Molecular Mechanisms of Postoperative Lymphopenia

Doctor of Medicine (Research) Thesis

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Foreword

A thank you to those who made me who I am today.

I thank my father for teaching me the importance of dedication and hard work,
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I dedicate this body of work to my family, whose love I cherish every day.

CONTENTS

CONTENTS – Tables

Contents – Figures

Chapter 6 Perioperative lymphokine production

Attestation

I, Pervez Sultan, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Low anaerobic threshold, a marker of decreased exercise capacity, is associated with poorer outcomes following surgery. In this thesis I demonstrate a relationship between low preoperative anaerobic threshold and preoperative lymphopenia (low lymphocyte count). I subsequently demonstrate that preoperative lymphopenia is associated with prolonged hospitalisation and increased postoperative complications using 2 surgical cohorts (n=240 and n=881).

Significant metabolic (bioenergetic) and functional changes occur in lymphocytes postoperatively, which may contribute to increased morbidity and mortality. Lymphocytes demonstrate a postoperative decrease in glycolysis and oxidative phosphorylation. Altered postoperative bioenergetic function are accompanied by an increase in mitochondrial reactive oxygen species production and a reduction in lymphocyte mitochondrial membrane potential, which are known to be associated with apoptosis or cell death. Increased apoptosis of lymphocytes following surgery is the likely mechanism for acquired lymphopenia postoperatively (reduction in lymphocyte count which occurs postoperatively).

A decrease in glycolysis is accompanied by increased CD8(+) lymphocyte cytokine production. Postoperative inflammasome activation as demonstrated by increased caspase-1 activity, appears to occur secondary to glucocorticoid release associated with the stress response to surgery. Caspase-1 is associated with glycolysis inhibition (decreased glycolysis postoperatively) and increased apoptosis (reduced lymphocyte count postoperatively). Increased Interleukin-1-beta expression, which is associated with activation of the inflammasome and increased cytokine production, is demonstrated following incubation of lymphocytes with glucocorticoid.

I hypothesise that postoperative changes in lymphocyte function occur secondary to increased glucocorticoid levels activating the inflammasome pathway during the stress response to surgery. This thesis provides translational data introducing the concept that lymphocyte metabolic abnormalities underlie the postoperative immune phenotype.

CHAPTER 1

Introduction

Chapter 1

Introduction

Lymphocytes are essential for effective innate and adaptive responses to trauma and inflammation.(Kim, Zhao et al. 2007, Palm and Medzhitov 2007) Preoperative lymphocyte function together with perioperative changes may play a vital role in the development of postoperative morbidity.

In this thesis I explore the relationship between aerobic capacity and lymphopenia and hypothesise that this contributes to the development of postoperative morbidity. I also explore the perioperative changes in human peripheral lymphocyte bioenergetics and functionality and elucidate potential mechanisms for the findings demonstrated.

1.1 Surgery and morbidity

An estimated 234 million major surgical procedures are performed world-wide per year.(Weiser, Regenbogen et al. 2008) Surgery is associated with significant patient morbidity and mortality. A 7-day European cohort study demonstrated a 4% in-hospital mortality rate within the United Kingdom (UK) following adult non-cardiac surgery.(Pearse, Moreno et al. 2012)

Postoperative complications are significant, impacting not only on recovery time but health-care expenditure and the subsequent risk of death. Prospectively collected data over 8 years in 105,000 patients has demonstrated that development of postoperative complications is a reliable predictor of long term survival.(Khuri, Henderson et al. 2005) Even apparently minor postoperative morbidity such as wound complications significantly increases the risk of mortality up to 8 years following surgery.

Mechanisms involved in the development of postoperative morbidity are poorly understood. Identification of the specific pathophysiological changes responsible for the development of postoperative morbidity may help to identify new targets for risk stratification and therapeutic manipulation.

1.2 Sepsis and surgery

Surgical patients account for up to 30% of patients with sepsis.(Angus, Linde-Zwirble et al. 2001) In the United States (US) alone, of the 40 million surgical operations performed annually, approximately 2 million operations are complicated by surgical site infection.(Vogel, Dombrovskiy et al. 2009) Surgical patients are therefore a high-risk group for developing sepsis. The immune system in the postoperative period may be transformed by multiple factors including: metabolic and haematological changes, bacterial infection, blood transfusion and anaesthesia, which all contribute to the development of sepsis.(Windsor, Klava et al. 1995, Desborough 2000, Angele and Chaudry 2005)

Most of the mechanistic research in patients with established sepsis lack robust control populations. Confounding factors such as age, gender, ethnicity and comorbidity together with therapeutic agents used in the treatment of sepsis alter physiology, therefore making interpretation of experiments and generalizability of findings difficult.(Cain, Gutierrez del Arroyo et al. 2015) Utilising endotoxin infusion in healthy volunteers as the control population for critical illness studies has been shown to mimic aspects of the systemic inflammatory response.(Andreasen, Krabbe et al. 2008) However this model has its limitations since, for obvious ethical reasons, this population cannot receive endotoxin at high enough doses to trigger end organ dysfunction. Furthermore endotoxin utilised in this study may result in differing immune response due to activation of non-infectious (damage associated molecular patterns-DAMPs) rather than infectious (pathogen associated molecular patterns-PAMPs) inflammatory response.

Major elective surgery offers a model for exploring mechanisms underlying sepsis.(Cain, Gutierrez del Arroyo et al. 2015) The inflammatory response to tissue trauma occurring during surgery is reproducible and surgery has a similar inflammatory effect as that caused by infection. Furthermore, surgical patients undergo extensive phenotyping allowing characterisation of baseline organ function, which can act as an individual's own control data.

1.3 The effect of surgery on immune response

Sterile inflammation is a process in which inflammation occurs in the absence of pathogen, for example inflammation associated with a burn.(Chen and Nunez 2010) The mechanisms by which tissue injury induces inflammation are less clear. It is believed that necrotic cells release endogenous danger signals known as alarmins, which act on innate immune cells to activate similar receptors and signalling

pathways to those used by pattern recognition receptors (PRRs) or pathogen-associated molecular patterns (PAMPs), leading to cytokine production.(Bianchi 2007, Rosin and Okusa 2011)

Major surgical interventions result in immune cell alterations, including reductions in peripheral T lymphocytes, cytotoxic T cells, and natural killer cells.(Slade, Simmons et al. 1975) This surgery-induced postoperative inflammatory response characterised by impairment of the cell-mediated immune system or short-term immune suppression, has since been demonstrated in several other studies.(Faist, Baue et al. 1983, Haupt, Riese et al. 1998)

Surgery is thought to have a greater effect on immune cells than anaesthesia alone. Peripheral lymphocyte count and CD3 counts do not significantly change 20 minutes following induction of anaesthesia with thiopentone, fentanyl and isoflurane, but do so following surgical incision.(Brand, Kirchner et al. 1997) Identifying the precise mechanisms, chronology of the changes, cause and effect relationships between molecular pathways demonstrated and evaluating in-vivo function however still remains a challenge.

While the precise mechanisms explaining how postoperative complications often occur in systems distant to the operative site are not fully understood, the immune system has been implicated as a significant contributor to such morbidity.(Bennett-Guerrero, Welsby et al. 1999) The sepsis literature may help identify potential mechanisms of complication development in the postoperative phase. Deficiencies in proinflammatory responses for example may be harmful in patients with sepsis. This deficient inflammatory response may be mediated by polymorphisms in the genes responsible for cytokine production such as TNF-α promoter,(O'Dwyer, Mankan et al. 2008) IL-23 and IL-27 gene expression.(O'Dwyer, Mankan et al. 2008) This suggests that a cytokine-based immune response is beneficial in sepsis.

Prolonged visceral ischemia during major surgery results in an increase in levels of certain cytokines including tumour necrosis factor-alpha, (TNF-α), IL-6, IL-8 and IL-10.(Wakefield, Carey et al. 1993) The degree of cytokine production appears to correlate with the frequency and magnitude of postoperative organ dysfunction.(Wakefield, Carey et al. 1993, Poeze, Ramsay et al. 2002) For example, cytokine gene expression (reduced interferon gamma mRNA) is altered immediately following surgery in patients with postoperative pneumonia.(White, Martin-Loeches et al. 2011) Genes encoding the cytokine interleukin-

6 (IL-6) also become activated during aortic surgery and the resultant systemic inflammatory reaction has been associated with subsequent impaired pulmonary function.(Adembri, Kastamoniti et al. 2004)

1.4 Effect of surgery on lymphocytes

An increase in peripheral leukocyte count, a decrease in lymphocyte count and increase in neutrophil to lymphocyte ratio (NLR) occurs postoperatively.(Hamid, Bancewicz et al. 1984, Lennard, Shenton et al. 1985, Yamauchi, Kobayashi et al. 1998) Studies examining postoperative trends in lymphocyte numbers have involved relatively small numbers (Table1.1; n=20-35), and few studies have explored trends in NLR perioperatively.

The decreases in lymphocyte count seen postoperatively in the general surgical and maxillofacial populations of these studies return to preoperative baseline levels by day 3-7 following surgery in patients not receiving perioperative steroid therapy.(Hamid, Bancewicz et al. 1984, Lennard, Shenton et al. 1985, Dietz, Heimlich et al. 2000)

Table 1.1. A summary of the prospective studies exploring postoperative trends in lymphocyte count

Author	Year	Cohort	Type of study	Number	Time points of sampling	Method	Decrease in lymphocyte count	Time to recovery to preoperative level
Hamid et al.(Hamid, Bancewicz et al. 1984)	1984	Major elective general surgery	Prospective	20	D-3 2 hrs postoperative D2, D4, D7, D29	Coulter Counter (Model ZF)	Lowest lymphocyte count on D2 (median value one-third of preoperative value)	D4
Lennard et al.(Lennard, Shenton et al. 1985)	1985	Elective minor and major*	Prospective	35	D0, D1, D3, D7, D14, D21	Coulter Counter	Minor - Lowest count D1 Major - lowest count D3	*Minor - D3 *Major - D7
Dietz et al.(Dietz, Heimlich et al. 2000)	2000	Primary head and neck SCC	Prospective	33	D-1 D1, D7, D21-28 (all patients received steroids either on D0 (n=8) or for 3 days postoperatively (n=25)	Not documented	Lowest count D1	Mean count recovered by D28

* Minor (general and vascular) and Major (general vascular, gynaecology and renal); SCC = squamous cell carcinoma; D-1 = 1 day before surgery; D0 = Day of surgery; D1 = 1 day following surgery etc.; No studies accounted for withdrawals or missed samples.

The cause for postoperative decrease in lymphocyte count is unknown. Redistribution to lymphoreticular structures(Westermann and Bode 1999) or lymphatic tissues(Toft, Lillevang et al. 1993, Toft, Svendsen et al. 1993) may play a role in development of postoperative lymphopenia, together with the extent of tissue trauma, volume of blood loss,(Cullen and van Belle 1975, Dietz, Heimlich et al. 2000) and neurohumoral and hormonal changes, which may also affect lymphocyte mobilization. Redistribution has previously been hypothesised to occur with cortisol release during the stress response to surgery,(Hamid, Bancewicz et al. 1984) however evidence supporting this hypothesis and mechanisms by which this occurs are lacking.

Immunosuppressive factors such as inflammatory cytokines,(van der Poll and Lowry 1995, Biffl, Moore et al. 1996) prostaglandin E_2(Grbic, Mannick et al. 1991) and nitric oxide(Jacob, Ochoa et al. 1993) adversely affect lymphocyte function which may be reflected in the decrease in numbers seen following major surgery. Following tissue trauma and injury, lymphocyte numbers and subset population alterations have been described in different lymphoid compartments but the mechanisms responsible for this remain unknown. The stress response to surgery which includes an increase in catecholamines and corticosteroid levels has been well described.(Desborough 2000) Cortisol results in a known shift in the leukocyte differential to a lower percentage of lymphocytes.(Thomson, McMahon et al. 1980) Since the adrenal axis is a sensitive feedback system for adverse physiological conditions, including post-surgical response, changes in the relative lymphocyte count may be a simple, early marker of neurohumoral activation. Utilizing a large animal model, Yamada et al. associated plasma cortisol levels and lymphocyte apoptosis with surgical trauma in dogs undergoing anaesthesia alone versus anaesthesia and laparotomy. The development of lymphopenia was hypothesised to be secondary to cortisol induced apoptosis occurring following surgical trauma.(Yamada, Tsuchida et al. 2002)

1.5 Causes of lymphopenia

The total human T-lymphocyte pool size is approximately 3×10^{11} cells with an approximate turnover of 3×10^{9} cells per day.(Hellerstein, Hanley et al. 1999, Neese, Misell et al. 2002) Peripheral T cell populations are maintained by naïve T cells produced in the thymus, cellular self-renewal (or homeostatic proliferation) and clonal expansion of activated cells. Various experimental techniques have attempted to quantify the relative contributions of these processes, however interpretation of such data in terms of the underlying rates of T cell production, division, and death has proven to be notoriously difficult and involves mathematical modelling.(De Boer and Perelson 2013) By utilizing

deuterium (^2H_2O) in healthy human adults, the expected life span of naïve T cells have been estimated to be almost a decade,(Vrisekoop, den Braber et al. 2008) and are largely maintained by peripheral renewal, leaving a fairly minimal role of thymic production.(Bains, Antia et al. 2009, den Braber, Mugwagwa et al. 2012) Total memory T cell populations (CD45RO+) tend to be stable, but turnover much more rapidly than naïve T cell populations (CD45RO-CD27+), with expected life spans of 30 to 52 weeks in humans.(Vrisekoop, den Braber et al. 2008) During normal homeostasis CD4+ T cells tend to be turning over more rapidly than CD8+ T cells, as demonstrated by the increased half-life of CD8+ cells.(Vrisekoop, den Braber et al. 2008, den Braber, Mugwagwa et al. 2012) The reason for the difference in turnover rates is unknown but is thought to be partly mediated by homeostatic cytokines, such as IL-7.(Puel, Ziegler et al. 1998)

Lymphopenia has been defined as an absolute lymphocyte count more than two standard deviations below the mean for a healthy age and sex-matched population. The absolute and relative (percentage of total white cell count) lymphocyte counts which constitute lymphopenia vary between laboratories based on local populations but usually lie between 1.0-1.5 x 10^9/L and 15-22% respectively in most institutions.(Cancer Research UK website. 2015) Consequently the cut-off values used to define lymphopenia in most contemporary studies are usually found within this range.

Established causes of lymphopenia (in the peripheral circulation) are summarised in Table 1.2. Absolute lymphocyte numbers within the circulation decrease following surgery, traumatic injury,(O'Mahony, Palder et al. 1984) acute illness requiring ICU admission(Feeney, Bryzman et al. 1995) and burns(Zapata-Sirvent and Hansbrough 1993) Normal CD4:CD8 ratio is quoted between 0.9–1.9. These conditions are associated with a greater decrease in proportion of CD4+ lymphocytes compared to CD8+ lymphocytes, resulting in an overall decrease in CD4+: CD8+ ratio. While lymphopenia has been related to mortality as demonstrated in Table 1.3, correlation between lymphocyte function and postoperative outcome / phenotype in humans is lacking.

Table 1.2. Recognised causes of lymphopenia

Cause of lymphopenia	Examples
Reduced production	Infection (HTLV-1); bone marrow defects (idiopathic CD4 lymphopenia)
Increased apoptosis	Surgery; infection (Mycobacteria, HIV); autoimmune (SLE – anti T-cell antibodies,); drugs (corticosteroids, chemotherapy, immunosuppressants; catecholamines); sepsis; cardiac failure; radiation
Redistribution to lymphoid organs	Catecholamines (surgery, sepsis, exercise)
Failed homeostasis	Infection (Influenza - T-reg suppression); autoimmune (RA)
Idiopathic	Autoimmune (Sjogren's syndrome); non-Hodgkin's lymphoma
Exhaustion (reduced function)	Infection (CMV, EBV); age

HTLV = human T-lymphotropic virus; HIV = human immunodeficiency virus; SLE = systemic lupus erythematosus; T-reg = T regulatory cell; RA = rheumatoid arthritis; CMV=cytomegalovirus; EBV=Epstein Barr virus; *Malignancy can cause reduced production (if bone marrow infiltration occurs), redistribution and exhaustion

1.6 Association between lymphopenia and mortality

Lymphopenia is associated with increased mortality in patients with trauma,(Cheadle, Pemberton et al. 1993) cancer,(Fogar, Sperti et al. 2006, Ray-Coquard, Cropet et al. 2009, Vicente Conesa, Garcia-Martinez et al. 2012, Zhang, Huang et al. 2013) and sepsis.(Boomer, To et al. 2011) Failure to normalise lymphopenia has also been associated with increased mortality following trauma.(Heffernan, Monaghan et al. 2012) Lymphopenia, utilising a cut-off absolute lymphocyte count value ranging from 1.0-1.5 x 10^9/L,(Fogar, Sperti et al. 2006, Clark, Connor et al. 2007{Kozak, 2015 #1656, Bhatti, Peacock et al. 2010,

Ozturk, Ozkan et al. 2010, Bhaskar and Parker 2011, Lomivorotov, Efremov et al. 2011, Laulund, Lauritzen et al. 2012, Vicente Conesa, Garcia-Martinez et al. 2012)} has also been associated with increased postoperative mortality following cancer and non-cancer surgery (Table 1.3).

1.7 Association between lymphopenia and morbidity

Lymphopenia is a common finding within the hospital environment. A prospective study involving over 1000 patients over a 3 month period within a single Australian institution identified that lymphopenia in hospital patients was most frequently reversible, and due to acute illness, notably surgery, sepsis and trauma. Malignancy, with or without chemotherapy, and steroid use were also found to be common causes.(Castelino, McNair et al. 1997) This study must be interpreted with caution however as it utilised an extremely low lymphocyte cut-off value of $<0.6 \times 10^9$/L to define lymphopenia, which was not referenced in their study. The authors state that this value was used due to the practical reason that a very large number of patients had absolute lymphocyte counts either less than the more commonly quoted "normal" lymphocyte values between 1.0×10^9/L to 1.5×10^9/L.

Although lymphopenia has been associated with delayed wound healing following hip surgery,(Pacheco-Haro and Chavez-Cadena 2012) few human studies thus far have prospectively examined the association between lymphopenia and postoperative morbidity or postoperative length of hospital stay.

Table 1.3. Summary of studies demonstrating worse survival with preoperative lymphopenia

Author, Year	Study design	Surgery	N	Lymphopenia definition (x 10^9/L)	Association with lymphopenia
Laulund 2012	Pooled data (3 observational studies)	Hip fracture	1689	<1.4 (Bhaskar) <1.5 (Koval) <1.0 (Ho)	Increased mortality OR 2.60 (95% CI = 1.61-4.20)
Lomivorotov 2011	Retrospective observational	Adult cardiac	1368	<1.5	Increased mortality (univariate OR, 3.53; CI, 1.98-6.28; multivariate OR, 2.06; CI, 1.02-4.15)
Bhaskar 2011	Prospective observational	Hip fracture	791	≤1.1	Increased 1 year mortality; 142/428 (33.2%) vs. 67/363 (18.5%); OR 1.79
Ozturk 2010	Prospective observational	Hip fracture	74	<1.5	Increased 1 year mortality; multivariate analysis*
Bhatti 2010	Retrospective observational	Pancreatic cancer	84	N/A	Lymphocyte count was a prognostic covariate; hazard ratio 1.56 (95% CI=1.02–2.39)
Kozak 2015	Retrospective	Colorectal cancer	129	<1.0	Worse overall survival (date of surgery to the date of death from any cause; multivariate analysis; HR, 0.34; 95% CI, 0.162-0.724)
Clark 2007	Retrospective observational	Pancreatic cancer	44	<1.5	Worse survival; 8.8 [5.3–13.3] months versus 15.0 [10.0–28.3] months and multivariate analysis

***** multivariate analysis also demonstrated that ASA score (III or IV), female gender and low haemoglobin levels on admission remained independent and significant risk factors associated with one-year mortality.

1.8 Lymphopenia - A mediator rather than a marker of increased mortality?

Inhibition of lymphocyte apoptosis utilizing a caspase-3-inhibitor has been shown to improve mortality in mice with sepsis.(Hotchkiss, Chang et al. 2000) The findings from this study highlight a potential mechanistic role for lymphocytes contributing to mortality secondary to infection. This therefore introduces the potential significance of lymphocytes in the development of postoperative morbidity and mortality. Other mutant lymphocyte models have also been explored which further support the concept of lymphocytes mediating survival outcomes.

Rag-1 (-/-) mice, which are completely deficient of mature B and T lymphocytes, demonstrate a 5-fold augmentation in gut epithelial apoptosis following caecal ligation and puncture (CLP) compared to WT mice. Reconstitution of lymphocytes in Rag-1 (-/-) mice via adoptive transfer also decreases intestinal apoptosis to levels similar to those seen in WT animals. Subset analysis indicates that CD4(+) but not CD8(+), are responsible for the anti-apoptotic effect of lymphocytes on the gut epithelium. Gut-specific overexpression of Bcl-2 in transgenic mice decreases mortality following CLP. This survival benefit is lymphocyte dependent since gut-specific overexpression of Bcl-2 fails to alter survival when the transgene is overexpressed in Rag-1(-/-) mice. Further, adoptively transferring lymphocytes to Rag-1(-/-) mice that simultaneously overexpress gut-specific Bcl-2 results in improved mortality following sepsis. Thus, sepsis appears to unmask CD4(+) lymphocyte control of gut apoptosis that is not present under homeostatic conditions, which acts as a key determinant of both cellular survival and host mortality.(Stromberg, Woolsey et al. 2009)

Results from murine studies must however be interpreted with some caution. Mice are considered to be the experimental tool of choice for many immunologists. Study of their immune responses has yielded great insight into the human immune system function, however fundamental differences exist between murine and human immune systems. Known discrepancies between humans and mice have been described in innate and adaptive immunity, including: leukocyte subset balance, Toll like receptors (TLR), inducible nitric oxide (NO) synthase, B cell and T cell signalling pathway components, cytokines and cytokine receptors, Th1/Th2 differentiation (the two functionally distinct subsets of mature T-helper cells), co-stimulatory molecule expression and function, and chemokine and chemokine receptor expression.(Mestas and Hughes 2004) The acute inflammatory stresses from different aetiologies may result in highly similar genomic responses in humans, however the responses in corresponding mouse models correlate poorly with human conditions and also, one another.(Seok, Warren et al. 2013)

Whether findings from Hotchkiss et al. reflect the importance of human lymphocyte number and function in preventing postoperative, or sepsis related morbidity and mortality, remains unclear. Altered cell surface expression of splenic CD4+ and CD8+ cells in patients dying from sepsis (Boomer, To et al. 2011) together with altered cytokine production in this population provide further supporting evidence to the hypothesis that lymphocytes play an important role in human sepsis and development of multiple organ failure. However it is difficult to ascertain from studies whether they are markers or mediators of poor outcomes in humans.

1.9 Neutrophil to lymphocyte ratio (NLR) and survival outcomes

High neutrophil to lymphocyte ratio (NLR)(Gibson, Croal et al. 2007, Bhutta, Agha et al. 2011, Kao, Klebe et al. 2011, Chen, Lin et al. 2012, Tomita, Shimizu et al. 2012, Vaughan-Shaw, Rees et al. 2012, Azab, Shah et al. 2013) has been described as a biological marker of subclinical inflammation associated with increased postoperative mortality. Raised serum NLR also predicts overall survival in patients with cancer.(Walsh, Cook et al. 2005, Gomez, Farid et al. 2008, Halazun, Aldoori et al. 2008, Sarraf, Belcher et al. 2009, Bhatti, Peacock et al. 2010, Shimada, Takiguchi et al. 2010, Dan, Zhang et al. 2013, Krane, Richards et al. 2013) Significantly higher NLR has also been associated with stage of colorectal cancer.(Satomi, Murakami et al. 1995) The majority of studies tend to utilise NLR > 5 as a cut-off value to define raised NLR(Walsh, Cook et al. 2005, Gomez, Farid et al. 2008, Halazun, Aldoori et al. 2008, Bhutta, Agha et al. 2011, Ayca, Akin et al. 2014) since this provides an index of normal laboratory values of neutrophil and lymphocyte counts as originally proposed by Zahorec et al.(Zahorec 2001)

1.10 Cardiovascular disease and the immune system

Chronic inflammation is a basic pathological mechanism that underlies various diseases including cardiac failure. The contribution of lymphocytes has been reported in this process.(Fujiu and Nagai 2013)

Relative lymphopenia is an independent risk factor for development of coronary artery disease in asymptomatic patients.(Sweetnam, Thomas et al. 1997) Even after correction for New York Heart Association (NYHA) class and peak oxygen consumption, relative lymphopenia (low lymphocyte percentage as a proportion of total leukocyte count) is an independent predictor of one and 4 year survival rates in patients with advanced heart failure.(Ommen, Hodge et al. 1998) Similarly, relative lymphopenia has been associated with worse survival in patients with chronic coronary artery disease,(Ommen, Gibbons et al. 1997) acute heart failure(Rudiger, Burckhardt et al. 2006) and elderly patients with congestive cardiac failure.(Acanfora, Gheorghiade et al. 2001) Cardiac failure is strongly

associated with increased postoperative mortality in patients undergoing major non-cardiac surgery.(Hernandez, Whellan et al. 2004)

NLR has also been reported as an independent predictor of outcome in stable coronary artery disease, as well as a predictor of short- and long-term mortality in patients with acute coronary syndromes.(Tamhane, Aneja et al. 2008, Ayca, Akin et al. 2014) It is linked with increased risk of ventricular arrhythmias during percutaneous coronary intervention (PCI) and higher long-term mortality in patients undergoing PCI irrespective of indication for procedure.(Bhat, Teli et al. 2013) NLR has also been reported as a prognostic marker for outcome following coronary artery bypass grafting(Gibson, Croal et al. 2007) and post coronary artery bypass graft associated atrial fibrillation.(Bhat, Teli et al. 2013) Additionally, NLR may be used as a simple and easy-to-measure marker for prediction of short-term prognosis and in-hospital mortality in patients with ischaemic and haemorrhagic stroke.(Gokhan, Ozhasenekler et al. 2013) In patients admitted with advanced heart failure, a high NLR is associated with greater inpatient mortality(Bhat, Teli et al. 2013) and NLR has also been associated with the severity of chronic heart failure.(Avci, Alizade et al. 2014) The relationship between NLR and cardiac failure, as defined by low anaerobic threshold determined by cardiopulmonary exercise testing, has not been previously explored as a potential mechanism for poorer outcomes in this population.

1.11 Association between lymphopenia and heart failure

Epidemiological studies demonstrate that occult heart failure (Hernandez, Whellan et al. 2004, Hammill, Curtis et al. 2008) and low anaerobic threshold (AT) measured by cardiopulmonary exercise testing (CPET) which is consistent with sub-clinical heart failure,(Older, Smith et al. 1993, Wilson, Davies et al. 2010) contribute to morbidity and mortality following non-cardiac surgery. Since original work undertaken by Older et al. poor exercise capacity in surgical patients, as measured objectively by preoperative CPET,(Lee, Chaloner et al. 2006, Murray, Whiting et al. 2007, Ausania, Snowden et al. 2012, Colson, Baglin et al. 2012, Hartley, Pichel et al. 2012, Junejo, Mason et al. 2012, Swart and Carlisle 2012) has repeatedly been associated with poorer postoperative outcomes, including infection, following surgery.(Wilson, Davies et al. 2010)

While immune cells can have protective effects to compensate for and overcome mechanical stressors (such as volume / pressure overload), hypoxia, and neurohumoral stimulation, they also contribute to sustained inflammation and development of cardiac failure and poor aerobic capacity.(Topkara, Evans et al. 2011) Cardiac failure is now being associated with neurohormonal activation and chronic systemic

inflammation.(Topkara, Evans et al. 2011) These are characterised by alterations in numbers, and function, of polymorphonuclear monocytes and T-cells.(Topkara, Evans et al. 2011) The immune component of heart failure is also demonstrated when innate immune signalling genes are analysed by a complex statistical analysis known as principal component analysis (PCA). The profiles differ depending on the nature of the pathological tissue injury pattern. For example there are distinct genetic expression profiles for innate immune genes in failing and non-failing hearts.(Mann, Topkara et al. 2010). Increasing severity of heart failure is associated with increased levels of circulating cytokine,(Rauchhaus, Koloczek et al. 2000) protein,(Frantz, Fraccarollo et al. 2003) endotoxin,(Niebauer, Volk et al. 1999), and lymphopenia.(von Haehling, Schefold et al. 2009, Nunez, Minana et al. 2011, Vaduganathan, Ambrosy et al. 2012)

The relationship between inflammation and postoperative outcome has been described previously. For example, patients with reduced levels of antibodies to endotoxin preoperatively, exhibit higher levels of proinflammatory cytokines and more perioperative morbidity.(Bennett-Guerrero, Ayuso et al. 1997, Bennett-Guerrero, Panah et al. 2001) The relationship between cardiomyocytes and inflammatory cells residing in or recruited to the heart,(LaFramboise, Scalise et al. 2007, Pedrotty, Klinger et al. 2009) and immune function is becoming an increasingly important target by researchers attempting to reduce mortality from cardiac failure.

While many patients with low AT demonstrate physiological parameters consistent with heart failure, few of these patients have a formal diagnosis of cardiac failure prior to surgery. (Weber, Kinasewitz et al. 1982, Weber, Janicki et al. 1987, Sullivan and Cobb 1990, Gitt, Wasserman et al. 2002) Mechanisms exploring why patients with heart failure have poorer postoperative outcomes are underexplored. Cardiac failure(Torre-Amione 2005) and malignancy(Proctor, McMillan et al. 2012) are associated with immunosuppression and chronic low-grade systemic inflammation, as indicated by readily available biomarkers such as lymphopenia and raised NLR. These inflammatory biomarkers appear to robustly predict an increased risk of morbidity following colorectal surgery.(Richards, Roxburgh et al. 2012) As previously highlighted preoperative NLR, in addition to absolute and relative lymphopenia have been independently associated with the development of complications following major surgery.(Proctor, McMillan et al. 2012) Thus, understanding the mechanisms that contribute to established preoperative inflammation may identify modifiable factors that enable reductions in postoperative morbidity(Davenport, Henderson et al. 2005) and hence improve longer-term outcomes.(Longo, Virgo et al. 2000, Khuri, Henderson et al. 2005) Accumulating evidence suggests that the cardiovascular and

immune systems work synergistically together with other organ systems. Chronic inflammation may result in impaired immune and cardiac function, thus increasing susceptibility to infection, complications, morbidity and mortality postoperatively.

For purposes of this thesis, sub-clinical heart failure is defined either as an asymptomatic patient with AT<11mL/kg/min or a patient with AT <11mL/kg/min without a formal diagnosis of heart failure. The high-risk preoperative patient is routinely phenotyped through routine investigations including full blood count, (identifying lymphopenia and high NLR); and in some centres, CPET (identifying low AT). The link between lymphopenia and low AT has not previously been explored. Worse outcomes in patients with low AT may be associated with presence of lymphopenia. The fact that lymphopenic, or "high risk" patients demonstrate worse survival following cancer and non-cancer surgery (Table 1.3) may be related to a greater prevalence of heart failure (low AT) in this patient cohort.

1.12. Bioenergetic function in lymphocytes - murine / cell line studies

Bioenergetics refers to metabolic processes resulting in production and utilization of ATP (adenosine triphosphate). T cell metabolism is intimately linked to T cell survival, function and differentiation.(Gerriets and Rathmell 2012) Cells generate ATP by glycolysis and by oxidative phosphorylation.(Jones and Thompson 2007, Vander Heiden, Cantley et al. 2009) Impaired mitochondrial function, reduced ATP production, increased oxidative stress and T cell necrosis have been identified as key contributors to the development of inflammation in patients diagnosed with systemic lupus erythematosus.(Gergely, Grossman et al. 2002, Doherty, Oaks et al. 2014) Morbidity secondary to sepsis and trauma has a bioenergetic component. Whether a similar inflammatory pattern occurs postoperatively resulting in lymphocyte bioenergetic dysfunction, apoptosis and patient morbidity remains unclear.

Buttgereit et al. described a hierarchy of energy-consuming processes within activated rat thymocytes which become affected in the face of impaired energy production.(Buttgereit, Burmester et al. 2000) The largest consumer of energy is calcium transport followed by Na^+K^+ATPase pump (sodium active transport) and macromolecule synthesis including proteins and DNA / RNA. Key functions that are adversely affected by inadequate energy production by lymphocytes are summarised in table 1.4.

Table 1.4. Key lymphocyte functions requiring ATP

Functions requiring ATP in lymphocytes	
Cytokine production	Antigen processing
Cell motility	Lymphocyte activation
Cell division	Antibody production
Antigen presentation	Cytotoxicity

Unlike tumours, T cells rapidly transition between resting catabolic states (naïve and memory T cells) to one of growth and proliferation (effector T cells) as part of normal development. In addition, as T cells differentiate during an immune response they also move from what are presumably nutrient-replete lymphoid organs to sites of cancer or infection, where oxygen, nutrients, and growth factors may become limiting.(Pearce, Poffenberger et al. 2013) Changes in peripheral T cell function are not only supported by but are dependent on metabolic reprogramming, and specific effector functions are dependent upon adoption of the correct metabolism.(Pearce, Poffenberger et al. 2013) During each stage of a T cell's life, whether it is naïve, activated, antigen-experienced, or anergic, for optimal function, metabolism must match the function of that particular T cell.

Mitochondrial ATP production is fuelled in murine naïve and memory (quiescent) lymphocytes by external glucose, amino acids and lipids or breakdown from intracellular components during autophagy.(Pearce 2010). In quiescent lymphocytes ATP is mainly used to drive protein synthesis and cation transport.

1.13 T cell activation and metabolism

In the two-signal model(Baxter and Hodgkin 2002), full T cell activation occurs when there is:

1) Binding of MHC-II associated antigen on antigen presenting cells (APCs) to the T-cell receptor (TCR) - CD3 complex.
2) A co-stimulatory signal provided by B7-1 (CD80) or B7-2 (CD86) binding on APC to the T-cell CD28 receptor, or by Interleukin-2 (IL-2) ligation.(Zheng, Delgoffe et al. 2009)

The T cell receptor (TCR, molecule found on the surface of T lymphocytes that is responsible for recognising antigens bound to major histocompatibility complex (MHC) molecules) signal is amplified through a protein tyrosine kinase cascade, involving a rise in intracellular calcium and activation of

protein kinase C (PKC). The subsequent activation of transcription factors leads to phospholipid turnover, ionic signals, alteration in cytoskeleton, gene transcription and the final increase of macromolecule synthesis. These processes result in cytokine synthesis and movement of the cell from G0 to G1 in the cell cycle. These processes require a rapid increase in cellular metabolism. Increased metabolism is a critical part of activation because if T cells fail to increase glucose metabolism due to inadequate nutrients or direct metabolic inhibition, activation and proliferation are suppressed.(Cham and Gajewski 2005, Jacobs, Herman et al. 2008, Shi, Wang et al. 2011) Furthermore increased glucose uptake can *enhance* T cell activation and proliferation.(Jacobs, Herman et al. 2008)

Most cells metabolise glucose to pyruvate via glycolysis and then oxidize pyruvate to carbon dioxide (CO_2) in the mitochondria, generating the large majority of their ATP through oxidative phosphorylation. To fuel the energetic and biosynthetic demands of rapid clonal expansion, activated T cells, even in the presence of adequate oxygen, generate a significant proportion of ATP by upregulation of glucose uptake and glycolysis.(Fox, Hammerman et al. 2005, Jones and Thompson 2007, Maciver, Jacobs et al. 2008) Lymphocytes activated in vitro preferentially convert pyruvate into lactate that is secreted from the cells rather than oxidize pyruvate in the mitochondria.(Vander Heiden, Cantley et al. 2009) This process, known as aerobic glycolysis, yields only 2 ATP per molecule of glucose, compared to a maximum of 36 ATP when glycolysis is coupled to oxidative phosphorylation (Figures 1.1 and 1.2 summarise ATP production and oxidative phosphorylation).

While it seems counterintuitive for cells to employ a low-efficiency pathway to produce ATP under conditions of high energy demand, it has been proposed that proliferating and rapidly growing cells preferentially utilise aerobic glycolysis to optimise ribose-5-phosphate and NADPH, needed for the synthesis of macromolecules during cell division, and the reducing equivalents needed to protect dividing cells from oxidative damage.(Brand and Hermfisse 1997, Vaughn and Deshmukh 2008, Vander Heiden, Cantley et al. 2009)

Individual complexes within the electron transport chain (ETC) can be inhibited in order to identify and compare changes in oxygen consumption rate (OCR- a marker of oxidative phosphorylation) and extracellular acidification rate (ECAR - a marker of glycolysis). By interrogating perioperative changes in lymphocyte metabolic profiles using extracellular flux analysis, the presence of ETC dysfunction (and individual ETC complex dysfunction) may be identified, which may account for perioperative

lymphopenia and ultimately postoperative morbidity. Detailed methodology of this technique, including the drugs utilised to inhibit ETC complexes, are summarised in Chapter 2.6.

1.14 Lymphocyte bioenergetic function and postoperative morbidity

Postoperative metabolic impairment may predispose lymphocytes to apoptosis, which may result in the acquired lymphopenia / decrease in lymphocyte count demonstrated following surgery (Table 1.1). Since lymphocytes play a vital role in defence against infection,(Hotchkiss, Chang et al. 2000) postoperative reduction in lymphocyte count secondary to metabolic impairment and apoptosis, may contribute to the increased risk of infectious complications which is known to occur following surgery.

Figure 1.1 ATP production in relation to glycolysis, Krebs cycle and oxidative phosphorylation

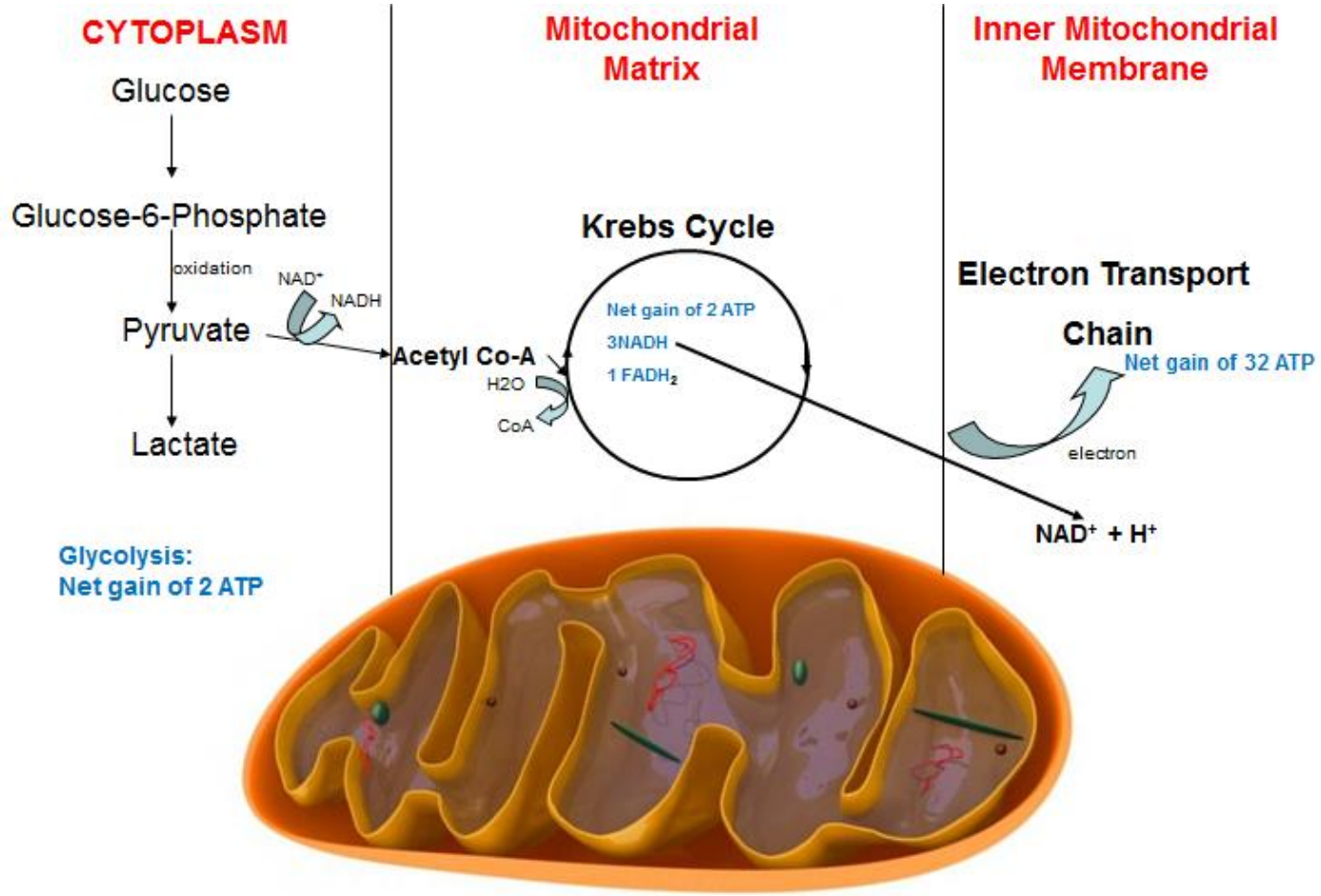
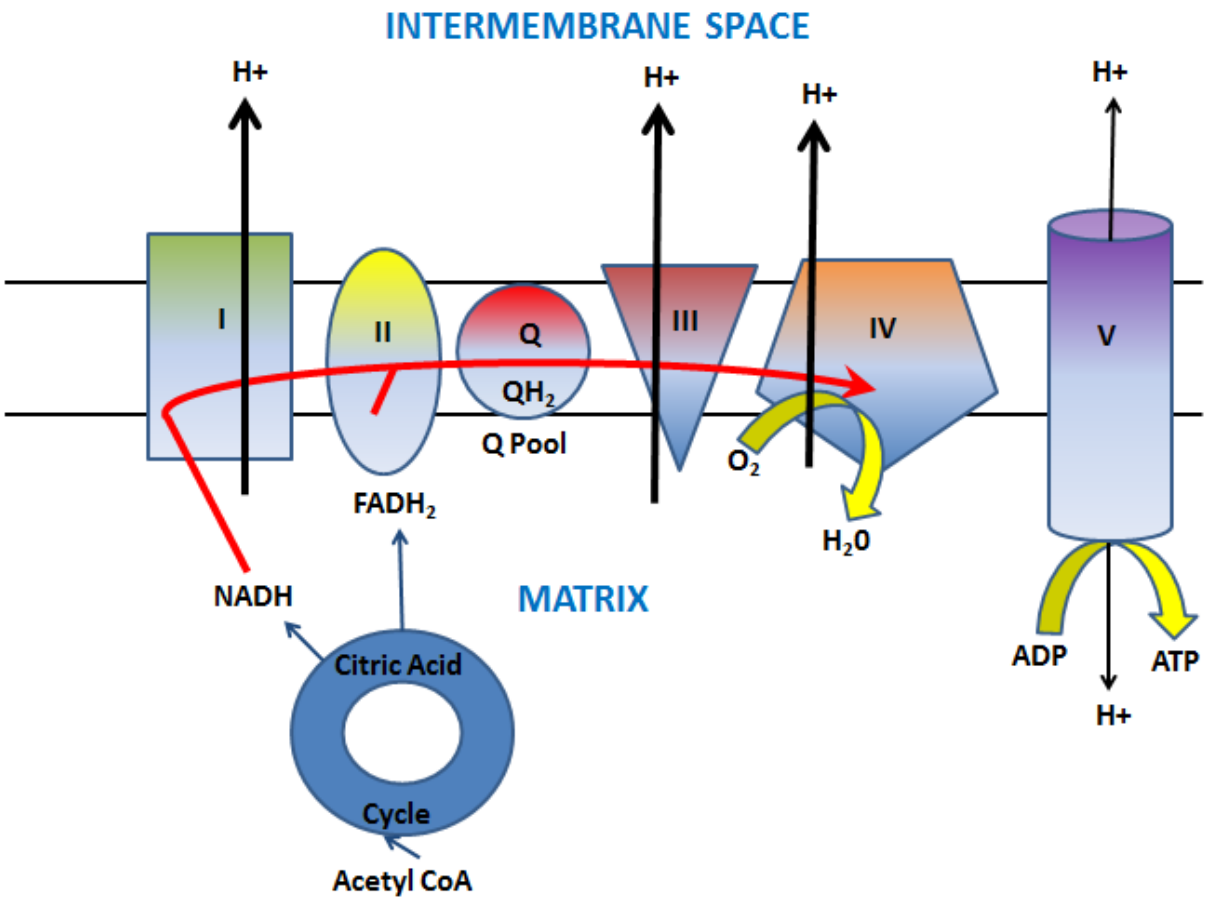


Figure 1.2 ATP production by oxidative phosphorylation within the electron transport chain



Electron transport depicted by red line.

1.15 Anergy

Anergy can be defined as unresponsiveness to antigenic stimulation leading to decreased cytokine production, glycolytic and mitochondrial ATP production and proliferation. If the TCR signal is received without CD28 co-stimulation, the T-cell becomes anergic, with a reduction in glucose metabolism.(Zheng, Delgoffe et al. 2009) This may be due to a lack of B7-CD28 binding (e.g. due to anti-B7 antibody), or to binding of B7 to inhibitory receptors such as cytotoxic T-lymphocyte antigen 4 (CTLA-4), programmed cell death 1 (PCD 1) or B and T lymphocyte attenuator (BTLA). (Chen 2004)

Although anergic lymphocytes are maintained in vivo, their precise role remains unclear.(Walker and Abbas 2002) Postoperative anergy has previously been described in peripheral blood mononuclear cells,(Faist, Baue et al. 1983, Haupt, Riese et al. 1998) and an excess of anergic T-cells seen in situations of physiological stress such as trauma has been associated with poorer outcomes.(Bandyopadhyay, De et al. 2007)

Defects in cellular energy production of alloreactive T cells have been shown to both reduce lymphocyte function and induce apoptosis resulting in lymphopenia.(Gatza, Wahl et al. 2011) Lymphocytes from lymphopenic patients have an increased propensity to apoptosis and reduced ATP content. Little is however known about the metabolic adaptations that occur in vivo to meet the increased demand for ATP in activated and proliferating lymphocytes in the postoperative state. Bioenergetic changes following anaesthesia and surgery, preceding exposure to various T-cell stimuli, may therefore be a cause of postoperative lymphocyte dysfunction, apoptosis and subsequent increased hospital length of stay. Study of perioperative changes in lymphocyte bioenergetic function may help to explain why lymphocyte counts drop following surgery.

1.16 Cytokine production by lymphocytes

Cytokines are low molecular weight proteins (<80kDa), which play a central role in the homeostasis of the immune system. Cytokine secretion and receptor expression on cellular membranes or in soluble forms, coordinate the immune response by inducing activation and/or inhibition of naive, memory and effector cells. Lymphocytes are an important source of cytokines (lymphokines) and play a significant role in mediating immunity and inflammation following tissue injury.(Sheeran and Hall 1997) Cytokines generally only require picomolar concentrations that act on target cell surface receptors and ultimately influence protein

synthesis within the cell. Most cytokines are synthesised as they are required and not stored. Some cytokines are however pre-synthesised and stored in cytoplasmic granules.(Gordon and Galli 1991)

1.17 Classification of lymphokines

According to the profile of cytokine synthesis, T cells can be subdivided into two functionally distinct subsets of mature TH cells: TH1 and TH2. TH1 cells are characterised by their regulation of cell-mediated immunity and the secretion of interleukin-2 (IL-2), interferon gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α). These cytokines are microbicidal and act on intracellular pathogens. In contrast, the TH2 subset is important for humoral immunity, B-cell proliferation and the production of most antibodies through IL-4-6, IL-I0, and IL- 13.(Mosmann and Coffman 1989) TH2 cell cytokines mainly act extracellularly, and suppress the TH1 response. Table 1.5 summarises TH1 cytokine function.

Table 1.5 Summary of TH1 cytokine function

Lymphokine	Cellular effects
Interleukin-2 (IL-2)	The major pleiotropic cytokine produced by stimulated CD4+ T cells. Stimulates T cell growth, promotes T helper cell activity, NK cytolytic activity, augments cytotoxicity. Stimulates B cell and T reg differentiation by activating STAT proteins through phosphorylation of JAK1 and JAK3, depending on the cytokine milieu after antigen stimulation. Mediates activation induced cell death. Modulates expression of key cytokine receptors controlling responsiveness to a range of cytokines following antigen encounter.
TNF-Alpha (TNF-α)	Found within endosomes and lysosomes and protects against organisms that infect macrophages. Can also be stored as membrane proteins, which provides an immediate cytokine source during tissue injury. (Massague and Pandiella 1993) Stimulates neutrophil and macrophage activity, inhibits collagen synthesis by fibroblasts, inhibits endothelial cell proliferation, stimulates fibroblast proliferation. Cytotoxic for tumour cells and is inhibited by IL-10.
Interferon-Gamma (IFN-γ)	Stimulates macrophage activity and B cell differentiation, stimulates and inhibits fibroblast proliferation, inhibits collagen production and collagen cross-linking, inhibits endothelial cell proliferation, increases endothelial cell adhesiveness for lymphocytes. Defence against microorganisms that establish intracellular infections including listeria, monocytogenes, leishmania, viruses and pathogenic inflammatory diseases. IFN-γ limits outgrowth of IL-4 producing T cells.

JAK = Janus kinase pathways; STAT = Signal Transducers and Activators of Transcription

Perioperative changes in cytokine expression and intracellular expression within lymphocytes may help further our understanding of cytokine production in sepsis.

1.18 Cytokine production in sepsis and surgery

Perioperative patterns of cytokine production may provide valuable insight into lymphokine production associated with sepsis. The importance of cytokine detection method and cytokine production site interrogated is demonstrated in the sepsis literature. While IFN-γ and TNF-α production have been shown to decrease in splenocytes when stimulated in vitro from patients who die from sepsis,(Boomer, To et al. 2011) higher serum TNF-α,(Calandra, Baumgartner et al. 1990, Kothari, Bogra et al. 2013) and IL-2,(Balc, Sungurtekin et al. 2003) cytokine levels have been associated with increased severity of sepsis. Furthermore the severity of sepsis or stage of illness may have profound effects on levels of cytokine production, however conflicting evidence exists. For example, Boomer et al. described a decrease in TNF-α production from splenocytes stimulated in vitro in patients who died from sepsis,(Boomer, To et al. 2011) however preserved secretion of TNF-α was described in acutely septic patients.(Boomer, Shuherk-Shaffer et al. 2012) Authors attributed these differences to demographic heterogeneity between the studies. Hence a standardised surgical model inducing lymphopenia, could aid our understanding of lymphokine production by potentially offering mechanistic insight and addressing the discrepancies seen in many sepsis studies.

1.19 Hypotheses

In this thesis, I hypothesise that:

1) Preoperative lymphopenia is associated with increased postoperative morbidity and and reduced exercise capacity.
2) Lymphocytes have impaired metabolism and functionality postoperatively (increased oxidative stress and altered cytokine production) resulting in increased apoptosis, and reduced lymphocyte count.
3) Postoperative changes in lymphocyte metabolism are driven by activation of the inflammasome (Chapter 7), secondary to increased glucocorticoid levels released during the stress response to surgery.

A summary of the hypotheses explored in this thesis is presented in Figure 1.3.

1.20 Aims

I aim to demonstrate the association between lymphopenia and postoperative morbidity. I also aim to demonstrate that the postoperative decrease in lymphocyte count occurs secondary to altered metabolic function and apoptosis, predisposing patients to infectious complications.

Ultimately this work will contribute further to our understanding of how surgery affects lymphocytes, immunity and patient outcomes.

1.21 Objectives

1) The association between impaired exercise capacity and lymphopenia will be explored by utilising preoperative cardiopulmonary exercise and leucocyte data in patients undergoing major colorectal surgery.

