrome

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Abstract

Multiple organ dysfunction syndrome (MODS) is the predominant cause of death in intensive care units worldwide but treatment remains supportive. Although mitochondrial dysfunction is thought to occur in sepsis related MODS, mitochondrial function in MODS remains poorly understood. An important barrier has been the requirement for organ biopsies to measure mitochondrial function because performing organ biopsies in patients with MODS poses significant risk of fatal bleeding and infection. A peripheral marker of mitochondrial function was therefore required. The hypotheses were that mitochondrial function can be measured from peripheral blood mononuclear cells, changes through the disease course and reflects disease severity in MODS. The aims were to develop an assay of mitochondrial function from peripheral blood and to apply it in patients in MODS through their disease course.

Mitochondrial respiration assay was developed in the laboratory using peripheral blood from healthy volunteers and healthy male Wistar rats. The assay was used in experimental models of hypertension and mild acute pancreatitis and in a clinical trial of mild acute pancreatitis. Finally, peripheral blood mitochondrial function was measured daily during the first week, at three weeks and at six months in patients with MODS.

Mitochondrial function from peripheral blood changed over the course of disease in MODS. Mitochondrial reactive oxygen species increased early and was followed by a decrease in mitochondrial respiration in MODS. Temporal mitochondrial respiration negatively correlated with temporal organ failure scores and mitochondrial respiration did not discriminate between septic and non septic causes of MODS. Mitochondrial respiration and mitochondrial superoxide correlated with each other throughout the first week. There were persistent features of mitochondrial dysfunction in septic MODS at six months.

Multiple aspects of mitochondrial dysfunction occurred in patients with MODS and correlated with the severity of MODS. The results imply that shutting down mitochondrial respiration may be an adaptive response in MODS and manipulating mitochondrial respiration in MODS may be beneficial. The results from testing the assay in other disease states enabled a broader understanding of mitochondrial function in MODS. These findings have opened up several avenues for further clinical and laboratory research.

To my husband, Andrew for your support and encouragement

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Abbreviations

ADP	Adenine diphosphate
ANOVA	Analysis of variance
AP	Acute pancreatitis
APACHE	Acute physiology and chronic health evaluation
ARDS	Acute respiratory distress syndrome
ATP	Adenine triphosphate
BP	Blood pressure
BPM	Beats per minute
BSA	Bovine serum albumin
BSL	Blood sugar level
CI	Complex I or NADH dehydrogenase
CII	Complex II or succinate dehydrogenase
CIII	Complex III or coenzyme Q:cytochrome C reductase
CIV	Complex IV or cytochrome c oxidase
CPD	Citrate phosphate dextrose
CS	Citrate synthase
Ct	Threshold cycle
CV	Complex V or ATP synthase
DAMP	Danger associated molecular pattern
DIC	Disseminated intravascular coagulation
DNA	Deoxyribose nucleic acid
Endo	Endogenous respiration
ETS	Electron transport system
FCCP	Trifluorocarbonylcyanide phenylhydrazone
FCR	Flux control ratio
G3P	Glycerol 3 phosphate
GpDH	Glycerol 3 phosphate dehydrogenase
HMGB-1	High mobility group box protein 1
HV	Healthy volunteers
ICU	Intensive care unit
IL-6	Interleukin-6
JC-1	Tetraethyl benzimidazol carbocyanine iodide
LSD	Least significant difference
MD	Mitochondrial dysfunction
MF	Mitochondrial function
MiR05	Mitochondrial respiration buffer
MODS	Multiple organ dysfunction syndrome
MR	Mitochondrial respiration
mtDNA	Mitochondrial DNA
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NFκβ	Nuclear factor kappa B
NICA	Normothermic ischaemic cardiac arrest
NO	Nitric oxide
NS	Patients with MODS unrelated to sepsis

OF	Patients with MODS
OXPHOS	Oxidative phosphorylation
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PDH	Pyruvate dehydrogenase
RANOVA	Repeated measures analysis of variance
RCR	Respiratory control ratio
ROS	Reactive oxygen species
S	Patients with MODS related to sepsis
S4o	State 4 respiration induced by oligomycin
SAPS	Simplified acute physiology score
SEM	Standard error of mean
SF-36	Short form 36
SHR	Spontaneous hypertensive rat
SIRS	Systemic inflammatory response syndrome
SOFA	Sequential organ failure assessment
SST	Serum separating tube
SUIT	Substrate uncoupler inhibitor titration protocol
TCA	Tricarboxylic acid cycle
TISS	Therapeautic intervention scoring system
TNFα	Tumour necrosis factor alpha
WBC	White blood cell

Chapter 1 Multiple organ dysfunction syndrome in critical illness

The leading cause of death in critical illness is multiple organ dysfunction syndrome (MODS) and managing MODS by supporting vital organs is the core business of the intensive care unit. MODS is the unwanted outcome of successful shock resuscitation (1) and develops only when patients live long enough after resuscitation from their initial injury. Hence, MODS was first described after significant improvements were made in the area of shock resuscitation. In 1973, Tilney (2) described eighteen patients who required treatment with haemodialysis for acute renal failure after repair of ruptured abdominal aortic aneurysms. Seventeen out of the eighteen patients eventually died with a mean survival time of eighteen days post operation. Tilney made two important observations: i) that patients in the study died from failure of organs which were initially not directly injured and, ii) there was a lag of days or weeks between initial events and development of distant organ failure (2).

Soon after, in 1975 Baue made the important observation that primary injuries of different aetiologies can result in MODS (3). Baue reported three patients who all died from MODS despite the primary injury being an anastomotic leak in the first patient, severe acute pancreatitis in the second patient and replacement of aortic and mitral valve in the third patient. Now, it is widely recognised that MODS can be the consequence of a diverse range of clinical conditions including trauma, acute pancreatitis, burns, sepsis and shock (4) (5). The two most common clinical predisposing factors for MODS is sepsis and shock, but processes that induce major inflammation without infection such as acute pancreatitis can also culminate in MODS. Baue highlighted that the sequence of events or the causative factors of MODS was not understood.

One of the significant factors that hindered progress in understanding of MODS in the 70s and 80s was a lack of unifying terminology. MODS has had many names in the last four decades: multiple systems failure, progressive systems failure, sequential systems failure, multiple organ failure (6, 7). The word “failure” was the common thread amongst these various terminologies and implied that organ failure is an all or none process. By contrast, in the last two decades it has become understood that organ failure is not an all or none phenomenon, but rather a range of organ dysfunction which leads to overt organ failure. So, in 1992, the term multiple organ dysfunction syndrome or MODS was developed at a consensus conference by the Critical Care Society and American College of Chest Physicians

(8). MODS better describes the continuum of changes that occur in more than one organ system following a significant insult such as trauma, burns, infections, aspiration, multiple blood transfusions, pulmonary contusion and pancreatitis (6).

At that consensus conference, MODS was defined as the presence of altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention (9). Clinically, a number of scores such as sequential organ failure score (SOFA) (10) or multiple organ dysfunction score (11) are used to stratify the level of organ dysfunction. These scores are obtained from easily available laboratory or physiological markers for each organ. The organ systems used for these scores are: respiratory, cardiovascular, renal, hepatic, gastrointestinal, haematological, endocrine and central nervous system.

Major interest in mitochondrial pathophysiology of MODS has arisen in the last decade. The four most important reasons for this has been the high incidence of MODS amongst patients with critical illness, the poor prognosis of MODS in this patient group, two decades of research interventions based around inflammation pathophysiology of MODS that have failed to improve outcomes of MODS and the revelation that mitochondrial dysfunction occurs in patients with MODS.

1.1 Growing incidence of multiple organ dysfunction syndrome in critical illness

Reporting the incidence of MODS is fraught with difficulty because of the use of numerous different terminologies for MODS in the last 40 years and the fact that MODS arise from many different insults. MODS has been estimated to play a role in up to 80% of all deaths in the intensive care unit (12). MODS is a major cause of morbidity in intensive care units and develops in about 15% of all intensive care unit (ICU) admissions (13). Furthermore, as more patients survive the initial insult with the improvement of initial resuscitation and advances in diagnostics and perioperative care, the incidence of MODS may be rising.

The incidence of MODS following surgery and trauma has been well described. In 1980, Fry et al.. described 553 patients from a surgical emergency unit and found that 7% had MODS with a mortality of 74% in patients that developed MODS (14). Similarly, in the early 1990s, Baue reported the incidence of MODS to be 4% in a surgical ICU with 49% mortality (15). Later on, in 1996 Moore studied trauma patients with an injury severity score >15 and found that 15% developed MODS with a mortality rate of 36% (16). Moore noted a bimodal distribution of mortality of MODS with 39% dying early of MODS and 61% dying late of MODS (16). In 2000, Offner et al.. reviewed the temporal trends of MODS incidence and

mortality over 5 years in a trauma centre in Denver and found the incidence of MODS to be 17% with a mortality rate of 37% (17). It is difficult to compare incidence of MODS between different intensive care units as the case mix in each unit may differ making comparisons hard to interpret.

To address this, researchers have reported incidences of MODS in the same ICU at different time spans. Zimmerman compared outcomes of patients in ICU between 1979-82 and 1988-90 and found an identical proportion (14%) developed MODS (18). The overall hospital mortality rate from MODS was unchanged during that time period. Pfeiffer et al. reviewed causes of death following trauma and found the incidence of MODS following trauma remained largely unchanged with the median being 8.7% in the 1990s and 6.5% in the 2000s (19). Pfeiffer stated that MODS is still one of the predominant causes of late deaths (>1 week) in patients with trauma. Regel et al. compared the decades 1972-81 with 1981-92 and found that the incidence of MODS had increased from 15.4 % to 28.2% and mortality from MODS increased from 13.8% to 18.6% (20).

1.2 Poor prognosis of MODS in critical illness

The main determinants of severity of MODS are the number of organs that failed and the duration of the organ failure (21). In sepsis, using the SOFA score, mortality increases from <20% when one organ fail to >80% when four organs fail (10) (Figure 1-1). Similarly, in trauma patients, mortality was 4.3% in single organ system failure, 32% with two, 67% with three and 90% with four (12). Also, the length of time the patient is in organ failure has a direct correlation with mortality rate (21) (Table 1-1 H). In patients with one organ failure, the mortality rate almost doubles from 22% on the first day to 41% on the seventh day (18).

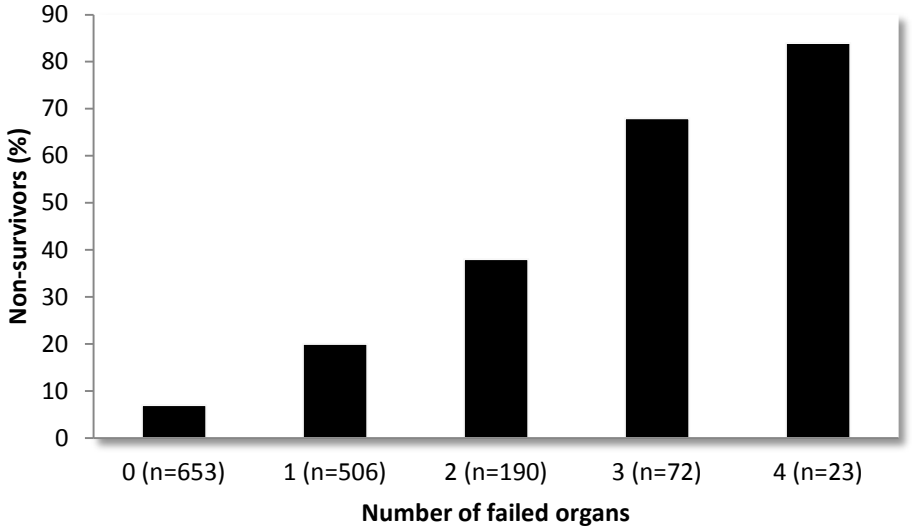


Figure 1-1 Relationship between number of organs failed and percentage of non-survivors in each group adapted from Vincent **et al.** (10).

		Day of failure						
Number of organ dysfunction		1	2	3	4	5	6	7
		% died	% died	% died	% died	% died	% died	% died
1	1979-1982	22	31	34	35	40	42	41
	1988-1990	23	36	42	36	37	42	42
2	1979-1982	52	67	66	62	56	64	68
	1988-1990	52	74	70	64	68	65	65
≥3	1979-1982	80	95	93	96	100	100	100
	1988-1990	85	94	94	87	86	87	89

Table 1-1 Hospital mortality according to the number and duration of organ system failure for 2405 ICU admissions during 1979-1982 compared to 7703 ICU admissions during 1988–1990 adapted from Knaus et al. (21). OSF [Organ system failure].

1.3 Summary of research interventions in MODS

The majority of research in MODS in the last three decades have occurred in the context of sepsis induced MODS. In the first decade, it was believed that the immune system was up-regulated in sepsis induced MODS and therefore trials of new therapies aimed to down

regulate the immune system (Table 1-2). None of these interventions decreased mortality and some increased mortality compared to placebo (Table 1-2). On the basis of these failed trials, Bone et al. (8) hypothesised that the development of sepsis induced MODS has several stages each of which has a pro and anti inflammatory component.

Table 1-2 Failed clinical trials of drugs in sepsis adapted from Bone et al.. (22)

Agent
Methylprednisolone
Glucocorticoid
E5 monoclonal antibody to endotoxin
Human monoclonal antibody HA-1A
Monoclonal antibody to human tumor necrosis factor alpha
Monoclonal antibody
Dimeric receptors
Recombinant human interleukin-1 receptor antagonist
Platelet activating factor receptor antagonist BN 52021

There are now over 100 randomised controlled clinical trials of therapies of sepsis and MODS largely based around the concept of dysregulation of the immune system (23). Despite the large number of trials, the outcomes have been equally poor. No specific therapy has led to improved outcomes in MODS (22-24) and none of these therapies are approved to be used clinically. These failed trials have prompted the research community to rethink the basis upon which these therapies were based. The fact that the pathophysiology of MODS remains incompletely understood has become increasingly obvious and now thought to be one of the main reasons for the failure of these trials.

1.4 Mitochondrial pathophysiology in MODS

One of the least understood aspect of pathophysiology in MODS is mitochondrial function. Mitochondria are cellular organelles that use over 90% of our inspired oxygen and generate the energy currency of the cell, adenine triphosphate (ATP). In 2002, Brealey et al. (25) analysed mitochondrial function from skeletal biopsies and reported mitochondrial

dysfunction in patients with sepsis and MODS. On the basis of largely experimental data and limited clinical data, mitochondrial dysfunction is thought to be a key cellular event in the development of MODS in critical illness (26, 27). However, there remains large gaps in our understanding of mitochondrial function in MODS: only MODS related to sepsis was studied; temporal mitochondrial function in MODS was not explored; different aspects of mitochondrial function was not correlated; whether mitochondrial dysfunction is reversible in the long term is unknown.

1.5 Conclusion

MODS occurs in 15% of patients in intensive care units and is the leading cause of death in intensive care units worldwide. Despite decades of research, there is no approved clinical therapy that alters outcomes in MODS. These reasons, along with incomplete understanding of the pathophysiology that leads to MODS (Chapter 2), are responsible for the growing realisation that understanding mitochondrial pathophysiology in MODS may lead to better understanding of the disease process and may help to prognosticate and treat MODS in the future.

Chapter 2 Pathophysiology of MODS

The pathophysiology of MODS is complex and may be likened to a complex dynamic non-linear system involving a large number of variables that are highly interdependent (28). The variables that will be discussed in this chapter are inflammation, apoptosis, neurohumoral, coagulation, macro/micro circulation dysfunction and specifically mitochondrial dysfunction. Some of these variables have been highly researched such as inflammation, whereas, others such as mitochondrial function in MODS is still incompletely understood. The objective of this chapter is to summarise what is widely known about each of these factors and highlight mitochondrial function in MODS.

Almost any acute disease process can act as the inciting event in MODS (Table 2-1). The inciting event may be a pathogen in the case of an infection or any process that invokes an injury to the host's tissue such as trauma/burns/severe acute pancreatitis. MODS is usually progressive and follows a predictable clinical pattern. The first organ that shows signs of dysfunction have universally been reported to be the lung (Table 2-2). In contrast, the second and third organs that are usually affected are either the liver or the kidney (Table 2-2). Myocardial and hematologic failures usually occur later and central nervous system alterations can occur either early or late. The pattern can differ depending on the patient and the clinical situation. For example, in a patient with myocardial infarction, the heart may well fail earlier. Regardless of the pattern of organ failure, as more organs fail the likelihood of death increases.

Table 2-1 Diverse inciting events that may result in MODS adapted from Oh's Intensive care. (29)

Sepsis
Non sepsis
Burns
Trauma
Haemorrhage
Acute Pancreatitis
Ischaemic perfusion injury
Aspiration
Extracoroporeal circulation (cardiac bypass)

Eclampsia
Poisoning/toxicity
Heat-induced illness
Multiple blood transfusion
Autoimmune diseases

Table 2-2 Patterns of organ failure described in MODS.

Author	**Year**	**First**	**Second**	**Third**
Baue (3)	1975	Lung	Kidney	Liver
Border (30)	1976	Lung	Heart	Liver
Cerra (31)	1980	Lung	Liver	Kidney
Fry (14)	1980	Lung	Liver	Intestine
McMenamy (32)	1981	Lung	Liver	Heart
Goris (33)	1985	Lung	Liver	Intestine
Deitch	1993	Lung	Liver	Intestine

The observation that organs involved in MODS are not necessarily the ones injured in the inciting event and there may be a lag of days to weeks between the inciting event and development of MODS suggests that MODS is a systemic process mediated by circulatory factors. Even though MODS may occur after a range of diverse inciting events, the clinical signs at presentation are similar with aberrations in temperature, respiratory rate, heart rate and white cell count. These signs are collectively known as the systemic inflammatory response syndrome (SIRS) and are associated with the proinflammatory response.

2.1 Proinflammatory response

The inflammatory response to the inciting event becomes dysregulated in MODS. The proinflammatory response is mediated by pathogen associated molecular patterns (PAMPs) from bacteria/fungi (35) or danger associated molecular patterns (DAMPs) from damaged host tissue (36). PAMPs/DAMPs bind pattern recognition receptors in innate immune cells to activate them (37). The activated immune cells produce a myriad of proinflammatory and anti-inflammatory cytokines. Increased levels of cytokines such as interleukin 6 (IL-6) or tumour necrosis factor alpha (TNF-α) are associated with organ dysfunction and increased

risk of death in sepsis (38-40). Cytokines interact with the immune, endocrine and nervous systems to mediate host defence and tissue repair (41). These responses include enhanced phagocytic activity, increased expression of inducible nitric oxide synthase and production of nitric oxide which leads to vascular endothelial injury with capillary leak, synthesis of acute phase proteins by the liver, chemotaxis of leukocytes to site of infection/inflammation, activation of the coagulation system (42) and myocardial depression.

The inflammatory cytokines are modulated by intracellular cell signalling molecules that bind to the pattern recognition receptors and are able to control production or activation of pro and anti inflammatory molecules. Nuclear factor kB (NF-kB) and high mobility group box protein -1 (HMGB-1) are examples of intracellular cell signalling molecules. NF-kB is a co-factor involved in the transcription of genes that encode inflammatory proteins, programmed cell death (apoptotic) signalling pathways and nitric oxide production (43). NF-kB levels are increased in sepsis in proportion with severity. Similarly, plasma HMGB-1increase in sepsis in proportion with severity (44). HMGB-1 potentiates binding of inflammatory molecules to inflammatory cells and also acts as a nuclear co-factor that enhances deoxyribose nucleic acid (DNA) transcription of inflammatory mediators (45).

2.2 Hypoinflammatory response

It has been observed that patients often also develop a hypoinflammatory response (46). There is now consensus that the inciting event may evolve into two phases, a hyperinflammatory response and a hypoinflammatory response (22). For ease of understanding, it is usually conceptualised that the hypoinflammatory response follows the hyperinflammatory response. But, in reality the two phases can occur simultaneously or occur several times in the same patient (47). The dysregulated hypoinflammatory response is often a feature of MODS and it increases the host's susceptibility for secondary infections such as ventilator associated pneumonia and opportunistic infections (48). The hypoinflammation is due to a variety of defects in the immune system including a dysfunctional adaptive immune system (49). The adaptive immune system undergoes programmed cell death called apoptosis culminating in a reduction in lymphocytes (50, 51). Hypoinflammation also involves decreased cytokines upon monocyte stimulation (52, 53), decreased number of human leukocyte antigen presenting receptors (54, 55) and expression of cytokines that suppress TNF-α expression (56).

In the last three decades, the inflammatory response in sepsis was thought to be the most important cause for mortality in sepsis and therefore most randomised clinical trials of sepsis

targeted this response. Despite seventy randomised clinical trials, there are no therapeutic interventions in current clinical use (23).

2.3 Apoptosis

Apoptosis is programmed cell death and occurs in the lymphoid organs, gut and vascular endothelium in sepsis and MODS (57) and a detailed discussion of the role of apoptosis in sepsis and MODS is beyond the scope of this thesis. Several reviews have been published highlighting the role of apoptosis in sepsis (46) and MODS (58).

Briefly, there are two main pathways involved in the induction of apoptosis. The extrinsic pathway is the death receptor mediated pathway involving caspase 8 and may be activated by cytokines such as TNF-α. The intrinsic pathway is the mitochondria mediated pathway involving caspase 9 and may be activated by ROS, chemotherapeautic agents and radiation. Caspase 8 and 9 are both capable of activating caspase 3 which is a crucial apoptotic protease involved in the final common pathway of the apoptotic cell death program. It is likely that both the extrinsic and the intrinsic pathway are involved in apoptosis (46).

Apoptosis may also be induced through glucocorticoids and granzymes (59). Proinflammatory molecules undergo cleaving by caspases and induce apoptosis of the adaptive immune system culminating in marked lymphocytopaenia (60). Apoptosis of cells leaves the patient susceptible to further insults due to immunoparalysis(61). The two main mechanisms that apoptosis can cause immunoparalysis is by decreasing the numbers of B and T cells and thus impairing the adaptive immune response and by inducing anergy in the helper T cells that survived (46).

Several animal studies have shown blockade of apoptosis to be effective sepsis therapies. When Bcl 2 was overexpressed, mice were three times more likely to survive as controls (62). Similarly, inhibition of CD95 (63) and inhibition of caspase (64) also decreased mortality in caecal ligation puncture models of sepsis. Despite these successful experimental therapies, none have been tested clinically and none are in routine clinical use at present. .

2.4 Gut

The gut has been hypothesised to be the “motor” of MODS (4). It was proposed that the enteric microorganisms provide an inflammatory focus during MODS. During critical illness and shock, the mucosal lining of the bowel is disturbed due to inflammation and microcirculation alterations (65). This may lead to translocation of the enteric organisms

followed by invasion and infection. Gut derived bacteria induce inflammatory molecules and perpetuate the inflammation. Although decontamination of the digestive tract has been studied in many clinical trials, it is not in routine use in Australia and New Zealand (66, 67).

2.5 Neurohormonal

The stress response in critical illness involves production of hormones by the adrenal gland. The adrenal gland is responsive to the hypothalamic secretion of the corticotrophin releasing hormone under direct neural control and is modulated by circulating cytokines. Inadequate hypothalamic-pituitary-adrenal response to critical illness or glucocorticoid resistance may contribute to MODS. Numerous studies of corticosteroids have failed to show any benefit and in some studies, corticosteroids have increased mortality compared to placebo (68-70). Additionally, changes to thyroid metabolism occur in critical illness. There is overproduction of inactive reverse tri-iodothyronine instead of the active tri-iodothyronine. The fall in active tri-iodothyronine is proportional to the severity of MODS and predicts mortality (71). Despite a fall in active tri-iodothyronine, treatment with thyroxine has shown no mortality benefit in critical illness (72).

2.6 Coagulation

Coagulation plays an important role in sepsis and MODS (73, 74). Tissue factor is the major initiator of the coagulation system but endotoxin, foreign bodies, negatively charged particles may also initiate coagulation (75). Tissue factor forms a complex with factor VIIa which then activates factor X and IX. Factor Xa binds to Va to make prothrombinase which converts prothrombin to thrombin. Thrombin enhances platelet adhesion, activates platelets and factors V, VIII and XI which are present in platelets which ultimately culminates in massive amplification of thrombin on the platelet surface. With mild inflammatory reactions, thrombin is produced at the site of infection or injury to isolate infectious agent and initiate tissue repair. Coagulation is controlled by anti-coagulants such as anti-thrombin III and protein C. Anti-thrombin III bind to thrombin and endothelium, releases prostacyclin and inhibits platelet aggregation whereas protein C forms a complex with protein S to inhibit factor Va and VIIIa. In sepsis and MODS, levels of anticoagulants are decreased and there is a dysregulation of coagulation which results in thrombin deposits, disseminated intravascular coagulation (DIC) and further propagation of proinflammatory molecules and adhesion mocecules (76). The adhesion molecules in the vasculature attract activated leukocytes and neutrophils (77). These produce lytic enzymes and oxidative stress leading to macro/microcirculatory and organ dysfunction (78). Despite wide knowledge of coagulation

pathways in sepsis, clinical trials targeting coagulation pathways have failed to show mortality benefit in sepsis and MODS. Activated protein C has now been taken off the market because clinical trials failed to show any benefit in sepsis and MODS (24, 79).

2.7 Macrocirculation

Vascular tone is often reduced in MODS resulting in a state of shock and decreased delivery of oxygen to vital organs/cells. Excessive nitric oxide is one of the well-characterised mechanisms responsible for the circulatory shock (80). Nitric oxide is produced in the vascular endothelium by inducible nitric oxide synthase by inflammatory mediators (81). Whilst nitric oxide is toxic to microorganisms and acts as an inflammatory signal (82), excessive nitric oxide relaxes vascular smooth muscle and results in venodilation, low systemic arterial pressures and altered blood flow into vital organs (83). However, clinical therapeautic studies of inhibitors of inducible nitric oxide synthase or antagonists of nitric oxide have increased mortality in septic shock (84).

2.8 Microcirculation

As part of this uncontrolled inflammatory response, coagulation response and increased nitric oxide production, the vascular endothelium is damaged (75). There is widespread fibrin deposition, development of tissue exudates composed of aggregated leukocytes and platelets, reduced red blood cell deformability and increased blood viscosity (85). The result is microvascular occlusion and an increase in microvascular permeability leading to interstitial oedema which compounds tissue and cell oxygenation further by restricting oxygen diffusion (86) (87). Decreased sublingual microcirculatory flow in patients with sepsis has been shown to be associated with organ failure and death (88).

2.9 Mitochondrial dysfunction

Over the past decade, it has become known that there is inadequate oxygen utilisation in MODS and cells are unable to use the oxygen that is delivered to them (27). This is based on the observation that non survivors of septic shock demonstrate an impaired capacity to increase tissue oxygen consumption despite adequate oxygen delivery (89),(90, 91). Since mitochondria are the organelles that use over 90% of the oxygen available to cells (92) (93), there may be an acquired derangement in mitochondrial respiration which is called cytopathic hypoxia (27). Mitochondrial dysfunction (MD) is now thought to be a key cellular event in the development of MODS in critical illness (26, 27).

2.9.1 Mitochondrial respiration

Mitochondria are subcellular organelles (Figure 2-1) whose predominant function is to exchange chemical energy into the cellular currency adenine triphosphate (ATP). Mitochondrial respiration describes the breakdown of nutrients such as carbohydrate, fat and protein into ATP. The majority of cellular ATP (~90%) is formed oxidatively by the process of oxidative phosphorylation within the densely packed inner mitochondrial membranes (94). Oxidative phosphorylation (OXPHOS) couples the electron transport system (ETS) to the phosphorylation system to produce ATP using oxygen as the terminal electron acceptor (Figure 2-2) (95). OXPHOS in the inner membrane of the mitochondria provides the most efficient cellular energy supply. 1g of glucose yields 32 mol of ATP through OXPHOS whereas, 1g of glucose only yields 4 mol of ATP anaerobically (27).

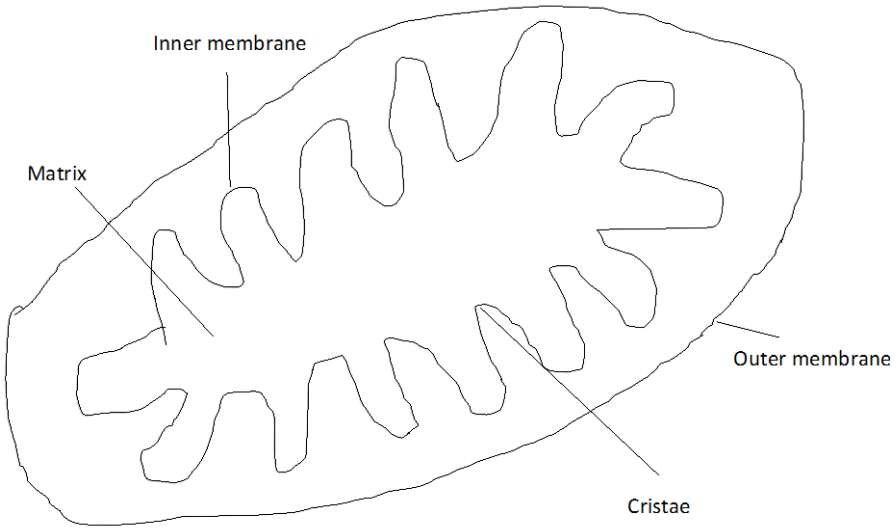


Figure 2-1 The structure of the mitochondria.

2.9.2 Electron transport system

The entire process takes place in the inner membrane of the mitochondria (Figure 2-2). The ETS comprises four main protein complexes named CI (NADH:ubiquinone oxidoreductase or NADH dehydrogenase), CII (succinate dehydrogenase), CIII (Coenzyme Q: cytochrome *c* oxidoreductase) and CIV (Cytochrome *c* oxidase). Other important respiratory complexes include the electron transfer flavoproteins 1-7 which are involved in lipid oxidation and located on the matrix surface of the inner mitochondrial membrane and glycerol 3-phosphate dehydrogenase (GpDH) located on the outer surface of the inner mitochondrial membrane (96), (97), (98).

Substrates from the mitochondrial matrix supply oxidizable fuels to CI, CII and electron transfer flavoproteins which all pass electrons to CIII by ubiquinone. GpDH is supplied with electrons from the cytosolic side of the inner mitochondrial membrane. Ubiquinone is oxidised by CIII and electrons are then passed to CIV, which reduces oxygen to water.

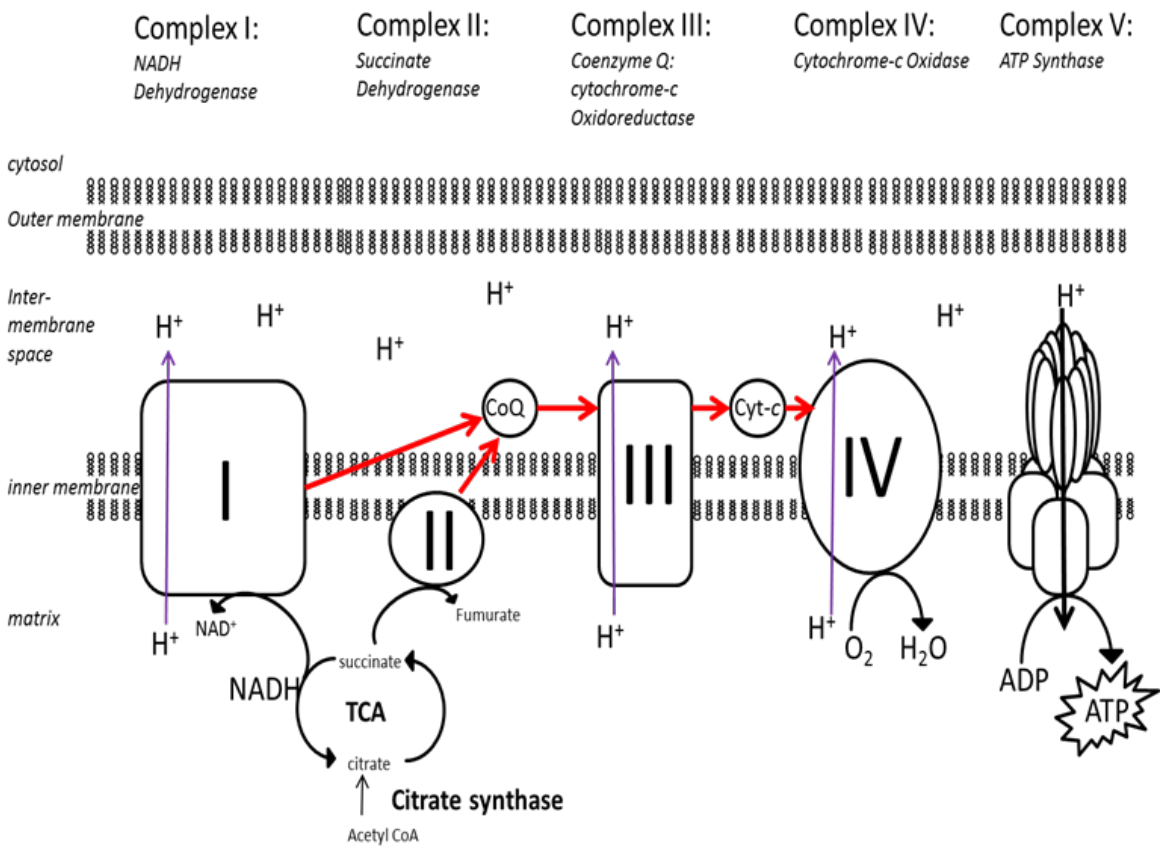


Figure 2-2 The Electron Transport System on the inner membrane of mitochondria. Reducing equivalents from the Tricarboxylic Acid Cycle (TCA) feed into Complex I and II. Electron transport is depicted with red arrows ending in Complex IV which reduces molecular oxygen. Proton translocation is depicted by purple arrows, producing a proton gradient in the intermembrane space driving ATP production by the F_1/F_0 ATP Synthase (CV).

2.9.3 The proton motive force

In this process only CI, CIII and CIV pump protons from the matrix to the inter membrane space. In each case, a drop in redox potential of the electron whilst passing through the complex is coupled to the extrusion of protons into the inter membrane space (99). Ten protons are extruded for each electron pair passing from CI to oxygen or six protons for each pair of electron passing from CIII to oxygen whilst no protons are extruded by CII (100), (101). This generates a proton motive force which then drives ATP production by proton re-entry through CV, the F_1/F_0 ATP Synthase. Under steady state condition the protons extruded into the intermembrane space and protons re-entering the matrix exactly balance. Apart from CV, there are also other ways that protons from the intermembrane space can re-enter the

mitochondrial matrix (102). These processes are referred to as ‘proton leak’ and are dominant in the absence of ATP synthesis. Proton leak serves an important purpose in limiting the electromotive force and restrict leakage of single electrons from the electron transport chain to form superoxide (103), (104).

2.9.4 Other mitochondrial function

However, energy supply is not the mitochondrion’s sole role. These organelles are also involved in modulating oxidative stress, critical metabolic cycles, calcium regulation, cell cycle regulation and apoptosis which is of great importance in pathological settings (94, 105, 106). Consequently mitochondrial dysfunction not only initiates apoptosis when intracellular homeostasis is disturbed but may also drive necrosis when ATP production is impeded.

2.9.5 Mitochondria and inflammation

Mitochondria plays an important role in inflammation and over the last decade a large body of work has emerged on this topic. However, a full discussion on this topic is beyond the scope of this thesis. In brief, several mitochondrial molecules have been implicated in inflammation. Some of these molecules are mitochondrial deoxyribose nucleic acid (mtDNA), cytochrome *c*, ATP, reactive oxygen species (ROS) and mitochondrial N formyl peptides (107).

MtDNA was found to be several thousand times higher in plasma of trauma patients compared to healthy volunteers (108). Intra-articular injection of mitochondrial DNA lysates in healthy mice induces arthritis that is mediated by monocytes/macrophages (109). The exact mechanism of how mtDNA activates inflammation is unknown but it has been shown that in neutrophils, mtDNA can phosphorylate mitogen activated protein kinase, which then interacts with toll like receptors (110).

Also, cytochrome *c* released into extracellular fluid leads to accumulation of neutrophils/ macrophages and release of proinflammatory cytokines and chemokines (111). Similarly, extracellular ATP from hepatocytes activates inflamasomes and alerts circulating neutrophils to adhere to liver sinusoids around foci of injury (112) and accumulation of damaged ROS generating mitochondria is capable of activating inflammation (113). ROS acts as signalling molecules to trigger proinflammatory cytokine production. Mitochondrial derived ROS influences transcription of proinflammatory cytokines such as interleukin 6. Finally, byproducts of mitochondrial translation, mitochondrial N formyl peptides interact with G protein coupled receptors of immune cells to provoke downstream inflammatory responses

(114), (115). A detailed review of the role of mitochondria in inflammation has been published by Krysko et al. (107)

2.9.6 Mitochondrial function in sepsis and MODS

Mitochondrial abnormalities were found in sepsis as far back as 30 years ago (116). Even though mitochondrial function in sepsis has been studied for three decades there is considerable variability amongst results of experimental studies. Some studies report increased mitochondrial function whilst others report decreased function or no change (92). These differences may be due to differences in species being studied, differences in the degree of injury or treatment, techniques used to measure mitochondrial function and the timing of mitochondrial function in relation to the timing of the injury. Overall the short term experimental models showed more variable results than the long term experimental models. The long term experimental models mainly showed a decrease in mitochondrial function. In these long term experimental models of septic shock and organ failure, mitochondrial respiration falls, ATP levels decrease and dysfunction of the electron transport system occurs (117). Since ATP is the rate limiting step of cellular metabolism, without sufficient ATP production cellular homeostasis is threatened and this may be observed clinically as MODS.

Clinical studies of mitochondrial respiratory function are relatively sparse in MODS (Table 2-3). These studies have measured mitochondrial respiration in muscle biopsies, organ biopsies, platelets and peripheral blood mononuclear cells in patients with septic shock (25, 118, 119). In studies involving muscle and organ biopsies, only one time point was studied because taking biopsies is an invasive procedure in already compromised patients. Mitochondrial dysfunction was found in leg muscle biopsies (25), liver (119) and intercostal muscle (118) in septic organ failure and was found to correlate with the amount of norepinephrine required in critically ill patients (Table 2-3). Decreased activity in CI was found in both skeletal muscle and liver, and a decreased CIV activity was also found in the liver (119).

Table 2-3 Clinical studies of mitochondrial respiration in sepsis.

Study	Year	Tissue	Number of samples per patient	Timing of sample	Inclusion	Scores used	No. (Patients/ Controls)	Results
Tissues								
Fredriksson	2006	Intercostal and leg muscle	Once	Not specified	Sepsis/septic shock	SOFA	10/10	CS, CI and IV all decreased.
Vanhorebeek	2005	Liver and muscle	Once	After death	Surgical ICU MOFs	APACHE II, TISS 28	18/18	Decreased CI and IV activity in liver in the BSL 10-11 group
Brealey	2002	Skeletal muscle biopsies	Once	Within 24hrs of admission into ICU	Sepsis/septic shock	SAPSII, SOFA	28/9	CI activity inversely correlated with NE, decreased ATP
Peripheral blood								
Japiassu	2011	PBMC	Twice	Within 48hrs of sepsis diagnosis and at day 7	Septic shock	SOFA	20/18	Decrease in State 3 respiration, recovery of RCR at d7
Garrabou	2012	PBMC (Blood)	Once	Not stated	Sepsis without shock	SAPS	19/20	CI,III,IV activity decreased CI O_2 consumption decreased
Sjovall	2010	Platelets (Blood)	Three	Within 48hrs of ICU admission	Severe sepsis/septic shock	SOFA	18/18	ETS increased at day6-7, State 4 respiration increased from day3-4
Belikova	2007	PBMC (Blood)	Once	Within 48 hrs of ICU admission	Sepsis/Septic shock	SAPSII, SOFA	18/32	Endo increased State 3 decreased

Footnote: SOFA [sequential organ failure assessment], APACHE [acute physiology and chronic health evaluation], SAPS [simplified acute physiology score), TISS [therapeautic intervention scoring system], CS [citrate synthase], Endo [endogenous respiration], BSL [blood sugar level], RCR [respiratory control ratio], State 3 respiration [respiration with substrates and adenine diphosphate]

2.9.7 The need for a peripheral marker of mitochondrial function

Mitochondrial respiratory function has been measured in animal tissues in experimental models and muscle or organ biopsies have been used in patients in clinical studies. The measurement of mitochondrial respiratory function by tissue biopsy is invasive and carries significant associated risks of haemorrhage, neurovascular damage, infection and associated anaesthetic risks. Moreover, tissue biopsies are generally painful and cannot be repeated at frequent intervals to understand temporal pathophysiology of MODS or to monitor progression of MODS in the clinical setting.

More recently, decreased State 3 mitochondrial respiration function has been described in peripheral blood mononuclear cells (PBMC) within 48 hrs of ICU admission in patients with septic shock (Table 2-3). PBMC are a subset of peripheral white blood cells (WBC) comprising lymphocytes and monocytes and are easily accessible from peripheral blood and contain mitochondria (120). In the circulation, these cells are exposed to all organ systems and may also therefore provide insight to general mitochondrial health in acute illness settings. PBMC are part of the inflammatory response which plays a significant role in the systemic inflammatory response in MODS. Lipopolysaccharide is an endotoxin found in gram negative bacterial cell wall and is found in serum from patients with sepsis and acute pancreatitis. Lipopolysaccharide stimulates circulating monocytes and releases proteins, free radicals and lipids and triggers the development of systemic inflammatory response (121). Whilst sepsis is associated with an increased expression of the CD14 receptor and toll like receptors compared to controls, death in sepsis is associated with down regulation of these receptors compared to patients that survived (122). In MODS, the severity of systemic inflammatory response is an important determinant of ICU survival (123). Given that mononuclear cells are intricately involved in the inflammatory response in MODS, it is plausible that measuring mitochondrial respiration from PBMC will give a further insight into pathophysiology of MODS.

Since undertaking the studies in this thesis, further two studies have reported PBMC respiration in sepsis/septic shock and found decreased State 3 mitochondrial respiration with the CII substrate succinate in septic shock (124), and decreased endogenous respiration and State 2 respiration with CI along with decreased CI, CIII and CIV activity in sepsis with no decrease in mitochondrial content as measured with citrate synthase (125). Additionally, platelet mitochondria have also been reported to uncouple in sepsis (126). Although informative, these studies have left several aspects of mitochondrial function in MODS

unanswered. Firstly, only MODS related to sepsis was studied, second, temporality of mitochondrial respiration was not fully explored, third, different aspects of mitochondrial function was not correlated and lastly, what happens to mitochondrial function in the long term after patients are discharged from hospital is not known. Therefore, what is required is a peripheral mitochondrial function assay which will measure PBMC mitochondrial function in patients with MODS over time and determine whether it correlates with severity of MODS.

2.10 Conclusion

Traditionally, research on MODS focussed on understanding inflammation. Decades of research and over 100 clinical trials of therapy in septic MODS failed to improve outcomes (23). Whilst a lot is known about inflammation in MODS, the role of mitochondria in MODS remains ill understood with several fundamental gaps in our knowledge.

Clearly, addressing these fundamental gaps in our knowledge is necessary to delineate the future potential for using mitochondrial function for prognosis and treatment in MODS. Since PBMC are crucially involved in MODS and can be easily obtained repeatedly from patients, PBMC may be used to understand mitochondrial function. In the next chapter, peripheral white blood cell mitochondrial respiratory function in health will be summarised and peripheral white blood cell mitochondrial respiratory function in disease states will be summarised systematically.

Chapter 3 White blood cell mitochondrial respiratory function in disease states

3.1 Introduction

In the last chapter, pathophysiology of sepsis and MODS was discussed and fundamental gaps in mitochondrial pathophysiology in sepsis and MODS were highlighted ending with the possibility of measuring mitochondrial function from white blood cells. Since mitochondrial dysfunction is involved in many common disease pathologies, this chapter will systematically review existing methodology employed to measure mitochondrial respiration from white blood cells in various disease states.

Mitochondrial dysfunction is not only found in acute diseases such as sepsis and MODS, but also in chronic diseases such as diabetes, hypertension, Alzheimer's, Parkinson's, many cancers, obesity and epilepsy. Given that mitochondrial dysfunction play a role in the pathophysiology of many disease states, use of various means of detecting mitochondrial dysfunction in the clinical setting is likely to be increasingly important.

In both acute and chronic diseases, mitochondrial function has most commonly been studied using tissue biopsies from skeletal muscle and organs. In patients with septic shock, CI activity from muscle biopsies correlated with amount of norepinephrine required to maintain mean arterial blood pressure (25). When CI activity of the electron transport system (ETS) was measured spectrophotometrically in patients with Parkinson's disease, CI activity was found to be decreased in muscle (127), (128) and brain biopsies(129). Furthermore, decreased CI activity was also found in skeletal muscle biopsies in type II diabetes (130).

In the course of monitoring these acute and chronic diseases it may be useful to regularly assess mitochondrial respiration (MR), however this is not currently done clinically. This is because tissue muscle biopsies are invasive and alternative less invasive options would be preferable. An alternative and readily accessible option is to measure MR in the blood because blood can be sampled repeatedly. The components of blood that contain mitochondria are platelets and all white blood cells (WBC) including monocytes, eosinophils and neutrophils. Red blood cells do not contain any mitochondria after the reticulocyte stage (131). WBC play a pivotal role as part of the inflammatory process in disease settings such as sepsis and MODS (132, 133). WBC are also exposed to the entire body therefore WBC mitochondrial function may provide insight to mitochondrial health in acute or chronic illness settings.

Since WBC contain mitochondria and can be readily isolated from peripheral blood (134), they may be a suitable tissue source of accessible mitochondria and hence measurements of mitochondrial function in WBC may well facilitate clinical management by helping with diagnosis, prognosis or to monitor efficacy of treatments.

To date there are various isolated reports of WBC mitochondrial assessment in a range of diseases but no comprehensive reviews. It is timely to review the current literature for peripheral blood mitochondrial assessment in order to determine what might be the current advantages and disadvantages of this approach as a new clinical tool. As discussed in Chapter 2, apart from the mitochondria's main function of generating ATP through OXPHOS, mitochondria have several other important roles within the cell. Mitochondria are involved in cell metabolism (135), apoptotic cell death (136), (137) calcium signalling (138), cell proliferation (139) , regulation of metabolism (140), regulation of membrane potential (135) and reactive oxygen species (ROS) (141) and as such, mitochondrial function can be measured in a number of ways. In this review the focus was on the mitochondrial respiration function of mitochondria in disease states and so the scope of this review was limited to oxidative phosphorylation (OXPHOS) function and electron transport system (ETS) enzyme activities which are the two key measures of MR and thus mitochondrial well being.

The aim of this review was therefore to systematically review mitochondrial function from white blood cells in various disease states and critically evaluate the methodology used to measure mitochondrial function from white blood cells. This study also served as a preamble for the development of methodology for the experimental and clinical studies in this thesis. This review comprises two parts, of which the first is an introductory summary of WBC mitochondrial respiration and the current methods to measure mitochondrial OXPHOS respiration and capacity. The second is a formal literature review of studies that measure MR in peripheral white blood cells in disease states.

3.2 Measuring WBC Electron Transport System Mitochondrial Function

Since Clarke et al. described a normal pattern of respiratory carriers in WBC in the 1960s, several authors have studied WBC mitochondria. Foster et al. (142) studied OXPHOS in mitochondrial preparations from human WBC and concluded that mitochondria are the major source of ATP in intact WBC. Jemelin et al. (134) performed experiments in 1970 and found that, glycerol-3-phosphate dehydrogenase (GpDH) was one of the most active enzymes in oxidative processes in WBC (98).

3.2.1 Subpopulations of WBC

Until the middle of the last century, there were contradictory findings in regards to WBC respiration. The two main reasons were unpurified or heterogenous cells were being used by researchers to study WBC respiration and the methods used for quantitative evaluation of mitochondrial respiration was not refined (143), (144).

In 1985, Venezelos studied the oxidation of pyruvate and acetate by separating leucocytes from venous blood into lymphocytes and granulocytes from 35 healthy volunteers aged between 20 and 50 years (145). Oxidation of both pyruvate and acetate by lymphocytes was highly dependent on the substrate concentration in the medium reaching a plateau between 0.5 and 1mmol/l and pyruvate should be added at an optimal concentration of 0.5 to 1mmol/l as oxidation of pyruvate is dependent on the substrate concentration. (145). Pyruvate dehydrogenase (PDH) complex is only partially activated in healthy lymphocytes and once electron transport system (ETS) was uncoupled from phosphorylation by adding 0.1umol/l carbonyl cyanide chlorophenolhydrazone (CCCP), oxidation of pyruvate, acetate and succinate improved. Therefore, adding CCCP enhances flux through PDH to facilitate detection of defects in pyruvate oxidation (145). CCCP had no effect below 0.01µmol/l and was inhibitory at 1µmol/l. Venizelos also found that oxidation of acetate by granulocytes was a third of that of lymphocytes and concluded that lymphocytes were better than mixed leukocytes for oxidative studies.

In 2001, Peachman et al.. found that although all white blood cells contain mitochondria, the functional role of mitochondria in eosinophils and neutrophils is not respiration (146). The net oxygen consumption for mitochondrial respiration in eosinophil and neutrophil is minimal compared to mononuclear cells (Figure 3-1) (146).

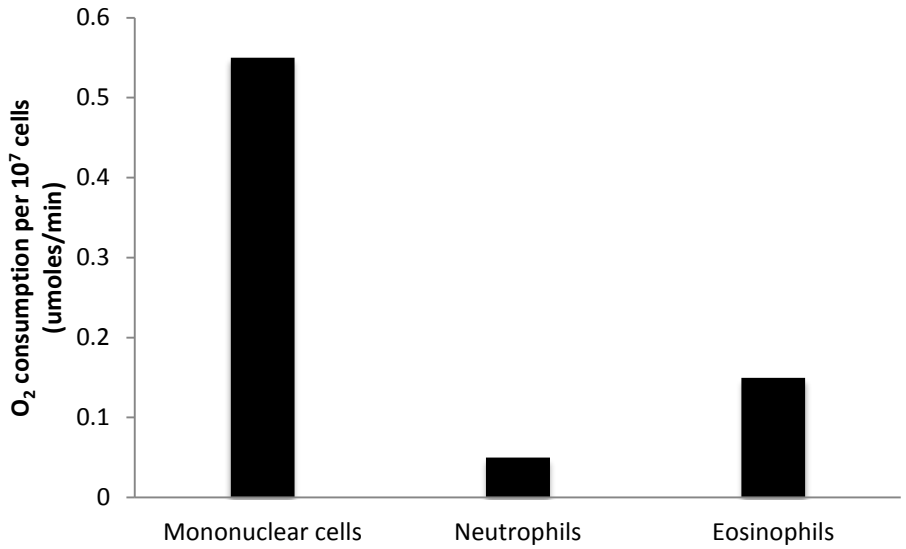


Figure 3-1 Measurement of respiration of mononuclear cells, neutrophils and eosinophils adapted from Peachman et al (146)

As positive controls, the hepatocyte cell line HepG2 and primary human mononuclear cells were used. Human neutrophils were used as a negative control. (146).

The basal oxygen consumption in quiescent peripheral blood mononuclear cells was reported to be 7.09+/-0.17 nmol O_2/min/10^7 cells measured with a Clark electrode (147). This basal oxygen consumption in cells reflect three things: 1) uncoupled respiration that is oxygen consumption not related to ATP production 2) extramitochondrial oxygen consumption and 3) the oxygen requirement for ATP consuming processes. In healthy PBMC the uncoupled oxygen consumption was calculated by blocking CV by oligomycin and was 1.17+/-0.22 nmol O2/min/10^7 cells. Some of the ATP consuming processes are Na+/K+/ATPase, Ca2+/ATPase, protein synthesis and RNA/DNA synthesis. Only about a 1/3 of the energy consumption of quiescent cells are attributable to these specific processes. From this, Schmid concluded that quiescent immune cells have a general feature to have a reserve capacity in order to be able to rapidly activate energy metabolism efficiently in response to antigenic stimulation (147) . Extramitochondrial respiration was found to be significantly less than 5% under all conditions.

3.3 Methods to measure mitochondrial function

The main function of mitochondria is ATP production via oxidative phosphorylation which couples the ETS to the phosphorylation system. This will be the focus of this review which encompasses two main methods of analysis; polarography to measure oxygen consumption and spectrophotometry to measure the activity of individual enzyme complexes.

3.3.1 Polarographic analysis

Polarographic measurement utilizes a long time established method of a Clark-type electrode to measure the oxygen concentration of a temperature-controlled medium containing respiring biological tissue or cells (148). The Clark type electrode consists of a silver anode and a platinum/gold cathode. Once a voltage is applied, the platinum/gold cathode becomes more negative relative to the silver anode. When a potential of 600-700mV is reached, oxygen is reduced to water at the platinum/gold cathode and this reduction causes a current to flow between the two electrodes. The electrical current is directly proportional to the O_2 concentration in the sample, and as respiring mitochondria or cells deplete oxygen concentration the change in oxygen relative to time provides a measurement of oxygen flux.

Endogenous or basal respiration is sometimes recorded, and the addition of ADP and substrates or specific poisons for the ETS complexes allows for functional analysis of each complex alone or in combination as a proxy for oxidative phosphorylation. It is acknowledged that Clark type electrodes require high cell concentrations to obtain reliable and reproducible results (147) and hence, researchers are limited to measuring a part of the ETS. Despite this, the Clark-type electrode remains the gold standard for many investigators but is seen by some as problematic due to a significant background respiration of the electrode (149).

3.3.2 Spectrophotometric analysis

Mitochondrial oxidative phosphorylation may also be investigated by measuring the activity of individual complexes (CI – CIV) using spectrophotometry. Spectrophotometers measure the amount of light that a sample absorbs by passing a beam of light into the sample and measuring the intensity (I)of light reaching a detector (Figure 3-2).

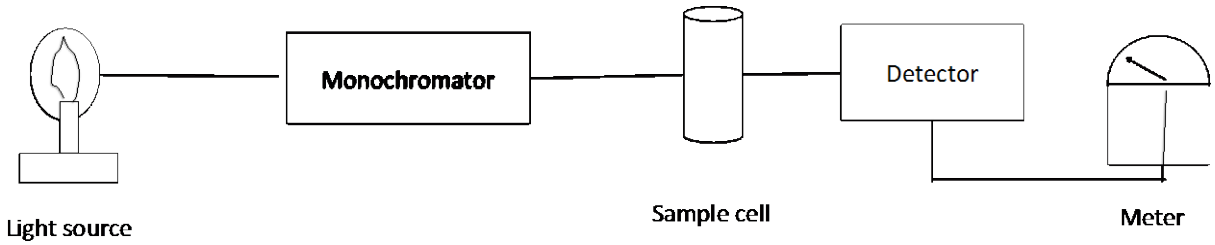


Figure 3-2 Schematic diagram of a spectrophotometer.

CI activity is commonly assayed by the addition of NADH and Coenzyme Q_{10} to cells by following the loss of absorbance at 340nm due to NADH oxidation (150). The subsequent addition of rotenone, a CI-specific inhibitor, allows for the calculation of CI specific activity. The ubiquinone-oxidoreductase activity of CII is measured by following the reduction of the

electron acceptor dye dichlorophenolindophenol (DCPIP) at 600nm. CIII is usually measured by the reduction of cytochrome-c which is followed at 550nm using a quinone as an artificial electron donor decyclubiquinone or ubiquinol. Reduced cytochrome c becomes oxidised by CIV leading to a loss of absorbance at 550nm. The difference following addition of the CIV inhibitor KCN corresponds to CIV activity (Table 3-1).

Table 3-1 The enzymes and proteins of the respiratory chain.

Enzyme	**Protein**	
NADH CoQ ubiquinone reductase	Complex I (CI)	Performed at 340nm following the decrease in absorbance resulting from the oxidation of NADH. NADH as donor addition of NADH and Co-Enzyme Q_{10} to cells by following the loss of absorbance at 340nm due to NADH oxidation (150). The subsequent addition of rotenone, a CI-specific inhibitor, allows for the calculation of CI specific activity.
NADH cytochrome c reductase	Complexes I-III (CI-III)	The assay is performed at 340nm following the increase in absorbance resulting from the reduction of cytochrome c using oxidised cytochrome c oxidase as the acceptor (151)
Succinate ubiquinone reductase	Complex II (CII)	The assay is performed at 600 nm following the decrease in absorbance resulting from the reduction of 2,6-dichlorophenolindophenol (151) measured by following the reduction of the electron acceptor dye dichlorophenolindophenol (DCPIP) at 600nm.

Enzyme	**Protein**	
Ubiquinol cytochrome c reductase	Complex III (CIII)	The assay is performed at 550nm following the increase in absorbance resulting from the reduction of cytochrome c (151) measured by the reduction of cytochrome-c which is followed at 550nm (500nm by Li) using a quinone as an artificial electron donor decyclubiquinone or ubiquinol.
Succinate cytochrome c reductase	Complexes II-III	Theassay is performed at 550 nmfollowing the increase in absorbance resulting from the reduction of cytochrome c (151)
Cytochrome c oxidase	Complex IV	The assay is performed at 550nm following the decrease in absorbance resulting from the oxidation of reduced cytochrome c (151). Reduced cytochrome c becomes oxidised by CIV leading to a loss of absorbance at 550nm. The difference following addition of the CIV inhibitor KCN corresponds to CIV activity

The activity of an enzyme is a better indication of function than the concentration of protein because of at least three reasons: 1) In some disease states such as hypertension (152) the concentration of the protein is upregulated as a compensatory factor when activity of the enzyme is decreased, 2) activity of an enzyme can change within minutes to hours whereas a change in concentration of the enzyme may require hours to days because protein synthesis has to occur first for the concentration of the enzyme to rise and 3) there may be similar concentrations of a protein but its functional activity state is changed through a conformation change that may not be readily detected by absolute protein quantification methods.

3.4 Method

A search of the Ovid MEDLINE and EMBASE databases from December 1970 through to January 2010 was carried out by two reviewers independently using the following search strategy.

Oxidative Phosphorylation OR
Electron Transport/ Physiology, Analysis OR
Electron Transport Chain Complex Protein/ Physiology, Metabolism and Analysis OR
Polarography OR
Mitochondria/Metabolism, Physiology, Pathology, Pathophysiology OR
Oxygen consumption/Physiology OR
Cell Respiration/Physiology OR
Clark type electrode.mp OR
Oxygen electrode.mp OR
High resolution respirometry.mp OR
Bioenergetics.mp

AND

White blood cells/ Abnormality, Analysis, Blood, Diagnosis, Diagnostic Use, Drug Effects, Enzymology, Immunology, Metabolism, Pathology, Pharmacology, Physiology OR

Peripheral blood cells.mp

Leucocytes

The references of primary and review articles were also examined to identify publications not retrieved by electronic searches. Abstracts were reviewed independently by the two primary reviewers and a proforma inclusion or exclusion criteria sheet filled out for each abstract.

3.5 Inclusion criteria

Mitochondrial function was defined as mitochondrial oxidative phosphorylative (OXPHOS) function assessed by measuring oxygen flux and/or by measuring electron transport chain enzyme activities. Full text articles were included in the review if the title and/or abstract reported 1) the use of peripheral white blood cells, 2) OXPHOS function was measured in a diseased state compared to appropriate controls and 3) the method used to measure OXPHOS function was oxygen flux or measurement of electron transport chain enzyme activities.

3.6 Exclusion criteria

Studies were excluded from the review if peripheral white blood cells were not used or OXPHOS function was not measured via oxygen flux or measuring ETS enzymes. Measuring OXPHOS function in white blood cells and comparing with another tissue in the same disease state was also considered grounds for exclusion of a study. The limits placed were English language and human studies.

3.7 Data extraction

A data extraction excel spreadsheet was filled out for each full text article that was included. Author, publication date, type of blood cells, method of separation of white blood cells, method of measuring mitochondrial OXPHOS function, disease model, type of control and reported primary and secondary outcomes were extracted from each original article and tabulated.

3.8 Results

2208 abstracts were identified; 33 of these met the inclusion criteria. In 27, PBMC were studied (82%), four (12%) studied granulocytes and two (6%) studied both. Five to 150 mls of whole blood was retrieved from each patient per study (Table 1), however, 10 (30%) studies did not mention how much blood they required. All studies separated whole blood using a density gradient method and 27 (82%) studies used cell pellet (homogenate) and six (18%) studies used a purified mitochondrial fraction. In 14 (42%) studies the assays were done at 37^{o}C, in nine (27%) the assays were done at 30^{o}C and eight (24%) did not report the temperature that was used.

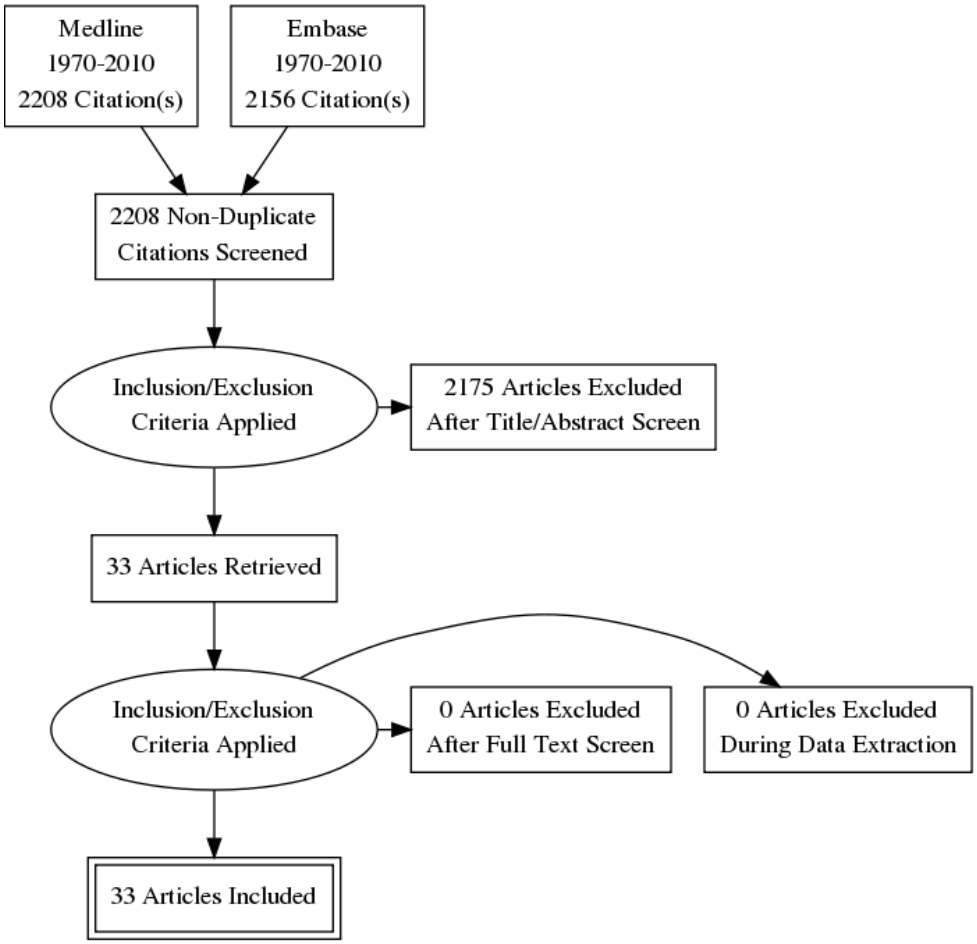


Figure 3-3 PRISMA flow chart for systematic review

For this review the disease states that have been investigated are divided into acute and chronic (Table 3-2). Chronic disease has been further subdivided into those affecting the brain (neurological), kidney (renal), liver (hepatic), lung (pulmonary), reproductive, infective, metabolic, hematological and inherited disorders.

