

**Extracorporeal Membrane Oxygenation
For Severe Systemic Inflammatory
Response:
Development Of A Rabbit Model**

Thesis submitted for the degree of

Doctor of Medicine

At the University of Leicester

By

Espeed Khoshbin

**Department of Cardiovascular Sciences
Division of Cardiac Surgery**

Mar 2008

UMI Number: U500642

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



UMI U500642

Published by ProQuest LLC 2013. Copyright in the Dissertation held by the Author.
Microform Edition © ProQuest LLC.

All rights reserved. This work is protected against
unauthorized copying under Title 17, United States Code.



ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

TABLE OF CONTENTS

ABSTRACT

ACKNOWLEDGEMENTS

ABBREVIATIONS

PUBLICATIONS & PRESENTATIONS

PREFACE

CHAPTER ONE

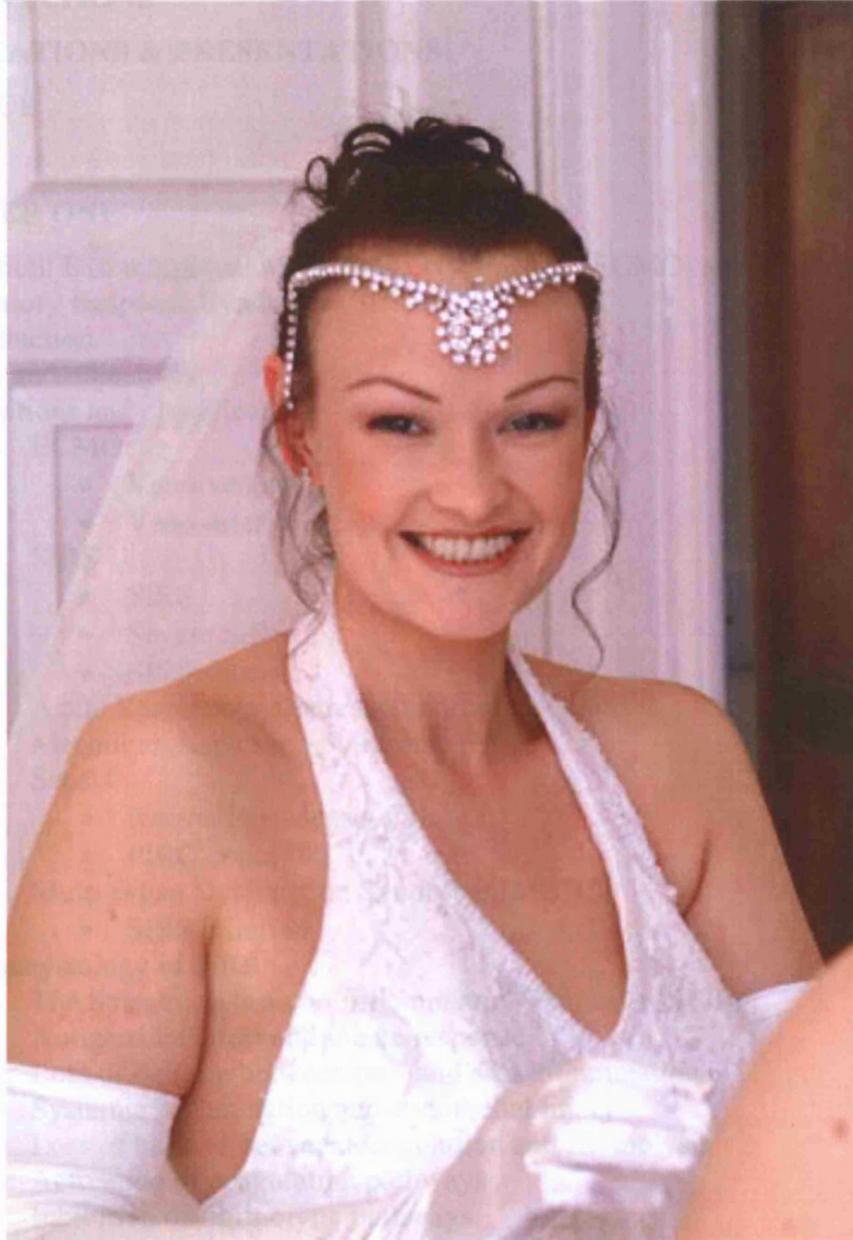
Introduction

1.1 Background

1.2 Introduction

1.3 Aim

1.4 Definition



This thesis is dedicated to my wife

Tracy MA Khoshbin

TABLE OF CONTENTS

ABSTRACT

ACKNOWLEDGEMENTS

ABBREVIATIONS

PUBLICATIONS & PRESENTATIONS

PREFACE

CHAPTER ONE

Page 1-37

Introduction: Extracorporeal Membrane Oxygenation (ECMO) and Systemic Inflammatory Response Syndrome (SIRS)