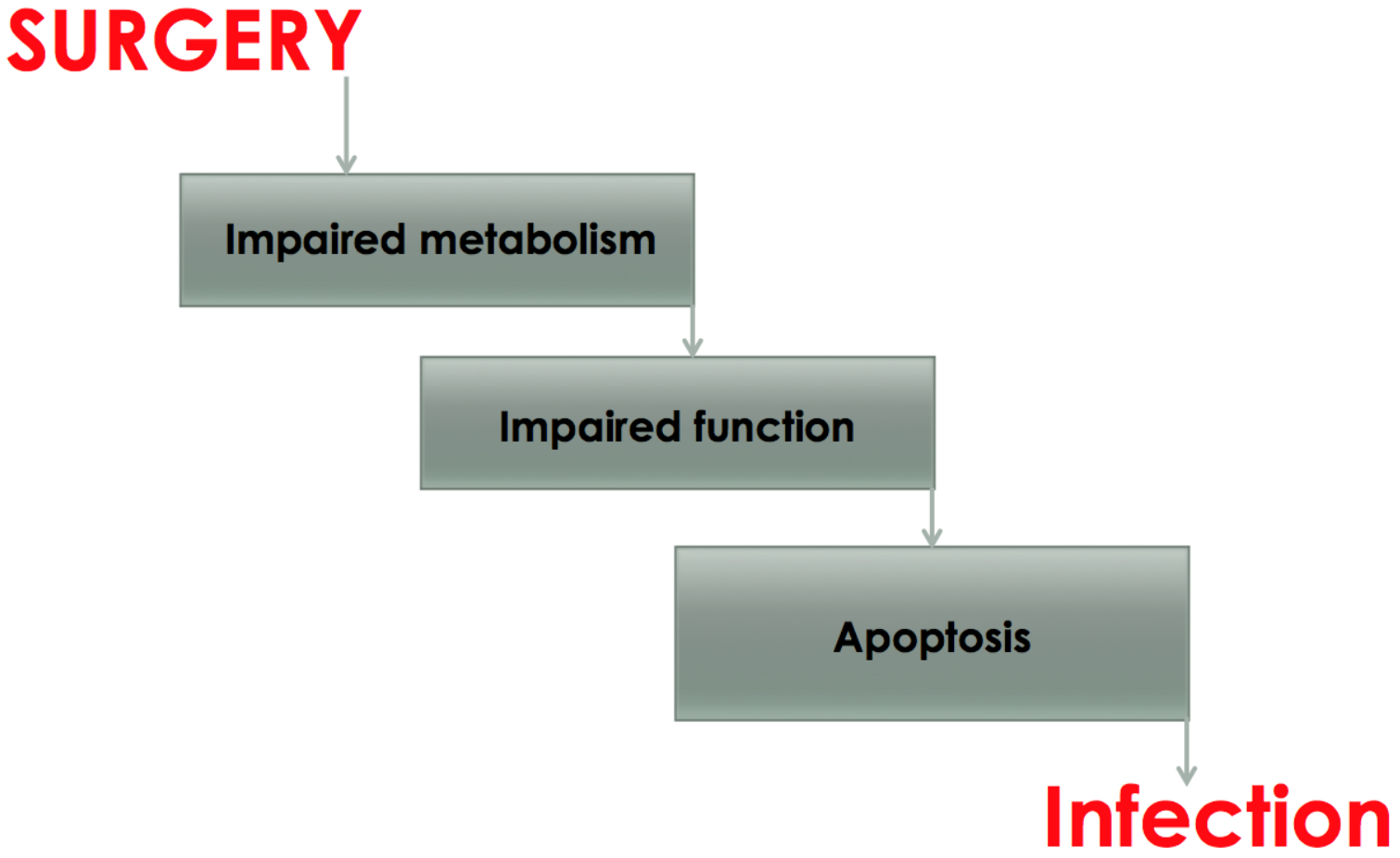
2) The relationship between preoperative lymphopenia and postoperative morbidity will be explored in 2 separate patient cohorts: major colorectal and mixed adult surgical population. Morbidity will be assessed utilising hospital length of stay in both cohorts, a validated postoperative morbidity scoring tool in the mixed adult surgical population and diagnosis of sepsis (≥2 systemic inflammatory response syndrome criteria and presence of positive culture) in the colorectal cohort.

3) The effects of surgery on metabolic function of lymphocytes will be assessed pre- and postoperatively using paired patient samples (pre vs. postoperative) of isolated lymphocytes interrogated with extracellular flux analysis.

4) The effects of surgery on lymphocyte function will be assessed in paired (pre vs. postoperative) patient samples using flow cytometry assays exploring mitochondrial function (reactive oxygen species production and mitochondrial membrane potential) and lymphocyte cytokine production.

5) Whether postoperative activation of an inflammatory pathway (inflammasome) occurs secondary to glucocorticoid, which account for postoperative changes seen in lymphocytes will be explored utilising flow cytometry and western blot analysis. Activated caspase 1 and Interleukin-1-beta expression (demonstrating inflammasome activation) will be assessed in lymphocytes incubated with a physiological concentration of glucocorticoid.

Figure 1.3. Summary of thesis hypothesis



CHAPTER 2

General Methods

Chapter 2

General Methods

2.1 Study Cohorts

The different studies utilised for clinical data analysis and blood sampling for laboratory experiments in this thesis are outlined in Table 2.1.

Table 2.1 Clinical studies utilised to obtain samples in this thesis

	Lymphopenia and AT length of stay. Perioperative changes in lymphocyte count (Chapter 3)	**Perioperative changes in bioenergetic function (Chapter 4)**	**Lymphopenia and length of stay and complications (Chapter 3). Perioperative changes in mitochondrial membrane potential, mROS production and cytokine production, (Chapters 5 and 6)**
Study Name	COMPETE-C	POM-E	VISION-UK
Ethics Reference	MREC 08/H0203/159 ISRCTN 14680495	MREC 09-H0805-59 ISRCT N90019424	MREC 10/WNo03/25 ISCTN NCT00512109
Study site	Derriford Hospital, Plymouth	University College Hospital, London	Multi-centre (University College Hospital, London)
Study date	03/2009 – 04/2010	04/2010 – 08/2014	09/2011 – 08/2013
Study cohort	Elective major colorectal	Elective hip and knee arthroplasty aged > 50 years old	Elective non-cardiac surgery. Overnight stay >45 years old

COMPETE-C = Cardiac Output Monitoring & Preoperative Exercise TEsting for Colorectal surgery; POM-E = Post-Operative Morbidity Exercise; AT = anaerobic threshold; VISION-UK = Vascular events In non-cardiac Surgery patIents cOhort evaluation; mROS = mitochondrial reactive oxygen species.

Two studies were utilised to analyse perioperative morbidity data (COMPETE-C and VISION-UK), 1 study was utilised to analyse perioperative trends in lymphocyte count (COMPETE-C) and 2 clinical studies were utilised to obtain blood samples for laboratory experiments (COMPETE-C and VISION-UK).

2.2 Summary of study cohorts used in thesis

a) <u>Cardiac Output Monitoring & Pre-operative Exercise TEsting for Colorectal surgery (COMPETE-C)</u> (Challand, Struthers et al. 2012)

Study type: Double-blind stratified randomised controlled trial

Ethics: This was approved by the Cornwall and Plymouth Research Ethics Committee (Ref: 08/H0203/159) and conducted at Derriford Hospital, Plymouth, UK, between March 2009 and April 2010 (UK NIHR CRN 7285, ISRCTN 14680495). Patients were provided with written information at the time of CPET and invited to consider their participation. Written informed consent was obtained from all participants before randomization. Serious adverse events were reported to the Ethical Committee.

Inclusion criteria: All adult patients undergoing major colorectal surgery at Derriford Hospital

Exclusion criteria: Individuals whose oxygen consumption at anaerobic threshold (AT) was undetectable or measured <8.0 mL/kg/min (considered too unfit to randomise) were excluded, as were those where cardiopulmonary exercise testing (CPET) was not performed.

Methods: This study aimed to compare intra-operative goal directed fluid therapy (GDT) to standard of care and determine the effects of GDT on length of stay, critical care admission, mortality, and hospital readmission rates.

Patients attended preoperative clinic for assessment. Demographics and Revised Cardiac Risk Index (RCRI) scores were recorded. In brief this scoring tool awards 1 mark for each of the following: high risk surgery; history of ischaemic heart disease; history of congestive cardiac failure; history of cerebrovascular disease; preoperative insulin requirement; serum creatinine >177mmol/l).(Lee, Marcantonio et al. 1999) A score ≥ 3 is considered high risk of perioperative cardiac event. Blood tests were taken in all patients preoperatively and depending on clinical need postoperatively.

Patients completed symptom-limited maximal CPET as part of their routine preoperative assessment on a stationary ergometer (Zan, nSpire, Colorado, USA). AT, measured in oxygen consumption relative to body mass (mL/kg/min) was used as the marker of aerobic fitness and determined according to published guidelines.(2003)

The majority of patients were admitted to hospital on the day of surgery. Bowel preparation was discouraged; those patients receiving bowel preparation were admitted for an intravenous (iv) infusion of 1–2 litres of Hartmann's solution in the 12 hours preceding their arrival in the operating theatre, according to the hospital protocol.(Sanders, Mercer et al. 2001) All patients received general anaesthesia, the conduct of which was left to the discretion of the consultant anaesthetist. Supplementary thoracic epidural anaesthesia was generally used for open procedures and intrathecal diamorphine with local anaesthetic or local anaesthetic field blocks were used for laparoscopic procedures. Intraoperative crystalloid, colloid, blood products, and inotropes/vasopressors were administered by the anaesthetist based on estimated maintenance fluid requirements, intraoperative losses, and the measurement of standard haemodynamic variables—aiming for a maintenance rate of 10 mL/kg/hr Hartmann's solution. Invasive arterial and central venous pressure monitoring was undertaken in selected patients.

Patients were risk-stratified as aerobically unfit (anaerobic threshold 8.0 – 10.9 mL/kg/min) or aerobically fit (AT ≥ 11.0 mL/kg/min) and within these strata were allocated to the intervention, goal directed therapy or standard fluid management (control) groups by random block allocation using sequentially numbered opaque sealed envelopes. The investigator had no involvement in perioperative decision-making or postoperative care. A researcher blinded to group allocation recorded postoperative outcomes. Adverse events were monitored by the local Research and Development service. A medically qualified investigator inserted an oesophageal doppler probe (CardioQTM, Deltex Medical, Chichester, UK) immediately after induction of anaesthesia and recorded haemodynamic variables every 15 minutes until the end of surgery. Patients allocated to GDT received supplementary colloid (VoluvenTM; Fresenius Kabi Ltd, Cheshire, UK) given by the investigator.

Standardised postoperative care was provided on a dedicated colorectal surgery ward. Admission to the critical care unit was at the discretion of the surgeon and anaesthetist. All patients were allowed free fluids, light diet, or both on the evening of surgery if tolerated. There was no formal protocol for postoperative fluid administration though local guidelines recommend a daily fluid intake of 2 litres.

Early mobilisation was encouraged, epidurals were discontinued at 48–72 h, and pain managed with oral analgesics at the earliest opportunity.

COMPETE-C study data used in thesis: The COMPETE-C study data is used in Chapter 3 to explore the relationship between:

I. Lymphocyte count and anaerobic threshold
II. Preoperative lymphopenia and postoperative length of hospital stay
III. Preoperative lymphopenia and postoperative incidence of sepsis
IV. Perioperative trends in leukocyte parameters (lymphocyte count, percentage and NLR

b) Post-Operative Morbidity-Exercise: preoperative exercise biology and postoperative outcomes (POM-E)

Study type: Observational cohort study

Ethics: This study was approved by the South-East London REC office (Ref: 09-H0805-59) and conducted at UCLH between April 2010 and August 2014 (ISRCTN 90019424). Informed written consent was obtained prior to elective major surgery at UCLH in patients who underwent CPET. Consent was obtained for perioperative morbidity data collection combined with blood sampling. Adverse events were monitored by the local Research and Development service. Serious adverse events were additionally reported to the Ethical Committee.

Inclusion criteria: Patients undergoing major elective major surgical procedures that are associated with a high incidence of postoperative morbidity (abdominal/oesophageal/hepatic resection/gynaecology/urological reconstructive surgery) and who meet the following criteria:

1. American Society of Anesthesiologists (ASA) risk grade 3 - 4
2. Aged greater than 50 years, either sex

Exclusion criteria: Patients were excluded if they: had evidence of acute myocardial ischaemia (contraindication for inotropic support); acute arrhythmias (contraindication for inotropic support); pregnancy (lithium-based cardiac output monitoring device); or if patients were receiving palliative treatment only.

Methods: This Observational cohort study of response to cardiopulmonary exercise testing in healthy volunteers and non-cardiac surgical patients, blood samples, microvascular and cardiopulmonary data were collected prior to and 60 minutes following exercise. Postoperatively, blood samples, microvascular and cardiopulmonary data were also collected. A researcher who was blinded to the CPET and blood test results, collected morbidity outcomes. The aim of the study was to determine immune, bioenergetic and microcirculatory responses to CPET in healthy volunteers and non-cardiac surgical patients. As part of this study venepuncture was performed preoperatively and on day 3 postoperatively.

The same team performed surgery and anaesthesia and standardised care was delivered according to local protocols. Low molecular weight heparin was administered routinely for thromboprophylaxis. Where performed, epidural analgesia was discontinued within 36 hours postoperatively to enable mobilisation and physiotherapy. Patient-controlled morphine analgesia was administered to patients who had undergone procedures under spinal anaesthesia. Resumption of oral fluid and solid intake was encouraged on the day of surgery.

POM-E study data used in thesis: Patient blood samples from POM-E study participants were utilised for:

I. Perioperative lymphocyte bioenergetic experiments performed in Chapter 4.

c) <u>The Vascular events In noncardiac Surgery patIents cOhort evaluatioN (VISION-UK)</u>

Study Type: Prospective cohort study

Ethics: This study was approved by the London REC office (MREC Ref: 10/WNo03/25) and national ethical approval was obtained (clinicaltrials.gov, identifier NCT 00512109). The multi-site study included UCLH recruitment where informed verbal and written consent were obtained prior to surgery at UCLH. Consent was obtained for perioperative morbidity data collection combined with blood sampling. Adverse events were monitored by the local Research and Development service. Serious adverse events were additionally reported to the Ethical Committee.

Inclusion criteria: Eligible patients for the VISION study had elective non-cardiac surgery during a weekday, were aged 45 years or older, received general or regional anesthesia, and underwent

elective or urgent/emergency surgery during the day or at night, during a weekday or the weekend requiring overnight hospital stay.

Exclusion criteria: Patients were excluded who were <45 years old, did not require an overnight hospital admission after surgery, who were previously enrolled in the VISION Study, or who declined informed consent.

Methods: The VISION study is a large, international, multi-centre, prospective cohort study evaluating complications after non-cardiac surgery in over 15,000 patients. The aim of the study was to establish diagnostic criteria, characteristics, predictors, and 30-day outcomes of myocardial injury after non-cardiac surgery. In patients undergoing non-cardiac surgery, troponin T was measured during the first 3 postoperative days. Patients therefore underwent venepuncture preoperatively and daily for the first 3 postoperative days. The primary outcome was 30-day mortality. Eligible patients for the VISION-UK study at University College Hospital, London received a general or regional anaesthesia.

An investigator reviewed and approved all data. Research personnel at participating centers submitted the case report forms and supporting documentation directly to the data management system (iDataFax; coordinating center, McMaster University, Hamilton, Ontario, Canada). Data monitoring in VISION consisted of central data consistency checks, statistical monitoring, and on-site monitoring for all centers. Postoperative morbidity data was collected in patients recruited into this study on day 3 and day 7 following surgery.

VISION-UK study data used in thesis: VISION-UK data is used throughout this thesis to explore:

I. Relationship between preoperative lymphopenia and postoperative length of hospital stay (Chapter 3)
II. Preoperative lymphopenia and postoperative morbidity (Chapter 3)
III. Postoperative changes in mitochondrial reactive oxygen species production and mitochondrial membrane potential within lymphocytes (Chapter 5)
IV. Postoperative changes in intracellular cytokine production within lymphocytes (Chapter 6)
V. Effect of glucocorticoids on lymphocyte reactive oxygen species production, and inflammasome activation (activated caspase 1 and interleukin 1-beta expression; Chapter 7)

2.3 Statistical analyses and power calculations

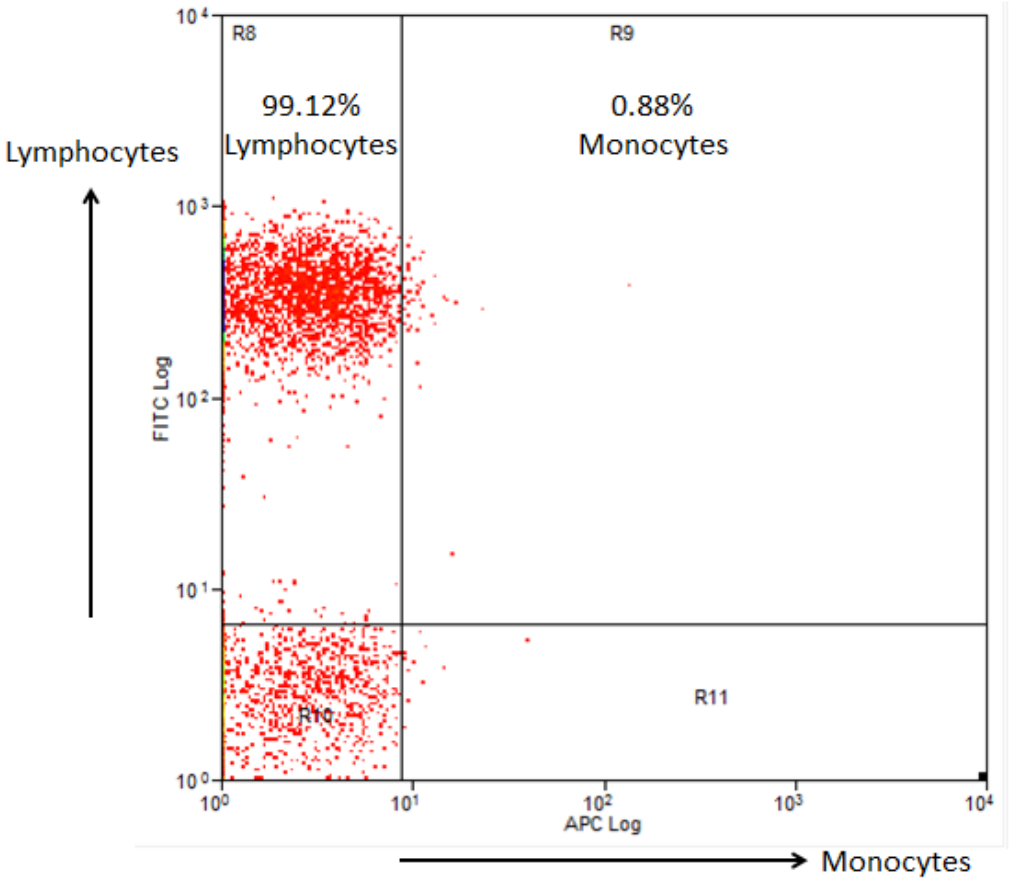
The analyses and power calculations for studies and experiments in chapters 3 to 7 are outlined within the relevant chapters.

2.4 Sample processing – Lymphocyte Isolation

Lymphocytes were obtained preoperatively from 20 mL of heparinised (final concentration 50 iu/mL) peripheral venous blood placed on ice and, within 1 hour, diluted 1:1 in phosphate-buffered saline (PBS) before undergoing density gradient centrifugation with Ficoll Plus (Amersham Biosciences, Chalfont St Giles, Beds, UK). This was centrifuged at 400 x g for 30 minutes at 20˚C with no centrifuge brake. The resulting plasma layer was carefully aspirated using a sterile Pasteur pipette. The layer containing lymphocytes and monocytes (mononuclear layer) was then aspirated and washed with PBS at 4˚C, centrifuged at 300g for 8 minutes and then washed again and centrifuged for 6 minutes. After a further wash with phosphate-buffered saline (BD Biosciences, Amersham, UK) to remove platelets, isolated PBMCs were resuspended at $1x10^6$ cells / mL in Dulbecco's Modified Eagle's Medium (DMEM) and placed in a plastic culture dish for 2 hours to allow monocytes to adhere to the plastic. Non-adherent lymphocytes were then gently resuspended using a pipette, counted again and resuspended in DMEM 4.5 g/L glucose. Cell count and cell viability was established by the Trypan Blue dye exclusion test. (De Luca 1965) A Neubauer-improved haemocytometer was used for manual cell counting.

Lymphocytes of purity > 98% were isolated following removal of monocytes (Figure 2.1). CD14 is expressed by macrophages, neutrophils, dendritic cells and monocytes. Following the cell isolation method described above, lymphocytes and monocytes are initially isolated. The supernatant remaining following monocyte adherence to culture dish consists of >95% CD4(+) and CD8(+) lymphocytes. Total numbers of isolated lymphocytes from each sample depended on volume of blood taken during venepuncture and absolute lymphocyte count of patient. The reagents, antibodies and equipment utilised for experiments in this thesis are summarised at the end of this chapter (Tables 2.3–2.5).

Figure 2.1 Flow cytometry plot demonstrating proportion of CD14(+) monocytes (APC) and CD3(+) T lymphocytes (FITC) in supernatant following lymphocyte isolation technique (monocyte adherence) (>99% viability demonstrated by Trypan blue haemocytometry)



2.5 Extracellular Flux Analysis

The Seahorse Bioscience XF24 extracellular flux analyser can be utilised to determine distinct bioenergetic profiles for human lymphocytes.(Chacko, Kramer et al. 2013) This system allows for real-time, non-invasive measurements of oxygen consumption rate (OCR) extracellular acidification rate (ECAR) / proton production rate (PPR), which can be correlated to mitochondrial function and glycolysis, respectively.(Dranka, Benavides et al. 2011)

Mitochondrial OCR (a measure of oxidative phosphorylation) and ECAR (a measure of glycolytic lactate production (Brand and Nicholls 2011)) were measured (Seahorse XF System, North Billerica, MA, USA) in freshly isolated lymphocytes from pre- and postoperative patients under identical experimental conditions, 3 days apart with at least 5 measures per patient sample, lymphocyte yield permitting. The technique is summarised in Table 2.2.

Table 2.2. Overview of extracellular flux analyzer (seahorse XF24) technique

Day before assay	Day of assay
Hydrate cartridge and store overnight at $37^{O}C$	Load cartridge, calibrate and add stress test compounds
Prepare assay medium stock	Change to assay medium and pre-incubate Run experiment and analyse data
Seed cells in XF microplate	

Isolated lymphocytes were stored at a concentration of 1million cells per mL in 4.5 g/L DMEM (Invitrogen, Paisley) in a humidified incubator at 37˚C with 5% CO_2, 21% O_2 and 74% N_2. This provides conditions ideal for tissue and cell culture due to the slightly acidic environment, which is designed to mimic the cell's natural conditions and maintain a constant pH 7.2-7.5. Lymphocytes were subsequently re-suspended for one hour prior to seeding in assay plate wells, to a concentration of 10 million cells per mL (1 million cells in 0.1 mL) and 25 µg/mL concanavalin A in unbuffered DMEM (without sodium bicarbonate or phenol red, supplemented with 5.6 mM glucose, 5 mM pyruvate and 2 mM L-glutamine). Sodium bicarbonate buffer has the ability to neutralise pH associated with H^+ ion production. Unbuffered solution (without sodium bicarbonate) is therefore utilised in

order for any changes in pH to be detectable to assess ECAR, a surrogate marker of glycolysis. The unbuffered solution allows for customization of the media for XF experimental design, permits greater control over substrates and enables the flux analyser to accurately measure ECAR and PPR.

Cell-Tak™ (BD Biosciences) is an extracellular matrix protein preparation isolated from the marine mussel Mytilus edulis. It is used to adhere cells to the plate well that do not naturally settle to the bottom in a microplate under gravity. The wells had been previously prepared with Cell-Tak. Lymphocytes were left to adhere for 90 minutes at 37ºC in humidified air before commencement of the assay.

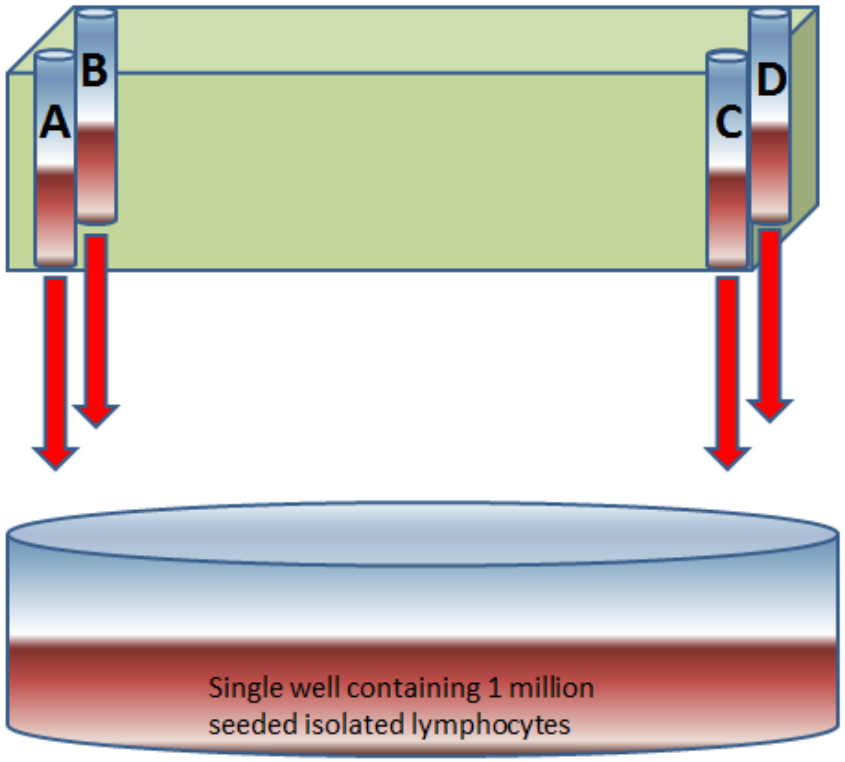
By means of a mechanical piston, the device creates a sealed transient micro-chamber of 7 µl volume above the lymphocyte monolayer. Oxygen and H^+ concentration in this microenvironment change according to cell metabolism. After a pre-determined period of time (90 seconds for these experiments) the piston rises and the media (0.6 to 1.0 mL) re-equilibrates with the ambient air. Two sets of analyte specific sensors are attached to the tip of the piston and lie 200 µm above the cells when the measurements are made. The sensors contain fluorescent particles embedded in a polymeric substrate that allows for selective diffusion of the specific analyte to contact with the fluorophores. Light-Emitting Diodes (LEDs) provide monochromatic light via fiber optic bundles to excite the sensors (λ=530 and λ=470 nm for oxygen and H^+, respectively). The fluorophores are quenched by oxygen and H^+ and the light emitted is correlated with the concentration of the analyte in the media. Each sensor is connected via the fiber optic bundle to a photodiode that converts the emitted light signal into current. The XF24 is a plate-based assay where up to 20 concomitant of these systems can be studied. 4 wells are not plated with cells and constitute controls for the assay conditions, namely temperature and media characteristics. Maintenance of the XF24 was undertaken in accordance with manufacturer guidelines by UCL Biomedical Sciences department. This involved daily calibration of sensors and LEDs to ensure reliable, accurate and consistent results between samples taken on different days.

The assay cartridge is first placed into the XF Analyzer to allow automatic calibration of optical sensors. Next, the cell culture plate is inserted into the instrument. The injection ports attached to the wells allow for injection of inhibitors of mitochondrial respiratory

chain to determine the defects in individual cellular respiration pathways or enzymes (Figure 2.2 and 2.3). Following baseline readings, four compounds were added in 3 separate injections with three further OCR and ECAR measurements after each compound (Figures 2.4 and 2.5). Experiments for isolated lymphocytes have previously determined that the optimal cell number required for accurate measurements of OCR is 1 million (100 µL of a 10 million/mL concentration).(Wang, Dillon et al. 2011) The optimum concentration of the inhibitors and activators to be used for the assessment of mitochondrial function were determined by titrating the individual compounds in separate experiments against the cell number determined. These concentrations were consistent with those recommended by the manufacturer.

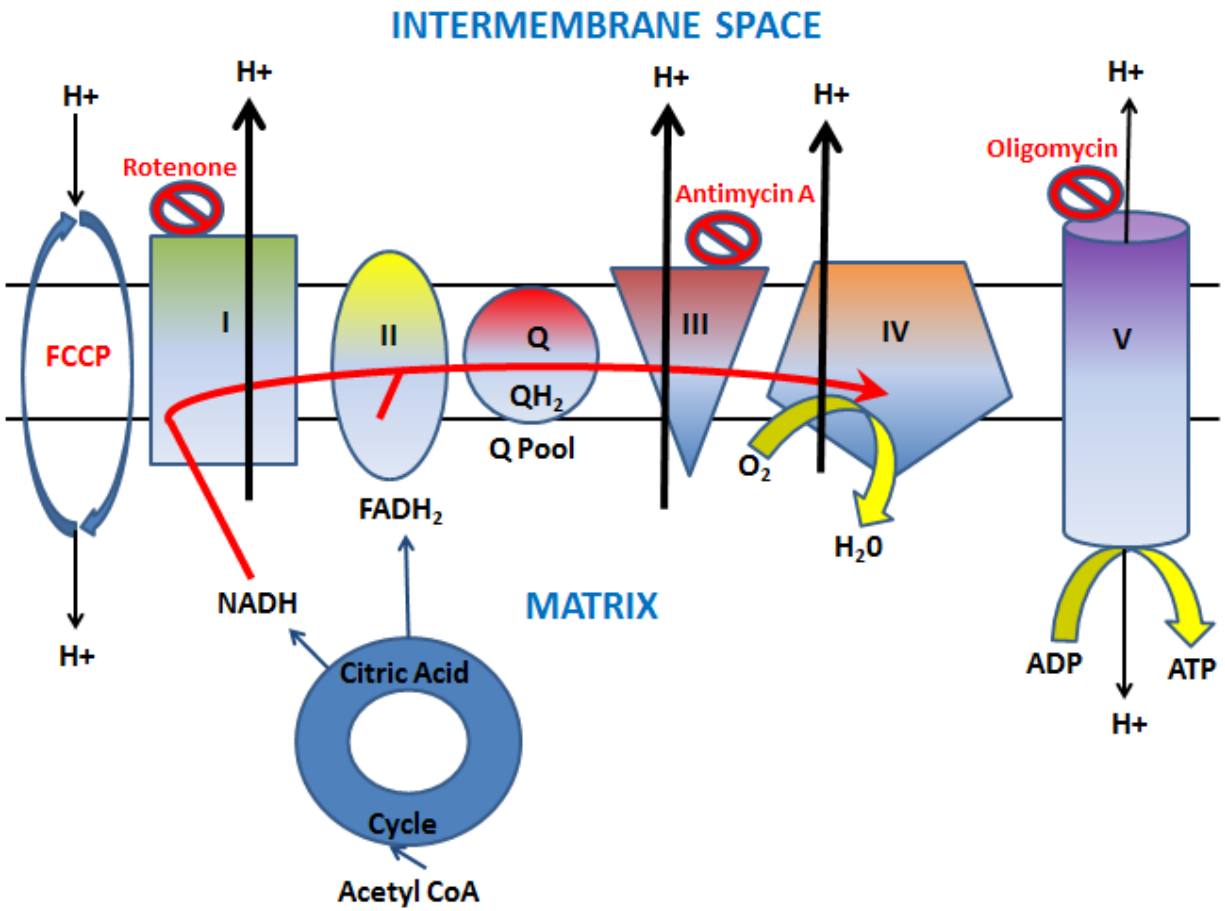
The mean basal respiration is determined by taking 3 OCR measurements before the addition of the inhibitors or activators. These 3 baseline readings taken 10 minutes apart are to demonstrate that the sensors within the machine read consistently and any subsequent changes occurring in OCR and ECAR occur secondary to drugs added (Drugs explained in section 2.6). Mitochondrial basal respiration, proton leak, and the maximal respiration were calculated after correcting for the non-mitochondrial OCR for each assay. Cells were allowed to attach to the XF24 plate for 45 minutes before measurement of mitochondrial function. Under these conditions, viability was over 90% for cells.

Figure 2.2 Diagram representing 4 injection ports in relation to each seahorse plate well



Measurements of OCR and ECAR taken every 10 minutes; injections A, B, C added to well after 30, 60 and 90 minutes respectively

Figure 2.3 Mitochondrial drugs utilised during extracellular flux analysis



= inhibitor (e.g. rotenone inhibits complex 1). Figure depicts site of action of each mitochondrial drug within the ETC.

2.6 Pharmacological interrogation of mitochondrial complex activity

(a) ATP synthase (Complex V) inhibitor - Oligomycin 0.25µM (0.2µg.mL^{-1})(Sigma Aldrich, Dorset, UK) Dose titrations for oligomycin were carried out in order to induce a maximal change in respiration without signs of cell death measured by Trypan blue haemocytometry.(Zaobornyj and Valdez 2005, 2013) Lymphocyte cell death was not affected with concentrations up to 0.25µM left for 60 minutes. ATP-linked OCR and proton leak were determined by adding oligomycin. The fall in OCR following oligomycin injection is the rate of oxygen consumption that corresponds to ATP synthesis, and the oligomycin- insensitive rate is considered as proton leak across the inner mitochondrial membrane. Oligomycin causes a reduction in ATP production by inhibiting the proton channel of the Fo portion of ATP synthase. With state 3 phosphorylation inhibited, remaining oxygen consumption is not coupled to ATP production, therefore oxygen consumption contributing to ATP synthesis can be distinguished from that being used to overcome the inner mitochondrial membrane proton leak. Blocking mitochondrial ATP production increases glycolysis in an effort to maintain overall cellular ATP production and the resultant increase in ECAR gives an indication of the glycolytic capacity of a cell (Figure 2.5).

(b) Electron transport chain coupling - Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) 2µM (Sigma Aldrich, Dorset, UK): ionophore.(Hoth, Fanger et al. 1997)

FCCP, an uncoupler of the electron transport chain, was used to determine the maximal respiration rate. This rate gives the theoretical maximum oxygen consumption that can take place at cytochrome c oxidase whether limited by availability of substrate or activity of the electron transport chain. The difference between the basal rate and this FCCP stimulated rate is termed the spare respiratory capacity of the mitochondrion, which is a measure of the maximal potential respiratory capacity the cell can utilise under conditions of stress and/or increased energetic demands e.g. occurring during activation and proliferation (Figure 2.4). This compound transports protons across the mitochondrial membrane. This results in a loss of electrical gradient across the membrane, which subsequently stimulates maximal electron transport chain (ETC) activity. This effort to regain the mitochondrial membrane results in an increase in oxygen consumption. This uncouples ETC activity from ATP production and gives an indication of the maximal respiratory capacity of the ETC, assuming substrate delivery is adequate (Figure 2.4). Prior administration of oligomycin ensures that the proton gradient cannot be regained by the reverse action of ATP synthase. As with oligomycin, glycolysis and consequently ECAR will further increase in an effort to maintain cellular ATP levels.

(c) Complex 1 activity - Rotenone (1µM) (Zaobornyj and Valdez 2005, 2013 , Lu, Wu et al. 2015) with **antimycin A** (1µM)(Vives-Bauza, Zhou et al. 2010{Garedew, 2010 #1703)}(Sigma Aldrich, Dorset, UK): ETC complex inhibitors.

Rotenone inhibits Complex I by preventing the transfer of electrons from the Fe-S centre in Complex I to ubiquinone (Coenzyme Q). Antimycin A, an inhibitor of Complex III, was used to completely inhibit mitochondrial electron transport. The OCR determined after antimycin A injection is attributable to non-mitochondrial oxygen consumption. Antimycin A inhibits Complex III by binding the Qi site of cytochrome c reductase, preventing the oxidation of ubiquinol. Stopping mitochondrial respiration in this way allows the contribution of non-mitochondrial processes (substrate oxidation and cell surface oxygen consumption) to be quantified and subtracted from basal and maximal OCR figures in order to express pure mitochondrial oxygen consumption.(Wu, Neilson et al. 2007)

(d) Glycolytic capacity - 2 Deoxy-Glucose (100mM administered as third injection for glycolysis experiments; see Figure 2.5)(2013)

To determine the extent to which ECAR represents glycolysis in this system, (rather than other cellular sources of acid), lymphocyte function was also assessed following 2-deoxyglucose (2-DG) 100nM administration.(2013) 2DG is a glucose analogue with the 2-hydroxyl group replaced by hydrogen. Due to its structural similarity to glucose it is transported into the cell by glucose transporters. It becomes bound to glucose hexokinase, but cannot be phosphorylated by this enzyme, and glycolysis is therefore competitively inhibited. The resultant decrease in basal ECAR demonstrates the proportion of baseline ECAR that is due to glycolysis.

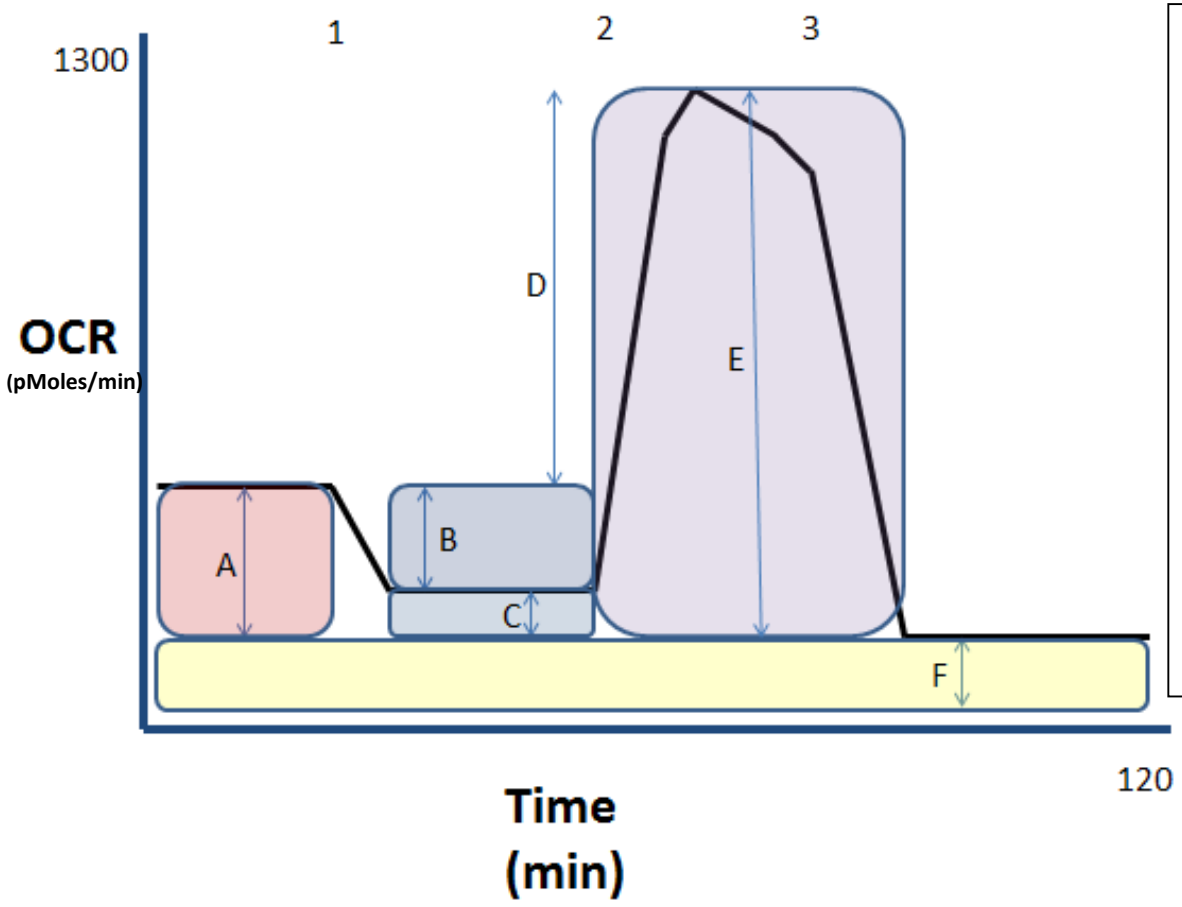
2.7 Spare respiratory capacity

The term reserve respiratory capacity or spare respiratory capacity (Figure 2.4 OCR following FCCP – baseline OCR) is used to describe the amount of extra ATP that can be produced by oxidative phosphorylation in case of a sudden increase in energy demand. Depletion of the reserve respiratory capacity has been related to a range of pathologies affecting high energy requiring tissues. During aging of an organism, and as a result of mitochondrial dysfunctions, the efficiency of oxidative phosphorylation declines. Postoperatively, lymphocytes may require a sudden burst of additional cellular energy in response to stress or increased workload for many reasons (Table 1.4). If the spare respiratory capacity of the cells is not sufficient to provide the required ATP, affected cells may be driven into senescence or cell death.(Desler, Hansen et al. 2012) Exhaustion of the reserve respiratory capacity has been correlated with a variety of pathologies including heart disease,(Sansbury, Jones et

al. 2011) neurodegenerative disorders,(Nicholls 2008) and cell death in smooth muscle.(Hill, Higdon et al. 2010) Changes in SRC in the postoperative phase may therefore be a contributing factor to development of postoperative morbidity and sepsis.

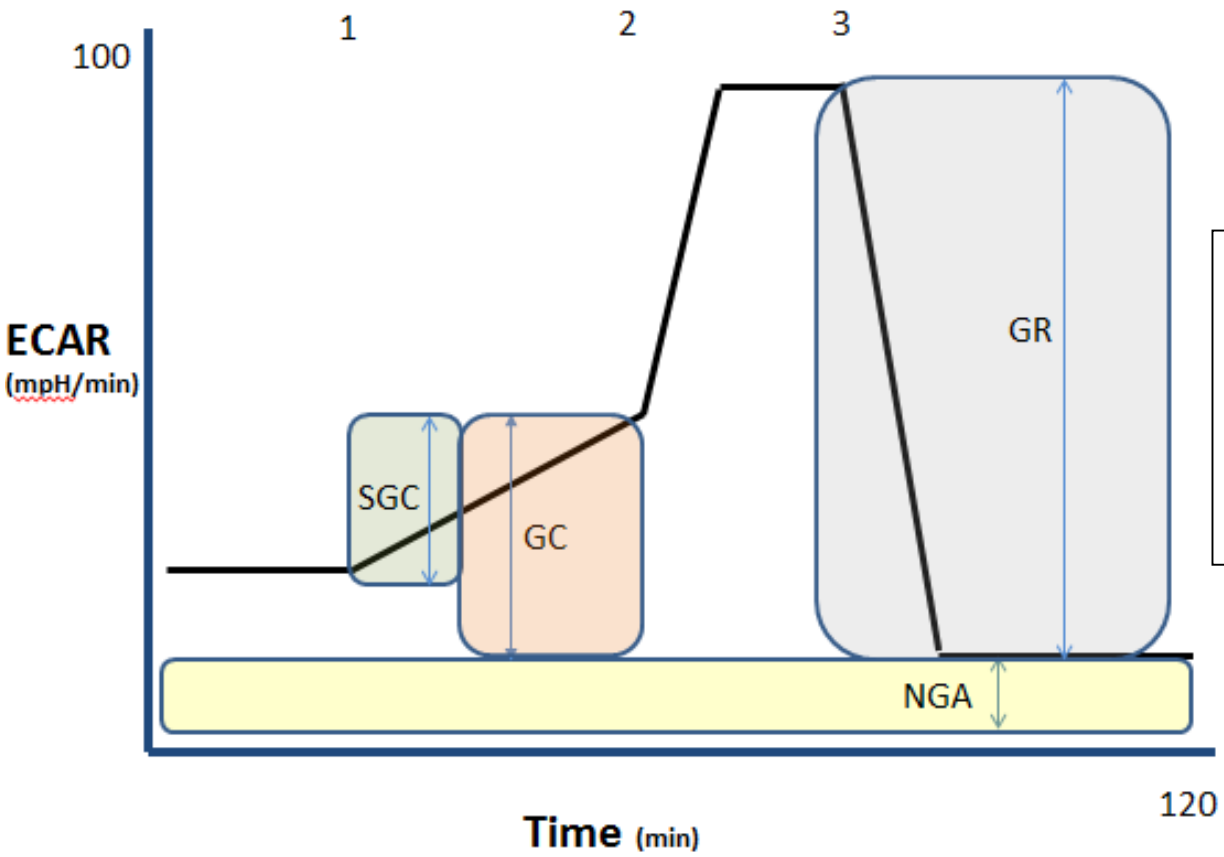
Glycolytic reserve and spare respiratory capacity reflect a cell's ability to respond to environmental perturbation. Some cell types operate closely to their maximum metabolic potential and are therefore less likely to survive a significant increase in metabolic demand or a decrease in substrate availability. For example, glioma stem cells have higher spare respiratory capacity than their progeny and this increase positively correlates with the ability to survive stressors like radiation.(Vlashi, Lagadec et al. 2011)

Figure 2.4 An example of oxygen consumption rate plot with labelled respiration parameters and drug administration points



OCR measured in pMoles/min, measured at 3 time points at 10 minute intervals before and after injection: 1= Oligomycin; 2= FCCP; 3= Rotenone and Antimycin A; A= Basal respiration; B= ATP turnover (coupling efficiency=Basal respiration – Respiration following oligomycin); C= H+ leak; D= Spare respiratory capacity=ETC Accelerator Response - Basal Respiration; E= Respiratory capacity; F= Non-mitochondrial respiration; Basal Response = 3rd basal measurement i.e. measurement before oligomycin
ETC Accelerator Response = Maximum rate after FCCP; ATP Coupler Response = Minimum rate after oligomycin

Figure 2.5 An example of extracellular acidification rate measurement plot demonstrating altered mitochondrial metabolism following injections of 1) oligomycin, 2) FCCP, and 3) 2-DG



1=oligomycin; 2= FCCP; 3= 2 Deoxyglucose; SGC = Spare glycolytic capacity (ECAR following oligomycin – Baseline ECAR); GC = Glycolytic capacity; GR = Glycolytic reserve; NGA = Non-glycolytic acidification

2.8 Measurement of mitochondrial membrane potential using a tetramethylrhodamine methyl ester (TMRM) whole blood assay

(a) Cell preparation for TMRM assay

Blood samples were processed on the same day as venepuncture. Mitochondrial membrane potential was measured using the following TMRM assay methodology.

TMRM is a cell-permeant, cationic, red-orange fluorescent dye that is readily sequestered by active mitochondria. It has been used to detect mitochondrial permeability and membrane depolarisation in lymphocytes.(Rasola and Geuna 2001) Preoperative CD4(+) and CD8(+) T cell populations were gated and analysed with and without an optimised concentration of FCCP. 2 µM concentration was found to reduce the mitochondrial membrane potential while ensuring that sufficient cells remained viable for flow cytometry analysis within 30 minutes of administration. FCCP was utilised as a positive control because it uncouples the ETC and therefore decreases mitochondrial membrane potential of lymphocytes. Providing patients were still hospitalised and consented to postoperative venepuncture, a further patient matched blood sample was taken on day 2 post surgery.

(b) Measurement of membrane potential using the TMRM assay

T-cell subsets (CD4 and CD8 cells) and their mitochondrial membrane potential were analysed by multiparametric flow cytometry. 100 µL of each heparinised blood sample was incubated with optimal concentrations of a APC conjugated CD4 antibody and vioblue conjugated CD8 antibody (Mylteni Biotec, Auburn) diluted in PBS with 0.2% bovine serum albumin for 30 min at 4^{o}C in the dark. The blood sample was then stimulated for 15 min at 37°C with or without 2 µM FCCP (Sigma Aldrich). After incubation with FCCP, 200 µM TMRM (made up in JC-1 buffer solution freshly prepared from a stock solution 2 mM in DMSO) was added and samples left for further 15-minute incubation. The JC-1 dye is used to determine to monitor mitochondrial membrane potential. It is predominantly a monomer that yields green fluorescence with emission of 530±15 nm. At high concentrations (due to higher mitochondrial membrane potentials) the dye aggregates yielding a red to orange coloured emission (590±17.5 nm). Consequently, mitochondrial depolarisation is indicated by a decrease in the red/green fluorescence intensity ratio and an increase in the aggregate fluorescent count is indicative of hyperpolarization. The potential-sensitive colour shift is due to concentration-dependent formation of red fluorescent J-aggregates. Treating cells with FCCP eliminates mitochondrial membrane potential and JC1 staining. JC1 is suitable for the labelling of mitochondria in live cells and is not compatible with fixation.

The samples subsequently underwent erythrocyte lysis with blood lysis buffer (Sigma Aldrich) for 15 min at room temperature. The cells were finally washed and centrifuged twice in PBS (Invitrogen) and re-suspended in 500µL PBS ready for flow cytometry. Isotype-matched mouse immunoglobulins were used as controls for CD4 and CD8 in each experiment. No isotype control was used for TMRM. Samples were immediately measured using a Cyan cytometer (BD Biosciences). The data were analysed using summit software (BD Biosciences). After the standard calibration of the flow cytometer, data were recorded until 10000 CD4+ T-cells were acquired or the cell suspension was exhausted.

2.9 Measurement of mitochondrial reactive oxygen species (ROS) production using a MitoSox assay on isolated lymphocytes

(a) Cell preparation for MitoSox assay

100,000 Isolated lymphocytes from patients were incubated with staurosporine (a proapoptotic microbial alkaloid) and mitochondrial ETC inhibitors (myxothiazol, rotenone and FCCP) for 30 minutes to increase mROS production by lymphocytes. Following incubation the MitoSox assay described below was performed in order to elucidate percentage of lymphocytes expressing mitochondrial ROS. Pre- and postoperative lymphocyte samples were also interrogated for presence of mitochondrial ROS at baseline (control) and following FCCP stimulation.

Staurosporine is a non-selective protein kinase inhibitor, which induces mitochondrial apoptosis through caspase dependent and independent pathways.(Zhang, Gillespie et al. 2004) Myxothiazol is an inhibitor of the mitochondrial cytochrome bc1 complex. It is a competitive inhibitor of ubiquinol, and binds at the quinol oxidation site of the bc1 complex, blocking electron transfer within the ETC of mitochondria.

(b) Measurement of mitochondrial ROS using MitoSox assay in isolated lymphocytes

The FlowCellect™ MitoStress Kit (Millipore, MA, USA) allows for the measurement of mitochondrial superoxide generation, which is detected by membrane permanent dye MitoSox Red using a single cellular sample.(Mukhopadhyay, Rajesh et al. 2007) Multiparametric evaluation of this marker allows the analysis of oxidative stress.(Aronis, Melendez et al. 2003)

The assay was performed as per manufacturer recommendations. 100,000 isolated lymphocytes (purity >99%) in 100 µL were added with and without 2 µL MitoSox Red working solution. Cells were incubated for 15 minutes in a 37°C CO_2 incubator and subsequently centrifuged at 300 x g for 5 minutes at room temperature. After aspirating off supernatant, one further wash was performed with 200 µL of 1x Assay

Buffer per well and cells were centrifuged at 300 x g for 5 minutes at room temperature, before being re-suspended with 200 µL of 1x Assay Buffer. Cells were analysed immediately on a flow cytometer equipped with blue and red lasers.

MitoSox Red is a fluorogenic dye that is live-cell permanent, and is selectively targeted to the mitochondria where it reacts with superoxide radicals and fluoresces yellow/red. MitoSox Red is excitable by a 488nm laser and fluoresces maximally at 580 nm (yellow fluorescence on the guava easyCyte 8HT).

2.10 Cell preparation of cells for intracellular cytokine assay

T-cell subsets and their intracellular cytokine production were analysed by multiparametric flow cytometry. 1 mL of a heparinised whole blood sample was stimulated for 16 hours at 37°C in RPMI medium (Gibco, New York, USA) at a ratio of 2:1 (RPMI : blood) with 10 µg/mL brefeldin A(Levings, Sangregorio et al. 2001{Gorden, 2005 #1705)} (control sample) (Sigma Chemical) with or without 100 ηM PMA (Tsukahara, Gordienko et al. 1993)(Sigma Chemical, Dorset, UK) and ionomycin (750mg/mL, Sigma Chemical, Dorset, UK).(Foster, Prussin et al. 2007) RPMI 1640 is a liquid medium utilizing a bicarbonate buffering system with amino acids and vitamins providing effective culture of lymphocytes. Brefeldin disaggregates the Golgi complex and enables the intracellular cytokine accumulation of proteins.(Pala, Hussell et al. 2000) PMA stimulates protein kinase C directly, the theta isoform of which is essential for T-cell activation.(Isakov and Altman 2002) Ionomycin is an ionophore produced by the bacterium *Streptomyces conglobatus*. It is used to raise the intracellular level of calcium (Ca^{2+}) and stimulate intracellular cytokine production in conjunction with PMA. After incubation, 100 µL of each blood sample was incubated with optimal concentrations of a FITC conjugated CD4(+) antibody and vioblue conjugated CD8(+) antibody (Mylteni Biotec, Auburn, California) diluted in PBS with 0.2% bovine serum albumin for 30 minutes at 4^{o}C in the dark. The samples were subsequently fixed and permeabilised with 200µL Cytofix/Cytoperm (BD Biosciences, San Diego) for 20 minutes at room temperature and then 1mL of BD Perm/Wash (BD Biosciences, San Diego) was added prior to centrifugation at 1500 rpm for 5 min and removal of the supernatant. Following centrifugation 4µL of APC conjugated cytokine (Miltenyi Biotec Ltd, Surrey) (IL-2, TNF-α or IFN-γ) were added to the cells and incubated at 4^{o}C in the dark for 30 minutes.