Table 3-2 Summary of studies included in systematic review

Disease	Subjects		Cells (Volume of blood in mls, Temperature ^{o}C)	Results		Reference
	Patients (n)	Controls (n)		Polarography (O_2 consumption)	Spectrophotometry (Enzyme complex activities)	
Acute						
Severe sepsis or shock	18	32	PBMC (NS, 37)	Baseline O_2 consumption **higher** (p=0.001) in septic patients and ADP-stimulated respiration **lower** (p=0.002) compared to controls		Belikova 2007
CO poisoning	5	32	PBMC (20, 37)		CIV activity was **decreased** (p<0.001) in patients compared to controls	Miro 1999
CO poisoning	3	12	PBMC (NS, 37)		CII and CIII activities were **not different** to controls, while CIV activity was **decreased** at 0d (p<0.01) and 3d (p<0.05), and had normalised by 12d	Miro 1998
Chronic						

Disease	Subjects		Cells (Volume of blood in mls, Temperature ^{o}C)	Results		Reference
	Patients (n)	Controls (n)		Polarography (O_2 consumption)	Spectrophotometry (Enzyme complex activities)	
Friedrich's Ataxia	12	25	PBMC (5, 30)		CI activity **decreased** ($p<0.001$) in patients compared to controls	Heidari 2009
Friedrichs ataxia	6	11	PBMC mitochondria[2] (30,30)		CII activity **not different** in patients vs controls	Stumpf 1981
Mild Cognitive Impairment and Alzheimers Disease	5+8	7	PBMC mitochondria[2] (40, 30)		No difference in CI, CIII, or CIV activity between groups	Valla 2006
Alzheimers disease	33	30	PBMC mitochondria[2] (NS, NS)		No difference in CI, CII, or CIV activity in patients vs controls	Molina 1997

Disease	Subjects		Cells (Volume of blood in mls, Temperature ^{o}C)	Results		Reference
	Patients (n)	Controls (n)		Polarography (O_2 consumption)	Spectrophotometry (Enzyme complex activities)	
Alzheimers disease (treated and un-treated)	13+13	26	PBMC (30, 37)	No difference between untreated patients and controls, while treated patients had **increased** CI ($p<0.001$), and non-significant increase in endogenous ($p=0.06$) and CII ($p=0.09$) O_2 consumption	CI, CIII, and CIV activity **not different** between untreated patients and controls, but **higher** in treated patients ($p=0.009$, 0.02 and 0.01, respectively). Citrate synthase activity did not differ between groups.	Casedemont 2003
Parkinsons disease	40	30	PBMC (15-20, 30)		CII and citrate synthase activity **not different**, while CI and CIV activity **decreased** in patients ($p<0.001$ and $p=0.014$, respectively)	Shinde 2006
Parkinsons disease *(parkin gene mutations and idiopathic parkinsons)*	10+20	17	PBMC[z] (20, NS)		CI activity **decreased** 65% in idiopathic PD and 64% in patients with parkin gene mutations relative to controls ($p<0.001$) while CIV activity **decreased** only in idiopathic PD ($p<0.01$)	Muftoglu 2003

Disease	Subjects		Cells (Volume of blood in mls, Temperature ^{o}C)	Results		Reference
	Patients (n)	Controls (n)		Polarography (O_2 consumption)	Spectrophotometry (Enzyme complex activities)	
Parkinsons disease	36	30	PBMC mitochondria (30mls, 30, 38 (CIV))		CI, CII, CIV and citrate synthase activity **not different** between patients and controls	Martin 1996
Parkinsons disease	20	17	PBMC (20, NS)		CI, CIII and CIV activity **not different** between patient and control, while CII activity **decreased** in patients ($p<0.05$)	Yoshino 1992
Parkinsons disease	16	15	PBMC (30, 38 (CIV))		CI and IV **decreased** ($p<0.05$) in patients, while CII and citrate synthase activity not different between patients and controls	Barosso 1993
Multiple sclerosis	17	17	PBMC (4, NS)		CIV activity **not different** between patients and controls	Steen 1987
Opthalmoplegia	5	8	PBMC (150, 37)	CI and CII oxygen consumption **decreased** 30-50% in patients vs controls (significance not reported)		Kunz 1995

Disease	Subjects		Cells (Volume of blood in mls, Temperature ^{o}C)	Results		Reference
	Patients (n)	Controls (n)		Polarography (O_2 consumption)	Spectrophotometry (Enzyme complex activities)	
Sporadic amyotrophic lateral sclerosis	21	21	PBMC (12-20, 37)	Basal and CIV oxygen consumption did **not differ** between patients and controls	CIV activity **not different** between patients and controls	Curti 1996
Chronic renal failure	18	15	Granulocytes (NS, 37)	Basal and stimulated O_2 consumption **decreased** ($p<0.01$) in patients vs controls		Kiersztejn 1992
Chronic renal disease and hemodialysis	6+6	6	PBMC (20, 30)		CIV activity **decreased** 65% ($p=0.003$) in chronic kidney disease and 46% ($p<0.01$) in hemodialysis patients compared to controls	Granata 2009
COPD	42	50	PBMC (7-9, NS)		CIV activity **increased** ($p<0.05$) in patients compared to controls	Hakhameneshi 2008
Smoking	35	35	PBMC (20, 37)	CI O_2 consumption did not differ, while CII **increased** ($p<0.01$) in smokers vs controls, and baseline O_2 consumption trended towards higher values ($p=0.07$)	CII, CIII activity not different, while CIV activity **decreased** (<0.01) in smokers vs controls	Miro 1999

Disease	Subjects		Cells (Volume of blood in mls, Temperature ^{o}C)	Results		Reference
	Patients (n)	Controls (n)		Polarography (O_2 consumption)	Spectrophotometry (Enzyme complex activities)	
Poly cystic ovary syndrome	20	20	Granulocytes (NS, NS-Polarography, 37)	Endogenous and CI O_2 consumption **decreased** ($p<0.01$ and 0.05, respectively) in patients, with no difference in CII O_2 consumption	CI activity 50% **decreased** in patients ($p<0.05$)	Victor 2009
Morbid obesity	59	40	PBMC mitochondria[a] (NS, 30)		CI, CIII and CIV activity increased ($p<0.01$) in morbidly obese patients compared to post-obese surgical calorie restricted patients	Li 2007
Obesity	60	151	PBMC (NS, 37)		CIV activity **increased** ($p<0.01$) in obese patients and citrate synthase activity **decreased** ($p<0.01$) compared to controls	Capkova 2002
Type II Diabetes	10	10	PBMC (NS, NS)		CIV and citrate synthase activity were **not different** in patients vs controls	Garcia-ramirez 2008

Disease	Subjects		Cells (Volume of blood in mls, Temperature ^{o}C)	Results		Reference
	Patients (n)	Controls (n)		Polarography (O_2 consumption)	Spectrophotometry (Enzyme complex activities)	
Malnourishment	15	30	PBMC (24, RT)		CI activity was **decreased** in malnourished patients before ($p<0.001$) and 7d ($p<0.05$) after refeeding compared to controls, and normalised by 14d	Briet 2004
Thyroid disorders *(Hashimotos, Graves disease, toxic adenoma)*	11+17+	14	Granulocytes (NS, NS)	Stimulated O_2 consumption was not different in Hashimotos compared to controls, but **higher** ($p<0.01$) in Graves disease and toxic adenoma		Szabo 1996
Phenylketonuria *(diet-restricted and non-diet restricted*	14+12	17	PBMC (10, 30)		CII+CIII activity not different between treated (diet-restrictive), untreated (diet-unrestricted) and control groups	Hargreaves 2002

Disease	Subjects		Cells (Volume of blood in mls, Temperature ^{o}C)	Results		Reference
	Patients (n)	Controls (n)		Polarography (O_2 consumption)	Spectrophotometry (Enzyme complex activities)	
HIV (typical progressors+long term non-progressors	27+26	31	PBMC (20, 37)		CII and CIII were **decreased** in the typical progressors ($p<0.001$, $p<0.01$, respectively) compared to controls, and CII activity **decreased** in the long term non-progressors ($p<0.05$). CIV activity was not different between groups.	Peraire 2007
Cystic Fibrosis	13	10	PBMC and granulocytes (15, NS)		CIV activity **decreased** ($p=0.02$) in patient mononuclear cells compared to controls but **not different** in neutrophils	Percival 1995
Cystic Fibrosis and heterozygotes	8+10	11	PBMC (10, 37)		CI activity **not different** between patients, heterozygotes and controls	Senguinetti 1982
Oxphos disease	24	87	PBMC (5, 37)	CI O_2 consumption **decreased** ($p<0.0001$) in patients compared to controls		Artuch 2000

Disease	Subjects		Cells (Volume of blood in mls, Temperature ^{o}C)	Results		Reference
	Patients (n)	Controls (n)		Polarography (O_2 consumption)	Spectrophotometry (Enzyme complex activities)	
Sideroblastic anemia	69	23	PBMC and granulocytes (separately) (15-20,25)		CIV activity **decreased** in patients granulocytes compared to conrols (30%, no significance reported), with no difference in lymphocytes, while citrate synthase activity did not differ in either cell type.	Aoki 1980
Pernicious anaemia	9	6	Granulocytes (NS, 37)	Baseline and stimulated O_2 consumption did **not differ** between patients and controls		Katka 1983

Footnote: NS not stated**; PBMC [peripheral blood mononuclear cells], O2 [oxygen], CO [carbon monoxide], ADP [adenine diphosphate], CI [Complex I], CII [Complex II], CIII [Complex III], CIV [Complex IV], PD [Parkinsons disease].**

3.9 Acute Diseases

3.9.1 Severe Sepsis

Belikova investigated oxygen consumption of PBMC in sepsis (153). Peripheral blood from patients with severe sepsis or septic shock (n=18) was obtained and compared with peripheral blood in healthy volunteers (n=32). PBMC were separated with a Ficoll gradient and oxygen consumption was measured in a Clark electrode. Basal respiration rates were measured followed by maximal respiration induced by ADP and then addition of antimycin A (a selective inhibitor of CIII) to determine how much of this maximal respiration was mitochondrial in origin. PBMC had a higher baseline oxygen consumption compared to healthy controls ($p=0.002$). Stimulation of ADP resulted in a significantly lower increase in consumption of oxygen from baseline in septic cells compared to healthy volunteers (25% vs 161% $p<.0001$).

3.9.2 Carbon monoxide (CO) poisoning

Carbon monoxide is a toxic, colourless, odourless, tasteless gas and hence difficult to detect. Carbon monoxide poisoning symptoms range from mild headaches, flu like symptoms to toxicity of the central nervous system, heart and death in severe poisoning (154). Lipid peroxidation and CIV activity was measured in lymphocytes obtained by separating 20mls of whole blood in 5 patients with acute CO poisoning and compared with 32 non-smoker healthy controls. There was a significant CIV inhibition as well as an increase in lipid peroxidation. The authors concluded that the CIV inhibition may be mediating the lipid peroxidation (155). In another study by the same group, CIV activity was measured in 3 patients with acute CO poisoning at the time of admission, on day 3 and day 12. CIV was decreased by 76% during acute CO poisoning and this decrease persisted at day 3, while on day 12 CIV was no longer decreased (156).

3.10 Chronic Diseases

3.10.1 Neurological Diseases

The neurological diseases studied were Friedrich's Ataxia (2), Alzheimers Disease (2), Parkinsons Disease (5), Multiple Sclerosis and Sporadic Amyotrophic Lateral Sclerosis (SALS).

3.10.1.1 Friedrich's Ataxia

Friedrich's Ataxia (FRDA) is an autosomal recessive disorder resulting in neurological and cardiac abnormalities. The chromosomal locus of FRDA is 9q13 and there is an expansion of an unstable GAA trinucleotide repeat in intron 1 in 97% of cases (157). In Friedrich's Ataxia excess accumulation of iron occurs in the mitochondria which can cause irreversible oxidative damage and result in mitochondrial dysfunction (157). NADH ferricyanide reductase activity and intracellular ATP were significantly lower in lymphocyte cell homogenates from 5mls of blood in a recent study with 12 patients and 25 controls (157). In 1981, Stumpf studied succinate cytochrome *c* reductase activity in purified mitochondria obtained from leukocytes from 30mls of blood in seven patients with Friedrich's Ataxia and compared these with healthy controls and found no difference (158).

3.10.1.2 Alzheimer's Disease

Alzheimer's Disease (AD) is the most common neurodegenerative disorder in the elderly which results in cognitive and memory decline. The pathology of AD involves protein aggregates (plaques and tangles) in the cerebral cortex surrounded by morphologically abnormal mitochondria (159). In platelets, depressed activity of cytochrome *c*-oxidase (CIV) has been measured (160-163), however this is disputed (164). CIV deficiency may be present in AD in certain brain regions such as the hippocampus (165) and the frontal and temporal cortices (166).

Three studies have measured ETS activity of PBMC in AD (167-169). Valla et al., Molina and Casademont found no difference in CIV compared to controls in PBMC. Measured activities of CI and CII of the ETS (168) appear unchanged in AD and endogenous respiration and CI and CII fuelled respiration did not differ in AD PBMC (167). The activity of citrate synthase was unchanged in AD, indicating no loss in mitochondria (167, 168). Valla and Molina used purified mitochondria from lymphocytes whereas Casademont used cell homogenate.

3.10.1.3 Parkinson's Disease (PD)

Parkinson's disease is a neurodegenerative disorder where motor skills are predominantly affected along with speech and other functions (170). The pathology lies in the death of cells in the substantia nigra resulting in a deficiency of dopaminergic neurons, and mitochondrial CI has been shown to be deficient in substantia nigra in patients with PD. Most studies of platelets mitochondria in PD patients have also shown a CI defect (171-173).

In lymphocytes, patients with PD had significantly depressed activities of rotenone sensitive NADH cytochrome-*c* reductase and cytochrome-*c* oxidase in lymphocytes by 9.5% and 26% respectively than age matched healthy subjects (174, 175) . Muftoglu (176) reported a 62.5% decrease in CI activity in leukocyte mitochondria in patients with Parkin mutations compared to controls and the mean activity of idiopathic PD patients CI was (~36% depressed 64.5% of that of the controls. Yoshino (177) also reported depressed CII activity in lymphocytes by 13.2%, and Barroso and Muftoglu found that there was decreased CIV in PD patients by 45% and 60% respectively (174, 176). In contrast Martin compared PD patients and healthy subjects and found no significant difference in respiratory chain enzyme activities of lymphocyte mitochondria (175) . Notably Martin, Shinde, Muftuoglu used isolated mitochondria, whereas Barosso and Yoshino used cell homogenates. This may account for the differences in results as isolation of mitochondria is prone to selection bias (152). In conclusion most studies have reported a decreased CI in lymphocytes in Parkinsons disease.

3.10.1.4 Multiple Sclerosis (MS)

MS affects young adults and is an autoimmune disease in which the axons in the brain and spinal cord become demyelinated causing a range of neurological symptoms. Steen suspected ubiquinone deficiency in MS after finding low ubiquinone in serum in patients with MS (178). Lymphocytes were isolated from seventeen patients with MS and from healthy controls from 4 mls of blood. Lymphocyte ubiquinone, cytochrome c oxidase activity and NADH cytochrome reductase activity were found to be the same in patients as controls. Steen concluded that the low serum ubiquinone was not due to lack of production but perhaps increased hepatic degradation of ubiquinone.

3.10.1.5 Sporadic Amyotrophic Lateral Sclerosis

Sporadic Amyotrophic Lateral Sclerosis (SALS) is a neurodegenerative disease where motor neurons controlling the voluntary muscle movement degenerate. Both upper and lower motor neurons are involved and the muscle gradually weakens, develops fasciculations and then gradually atrophies (179). In SALS, there are heightened markers of oxidative injury and glutathione peroxidase in blood and superoxide dismutase in cerebrospinal fluid is decreased (180). Curti measured basal oxygen consumption rate polarographically using a Gilson oxygraph chamber with a Clark type electrode in 21 patients with SALS and 21 controls from lymphocytes obtained from 12-20 mls of whole blood (181). Samples were processed and lymphocytes separated after 3 hrs of withdrawal of blood and found that the basal oxygen consumption rate did not differ between the groups. The addition of FCCP

(trifluorocarbonylcyanide phenylhydrazone, an uncoupler of ETS from phosphorylation) increased the oxygen consumption rate significantly in controls ($p<0.01$) but not significantly in patients. The activity of CIV did not differ between patients and controls. They concluded that overall oxidative metabolism is unchanged, however, the ability to match a stressful situation such as with FCCP induced increase in oxygen consumption, is depressed in patients (181).

3.10.2 Renal disease

Progression of renal dysfunction is contributed by oxidative stress from renal mitochondria (182). Mitochondrial oxygen consumption was measured in granulocytes obtained from whole blood polarographically in a Clark type oxygen electrode and was significantly lower in patients undergoing hemodialyis (n=18) for renal failure compared to healthy controls (n=15) (183). In another study, peripheral blood mononuclear cell Complex IV activity was measured spectrophotometrically between chronic kidney disease (CKD) patients (n=6), patients on hemodialysis (HD) (n=6) for CKD and controls (n=6), complex IV activity was significantly decreased by 65% in the CKD and 46% in the HD group compared to controls (184). There was no significant difference between the CKD and HD groups. ROS was also found to be significantly higher in the CKD and HD group compared to controls. In sharp contrast to Complex IV activity, when the protein expression of COXI and COXIV subunits of Complex IV were measured, they were both found to be higher in CKD and HD patients compared to healthy controls. This was taken to mean that a dysregulation was occurring where increased protein translation did not equate to increased mitochondrial respiration.

3.10.3 Pulmonary diseases

Chronic obstructive pulmonary disease (COPD) encompasses chronic bronchitis and emphysema and causes narrowing of the airway and is a common pulmonary disease (185). Mitochondrial CIV activity was spectrophotometrically measured in white blood cell homogenate obtained from separating whole blood in 42 COPD patients and 50 healthy controls (186). Mitochondrial CIV activity was significantly increased in WBCs of patients with COPD compared to healthy controls ($p<0.05$). The authors were unable to determine whether this change was a primary change or a secondary change due to hypoxia.

3.10.4 Chronic smoking

Smoking is one of the biggest risk factors for heart disease (187). When mitochondrial respiration was measured in 35 **chronic** smokers and compared to 35 healthy controls in

lymphocytes obtained from whole blood, complex IV measured spectrophotometrically was significantly decreased in the smokers. Amongst the smokers, there was a trend towards increased baseline respiration and a statistically significant increased mitochondrial respiration polarographically with succinate (188). Lipid peroxidation of lymphocyte membranes was significantly increased in smokers compared to non-smokers in this study whereas when lipid peroxidation was measured in smokers that have never smoked before, lipid peroxidation was not altered in lymphocyte membranes (189).

3.10.5 Obesity and Type 2 Diabetes Mellitus (T2DM)

Obesity, defined as Body Mass Index (BMI) >30kg/m^2, is a global epidemic and increases the risk for other diseases such as Type 2 Diabetes Mellitus (T2DM), coronary heart disease, hypertension, osteoarthritis, and types of cancer (190), (191), (192). T2DM, characterized by high blood glucose and insulin resistance is consequently a highly prevalent disease and a large risk factor for myocardial infarction (193).

Citrate synthase and CIV activity were measured in PBMC in 60 obese (BMI=37.7 ± 7.7kg/$m^{2)}$ and 161 non-obese subjects (194). In obese subjects, higher CIV activity (1.2 fold, $p<0.01$) and lower CS activity (1.3 fold, $p>0.01$) was measured, suggesting that in obesity a loss of mitochondria occurs, but an increase in the oxidative capacity. ETS enzyme activities were compared in 59 morbidly obese women (BMI=49.6±1.7kg/m^2) with 40 *previously* obese women subjected to surgical caloric restriction (BMI=28.9±1.1kg/m^2) (195). The activity of ETS enzymes CII-IV was significantly lower in the lean group ($p<0.05$), and trended towards lower activities for CI. CII-IV activity also correlated positively with BMI and caloric intake from carbohydrate (but not for protein, fat or energy), and all enzymes positively correlated with fasting plasma insulin.

When ten men with T2DM and late complications were compared with healthy age-matched controls, lymphocytes from T2DM subjects did not differ significantly in CS or CIV activity when compared to healthy subjects (196).

3.10.6 Malnutrition

According to the World Health Organisation, malnutrition is the biggest contributor to child mortality in the world (197). Upon refeeding, classical markers of nutritional status such as body water composition and albumin take weeks to change (198). When CI activity was measured in thirteen malnourished patients and compared to 42 healthy controls, CI activity significantly increased in the patients by day 7 upon refeeding (198). In another study,

complex I activity was measured in 15 malnourished patients (>10% body weight loss over the previous six months) on admission to hospital (t=0 days), and compared with 30 healthy volunteers, and followed during re-feeding (t=7, 14 and 30 days after admission). On admission, CI activity was 43% lower in malnourished patients ($p<0.05$) compared to controls, increased significantly by 7 days ($p<0.02$), and normalized by 14 days (199).

3.10.7 Genitourinary diseases

Polycystic ovarian syndrome affects 5-10% of premenopausal women (200) and symptoms include premenopausal irregularities, hyperandrogenism and infertility (201). Insulin resistance plays an important role in the pathogenesis and increases the risk of developing type 2 diabetes. In patients with PCOS acute hyperglycaemia results in increased reactive oxygen species by peripheral blood leukocytes (202).

Victor (203) studied 20 women of fertile age with PCOS and 20 fertile healthy women. Blood was collected during the second or third day of the menstrual cycle during the follicular phase and granulocytes were separated. In the granulocytes, CI was assayed spectrophotmetrically by calculating the reduced nicotinamide adenine dinucleotide (NADH) oxidation rate. The rate of oxygen consumption was lower in the PCOS patients measured in the clark type electrode ($p<0.01$). CI activity was inhibited in PCOS patients calculated as the rate of NADH oxidation ($p<0.05$).

3.10.8 Thyroid Disorders

Grave's disease and Hashimoto's thyroiditis are autoimmune thyroid disorders where the activity of T suppressor cells are decreased. Female patients with Grave's Disease (n=17), Hashimoto's thyroiditis (n=11) and toxic adenoma (n=8) had granulocytes isolated and the respiratory burst stimulated with zymosan, a toll like receptor activator. To measure the contribution of mitochondrial respiration to the respiratory burst, mitochondrial respiration was blocked by antimycin A, a CIII inhibitor. In healthy individuals, the majority of oxygen consumption by zymosan-stimulated cells was non-mitochondrial, as antimycin-A exhibited no inhibition on oxygen flux. In both hyperthyroid groups (graves disease and toxic adenoma), oxygen flux was higher ($p<0.01$) than controls, and with the addition of antimycin-A, oxygen flux was a similar level to controls. Although the methodology is unclear, it appears that in euthyroid states, the respiratory burst consists of non-mitochondrial oxygen consumption (eg. NADPH oxidase), and in hyperthyroid states mitochondrial respiration (204).

3.10.9 HIV

Human Immunodeficiency Virus (HIV) targets immune cells and is considered a global pandemic by the WHO. HIV RNA has been found in the mitochondria of infected individuals, and appears to influence apoptosis (205). Mitochondrial DNA is involved in the translation of ETS complexes, therefore HIV and HAART treatment have the potential to inhibit ETS activity.

CII, III, IV and GpDH activity was measured relative to citrate synthase and was decreased in twenty-five patients with HIV compared to twenty-five age and sex matched non-HIV infected controls (206). There was no difference in citrate synthase activity between HIV infected subjects and controls pointing to similar numbers of mitochondria in between the groups. Peraire investigated mtDNA and ETS CII, III and IV activity in healthy controls and two groups of untreated HIV-infected individuals (1) typical progressors (immunological decline and high HIV-viremia) and (2) long term non progressors (self-control of infection, low HIV-viral burden). In typical progressors, mtDNA decreased 25% ($p<0.05$) and CII and CIII decreased by ~30% ($p<0.01$ and $p<0.001$, respectively) relative to healthy controls. No change in mtDNA was found in non-progressors, with a 25% drop in CII ($p<0.01$), and no change in other ETS enzymes (207)

3.10.10 Hereditary

3.10.10.1 Oxidative Phosphorylation (OXPHOS) Diseases

Inherited mitochondrial disorders have an incidence of 1 in 10,000 live births. The diagnosis is complicated as the oxidative phosphorylation system requires polypeptides from both the nuclear genome and the maternally-inherited mitochondrial genome (208). Polarography was performed on PBMC of 100 pediatric patients (1 month to 17 years of age) with a suspicion of an OXPHOS disease and 87 healthy controls (209). This diagnosis was confirmed in 24 patients, who exhibited significantly lower CI-stimulated oxygen flux compared to a healthy pediatric population (median 47% lower, $p<0.00005$), and a non-significant 15% drop in CII-stimulated oxygen flux.

3.10.10.2 Cystic Fibrosis

Cystic fibrosis is an autosomal recessive inherited disorder characterised by high concentrations of sodium and chloride in the sweat, respiratory difficulties from acute and chronic infections, pancreatic insufficiency resulting in persistent malabsorption and sometimes liver disease and diabetes mellitus (210).

When 13 adolescents with pancreatic insufficiency were compared with 10 healthy controls they found COX activity was significantly lower in the mononuclear cells in the patients (p=0.020) (211). In an older study (Serengetti 1982) when white blood cells from 11 controls were compared to white blood cells from eight patients with CF there were no significant differences in the level of CI (212). This latter group used a mixture of all types of white blood cells whereas Percival used mononuclear cells only.

3.10.11 Hematologic Diseases

3.10.11.1 Primary Sideroblastic Anaemia

In sideroblastic anaemia the body has iron available but fails to incorporate iron into haemoglobin. Patients show hypochromic or dimorphic anaemia, elevated serum iron with high saturation of total iron binding capacity and erythroid hyperplasia in the bone marrow with a large number of sideroblasts (213). Mitochondrial enzyme activities in granulocytes and lymphocytes from 15-20 mls of peripheral blood in patients with primary sideroblastic anaemia (69 patients) were measured and compared to blood from healthy controls (number not given). Patients were found to have decreased CIV activity in granulocytes but not in lymphocytes (p value not given). Oligomycin sensitive ATPase activity was also decreased in granulocytes but not in lymphocytes in patients. Citrate synthase activity in granulocytes and in lymphocytes had no differences between patients and controls (214).

3.10.11.2 Pernicious anaemia

Pernicious anaemia is caused by the autoimmune destruction of gastric parietal cells leading to a lack of intrinsic factor which then leads to vitamin B_{12} defeciency (215). Neutrophil oxygen consumption was measured in 9 patients with pernicious anaemia from whole blood samples. Neutrophils were separated from whole blood using Boyum's method and baseline oxygen consumption was measured in a Clark type electrode. There were no significant alterations in the oxygen consumption between patients and healthy controls (216). They concluded that this fits the clinical impression that infections are not more common or more severe in patients with untreated pernicious anaemia.

3.11 Discussion

Mitochondrial respiration (MR) has been studied in many disease states most of which have been chronic disease states. PBMC MR was most commonly studied whilst granulocytes were the second most common cell type studied. The volume of blood used was variable but ranged from five to 150mls. There was considerable heterogeneity with regard to the temperature at

which assays were done ranging from room temperature to 37^{o}C. Whilst most studies used cell homogenates, five studies used isolated mitochondria.

MR function was most commonly studied in chronic diseases, with chronic neurological diseases being the most common pathologies studied (13/33 studies). Common chronic diseases such as hypertension (217), Type II diabetes and obesity were under represented. Amongst common chronic diseases, Type II diabetes was studied once (196) and obesity was studied twice (194, 195). Although, hypertension is a common disease worldwide and mitochondrial dysfunction occurs in the heart in hypertension (218), PBMC MR function has never been studied in hypertension.

There were only three out of thirty three studies focusing on acute disease states (153), (156), (155). This may be because, chronic diseases are logistically easier to study compared to acute diseases. Chronic diseases are more easily followed up in clinics as they progress slowly and therefore round the clock research personnel or laboratory instruments are not required leading to a substantial cost reduction. However, there are changes in white blood cell mitochondrial function in acute disease states which need to be understood in order to decipher any potential clinical relevance of WBC MR in these diseases. With such limited data on acute disease states, it is impossible to gauge whether MR function differs between chronic and acute disease states or whether MR function reflects the severity of the acute disease.

3.11.1 Diagnostic and prognostic relevance of WBC MR

Even though, WBC MR has diagnostic and prognostic potential in many of these disease states (Table 3-3) MR function in WBC have not been fully evaluated to fulfil such a role. In paediatric OXPHOS diseases, measuring PBMC MR revealed significantly lower CI stimulated oxygen flux in 24 patients and confirmed the diagnosis (209). To diagnose OXPHOS disorders muscle and skin biopsies are usually used but comparatively PBMC MR may be a much less invasive option (219) .

Typical progressors of HIV revealed significantly decreased CII and CIII compared to non-progressors of HIV and healthy controls (207). Since the pattern of MR function is different in progressors compared to non-progressors of HIV, this may have the potential to aid prognosis if confirmed in future studies. In polycystic ovarian syndrome the CI activity was decreased, but, whether this correlated with the degree of insulin resistance is not known

(220). CIV activity is up regulated in COPD patients, however, has not been correlated with any clinical measures of lung function to know whether this may aid prognosis (186).

Table 3-3 Diseases where white blood mitochondrial respiratory function has clinical potential.

Disease	**Diagnostic potential**	**Prognostic potential**	**Potential to monitor efficacy of therapy**	**Statistically powered clinical studies required**
Severe sepsis/shock		✓		To correlate with outcomes
CO poisoning		✓	✓	To monitor CIV activity with oxygen treatment
Friedrich's Ataxia				To confirm current findings
Parkinson's disease				For a definitive conclusion
Chronic renal disease				To confirm current findings
Polycystic ovary syndrome		✓		To correlate with outcome of glucose intolerance
Malnourishment			✓	
HIV		✓		
Mitochondrial OXPHOS disease	✓			

CIV activity in PBMC is significantly decreased in chronic renal disease compared to controls and this has the potential to delineate the mortality risk in this population which may also aid in renal transplant decision making (221). Even though decreased PBMC CI activity is

evident in Parkinson's disease no correlation with clinical outcomes has been done to date (222), (176), (175), (177), (174).

Even though WBC mitochondrial respiration (MR) has been studied in many chronic diseases many questions still remain unanswered. How does WBC MR relate to tissue MR in disease states? Does WBC MR correlate with disease outcomes and thus aid in prognosis? Does WBC MR change with current treatment and therefore be used to monitor therapy?

Mitochondrial function in acute diseases has only been studied fleetingly. Since there is now evidence that mitochondrial dysfunction is a key event in multiple organ dysfunction syndrome and sepsis (27), WBC MF needs to be studied in more detail in these disease states. In carbon monoxide poisoning there was a significant decrease in CIV but whether this decrease correlated with clinical outcome of the patient was not measured (156), (155).

3.11.2 PBMC are a better choice for studies of MR

PBMC are better than mixed white blood cells or granulocytes for oxidative studies (145) and this is highlighted by the fact that 27 out of the 33 studies chose PBMC as the cell to study mitochondrial function. Granulocytes had to be stimulated artificially before oxygen consumption could be measured. This is because the main subset of granulocytes are neutrophils and the main function of mitochondria in neutrophils is to maintain membrane potential rather than generate ATP through OXPHOS (223). Thus granulocytes may not be appropriate to study OXPHOS function in disease states.

3.11.3 Drawbacks of spectrophotometry

In the included studies, mitochondrial OXPHOS was investigated by measuring the activity of individual complexes using spectrophotometry or polarography. The included studies have measured parts of the ETS and have not been able to evaluate WBC ETS as a whole in these disease states. The limiting factor was most likely the large volume of blood that is required to measure all of the WBC ETS complexes using spectrophotometry or polarography. The studies that used spectrophotometry were limited by having to use different assays to measure the various complexes and hence requiring a large sample of blood. One of the prominent difficulties in measuring OXPHOS enzyme activities is that of Complex I in lymphocytes (224), (225). Complex I in lymphocytes is difficult to measure spectrophotometrically as there is both rotenone sensitive and rotenone insensitive NADH oxidation within mitochondria in lymphocytes. NADH is oxidised by NADH:ubiquinone oxidoreductase (Complex I) but NADH can also be oxidised by a rotenone insensitive manner by NADH: cytochrome b5

oxidoreductase which is located in the mitochondrial outer membrane. Hence measurement of Complex I is based on estimation of the specific rotenone-sensitive enzyme activity. Dyes such as MTT3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) and resazurin have been used to assess the activity of reductases of the ETS , where fluorescence of the reduced dye can be measured spectrophotometrically. However, non-mitochondrial reductase activity is a drawback of this method, and in the case of MTT it was found that the majority of measured fluorescence was non-mitochondrial (226). These fluorescent dyes are now used as a measure of bacterial metabolic activity in anti-bacterial drug screens (227). Spectrophotometric measurements of CIV activity revealed three fold higher reserve capacity than polarographic measurements in human skeletal muscle. This difference is due to the detergent used in the spectophotometric method which activates CIV (228).

3.11.4 Advantage of polarography over spectrophotometry

The advantage of the polarographic method over spectrophotometry is that it provides the possibility of assessing the activity of all complexes of the oxidative phosphorylation system in intact mitochondrial membranes under conditions which reflect the 'in vivo' situation (228). However, this advantage is lost when using traditional Clark type electrodes and all of the included studies except one (229) using polarography used the simple Clark type oxygen electrode. The simple Clark electrode has several downfalls :1) oxygen diffusion through the electrode 2) small changes in respiration, respiratory control ratios and subtle differences in respiratory effects of inhibitors are hard to detect 3) large quantities of biological samples (10^7 cells or more) are required to evaluate the whole ETS and obtain a stable signal (149).

3.11.5 Advantages of high resolution oxygraphy over standard polarography

More recently, a new standard of measuring mitochondrial respiration was established with the Oroboros Oxygraph. The Oroboros Oxygraph provides high resolution respirometry based on highly sensitive electrodes, minimisation of oxygen diffusion and software that enables on line recording of oxygen consumption rates with minimal quantities of biological samples (10^6 cells) (149). At low respiratory flux per volume such as white blood cells, the oxygen capacity of the system provides sufficient time for evaluation of slow approaches of the biological sample to a steady state and allows for application of complex titration regimes in intact or permeabilised cells. This feature enables the ETS to be looked as a whole system from a small number of white blood cells (149). Even though high resolution respirometry has these advantages, the drawback of high resolution respirometry is a lack of high throughput and specialised equipment. If high resolution respirometry is to be used clinically in hospital

laboratories, it will need to have higher through put than what is possible currently. Recently, a high through put method for oxygen consumption in permeabilised skin fibroblasts have been optimised but it is fluorescent based and therefore lacks the accuracy that high resolution respirometry has to offer (230).

3.11.6 Temperature alters enzyme kinetics

The temperature at which oxygen consumption or complex activities were measured varied from room temperature to 37^{o}C. The rate of an enzyme-catalysed reaction increases as the temperature is raised (231). For example, the relative increase in the rate of reactions catalysed by Complex V is around two fold in the range from 25^{o}C to 37^{o}C (232). Variations in reaction temperature as small as one or two degrees may introduce changes in results. The temperature of peripheral blood in-vivo is 37^{o}C and hence the studies that measured oxygen consumption or complex activities at this temperature reflected in-vivo conditions.

3.11.7 Use of isolated mitochondria versus intact cells

Six of the 33 studies isolated mitochondria. In Parkinson's disease purified mitochondria from PBMC showed no difference in CIV activity (175), (176) compared to controls whereas studies that did not purify mitochondria found differences in CIV activity(222), (174). There are advantages and disadvantages in using both isolated mitochondria or intact cell (99) (Table 3-4). But, the method of isolation of mitochondria can have a large effect on accuracy and reproducibility of polarographic studies (151). The vigorous process of isolating purified mitochondria may only render the "best mitochondria" viable, thus producing a selection bias (152). Additionally, in order to use cell homogenates a relatively small volume of blood (5-10 mls) is required compared to when isolated mitochondria is used. If these tests are to be used clinically in the future it will be ideal if the test requires a small volume of blood.

Table 3-4 Advantages and disadvantages of working with isolated mitochondria and intact cells adapted from Brand et al. (99)

Isolated mitochondria		**Intact cells**	
Advantage	**Disadvantage**	**Advantage**	**Disadvantage**

Isolated mitochondria		Intact cells	
Advantage	**Disadvantage**	**Advantage**	**Disadvantage**
3.11.7.1.1.1.1.1 Simple, well understood. 3.11.7.1.1.1.1.2 Better for studies of mechanism. 3.11.7.1.1.1.1.3 Factors from cytoplasm do not interfere	Lack cellular context	Greater physiological relevance as cellular environment is not tampered with. Cell to cell interactions preserved.	. Difficult to interprete results due to complexity Lack organismal context
Easy to isolate from many animal tissues	Only mitochondria that survive the isolation process is studied resulting in selection bias. Isolation from tough tissues may be a problem	No artefacts due to mitochondrial isolation.	Can be difficult to isolate viable cells to study

Isolated mitochondria		Intact cells	
Advantage	**Disadvantage**	**Advantage**	**Disadvantage**
3.11.7.1.1.1.1.4 Reagents and substrate can be added directly and conditions may be controlled tightly	Necessary to choose appropriate experimental conditions	The cell sets the mitochondrial environment	Many reagents and substrates cause cell damage which restricts experiment options. , Extracellular substrates and conditions need to be chosen carefully
Methods well established	Often need large amounts of sample Mitochondria from different cell types may become mixed	Relatively small amounts of sample required.	Many methods not well established yet
Meaningful to normalize to protein or cytochrome content	Effects due to mitochondrial proliferation, localization lost during isolation	Effects due to mitochondrial proliferation and localization retained	The meaning of results changes with normalization (cell number, cell mass, DNA)

None of the included studies used glycerol-3-phosphate (G3P) as a substrate. Glycerol-3-phosphate dehydrogenase (GpDH) oxidises G3P to dihydroxyacetone phosphate, reducing a flavin prosthesic group that donates its reducing equivalents to the electron transport chain at the level of the CoQ (97). Activity of GpDH increases under conditions of enhanced

metabolic activity and therefore it is expected that activity of GpDH will be altered in disease states (98).

This systematic review has several important strengths. We chose to amalgamate studies that investigated direct ETS function so that we could summarise multiple studies looking at the same disease state and comment on homogeneity and heterogeneity of the results . The nature of the test means that all the studies could only be carried out prospectively and hence this study is rid of any retrospective biases. Our study employed explicit eligibility criteria and a comprehensive search in three major electronic databases as well as the grey literature.

This systematic review also has several weaknesses. The studies were too small to come to any significant conclusion about the clinical relevance of MR function in any specific disease state. Like any other systematic review, there remains a chance of publication bias as positive results are more often published than negative results. However, in the studies included MR function is not always the primary endpoint of those studies and therefore this limits publication bias.

This study highlighted the gap in literature with regard to white blood cell mitochondrial pathophysiology in common chronic disease states such as hypertension and acute disease states such as sepsis, MODS and acute pancreatitis. Additionally, due to the dearth of PBMC MR studies in acute disease states, the differences in MR function between acute and chronic diseases are unknown and whether severity of acute disease states alters MR function is also unknown. This study also shed light on white blood cell mitochondrial pathophysiology in various acute and chronic disease states and critically evaluated the methodology used to measure mitochondrial respiration in WBC. Specific mitochondrial function assays in white blood cells may be useful in the clinical context for diagnosis and prognosis for some of these diseases. Moreover, tests of MR need not be limited in aiding diagnosis or prognosis, but may also be used to monitor therapies. Notably several mitochondrial targeted drugs or compounds that fuel or substantially influence mitochondria are known (233), and the measurement of mitochondrial function will provide a therapeutic gauge to test efficacy of such drugs or compounds.

3.12 Conclusion

This study has identified an important gap in knowledge with regard to mitochondrial function in common chronic disease states such as hypertension and acute illnesses such as severe sepsis/MODS/acute pancreatitis. It is not known whether mitochondrial function

differs between acute and chronic disease states and with increasing severity of acute illness. In summary, white blood cell mitochondrial respiratory function in disease states have the potential to be a widely used clinical assay for diagnosis in diseases such as mitochondrial OXPHOS diseases, prognosis in diseases such as MODS, severe sepsis/shock, CO poisoning, polycystic ovary syndrome and HIV, and aid in monitoring efficacy of existing treatments in CO poisoning and malnourishment. Mitochondrial function in white blood cells in disease states was systematically evaluated and it became apparent that, the method in which white blood cell mitochondrial respiration is evaluated may be further optimized with the use of new technologies such as high resolution oxygraphy. This systematic review provided a solid base, upon which methodology for evaluating mitochondrial function in peripheral blood mononuclear cells in acute disease settings may be further developed.

Chapter 4 Thesis

4.1 The central hypothesis

The first three Chapters have laid the foundation for this thesis. It has been clearly stated that understanding mitochondrial pathophysiology in MODS is important. The reasons for this were discussed (Chapter 1). Current understanding of pathophysiology of MODS was discussed and a fundamental gap in knowledge of mitochondrial pathophysiology in MODS was identified (Chapter 2). The first step in filling this gap was to systematically review the methodology used to evaluate peripheral blood mitochondrial function in acute and chronic disease states (Chapter 3). The systematic review found a dearth of studies evaluating mitochondrial function in acute disease states such as MODS/sepsis/acute pancreatitis. It was also discussed that peripheral white blood cells are involved in inflammation in MODS (Chapter 2) and may provide an easily obtainable source for mitochondrial studies (Chapter 3). It was therefore hypothesised that:

Mitochondrial function can be measured from peripheral blood mononuclear cells, changes over time through the course of the disease and reflects disease severity in patients with MODS.

4.2 Aims

The aims were to:

- Measure PBMC mitochondrial function in patients with MODS during the first week of admission to ICU
- Determine whether mitochondrial respiration correlates with severity of organ failure scores
- Determine whether PBMC mitochondrial function differs in those with septic or non-septic causes of MODS
- Measure longitudinal PBMC mitochondrial function in patients with MODS at 3 weeks and 6 months from inclusion

4.3 The structure of the thesis

To test the central hypothesis, it was necessary to first optimise the assay for mitochondrial respiration in peripheral blood mononuclear cells (Chapter 5). Additionally, mitochondrial respiration results needed to be interpreted in light of other mitochondrial function. Chapter 5 will therefore stipulate methodology concerning different aspects of mitochondrial function used in subsequent experimental and clinical trials.

An important gap discovered in the systematic review (Chapter 3) was the dearth of peripheral blood mitochondrial function studies in common chronic disease states such as hypertension as well as in acute disease states such as MODS/ severe sepsis/ acute pancreatitis. As a result, whether mitochondrial function differs between acute and chronic disease states is unknown and whether severity of acute illness alters mitochondrial function is also unknown.

To test the central hypothesis it was important to gain an in-depth understanding of peripheral blood mitochondrial function in MODS. Therefore, it was also necessary to understand how mitochondrial function in MODS differs from common chronic disease states such as hypertension. Additionally, it was important to understand whether the pattern of mitochondrial function in severe acute illness such as MODS differs from mild acute illness such as mild acute pancreatitis.

To this end, the newly developed assay in Chapter 5 was applied to a chronic experimental model of hypertension (Chapter 6), an acute experimental model of acute pancreatitis (Chapter 7), clinical trial of mild acute pancreatitis (Chapter 7) and finally the central hypothesis of this thesis was tested in Chapter 8 and Chapter 9 (Figure 4-1).

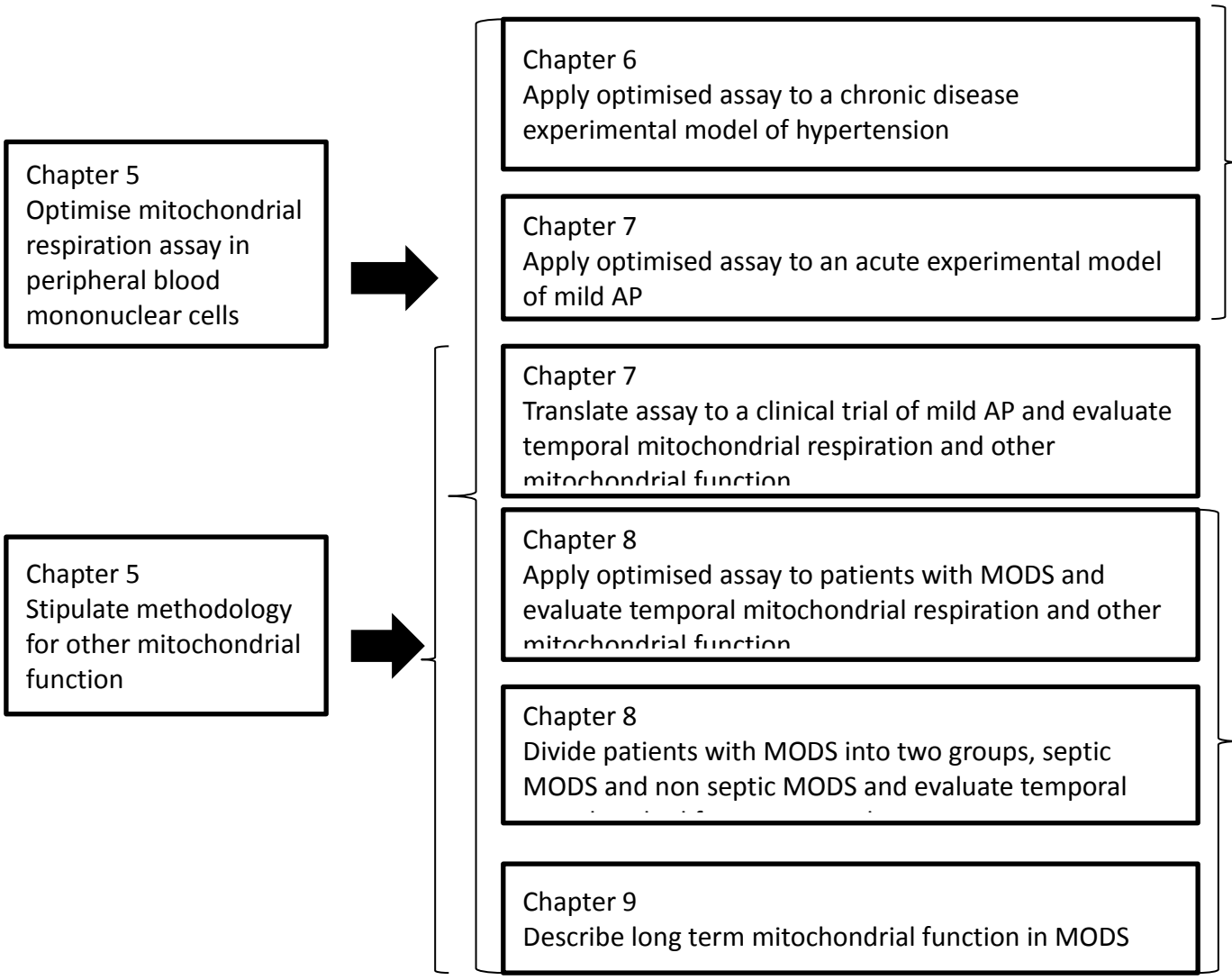


Figure 4-1 Structure of the thesis.

4.3.1 Hypotheses and aims for each chapter

Chapter 6 applied the assay in an established chronic experimental model of hypertension and compared peripheral blood mononuclear cell mitochondria and heart mitochondria. Chapter 6 was designed to test the hypothesis that mitochondrial respiration function is altered in peripheral blood mononuclear cells in hypertension. The aim of this study was to measure blood mononuclear cell mitochondrial respiratory function in spontaneously hypertensive male rats (SHR) at an early hypertensive stage and a compensated hypertrophic stage and compare these two groups with age matched male Wistar rats.

Chapter 7 applied the assay in an experimental model of mild acute pancreatitis (AP) and took the assay from the bench to the bedside to a clinical trial of mild AP. This study was designed to test the hypothesis that PBMC mitochondrial respiration is altered in AP and the nature of this change is reversible. The aims of this study were to measure early mitochondrial function

in an experimental model of AP and to measure mitochondrial function in patients with AP from admission to discharge.

Finally, in chapters 8 and 9 the hypothesis of this thesis was directly tested by employing the optimised assay as well as measuring other aspects of mitochondrial function in patients with septic and non septic MODS. The aims were to measure PBMC mitochondrial function in patients with MODS during the first week of admission to ICU and determine whether it correlates with scores of the severity of organ failure scores and with mortality, to determine whether PBMC mitochondrial function differs in those with septic or non-septic causes of MODS and to measure the longitudinal changes in PBMC mitochondrial function at 3 weeks and 6 months after inclusion.

In Chapter 10 the findings of the experimental chapters were summarised and future work was discussed. Mitochondrial function in MODS was compared with hypertension and the pattern of mitochondrial function recovery from mild acute illness (mild acute pancreatitis) was contrasted with the pattern of mitochondrial function recovery from MODS. This enabled an in-depth understanding of mitochondrial function in MODS.

Hypothesis (Chapter 6)
Mitochondrial respiration function is altered in peripheral blood mononuclear cells in hypertension
Aim
Measure blood mononuclear cell mitochondrial respiratory function in spontaneously hypertensive male rats (SHR) at an early hypertensive stage and a compensated hypertrophic stage and compare these two groups with age matched male Wistar rats

Hypothesis (Chapter 7)
PBMC MR is altered in AP and the nature of this change is reversible
Aims
Measure early mitochondrial function in an experimental model of AP
Translate assay of peripheral blood mitochondrial function from experimental models to patients
Measure mitochondrial function in patients with AP from admission to discharge

Hypothesis (Chapter 8)
Mitochondrial function can be measured from peripheral blood mononuclear cells, changes over time through the course of the disease and reflects disease severity in patients with MODS.
Aims
Measure PBMC mitochondrial function in patients with MODS during the first week of admission to ICU
Determine whether mitochondrial respiration correlates with severity of organ failure scores
Determine whether PBMC mitochondrial function differs in those with septic or non-septic causes of MODS

Hypothesis (Chapter 9)
Mitochondrial dysfunction persists long term in patients with MODS
Aim
Measure longitudinal changes in PBMC mitochondrial function at 3 weeks and 6 months after inclusion

Figure 4-2 Hypotheses and aims for each chapter

Chapter 5 Method

This thesis incorporated laboratory protocols, animal experimental models and clinical studies. As such, the methodology for this thesis will be described under individual sections within this chapter. As discussed in Chapter 4 of this thesis, mitochondrial respiration assay will be developed in the first section of this chapter. The subsequent three sections will describe the other laboratory protocols, animal experimental models and the clinical studies.

5.1 Development of mitochondrial respiration assay in peripheral blood mononuclear cells

In Chapter 3, the methodologies used to measure white blood cell mitochondrial respiration were systematically reviewed. Specifically, the type of cells to be used, whether isolated mitochondria or cell homogenates should be used, types of substrates to be used, the temperature that the assays should be carried out in and the type of technology to be used were discussed.

Further, it became apparent that PBMC are better than mixed white blood cells or granulocytes for measuring mitochondrial respiration. PBMC are even more appropriate in the context of MODS because of their significant role in immunopathogenesis of sepsis (234, 235). Use of cell homogenates requires less blood for evaluation and has the advantage of avoiding selection bias that isolated mitochondria undergo during processing (152). The substrates that were used in the polarographic studies were CI linked substrates glutamate, malate, pyruvate and CII linked substrate succinate (Chapter 3, Table 3-2). None of the studies used glycerol-3-phosphate even though it donates its reducing equivalents to the electron transport chain at the level of the ubiquinone (98) and the activity of glycerol 3 phosphate dehydrogenase may alter in disease states. A temperature of 37^{o}C resembles *in vivo* temperature and therefore was felt to be most appropriate for measurement of mitochondrial respiration since temperature alters the rate of enzyme activity.

Polarography has an advantage over spectrophotometry in that it provides the possibility of assessing the activity of all complexes of the oxidative phosphorylation system in intact mitochondrial membranes under conditions which reflect the ‘in vivo’ situation (228). However, the simple Clark electrode used in polarography has several downfalls. Oxygen may diffuse through the electrode, small changes in respiration are difficult to detect and large

biological samples are required (236). High resolution respirometry minimises some of these disadvantages.

However, using high resolution respirometry to measure white blood cell mitochondrial respiration required optimisation of existing techniques. Since multiple parameters of peripheral blood mitochondrial function needed to be assessed daily in critically ill patients to address the hypothesis of this thesis, it was important to obtain the smallest volume of blood possible to do the assays. Further, it was important to ensure that the process of isolating PBMC does not affect mitochondrial respiration parameters. The strength of high resolution respirometry is that it enables multiple substrates, inhibitors and uncouplers to be titrated within the same sample (236). After preliminary optimisation of the assay was done in healthy rats, the assay required translation to healthy volunteers. In this chapter, the development of methodology for PBMC mitochondrial respiration using high resolution respirometry will be discussed. Each section below describes the final protocol followed by the optimisation steps required to reach the final protocol.

5.1.1 Blood tube

Blood was obtained and immediately transferred to vacutainers containing citrate phosphate dextrose (CPD) (Figure 5-1) tubes and serum separating tubes (SST).

Figure 5-1 Transfer of blood into citrate phosphate dextrose (CPD) tubes.

After SST tube had clotted, it was centrifuged at 4^{o}C, 3000rpm for 5 mins and aliquot of serum was stored in labelled eppendorf tubes at -80^{o}C. Serum was sent to Auckland City Hospital's laboratory for batch analysis.

5.1.1.1 Optimisation step

For isolation of PBMC from whole blood CPD (citrate phosphate dextrose), K_2EDTA (potassium ethylenediaminetetraacetic acid), and Heparin blood tubes were compared (n=3). Although not statistically significant, after separation of PBMC, it was found that CPD tubes gave the greatest viable yield of PBMC per millilitre of whole blood (Table 5-1).

Table 5-1 Retrieval of peripheral blood mononuclear cells (PBMC) from various blood tubes. CPD citrate phosphate dextrose; K_2EDTA potassium ethylenediaminetetraacetic acid.

	CPD (n=3)	**K_2EDTA (n=3)**	**Heparin (n=3)**
Viable PBMC (* 10^6cells/ml of blood)	1.9+/-0.2*	1.8+/-0.3*	1.4+/-0.4*

*p>0.05 on ANOVA.

5.1.2 Separating PBMC from whole blood

The citrate phosphate dextrose (CPD) tubes were used to retrieve peripheral blood mononuclear cells (PBMC). PBMC were retrieved from whole blood using the density gradient separator Ficoll-Paque^{TM} Plus (GE Lifesciences). Ficoll-Paque^{TM} Plus is a sterile density centrifugation medium which separates mononuclear cells from whole blood. Blood from the CPD tubes were transferred into 50ml falcon tubes and diluted at a 1:1 ratio with phosphate buffered saline (PBS). The same volume of Ficoll density gradient as the whole blood was injected gently to the bottom of the tube over 1 minute so that the diluted blood was on top of the Ficoll-Paque^{TM} Plus gradient (Figure 5-2). The tube was centrifuged at 1600G for 10mins at 22^{o}C with brake set over 1 minute.

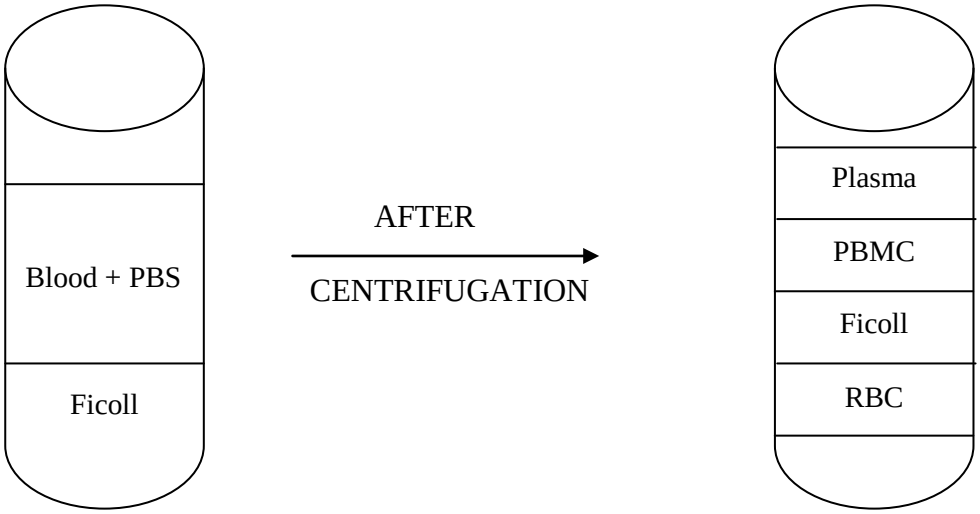


Figure 5-2 Retrieving mononuclear cells from whole blood.

The PBMC layer was retrieved into a 15ml falcon tube and topped up fully with PBS and then centrifuged at 650G for 10mins at 22^{o}C. The cells were then washed with PBS.

5.1.2.1 Optimisation steps

Ficoll paque (GE Healthcare) was compared with Histopaque 1077 (Sigma Aldrich) for separation of PBMC from whole blood (n=3 healthy volunteers (HV)). Both resulted in comparable isolation of PBMC per millilitre of whole blood 1.6+/- 0.2 million cells/ml of whole blood (Ficoll paque) and 1.5+/- 0.3 (Histopaque 1077) million cells/ml of whole blood. Ficoll paque was easily obtainable compared to Histopaque and therefore used in the experimental and clinical trials.

Mitochondrial respiration was measured (n=3 healthy rats/group) straight after the PBMC layer was retrieved without any washing, after one, two and three washes with PBS at 650G for 10mins at 22 ^{o}C. The number of washes did not make any significant difference to mitochondrial respiration measured. The same study was repeated in patients with mild acute pancreatitis to decipher whether “sick” PBMC would withstand repeated washes (n=4 patients with mild acute pancreatitis). No significant differences were found in respiration states when one, two and three washes were compared (Figure 5-3).

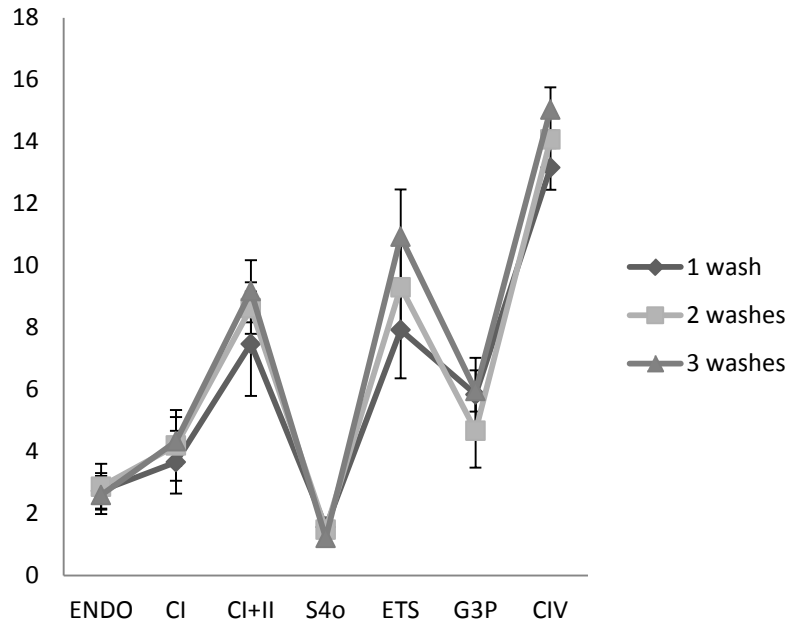


Figure 5-3 Mitochondrial respiration state with one, two or three pre-washes with phosphate buffered saline (n=4 mild AP patients/group). p>0.05 on ANOVA for all respiratory states when 1, 2 and 3 washes compared. ENDO [endogenous], CI [Complex I], CI+II [Complex I and II], S4o [Complex I and II leak], ETS [Electron Transport System], G3P [Glycerol-3-Phosphate], CIV [Complex IV].

5.1.3 Preparing mononuclear cells

After washing with PBS cells were resuspended in mitochondrial respiration buffer (MiR05) buffer containing (in mmol/l (final)) 0.5 EGTA [ethylene glycol tetraacetic acid], 3 $MgCl_2$ [magnesium chloride], 60 potassium-lactobionate, 20 taurine, 10 KH_2PO_4 [monopotassium phosphate], 110 sucrose, and 1 mg/ml BSA [bovine serum albumin] in 20 mmol/l HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] (236). A cell count was performed using trypan blue and the Countess Automated Cell Counter (Invitrogen), and respiration assays normalized to million live cells (Figure 5-4).

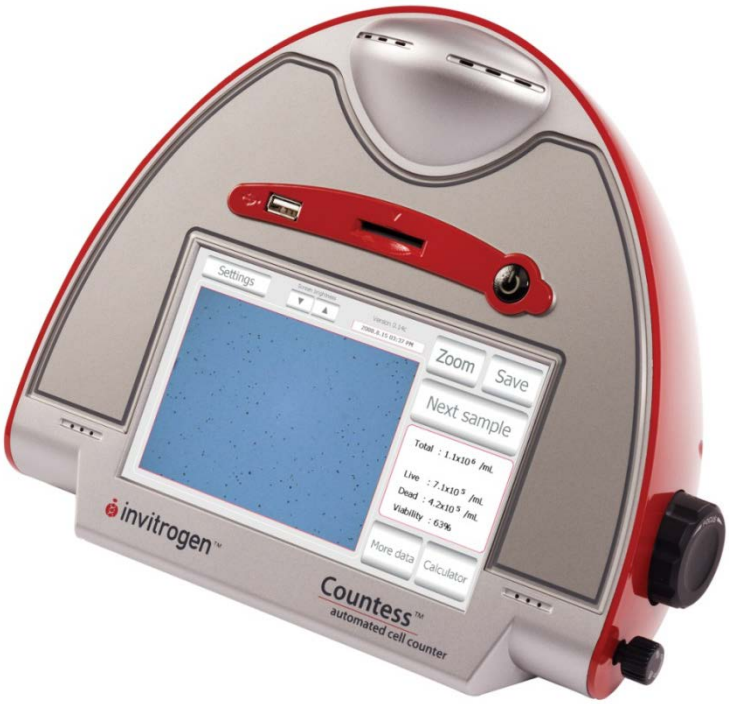


Figure 5-4 Countess automated cell counter to count mononuclear cells.

5.1.4 Mitochondrial respiration assay

An OROBOROS Oxygraph-2K (Oroboros Instruments, Innsbruck, Austria) was used for oxygraphy (Figure 5-5). The objective of high resolution oxygraphy is to quantify low levels of oxygen and rate of oxygen consumption (oxygen flux) from cells and tissues so that respiratory activity can be measured from very small biological samples (10^6 cells). The OROBOROS Oxygraph-2k achieves this with its unique chamber design, using oxygen-tight materials and highly sensitive electrodes and electronics to avoid oxygen back diffusion that is common in traditional polarography, tight control of temperature with peltier-temperature control and software that calculates oxygen flux continuously so that oxygen flux of the biological sample can be displayed on the monitor along with oxygen concentration (149). The strength of high resolution respirometry is the limit of detection of oxygen flux as low as 1 pmol $O_2.s^{-1}.ml^{-1}$ and oxygen back diffusion at zero oxygen is <4 $pmol.s^{-1}.ml^{-1}$ (236).

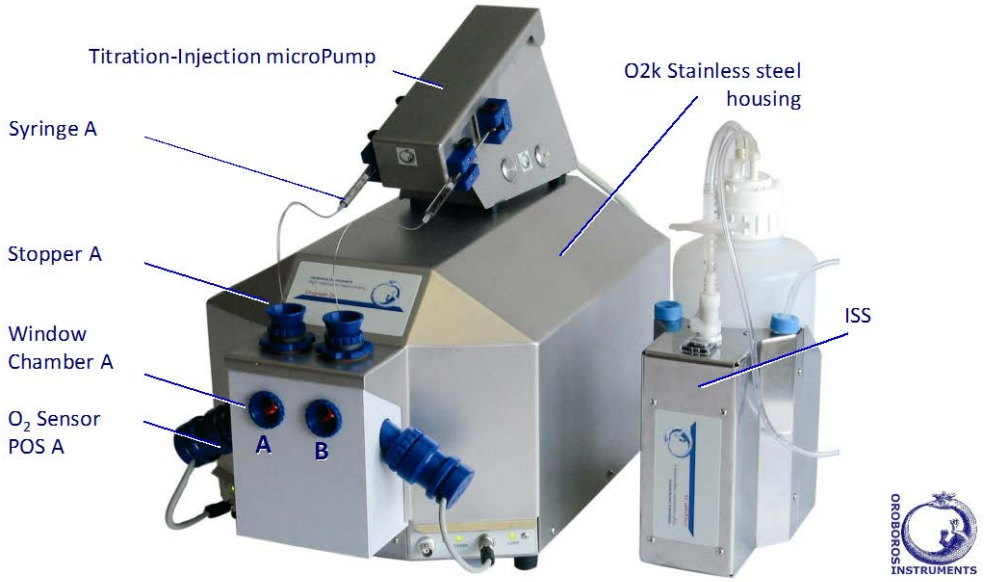


Figure 5-5 OROBOROS Oxygraph-2k for high-resolution respirometry as provided by OROBOROS INSTRUMENTS. O2k [Oroboros oxygraph 2k], POS [polarographic oxygen sensor], ISS [integrated suction system]. (Copyright permission sought from oroboros.at)

At low respiratory flux per volume such as peripheral blood mononuclear cells, the oxygen capacity of the system provides sufficient time for evaluation of slow approaches of the biological sample to a steady state and allows for application of complex titration regimes in intact or permeabilised cells. This feature enables the ETS to be analysed as a whole system from a small number of white blood cells (149).

Respiratory measurements were performed at 37°C in 2 ml incubation assay medium, and the oxygen concentration at air saturation of the medium was 215 nmol O_2/ml at 95 kPa barometric pressure. Oxygen flux was calculated as the time derivative of oxygen concentration using the DatLab 4 Analysis Software, OROBOROS (Oroboros Instruments, Innsbruck, Austria).

Approximately 10 million mononuclear cells were added per 2ml chamber and endogenous respiration was measured. The endogenous cellular respiration (Endo) is the sum of oxidative phosphorylation, respiration due to proton leak across mitochondrial inner membranes (which do not contribute to ATP synthesis), and non mitochondrial oxygen consumption by numerous oxidases. Digitonin (25µg/ml) was then added to permeabilise the cholesterol rich plasma membrane, thereby leaving mitochondrial membranes intact and permitting the diffusion of cytosolic components out of cells and mitochondrial substrates into cells. A

substrate uncoupler inhibitor titration (SUIT) protocol was then employed (Table 5-2). Table 5-2 summarises the SUIT protocol used.

Table 5-2 Substrates, uncouplers, and inhibitors used in permeabilised mononuclear cells with abbreviations (Abbr.), and site of action (electron entry, substrate entry, or inhibition of ATP synthase).

Substrates	**Abbr.**	**Site of action**	**Concentration in syringe (solvent)**	**Storage (°C)**	**Final concentration in 2-ml O2k-chamber**	**Titration (µl) into 2 ml**	**Syringe (µl)**
Glutamate	G	CI	2 M (H_2O)	−20	10 mM	10	25
Malate	M	CI	0.8 M (H_2O)	−20	2 mM	5	25
ADP	D	CV	0.5 M (H_2O)	−80	1.25 mM	2.5-10	10
Cytochrome c.	c	CIV	4 mM (H_2O)	−20	10 µM	5	25
Pyruvate	P	CI	2 M (H_2O)	Fresh	5 mM	5	25
Succinate	S	CII	1 M (H_2O)	−20	10 mM	20	25
Glycerol-3-phosphate	G3P	GpDH	1M (H_2O)	-20	10mM	20	25
Ascorbate	A	CIV	0.8 M (H_2O)	−20	2 mM	5	25
TMPD [a]	T	CIV	0.2 M (H_2O)	−20	0.5 mM	5	25
Uncoupler							
FCCP[b]	F	$\Delta\mu_{H^+}$	1 mM (EtOH)	−20	0.5 µM steps	1 µl steps	10
Inhibitors							
Rotenone Malonic acid Antimycin A	rot mna ama	CI CII CIII	1.0 mM (EtOH) 2 M (H_2O) 5 mM (EtOH)	−20 Fresh −20	0.5 µM 5.0 mM 2.5 µM	1 5 1	10 25 10

Substrates	Abbr.	Site of action	Concentration in syringe (solvent)	Storage (°C)	Final concentration in 2-ml O2k-chamber	Titration (µl) into 2 ml	Syringe (µl)
Oligomycin	olig	CV	4 mg/ml (EtOH)	−20	2 µg/ml	1	10

[a] N,N,N,N ¢,N ¢-tetramethyl-p-phenylenediamine dihydrochloride, [b]Carbonyl cyanide p-trifluoromethoxyphenylhydrazone, H2O [water], EtOH [alcohol], CI [Complex I], CII [Complex II], CIII [Complex III], CIV [Complex IV], CV [Complex V], GpDH [Glycerol-3-phosphate dehydrogenase].

After waiting for oxygen flux to decline to a low steady state, Complex I (CI) mediated leak (CI Leak, also known as State 2) was stimulated by the addition of CI substrates (10 mmol/l glutamate, 2 mmol/l malate). Addition of excess adenine diphosphate (ADP) (1.25 mmol/l for PBMCs) then stimulated oxidative phosphorylation (CI OXPHOS, also known as State 3). Addition of cytochrome *c* (10 µmol/l) was used to test the functional integrity of mitochondrial inner membranes, and pyruvate (5mmol/l) was then added to ensure saturation of CI OXPHOS from the tricarboxylic acid cycle (TCA). Phosphorylating respiration with Complex I and II substrates (CI,II OXPHOS, also known as State 3) was then assessed by addition of succinate (10 mmol/l). Leak respiration with CI and Complex II (CII) substrates (CI,II Leak, also known as State 4o) was measured after adding the ATP synthase inhibitor oligomycin (5µmol/l), resulting respiration in this state is attributable to proton leakage (103). Once a steady CI,II Leak was achieved, multiple titrations (generally three) of carbonyl cyanide *p*-(trifluoromethoxy)phenyl-hydrazone (FCCP, 0.5 µmol/l) were used to maximally chemically uncouple the ETS from the OXPHOS. Complex I and then CII activity were selectively inhibited by the addition of rotenone (1 µmol/l) and malonate (15mmol/l). Glycerol-3-phosphate (G3P) (10mmol/l) was then added to activate glycerol-3-phosphate dehydrogenase (GpDH). Antimycin A (1 µmol/l) was added to inhibit Complex III (CIII), and provides a measure of residual (non-mitochondrial) oxygen consumption (149). Finally, the activity of Complex IV (CIV) was measured by the addition of the electron donor couple *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD, 0.5 mmol/l) and ascorbate (2 mmol/l).

Respiration flux was measured as O_2 flux per million live cells (pmol/s/million cells) and chemical backgrounds were measured and subtracted from all experiments. Figure 5-6 is a representative oxygraph respiratory trace for mononuclear cells.