- Introduction
- Aims and objectives
- Definitions and classifications
 - ECMO
 - Venovenous
 - Venovenous-arterial
 - SIRS
 - SIRS
 - Severe SIRS
 - SIRS shock
 - Acute Respiratory Distress Syndrome (ARDS)
 - Meconium Aspiration Syndrome (MAS)
 - Sepsis
 - International sepsis forum
 - PIRO concept
 - Multi-organ Dysfunction Syndrome (MODS)
 - SIRS criterion
- Pathophysiology of SIRS
 - The Systemic Thrombo-inflammatory Pathways (STIP)
 - Antigen challenge and innate response
 - Loss of balance between pro- and anti-inflammation
 - Systemic inflammation and endothelial injury
 - Loss of balance between coagulation and fibrinolysis
 - Activation of coagulation pathways
 - Inhibition of fibrinolytic pathways
 - Deficiency in protein C anticoagulant pathway
 - Mechanism of organ dysfunction and MODS
 - Endothelial cell dysfunction
 - Oxidative stress and DNA damage
 - Apoptosis
 - Heat shock response
- The haematic response to ECMO
- The way forward
 - Need for an animal model

CHAPTER TWO**Page 38-100****Materials and Methods**

- Introduction
- Thesis design
- Literature review
- Clinical experiments
 - Institutional report
 - Experimental methodology (PIRO)
 - Patients
 - Comparative performance review
 - Experimental methodology
 - Patients
- In-vivo animal experiments
 - Methodology
 - Animal scientific act
 - Animal welfare
 - The model
 - Rabbit anaesthesia
 - Induction of surgical anaesthesia
 - Maintenance
 - Management of anaesthetised rabbits
 - Optimal anaesthesia
 - Surgical procedures
 - Tracheostomy
 - Femoral cannulations
 - Supra-pubic catheterisation
 - Endotoxin preparation
 - Management of anaesthetised rabbit
 - Post mortem examination
 - Technical points / lessons learned
 - Wet to dry ratio
- In-vitro laboratory experiments:
 - Standard laboratory investigations
 - Enzyme linked immuno-sorbent assay (ELISA)
 - Quality control
 - Determination of plasma/serum concentrations
 - Storage
 - Rabbit ELISA protocol
 - Antibody purification
 - Biotinylation of antibodies
 - Optimisation
 - Results
 - Standard curve
 - Calculation of antigen concentration
 - Ready to use Kits
 - CRP
 - Nitric Oxide
 - TGF- β 1

- Optimised rabbit ELISAs
 - TNF α
 - IL-1 β
 - IL-5
 - Inhibition ELISA assay
 - IL-6
 - IL-8
 - IL-10
 - Neutrophil Elastase
 - Tissue Factor
 - Protein C
 - PAI-1
 - Thrombomodulin
 - Fibrinogen
 - Lectin pathway activation
 - C3 activation assay
- Automated analysis
 - Total anti-oxidant status
- Histology
 - Hematoxylin and eosin
- Immunohistochemistry
 - Animal research kit (ARK)
 - Rabbit ABC immunohistology protocol
- Cellular components of STIP
 - Neutrophils
 - Mononucleated phagocytes
 - T lymphocytes
 - T helper cells
- Histological and immunohistochemical scoring system
- CytoDEATH M30
- Immunofluorescence
- Rabbit immunofluorescence protocol
- Malondialdehyde deoxyguanosine adduct (M1G)
- Anti 4-hydroxy-2-nonenal monoclonal antibody (Genox)
- Data acquisition
- Oxidative stress and DNA damage
- Controls
- Apoptosis
- Statistical analysis
 - Comparing variables
 - Regression plot
 - Sample size measurements
- Bibliography

CHAPTER THREE**Page 101-122**

Institutional report of ECMO for severe SIRS and MODS

- Epidemiology
- Aims and objectives
- Patients and methodology
- Results
 - Neonates
 - Children
 - Adults
- Discussion
- Conclusion

CHAPTER FOUR**Page 123-151**

Reduction in the haematic response to ECMO: The state of the art in oxygenator technology

- Introduction
- Materials and methods
- Results
 - Adults
 - Referral details
 - Oxygenator efficiency
 - Coagulation data
 - Consumption of blood products
 - Oxygenator pressure and resistance
 - Chest x-ray scores
 - Days to complete subsidence
 - ECMO run
 - Clots and oxygenator failure
 - Survival outcome
 - Neonates
 - Referral details
 - Oxygenator efficiency
 - Coagulation data
 - Consumption of blood products
 - Oxygenator pressure and resistance
 - Chest x-ray scores
 - Days to complete subsidence
 - Clots and oxygenator failure
 - ECMO run
 - Case report of a fatal neonatal oxygenator failure
 - Survival outcome
- Discussion
 - Surface area of oxygenators and gas exchange efficiency
 - Surface area of oxygenators and blood path resistance
 - Surface area of oxygenators and haematic response
 - Safety of oxygenators
 - Prime volume
 - Heat exchange
- Conclusion