The cells were then fixed with 100 µL 2% PFA and erythrocyte lysis (Sigma Aldrich, Dorset, UK) was performed with blood lysis buffer for 15 minutes. The cells were finally washed and centrifuged twice in PBS and resuspended in 500 µL PBS ready for flow cytometry. This whole blood cytokine assay was

developed to optimise intracellular cytokine detection using flow cytometry. Isotype-matched mouse immunoglobulins were used as controls for each experiment.

Samples were immediately measured using a Cyan cytometer (BD Biosciences). After the standard calibration of the flow cytometer, data were recorded until 10000 CD4(+) T-cells were acquired or the cell suspension was exhausted.

2.11 Flow cytometry assays

(a) Preparation of cells for measurement in flow cytometer

Flow cytometry methodology for analysis of mitochondrial membrane potential, mitochondrial reactive oxygen species production and intracellular cytokine production by lymphocytes are described in the methods sections of the relevant chapters (Chapters 5 and 6).

For all experiments, the cytometer was maintained and calibrated on a daily basis using calibrant samples to ensure the laser transmitters and detectors produced consistent and reliable frequency and intensity readings. All flow cytometry data were analysed using summit software (BD Biosciences).

(b) Control Samples utilised in flow cytometry experiments

The following control samples were prepared for the flow cytometry experiments performed within this thesis, as recommended by Tung et al.(Tung, Heydari et al. 2007)

I. Unstained control

Unstained cells were utilised as control samples due to autofluorescence, a phenomenon which is dependent on multiple factors including: incubation medium used, activation status of cells and laser channel utilised.

II. Isotype control

The reagents utilised to identify cell types in this thesis rely on reagent binding to antigen binding sites containing variable regions (within Fab portion of antibody). Binding of reagents can however also occur to constant regions (within Fc portion of antibodies). Isotype controls elucidate the binding occurring to constant regions / Fc portions of antibodies, therefore allowing subsequent determination of amount of fluorescence occurring from variable / Fab regions being studied.

III. Fluorescence Minus One (FMO)

For all flow cytometry experiments performed in this thesis utilising greater than one fluorophore, a FMO sample was utilised as a control in order to determine the effect that each channel had on fluorescence. Since the compensation corrections differ according the amounts of the various reagents present on cells in different subsets, it is important to independently determine the boundary between positive and negative cells for each subset. This is done by including FMO controls for all fluorescence channels within this boundary. The FMO control samples were treated with the same medium and protocol as other samples, but were stained with one less reagent in the stain set. This aided discrimination between poorly defined cell populations and increased accuracy of results.

Furthermore in all flow cytometry experiments, data was always collected prior to application of fluorescence compensation correction.

Table 2.3. Chemicals and reagents used in thesis experiments

Reagent	Supplier	Address	Batch No.	Lot No.
Dulbeccos's Phosphate-Buffered Saline (Calcium and Magnesium-free) (PBS)	Invitrogen Ltd	Paisley, UK	14190-094	1250143
Ficoll-Paque PLUS (Ficoll sodium diatrizoate)	GE Healthcare	Buckinghamshire, UK	17-1440-03	10115414
Dulbecco's Modified Eagle Medium with GlutaMAX, Sodium Pyruvate and 1g/L or 4.5 g/L D-glucose (DMEM)	Invitrogen Ltd	Paisley, UK	21885-025	1038505
Blood Lysis Buffer: 0.84 g $NaHCO_3$ 7.7g NH_4CL 1000 mL distilled water	Chemicals from Sigma Aldrich	Dorset, UK		
Paraformaldehyde (diluted to 2% concentration in PBS)				
Dexamethasone				
Trypan Blue solution	Sigma Aldrich	Dorset, UK		
Heparin sodium 1000 iu/mL	Leo Laboratories Ltd	Buckinghamshire, UK		
Concanavalin A, from *Canavalia ensiformis* (Jack bean), Type IV 097K670	Sigma Aldrich	Dorset, UK		
Oligomycin	Sigma Aldrich	Dorset, UK		
FCCP	Sigma Aldrich	Dorset, UK		
Rotenone	Sigma Aldrich	Dorset, UK		
Antimycin A	Sigma Aldrich	Dorset, UK		
2-deoxyglucose (2-DG)	Sigma Aldrich	Dorset, UK		
MitoStress kit	Millipore	Hayward, CA, USA	FCCH100109	12-0525
Dimethyl Sulfoxide	Sigma Aldrich		03363MD	200-664-3
Bovine Serum Albumin (diluted to required concentration in PBS then sterile filtered)	Sigma Aldrich	Dorset, UK		

Lot and batch numbers provided where available

Table 2.4. Monoclonal antibodies and isotype controls used in thesis experiments

Antibody-fluorochrme	Isotype	Clone	Supplier	Utility	Batch No.	Lot No.
CD3-APC CD3-FITC	Mouse IgG2a	BW264/56	1	T-lymphocytes		
CD4-FITC	Mouse IgG1	M-T466	1	T helper lymphocytes (and at lower levels on monocytes / dendritic cells)	130-080-501	5120321133
CD8-VioBlue	Mouse IgG2a		1	T cytotoxic lymphocytes and NK cell subset		5130226124
CD14-APC	Mouse IgG2a	TUK4	1	Monocytes / macrophages. Low level expression on neutrophils.		
TNF-A- APC	Rat Ig2b		1	Cytokine	130-091-649	5120725162
IFN-G- APC	Rat Ig2b		1	Cytokine	130-091-640	5120614130
IL-2- APC	Rat Ig2b		1	Cytokine	130-091-644	5120614131

1. Miltenyi Biotec Ltd, Surrey, UK. 2. Abcam plc, Cambridge, UK 3. Biolegend UK Ltd, Cambridge, UK. 4. Cayman Chemical Company, Ann Arbor, USA. Lot and batch numbers provided where available

Table 2.5. Scientific machines and disposable equipment utilised

Equipment	Supplier	Address	Serial No.	Lot No.
Centrifuge: Haraeus Megafuge 1.0R	Kendro Laboratory products	Langenselbold, Germany	232128	
Centrifuge: ALC PK120	ALC	Cologno Monzese, Italy	79909574	
Centrifuge: Eppendorf 5415 C	Eppendorf	Hamburg, Germany		
Heated water bath Grant model JB4	Grant Instruments	Cambridge, UK	629640002	
Vortex – Rotamixer	Hook and Tucker instruments	Croyden, UK	7725	
Flow Cytometer: CyAn ADP 9 colour with Summit 4.3.1 software	Beckman Coulter Inc	Fullerton, USA	626	
Scales				
15 mL Falcon polypropylene tubes	BD Biosciences	Oxford, UK		
50 mL Falcon polypropylene tubes	BD Biosciences	Oxford, UK	8362220	3009676
10, 200 and 1000 µL Diamond sterilised pipette tips	Gilson Inc.	Middleton, USA	REFF167101 REFF167103 REFF167104	GCDE0/12314 UBBF2/52780 UZBC2/20080
24-well plates: Costar 24 well culture cluster	Corning International	NY, USA		
Flow cytometer test tubes	Elkay			
3 mL sterile Pasteur pipettes	Ramboldi Ltd	Limassol, Cyprus		
Cell culture incubator Galaxy R 170litre	RS Biotech Laboratory Ltd	Irving, UK	2154	
Biological safety cabinet Class II Holten LaminAir Model 1,2	Thermo Scientific	Waltham, USA		
Bench microscope: Zeiss Axiovert 25	Zeiss	Oberkochen, Germany	606731	451200
Neubauer improved haemocytometer	Assistant	Germany		
24 Well culture plate: Falcon	Becton Dickinson	Franklin Lakes, NJ, USA	353047	

Lot and serial numbers provided where available

CHAPTER 3

Lymphopenia is associated with lower anaerobic threshold and increased postoperative morbidity

Chapter 3

Lymphopenia is associated with lower anaerobic threshold and increased postoperative morbidity

3.1 Introduction

The relationship between lymphopenia and mortality in patients with cardiac failure highlights the need to explore preoperative immunological status as a plausible mechanism contributing to patients with low AT developing postoperative morbidity and requiring a prolonged hospital stay (Chapters 1.10 and 1.11).

Exercise data has previously been utilised to distinguish patients with varying severity of heart failure. (Hansen, Sun et al. 2012) Hansen et al. demonstrated distinctive patterns for New York Heart Association (NYHA) classification, reflecting sequential changes in cardiac output, arterial and mixed venous Oxygen (O_2) and carbon dioxide (CO_2) content differences, and ventilation and developed reference formulas (based on oxygen consumption, CO_2 production and ventilation) on the basis of exercise tests of healthy subjects.

While lymphopenia has been related to cardiac morbidity, the relationship between lymphopenia and AT measured by cardiopulmonary exercise testing (CPET) has thus far not been explored. I also determine perioperative changes in peripheral lymphocyte count and NLR occurring within the first 5 days following surgery (and until hospital discharge) using prospectively collected data with large patient numbers.

3.2 Hypothesis

In patients undergoing elective colorectal surgery (COMPETE-C) and mixed surgery (VISION-UK), preoperative lymphopenia is associated with impaired aerobic capacity, postoperative morbidity and increased length of hospital stay.

Methods

3.3 Study populations

Patients undergoing major colorectal surgery that were screened or recruited to participate in the COMPETE-C randomised controlled trial, conducted at Derriford Hospital, Plymouth, UK, between March 2009- April 2010 (ISRCTN 14680495), were included in the following analyses:

1) AT / lymphocyte count analysis
2) Preoperative lymphopenia and hospital length of stay
3) Preoperative lymphopenia and incidence of sepsis (≥ 2 SIRS criteria and positive culture)
4) Differences in preoperative lymphocyte counts in patients diagnosed with postoperative sepsis
5) Postoperative trends in lymphocyte count (absolute and %) and NLR

Absolute lymphocyte count, relative lymphocyte count and NLR were analysed from 7 time points during the patients' hospital stay where available form the COMPETE –C study data. Time points analysed included preoperative, day 1-5 post surgery and day of discharge (if > day 5 post surgery). Cut-off values for lymphopenia were defined as < 1.2 x 10^9/L and NLR >5. Justification for utilising these values is provided in Chapters 1.5 and 1.6.

Patients undergoing major elective surgery across all specialties at UCLH recruited into the VISION-UK study were included in the following analyses:

1) Preoperative lymphopenia and hospital length of stay
2) Preoperative lymphopenia and incidence of postoperative morbidity (POMS scoring)

3.4 Data collection

Different team members collected all data in a blinded manner. Those performing surgery and investigators were unaware of the hypothesis in question.

COMPETE-C: Cardiopulmonary Exercise Testing. Patients attended preoperative clinic for assessment and CPET testing. Patients completed symptom-limited maximal cardiopulmonary exercise testing (CPET) as part of their routine preoperative assessment. Following a period of 2min rest and 2 min unloaded pedalling, patients began ramped, continuous incremental, and symptom limited exercise on a stationary ergometer (Zan, nSpire, CO, USA), with a ramp gradient 15–20 W/minute. CPET was

stopped as determined by the patients' tolerance. Ventilation and gas exchange variables were measured using a metabolic cart (Zan, nSpire, CO, USA). 12-lead electrocardiogram, non-invasive blood pressure, and pulse oximetry were monitored throughout the test period. Anaerobic threshold (AT) was used as the marker of aerobic fitness. AT was determined by two independent assessors according to the published guidelines using V-slope and confirmed by ventilatory equivalents.(Pina, Balady et al. 1995)

VISION-UK: Postoperative morbidity survey (POMS) scoring. All patients recruited in to the VISION-UK study at University College London Hospital were followed up by research nurses blinded to the morbidity scores and blood test results. Prospective POMS scoring,(Bennett-Guerrero, Welsby et al. 1999, Sanders, Keogh et al. 2012) a validated tool used to assess development of postoperative morbidity (complications) was performed on day 3 and day 7 postoperatively by performing chart and patient review where indicated. The categories and definitions of morbidity information collected using this survey are outlined below:

Table 3.1 POMS Survey – performed on day 3 and day 7 following surgery in VISION-UK patients

Morbidity type	Criteria
Pulmonary	The patient has developed a new requirement for oxygen* or respiratory support*
Infectious	Currently on antibiotics* and/or has had a temperature of >38°C in the last 24 h*
Renal	Presence of oliguria <500 mL/24 h*, increased serum creatinine (>30% from preoperative level)*; urinary catheter in situ for nonsurgical reason*
Gastrointestinal	Unable to tolerate an enteral diet for any reason*, nausea and vomiting or abdominal distension*
Cardiovascular	Diagnostic tests or therapy within the last 24 h for any of the following: (1) new MI or ischemia*, (2) hypotension (requiring fluid therapy >200mL/h or pharmacological therapy*, (3) atrial or ventricular arrhythmias*, (4) cardiogenic pulmonary edema and thrombotic event (requiring anticoagulation)*.
Neurological	New focal neurological deficit*, confusion or delirium*
Hematological	Requirement for any of the following within the last 24 h: packed erythrocytes, platelets, fresh-frozen plasma, or cryoprecipitate*
Wound	Wound dehiscence requiring surgical exploration or drainage of pus from the operation wound with or without isolation of organisms*
Pain	New postoperative pain significant enough to require parenteral opioids or regional analgesia*
Mobility	Unable to mobilise in same manner as preoperatively*
Drain	In situ*

* indicates one point, maximum possible score = 20

3.5 Study Outcomes

Full details of the COMPETE-C (Challand, Struthers et al. 2012) and VISION-UK (Botto, Alonso-Coello et al. 2014) protocols have previously been published.

Outcomes for this chapter are as follows:
Primary outcome – Demonstrate relationship between AT <11 mL/kg/min and lymphopenia relative to AT >11mL/kg/min (COMPETE-C).

Secondary outcomes - Demonstrate relationship between lymphocyte count and development of postoperative sepsis (COMPETE-C cohort: defined by ≥ 2 of the following: temperature < 36^{o}C / >38^{o}C; heart rate >90/min; respiratory rate >20/min or $PaCO_2$ <4.3KPa; White cell count <4 or >12 x 10^9/L *and* a positive culture) and postoperative morbidity (VISION-UK cohort: POMS criteria) and the association between lymphopenia (<1.2 x 10^9/L) and length of hospital length of stay (COMPETE-C and VISION-UK cohorts). I also explore the impact of age, gender and co-morbidity (RCRI ≥ 3, AT< 11mL/kg/min) on LOS and the postoperative recovery of lymphocyte count (and NLR) until day of hospital discharge (COMPETE-C cohort).

3.6 Sample size and statistical analysis

I powered the primary outcome on the basis that approximately 30% colorectal patients with low AT (<11mL/kg/min)(Wilson, Davies et al. 2010) would exhibit a prognostically significant difference in lymphocyte count <1.2 x 10^9/L,(Halazun, Aldoori et al. 2008, Ding, An et al. 2010, Proctor, McMillan et al. 2012) with an anticipated standard deviation of 1.0.Thus, ≥185 patients with AT > 11mL/kg/min, would be required compared with ≥ 56 patients with low AT (alpha = 0.05; power = 90%). Kolmogorov–Smirnov was used to test continuous data for normality of distribution. Mean values between AT groups were analysed using independent samples t-test. One way MANOVA (multivariate) analysis was used to determine NLR and lymphopenia in low and normal AT groups adjusted for age and malignancy (known causes of lymphopenia(Peron, Cropet et al. 2013, Brass, McKay et al. 2014) and reduced AT(Matsumura, Nishijima et al. 1983, Williams, Nyasavajjala et al. 2014)) Length of stay analyses between normal and lymphopenic patients, high and low AT and high and low NLR were performed using log rank analysis and cox regression analysis was utilised to determine the effect of lymphopenia (<1.2 x 10^9 cells/L) and other potential confounding variables, once again corrected for age and malignancy. Related samples Friedman's two-way analysis of variance by ranks was used to compare whether differences existed between non-parametric data at different perioperative time points. Post-

hoc analysis was performed with Friedman pairwise test. All statistical analyses were performed using IBM SPSS Version 20 (IBM Corporation, Somers, New York, USA).

Results

3.7 Demographics

Demographics, and associated cardiopulmonary test parameters, of patients undergoing preoperative CPET are shown in Table 3.2.

Table 3.2. CPET demographics in COMPETE-C colorectal surgery cohort, stratified according to the prognostically relevant anaerobic threshold of 11mL $kg^{-1}min^{-1}$

	AT <11mL/kg/min	AT>11mL/kg/min	P-value
Number	79	161	
Age (Years)	71 (69-73)	63 (61-66)	0.0001
Male : Female	1.05	1.47	0.3
Cancer n(%)	66 (86)	114 (72)	0.02
Chemotherapy (n,%)	21 (13)	11 (14)	0.82
Body mass index (kg/m^2)	29.6 (28.5-30.6)	27.4 (26.7-28.0)	0.0004
AT (mL/kg/min)	9.4 (8.23-10.33)	13.75 (12.2-15.78)	<0.001
VE/VCO2	30.3 (28.9-31.7)	27.8 (27.2-28.5)	0.0004
Peak VO_2 (mL/kg/min)	14.8 (13.9-15.6)	22.5 (21.5-23.4)	<0.0001

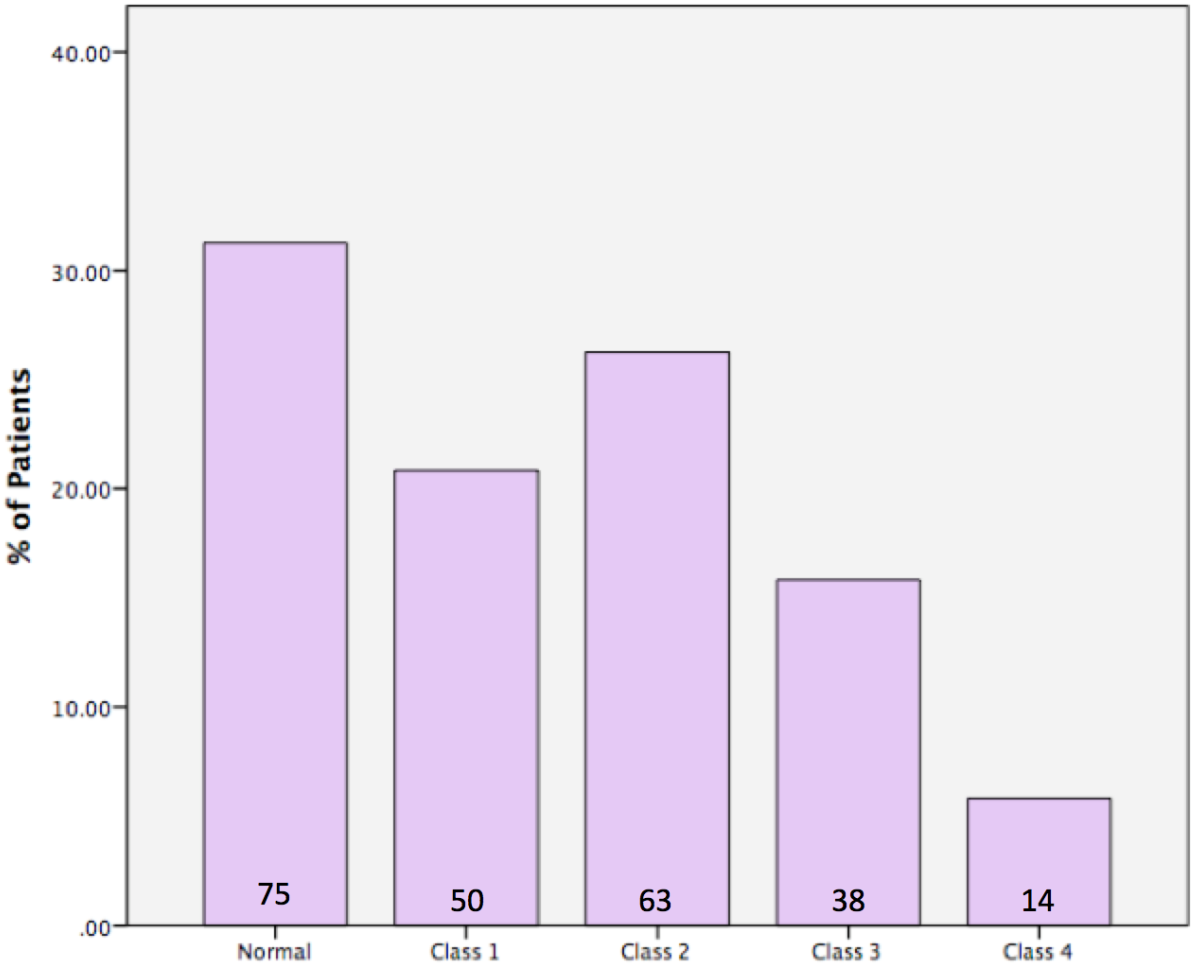
Data are presented as mean (95% confidence intervals), unless stated otherwise.

Patients included in the analysis for this study were randomised to receive either goal directed colloid therapy or standard of care fluid therapy. There was no difference in mean AT (mean AT in both groups >11mL/kg/min; p = 0.589), preoperative lymphocyte count (SD) (1.75 (0.67) vs. 1.80 (0.80) x 10^9/L; p=0.657) or preoperative relative lymphocyte count (24.6 (8.7) vs. 24.5 (8.7) %; p=0.96) between the randomised and non-randomised groups. Length of stay was also similar between groups (p=0.07).

3.8 CPET physiological characteristics and leukocyte subsets

69 % of patients exhibited an AT associated with New York Heart Association class 1-4 heart failure,(Lang, Karlin et al. 2009, Hansen, Sun et al. 2012)(Figure 3.1), with the majority of anaerobic threshold values consistent with Normal and NYHA Class 1-2.

Figure 3.1 Histogram showing numbers of patients (n=240) stratified by AT-defined NYHA class



Cut-off values for AT estimated from recent published series (Normal >14 mL/kg/min; NYHA 1 = 12.1-14 mL/kg/min; NYHA 2 = 10.1-12 mL/kg/min; NYHA 3 = 7.6-10 mL/kg/min; NYHA 4 = <7.5 mL/kg/min).(Lang, Karlin et al. 2009, Hansen, Sun et al. 2012). Numbers of patients in each category shown in each bar.

3.9 Anaerobic threshold and leukocyte subsets (COMEPETE-C)

Unadjusted, the AT cut-off value <11mL/kg/min was associated with absolute and relative lymphopenia and higher NLR, (Table 3.3). Adjusted for the presence of malignancy and age, impaired cardiovascular performance was also associated independently with higher NLR (p=0.022), absolute (p=0.001) and relative lymphopenia (p=0.001). Controlling for age and the presence of malignancy (present in 77% of colorectal surgical patients), no significant interaction was observed between malignancy and AT <11mL/kg/min (P=0.29). Multiple regression analysis identified AT as the sole factor associated with higher NLR (P=0.033).

Table 3.3 Leukocyte subsets according to low or normal AT groups

	≥ 11mL/kg/min (n=159)	**< 11mL/kg/min (n=77)**	**P-value**
WCC (x 10^9/L)	7.39 (2.09)	7.99 (2.20)	0.043*
NLR	3.05 (1.55)	3.59 (1.65)	0.017*
Neutrophil count	4.90 (1.85)	5.23 (1.73)	0.208
Lymphocyte count (x 10^9/L)	1.83 (0.76)	1.63 (0.55)	0.043*
Monocyte count %	0.57 (0.21)	0.63(0.27)	0.07
Lymphocyte %	25.74 (9.02)	22.32 (7.31)	0.005*
Neutrophil %	66.31 (9.36)	69.25 (7.72)	0.02*
Monocyte %	7.95 (2.51)	8.43 (3.04)	0.209

All data represented mean (SD); n=236 patients. * = p<0.05; Missing AT data from 4 patients and missing preoperative leucocyte data from 11 patients.

3.10 Sepsis Outcomes related to lymphopenia (COMEPETE-C)

Patients that developed postoperative sepsis in the colorectal population had lower preoperative lymphocyte counts compared to patients who did not develop sepsis (n=30 vs. 142; 1.61(0.45) vs. 1.85(0.77) x 10^9/L respectively; p=0.022; independent t-test). Preoperative lymphopenia was not associated with increased sepsis (4/15 in lymphopenic group vs. 26/157 in normal lymphocyte group; p=0.324; Chi-squared test).

3.11 Postoperative development of morbidity (VISION-UK)

In the VISION-UK patients recruited at UCLH undergoing surgery from a variety of surgical specialties, preoperative lymphopenia (patients with preoperative lymphocyte count <1.2 x 10^9/L; mean count (SD) = 1.00 (0.46) x 10^9/L in lymphopenic group and 2.14 (0.81) x 10^9/L in normal count group) was associated with increased postoperative morbidity at Day 3 (antibiotic requirement, catheter requirement, no enteral feeding, blood transfusion requirement and pain), and at Day 7 postoperatively (pulmonary, antibiotic requirement, no enteral feeding, wound complications and pain; Table 3.4).

Table 3.4 Incidence of POMS defined complications stratified to preoperative lymphopenia (VISION-UK)

		n	Normal (n=773)	Lymphopenia (n=96)	Relative Risk [95% CI]	P-value
DAY 3 POMS	**Pulmonary**	161	137	24	1.41[0.97-2.06]	0.075
	Antibiotics	89	72	17	1.90 [1.17-3.09]	0.009*
	Fever	19	15	4	2.15[0.73-6.34]	0.17
	AKI	8	7	1	1.15[0.14-9.25]	0.90
	Oliguria	3	3	0	1.14[0.06-21.90]	0.087
	Catheter	68	55	13	1.90[1.08-3.35]	0.03*
	No Enteral feed	69	27	42	12.53[8.11-19.34]	<0.0001*
	N+V / distension	120	103	17	1.33[0.84-2.13]	0.23
	Hypotension	5	5	0	0.73[0.04-13.02]	0.83
	ACS	3	3	0	1.14[0.06-21.90]	0.93
	Anticoagulation	3	3	0	1.14[0.06-21.90]	0.93
	Arrhythmia	4	4	0	0.89[0.05-16.34]	0.94
	Confusion	4	3	1	2.01[0.23-17.83]	0.53
	Focal neurological deficit	1	1	0	2.66[011-64.84]	0.55
	Wound	39	31	8	2.08[0.98-4.39]	0.06
	Blood	27	19	8	3.39[1.53-7.53]	0.003*
	Pain	153	129	24	1.50[1.02-2.19]	0.04*
	Drain	24	19	5	2.11[0.81-5.55]	0.13
			N=771	**N=96**		
DAY 7 POMS	**Pulmonary**	41	31	10	2.59[1.31-5.12]	0.006*
	Fever	5	4	1	2.01[0.23-17.78]	0.53
	Antibiotics	49	38	11	2.32[1.23-4.39]	0.009*
	AKI	4	3	1	2.68[0.28-25.48]	0.39
	Oliguria	6	4	2	4.02[0.75-12.63]	0.11
	Catheter	71	59	12	1.63[0.91-2.93]	0.10
	No Enteral feed	32	23	9	3.14[1.50-6.60]	0.002*
	N+V / distension	37	30	7	1.87[0.85-4.15]	0.12
	Hypotension	2	1	1	8.03[0.51-127.37]	0.14
	ACS	1	1	0	2.65[0.11-64.67]	0.55
	Anticoagulation	4	4	0	0.88[0.05-16.30]	0.93
	Arrhythmia	1	0	1	23.88[0.98-582.06]	0.05
	Confusion	2	1	1	8.03[0.51-127.37]	0.14
	Focal neurological deficit	2	1	1	8.03[0.51-127.37]	0.14
	Wound	20	13	7	4.32[1.77-10.57]	0.001*
	Blood	7	5	2	3.18[0.63-16.17]	0.16
	Pain	27	20	7	2.81[1.22-6.47]	0.02*
	Drain	10	8	2	2.01[0.43-9.32]	0.37

*= Demonstrates increased incidence of morbidity in lymphopenic group; AKI=acute kidney injury; N+V=nausea and vomiting; ACS=acute coronary syndrome

3.12 Lymphopenia and Hospital Length of stay

The absolute lymphocyte count, <1.2 x 10^9/L (COMPETE-C; n= 228; p=0.035-Figure 3.2), but not relative lymphocyte count <15% (p=0.243) nor NLR>5 (p=0.193), was associated with increased hospital length of stay. Preoperative lymphopenia was also associated with prolonged hospital length of stay in the VISION-UK patients (n=881; Figure 3.3).

Cox regression (or proportional hazards regression) is a method for investigating the effect of several variables upon the time a specified event such as hospital discharge takes to happen. Cox regression analysis was performed on the clinical and demographic data available from the COMPETE-C data set in order to determine which factors influenced LOS. Results from this analysis are summarised in Table 3.5.

Table 3.5 Cox regression model showing the effect of variables on length of stay

Variable	Co-efficient (B)	Standard Error	Wald (X^2)	P-Value	Risk [95%CI]
Age	-0.015	0.007	0.504	0.478	0.995 [0.981, 1.009]
Cancer diagnosis	0.012	0.192	0.004	0.950	1.012 [0.694, 1.475]
RCRI score	-0.156	0.094	2.759	0.097	0.856 [0.712, 1.028]
AT	0.041	0.019	4.995	0.025*	1.042 [1.005, 1.081]
Lymphopenia	-0.479	0.204	5.526	0.019*	0.620 [0.416, 0.924]

*= p<0.05

The association between absolute lymphocyte count < 1.2 x 10^9/L and LOS in the COMEPETE-C cohort was found to be independent of age, RCRI score and diagnosis of cancer. Absolute lymphocyte count also did not independently predict LOS > 7 days (ROC analysis; p = 0.367) therefore indicating that alone it is not a reliable predictor of prolonged length of hospital stay in this patient population.

Figure 3.2 Kaplan Meier Plot demonstrating length of stay and preoperative lymphopenia (COMPETE—C; n=240; <1.2 x 10^9/L; log rank test)

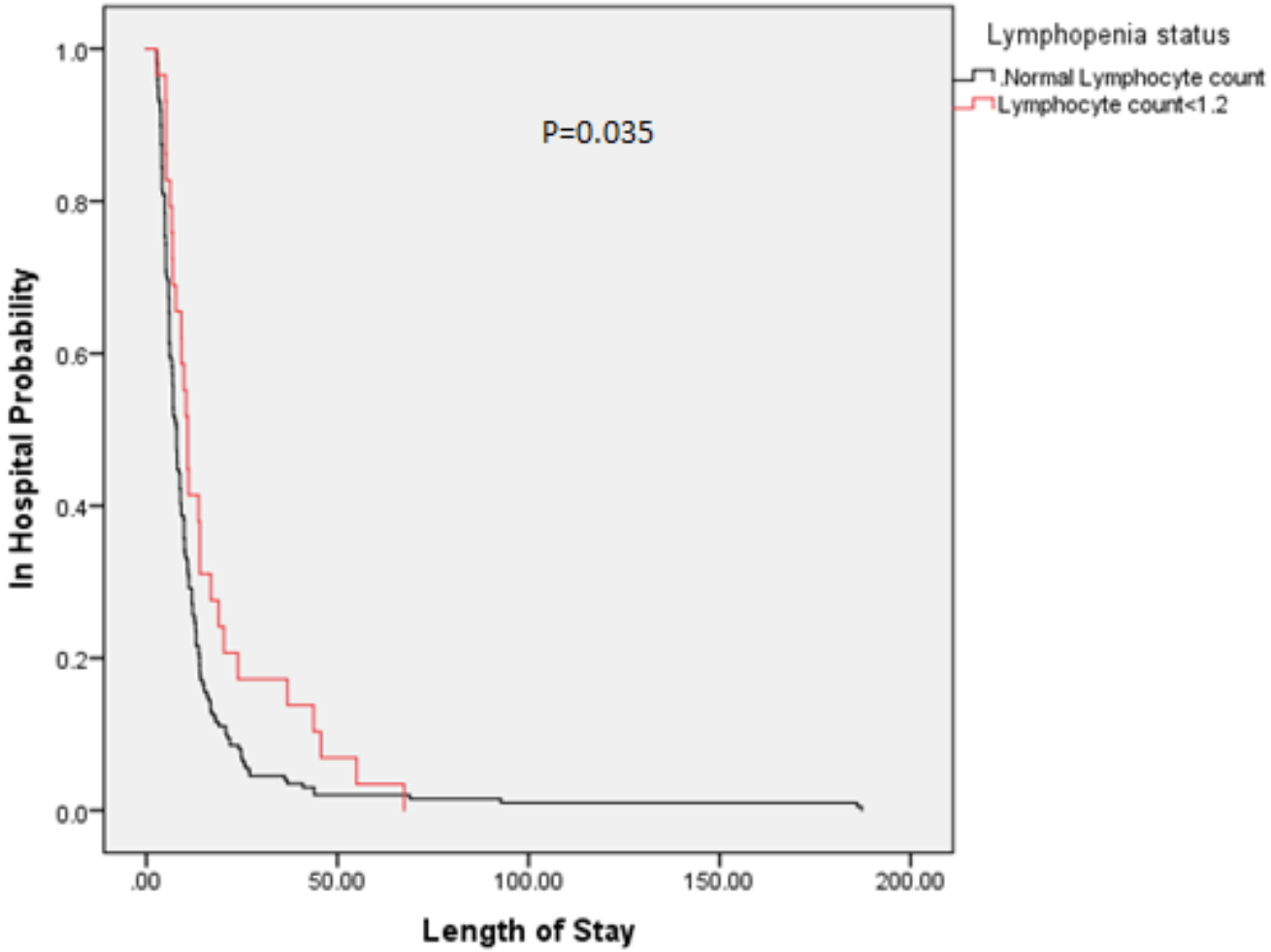
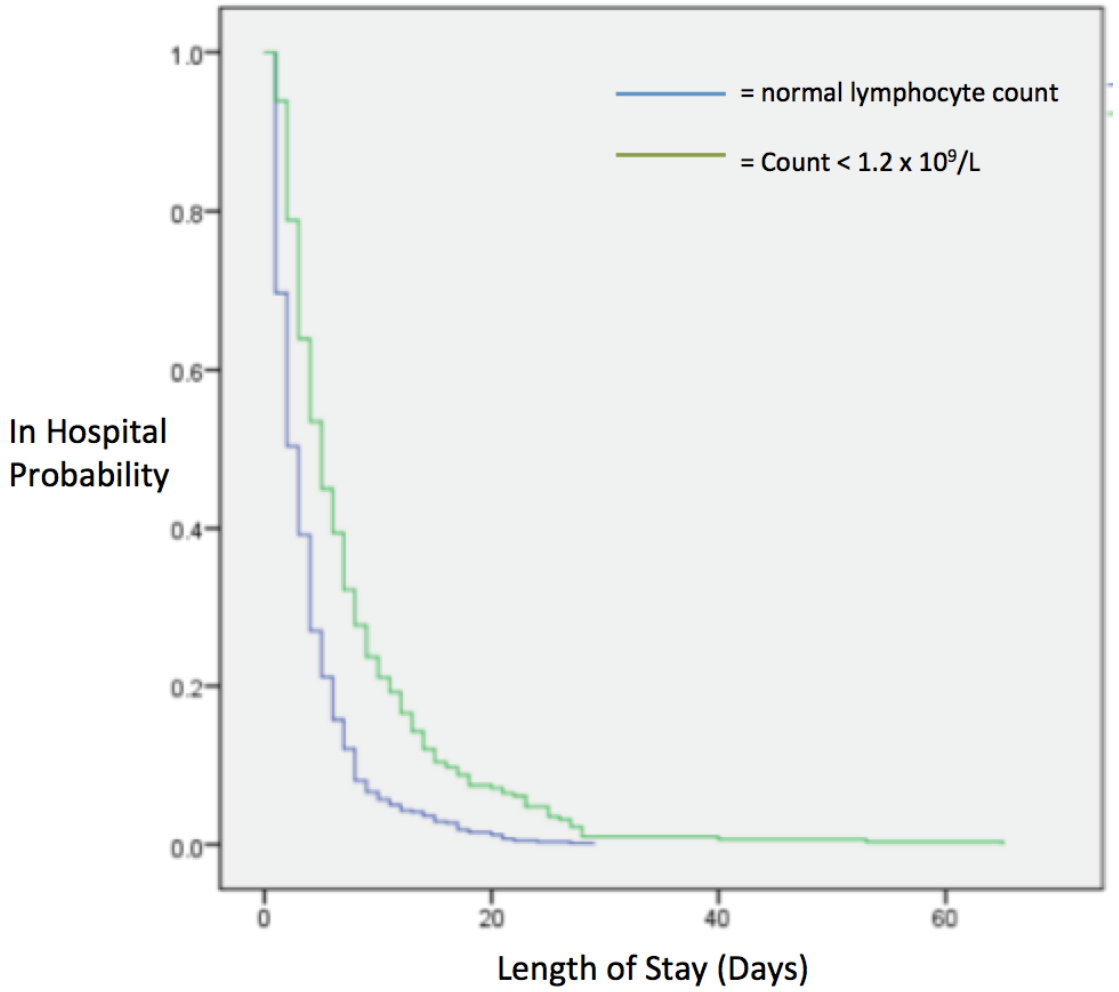


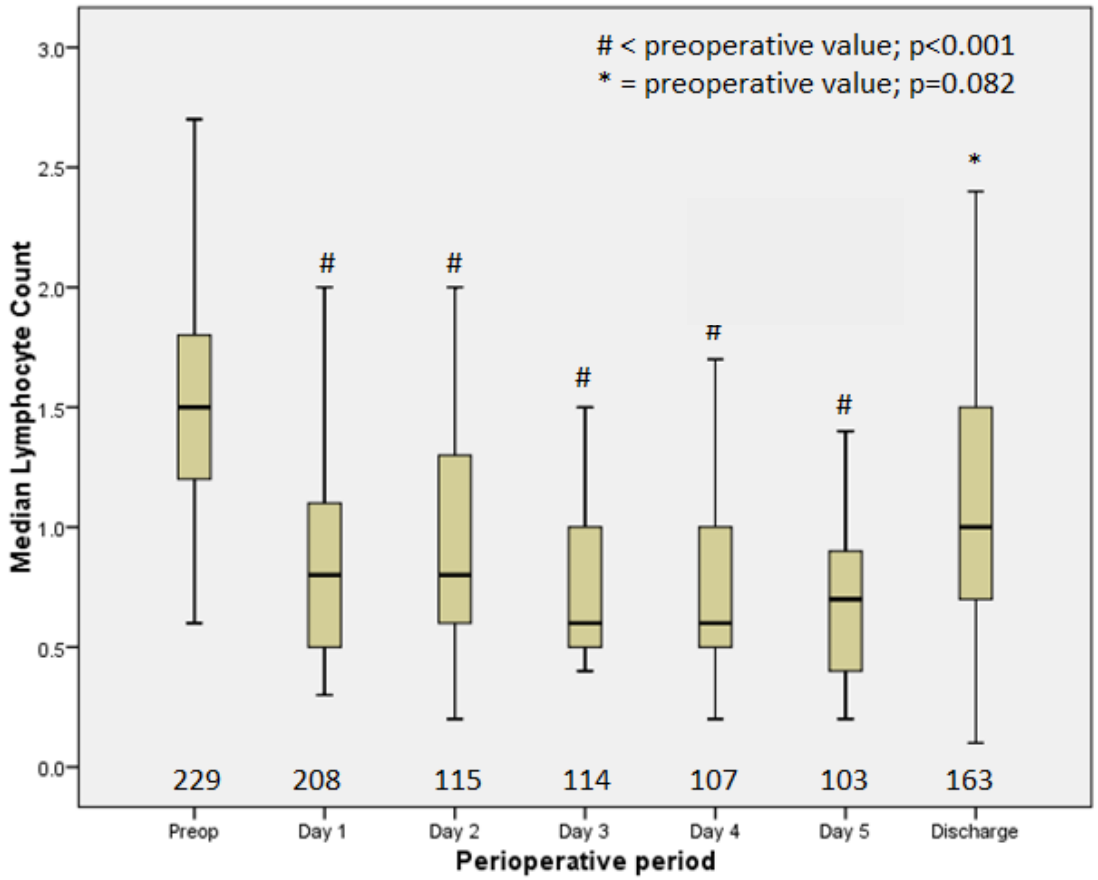
Figure 3.3 Kaplan Meier plot demonstrating length of stay and preoperative lymphopenia (VISION-UK; n=881; log-rank test; p<0.001)



3.13 Perioperative trends in lymphocyte count and NLR (COMPETE-C)

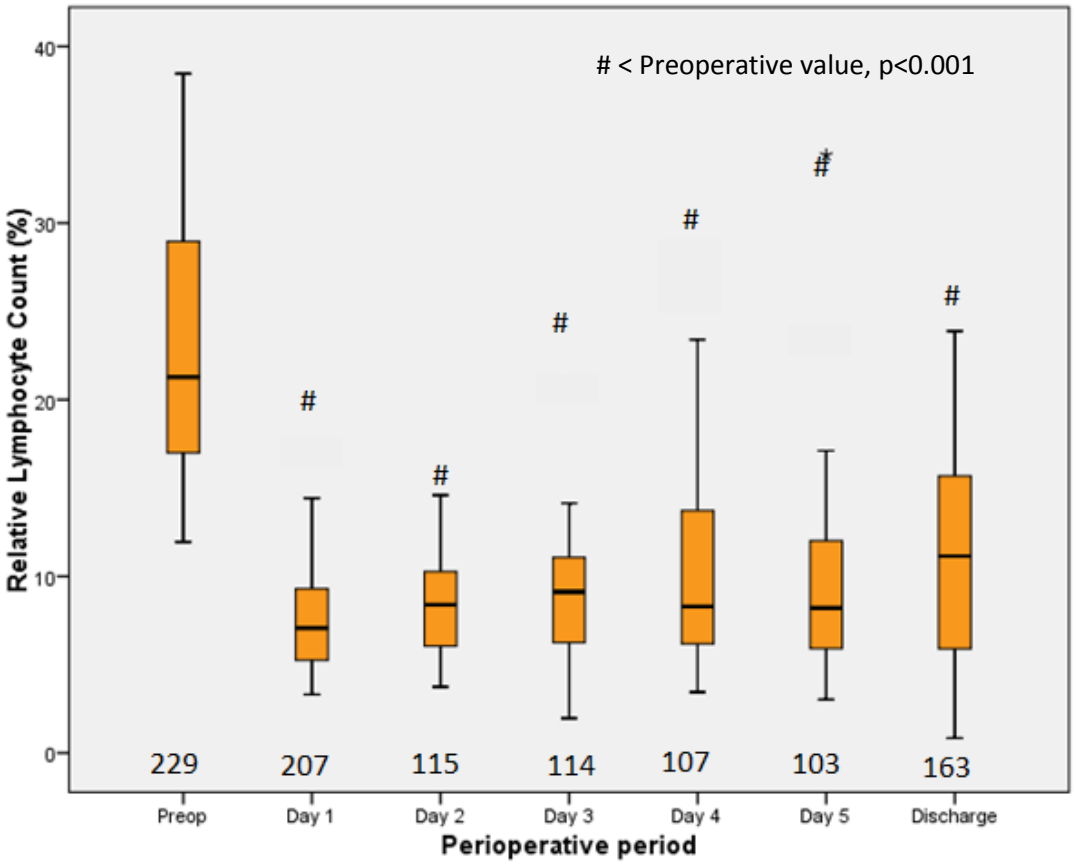
Day 1 to 5 postoperative counts are summarised in Figures 3.4-3.6. Absolute count, but not relative lymphocyte count or NLR, recovered to preoperative level by day of hospital discharge (p=0.082, p<0.001, p<0.001 respectively). Median length of stay (LOS) was 8 (IQR 5-13) days.

Figure 3.4 Median lymphocyte count (x 10^9/L) in the perioperative period



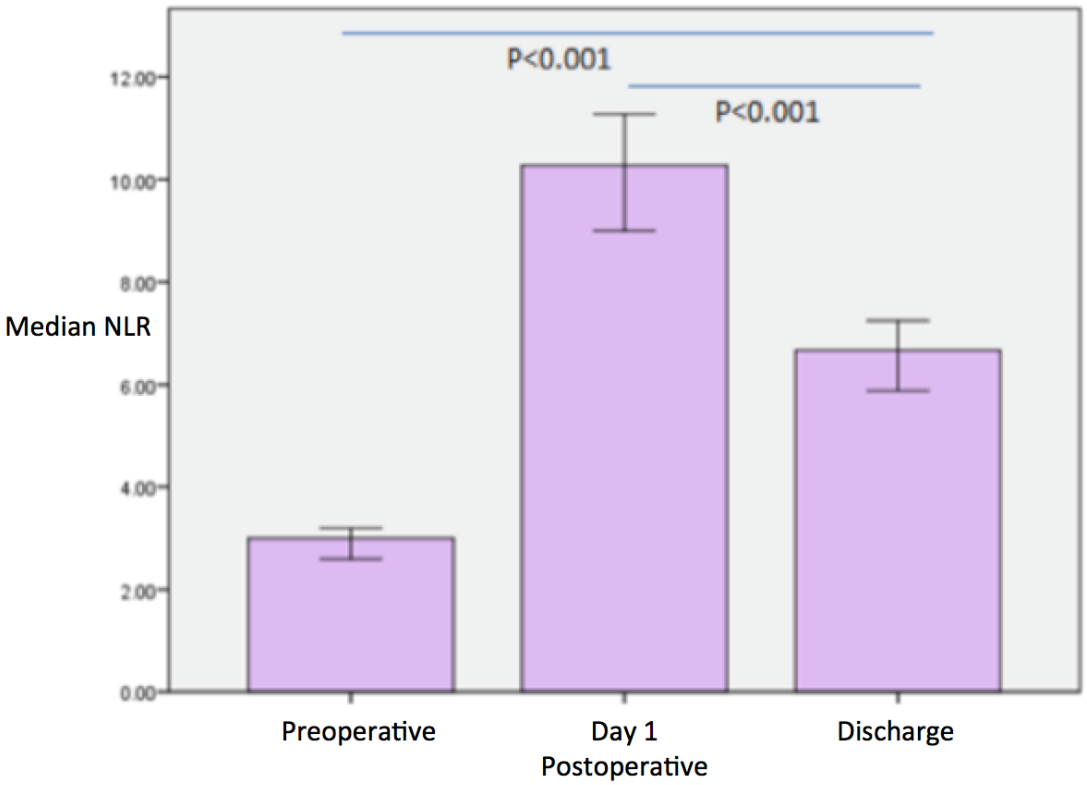
Numbers above x-axis demonstrate number of patients with lymphocyte counts performed. Proportion of patients remaining in hospital: Day 1 = 100% (n=239); Day 2 100% (n=239); Day 3 93% (n=222); Day 4 83% (n=198); Day 5 72% (n=172). Significant difference between groups evaluated with related samples Friedman's two-way analysis of variance by ranks (p<0.001; n=28) and post-hoc analysis performed with Friedman pairwise test.

Figure 3.5 Perioperative relative lymphocyte count (COMPETE-C)



Numbers above x-axis demonstrate number of patients with lymphocyte counts performed. Differences between groups evaluated with related samples Friedman's two-way analysis of variance by ranks (n=28; p<0.001) and post-hoc analysis with Friedman pairwise test. No differences were demonstrable between Day 1-Day 5 and discharge counts.

Figure 3.6 Perioperative changes in NLR (COMPETE-C)



Preoperative n= 229; D1 n=208 and discharge n=163

Discussion

3.14 Anaerobic threshold and leukocyte subsets

The relationship between immune response and exercise capacity has previously been described.(Mathur and Pedersen 2008) Habitual exercise for example, is associated with improved immune responses to vaccination(Pascoe, Fiatarone Singh et al. 2014) and outcomes following viral infections(Simpson 2011) and malignancy.(Ballard-Barbash, Friedenreich et al. 2012)

These data show that cardiac failure, stratified by AT (<11mL/kg/min measured by CPET), is associated with lymphopenia and raised NLR. These biomarkers have emerged as robust prognostic indicators of colorectal surgical outcome.(Satomi, Murakami et al. 1995, Walsh, Cook et al. 2005, Chu-Yuan, Jing et al. 2013, Kozak, von Eyben et al. 2015) Though many studies have reported the association between preoperative NLR and postoperative complications (Cook, Walsh et al. 2007, Halazun, Aldoori et al. 2008, Ding, An et al. 2010, Proctor, McMillan et al. 2012, Jankova, Dent et al. 2013) and low AT and postoperative complications,(Lee, Chaloner et al. 2006, Murray, Whiting et al. 2007, Ausania, Snowden et al. 2012, Colson, Baglin et al. 2012, Hartley, Pichel et al. 2012, Junejo, Mason et al. 2012, Swart and Carlisle 2012) none have thus far linked lymphopenia (or NLR) to aerobic performance.

These findings suggest that low preoperative AT, often found in patients who have not received a formal diagnosis of heart failure, is associated with lymphopenia which is indicative of a chronic inflammatory immune state. This pre-existing immune state appears to be associated with subsequent development of complications from all body systems resulting in prolonged hospital length of stay. Prolonged hospital stay in patients with preoperative lymphopenia has been demonstrated in two separate cohorts (colorectal and different types of non-cardiac surgery). This suggests that this is a phenomenon, which is consistently seen in lymphopenic patients undergoing major elective surgery. Since the presence of overt cardiac failure is an important prognostic factor in determining postoperative outcome,(Hernandez, Whellan et al. 2004, Hammill, Curtis et al. 2008) this data suggests that dysregulation of inflammatory control may play a contributory role in developing postoperative morbidity.

Cardiac failure is associated with neurohormonal activation and chronic systemic inflammation,(Topkara, Evans et al. 2011) characterised by marked alteration in absolute numbers, and function, of polymorphonuclear, monocytes and T-cells.(Topkara, Evans et al. 2011) More severe heart failure is associated with higher levels of circulating endotoxin(Niebauer, Volk et al. 1999), cytokines(Rauchhaus, Koloczek et al. 2000) and lymphopenia.(von Haehling, Schefold et al. 2009, Nunez, Minana et al. 2011, Vaduganathan, Ambrosy et al. 2012) Immune dysregulation has been traditionally thought to be a marker of cardiac disease progression. However recent evidence suggests that cardiac failure and poor aerobic capacity are in fact *caused* by immune dysfunction.(Topkara, Evans et al. 2011) There is also evidence demonstrating patients with preoperative depletion of antibodies to endotoxin exhibit higher levels of proinflammatory cytokines and sustaining more perioperative morbidity.(Bennett-Guerrero, Ayuso et al. 1997, Bennett-Guerrero, Panah et al. 2001)

Whether lymphopenia is a mediator, or a marker, of poorer perioperative outcomes requires further research. Data in this chapter demonstrates a significantly lower preoperative lymphocyte count in patients who subsequently develop sepsis, which supports previous studies highlighting the important role of lymphocytes in murine(Hotchkiss, Tinsley et al. 1999) and human(Heffernan, Monaghan et al. 2012) models of injury/critical illness. A substantial proportion of patients exhibit lymphopenia preceding surgery, which suggests the presence of an “at risk” population of patients susceptible to developing postoperative morbidity. This is also consistent with recent data showing increased mortality in patients failing to reverse lymphopenia during critical illness.(Heffernan, Monaghan et al. 2012)

There are several limitations of this exploratory study. Matching NYHA class to CPET variables may be inaccurate given the fact that inter-observer variability exists among clinicians grading severity of cardiac failure according to clinical symptoms using the NYHA system.(Raphael, Briscoe et al. 2007) Validation of these cut-off values analysing lymphocyte functionality and AT may be warranted together with measurement of endotoxin levels. The association between NLR and coronary artery disease (Demir 2013, Fowler and Agha 2013) may reveal mechanistic links to perioperative outcomes, including myocardial injury. Ultimately, detailed outcome data- including propensity to infectious complications- would test the hypothesis further that low AT may be associated with defective innate and/or adaptive immune functionality.

In summary, lymphopenia is associated with impaired cardiorespiratory reserve. These observations suggest that preoperative inflammation, associated with impaired cardiorespiratory performance, may contribute a pathophysiological role in determining postoperative outcome.

3.15 Lymphopenia and length of stay

This data demonstrates an association between lymphopenia (<1.2 x 10^9/L) and prolonged length of hospital stay following elective colorectal surgery. This cut-off value of lymphopenia has also previously been related to worse survival in patients with pancreatic cancer.(Fogar, Sperti et al. 2006) The effect of lymphopenia on LOS in this cohort was found to be independent of age, RCRI and diagnosis of cancer. Assessing hospital stay in this cohort reflects the acquisition of postoperative morbidity, and thus serves a useful time-dependent measure enabling the most robust statistical assessment – Kaplan-Meier/log-rank tests- to interrogate the data, as previously reported.(Visser, Keegan et al. 2009) Development of sepsis in this cohort was also associated with a lower preoperative lymphocyte count. It should be noted that while the Compete-C trial was a study demonstrating no differences in outcome between goal-directed therapy and control groups,(Challand, Struthers et al. 2012) there are clear differences between lymphopenic and normal lymphocyte groups with regards to length of stay.