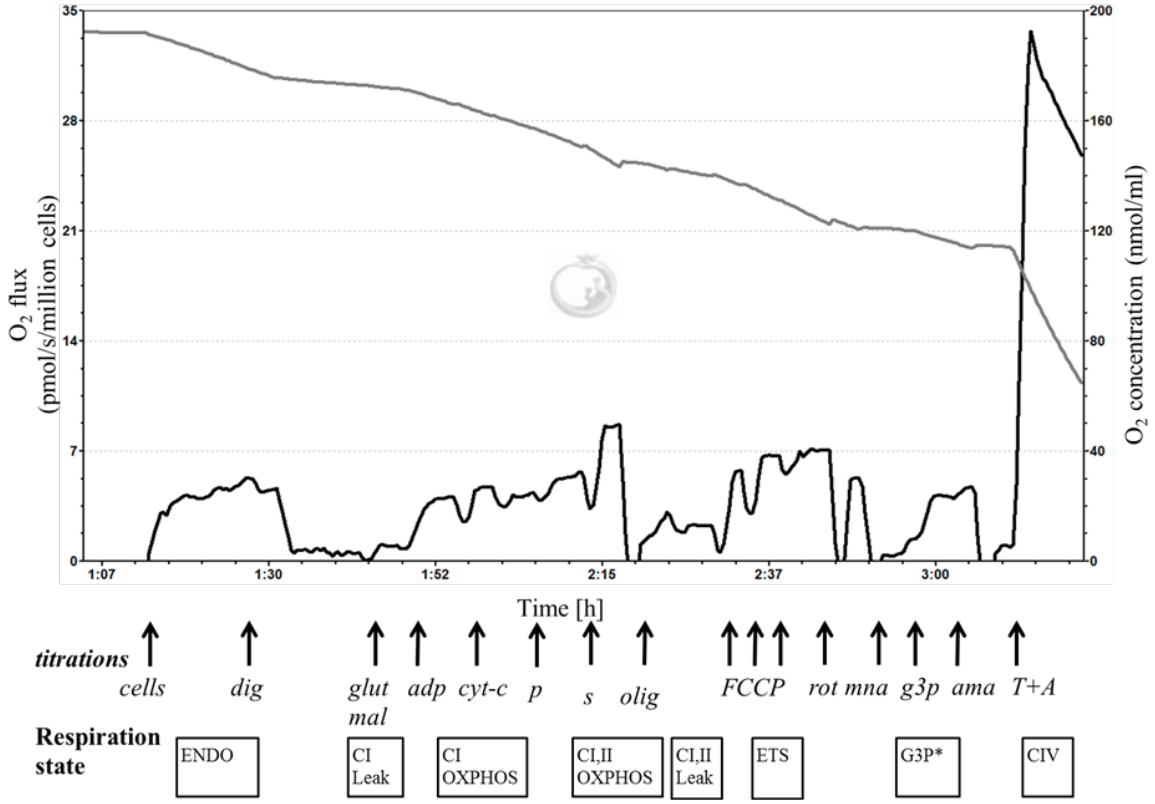


Figure 5-6 Representative oxygraph mitochondrial respiration traces of mononuclear cells showing oxygen concentration (gray line, right Y-axis) and oxygen flux (black line, left Y-axis) over time. Titrations are italicised and the corresponding respiration state shown beneath. dig [digitonin], glut [glutamate], mal [malate], adp =[adenosine di-phosphate], cyt-c [cytochrome-c], p [pyruvate], s [succinate], olig [oligomycin], FCCP [carbonyl cyanide p-(trifluoromethoxy) phenyl-hydrazone], rot [rotenone], mna [malonic acid], g3p [glycerol-3-phosphate], ama [antimycin-a], T+A [N, N, N', N'-tetramethyl-Phenylenediamine + ascorbate], ENDO [endogenous], CI [Complex I], CI,II[Complex I and II], OXPHOS [oxidative phosphorylation], ETS [Electron Transport System], CIV [Complex IV].

Table 5-3 provides a summary of mitochondrial respiration functions measured.

Table 5-3 Mitochondrial respiration function measurements: direct mitochondrial respiration measurements and derived flux control ratios (FCR).

State	Abbreviation	Meaning
Direct mitochondrial respiration measurements		
Endogenous	Endo	Respiration is based on endogenous substrates present in the intact cell (237)
Complex I Leak	CI Leak, State 2	After permeabilising the cytoplasmic cell membrane and adding in CI substrates, respiration mainly compensating for passive proton leak, proton slip, electron slip and cation cycling through inner membrane ion channels such as uncoupling proteins or permeability transition pores (238), (237)
Complex I OXPHOS	CI OXPHOS, State 3	Respiration controlled by ATP turnover and substrate oxidation through CI (99)
Complex I and Complex II OXPHOS	CI,II OXPHOS, State 3	Respiration controlled by ATP turnover and substrate oxidation through CI and CII (99)
Complex I and Complex II Leak	CI,II Leak, S4o	Respiration mainly compensating for passive proton leak, proton slip, electron slip and cation cycling through inner membrane ion channels such as uncoupling proteins or permeability transition pores (238), (237)

State	Abbreviation	Meaning
Electron transport system capacity	ETS, State 3u	Uncoupler titration removes electrochemical backpressure and therefore stimulates maximal respiration (237). Respiration is controlled by substrate oxidation (99)
Glycerol-3-phosphate	G3P	Reflects ETS capacity with the enzyme glycerol-3-phosphate dehydrogenase (GpDH)
Cytochrome-c-oxidase	CIV	Reflects ETS capacity with the enzyme cytochrome *c* oxidase (237)
Derived parameters – Flux control ratios		
CI OXPHOS/CI Leak		Measure of phosphorylation efficiency with CI substrates
CI,II OXPHOS/CI,II Leak (RCR)		Measure of combined phosphorylation efficiency, otherwise known as the respiratory control ratio (RCR) and is a sensitive indicator of overall mitochondrial function
CI,II OXPHOS/ETS		Expression of the limitation of OXPHOS capacity by the phosphorylation system
%CI ETS		Percentage of CI as a function of ETS capacity
CI,II Leak/ETS		Estimate for normalised uncoupled respiration

From the direct mitochondrial respiration measurements, the following flux control ratios (FCR) of MR function were derived (149):

- CI OXPHOS/CI Leak was calculated as a measure of phosphorylation efficiency with CI substrates

- CI,II OXPHOS/CI,II Leak was calculated as a measure of combined phosphorylation efficiency, otherwise known as the respiratory control ratio (RCR) and is a sensitive indicator of overall mitochondrial function (149), (99)

- CI,II OXPHOS/ETS is an expression of the limitation of OXPHOS capacity by the phosphorylation system. This ratio decreases with decreasing capacity of the phosphorylation system (239).

- %CI ETS is the percentage of CI as a function of ETS and was calculated using the formula: ((ETS-ETSrot)/ETS)* 100 for an estimate of flux through CI alone relative to total ETS flux (239).

- CI,II Leak/ETS is the leak flux control ratio and reflects the level of leak respiration relative to ETS capacity and provided an estimate for normalised uncoupled respiration (237).

5.1.4.1 Optimisation steps

Respiration media

Mitochondrial respiration media MiR05 and plasma were both considered for the analysis of mitochondrial respiration. MiR05 was designed specifically as a medium for mitochondrial respiration analysis for high resolution respirometry (Table 5-4) (240). The reasoning for each ingredient in the buffer is provided in Table 5-4.

Table 5-4 Components of MiR05 and their function within the buffer.

Component	**Function**
EGTA [ethylene glycol tetraacetic acid]	Chelator for heavy metals with high affinity for calcium and low affinity for magnesium
Magnesium	Activation of ATP due to ATPase activity is Magnesium dependent. Mitochondrial preparations cannot be tested in the absence of magnesium.
potassium-lactobionate and KH_2PO_4 [monopotassium phosphate]	To match the high intracellular concentration of potassium
Taurine	Biological membrane stabiliser and ROS scavenger (241)
Sucrose	Stabilises membrane bound cytochrome c
BSA [bovine serum albumin]	Membrane stabiliser, oxygen radical scavenger, binding of calcium and free fatty acids

MiR05 was compared with patient's own plasma as a choice for mitochondrial respiration medium. Blood was taken from healthy volunteers (n=5) and assays done in both MiR05 and plasma from each volunteer. There were no significant differences in any respiration states between MiR05 and plasma (Figure 5-7). However, when this assay was being developed, it became evident that plasma from patients with sepsis was capable of altering mitochondrial function (242). If the patient's plasma was used as media, it would be difficult to ascertain whether any changes seen in mitochondrial function was due solely to the "diseased" plasma or whether the changes were due to a difference in mitochondrial function. It was therefore decided that MiR05 was the best option to be used as respiration media because use of patient's plasma may add bias to the analysis of mitochondrial respiration.

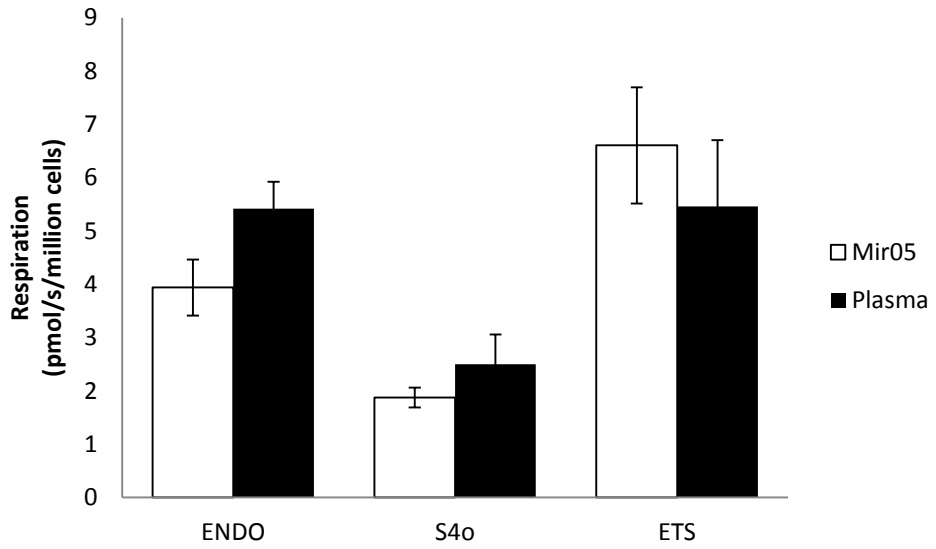


Figure 5-7 Comparison of MiR05 and plasma as buffers for mitochondrial respiration (n=5 healthy volunteers/group). p>0.05 for all respiratory states on student's *t* test when Mir05 and plasma were compared. ENDO [endogenous], S4o [Complex I and Complex II leak], CIV [Complex IV].

Permeabilised cells/isolated mitochondria

As highlighted in Chapter 3, mitochondrial respiration can be analysed from permeabilised cells or isolated mitochondria. In order to choose between these two methods, factors that were considered were the amount of sample required, the time that the assay will take and avoiding selection bias.

To choose one of these methods it was prudent to have in mind the ultimate aim which was to use this assay clinically in critically ill patients with MODS frequently through out their disease course. Therefore it was paramount that the assay required the least amount of sample possible to avoid unnecessary harm to patients. Using permeabilised cells has the advantage of using much smaller samples compared to isolated mitochondria. Additionally, isolation of mitochondria also adds time to the assay and the process of isolation itself may selectively loose damaged mitochondria creating a bias in the analysis. This is in contrast to permeabilised cells where all cells are analysed and therefore there is no selection bias. Taking all of the above into consideration, permeabilised cells were chosen for analysis of mitochondrial respiration.

Permeabilisation of cells with digitonin

Digitonin was used to permeablise cells to enable analysis of ETS. First, the optimum concentration of digitonin had to be calculated. 10 million cells were suspended in MiR05 within the chamber. After obtaining routine respiration, rotenone was titrated until routine

respiration was obliterated. Succinate and ADP was then titrated in. No effect was seen at this point because succinate cannot penetrate the cell membrane. Stepwise digitonin titrations of 10µg were then done to derive the optimum digitonin concentration until maximal respiration with succinate was reached. Optimum digitonin concentration for PBMC was found to be 5µg/million cells (Figure 5-8).

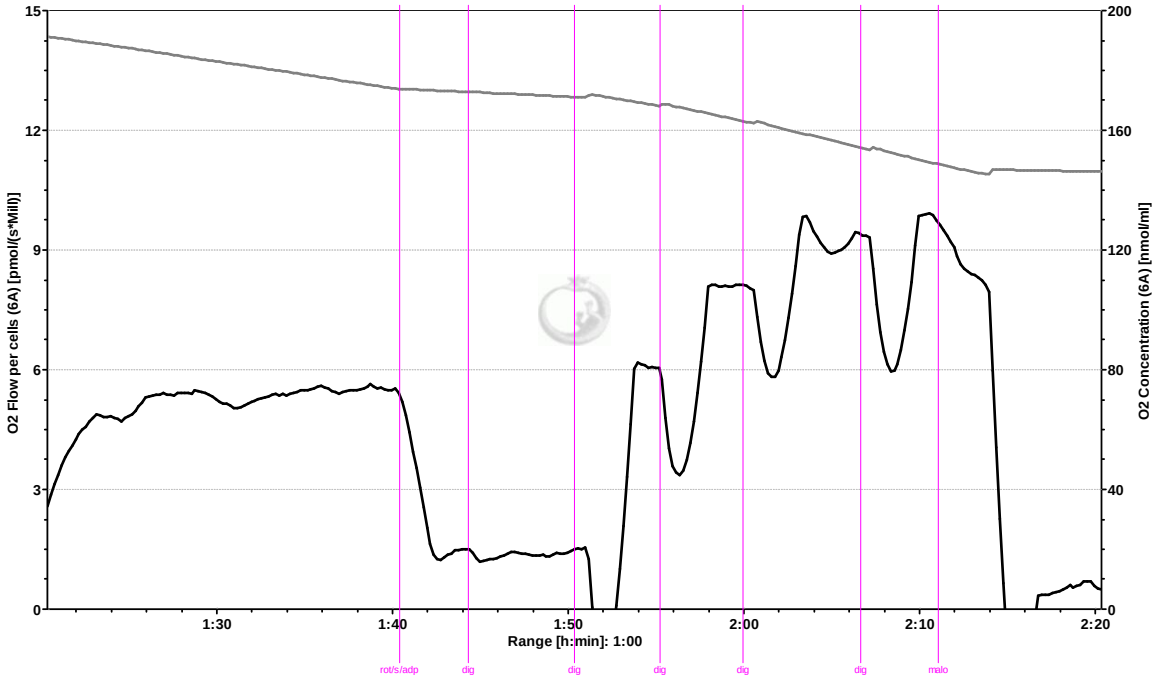


Figure 5-8 Optimisation of mononuclear cell permeabilisation with digitonin by stepwise titrations of digitonin. Oxygen flux in pmol/s/million cells (left Y-axis) and oxygen concentration in chamber (right Y-axis) over time. dig [titrations of digitonin].

Intact cells vs permeabilised cells

The option of using intact cells for additional analysis of mitochondrial respiration remained when the decision was made to use permeablised cells to enable analysis of ETS. The question was whether additional analysis of mitochondrial respiration with intact cells will confer useful information above and beyond using permeablised cells. Mitochondrial respiration were measured from intact "unpermeabilised" cells and compared with permeabilised cells (n=3 healthy volunteers) and found to have no significant difference (Figure 5-9). The same experiment was repeated in patients with MODS (n=5) and no significant difference was found (Figure 5-9, Figure 5-10).

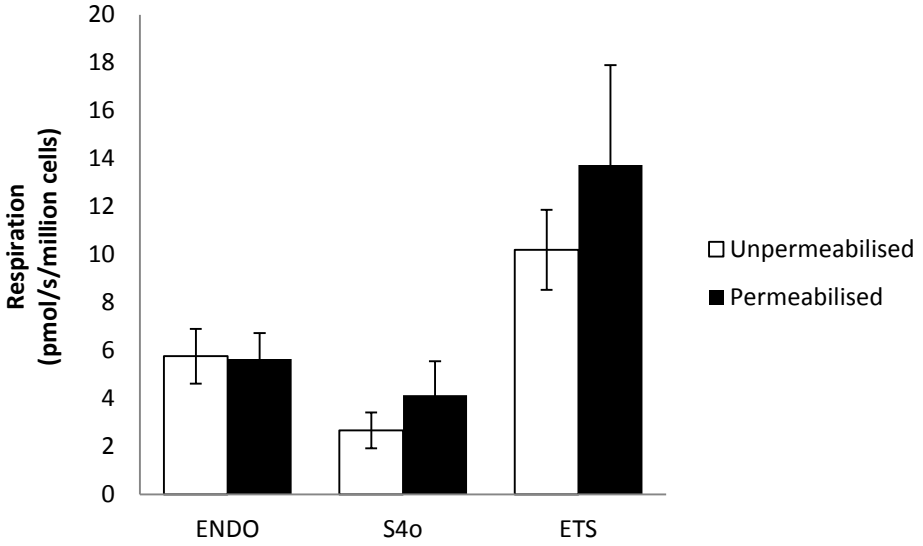


Figure 5-9 Mitochondrial respiration in unpermeabilised and permeabilised cells (n=3 healthy volunteers). p>0.05 for all states of respiration on student's *t* test when unpermeabilised compared to permeablise. ENDO [endogenous], S4o [Complex I and II leak], ETS [Electron Transport System].

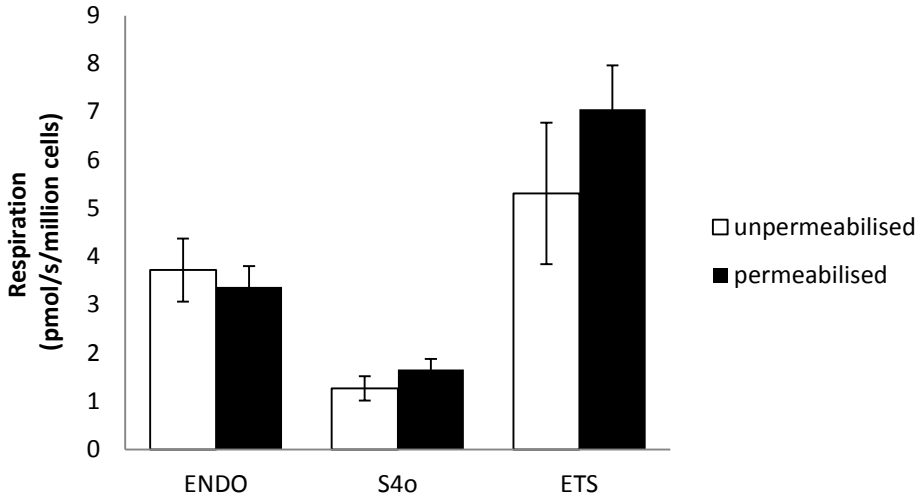


Figure 5-10 Mitochondrial respiration in unpermeabilised and permeabilised cells (n=5 patients with MODS). p>0.05 for all states of respiration on student's *t* test when unpermeabilised compared to permeablise. ENDO [endogenous], S4o [Complex I and II leak], ETS [Electron Transport System].

Amount of PBMC required for an optimum assay

To optimise the amount of blood required, respiration at different concentration of PBMC was measured (Figure 5-11). In the range of 6-12 * 10^6 million cells per chamber, there were little difference in mitochondrial respiration values. Although there were no statistical significant differences found, there was a significant time delay in obtaining a stable signal due to a large variation in the values measured when there were less than 6 million cells per chamber.

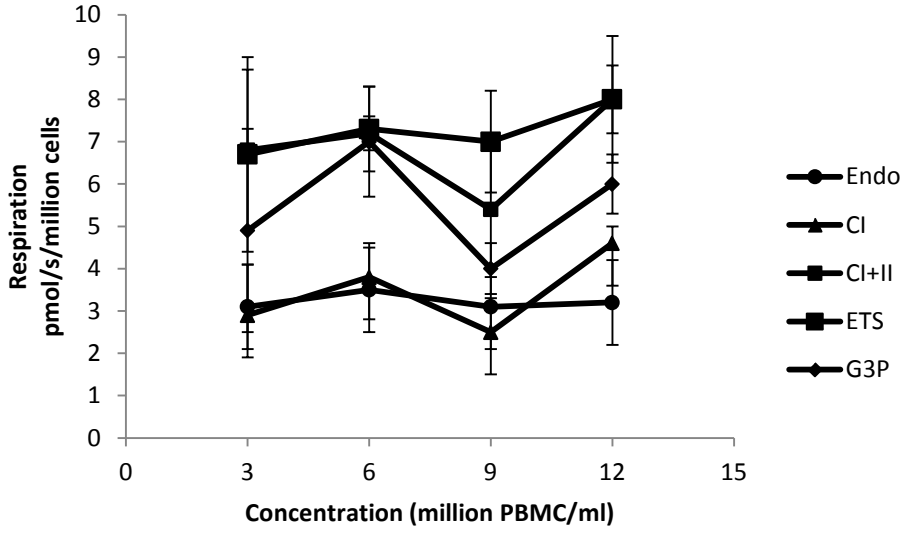


Figure 5-11 Mitochondrial respiration states using different concentration of PBMC (n=3 HV/group). p>0.05 for all states of respiration on ANOVA when different concentrations of peripheral blood mononuclear cells compared. PBMC [peripheral blood mononuclear cells], ENDO [endogenous], CI [Complex I], CI+II [Complex I and II], ETS [Electron Transport System], G3P [Glycerol-3-phosphate].

Time frame that a sample may be used by

It became evident that there may be delays in processing the sample due to factors that are uncontrollable for example resource allocation in the laboratory or transporting the sample from the hospital/patient's home to the laboratory. Therefore it was prudent to understand whether a time delay in processing the sample would alter mitochondrial respiration. In the experimental study (n=4/group) mitochondrial respiration was either measured immediately upon blood collection (t=0) and 3.5 hrs after (t=3.5). There were no differences in mitochondrial respiration found (Figure 5-12). This experiment was repeated in patients with mild AP (n=5) to ensure that "diseased" mitochondria behave similarly to "non-diseased" mitochondria (Figure 5-13). In patients with mild AP, t=0 was defined as the time when blood processing was being started at the laboratory. The average time for transportation between the hospital and the laboratory was 27+/- 6 mins. T=3.5 was defined as 3.5 hrs past the time when blood processing was started (t=0). No significant differences were found in any of the mitochondrial respiration states.

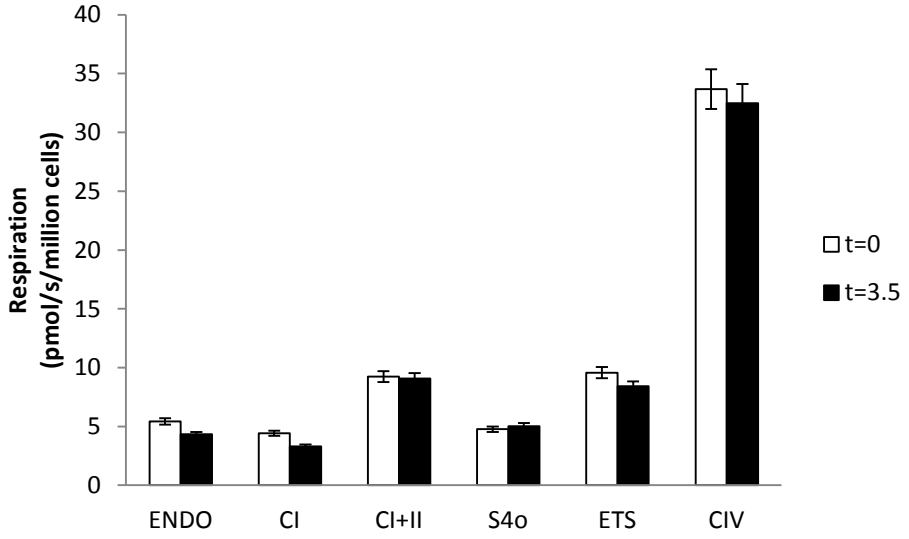


Figure 5-12 Mitochondrial respiration measured in healthy rats (n=4) immediately after blood collection (t=0) and 3.5 hrs after (t=3.5). p>0.05 for all states of respiration on student's *t* test when the two times were compared. ENDO [endogenous], CI [Complex I], CI+II [Complex I and II], S4o [Complex I and II leak], ETS [Electron Transport System],CIV [Complex IV].

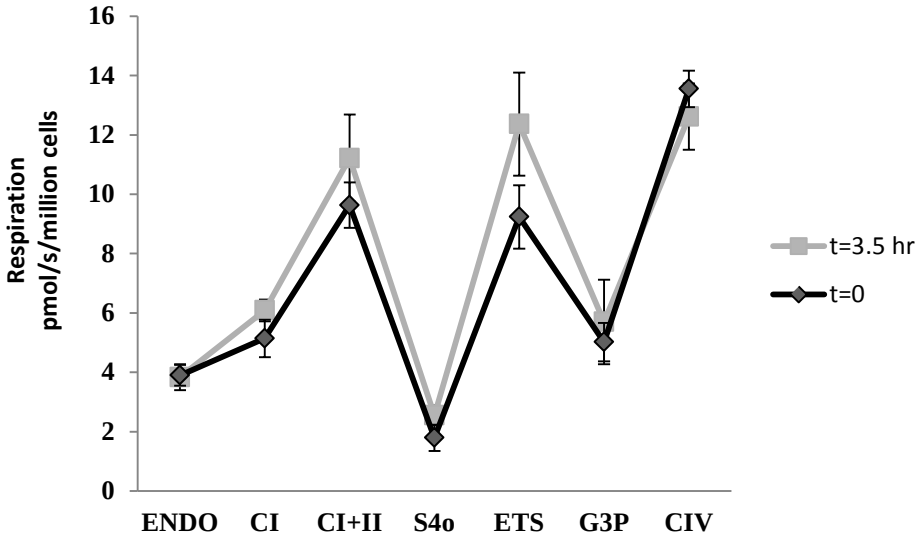


Figure 5-13 Mitochondrial respiration measured in patients with mild AP (n=5) immediately after blood collection (t=0) and 3.5 hrs after (t=3.5). p>0.05 for all states of respiration on student's *t* test when the two times were compared. ENDO [endogenous], CI [Complex I], CI+II [Complex I and II], S4o [Complex I and II leak], ETS [Electron Transport System],G3P [Glycerol-3-phosphate], CIV [Complex IV].

Influence of gender on mitochondrial respiration

Amongst fourteen healthy volunteers, there were no differences in mitochondrial respiration found between males and females (Table 5-5).

Table 5-5 Comparison of mitochondrial respiration between males and females.

	Endo	CI	CI+II	S4o	ETS	G3P	CIV
Male (n=6)	3.1+/-0.3	3.5+/-0.7	6.7+/-1.2	1.6+/-0.3	7.3+/-1.4	5.8+/-1.3	11.8+/-1.4
Female (n=8)	3.4+/-0.2	3.6+/-0.6	6.8+/-0.8	1.4+/-0.2	7.4+/-0.6	4.4+/-0.7	20.6+/-4.8

Mitochondrial respiration measured in pmol/s/million cells. p>0.05 for all states of respiration on student's *t* test when males and females were compared. ENDO [endogenous], CI [Complex I], CI+II [Complex I and II], S4o [Complex I and II leak], ETS [Electron Transport System],G3P [Glycerol-3-phosphate], CIV [Complex IV].

Influence of age on mitochondrial respiration

The cohort of healthy volunteers was separated by age less than 50 years (n=7) and more than 50 years of age (n=7). There were no differences in mitochondrial respiration found between these groups (Table 5-6). Even though the average CIV respiration was 20.0+/-5.7 in the <50 age group compared to 13.4+/-1.4 in the >=50 age group, the variance rendered this statistically insignificant.

Table 5-6 Influence of age in mitochondrial respiration in healthy volunteers.

	Endo	CI	CI+II	S4o	ETS	G3P	CIV
<50 (n=7)	3.2+/-0.2	3.4+/-0.4	7.0+/-1.4	1.4+/-0.2	6.8+/-0.6	5.2+/-0.9	20.0+/-5.7
>=50 (n=7)	3.3+/-0.3	3.7+/-0.8	6.5+/-1.3	1.6+/-0.4	8.0+/-1.2	4.8+/-1.1	13.4+/-1.4

Mitochondrial respiration measured in pmol/s/million cells. p>0.05 for all states of respiration on student's *t* test when the two groups were compared. ENDO [endogenous], CI [Complex I], CI+II [Complex I and II], S4o [Complex I and II leak], ETS [Electron Transport System],G3P [Glycerol-3-phosphate], CIV [Complex IV].

Mitochondrial respiration within same healthy volunteer at two different time points

Healthy volunteers (n=7) underwent blood taking twice at least one week apart to establish whether there are interpersonal differences in mitochondrial respiration measured at two different time points (Figure 5-14). On paired t-test there were no significant differences

found in any of the mitochondrial respiration parameters measured. Whilst not statistically significant, the large variance in CIV was noted.

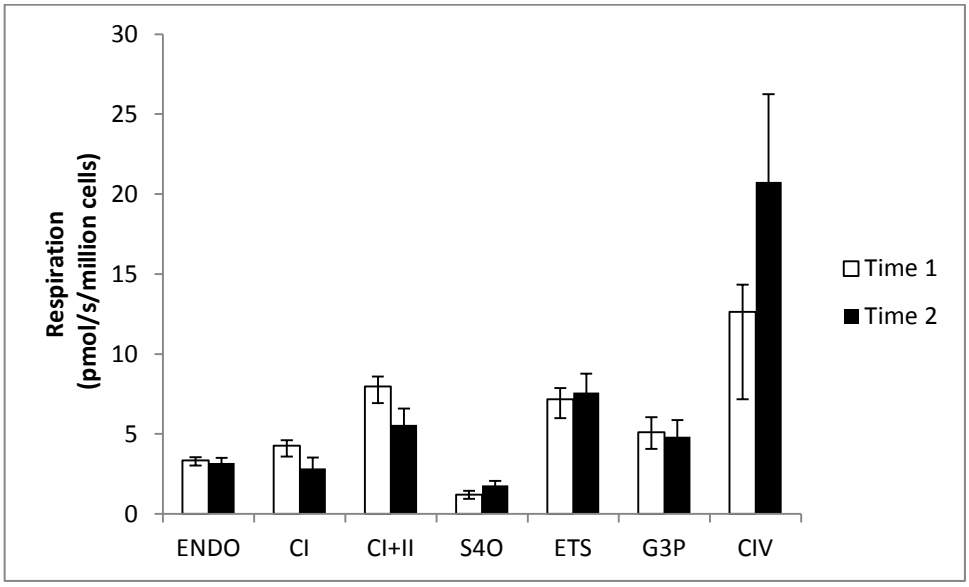


Figure 5-14 Interpersonal differences in mitochondrial respiration from blood taken at least 1 week apart in healthy volunteers (n=7). p>0.05 for all states of respiration on student's *t* test when the two time points were compared. ENDO [endogenous], CI [Complex I], CI+II [Complex I and II], S4o [Complex I and II leak], ETS [Electron Transport System],G3P [Glycerol-3-phosphate], CIV [Complex IV].

Principle behind substrate-uncoupler-inhibitor titration (SUIT) protocols

A multiple substrate-uncoupler-inhibitor titration (SUIT) protocol was employed (Figure 5-6, Table 5-2) so that multiple respiratory states could be measured from a limited amount of biological sample. Multiple substrates are used on the basis that electron flow converges at the Q junction and then follows a linear pathway through CIII and ultimately to CIV via cytochrome c (Figure 5-15). Under physiological circumstances, the TCA cycle is in full operation in the intact cell with influx of pyruvate, electron flow into the Q-junction (Figure 5-15) converges according to a NADH:succinate ratio of 4:1. Thus to reconstitute the TCA cycle, in permeabilised cells, the simultaneous application of CI and CII linked substrates is necessary to exert an additive effect on respiratory flux. Analysing maximum oxidative phosphorylation capacities avoids discrepancies between intact cells and permeabilised cells. Substrates feeding into the TCA cycle are generally added at saturating concentrations for measuring mitochondria respiratory capacity providing a buffer against substrate depletion in the course of the experiment. Compared to traditional MR assays, SUIT protocols are therefore known to better reflect respiration flux in vivo and are able to explore the relative capacity of the electron transport system (ETS) and phosphorylation system (OXPHOS)

components (239). In contrast to separate assays used traditionally, SUIT protocols enable calculations of statistically robust flux control ratios (FCR) (239).

G3P requires a specific mention as it is not an usual substrate that has been traditionally used. G3P is a substrate used by glycerol-3-phosphate dehydrogenase (GpDH) in mononuclear cell mitochondria and it feeds electrons directly into the Q junction (243). GpDH provides an alternate shuttle to the malate aspartate shuttle and is found in fast glycolytic skeletal muscle and PBMC. In four separate experiments, the saturating concentration of G3P was found to be 2-3µmol/million PBMC (n=4 HV).

The uncoupler used in this SUIT protocol was FCCP. FCCP generates an artificially increased proton leak as a bypass of ATP synthesis. By dismantling the transmembrane proton gradient, the maximum electron transport system capacity can be measured. Inhibitors that were used in this SUIT protocol were rotenone, malonate and antimycin. Rotenone inhibits CI to provide a measure of flux through CII alone, malonate inhibits CII and antimycin A inhibits CIII. The final concentrations of substrates, inhibitors and uncouplers were stated in Figure 5-2.

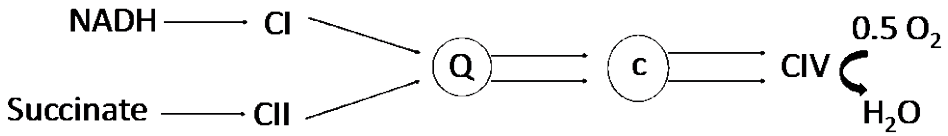


Figure 5-15 Convergent CI+II linked electron flow at the Q junction adapted from Gnaiger *et al.*.(96).

5.2 Other laboratory protocols

5.2.1 Mitochondrial DNA analysis

Mitochondrial DNA (mtDNA) was quantified using real-time polymerase chain reaction (PCR). DNA was isolated from 50µL of frozen (-80°C) buffy coat samples using the MagMax 96 DNA multi-sample Kit from Ambion using the manufacturer's protocol. DNA concentrations were determined using a Nanodrop spectrophotometer. Multiplex Quantitative PCR was carried out with an Applied Biosystems 7500 Real time PCR System using the Applied Biosystems assays for the mitochondrial gene MTCO1 (0188166_m1) and the nuclear gene RN18S1 (03928985_s1) was used as the internal control gene. PCR reactions were carried out in triplicate in a 96 well plate using Taqman Universal Master Mix II (no UNG) in a total volume of 10uL including 2uL of DNA. Threshold cycle (Ct) values for both genes were determined and values above 35 were removed from the analysis. $2^{-\Delta Ct}$ was derived by first calculating ΔCt as (Ct_{MTCO1}- Ct_{RN18S1}).

5.2.2 Mitochondrial superoxide using fluorescent dye MitoSOX red and flow cytometry

MitoSOX red (Invitrogen, Auckland, NZ), a mitochondrial targeted superoxide indicator (16) was used to detect superoxide in PBMCs. Approximately 1 million cells were incubated with 1µM MitoSOX red for 30mins at 37^{o}C in Hanks' balanced salt solution with calcium and magnesium, then washed twice in the same buffer and analysed by FACS Calibur flow cytometer excitation at 510nm and fluorescence detection at 580nm (FL 2) and data was analyzed using FlowJo software (version 7.6.4; Tree Star Inc.).

5.2.3 Mitochondrial membrane potential using JC-1 and flow cytometry

To measure the mitochondrial membrane potential ($\Delta\Psi$), PBMCs were incubated with J-aggregate forming cationic dye 5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (Invitrogen, Auckland, NZ) at a concentration of 10 µg/mL for 10 minutes at 37°C and then 20 minutes at 37^{o}C 5% CO2, then washed twice in PBS. Each time a separate positive control was set up by incubating the PBMCs with carbonyl cyanide m-chlorophenyl hydrazone (CCCP) and the dye JC-1 as per kit instructions. JC-1 fluorescence was detected by FACS Calibur flow cytometer in two channels FL 1 and FL 2. JC-1 at relatively low concentration exists as a monomeric form that fluoresces at 527nm (FL 1). When concentrated within polarized membranes (e.g. coupled respiring mitochondria) aggregates form, which fluoresce at 590nm (FL 2), and this fluorescence is proportional to the mitochondrial membrane potential (17). Dissipation of the mitochondrial membrane potential (e.g. due to permeability transition pore opening or increase proton conductance) prevents the formation of JC-1 aggregate and diminishes the 590nm (FL 2) fluorescence relative to the monomer at 527nm (FL 1) (17). Fluorescence at both 590nm and 527nm was measured and a ratio of aggregate to monomer calculated. Data was analysed using FlowJo software (version 7.6.4; Tree Star Inc.).

5.2.4 Assessment of intracellular ATP

Intracellular ATP was detected using the luminescence ATPLite detection assay system (PerkinElmer, Christchurch, NZ). Cells were treated according to the manufacturer's instructions, and luminescence was measured using an Envision microplate reader (PerkinElmer, Christchurch, NZ).

5.2.5 Assessment of intracellular protein

Intracellular protein extracts were quantified by Bicinchoninic acid (BCA) assay. BSA was used to generate a standard curve used in the determination of protein concentration. BSA was initially dissolved in MilliQ-water to make up a stock solution of 10 mg/ml and stored at -20 °C. The stock was diluted in the same buffer as the unknown sample in order to set up standards of concentrations 1, 0.5, 0.25 and 0.125 mg/ml by carrying out a serial dilution. A 96 well microplate was used in which 25 µl of standards and protein samples were added to 200 µl of BCA working solution (50 parts BCA solution and 1 part 4% Copper (II) sulphate solution) in each well. The samples in each well were mixed together. The plate was then left to incubate at 37 °C for 30 min, following which the absorbance of each sample was measured at 562 nm using a microplate reader (SoftMax Pro 4.1.7, Spectra Max 340, Molecular Devices, USA). Protein concentrations of the unknown samples were determined using the standard curve.

5.2.6 Assessment of cytochrome *c*

Cytochrome *c* was measured as an assessment of mitochondrial content using a human cytochrome *c* sandwich enzyme linked immunosorbent assay (ELISA) kit (Enzo Lifesciences, Hamilton, NZ). Frozen cells were thawed, washed with PBS lysed and resuspended to a concentration of 1.5 x 10^6 cells/ml. Human cytochrome c was measured as per the instructions and absorbance was read using a microtitre-plate format and spectrometer (SoftMax Pro 4.1.7, Spectra Max 340, Molecular Devices, USA) at 450nm.

5.2.7 Characterising mononuclear cells using flow cytometry

PBMCs were stained with the following monoclonal antibodies: CD3+ FITC, CD4+ PE, CD8+ APC and 7-aad in one test tube (244) and CD16+ FITC, CD14+ PE, HLA-DR APC and 7-aad in another test tube (245). Antibodies for CD3+ FITC, HLA-DR APC were sourced from Biolegend (San Diego, USA), CD14+PE from AbDSerotec (Kidlington, UK) and the remainder from BD Biosciences (Kidlington, UK). Once stained, the cells were incubated, washed and analysed in a FACS Calibur (BD Biosciences, San Jose, USA) flow cytometer. Data was analysed using FlowJo software (version 7.6.4; Tree Star Inc.).

5.3 Animal experimental models

To further validate the mitochondrial respiration protocol developed in Chapter 5.1, this thesis incorporated two experimental models: spontaneous hypertensive rats (SHR) and acute pancreatitis (AP). All animal experiments were approved by the Animal Ethics Committee of

the University of Auckland and fulfilled requirements of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.(246) Male Wistar rats and SHR were housed in pairs (Figure 5-16) and kept under a constant 12 h light: dark cycle with 50-70% humidity at a temperature of 19-21°C. All rats were fed standard Harlan Tekland 2018 rodent diet (Madison, WI, USA) and allowed tap water ad libitum.

Figure 5-16 Wistar rats housed in standard plastic boxes.

5.3.1 Anaesthesia

Anaesthesia was induced within a fume hood in a clear Perspex box with 5% Isoflurane + 5L/min of oxygen delivered by a vapouriser (Datex-Ohmeda, Tewksbury, MA, USA) (Figure 5-17). Animals were then transferred onto a heated (37^{o}C) operating pad and maintenance anaesthesia was delivered by a nose cone with Isoflurane 1-3% and 2-3L/min of oxygen. The fraction of oxygen/air was kept at 38%.

A vacuum scavenger was attached to the nose cone by plastic tubing to scavenge any excess Isoflurane vapours. The depth of anaesthesia was judged by monitoring respiratory rate and

assessing the pedal withdrawal reflex. The pedal withdrawal reflex was tested by extending the hind limb and pinching the skin between the digits and a lack of withdrawal reflects deep anaesthesia (247).

Figure 5-17 Fumehood used to induce anaesthesia.

5.3.2 Blood sampling

Once deep anaesthesia was ensured on the operating pad, an injection of heparin (100IU/kg) was given through a 24G angiocath in the tail vein. Two minutes after injection of heparin, a midline thoracotomy was done and blood was retrieved through a euthanasing cardiac puncture with a 21G needle and a 20 ml syringe (Figure 5-18).

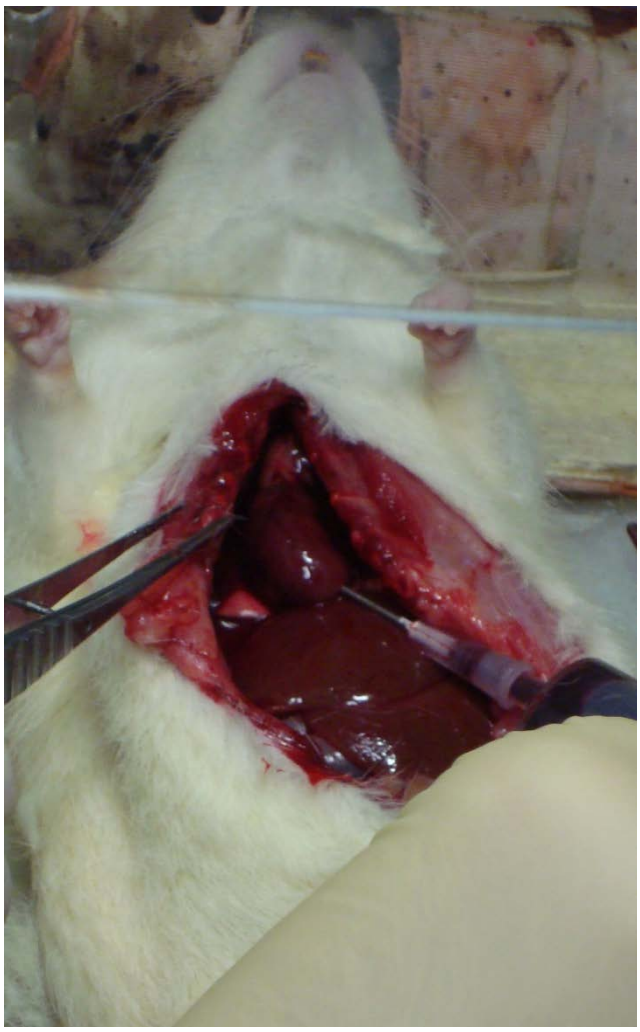


Figure 5-18 Midline thoracotomy and blood retrieval through cardiac puncture.

5.3.3 Experimental model of spontaneous hypertensive rats (SHR)

The SHR model was first described in 1963 by Okamoto and Aoki (248). A male Wistar rat with spontaneous hypertension was mated with a female Wistar rat whose blood pressure was above average to obtain the first generation (F1). From F1, a pair with spontaneous hypertension was selected and mated again. By F3, 100% had spontaneous hypertension. In F4, blood pressures steadily rose from 136 mmHg at 5 weeks to 190 mmHg at 15 weeks, 200 mmHg at 35 weeks.

Similar to human essential hypertension, SHR exhibit spontaneous hypertension without treatment, there is a gradual increase in blood pressure and blood pressure increases with age (249). They also exhibit end organ damage with cardiac hypertrophy, cardiac failure and renal dysfunction. Cardiac failure usually develops from 18 to 24 months (250). However, they tend not to exhibit the gross vascular problems of stroke or myocardial infarction (250).

5.3.3.1 Experimental groups

Twenty-eight animals were divided into four groups (i) Wistar 6 months (mo), n=6 (ii) SHR 6 mo, n=6 (iii) Wistar 14 mo, n=8 and (iv) SHR 14 mo, n=8.

5.3.3.2 Animal characterization and Doppler ultrasonography

The following signs of overt heart failure: 1) pericardial effusions, 2) pleural effusions, 3) ascites and 4) dyspnea at rest were monitored in all rats.(251) To avoid a stress related immune response, blood pressure (BP) was measured in a different cohort of 6 mo and 14 mo SHR and Wistars that were co-bred under identical conditions in our laboratory to the animals used in this experiment. BP was measured indirectly by tail cuff (MC4000 Blood Pressure Analysis System, Hatteras Instruments) (n=3 in each group). Body weight was measured in isoflurane-anesthetized rats and for fundamental measures of heart function an 8-mHz ultrasonic Doppler probe (Sonotrax A, Contec Medical Systems) was used with lubricating gel to record heart rate and heartbeat duration under constant flow rates of 3 l/min of 3% vol/vol isoflurane (218). Once a clear heart beat was detected, sonograms were generated for at least one minute (252). Heart rate, beats per minute (BPM) and beat duration (period of an individual beat) was determined directly from sonograms using Audacity 1.2.6.

5.3.3.3 Preparation of heart and skinned myofibers

After retrieval of blood, the heart was stopped by exsanguination then excised and immediately immersed in ice-cold saline (0.9% NaCl). Heart weight was recorded after trimming the major vessels and blot drying. The heart was transacted midway between the base and apex and a mid-point cross-section was removed and placed in saline in a petridish under a low-resolution microscope (Leica Microsystems) for analysis of heart dimensions (LV, RV and septum thicknesses) (253). Approximately 25mg of myocardium from the apex of the LV was trimmed and dissected into fibre bundles 0.5 × 1 mm after placement in 1 ml ice-cold, high-energy relaxing solution {in mmol/l, 10 Ca-EGTA buffer, 0.1 free calcium, 20 imidazole, 20 taurine, 50 K-MES [potassium 2-(*N*-morpholino)ethanesulfonic acid], 0.5 dithiothreitol, 6.56 $MgCl_2$, 5.77 ATP, 15 phosphocreatine, at pH 7.1}. To permeabilize fibres, fibre bundles were transferred into 2 ml fresh high-energy relaxing solution with saponin (50 μg) and agitated for 30 min at 4°C, as described previously (253). To remove saponin and adenine nucleotides, fibres were washed three times for 10 min in ice-cold incubation assay medium (in mmol/l, 0.5 EGTA, 3 $MgCl_2$, 60 K-lactobionate, 20 taurine, 10 KH_2PO_4, 110 sucrose, and 1 mg/ml BSA in 20 mmol/l HEPES, pH 7.1 at 30°C). Fibre bundles were blot dried on lint-free lens paper, and ~2 mg of LV was weighed for respiration assays

5.3.3.4 Statistical tests

Data were presented as mean ± SE (standard error), and analyses were performed using the statistical package Systat SigmaPlot 11 and graphs produced using Graphpad Prism 5.

Characteristics of the animal model as well as Doppler measurements were analyzed using two-way ANOVA with post hoc Tukey tests. Where appropriate, log transformations were applied to the data before model fitting. Differences were considered significant at $P < 0.05$.

5.3.4 Experimental model of acute pancreatitis

Eighteen male wistar rats were randomised to one of 2 groups: 1) control group (n=9) and 2) caerulein group (n=9). Group 1 received five, one hourly subcutaneous injection of 0.5ml saline and Group 2 received 50µg/kg of caerulein instead of saline (Figure 5-19). The subcutaneous injections were delivered into a skinfold on the back of the animal's neck whilst the animal remained awake (254). Analgesia was given with buprenorphine (0.05mg/kg) 5 mins prior to the first caerulien injection or saline injection. After 6hrs from the initial injection, anaesthesia was induced and blood was retrieved as described in sections 5.3.1 and 5.3.2. For assessment of acute pancreatitis, the pancreatic oedema index and serum amylase were measured. The pancreas wet weight was measured and divided by total body weight as a measure of the pancreatic oedema index (255). Two mls of the collected blood was allowed to clot and centrifuged and serum was sent to the Auckland City Hospital laboratory for measurement of amylase.

5.3.4.1 Statistical tests

Statistical analysis was carried out using Graph Pad Prism version 4.00 and SPSS version 18 for Windows (GraphPad Software , San Diego, California, USA). Values are expressed as the median and interquartile range or mean ± standard error of mean (SEM) where appropriate. Student *t* tests were used to evaluate statistical significance when the data fit a normal distribution and a Mann Whitney U test was carried out otherwise.

Figure 5-19 Subcutaneous injection on the back of the neck.

5.4 Clinical Studies

This section describes the two clinical studies that were used to investigate the hypotheses of this thesis. After conducting a study in an experimental model of acute pancreatitis, the mitochondrial respiration assay was employed in patients with acute pancreatitis in the first clinical study. Secondly, the central hypothesis of this thesis was investigated in a clinical study involving patients with MODS.

5.4.1 Clinical study involving patients with acute pancreatitis

5.4.1.1 Study design

The study was a prospective observational case-control study comparing peripheral blood mitochondrial function between patients with mild to moderate AP and healthy volunteers. Patients for this study were the control group in a randomized controlled trial in patients with mild to moderate acute pancreatitis (AP) (256). Patients were recruited in the Emergency Department at the Auckland City Hospital. Written informed consent was obtained from all participants. The enteral feeding study was approved by the New Zealand Regional Ethics Committee (NZREC) (NorthernX), NTX/08/11/107 [NCT01128478] and blood taking from matched controls was approved separately by the NZREC, NTX/10/11/119 [ACTRN12612000047897]. Patients were clinically managed by the usual clinicians independent of research staff's input.

5.4.1.2 Patients and healthy volunteers

A diagnosis of acute pancreatitis required at least two of the following three criteria: 1) abdominal pain typical of acute pancreatitis, 2) serum amylase and/or pancreatic amylase more than 3 times the upper limit of normal, 3) findings consistent with acute pancreatitis on abdominal imaging (257). Mild acute pancreatitis was defined as the absence of any (peri)pancreatic complications and the absence of organ failure. Moderate acute pancreatitis was defined as either sterile (peri)pancreatic complication or transient organ failure (258). Organ failure was defined in accordance with the SOFA score as a score ≥ 2 for at least one of three organ systems, i.e., respiratory, renal without pre-existing renal disease, and cardiovascular (259). Pancreatic infection was defined as one of the following: gas bubbles within (peri)pancreatic necrosis on computerised tomography, or a positive culture from (peri)pancreatic necrosis material obtained by image-guided fine needle aspiration during the first percutaneous/endoscopic drainage, minimally invasive or open surgery procedure of the admission.(237). Patients were excluded when they failed to present to hospital within 96 hours from the onset of symptoms. Other exclusion criteria were severe or critical AP, chronic pancreatitis, post-ERCP (endoscopic retrograde cholangiopancreatography) pancreatitis, intraoperative diagnosis of acute pancreatitis, pregnancy, malignancy and known mitochondrial disorders.

Progression of severity was defined as a patient's upstaging (i.e., from "mild" to "moderate" or from "moderate" to "severe" during hospitalisation) according to the determinants-based classification of severity (258). Hospital interventions recorded in the study were therapeutic ERCP, cholecystectomy, percutaneous drainage, or necrosectomy. Hospital readmission was defined as readmission due to exacerbation of abdominal pain within 3 months of hospital discharge (260).

The control group consisted of healthy volunteers from the community who responded to advertisements placed in the hospital, who were approximately gender and age matched (within 5 years of the patients). There was no remuneration offered for participating as a volunteer to this study. Healthy volunteers were eligible to be included in the study if there was no present or past history of acute pancreatitis, no concurrent acute illness, no known chronic illness and if they were on no regular medications.

The primary outcome was to measure temporal MR from PBMC in AP and compare with healthy volunteers (HV). The secondary outcomes were to evaluate other mitochondrial

function (MF) - mitochondrial superoxide, mitochondrial membrane potential and intracellular cytochrome *c* from PBMC.

5.4.2 Clinical study involving patients with MODS

5.4.2.1 Study design

The study was a prospective observational case-control study comparing peripheral blood mitochondrial function between patients with MODS and healthy volunteers. Patients were recruited as they presented to Department of Critical Care Medicine, Auckland Public Hospital consecutively between February 2011 and September 2011 and were followed up for 6 months. Clinical data was collected from patient charts and hospital notes in an unidentifiable format into Microsoft Excel 2007.

5.4.2.2 Study size

Given that this was the first study analysing mitochondrial function in MODS compared to healthy volunteers, a direct power analysis was not possible for this study. A biostatistician at Department of Surgery, Auckland Medical School was consulted for a-priori sample size calculation for this study. The only similar published study at the time which measured mitochondrial respiration function in sepsis and compared to healthy volunteers was noted (242). State 3 respiration with succinate was measured in eighteen patients with sepsis and eighteen healthy volunteers at one time point and a 30% decrease in patients was reported (P=0.001) (242). There was an important difference in the protocol of our study: mitochondrial function was measured eight times in the same patient which made the measurements more reliable in our study. The repeated measure design reduces the variance of estimates allowing statistical inference to be made from fewer subjects whilst keeping the validity of the results high. The intended power of the study and the p-value was set at 0.8 and 0.05 respectively. The study was designed to measure a 30% difference in CI,II Oxphos between healthy volunteers and patients with MODS. After discussion, 25 patients with MODS (approximately 50% with sepsis and 50% with non-sepsis) and 15 healthy volunteers were derived upon. An additional attrition rate of 8% was accounted for bringing the total number of patients to 27.

5.4.2.3 Patients and healthy volunteers

In order to test the hypothesis that mitochondrial function in MODS does not discriminate between septic and non septic MODS, two groups of patients with MODS were included in

the study: i) patients with septic shock and ii) patients with non septic shock. The inclusion criteria were patients with MODS as defined as sequential/sepsis related organ failure assessment score (SOFA) ≥2 for ≥2 organ and septic/non septic shock within 36 hrs of first organ dysfunction as defined as SOFA≥2 (Table 5-7). The exclusion criteria were > 36 hours after SOFA≥2 of first organ dysfunction, age <15 years, known mitochondrial disorder and patient's personal circumstances unamenable for follow up.

Table 5-7 Sequential/sepsis related organ failure assessment score (SOFA) as published by Vincent *et a.l* (10)

Organ system	0	1	2	3	4
Respiratory PaO_2/FiO_2	>400	≤400	≤300	≤200	≤100
Renal Creatinine (µmol/L)	<106	106-168	169-300	301-433 Urine output <500 mL/day	>433 Urine output <200 mL/day
Hepatic Bilirubin (µmol/L)	<20	20-32	33-100	101-203	>203
Cardiovascular Hypotension	No hypotension	MAP <70 mm Hg	Dopamine ≤5 µg/kg/min	Dopamine >5 or epinephrine ≤0.1 or norepinephrine ≤0.1 µg/kg/min	Dopamine >15 or epinephrine >0.1 or norepinephrine >0.1µg/kg/min
Haematological Platelet count ($x10^3/mm^3$)	>150	≤150	≤100	≤50	≤20
Neurological Glasgow Coma Scale score	15	13-14	10-12	6-9	<6

Shock was defined as hypotension with systolic blood pressure (SBP) <90 mm Hg despite adequate fluid resuscitation along with presence of perfusion abnormalities. Hypoperfusion abnormalities include but are not limited to lactic acidosis, oliguria or an acute alteration of mental status. SIRS was defined as any of two of the following: temperature <36^{o}C or >38^{o}C, WBC <4000 or >12,000 per mm^3, respiration>20/min or PCO_2 <32 mm Hg or mechanically ventilated and presence of >10% immature neutrophils. Sepsis was defined as when SIRS was the result of a confirmed infectious process. Organ failure was defined as a SOFA score > 1 for at least one of the four organ systems: respiratory, hepatic, cardiovascular or renal. The definitions above were adapted from the American College of Chest Physicians/Society of Critical Care Medicine consensus criteria for sepsis syndromes (9) (Table 5-8).

Table 5-8 Definitions of systemic inflammatory response syndrome, sepsis, severe sepsis, septic shock and multiple organ dysfunction syndrome used in clinical trial as published by Bone *et al.* .(9)

Term	Definition
Systemic inflammatory response syndrome (SIRS)	Response is manifested by 2 or more of the following conditions: • Temperature > 38°C (100.4°F) or < 36°C (96.8°F) • Heart rate > 90 beats per minute • Respiration rate > 20 breaths per minute or $Paco_2$ < 32 mm Hg • White blood cell count > 12,000/μL, < 4000/μL, or > 10% immature (band) forms
Sepsis	Systemic response to an infection defined by 2 or more SIRS criteria as a result of an infection
Severe sepsis	Sepsis associated with organ dysfunction, hypoperfusion or hypotension
Septic shock	Sepsis-induced hypotension despite adequate fluid resuscitation along with presence of perfusion abnormalities
Multiple organ dysfunction syndrome (MODS)	Presence of altered organ dysfunction in an acutely ill patient

The control group consisted of healthy volunteers from the community who responded to advertisements placed in the hospital, who were approximately gender and age matched (within 5 years of the patients). Healthy volunteers were eligible to be included in the study if there was no present or past history of organ failure, no concurrent acute illness, no known chronic illness and if they were on no regular medications. Written informed consent was obtained from all participants. There was no remuneration offered for participating in this study. The New Zealand Regional Ethics Committee (NZREC) (NorthernX) approved this study NTX/10/11/119 [ACTRN12612000047897].

The primary outcome was to measure temporal MR from PBMC in MODS and compare with healthy volunteers (HV). The secondary outcomes were to evaluate other mitochondrial function (MF) - mitochondrial superoxide, mitochondrial membrane potential and intracellular cytochrome *c* from PBMC and correlate MF with sequential organ failure assessment (SOFA).

5.4.2.4 Patient management and follow up

All patients were managed by the same physician/surgeons, anesthetists and intensive care specialists according to the standard protocols. Clinical practice was not modified for the purpose of the study. Once the patients were discharged home, blood was collected at the Department of Surgery, Auckland City Hospital or at the patient's home depending on patient preference.

Baseline data collected for all patients included age, gender, height, weight, aetiology of MODS, co-morbid conditions, hospital admission date, ICU admission date, lactate, blood sugar level, APACHEII, SOFA, SIRS criteria. Requirements for amount of fluid, number of organs failed including vasopressors, ventilation, renal replacement therapy and enteral/parenteral feeding in the last 48hrs before recruitment to study was also collected. Additionally, daily clinical data was collected each day blood was taken and included SOFA score, lactate, blood sugar level, white cell count and amount of fluid, vasopressors, intubation/ventilation, renal replacement therapy and enteral/parenteral feeding in the 24 hrs preceding the blood sample.

5.5 Conclusion

In this chapter, the methodology pertaining to this thesis was discussed. At the beginning of this chapter, mitochondrial respiration assay protocol was developed and optimized to be used in clinical studies. Other mitochondrial function assays and the protocols for the experimental studies and clinical studies were also described. In the next chapter, the mitochondrial respiration assay protocol will be tested in an experimental model of hypertension.

Chapter 6 Mononuclear Cell Mitochondrial Function in Hypertension – a Chronic Disease State

6.1 Introduction

In the last chapter of this thesis, a mitochondrial respiration protocol using peripheral blood mononuclear cells was developed. The ultimate aim of this thesis is to understand mitochondrial respiration in MODS by measuring mitochondrial respiration from PBMC in patients with MODS. Prior to using this assay in critically ill patients with MODS, it was important to test this assay in a variety of chronic and acute disease settings to gain an in-depth understanding of the continuum of mitochondrial dysfunction in disease states. The next two chapters in this thesis are dedicated to that purpose. Chapter 7 investigates mitochondrial function in mild acute pancreatitis and mitochondrial respiration assay is validated in the chronic disease state of hypertension in this chapter.

6.1.1 Hypertension and end organ damage

Hypertension is a common disease which affects 972 million people worldwide (217) and increases the risk of coronary artery disease, congestive heart failure, stroke and renal insufficiency (261). Hypertensive end organ damage is mediated by oxidative stress (262) and in experimental models of hypertension, reactive oxygen species (ROS) is increased in vessels, heart and kidneys (263).

6.1.2 Hypertension and mitochondrial function

Accumulation of oxidative damage cause mitochondrial damage and alter mitochondrial respiration (264). This perturbed mitochondrial state can in turn produce more ROS (265). In the pre-failing spontaneously hypertensive rat (SHR) heart ATP supply and handling is impaired (218) and in failing heart fibres a 40% depression in mitochondrial respiration has been demonstrated (152). In vascular endothelial and smooth muscle cells, ROS induces sustained mtDNA damage, alter mitochondrial transcript levels and mitochondrial protein synthesis, and lowered mitochondrial redox potential (266).

6.1.3 Hypertension and the immune system

In addition to being an oxidative pathology there is a role for the immune system in hypertension (267). The average count of lymphocytes and monocytes are significantly higher in SHR than Wistar Kyoto rats (268) and these lymphocytes and monocytes infest the

perivascular fat immediately adjacent to adventitia of aorta and mesenteric vessels (267). A significantly lower incidence of hypertension was also found in AIDS patients with low CD4+ lymphocyte count when compared with matched non-infected individuals (269). Mice which lack both B and T cells have blunted hypertensive responses to prolonged angiotensin II infusion or deoxycorticosterone acetate salt challenge (270). Endothelium dependent vasodilation and vascular superoxide production which are generally abnormal in hypertension are not affected in these mice (270).

Patients with essential hypertension have altered circulating levels of pro and inflammatory cytokines which suggests activation of monocytes (271). Also, oxidative stress is evident in whole blood and peripheral mononuclear cells from hypertensive patients with an increased ratio of oxidised to reduced glutathione and lower activity of anti-oxidant enzymes (272). ROS produced from the blood cells may be a contributory factor to the endothelial dysfunction in vascular cells (272).

6.1.4 Hypothesis

There is extensive research on the immune system, oxidative stress and mitochondrial function in various experimental and clinical settings in hypertension. However, what happens to mitochondrial respiratory function in immune cells in hypertension has not been studied to date. On the basis of the above, the hypothesis is that mitochondrial respiration function is altered in peripheral blood mononuclear cells in hypertension.

6.1.5 Aims

This study aims to address this gap in knowledge by using the SHR model. The SHR model is considered a good model of essential human hypertension, involving an early increase in BP, a long progression to compensated hypertrophy and eventual cardiac failure late in life (273).

The aim of this study was to measure blood mononuclear cell mitochondrial respiratory function in male SHR at an early hypertensive stage and a compensated hypertrophic stage and compare these two groups with age matched male Wistar rats.

6.2 Methods

6.2.1 Animals

Animals were prepared and handled as described in Chapter 5.3.

Six and 14 month (mo) old hypertensive rats with no overt signs of cardiac failure were used in this study and compared with six and 14mo old wistar rats as described in Chapter 5.3.

6.2.2 Blood Pressure

Blood pressure was measured indirectly by tail cuff as described in section 5.3.3.2.

6.2.3 Preparation of blood mononuclear cells

Two minutes after an injection of heparin (100 IU/kg) a midline thoracotomy was done and blood was retrieved through a euthanasing cardiac puncture as described in section 5.3.1 and 5.3.2. Blood was collected and PBMC were retrieved as described in section 5.1.1 and 0

6.2.4 Preparation of heart and skinned myofibres

The heart and skinned myofibres were prepared as per section 5.3.3.3.

6.2.5 Respiration assays

A multiple substrate-inhibitor titration protocol was employed because it is known to better reflect respiration flux in vivo and to explore relative capacity of the electron transport system (ETS) and phosphorylation system (OXPHOS) components (218). Two OROBOROS Oxygraph-2K (Anton Paar, Graz, Austria) were employed for oxygraphy, respiratory measurements were performed at 37°C in 2 ml incubation assay medium, and the oxygen concentration at air saturation of the medium was 215 nmol O_2/ml at 95 kPa barometric pressure. Respiration was measured as oxygen flux per million live cells for blood mononuclear cells (pmol/s/million cells), and as weight-specific oxygen flux (pmol O_2 s^{-1}·mg wet wt^{-1}) for the heart fibres. Oxygen flux was calculated as the time derivative of oxygen concentration using the DatLab 4 Analysis Software, OROBOROS (Innsbruck, Austria).

6.2.6 Respiration assays in mononuclear cells

The mononuclear cell mitochondrial respiration assay was described in section 5.1.4. The only alteration in the mononuclear cell mitochondrial respiration assay was a titration of NADH (0.5mmol/l), which was added after adding pyruvate and before adding succinate. NADH was added to test inner mitochondrial membrane integrity; this molecule will only cross the inner membrane if damage is present and it may then fuel CI (274).

6.2.7 Respiration assay in myocardial fibres

In parallel, from the same animal, 2–3 mg of permeabilized heart fibers were added to chambers in another oxygraph and allowed to equilibrate. Oxygen was then added to

chambers and maintained above 280 nmol/ml to ensure saturation. In heart fibre assays, CI leak was measured by the addition of CI substrates alone (10 mmol/l glutamate, 2 mmol/l malate). Subsequent addition of excess ADP (2.5mmol/l) stimulated oxidative phosphorylation (CI OXPHOS, state 3 respiration), and by then adding cytochrome *c* (10 μmol/l), the functional integrity of mitochondria was measured. To assess for any additional effect of CI OXPHOS, pyruvate (5mmol/l) was added. CI,II OXPHOS was assessed by addition of succinate (10 mmol/l) and CI,II Leak was measured by addition of atractyloside (1 mmol/l), followed with repeated titrations of carbonyl cyanide *p*-(trifluoromethoxy)phenyl-hydrazone (FCCP, 0.5 μmol/l) to uncouple mitochondria (ETS). Complex I and then Complex II activity were selectively inhibited by the addition of rotenone (1 μmol/l) and malonate (15mmol/l). Antimycin a (1 μmol/l) was then added to block Complex III. This gave a measure of non-mitochondrial oxygen consumption (236). Finally, the activity of cytochrome *c*-oxidase (CIV) was measured by the addition of the electron donor couple *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD, 0.5 mmol/l) and ascorbate (2 mmol/l). Activities were determined relative to wet mass (mg) of tissue for the heart fibres. Figure 6-1 is representative of an oxygraphy trace of myocardial fibres. The respiratory control ratio and CI as a percentage of ETS was calculated as described in Table 5-3.

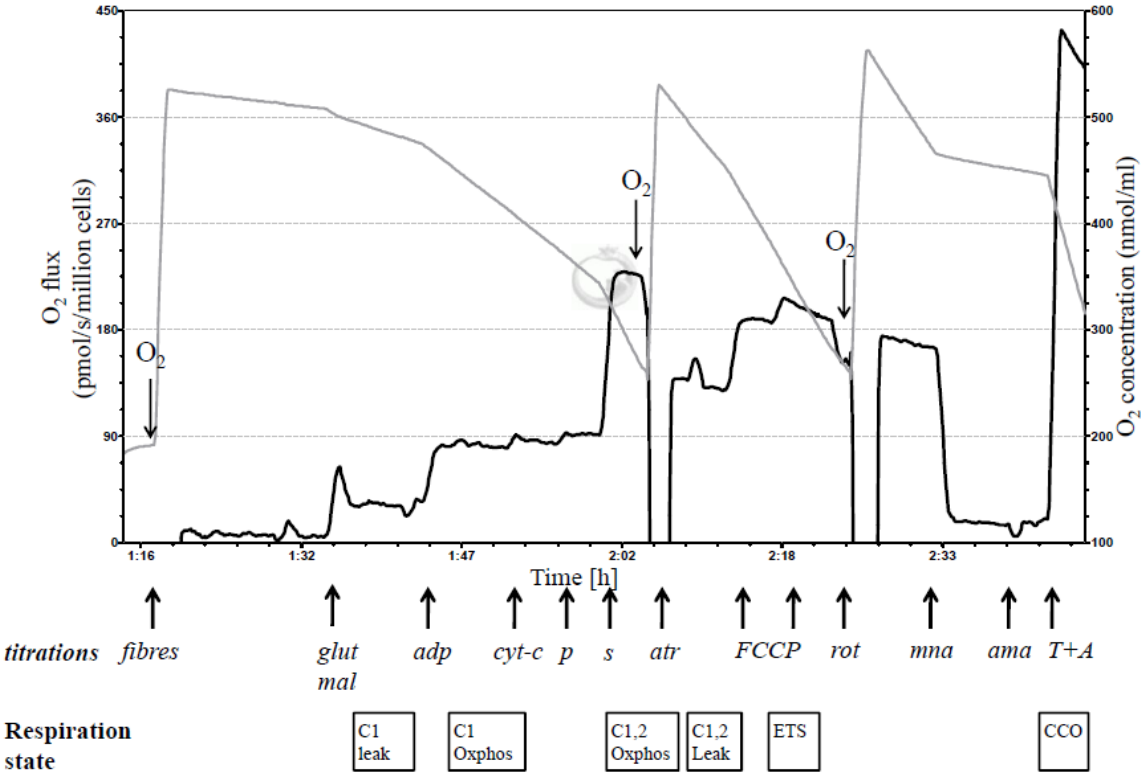


Figure 6-1 Representative oxygraphic trace of permeabilised heart fibres from SHR showing oxygen concentration (gray line, right Y-axis) and oxygen flux (black line, left Y-axis) over time. Titrations are italicised and the resulting respiration state shown beneath. See methods for further detail. *dig* [digitonin], *glut* [glutamate], *mal* [malate], *adp* =[adenosine di-phosphate], *cyt-c* [cytochrome-c], *p* [pyruvate], *s* [succinate], *olig* [oligomycin], *atr* [atractyloside], *FCCP* [carbonyl cyanide p-(trifluoromethoxy) phenyl-hydrazone], *rot* [rotenone], mna [malonic acid], ama [antimycin-a], T+A [N, N, N', N'-tetramethyl-Phenylenediamine + ascorbate], ENDO [endogenous], CI [Complex I], Oxphos [oxidative phosphorylation], ETS [Electron Transport System], CCO [Cytochrome-*c* Oxidase].

6.2.8 Statistical tests

Statistical tests were described in section 5.3.3.4.

6.3 Results

6.3.1 Animals and cardiac function

SHRs weighed significantly less, 38% at 6 mo (*n*=6 in each group) and 27% at 14 mo (*n*=8 in each group) compared to the Wistars (Table 6-1). There was no difference in heart weight between Wistars and SHR at 6 mo of age but at 14 mo of age the SHR heart was markedly heavier (P<0.001). SHR hearts weighed significantly less at 6 months compared to SHR hearts at 14 months. As a percentage of body weight the heart weighed more in SHR at both ages compared to Wistars (P<0.001 ; both ages). For SHR the systolic blood pressure was

176+/-6 mmHg and 189+/-11 mmHg for 6mo and 14mo respectively. For the Wistars, the systolic blood pressure was 132+/-8 and 154+/-9 mmHg (P<0.05; both ages).

Table 6-1 Physiological data for Spontaneously Hypertensive Rats (SHR) and their age-matched controls at 6 months and 14 months of age.

Age	**6 months (n=6)**		**14 months (n=8)**	
Exp. Group	**Wistar**	**SHR**	**Wistar**	**SHR**
body weight (g)	596 ± 15	372 ± 5***	610 ± 44	445 ± 10**
heart weight (g)	1.44 ± 0.03	1.31 ± 0.01	1.29 ± 0.05	1.64 ± 0.05***###
% heart of body weight	0.242 ± 0.003	0.351 ± 0.003***	0.215 ± 0.012	0.370 ± 0.008***

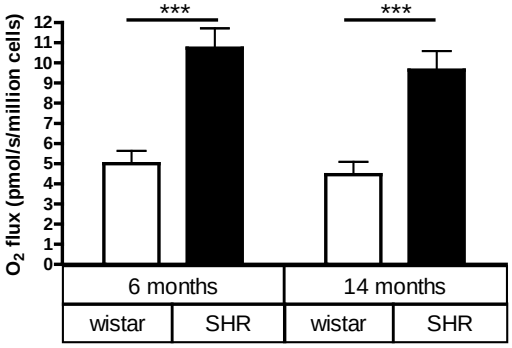
Footnote*p<0.05, **p<0.01, ***p<0.001 for a statistically significant difference between Wistar and SHR within their respective age groups, and #p<0.05, ##p<0.01, ###p<0.001 for statistically significant difference within Wistar or SHRs at different ages (two-way ANOVA followed by post-hoc tukeys).

6.3.2 Effect of hypertension on mononuclear cell mitochondrial function

Endogenous respiration in SHR mononuclear cells was increased two fold (P<0.001) compared to age-matched Wistars at both 6 and 14 months of age (Figure 6-2A). Phosphorylating respiration with CI substrates was not different between groups (data not shown). Exogenous NADH addition resulted in similar rates in SHR and wistar rats at 6 mo, however at 14mo SHRs had 2.3 fold (P<0.01) higher flux compared to Wistars (Figure 6-2B). After addition of the CII substrate, respiration was significantly higher in SHRs at both ages, 1.9 fold at 6 mo (P<0.01) and 2.8 fold at 14 mo (P<0.001) (Figure 6-2C). Similarly in SHRs at both 6mo and 14mo, ETS capacity, G3P fuelled respiration and flux through CIV was significantly higher compared to age matched Wistars (Figure 6-2D, Figure 6-2E and Figure 6-2F).

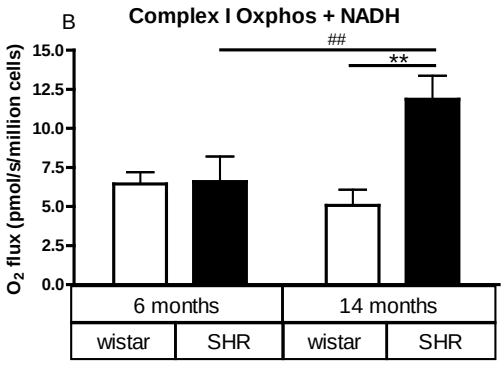
The CI,II OXPHOS/CI,II Leak was significantly diminished at both 6 mo and 14 mo in SHRs compared to corresponding Wistars (Figure 6-2G). The percentage of C I as a function of ETS showed a drop of 37% in SHR at 6mo (NS), and in 14mo SHRs a 52% drop (p<0.01) compared to age-matched Wistars (Figure 6-2H).

A



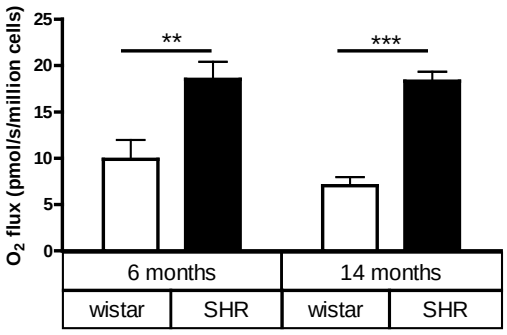
two-way anova effects	p-value
disease	<0.001
age	NS
disease x age	NS

B



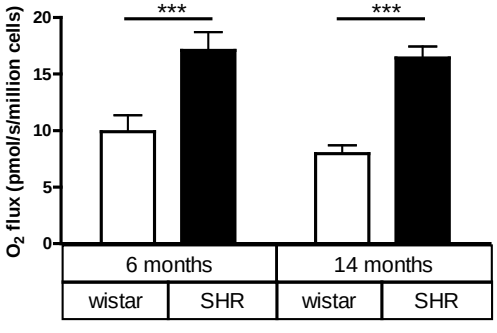
two-way anova effects	p-value
disease	0.014
age	NS
disease x age	0.018

C



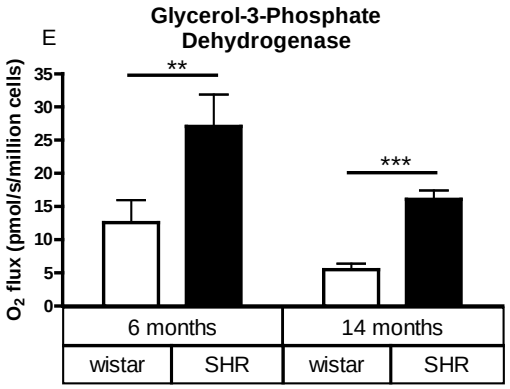
two-way anova effects	p-value
disease	<0.001
age	NS
disease x age	NS

D



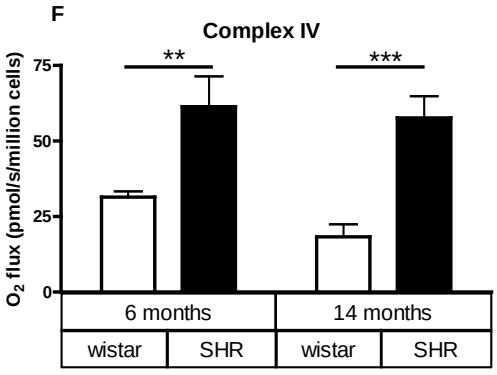
two-way anova effects	p-value
disease	<0.001
age	NS
disease x age	NS

E

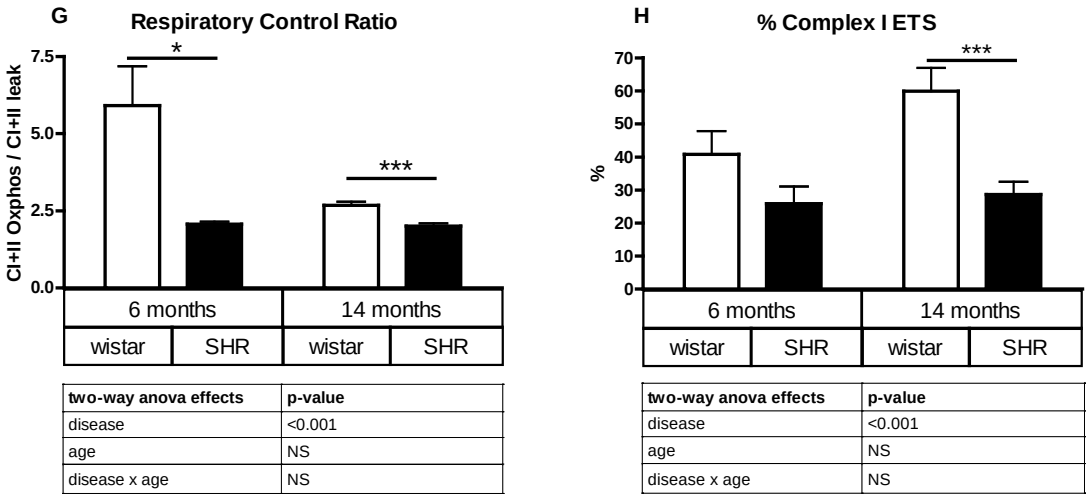


two-way anova effects	p-value
disease	<0.001
age	0.006
disease x age	NS

F



two-way anova effects	p-value
disease	<0.001
age	NS
disease x age	NS



two-way anova effects	p-value
disease	<0.001
age	NS
disease x age	NS

two-way anova effects	p-value
disease	<0.001
age	NS
disease x age	NS

Figure 6-2 Effect of hypertension on peripheral blood mononuclear cell mitochondrial function**.** Mononuclear cell respiration of SHR and Wistar rats at 6 mo and 14 mo of age (mean±SE). Different respiratory states measured were: ENDO [Endogenous respiration] (A), Complex I Oxphos + NADH [Complex I oxidative phosphorylation rate with added NADH] (B), CI+II Oxphos [Complex I,II oxidative phosphorylation rate] (C), ***ETS*** [Electron Transport System capacity] (D), Glycerol-3-phosphate dehydrogenase [glycerol-3-phosphate stimulated respiration] (E), CIV [Complex IV] (F). Ratios of respiratory states were also calculated, including the RCR [Respiratory Control Ratio] (G) and %CI *ETS* [percentage Complex I relative to total electron transport system capacity] (H). Two-way ANOVA effects and interactions are shown underneath each respiratory state, and results of post-hoc Tukeys are shown in the figure, *p<0.05, **p<0.01, ***p<0.001 for a statistically significant difference between SHR and Wistar within an age group and #p<0.05, #p<0.01, ###p<0.001 for a statistically significant difference with age within SHR or Wistar.

Permeabilized fibres from SHR and Wistar hearts showed similar combined respiratory flux through CI and CII and there were no differences in electron transport system capacity or CIV flux between any of the groups (Figure 6-3A–C). Similarly, the CI,II OXPHOS/CI,II Leak and percentage of Complex I as a function of ETS did not differ between groups (Figure 6-3D and Figure 6-3E).

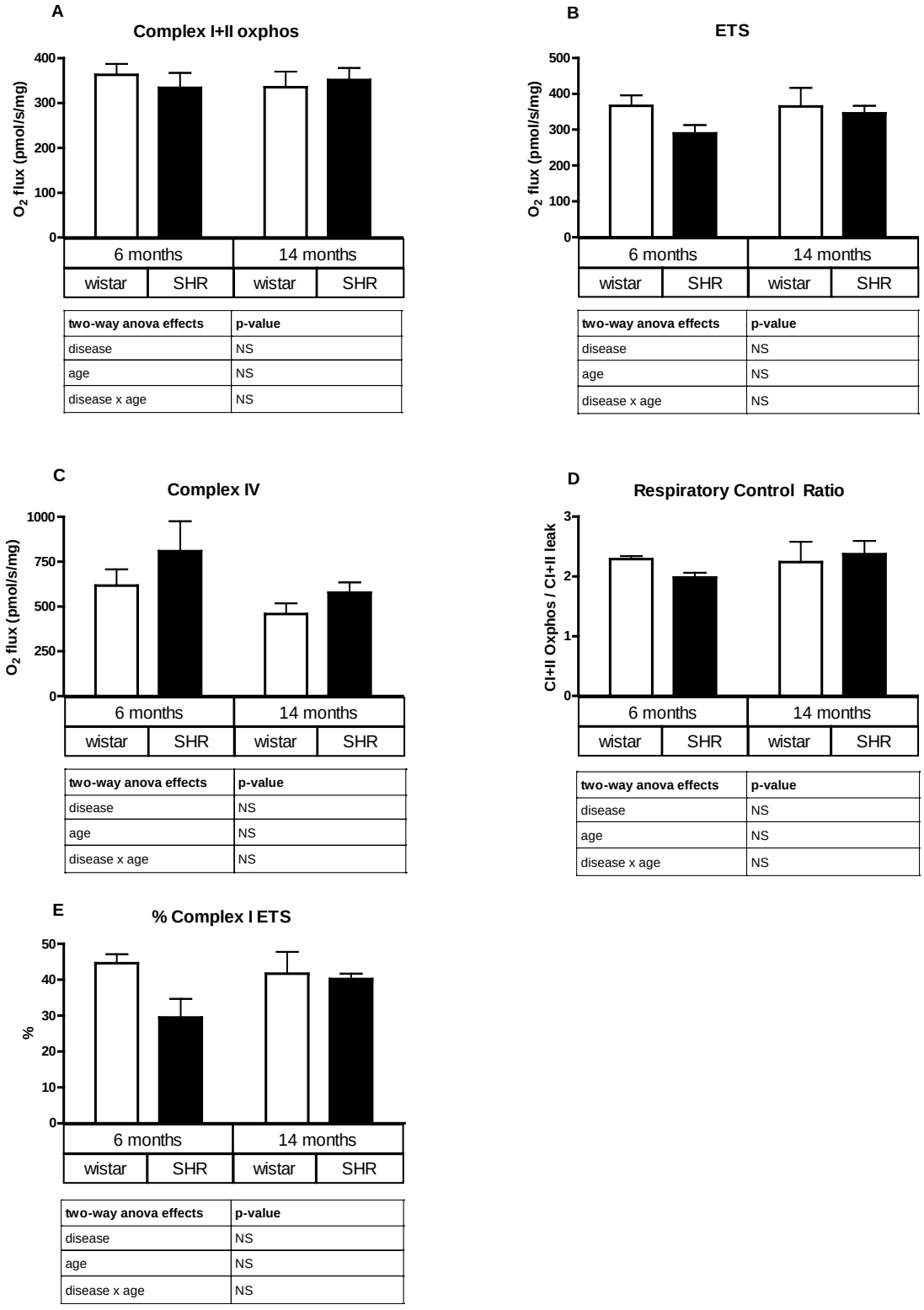


Figure 6-3 Effect of hypertension on heart tissue mitochondrial function. Permeabilized heart fiber respiration of SHR and Wistar rats at 6 mo and 14 mo of age (mean±SE) The different respiratory states measured were: CI+II Oxphos [Complex I,II oxidative phosphorylation rate] (A), ETS [Electron Transport System capacity] (B), CIV [Complex IV] (C). Ratios of respiratory states were calculated, including the RCR [Respiratory Control Ratio] (D) and %CI ETS [percentage Complex I relative to total electron transport system capacity] (E). Two-

way ANOVA effects and interactions are shown underneath each respiratory state, and results of post-hoc Tukeys are shown in the figure, NS p>0.05.

6.3.3 Effect of age on mononuclear cell mitochondrial function

There was a drop in G3P fuelled respiration with age (Figure 6-2E). In Wistars a 57% depression (p=0.014) with G3P was seen at 14 mo compared to 6 mo old rats, and in SHR a non significant drop of 41% was apparent with increasing age.

6.3.4 Effect of age on heart mitochondrial function

There were no statistically significant changes in heart mitochondrial function when comparing 6mo and 14mo Wistar or SHRs (Figure 6-3).

6.3.5 Hypertension-age interaction in mononuclear cells

Addition of exogenous NADH caused higher flux in SHR, and this increase was dependent on age (disease age interaction p=0.018) (Figure 6-2B)

6.3.6 Hypertension-age interaction in heart

There was no disease and age interaction in the heart (Figure 6-3).

6.4 Discussion

6.4.1 Main findings

This study examined mitochondrial function in two tissues, circulating PBMC and cardiac myofibers, taken from young (6 mo) and older (14 mo) SHR and control Wistar rats. Heart weights as a percentage of body weight increased in SHR rats with age compared to Wistars and the lack of systemic heart failure signs was consistent with compensated cardiac hypertrophy in SHR rats. While the mitochondrial respiration in permeabilized heart fibers did not differ between either rat strains or ages, the mitochondrial function of PBMC differed for several parameters. There was an increase in respiration globally in the SHR mononuclear cells compared to Wistars. Mononuclear cell endogenous respiration was increased two fold relative to age-matched Wistars at both ages examined. After cell permeabilization, phosphorylating flux was higher with all substrates in SHR of both ages relative to controls, with the only exception being Complex I linked substrates (glutamate, malate, and pyruvate) where there was no difference at 6 or 14 mo. While there was a higher ETS capacity, the %CI ETS was lower in SHR at 14 mo.

6.4.2 Spontaneous hypertensive rats as a disease model of hypertension

Spontaneous hypertensive rats are a well-established model of genetic hypertension that produces an increase in systolic wall stress resulting in concentric hypertrophy. Although the Wistar-Kyoto (WKY) rat is often the control for the SHR model, it has a number of deficiencies that precluded its use in this experiment. Wistar-Kyoto rats are genetically variable (275) and have been reported to have left to right ventricular shunts in up to 10% of rats (276). Papillary muscles isolated from the left ventricles of WKY were stiffer than Wistar rats and there was a greater level of myocardial fibrosis in the WKY. These findings are consistent with an underlying blood pressure independent cardiac hypertrophy in normotensive WKY rats (277) . Additionally, Wistar-Kyotos reportedly manifest increased infection rates (278) which could potentially confound the results of this study, as this study was directed at assessment of immune cell mitochondrial function. Due to these reasons male Wistars was the most appropriate controls in this study.

6.4.3 Mononuclear cell mitochondrial respiration changes early in hypertension

The resting or endogenous respiration in mononuclear cells of SHR was elevated two fold at both ages compared to age-matched Wistars (Figure 6-2A). The endogenous respiration of a cell reflects the sum effect of the leak of protons which does not generate ATP (CI,II Leak), non-mitochondrial oxygen consumption, and oxygen requirement for ATP consuming processes. Since the respiration attributable to proton leak and non mitochondrial oxygen fluxes were similar in SHR and Wistar mononuclear cells (data not shown) the increase in endogenous respiration is most likely from an increase in respiration by OXPHOS and this is in fact what we have found in this study. In a separate study, when PBMC were activated with Concavalin A, endogenous respiration increased by 53% and this increase was associated with increased ATP demands (147). Taken together it appears that the hypertensive state in SHR chronically activates mononuclear cells probably through stimuli such as angiotensin II and the result is increased endogenous mitochondrial respiration and OXPHOS to cope with the increased ATP demand. (270).

In parallel with high levels of endogenous respiration, CI,II OXPHOS was also two fold higher in SHR at both ages relative to Wistars. This is in contrast with respiration with CI substrates alone (glutamate, malate and pyruvate) which were similar between SHR and Wistars at both ages. Therefore the increased CI,II OXPHOS appears to be primarily driven by increased CII flux. Elevated levels of the CII substrate succinate has been reported in SHR plasma when compared to Wistar Kyoto rats (279). It is known that CII requires more oxygen

to produce the equivalent ATP from CI substrates and is therefore less efficient at ATP production than CI. This is reflected by the decreased RCR which was used to quantify coupled OXPHOS capacity (239). Although the RCR has not been studied before in mononuclear cells in hypertension, the RCR in 4 mo old SHR brain mitochondria measured as CI OXPHOS / CI leak was also found to be decreased compared to those from WKY, which is consistent the findings described in mononuclear cells in this study (280).

6.4.4 Decreased ETS capacity through CI in hypertensive PBMC

Additionally, the %CI ETS flux trended 37% lower (P=0.106) in 6 mo SHR than 6 mo Wistars and was 52% lower in 14 mo SHR relative to age-matched Wistars (p=0.001). This suggests a defect in CI linked respiration or organisation of Complex I within supercomplexes, when evaluated under conditions when any limitation by the phosphorylation system is removed by chemical uncoupling. CI has been studied in many tissue types in SHRs such as brain (280) and heart (152) and is well known for its involvement in cardiovascular disease (266). It is also a source of superoxide release if electron flow is blocked or reversed (281). In 12 week old SHR, brain mitochondrial CI enzyme activity assays confirmed decreased level of CI activity (280) and in 18 mo SHR hearts there was decreased CI supported flux (152).

Also, consistent with this study's finding, a progressive decrease in %CI ETS was recently reported in heart mitochondria from patients with heart disease and congestive heart failure compared to controls.(282) CI linked respiration is more coupled to the generation of a proton gradient, with CI based respiratory chains producing approximately 40% more ATP than CII based chains. Therefore, a drop in the relative contribution of CI in SHR should depress the overall efficiency of ATP production and this is observed in the lower %CI ETS in the SHR.

6.4.5 Increase in other respiration states in hypertension

In keeping with the overall increase in respiration, G3P fuelled respiration, CIV respiration and ETS were significantly higher in both 6 mo and 14 mo SHR compared to age-matched Wistars. In both 6 mo and 14 mo SHRs, G3P fuelled respiration was significantly higher compared to age-matched Wistars (Figure 6-2E). GpDH resides on the outer surface of the inner membrane of the mitochondria and oxidises G3P to dihydroxyacetone phosphate reducing a flavin prosthesic group that donates its reducing equivalents to the electron transport system at the level of CoQ (96). Similar to CII, GpDH does not itself directly contribute to the membrane potential. In SHR, the high levels of GpDH may reflect greater demands on rapid ATP production. However, as with Complex II, GpDH is less efficient at

producing ATP than CI, indicating a general compromise of efficiency for increased ATP production rate.

Increased activity of GpDH have been reported in a variety of disease states under conditions of enhanced metabolic activity (98). In pulmonary hypertension there is increased expression for the GpDH gene and metabolism via this pathway is increased during active remodelling of blood vessels (283). A change from fatty acid to glucose oxidation has been reported in cardiac hypertrophy and the use of the fatty acid octanoate as substrate was significantly decreased in cardiac muscle from 15w SHRs (284). Previous data have also shown a decreased contribution of exogenous oleate to acetyl CoA formation in 15 week old SHRs which would ultimately result in decreased fatty acid oxidation (285). Taken together, the increase in GpDH may be an early metabolic adaptation in mononuclear cells in SHR.

The increase in flux through CIV may be a result of elevated CIV concentration/abundance which can increase the mitochondrial oxygen affinity(286). Elevated CIV may be a compensation response to increased proportion of inhibited CIV molecules *in vivo*. Increased flux through CIV was also found in a Ren-2 rat model of hypertension when they were compared to Ren-2 rats treated with anti-hypertensives.(287) The maximal uncoupled respiration reflects an overall increase in ETS capacity.

In PBMC, the increase in NADH-fuelled respiration at 14 mo is most likely mitochondrial in origin since background non-mitochondrial respiration was not significantly different in any of the groups after adding inhibitors specific for the various mitochondria ETS complexes (rotenone, malonate and antimycin-A, data not shown). In addition, the respiration changes observed with NADH were dependent on both underlying disease and age (disease-age interaction, p=0.018) with the older SHR rats having the greatest response. This suggests decreased mitochondrial outer membrane integrity in SHR that is progressive with disease and age.

6.4.6 Effect of age on mitochondrial respiration in PBMC

Age had an independent effect in mononuclear cells with a drop in G3P fuelled respiration in both SHR and Wistars. Interestingly, a 57% depression (p=0.014) was seen at 14 mo compared to 6 mo old Wistar rats, while in SHR no significant drop was apparent with age (p=0.104). This is reflective of a more rapid decrease in G3P fuelled respiration with age in normotensive rats compared to hypertensive rats and this is consistent with an increase in rapid glucose oxidation in SHR (284).

6.4.7 Mitochondrial respiration in heart fibres remain unchanged in the hypertensive and compensated hypertrophy stage

The mitochondrial dysfunction in mononuclear cells anticipates dysfunction in the heart fibers, but in contrast to mononuclear cells, the mitochondrial respiration of SHR permeabilized heart fibers was comparable to age matched Wistars at both early hypertensive (6 mo) and compensated hypertrophic non failing stage (14 mo) (Figure 6-3). Similar to the current study, no difference in CI,II OXPHOS or CIV flux was found in 12 mo SHR permeabilized heart fibers by an earlier study in our lab compared to WKY rats of same age (218). Rimbaud et al. also found no difference in basal and maximal mitochondrial respiration in skinned heart fibers between 15 and 25 week SHR and WKY controls(284). Unlike the present study however, a marginal elevation in CI OXPHOS and ETS (both $p<0.05$) was reported previously in SHR (218). This discrepancy may relate to the differing methodology between the two studies. In our current study the control littermates were Wistars, whereas Hickey *et al.*. used Wistar-Kyotos. Additionally respiration assays were performed at 37^{o}C in this study whereas Hickey et al. used 30^{o}C (218). In that study, mitochondrial function differences between WKY and SHR was minimal at rest but following maximal workloads, SHR hearts revealed considerable depression in ETS and OXPHOS capacities relative to WKY hearts. This suggested that while SHR heart mitochondria appear normal at rest, under a stressful workload they may be more susceptible to oxidative damage. In this study we were specifically interested in how the resting heart mitochondrial function compared to mitochondrial function measured in circulating mononuclear cells.

6.4.8 Limitations

A consideration of this study is that the global differences in mitochondrial respiration in mononuclear cells were due to the differences between the SHR and Wistar strains. However the relative trajectory of these changes were not the same between these two species at different ages, for example % CI ETS (Fig.2H) showed no difference at 6 mo between the SHR and the Wistars but there was a significant increase in respiration at 14 mo. This suggests there was no specific underlying species difference that were accounting for the results.

The differences in mononuclear cell mitochondrial respiration in this study were found to be present from as early as 6 mo in SHR whereas no significant differences were seen in the heart tissue at either ages. However, a recent human heart study has shown similar mitochondrial findings of decreased %CI ETS in heart disease and congestive heart

failure(282) as seen in mononuclear cells in this study. The early mononuclear cell mitochondrial dysfunction compared to those in the compensated heart may be due to increased sensitivity of the mononuclear cell to the hypertensive state.

6.4.9 Proposed hypothetical mechanism for altered PBMC mitochondrial respiration in SHR

Although the aim of this study was not to establish the mechanism, one hypothesis for the observed altered mitochondrial respiration in mononuclear cell is that they are exposed to increased oxidative stress (263) (263) as a result of endothelial dysfunction Figure 6-4. Endothelial dysfunction is one of the hallmarks of hypertension and one of the main characteristics of endothelial dysfunction is an impaired vasomotor response.(288) The normal vascular endothelium plays a pivotal role in regulating vascular tone by releasing various relaxing and contracting factors such as catecholamines, vasoactive peptides, reactive oxygen species (ROS) and nitric oxide (NO). NO is particularly important and when released from endothelial cells works in concert with prostacyclin to inhibit platelet aggregation, attachment of neutrophils to endothelial cells and expression of adhesion molecules. ROS such as superoxide rapidly react with NO limiting its bioavailability and its protective effects. Excessive scavenging of NO by ROS is one of the important mechanisms that result in endothelial dysfunction in hypertension.(289) NO plays a crucial role in signaling to mitochondria, and recently, NO was shown to play a role in basal and adaptive mitochondrial biogenesis in the vasculature and regulation of mitochondrial turnover in hypertension (290). In hypertension, ROS is produced excessively by vascular endothelial cells (291) which rapidly react with NO limiting its bioavailability and its protective effects. Oxidative stress has been reported to alter mtDNA copy numbers in leukocytes (292) and to inhibit mitochondrial CI (293) as witnessed by a decreased %CI ETS in mononuclear cell mitochondria in this study. In addition, ROS is also produced in inflammatory leukocytes such as PBMC mitochondria and nicotinamide adenine dinucleotide phosphate oxidase which may contribute to the endothelial dysfunction (Figure 6-4) (291).

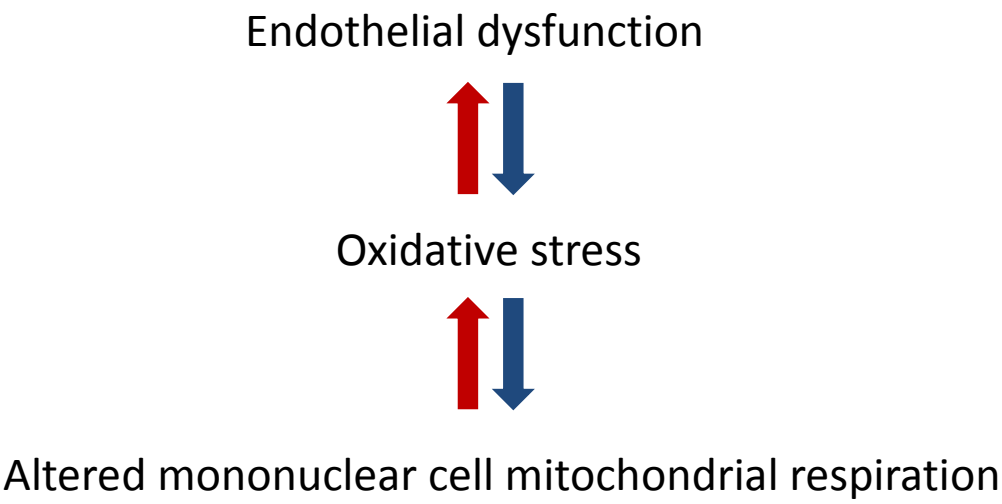


Figure 6-4 Proposed hypothetical mechanism for the observed altered mitochondrial respiration in mononuclear cells from spontaneous hypertensive rats.

6.4.10 Conclusion and future work

In this chapter, mitochondrial respiration assay was validated in an experimental model of chronic disease. In hypertension, PBMC were less efficient at producing ATP with a decreased CI as a function of ETS and a lower RCR in SHR compared to Wistars. From an early stage in the disease, the global increase in OXPHOS and ETS capacity is suggestive of increased ATP demands in hypertensive mononuclear cells. The differences in mononuclear cell mitochondrial respiration were present at a stage in the disease when there were no detectable heart tissue mitochondrial respiration differences. Mononuclear mitochondrial respiration may now be measured using this protocol in patients with hypertension. PBMC mitochondrial respiration will now need to be correlated with indices of subclinical organ damage to assess its potential to be a marker of subclinical multiple organ damage in hypertensive patients. This chapter has enabled an understanding of mitochondrial function in a chronic disease state. To broaden the understanding of mitochondrial function to acute disease states, the next chapter will focus on mitochondrial function in acute pancreatitis.

Chapter 7 Mononuclear Cell Mitochondrial Function in Mild Acute Pancreatitis

7.1 Introduction

To test the hypotheses of this thesis, a mitochondrial respiration protocol was developed in Chapter 5 and applied in an experimental model of hypertension in Chapter 6. It was established that mitochondrial respiration is altered early in PBMC in hypertension. Since the ultimate aim is to gain a fuller understanding of mitochondrial function in critically ill patients with MODS, the next step is to understand mitochondrial function in an acute disease state. This chapter measures mitochondrial function in acute pancreatitis and translates the mitochondrial respiration assay protocol to patients with mild acute pancreatitis.

7.2 Acute pancreatitis management is imprecise

Acute pancreatitis (AP) remains a challenging disease to manage, primarily because disease identification, severity prediction and the monitoring of responses to interventions are imprecise (294). This reflects the need to better characterise the critical outcome determining AP pathophysiology. Patients die with AP from MODS (295), and one of the features of MODS is mitochondrial dysfunction and the failure of cellular bioenergetics (27).

7.2.1 Mitochondrial dysfunction in AP

The main function of mitochondria is to efficiently generate adenine triphosphate (ATP) by oxidative phosphorylation (296). In addition, mitochondria are also involved in generation and detoxification of free radicals, apoptosis, regulation of cytoplasmic and mitochondrial matrix calcium, synthesis and catabolism of metabolites and transport of organelles to correct locations within the cell (296). Abnormalities in any of these functions can result from mitochondrial and cellular dysfunction (296). Recently, significant organ specific dysfunction of mitochondrial OXPHOS in the pancreas and in jejunum early in the course of experimental AP was demonstrated (254). Measuring mitochondrial respiration (MR) prior to the onset of organ failure might provide a useful way to predict disease severity and tracking disease course in AP.

7.2.2 Peripheral blood marker of mitochondrial function

An obvious problem is that organ biopsies would never be a practical, or an ethical approach in AP. Peripheral blood could provide an alternative and less invasive approach to access the

bioenergetic state of the patient. This is on the basis that mononuclear cells are exposed to pathological processes in the organs that are most prone to failing in critical illness, as they pass through different circulatory beds (297).

PBMC play an important role in the systemic inflammatory response, with their release of pro inflammatory cytokines which correlates with the severity of AP (298). However, it is not known whether MR changes in PBMC in AP.

7.2.3 Hypothesis

This study was designed to test the hypothesis that PBMC MR are altered in AP and to describe the nature of this change in MR. We tested this hypothesis in an experimental rodent model first and then translated this study to patients with AP in a separate clinical trial.

7.2.4 Aim

The aims of this study were two fold.

1) To measure early mitochondrial function in an experimental model of AP.

2) To measure mitochondrial function in patients with AP from admission to discharge.

7.3 Method

7.3.1 Animal study

Animals were prepared and handled as described in section 5.3. Injections of caerulein was used to induce acute pancreatitis as discussed in section 5.3.4.

7.3.2 Clinical study

The detailed description including inclusion and exclusion criteria is given in section 5.4.1.

7.3.3 Preparation of blood mononuclear cells

For the animal study, blood was sampled as described in section 5.3.2.

For the clinical study, blood samples were taken within 24 hrs of presentation to the hospital (day 1) and then on days 2, 3 and discharge and 20 mls of blood was drawn once from the HV. Blood was collected as described in section 5.1.1.

PBMC were retrieved and processed as described in section 0 and 5.1.3.

7.3.4 Characterising mononuclear cells using flow cytometry

PBMC were characterised as described in section 5.2.7 for the clinical study.

7.3.5 Mitochondrial respiration assays

A multiple substrate-inhibitor titration protocol was employed (Table 5-2 and Figure 5-6) as described in section 5.1.4 for both the animal study and the clinical study.

7.3.6 Assessment of mitochondrial DNA

Mitochondrial DNA was quantified using real-time PCR as described in section 5.2.1 for the clinical study.

7.3.7 Measurement of superoxide using fluorescent dye MitoSOX red

Mitochondrial superoxide was measured as described in section 5.2.2 for the clinical study.

7.3.8 Mitochondrial membrane potential using JC-1 using flow cytometry

Mitochondrial membrane potential was measured as described in section 5.2.3 for the clinical study.

7.3.9 Assessment of intracellular ATP

Intracellular ATP was detected as described in section 5.2.4 for the clinical study.

7.3.10 Intracellular protein

Intracellular protein was measured as described in section 5.2.5 for the clinical study.

7.3.11 Cytochrome *c*

Cytochrome *c* was measured as described in section 5.2.6 for the clinical study.

7.3.12 Statistical analysis

Statistical analysis was carried out using Graph Pad Prism version 4.00 and SPSS version 18 for Windows (GraphPad Software , San Diego, California, USA). Values are expressed as the median and interquartile range or mean ± standard error of mean (SEM) where appropriate. For the animal study and comparisons between HV and patients at day 1, Student *t* tests were used to evaluate statistical significance when the data fit a normal distribution and a Mann Whitney U test was carried out otherwise. For temporal mitochondrial function, repeated measures of analysis of variance (ANOVA) with a post-hoc least significant difference (LSD) correction was used to evaluate statistical significance in patients with mild acute pancreatitis

from day 1 to discharge. A student *t*-test was used to detect statistical significance between HV and each day of mitochondrial function in the patient group followed by a sequential Bonferroni correction for multiple comparisons. Significance was established at $P < 0.05$ and is reported as $p<0.05$, $p<0.01$, $p<0.005$ and $p<0.001$ where appropriate.

7.4 Results

Results for the experimental and clinical studies are presented separately. The mitochondrial respiration assay was done in both the experimental and clinical studies in permeabilised PBMC. In addition, within the clinical study PBMC receptors were characterized, mitochondrial membrane potential, mitochondrial superoxide, cytochrome *c* and ATP were measured.

7.4.1 Experimental

7.4.1.1 Animal characteristics

The average weight was 401+/- 8g and 411+/- 5g for the control and caerulein group respectively and there was no significant difference between the groups. The pancreatic oedema index (POI) was on average 1.8 fold elevated in the caerulein group ($p=0.008$) (Table 7-1). There was hyperamylasaemia in the caerulein group with an average amylase of 31060 ± 3211 IU relative to 2671 ± 173 IU in the control group, $p<0.0001$ (Table 7-1). There was no significant difference between the groups for alkaline phosphatase (ALP), alanine transaminase (ALT) activities, or in bilirubin, albumin, or creatinine. Table 7-1 shows that the average number of white blood cells in the caerulein group (4.86 ± 0.64 E+9/L) did not differ significantly from the control group (5.15 ± 0.32 E+9/L). The numbers of lymphocytes were lower in the caerulein group than the control (2.61 ± 0.31 E+9/L vs. 3.86 ± 0.19 E+9/L, $p = 0.01$). Monocyte number remained statistically similar between groups (0.20 ± 0.04 E+9/L in the control group vs. 0.33 ± 0.17 E+9/L in the caerulein group).

Table 7-1 Pancreatic oedema index and biochemical characteristics of control wistar rats and rats with mild acute pancreatitis (caerulein)

	Control (n=9)	**Caerulein (n=9)**	***P-value***
Pancreatic Oedema Index	2.73±0.23	4.85±0.78	**0.01**
Amylase (U/L)	2671±173	31060±3211	< **0.00**
Bilirubin (µmol/L)	0.86±0.14	0.88±0.13	0.93
ALP (U/L)	120±8	113±8	0.52

ALT (U/L)	47±4	49±4	0.78
Albumin (g/L)	37±0.46	38±0.42	0.19
Creatinine (mmol/L)	0.02±0.00	0.03±0.00	0.22
WBC (E+9/L)	5.15±0.32	4.86±0.64	0.70
Lymphocyte (E+9/L)	3.86±0.19	2.61±0.31	**0.01**
Monocyte (E+9/L)	0.20±0.04	0.33±0.17	0.40

Footnote: Values listed are mean±SEM. P-values to two significant figures.

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; WBC, white blood count.

7.4.1.2 Peripheral mononuclear cell MR

Endogenous respiration in the caerulein group (8.32 ± 1.7 pmol/s/million cells) was twofold higher than the control group (3.91 ± 0.35 pmol/s/million cells), (P = 0.03, d.f=14) (Figure 7-1A). CI Leak with Complex I substrates, and CI OXPHOS respiration flux were similar in both groups (p=0.99 and 0.26 respectively). After addition of the Complex II substrate succinate, CI,II OXPHOS respiration was twofold higher in the caerulein group, p = 0.03, d.f=15 (Figure 7-1B). However, after addition of oligomycin, CI,II Leak respiration was 2.7 fold higher in the caerulein group (8.60±2.05 pmol/s/million cells) compared to the control group (3.56±0.30 pmol/s/million cells), (P = 0.04, d.f=15) (Figure 7-1C). Flux through CIV was significantly higher in the caerulein group compared to the control group, (P = 0.03, d.f=15 and 0.02, d.f=15 respectively, Figure 7-1D and Figure 7-1E). The percentage of CI as a function of ETS (%CI ETS) also did not change significantly between the caerulein (23.5 ± 6.3%) and the control group (25.8 ± 3.9%), (P=0.77, d.f=14). Overall these data suggest a global increase in all respiration states, but no change in CI Leak or CI OXPHOS at 6 hrs from induction of AP in this experimental model.

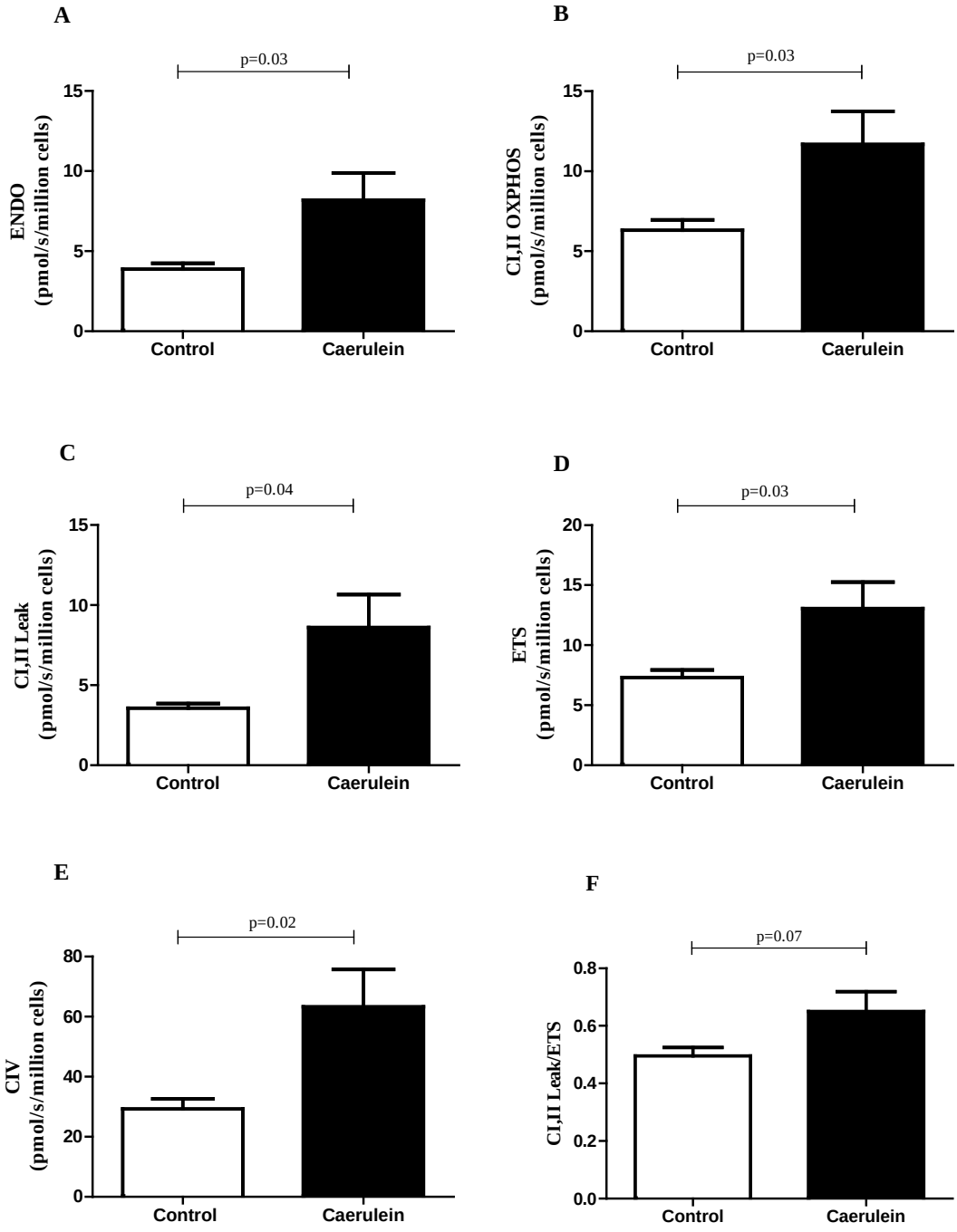


Figure 7-1 Mononuclear cell mitochondrial respiration in an experimental model of acute pancreatitis. Mononuclear cell mitochondrial respiration flux of controls, white bars (n=9) and caerulein rats with induced mild acute pancreatitis, black bars (n=9). Different respiratory states measured were: A) Endo [Endogenous respiration], B) CI,II OXPHOS [Complex I and II oxidative phosphorylation], C) CI,II Leak [leak respiration after complex V is blocked with oligomycin], D) ETS [Electron Transport System capacity] and E) Complex IV and F) CI,II Leak/ETS. Values are mean ± standard error of mean.

7.4.2 Clinical AP

7.4.2.1 Patient characteristics

There were 18 patients with acute pancreatitis and 15 healthy volunteers recruited for this study. Their baseline characteristics are given in Table 7-2 and there was no statistically significant difference between the groups for age or gender. The control group had no chronic diseases and were on no regular medications. There were significant differences in the baseline blood tests in patients compared to the HV, with a significant elevation in pancreatic amylase, alkaline phosphatase, gamma glutamyl transferase, alanine transferase, albumin and creatinine.

Table 7-2 Baseline characteristics, liver and kidney function tests of healthy volunteers (HV) and patients with acute pancreatitis (AP).

	Reference range	**HV**	**Acute pancreatitis**	**p**
Number		15	18	
Age (years)		51 (38-59)	55 (36-70)	0.6
Male : female ratio		6:9	8:10	0.35
Amylase (U/L)	25 - 135	35 (29-39)	848 (497–1142)	<0.0001*
ALP (U/L)	40 - 130	60 (43-71)	90 (54-134)	<0.0001*
GGT (U/L)	<60	5 (5-7)	114 (40-411)	0.02*
ALT (U/L)	<45	7 (6-8)	49 (19-235)	0.01*
Albumin (g/L)	36 - 52	16 (15-18)	44 (41-45)	<0.0001*
Creatinine (mmol/L)	0.02 - 0.1	0.02 (0.015-0.04)	0.072 (0.065-0.10)	<0.0001*

Footnote: Values are mean and SEM unless otherwise stated. P-values to two significant figures, * denotes P value on *Mann Whitney* test.

Abbreviations: ALP, alkaline phosphatase; GGT, gamma glutamyl transferase; ALT, alanine transferase.

7.4.2.2 Clinical course of acute pancreatitis in the patient group

The median APACHE II score on admission was 6 (3-11) (Table 7-3) and the median duration of symptoms prior to admission was 11 (3-24) hrs. The total length of hospital stay was 8.5 (6–13) days in the AP group. Nine patients required a total of 10 interventions and all

10 interventions were therapeutic ERCP and cholecystectomy. Progression of severity occurred in two patients and there was no in-hospital mortality and two patients required hospital readmission.

Table 7-3 Aetiology, inflammatory markers, blood count and severity scores of patients with acute pancreatitis (AP).

	Reference range	**Acute pancreatitis**
Aetiology		
Biliary(%)		10(56%)
Alcohol(%)		5(28%)
Unknown(%)		3(16%)
CRP (mg/L)	0 - 5	66 (16-145)
WBC (E+9/L)	4 - 11	12 (8-16)
Lymphocyte count (E+9/L)	1 - 4	1.2 (1.1-1.7)
Monocyte count (E+9/L)	0.2 - 1	0.7 (0.5-1.1)
APACHE II		6 (3-11)
BISAP score		0 (0-2)
SOFA score		0 (0-2)
Glasgow score		2 (0-2)
Co-morbidity class*		
I		6
II		8
III		4

Footnote: *As defined by the American Society of Anaesthesiologists guidelines. Values are expressed as the median and interquartile range.

Abbreviations: CRP, C-reactive protein; WBC, white blood cell count; APACHE, Acute Physiology and Chronic Health Evaluation; BISAP , Bedside Index Severe Acute Pancreatitis; SOFA, Sepsis-related Organ Failure Assessment.

7.4.2.3 Baseline and temporal changes in lymphocyte and monocyte subsets

The average total WBC (white blood count), lymphocyte and monocyte counts remained statistically unchanged through out admission (Table 7-4). However when monocyte and lymphocyte subsets were analysed, differences were seen compared to HV and within patients over the length of hospital stay (

Table 7-5). Firstly, compared to HV, the monocyte subsets CD14+, CD14+CD16-, HLADR and CD16+CD14-HLADR were all significantly higher ($p<0.001$ for all) and the lymphocyte subsets CD4+ and CD4+8+ was significantly decreased in the AP d1 group ($p<0.01$ for both) (

Table 7-5). Secondly, all subsets of monocytes studied with the exception of CD16+CD14- decreased statistically significantly at discharge compared to AP d1, AP d2 and d3 (

Table 7-5).

Table 7-4 Temporal APACHE II and blood counts in patients with mild acute pancreatitis (AP).

	Reference range	**AP d1**	**AP d2**	**AP d3**	**AP discharge**	***P-value***
APACHE II		6 (3-11)	2 (1-7)	2 (0-6)	2 (0-8)	0.378
WBC (E+9/L)	4 - 11	12 (8-16)	11 (7-14)	7 (6-12)	9 (6-12)	0.425
Lymphocyte count (E+9/L)	1 - 4	1.2 (1.1-1.7)	1.5 (1.1-1.9)	1.8 (1.1-2.2)	1.4 (1.1-2.1)	0.661
Monocyte count (E+9/L)	0.2 - 1	0.7 (0.5-1.1)	0.7 (0.6-1.0)	0.7 (0.4-0.9)	0.6 (0.5-0.9)	0.495
Creatinine (mmol/L)	0.02 - 0.1	0.07 (0.06-0.10)	0.06 (0.05-0.07)##	0.06 (0.05-0.08)##	0.07 (0.06-0.08)#	0.001
Amylase (U/L)	25 - 135	848 (497–1142)	618 (488-1112)##	258 (195-326)###zz	119 (64-145)###zzz ^^	<0.001

Footnote: Values are expressed as the median and interquartile range. Differences between days were analysed with repeated measures ANOVA with post hoc LSD. # indicates p<0.05 compared to AP day 1 (d1), ## indicates p<0.01 compared to AP day 1 (d1), ### indicates p<0.001 compared to AP d1, zz indicates p<0.01 compared to AP d2, zzz indicates p<0.001 compared to AP d2, ^ ^indicates p<0.01 compared to AP d3.

Abbreviations: APACHE, Acute Physiology and Chronic Health Evaluation. WBC, white blood cell count.

Table 7-5 Temporal monocyte and lymphocyte subsets in patients with mild acute pancreatitis (AP).

	Healthy volunteers	**AP d1**	**AP d2**	**AP d3**	**AP discharge**
Monocytes					
CD14+	16.7± 1.0	34.09±4.02***	24.94±3.29#	20.50±2.04##	15.21±2.02##ƨƨ
CD14+CD16-	4.1±0.8	18.80±3.51***	20.54±2.80***	16.37±1.60***	13.56±2.00***ƨ
CD16+	23.2±2.2	36.13±6.245	21.46±5.52#	20.03±5.14*#	12.28±3.48**##ƨ^
CD16+CD14+	1.8±0.2	5.21±1.55	5.14±1.38~	5.39±0.98~~	2.43±0.47
CD16+CD14-	5.1±1.3	8.30±3.02	9.29±2.16	10.49±2.55	6.02±1.07
HLADR+	61.2±2.5	79.59±2.50***	67.47±4.14##	84.71±1.40**ƨƨ	82.13±4.37***
CD14+CD16-HLADR	98.9±0.3	98.79±0.73	98.44±0.23	98.54±0.511	97.66±0.90
CD14+CD16+HLADR	97.5±0.6	96.94±1.15	97.30±1.21	97.47±0.75	96.38±1.08
CD16+CD14-HLADR	63.6±2.9	86.06±2.95***	71.76±4.00##	87.61±1.52**ƨƨ	85.88±3.30***ƨ
Lymphocytes					
CD4+	56.9±1.8	46.3±3.0**	43.6±3.3**	36.7±4.1***	35.6±4.2***
CD4+8+	7.4±0.9	2.7±0.5**	2.5±0.4***~~	1.6±0.3***~	1.1±0.1***

Footnote: Values represent mean percentage of total mononuclear cells ± SEM. * indicates p<0.05 compared to HV, ** indicates p<0.01 compared to HV, *** indicates p<0.001 compared to HV (students *t* test).).# indicates p<0.05 compared to AP day 1 (d1), ## indicates p<0.01 compared to AP d1, ƨ indicates p<0.05 compared to AP d2, ƨƨ indicates p<0.01 compared to AP d2, ^ indicates p<0.05 compared to AP d3, ~ indicates p<0.05 compared to discharge, ~~ indicates p<0.01 compared to AP discharge. Differences between days were analysed with repeated measures ANOVA with post hoc LSD.

7.4.2.4 Mitochondrial function in patients with AP at day 1

Endogenous respiration in the patients with AP was 1.5 times higher than the HV (4.44±0.48 vs. 3.27±0.10 pmol/s/million cells, P=0.04, d.f=28) (Figure 7-2A). CI Leak with Complex I substrates, and CI OXPHOS were similar in both groups (data not shown). After addition of the Complex II substrate succinate, CI,II OXPHOS respiration was 1.5 fold higher in the AP group (10.30±1.31 pmol/s/million cells) compared to HV (6.77±0.67 pmol/s/million cells, P=0.02, d.f=26) (Figure 7-2B). Following oligomycin addition CI,II Leak respiration was increased 1.8 fold in the AP group (2.76±0.50 pmol/s/million cells compared to HV (1.50±0.19 pmol/s/million cells, P=0.03, d.f=27) (Figure 7-2C). Flux through the ETS, G3P and CIV were similar between the groups 8.69±1.43, 4.97±0.68, 20.87±4.48 pmol/s/million cells for AP respectively and 7.38±0.66, 5.57±1.44 and 16.70±2.98 pmol/s/million cells for HV respectively (P=0.44, d.f=25, P=0.70, d.f=25 and P=0.47, d.f=27 for ETS, G3P and CIV respectively). The % CI ETS was not statistically significantly different between the two groups, (29.67+/-5.57% in HV and 29.04±7.48% in AP, P=0.95, d.f=26) (Figure 7-2D). The CI,II Leak/ETS was significantly higher in AP (0.60±0.18) compared to HV (0.21±0.02, P=0.04, d.f=26) (Figure 7-2E) indicating that there is a greater capacity to uncouple the ETS from OXPHOS in patients with AP than HV which can lead to increased oxygen consumption without additional ATP production. The cytochrome c concentration in AP was almost four fold lower than in the HV, P<0.0001 MW (Figure 7-3). The findings indicate an overall increased mitochondrial respiration including leak respiration and CI,II Leak/ETS in AP.

To understand whether the altered mitochondrial respiration can be explained by a change in quantity of mitochondria in AP, mitochondrial DNA was measured (299). There was no difference in the quantity of mitochondrial DNA normalised to a nuclear gene between AP and HV (P=0.63) indicating that the altered respiration findings are independent of mitochondrial quantity (Figure 7-2F).

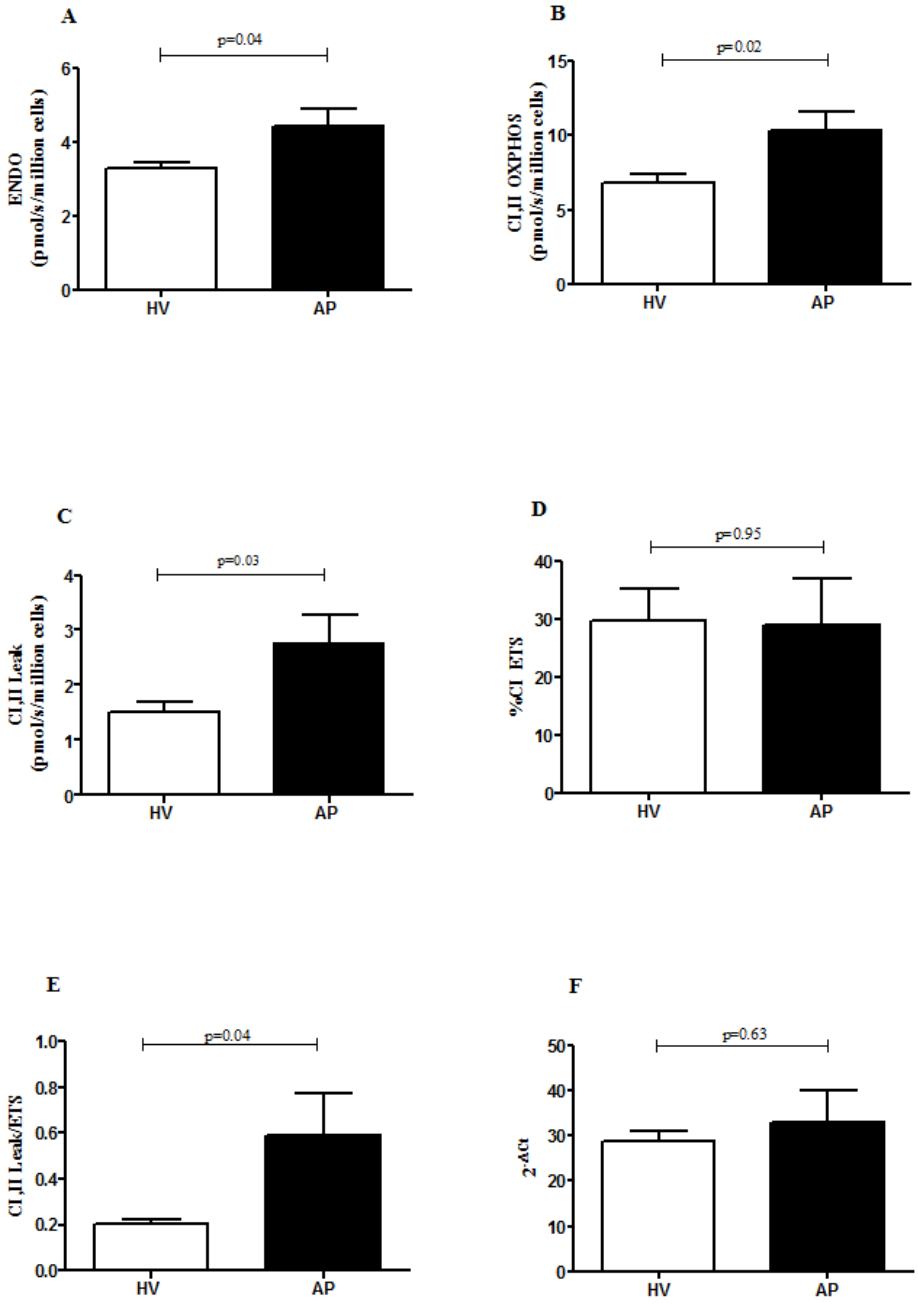


Figure 7-2 Mononuclear cell mitochondrial respiration in patients with mild acute pancreatitis**.** Mononuclear cell respiration of healthy volunteers (HV, white bars), n=15 and patients with mild acute pancreatitis (AP, black bars), n=18. A) ENDO [Endogenous respiration], B) CI,II OXPHOS[Complex I and II oxidative phosphorylation], C) CI,II Leak [leak, respiration after complex V is blocked with oligomycin], D) %CI ETS [percentage of CI as a function of ETS], E) CI,II Leak/ETS, F) Relative quantification of mtDNA using $2^{-\Delta Ct}$. The mitochondrial gene MTCO1 (0188166_m1) and the nuclear gene RN18S1 (03928985_s1) was used as the internal control gene. Values are mean ± standard error of mean.

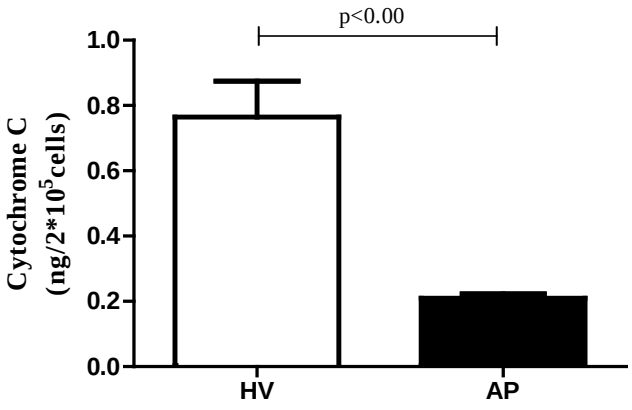


Figure 7-3 Mononuclear cell cytochrome C in mild acute pancreatitis. Mononuclear cell cytochrome C in healthy volunteers, white bars (HV, n=15) and patients with mild acute pancreatitis, black bars (AP, n=18). The *Mann Whitney* test was employed. Values are mean ± standard error of mean.

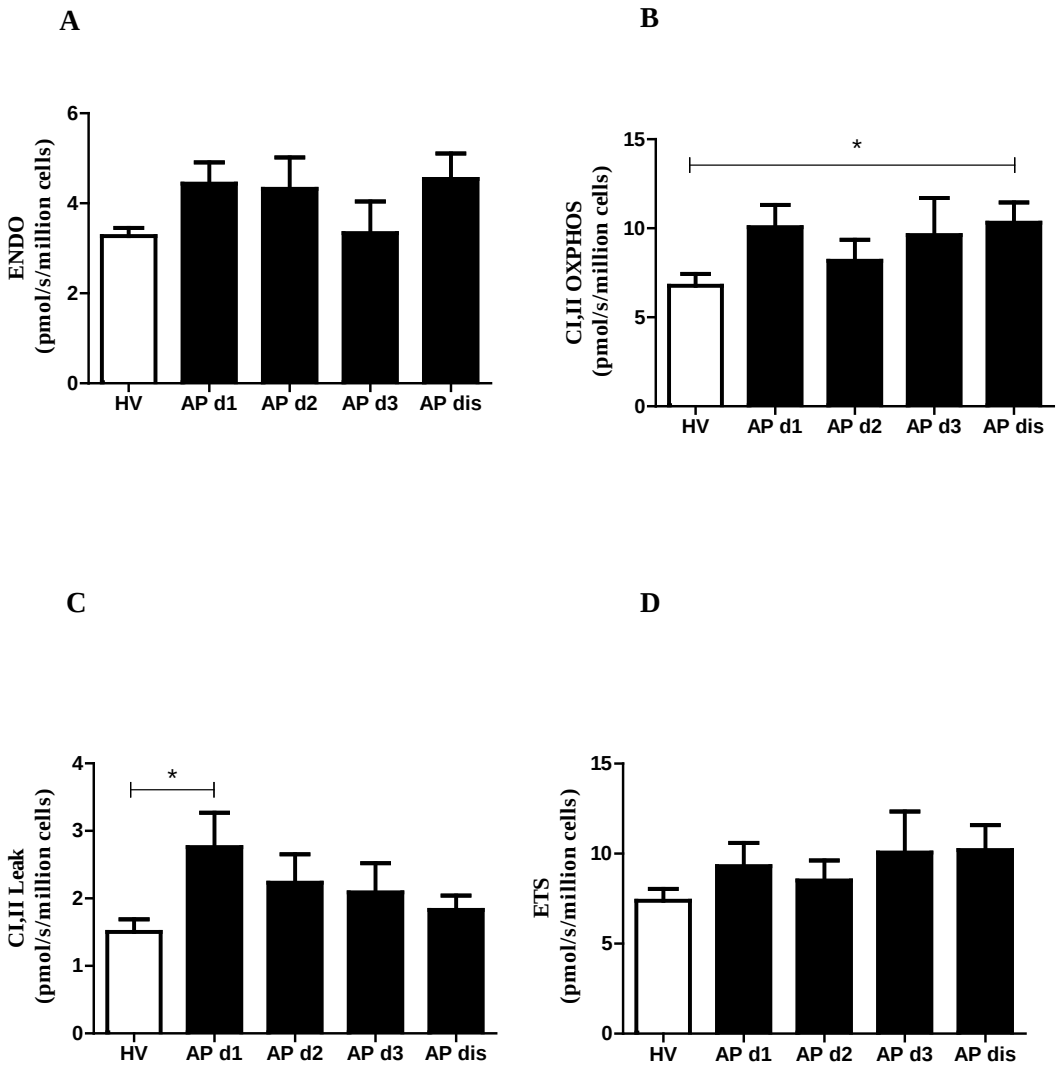


Figure 7-4 Temporal mononuclear cell mitochondrial respiration in mild acute pancreatitis. Healthy volunteers (HV, white bars), n=15 and patients with mild acute pancreatitis (AP, black bars), n=18 at day 1 (d1), day 2 (d2), day 3 (d3) and discharge (dis). Different respiratory states measured were: A) ENDO

[Endogenous respiration], B) CI,II OXPHOS[Complex I and II oxidative phosphorylation], C) CI,II Leak [leak, respiration after complex V is blocked with oligomycin] and D) ETS [electron transport system].

7.4.2.5 Differences in temporal mitochondrial respiration between HV and AP

After correcting for multiple comparisons with sequential Bonferroni CI,II OXPHOS was significantly increased at discharge (10.31±1.13 pmol/s/million cells in AP vs. 6.77±0.67 pmol/s/million cells, P=0.01, d.f.=25) (Figure 7-4B) as was CI,II Leak at d1 (2.76±0.50 pmol/s/million cells in AP compared to HV (1.50±0.19 pmol/s/million cells, P=0.03, d.f=27) (Figure 7-4C). However, Endo and ETS were no longer statistically significantly different on any of the days compared to HV after correction for multiple comparisons with sequential Bonferroni (Figure 7-4A and Figure 7-4D).

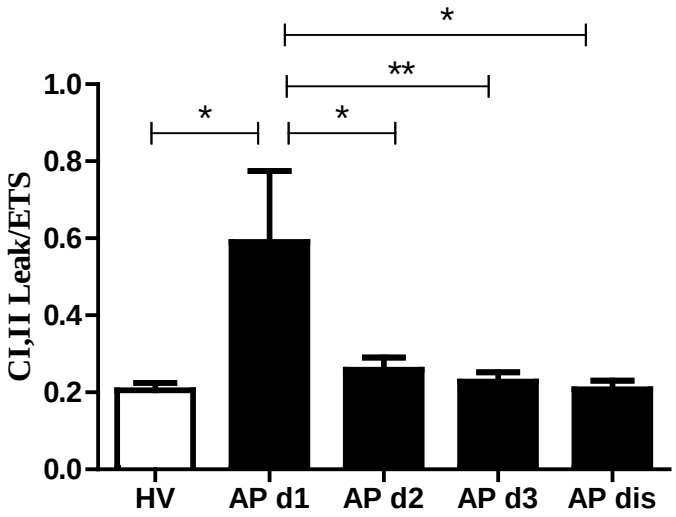


Figure 7-5 Ratio of leak to electron transport system capacity in mononuclear cells in mild acute pancreatitis**.** Mononuclear cell mitochondrial CI,II Leak/ETS [ratio of CI,II Leak to electron transport system (ETS)] and reflects the level of leak normalised to maximum ETS capacity in healthy volunteers (HV, white bars), n=15 and patients with mild acute pancreatitis (AP, black bars), n=18 at day 1 (d1), day 2 (d2), day 3 (d3) and discharge (dis). *Significantly different by at least p<0.05; ** Significantly different by at least p<0.01.