Consistent with findings from previous studies, this study demonstrates that there is a significant decrease in absolute and relative lymphocyte counts and a rise in NLR seen postoperatively. Previous studies have reported normalisation of lymphocyte count by the 4th-7th day following major general surgery.(Hamid, Bancewicz et al. 1984, Lennard, Shenton et al. 1985) This data is consistent with previously published data since absolute lymphocyte counts did not return to preoperative levels by the 5th postoperative day, but did so by the day of hospital discharge (median LOS [IQR] = 8 [8] days).

Further work is required to elucidate the factors, which influence recovery of leukocyte numbers. This data has not determined the individual or combined effects of anaesthesia and surgery upon leukocyte subset values. The advantage of using a homogenous tissue injury model such as colorectal surgery in a single institution was the limitation of a number of potential confounding factors including: pre-existing surgical comorbidity, surgical type,

operator, anaesthesia provider care and postoperative care received. The reproduction of results in other surgical specialties validates these findings.

In summary, preoperative lymphocyte count has emerged as a robust prognostic marker of surgical outcome, which raises the clinically relevant possibility that preoperative immune characteristics contribute to the pathophysiological mechanisms determining postoperative outcome. Furthermore even at the time of hospital discharge NLR and relative lymphocyte levels do not return to pre-surgery level, which implies that immunological recovery from surgery may take longer than previously appreciated.

CHAPTER 4

Postoperative lymphopenia is associated with a hypometabolic phenotype

Chapter 4
Postoperative lymphopenia is associated with a hypometabolic phenotype

4.1 Heart failure and lymphocyte bioenergetic function

Mitochondrial physiology of lymphocytes has previously been associated with metabolism of other cells including cardiomyocytes in murine studies.(Cortez, Neves et al. 2012) This concept is supported by studies demonstrating increased apoptosis of peripheral lymphocytes following myocardial infarction,(Konstantinova, Khomyakova et al. 2011) and myocardial mitochondrial dysfunction being demonstrated as a major contributing factor to cardiac failure.(Rosca, Minkler et al. 2011) Consequently lymphopenia may result in reduced AT as described in Chapter 3. Development of pre or postoperative lymphopenia may be secondary to impaired metabolic function.

Most studies thus far exploring lymphocyte metabolism have been either murine studies or experiments performed on human leukaemic cell lines. In this chapter I explore the bioenergetic changes associated with postoperative lymphopenia, (which has been unequivocally demonstrated to occur in Chapter 3).

4.2 Surgery and bioenergetic function of lymphocytes

Decreased lymphocyte proliferation in response to mitogenic stimulation occurs after tissue trauma,(Faist, Schinkel et al. 1993) which can last for days.(Horgan, Mendez et al. 1994) Impaired T cell proliferation is associated with increased susceptibility to infection and death.(Keane, Birmingham et al. 1983) Bioenergetic changes inducing lymphocyte apoptosis following anaesthesia and surgery may be a possible cause of postoperative immune dysfunction and increased morbidity. There is thus far no published human data describing the effect of surgery on bioenergetic function of lymphocytes.

Despite the importance of having sufficient ATP available for the energy-dependent processes involved in immune activation, little is known about the metabolic adaptations that occur in vivo to meet the increased ATP requirements during activation of lymphocytes in the postoperative state and in the presence of other pathologies including heart failure.(Gatza, Wahl et al. 2011)

Lymphocyte functions required for a successful response to antigenic stimulation are dependent on an increase in ATP via glycolysis.(Jones and Thompson 2007) Defects in cellular energy production can lead to both reduced lymphocyte function, and apoptosis with consequent lymphopenia. Bioenergetic failure is therefore a potential unifying defect consistent with both postoperative lymphopenia and a failure to respond appropriately to lymphocyte activation induced by the range of perioperative insults encountered. Postoperative tendency of lymphocytes to undergo apoptosis may also be secondary to bioenergetic dysfunction.

4.3 Hypothesis

Metabolic dysfunction develops in lymphocytes postoperatively.

Methods

4.4 Patients

For study, I selected a stable, well-defined population of patients free of pathologies associated with lymphopenia, undergoing scheduled, standardised tissue trauma (elective joint replacement surgery),(Bennett-Guerrero, Welsby et al. 1999, Ghaferi, Birkmeyer et al. 2009, Kirksey, Chiu et al. 2012) and characterised metabolic changes in lymphocytes postoperatively.

Orthopaedic patients undergoing elective hip and knee arthroplasty for degenerative arthropathy were recruited into the single-centre POM-E study at University College London Hospital. The same teams performed surgery and anaesthesia, and standardised care was delivered according to local protocols. Patient recruitment and protocol is summarised in Chapter 2.2.

4.5 Extracellular flux analyzer (Seahorse XF24) technique

Methods of cell separation and bioenergetic analysis of lymphocytes is summarised in Chapter 2.

4.6 Statistical Analysis

Data were analysed by descriptive and comparative approaches. All experiments were performed in replicates of 4-5 (lymphocyte yield permitting). Laboratory studies were powered on the basis that previous work has reported that ≥30% fall in ATP production result in altered cellular function.(Leist, Single et al. 1997) Given that normal ATP content in lymphocytes is approximately 3 nmol per million cells(Karlsson, DePierre et al. 1997, Gergely, Grossman et al. 2002) an anticipated difference in ATP content of 1±0.5 nmol (mean±SD) between lymphocyte groups would require at least 7 pre- and postoperative matched patient samples per experiment or lymphocyte count group (α=0.05; β=0.8). Therefore to account for approximately 45% dropout or withdrawal from the study or discharge before day 3 postoperatively a sample size of 13 would be needed. All results are expressed as absolute numbers and percentages (means ±SD). Fisher's exact test was used to compare categorical data distributions, and Kolmogorov–Smirnov to test continuous data for normality of distribution of continuous data. Parametric methods and

tests were used as indicated to analyse normally distributed data, and two-group non-parametric Wilcoxon signed-rank and Mann–Whitney U tests to analyse data deviating from a normal distribution. Statistical analyses were undertaken with IBM SPSS Version 20 (IBM Corporation, Somers, New York, USA). Reported P values are two-sided, and a p value ≤0.05 was considered to indicate statistical significance.

Results

4.7 Demographics

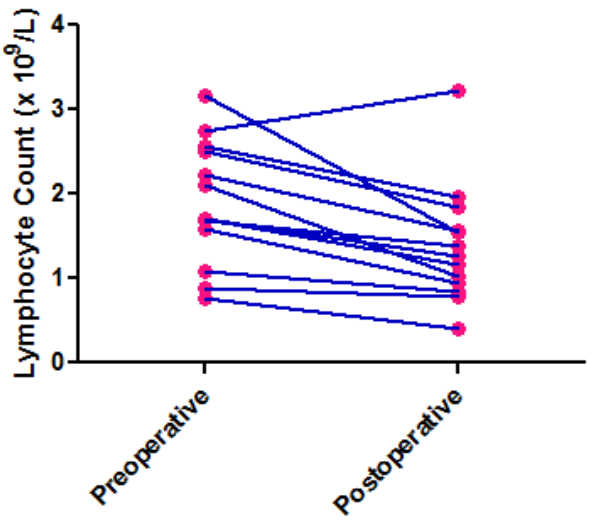
13 patients undergoing elective orthopaedic surgery (POME-E) were recruited into this study. Patient characteristics are summarised in Table 4.1. Perioperative changes in lymphocyte count are shown in Figure 4.1.

Table 4.1 Pre vs. postoperative (paired sample) orthopaedic patient characteristics

	Patient Cohort (n=13)
Age (years)	74 (69 - 78)
Preoperative lymphocyte count (x 10^9/L)	1.77 (1.30-2.23)
Preoperative relative lymphocyte count (%)	26.85 (20.45 – 33.25)
Postoperative lymphocyte count (x 10^9/L)	1.38 (0.95-1.81)

Values presented as mean (95%CI)

Figure 4.1 Perioperative changes in lymphocyte count in patients recruited for bioenergetic analysis



n=13; p<0.0001; Wilcoxen signed rank test

4.8 Oxygen consumption rate

Figure 4.2 demonstrates the postoperative median (IQR) reduction in oxygen consumption of number matched lymphocytes taken preoperatively and day 3 postoperatively. ETC accelerator response (defined as maximum response following FCCP – non-mitochondrial baseline oxygen consumption; see Figure 2.4) decreased postoperatively (383.44 (147.77-619.10) vs. 142.28 (107.62-176.93) pMoles/min; p=0.038). This rate is indicates the maximal oxygen consumption by mitochondria (the difference between baseline oxygen consumption from non-mitochondrial sources and maximal consumption following FCCP). It gives an indication of the oxygen consumption that can take place at complex IV, whether limited by availability of substrate or activity of the electron transport chain. This is a measure of the maximal potential respiratory capacity the cell can utilise under conditions of stress and/or increased energetic demands e.g. occurring during activation and proliferation

Median coupling efficiency (OCR basal – OCR Oligomycin; (IQR; indicating the amount of cellular oxygen consumption coupled to ATP synthesis) decreased postoperatively (126.61 (72.54 – 180.68) vs. 50.33 (36.72 – 63.95) pMoles/min; p=0.005), spare respiratory capacity (maximal potential respiratory capacity the cell can utilise under conditions of

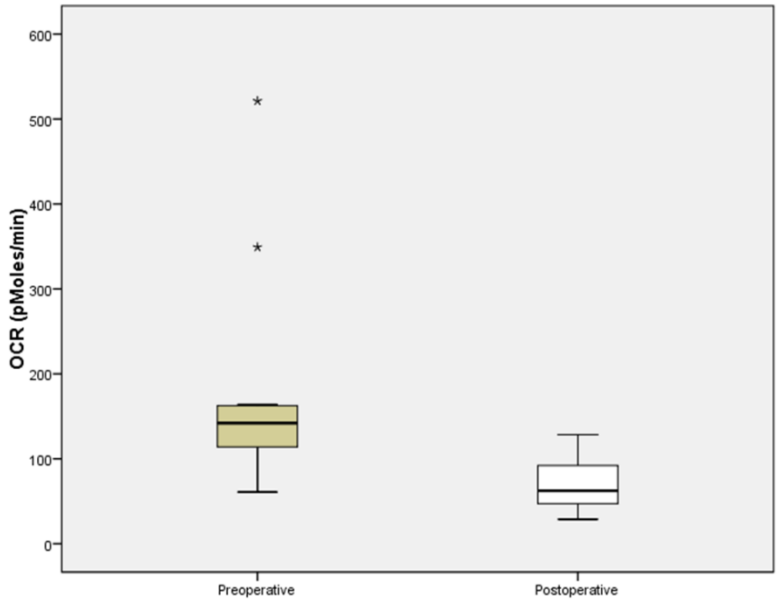
increased energetic demands) decreased postoperatively (OCR FCCP – OCR basal; 235.77 (63.81 – 407.74) vs. 80.57 (58.33 – 102.82) pMoles/min; p=0.007; IQR; Related samples Wilcoxen signed rank test).

4.9 Extracellular acidification rate

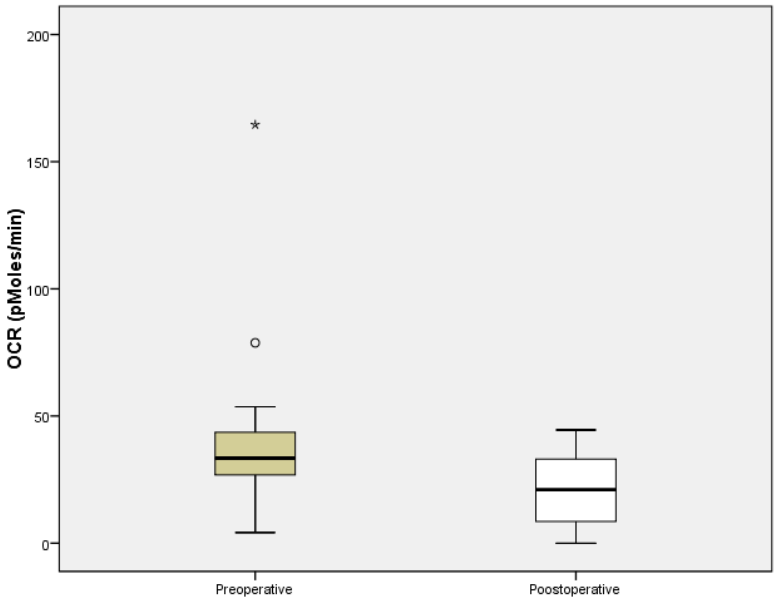
The effect of 2 deoxyglucose on ECAR in shown in Figure 4.3. The changes in extracellular acidification rate are summarised in Table 4.2. There was a decrease in postoperative ECAR values at baseline and following administration of each drug. Mean glycolytic reserve (FCCP ECAR - 2DG ECAR; 95% CI) decreased postoperatively (106.61 (73.52 - 139.70) vs. 75.38 (56.03 – 94.73); p=0.016).

Figure 4.2 Median oxygen consumption rate of lymphocytes preoperatively and day 3 postoperatively

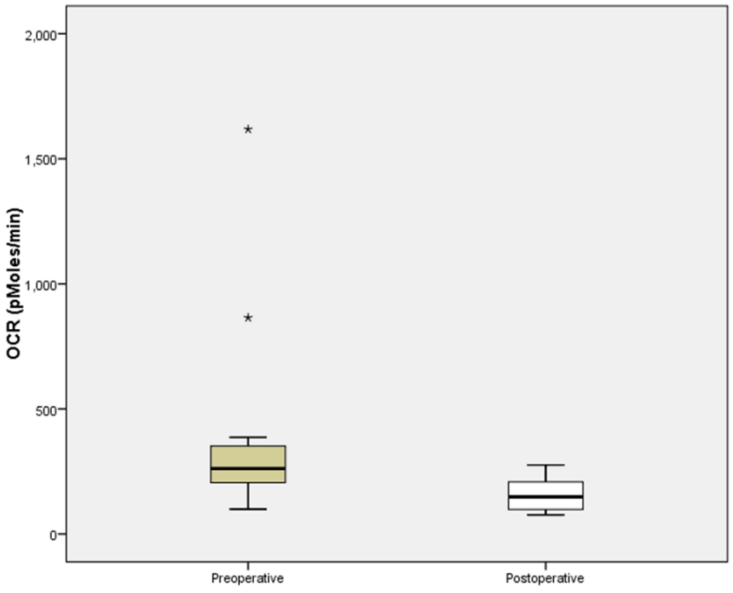
A) **Baseline OCR** (n=13; p=0.002)



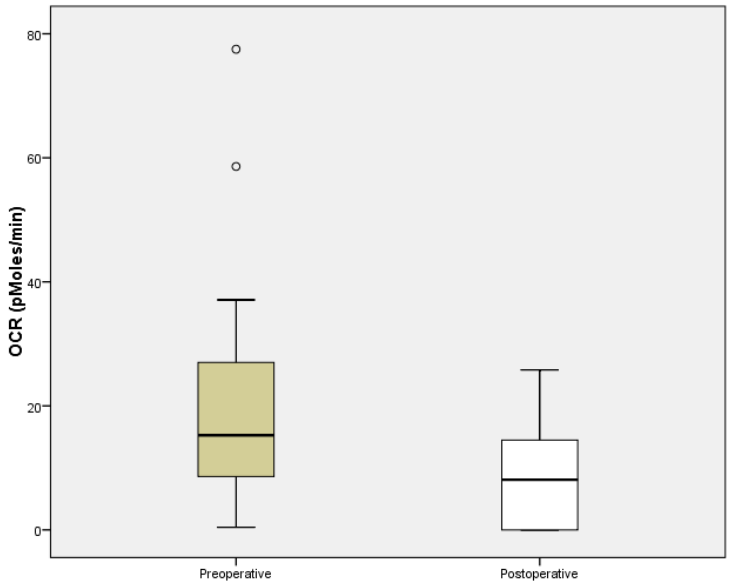
B) **OCR following oligomycin** (n=13; p=0.016)



C) **OCR following FCCP** (n=13; p=0.004)



D) **OCR following rotenone and antimycin-A** (n=13; p=0.039)

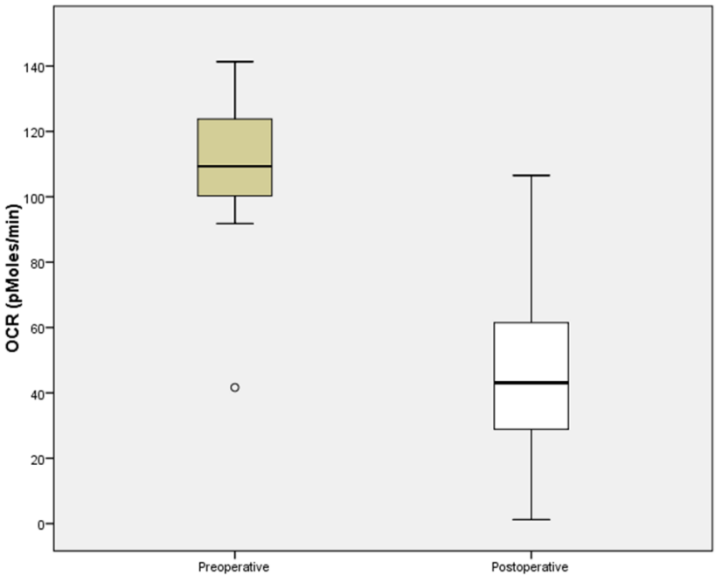


= Preoperative

= Postoperative

All paired values analysed using related samples Wilcoxen Signed rank test

Figure 4.3 Effect of 2DG (2 deoxyglucose) on oxygen consumption rate in the perioperative period (n=7; p=0.028)



= Preoperative

= Postoperative

All paired values analysed using related samples Wilcoxen Signed rank test

Table 4.2 Perioperative extracellular acidification rate values

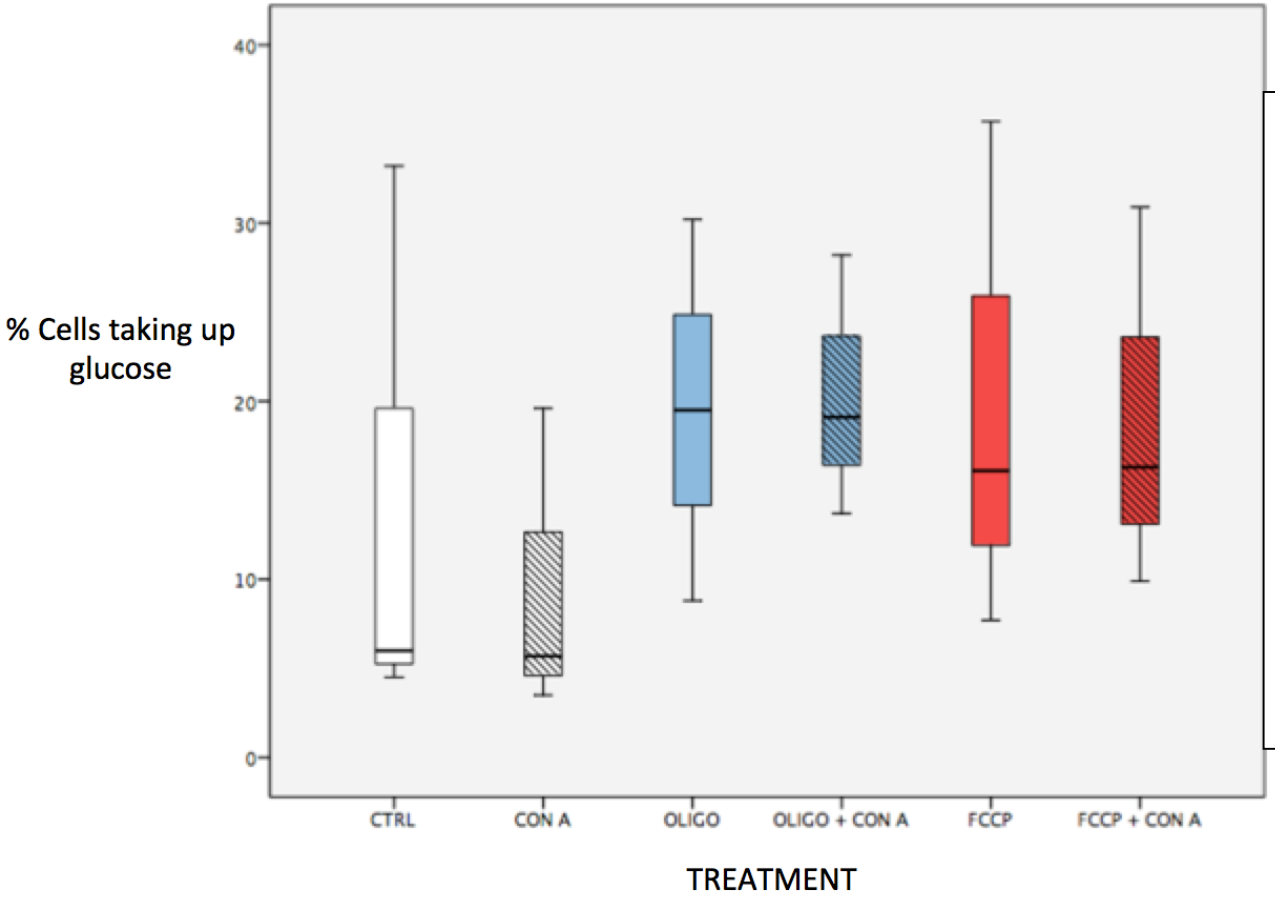
Treatment	Preoperative ECAR mpH/min (95% CI)	Postoperative ECAR mpH/min (95% CI)	p-value
Basal	60.10 (30.88-89.32)	37.41 (22.28-52.54)	0.0029*
Oligomycin	103.96 (51.95-155.96)	73.12 (47.33-98.91)	0.0236*
FCCP	132.94 (70.24-195.64)	92.11 (60.49-123.74)	0.0225*
Rotenone / Anti-A	106.56 (56.74-156.37)	72.15 (48.27-96.02)	0.0260*
2DG	17.16 (12.78-21.54)	12.12 (5.92-18.33)	0.1075

* Represents p value < 0.05. Values presented as mean (95%CI)

4.10 Glucose uptake 2-NBDG

I next aimed to demonstrate that mitochondrial inhibitors increased glucose uptake, and addition of concanavalin did not affect glucose uptake, implying cell viability was not affected. Mitochondrial inhibitors utilised were: oligomycin (0.25 µM) and FCCP (2 µM); in medium containing physiologically normal concentrations of glucose to mimic normoglycaemia (6 mmol/L). Isolated lymphocytes obtained from 3 non-diabetic preoperative patients. Uptake of 2-NBDG (2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose), a fluorescent glucose analogue was measured using flow cytometry, following activation of lymphocytes with concanavalin-A for 2 hrs to monitor glucose uptake in live cells, as an indicator of cell viability. Glucose uptake occurred following administration of concanavalin-A, oligomycin and FCCP, indicating cell viability in the methodology used in this study (Figure 4.4).

Figure 4.4 Effect of Concanavalin A and mitochondrial inhibitors on glucose uptake (2NBDG)



CTRL = Control

CON A = Concanavalin A

OLIGO = Oligomycin

Cell viability confirmed by the uptake of 2-NDBG in lymphocytes treated with Con-A, oligomycin and FCCP. Addition of Concanavalin A did not affect glucose uptake in control sample (p=0.285), Oligomycin (p=1.00), or FCCP (p=1.00).

No significant difference in 2-NDBG uptake with addition of oligomycin or FCCP.

Discussion

4.11 Discussion

This study demonstrates that there is a postoperative decrease in oxygen consumption in lymphocytes following brief activation with concanavalin A. Concanavalin stimulation was limited to 1 hour as further stimulation has been associated with cell apoptosis.(Barbosa, Arruda et al. 2001) Cell viability was confirmed in this study with positive uptake of glucose in the presence of concanavalin A and mitochondrial inhibitors. The decrease in lymphocyte cell metabolic function on day 3 following surgery corresponds with the ongoing presence of postoperative lymphopenia (both absolute and relative) seen in these patients.

Bioenergetic failure is a core feature of failure to recover from critical illness.(Brealey, Brand et al. 2002, Carre, Orban et al. 2010, Boomer, To et al. 2011, Pan, Yamada et al. 2011, Kubota, Chida et al. 2012) This data demonstrates that lymphocytes taken from patients postoperatively exhibit a distinct bioenergetic profile characterised by low metabolic capacity. The maximum OCR achieved following FCCP (ETC accelerator response) injection and spare respiratory capacity (indicator of maximal respiratory capacity of the lymphocyte under conditions of increased energetic demand e.g. antigen stimulation, cytokine / antibody production, proliferation Table 1.4), were lower postoperatively suggesting that there may be an inability for lymphocytes to meet the demand of energy production via oxidative phosphorylation required for immune activation and proliferation in the postoperative period.

Lymphocytes activated in vitro preferentially convert pyruvate into lactate that is secreted from the cells rather than oxidize pyruvate in the mitochondria, a process known as aerobic glycolysis.(Vander Heiden, Cantley et al. 2009) The decrease in ECAR at baseline following surgery and after administration of each mitochondrial inhibitor also shows that there is a decrease in lymphocyte glycolysis postoperatively. Regulatory genetic variants involving key mediators of gene networks have been implicated in the switch to glycolysis that occurs in sepsis, including HIF1α and mTOR, and mediators of endotoxin tolerance, T-cell activation, and viral defence.(Davenport, Burnham et al. 2016) Furthermore

transcriptome data in a study by van Vught et al. suggests a role for impaired glycolysis in immune suppression in patients with sepsis and susceptibility for ICU-acquired infections.(van Vught, Klein Klouwenberg et al. 2016) Genetic variants may be important in postoperative lymphocyte metabolic function and patient outcome and therefore warrants further exploration. The rate of change in pH in tissue culture extracellular fluid is a function of the cell concentration and rate of proton excretion per cell. Therefore inhibition of ECAR may have been related to the number of viable cells from cellular damage during the experiments. Cell viability was assessed prior to commencing experiments and was found to be >90% in all cases. Addition of Cell Tak and plating did not affect viability.(Edwards 2012) Loss of cell viability during extracellular flux analysis may have occurred but would be in keeping with the hypothesis of increased apoptosis of postoperative lymphocytes resulting from impaired bioenergetic function causing lymphopenia. Other causes for reduced ECAR include reduced lactate production secondary to inhibition of pyruvate to lactate conversion.

This study demonstrates postoperative global mitochondrial impairment and impaired glycolysis. Increased metabolism is a critical part of activation because if T cells fail to increase glucose metabolism due to inadequate nutrients or direct metabolic inhibition, activation and proliferation are suppressed.(Cham and Gajewski 2005, Jacobs, Herman et al. 2008, Shi, Wang et al. 2011). Since lymphocyte metabolism is closely related to survival, function and differentiation, these findings are consistent with the hypothesis that decreased lymphocyte proliferation in response to mitogenic stimulation occurs after tissue trauma (surgery),(Faist, Schinkel et al. 1993) which can last for days.(Horgan, Mendez et al. 1994) Therefore postoperative bioenergetic dysfunction in lymphocytes could be a contributing mechanism leading to increased lymphocyte apoptosis and reduced absolute and relative lymphocyte counts that occurs in the postoperative period (Chapter 3). Parameters describing cellular function of peripheral blood mononuclear cells in bioenergetic terms have been shown to semi-quantitatively correlate with the activity of rheumatoid disease and proved useful in assessing the therapeutic effect of the disease.(Kuhnke, Burmester et al. 2003, Doherty, Oaks et al. 2014) Mitochondrial hyperpolarization and the resultant ATP depletion have been shown to sensitise T cells for necrosis in patients with SLE, which may contribute to inflammation in these patients.(Gergely, Grossman et al. 2002) ATP depletion has been related to increased apoptosis in human T cells.(Leist, Single et al. 1997) The clinical effects of decreasing

baseline OCR to one-third (as seen in these patient samples) of preoperative values, warrant further investigation. These perioperative bioenergetic changes may however contribute to increased susceptibility to infection following surgery (Chapter 3).

Since I controlled for the number of lymphocytes interrogated using the flux analyser there is a clear postoperative decline in oxidative phosphorylation and glycolysis of lymphocytes. Although this is consistent with the concept of postoperative immune cell anergy which has previously been described in T lymphocytes(Maciver, Jacobs et al. 2008) and immune regulatory cells,(Faist, Baue et al. 1983, Haupt, Riese et al. 1998) the stage of lymphocyte cell cycle was not formally interrogated in this study, which would further support this hypothesis . A similar immune phenotype of decreased oxidative phosphorylation has been demonstrated in sepsis, (Crouser 2004, Carre and Singer 2008, Exline and Crouser 2008) chronic immune states and associated with organ dysfunction occurring in critical illness.(Banz, Jakob et al. 2011) Mitochondrial dysfunction in sepsis is linked with derangements in energy homeostasis and may involve impairment of electron flow through the ETC complexes(Crouser, Julian et al. 2002, Callahan and Supinski 2005, Belikova, Lukaszewicz et al. 2007) or increase in the proton conductance through the inner mitochondrial membrane.(Crouser, Julian et al. 2002, d'Avila, Santiago et al. 2008) These changes can affect mitochondrial oxygen consumption, resulting in bioenergetic failure as a consequence of impaired oxidative phosphorylation.(Crouser 2004, Carre and Singer 2008, Exline and Crouser 2008) Reduced ATP content and complex I activity from skeletal muscle have also been associated with increased severity of sepsis,(Brealey, Brand et al. 2002) compared to asymptomatic surgical patients.(Brealey, Brand et al. 2002, Edwards, Sultan et al. 2015) This highlights that a proportion of patients undergoing elective surgery may be predisposed to the increased risk of development of postoperative lymphocyte apoptosis, subsequent lymphopenia and morbidity.

The post-surgical alteration in oxygen consumption and glycolysis demonstrated in this study may be associated with alterations in immune response, including impaired T cell proliferation which has previously been associated with increased susceptibility to infection.(Keane, Birmingham et al. 1983) Therefore the decreases seen in OCR and ECAR postoperatively may result in diminished lymphocytic function dependent on ATP production. This may impair host responses to subsequent pathological and inflammatory insults and thus impact on multiple organ systems in the postoperative period as

described in a two-hit hypothesis. This may explain the increased complication rates and resultant prolonged hospital length of stay demonstrated in patients with preoperative lymphopenia (Chapter 3).

4.12 Differences in methodology compared to other lymphocyte bioenergetic studies

Four times the number of lymphocytes were utilised per experiment in this study (1 million vs. 250,000) compared to in the methodology published by Chacko et al.(Chacko, Kramer et al. 2013) and also lymphocytes were stimulated with concanavalin A for 1 hour prior to flux analysis. Concanavalin is a commonly utilised T cell mitogen which,(Dwyer and Johnson 1981), which was administered to stimulate activation of T cells and thereby increase ATP utilisation and production in order to maximise the signal demonstrated during bioenergetic profiling, without affecting cell viability. The difference in methodology is likely to be related to the effect of phosphate buffered saline (PBS) on metabolic function of lymphocytes, as Chacko et al. found increased oxidative phosphorylation with the use of RPMI compared to PBS. This may be due to presence of glutamine, which is a nutrient known to increases oxidative phosphorylation.(Lanning, Looyenga et al. 2014) Murine studies suggest that CD8(+) cells may have increased ability compared with CD4(+) cells to oxidise glutamine as an alternative fuel source, (Cao, Rathmell et al. 2014) which may alter metabolic profiles and results from lymphocytes interrogated. Utilising a greater number of lymphocytes and resuspension with PBS rather than RPMI may therefore result in altered metabolic profiles of lymphocytes due to it RPMI being a more effective culture medium than PBS.(Chacko, Kramer et al. 2013) Additionally I incubated cells with concanavalin A to increase oxygen consumption and metabolic activity prior to analysing bioenergetic profile which was not done in the Chacko et al. study perhaps because magnetic bead cell separation was used, which itself can activate cells.(Onlamoon, Boonchan et al. 2013) Instead I chose to utilise a Ficoll separation technique, which resulted in different timings before extracellular flux analysis could be commenced. The method utilised to isolate lymphocytes varied. In this study, monocytes were separated from mononuclear cells by adherence following isolation after Ficoll separation (98% purity), whereas Chacko et al. only obtained >80% purity by utilizing bead separation, which is substantially less than the 97% that would usually be expected with the use of such a technique.(Semple, Allen et al. 1993) However regardless of numbers of cells, and medium utilised to incubate cells, the methodology in this study was identical pre- and postoperatively with matched patient samples.

4.13 Study strengths

While surgical studies are often flawed due to presence of heterogeneity and confounding factors such as cancer within recruited patients, this study was undertaken on a relatively homogenous major surgical population undergoing elective hip and knee arthroplasty surgery. I studied orthopaedic patients as they represent the majority of elective surgery across different healthcare systems,(Hagen, Vaughan-Sarrazin et al. 2010) involving an increasingly aged population (mean age of 74 years in this study) with multiple comorbidities. The surgical procedures in this study were also standard models of tissue injury and recovery profiles without confounding factors such as sepsis and malignancy. Furthermore, a significant burden of clinical morbidity is not uncommon in these patients, as demonstrated by hospital readmission rates.(Jencks, Williams et al. 2009) Finally, there is an association between anergy, failure of delayed hypersensitivity response, and lymphopenia(Gibson, Croal et al. 2007) with adverse outcomes following surgery(Christou, Meakins et al. 1995), trauma(Puyana, Pellegrini et al. 1998, Bandyopadhyay, De et al. 2007) and sepsis.(Venet, Chung et al. 2009) However, the paucity and heterogeneity of patient studies, plus the complexity of the surgical procedures (chiefly in patients undergoing cancer surgery), limit robust conclusions from being drawn. The serial analysis of perioperative samples, enabling each patient to act as their own control, is a robust model that circumnavigates the significant challenges of appropriate control samples and minimises the potential for confounding factors within the study.

4.14 Study limitations

Limitations include the limited numbers of cells that could be obtained from postoperative and lymphopenic patients, which limited the number of experiments that could be performed rather than the validity of findings. The use of isolated lymphocytes may be considered a strength of this ex-vivo study, by allowing interrogation of cell metabolic profiles. However, since isolated lymphocytes were not in their natural milieu found in-vivo, the lymphocytes studied may have demonstrated different metabolic profiles in response to cytokines and afferent signals from other cells and neurohumoral systems. Also differences in metabolic profiles between different lymphocyte subsets were not interrogated as this was limited by lymphocyte yield. Ideally, one would combine the information obtained from cellular and in-vivo/ex-vivo studies to obtain a more complete characterization of metabolism. This would be extremely difficult to perform in practice due to limited lymphocyte yield from human blood sampling and inability to

administer mitochondrial complex inhibitors in vivo. Murine studies could be performed, however these do not necessarily translate to human responses as discussed in Chapter 1.8 and serial blood sampling would be difficult to perform.

All patients in this cohort underwent hip or knee arthroplasty under general anaesthesia with inhalation agent for maintenance, with or without peripheral nerve blockade. It remains unclear whether undergoing surgery utilising a regional technique alone e.g. neuraxial anaesthesia would alter postoperative bioenergetic function in any way, however when combined with regional the effect on bioenergetic function of lymphocytes appeared to be ubiquitous. None of the patients in this cohort underwent surgery under regional anaesthesia alone and therefore this question cannot be answered in this study. It remains unclear whether the effects demonstrated on lymphocyte metabolic profiles in this study are the result of tissue trauma and subsequent neurohumoral responses. Therefore further work is needed to elucidate the effect of age, type of surgery, site of surgery, postoperative analgesia regimens and postoperative infection on bioenergetic function of lymphocytes.

In summary lymphocytes demonstrate a hypometabolic phenotype postoperatively (decreased oxidative phosphorylation and glycolysis). Reduced ATP production has previously been related to T cell apoptosis, however findings from this study introduce the idea of postoperative lymphopenia occurring secondary to ETC dysfunction induced apoptosis. To explore this hypothesis further I next interrogate perioperative lymphocytes for changes in mitochondrial ROS production and mitochondrial membrane potential.

CHAPTER 5

Perioperative lymphocyte mitochondrial membrane potential and reactive oxygen species production

Chapter 5

Perioperative lymphocyte mitochondrial membrane potential and reactive oxygen species production

5.1 Reactive oxygen species and oxidative stress

Reactive oxygen species (ROS), formed as a natural by-product of oxygen metabolism include free radicals, oxygen ions and peroxides. Although ROS plays an important role in the immune system and is involved in key signalling events, excess levels can cause damage to lipids, DNA and proteins. This phenomenon is known as oxidative stress.

Mitochondria play a critical role in energy balance within cells, regulation of apoptotic proteins and are also the main source of reactive oxygen species (ROS). ROS are considered pathogenic in many disease states.(Aronis, Melendez et al. 2003) Superoxide production occurs during the oxidative phosphorylation process of cellular respiration in the process of reduction of molecular oxygen by the ETC.(Lambert and Brand 2009). The superoxide produced in turn can initiate a cascade of reactions that produce other ROS including peroxynitrite, hydrogen peroxide, and hydroxyl radical. A vicious circle of oxidative stress and damage to cellular structures can lead to either cell death by apoptosis or to a cellular energetic decline and aging.(Cadenas and Davies 2000) Mitochondrial dysfunction caused by oxidative stress has been implicated in numerous disease stages including neurodegeneration, cancer, diabetes and aging.(Griendling, Sorescu et al. 2000)

5.2 Mitochondrial reactive oxygen species and membrane potential

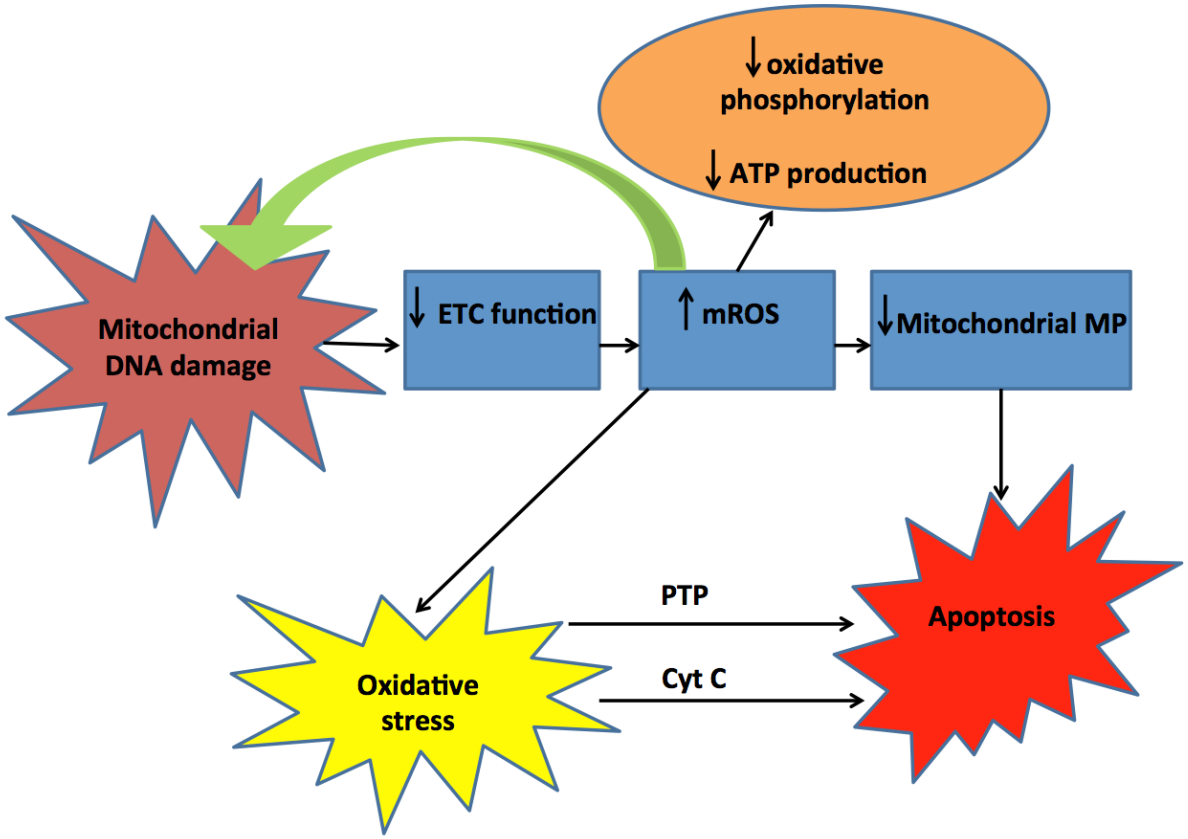
Decreased bio-energetic function and altered postoperative ETC function have been demonstrated in postoperative patients (Chapter 4). Impaired ETC function can decrease mitochondrial membrane potential,(Peng, Yu et al. 2006) and is associated with increased mitochondrial ROS (mROS) production (in leukaemic THP-1 cell lines) (Zhou, Yazdi et al. 2011) and increased apoptosis (in astrocytes).(Jou 2008) While the association between impaired ETC and increased mROS has not been reproduced in human lymphocytes, or explored in the perioperative period, THP-1 cell line is a human cell line which does

provide insight into potential mechanisms of postoperative immune cell dysfunction which could explain the ETC dysfunction demonstrated in this thesis.

Results from experiments utilising cell lines must be interpreted with some caution however. For example, because the THP-1 cell line was derived from the blood of a patient with acute monocytic leukaemia, the extent to which THP-1 cells mimics lymphocytes in vivo is not entirely known.(Qin 2012) THP-1 are cancer cells, which undergo increased levels of glycolysis(Gatenby and Gillies 2004) and oxidative phosphorylation(Zheng 2012) due to their constant proliferative state. In THP-1 cells, metabolites in the TCA cycle are not decreased to the same extent by 2-DG, which indicates that THP-1 utilises energy sources other than glucose. TCA cycle metabolites in THP-1 cells may be derived from acetyl-CoA by fatty acid β-oxidation.(Miwa, Shikami et al. 2013)

While reduced oxidative phosphorylation can result in reduced ROS production, mitochondrial DNA damage can result in a state of increased mROS production, reduced ATP production, reduced oxidative phosphorylation and mitochondrial dysfunction.(Kirkinezos and Moraes 2001) The relationship between ETC dysfunction, mROS production and mitochondrial membrane potential are outlined in Figure 5.1.

Figure 5.1 Mechanism by which ETC dysfunction may result in increased mitochondrial ROS, reduced oxidative phosphorylation and reduced membrane potential



ETC= electron transport chain; mROS= mitochondrial reactive oxygen species; MP= membrane potential; mitochondrial oxidative damage can result in release of intermembrane space proteins such as cytochrome c (Cyt C) to the cytosol by mitochondrial outer membrane permeabilisation and thereby activate cell apoptosis. mROS also induces mitochondrial permeability transition pore (PTP), rendering the inner membrane permeable to small molecules during ischaemia / reperfusion injury.

In this chapter I aim to demonstrate increased mROS production and reduced mitochondrial membrane potential secondary to iatrogenic / induced ETC dysfunction mimicking the postoperative phenotype (Figure 5.1). Postoperative reduction in bio-energetic function may occur due to impairment of the ETC which results in increased oxidative stress, increased mROS, reduced mitochondrial membrane potential, ultimately increasing propensity for lymphocyte apoptosis. This could provide a unifying mechanism for postoperative reduction in oxidative phosphorylation and OCR.

5.3 Hypothesis

Oxidative stress and decreased mitochondrial membrane potential develops within lymphocytes postoperatively.

Methods

5.4 Mitochondrial membrane potential (Tetramethylrhodamine, Methyl Ester; TMRM); whole blood assay

(a) Patients

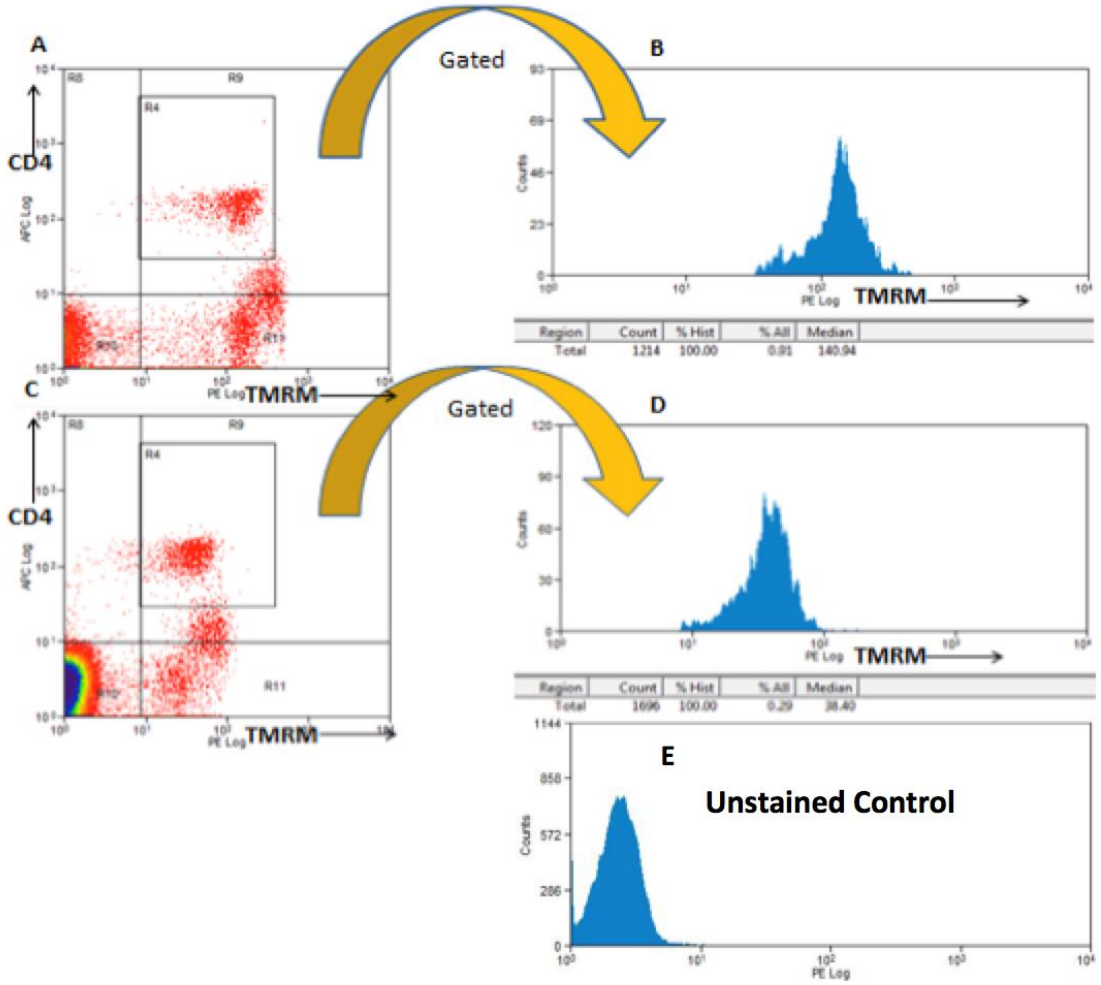
Patients undergoing general, urological, breast and orthopaedic surgery (VISION-UK) at University College London Hospital were recruited into this single centre study. All prospectively recruited subjects provided written informed consent for data collection combined with blood sampling, following ethical committee approval. Surgery and anaesthesia were performed and standardised care was delivered according to study protocols outlined in Chapter 2.

Preoperative and day 2 postoperative paired patient blood samples were taken from individuals. Day 2 samples were taken to standardise pharmacological interventions among the majority of patients and most patients were still in hospital on this day.

(b) Flow cytometry analysis of TMRM assay

Preparation of cells for the TMRM assay is summarised in the General Methods chapter (Chapter 2.8). Mitochondrial membrane potentials (Median FIuorescence Intensity of TMRM) of CD4(+) and CD8(+) cells were recorded as demonstrated in Figure 5.2 and 5.3.

Figure 5.2 Flow cytometry analysis of lymphocyte mitochondrial membrane potential



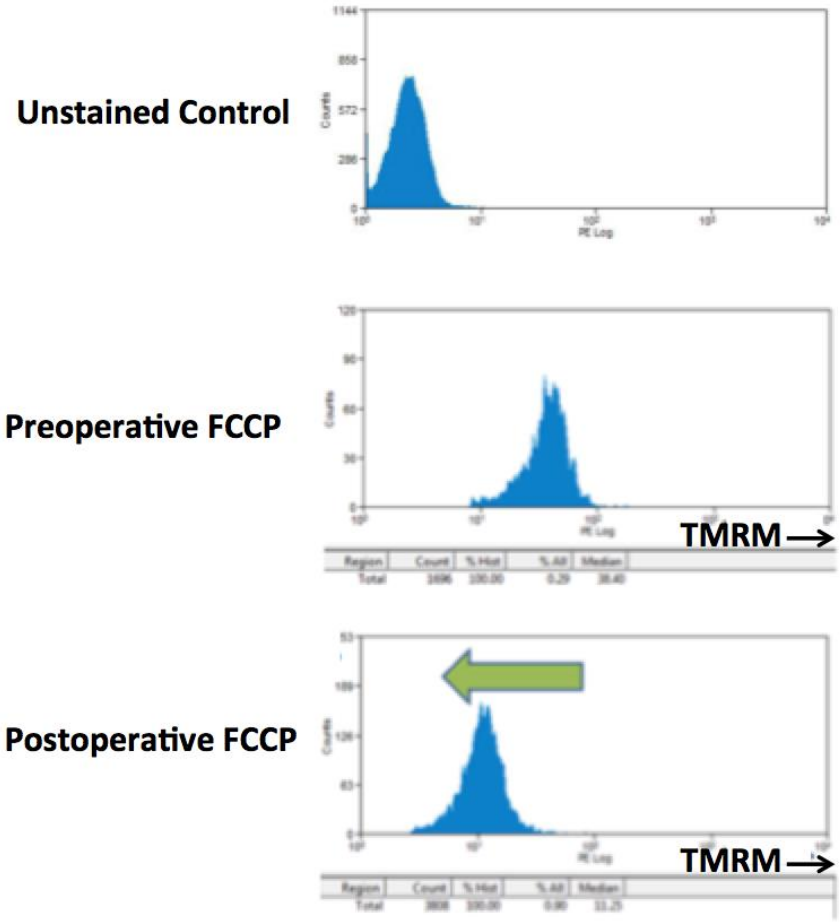
Lymphocyte population gated onto scatter plots A and C as previously described. CD4(+) cells gated onto histograms B and D in order to elucidate MFI. FCCP is a positive control (C-D), which decreases mitochondrial membrane potential, resulting in a decrease in MFI.

Isotype control sample used to determine CD4(+) cells as previously described.

No isotype control for TMRM.

Unstained control shown in E.

Figure 5.3 Changes in perioperative mitochondrial membrane potential of CD4 cells as demonstrated by PE (TMRM) MFI



Decrease in TMRM MFI demonstrated in CD4(+) cells from one patient sample. Measurements at different time points controlled for by utilising same technique, sample preparation, reagents and flow cytometry laser settings and protocols within the summit programme.

5.5 Mitochondrial ROS production in isolated lymphocytes

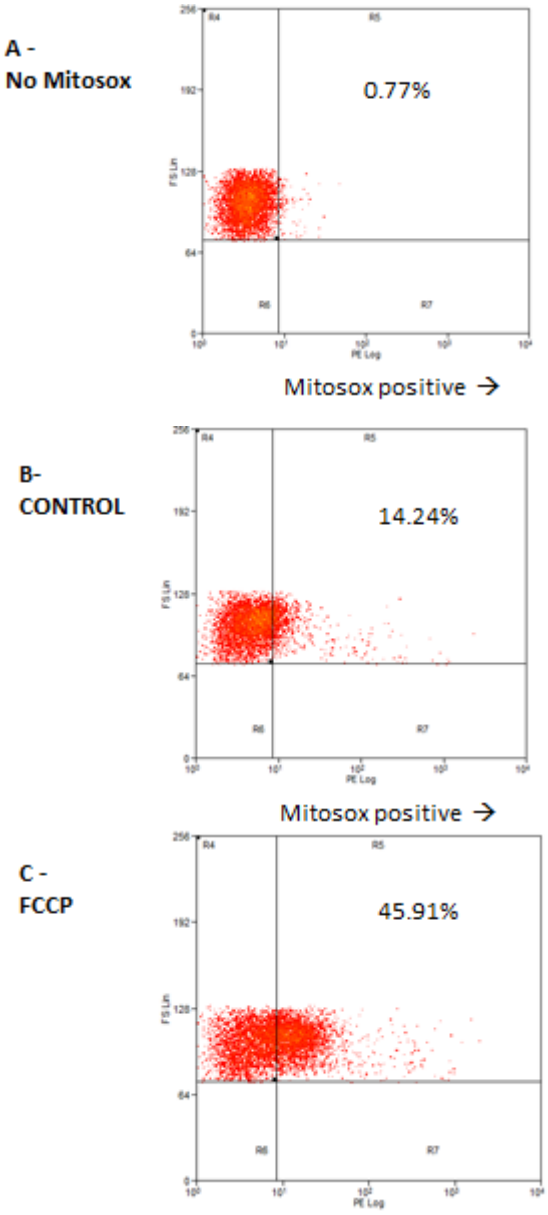
(a) Patients

Patients undergoing general, urological, gynaecological and orthopaedic surgery (VISION-UK) at University College London Hospital were recruited into this single centre study. Preoperative and day 2 postoperative paired patient blood samples were taken from individuals.