The CI,II Leak/ETS was significantly higher at d1 in AP (0.60±0.18) compared to HV (0.21±0.02, P=0.04, d.f=26) after correction for multiple comparisons but not thereafter (Figure 7-5). This is in sharp contrast with %CI ETS which was not different at d1 (p=0.95), but was significantly higher from d2 to discharge compared to HV, p<0.005 for d2, d3 and discharge after correcting for multiple comparison with sequential Bonferroni (Figure 7-6).

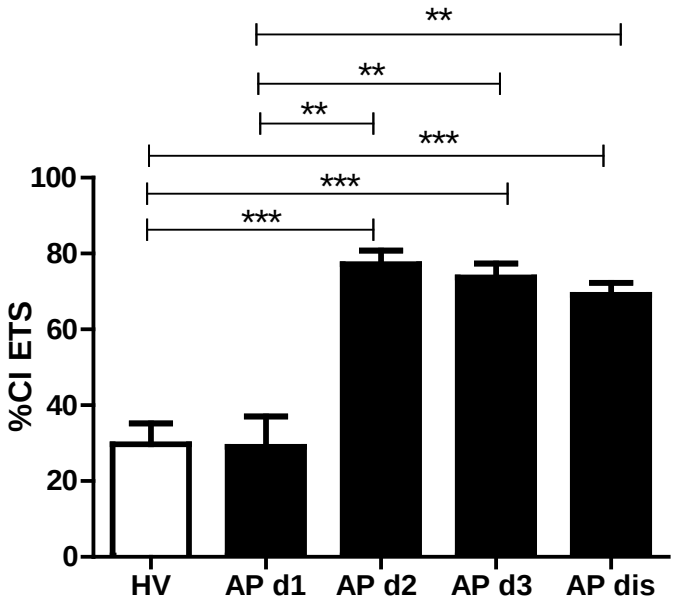


Figure 7-6 Complex I as a percentage of electron transport system capacity in patients with mild acute pancreatitis over time. Mononuclear cell mitochondrial CI as a percentage of total ETS capacity in healthy volunteers (HV, white bars), n=15 and patients with mild acute pancreatitis (AP, black bars), n=18 at day 1 (d1), day 2 (d2), day 3 (d3) and discharge (dis). %CI ETS is measured as [(ETS-rot)/ETS]*100 and indicates how much CI is contributing towards total ETS capacity. ** Significantly different by at least $p<0.01$; *** Significantly different by at least $p<0.005$.

Mitochondrial superoxide was increased from AP d1 to AP discharge compared to HV (Figure 7-7). The average fluorescent intensity in HV was 82.69+/-12.94 whereas at d1 in patients with acute pancreatitis, mitochondrial superoxide levels peaked and was 8 fold higher, 696+/-144 ($p<0.005$). At d2 the fluorescent intensity was still five fold higher than HV ($p<0.005$) and at day 3 and discharge, mitochondrial superoxide was trending downwards but was still significantly higher than HV, $p<0.005$ and $p<0.01$ respectively (Figure 7-7).

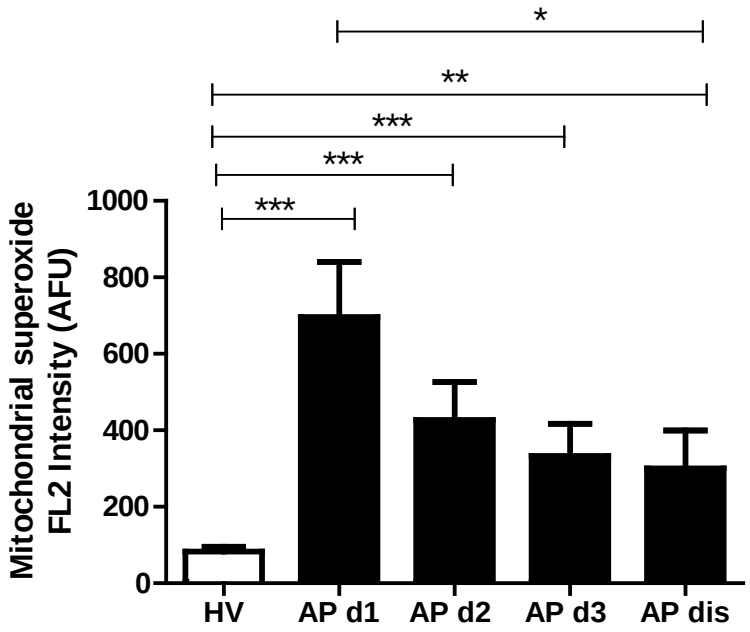


Figure 7-7 Mononuclear cell mitochondrial superoxide in patients with mild acute pancreatitis over time. Mitochondrial superoxide was measured with the dye MitoSOX in channel 2 (FL2) of a FACS Calibur flow cytometer in healthy volunteers (HV, white bars), n=15 and patients with mild acute pancreatitis (AP, black bars), n=18 at day 1 (d1), day 2 (d2), day 3 (d3) and discharge (dis). * Significantly different by at least $p<0.05$; ** Significantly different by at least $p<0.01$; ***Significantly different by at least $p<0.005$.

When JC-1 was used to measure mitochondrial membrane potential, the ratio of aggregate to monomer increased by two fold in the AP group on d1 (4.7+/-0.89) compared to the HV (2.19+/-0.33), (P=0.02), peaked at d2 (6.54+/-0.66) and remained raised until discharge (4.22+/-0.59) (Figure 7-8). The ATP to protein ratio did not differ significantly between patients with AP and HV at d1 (p=0.054) but was significantly increased at d2 (p=0.003) followed by slight but significant decreases at d3 (p=0.049) and discharge (p=0.017) (Figure 7-9).

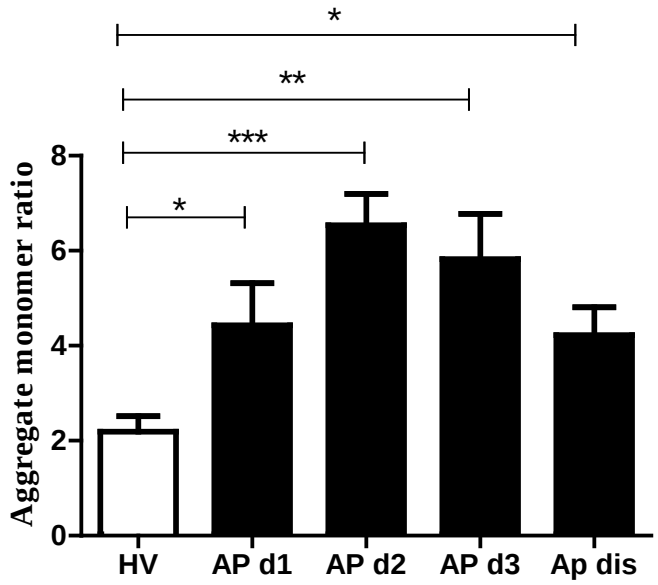


Figure 7-8 Mononuclear cell mitochondrial membrane potential in patients with mild acute pancreatitis over time. Mitochondrial membrane potential was measured with the dye 5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolylcarbocyanine iodide (JC-1). JC-1 fluorescence was detected by flow cytometry in two channels FL 1 and FL 2 in healthy volunteers (HV, white bars), n=15 and patients with mild acute pancreatitis (AP, black bars), n=18 at day 1 (d1), day 2 (d2), day 3 (d3) and discharge (dis). JC-1 exists as a monomeric form which fluoresces at FL 1 and when concentrated by actively respiring mitochondria exists as an aggregate which fluoresces at FL 2. An increase in the ratio of aggregate to monomer suggests higher mitochondrial membrane potential which in turn generates increased mitochondrial reactive oxygen species such as mitochondrial superoxide. * Significantly different by at least $p<0.05$; ** Significantly different by at least $p<0.01$; ***Significantly different by at least $p<0.005$.

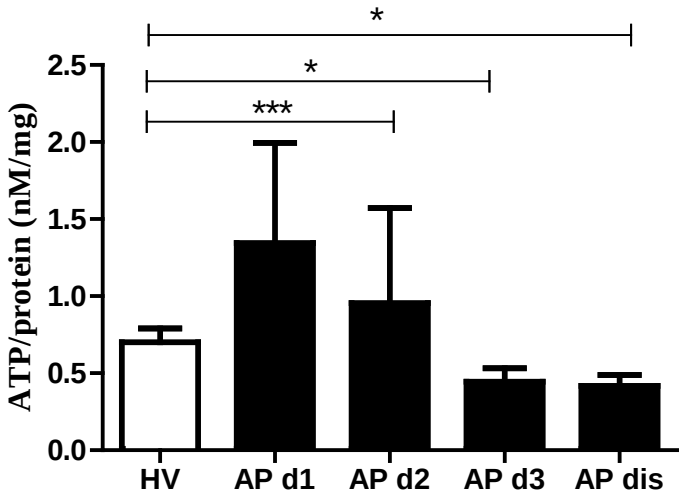


Figure 7-9 Adenine triphosphate to protein ratio in patients with mild acute pancreatitis**.** Mononuclear cell adenine triphosphate (ATP) to protein ratio in healthy volunteers (HV, white bars), n=15 and patients with mild acute pancreatitis (AP, black bars), n=18 at day 1 (d1), day 2 (d2), day 3 (d3) and discharge

(dis).. * Significantly different by at least p<0.05; ** Significantly different by at least p<0.01; ***Significantly different by at least p<0.005.

7.4.2.6 Temporal changes in mitochondrial function in AP – from day 1 to discharge

The CI,II Leak/ETS reflects the level of maximum leak relative to ETS and decreased three fold between d1 and discharge (0.655+/-0.369 vs. 0.195+/- 0.043, F=5.970, d.f 3.15, p=0.007) (Figure 7-5). This means that as the patients recovered, their electron transport system and their phosphorylation system was well coupled compared to earlier in the disease state and this change was able to be followed over time through this blood test.

The CI,II OXPHOS/CI,II Leak represents combined phosphorylation efficiency and phosphorylation and is a sensitive indicator of overall mitochondrial function and is otherwise known as the respiratory control ratio. The CI,II OXPHOS/CI,II Leak doubled between admission and discharge (3.68+/- 1.56 vs 6.83+/-2.63, F=5.267, df 3,18, p=0.009) (Figure 7-10). This is indicative of an electron transport system that is capable of progressively generating more ATP as the patient recovers.

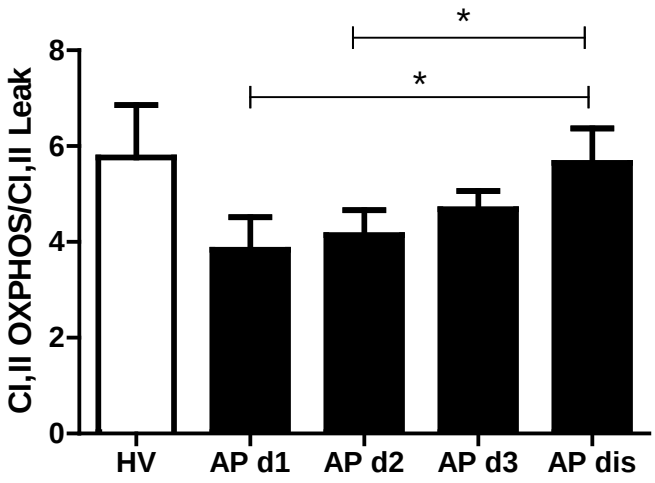


Figure 7-10 Temporal mononuclear cell mitochondrial respiratory control ratio (CI,II OXPHOS/CI,II Leak) in healthy volunteers (HV, white bars), n=15 and patients with mild acute pancreatitis (AP, black bars), n=18 at day 1 (d1), day 2 (d2), day 3 (d3) and discharge (dis). This ratio represents the degree of coupling between oxidation and phosphorylation and is a sensitive indicator of overall mitochondrial function. * Significantly different by at least p<0.05.

The %CI ETS gives an indication of how much CI is contributing to the total electron transport system capacity (Figure 7-6). The %CI ETS increased 7 fold from d1 to d2 and then remained steady until discharge (d1 10.47+/- 18.3, d2 72.50+/- 12.88, d3 72.30+/- 17.08, d4 65.87+/-13.91; F=21.530, d.f 3.15, p=0.000). This suggests that CI is able to increase its

electron transport capacity over a small period of time (24 hrs) and this change can be traced in the blood test.

There was a trend towards a decrease in mitochondrial superoxide from d1 to discharge, d1 696+/-144, discharge 300+/-99, F=4.637, d.f. 2.8, p= 0.052 (Figure 7-7). On post hoc least significant difference (LSD) testing there was significantly decreased mitochondrial superoxide between d1 (696+/-144 AFU) and discharge (300.10+/-99.20 AFU), p=0.016 (Figure 5). This decrease in mitochondrial superoxide followed the increase in CI,II OXPHOS/CI,II Leak (Figure 7-10) and %CI ETS compensation (Figure 7-6) in PBMC.

Mitochondrial membrane potential ($\Delta\Psi$) measured as the aggregate to monomer ratio with the dye JC-1 were similar in patients from admission to discharge (p=0.287) (Figure 7-8). ATP to protein ratio is almost double at d1 compared to HV and at d2 was significantly increased (P<0.005) but decreased at d3 and discharge compared to HV (P<0.05) (Figure 7-9).

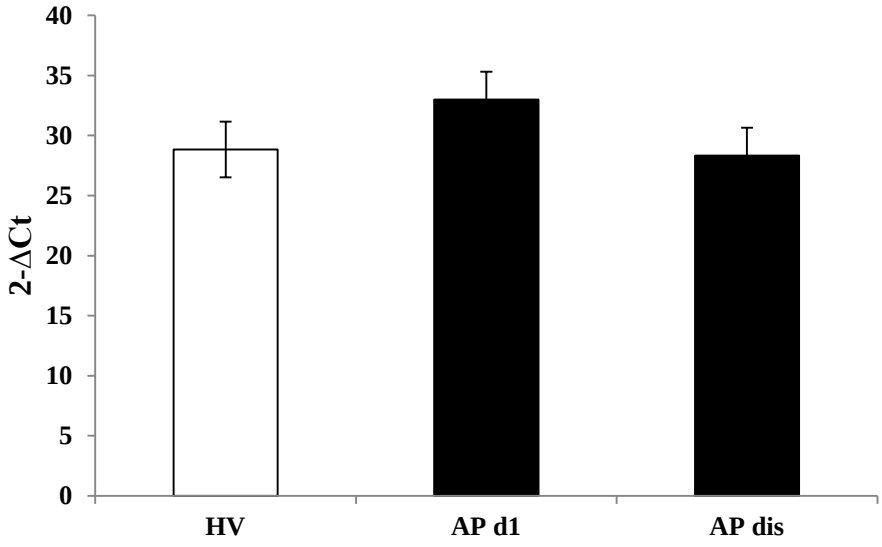


Figure 7-11 Relative quantification of mtDNA using $2^{-\Delta Ct}$. The mitochondrial gene MTCO1 (0188166_m1) and the nuclear gene RN18S1 (03928985_s1) was used as the internal control gene. Temporal mononuclear cell mitochondrial mtDNA (HV, white bars), n=15 and patients with mild acute pancreatitis (AP, black bars), n=18 at day 1 (d1), and n=10 at discharge (dis). Values are mean ± standard error of mean.

To understand whether the altered mitochondrial respiration can be explained by a change in quantity of mitochondria in AP, mt DNA was measured (299). There were no differences in the quantity of mitochondrial DNA normalised to a nuclear gene between AP d1, discharge and HV (P=0.35, *RANOVA* Figure 7-11) indicating that the altered respiration findings are independent of mitochondrial quantity.

7.5 Discussion

This was the first study to measure mitochondrial function (MF) in peripheral blood mononuclear cells in AP. Both experimental and clinical studies demonstrated altered MF early in mild AP. Augmented mitochondrial respiration (MR) was apparent for endogenous PBMC respiration and leak respiration in both experimental and clinical setting early in mild AP. The clinical study also revealed that in comparison to healthy volunteers there was an elevated CI,II Leak/ETS, an eight fold increase in mitochondrial superoxide, hyperpolarized mitochondrial membrane and a fourfold decrease in the mitochondrial protein cytochrome *c* at d1.

There were also temporal changes in MF as patients with AP recovered in hospital (Figure 7-4 to Figure 7-11). The CI,II Leak/ETS normalised over time and was no different from the healthy volunteers by discharge whilst the mitochondrial superoxide and the mitochondrial membrane potential decreased over time but still remained elevated compared to HV even at discharge. Even though there was no compensation by CI as seen by %CI ETS at d1, there was compensation by CI from d2 onwards as evident by the increased %CI ETS compared to HV and at d1. The increase in %CI ETS was followed by a statistically significant increase in the CI,II OXPHOS/CI,II Leak by discharge. Net ATP was raised early on and then decreased by d3 and discharge. These findings in the clinical study suggest altered mitochondrial function in patients with AP with some features of mitochondrial dysfunction.

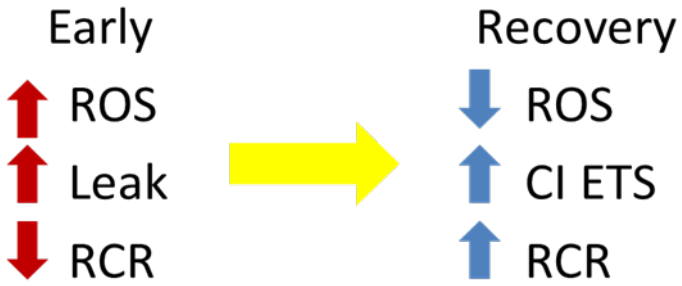


Figure 7-12 Pattern of mitochondrial function in early and at recovery phase of mild acute pancreatitis.

7.5.1 Altered mitochondrial function in mild AP

This study was designed to determine whether MF could be measured from PBMC early in patients with mild AP. It was also designed to determine the temporal pattern of mitochondrial function as patients with mild AP recovered. The finding that MF is altered in mild AP now need to be repeated in studies of severe AP to evaluate whether PBMC

mitochondrial respiratory function becomes further dysfunctional as found in severe sepsis (23) and whether the degree of dysfunction correlates with the severity of AP.

7.5.2 PBMC subsets in AP are altered even though absolute counts are not

PBMC comprised of lymphocytes and monocytes, were selected for evaluating MF because they are known to be altered in the pathophysiology of AP. It has been reported that a decrease in CD4+ and CD8+ subsets of lymphocytes is inversely proportional to the severity of AP (300) and risk of subsequent infection (301). The decreased lymphocyte numbers together with a decrease in cytochrome *c* and preserved ATP most likely represents apoptosis. In experimental pancreatitis, peripancreatic cells were shown to undergo apoptosis and induction of apoptosis in pancreatic acinar cells reduced the severity of AP.

It is also known that monocyte secretion of the proinflammatory cytokines (e.g. TNFα, IL-6 and IL-8) increases in patients with AP and that the extent of elevation in cytokines correlates with disease severity (298). We analysed subsets of PBMC to determine the cell populations and characteristics present in our experimental samples. Even though there was no net increase in the number of monocytes or lymphocytes in patients with mild AP in this study, within these cells types there were increases in specific monocyte subsets and decreases in specific lymphocyte subsets (Table 7-5), which is consistent with other reports (300), (302). The CD14+CD16- subset of monocytes has been shown to significantly increase in patients with AP indicative of their increased antigen presenting activity and elevated HLA-DR expression, a marker of cell activation (303), (304). Lymphopenia in the CD4+ and CD4+CD8+ subsets of lymphocytes has also been reported in AP (302). The monocyte and lymphocyte profiles in patients with AP in the present study are therefore consistent with an activated state in these PBMCs.

7.5.3 Mitochondrial function in related diseases

Until this present study, the MF of PBMC had not been previously studied in AP or in other acute mild inflammatory diseases and temporal mitochondrial function in PBMC have never been studied in any acute disease state. However, PBMC MF has been studied in severe sepsis and septic shock and at one (242), (125) or two (305) time points early on in the disease state. Endogenous PBMC respiration was higher in patients with sepsis in one study (242), which is consistent with our findings for rodents and patients with mild AP (Figure 7-1A and Figure 7-2A). This elevated respiration flux is thought to be due to elevated ATP demands in the activated state (306). Other reported findings in sepsis and septic shock include decreased respiration after stimulation with ADP. This suggests decreased

phosphorylation efficiency (242, 305) due to an increased relative contribution of the leak respiration after adding oligomycin when septic plasma was incubated with healthy cells (242). In addition, a 22% decrease in CI stimulated oxygen consumption relative to healthy volunteers has been reported (125). Taken together these various changes in MR were thought to reflect mitochondrial dysfunction (99).

Consistent in part with previous studies of sepsis (242) which measured leak respiration by adding oligomycin to PBMC from HV incubated with septic plasma, this present study also showed an increased leak respiration which infers to uncoupling of OXPHOS from phosphorylation in mitochondria in mild AP. None of these previous studies (242),(305), (125) measured combined respiration flux after substrates for both CI and CII (CI,II OXPHOS) but found decreased stimulation of flux after adding ADP with either no exogenous substrates (242) or substrates for CI or CII separately (305), (125) . It is known that combining substrates as done in the present study better reflects respiration in vivo (96). However, in contrast to sepsis, patients with mild AP showed an increased respiration after stimulation with ADP (CI,II OXPHOS, Figure 7-2B) and no significant change in the respiration flux through CI (%CI ETS, Figure 7-6) or ATP (Figure 7-9) at d1 compared to HV. This suggests that the elevated CI,II OXPHOS in AP PBMC may act to maintain the cell in net ATP balance in the face of elevated cellular ATP demands. The increase in CI,II OXPHOS with no increase in %CI ETS at d1 is suggestive of enhanced flux through CII. CI and CII electron inputs contribute to 10 and 6 proton transfer across the inner mitochondrial membrane respectively. CII inputs therefore have a theoretical 40% lower yield of ATP for a given amount of oxygen (307).

7.5.4 Late compensation by CI in mild AP

For effective compensation it would have been expected to observe an increase in %CI ETS but, there was no compensation by CI which is suggestive of a compromise of CI at least relative to the ETS as one aspect of the mitochondrial dysfunction early in mild AP. This ineffective compensation by CI is manifest by the lowest CI,II OXPHOS/CI,II Leak at day 1 in patients with AP even though this ratio did not reach statistical significance when compared with HV (Figure 7-10). Remarkably, from d2 onwards there is a significant increase in %CI ETS compared to HV and patients at d1 which suggests that compensation by CI occurs from d2 (Figure 7-6).

7.5.5 Improvement of mitochondrial respiration flux control ratios as patients recuperate

The CI,II Leak/ETS and the CI,II OXPHOS/CI,II Leak improved over time and were similar to HV by discharge which suggests an improvement of mitochondrial function as patients recuperated. The dynamic nature of these temporal findings mirrored the clinical state of the patient's condition from admission to discharge. These differences in respiration findings between sepsis and mild AP likely reflect the more benign nature of mild AP mediated inflammation compared to severe sepsis and septic shock.

7.5.6 High mitochondrial membrane potential driving mitochondrial superoxide production

Another notable aspect of mitochondrial dysfunction in mild AP evident in our study is the eight fold increase in mitochondrial superoxide in PBMC from AP patients early on in the disease at d1 (Figure 7-7). Reactive oxygen species are known to be intimately involved in the pathophysiology of AP and markers of oxidative stress correlate with the severity of AP (308). Previously superoxide radicals were reported to be increased in blood from patients with AP and correlated with disease severity (309). While mitochondrial ROS production is disputed in health (310), in pathological states mitochondria can be significant producers of ROS (308). A high mitochondrial membrane potential compared to HV as seen in this study by the increase in aggregate to monomer ratio (Figure 7-8) can also lead to increased ROS by strongly stimulating reverse electron transfer from CII to CI via ubiquinone (141). From d2 onwards there was a decrease in mitochondrial superoxide (Figure 7-7) which only became statistically significant at discharge compared to d1 and this decrease in mitochondrial superoxide coincided with the more effective compensation by CI as reflected by the increase in %CI ETS from d2 (Figure 7-6) and an improved CI,II OXPHOS/CI,II Leak (Figure 7-10) by discharge.

However, mitochondrial superoxide and the mitochondrial membrane potential still remained elevated at discharge compared to HV (Figure 7-7 and Figure 7-8). Whilst mitochondria are producers of ROS in pathological states, they can also be the target of ROS damage. When pancreatic acinar cells were exposed to oxygen radical generating systems, the organelles most susceptible to oxidative damage were the mitochondria(311). In addition, superoxide increases proton conductance in mitochondria by interacting with uncoupling proteins 1, 2 and 3 (312) and indeed, we observed an increased proton leak at d1 and an increase in CI,II Leak/ETS at d1 in patients with AP in this study.

7.5.7 Decreased cytochrome *c*

The decrease in cytochrome *c* in patients with AP may be due to several reasons. It may be due to release of cytochrome *c* from PBMC mitochondria into the serum or it may reflect a decrease in mitochondrial content in mild AP. Cytochrome c is a mitochondrial marker (307, 313) and in sepsis mitochondrial content is decreased (314). In AP it is known that pancreatic acinar cells release cytochrome c (315) and it has been shown that ROS play a key role in releasing cytochrome c from mitochondria (316) and to scavenge ROS (317).

7.5.8 Decreased ATP at discharge

The ATP increased early on in AP with significantly increased ATP at d2 compared to HV, which coincided with the compensation by CI and a decreased ROS. However, at d3 and discharge there was a slight but significant decrease in ATP compared to healthy volunteers and this suggests either a decreased supply of ATP which does not appear to be the case as the CI,II OXPHOS/CI,II Leak and CI,II Leak/ETS ratios have improved by discharge or an increased demand of ATP which is most likely the case given that the endogenous respiration is still slightly but not statistically significantly increased at discharge. The decrease in ATP and the increased mitochondrial superoxide at discharge are both markers of ongoing mitochondrial dysfunction.

7.5.9 Blood mitochondrial function compared to organ mitochondrial function in AP

Although MF has not been measured in peripheral blood or organs in patients with AP, MF has been measured in the pancreas and extra-pancreatic organs in experimental studies in AP. A high CI,II OXPHOS was also found in isolated pancreatic mitochondria at 2 hours following injection with caerulein (318). Leak respiration measured following oligomycin, increased by around 66% in pancreatic acinar cells in another caerulein model of AP (319). The findings of these studies are consistent with findings from peripheral blood in the present experimental and clinical studies. Another study found a decrease in respiration flux attributable to CI in the pancreas and jejunum in caerulein induced AP (254). In the present study, even though we did not see an outright decrease in CI in peripheral PBMC, it was noted that CI did not participate in the compensatory increase of respiratory flux until later in the disease course (Figure 7-6).

7.5.10 Limitations

Not all of the findings were identical between the experimental study and the clinical study. In the experimental study, there was an increase in ETS and flux through CIV (Figure 4.2D, 4.2E) but not in the clinical study. Aside from the differences between species, this may also be because the timing of blood taking in the experimental study was exactly 6 hrs after the first injection of caerulein and reflected an early time point in the disease course, whereas in the clinical study the median duration of symptoms prior to admission for the patients was 11 hrs (range 1-96 hrs) and this more heterogenous and later blood sampling may account for some of this difference. This later blood sampling may also be the reason for a significantly increased CI,II Leak/ETS in patients with AP compared to HV at day 1 (Figure 7-5) whereas in the experimental study there was no statistically significant difference in the CI,II Leak/ETS (Figure 7-1F) at 6 hrs from induction of AP between controls and rats with AP, which points to a comparatively more efficient ETS in the experimental study.

Only two patients progressed in severity and two patients were readmitted to hospital and therefore no statistical analysis was possible to investigate mitochondrial function differences between these groups and patients with mild AP without complications. This will need to be a topic for future studies.

Although it might be considered a limiting factor in the present study, monocytes and lymphocyte groups were not separated to measure MF because cell sorting may have disrupted the cell to cell interaction necessary for activation of lymphocytes (147). In addition sorting cells into separate groups would have increased the time before measuring MF. This is important in seeking to develop a practical and repeatable measure of MF that may be used to assess the clinical trajectory of patients and response to treatments.

7.6 Conclusion

This is the first study that has measured mitochondrial function in PBMC in mild AP, in both experimental and clinical studies. It shows that there is altered MF early in experimental mild AP. In the clinical study there are similar changes, but in addition some evidence of mitochondrial dysfunction with increased proton leak, increased CI,II Leak/ETS, increased superoxide release, and decreased cytochrome c in patients with AP. There appears to be a compensatory increase in specific mitochondrial respiration states for the cell to maintain ATP concentrations early on at d1 in AP. Temporally, there is an improvement of MR with an improved %CI ETS, CI,II OXPHOS/CI,II Leak and CI,II Leak/ETS but dysfunction lingers

with increased mitochondrial superoxide compared to HV at discharge. With a peripheral marker of mitochondrial function it is now possible to extend these studies to determine whether changes in mitochondrial function predicts the onset of organ failure in severe and critical AP, correlates with disease severity, and responses to fluid resuscitation and other interventions.

This chapter of the thesis translated the mitochondrial respiration protocol to an acute disease setting of mild acute pancreatitis. The pattern of mitochondrial function in early inflammation as well as in the recovery phase was established. In the next chapter, the central hypotheses of this thesis will be directly examined by measuring peripheral blood mitochondrial function in critically ill patients with MODS.

Chapter 8 Peripheral Blood Mononuclear Cell Mitochondrial Function in Multiple Organ Dysfunction Syndrome

8.1 Introduction

In Chapters 6 and 7, mitochondrial function from peripheral blood was successfully measured and described in a chronic disease state and in a mild acute inflammatory disease. In this chapter, the central hypotheses of this thesis will be tested by evaluating peripheral mitochondrial function in patients with MODS.

8.1.1 MODS and mitochondrial function

The relevant literature was described in Chapter 2. In brief, mitochondrial abnormalities were found in sepsis as far back as 30 years ago (116). The long term (3 days) experimental models mainly showed a decrease in mitochondrial function. In these long term experimental models of septic shock and organ failure, mitochondrial respiration falls and ATP levels decrease (117). Since ATP is the rate limiting step of cellular metabolism, without sufficient ATP production cellular homeostasis is threatened and this may be observed clinically as MODS.

Human data of mitochondrial respiratory function is relatively sparse in MODS and was summarised in Chapter 2. In summary, these studies have measured mitochondrial respiration in muscle biopsies, organ biopsies, platelets and peripheral blood mononuclear cells in patients with sepsis and/or septic shock (Table 2-2) (25, 118, 119). In studies involving muscle and organ biopsies, only one time point was studied because taking biopsies is an invasive procedure in already compromised patients. Mitochondrial dysfunction was found in leg muscle biopsies (25), liver (119) and intercostal muscle (118) in septic organ failure and was found to correlate with the amount of norepinephrine required in critically ill patients. Decreased activity in Complex I was found in both skeletal muscle and liver, and a decreased CIV activity was also found in the liver (119). Platelet mitochondria have been reported to uncouple in sepsis (126). More recently, decreased State 3 mitochondrial respiration function has been described in PBMC within 48 hrs of ICU admission in patients with septic shock (242). Since undertaking the studies in this thesis, further two studies have reported PBMC respiration in sepsis/septic shock and found decreased State 3 mitochondrial respiration with the CII substrate succinate in septic shock (124), and decreased endogenous respiration and State 2 respiration with CI along with decreased CI, CIII and CIV activity in sepsis with no decrease in mitochondrial content as measured with citrate synthase (125). Although

informative, these studies have left several aspects of mitochondrial function in MODS unanswered.

8.1.2 Gap in knowledge

Firstly, these studies all focused on sepsis rather than MODS as an entity and therefore the inclusion criteria was either with reference to diagnosis of sepsis or the time of admission to ICU. The time at which organ dysfunction was first noted or the degree of organ dysfunction was not part of the inclusion criteria in these studies. Secondly, all of these studies with the exception of Japiassu and Sjovall, measured mitochondrial function at one time point. Jaipassu reported on one aspect of mitochondrial function: respiratory control ratio at day 7 and Sjovall measured mitochondrial function in platelets three times during the first week of sepsis and was the most comprehensive report thus far of mitochondrial respiration. Since sepsis and septic shock is usually accompanied with evolving organ dysfunction, repeated measures of mitochondrial function are necessary in order to gain an understanding as the clinical situation changes. Thirdly, with the exception of Sjovall, all the other studies used traditional Clark type electrodes to measure mitochondrial respiration and as such only partial mitochondrial respiration either with CI or with CII have been described in these studies. Clark type electrodes have a number of disadvantages over high resolution oxygraphy including the requirement for large amounts of samples which is a significant barrier in clinical studies, as discussed in Chapter 3. Fourth, none of the studies measured mitochondrial ROS directly and regulating ROS is an important mitochondrial function. Therefore, whether mitochondrial ROS and mitochondrial respiration function has an association is not currently known. Last, mitochondrial function has never been described in MODS unrelated to sepsis even though severely injured trauma patients with MODS display indirect evidence of mitochondrial oxidative dysfunction (320). By addressing these gaps in knowledge, the central hypotheses of this thesis will be tested.

8.1.3 Hypotheses

This chapter reports a clinical study that tests the central hypothesis that mitochondrial dysfunction occur in PBMC of patients with MODS and correlates with severity of MODS. Further, that temporal mitochondrial respiration negatively correlates with temporal organ failure scores, mitochondrial respiration correlate with mitochondrial ROS and that it does not discriminate between septic and non-septic causes of MODS.

8.1.4 Aims

This clinical study had two aims:

Measure PBMC mitochondrial function in patients with MODS during the first week of admission to ICU and determine whether it correlates with scores of the severity of organ failure scores and with mortality.

Determine whether PBMC mitochondrial function differs in those with septic or non-septic causes of MODS

8.2 Method

8.2.1 Clinical study

The clinical study was described in section 5.4.2.

8.2.2 Preparation of blood mononuclear cells

20 mls of blood was taken six times over a period of 1 week. The time points for blood taking were daily for the first 4 days (1d, 2d, 3d, 4d), once either on the 5^{th} or 6^{th} day (5/6d) and at 1 week (1w). Blood was taken once from healthy volunteers.

Blood was collected as described in section 5.1.1 and peripheral blood mononuclear cells (PBMC) were retrieved as described in section 0.

8.2.3 Respiration assays - Assays of the mitochondrial electron transport system

A multiple substrate-inhibitor titration protocol that was developed and described in section 5.1.4 and was tested in a chronic experimental model of hypertension and acute experimental model and clinically in acute pancreatitis (Chapter 6 and Chapter 7) was employed.

8.2.4 Assessment of mitochondrial DNA

Mitochondrial DNA was quantified using real-time PCR as described in section 5.2.1

8.2.5 Measurement of superoxide using fluorescent dye MitoSOX red

MitoSOX red (Invitrogen, Auckland, NZ), a mitochondrial targeted superoxide indicator (20) was used to detect superoxide in PBMC as described in section 5.2.2.

8.2.6 Measurement of mitochondrial membrane potential using JC-1 using flow cytometry

Mitochondrial membrane potential ($\Delta\Psi$) was measured in PBMC as described in section 5.2.3.

8.2.7 Assessment of intracellular ATP

To determine whether ATP fluctuated over the disease state, intracellular ATP was measured as described in section 5.2.4.

8.2.8 Intracellular protein

To normalise the ATP measurements, intracellular protein was measured as described in section 5.2.5.

8.2.9 Cytochrome c

Cytochrome *c* was measured was used as a marker of mitochondrial content and measured as described in section 5.2.6.

8.2.10 Statistical analysis

Statistical analysis was carried out using Graph Pad Prism version 4.00 and SPSS version 18 for Windows (GraphPad Software , San Diego, California, USA). Values are expressed as the median and interquartile range or mean ± standard error of mean (SEM) where appropriate. For temporal mitochondrial function, repeated measures of analysis of variance (RANOVA) with a post-hoc least significant difference (LSD) correction was used to evaluate statistical significance from day 1 to discharge. A student *t*-test was used to detect statistical significance between HV and each day of mitochondrial function in the patient group followed by a sequential Bonferroni correction for multiple comparisons. Where the data did not fit normality, the Mann Whitney U test was used when two groups were compared and Kruskal Wallis was used when more than two groups were compared. To analyse cytochrome *c* data within patients at admission and at 6 months, a paired student *t* test was used. Correlation of mitochondrial function with SOFA scores were done with Pearson's correlation. A one way analysis of variance (ANOVA) was used to compare mitochondrial function at day 1 between healthy volunteers, survivors and non-survivors. Significance was established at $P < 0.05$ and is reported as $p<0.05$, $p<0.01$, $p<0.005$ and $p<0.001$ where appropriate.

8.3 Results

8.3.1 Clinical course of MODS in the patient group

There were 27 patients with MODS (OF) and 15 HV recruited for this study. Their baseline characteristics are given in Table 8-1 and there were no statistically significant differences between the groups for age and gender. The HV had no chronic diseases and were on no

regular medications. The median APACHE II score on admission was 24 (13-47) and the SOFA score on admission was 10 (4-15) (Table 8-2) and the median SOFA scores decreased over the week (Table 8-3). The duration of organ failure (in hrs) before the first bloods were taken was 18.5 hours (7-35) and the median number of organs failed was 2 (2-4). 5/27 (18.5%) patients died within three weeks and the mortality rate stayed the same at the 6 month follow up. The median ICU stay in days was 3 (1-100), and the median hospital stay in days was 12 (3-200) (Table 8-2).

Table 8-1 Patient and healthy volunteer demographics.

	HV(15)	**OF(27)**	**P**
Age (years) median (range)	51 (25-64)	59 (19-74)	0.65
Male : female ratio	6:9	16:11	0.24
Ethnicity	NZ European 13 Other 2	NZ European 17 Maori/Pacific 8 Other 2	0.08*

Footnote: Healthy volunteer (HV), Patients with multiple organ dysfunction syndrome (OF), P-value student *t-test* (P), *Ethnicity divided up into NZ European and non NZ European for *t-test*.

Table 8-2 Patient organ failure scores.

	OF (27)
SOFA (first 24 hrs)	10 (3-15)
APACHEII	24 (13-47)
APACHEII Chronic component	0 (0-5)
SAPS	39 (15-98)
Duration of OF in hrs prior to first blood	18.5 (7-35)
No. of failed organs	2 (2-4)
Vasopressor therapy (no of patients)	25
Renal replacement therapy (no of patients)	3
Mechanical ventilation (no of patients)	14
Mortality 1 week	3
Mortality 3 weeks	5

ICU length of stay (days)	3 (1-100)
Hospital length of stay (days)	12 (3-200)

Footnote: Patients with multiple organ dysfunction syndrome (OF), Sequential organ failure assessment (SOFA), Acute Physiology and Chronic Health Evaluation II (APACHE II), Simplified acute physiology score (SAPS).

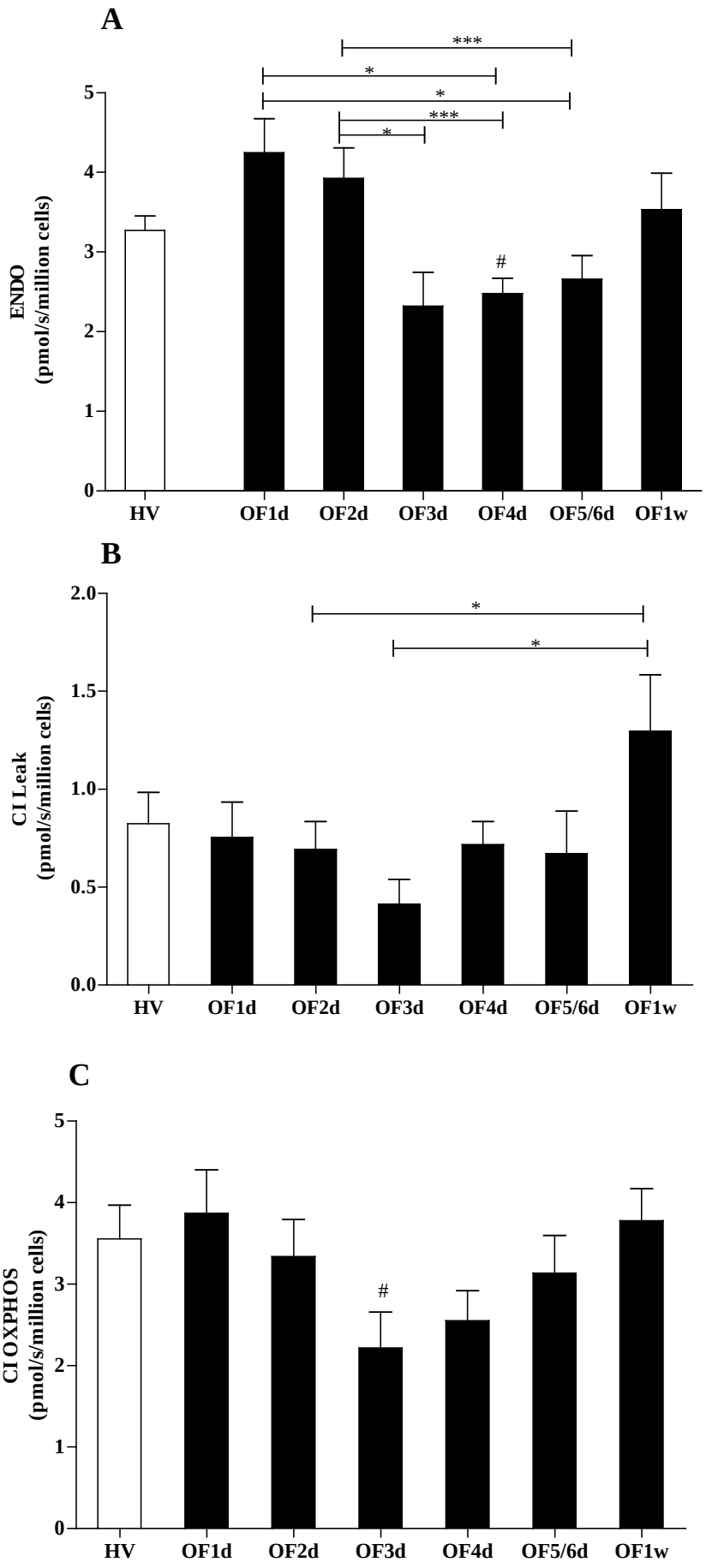
Table 8-3 SOFA scores over time in patients with MODS.

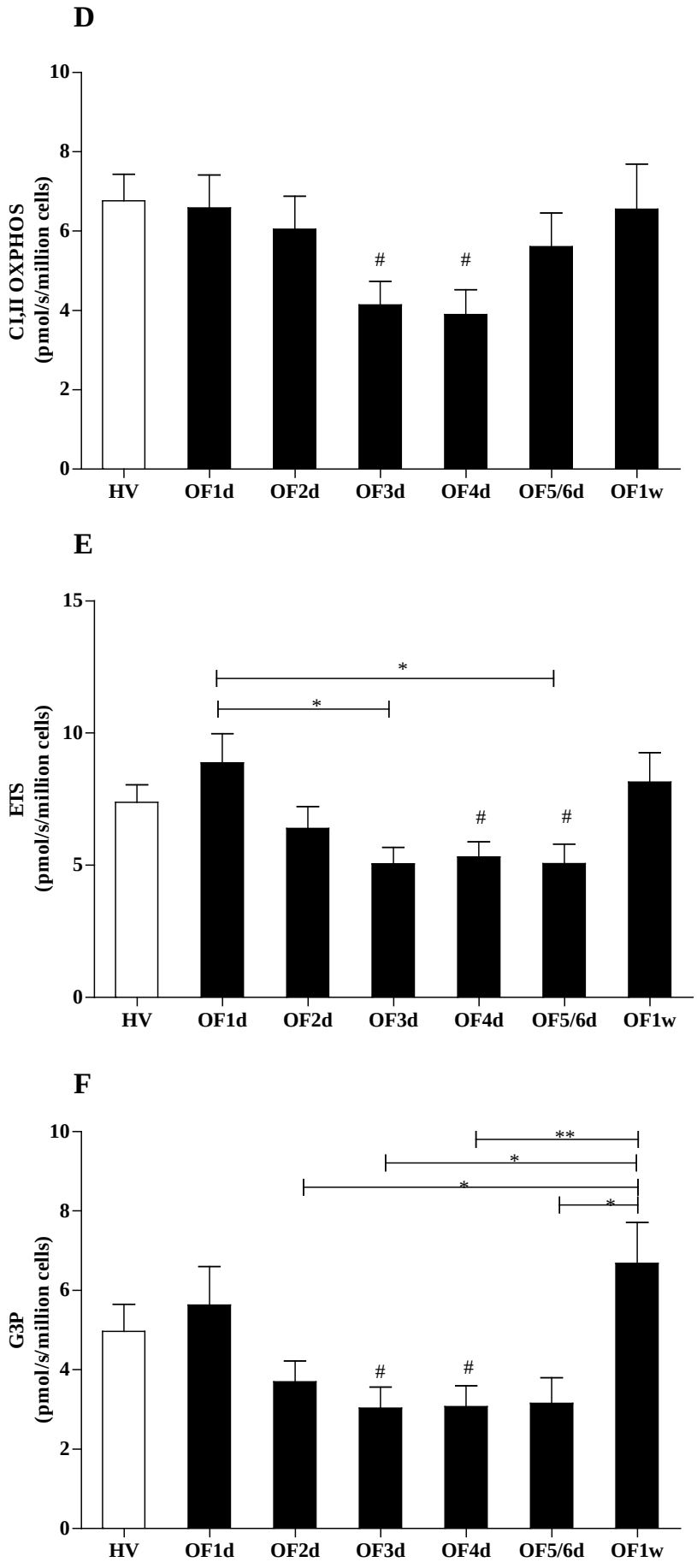
SOFA	OF1d (n=27)	OF2d (n=25)*	OF3d (n=22)#	OF4d (n=22)	OF5/6d (n=22)	OF1w (n=22)
Median	10	8	4	3	2	1
Min	4	1	0	0	0	0
Max	15	18	18	17	15	16
1st quartile	8	4	2	1	0	0
3rd quartile	12	10	10	4	6	3

Footnote: Sequential organ failure assessment (SOFA), Patients with multiple organ dysfunction syndrome (OF), day (d), week (w), number of patients (n). *2 patients died by day 2, #3 patients died by day 3, 1 patient was discharged at day 3 and 1 patient refused further participation at day 3.

8.3.2 Peripheral mitochondrial function in MODS compared to HV

Differences in mitochondrial function parameters in MODS (OF) compared to HV was only apparent from 3rd day on (Figure 8-1A–I). On the 3rd day, CI OXPHOS, CI,II OXPHOS and G3P were significantly decreased by 37% (P=0.043, Figure 8-1C), 39% (P=0.007, Figure 8-1D) and 38% (P=0.03, Figure 8-1F) compared to HV. On the 4th day, CI,II OXPHOS and G3P remained decreased by 42% (P=0.005, Figure 8-1D) and 38% (P=0.03, Figure 9-1F) and endogenous respiration and ETS decreased by 20% (P=0.007, Figure 8-1A) and 28% (P=0.003, Figure 8-1E) respectively. ETS remained decreased by 26% on 5/6d compared to HV (P=0.03, Figure 8-1E). Taken together, there was a generalised decrease in mitochondrial respiration in MODS but not until the 3rd day. Diminished endogenous respiration and electron transport system capacity prevailed at the end of the week (5/6th day).


A
***
*
*
***
*
5
4
3
2
1
0
ENDO
(pmol/s/million cells)
#
HV
OF1d
OF2d
OF3d
OF4d
OF5/6d
OF1w
B
2.0
1.5
1.0
0.5
0.0
*
*
CI Leak
(pmol/s/million cells)
HV
OF1d
OF2d
OF3d
OF4d
OF5/6d
OF1w
C
5
4
3
2
1
0
CI OXPHOS
(pmol/s/million cells)
#
HV
OF1d
OF2d
OF3d
OF4d
OF5/6d
OF1w


D
CI,II OXPHOS (pmol/s/million cells)
10
8
6
4
2
0
#
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HV
OF1d
OF2d
OF3d
OF4d
OF5/6d
OF1w
E
ETS (pmol/s/million cells)
15
10
5
0
*
*
#
#
HV
OF1d
OF2d
OF3d
OF4d
OF5/6d
OF1w
F
G3P (pmol/s/million cells)
10
8
6
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HV
OF1d
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OF5/6d
OF1w

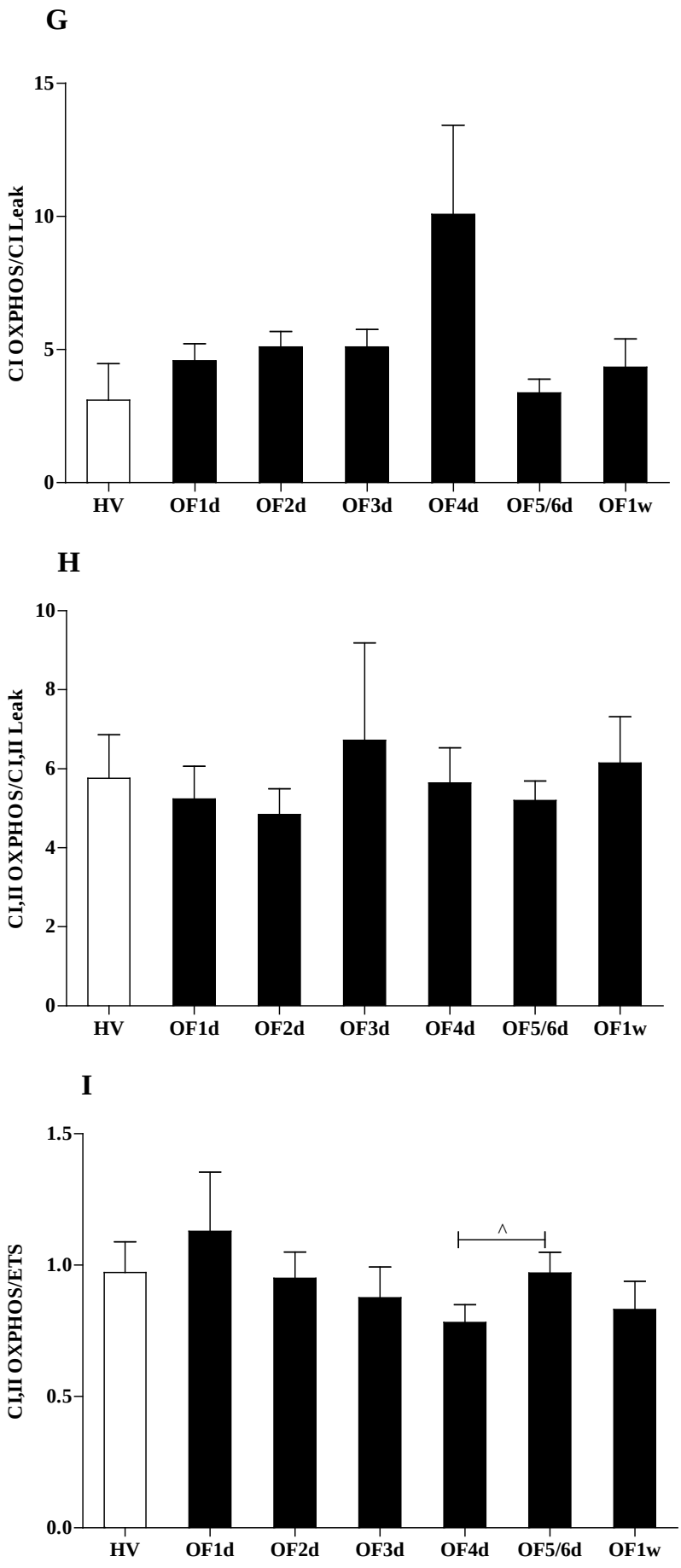

G
CI OXPHOS/CI Leak
15
10
5
0
HV
OF1d
OF2d
OF3d
OF4d
OF5/6d
OF1w
H
CI,II OXPHOS/CI,II Leak
10
8
6
4
2
0
HV
OF1d
OF2d
OF3d
OF4d
OF5/6d
OF1w
I
CI,II OXPHOS/ETS
1.5
1.0
0.5
0.0
^
HV
OF1d
OF2d
OF3d
OF4d
OF5/6d
OF1w

Figure 8-1 A–I Mononuclear cell mitochondrial respiration in patients with MODS. Mononuclear cell mitochondrial respiration and flux control ratios in HV, white bars (n=15) and patients with multiple organ dysfunction at different time points, black bars (n=27 at 1d, n=25 at 2d, n=22 for rest of the days). Different respiratory states measured were: A) Endo [Endogenous respiration], B) CI Leak C) CI OXPHOS [Complex I oxidative phosphorylation] D) CI,II OXPHOS [Complex I and II oxidative phosphorylation], E) ETS [Electron Transport System capacity] and F) G3P [ETS capacity with Glycerol-3-phosphate]. Flux control ratios measured were: G) CI OXPHOS/CI Leak [measure of phosphorylation efficiency with CI substrates], H) CI,II OXPHOS/CI,II Leak [measure of phosphorylation efficiency with CI+II substrates] and I) CI,II OXPHOS/ETS [ratio of phosphorylation to electron transport system capacity].Values are mean ± standard error of mean. #comparison to HV (student's *t* test with sequential *Bonferroni* correction), *comparison within patients at different time points (*RANOVA* with least significant different correction (*LSD*)), ^ comparison within patients at different time points (least significant difference (*LSD*)) #/^/*P<0.05, ##/^^/** P<0.01, ###/^^^/*** P<0.005.

8.3.3 Temporal mitochondrial function changes in MODS

There was a significant difference in endogenous respiration over time within the OF group (P=0.044, d.f.=3.903, *RANOVA*) (Figure 8-1A). Compared to 1d and 2d, there was a decrease in endogenous respiration by 3d (2d vs. 3d P=0.025), 4d (1d vs. 4d P=0.025) and 5/6d (2d vs. 5/6d P=0.004, Figure 8-1A). There was a significant difference in CI Leak over time within the OF group (P=0.002, d.f.=594, *RANOVA*) (Figure 8-1B). CI Leak was increased at 1w compared to days 1-6. Overall ETS changed over time within the OF group (P=0.044, d.f.=3.903, *RANOVA*). ETS capacity was decreased by 3d compared to 1d (1d vs. 3d P=0.042) and recovered by 1w (Figure 8-1E). There was an overall difference in G3P over time within the OF group (P=0.043, d.f.=2.497, *RANOVA*, Figure 8-1F). G3P recovered at 1w (2d vs. 1w P=0.041, 3d vs. 1w P=0.012, 4d vs. 1w P=0.006, 5/6d vs. 1w P=0.014). Taken together, temporal changes of mitochondrial respiration in MODS can be likened to a "U" shape pattern where the trough was at day 3 and day 4. At the end of the first week, there were no significant differences in mitochondrial respiration states compared to the beginning of the week.

With regard to flux control ratios, there were no overall temporal changes in CI OXPHOS/CI Leak, CI,II OXPHOS/CI,II Leak with RANOVA (Figure 8-1G-I). Although not significant on RANOVA, CI,II OXPHOS/ETS troughed at 4d and was significantly increased at 5/6d (4d vs. 5/6d P=0.019 *pairwise LSD*, Figure 8-1I). The decrease in CI,II OXPHOS/ETS at day 4 is evidence that mitochondrial OXPHOS was being limited by the phosphorylation system and the subsequent increase at day 5/6 is evidence of some recovery of the phosphorylation system.

8.3.4 Correlation of mitochondrial function with SOFA in MODS

Temporal mitochondrial function correlated with temporal SOFA scores and the majority of the correlations occurred from day 3 (3d) onwards (Table 8-4). 1d SOFA correlated with 1d

CI OXPHOS/CI,II OXPHOS (Pearsons r=-0.457, P=0.025) and most likely indicates that patients with higher SOFA scores have poor CI OXPHOS function early on in the disease state. By day 3, multiple mitochondrial respiration parameters correlated with SOFA. The 3d SOFA negatively correlated with 3d endogenous respiration (r=-0.542, P=0.03), 3d CI OXPHOS (r=-0.651, P=0.006), 3d CI,II OXPHOS (r=-0.705, P=0.002) and 3d ETS (r=-0.693, P=0.003). 4d SOFA correlated negatively with 4d ETS (r=-0.515, P=0.029). 5d SOFA correlated with 5d CI OXPHOS (r=-0.601, P=0.023), 5d ETS (r=-0.593, P=0.025) and G3P/S (r=0.567, P=0.043). 6d SOFA correlated with 6d CII OXPHOS/CI,II OXPHOS (r=-0.71, P=0.022) and 6d CI OXPHOS/CI Leak (r=-0.633, P=0.049). 7d SOFA correlated with 7d CI OXPHOS (r=-0.660, P=0.038). Overall, the majority of correlations between SOFA and mitochondrial respiration occurred from 3d onwards.

Table 8-4 Correlation of daily mitochondrial respiration parameters with daily sequential organ failure assessment (SOFA) scores.

Respiration state	SOFA1d	SOFA2d	SOFA3d	SOFA4d	SOFA5d	SOFA6d	SOFA1w
1d (n=27)							
Endo							
CI Leak							
CI OXPHOS							
CI,II OXPHOS							
CI,II Leak							
ETS							
G3P							*
2d (n=25)							
Endo			*				
CI Leak			*				
CI OXPHOS							
CI,II OXPHOS							
CI,II Leak			*				
ETS							
G3P	*						
3d (n=22)							
Endo			*		*		
CI Leak							
CI OXPHOS			**	*	*	*	
CI,II OXPHOS			***		**	*	
CI,II Leak							
ETS			***	*	***	*	
G3P							
4d (n=22)							
Endo			*		**		
CI Leak			*				
CI OXPHOS		*	*		*		
CI,II OXPHOS		*	*		*		
CI,II Leak			*		**		
ETS			***	*	***	*	*
G3P	*	*	*		*		
5/6d (n=22)							
Endo							
CI Leak							
CI OXPHOS			*		*		
CI,II OXPHOS			*				
CI,II Leak					*		
ETS					*		
G3P							
1w (n=22)							
Endo							
CI Leak	***	*	*	*		*	
CI OXPHOS							
CI,II OXPHOS							
CI,II Leak							
ETS							
G3P							

Footnotes CI [Complex I], CI,II[Complex I and II], OXPHOS [oxidative phosphorylation], ETS [Electron Transport System],G3P[glycerol-3-phosphate]. Pearson's correlation coefficients colour coded: -0.199 - 0.199 ≤ -0.2, ≥ 0.2 ≤ -0.3, ≥ 0.3 ≤ -0.4, ≥ 0.4 ≤ -0.5, ≥ 0.5 ≤ -0.6, ≥ 0.6 ≤ -0.7, ≥ 0.7 ≤ -0.8, ≥ 0.8 ≤-0.9, ≥ 0.9 *P<0.05, **P<0.01, ***P<0.005

8.3.5 Other mitochondrial function in MODS

8.3.5.1 Cytochrome c

Cytochrome *c* was decreased by 61% at OF1/2d (P=0.0002) compared to HV (Figure 8-2A). This decrease in cytochrome *c* from mitochondria most likely represents permeabilisation of the outer membrane of the mitochondria (321).

8.3.5.2 ATP

In OF, net ATP was decreased by 41% at 4d (P=0.0085) and further decreased by 65% at 1w (P<0.0001) compared to HV (Figure 8-2B). At day 4, the decrease in net ATP co-occurred with a decrease in CI,II OXPHOS and ETS and therefore most likely represents decreased production of ATP. The cellular demand for ATP during the first week of MODS is significantly raised and therefore the decrease in net ATP at day 4 and at 1week also represent the increased consumption of ATP by cellular processes.

8.3.5.3 Mitochondrial superoxide

Compared to HV, mitochondrial superoxide was increased by 71% at 1d (P=0.004) and by 98% at 1w (P=0.003) in patients with MODS (Figure 8-2C). The rise in mitochondrial ROS was significant right from the outset of MODS and mitochondrial ROS was decreased when mitochondrial respiration decreased at day 3/day 4. It is noteworthy, that as mitochondrial respiration recovers at the end of the week, mitochondrial ROS increased again.

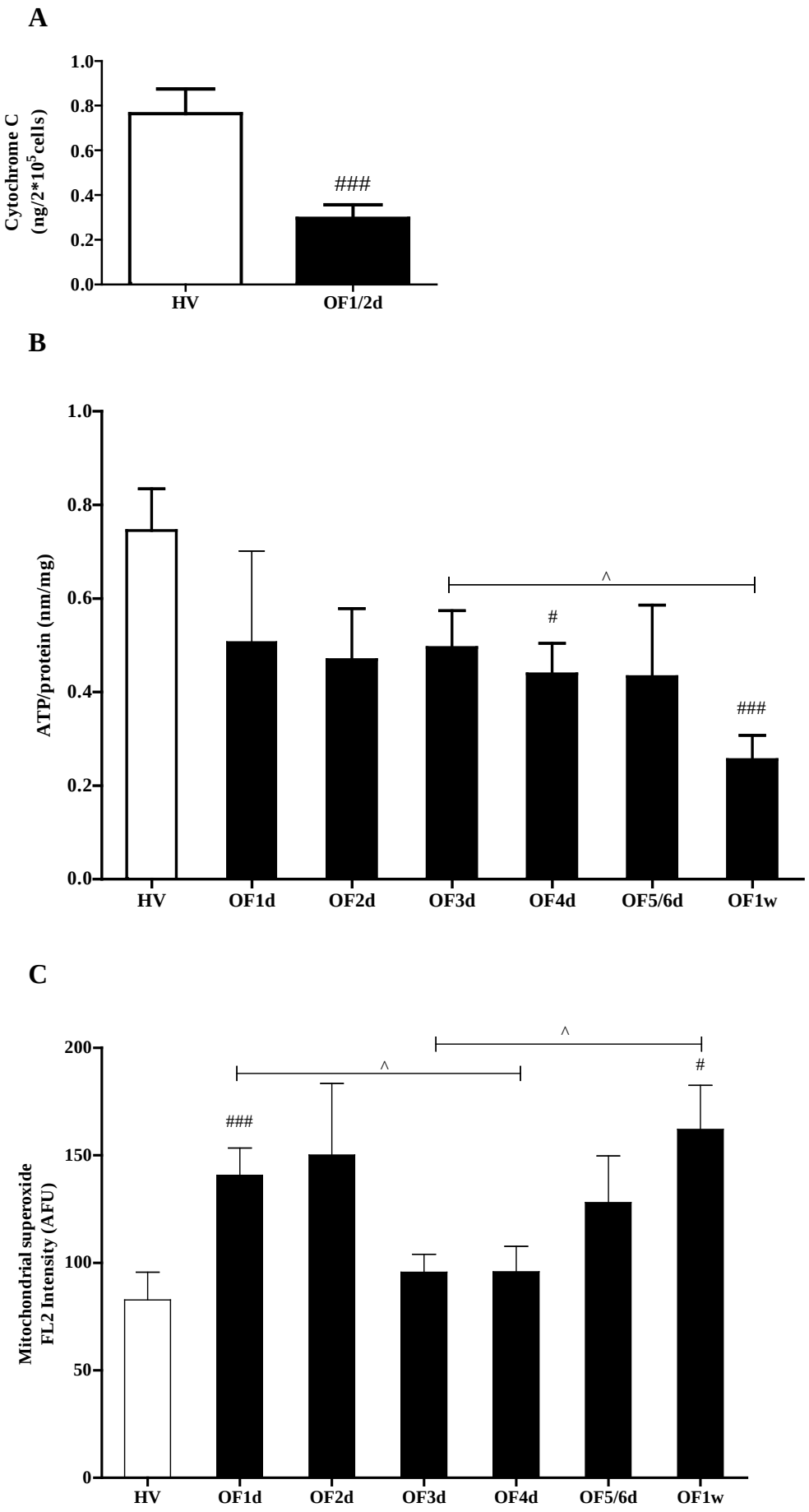
8.3.5.4 Mitochondrial membrane potential

Compared to HV, the aggregate monomer ratio was increased by 73% at 2d (P=0.007) and by 83% at 3d (P=0.027, MWU) in OF (Figure 8-2D). In MODS, there were significant differences in the aggregate monomer ratio within patients over time (P= 0.010, RANOVA). During the first week, the aggregate monomer ratio peaked on the 3d then decreased on the 4d and was followed by an increase within patients (Figure 8-2D). By and large, the mitochondrial membrane potential and mitochondrial superoxide follow each other's trajectory in MODS.

8.3.5.5 Mitochondrial DNA

To understand whether the altered mitochondrial respiration can be explained by a change in quantity of mitochondria, mitochondrial DNA was measured (299). There was no difference in the quantity of mitochondrial DNA normalised to a nuclear gene between OF and HV

(P=0.71, *RANOVA* Figure 8-2E) indicating that the altered respiration findings are independent of mitochondrial quantity.