(b) Flow cytometry analysis of MitoSox assay

Preparation of cells for the MitoSox assay is summarised in the General Methods chapter (Chapter 2.9). The flow cytometry analysis method is summarised in Figure 5.4.

Figure 5.4 Flow Cytometry Analysis of lymphocyte mitochondrial ROS production



Quadrant position established by using a sample containing A) no MitoSox dye (i.e. unstained or isolated cells only).B) Control sample with MitoSox dye and C) sample with MitoSox dye and FCCP. Percentage of cells in right upper and lower quadrants represents the percentage of lymphocytes expressing MitoSox (mitochondrial ROS). Not all cells positive for MitoSox following FCCP administration possibly due to: 1) inadequate incubation time of cells in FCCP 2) cell death 3) Insensitivity of assay.

Mitochondrial ROS production was interrogated in preoperative lymphocytes following addition of: staurosporine (protein kinase C inhibitor; proapoptotic microbial alkaloid) and mitochondrial ETC inhibitors (myxothiazol, rotenone and FCCP) for 30 minutes to increase mROS production by lymphocytes.

Pre- and postoperative lymphocyte samples were also interrogated for presence of mitochondrial ROS at baseline (control) and following FCCP stimulation.

5.6 Statistical analysis

The study was powered based on a 25% difference in mitochondrial membrane potential being interpreted as clinically significant in patients diagnosed with SLE as mitochondrial membrane potential and apoptosis were increased in SLE patients compared to healthy controls.(Gergely, Grossman et al. 2002) This degree of change in mitochondrial membrane potential impacts on ATP synthesis, T cell activation and apoptosis.(Fiers, Beyaert et al. 1999, Skulachev 1999) In order to achieve an alpha error of 0.05 and power 0.8, a sample size of 8 paired samples would be required to demonstrate differences between pre and postoperative TMRM. In order to account for withdrawals, early hospital discharge and difficult venepuncture a sample size of 19 was recruited for analysis.

For the assessment of changes in mROS production, since >10,000 lymphocytes were gated during flow cytometry analysis of each sample, in order to achieve an alpha error of 0.05 and power 0.8, patient numbers of 3 (matched pre- and postoperative samples) were needed to demonstrate differences in MFI (>25% increase considered significant) secondary to FCCP, myxothiazol, rotenone and staurosporine.

Results

5.7 Demographics – Membrane potential

Preoperative samples were taken from 19 patients undergoing general, urological, breast and orthopaedic surgery (demographics in Table 5.1). Preoperative CD4 mitochondrial membrane potential was interrogated on all of these patients and CD8 mitochondrial membrane potential on 13 of these patients. 8 patients were available and also had postoperative samples taken on day 2 following surgery (Figure 5.5).

Figure 5.5 Patient samples used in TMRM analysis

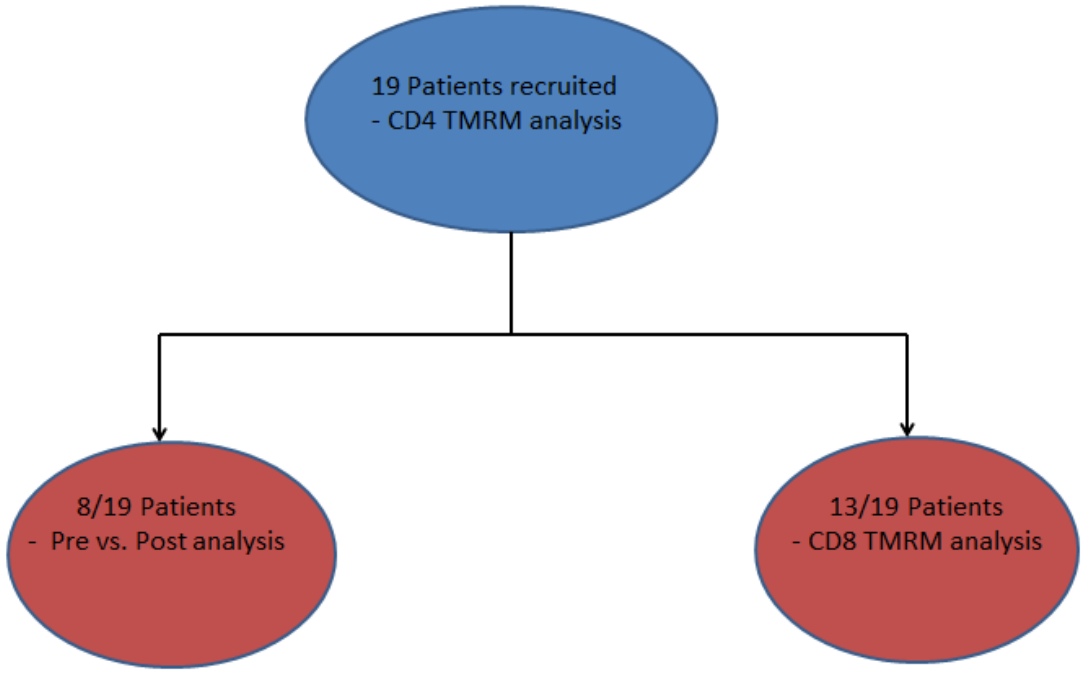


Table 5.1 Demographics of TMRM study cohorts

	CD4 TMRM cohort (n=19)	CD8 TMRM cohort (n=13)	Pre vs. Postoperative (n=8; matched)
Age (years)	61 (56 - 67)	61 (53 – 69)	60 (55 – 65)
Absolute lymphocyte count (x 10^9/L)	1.87 (1.50 – 2.24)	1.91 (1.45 – 2.37)	2.05 (1.24 – 2.80)
Relative Lymphocyte count (%)	26.21 (21.31 – 31.10)	26.02 (19.62 – 32.42)	27.96 (18.08 – 37.84)
Gender n (%)	Male 11 (58) Female 8 (42)	Male 7 (54) Female 6 (46)	Male 6 (75) Female 2 (25)
Type of surgery n (%)			
General	11 (58)	6 (46)	4 (50)
Urology	6 (32)	5 (38)	3 (38)
Breast	1 (5)	1 (8)	0
Orthopaedic	1 (5)	1 (8)	1 (12)

Numbers unless stated otherwise presented as mean (95% CI)

5.8 FCCP in TMRM Assay

FCCP decreased the mitochondrial membrane potential in CD4(+) and CD8(+) cells (Table 5.2).

Table 5.2 Mitochondrial membrane potential in preoperative samples

	TMRM MFI CTRL	TMRM MFI FCCP	P value
CD4 (n=19)	157.53 (121.37 – 193.69)	60.59 (48.36 – 72.81)*	<0.0001
CD8 (n=13)	211.93 (161.69 – 262.17)	74.61 (57.45 – 91.78)*	<0.0001

Values presented as mean (95% CI); *P < 0.0001

5.9 Perioperative changes in mitochondrial membrane potential

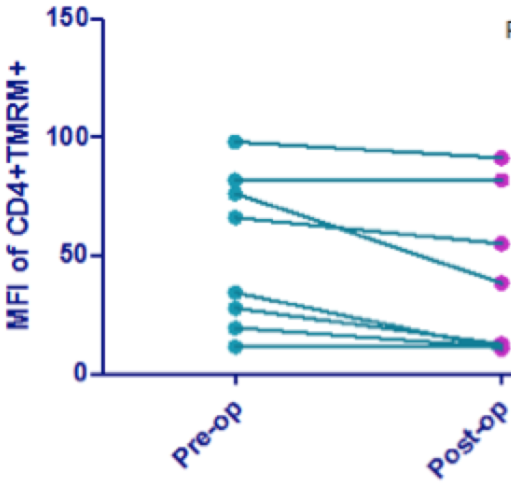
Changes in CD4(+) (Figure 5.6) and CD8(+) cell mitochondrial membrane potential are shown in table 5.3.

Table 5.3 Pre- vs. postoperative mitochondrial membrane potential of CD4 and CD8 cells

	PREOPERATIVE TMRM MFI		POSTOPERATIVE TMRM MFI	
	CTRL	**FCCP**	**CTRL**	**FCCP**
CD4	133.70	86.36	50.24	25.47
(n=8)	(51.3 – 186.48)	(40.56 – 186.48)	(21.43 – 80.55)	(11.25 – 75.26)*
CD8	205.16	84.76	269.03	120.60
(n=8)	(130.52 – 279.80)	(54.84 – 114.68)	(158.74 – 379.33)	(68.26 – 172.95)

CD 8 values presented as mean (95%CI) pre vs. postoperative samples analysed with paired t-tests; CD4 values presented as median (IQR) pre vs. postoperative samples analysed with Related samples Wilcoxen signed rank test. * p=0.028 (see Figure 5.6)

Figure 5.6 Change in CD4 mitochondrial membrane potential with FCCP administration



Related samples Wilcoxen signed rank test; n=8; p=0.028

There was no difference in control CD4(+) mitochondrial membrane potential postoperatively in the control samples (p=0.237), however postoperative membrane potential with FCCP was lower than preoperative (p=0.028). There was no decrease in CD8(+) mitochondrial membrane potential (control or FCCP) postoperatively.

5.10 ROS production with mitochondrial inhibitors

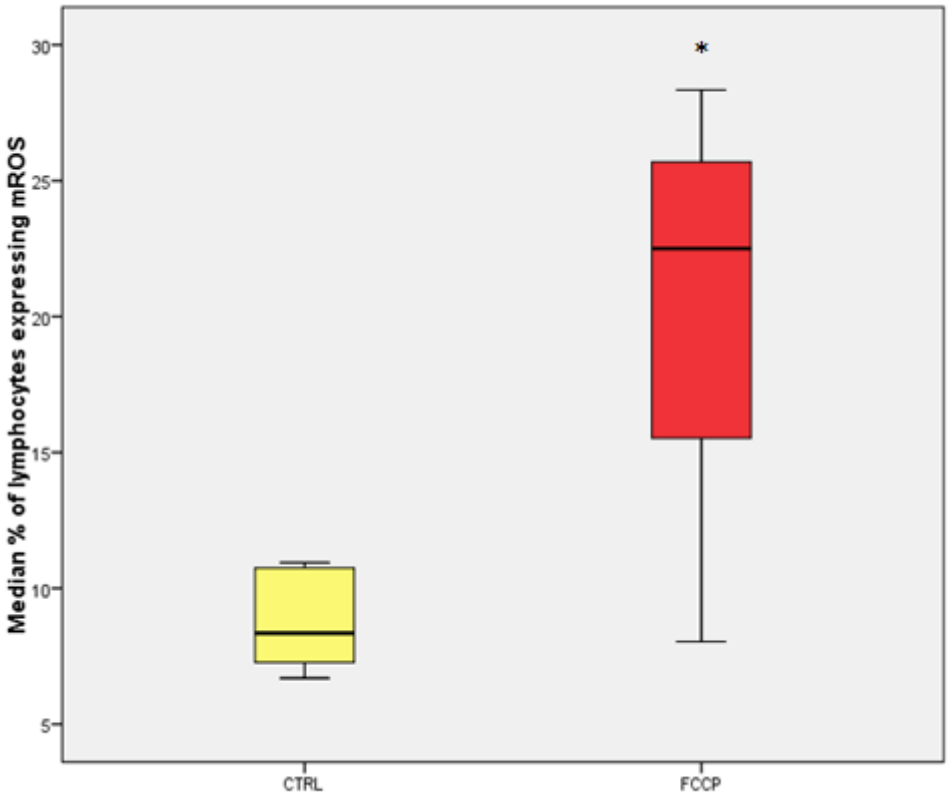
Preoperative blood samples were taken to investigate the effect of FCCP (n=7), myxothiazol (n=4), rotenone and staurosporine (n=3) on lymphocyte mROS production in this separate study. Mitochondrial ROS production was interrogated on isolated lymphocyte samples (>99% purity).

5.11 Demographics – ROS production with mitochondrial inhibitors

7 patients (5 male, 2 female) with a mean age (95% CI) of 48 years (34 – 62) were recruited into this study and preoperative samples were interrogated to assess mROS production (MitoSox) following FCCP (n=7), myxothiazol (n=4), rotenone (n=3) and staurosporine (n=3).

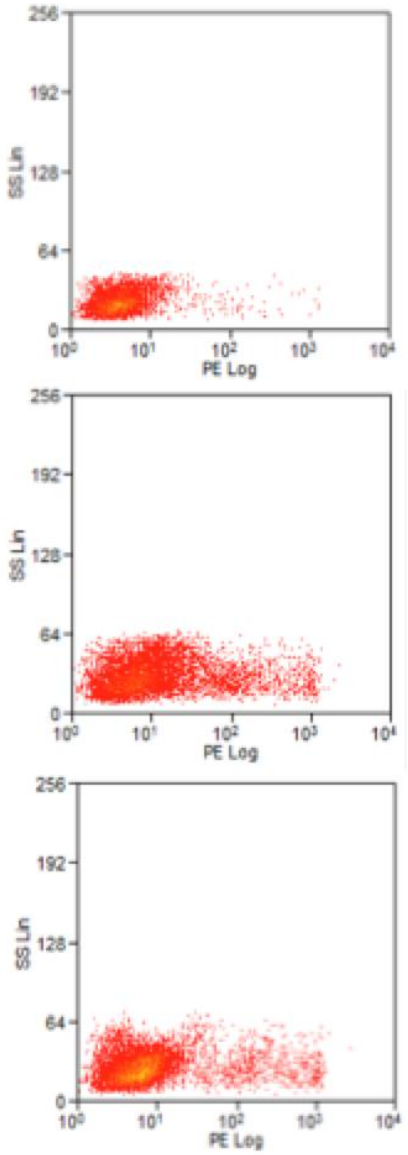
FCCP increased percentage of lymphocytes expressing mROS (Figure 5.7). Staurosporine, rotenone (Figure 5.8) and myxothiazol (Figure 5.9) also increased percentage of lymphocytes expressing mROS.

Figure 5.7 Effect of FCCP on mROS production in preoperative lymphocytes



*n=7; p=0.018; Related samples Wilcoxen signed rank test.

Figure 5.8 Effect of staurosporine and rotenone on mROS production by lymphocytes



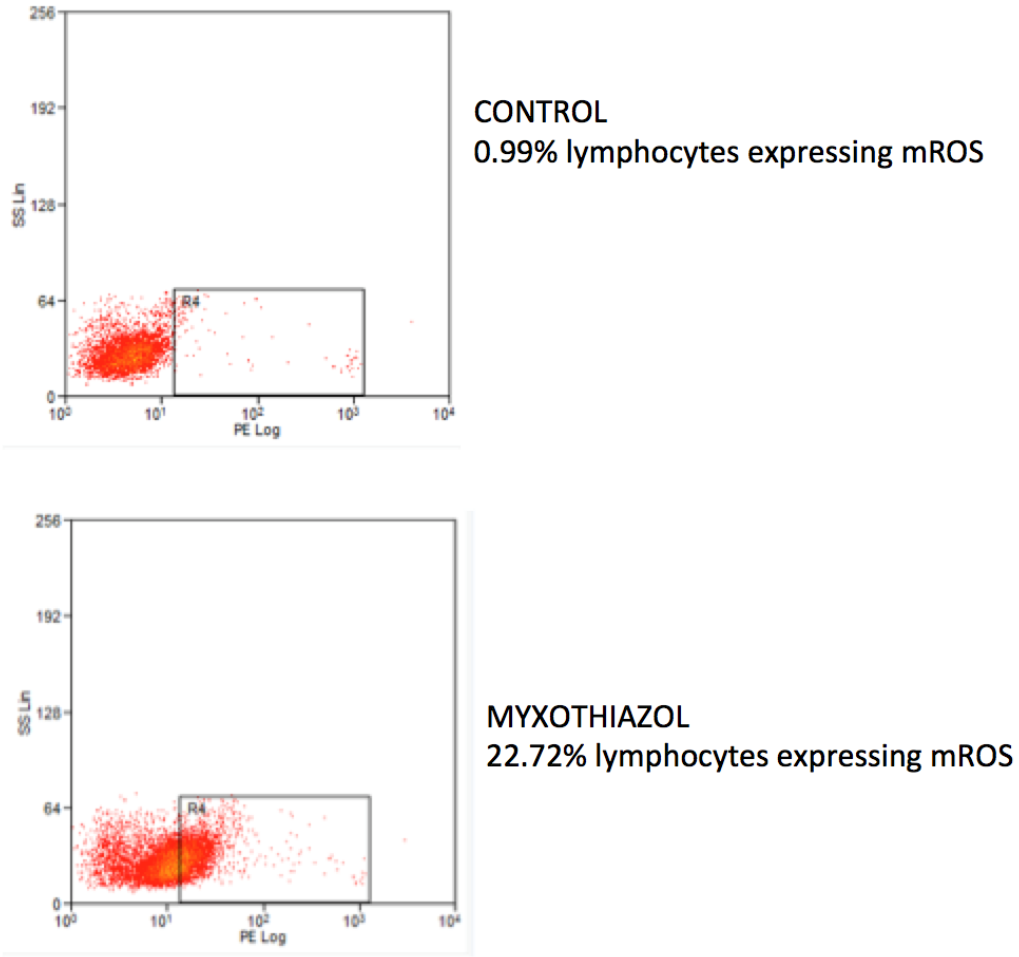
CONTROL
3.23% lymphocytes expressing mROS

STAUROSPORINE
13.56% lymphocytes expressing mROS

Rotenone
10.32% lympocytes expressing mROS

Lymphocyte population gated onto SS vs. PE plots shown above

Figure 5.9 Effect of myxothiazol on lymphocyte mROS production



5.12 Perioperative mitochondrial ROS expression

Demographics

9 patients were recruited into this study (6 male, 3 female) with a mean (95% CI) age of 71 (64-78) years. 6 patients underwent urology procedures and the remaining 3 patients had general, gynaecology and orthopaedic procedures performed.

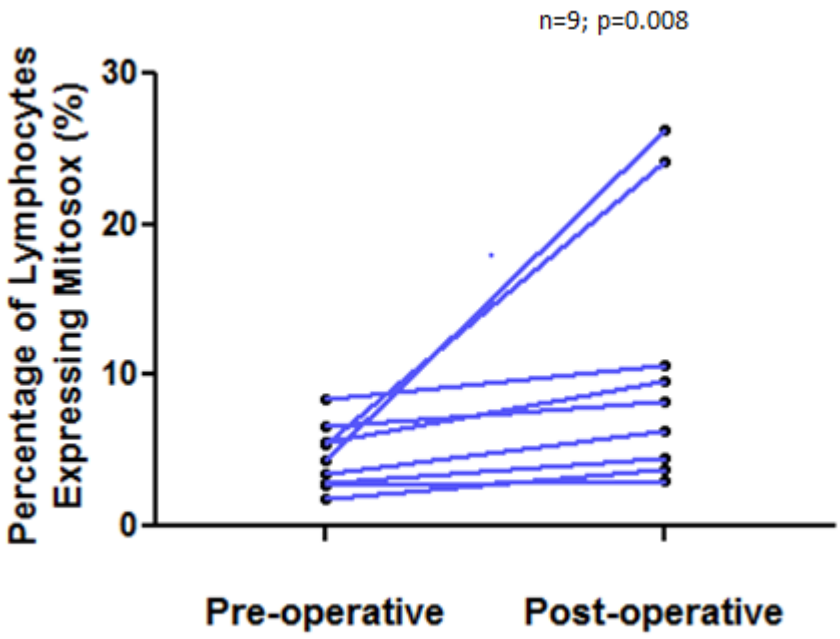
Table 5.4 Perioperative MitoSox expression

	Median % (IQR) Lymphocytes expressing MitoSox
Preoperative Control (n=9)	4.90 (2.50 – 7.07)
Postoperative Control (n=9)	9.45 (3.62 – 24.68)
Preoperative FCCP (n=6)	8.43 (2.26 – 21.08)
Postoperative FCCP (n=6)	7.53 (4.50 – 21.50)

Values analysed using related samples Wilcoxen signed rank test

Percentage of lymphocytes expressing MitoSox postoperatively are summarised in Table 5.4. Percentage of lymphocytes expressing mROS increased postoperatively in control samples (p=0.008; Figure 5.10) but not following FCCP (p=0.917).

Figure 5.10 Perioperative baseline expression of MitoSox by lymphocytes

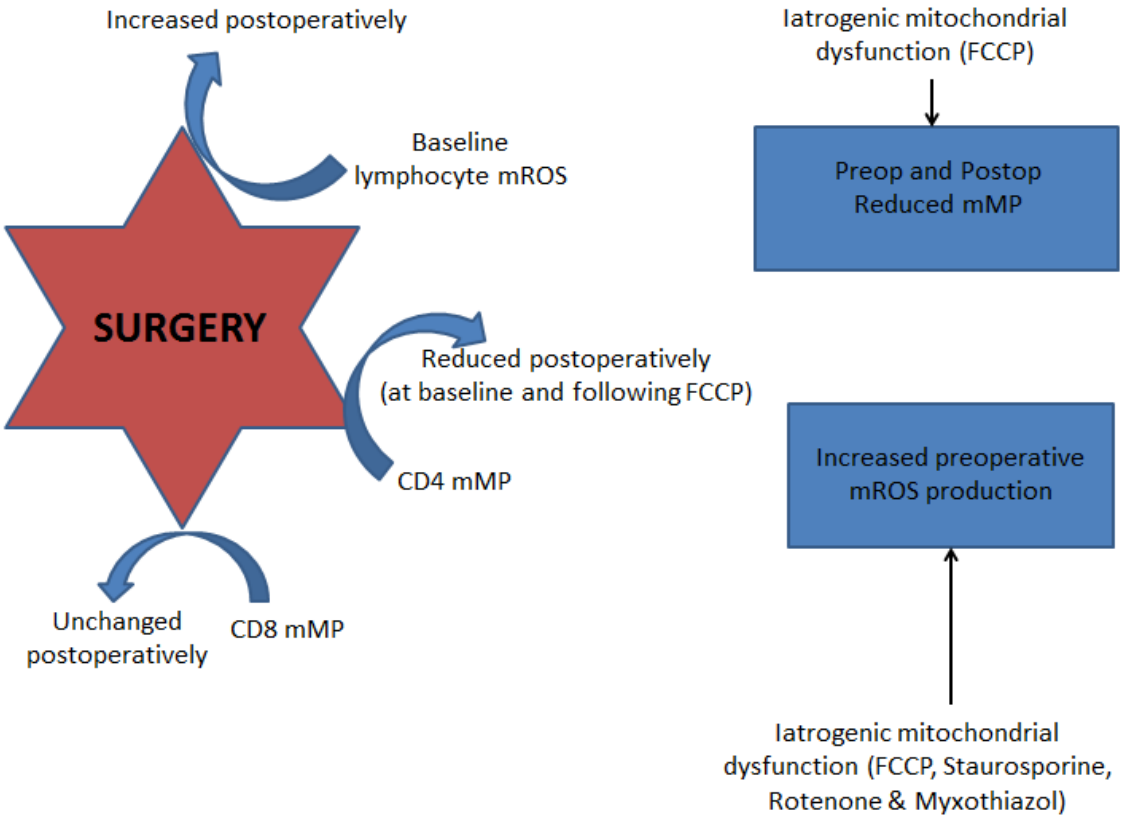


Discussion

This study demonstrates perioperative changes in mitochondrial membrane potential (Figure 5.6) and mROS production (Figure 5.10) in human lymphocytes. A postoperative decrease in CD4(+) cell mitochondrial membrane potential is demonstrated in cells challenged with FCCP. FCCP also impairs ATP synthesis by transporting H+ ions through the cell membrane before they can be utilised in oxidative phosphorylation, which is related to increased mROS production. This phenotype of mitochondrial dysfunction occurs postoperatively in lymphocytes, which demonstrate increased baseline mROS production and decreased mitochondrial membrane potential.

There was however no difference in CD8(+) FCCP mitochondrial membrane potential demonstrated postoperatively compared to paired preoperative FCCP samples. These findings suggest that there may be functional differences in how CD4(+) and CD8(+) cells respond to surgery. The findings of this chapter are summarised in Figure 5.11.

Figure 5.11 Summary of perioperative TMRM and MitoSox results



mMP= mitochondrial membrane potential; mROS= mitochondrial membrane potential; ETC= electron transport chain; preop=preoperative; postop=postoperative

5.13 Established metabolic differences between CD4+ and CD8+ cells

Cao et al. compared activation-induced proliferation and metabolic reprograming of CD4(+) and CD8(+) T cells isolated from mice spleen and lymph nodes. Resting CD4(+) and CD8(+) T cells were found to be metabolically similar and used a predominantly oxidative metabolism. Following activation CD8(+) T cells proliferated more rapidly. Stimulation led both CD4(+) and CD8(+) T cells to sharply increase glucose metabolism and adopt aerobic glycolysis as a primary metabolic program. Activated CD4(+) T cells, however, remained more oxidative and had greater maximal respiratory capacity than activated CD8(+) T cells. CD4(+) T cells were also associated with greater levels of ROS and increased mitochondrial content, irrespective of the activation context. CD8(+) cells were better able, however, to oxidize glutamine as an alternative fuel source. The more glycolytic metabolism of activated CD8(+) T cells correlated with increased capacity for growth and proliferation, along with reduced sensitivity of cell growth to metabolic inhibition. These specific metabolic programs may therefore promote greater growth and proliferation of CD8(+) T cells and enhance survival in diverse nutrient conditions such as the postoperative state.(Cao, Rathmell et al. 2014) The decreased glycolysis seen during extracellular flux analysis (Chapter 4) may represent increased susceptibility to lose mitochondrial membrane potential resulting in decreased CD4(+) glycolysis. Maintenance of CD8(+) glycolysis postoperatively may result in greater CD8 cytokine production postoperatively compared with CD4 cells. These findings would also be in keeping with the reduction in CD4:CD8 ratio described in the postoperative phase and inflammatory states such as sepsis.(Xia, Liu et al. 2012)

While differences have been shown to remain between CD4(+) and CD8(+) T cells irrespective of activation context, ROS production in each population is dependent on both the strength of the activating stimuli and cytokine context.(Cao, Rathmell et al. 2014) Metabolic reprogramming is therefore partially dictated by the environment and by co-stimulatory signals. This is likely to be important in vivo, where T cells are presented with antigens of varied avidity in the presence of varied cytokine milieus, oxygen tensions, and nutrient environments. Recent studies using nutrient transporter knockout T cells(Sinclair, Rolf et al. 2013, Macintyre, Gerriets et al. 2014, Nakaya, Xiao et al. 2014) indicate that activation-induced metabolic reprogramming is critical for many inflammatory responses in vivo; however, metabolic comparison of in vivo reprogrammed CD4 and CD8 cells has not yet been performed.

The importance of glycolysis in cytokine production has been demonstrated in murine models.(Chang, Curtis et al. 2013) These concepts are summarised in Figure 5.12 and perioperative cytokine production is explored further in Chapter 6.

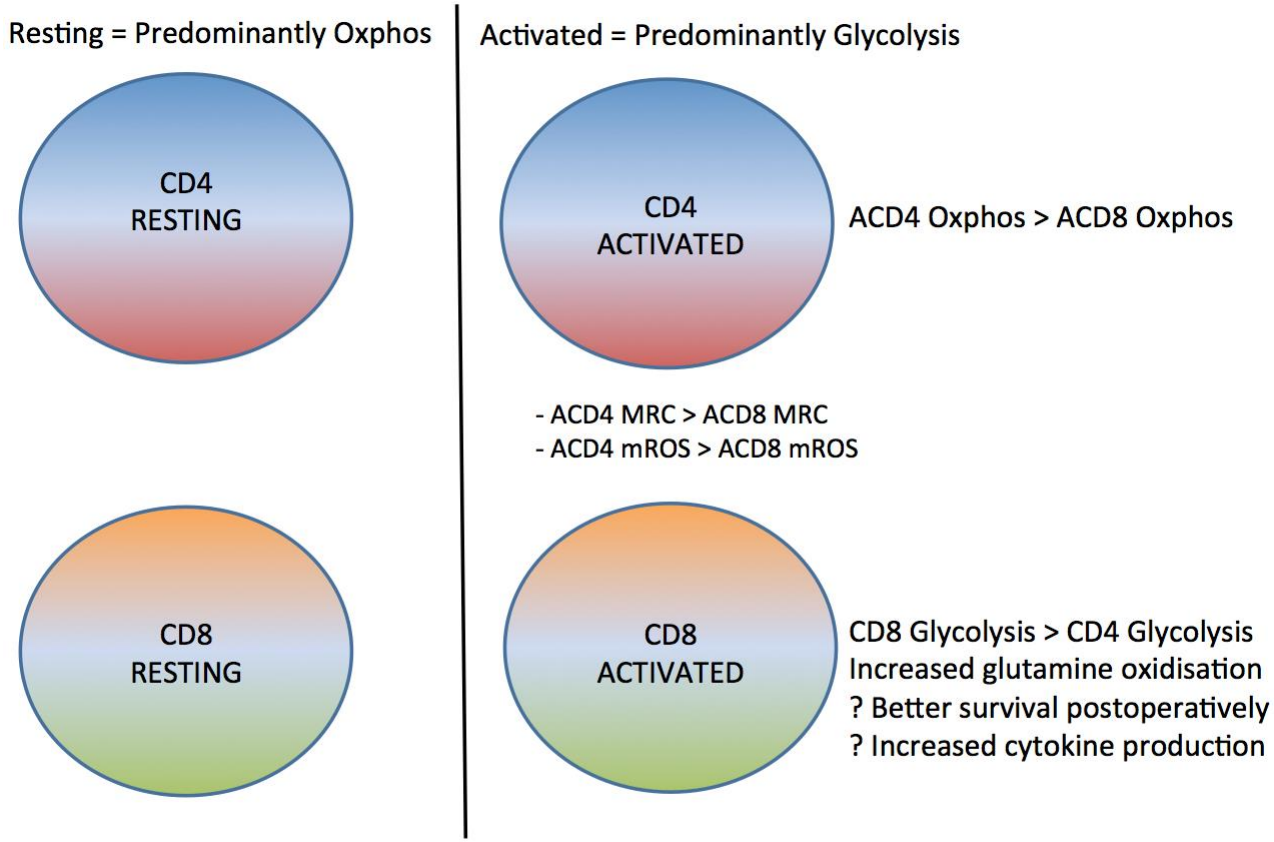
5.14 Study Limitations

This study has several limitations. The effects of surgery on mROS production by all lymphocytes, rather than CD4(+) and CD8(+) cells separately, were explored due to experiments being limited by lymphocyte yield. It would be important to co-stain CD4(+), CD8(+) cells and MitoSox following FCCP treatment in order to determine whether perioperative differences in mROS production exist between these populations. This could be assessed in further detail utilising confocal microscopy, which would allow imaging of living cells, with requirement of fewer cells to demonstrate an effect.

There are two outlying results in Figure 5.10 (postoperative expression of MitoSox by isolated lymphocytes). Whether this is due to experimental error or true physiological differences in these patients in the postoperative period is not clear, however these two results could have skewed the results of the mROS production analysis and should therefore be interpreted with some caution. It should however be noted that since a significant number of lymphocytes in the postoperative period may already have undergone apoptosis (lymphocyte counts hypothesised to drop postoperatively due to apoptosis), this may explain why changes in mROS and mMMP, while are significant may not appear as great as bioenergetic data in Chapter 4. The lymphocytes that are likely to show the greatest differences in the membrane potential and ROS may have already undergone apoptosis in vivo prior to performing experiments.

In summary pharmacologically induced mitochondrial dysfunction in lymphocytes results in reduced mitochondrial membrane potential and increased mROS production. Surgery results in a similar phenotype suggesting that postoperative increase in CD4 lymphocyte mROS production is attributable to loss of mitochondrial membrane potential. These findings are consistent with the reduced bioenergetic function demonstrated in postoperative lymphocytes (Chapter 4).

Figure 5.12 Proposed differences in metabolic profiles between CD4 and CD8 cells



ACD4 = activated CD4 cells; ACD8= activated CD8 cells; MRC = maximum respiratory capacity; oxphos= oxidative phosphorylation; based on findings from murine study(Cao, Rathmell et al. 2014)

CHAPTER 6

Perioperative lymphokine production

Chapter 6
Perioperative Lymphokine production

6.1 Lymphocytes and inflammation

In addition to their "traditional" roles in the immune system, lymphocytes are becoming increasingly recognised as regulators of inflammation. Transgenic models of wound infection demonstrate that CD4+ lymphocytes are pivotal in modulating the function of polymorphonuclear cells at Staphylococcus Aureus (S. Aureus) infected wound sites.(McLoughlin, Solinga et al. 2006) Compared to wild type (WT) mice, alphabeta T cell receptor knockout mice demonstrate significantly lower concentrations of polymorphonuclear specific CXC chemokines (a small family of cytokines acting on CXC chemokine receptors, which recruit leukocytes) in wound tissue. Severity of wound infection is enhanced by administration of a CXC chemokine and abrogated by antibodies that blocked the CXC receptor. These data reveal an important and underappreciated role for CD4(+) alphabeta T cells in S. Aureus infection in orchestrating immune responses including: controlling local CXC chemokine production; neutrophil recruitment to the site of infection and subsequent bacterial replication. Other murine models have shown that T-regulatory cells reduce bowel inflammation secondary to innate immune cells via IL-10 and TGF-β.(Maloy, Salaun et al. 2003) Furthermore in Rag-1-deficient mice, injected CD4 and CD8 cells result in tempering of TLR induced cytokine storm in an antigen-independent fashion.(Kim, Zhao et al. 2007)

6.2 Advances in method of lymphokine detection

Intracellular cytokine expression within lymphocytes in the perioperative period is thus far an unexplored area of research. (Pala, Verhoef et al. 2000) Studies have previously used ELISA to evaluate cytokine expression from isolated lymphocytes(Berguer, Bravo et al. 1999, Franke, Lante et al. 2009) and supernatant following surgery.(Evans, Galustian et al. 2009) This method does not however identify the cellular source of cytokines secreted into plasma and serum. Whole blood assays have the advantage of not requiring lymphocyte isolation, requiring less preparation time and manipulation (Rodriguez-Caballero, Garcia-Montero et al. 2004) while retaining host lymphocytes in a microenvironment more similar to that *in vivo*.(Prussin 1997, Pala, Hussell et al. 2000) Protein

transport inhibitors can also be utilised in conjunction with this method in order to determine which cells produce the cytokine.

6.3 Glycolysis and cytokine production

When activated murine T cells are provided with co-stimulation and growth factors but are blocked from engaging glycolysis using galactose, their ability to produce TH1 cytokines (IFN-γ) is markedly compromised,(Chang, Curtis et al. 2013) with decreased IFN-γ mRNA in cells utilising oxidative phosphorylation. These results demonstrate that the engagement of aerobic glycolysis specifically permits the translation of IFN-γ mRNA in activated T cells and thereby regulates the ability of the cells to attain full effector status.(Chang, Curtis et al. 2013) A decrease in lymphocyte glycolysis (Chapter 4) may therefore be accompanied by a decrease in cytokine production postoperatively in human lymphocytes.

6.4 Cytokines and surgery

Cytokines have local effects of mediating and maintaining the inflammatory response to tissue injury and are also responsible for initiating some of the systemic responses, which occur following trauma. For example, prolonged visceral ischemia during major surgery has been shown to result in increased levels of TNF-α,(Wakefield, Carey et al. 1993) the magnitude of which correlates with the frequency and degree of postoperative organ dysfunction.(Wakefield, Carey et al. 1993, Poeze, Ramsay et al. 2002) It has become clear that an adequate host immune response against infection is largely dependent on the activation of TH1 and TH2 cells.(Del Prete, Maggi et al. 1994, Romagnani 1997) Host immune responses following major surgical trauma is associated with down-regulation of cellular immunity in the early postoperative period,(Salo 1992) which may contribute to infectious complications following surgery.(Christou, Meakins et al. 1995)

6.6 Hypothesis

Reduced glycolysis impairs TH1 cytokine production in T-lymphocytes postoperatively.

Methods

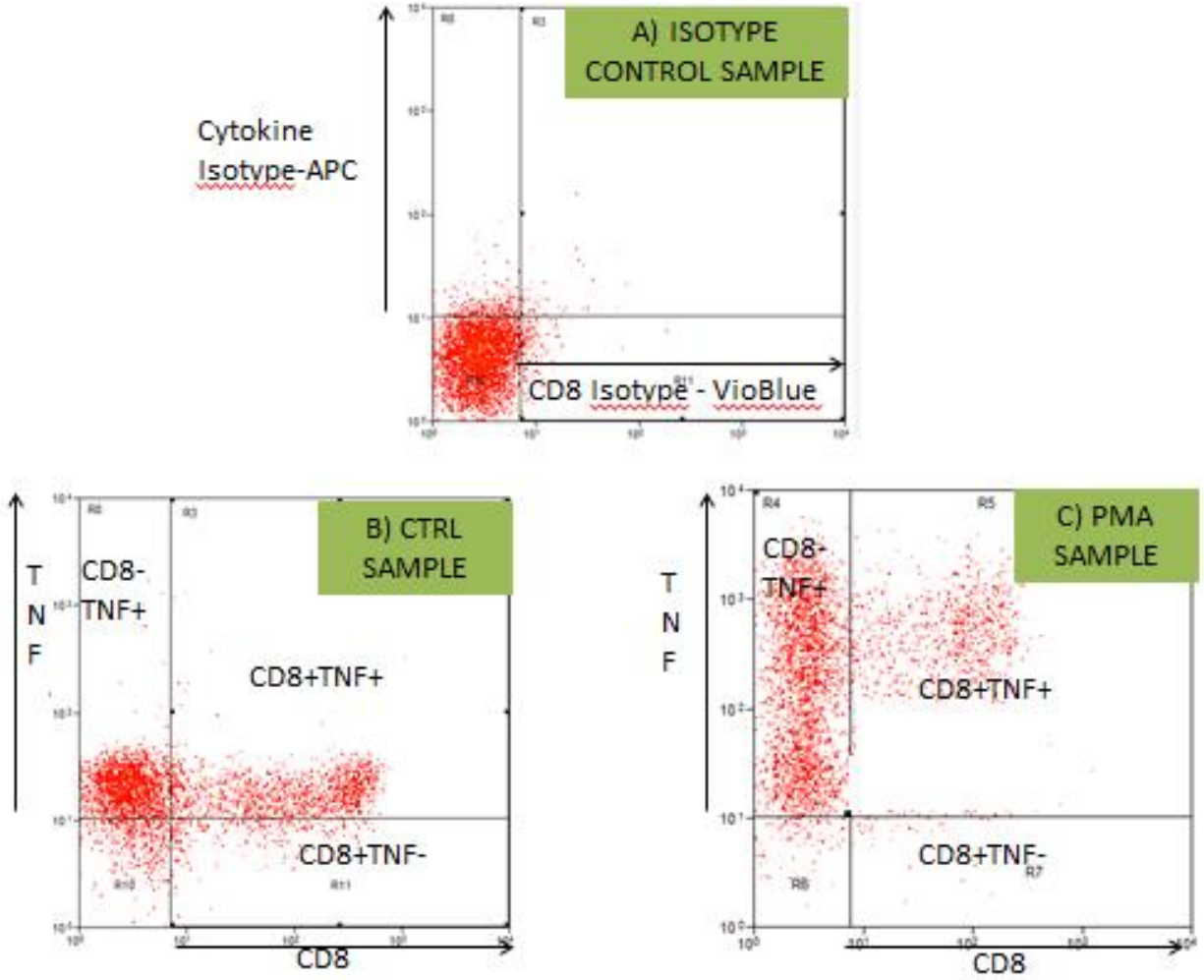
6.7 Patients

Patients undergoing general, urology, orthopaedic and gynaecology surgery (VISION-UK) were recruited into this study. Blood samples were taken preoperatively on the morning of surgery through a 20 G IV cannula. Postoperative samples were taken in a subset of patients on day 3 following surgery.

6.8 Flow cytometry analysis of intracellular cytokine assay

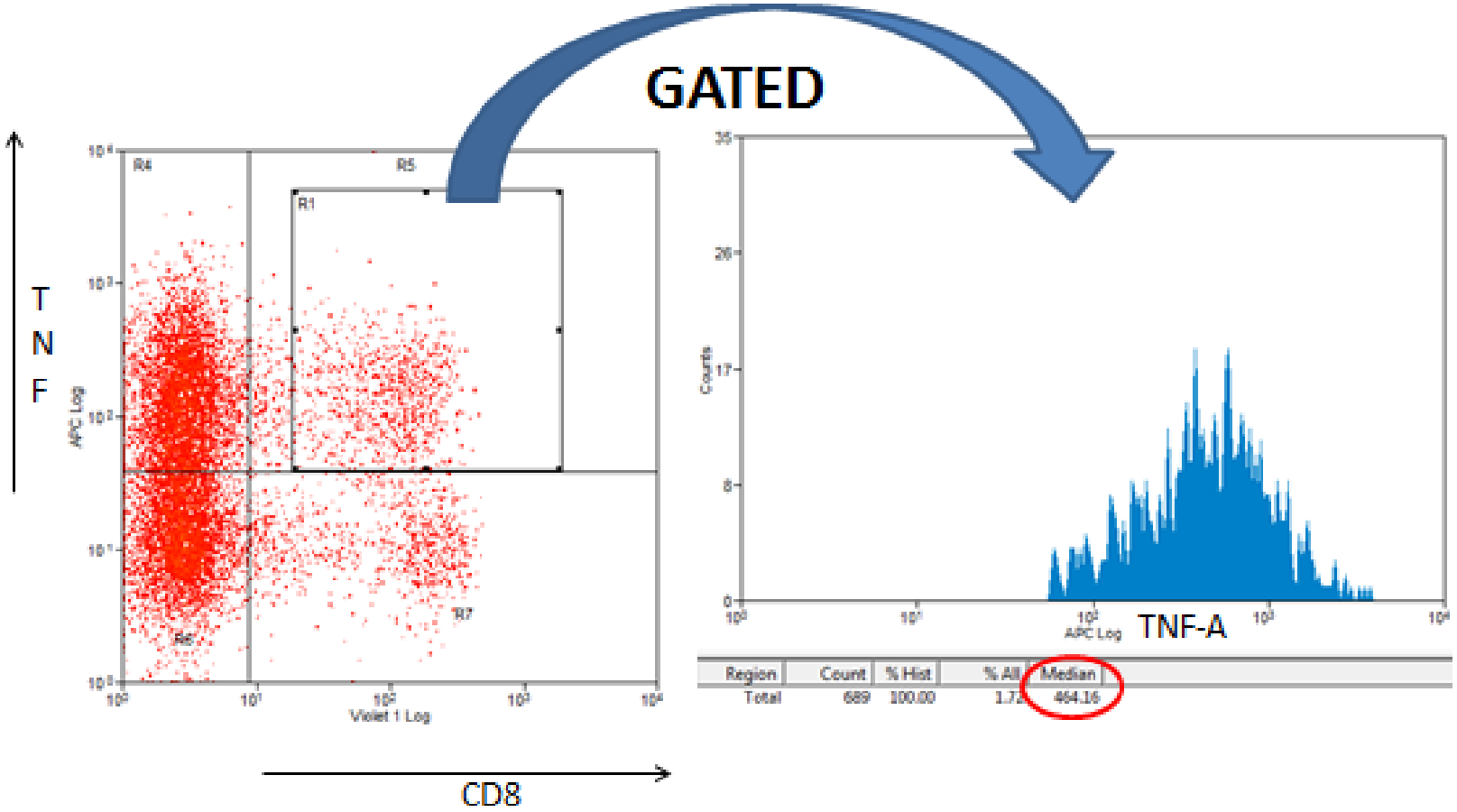
The proportion of CD4(+) or CD8(+) cells expressing IL-2, TNF-α or IFN-γ was calculated (Figure 6.1) and corresponding MFI recorded (Figure 6.2).

Figure 6.1 Flow cytometry analysis of intracellular cytokine production by lymphocytes



Lymphocytes gated onto density plots. Quadrants set with isotype control sample (A). Percentage of CD8+TNFα+ cells presented in summit software as percentage of gated lymphocyte population. Median Frequency Intensity subsequently elucidated by transferring CD8+TNFα+ gated population onto histogram (Figure 6.2)

Figure 6.2 Method utilised to obtain MFI of CD8(+)TNFα(+) cells



CD8(+)TNFα(+) population gated onto histogram to determine MFI (circled in red)

In this chapter I explore the effect of:

1) PMA and ionomycin activation on CD4(+) and CD8(+) cell cytokine production pre-and postoperatively
2) Surgery on cytokine production in unstimulated CD4(+) and CD8(+) cells

6.9 Statistical Analysis

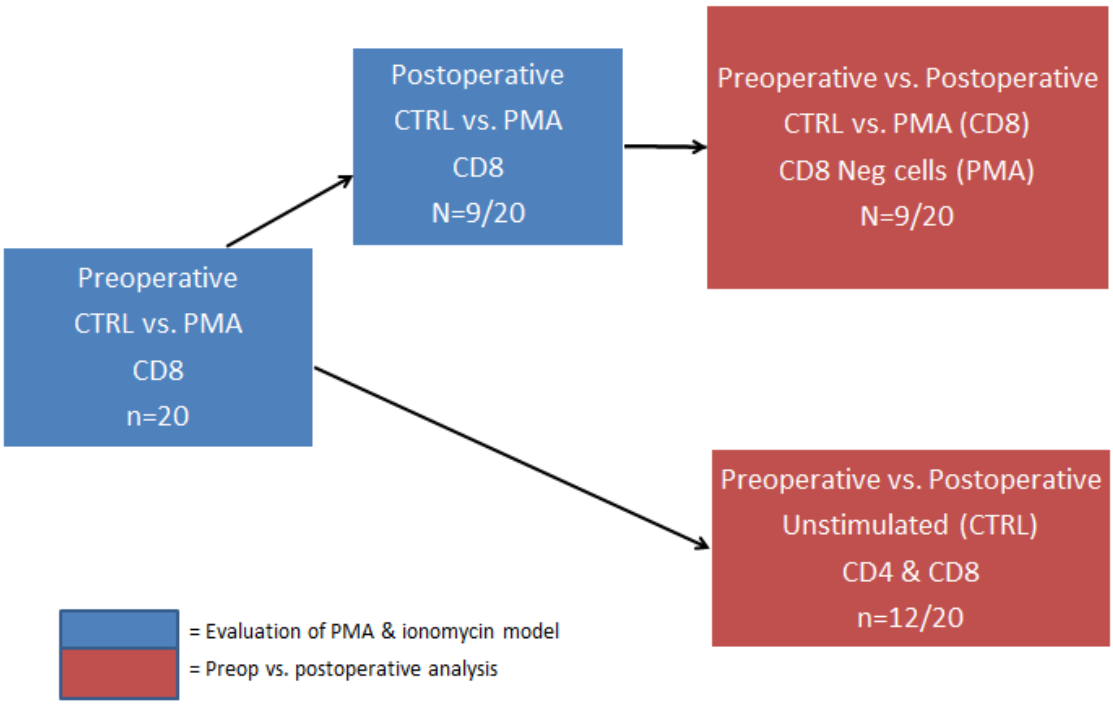
A 10% difference in median fluorescence intensity (MFI; the median total amount of cytokine present in each cell averaged from 10,000 cells analysed using flow cytometry) has previously been described between septic and non-septic patients.(Boomer, To et al. 2011) To achieve a power of 0.8 and alpha error of 0.05, a sample size of 9 pre- and postoperatively matched patients would be required. 12 matched samples were obtained to account for a 30% withdrawal rate from the study or early hospital discharge. Data were analysed by descriptive and comparative approaches. All results are expressed as absolute numbers and percentages (means (95%CI); medians (IQR)).

Results

6.10 Patient cohorts

20 Patients were recruited into this study from VISION-UK. Of these, 12 patients also had postoperative blood samples taken for further evaluation of perioperative changes of lymphokine production in unstimulated CD4(+) and CD8(+) cells. 9 patients also had evaluation of lymphokine production from CD8 cells activated with PMA and ionomycin. Patient groups recruited and experiments performed are outlined in Figure 6.3 below.

Figure 6.3 Patient cohorts recruited for lymphokine study



CTRL = cells + brefeldin A; PMA = cells + brefeldin A + PMA + ionomycin

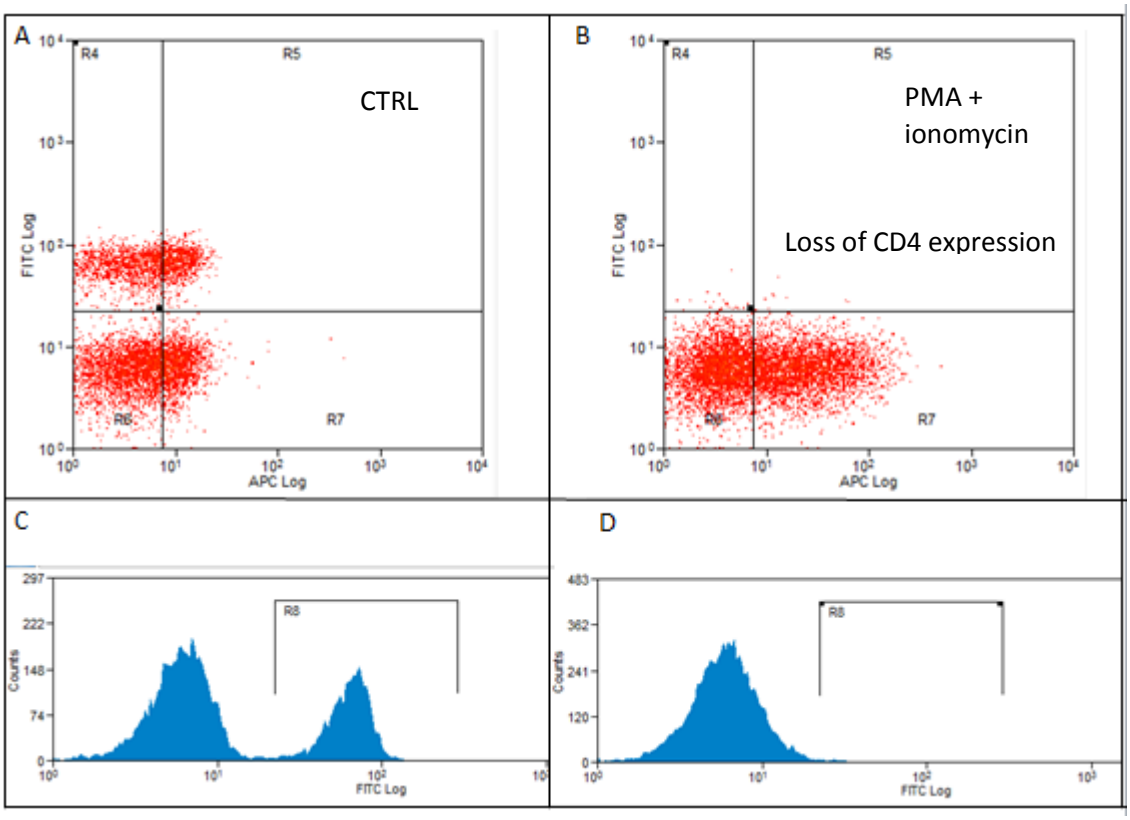
Results are summarised as follows:

1) Effect of PMA and ionomycin stimulation on CD4(+) cytokine production
2) Evaluation of PMA and ionomycin stimulation on CD8(+) cells pre- (n=20) and postoperatively (n=9)
3) Evaluation of perioperative changes in unstimulated CD4(+) and CD8(+) cells (n=12)
4) Evaluation of perioperative changes in PMA and ionomycin stimulated CD8(+) cells (n=9)

6.11 Effect of PMA and ionomycin stimulation on CD4(+) cytokine production

PMA and ionomycin caused down-regulation (shedding) of CD4(+) cells. Consequently it was not possible to ascertain cytokine production from CD4(+) cells following treatment with PMA. (Figure 6.4)

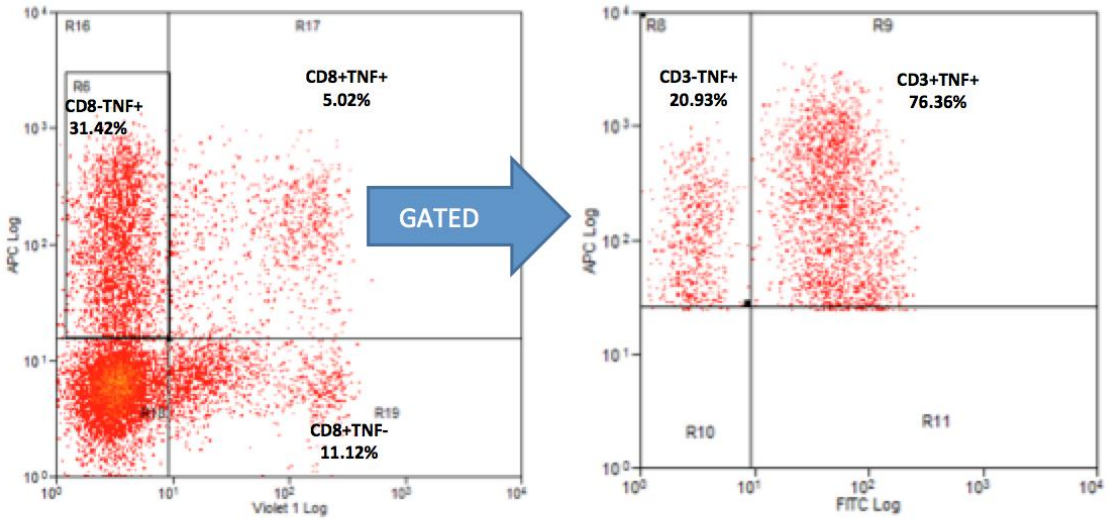
Figure 6.4 Density plots (A and B) and histograms (C and D) demonstrating the down-regulation of CD4 (FITC) secondary to PMA and ionomycin stimulation (B and D)



Flow cytometry figure of CD4(+) (FITC) co-stained with TNF-A (APC). Density plot A and histogram C represent control samples and density plot B and histogram D are following incubation with PMA and ionomycin.

Following stimulation with PMA, the lymphocyte populations expressing cytokine consisted of predominantly CD8(+) and CD3(+) cells as demonstrated in Figure 6.5.

Figure 6.5 Density plots of CD8(-)TNF(+) cells gated onto CD3TNF plot following incubation with PMA and ionomycin



CD8(-) (vioblue)TNF(+) (APC) cells gated onto CD3(+) (FITC) TNF(+) (APC) density plot. This demonstrates that the majority of the cytokine expressing lymphocytes that do not express CD8 are CD3(+) (and likely to be CD8(-)CD3(+)CD4(+) cells). Lymphocytes were also co-stained with antibodies for CD19(+) and CD14(+) and did not show presence in the CD8(-)TNF(+) group.

6.12 Evaluation of PMA and ionomycin stimulation pre- and postoperatively on CD8 cells

Cytokine expression was assessed following stimulation with PMA and ionomycin:

A) Preoperatively (n=20)

B) Postoperatively (n=9)

Demographics of patients recruited into this study are summarised in Table 6.1.

Table 6.1 Summary of patient demographics of the cytokine expression with PMA and ionomycin

	Preoperative (n=20) Cohort	**Postoperative (n=9) Cohort**
Age (Years)	61 (54 – 72)	59(52-68)
Gender (M/F)	12 / 8	4 / 5
Absolute lymphocyte count (x 10^9/L)	1.76 (1.46-2.06)	2.36 (1.29-3.43)
Relative lymphocyte count (%)	25.02 (21.20-28.84)	26.61(17.30-35.92)
Type of surgery, n(%)		
General	7 (35)	3(33)
Gynaecology	2 (10)	0 (0)
Urology	8 (40)	1(11)
Orthopaedic	3 (15)	5(56)

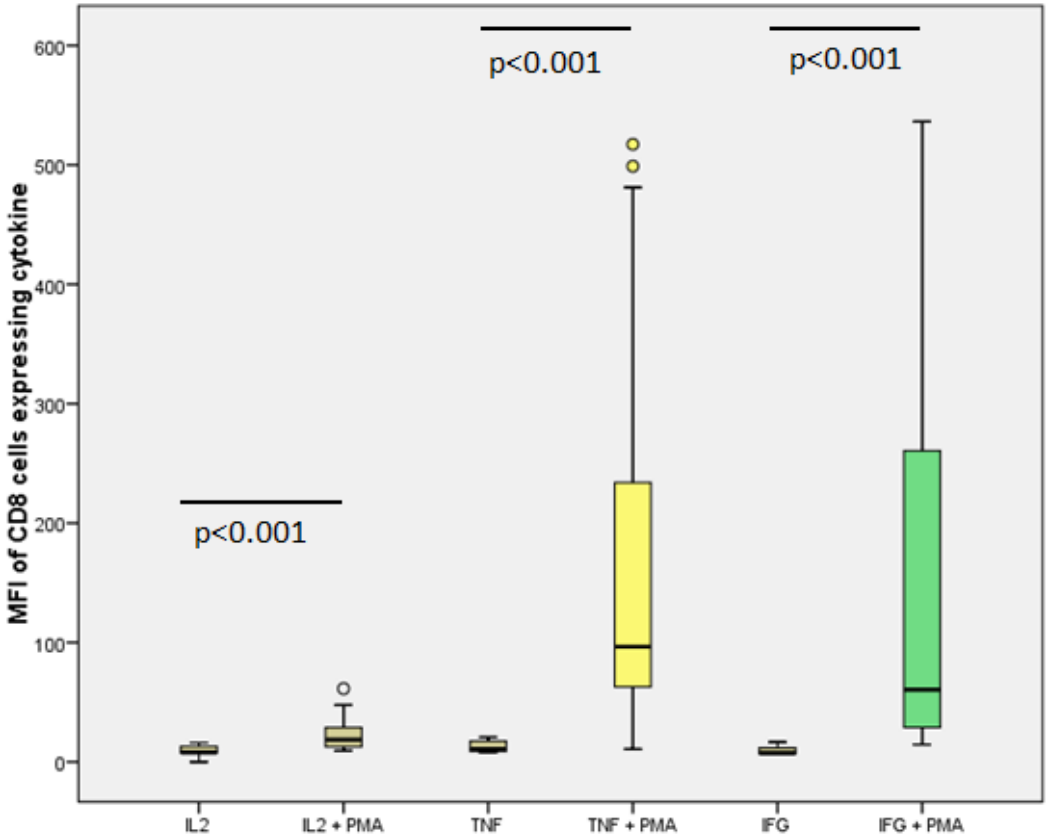
Mean and 95% CI unless stated otherwise

Effect of PMA and ionomycin on CD8 cell cytokine production

PMA and ionomycin consistently increased CD8(+) intracellular cytokine production demonstrated by increased MFI (Figure 6.6) and percentage (Figure 6.7) of IL-2, TNF-α and IFN-γ production preoperatively and postoperatively.

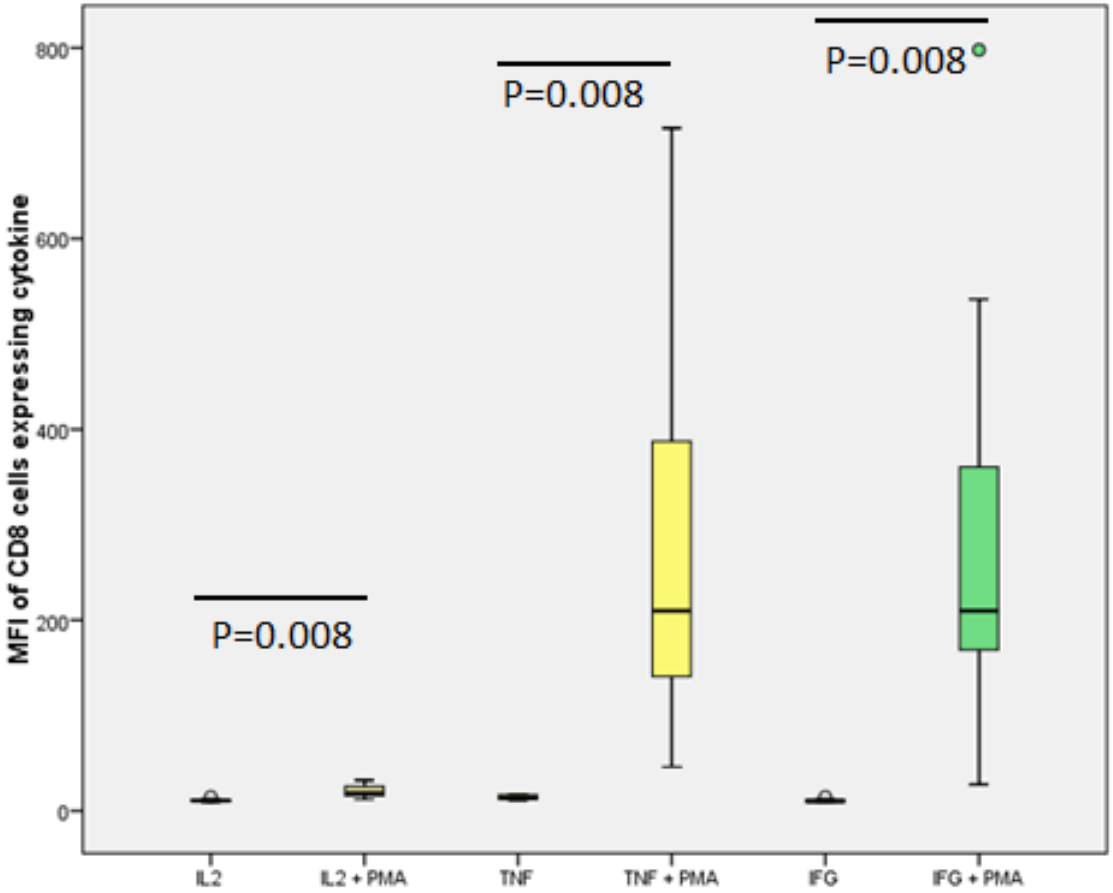
Figure 6.6 Effect of PMA on MFI of CD8(+) cells expressing cytokine A) Preoperatively and B) Postoperatively

A) Preoperative effect of PMA and ionomycin on CD8(+) MFI



n=20; Control samples indication by "cytokine" and cells treated with PMA and ionomycin indicated by "Cytokine + PMA". Results represent whole blood treated with PMA and ionomycin in addition to brefeldin alone (CTRL); Related samples Wilcoxen signed rank test.

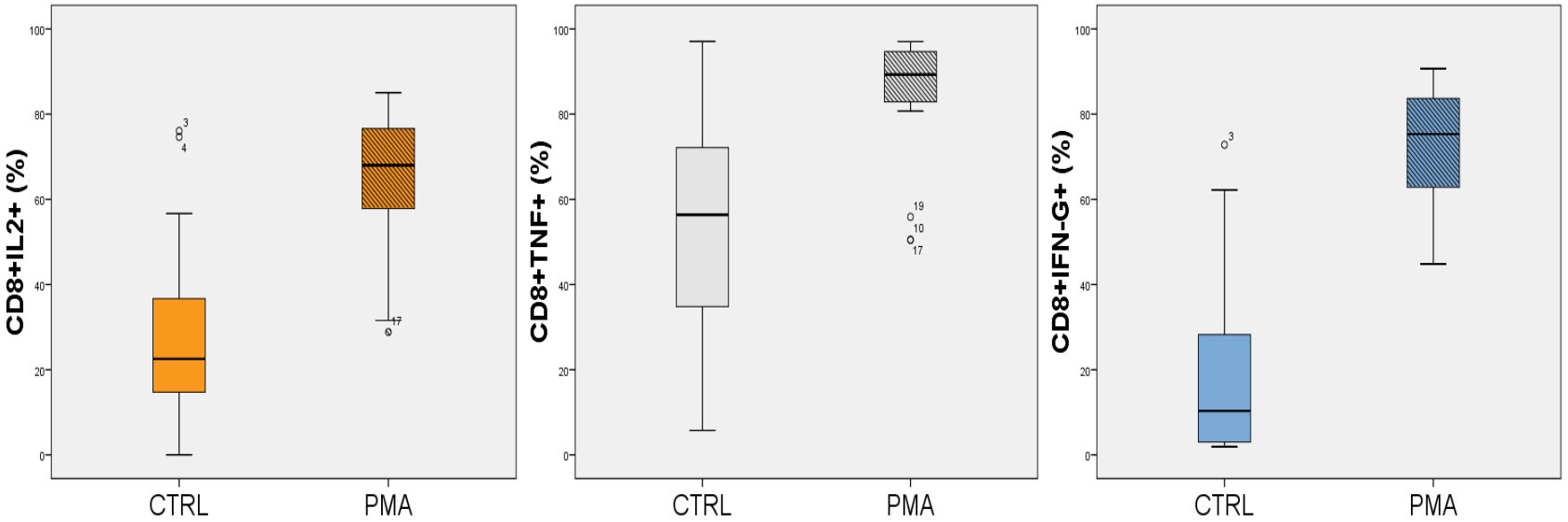
B) Postoperative PMA and ionomycin on CD8(+) MFI



n=9; Control samples indication by "cytokine" and cells treated with PMA and ionomycin indicated by "Cytokine + PMA". Results represent whole blood treated with PMA and ionomycin in addition to brefeldin alone (CTRL); Related samples Wilcoxen signed rank test.

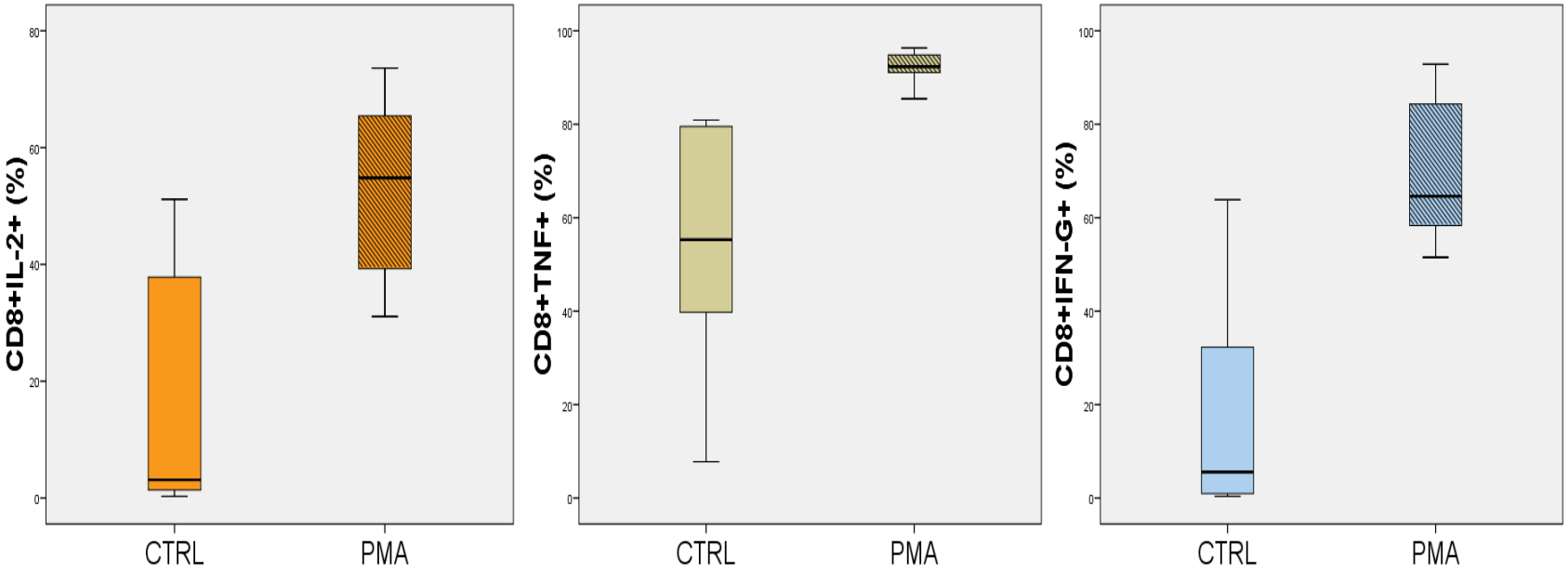
Figure 6.7 Effect of PMA on percentage of CD8(+) cells expressing cytokine A) Preoperatively and B) Postoperatively

A) Preoperative



n=20; all p<0.001; related samples Wilcoxen signed rank test; CTRL = control. Shaded bars represent whole blood treated with PMA and ionomycin in addition to brefeldin alone (CTRL)

B) Postoperatively



n=9; all p=0.008; CTRL = control. PMA = PMA + ionomycin. Shaded bars represent whole blood treated with PMA and ionomycin in addition to brefeldin alone (CTRL)

6.13 Evaluation of perioperative changes in cytokine production of unstimulated (without PMA and ionomycin) CD4(+) and CD8(+) cells (n=12)

Table 6.2 summarises the patients recruited into this study.

Table 6.2 Summary of patient demographics in the paired pre- vs. postoperative cytokine expression cohort

	Preoperative values of recruited patients (n=12)
Age (Years)	59 (49-68)
Gender (M/F)	7 / 5
Absolute lymphocyte count (x 10^9/L)	2.32 (1.53-3.10)
Relative lymphocyte count (%)	28.08 (20.70 – 35.47)
Type of surgery, n(%)	
General	5 (42)
Urology	2 (16)
Orthopaedic	5 (42)

Values in mean (95%CI) unless stated otherwise

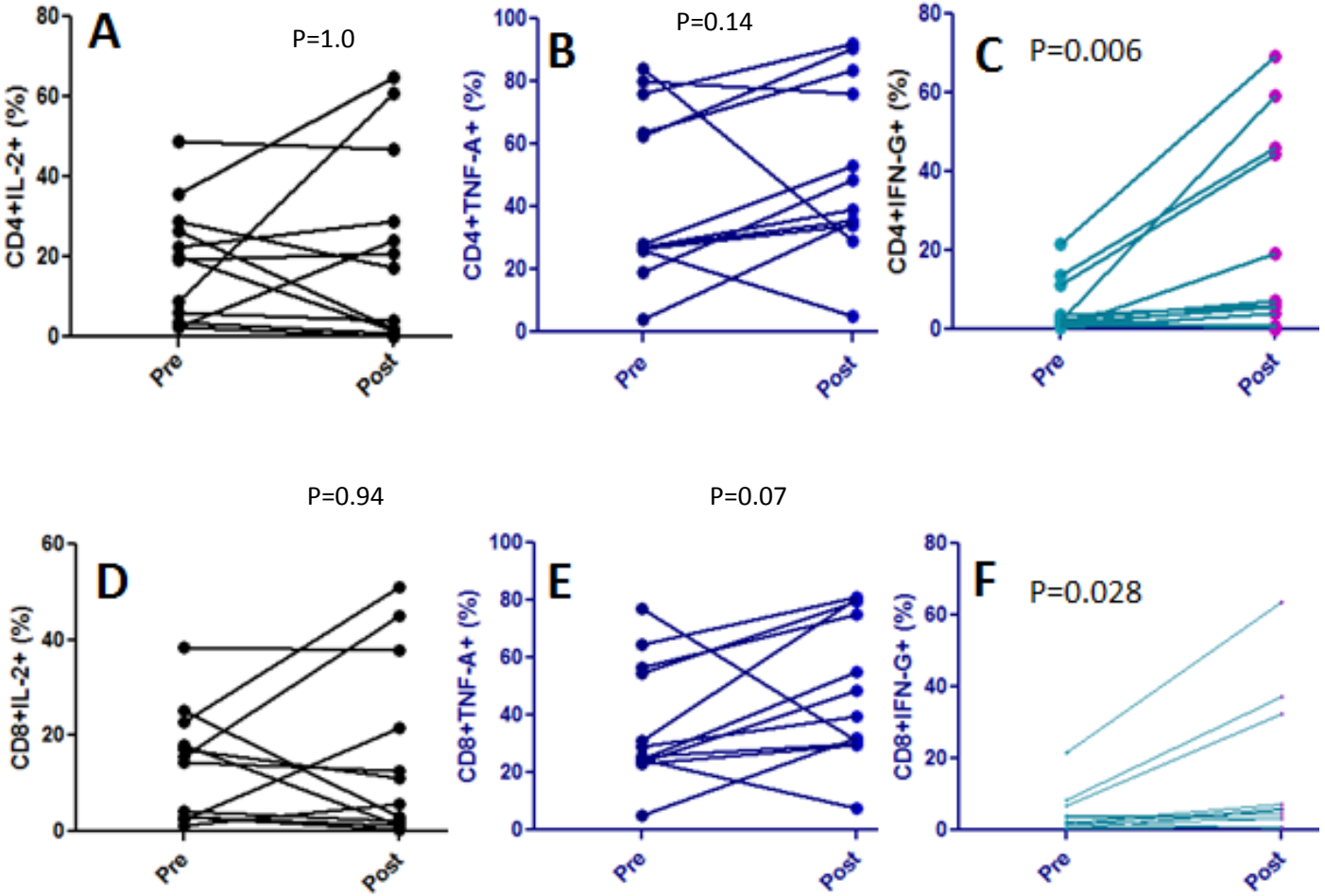
Unstimulated CD4(+) cytokine expression

There was a postoperative increase in median (IQR) percentage of CD4(+) cells expressing IFN-γ (n=12; p=0.006; Figure 6.8).There was no difference in MFI of CD4(+) cells expressing cytokine postoperatively (IL-2 p=0.308; TNF-α p=0.182; IFN-γ p=0.110; related samples Wilcoxen signed rank test).

Unstimulated CD8(+) cytokine expression

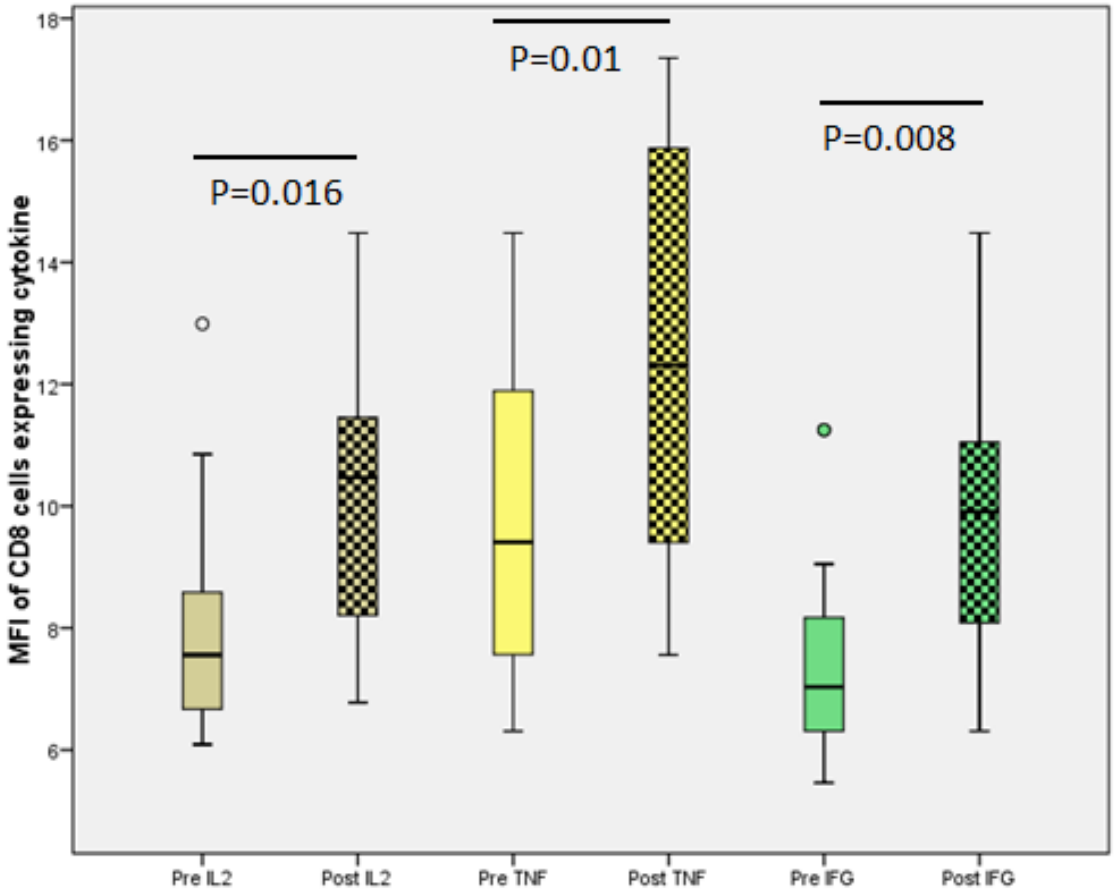
There was an increase in median (IQR) percentage of CD8(+) cells expressing IFN-γ (Figure 6.8). There was an increase in MFI of CD8(+) cells expressing IL-2, TNF-α and IFN-γ (Figure 6.9).

Figure 6.8 Perioperative percentage cytokine expression by CD4(+) (A-C) and CD8(+) (D-F) cells



Perioperative changes (Pre = preoperative; Post = postoperative) in percentage of CD4+ (A-C) and CD8+ (D-F) cells expressing cytokine (n=12)

Figure 6.9 Perioperative changes in cytokine production from unstimulated CD8(+) cells

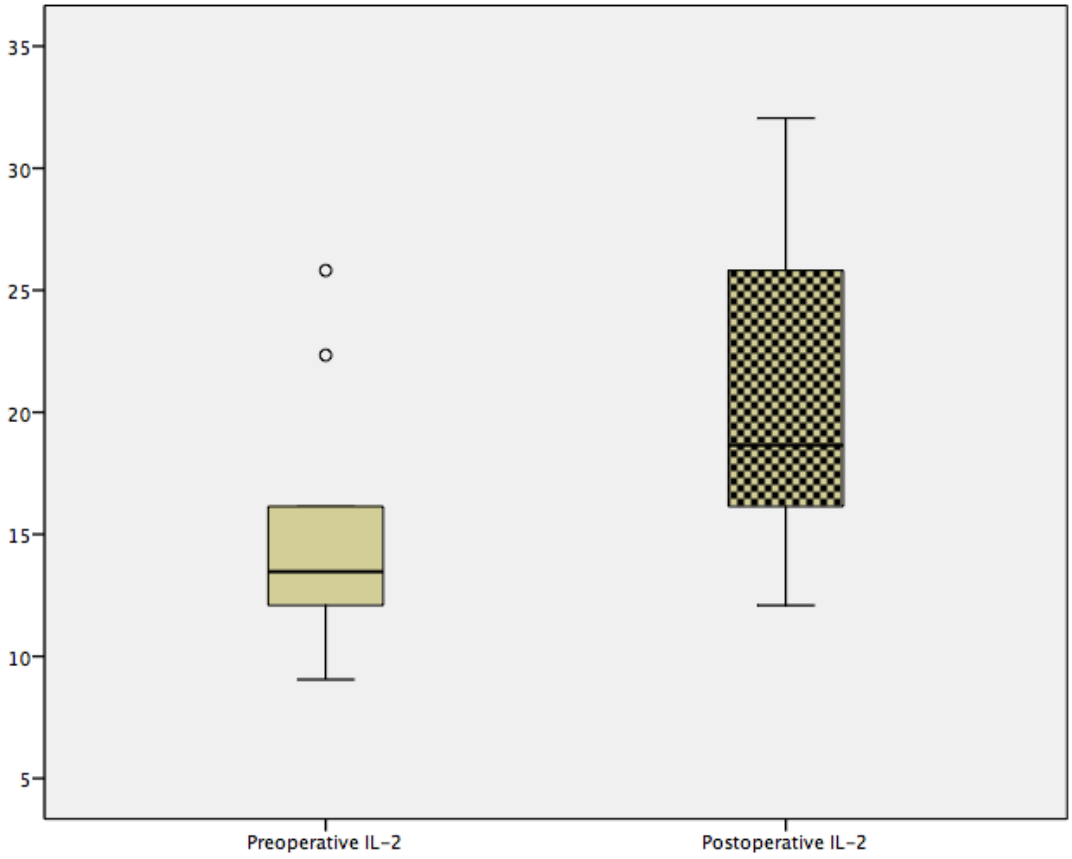


6.14 Evaluation of perioperative changes in cytokine production from PMA and ionomycin stimulated CD8(+) cells (n=9)

Compared to preoperative samples paired samples, when stimulated with PMA, a rise was noted postoperatively in MFI of CD8(+) cells expressing IL-2 and IFN-γ (Figure 6.10 A and C). There was no difference in median (IQR) percentage of CD8(+) cell expression of TNF-α or IFN-γ postoperatively with PMA stimulation, however an increase in median (IQR) percentage of CD8(+) cells producing IL-2 was also demonstrated (Figure 6.11).

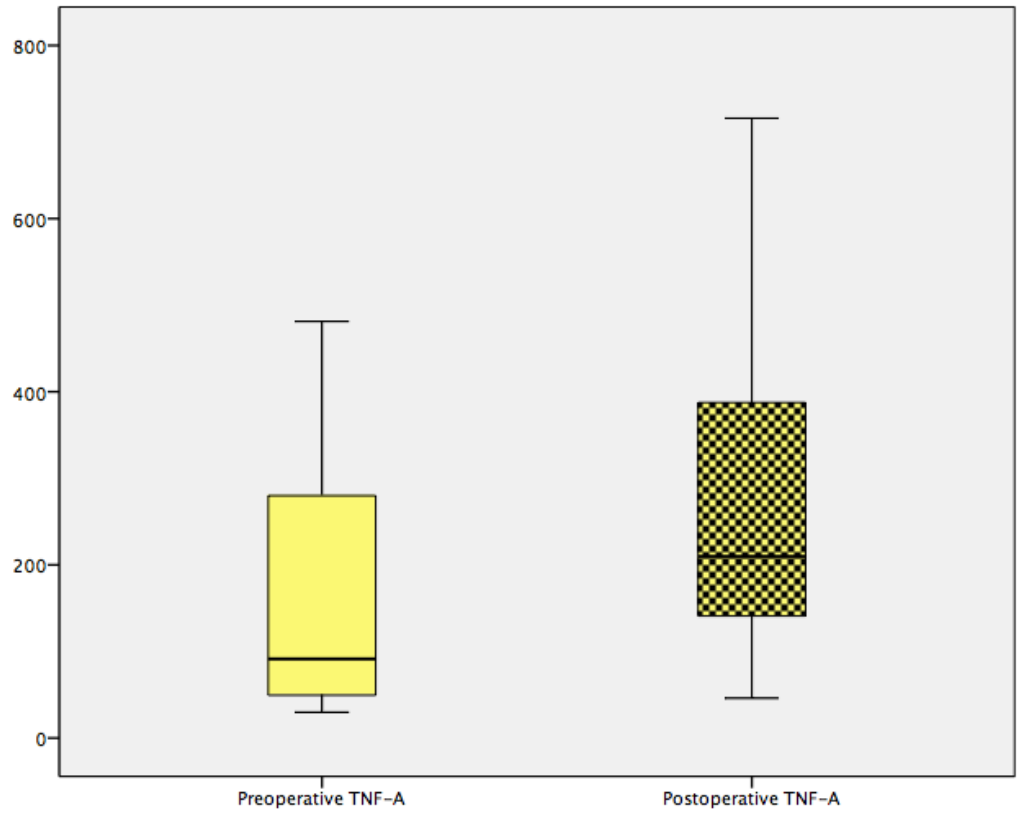
Figure 6.10 Perioperative changes in MFI (A-C) and percentage (D) of CD8+ cells stimulated with PMA and ionomycin expressing cytokines

A) IL-2



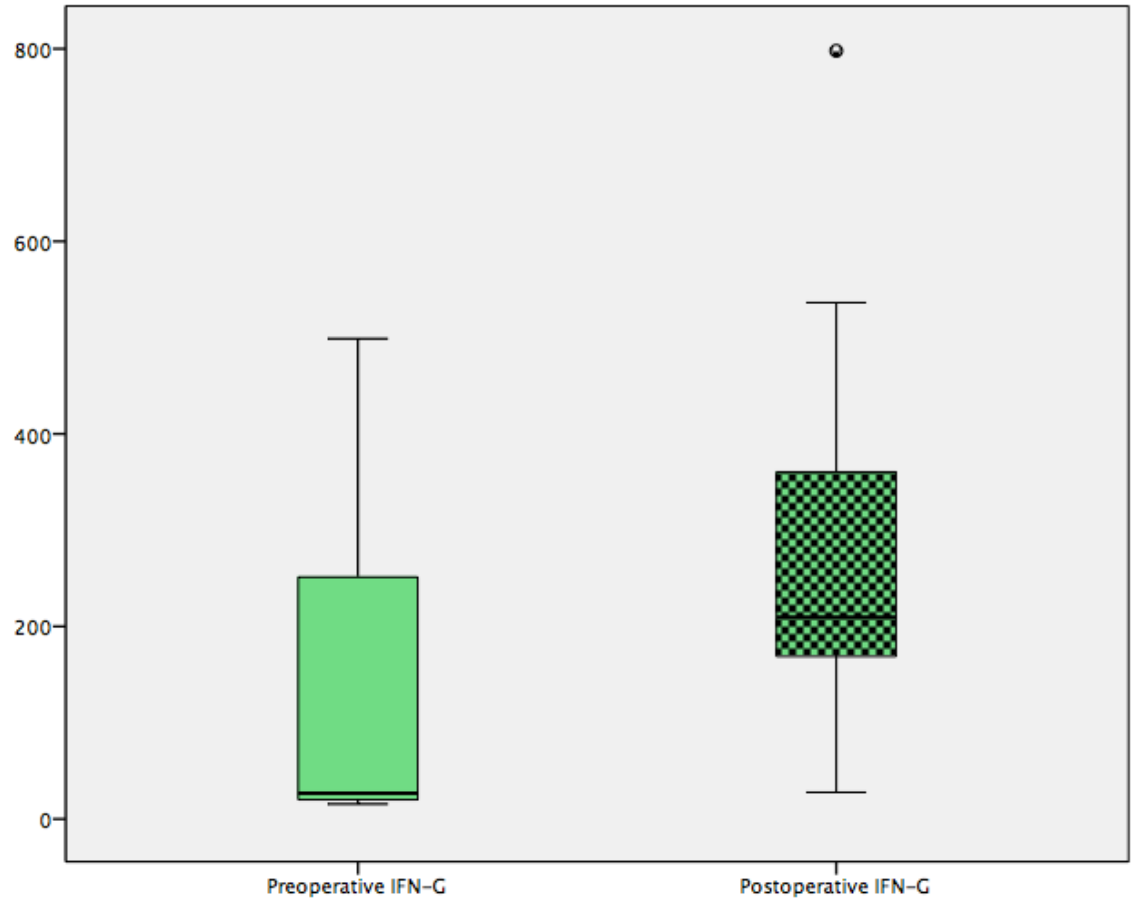
Shaded bar = postoperative; n=9; p=0.008

B) TNF-α



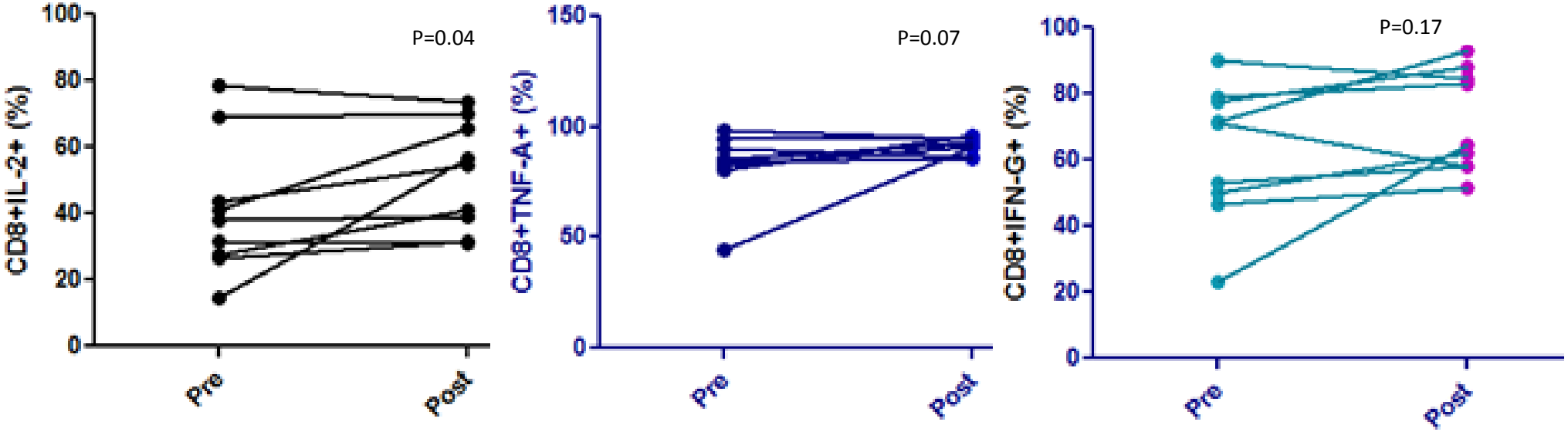
Shaded bar = postoperative; n=9; p=0.066

C) IFN-γ



Shaded bar = postoperative; n=9; p=0.021

Figure 6.11 Perioperative changes in percentage CD8(+) cytokine expression with PMA and ionomycin stimulation



6.15 CD4 cell (CD8 negative cell) response to PMA pre and postoperatively

Percentage change in CD4(+) cells expressing cytokine did not vary perioperatively (IL-2 p=0.767; TNF-α p=0.515; IFN-γ p=0.173).

MFI of CD4(+) cells (identified by CD8(-) cell population) increased with PMA stimulation for each cytokine (IFN-γ p=0.008; IL-2 p=0.008; TNF-α p=0.011; Figure 6.12).

6.16 Relative Gain in MFI with PMA

Relative gain (% increase in MFI) in MFI with PMA was not different pre- versus postoperatively for IL-2 (p=0.859; n=9) or TNF-α (p=0.678; n=9), but was higher with PMA in postoperative samples for IFN-γ (Figure 6.13).

Figure 6.12 MFI of CD8(-) cells (CD4 cells) following PMA and ionomycin stimulation in the perioperative period

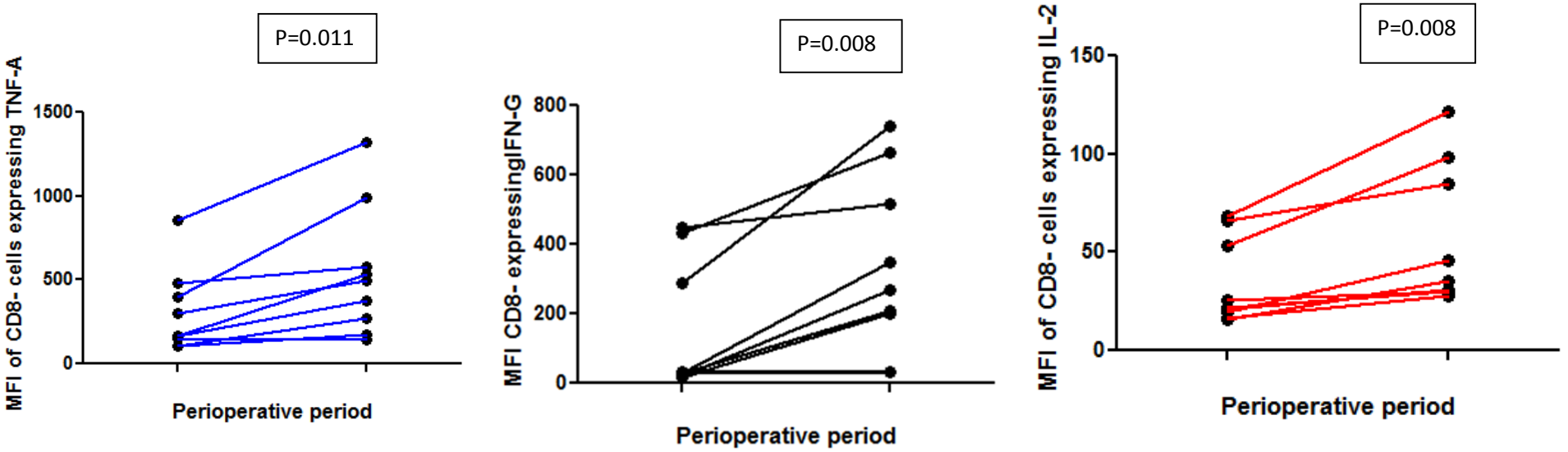
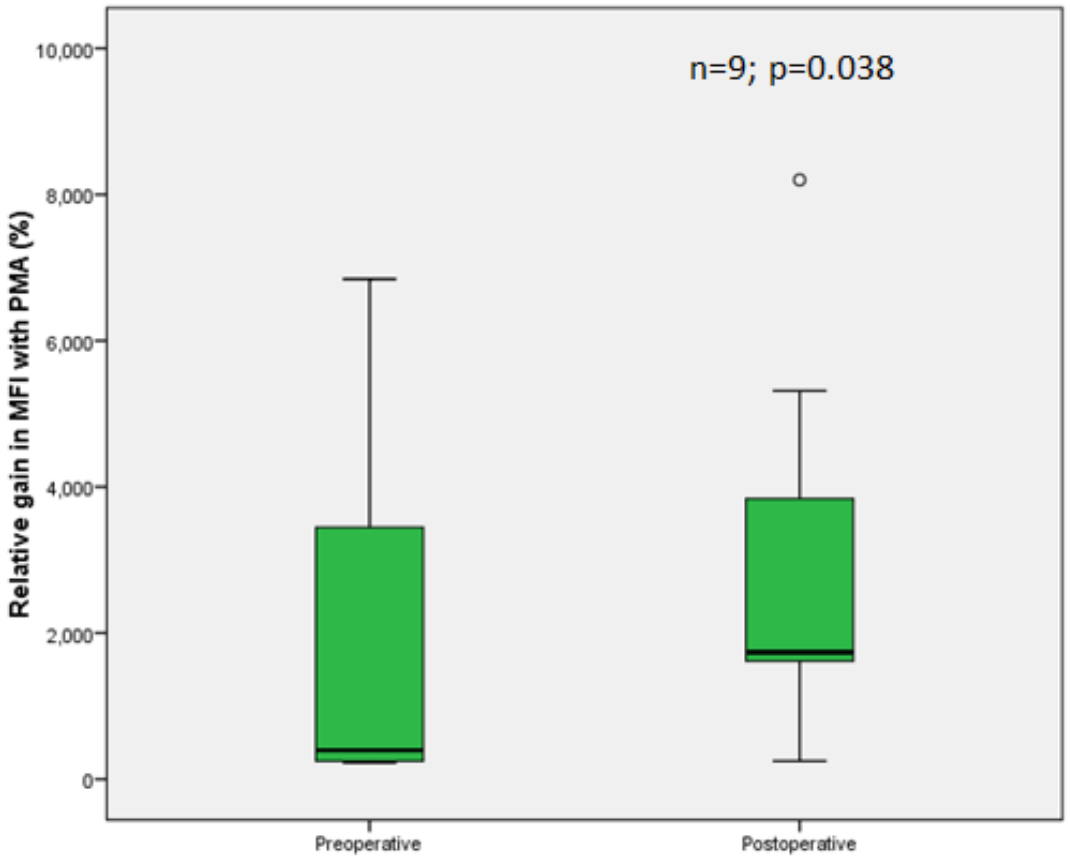


Figure 6.13 Fold change in MFI of CD8(+) cells expressing IFN-γ caused by PMA compared in the perioperative period (related samples Wilcoxen signed rank test)



Discussion

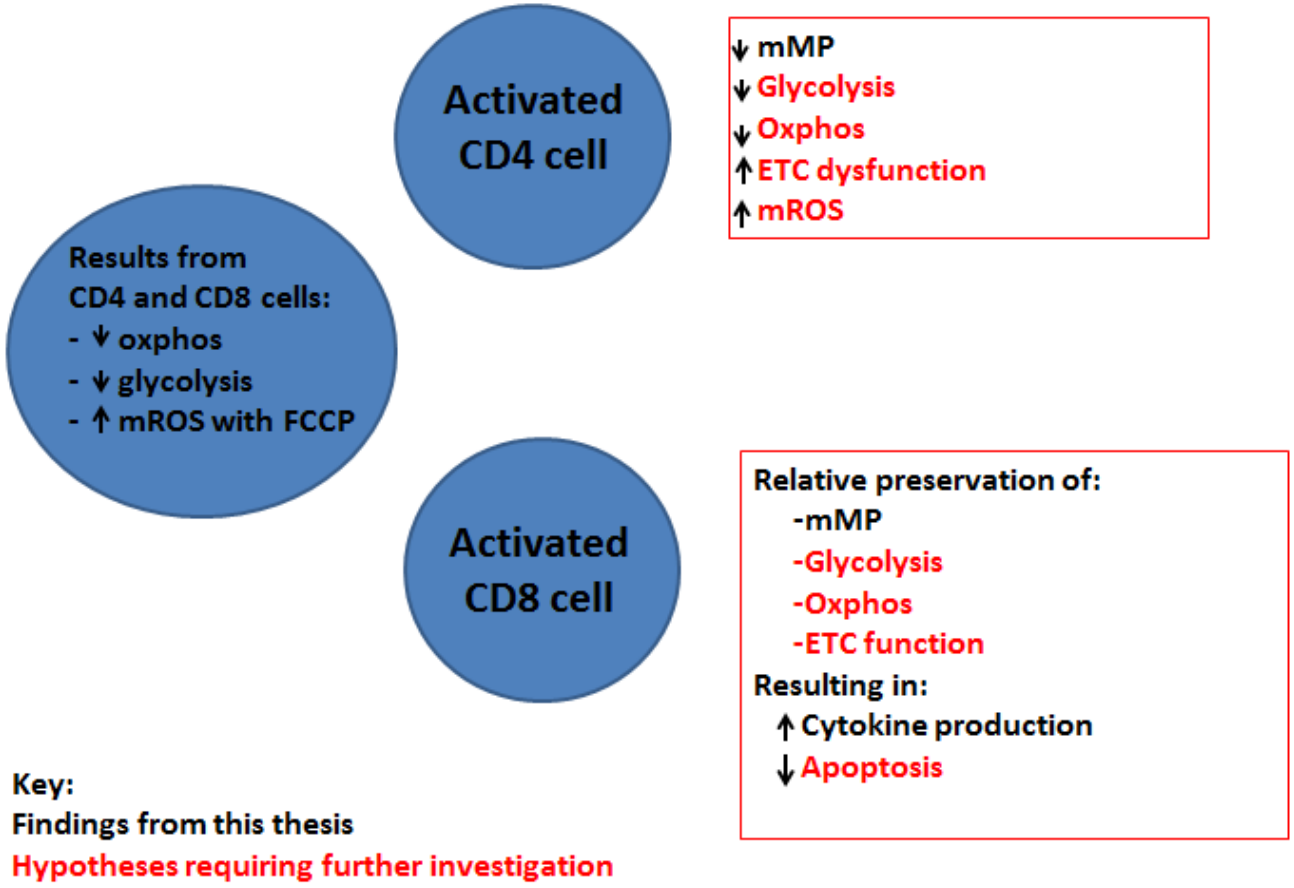
6.17 Discussion

TH1 lymphokine production by stimulated and unstimulated CD8(+) cells increases postoperatively. Cytokine production by unstimulated CD4(+) cells on the other hand does not increase postoperatively.

The main finding of this study is that perioperative differences are demonstrated between CD4(+) and CD8(+) cells. CD8(+) cells appear to have an increased ability compared to CD4(+) cells to be activated and produce cytokine following anaesthesia and surgical trauma. There was no demonstrable difference in CD8(+) FCCP mitochondrial membrane potential postoperatively compared to preoperative FCCP sample which is consistent with cytokine findings of this Chapter i.e. maintained ability to produce cytokine which may indicate less ETC dysfunction or relatively preserved glycolytic function of CD8(+) cells. The reduced cytokine production demonstrated by CD4(+) cells postoperatively may therefore be related to the reduced membrane potential seen with ETC dysfunction which may be due to increased propensity to apoptosis, either induced by FCCP, or occurring postoperatively. Figure 6.14 summarises potential mechanisms for increased CD8(+) cytokine production in relation to other findings in this thesis.

Normal CD4:CD8 ratio is approximately 1:2. Surgery results in a reduction of CD4:CD8 ratio,(Dietz, Heimlich et al. 2000). Decreased CD4:CD8 ratio has also been related to increased incidence of postoperative sepsis following abdominal surgery in HIV infected patients.(Xia, Liu et al. 2012) This is in keeping with the hypothesis of more pronounced CD4(+) bioenergetic function impairment occurring compared to CD8(+) cells, predisposing CD4(+) cells to apoptosis (greater ETC dysfunction and increased mROS) postoperatively, and therefore decreasing CD4:CD8 ratio. This study also suggests that the ratio change following surgery may be accompanied by altered CD4 function. Since activation and cytokine production are energy dependent processes, the inability to increase TH1 cytokine production of postoperative CD4 cells may be related to altered lymphocyte metabolic and bioenergetic function (Chapter 4) along with CD4 mitochondrial membrane potential reduction postoperatively.

Figure 6.14 Potential mechanisms explaining increased CD8(+) cytokine production, related to other findings in this thesis. (Cells presumed to be more likely to be activated in the postoperative period)



Since cytokine production is dependent on glycolysis,(Chang, Curtis et al. 2013) it was hypothesised that cytokine production would reduce postoperatively. This study demonstrates that when lymphocytes are interrogated postoperatively, a time when patients are often lymphopenic, they do not exhibit a decrease in TH1 cytokine production. These findings suggest that the lymphopenia induced by surgery may be part of a spectrum of change in lymphocyte function which when severe leads to the immune suppressed state described in acute sepsis.(Boomer, Shuherk-Shaffer et al. 2012) The "exhausted" and anergic immune phenotypes seen in sepsis have previously been associated with failure to secrete cytokines, reduced proliferation in response to antigen, increased expression of certain cell surface receptors (e.g. CD69) and decreased expression of the IL-7 receptor (cd127).(Yi, Cox et al. 2010)

6.18 Comparison to other perioperative cytokine study findings

Matsuda et al. demonstrated no significant change in percentage of CD4 cells expressing IFN-γ on day 3 postoperatively following stimulation in 53 colorectal cancer surgical patients.(Matsuda, Furukawa et al. 2007) I found an increase in percentage of CD4 cells expressing IFN-G in unstimulated cells, which may be due to differences in experimental design. Firstly Matsuda did not report their findings in unstimulated cells (without PMA and ionomycin stimulation) and appeared not to demonstrate the downregulation of CD4 secondary to PMA and ionomycin that was apparent in my experiments. This may be due to their shorter incubation period of 4 hours compared to the 16 hours that I used with PMA and ionomycin. Their methodology does not however describe how CD4 expression was determined or how they overcame the downregulation. The increase in IFN-γ expression also seen in this study could be related to a longer incubation times in brefeldin A at 37^{o}C (16 hours versus 4 hours) prior to intracellular staining and flow cytometry analysis. Brefeldin has been shown to induce endoplasmic reticulum stress,(Islam, Hassan et al. 2006) activate apoptotic caspases.(Lee, Kim et al. 2013) Brefeldin may have therefore influenced cytokine production by activating cells. Finally, Matsuda's colorectal cancer population may demonstrate differences in cytokine production compared to the patients in this study since none of the patients recruited in this chapter were diagnosed with cancer.

Ishikawa *et al.* reported an increase in percentage of CD4 cells expressing IFN-γ on day 2 post gastric and colorectal surgery but a decrease following hepatic resection.(Ishikawa, Nishioka et al. 2004) The discrepancy between these results may be due to the differences in background malignancies, the method of cell stimulation (PMA and ionomycin versus nil) and the use of a whole blood assay rather

than enzyme-linked immunosorbent assay (ELISA). Limitations of other previous studies which have evaluated cytokine production postoperatively include: failure to report the MFI (the absolute amount of cytokine present within each cell),(Berguer, Bravo et al. 1999, Stalder, Birsan et al. 2005, Matsuda, Furukawa et al. 2007) failure to assess accumulation of intracellular cytokine utilising protein transport inhibitor,(Stalder, Birsan et al. 2005) and presenting cytokine production from a combination of cell types.(Berguer, Bravo et al. 1999, Franke, Lante et al. 2009)

6.19 Study strengths and limitations

A limitation of this study was the downregulation of CD4 seen with PMA stimulation.(Kemp and Bruunsgaard 2001) CD8(-) cells (CD4 cells) were analysed following PMA stimulation, however relative gain of CD8 negative cells could not be calculated because without PMA stimulation, CD8(-) cytokine(+) populations were insufficient in number to analyse.(i.e. CD8-cytokine+ populations were more pronounced following PMA stimulation). Despite this, cytokine production in unstimulated CD4 and CD8 cells clearly show differing responses to surgery.