A
Cytochrome C
(ng/2*10^5 cells)
1.0
0.8
0.6
0.4
0.2
0.0
###
HV
OF1/2d
B
ATP/protein (nm/mg)
1.0
0.8
0.6
0.4
0.2
0.0
^
#
###
HV
OF1d
OF2d
OF3d
OF4d
OF5/6d
OF1w
C
Mitochondrial superoxide
FL2 Intensity (AFU)
200
150
100
50
0
^
^
###
#
HV
OF1d
OF2d
OF3d
OF4d
OF5/6d
OF1w

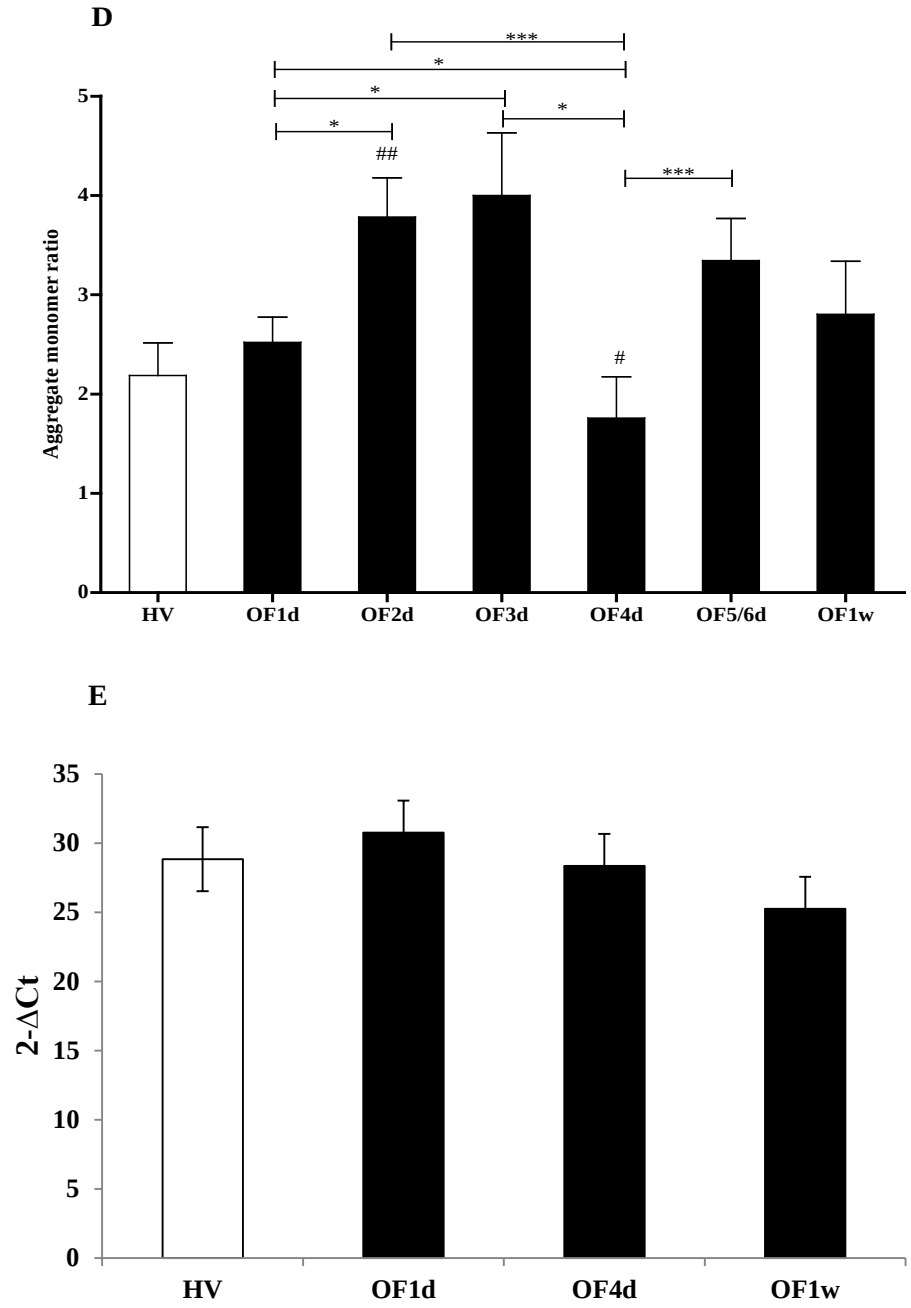
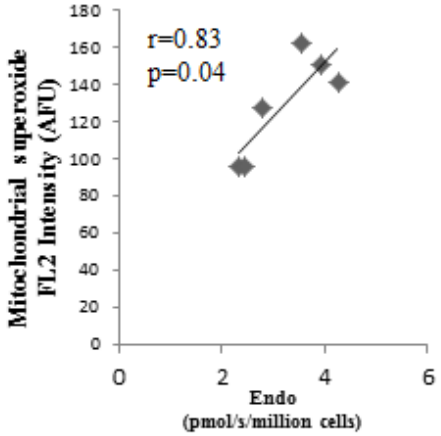
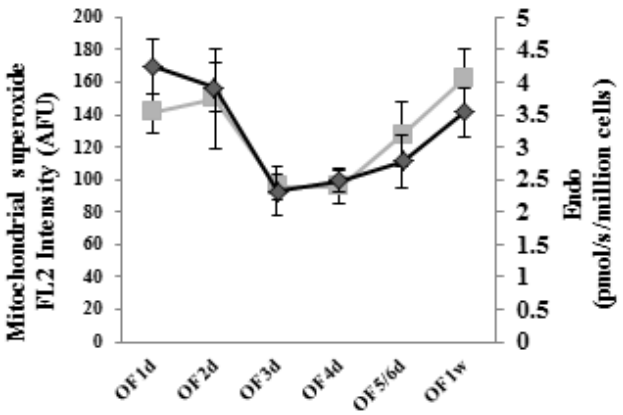


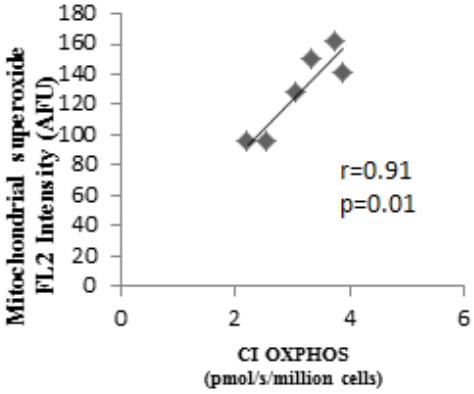
Figure 8-2 A-E. Other mitochondrial function in patients with MODS (black bars, n=27 at 1d, n=25 at 2d, n=22 from 3d onwards) compared to healthy volunteers (white bars, n=15) at different time points**.** A) Mononuclear cell Cytochrome C B) Mononuclear cell adenine triphosphate (ATP) to protein ratio C) Mononuclear cell mitochondrial superoxide and D) Mononuclear cell mitochondrial membrane potential measured with the dye 5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolylcarbocyanine iodide (JC-1). JC-1 fluorescence was detected by flow cytometry in two channels FL 1 and FL 2. JC-1 exists as a monomeric form which fluoresces at FL 1 and when concentrated by actively respiring mitochondria exists as an aggregate which fluoresces at FL 2. An increase in the ratio of aggregate to monomer suggests higher mitochondrial membrane potential which in turn generates increased mitochondrial reactive oxygen species such as mitochondrial superoxide. E) Relative quantification of mtDNA using $2^{-\Delta Ct}$. The mitochondrial gene MTCO1 (0188166_m1) and the nuclear gene RN18S1 (03928985_s1) was used as the internal control gene. Values are mean ± standard error of mean. #comparison to HV (student's *t* test), *comparison within patients at different time points (paired student's *t* test). #/* P<0.05; ##/** P<0.01; ###/***P<0.005.

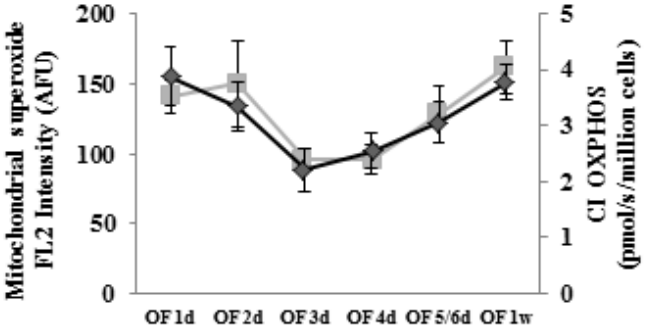
8.3.6 Relationship between mitochondrial respiration and mitochondrial superoxide over the course of the week

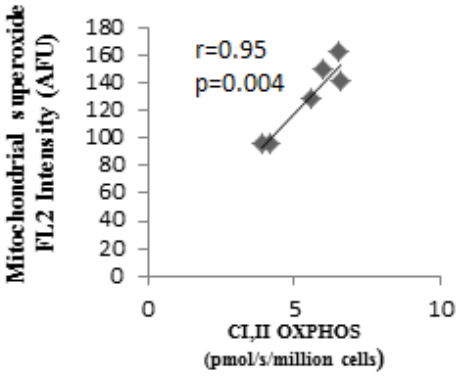
Trajectories of mitochondrial respiration states and mitochondrial superoxide closely followed each other over the course of the week in MODS. When mitochondrial respiration states and mitochondrial superoxide were plotted in the same graph over time, it became apparent that the trajectories in these variables were very similar (Figure 8-3 B, D, F, H and J). The average mitochondrial superoxide and mitochondrial respiration from each day was then correlated over the week (Figure 8-3 A, C, E, G and I). There were significant correlations between mitochondrial superoxide and the mitochondrial respiration states Endo, CI OXPHOS and CI,II OXPHOS ($P<0.05$).

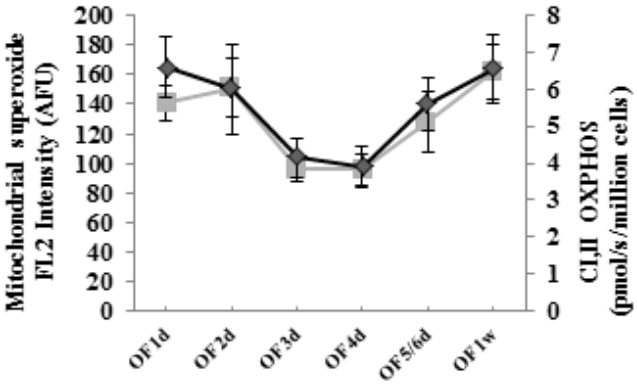

r=0.83
p=0.04
Mitochondrial superoxide FL2 Intensity (AFU)
Endo (pmol/s/million cells)


Mitochondrial superoxide FL2 Intensity (AFU)
Endo (pmol/s/million cells)
OF1d
OF2d
OF3d
OF4d
OF5/6d
OF1w


r=0.91
p=0.01
Mitochondrial superoxide FL2 Intensity (AFU)
CI OXPHOS (pmol/s/million cells)


Mitochondrial superoxide FL2 Intensity (AFU)
CI OXPHOS (pmol/s/million cells)
OF1d
OF2d
OF3d
OF4d
OF5/6d
OF1w


r=0.95
p=0.004
Mitochondrial superoxide FL2 Intensity (AFU)
CI,II OXPHOS (pmol/s/million cells)


Mitochondrial superoxide FL2 Intensity (AFU)
CI,II OXPHOS (pmol/s/million cells)
OF1d
OF2d
OF3d
OF4d
OF5/6d
OF1w

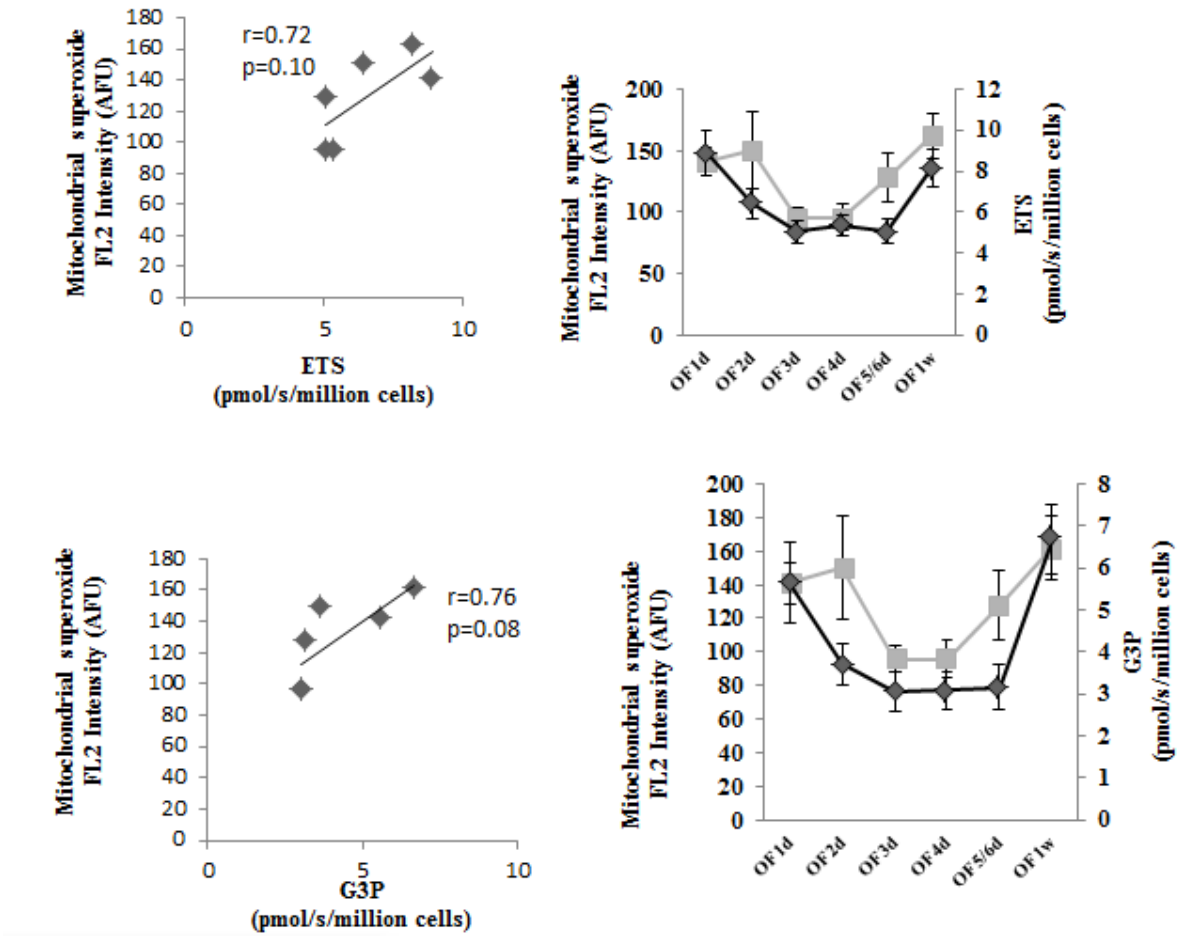


Figure 8-3A-J. Temporal relationship between mitochondrial respiration states and mitochondrial superoxide in MODS. The mitochondrial respiration states are A,B) Endo [Endogenous respiration], C,D) CI OXPHOS [Complex I oxidative phosphorylation], E,F) CI,II OXPHOS [Complex I and II oxidative phosphorylation], G,H) ETS [Electron Transport System capacity] and I,J) G3P [ETS capacity with Glycerol-3-phosphate]. In figures B, D, F, H and J mitochondrial superoxide (left y-axis) and mitochondrial respiration states (right y-axis) were plotted over time (x-axis) in MODS. In figures A, C, E, G and I the average mitochondrial superoxide and mitochondrial respiration from each day were correlated over the week; r=Pearson's correlation co-efficient, p= 2 tailed p value. There were significant correlation between mitochondrial superoxide and the mitochondrial respiration states Endo, CI OXPHOS and CI,II OXPHOS.

8.3.7 Mitochondrial function in non survivors

Mitochondrial function measured at day 1 was compared between survivors (n=22) and eventual non-survivors (n=5) and healthy volunteers. Nonsurvivors utilised double the G3P as a fuel over substrates for CI and CII compared to survivors (Figure 8-4A). ATP was decreased by 32% in non survivors (0.39+/-0.07 nM/mg) compared to survivors (0.57+/-0.27 nM/mg) and by 48% compared to HV (0.75+/-0.09 nM/mg) (P=0.002, *KW*) (Figure 8-4B). Mitochondrial superoxide was increased by 132% in non survivors (190+/-26.56 AFU, P=0.004, d.f.=33) compared to HV (82.69+/-12.94 AFU) (P=0.004) (Figure 8-4C). Even though the difference was not statistically significant, aggregate monomer ratio with JC-1 was 18% lower in the non survivors (1.79+/-0.39, P=0.423, d.f.=33) compared to HV (2.19+/-

0.33) (Figure 8-4D). Cytochrome *c* content was elevated by 240% in non survivors (0.75+/-0.31 ng/2*10^5 cells) compared to survivors (0.22+/-0.03 ng/2*10^5 cells, P<0.0001, d.f.=40) (Figure 8-4E). These findings are consistent with mitochondrial dysfunction in non survivors of MODS.

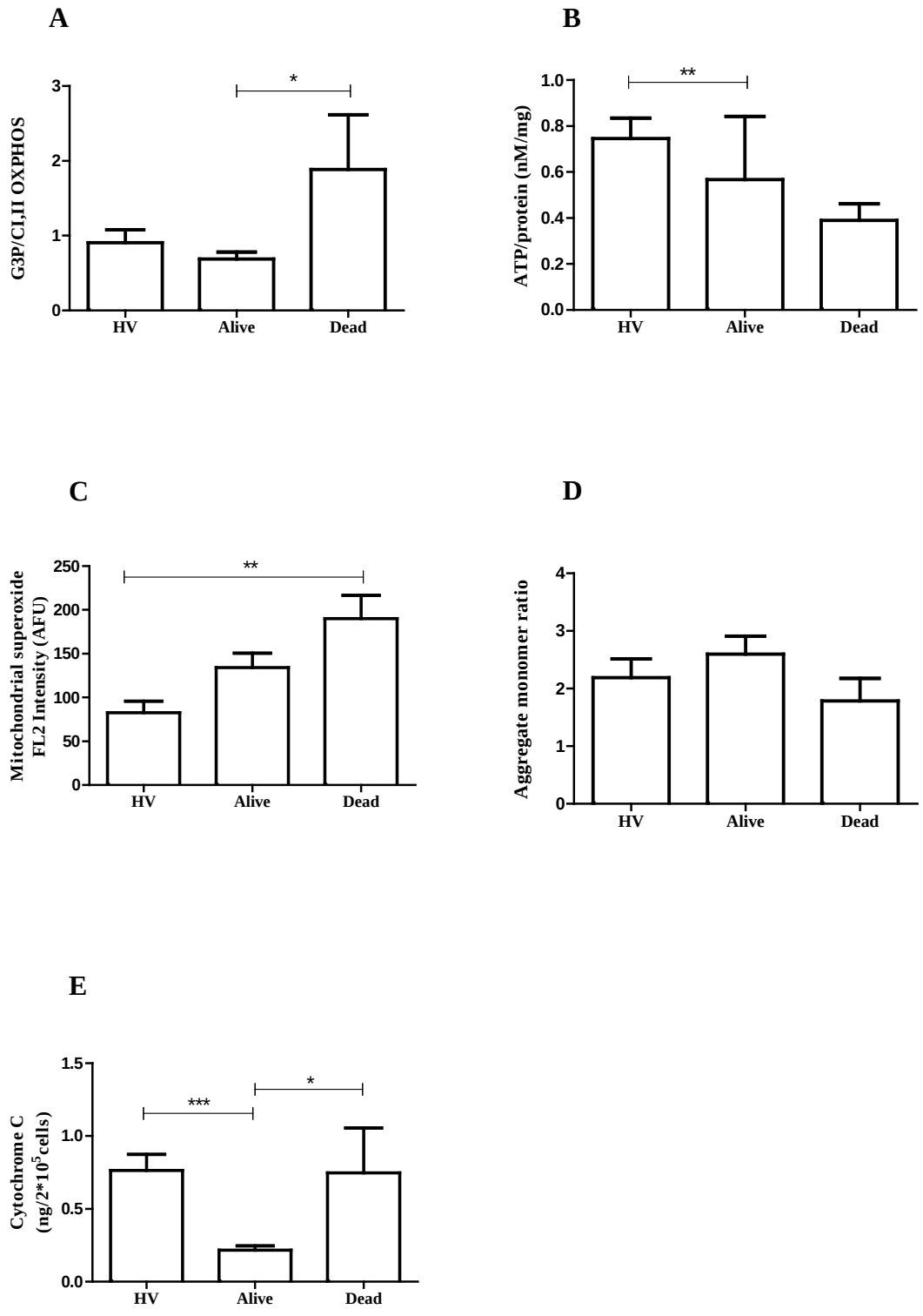


Figure 8-4 Mitochondrial function in survivors, non-survivors and healthy volunteers. Day 1 mitochondrial function was compared between patients who survived (Alive, n=22) and eventual non

survivors (Dead, n=5) and healthy volunteers (HV, n=15). A) Ratio of electron transport system capacity with glycerol-3-phosphate and oxidative phosphorylation with CI+II substrates, B) Mononuclear cell adenine triphosphate (ATP) to protein ratio, C) Mononuclear cell mitochondrial superoxide D) Mononuclear mitochondrial membrane potential and E) Mononuclear cytochrome c content. Comparison between groups (One way ANOVA with Tukey's correction), *P<0.05, **P<0.01, ***P<0.005.

8.3.8 Interim conclusion

Mitochondrial dysfunction occurred in MODS and is dynamic in nature. Severity of organ failure as measured by SOFA inversely correlated with mitochondrial respiration states from day 3. Increase in mitochondrial superoxide occurred first and was followed by a decrease in mitochondrial respiration later in the disease course. There were significant correlations between mitochondrial respiration and mitochondrial superoxide over time. Non survivors had several key differences in early (day 1) mitochondrial respiration. In non survivors, the electron transport system capacity with G3P was twice that of CI,II OXPHOS compared to patients that survived. Non survivors had almost half the ATP and a rise in mitochondrial superoxide by 130% compared to healthy volunteers.

8.3.9 Clinical course of patients with sepsis (S) and non sepsis (NS) MODS

The median APACHE II score on admission was 24 (13-47), 23.5 (13-47) and 24 (15-41) in OF, S and NS respectively (Table 8-5). The SOFA score on admission was 10 (4-15), 10.5 (4-15) and 10 (6-15) in OF, S and NS. Amongst the 15 patients with sepsis, the site was abdominal in five, lung in two, soft tissue in three, urogenital in four and other in one patient. In the NS group, the cause of MODS was hemorrhage from aneurysms or post operative bleeding in three, trauma in three, cardiac arrest in three and other in two. The duration of organ failure (in hrs) before the first bloods were taken was 18.5 (7-35), 18.5 (7-35) and 20 (7-24) and the median number of organs failed was 2 (2-4) in all groups. 5/27 (18.5%) patients died within three weeks, three with S and two with NS. The mortality rate stayed the same at the 6 month follow up. The median ICU stay in days was 3 (1-100), 3 (1-42) and 3 (1-100) in OF, S and NS respectively and the median hospital stay in days was 12 (1-200), 10 (1-51) and 12 (3-200) (Table 8-5).

Table 8-5 Patient demographics in septic and non-septic MODS

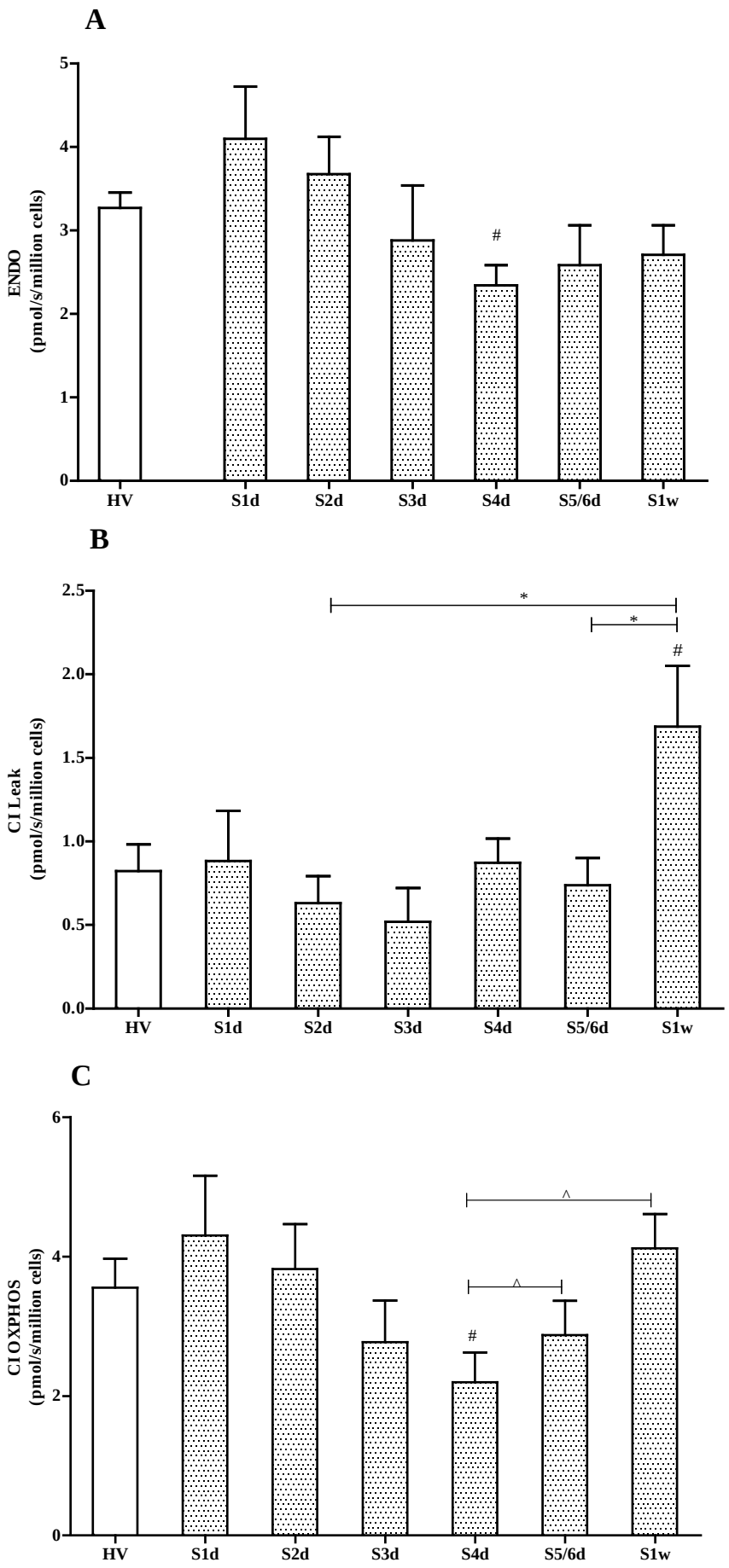
	Septic (S)	**Non septic (NS)**
Number	15	12
Age (years) median (range)	59.5(21-69)	59 (19-74)
Male : female ratio	9:6	7:5
Cause of OF	**Source of sepsis** Abdominal 5 Pneumonia 2 Soft tissue 3 Urogenital 4 CNS sepsis 1	Ruptured aneurysm 2 Trauma+hemorrhage 3 Cardiac arrest 4 Intraoperative hemorrhage 2 Drug induced 1
SOFA (first 24 hrs)	10.5 (3-15)	10 (4-15)
APACHEII	22 (13-35)	24 (15-47)
APACHEIIC Chronic	0 (0-5)	0 (0-5)
SAPS	35(15-81)	47.5 (28-98)
Duration of MODS in hrs prior to first blood	18.5 (7-35)	20 (7-24)
No. of failed organs	3 (2-4)	2 (2-4)
Vasopressor therapy (no of patients)	14	11
Renal replacement therapy (no of patients)	2	1
Mechanical ventilation (no of patients)	4	10
Mortality 1 week	1	2

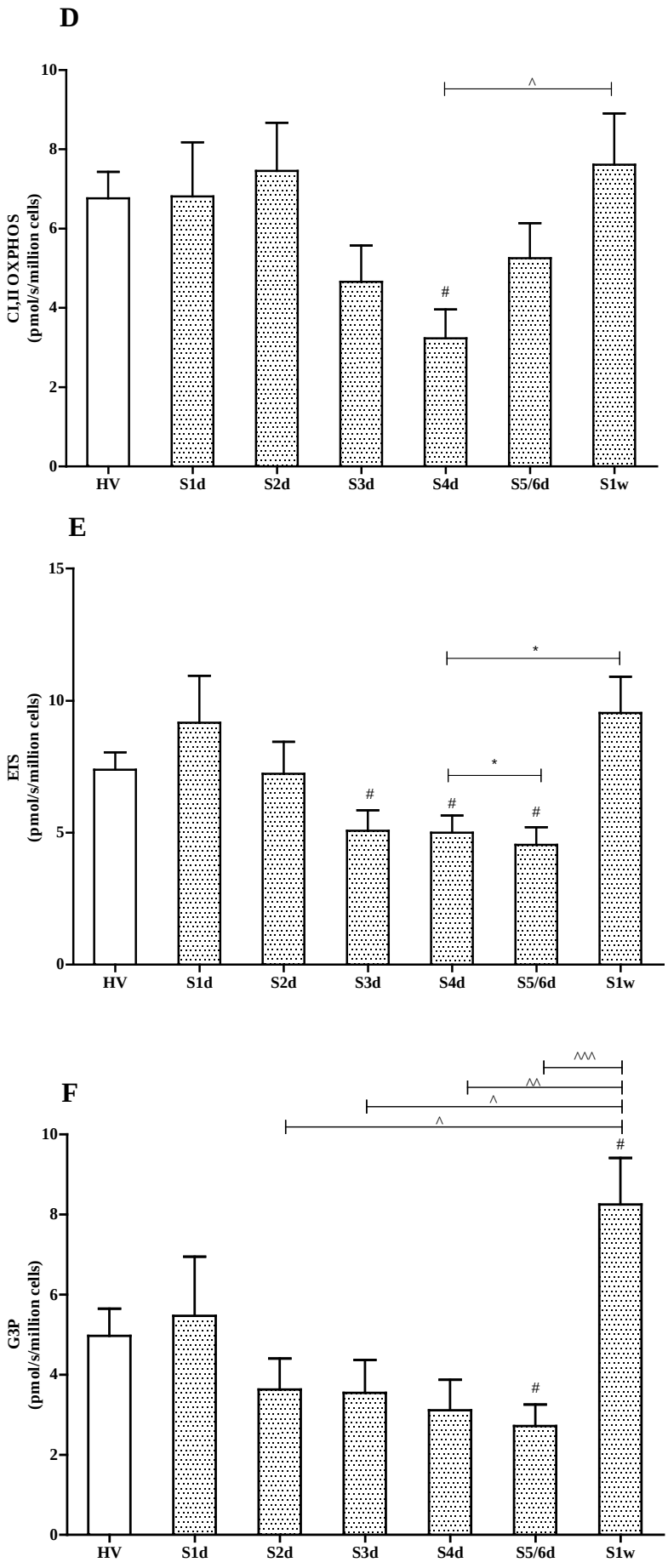
Mortality 3 weeks	3	2
ICU length of stay (days)	3 (1-42)	3 (2-100)
Hospital length of stay (days)	10 (1-51)	12 (3-200)

Footnote: Multiple organ dysfunction syndrome (MODS), Sequential organ failure assessment (SOFA), Acute Physiology and Chronic Health Evaluation II (APACHE II), Simplified acute physiology score (SAPS), Intensive care unit (ICU)

8.3.10 Peripheral mitochondrial function in septic MODS (S) compared to HV

Endogenous respiration was decreased by 21% at 4d compared to HV (P=0.005, Figure 8-5A). CI Leak was increased by 48% at 1w compared to HV (P=0.03, Figure 8-5B). CI OXPHOS was decreased by 32% at 4d compared to HV (P=0.03, Figure 8-5C). CI,II OXPHOS is decreased 43% at 4d (P=0.002) and then recovers by 1w compared to HV (Figure 8-5D). ETS capacity is decreased by 23% at 3d (P=0.03) through to 5/6d (P=0.007) compared to HV (Figure 8-5E). G3P was decreased by 27% at 5/6d (P=0.02) but is increased by 55% at 1w (P=0.04) compared to HV (Figure 8-5F). Within the first week, there were no differences in CI OXPHOS/CI Leak, CI,II OXPHOS/CI,II Leak and CI,II OXPHOS/ETS between HV and S (Figure 8-5G-I). Overall, mitochondrial respiration was depressed at day 3 and day 4 followed by increased leak and supranormal respiration with G3P at the 1 week mark.


A
ENDO
(pmol/s/million cells)
5
4
3
2
1
0
#
HV
S1d
S2d
S3d
S4d
S5/6d
S1w
B
CI Leak
(pmol/s/million cells)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
#
HV
S1d
S2d
S3d
S4d
S5/6d
S1w
C
CIOXPHOS
(pmol/s/million cells)
6
4
2
0
^
^
#
HV
S1d
S2d
S3d
S4d
S5/6d
S1w


D
CI,II OXPHOS
(pmol/s/million cells)
10
8
6
4
2
0
^
#
HV
S1d
S2d
S3d
S4d
S5/6d
S1w
E
ETS
(pmol/s/million cells)
15
10
5
0
*
*
#
#
#
HV
S1d
S2d
S3d
S4d
S5/6d
S1w
F
G3P
(pmol/s/million cells)
10
8
6
4
2
0
^^^
^^
^
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#
#
HV
S1d
S2d
S3d
S4d
S5/6d
S1w

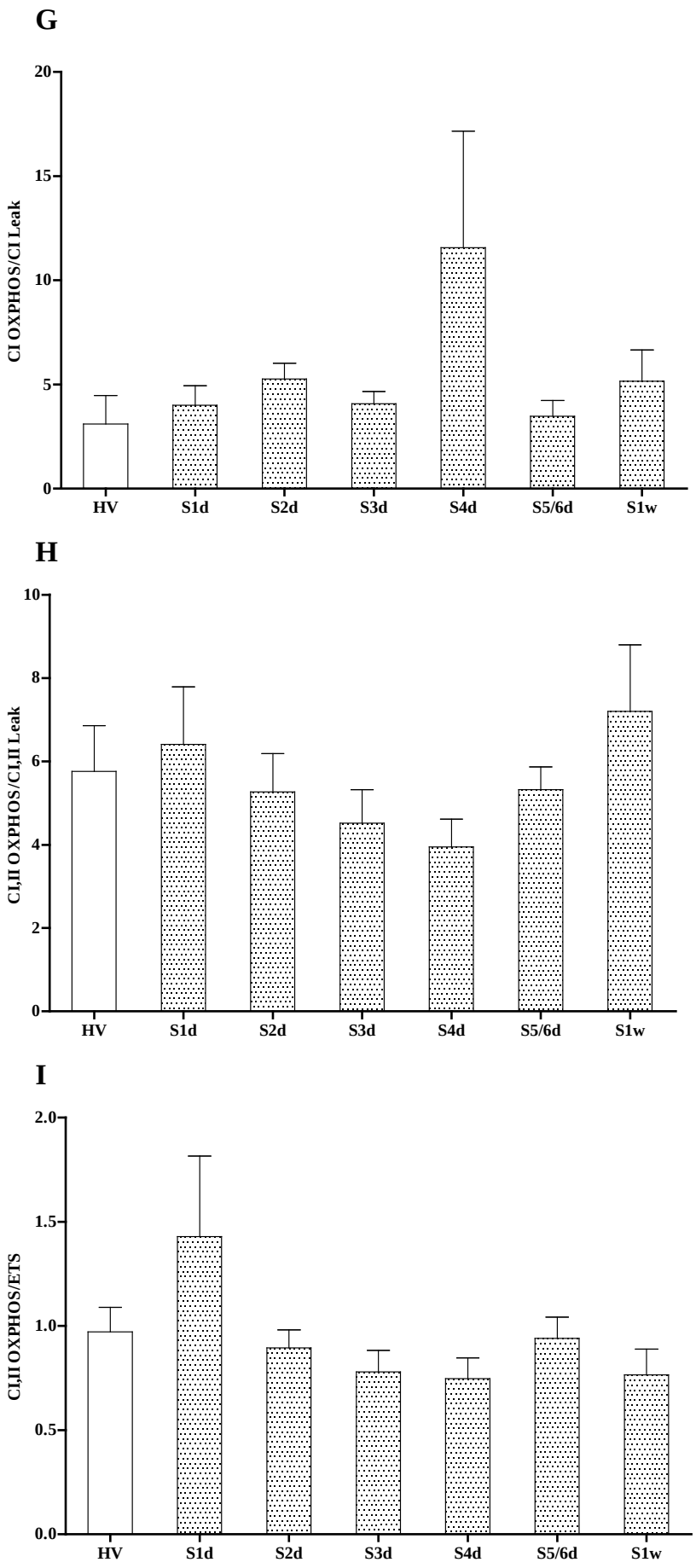

G
CI OXPHOS/CI Leak
20
15
10
5
0
HV
S1d
S2d
S3d
S4d
S5/6d
S1w
H
CI,II OXPHOS/CI,II Leak
10
8
6
4
2
0
HV
S1d
S2d
S3d
S4d
S5/6d
S1w
I
CI,II OXPHOS/ETS
2.0
1.5
1.0
0.5
0.0
HV
S1d
S2d
S3d
S4d
S5/6d
S1w

Figure 8-5A–I Mononuclear cell mitochondrial respiration and flux control ratios in patients with septic MODS. HV, white bars (n=15) and patients with septic MODS (S) at different time points, white dotted (n=15 at 1d and 2d, n=13 from 3d onwards). Different respiratory states measured were: A) Endo [Endogenous respiration], B) CI Leak C) CI OXPHOS [Complex I oxidative phosphorylation] D) CI,II OXPHOS [Complex I and II oxidative phosphorylation], E) ETS [Electron Transport System capacity] and F) G3P [ETS capacity with Glycerol-3-phosphate]. Flux control ratios measured were: G) CI OXPHOS/CI Leak [measure of phosphorylation efficiency with CI substrates], H) CI,II OXPHOS/CI,II Leak [measure of phosphorylation efficiency with CI+II substrates] and I) CI,II OXPHOS/ETS [ratio of phosphorylation to electron transport system capacity].Values are mean ± standard error of mean. #comparison to HV (student's *t* test with sequential *Bonferroni* correction), *comparison within patients at different time points (*RANOVA* with least significant different correction (*LSD*)), ^ comparison within patients at different time points (least significant difference (*LSD*)) #/^/*P<0.05, ##/^^/** P<0.01, ###/^^^/*** P<0.005.

8.3.11 Temporal mitochondrial function changes in septic MODS

There were significant temporal differences in CI Leak (P=0.03, *RANOVA*) and ETS (P=0.04, *RANOVA*) in patients with septic MODS (Figure 8-5B, and Figure 8-5E). CI Leak was raised at 1w compared to earlier in the disease state (Figure 8-5B). ETS decreased from d3 onwards and troughs at d5/6d followed by an increase at 1w (Figure 8-5E). Although not significantly different on *RANOVA*, there were some statistically significant differences seen on *pairwise LSD* in the following parameters. CI OXPHOS troughed at 4d and was increased on 5/6d and 1w compared to 4d (4d vs 5/6d P=0.037, 4d vs 1w P=0.013) (Figure 8-5C). CI,II OXPHOS was increased at 1w compared to 4d (4d vs. 1w P=0.049) (Figure 8-5D). G3P was significantly increased at 1w compared to d2 onwards (1w vs. 2d P=0.023, 1w vs. 3d P=0.033, 1w vs. 4d P=0.006, 1w vs. 5/6d P=0.003) (Figure 8-5F). Within the first week, there were no temporal changes in CI OXPHOS/CI Leak (Figure 8-5G), CI,II OXPHOS/CI,II Leak (Figure 8-5H) and CI,II OXPHOS/ETS (Figure 8-5I). Taken together, mitochondrial respiration changes were U shaped over time, with the trough at day 3 and day 4 in septic MODS. In keeping with the U shape, most respiration parameters (except CI Leak and G3P) did not differ significantly between early (1d and 2d) and later (7d) in the week.

8.3.12 Other mitochondrial function in septic MODS

8.3.12.1 Cytochrome C

In septic MODS, cytochrome *c* was decreased by 64% in 1/2d compared to HV (P=0.0023, Figure 8-6A) and most likely represents mitochondrial outer membrane permeabilisation in septic MODS (321).

8.3.12.2 ATP

In sepsis, ATP was decreased by 8% at 1d (P=0.02, *MW*), by 55% at 2d (P=0.0017) and by 61% at 1w (P=0.001) compared to HV (Figure 8-6B). At 1d, 2d and 1w, mitochondrial

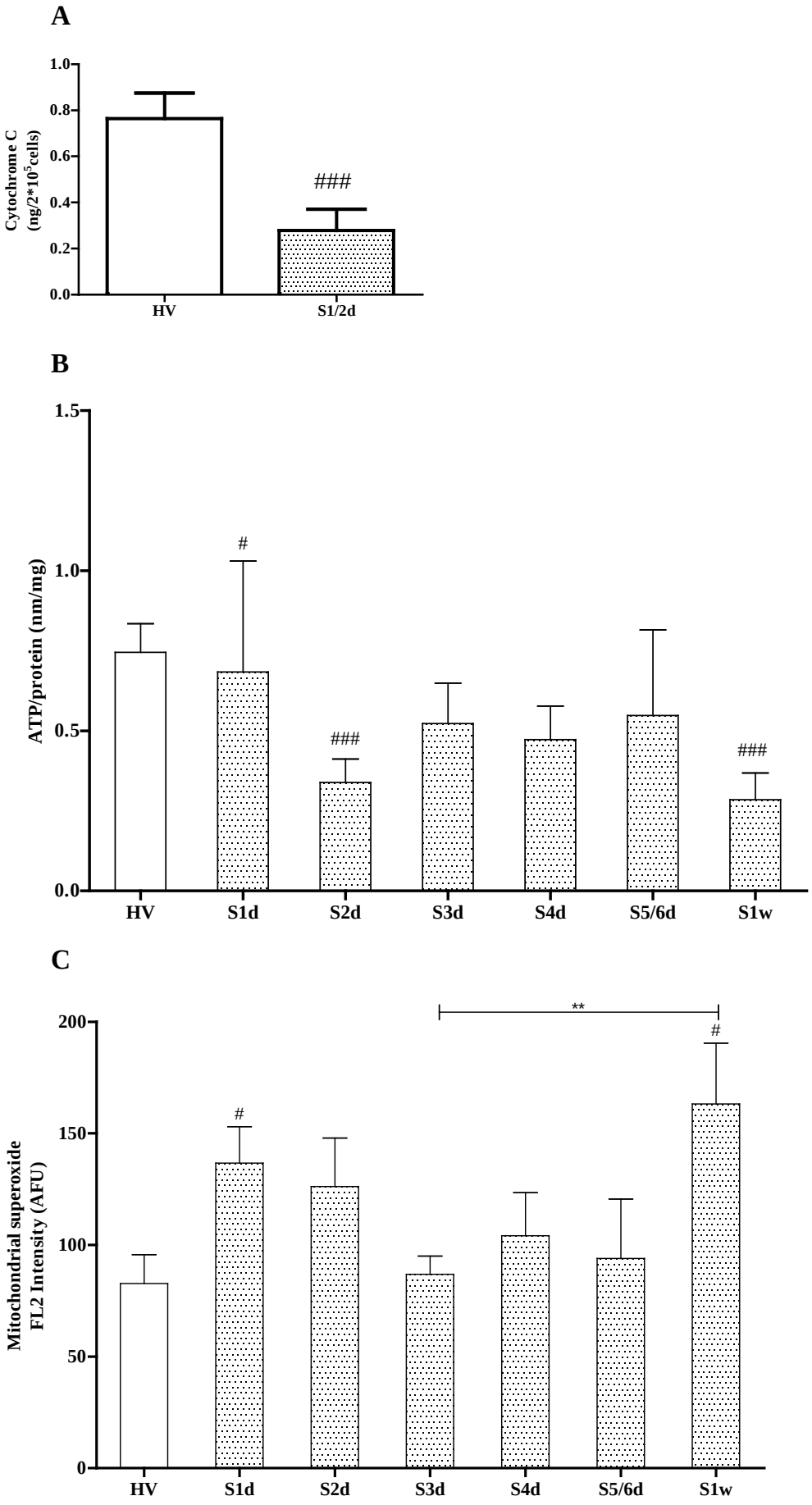
respiration (OXPHOS and ETS) was no different to HV in septic MODS and therefore the decrease in net ATP at these time points was most likely due to increased consumption of ATP due to raised metabolic demands in septic MODS.

8.3.12.3 Mitochondrial superoxide

Compared to HV, mitochondrial superoxide was increased by 66% at 1d (P=0.014) and by 99% at 1w (P=0.013, *MWU*) for septic MODS (Figure 8-6C). In sepsis, there were significant differences in mitochondrial superoxide within patients over time (P=0.035, *RANOVA*) (Figure 8-6C). Mitochondrial superoxide decreased at 3d and then increased again at the 1w mark. Tying in with the mitochondrial respiration results, it was evident that the significantly raised mitochondrial superoxide early in septic MODS was only decreased at day3/4 alongside a decrease in mitochondrial respiration at day3/4. Once mitochondrial respiration increased at 1w, mitochondrial superoxide levels also increased at the end of the week.

8.3.12.4 Mitochondrial membrane potential

Compared to HV, the aggregate monomer ratio was increased in septic MODS by 78% at 2d (P=0.005), by 108% at 3d (P=0.010, *MWU*) and by 86% at 5/6d (P=0.004) (Figure 8-6D) and was consistent with changes in mitochondrial respiration and mitochondrial superoxide.


A
Cytochrome C
(ng/2*10^5cells)
1.0
0.8
0.6
0.4
0.2
0.0
###
HV
S1/2d
B
ATP/protein (nm/mg)
1.5
1.0
0.5
0.0
#
###
###
HV
S1d
S2d
S3d
S4d
S5/6d
S1w
C
Mitochondrial superoxide
FL2 Intensity (AFU)
200
150
100
50
0
**
#
#
HV
S1d
S2d
S3d
S4d
S5/6d
S1w

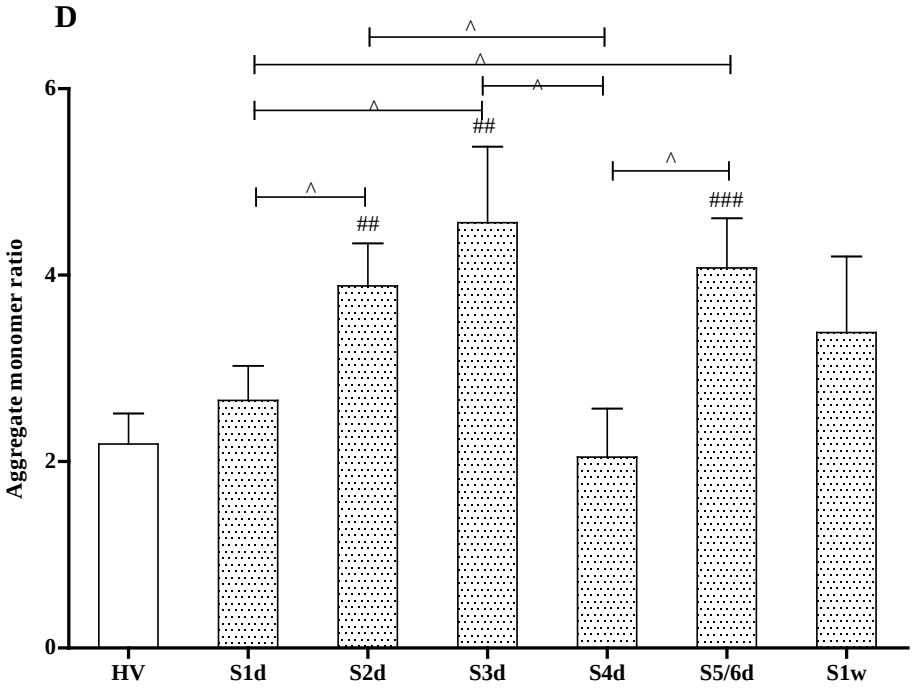


Figure 8-6A-D. Other mitochondrial function in patients with septic MODS (white bar with dots, n=15 at 1d and 2d, n=13 from 3d onwards) compared to healthy volunteers (white bars, n=15) at different time points. A) Mononuclear cell Cytochrome C B) Mononuclear cell adenine triphosphate (ATP) to protein ratio C) Mononuclear cell mitochondrial superoxide and D) Mononuclear cell mitochondrial membrane potential measured with the dye 5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolylcarbocyanine iodide (JC-1). JC-1 fluorescence was detected by flow cytometry in two channels FL 1 and FL 2. JC-1 exists as a monomeric form which fluoresces at FL 1 and when concentrated by actively respiring mitochondria exists as an aggregate which fluoresces at FL 2. An increase in the ratio of aggregate to monomer suggests higher mitochondrial membrane potential which in turn generates increased mitochondrial reactive oxygen species such as mitochondrial superoxide. Values are mean ± standard error of mean. #comparison to HV (student's *t* test), *comparison within patients at different time points (paired student's *t* test). #/* P<0.05; ##/** P<0.01; ###P<0.005.

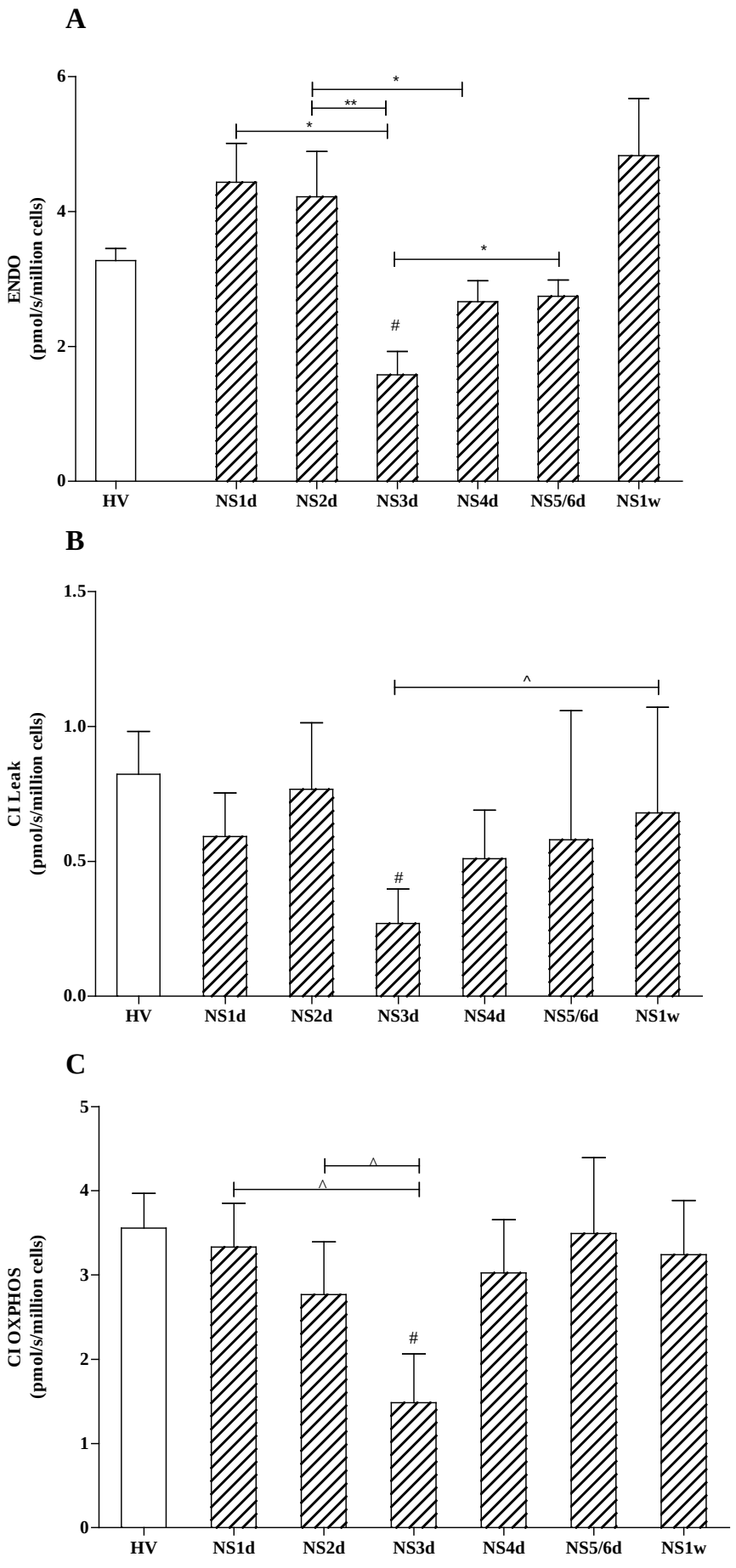
8.3.12.5Interim conclusion

The pattern of mitochondrial function in septic MODS closely resembled MODS. Mitochondrial respiration changes were U shaped over time, with the trough at day 3 and day 4 in septic MODS but a supranormal CI Leak and respiration with G3P persisted at the end of the week. Net ATP was not decreased compared to HV between days 3-5/6 when mitochondrial respiration was depressed.

8.3.13 Peripheral mitochondrial function changes in non septic MODS (NS) compared to HV

At day 3, there was a decrease in endogenous respiration, CI Leak, CI, OXPHOS, CI,II OXPHOS and G3P in non septic MODS compared to HV. Endogenous respiration decreased by 52% (P=0.02, Figure 8-7A), CI Leak decreased by 67% (P=0.02, Figure 8-7B) and CI

OXPHOS decreased by 58% (P=0.007, Figure 8-7C). CI,II OXPHOS and G3P were also decreased by 49% and 52% at day 3 compared to HV (P=0.003, Figure 8-7D and P=0.01, Figure 8-7F respectively). There were no differences in ETS, CI OXPHOS/CI Leak, CI,II OXPHOS/CI,II Leak or CI,II OXPHOS/ETS between HV and NS (Figure 8-7E, Figure 8-7G, Figure 8-7H and Figure 8-7I).


A
ENDO
(pmol/s/million cells)
6
4
2
0
*
**
*
*
#
HV
NS1d
NS2d
NS3d
NS4d
NS5/6d
NS1w
B
CI Leak
(pmol/s/million cells)
1.5
1.0
0.5
0.0
^
#
HV
NS1d
NS2d
NS3d
NS4d
NS5/6d
NS1w
C
CI OXPHOS
(pmol/s/million cells)
5
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HV
NS1d
NS2d
NS3d
NS4d
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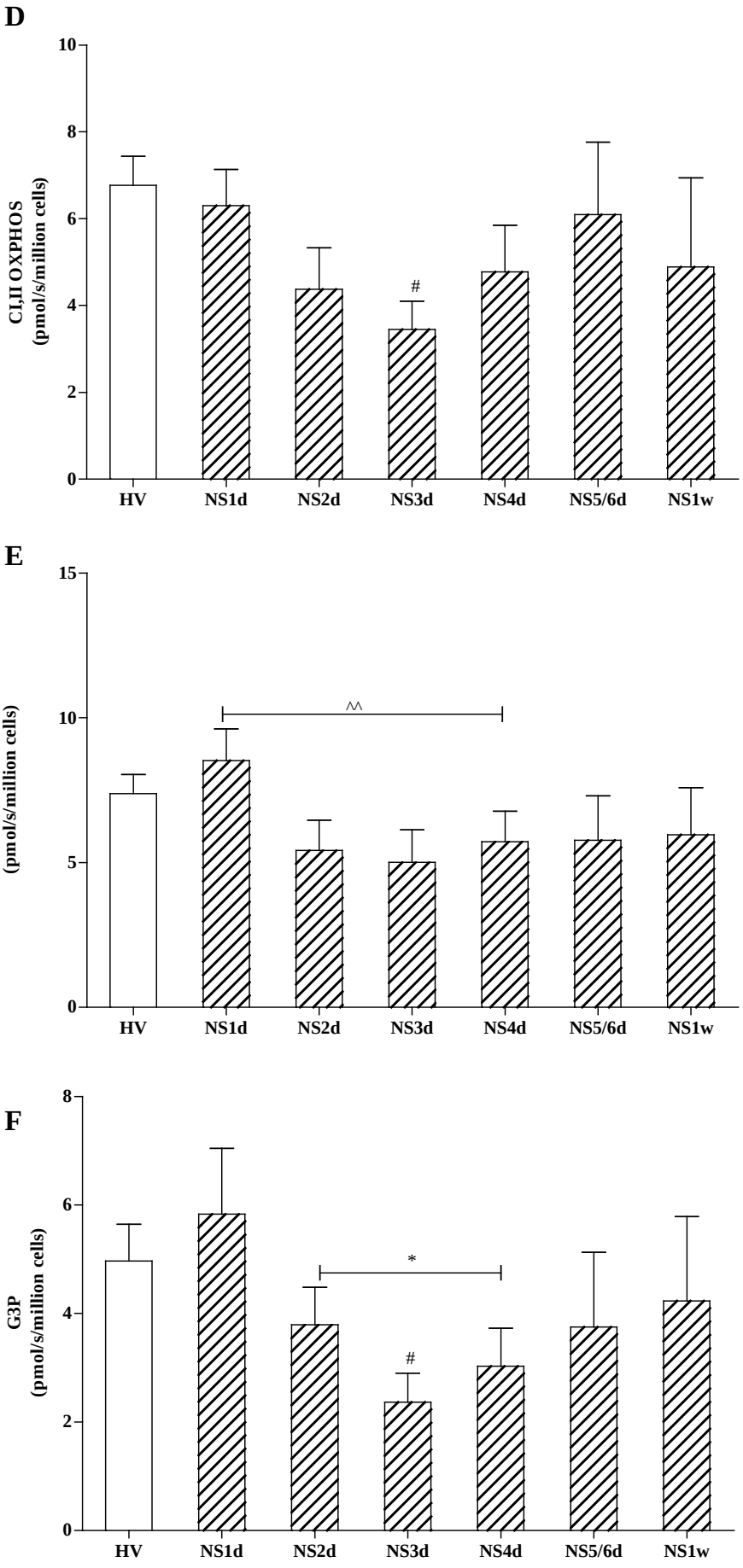
NS1w


D
CI,II OXPHOS
(pmol/s/million cells)
10
8
6
4
2
0
#
HV
NS1d
NS2d
NS3d
NS4d
NS5/6d
NS1w
E
ETS
(pmol/s/million cells)
15
10
5
0
^^
HV
NS1d
NS2d
NS3d
NS4d
NS5/6d
NS1w
F
G3P
(pmol/s/million cells)
8
6
4
2
0
*
#
HV
NS1d
NS2d
NS3d
NS4d
NS5/6d
NS1w

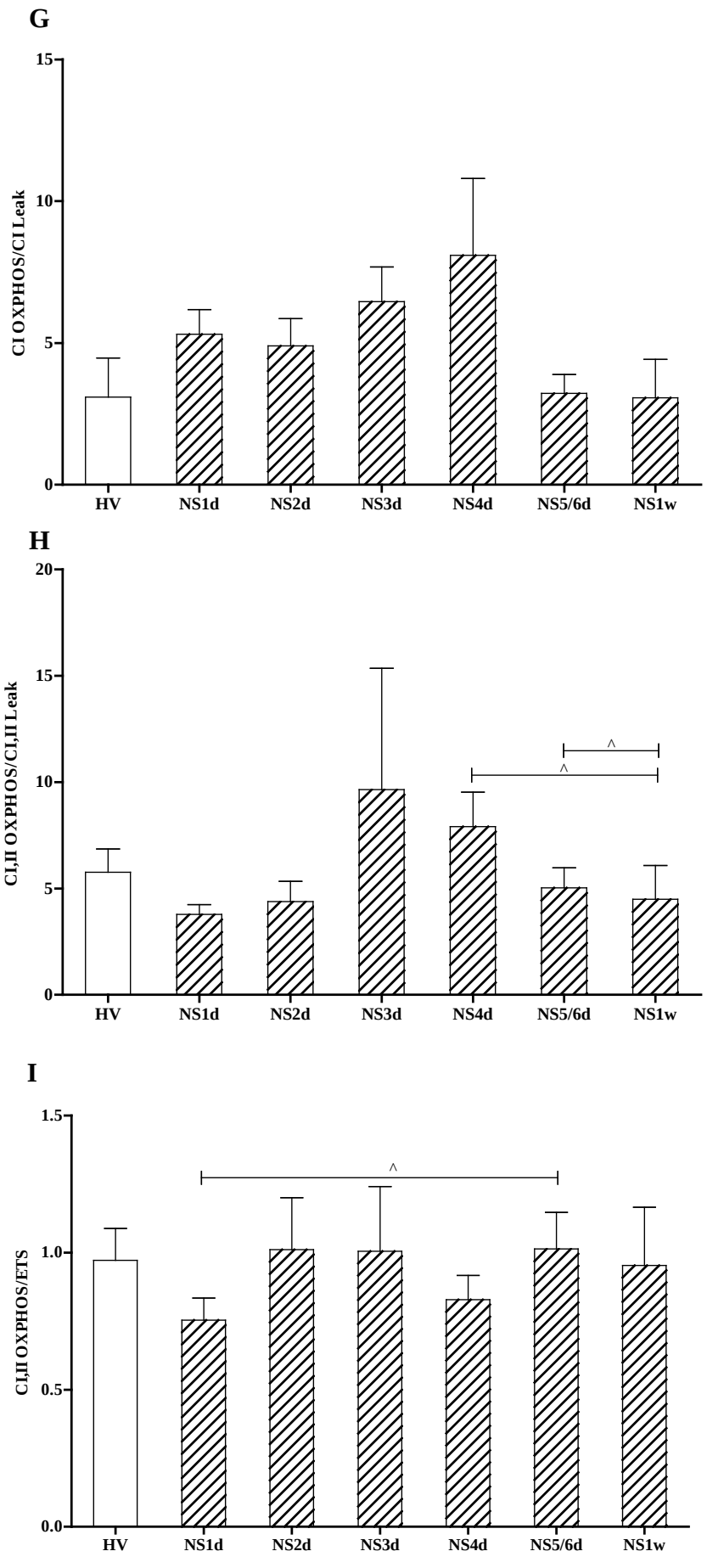

G
CI OXPHOS/CI Leak
15
10
5
0
HV
NS1d
NS2d
NS3d
NS4d
NS5/6d
NS1w
H
CI,II OXPHOS/CI,II Leak
20
15
10
5
0
^
^
HV
NS1d
NS2d
NS3d
NS4d
NS5/6d
NS1w
I
CI,II OXPHOS/ETS
1.5
1.0
0.5
0.0
^
HV
NS1d
NS2d
NS3d
NS4d
NS5/6d
NS1w

Figure 8-7 A–I Mononuclear cell mitochondrial respiration and flux control ratios in patients with non septic MODS. Healthy volunteers (HV), white bars (n=15) and patients with non septic MODS (NS) at different time points, hatched bars (n=12 at 1d, n=10 at 2d and n=9 from 3d onwards). Different respiratory states measured were: A) Endo [Endogenous respiration], B) CI Leak C) CI OXPHOS [Complex I oxidative phosphorylation] D) CI,II OXPHOS [Complex I and II oxidative phosphorylation], E) ETS [Electron Transport System capacity] and F) G3P [ETS capacity with Glycerol-3-phosphate]. Flux control ratios measured were: G) CI OXPHOS/CI Leak [measure of phosphorylation efficiency with CI substrates], H) CI,II OXPHOS/CI,II Leak [measure of phosphorylation efficiency with CI+II substrates] and I) CI,II OXPHOS/ETS [ratio of phosphorylation to electron transport system capacity].Values are mean ± standard error of mean. #comparison to HV (student's *t* test with sequential *Bonferroni* correction), *comparison within patients at different time points (*RANOVA* with least significant different correction (*LSD*)), ^ comparison within patients at different time points (least significant difference (*LSD*)) #/^/*P<0.05, ##/^^/** P<0.01, ###/^^^/*** P<0.005.

8.3.14 Temporal mitochondrial function changes in non septic MODS

There were significant temporal differences in endogenous respiration (P=0.04, *RANOVA*) and G3P (P=0.026, *RANOVA*) within the NS group (Figure 8-7A, Figure 8-7F). Endogenous respiration decreased at days 3 and 4 compared to days 1 and 2 and then increased at day 5/6 compared to day 3 (Figure 8-7A). G3P also troughed at 3d/4d and then progressively increased (Figure 8-7F). Although not significantly different on *RANOVA*, there were some statistically significant differences seen on *pairwise LSD* in the following parameters: CI Leak (3d vs. 1w P=0.037) (Figure 8-7B) and CI OXPHOS (1d vs. 3d P=0.028, 1d vs. 4d P=0.042) (Figure 8-7C) all troughed at 3d. CI,II OXPHOS/CI,II Leak was significantly decreased at 1w compared to 4d (4d vs. 1w P=0.012) and 5/6d (5/6d vs. 1w P=0.041) (Figure 8-7H) and reflects the non statistically significant increase in CI,II leak at day 4 and day 5/6. CI,II OXPHOS/ETS was increased at 5/6d compared to 1d (1d vs. 5/6d P=0.019) (Figure 8-7I) and is most likely due to the recovery of both OXPHOS and ETS during that time. Once again, the overall pattern of mitochondrial respiration was a U shaped curve with troughs at day 3 and day 4.

8.3.15 Other mitochondrial function in non septic MODS

8.3.15.1 Cytochrome C

In non septic MODS, cytochrome *c* was decreased by 58% at 1/2d (P=0.003,Figure 8-8A) compared to HV most likely reflecting mitochondrial outer membrane permeabilisation (321).

8.3.15.2 ATP

In non septic MODS, net ATP was decreased at 1d by 55% (P<0.0001), 3d by 40% (P=0.02, d.f.=20), 45% by 4d (P=0.007), 62% by 5/6d (P=0.0005) and by 70% at 1w (P=0.0002) compared to HV (Figure 8-8B). The decrease in net ATP at 1d and 1w was not accompanied by a decrease in mitochondrial respiration and therefore is most likely due to increased

consumption of ATP due to an increased metabolic demand. Whereas, at day3/4 the decrease in ATP was most likely multifactorial from decreased respiration leading to decreased production of ATP and also increased consumption of ATP due to increased metabolic demand.

8.3.15.3Mitochondrial superoxide

Compared to HV, mitochondrial superoxide was increased by 78% at 1d (P=0.011) and by 104% at 5/6d (P=0.005, *MWU*) for NS (Figure 8-8C). The increased mitochondrial superoxide at day 1/2 is decreased by a decrease in mitochondrial respiration at day 3/4. A rise in mitochondrial superoxide is evident at the end of the week when mitochondrial respiration recovers.

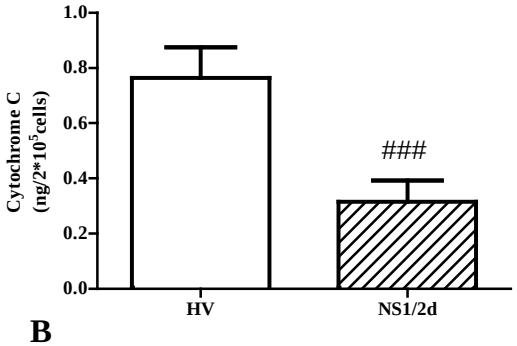
8.3.15.4Mitochondrial membrane potential

There were no differences in mitochondrial membrane potential between patients and HV in non septic MODS. Within patients, mitochondrial membrane potential decreased at day 4 compared to day 2 on *pairwise LSD* but not on *RANOVA* (P<0.005, Figure 8-8D). Consistent with the mitochondrial membrane potential at day 4, mitochondrial superoxide also reached its nadir at day 4.

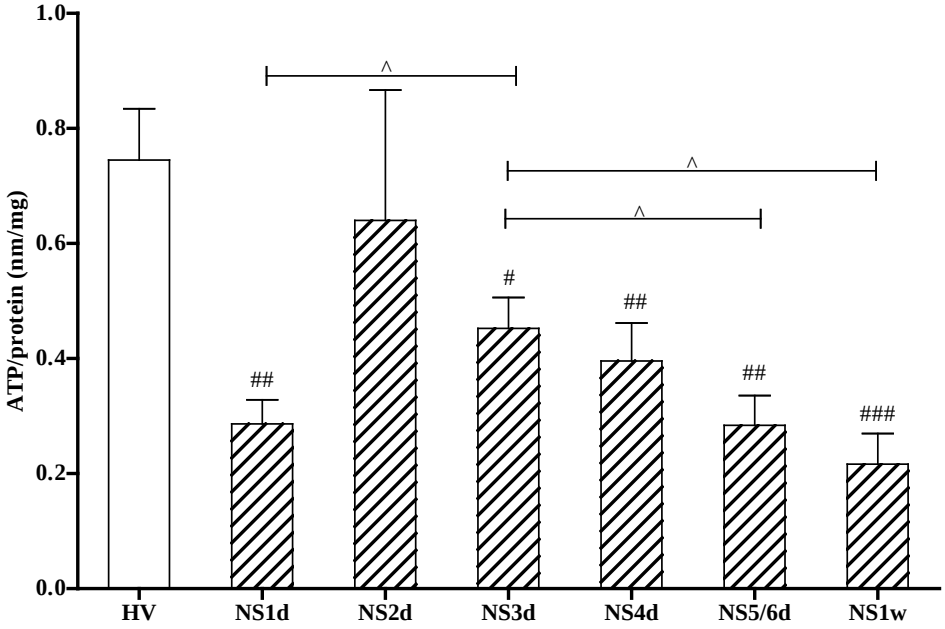
8.3.15.5Interim conclusion

The pattern of mitochondrial function in non septic MODS was predominantly similar to MODS and septic MODS with a few exceptions. Once again, the overall pattern of mitochondrial respiration was a U shaped curve with troughs at day 3 and day 4 but unlike septic MODS there were no significant abnormalities left at the end of the week. Unlike septic MODS, net ATP remained decreased through the week.