The serial analysis of perioperative samples, enabling each patient to act as their own control, is a robust model that circumnavigates the significant challenges of appropriate control samples. Since patients were recruited from different surgical specialties, increased CD8 cytokine production appears to be independent of site of surgery. Whether patients had clinical signs of infection on the day of postoperative blood sampling was not factored into the data analysis, however this is unlikely given the fact that the team proceeded with elective surgery. Furthermore, the postoperative sample was chosen on day 3 following surgery because patients are usually still lymphopenic at this time point compared to preoperative values (Chapter 3). Furthermore due to the elimination half-life of the anaesthetic agents utilised in this study, anaesthetic drugs would have been eliminated by this postoperative day.(Smith, Scarth et al. 2011) Further work is required to elucidate how intracellular cytokine production correlates with serum levels, how intracellular cytokine production changes until day of hospital discharge and its relationship to bioenergetic function and leukocyte counts (including CD4 and CD8 levels). Furthermore the relationship between intracellular cytokine production and development of signs of sepsis requires investigation. Cytokine production in relation to site and severity of surgery and pre-existing co-morbidities requires investigation. Further work is needed to elucidate whether bio-energetic changes exist in CD4(+) and CD8(+) cells postoperatively relate to differences in intracellular cytokine production. Finally, apoptosis assays of different lymphocyte

subsets would help to determine whether cells undergoing apoptosis are less likely to produce cytokine.

Postoperative cytokine production may vary from baseline on a daily basis following surgery and it must be appreciated that I only evaluated production on day 3 following surgery. In primates undergoing nephrectomy for example, intracellular production of cytokines by T (CD3) cells treated in a whole blood assay with PMA, ionomycin and concanavalin A, decreased on day 1 postoperatively compared to control animals not undergoing surgery (reduction from baseline of: 50% for IL-2, 29% for IFN-G and 22% for TNF-A).(Stalder, Birsan et al. 2005) Although this decrease was observed on postoperative day 1, in subsequent days the production of IL-2 and IFN-γ increased, reaching levels similar to those observed before surgery. This primate study did not however compare paired samples pre- and postoperatively from the same individual.

6.20 Summary

In summary intracellular cytokine production is lower in unstimulated CD4+ cells postoperatively. A decrease in lymphocyte glycolysis and increase in CD8(+) cytokine production seen postoperatively may be related to the stress response to surgery causing mitochondrial dysfunction leading to mitochondrial ROS production and activation of the inflammasome. The proposed relationship and mechanisms linking metabolic failure and activation of the inflammasome are explored further in the following chapter.

CHAPTER 7

Stress induced hypometabolism in lymphocytes via activation of the inflammasome

Chapter 7

Stress induced hypometabolism in lymphocytes via activation of the inflammasome

7.1 Introduction

In this chapter I aim to explore a potential unifying mechanism, which would explain the postoperative immune phenotype demonstrated in this thesis so far:

Decreased oxidative phosphorylation

Decreased glycolysis

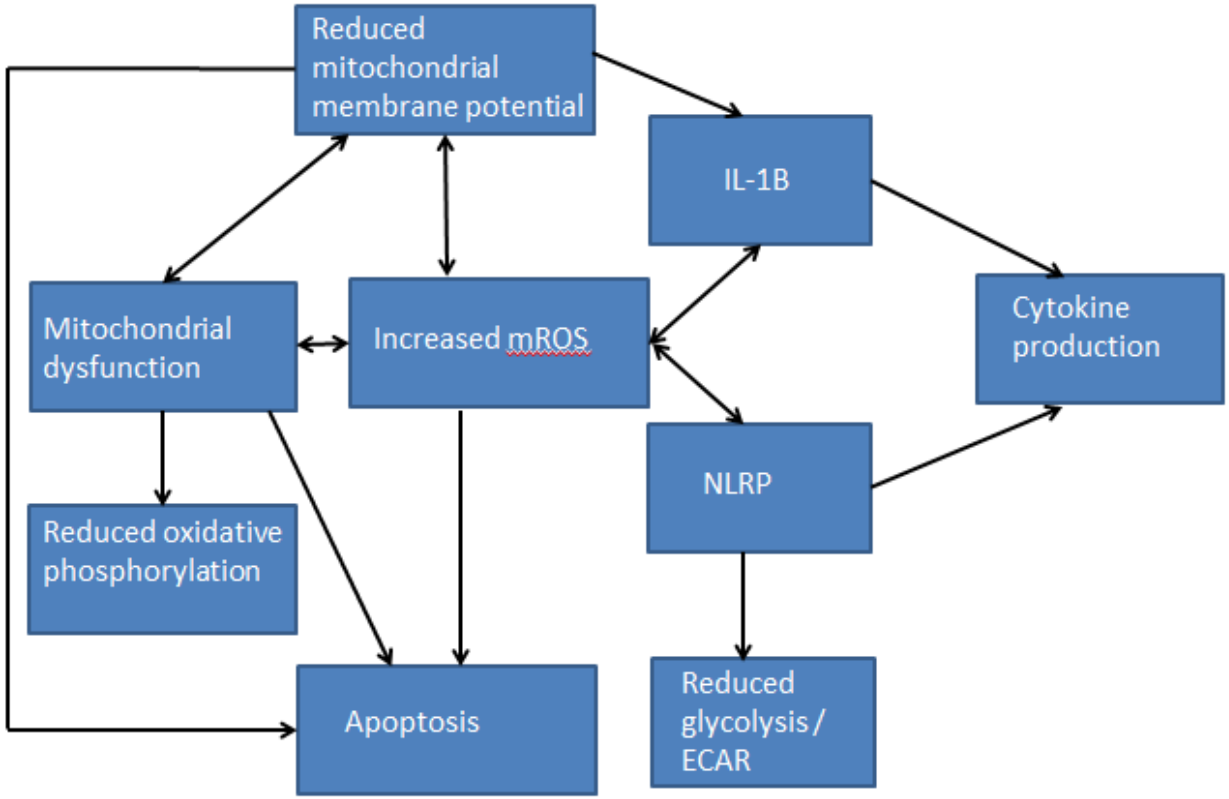
Increased mROS production in lymphocytes

Decreased mitochondrial membrane potential (CD4 cells)

Increased TH1 lymphokine production (unstimulated CD8 cells)

One possible unifying hypothesis which could explain the findings of reduced glycolysis, reduced mitochondrial membrane potential and increased mROS is through the activation of the NLRP inflammasome, outlined in Figure 7.1.

Figure 7.1 Figure demonstrating hypothesis of inflammasome activation secondary to postoperative mitochondrial dysfunction



7.2 Inflammasome

Impairment of oxidative phosphorylation and glycolysis demonstrated in the postoperative period, results in reduced lymphocyte ATP production, which may impair lymphocyte immune response in the postoperative period.

The inflammasome is a collection of multi-protein complexes, which mediate the processing and maturation of the inflammatory cytokines interleukin-1β (IL-1β) and IL-18. Interleukin 1 Beta (IL-1β) is an important mediator of the inflammatory response (associated with increased cytokine release), and is involved in a variety of cellular activities, including cell proliferation, differentiation, apoptosis and turnover of memory CD4(+) T cells.(Shive, Mudd et al. 2014)

The constituents of the inflammasome vary depending on the activator initiating its assembly. It is consequently a critical checkpoint for the induction of other cytokines. (Contassot, Beer et al. 2012) It is responsible for detecting and responding to a large range of PAMPs (pathogen associated molecular patterns e.g. bacterial flagelli) and DAMPs (damage associated molecular patterns e.g. uric acid crystals).

7.3 Caspase inhibits glycolysis

In vivo, stimuli that fully activate caspase-1, inhibit glycolysis in wild-type cells compared with caspase-1-deficient cells (Shao, Yeretssian et al. 2007) at several glycolytic checkpoints (Shao, Yeretssian et al. 2007, Munoz-Planillo, Kuffa et al. 2013). Caspase-1 also regulates lymphocyte homeostasis through pyroptosis (a form of programmed cell death associated with antimicrobial responses during inflammation) as observed in Human Immunodeficiency Virus-associated CD4 depletion.(Gibbison, Angelini et al. 2013, Doitsh, Galloway et al. 2014) Figure 7.2 summarises a potential mechanism relating hypometabolism and inflammasome activation. Figure 7.3 summarises factors involved in the activation and regulation of the inflammasome.

7.4 Caspase requires activation of inflammasome

In 2004 Agostini et al. (Agostini, Martinon et al. 2004) demonstrated that nucleotide-binding oligomerisation domain (NOD) like receptor family, pyrin domain containing 3 (NLRP3), which is mutated in auto-inflammatory hereditary periodic fever syndromes, and homologous to NLRP1, forms an inflammasome complex comprising an adaptor protein (ASC), the caspase-recruitment and activation domain (CARD) containing protein cardinal and caspase-1. Caspase-1-dependent cell death is

initiated by infection, whereas apoptosis is induced by mitochondria. Both release stimulatory products into the cytosol to activate sensors that undergo oligomerisation to form an activation platform (inflammasome and apoptosome).(Labbe and Saleh 2008) Caspase-1 activation is therefore largely dependent on the inflammasome.(Schroder and Tschopp 2010)

Activation of the inflammasome leads to the cleavage and activation of caspase-1 and subsequent cleavage of pro-IL-1β to its mature, active form, which is then released from the cell. Once released, IL-1β binds to the IL-1R, leading to downstream signalling and a cascade of inflammation involving other proinflammatory cytokines. TLR triggering and autocrine IL-1R activation lead to pro-IL-1β transcription.(Man and Kanneganti 2015) Targets that may inhibit IL-1-mediated inflammation include specific inflammasome triggers, activation mechanisms, specific components of the inflammasome, caspase-1, IL-1β release, binding of IL-1β to the IL-1R, IL-1R signalling transduction, and downstream proinflammatory cytokines and superoxide ion ($O2^-$).(Bulua, Simon et al. 2011) (Shao, Yeretssian et al. 2007)

7.5 Proposed ligands, which activate the inflammasome

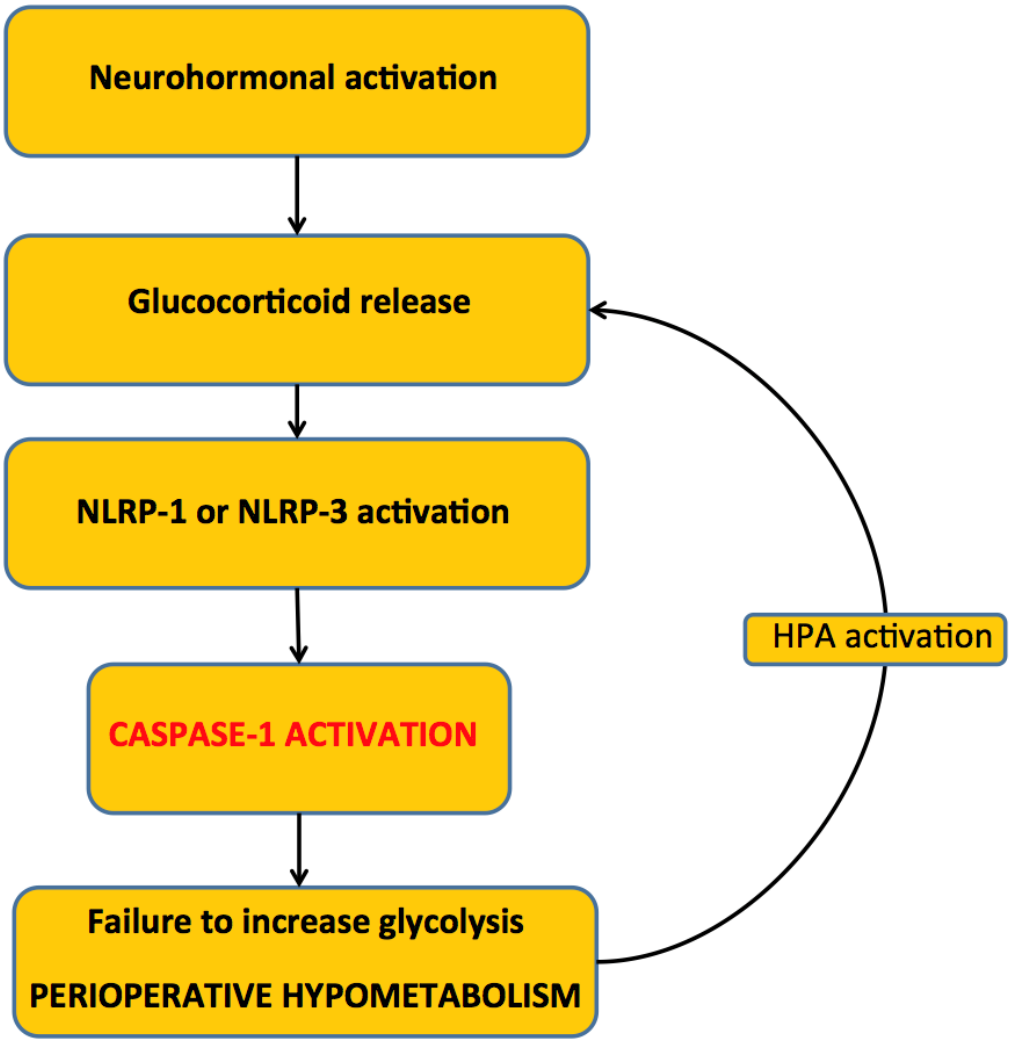
Inflammasome activation involves several hypothesised mechanisms, including potassium (K+) efflux secondary to ATP-gated channels,(Munoz-Planillo, Kuffa et al. 2013) reactive oxygen species (ROS), monosodium urate (MSU), and damage to cell membranes.(Schroder and Tschopp 2010) Both NLRP1 and NLRP3 inflammasomes are present in human T-cells. Zhou et al. demonstrated that complex 1 inhibition (rotenone) and complex III inhibition (antimycin) is associated with increased mROS production.(Zhou, Yazdi et al. 2011) Mitochondrial dysfunction (increased mROS as seen in Chapter 5) is therefore strongly implicated- although not obligatory(Munoz-Planillo, Kuffa et al. 2013)- for the activation of the NLRP inflammasome, through the generation of mitochondrial ROS.(Zhou, Yazdi et al. 2011) Increased mROS production in leukaemic cell lines has been associated with activation of the NLRP3 inflammasome and increased IL-1B expression.(Zhou, Yazdi et al. 2011)

Despite their anti-inflammatory uses, glucocorticoids activate the inflammasome. This has been demonstrated in activation of monocyte NLRP3 mRNA and protein in TH1P monocyte like cells.(Busillo, Azzam et al. 2011) A recent rat brain study demonstrated that low dose glucocorticoids cause a common inhibition of oxidative phosphorylation (State 3) and of complex V activity and modify proton-flux through the mitochondrial inner membrane. Dexamethasone was also able to induce a specific inhibition of complex I activity and to decrease the superoxide anion radical generation. Based on data

from this rat study, inhibition of complex V and partial reversion of uncoupling appear to be common properties of glucocorticoids.(Morin, Zini et al. 2000) The theoretical consequence of such inhibitions could be the modulation of the mitochondrial function, oxygen consumption rate, ATP synthase activity and superoxide anion radical production, involved in many patho-physiological phenomena.

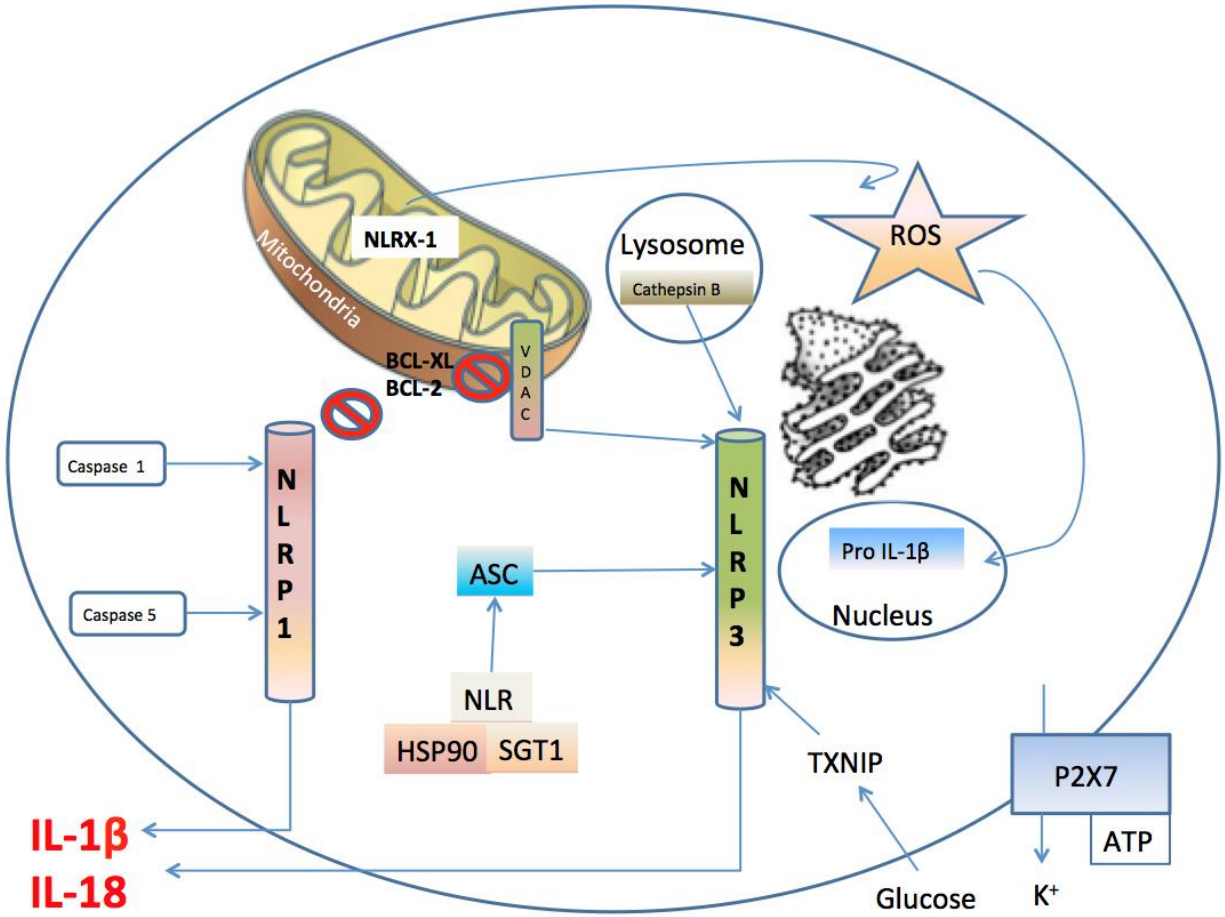
Reduced glycolysis postoperatively results in activation of hypothalamic pituitary adrenocortical pathway stimulating production of glucocorticoid from the adrenal cortex. The prototypical neurohormonal response to surgery and trauma is characterised by substantial and prolonged elevations in circulating glucocorticoids.(Naito, Tamai et al. 1992) Glucocorticoids (dexamethasone) result in caspase-1 activation in murine models.(Chua, Chua et al. 2003)

Figure 7.2 Hypothesis of inflammasome activation following postoperative hypometabolic state



HPA = hypothalamic pituitary adrenocortical pathway

Figure 7.3 Molecular mechanisms involved in activation of inflammasome



= inhibition

Nucleotide-binding oligomerisation domain, leucine rich repeat containing X1 (NLRX1) within mitochondria modulates ROS generation. Mitochondrial ROS causes transcriptional induction of IL-1β and NLRP3 through mitogen-activated protein kinases (MAPKs), NF-KB (nuclear factor kappa-light-chain-enhancer of activated B cells; a protein complex that controls transcription of DNA) and hypoxia-inducible factor 1 (HIF-1), priming the cell for inflammasome activation. NLR is bound by heat shock protein 90 (HSP90; a chaperone protein that assists other proteins to fold properly, stabilises proteins against heat stress, and aids in protein degradation) and suppressor of G-two allele of SKP1 (SGT1; important gene in resisting pathogens) maintaining them in an active state, upon which oligomerisation with ASC (Apoptosis-associated speck-like protein containing a CARD) and caspase-1 form a functional inflammasome leading to release of IL-1B and IL-18. Phagocytosis causes lysosomal rupture followed by release of cathepsin B into the cytosol and NLRP3 inflammasome activation. Microbial toxins and ATP ionotropic Purinergic Receptor P2X, Ligand-Gated Ion Channel, 7 (P2X7; purinergic receptor that functions as an ATP activated cation channel) lead to K+ efflux, which activates the NLRP3 inflammasome. High glucose concentrations activate NLRP3 through Thioredoxin-interacting protein (TXNIP) binding.

7.6 Hypothesis

Glucocorticoids increase primary human lymphocyte mitochondrial ROS production, which causes activation of caspase-1 via the NLRP1 inflammasome.

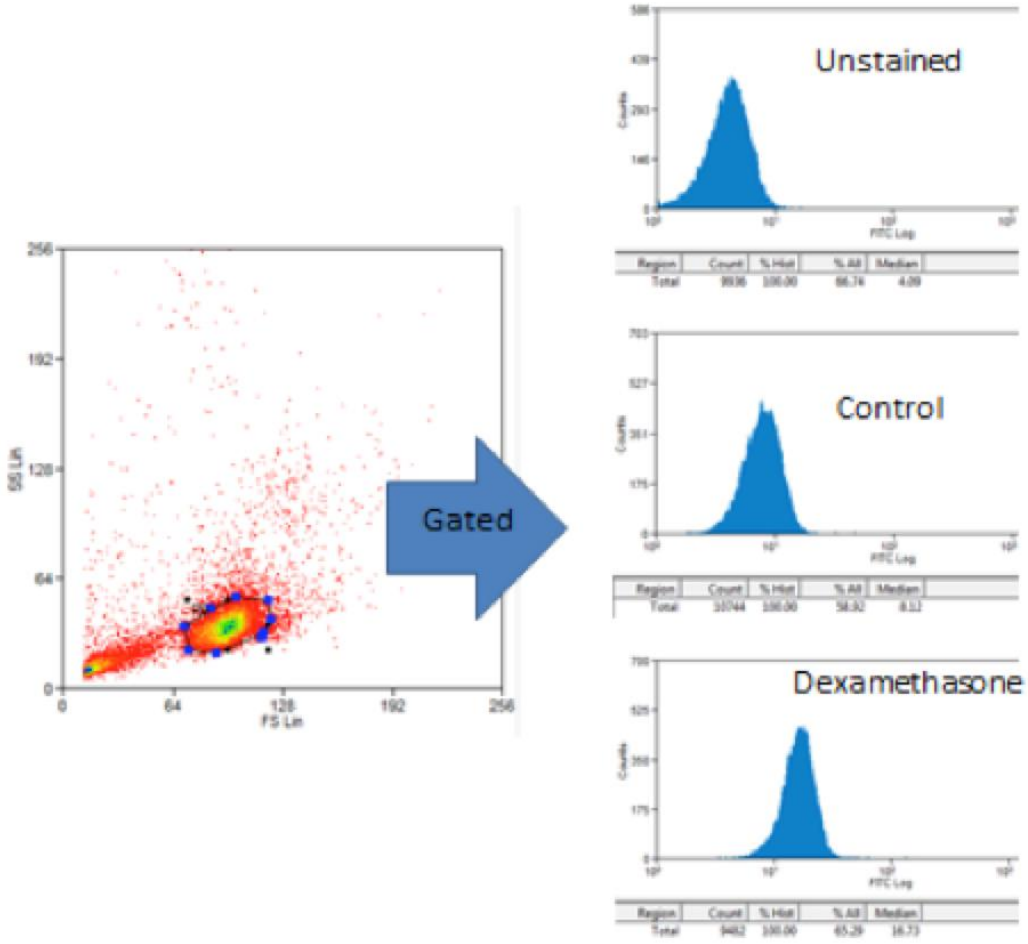
Methods

Five patients with normal preoperative lymphocyte counts with no co-morbidities undergoing elective urological procedures (VISION-UK) were recruited for this study. Isolated lymphocytes were incubated with dexamethasone 1µM for 60 minutes as these levels are close to human physiological levels postoperatively(Roth-Isigkeit and Schmucker 1997) while resulting in <10% lymphocyte apoptosis. mROS and caspase-1 expression were subsequently interrogated; mROS production was quantified using the previously described MitoSox assay in Chapter 2.; caspase-1 activity quantified using a FLICA™ Caspase 1 Assay Kit (ImmunoChemistry Technologies,ABD Serotec, Kidlington, UK).

The *in vitro* caspase-1 assay employs the fluorescent inhibitor probe FAM-YVAD-FMK to label active caspase 1 enzyme in living cells or tissue samples. FLICA™ is cell permeant and non-cytotoxic. FLICA (Fluorescent Labeled Inhibitor of Caspases) probes are comprised of an inhibitor peptide sequence that binds to active caspase enzymes, a fluoromethyl ketone (FMK) moiety that results in an irreversible binding event with the enzyme, and a fluorescent tag (either carboxyfluorescein or sulforhodamine B) reporter. For a caspase 1 inhibitor, the multi-enzyme recognition sequence is tyrosine-valine-alanine-aspartic acid (YVAD). The FLICA™ FAM-YVAD-FMK probe interacts with the enzymatic reactive center of activated caspase 1 via the YVAD recognition sequence, forming a covalent thioether adduct with the enzyme through the FMK moiety.

50, 000 isolated lymphocytes were stained with FLICA reagent as per protocol (ImmunoChemistry Technologies, ABD Serotec, Kidlington, UK). Reagent was constituted with 50µL DMSO to form the stock concentrate. The stock concentrate was diluted with 200µL PBS to form the working solution. 10µL of the working solution was added directly to a 500µL aliquot of your cell culture for labeling. Cells and reagent were incubated for 30 minutes. Unbound FLICA™ FAM-YVAD-FMK reagent was washed twice using PBS. The remaining green fluorescent signal is a direct measure of caspase 1 activity at the time the probe was added. The fluorescent signal was subsequently analyzed using flow cytometry. See Figure 7.4 below.

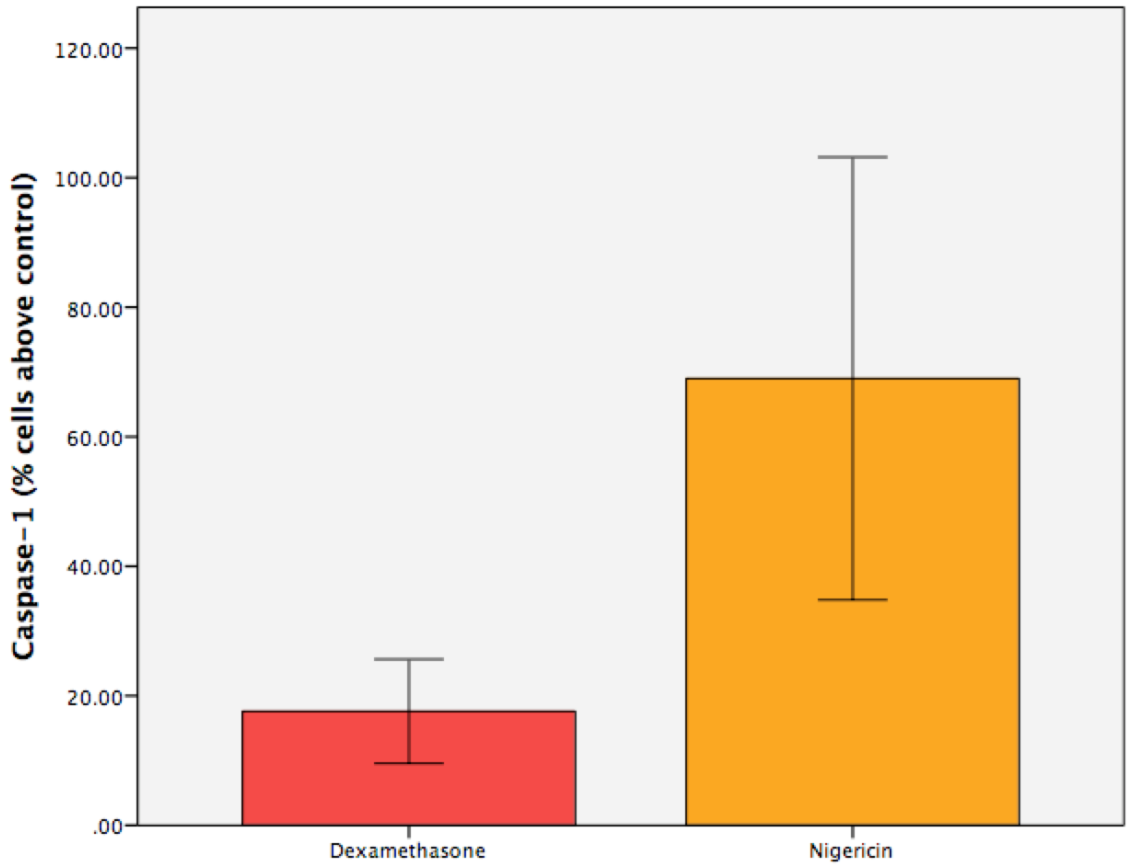
Figure 7.4 Method used to determine Caspase-1 activity in vitro



FITC MFI recorded from above histogram plots; MFI values taken from greater than 10,000 isolated lymphocytes.

Addition of nigericin was performed in order to demonstrate that dexamethasone resulted in caspase-1 activation (a positive control - Figure 7.5). Caspase expression in isolated lymphocytes was also compared between cells incubated in dexamethasone and nigericin, an activator of the NLRP3 inflammasome. (Figure 7.5).

Figure 7.5 Nigericin utilised as a positive control for NLRP3 inflammasome activation



n=5; Caspase expression increased in isolated lymphocytes treated with dexamethasone (1µM) compared to control sample (p=0.04) and treated with Nigericin, a NLRP3 inflammasome activator; p=0.04); Error bars represent 95% CI

Immunoblots were performed using protein from 2.5×10^6 lymphocytes loaded per well of a 10% acrylamide gel from preoperative patients (n=3) with normal lymphocyte count and no co-morbidity. 2.5 million lymphocytes were utilised for these experiments because this number resulted in a reproducible amount of protein, approximately 15 mcg per well. Immunoblots were performed with polyclonal goat anti-human IL-1 beta/IL-1F2 Antibody (1:1000; AF-201-NA; R and D Systems, Abingdon, UK). Cell lysates were probed with anti–β-actin antibodies (Santa Cruz Biotechnology, Inc.), which was utilised as a loading control.

7.7 Statistical Analyses

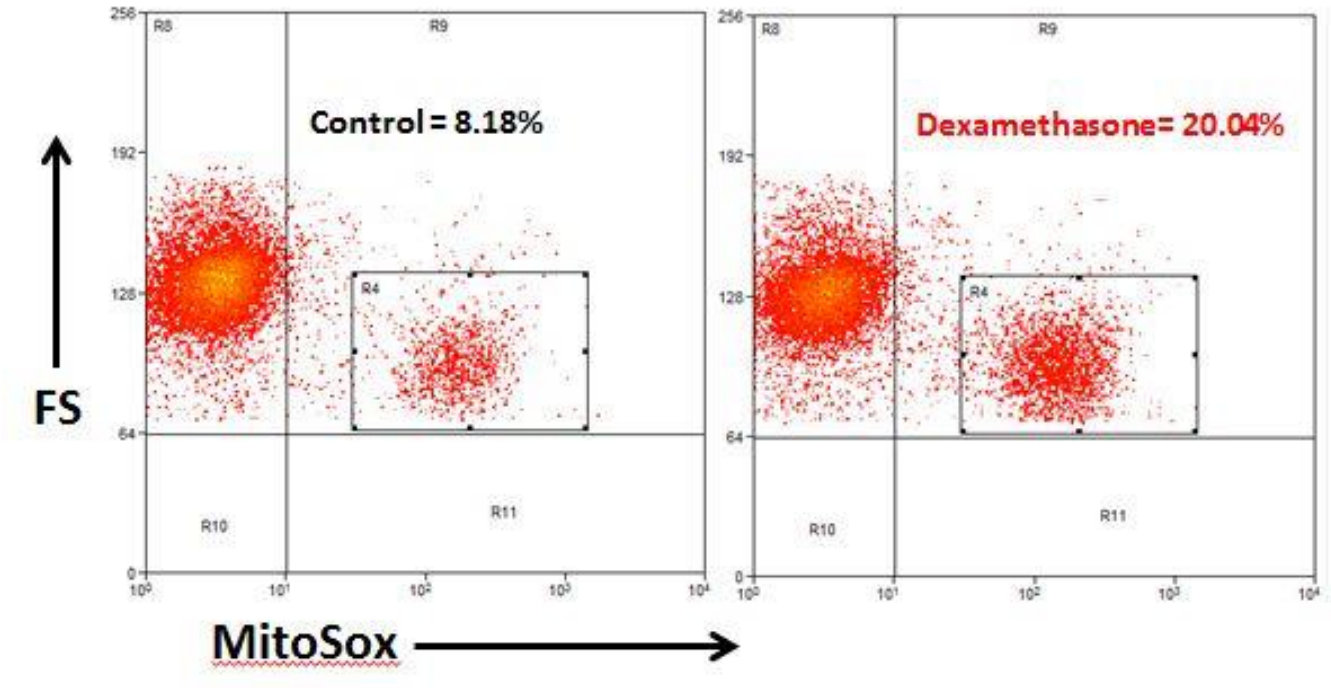
All results are expressed as absolute numbers and percentages (means 95%CI). Parametric methods and tests were used as indicated to analyze normally distributed data, while two-group nonparametric Wilcoxon signed-rank and Mann–Whitney U tests were used to analyze data deviating from a normal distribution. Statistical analyses were undertaken using SPSS IBM SPSS Version 20 (IBM Corporation, Somers, New York, USA). Reported p values are two-sided; a p value ≤0·05 was considered to indicate statistical significance.

Results

Incubation with dexamethasone generated an increase in mitochondrial reactive oxygen species (Figure 7.6 A, B) and was associated with increased caspase-1 activity as assessed by FLICA assay (Figure 7.6 C).

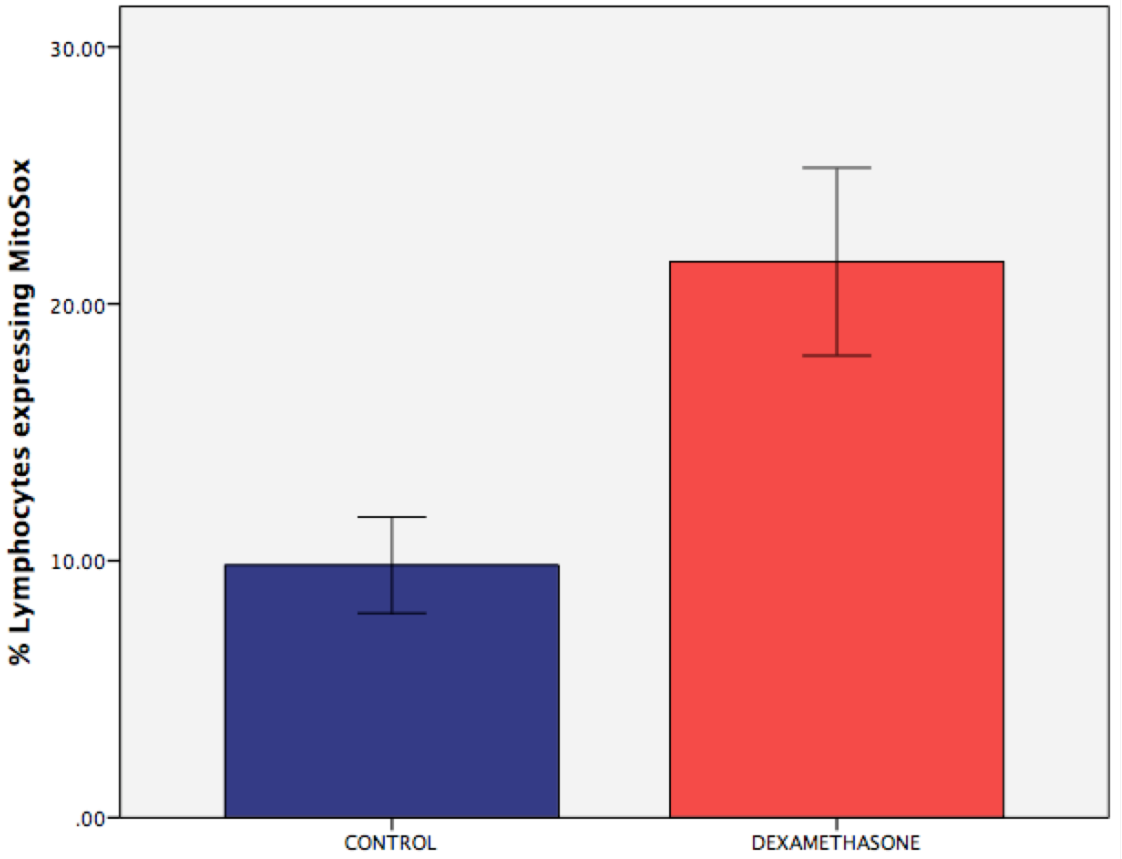
Figure 7.6 Mitochondrial ROS production in isolated lymphocytes (a, b) and caspase-1 activity (c) following incubation with dexamethasone

a)



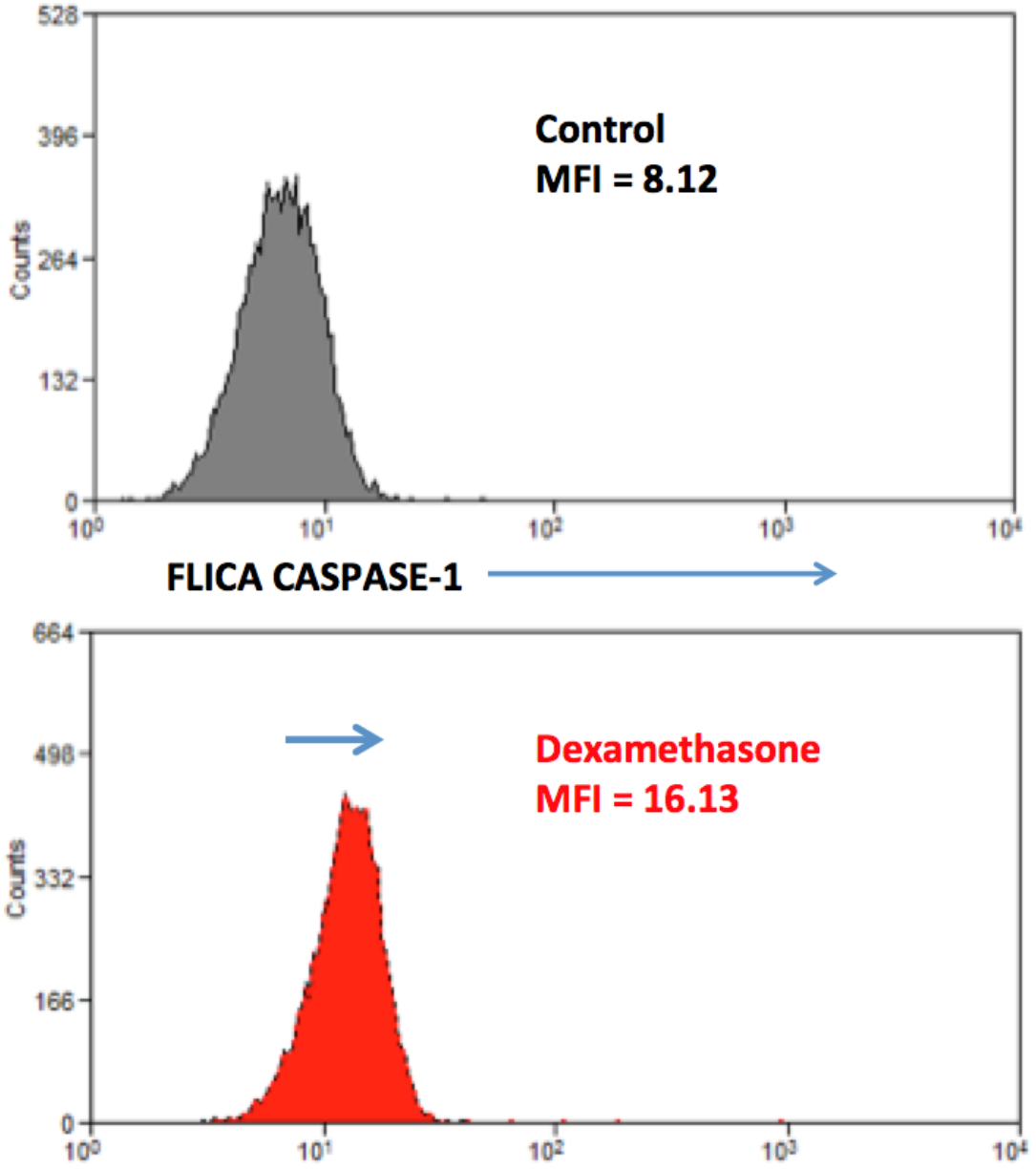
>10,000 isolated lymphocytes interrogated per sample

b)



n=5; p=0.007; incubation with 1µM dexamethasone; error bars indicate 95% CI

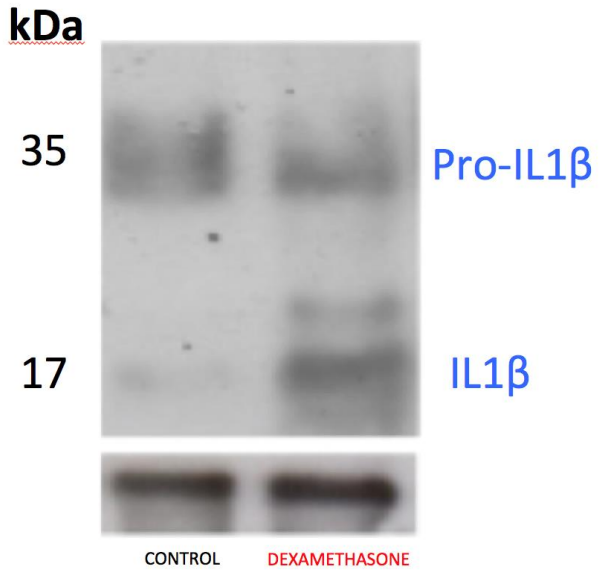
c)



Dexamethasone results in increased caspase activity compared to control as demonstrated by increased FITC MFI (of FLICA caspase-1).

Immunoblot analysis following dexamethasone demonstrated an increase in IL-1β production. (Figure 7.7)

Figure 7.7 Immunoblot analysis demonstrating IL-1β production with dexamethasone



I performed the above Western blot with Dr. John Whittle, β-actin utilised as loading control.

Discussion

The mechanism underlying a global decrease in metabolic status postoperatively is consistent with increased activity of caspase-1, a pivotal executioner caspase that mediates the inflammatory response to a plethora of pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs). Activation of a ubiquitous sensor of danger-molecules in T cells is consistent with the lack of clear association between preoperative lymphocyte count and clinical presentation or differences in parameters (Chapter 3). Therefore pre-existing lymphopenia does not necessarily result in identifiable morbidity preoperatively, however following surgical insult, the differences in Immune phenotype and outcomes secondary to inflammasome activation become unmasked. This data supports the hypothesis that impaired glycolysis occurs postoperatively secondary to a glucocorticoid driven activation of the inflammasome via increased caspase-1 activity. The impairment in glycolysis and activation of the inflammasome (demonstrated by increased IL-1β and increased caspase-1 activity) also appear to be consistent with the postoperative increase in lymphokine production demonstrated in Chapter 6.

Activation of the NLRP1 inflammasome appears to be the most likely pathway resulting in the postoperative phenotype demonstrated in this thesis. Splenocytes from NLRP3 deficient mice and wild type controls both undergo increased levels of apoptosis and caspase activity compared following dexamethasone exposure. Splenocytes from NLRP3 deficient mice also express more NLRP1 protein following incubation with 1μM dexamethasone for 16 hours, indicating activation of caspase-1 via the NLRP1 inflammasome.(Edwards, Sultan et al. 2015) Acute (postoperative) or chronic (preoperative lymphopenia) is associated with impaired lymphocyte functionality resulting from glucocorticoid activity and is associated with increased postoperative morbidity and mortality in these populations.

Nigericin is an inflammasome activator and an inhibitor of lymphocyte proliferation.(Daniele, Holian et al. 1978) Inflammatory gene upregulation has been demonstrated in human lung cells by glucocorticoids and TNFα.(Lannan, Galliher-Beckley et al. 2012) Glucocorticoid related decrease in glycolysis and mitochondrial membrane potential of lymphocytes from patients with Chronic lymphocytic leukaemia (CLL), appears to recreate the postoperative phenotype shown in this thesis.(Tung, Shi et al. 2013) Glucocorticoid activity is likely to be just one factor responsible for these

postoperative changes seen. For example programmed cell death 1 and its inhibitory receptor, have been associated with T cell exhaustion,(Boomer, To et al. 2011) neutrophil related T cell suppression has been described in human sepsis.(Pillay, Kamp et al. 2012)

Taken together, the data from this thesis provides translational data supporting the role of impaired bioenergetic function in development of complex and multifactorial pathology. Clinical and immune function has also been directly associated with metabolic function. The fact that a substantial proportion of patients are lymphopenic prior to surgery, suggests that a population exists with an increased risk of developing critical illness.

7.8 Mitochondria and inflammasome

Reed and colleagues(Bruey, Bruey-Sedano et al. 2007) have also demonstrated that B-cell lymphoma-2 (BCL-2) and BCL-XL genes, which inhibit apoptosis by controlling mitochondrial outer membrane permeabilisation (MOMP), block the NLRP1 inflammasome. Tschopp et al. extended the links between cell death and inflammation by postulating that mitochondria not only produce ATP, but are also vital for inflammation.(Tschopp 2011) Specifically, it was demonstrated that NLRP3 localises to the endoplasmic reticulum and translocates to the mitochondria upon stimulation, and that assembly of the NLRP3 inflammasome occurs in a MOMP-dependent manner.(Zhou, Yazdi et al. 2011) Consistently, inhibition of voltage-dependent anionic channels or overexpression of BCL-2, which blocks MOMP, dampens this response.

Whether ROS is upstream or downstream of NLRP induction remains unclear. Bauernfeind et al. demonstrated that although NLRP3 inflammasome activation is unique among other known inflammasomes in its sensitivity to ROS inhibition, this phenomenon is attributable to the fact that NLRP3 strictly requires priming by a proinflammatory signal, a step that is blocked by ROS inhibitors.(Bauernfeind, Bartok et al. 2011) Although these data do not exclude a general role for ROS production in the process of NLRP3-triggered inflammation, they suggest that ROS is upstream of NLRP3 induction, but not activation.

The stress response to surgery results in the release of corticotrophin from the pituitary stimulating cortisol secretion from the adrenal cortex.(Desborough 2000) Caspase-1 related inhibition of glycolysis (Shao, Yeretssian et al. 2007) may result in postoperative lymphocyte bioenergetic dysfunction. What

remains unclear is whether the metabolic changes demonstrated postoperatively (reduction in glycolysis and oxidative phosphorylation) are solely due to CD4 cell dysfunction.

Data from my thesis suggests that the stress response to surgery rather than inducing individual complex inhibition in humans, may result in a more global impairment in ETC function. Therefore it is not clear whether glucocorticoids alone recreate the same postoperative bioenergetic phenotype in humans. I surmise that a ubiquitous stress-glucocorticoid response mechanism may promote postoperative lymphopenia by inducing bioenergetic impairment,(Stahn and Buttgereit 2008) increasing cytokine production and promoting apoptosis. This hypothesis would fit with the neurohormonal response to surgery and trauma, which is characterised by substantial and prolonged elevations in circulating glucocorticoids.(Naito, Tamai et al. 1992) This hypothesis would also explain the bioenergetic phenotype seen in postoperative lymphocytes, and also account for a failure to increase glycolysis in the face of lower oxidative phosphorylation.

In summary, these findings suggest that perioperative glucocorticoid driven activation of the inflammasome may represent an under-recognised, pathologic mechanism that may result in a hypometabolic postoperative phenotype, possibly driven by caspase-1 mediated inhibition of glycolysis. This new paradigm suggests that manipulation of this inflammasome pathway through caspase-1 and inflammasome inhibitors such as VX-765 or parthenolide for example, may offer a novel strategy to reduce postoperative length of hospital stay and morbidity following the acute inflammatory challenges of surgery.

Chapter 8
Discussion

Discussion

8.1 Thesis summary

This is the first study to demonstrate a link between low AT and lymphopenia. Lymphopenia and low AT are independently associated with prolonged hospital stay following elective colorectal surgery. Immune dysfunction may therefore be a mediator (and low AT may be a marker) of poor perioperative outcomes. Postoperative alterations in lymphocyte count and NLR do not recover to baseline levels by day of hospital discharge following colorectal surgery.

Significant bioenergetic and functional changes occur in lymphocytes postoperatively, which may explain why morbidity and mortality are increased following surgery. Ex-vivo analysis of lymphocytes demonstrates a decrease in glycolysis and oxidative phosphorylation consistent with global impairment of the ETC within lymphocytic mitochondria. Altered metabolic function postoperatively is also accompanied by a reduction in postoperative lymphocyte mitochondrial membrane potential, increased mitochondrial ROS production and increased lymphokine production. Since impaired bioenergetic and immune function occur postoperatively, these changes may have a greater clinical impact on patients who are lymphopenic preoperatively, which may explain the prolonged hospitalization demonstrated following surgical insult in this cohort.

I hypothesised that the postoperative bioenergetic and immune functional changes demonstrated in lymphocytes may occur secondary to increases in glucocorticoids, associated with the stress response to surgery. These may subsequently result in activation of the inflammasome pathway. This hypothesis is supported by demonstration of increased caspase-1 activity (known to be associated with inhibition of glycolysis) and IL-1β expression (associated with activation of the inflammasome and increased cytokine production) following incubation of isolated lymphocytes with dexamethasone.

This thesis provides translational data regarding postoperative changes in immune function, which potentially predispose patients to increased morbidity and mortality following surgery. I introduce the concept that metabolic abnormalities underlie the postoperative immune phenotype. These findings introduce new potential therapeutic targets, which may help to reduce hospital length of stay.

8.2 Summary of key findings

Reduced aerobic capacity is associated with lymphopenia and preoperative lymphopenia is associated with prolonged hospital stay following major elective colorectal surgery

Patients with more advanced heart failure, as reflected by low AT, demonstrate an increased number of neutrophils. This relative neutrophilia is accompanied by a decreased number of lymphocytes, characterised by higher proportions of terminally differentiated CD4(+) and CD8(+) T-subsets.(Moro-Garcia, Echeverria et al. 2014) These data are consistent with molecular mechanisms driving a T-cell immunosenescent state.(Macaulay, Akbar et al. 2013)

Importantly, treatment of cardiac failure with several commonly available evidence-based treatments (e.g. angiotensin-converting enzyme inhibitors(Gage, Fonarow et al. 2004), cardioselective beta blockade (Barnes and Ackland 2010) not only improves cardiovascular performance and reduces mortality, but also reverses the associated immune dysfunction. Experimental and clinical data suggest that established immune dysfunction is instrumental in promoting further cardiac failure, rather than merely being a marker of disease progression.(Topkara, Evans et al. 2011) Data from the perioperative literature supports this concept: patients with evidence for preoperative depletion of antibodies to endotoxin exhibit higher levels of pro-inflammatory cytokines and sustain more perioperative morbidity.(Bennett-Guerrero, Ayuso et al. 1997, Bennett-Guerrero, Panah et al. 2001, Moretti, Newman et al. 2006)

Data from this thesis shows that a low preoperative AT measurement as assessed by CPET (<11 mL/kg/min), is associated with lymphopenia and increased NLR. These factors have been independently demonstrated to be robust prognostic indicators following colorectal surgery. This data therefore supports the hypothesis that subclinical heart failure (as defined by poor aerobic capacity or AT <11 mL/kg/min) is associated with leucocyte markers of chronic systemic inflammation (lower lymphocyte count and higher NLR). This alteration in immune phenotype may be a significant factor explaining why patients with lymphopenia (who also have a lower AT) have prolonged hospital stay and increased medical and surgical complication rates following elective surgery. Therefore this thesis introduces a novel concept that immune dysregulation rather than being a marker of disease progression is considered to be a pivotal feature driving poor aerobic capacity,(Topkara, Evans et al. 2011) and cardiac failure. Lymphopenia is therefore a biological marker, which highlights a group of patients at increased risk of adverse outcomes. This can be easily identified before major surgery in

most patients and points to underlying pathophysiological mechanisms, which may be amenable to therapeutic manipulation to improve perioperative outcome.