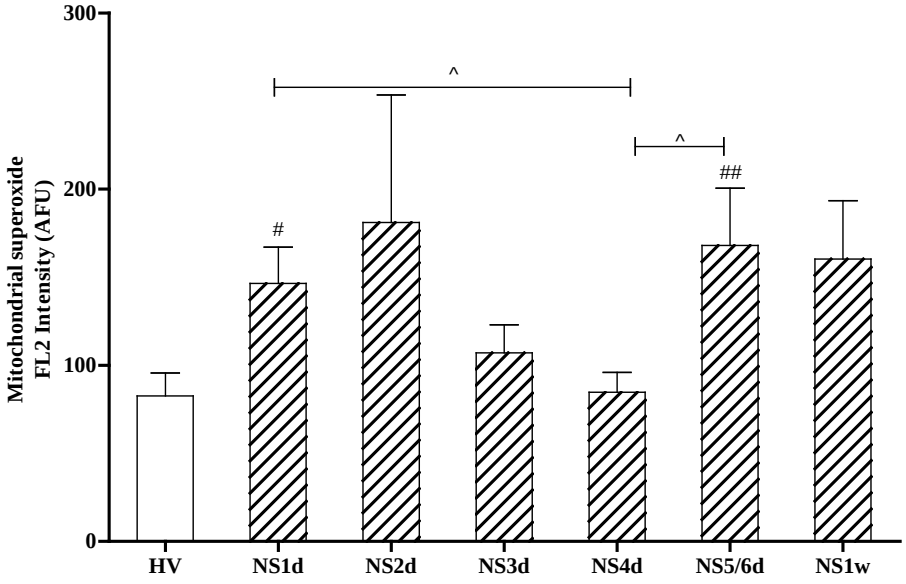
A


Cytochrome C
(ng/2*10^5cells)
1.0
0.8
0.6
0.4
0.2
0.0
###
HV
NS1/2d

B


ATP/protein (nm/mg)
1.0
0.8
0.6
0.4
0.2
0.0
^
^
^
#
##
##
##
###
HV
NS1d
NS2d
NS3d
NS4d
NS5/6d
NS1w

C


Mitochondrial superoxide
FL2 Intensity (AFU)
300
200
100
0
^
^
#
##
HV
NS1d
NS2d
NS3d
NS4d
NS5/6d
NS1w

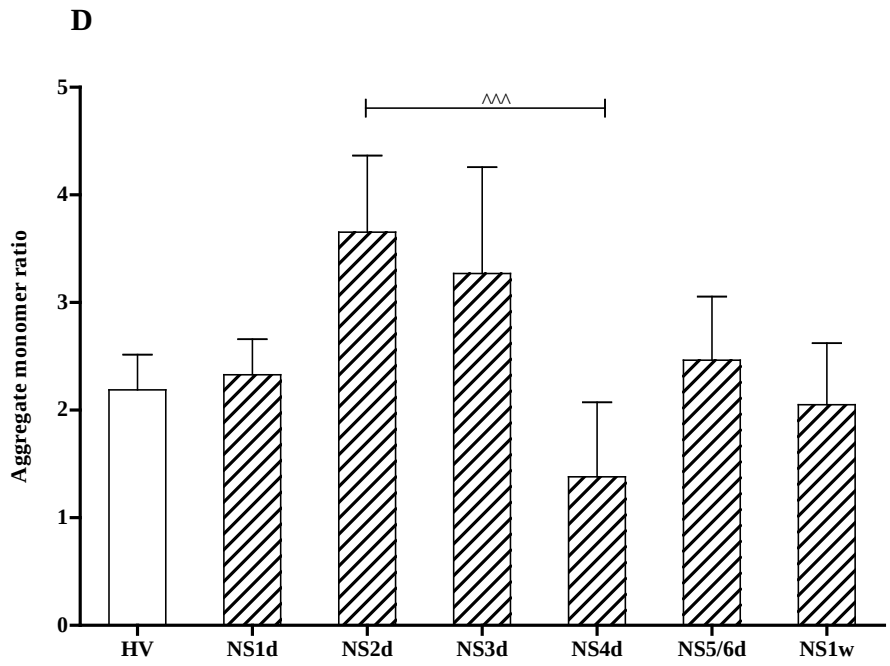


Figure 8-8 A-D. Other mitochondrial function in patients with non septic MODS (hatched bars, n=12 at 1d, n=10 at 2d and n=9 from 3d onwards) compared to healthy volunteers (white bars, n=15) at different time points. A) Mononuclear cell Cytochrome C B) Mononuclear cell adenine triphosphate (ATP) to protein ratio C) Mononuclear cell mitochondrial superoxide and D) Mononuclear cell mitochondrial membrane potential measured with the dye 5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolylcarbocyanine iodide (JC-1). JC-1 fluorescence was detected by flow cytometry in two channels FL 1 and FL 2. JC-1 exists as a monomeric form which fluoresces at FL 1 and when concentrated by actively respiring mitochondria exists as an aggregate which fluoresces at FL 2. An increase in the ratio of aggregate to monomer suggests higher mitochondrial membrane potential which in turn generates increased mitochondrial reactive oxygen species such as mitochondrial superoxide. Values are mean ± standard error of mean. #comparison to HV (student's *t* test), *comparison within patients at different time points (paired student's *t* test). #/* P<0.05; ##/** P<0.01; ###P<0.005.

8.3.16 Temporal PBMC mitochondrial function in MODS associated with septic compared to non septic MODS

Even though the temporal pattern of net ATP appeared different in septic compared to non septic MODS, this was not statistically significant (P=0.10). The only statistically significant difference seen between septic and non septic MODS is at 7d between septic and non septic endogenous respiration. Septic endogenous respiration was 2.71+/- 0.35 pmol/s/million cells and non septic endogenous respiration was 4.83+/-0.85 pmol/s/million cells at 7d (P=0.041, *RANOVA*, Figure 8-9).

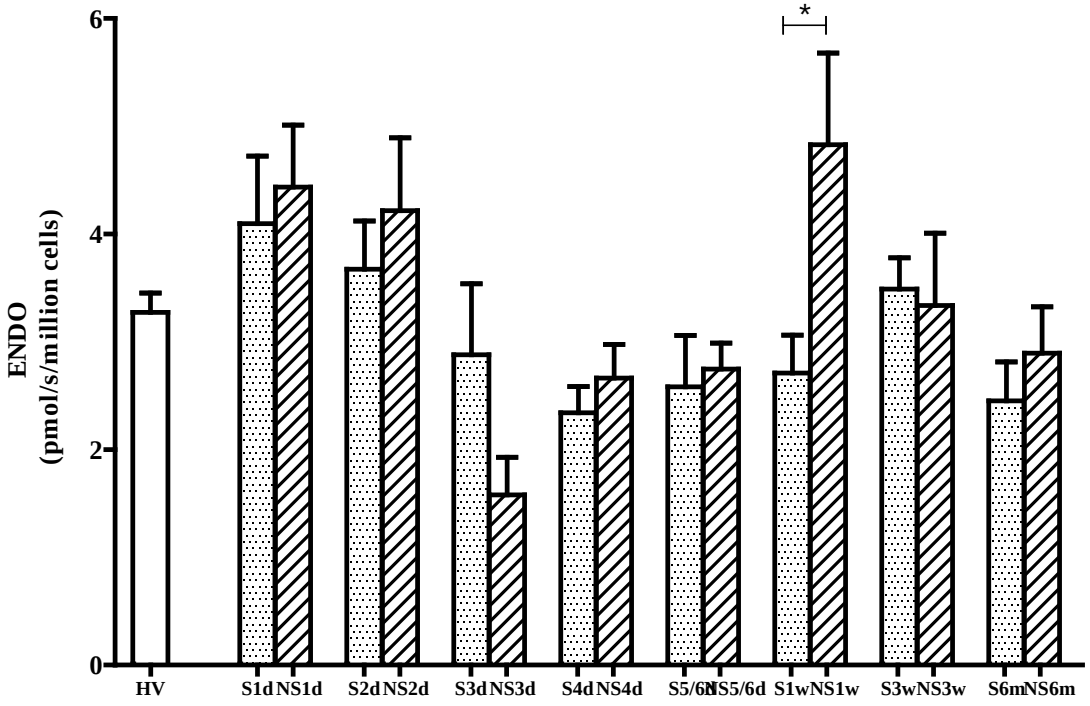


Figure 8-9 Mononuclear cell endogenous respiration between septic and non septic MODS. Healthy volunteers HV, white bar (n=15) and patients with septic (white bars with dots, n=15 at 1d and 2d, n=13 from 3d onwards) and non septic organ failure (hatched bars, n=12 at 1d, n=10 at 2d and n=9 from 3d onwards) at different time points. *P<0.05, RANOVA *with LSD correction*.

8.4 Discussion

8.4.1 PBMC mitochondrial dysfunction occur in MODS

The central hypotheses of this thesis were successfully tested. It was shown that multiple aspects of mitochondrial dysfunction occurred in PBMC of patients with MODS. Further, that temporal mitochondrial respiration negatively correlated with temporal organ failure scores and temporal mitochondrial respiration did not discriminate between septic and non-septic causes of MODS.

Mitochondrial dysfunction occurred in MODS and this study highlighted the dynamic nature of mitochondrial function as the disease evolved over time. Severity of organ failure as measured by SOFA inversely correlated with mitochondrial respiration from day 3 onwards. Increase in mitochondrial superoxide occurred first and was followed by a decrease in mitochondrial respiration later (from day 3) in the disease course. The average mitochondrial respiration and mitochondrial superoxide correlated with each other over time. Non survivors had several key differences in mitochondrial respiration at day 1. In non survivors, the electron transport system capacity with G3P was twice that of CI,II OXPHOS compared to

patients that survived. Non survivors had almost half the ATP and a rise in mitochondrial superoxide by 130% compared to healthy volunteers. Mitochondrial dysfunction occurred in MODS regardless of whether MODS was associated with sepsis or non sepsis and the pattern of mitochondrial dysfunction was similar.

8.4.2 What this study adds in relation to previous clinical studies of PBMC mitochondrial respiration

This was the first clinical study that characterised temporal PBMC mitochondrial function in patients with multiple organ dysfunction syndrome as an entity. No studies have measured PBMC mitochondrial respiration in non septic MODS. As such, the findings from this study will be reviewed in relation to previous clinical studies which were limited to sepsis/septic shock and analysed mitochondrial function in PBMC at one or two time points (242),(305),(125). In sepsis and septic shock, decreased CII OXPHOS (305) and CI OXPHOS (125) were reported and is consistent with our findings. Unlike this study, none of these studies (242),(305),(125) measured combined respiration flux after substrates for both CI and CII (CI,II OXPHOS) even though combining substrates better reflects respiration in vivo compared to providing substrates separately for either CI or CII (96). Electrons from CI and CII converge at the Q junction thereby producing an additive effect which represents maximal mitochondrial respiration in vivo (96).

In this study, patients with septic MODS had significantly decreased CI,II OXPHOS at day 4 compared to HV. Endogenous respiration was reported to be decreased in one study (125) and increased in another (242). The discrepancy between those studies was most likely because respiration was measured once in those studies at different time points and their patient population differed. Whilst Belikova (242) measured respiration from blood in patients with severe sepsis or septic shock within 48hrs after intensive care admission, Garrabou (125) did not mention the timing of blood taking and included patients only with sepsis but not with septic shock. Our findings show that endogenous respiration tended to increase in the first 48 hrs compared to HV (although not statistically significant) and then decreased from 3d onwards, reaching statistical significance on day 4 compared to HV (Figure 8-1). Similarly, CII Leak respiration was reported to be increased in severe sepsis or septic shock in one study (242) but another study found no difference between their controls and patients with septic shock (305). Again, those discrepancies may be due to the patients and controls included in those studies and also due to temporal changes that may have been missed because those studies only measured respiration at one time point. The first study included patients with both severe sepsis and septic shock and had healthy volunteers as controls, whereas, the

second study measured respiration in patients with septic shock only and had critically ill patients without sepsis or shock as their controls. In this study, there was no difference in CI,II Leak respiration in patients with sepsis compared to HV at any time point, but, there was an increase in CI Leak at 1w in sepsis compared to HV ($P<0.05$). ETS capacity was reported to decrease by Belikova (242) but no change was found by Japiassu (305) and may be due to difference in timing of blood taking in those studies. In this study, the ETS capacity did not decrease significantly until day 3 and then remained decreased significantly until 1 week.

8.4.3 How this study compares to previous studies of mitochondrial respiration in other tissues in patients with MODS

There is sparse human data with regard to mitochondrial respiration in other tissues in MODS. The published studies have focused only on sepsis/septic shock and there are no studies of mitochondrial respiration function from other tissues in non septic MODS. In septic MODS, mitochondrial respiration function in skeletal muscle and liver have been described at one time point in the disease and platelet mitochondrial respiration has been described three times within the first seven days.

In 1985, cytochrome oxidase activity was found to be decreased in skeletal muscle from needle biopsies in patients with circulatory shock (322). In 2002, skeletal muscle biopsies were taken from the leg within 48hrs of admission to the ICU from 16 sepsis survivors, 12 sepsis non survivors and 9 controls who were undergoing elective hip joint replacement. CI activity expressed as a ratio of citrate synthase was decreased in patients compared to controls ($P=0.03$) and CI activity negatively correlated with the dose of norepinephrine required in patients (25) . Fredrikson studied ten patients with sepsis and ten controls and also reported decreased citrate synthase, CI and CIV in skeletal muscle biopsy from the leg. Based on those studies, skeletal muscle mitochondrial respiration appears to behave similarly to PBMC mitochondrial respiration reported in this study. This is because in PBMC, CI OXPHOS decreased at day 4 in septic MODS (Figure 8-5) and CI OXPHOS at day 3 correlated negatively with SOFA score at day 3 in MODS ($r=-0.651$, $P=0.006$) (Table 8-4).

Liver mitochondrial respiration has only been described from post mortem liver biopsies in patients with sepsis in one study (323). Liver mitochondrial respiration was compared between non survivors who had conventional insulin therapy and non survivors who had intensive insulin therapy for blood sugar control in sepsis. There were no non septic controls in this study and therefore it is difficult to draw any comparison between PBMC mitochondrial function and liver mitochondrial function in sepsis. Even though the intensive

insulin therapy group had increased CI and CIV activities compared to conventional insulin therapy group, there were two key differences in the composition of patients between those groups that made direct comparison of mitochondrial function difficult in that study between the two groups (323). The two key differences were that the conventional group had fewer patients with APACHEII>15 compared to the intensive insulin therapy group (P=0.04) and the median days in ICU before death in the conventional group was 34 days compared to 12 days in the intensive insulin group (P=0.03). Since this study, other clinical studies comparing tight glycaemic control compared to conventional glycaemic control have shown detrimental effects of tight glycaemic control in sepsis (324).

More recently, platelet respiration function was studied three times during the first week in septic shock (126). Patients with severe sepsis/septic shock were recruited within 48 hrs of ICU admission. In contrast to PBMC and skeletal muscle, platelet mitochondrial respiration was increased in sepsis compared to controls. Maximum ETS capacity (ETS) and CI OXPHOS were both increased from day 3 onwards and respiration after oligomycin (State 4 or CI+II Leak) and ratio between ETS and State 4 were increased at day 6/7 compared to controls. Moreover, non survivors had significantly higher ETS compared to survivors and controls. The authors concluded that there was no detectable functional inhibition of respiratory complexes in septic patients in platelets and that temporal increase of platelet mitochondrial respiration is negatively associated with clinical outcome in patients with sepsis (126).

Whilst platelets have been reported to have enhanced mitochondrial respiration in sepsis/septic shock (126), PBMC mitochondrial respiration function has similarities to skeletal muscle mitochondrial respiration in patients with septic MODS (25), (325). This is relevant for translational studies in patients because peripheral blood is more readily and safely accessible than vital organs such as liver biopsies (326) and skeletal muscle biopsies and may be used as a proxy for mitochondrial respiration in vital organs.

8.4.3.1 This study in relation to mitochondrial respiration in experimental models of MODS

In this study, MODS was divided into septic and non septic MODS. Hence, this section will discuss experimental models of septic and non septic MODS in relation to this study's findings. Although, no experimental model of septic MODS exists, several experimental studies have been done in sepsis. In sepsis, mitochondrial abnormalities were reported in experimental studies as far back as 30 years but there is still considerable variability in

findings from these studies (92). In sepsis, some studies reported decreased mitochondrial respiration function whereas others reported increased function or no change. Studies that reported increased mitochondrial respiration function tended to be short term (327), (328), (329). Dawson *et al.*. gave a 70mg/kg injection of E.coli endotoxin to rats and serially measured mitochondrial respiration until a median duration of 4hrs. He reported either no change or enhanced mitochondrial respiration from liver, skeletal muscle and cardiac muscle (328).

There are many reasons why these discrepancies exist for example, differences in species studied, the degree of injury in the experimental model, different organs used to measure mitochondrial function, varied techniques used to measure mitochondrial function (330), and the time of the organ biopsy in relation to evolving organ dysfunction (92), (331). Whilst this variability in findings exist, most long term models of sepsis have shown decreased mitochondrial respiratory function (331).

The two main causes for non septic MODS in this study were haemorrhage and cardiac arrest. In animal models of hemorrhagic shock, decreased State 3 respiration in the presence of CI (glutamate, malate) and CII (succinate) substrates was described in 1971 (332). Haemorrhagic shock was induced in rats by bleeding through a cannula in the femoral artery until blood pressure reached 30mm Hg. Mitochondria was obtained from livers from 30mins to 6hrs after onset of haemorrhagic shock and assays were performed at 25^{o}C. Oxygen uptake was measured in mitochondrial suspensions with either glutamate, malate or succinate and rotenone. State 4 respiration in the absence of ADP and State 3 respiration in the presence of ADP was measured and respiratory control ratio (RCR) was reported as State3/State 4. Initially, RCR with both CI and CII linked substrates were stable or even slightly increased but a decrease was apparent at 1hr, with continued decline to the end of the experiment at 6hrs (332).

Although there are no experimental models of cardiac arrest induced MODS, there are experimental in vivo models of cardiac arrest (333) (334) and in vitro laboratory models of cardiac arrest (335) and models of myocardial ischaemia induced by occluding one of the coronary arteries (336). In a mouse model of cardiac arrest, 7-12 week old male mice were anaesthetised and asystole was induced for 12 minutes with intravenous potassium chloride (334). Cardiopulmonary resuscitation was performed with rapid finger compressions and resumption of mechanical ventilation and intravenous adrenaline. A sham control group received surgery but did not receive cardiac arrest. Heart mitochondria were isolated and CI

activity was measured by NADH oxidation pre-arrest, 5 minutes and 60 minutes post CPR and no difference was found in CI activity at 5 or 60 minutes of CPR compared to pre-arrest (334). In an in vitro rat heart model of normothermic cardiac arrest, rat hearts were retrieved under anaesthesia and then perfused uniformly by the Langerdorff technique (335). The left atrium was then cannulated to allow atrial perfusion according to the working rat heart model. Normothermic ischaemic cardiac arrest (NICA) was induced by simultaneous clamping of both aortic and atrial supply tubes. Reperfusion was initiated by unclamping the supply of buffer through the aortic tube. Mitochondria were isolated from rat heart at the end of the experiment and oxidative phosphorylation was measured using the substrate glutamate. 10-20 minutes of NICA did not show any significant oxidative phosphorylation abnormality. In contrast, after 25 minuts of NICA, both subendocardial and subepicardial layers had decreased State 3 respiration with glutamate and prolongation of NICA caused a further decline in State 3 respiration with glutamate most markedly seen in mitochondrial from subendocardium. Increasing periods of NICA caused a progressive decrease in tissue ATP levels and reperfusion after 25-30 minutes caused a significant increase in tissue ATP levels even though it did not reach pre ischaemic levels (335). Similar to the above studies, this study also found decreased CI OXPHOS and CI,II OXPHOS in non-septic MODS.

8.4.4 Timing of mitochondrial dysfunction in MODS during the first week – this study's findings in relation to existing clinical and experimental studies

This study shows that the classically reported decrease in mitochondrial respiration function does occur in septic as well as non septic MODS. However, mitochondrial respiration does not decrease until day 3 or day 4 in septic and non septic MODS. In contrast, platelets were not found to have inhibition of mitochondrial respiration within the first week of septic shock and in fact, had increased ETS capacity and CI OXPHOS compared to controls (126). Given that platelets are the only tissue that have enhanced mitochondrial respiration in sepsis, it is likely that platelet mitochondria behave differently to PBMC, skeletal muscle and liver in sepsis and septic shock.

Whilst no other detailed temporal study of mitochondrial respiration in MODS exists in humans, Brealey *et al.* (326) designed a 3 day faecal peritonitis rat model of sepsis to simulate the human condition and produced a spectrum of disease from mild to severe. Mitochondrial complex activities (CI, CII/III, CIV) was measured spectrophotometrically at 30^{o}C from liver and skeletal muscle and demonstrated decreased CI activity from both tissues at 24 hours but not at 4 hours and no difference in CIV activity in severe sepsis. The authors concluded that mitochondrial dysfunction is progressive and relates to disease severity in both vital (liver)

and non vital (skeletal muscle) organs. The limitation of the experimental study was that it was comparatively shorter in time than the current clinical study and none of the rats with severe sepsis survived to the 72h time point. Likewise, in this study CI OXPHOS was significantly decreased at day 4 in septic MODS. CI related mitochondrial respiration has been previously correlated with the severity in sepsis in PBMC(125) and in skeletal muscle (25) and findings from this study provide further evidence that CI is affected in septic MODS. Postulated mechanisms of inhibition of CI involve damage by reactive oxygen and nitrogen species (337), (338), (293).

8.4.5 Relationship between mitochondrial ROS and mitochondrial respiration over time in patients with MODS

In this study, mitochondrial respiration and mitochondrial superoxide correlated with each other over the course of the week in patients with MODS. Mitochondria are the major producers of intracellular reactive oxygen species (ROS) in the form of mitochondrial superoxide and hydrogen peroxide, which are released from respiratory complexes I and III of the ETS (339). Although ROS may provide regulatory feedback from mitochondria in health (340), in pathological settings ROS may increase to outstrip cellular antioxidant defences (340), resulting in cellular and tissue damage (341-343). Paradoxically ROS producing mitochondria become targets of their own ROS, which ultimately may impact oxidative ATP production from the ETS. ETS dysfunction can further enhance ROS production, establishing a vicious cycle of cellular damage (340).

At day 3, ROS returned to the level of healthy volunteers for the first time (Figure 8-2) and concurrently, there was also a decrease in mitochondrial respiration seen in MODS at day 3 (Figure 8-1). When the relationship between ROS and respiration was further investigated, it became apparent that mitochondrial respiration and mitochondrial ROS strongly correlate with each other over time (Figure 8-3). It is plausible that the high levels of ROS at day 1/day 2 leads to the decrease in mitochondrial respiration from day 3 and decreased mitochondrial respiration at day 3 in turn leads to decreased levels of ROS (Figure 8-3). This would not be surprising as mitochondrial respiration dysfunction is known to be intimately linked to oxidative stress. Superoxide can inhibit complex I by oxidative damage to cardiolipin which is required for complex I functioning (344) and the raised superoxide is capable of reacting with nitric oxide to generate peroxynitirite and other nitrogen species that are able to alter the structure and function of several other mitochondrial proteins particularly complex I (293).

The level of mitochondrial superoxide at day 1 also negatively correlated with the proportion of total OXPHOS attributable to CII (CII OXPHOS/CI,II OXPHOS) at day 4 and a similar trend was evident with day 3 G3P in septic MODS (Figure 8-10A, Figure 8-10B). Thus, we have shown that the level of insult created by ROS early in septic MODS is inversely proportional to specific mitochondrial respiration states later in septic MODS (day 3 and day 4).

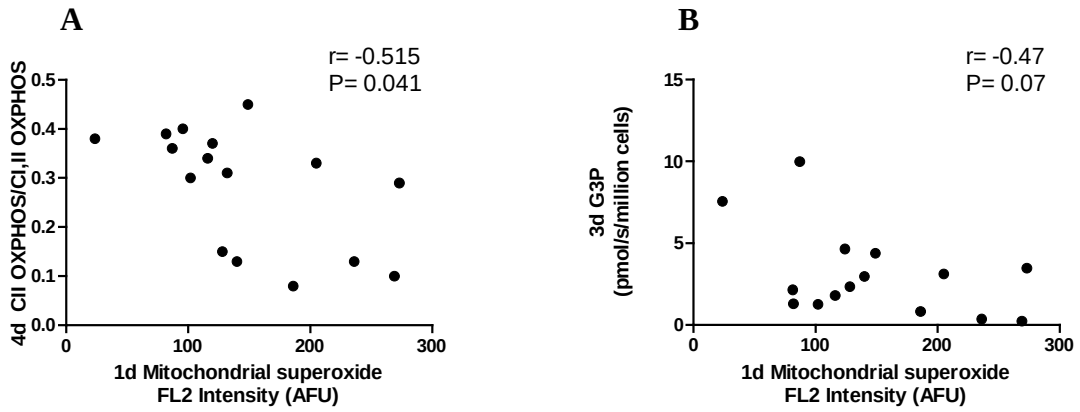


Figure 8-10A-B. Relationship between early mitochondrial superoxide and mitochondrial function later in the disease state in patients with septic MODS**.** Correlation between day 1 mitochondrial superoxide with fraction of OXPHOS attributable to CII at day 4 (A) and day 3 electron transport system capacity with glycerol-3-phosphate (B). 'r' Pearson's correlation coefficient.

8.4.6 Change in ROS over time may partly explain the failure of antioxidant interventions in patients with sepsis and MODS

Despite numerous experimental animal trials reporting antioxidants to be beneficial in sepsis (233), clinical trials of antioxidants in the critically ill have generally failed to show improved mortality and a recent large randomized clinical trial have even reported a non statistically significant increase in mortality when critically ill patients were supplemented with glutamine (345). The temporal findings from this study highlight that levels of mitochondrial superoxide change during the course of the disease. Since the clinical trials did not measure oxidative stress in patients prior to inclusion, it is possible that patients that were included had low levels of oxidative stress prior to commencing therapy with anti-oxidants. Future studies could use PBMC mitochondrial ROS measurements to confirm the status of study subjects before commencing treatment.

The dynamic nature of oxidative stress in MODS and its relationship to mitochondrial respiration may offer further explanation for the failure of antioxidants to improve mortality in patients with MODS. Experimental studies have reported that antioxidants decrease oxidative stress and augment mitochondrial respiration (233). In contrast, it was only when mitochondrial respiration was depressed at day 3 and day 4 that ongoing levels of ROS

decreased for the first time to HV levels (Figure 8-2). The shut down of mitochondrial respiration along with its low ROS output was associated with recovery from MODS in patients. Therefore, it is possible that augmenting mitochondrial respiration by antioxidant therapy may pose harm to patients.

8.4.7 Depressed mitochondrial respiration is an adaptive response in MODS?

It appears that the depressed mitochondrial respiration is a response to MODS rather than a causative factor for MODS. As set out by Hill's criteria for causation, one of the minimal conditions necessary for a causal relationship to exist is temporality. In applying this principle, if depressed mitochondrial respiration was causal for MODS, mitochondrial respiration would be expected to decrease before MODS occurred. As shown by the data presented here, this is clearly not the case. It is not until day 3 of MODS that mitochondrial respiration was depressed.

Interestingly, whilst mitochondrial respiration was depressed, mitochondrial membrane potential was maintained at the level of HV and ROS was no different to HV. Taken together, these findings in PBMC at day 3/ day 4 is consistent with an adaptive state in which affected organs shut down and the cell shifts its focus to survive as a defense mechanism (92),(346). Although this is the first human study describing temporal mitochondrial respiration function in MODS, the hypothesis that MODS is a state of hibernation/asestivation has been prevalent for a decade (347). It is hypothesised that prolonged systemic inflammation with overproduction of cytokines, reactive oxygen species, reactive nitrogen species production and associated tissue hypoperfusion leads to the organs switching off their energy consuming processes and a decrease in mitochondrial respiration may be an attempt of the organs to prevent irreversible damage (326). It is also known that excessive ROS can cause damage to the protein complexes within the electron transport system particularly CI which would also in turn cause a "shut down" (281). It is plausible that the state of shut down may be beneficial in MODS but whether the timing of the shut down may be manipulated or whether patients may benefit from instituting a state of shut down earlier than day 3/day 4 in MODS remain unanswered.

8.4.8 Short lived shut down of mitochondrial respiration within the first week in MODS

Although decreased net ATP and increased mitochondrial superoxide were evident at the end of the week, the shut down in mitochondrial respiration was short lived in MODS. The shut down of mitochondrial respiration at day 3/day 4 was followed by an increase in CI OXPHOS

and CI,II OXPHOS back to HV levels towards the end of the first week. Whether this recuperation of mitochondrial respiration is permanent is unknown and will be addressed in the next Chapter.

The short duration of the shut down of mitochondrial respiration during the first week of MODS reported in this study may be due to the nature of the cohort of patients within this study with a median ICU stay of three days. At the end of the week, the increase in CI OXPHOS and CI,II OXPHOS coincided with an increase in ROS and an increase in mitochondrial membrane potential. This recuperation of cellular bioenergetics occurred at the same time as patients improved from MODS clinically and may represent the arousal of cells from a state of shut down (326). Further studies now need to evaluate whether implementing this reversal artificially is feasible and whether it has the potential to improve outcomes in MODS.

8.4.9 Mitochondrial respiration is similar between septic and non septic MODS

We have also shown in this study that mitochondrial dysfunction not only occurs in septic MODS but also occurs in non septic MODS. Moreover, the temporal pattern of mitochondrial dysfunction in non septic MODS is similar to septic MODS. Even though there were some differences in mitochondrial respiration with increased CI Leak and increased respiration with G3P in septic MODS but not in non-septic MODS, these were not statistically significant Although mitochondrial function has not been studied in non septic MODS, these results are not too surprising because the clinical syndrome of MODS and pathophysiology of MODS is similar regardless of whether it is septic or non septic in origin. Over many years of research, it is now becoming evident that the inflammatory response in septic and non septic MODS is similar (348).

8.4.10 Limitations

There were several limitations for this study and they will be discussed under assay related limitations and clinical study related limitations. Although it might be considered a limiting factor in the present study, monocytes and lymphocyte groups were not separated to measure mitochondrial function because cell sorting may have disrupted the cell to cell interaction necessary for activation of lymphocytes (147). In addition sorting cells into separate groups would have increased the time before measuring mitochondrial function. This is important in seeking to develop a practical and repeatable measure of mitochondrial function that may be used to assess the clinical trajectory of patients and response to treatments.

The mitochondrial respiration, mitochondrial superoxide and mitochondrial membrane potential could only be measured on fresh cells and took approximately 4 hrs per specimen. Addtionally, a maximum of three oxygraphs were available at any one time for measuring mitochondrial respiration. These constraints resulted in a maximum of three blood samples to be processed per day limiting the number of patients that could be enrolled at any one time in this study.

Another limitation of this study was that only peripheral blood mitochondrial function was studied and not organ mitochondrial function and therefore extrapolating PBMC findings to other organs should be done with caution. Obtaining daily organ biopsies from vital organs in critically ill patients will have been unethical due to the high risks posed to those patients. Decreased mitochondrial respiration has been described previously by other authors (25, 118) at one time point in patients with MODS and since MODS is a systemic process it is likely that the other organs will also have similar temporal trajectories in MODS.

There were several limitations with regard to the clinical study. The inclusion of this study required patients to have established MODS therefore what happens to mitochondrial respiration pre MODS remains unknown. This limitation was partly overcome by understanding mitochondrial respiration in a mild inflammatory disease (Chapter 7) which included patients with mild acute pancreatitis without MODS. Whilst the non-septic MODS was a heterogenous group of patients with haemorrhagic shock and cardiogenic shock and may be seen as a limitation, studies of critical illness often enrol patients with variety of different diseases which is thought to increase the external validity of these studies ((324, 349, 350).

Overall, there were a limited number of patients in this study and as such, even when there were strong trends in some findings, statistical significance was not achieved due to multiple comparisons. The number of non surviving patients in this study was only five and therefore the differences between survivors and non survivors found in this study need to be reproduced. The role of G3P as a mitochondrial fuel has never been studied in sepsis. In non-survivors, respiration with G3P was almost double compared to CI+II OXPHOS (Figure 9-1A). If this finding is reproduced, respiration with G3P has the potential to be an important predictor of early mortality in patients with MODS. In addition, patients that died within the first week (n=3) unavoidably had missing data and therefore could not be included from the temporal data analysis which may have added a selection bias to the temporal analysis.

The healthy volunteers were unable to be matched by ethnicity to the study group. The healthy volunteers were mainly European in descent whereas there were significant Maori/Pacific Islanders in the patient group. This was despite considerable effort to recruit healthy volunteers from the hospital and university campus. This most likely reflects the over representation of Pacific/Maori in intensive care units in NZ (351).

8.4.11 Conclusion

In this study, for the first time, temporal mitochondrial function was comprehensively described in patients with MODS and was shown to correlate with severity of MODS. Mitochondrial respiration was depressed but not until day 3/day 4 in MODS. In contrast, mitochondrial superoxide was raised from day 1 of the study. There was a strong association between mitochondrial respiration and mitochondrial superoxide over time in MODS. The pattern of mitochondrial function over the first week was found to be similar in both septic and non septic MODS. Mitochondrial respiration improved at the end of the first week in non septic MODS but not in septic MODS.

Chapter 9 Long term PBMC mitochondrial function in patients with MODS

9.1 Introduction

In Chapter 8, the hypotheses that mitochondrial dysfunction occur in PBMC in MODS was successfully tested and it was also shown that mitochondrial respiration correlated with mitochondrial superoxide over time and that temporal mitochondrial respiration correlated with the severity of MODS. At the end of the week, there was some recovery of mitochondrial respiration but there were still features of mitochondrial dysfunction in MODS with decreased net ATP and increased mitochondrial superoxide. Particularly in septic MODS, supranormal CI Leak and respiration with G3P persisted at the end of the week. However, what happens to mitochondrial function in the mid and long term as patients recover from MODS is not known.

Although no experimental or clinical studies have measured mitochondrial function in the long term, there may be persistent mitochondrial dysfunction in the long term as patients recuperate from MODS. This is because, ICU survivors have important long term sequelae such as reduced physical and emotional functioning, decreased quality of life, increased use of healthcare resources and persistently abnormal pulmonary function tests (352). Patients admitted to ICU due to severe sepsis have worse QOL and physical functioning as measured by the Short Form 36 (SF-36) after discharge over time compared with sex and age matched controls (353) and another review which included 7320 ICU survivors also reported lower QOL including physical functioning (354). When patients were followed up 5 years after their admission to ICU with acute respiratory distress syndrome, the mean score on the physical component of the SF-36 at 5 years remained approximately 1 standard deviation below the control group (355).

Although there is no direct evidence implicating mitochondrial dysfunction to decreased long term quality of life and decreased physical functioning in patients with MODS, there may be an indirect link through ICU acquired weakness. ICU acquired weakness is associated with decreased long term function and quality of life outcomes in survivors of acute respiratory distress syndrome (ARDS) (355). One of the postulated molecular mechanisms of ICU acquired weakness is mitochondrial dysfunction (356).

9.2 Hypothesis

To evaluate whether long term physical functioning and quality of life is associated with mitochondrial dysfunction, the first step was to establish whether mitochondrial dysfunction persists in the long term in critically ill patients with MODS. Therefore, the hypothesis for this study was that mitochondrial dysfunction persists long term in patients with MODS.

9.3 Aim

The aim of this study was to measure and describe the longitudinal changes in PBMC mitochondrial function at 3 weeks and 6 months in the same patients with MODS that were included in Chapter 8.

9.4 Method

9.4.1 Clinical study

Patients were included in this study from Auckland City Hospital, Department of Critical Care Medicine and the clinical study is described in detail in Chapter 5.4.2. Patients included were followed up once at 3 weeks and once at 6 months from inclusion.

9.4.2 Preparation of blood mononuclear cells

The time points for blood taking were 3 weeks (3w) and at 6 months (6m). Blood was taken once from healthy volunteers.

Blood was collected as described in section 5.1.1 and peripheral blood mononuclear cells (PBMC) were retrieved as described in section 0.

9.4.3 Respiration assays - Assays of the mitochondrial electron transport system

A multiple substrate-inhibitor titration protocol described in section 5.1.4 was employed.

9.4.4 Assessment of mitochondrial DNA

Mitochondrial DNA was quantified using real-time PCR as described in section 5.2.1.

9.4.5 Measurement of superoxide using fluorescent dye MitoSOX red

MitoSOX red (Invitrogen, Auckland, NZ), a mitochondrial targeted superoxide indicator (20) was used to detect superoxide in PBMC as described in section 5.2.2.

9.4.6 Measurement of mitochondrial membrane potential using JC-1 using flow cytometry

Mitochondrial membrane potential ($\Delta\Psi$) was measured in PBMC as described in section 5.2.3.

9.4.7 Assessment of intracellular ATP

To determine whether ATP fluctuated over the disease state, intracellular ATP was measured as described in section 5.2.4.

9.4.8 Intracellular protein

To normalise the ATP measurements, intracellular protein was measured as described in section 5.2.5.

9.4.9 Cytochrome c

Cytochrome *c* was measured was used as a marker of mitochondrial content and measured as described in section 5.2.6.

9.4.10 Statistical analysis

Statistical analysis was carried out using Graph Pad Prism version 4.00 and SPSS version 18 for Windows (GraphPad Software , San Diego, California, USA). Values are expressed as the median and interquartile range or mean ± standard error of mean (SEM) where appropriate. For temporal mitochondrial function, repeated measures of analysis of variance (RANOVA) with a post-hoc least significant difference (LSD) correction was used to evaluate statistical significance from day 1 to discharge. A student *t*-test was used to detect statistical significance between HV and each day of mitochondrial function in the patient group followed by a sequential Bonferroni correction for multiple comparisons. Where the data did not fit normality, the Mann Whitney U test was used when two groups were compared and Kruskal Wallis was used when more than two groups were compared. To analyse cytochrome *c* data within patients at admission and at 6 months, a paired student *t* test was used. Significance was established at $P < 0.05$ and is reported as $p<0.05$, $p<0.01$, $p<0.005$ and $p<0.001$ where appropriate.

9.5 Results

9.5.1 Patient characteristics in MODS

20 out of 22 survivors were followed up at 3 weeks and at 6 months (Table 9-1). 3/22 patients had APACHEIIC (chronic health portion of APACHEII score) of greater than zero, two with APACHEIIC of 5 and one with APACHEIIC of 2 (Table 9-2).

Table 9-1 Patient and healthy volunteer demographics

	HV (15)	**MODS (20)**	**P**
Age (years) median (range)	51 (25-64)	59 (21-72)	0.48
Male : female ratio	6:9	13:7	0.15
Ethnicity	NZ European 13 Other 2	NZ European 12 Maori/Pacific 6 Other 2	0.09*

Footnote: Healthy volunteer (HV), Patients with multiple organ dysfunction syndrome (OF), P-value student *t-test* (P), *Ethnicity divided up into NZ European and non NZ European for *t-test*.

Table 9-2 Organ failure scores and characteristics of patients who were followed up

	OF (20)
SOFA (first 24 hrs)	8.5 (3-11)
APACHEII	23.5 (13-41)
APACHEIIC Chronic	0 (0-5)
SAPS	40.5 (15-86)
No. of failed organs	2 (2-4)
ICU length of stay (days)	3 (1-100)
Hospital length of stay (days)	11 (1-200)

Footnote: Patients with multiple organ dysfunction syndrome (OF), Sequential organ failure assessment (SOFA), Acute Physiology and Chronic Health Evaluation II (APACHE II), Simplified acute physiology score (SAPS), Intensive care unit (ICU).

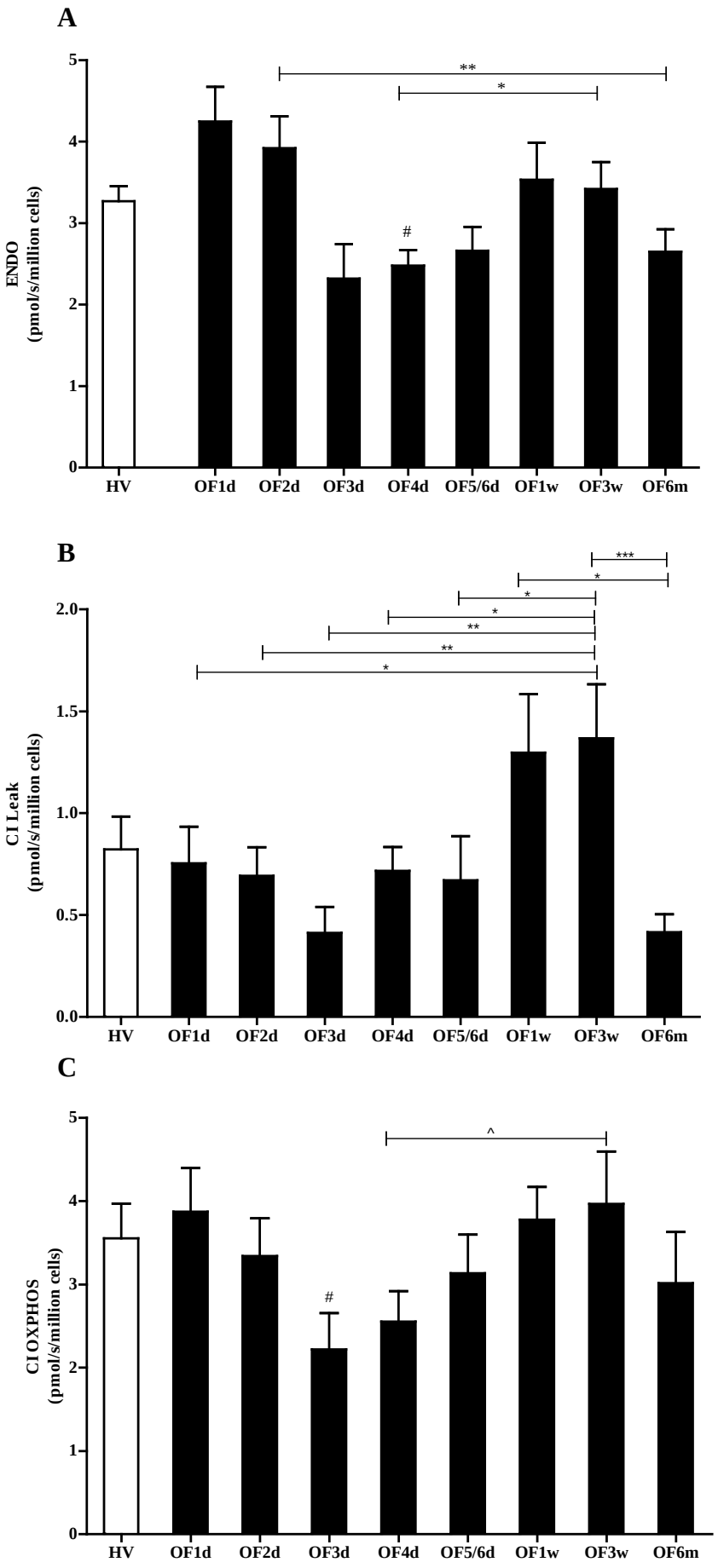
9.5.2 Intermediate and long term mitochondrial function changes in MODS

There were no significant differences between patients with MODS and HV at 3 weeks or 6 months in any of the mitochondrial respiration parameters (Figure 9-1). But, there were significant temporal differences in mitochondrial respiration within patients over time. Endogenous respiration varied over time within the OF group (P=0.044, d.f.=3.903, *RANOVA*) (A). By 3w, endogenous respiration was no different from 1d and 2d but was increased compared to 4d and at 6m, endogenous respiration was decreased compared to 2d (Figure 9-1A).

There was a significant difference in CI Leak over time within the OF group (P=0.002, *RANOVA*) (Figure 9-1B). CI Leak was increased at 3w compared to days 1-6. At 6m, CI Leak was decreased compared to 3w. Although not significant on *RANOVA* testing, CI OXPHOS was decreased at 4d compared to 3w (4d vs. 3w P=0.021, *pairwise LSD*) (Figure 9-1C). ETS changed over time within the OF group (P=0.044, d.f.=3.903, *RANOVA*). At 3w, ETS capacity is no different from 1d and is increased compared to 3d, 4d and 5/6d. However at 6m, ETS capacity was decreased compared to 3w.

There was an overall difference in G3P over time within the OF group (P=0.043, *RANOVA*) (Figure 9-1F). G3P was raised at 3w compared to days 1-6.

CI OXPHOS/CI Leak was temporally significantly different within the OF group (P=0.025, *RANOVA*) (Figure 9-1G). CI OXPHOS/CI Leak is decreased at 3w compared to 2d and 3d, but at 6m CI OXPHOS/CI Leak is increased compared to 3w. The changes seen in CI OXPHOS/CI Leak was consistent with the changes found in CI Leak over time. Although there were no overall temporal changes in CI,II OXPHOS/CI,II Leak with *RANOVA*, on pairwise LSD testing CI,II OXPHOS/CI,II Leak was significantly decreased at 6m compared to 5/6d (5/6d vs. 6m P=0.037, Figure 9-1H) and relates to the persistent decrease in CI,II OXPHOS at 6m in patients with MODS which was not statistically significant (Figure 9-1D). Similarly, on *pairwise LSD* but not on *RANOVA*, CI,II OXPHOS/ETS was decreased at 3w compared to 2d (2d vs. 3w P=0.022, *pairwise LSD*) and at 6m compared to 5/6d (5/6d vs. 6m P=0.010, *pairwise LSD*) (Figure 9-1I).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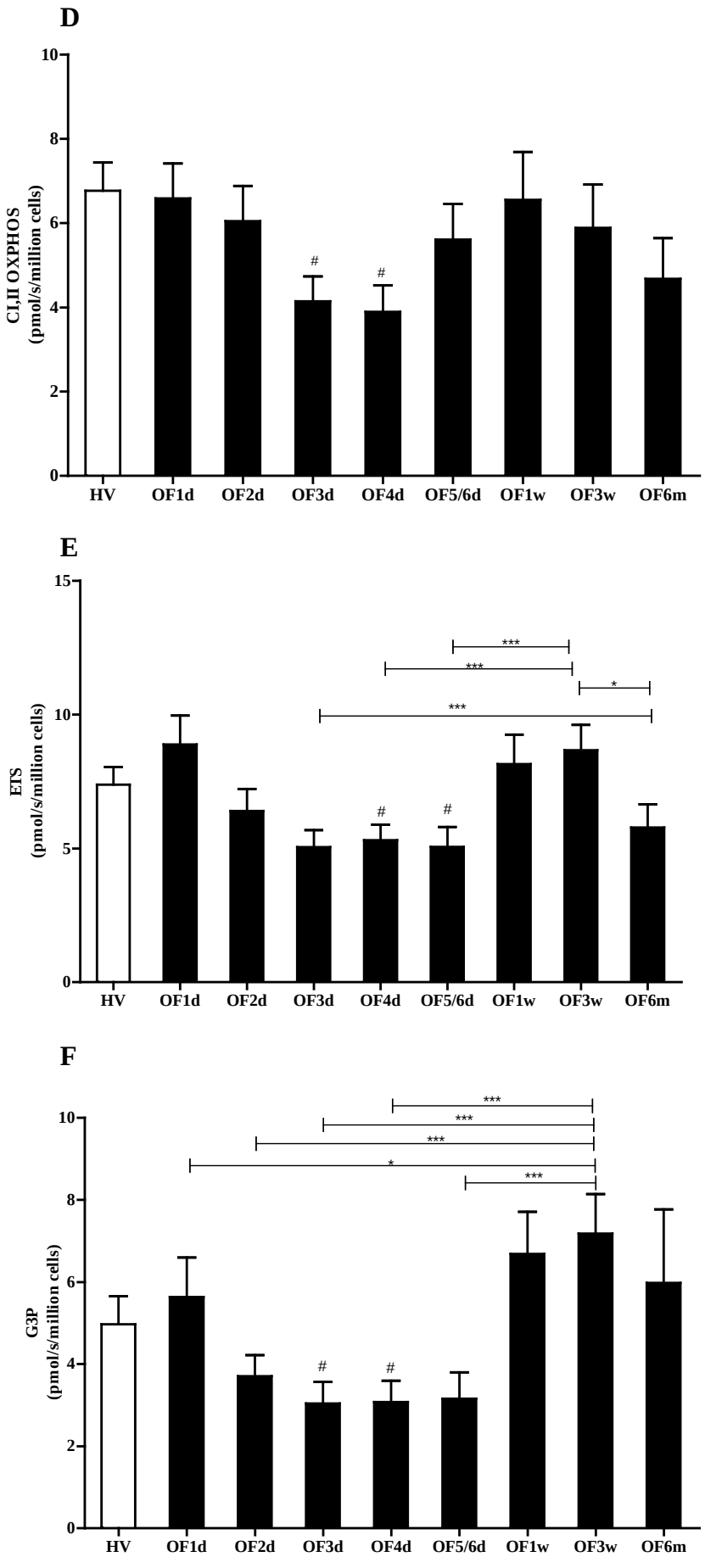
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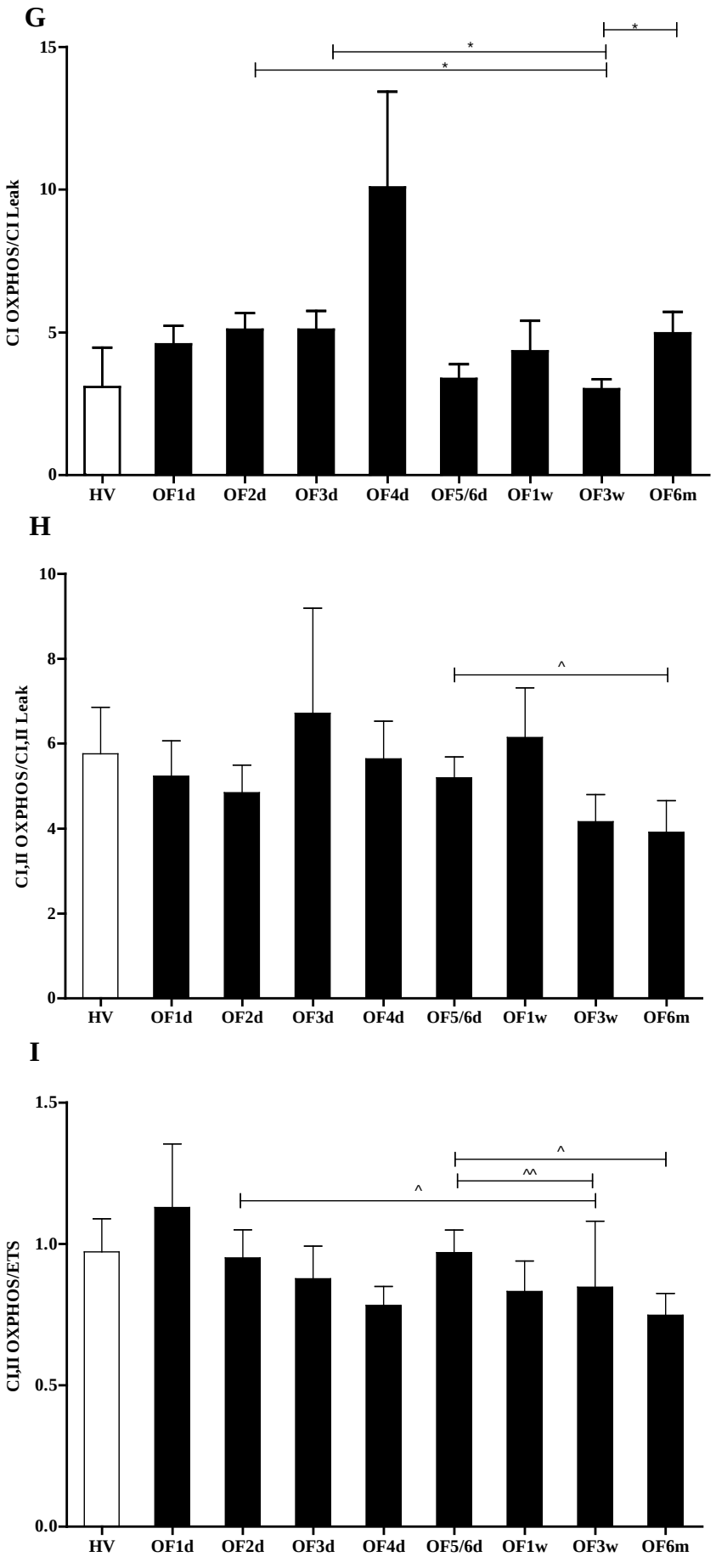
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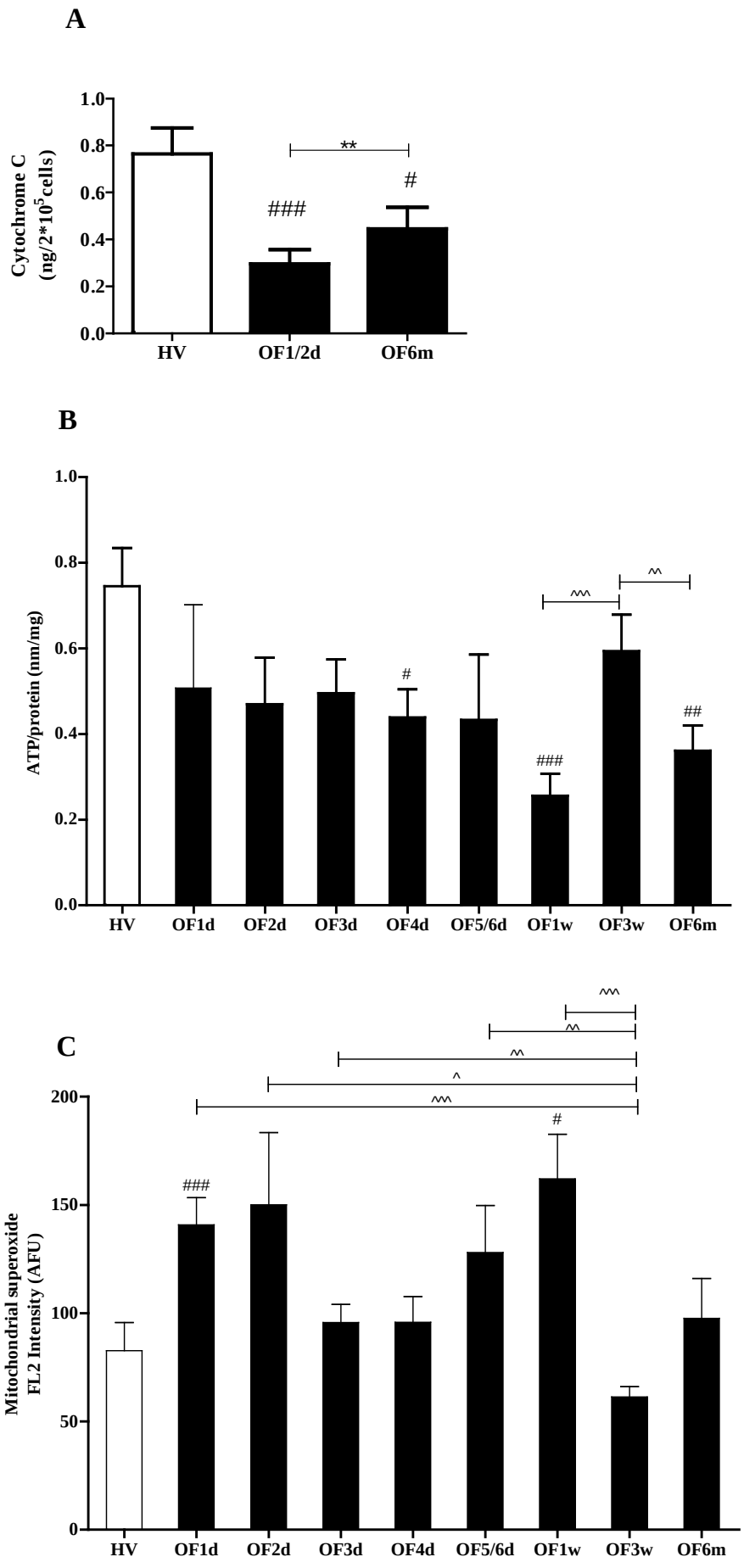
Figure 9-1A-I. Long term mononuclear cell mitochondrial respiration and flux control ratios in patients with MODS. Healthy volunteers (HV, white bars, n=15) and patients with multiple organ dysfunction at different time points, black bars (n=27 at 1d, n=25 at 2d and n=22 from 3d-1w, n=20 at 3w and 6m). Different respiratory states measured were: A) Endo [Endogenous respiration], B) CI Leak C) CI OXPHOS [Complex I oxidative phosphorylation] D) CI,II OXPHOS [Complex I and II oxidative phosphorylation], E) ETS [Electron Transport System capacity] and F) G3P [ETS capacity with Glycerol-3-phosphate]. Flux control ratios measured were: G) CI OXPHOS/CI Leak [measure of phosphorylation efficiency with CI substrates], H) CI,II OXPHOS/CI,II Leak [measure of phosphorylation efficiency with CI+II substrates] and I) CI,II OXPHOS/ETS [ratio of phosphorylation to electron transport system capacity].Values are mean ± standard error of mean. #comparison to HV (student's *t* test with sequential *Bonferroni* correction), *comparison within patients at different time points (*RANOVA* with least significant different correction (*LSD*)), ^ comparison within patients at different time points (least significant difference (*LSD*)) #/^/*P<0.05, ##/^^/** P<0.01, ###/^^^/*** P<0.005.

9.5.3 Other Mitochondrial function in MODS –long term follow up

Cytochrome *c* was decreased by 42% at 6m (P=0.03) in patients with MODS compared to HV. On paired *t* test, cytochrome *c* increased at 6m by 50% compared to 1/2d (p=0.0048) in MODS (Figure 9-2A). In MODS, net ATP was still decreased by 52% at 6m (P=0.0007) compared to HV (Figure 9-2B). Compared to HV, there were no differences in mitochondrial superoxide in MODS at 3 weeks or 6 months. Even though mitochondrial superoxide was decreased during the 3 week time point compared to the first week in MODS, these changes were only found on *pairwise LSD* and not on *RANOVA* (Figure 9-2C). Compared to HV (2.19+/-0.33), the aggregate monomer ratio was increased by 143% at 6m (5.31+/-0.90, P=0.008, MWU) in OF (Figure 9-2D). In MODS, there were significant differences in the aggregate monomer ratio within patients over time (P= 0.010, *RANOVA*). In MODS, during the first three weeks, the aggregate monomer ratio peaked on the 3d, then decreased on the 4d and was followed by an increase (4d vs. 5/6d P=0.003, 4d vs. 3w P=0.008, 4d vs. 6m P=0.003) within patients. There were no significant temporal differences in mtDNA during the study period (Figure 9-2E).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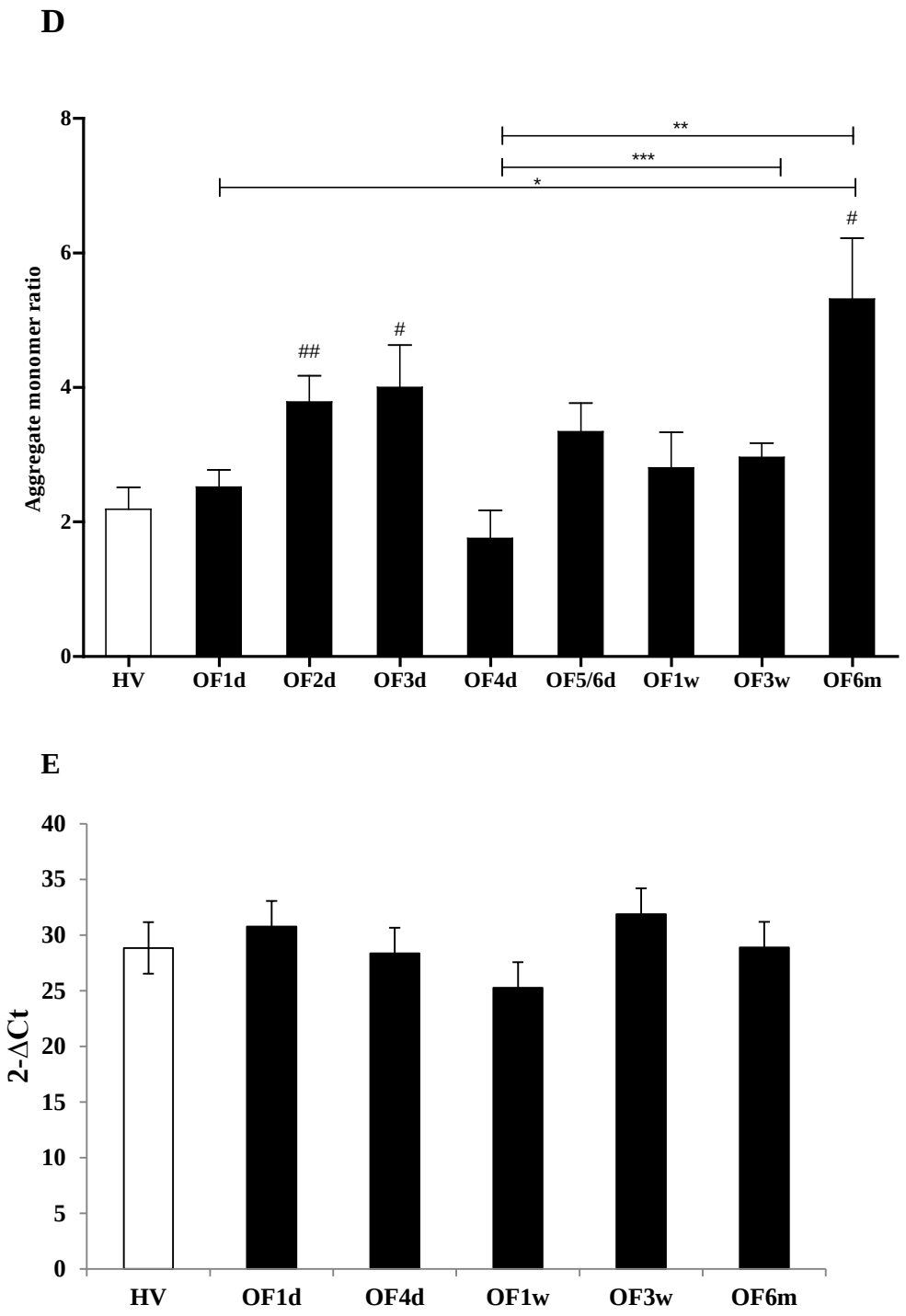


Figure 9-2A-E. Other long term mitochondrial function in MODS (black bars, n=27 at 1d, n=25 at 2d and n=22 from 3d-1w, n=20 at 3w and 6m) compared to healthy volunteers (white bars, n=15) at different time points. A) Mononuclear cell Cytochrome C B) Mononuclear cell adenine triphosphate (ATP) to protein ratio C) Mononuclear cell mitochondrial superoxide, D) Mononuclear cell mitochondrial membrane potential measured with the dye 5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolylcarbocyanine iodide (JC-1). JC-1 fluorescence was detected by flow cytometry in two channels FL 1 and FL 2. JC-1 exists as a monomeric form which fluoresces at FL 1 and when concentrated by actively respiring mitochondria exists as an aggregate which fluoresces at FL 2. An increase in the ratio of aggregate to monomer suggests higher mitochondrial membrane potential which in

turn generates increased mitochondrial reactive oxygen species such as mitochondrial superoxide. E) Relative quantification of mtDNA using $2^{-\Delta Ct}$. The mitochondrial gene MTCO1 (0188166_m1) and the nuclear gene RN18S1 (03928985_s1) was used as the internal control gene. Values are mean ± standard error of mean. #comparison to HV (student's *t* test), *comparison within patients at different time points (paired student's *t* test). #/* P<0.05; ##/** P<0.01; ###P<0.005.

9.5.4 Intermediate and long term follow up in septic and non-septic MODS – patient demographics

At both 3 weeks and 6 months, 11 patients were from the septic MODS and 9 patients were from the non septic MODS group (Table 9-3). There was one patient in the septic MODS with APACHEIIC (chronic health points) of 5 and there were two patients in the non septic MODS group with APACHEIIC of 2 and 5.

Table 9-3 Demographics of patients that were followed up in septic and non-septic MODS.

	Septic (11)	**Non septic (9)**
Age (years) median (range)	57(21-72)	60 (21-69)
Male : female ratio	7:4	6:3
Cause of OF	**Site of sepsis** Abdominal 3 Lung 2 Soft tissue 1 Urogenital 4 CNS 1	Ruptured aneurysm 2 Trauma+hemorrhage 3 Cardiac arrest 2 Intraoperative hemorrhage 2
SOFA (first 24 hrs)	9 (3-11)	7 (4-10)
APACHEII	22 (13-35)	24 (15-41)
APACHEIIC Chronic	0 (0-5)	0 (0-5)
SAPS	35 (15-81)	56 (28-86)
No. of failed	3 (2-4)	2 (2-4)

organs		
ICU length of stay (days)	3 (1-42)	3 (2-100)
Hospital length of stay (days)	10 (1-51)	12 (3-200)

Footnote: Multiple organ dysfunction syndrome (MODS), Sequential organ failure assessment (SOFA), Acute Physiology and Chronic Health Evaluation II (APACHE II), Simplified acute physiology score (SAPS), Intensive care unit (ICU).

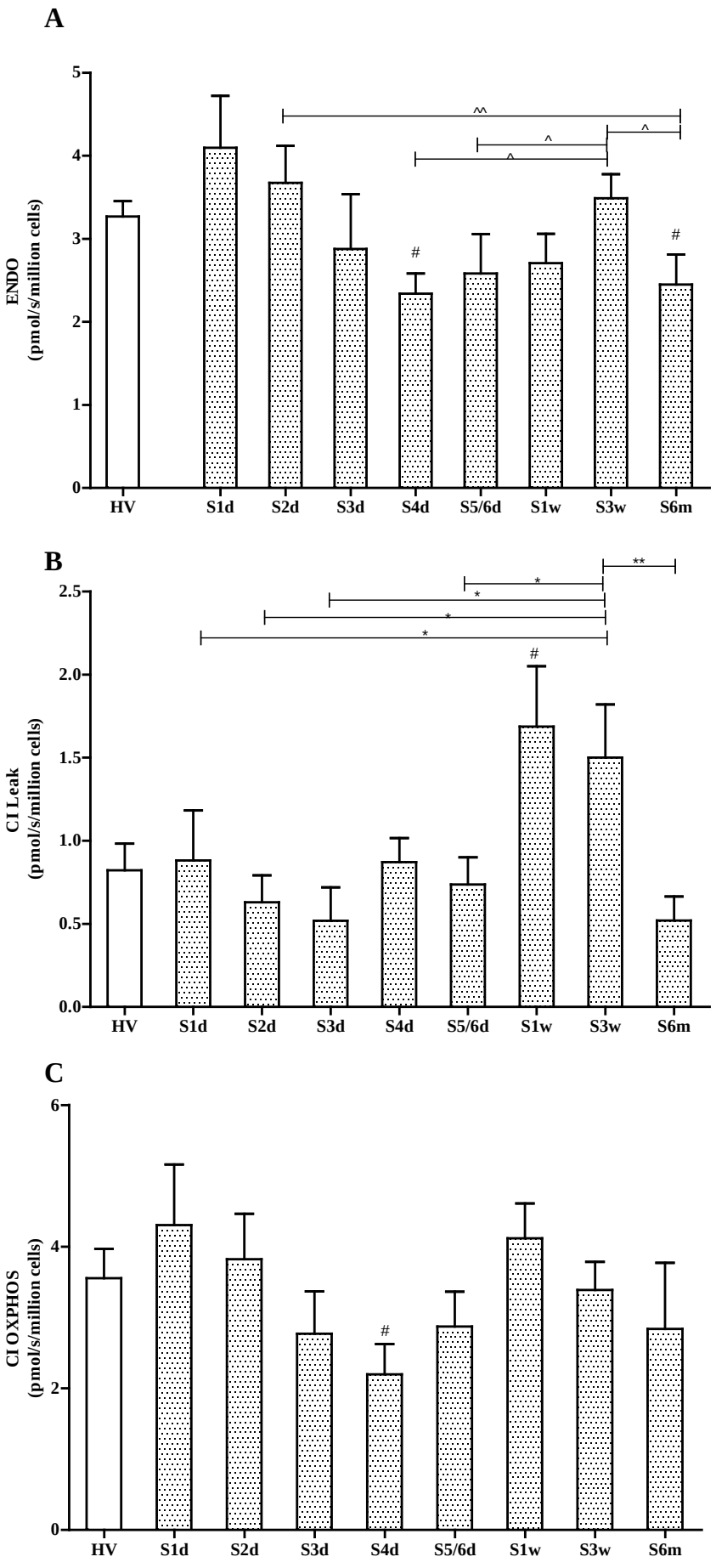
9.5.5 Septic MODS vs HV – intermediate and long term follow up

At 6m, endogenous respiration was still decreased by 19% compared to HV (P=0.04, Figure 9-3A) and CI,II OXPHOS was significantly decreased by 30% at 3w (P=0.03) and by 47% at 6m (P=0.02) compared to HV (Figure 9-3D). In keeping with this, CI,II OXPHOS/CI,II Leak was significantly decreased by 62% at 6m compared to HV (P=0.01, *MWU*, Figure 9-3H) and CI,II OXPHOS/ETS was significantly decreased by 44% at 3w (P=0.02) and by 35% at 6m (P=0.03) compared to HV (Figure 9-3I).

9.5.6 Long term mitochondrial function changes in septic MODS – within patients

There were significant temporal differences in CI Leak (P=0.03, *RANOVA*) and ETS (P=0.04, *RANOVA*) in patients with septic MODS (Figure 9-3B, and Figure 9-3E). CI Leak was raised at 1w and 3w compared to earlier in the disease state (Figure 9-3B). At 6m, CI Leak was decreased compared to 3w. ETS was decreased from 3d onwards and troughs at 5/6d followed by increases at 1w and 3w. At 6m ETS is significantly decreased compared to 3w (Figure 9-3E).

Although not significantly different on *RANOVA*, there were some statistically significant differences seen on *pairwise LSD* in the following parameters. Endogenous respiration was decreased at 6m compared to 2d and 3w (2d vs. 6m P= 0.008, 3w vs. 6m P=0.025) (Figure 9-3A). At 3w, endogenous respiration is significantly increased compared to 4d and 5/6d (4d vs. 3w P=0.049, 5/6d vs. 3w P=0.048). G3P was significantly increased at 3w compared to day 2 and 4 (3w vs. 2d P=0.023, 3w vs. 4d P=0.022) (Figure 9-3F). CI,II OXPHOS/CI,II Leak was significantly decreased at 6m compared to days 2, 3 and 5/6 (6m vs. 2d P=0.015, 6m vs 3d P=0.033, 6m vs. 5/6d P=0.002) (Figure 9-3H). CI,II OXPHOS/ETS was decreased at 3w compared to 3d and 5/6d (3w vs. 3d P=0.041, 3w vs. 5/6d P=0.021). CI,II OXPHOS/ETS was decreased at 6m compared to 5/6d (5/6d vs. 6m P=0.01) (Figure 9-3I).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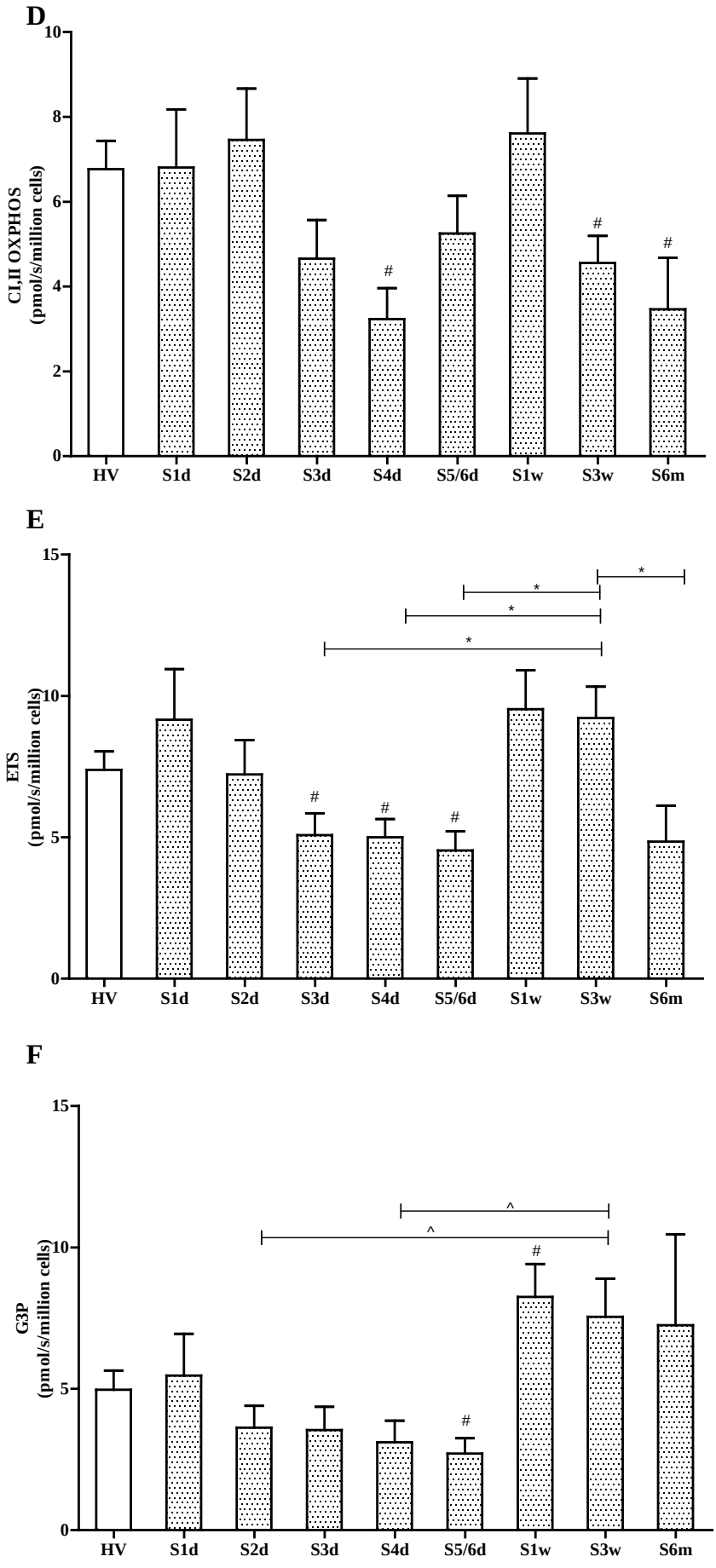
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*
*
*
#
#
#
HV
S1d
S2d
S3d
S4d
S5/6d
S1w
S3w
S6m
F
G3P
(pmol/s/million cells)
15
10
5
0
^
^
#
#
HV
S1d
S2d
S3d
S4d
S5/6d
S1w
S3w
S6m

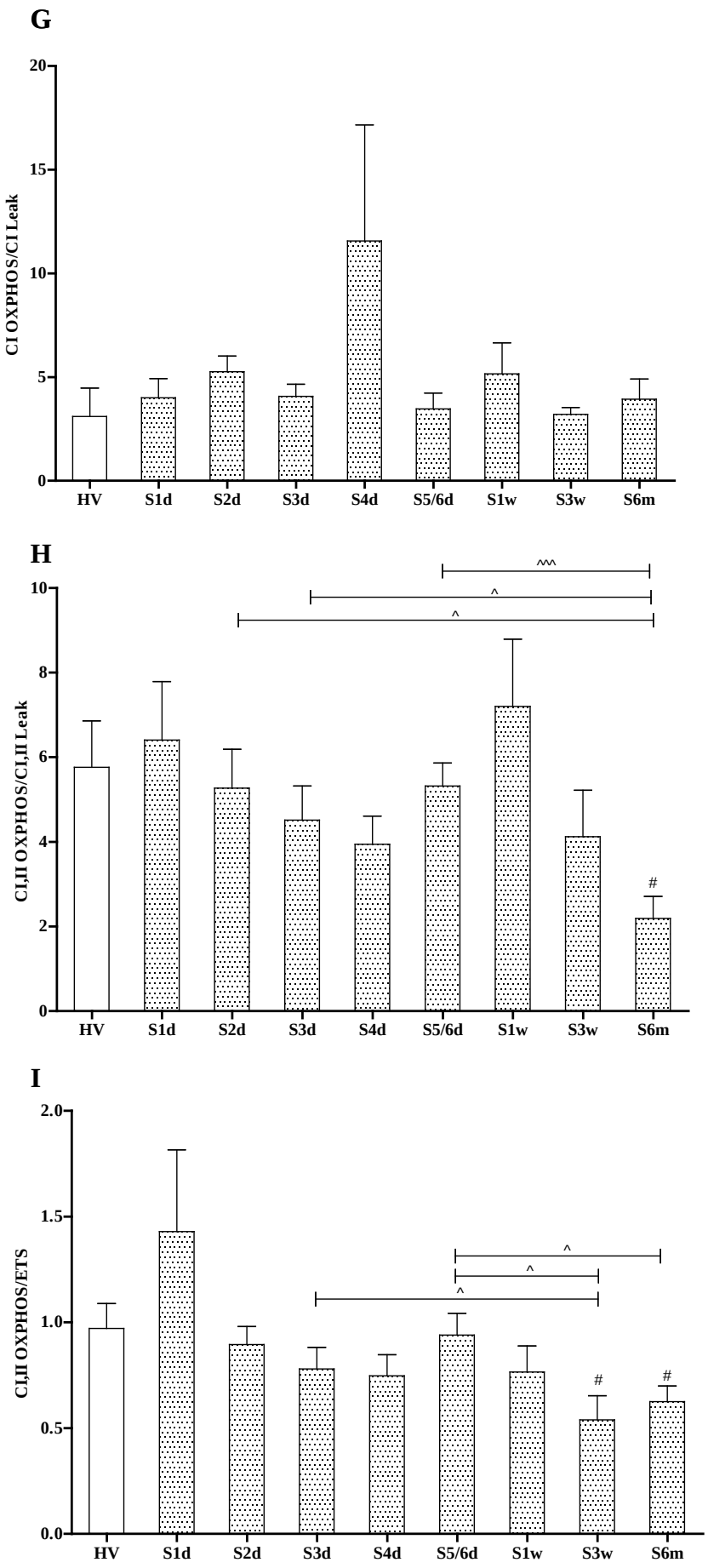
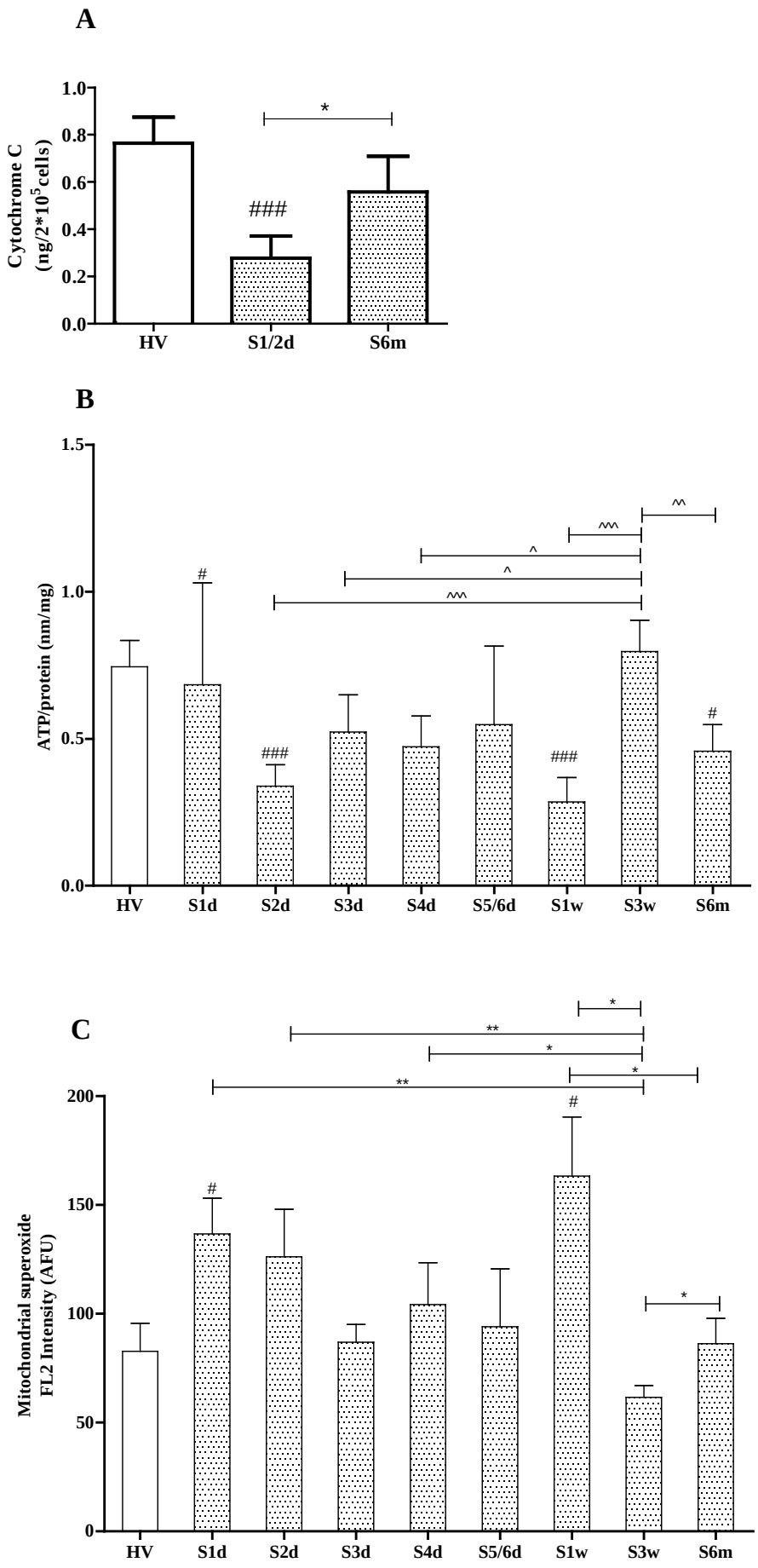

G
CI OXPHOS/CI Leak
20
15
10
5
0
HV
S1d
S2d
S3d
S4d
S5/6d
S1w
S3w
S6m
H
CI,II OXPHOS/CI,II Leak
10
8
6
4
2
0
^^^
^
^
#
HV
S1d
S2d
S3d
S4d
S5/6d
S1w
S3w
S6m
I
CI,II OXPHOS/ETS
2.0
1.5
1.0
0.5
0.0
^
^
^
#
#
HV
S1d
S2d
S3d
S4d
S5/6d
S1w
S3w
S6m

Figure 9-3A-I. Long term mononuclear cell mitochondrial respiration and flux control ratios in patients with sepsis related multiple organ dysfunction syndrome. Healthy volunteers (HV, white bars, n=15) and patients with septic multiple organ dysfunction at different time points, white bars with dots (n=15 at 1d and 2d, n=13 from 3d-1w, n=11 at 3w and 6m). Different respiratory states measured were: A) Endo [Endogenous respiration], B) CI Leak C) CI OXPHOS [Complex I oxidative phosphorylation] D) CI,II OXPHOS [Complex I and II oxidative phosphorylation], E) ETS [Electron Transport System capacity] and F) G3P [ETS capacity with Glycerol-3-phosphate]. Flux control ratios measured were: G) CI OXPHOS/CI Leak [measure of phosphorylation efficiency with CI substrates], H) CI,II OXPHOS/CI,II Leak [measure of phosphorylation efficiency with CI+II substrates] and I) CI,II OXPHOS/ETS [ratio of phosphorylation to electron transport system capacity].Values are mean ± standard error of mean. #comparison to HV (student's *t* test with sequential *Bonferroni* correction), *comparison within patients at different time points (*RANOVA* with least significant different correction (*LSD*)), ^ comparison within patients at different time points (least significant difference (*LSD*)) #/^/*P<0.05, ##/^^/** P<0.01, ###/^^^/*** P<0.005.

9.5.7 Other Mitochondrial function in septic MODS –long term follow up

In septic MODS, cytochrome *c* doubled at 6m compared to 1/2d (P=0.01) (Figure 9-4A). In sepsis, ATP was decreased by 39% at 6m (P=0.03) compared to HV (Figure 9-4B). Compared to HV, there were no differences in mitochondrial superoxide in septic MODS at 3 weeks or 6 months. In sepsis, there were significant differences in mitochondrial superoxide within patients over time (P=0.035, *RANOVA*) (Figure 9-4C). Mitochondrial superoxide decreased in the middle of the week at 3d and then increased again at the 1w mark. At 6m, mitochondrial superoxide increased compared to 3w. Compared to HV (2.19+/-0.33), the aggregate monomer ratio was increased in septic organ failure by 145% at 6m (5.37+/-0.32, P=0.015,MWU) (Figure 9-4D).


A
Cytochrome C
(ng/2*10^5 cells)
1.0
0.8
0.6
0.4
0.2
0.0
*
###
HV
S1/2d
S6m
B
ATP/protein (nm/mg)
1.5
1.0
0.5
0.0
#
###
^^
^
^^
^^
###
#
HV
S1d
S2d
S3d
S4d
S5/6d
S1w
S3w
S6m
C
Mitochondrial superoxide
FL2 Intensity (AFU)
200
150
100
50
0
*
**
*
*
**
#
#
*
HV
S1d
S2d
S3d
S4d
S5/6d
S1w
S3w
S6m

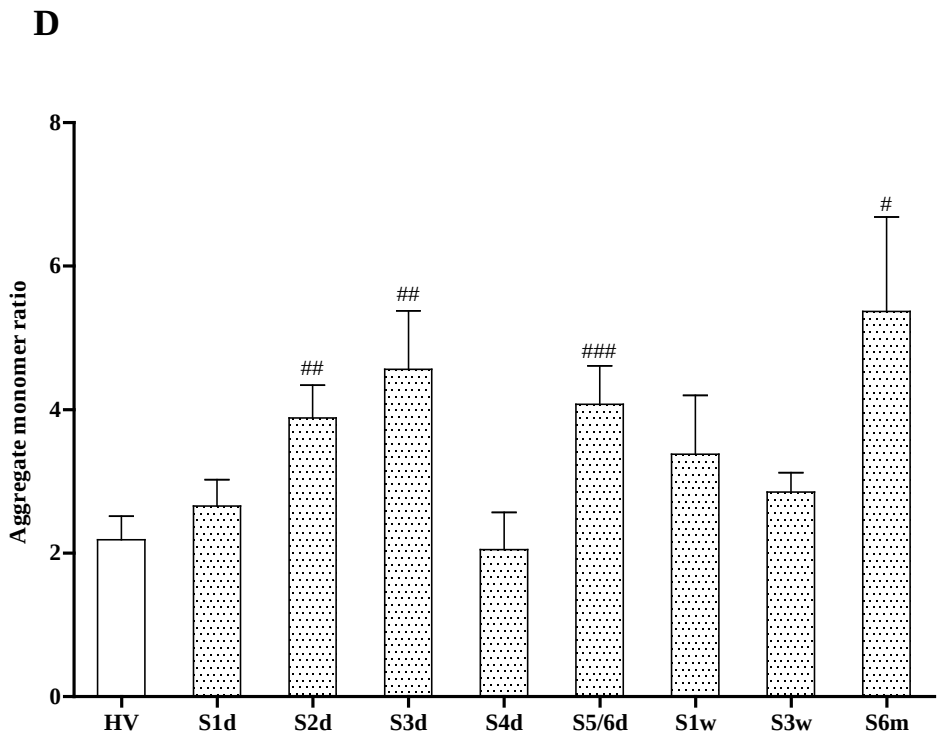


Figure 9-4 A-D. Other long term mitochondrial function in sepsis related multiple organ dysfunction syndrome (white bars with dots, n=15 at 1d and 2d, n=13 from 3d-1w, n=11 at 3w and 6m)) compared to healthy volunteers (white bars, n=15) at different time points. A) Mononuclear cell Cytochrome C B) Mononuclear cell adenine triphosphate (ATP) to protein ratio C) Mononuclear cell mitochondrial superoxide and D) Mononuclear cell mitochondrial membrane potential measured with the dye 5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolylcarbocyanine iodide (JC-1). JC-1 fluorescence was detected by flow cytometry in two channels FL 1 and FL 2. JC-1 exists as a monomeric form which fluoresces at FL 1 and when concentrated by actively respiring mitochondria exists as an aggregate which fluoresces at FL 2. An increase in the ratio of aggregate to monomer suggests higher mitochondrial membrane potential which in turn generates increased mitochondrial reactive oxygen species such as mitochondrial superoxide. Values are mean ± standard error of mean. #comparison to HV (student's *t* test), *comparison within patients at different time points (paired student's *t* test). #/* P<0.05; ##/** P<0.01; ###P<0.005.

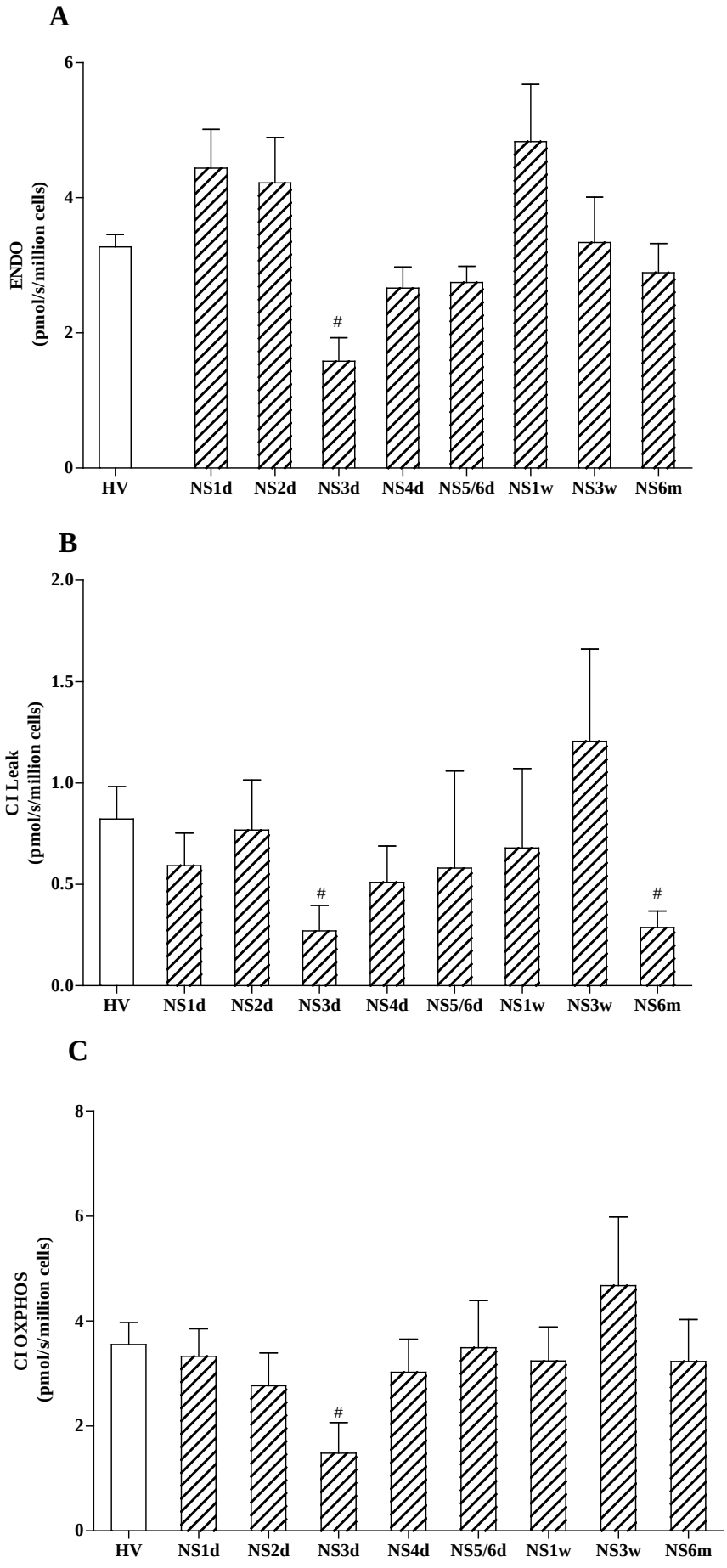
9.5.8 Intermediate and long term peripheral mitochondrial function changes in non septic organ failure compared to HV

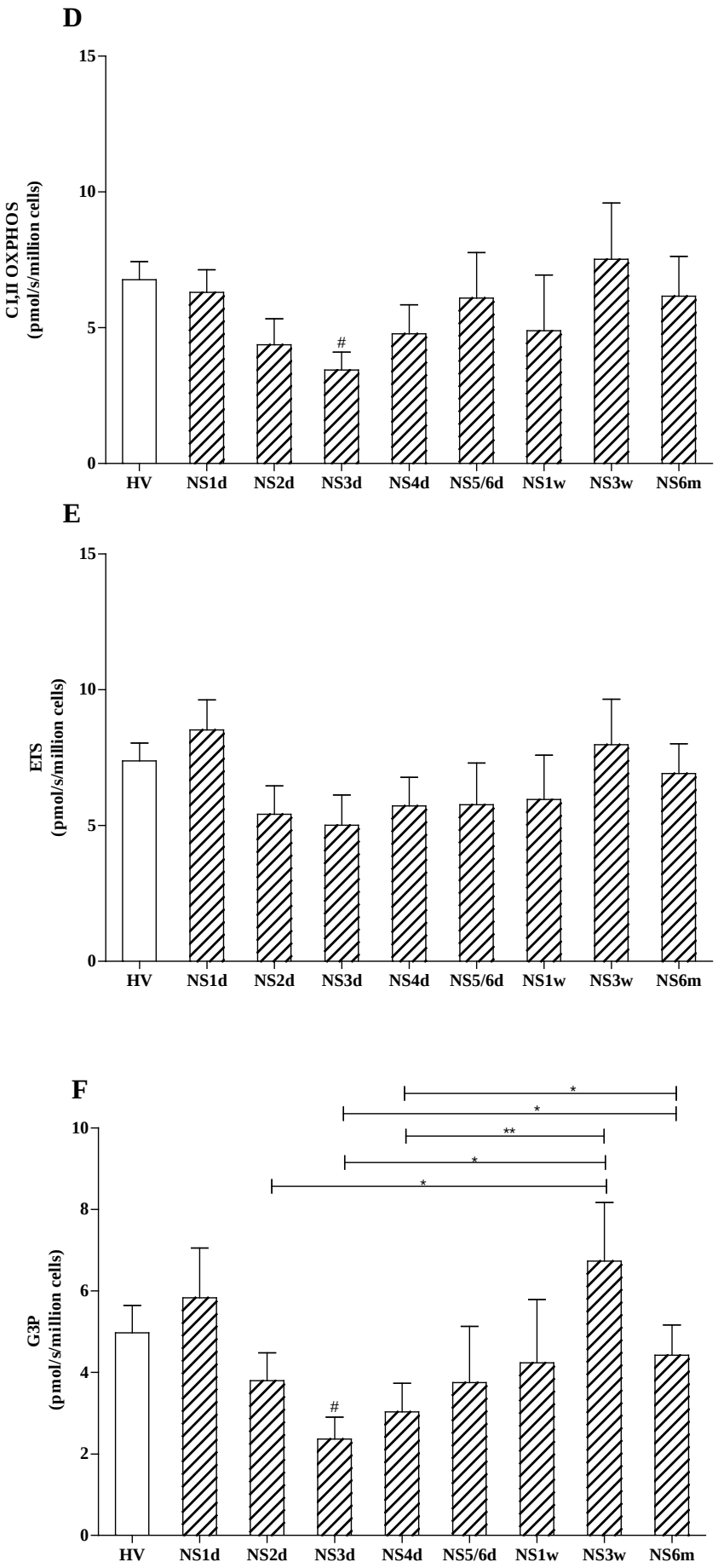
CI Leak was decreased at 6m (0.29+/-0.08 pmol/s/million cells, P=0.02, d.f.=21) compared to HV (0.82+/-0.16 pmol/s/million cells) (Figure 9-5B).

9.5.9 Intermediate and long term mitochondrial function changes in non septic organ failure within patients

There were significant temporal differences in G3P (P=0.026, *RANOVA*) within the NS group (Figure 9-5F). G3P troughed at 3d/4d and then progressively increased with a peak at 3w (3w vs. 3d P=0.033, 3w vs. 4d P=0.037).

Although not significantly different on *RANOVA*, there were some statistically significant differences seen on *pairwise LSD* in the following parameters. ETS troughed at 3d and progressively increased by 3w compared to 4d (4d vs. 3w P=0.007) (Figure 9-5E). CI OXPHOS/CI Leak is significantly decreased at 3w compared to day 2 (2d vs. 3w P=0.016) (Figure 9-5G), but is increased at 6m compared to 3w (3w vs. 6m P=0.027).


A
ENDO
(pmol/s/million cells)
6
4
2
0
#
HV
NS1d
NS2d
NS3d
NS4d
NS5/6d
NS1w
NS3w
NS6m
B
CI Leak
(pmol/s/million cells)
2.0
1.5
1.0
0.5
0.0
#
#
HV
NS1d
NS2d
NS3d
NS4d
NS5/6d
NS1w
NS3w
NS6m
C
CI OXPHOS
(pmol/s/million cells)
8
6
4
2
0
#
HV
NS1d
NS2d
NS3d
NS4d
NS5/6d
NS1w
NS3w
NS6m


D
CI,II OXPHOS
(pmol/s/million cells)
15
10
5
0
#
HV
NS1d
NS2d
NS3d
NS4d
NS5/6d
NS1w
NS3w
NS6m
E
ETS
(pmol/s/million cells)
15
10
5
0
HV
NS1d
NS2d
NS3d
NS4d
NS5/6d
NS1w
NS3w
NS6m
F
G3P
(pmol/s/million cells)
10
8
6
4
2
0
*
*
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HV
NS1d
NS2d
NS3d
NS4d
NS5/6d
NS1w
NS3w
NS6m

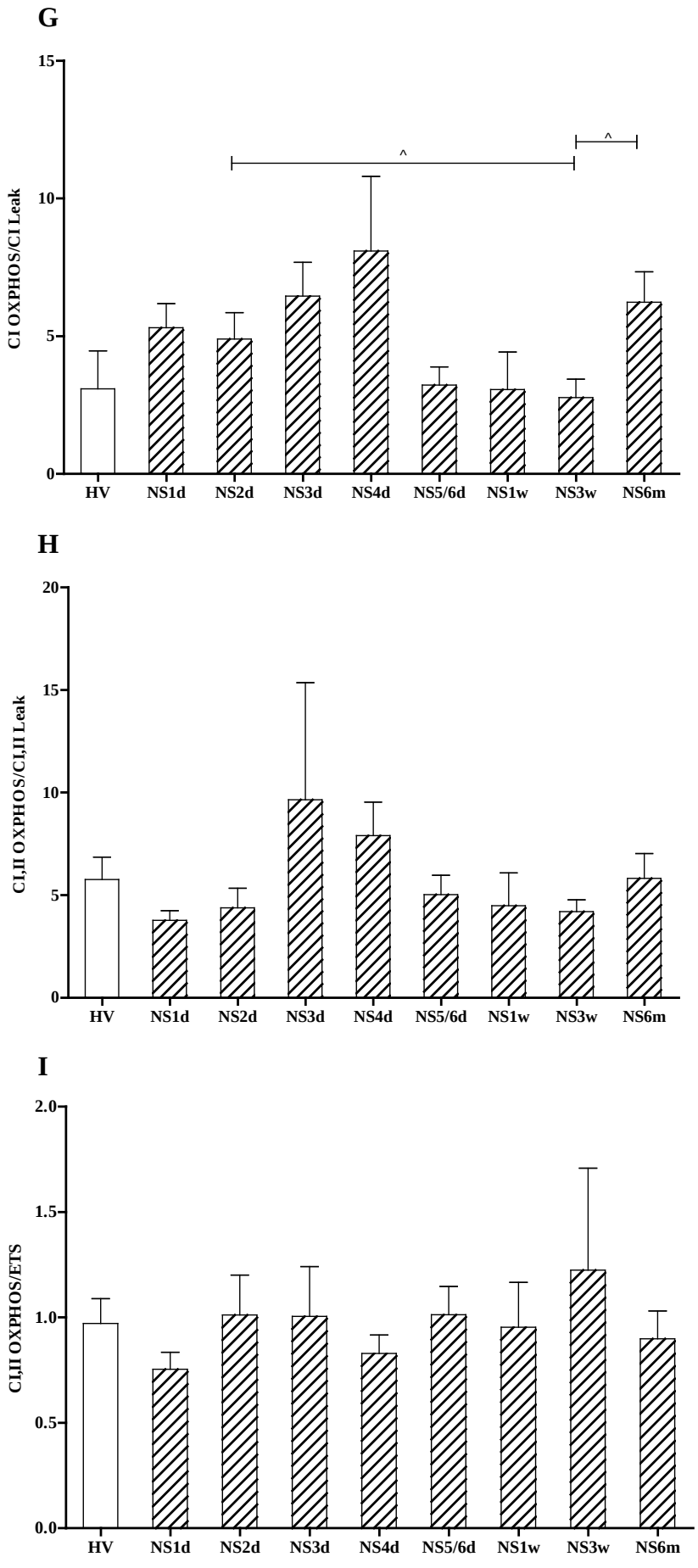
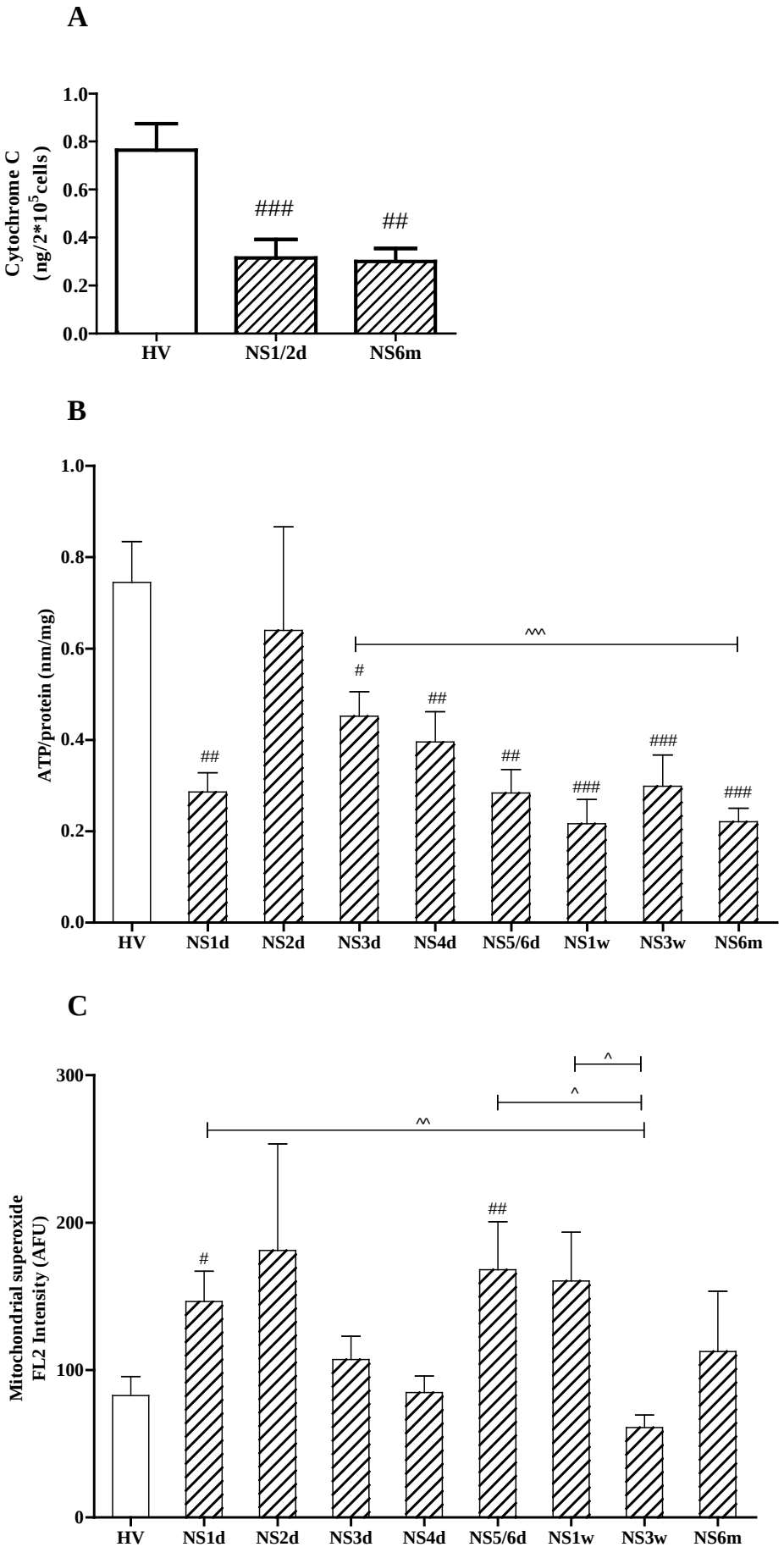

G
CI OXPHOS/CI Leak
15
10
5
0
^
^
HV
NS1d
NS2d
NS3d
NS4d
NS5/6d
NS1w
NS3w
NS6m
H
CI,II OXPHOS/CI,II Leak
20
15
10
5
0
HV
NS1d
NS2d
NS3d
NS4d
NS5/6d
NS1w
NS3w
NS6m
I
CI,II OXPHOS/ETS
2.0
1.5
1.0
0.5
0.0
HV
NS1d
NS2d
NS3d
NS4d
NS5/6d
NS1w
NS3w
NS6m

Figure 9-5A-I. Long term mononuclear cell mitochondrial respiration and flux control ratios in patients with non septic multiple organ dysfunction syndrome. Healthy volunteers (HV), white bars (n=15) and patients with non-septic multiple organ dysfunction at different time points, hatched bars (n=12 at 1d, n=10 at 2d and n=9 from 3d onwards). Different respiratory states measured were: A) Endo [Endogenous respiration], B) CI Leak C) CI OXPHOS [Complex I oxidative phosphorylation] D) CI,II OXPHOS [Complex I and II oxidative phosphorylation], E) ETS [Electron Transport System capacity] and F) G3P [ETS capacity with Glycerol-3-phosphate]. Flux control ratios measured were: G) CI OXPHOS/CI Leak [measure of phosphorylation efficiency with CI substrates], H) CI,II OXPHOS/CI,II Leak [measure of phosphorylation efficiency with CI+II substrates] and I) CI,II OXPHOS/ETS [ratio of phosphorylation to electron transport system capacity].Values are mean ± standard error of mean. #comparison to HV (student's *t* test with sequential *Bonferroni* correction), *comparison within patients at different time points (*RANOVA* with least significant different correction (*LSD*)), ^ comparison within patients at different time points (least significant difference (*LSD*)) #/^/*P<0.05, ##/^^/** P<0.01, ###/^^^/*** P<0.005.

9.5.10 Other Mitochondrial function in non septic MODS –intermediate and long term follow up

In non septic MODS, cytochrome *c* decreased by 61% at 6m (P=0.01, MW) compared to HV (Figure 9-6). There was no difference in cytochrome *c* between 1/2d and 6m in non septic MODS (P=0.08). In non septic MODS, ATP was decreased by 60% at 3w (P=0.0001) and by 71% at 6m (P=0.0001) compared to HV (Figure 9-6B). Compared to HV, there were no differences in mitochondrial superoxide in non septic MODS at 3 weeks or 6 months. Even though mitochondrial superoxide was decreased during the 3 week time point compared to the first week in non septic MODS, these changes were only found on *pairwise LSD* and not on *RANOVA* (Figure 9-6C). At 6m (5.24+/-1.22, P=0.049, *MWU*) the aggregate monomer ratio was increased in non septic organ failure by 139% compared to HV (2.19+/-0.33) (Figure 9-6D).


A
Cytochrome C
(ng/2*10^5cells)
1.0
0.8
0.6
0.4
0.2
0.0
###
##
HV
NS1/2d
NS6m
B
ATP/protein (nm/mg)
1.0
0.8
0.6
0.4
0.2
0.0
^^
#
##
##
###
###
###
HV
NS1d
NS2d
NS3d
NS4d
NS5/6d
NS1w
NS3w
NS6m
C
Mitochondrial superoxide
FL2 Intensity (AFU)
300
200
100
0
^
^
^^
#
##
HV
NS1d
NS2d
NS3d
NS4d
NS5/6d
NS1w
NS3w
NS6m

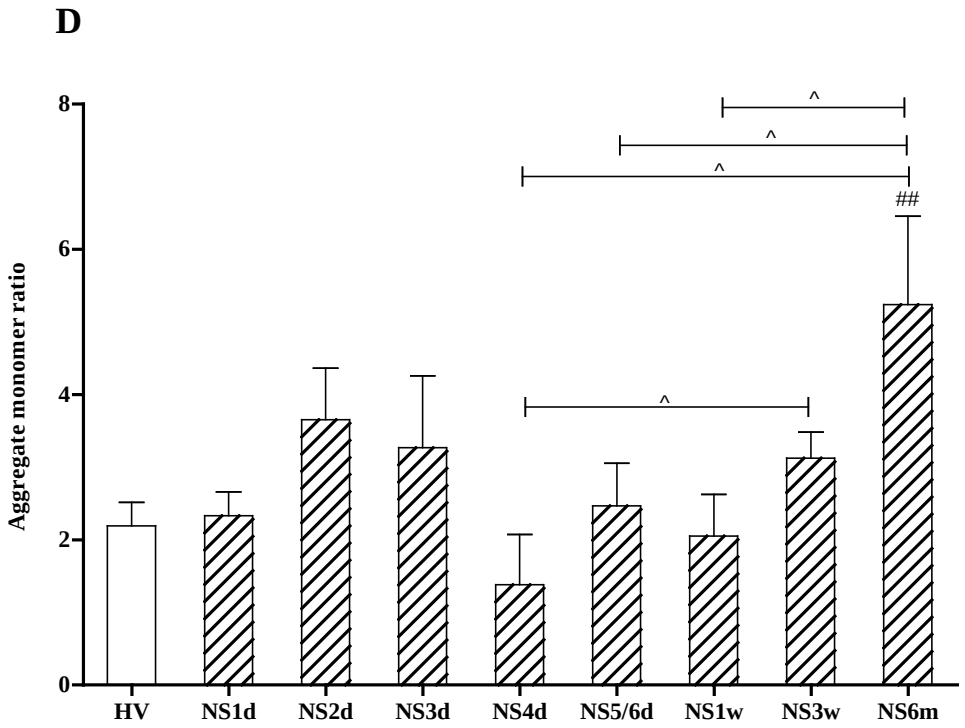


Figure 9-6A-D. Other mitochondrial function in patients with non septic MODS (hatched bars, n=12 at 1d, n=10 at 2d and n=9 from 3d onwards) compared to healthy volunteers (white bars, n=15)) at different time points. A) Mononuclear cell Cytochrome C B) Mononuclear cell adenine triphosphate (ATP) to protein ratio C) Mononuclear cell mitochondrial superoxide and D) Mononuclear cell mitochondrial membrane potential measured with the dye 5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolylcarbocyanine iodide (JC-1). JC-1 fluorescence was detected by flow cytometry in two channels FL 1 and FL 2. JC-1 exists as a monomeric form which fluoresces at FL 1 and when concentrated by actively respiring mitochondria exists as an aggregate which fluoresces at FL 2. An increase in the ratio of aggregate to monomer suggests higher mitochondrial membrane potential which in turn generates increased mitochondrial reactive oxygen species such as mitochondrial superoxide. Values are mean ± standard error of mean. #comparison to HV (student's *t* test), *comparison within patients at different time points (paired student's *t* test). #/* P<0.05; ##/** P<0.01; ###P<0.005.

9.6 Discussion

9.6.1 Main findings

There were several features of persistent mitochondrial dysfunction in patients with MODS with decreased cytochrome *c*, decreased net ATP and increased aggregate to monomer ratio compared to HV at 6 months. In septic MODS, mitochondrial respiration abnormalities were still present with decreased CI,II OXPHOS and the respiratory control ratios CI,II OXPHOS/CI,II Leak and CI,II OXPHOS/ETS were also depressed at 6 months. Additionally, in both septic and non septic MODS there was a decrease in net ATP and elevated aggregate to monomer ratio.

9.6.2 What this study adds in relation to previous clinical studies of PBMC mitochondrial function

This is the first study to have measured long term mitochondrial function in patients with MODS. Traditionally, MODS was thought of as a reversible process with normal histological appearances in organs (51) for example, patients with acute tubular necrosis who survive seldom require long term renal replacement (357). By contrast, in the last decade, there were numerous reports of poor long term function and quality of life in critically ill patients but no studies have evaluated long term mitochondrial function as a possible molecular mechanism for this. Mitochondrial function has been postulated to be an important mechanism for ICU acquired weakness and ICU acquired weakness is associated with poor long term physical function in patients. Therefore, it stands to reason that persistent long term mitochondrial dysfunction may be associated with poor physical functioning in this patient group.

Moreover, survivors of acute respiratory distress syndrome complained of fatigue, muscle weakness and decreased physical function measured by 6-min walking distance at one year (358) and five years (355). Whilst mitochondrial dysfunction has not been associated with long term fatigue in critically ill patients, mitochondrial dysfunction has been implicated to chronic fatigue syndrome (359, 360). Additionally, structural mitochondrial abnormalities have been described in post viral fatigue syndrome (361). Therefore, it is plausible that mitochondrial dysfunction is associated with decreased physical functioning in the long term in patients with MODS.

9.6.3 Long term mitochondrial respiratory function in MODS

Also, for the first time, in our study patients were followed until 6 months after their admission to ICU and mitochondrial respiration measured at that time point. At the 6 month follow up there were still derangements in mitochondrial function. The key differences in mitochondrial respiration were in septic MODS. In septic MODS, endogenous respiration, CI,II OXPHOS, CI,II OXPHOS/CI,II Leak and CI,II OXPHOS/ETS were still decreased compared to HV and ATP remained decreased in all patients with MODS. This decrease in mitochondrial respiration and ATP in the long term may account for the reported decrease in vitality and quality of life in patients after 1 year following discharge from ICU with MODS (362). Other studies have also reported decreased quality of life after discharge from ICU (362-364). Quality of life have been found to be associated with fatigue in cancers (365), (366) and since many definitions of fatigue involve a sensation of low energy, fatigue could be a disorder of energy balance (367). Since mitochondria produce the most energy in cells, it

is plausible that mitochondrial dysfunction cause fatigue and may partly be the reason for the decreased quality of life post discharge from ICU (367).

9.6.4 Limitations

There were a limited number of patients in this study and as such, even when there were strong trends in some finding, statistical significance was not achieved due to multiple comparisons. The other limitation in this study was a lack of patient oriented outcome measurements such as fatigue or quality of life. Although, a questionnaire was initially trialled, lack of time and personnel prevented the questionnaires to be finished and returned.

9.6.5 Conclusion and future work

Mitochondrial dysfunction persists in the long term in patients with MODS. Patients were followed until 6 months and mitochondrial respiration states still remained depressed in septic MODS at 6 months and decreased net ATP were found both in septic and non septic MODS at 6 months. Depressed mitochondrial respiration in the long term may be partly responsible for the decreased quality of life that is reported in patients with critical illness.

Chapter 10 Summary of thesis, implications of research and future work

10.1 Importance of MODS

MODS is the predominant cause for death in intensive care units world wide. The mortality from organ failure is up to 80% and there is considerable financial burden with ICU admissions (368). Despite decades of research in MODS, the pathophysiology is still not fully understood and treatments remain mainly supportive. The central paradigm in MODS used to be inadequate oxygen delivery to meet the demands of vital organs, but, now it is thought that impaired use of oxygen by the cells play a more important role (25).

10.2 Importance of mitochondrial function in MODS

Since most of the oxygen delivered to cells is used by mitochondria, mitochondria have a prominent role in the pathophysiology of organ failure. Even though the short term animal models of sepsis have shown mixed results, long term models have consistently shown decreased mitochondrial function. However, there have only been a handful of studies which measured mitochondrial function in patients with MODS. Mitochondrial dysfunction was found in leg muscle biopsies (25), liver and intercostal muscle (118) at one time point in septic organ failure and was found to correlate with the amount of norepinephrine required in critically ill patients (25). In order to measure mitochondrial function clinically, we needed a more accessible tissue rather than muscle biopsies, since muscle biopsies are impractical in acute illness settings and cannot be repeated easily to monitor the status of the disease.

10.3 Measuring mitochondrial function in MODS may be useful in clinical practice

So, more recently mitochondrial function was measured in peripheral blood mononuclear cells in septic organ failure and also found to negatively correlate with SOFA score and thus the severity of organ failure (305). But, the timing of this mitochondrial dysfunction in relation to timing of MODS was not investigated in septic MODS. In non septic MODS, PBMC mitochondrial respiratory function was never studied. This thesis comprehensively described peripheral blood mononuclear cell mitochondrial function in septic and non septic MODS, mild AP and hypertension in order to understand mitochondrial function in a broad spectrum of disease states.

10.4 Mitochondrial function in MODS vs. acute illness without MODS vs. chronic illness

Table 10-1 summarises the similarities and differences in mitochondrial respiration in each of the three diseases studied in this thesis. In experimental models of hypertension, there was raised OXPHOS and ETS. In sharp contrast to mild AP, in a chronic illness such as hypertension, the electron transport system attributable to CI (%CI ETS) halved by 14 months in SHR whereas there was an increase in %CI ETS as patients recuperated from their illness in AP.

In mild AP, PBMC mitochondrial function was slightly but significantly raised early in the disease course in order to keep up with the cell's ATP demands. This is in contrast with MODS where no significant increases in any respiration states were seen early in the disease state.

Table 10-1 Comparison of mitochondrial respiration states and flux control ratios in hypertension, mild acute pancreatitis and multiple organ dysfunction syndrome.

	MODS	**Mild AP**	**Hypertension**
Endo	↓	↑	↑
CI Leak	--	--	--
CI OXPHOS	↓	--	--
CI,II OXPHOS	↓	↑	↑
CI,II Leak	--	↑	--
ETS	↓	↑	↑
CI,II OXPHOS/CI,II Leak	--	--	↓
%CI ETS	--	↑	↓

Footnote: Endo [Endogenous respiration],CI OXPHOS [Complex I oxidative phosphorylation], CI,II OXPHOS [Complex I and II oxidative phosphorylation], ETS [Electron Transport System capacity] CI,II OXPHOS/CI,II Leak [measure of phosphorylation efficiency with CI and CII substrates] and %CI ETS [measure of contribution of CI to total electron transport system capacity].

10.5 Pattern of mitochondrial function as patients recovered from mild AP compared to MODS

The pattern of mitochondrial respiration from a mild acute illness (mild AP) differed significantly from MODS. In contrast to AP where there were no depression in mitochondrial respiration states as patients recovered, MODS had significant mitochondrial depression in all respiration states from day 3 onwards. The trough in mitochondrial respiration in the mid phase (at 3d and 4d) correlated with SOFA and thus the severity of MODS. Additionally, compensation of ETS by CI (%CI ETS) was present from day 2 in mild AP whereas there was no such compensation in MODS. Interestingly, levels of ROS remained higher than HV levels as patients recuperated from mild AP and this may have been a consequence of the compensation by CI (281). In contrast to the higher than HV levels of ROS as patients recuperated from AP, ROS was decreased to HV levels when mitochondrial respiration was depressed in MODS. This is indirect evidence that higher levels of ROS compared to HV may not necessarily lend towards a bad outcome and ROS levels need to be interpreted within the context of the disease as well as the disease course.

10.6 Long term mitochondrial dysfunction in septic MODS

Interestingly, even at 6 months there was persistent abnormality in mitochondrial respiration function in septic MODS with decreased endogenous respiration, CI,II OXPHOS, decreased CI,II OXPHOS/CI,II Leak and decreased net ATP compared to HV which equated to less efficient mitochondria in producing ATP. Until now, organ dysfunction in sepsis was thought to have no long term sequel, but our research has revealed persistent mitochondrial dysfunction even at 6 months. Decreased vitality and quality of life was reported (362) even after 1 year in patients who suffered from septic MODS. Whether level of vitality in this clinical setting is associated with mitochondrial function will need to be a topic of future research.

10.7 Implications of the research findings

The knowledge gained in this thesis has several important implications for clinical and basic research for the future. With regard to clinical research, the importance of evolving pathophysiology over time has been highlighted in this thesis. Therefore, results of multi-national clinical trials which recruit patients on the basis of the time of ICU admission without regard for the patient's evolving pathopysiology need to be interpreted with caution. To some extent this may also partly explain the failure for so many clinical trials in critical illness (22, 369).

The work in this thesis has highlighted the levels of ROS and mitochondrial respiration over time and the inter-relationship between them through the course of the illness. This has important implication for further clinical research as numerous clinical trials have been done on antioxidants in critically ill patients (370). None of these trials measured ROS prior to initiating treatment with antioxidants and therefore were unaware of whether the patient had increased ROS or normal levels of ROS when initiating treatment with antioxidants. Additionally, these trials also did not take mitochondrial respiration into account. Since the data presented here shows that mitochondrial respiration is most likely the greatest source of mitochondrial superoxide, it is possible that mitochondrial targeted antioxidants such as MitoQ (371) will be more effective than the antioxidants which have been used in these clinical trials which do not target the mitochondria.

The clinical study in patients with MODS raised several questions: Is shut down of mitochondrial respiration a protective response for MODS? If so, is augmenting mitochondrial respiration harmful in MODS? If depression of mitochondrial respiration is protective, should we induce depression of mitochondrial respiration early in MODS? Does mitochondrial targeted treatments have any role in MODS?

Until now, timing of intervention in MODS has largely been ignored (22). Therefore, another avenue of research is the optimum timing of intervention to alleviate organ dysfunction. Given the dynamic nature of mitochondrial function in MODS, it is plausible that different treatment approach will work at different times depending on what the mitochondrial function is in an individual patient at that time. This assay of PBMC mitochondrial function has the potential to tailor treatments for individual patients by measuring mitochondrial function upon admission and then deciding upon optimal treatment.

The knowledge gained from the temporal aspect of the clinical study highlighted the inadequacy of current experimental models of MODS. Experimental models need to better reflect the clinical course of patients in modern intensive care units by provision of supportive treatments such as antibiotics, mechanical ventilation and cardiovascular support. This may underpin the reason for so many experimental trials of treatments with positive results which ultimately failed as clinical trials (369).

In the next section, the above implications will be further developed into future clinical, laboratory and basic research.

10.8 Future research

Our work has elucidated the evolving nature of mitochondrial function in mild AP, hypertension and in organ failure from peripheral blood which is easily accessible. Thus, this method of assessing mitochondrial function can be applied in future laboratory studies, clinical studies and ultimately in the clinic setting. In this section, future clinical studies will be discussed first followed by laboratory studies. The mitochondrial function protocol validated in this thesis may be used for prognostication in AP, hypertension and MODS. Also, the same protocol may be used to monitor and evaluate efficacy of treatments in these disease states. Future laboratory studies including computational and experimental studies will then be discussed.

10.8.1 Clinical Studies

10.8.1.1 Clinical studies of prognosis in AP

Rationale

Currently the prognostic systems commonly used for predicting AP severity take too long or have a poor positive predictive value (372) (373) (294) (374). These systems use combinations of patient demographics and various simple biochemical measurements and are suitable only as a one-off prediction of severity rather than repeated assessments.

It has been common to rely on daily clinical exam and CRP measurements in ward patients or, APACHE II scores in intensive care patients with CT scanning to provide intermittent assessment of severity. While useful, there remains an unmet need for a severity marker that would allow prediction of severity in AP. In the ideal situation this would be rapid, reproducible, cheap and without causing discomfort to patients (372).

As seen in Chapter 4, in mild AP there is an increased mitochondrial respiration flux on admission with increased endogenous respiration flux, CI,II OXPHOS and G3P whereas %CI ETS increases later on. In contrast, in MODS which is a determinant feature of severe AP (375) there is no increase in respiration flux in any state early on and no increase in %CI ETS. We have also shown that mitochondrial respiration parameters correlate with the sequential organ failure assessment scores in patients with organ failure in Chapter 8 of this thesis. In the future, larger studies including patients with severe AP will need to correlate outcomes of AP with these mitochondrial respiration parameters to determine whether this assay will aid prognosis in AP.

Significance

20-25% of patients with AP develop a severe form of the disease that is characterised by infected necrosis of the pancreas and MODS (375), and this is associated with a protracted hospital stay and an increased morbidity and mortality (376), (377). The assessment of severity is very important in the management of patients with AP because rapid changes can occur with the onset of necrosis, infection and MODS (374) and this mitochondrial respiration assay has the potential in aiding prognosis.

10.8.1.2 Clinical studies of prognosis of end organ damage in hypertension

Rationale

End organ damage in hypertension include hemorrhagic stroke, retinopathy, coronary heart disease/myocardial infarction, heart failure, renal failure and in the vasculature atherosclerotic changes including the development of stenoses and aneurysms (378). The presence of end organ damage provides prognostic information of the hypertensive state for example concentric cardiac hypertrophy is associated with an increased number of cardiovascular events (379) and a thickened carotid wall is linked with an increased risk of developing myocardial infarct and an acute cerebral ischaemic event (380). Currently the recommended assessment of organ damage includes evaluation of cardiac structural changes, the thickness of carotid artery wall and alterations in renal function with an increased creatinine and presence of microalbuminuria. However the current recommended assessments of organ damage are expensive and this is reflected by the American guidelines which do not recommend the search for target organ damage as an essential part of management of hypertension (381).

Measuring peripheral blood mitochondrial function may be a viable alternative. As shown in Chapter 5 of this thesis, the %CI ETS halved as hypertension progressed to compensated cardiac hypertrophy in peripheral blood mononuclear cells in an experimental model of hypertension (252). Future studies need to measure %CI ETS in patients with hypertension and correlate with severity of end organ damage in order to assess its prognostic relevance in hypertension. The hypothesis is that %CI ETS correlates with severity of end organ damage in patients with hypertension.

Significance

Hypertension is a common disease and a leading risk factor for morbidity and mortality throughout the world (382). If %CI ETS correlates with severity of end organ damage then this assay will aid hypertension prognosis.

10.8.1.3 Clinical studies measuring PBMC mitochondrial function with known treatments in MODS

Enteral feeding

Rationale

In critically ill patients, early enteral nutrition lowers incidence of pneumonia and reduces mortality (383). It has also been noted that enteral feeding improves mitochondrial function in muscle and lymphocytes (384), most likely by supplying reducing equivalents (fuel) to drive ATP production, or by maintaining mitochondrial membrane potentials. In patients with malnutrition, Complex I activity recovered before the other traditional markers of nutrition assessment upon refeeding (198).

It is not known whether enteral feeding improves outcome in severe AP and MODS by alleviating mitochondrial dysfunction. Future clinical studies can now be done to assess the new assay of PBMC mitochondrial respiratory function to determine whether mitochondrial function changes in response to treatment with enteral feeding in patients with AP and MODS. The hypothesis is that enteral feeding improves tissue mitochondrial function in severe AP and organ failure and that this change in function can be detected in peripheral blood.

Significance

Enteral nutrition has been shown to be an important part of treatment in severe AP (385), (386), (387) and organ failure (388) (389) and yet the exact mechanism why enteral feeding improves outcomes in these disease states is not known. This study will provide mechanistic understanding about how enteral feeding improves outcomes in these critical illnesses.

Insulin therapy

Rationale

Intensive insulin therapy to control hyperglycaemia is associated with higher activities of CI and CIV in liver biopsies taken *after death* in critically ill patients (119). We hypothesise that intensive insulin therapy improves outcomes in critical illness by alleviating mitochondrial

dysfunction. Future studies will be able to utilise this methodology of PBMC mitochondrial function to investigate whether intensive insulin therapy has a mitochondrial mode of action.

Significance

Insulin therapy is an important treatment in MODS and yet how it affects mitochondria is not known (390).

10.8.1.4 Tailoring mitochondrial targeted treatments based on evolving mitochondrial function in organ failure

Rationale

Clinical therapeutic trials in sepsis and organ dysfunction have had a high failure rate (22). One reason for the failure of these trials is that these trials have largely ignored the multiple phases in sepsis (22). Before instituting therapy, it is prudent to understand which phase of sepsis the patient is in.

There is a growing body of evidence that mitochondrial targeted treatments are effective in sepsis and organ dysfunction in animal trials (233). But, experimental studies of mitochondrial treatments have not taken into account the temporal nature of mitochondrial dysfunction in organ failure. If clinical trials of mitochondrial targeted treatments are to be successful we must first determine in each patient which phase of mitochondrial dysfunction they are in, before instituting therapy. Some mitochondrial treatments e.g. antioxidants may be effective during the early phase of organ failure when ROS is high and other mitochondrial treatments to modulate mitochondrial function may be more effective in the mid phase when mitochondrial respiration function is depressed. The hypothesis is that the PBMC mitochondrial function assay will enable assessment of mitochondrial function and therefore aid in choosing appropriate mitochondrial treatments. This assay may also be helpful in evaluating efficacy of mitochondrial targeted treatments once treatment is commenced.

Significance

Organ dysfunction from sepsis and shock is a major health care problem resulting in considerable mortality (368), (391). Despite this, treatments for organ dysfunction is predominantly supportive and many attempted therapeutic strategies have failed to improved outcome in large randomised controlled trials (22).

10.8.1.5 Fatigue following MODS

Rationale

Three small studies have reported fatigue in ICU patients and associated fatigue with long term ventilator assistance (367) (392) (393). Many definitions of fatigue involve a sensation of low energy, suggesting that fatigue could be a disorder of energy balance (367). Since mitochondria produce the most energy in cells, it is plausible that mitochondrial dysfunction may be an important factor for post ICU fatigue (367).

In a small study, patients with post-infective fatigue after infection with Epstein Barr virus had peripheral blood samples taken at various time points (394). The PBMC gene expression profiles were indicative of an altered host response. Several of the genes were mitochondrial related including fatty acid metabolism and the cell cycle (394). Mitochondrial respiration dysfunction is still evident at 6 months after discharge in septic organ failure as seen in Chapter 9. The hypothesis is therefore that post ICU discharge fatigue correlates with persistent mitochondrial dysfunction after ICU discharge.

Significance

The PBMC MF assay will enable a mechanistic understanding of fatigue after ICU discharge and may provide a basis for treatment of post ICU fatigue in these patients.

10.8.2 Laboratory studies

10.8.2.1 Towards better computer modelling of acute inflammation and organ dysfunction in critical illness

Rationale

Due to the complex nature of acute inflammation with its plethora of molecular interactions, mathematical models of acute inflammation have emerged in the past decade (395), (396) (397). In the future, these models have the potential to be applied to simulate outcomes of placebo controlled randomised clinical trials to streamline design and execution of those trials and may lead to personalised medicine (397).

However, these mathematical models have not taken into account the role of mitochondrial respiration function in organ dysfunction (395), (396), (397). This is most likely because temporal mitochondrial respiration function in organ dysfunction in humans was not known until now. The hypothesis is that incorporating temporal mitochondrial function data in these complex mathematical models may increase accuracy of these models to predict organ dysfunction.

Significance

Organ dysfunction from sepsis and shock is a major health care problem resulting in considerable mortality (368), (391). Despite this, treatments for organ dysfunction is predominantly supportive and many attempted therapeutic strategies have failed to improve outcome in large randomised controlled trials (22).

10.8.2.2 S-S peptides as mitochondrial targeted treatment in experimental studies of severe AP andMODS

Rationale

Mitochondrial dysfunction is associated with enhanced oxidative stress (398), and although there are endogenous defences, antioxidant reserves can be overwhelmed (399) such that administration of exogenous antioxidants may be required to replenish depleted reserves and reduce oxidative damage. Szeto-Schiller-31 (SS-31) peptides are novel cell permeable peptides which can act as antioxidants. These peptides directly target mitochondria to scavenge ROS at the site of production and reduce oxidative damage (400) (401).To date SS-peptides have been evaluated in experimental models of cardiac ischemic-reperfusion (402), (403) neurodegenerative disorders (404) and islet cell transplantation (405), and show beneficial effects on function and integrity of stressed cells and organs. The hypothesis is that these peptides will alleviate oxidative stress and the PBMC mitochondrial function assay will detect changes induced by these novel treatments.

Significance

SS peptides will be a novel treatment in AP and MODS and the PBMC MF assay may provide an opportunity to monitor efficacy of these peptides.

10.8.2.3 Drug discovery in MODS – Use of PBMC mitochondrial function assay to identify bioenergetic alterations by experimental drugs

Rationale

Novel compounds must be tested at an early stage to assess any adverse effects on mitochondria (406). One of the challenges of drug discovery is to develop cell based assays that can detect changes in response to compounds (407). Monitoring the rate of oxygen consumption gives valuable insight into the state of the living cell's bioenergetics to experimental drugs and has so far been done using traditional Clark type electrodes (148). The hypothesis is that PBMC mitochondrial respiration assay using high resolution respirometry will offer detailed information about mitochondrial respiration alterations by new compounds than the traditional Clark type electrodes thereby aiding drug discovery.

Significance

Despite decades of research, there are no specific treatments in MODS even though MODS is a major health care problem (408). The peripheral blood mononuclear cell based assay of mitochondrial respiration using high resolution respirometry described in this thesis may be used to study future drugs and their interactions with mitochondria and will therefore aid in drug discovery.

10.8.2.4 Future high throughput assays for prognosis in MODS, AP and hypertension for clinical use

Rationale

One disadvantage of the method we have used to measure mitochondrial respiration in these disease states is the length of time and the labour required per assay per patient. The method of high resolution respirometry was used because of the small amount of sample required to ascertain a large number of mitochondrial respiration states and flux control ratios (238). However, to use these assays clinically, the assays must now be refined to require less time and labour and have a high throughput. An example of a high throughput method is the quenched fluorescence oxygen sensing based 96 well plate assay for mitochondrial respiration in digitonin permeabilised fibroblasts for patients with suspected mitochondrial cytopathy (230). In patients, ADP stimulated respiratory activity with CI linked substrates pyruvate and malate was decreased compared to control cells. The results confirmed the validity of the assay as a high throughput screening method for mitochondrial function in digitonin permeabilised cells in patients with suspected mitochondrial cytopathy (230).

The hypothesis is that high throughput mitochondrial respiration function assays can be used clinically to aid in prognosis in AP, organ failure and hypertension. Future studies need to build on our findings and focus on measuring specific states of mitochondrial respiration or flux control ratios in a high throughput way in each of these disease states.

Significance

Aberrations of mitochondrial function is central to many disease states (99) . But, measuring mitochondrial function in patients is not done clinically due to the invasive nature of the test. Peripheral blood is easily accessible and therefore the assay of mitochondrial function described in this thesis can be applied in a broad range of clinical scenarios if made into a high throughput plate format.

10.9 Conclusion

In conclusion, the central hypotheses were successfully tested. In addition, the work presented in this thesis has successfully investigated mitochondrial function from peripheral blood in a broad spectrum of diseases and disease severity. Several contributions to existing knowledge were made including the first descriptions of mitochondrial respiration in mild acute pancreatitis, hypertension and non septic MODS. The key findings were that multiple aspects of mitochondrial dysfunction occurred in PBMC of patients with MODS and correlates with severity of MODS. Further, that temporal mitochondrial respiration negatively correlated with temporal organ failure scores and did not discriminate between septic and non septic causes of MODS. An increase in mitochondrial reactive oxygen species was evident early in MODS followed by a decrease in mitochondrial respiration later in the disease course. Mitochondrial respiration and mitochondrial superoxide correlated with each other over time during the acute phase of MODS. Although mitochondrial respiration recovered in the short term, there were features of mitochondrial dysfunction particularly in septic MODS in the long term at 6 months.

The important findings in AP and hypertension were that mitochondrial respiration was increased early in AP with CI compensation for the electron transport system later on from day 2 and that the CI attribution for the electron transport system decreases as hypertension progresses to compensated cardiac hypertrophy. As discussed in this chapter, these findings have opened up several avenues for further clinical and laboratory research.

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