Postoperative changes in relative lymphocyte count and NLR do not recover by day of hospital discharge

I also present the largest prospective study evaluating trends in peripheral lymphocyte count and NLR conducted in a perioperative setting. The development of postoperative lymphopenia demonstrated in this colorectal cohort of patients is consistent with findings from previous studies. The recovery profile demonstrates that NLR and relative lymphocyte counts do not return to baseline levels by day of hospital discharge. This suggests that patients have a degree of ongoing inflammation (high NLR) and potentially impaired immune function following discharge from hospital, which may confer increased susceptibility to morbidity until levels return to baseline values. I have also shown that preoperative lymphopenia is an independent risk factor for prolonged length of hospital stay and development of complications following surgery in 2 patient cohorts.

Bioenergetic function of lymphocytes is impaired following elective orthopaedic surgery

In patients with sepsis, cellular bioenergetic dysfunction has also been demonstrated in muscle(Brealey, Brand et al. 2002) and is proposed as a key mechanism of organ dysfunction in this setting. Cardiomyocytes and lymphocytes have previously been reported to have similar metabolic profiles in murine studies.(Cortez, Neves et al. 2012) Given that subclinical heart failure (low AT) has already been associated with lymphopenia and previous studies have described impaired bioenergetic function in cardiomyocytes from patients with heart failure, I next explored lymphocyte bioenergetics as a possible mechanism for impaired postoperative immunity. This could potentially explain why lymphopenic patients have an increased hospital length of stay.

Bioenergetic analysis of lymphocyte function demonstrates that decreased oxidative phosphorylation and glycolysis occur in the postoperative phase at day 3 following elective orthopaedic surgery. Change in lymphocyte bioenergetic function may be a cause of or related to the increased propensity to apoptosis demonstrated in lymphopenic patients (as seen in the postoperative state).(Edwards, Sultan et al. 2015) The bioenergetic dysfunction observed in the

lymphocytes of these postoperative patients may represent a more global abnormality, resulting in reduced energy production and impaired organ function.

Postoperative lymphocyte changes are consistent with mitochondrial dysfunction and activation of the inflammasome

ETC dysfunction as demonstrated by reduced oxidative phosphorylation is also accompanied by other evidence of altered mitochondrial function postoperatively including an increase in mitochondrial ROS production and decrease in mitochondrial membrane potential seen postoperatively in a variety of types of surgery. There is an increase in IL-1β, which is consistent with the hypothesis that there is activation of the NLRP inflammasome. There is also increased caspase-1 activity, which has previously been linked to inhibition of glycolysis. The trigger for this may be due to the increase in cortisol, which is seen postoperatively during the well-described stress response to surgery. The increase in circulating cortisol seen perioperatively may therefore result caspase related decrease in glycolysis and ETC dysfunction, which leads to the activation of the inflammasome and subsequent increase in lymphokine production. The precise mechanism however by which ETC function alters within lymphocytic mitochondrion postoperatively remains unclear.

In active rheumatoid arthritis and SLE, increased lymphocyte mitochondrial oxidative phosphorylation is seen which can be ameliorated by high dose glucocorticoids,(Kuhnke, Burmester et al. 2003) presumably via their non-genomic effects reducing mitochondrial transmembrane potential and decreasing ATP availability.(Buttgereit, Burmester et al. 2000) These studies are consistent with findings from my thesis suggesting that postoperative increases in glucocorticoid levels result in reduced mitochondrial membrane potential and bioenergetic function of lymphocytes.

Given the presence of postoperative defects seen in lymphocytes, it is plausible that this group of immune cells plays an integral role in the development of postoperative morbidity resulting in prolonged hospital stay for patients with pre-existing lymphopenia. The ex-vivo postoperative functional impairment seen in lymphocytes may reflect in-vivo immune deficits of the adaptive immune response and the early control of inflammation and innate immunity, which may be more pronounced in patients with preoperative lymphopenia.

8.3 New aspects of work undertaken in this thesis

This study establishes:

- A relationship between low AT and lymphopenia.
- Perioperative changes in leucocyte counts in patients undergoing colorectal surgery.
- A relationship between preoperative lymphopenia and prolonged length of stay
- The successful use of extracellular flux analysis to demonstrate reduced oxidative phosphorylation and glycolysis postoperatively in mitochondrial of lymphocytes. This study identifies extracellular flux analysis as a valid technique in assessing human disease states.
- Perioperative changes in human lymphokine production utilising a whole blood assay.
- Perioperative decreases in human lymphocyte mitochondrial membrane potential and increased mROS production in lymphocytes.
- The changes demonstrated in membrane potential nd mROS production are reproducible through use of mitochondrial inhibitors.
- That caspase-1 related inhibition of glycolysis may result in postoperative lymphocyte bioenergetic dysfunction.
- Activation of the inflammasome (evidenced by increased IL-1β) in response to dexamethasone.

8.4 Strengths and Limitations

Although there was an upward trend in lymphocyte count postoperatively, the time taken to return to baseline levels could not be fully elucidated from this study as patients were only followed up during their hospital stay. Furthermore the effect of preoperative phenotype (including cancer and preoperative lymphocyte count) and type of surgery on the recovery profile of lymphocyte and NLR warrants further investigation.

The conclusions drawn from each chapter of this thesis should ideally be demonstrated on individual patients during their perioperative course. By demonstrating a postoperative decrease in patient lymphocyte count, associated with decrease in postoperative bioenergetic function, increased lymphokine production, caspase-1 and IL-1β expression and cortisol levels, this would further support the overall hypothesis outlined in this thesis. However, practically this would be extremely difficult to undertake since the yield of lymphocytes required to perform many of the experiments involving isolated lymphocytes (extracellular flux analysis, mitochondrial membrane potential, mROS production, western blot analysis) would be significantly more than could otherwise be taken from a venepuncture of 15-20 mLs of patient whole blood. This would be particularly difficult to achieve in lymphopenic patients, especially postoperatively when the lymphocyte count would be expected to drop further. Similarly, though it would be interesting to repeat the experiments in this thesis on individual subsets of lymphocytes, the yield would make it difficult to perform this with the same lymphocyte numbers and therefore the power of the study would be compromised.

One of the main methodological strengths of the experiments performed in this study was that individuals' own samples were compared pre- and postoperatively, which reduced the number of confounding factors and allowed for elucidation of effects of perioperative management upon lymphocyte changes. While the most likely cause for the changes seen is surgery and tissue injury, the data from this study cannot exclude effects of pharmacological therapies administered during the perioperative period, particularly anaesthetic and analgesic agents. Day 3 samples were chosen in the bioenergetic study in order to minimise the effects of anaesthetic agents on the results. While obtaining day 3 samples was practical following arthroplasty, (where the mean length of stay 5 is days) comparing day 3 lymphocytes to preoperative samples would have been more challenging for other surgical specialties due to the shorter length of hospital stay. Therefore for several other experiments (mitochondrial ROS and mitochondrial membrane

potential) day 2 postoperative samples were taken. Further work is required to determine the timeline of immune phenotype changes which occur from administration of anaesthetic agent, to start of surgical trauma, to each day following surgery to identify when peak changes occur and then identify recovery profiles in relation to preoperative patient phenotype and type of surgery / size of surgical trauma. Furthermore comparisons of immune phenotype following general and regional anaesthesia are warranted in order to elucidate whether anaesthetic agents are contributing factors to bioenergetic and functional lymphocyte changes seen postoperatively.

It is not clear what difference in lymphocyte bioenergetic function, cytokine or mROS production correlates with significant clinical differences in the perioperative period. Further work is therefore required to associate these changes with clinical morbidity and mortality.

Although a postoperative increases in ROS and decreases in mitochondrial membrane potential have been demonstrated, these are in separate samples to those in which cytokine production and altered oxidative phosphorylation and IL-1β secondary to stimulation with cortisol have been demonstrated and from different surgical specialties. It would therefore be prudent to demonstrate whether differences exist in lymphocyte bioenergetics and functionality between surgical specialties and what degree of tissue trauma would be required to institute these changes. For example exploration of whether changes demonstrated following minor dental surgery are comparable to major general surgery is warranted. Additionally comparison of samples from patients with postoperative normal and lymphopenic counts would help determine whether these differences are more pronounced in the postoperative lymphopenic patients. These analyses were not performed due to the fact that most patients were found to be lymphopenic postoperatively.

While results from this thesis introduce the concept of low AT being a marker rather than a mediator of perioperative poor outcome, further work is needed to explore this hypothesis. Postoperative samples should be interrogated for other immune signals, which have been associated with cardiac failure including endotoxin,(Niebauer, Volk et al. 1999) catecholamines(Tank and Lee Wong 2015) and BNP.(Miller, Saenger et al. 2016)

8.5 Supporting evidence for hypotheses outlined in this thesis

Preoperative lymphopenia is associated with increased postoperative morbidity and length of hospital stay

Established preoperative lymphopenia (prevalence: 15-18%) is associated with excess postoperative morbidity (as assessed by postoperative morbidity scoring), including infections, and prolonged hospital stay as demonstrated in two cohorts of orthopaedic patients (n=417 and 328).(Edwards, Sultan et al. 2015) This data supports findings from colorectal patients in this thesis, which demonstrates prolonged hospital stay in patients with preoperative lymphopenia.

Preoperative lymphopenia has a similar immune phenotype to postoperative lymphocytes

Lymphocytes taken from lymphopenic patients demonstrate reduced proliferation, higher levels of apoptosis, reactive oxygen species production and cytokine production (all $p<0.05$), and lower levels of intracellular ATP, glycolytic capacity and mitochondrial oxygen consumption (all $p<0.01$) when compared to lymphocytes taken from patients with normal preoperative counts.(Edwards, Sultan et al. 2015) This is consistent with the decreased functionality demonstrated in postoperative lymphocytes, which were compared to individuals' own preoperative samples. Lymphocytes from lymphopenic patients also therefore show decreased functionality associated with impaired bioenergetic capacity. This at-risk patient population can thus be identified preoperatively and may potentially benefit from immune-enhancing strategies.

Systemic markers of inflammation and heart failure

In patients with chronic systolic heart failure, the non-classical monocyte CD14(dim)CD16(+) subset is more prevalent and inversely associated with worsening cardiac performance.(Amir, Spivak et al. 2012) By contrast, reduced levels of the classical CD14(++)CD16(−) monocyte subset are reported in heart failure, compatible with remodelling roles for different subsets.(Apostolakis, Lip et al. 2010) CD14(+) monocyte subset analysis showed that the CD14(++)CD16(−) subset was reduced in patients with low cardiopulmonary reserve (colorectal patients; median AT = 10 (9-11) mL/kg/min; n=38) compared to age matched controls with higher mean AT undergoing arthroplasty (orthopaedic cohort, median AT >11 mL/kg/min; n=31).(Sultan, Edwards et al. 2014) These findings are in keeping with the hypothesis that low AT could be a marker rather than a mediator of poorer perioperative outcomes due to impaired immune function associated with subclinical heart failure.

Lymphocyte apoptosis postoperatively

Lymphocytes taken from patients with preoperative lymphopenia demonstrate a similar bioenergetic profile to postoperative lymphocyte samples. The hypometabolic phenotype of lymphopenic patients, characterised by mitochondrial dysfunction leading to increased apoptosis through the intrinsic pathway, would explain the susceptibility of these patients to postoperative infection and therefore prolonged hospital stay. Freshly isolated preoperative lymphocytes were incubated with common perioperative stressors at physiologically relevant doses. Lymphocytes obtained from postoperative patients expressed higher levels of the early apoptosis marker annexin V than matched preoperative samples.(Edwards, Sultan et al. 2015)

Murine NLRP3 KO study

Genetic ablation of the murine NLRP3 inflammasome fails to prevent glucocorticoid-induced splenocyte apoptosis and caspase-1 activity, but was associated with increased NLRP1 protein expression. (Edwards, Sultan et al. 2015) Given the fact that dexamethasone results in increased IL-1β production, these findings suggest that activation of the NLRP1 (not the NLRP3) inflammasome is responsible for the hypometabolic phenotype seen. Therefore cortisol may be a novel activator of the NLRP1 inflammasome perioperatively. Targeting the hypometabolic phenotype of postoperative lymphocytes offers a novel strategy to reduce postoperative infections and sepsis. Inflammasome inhibition utilising caspase-1 and inflammasome inhibitors such as VX-765 or parthenolide for example may affect postoperative lymphopenia and morbidity.

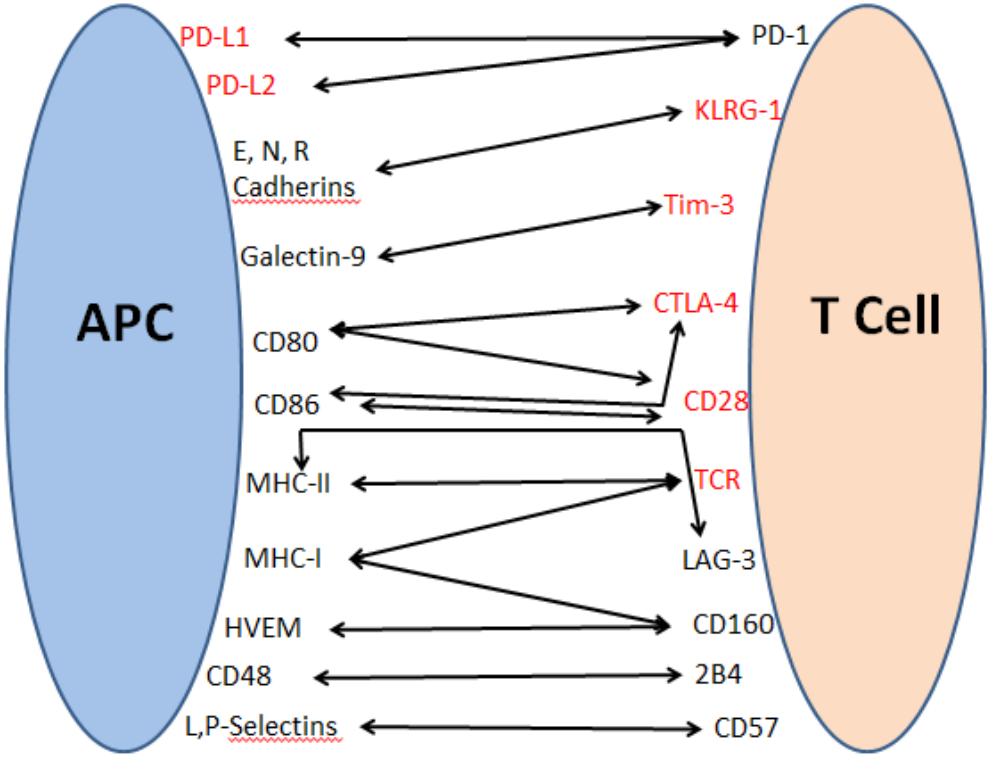
8.6 Hypometabolic functional states of lymphocytes

Effector T cells play a vital role in adaptive immunity. While advances have been made in characterization of effector T cells in infectious disease models (particularly mouse models), the nature of these cells is not well understood in postoperative patients. The phenotype and functional profile of postoperative T cells is likely to be dramatically impacted by the different microenvironment.

Successful activation of resting T-cells requires two signals.(Baxter and Hodgkin 2002) Firstly, this is produced by binding of MHC-II associated antigen on APCs to the TCR - CD3 complex. The second, co-stimulatory signal is provided by binding of B7-1 (CD80) or B7-2 (CD86) on the APC to the T-cell CD28 receptor, or by IL-2 ligation. The TCR signal is amplified through a protein tyrosine kinase cascade, involving a rise in intracellular calcium and activation of protein kinase C. The subsequent activation of transcription factors leads to movement of the cell from G0 to G1 in the cell cycle, IL-2 and other cytokine synthesis and RNA and protein production. These processes require a rapid increase in cellular metabolism.

Interaction between co-stimulatory molecules CD80, CD86 and CD28 is crucial for appropriate T cell activation and immunoregulatory receptors such as CTLA-4 and PD-1 fine tune T cell activation (Figure 1).(Crespo, Sun et al. 2013) Further research is needed to determine whether higher levels of immunoregulatory receptors (e.g. LAG-3, CD160, KLRG-1, Tim-3, CTLA-4 and CD57) are present in the postoperative microenvironment of T cells. Some of the T cell immunoregulatory receptors and their ligands are summarised in Figure 8.1.

Figure 8.1 T cell immunoregulatory receptors and their ligands



Receptors in red; ligands in black; PDL1-2 = Programmed death ligand; PD-1 = Programmed cell death protein 1; MHC = major histocompatibility complex; HVEM = herpesvirus entry mediator; KLRG-1 = co-inhibitory receptor killer-cell lectin like receptor G1; Tim-3 = T cell immunoglobulin mucin-3; CTLA-4 = cytotoxic T-lymphocyte-associated protein 4 (CD152); TCR = T cell receptor; LAG-3 = Lymphocyte activation gene-3; 2B4 = Natural Killer Cell Receptor 2B4 (CD244)

Several distinct states have been described in which reduced lymphocyte functionality may be seen (Table 8.1). Although there are similarities between these states they are thought to represent distinct lymphocyte phenotypes. The postoperative lymphocytes appear to demonstrate in part, an exhaustive phenotype as apoptosis and metabolism are decreased. The alteration in cytokine production in this states decreases, however the interaction between lymphocytes and the inflammasome is not taken into account in this simplified classification system. It is likely that anergy, exhaustion and senescence are all present in elderly surgical patients studied in this thesis.

Table 8.1 Terms describing T cell unresponsive, hyporesponsive or dysfunctional T cells

Ignorance		T cell Unaware or ignorant of (self-) antigen due to physical sequestration or low level expression of antigen
Tolerance	**Central**	Deletion of self-reactive thymocytes expressing TCR with too high affinity for self-antigen/MHC complexes
	Peripheral (self)	To prevent auto-immunity, self-reactive T cells that escape negative selection are inactivated in the periphery by: deletion; suppression by T reg cells; induction of cell –intrinsic program mediating cell unresponsiveness
Anergy	**In vitro**	Dysfunction of T cells from in vitro stimulation in absence of co-stimulatory signals. Decreased cytokine production, metabolism and apoptosis unchanged
	In vivo (adaptive tolerance)	Dysfunction of T cells induced by sub-optimal in vivo stimulation
Exhaustion		Persistent antigen and inflammation during chronic infection induces progressive loss of effector function in virus specific T cells. A state of functional hyporesponsiveness. Decreased cytokine production and increased apoptosis
Senescence		Irreversible, permanent cell cycle arrest commonly reflected by telomere shortening. Decreased cytokine production and unchanged metabolism
Quiescence		Reversibly arrested cell cycle state (G0 phase)

As Figure 8.1 suggests there is an extremely complex network of immunoregulatory receptors and their ligands expressed on APCs which interact with infiltrating T cells.(Crespo, Sun et al. 2013) Some of the key features of the hyporesponsive states are and their interactions are outlined in Table 8.2.

Table 8.2 Features of key hyporesponsive states

	Initiation	Phenotype	Regulation
Anergy	Reduced CD28 co-stimulation Increased co-inhibition Defective mTOR and Ras/MAPK signalling Ca2+ imbalance results in retention of active RAP-1 in cytosol	Reduced IL-2 Cell cycle arrest at G1/S phase	Transcription factor (Erg2) Epigenetic factors (IKAROS and Sirt 1) result in histone modifications
Exhaustion	Increased PD-1 expression May also express inhibitory receptors (CD2444, BTLA, CTLA-4, CD160, LAG-3, Tim-3)	Reduced IL-2, IFN-G, TNF-A Cell cycle arrest	
Senescence	Reduced CD28 expression Increased Tim-3, CD57, KLRG-1 Increased cell cycle controlling proteins (p16, p21, p53)	Telomere shortening Cell cycle arrest in G1/S phase	

8.7 Anergy

An excess of anergic T-cells is seen in trauma, where it is thought to be an indicator of pathological immune suppression and has also been associated with poorer outcomes.(Bandyopadhyay, De et al. 2007) The phenotype of the lymphocytes from postoperative patients studied in this thesis is only partly consistent with anergy. A failure to become activated when stimulated may lead to lower levels of both glycolytic and mitochondrial ATP production. However, anergic lymphocytes do not have increased apoptosis in vivo,(Walker and Abbas 2002), so widespread T-cell anergy is unlikely to explain the increase in apoptosis which occurs postoperatively.

8.8 Exhaustion

T cell exhaustion is a state of T cell dysfunction that arises during chronic infection and cancer. It is defined by poor effector function, sustained expression of inhibitory receptors and a transcriptional state distinct from that of functional effector or memory T cells.(Wherry 2011) Exhaustion prevents optimal control of infection and tumours. Both extrinsic negative regulatory pathways (such as immunoregulatory cytokines) and cell-intrinsic negative regulatory pathways (such as PD-1) have key roles in exhaustion.

Hierarchical T cell exhaustion occurs during chronic infection. During initial infection, naive T cells are primed by antigen, co-stimulation and inflammation and differentiate into effector T cells. Clearance of

infection and antigen allows a subset of these functional effector T cells to further differentiate into highly polyfunctional memory (CD8) T cells able to coproduce many cytokines (such as IFN-γ, tumor necrosis factor (TNF) and IL-2), becoming cytolytic and proliferating vigorously. These cells also have considerable survival capacity and are maintained long term without antigen. During chronic infection, infection persists after the effector phase. As antigen and/or viral load increases, T cells progress through stages of dysfunction, losing effector functions and other properties in a hierarchical manner. T cell exhaustion is also accompanied by a progressive increase in the amount and diversity of inhibitory receptors expressed (including PD-1, LAG-3, 2B4, CD160, Tim-3, CTLA-4).(Crawford and Wherry 2009) In addition, altered inflammation and changes in immunoregulatory cytokines such as IL-10 and/or TGF-β can have an increasingly important role. Ultimately, if the severity and/or duration of the infection is high and/or prolonged, virus-specific T cells can be completely eliminated, leading to loss of virus-specific T cell responses. The severity of T cell exhaustion is correlated with increasing inhibitory receptor expression, high viral (or antigen) load, loss of CD4+ T cell help and prolonged infection.(Wherry 2011) PD-1 may define and maintain T cell exhaustion, which is supported by evidence that PD1 blockade may rescue T cell effector function and result in improved effector responses such as cytokine expression, cell cycle progression and cytotoxicity.(Blackburn, Shin et al. 2009, Jin, Anderson et al. 2010)

The postoperative phenotype described in this thesis has several similarities with the exhaustion phenotype. Firstly, lymphocyte function is impaired in both groups. Secondly, lymphocyte apoptosis is increased in the advanced exhaustion phenotype, mediated by upregulation in Bim and downregulation of Bcl-2, consistent with the increases in intrinsic mitochondrial ROS observed.(Akbar and Henson 2011) Lastly, widespread transcriptional profiling of memory, effector and exhausted T-cells has revealed metabolic abnormalities in exhausted lymphocytes, with downregulation of several genes involved in energy metabolism and the citric acid cycle.(Wherry, Ha et al. 2007) These transcriptional changes could potentially be responsible for reductions in glycolysis and oxidative phosphorylation in exhausted T-cells as seen in Chapter 4, however this requires further study. Despite these similarities, our patient cohort had an extremely low incidence of HIV, HBV and HCV from which most studies describing T cell exhaustion have described observations.

8.9 Senescence

At a critical point telomere shortening due to repeated antigenic exposure triggers DNA damage which inhibits cell cycling. This results in large numbers of highly differentiated (CD27(-) CD28(-)) T-cells with reduced function and proliferative ability.(Akbar and Henson 2011) Senescence is associated with advanced age and CMV.

The features of the postoperative lymphocytes may in part, fit with the senescent T-cell phenotype. Postoperative lymphocytes have a hypometabolic (and pro-apoptotic) phenotype with reduced glycolytic and/or mitochondrial oxidative phosphorylation. There is however no consensus on the apoptotic predisposition of senescent lymphocytes; long term cultures used to study senescent cells tend to be resistant to cell death, whereas more freshly isolated highly differentiated T-cells appear sensitive to apoptosis.(Plunkett, Franzese et al. 2007)

In summary, whilst the phenotypes described in anergy, exhaustion and senescence do not fit precisely with the abnormalities found in postoperative lymphocytes, some similarities do exist. This is particularly the case for increased activity of co-inhibitory receptors such as PD-1 or dysfunction in other parts of the co-stimulatory pathway. The lymphocyte stimulation performed in the bioenergetic study within this thesis consisted of concanavalin A, which mimics TCR ligation by activating PKC, (Krauss and Brand 2000) without direct effects on the co-stimulatory pathway. Lymphocytes from preoperative samples demonstrated adequate co-stimulation by displaying glycolysis, whereas postoperative lymphocytes were unable to do this perhaps due to an abnormality in the co-stimulatory pathway (IL-2 or CD28 and CD80 / CD86). Inadequate co-stimulatory receptor activity (CD28 or IL-2) or increased co-inhibitory receptor activity (PD-1, CTLA-4) may be responsible for the altered metabolic profile demonstrated postoperatively. Surgery may also alter the ability of MHC class II molecules to bind with TCRs, or inhibit PKC activation following Concanavalin A activation.

The two co-stimulatory pathways which may be implicated in the impaired postoperative bioenergetic function are the: 1) PI3K/Akt/mTOR pathway (CD28) and the 2) JAK/STAT/PIM pathway (IL-2). These are discussed as potential therapeutic targets in the next part of this discussion.

8.10 Future work and potential therapeutic targets

Future bioenergetic studies and therapeutic targets

It would be important to identify whether differences exist in postoperative inhibitory receptor expression (PD-1, CTLA-4, LAG-3), markers of senescence (differentiation markers CD27, CD28 and CD58, plus KLRG-1) in order to gain further understanding of the likely mechanism for lymphocyte hypometabolism.

Since differences appear to exist between lymphokine production of CD4(+) and CD8(+) cells, it would be important to determine whether differences also exist between different subsets of lymphocytes in terms of bioenergetic function including:

- Naive Th cells (CD3+ CD4+ CD45RA+ CD62L+)
- Effector Th cells (CD3+ CD4+ CD69+)
- Memory Th cells (CD3+ CD4+ CD45RO+)
- As above for Tc cells (CD8 not CD4)
- B-cells (CD3- CD19+)
- NK cells (CD3- CD56+ CD16+)

Given the fact that CD4 cells were unable to increase Th1 lymphokine production postoperatively, these cells may demonstrate reduced oxidative phosphorylation and glycolysis compared to CD8(+) cells.

This thesis has identified an association between the postoperative state and reduced bioenergetic function of lymphocytes. What remains unclear is the precise molecular mechanism causing the altered mitochondrial function. The physiological levels of glucocorticoid, types of glucocorticoid, cellular pathways and receptors which are activated, warrant further investigation in order to identify potential therapeutic targets to maintain baseline lymphocyte function.

An accumulating body of evidence highlights the diverse array of metabolic functions regulated by the steroid receptor co-activator (SRC), including systemic metabolite homeostasis, inflammation, and energy regulation.(Stashi, York et al. 2014) The cooperative and unique functions among the SRCs resulting in bioenergetic functional changes require investigation. Furthermore deciphering the fractional and synergistic contributions of the SRCs to metabolic homeostasis is crucial to

understanding fully the networks underlying metabolic transcriptional regulation in the perioperative period. Cytochrome c oxidase may be a therapeutic target of the glucocorticoid effect on the respiratory chain in order to limit its effect on postoperative change in oxidative phosphorylation based on data from rat kidneys.(Simon, Jolliet et al. 1998)

By preventing reduction in oxidative phosphorylation and glycolysis seen in lymphocytes postoperatively with any given therapy, it would be prudent to also demonstrate a concomitant increase in lymphokine production showing that they are indeed related. Some important pathways involved in lymphocyte bioenergetics are outlines in the Table 8.3. The mediators identified in Table 8.3 identify potential therapeutic targets for investigating whether postoperative changes in lymphocyte bioenergetic function can be modified.

Table 8.3 Key pathways controlling lymphocyte energy metabolism

Stimulating factor	Pathway	Mediator	Effects
Co-stimulation (CD28 & CD80/CD86)	PI3K	AKT	Links signal transduction to anabolic and glycolytic metabolism GLUT-1 receptor insertion at cell membrane, activates hexokinase, phosphofructokinase Inhibition of pro-apoptotic BAD
		mTOR	Promotes protein synthesis if insufficient intracellular supply of amino acids
Low ATP (High AMP: ATP)		AMPK	Negative regulation of Akt / mTOR Inhibits growth and proliferation during nutrient deficiency / physiological stress Induces p53-mediated cell cycle arrest at the junction of G1 and S.
IL-2	JAK and STAT pathways (via	PIM1 / PIM2	Functions overlap those of Akt and mTOR

PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B; mTOR, mammalian target of rapamycin; AMPK, 5' AMP-activated protein kinase; PIM1 /PIM2, proviral integration site 1 / 2 =oncogene, ; JAK, Janus kinase; STAT, signal transducer and activator of transcription; BAD, B-cell lymphoma-2-antagonist-of-cell-death.

Proto-oncogenes / tumour suppressors are also known to modulate energy metabolism. Many regulate glucose metabolism directly,(DeBerardinis and Thompson 2012) and activate the PI3K/Akt/mTOR metabolic signalling pathway, which is important for cell survival, metabolism and proliferation. Manipulation of these pathways can affect lymphocyte fate. For example, during T-cell activation, inhibition of mTOR can prevent proliferation and leads to formation of CD8 memory T-cells.(Pearce 2010) Some key oncogenes, which are also potential therapeutic targets, are described in Table 8.4.

Table 8.4 Key oncogene effects on metabolism

Oncogene	Effects
Akt	Glucose uptake (via GLUT-1 transporter)
	Aerobic glycolysis
	Lipid synthesis (utilising mitochondrial citrate metabolised to Acetyl CoA)
mTORC1	Protein synthesis
	Lipid synthesis
	Mitochondrial metabolism (anabolic precursors)
	Decreased autophagy
Myc	Glutamine uptake
	Glutaminase conversion of glutamine to glutamate (for mitochondria)

In many cell systems, mTORC1 couples PI3K (phosphoinositide 3-kinase) and Akt (or protein kinase B) with the control of glucose uptake and glycolysis. However, this is not the case in activated CD8(+) T-lymphocytes responding to the cytokine IL-2 where PI3K/Akt signalling is dispensable for the elevated levels of glycolysis that is characteristic of activated T-cells.(Finlay, Rosenzweig et al. 2012, Finlay 2013) mTORC1 is essential for glycolytic metabolism in CD8(+) T-cells, and this reflects the fact that mTORC1 does not lie downstream of PI3K/PKB signalling in CD8(+) T-cells, as is the case in many other cell systems. Thus differences in cytokine production demonstrated between CD4(+) and CD8(+) cells postoperatively may be due to alterations in P13K/Akt/mTOR pathway activity.

mTORC1 activity is required for TCR-induced c-Myc expression and the switch to increased glycolysis.(Finlay, Rosenzweig et al. 2012) Accordingly, deletion of c-Myc in naive T-cells prevents TCR-induced glucose uptake and glycolysis, (Wang, Dillon et al. 2011) A successful T-cell activation response involves expression Myc, an oncogene which plays a role in glutamine uptake, mitochondrial activity and mitochondrial biogenesis.(Altman and Dang 2012) A failure of adequate lymphocyte activation could therefore impair mitochondrial formation resulting in reduced postoperative respiratory capacity. This is described as an important factor in ongoing memory T-cell survival.(van der Windt, Everts et al. 2012) Evaluation of Myc and mTORC1 levels is needed in the perioperative period to determine whether these result in reduced glycolysis in lymphocytes postoperatively.

mTORC1 also regulates glucose metabolism in CD8 lymphocytes through regulating the expression of the transcription factor HIF1α (hypoxia-inducible factor 1α). Strikingly, HIF1α functions to couple

mTORC1 with a diverse transcriptional programme that among other roles controls glucose metabolism.(Laplante and Sabatini 2012) Therefore changes in HIF1α levels in the perioperative period should be explored as a potential mechanism for altering bioenergetic function and may explain reductions in oxidative phosphorylation and glycolysis seen.

In order to identify whether surgery alters these signalling pathways, which could result in the metabolic alterations demonstrated, further studies are also needed to assess and quantify Akt and mTor levels in lymphocytes following stimulation in the perioperative period. Additionally, assessment of the integrity of downstream TCR activation events after stimulation e.g. measuring IL-2 production in patient cells following mitogenic stimulation should be explored. The postoperative phenotype can be recreated in healthy control lymphocytes by performing co-stimulatory pathway blockade. The following pathways should be inhibited in order to demonstrate the likely cause of postoperative hypometabolism:

- CD28 receptor antagonism
- mTOR inhibition with rapamycin
- AMPK activation (inhibits mTOR) with metformin
- PD-1 / CTLA-4 agonism

The PI3K/PKB/FOXO (forkhead box O) pathway also controls the expression of these key trafficking molecules. Defects in transcription or inhibition of the kinases involved in these pathways may be mechanistic in the metabolic and functional abnormalities seen in postoperative lymphocytes. (Finlay and Cantrell 2010)

The Foxo family of transcription factors is a well-defined target of the Akt kinase. Aside from evolutionarily functions in nutrient sensing (via the P13K/Akt/mTOR pathway) and stress responses (via C-Jun N-terminal kinase), Foxo proteins also regulate the expression of target genes involved in the control of T cell homeostasis and tolerance.(Hedrick, Hess Michelini et al. 2012) Some of the effects of Foxo1 stimulation include:

- IL-7Rα expression – discussed further in the future cytokine target section.
- Sphingosine-1-phosphate receptor 1 (S1P1) - involved in T-lymphocyte trafficking. Deficiency of S1P1 leads to T-cell lymphopenia in the peripheral lymphoid organs.(Matloubian, Lo et al. 2004)

- CCR7 – a chemokine receptor required for the migration of circulating lymphocytes to lymphoid organs.(Kerdiles, Beisner et al. 2009)
- CD62L (L-selectin) – an adhesion molecule necessary for lymphocyte recirculation via adhesion to lymphoid tissue high endothelial venules and lymph node homing.

Foxo1 deficiency leads to decreased lymphopenia-induced homeostatic proliferation due to reduced IL-7RαR expression, reduced egress of naive thymic T-cells due to S1P1 deficiency, and reduced lymph node lymphocyte numbers, with consequent total organism lymphopenia.(Gubbels Bupp, Edwards et al. 2009, Kerdiles, Beisner et al. 2009, Ouyang, Beckett et al. 2009) Therefore Foxo deficiency could be affected by the stress response to surgery and may be a contributing factor to peripheral lymphopenia.

Further work should assess the impact of Foxo-1 dysregulation on the metabolic and functional profiles seen postoperatively. Useful things to identify perioperatively include:

- Levels of lymphocyte S1P1 and CD127 (IL7) receptors
- Effect of S1P1 and CD127 receptor agonists on lymphocyte metabolism

<u>Autophagy targets</u>

Autophagy is an intracellular catabolic process involving the generation of a double-membraned structure, sequestration of cytoplasmic components, and delivery of bound cellular matter to lysosomal compartments for degradation. This process is conserved in all eukaryotic cell types, and has been implicated in cellular survival during periods of cellular or cytotoxic stresses.(Kroemer, Marino et al. 2010, Levine, Mizushima et al. 2011) Autophagy can be divided into three main classes:

1) Macroautophagy -de novo synthesis of autophagosomal membranes around bulk substrates to be degraded and the stepwise fusion of these autophagosomes with degradative vesicles to form autolysosomes.
2) Selective autophagy, (subclass of macroautophagy) involves binding of substrates to adapter molecules for inclusion into the nascent autophagosome.
3) Microautophagy involves the small-scale blebbing and sequestration of soluble components within the immediate microenvironment of previously formed autophagosomes generated during macroautophagy.

The role of autophagy and mitophagy in the postoperative phase is an under explored area. Autophagy provides an alternative source of nutrients scavenged from the cytoplasm, an alternative form of cellular degradation that augments the proteasomal system, and a major pathway for intracellular remodeling to reflect changes in the developmental or metabolic requirements of the cell.(Yorimitsu and Klionsky 2005)

Autophagy genes including: Vps34, Atg3, Atg5, Atg7, Beclin-1, LC3, and p62 are expressed in both T and B lymphocytes.(McLeod, Jia et al. 2012) The expression of these genes in the perioperative period warrants further investigation in conjunction with clinical outcome.

Methyl pyruvate can restore cytokine production by autophagy-deficient T cells,(Hubbard, Valdor et al. 2010) suggesting a function of autophagy in energy production and other essential processes in T lymphocytes. Much work remains to identify the soluble, cytoplasmic components that require autophagy for turnover and to determine whether their autophagy mediated turnover requires specific adapter proteins. The downstream targets of the T-cell receptor, which are responsible for activation-mediated autophagy, are unknown. The coordinated activity of NFAT, NFκB, or MAPK signals may be required. Furthermore, the contribution of autophagy to a robust T-cell proliferative response remains unclear. Further work is necessary to determine which targets are processed by autophagy and which are proteosomally degraded. Targeting autophagy may provide interesting therapies for T cells to overcome anergy induced by surgery.

Future cytokine targets

The data from this thesis examined Th1 cytokines and explored CD4 and CD8 subsets of lymphocytes. Limiting investigation to these 2 subsets of lymphocyte allowed me to demonstrate that cytokine production increased in CD8(+) cells more than CD4(+) cells. However, further investigation is required to elucidate not only how cytokine production from other subsets of lymphocytes changes postoperatively but also whether Th2 cytokine production is altered in different subsets postoperatively.

In myeloid immune cells, PI3K and mTOR seem to constrain full immune cell activation by upregulation of the key anti-inflammatory cytokine interleukin 10 and inhibition of proinflammatory cytokines.(Weichhart and Saemann 2008) Decreased activity of these signalling pathways may be in part responsible for postoperative changes in TH1 cytokine production.

Manipulation of these pathways could therefore prove to be potential therapeutic targets to affect postoperative immune cell function via manipulation of cytokine production.
IL-7 has several important roles in lymphocyte homeostasis:

- naïve T-cell homeostatic proliferation
- T-cell lymphopoesis in the bone marrow
- anti-apoptotic actions via Bcl-2 upregulation
- support of bioenergetic function through transcriptional regulation of the Glut-1 and hexokinase II genes or via the Akt pathway(Jacobs, Michalek et al. 2010)

T-cell response to IL-7 is likely to be regulated by varying expression of IL-7 receptor α-chain (IL-7Rα, or CD127) (Ouyang and Li 2011) The widespread pro-energetic and pro-survival activity of IL-7 on lymphocytes means that reduced IL-7 activity due to reduced IL-7 lymphocyte exposure, or reduced IL-7Rα expression, may explain the postoperative lymphocyte phenotype.

Cynomolgus monkeys receiving 10 days of exogenous IL-7 (rhIL-7) showed substantial, reversible increases in T-cell numbers involving a dramatic expansion of both naive and non-naive phenotype CD4(+) and CD8(+) subsets.(Fry, Moniuszko et al. 2003) The use of this therapy for humans in the perioperative phase has not been performed. Additionally, whether increases in T-cell numbers postoperatively secondary to rhIL-7 result in improved outcome remains unclear.

Pre-clinical and phase 1 human studies have demonstrated acceptable tolerability of subcutaneous recombinant human IL-7 (rhIL-7) administered in subjects with refractory malignancy for its anti-tumour responses.(Sportes, Babb et al. 2010) The lymphocyte effects of IL-7 include an increase in T-cell cycling, upregulation of bcl-2 and major sustained increases in $CD4^+$ and $CD8^+$ lymphocyte circulating numbers (thought to be due to increased production and peripheral proliferation). In a recent murine peritonitis model, rhIL-7 treatment started 90 minutes after caecal ligation and puncture improved survival, reduced T-cell apoptosis, restored IFN-γ production and improved immune effector cell recruitment to the infected site.(Unsinger, McGlynn et al. 2010) This appears to be a promising potential treatment to improve immune function and reduce hospital length of stay secondary to morbidity for humans in the perioperative period.

To combat postoperative immunodepression, perioperative IL-2 administration has been tested in a phase II randomised trial (n = 39) to analyse efficacy on patients with radically operable gastric cancer.(Romano, Piacentini et al. 2004) In this study patients who received IL-2 had a significant increase in total and CD4(+) lymphocytes and these patients did not experience any anaesthesia-related or surgical complications. The treatment group however did experience a greater incidence of fever than the control group. The wide range of side-effects resulting from IL-2 keeps it from widespread use at present. The prognostic impact of IL-2 treatment remains unclear.

Future inflammasome and IL-1Beta studies and therapeutic targets

During the past few decades, it has been widely recognised that Reduction-Oxidation (redox) responses occurring at the intra- and extra-cellular levels are one of most important biological phenomena and dysregulated redox responses are involved in the initiation and progression of multiple diseases. Thioredoxin1 (Trx1) and Thioredoxin2 (Trx2), mainly located in cytoplasm and mitochondria, respectively, are ubiquitously expressed in variety of cells and control cellular reactive oxygen species by reducing the disulfides into thiol groups. Thioredoxin interacting protein (Txnip/thioredoxin binding protein-2/vitamin D3 upregulated protein) directly binds to Trx1 and Trx2 (Trx) and inhibit the reducing activity of Trx through their disulfide exchange.(Yoshihara, Masaki et al. 2014) Recent studies have revealed that Trx1 and Txnip are involved in some critical redox-dependent signal pathways including NLRP-3 inflammasome activation in a redox-dependent manner.(Zhou, Tardivel et al. 2010) Therefore, Trx/Txnip, which exist in mitochondria are a redox-sensitive signalling complex regulating cellular redox status and has emerged as a key component in the link between redox regulation and the pathogenesis of diseases. This may be important in the regulating the increase in mROS seen in lymphocytes postoperatively and may therefore be an effective therapeutic target. While antioxidant treatment is associated with improvements in isolated aspects of lymphocyte function in animal and ex-vivo models these have not been explored adequately in clinically relevant studies.(Kurihara, Nagoshi et al. 2007, De la Fuente, Cruces et al. 2011)

Much research over the past few decades has focused on determining the function of members of the cytosolic Nod-like receptor (NLR) family in terms of their triggers and the signalling pathways that they control. As previously described in chapter 7, NLRP proteins play a role in sensing both microbial and danger signals and triggering the caspase-1 dependent inflammasome. The Nod subfamily characterised by proteins with a caspase-activating and

recruitment domain (CARD) or a so-called 'X' domain. Nod1, Nod2, NLRX1 and NLRC5 are all members of this subfamily and there has been recent work demonstrating the importance of these molecules in both pathogen sensing and regulation of innate and adaptive immunity.(Magalhaes, Sorbara et al. 2011) The changes in expression of these molecules within lymphocytes in response to perioperative increases in cortisol also require further investigation.

Ginsenoside Rg1 protects cells by antagonizing neuronal apoptosis.(Wu, Pan et al. 2012) The phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signalling pathway may effectively reduce mitochondrial pathway-induced cell apoptosis.(Huang, Wu et al. 2014) Pre-treatment of rat chondrocytes with Rg1 has been shown to decrease the activity of IL-1β that reduces expression of Bcl-2 and level of phsophorylated-Akt, and increases Bax (Bcl-2-associated X protein) activity, Cytochrome c release, and caspase-3 activation. It also reverses the activity of IL-1β. These results indicate that Rg1 may protect chondrocytes from IL-1β-induced apoptosis via the phosphatidylinositol 3-kinase/protein kinase B signalling pathway, through preventing caspase-3 release.(Huang, Wu et al. 2014) The protective effect of Rg1 on interleukin 1β (IL-1β)-induced lymphocyte apoptosis and the underlying molecular mechanisms has not been investigated. This could potentially be explored as a therapy to reduce postoperative lymphocyte apoptosis and inflammasome activation.

Follistatin-like protein 1 (FSTL-1) is a secreted g protein produced mainly by cells of the mesenchymal lineage, such as cardiomyocytes and endotheliocytes.(Oshima, Ouchi et al. 2008) FSTL-1 inhibits apoptosis in cardiomyocytes but also may have a proinflammatory function. FSTL-1 is overexpressed in a number of inflammatory conditions characterised by elevated IL-1β. Whether its expression by lymphocytes changes postoperatively as has been shown in patients with septic shock (Chaly, Fu et al. 2014) requires investigation. Similarly whether this contributes to postoperative lymphocyte apoptosis requires investigation.

Future cohort studies

Longitudinal population studies determining life expectancy, incidence of morbidity and cause of mortality, are required to determine long-term outcomes in asymptomatic non-surgical patients with lymphopenia.

8.11 Surgery as a model for cardiac failure, exercise and sepsis

Cardiac failure

The role of autonomic imbalance on lymphocyte distribution and peripheral number was highlighted by a study demonstrating marked lymphopenia - particularly of Th- and B-cells - in beta-blocker-naïve patients with cardiac failure, whereas beta-blocker treated patients had only a mild relative lymphopenia compared with healthy controls(von Haehling, Schefold et al. 2009). The close parallels between lymphocyte and cardiac responses to catecholamines have been highlighted by the elevation of GRK2 (G protein-coupled receptor kinase) expression and activity in lymphocytes in patients with heart failure, leading to failure of β-adrenoceptor mediated cAMP response. Abnormally high levels of kinase activity in lymphocytes are mirrored in cardiomyocytes, and have been strongly related to poorer ventricular ejection fraction and NYHA symptom severity.(Iaccarino, Ciccarelli et al. 2005) Apoptosis may also play a role in the lymphopenia of chronic cardiac failure,(Agnoletti, Boudjemline et al. 2004) which may be induced ex-vivo by circulating mediators such as catecholamines (Bergquist, Josefsson et al. 1997) or natriuretic peptides.(Shaw, Critchley et al. 2012) The effect of heart failure therapy on propensity to lymphocyte apoptosis has not been explored. Similarly, measurement of NT-proBNP, endotoxin and kinase levels in relation in the postoperative period may provide additional support of the similarities between heart failure and the postoperative state.

Exercise

Exercise may be used as a model to recreate some of the physiological and neuroendocrine changes, which occur during surgery. Episodes of exercise cause a prompt increase in circulating lymphocytes - particularly memory T-cells and NK cells – the magnitude of which is related to exercise intensity and duration.(Mignini, Traini et al. 2008) Within an hour of ceasing exercise, lymphocyte numbers fall to levels lower than pre-exercise, a reduction, which may last for several hours and this particularly affects $CD4^+$ and NK subsets. Redistribution of lymphocytes from the spleen to other sites of potential antigen encounter can be mimicked in murine models by catecholamines(Kradin, Rodberg et al. 2001) and abolished by pharmacological sympathetic blockade.(Kruger, Lechtermann et al. 2008). Exhaustive exercise induces lymphocyte apoptosis in approximately 60% and after 24 hours 86% still demonstrate an apoptotic pattern of DNA distribution, but the precise mechanism by which this occurs remains unclear.(Mars, Govender et al. 1998) A healthy volunteer study also demonstrated that exhaustive exercise induces apoptosis in peripheral blood lymphocytes whereas moderate exercise does not.(Mooren, Bloming et al. 2002)

Further work is required to elucidate the similarities between the postoperative and post-exercise states in terms of bioenergetic function and inflammasome activation.

ROS generated temporarily during episodes of exercise have been shown to induce the transcription of antioxidant genes, such that chronic exercise improves cellular redox status.(Los, Droge et al. 1995) The effect of instituting an exercise programme prior to elective surgery and its effect on lymphocyte mitochondrial function and perioperative outcome is an area, which needs further research.

Sepsis

Failure of ATP production underlies organ dysfunction in critical illness. Using muscle biopsies from septic and healthy preoperative controls, severity of sepsis has been correlated with reduced ATP concentration, reduced complex I activity, increased nitric oxide production and reduced levels of glutathione.(Brealey, Brand et al. 2002) This failure of energy production in sepsis has been further demonstrated in immune cells. Reduced mitochondrial oxygen consumption due to ATP production (reduced state III respiration) has also been demonstrated in peripheral blood mononuclear cells from patients with septic shock.(Belikova, Lukaszewicz et al. 2007, Japiassu, Santiago et al. 2011) A further limitation common to studies in the septic patient population is the difficulty allowing for potential confounding factors in the association between defective energy production and worse clinical outcomes. In particular, the extent of pre-insult bioenergetic dysfunction is unknown, and the influence of common treatments, which may alter bioenergetic profiles such as glucocorticoids, antibiotics and insulin is hard to control for in these small studies. Further work is required to compare sepsis and the postoperative states.

Since similarities appear to exist between lymphocytes taken from different diseases, comparison of immune phenotypes of each of these disease states (including sepsis, following exercise and postoperatively) would provide further insight into general and disease specific patterns, which may exist.

8.12 Conclusion

Lymphopenia is common in the elective surgical population and is associated with other markers of increased perioperative risk such as low AT. Exploration of the postoperative immune and bioenergetic features of lymphocytes demonstrates a phenotype of reduced cellular function compared to preoperative baseline levels, which may have important implications for acquired lymphopenia, morbidity and recovery following surgery.

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Appendix 1 UCL trainee research forum 2012 presentation

Metabolic Dysfunction Underlies Lymphopenia-Associated Postoperative Morbidity

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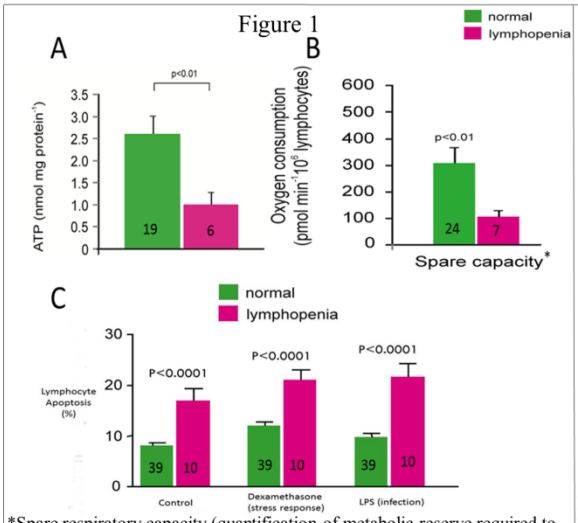
BACKGROUND: The development of lymphopenia in seemingly disparate pathologies, including critical illness,[1] cancer[2] and chronic heart failure[3] is associated with poor outcomes. We hypothesized that bio-energetic dysfunction provides a unifying mechanistic link underlying this lymphopenic phenotype.

METHODS: In patients free of pathologies linked to low lymphocyte count (≤1.5 x109 cells L-1; <20% total leukocyte count), we characterized ex-vivo bio-energetic and immune functionality in lymphocytes isolated from lymphopenic and non-lymphopenic patients. We then evaluated the association between the metabolic phenotype of lymphocytes with postoperative morbidity, using elective orthopedic surgery as a standardized model of tissue injury.

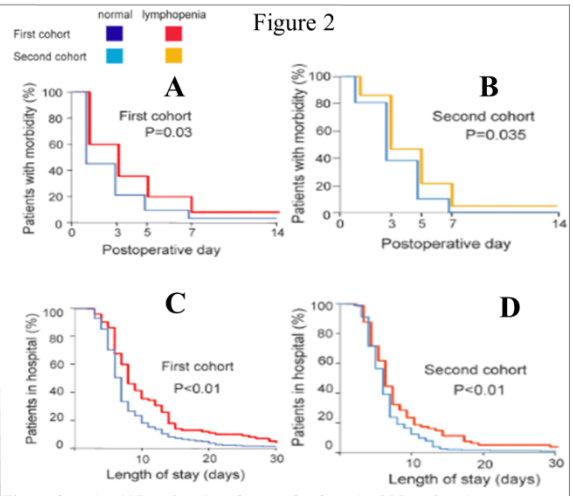
RESULTS: Lymphocytes taken from patients with established lymphopenia demonstrated lower levels of ATP (Fig 1A) and mitochondrial oxygen consumption (Fig. 1B), accompanied by increased propensity for cellular death (apoptosis; Fig. 1C) induced by common cellular stressors (all p<0.05). In two separate orthopedic cohorts (n=417 and 328 respectively), preoperative lymphopenia was present in 15-18% of patients and associated with prolonged postoperative morbidity (hazard ratio (HR): 1.25 (95% CI 1.03–1.52); p=0.002; Fig. 2A and 2B) and length of hospital stay (HR: 1.47 (95% CI 1.20–1.8); p<0.0001; Fig. 2c and 2D).

Figure 1



*Spare respiratory capacity (quantification of metabolic reserve required to prevent cellular death), in isolated lymphocytes taken from normal and lymphopenic patients pre-operatively.
Numbers in bars indicate number of patients.

Figure 2



First cohort (n=417 patients) and second cohort (n=328 patients).
Panels A and B show the time taken for patients to become morbidity-free, as defined by POMS collection timepoints.
Panels C and D show the time taken to hospital discharge for both cohorts.
P-values for log rank test.

DISCUSSION: Lymphocytes from patients with established lymphopenia show bio-energetic dysfunction; this is associated with greater postoperative morbidity following elective orthopaedic surgery. Mitochondrial dysfunction may be an important mechanism and a putative therapeutic target to counteract maladaptive responses to inflammation/tissue injury.

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