CHAPTER FIVE**Page 152-181****Rabbit model of graded SIRS and MODS**

- Aims and objectives
- Materials and methods
- Results
 - Phase one experiments: Dose response relationship
 - Primary outcome
 - Dose response curve
 - Secondary outcome measures
 - Cardiovascular
 - Clinical
 - Heart rate
 - Mean arterial pressure
 - Central venous pressure
 - Two peaks hypothesis
 - Wet to dry ratio
 - Respiratory
 - P/F ratio
 - Oxygenation Index (OI)
 - Wet to dry ratio
 - Renal
 - Creatinine
 - Urine output
 - Wet to dry ratio
 - Hepatic
 - LFT
 - Wet to dry ratio
 - Gastrointestinal
 - Wet to dry ratio
 - Leukocytes
 - Coagulation, platelets and fibrinogen
 - Phase two experiments: Inter-individual variation
 - Primary outcome
 - Secondary outcome measures
 - Cardiovascular
 - Mean arterial pressure
 - Respiratory
 - P/F ratio
 - OI
 - Renal
 - Creatinine
 - Hepatic
 - LFT
 - Gastrointestinal
 - Leukocytes
 - Coagulation

- Discussion
- Conclusion

CHAPTER SIX**Page 182-217****Biomarkers of the innate immune response in rabbits**

- Introduction
- Material and methods
- Results
 - Acute phase proteins
 - CRP
 - Fibrinogen
 - Cytokines
 - TNF α
 - IL-1 β
 - IL-5
 - IL-6
 - IL-8
 - IL-10
 - Complements
 - C3
 - Lectin pathway activation
 - Other inflammatory mediators
 - Nitric oxide
 - TGF- β 1
 - Neutrophil Elastase
 - Coagulative mediators
 - Tissue Factor
 - Thrombomodulin
 - Fibrinolytic mediators
 - PAI-1
 - Fibrinogen
- Discussion
 - Acute phase proteins
 - Cytokines
 - Complements
 - Other inflammatory mediators
 - Coagulative mediators
 - Fibrinolytic mediators
- Conclusion

CHAPTER SEVEN**Page 221-242**

Cellular components of the innate response in rabbits

- Introduction
- Materials and methods
- Results
 - Perivascular oedema
 - Alveolar haemorrhage
 - Thrombosis of pulmonary vessels
 - Alveolar consolidation
 - Goblet cell hyperplasia
 - Macrophages in alveolar spaces
 - Lymphoid aggregates
 - Multinucleated giant cells
 - Eosinophils
 - Eosinophilic intracytoplasmic inclusions
 - Mononuclear and poly-nuclear leukocytes
 - Total score
- Discussion
- Conclusion

CHAPTER EIGHT**Page 243-257**

Oxidative stress, DNA damage and apoptosis in a rabbit model of severe systemic inflammatory response syndrome

- Introduction
- Results
 - Total antioxidant status
 - DNA damage
 - Apoptosis
- Discussion
- Conclusion

CHAPTER NINE**Page 258-273***Conclusion.*

- ECMO is superior to conventional ICU management only in a selected group of severe SIRS patients
- Limitations to the management of severe SIRS on ECMO
- The rabbit model
- The next phase of animal experiments
- Thrombin; a common biochemical component for feedback escalation and progression of severe SIRS
- Closing remarks

APPENDIX ONE**Page 274-276****APPENDIX TWO****Page 277-278****BIBLIOGRAPHY****Page 279-310**

ABSTRACT

Introduction: Extracorporeal Membrane Oxygenation (ECMO) is a relatively new supportive therapy for severe Systemic Inflammatory Response Syndrome (SIRS). However ECMO itself exacerbates inflammatory, coagulative and anti-fibrinolytic responses in the blood (Haematic response).

Hypothesis: ECMO is an acceptable supportive therapy for patients with severe SIRS despite triggering haematic response.

Objectives: To develop a reliable and reproducible animal model of SIRS to investigate strategies for reducing the haematic response.

Materials and Methods: *Literature review:* (I) The Systemic Thrombo-inflammatory Pathway (STIP). (II) The haematic response. *Clinical studies:* (I) Institutional review of ECMO for severe SIRS. (II) Comparative review of oxygenators performance. *In-vivo studies:* Animal experiments, (I) Development of a rabbit model of graded SIRS. (II) Dose response relationship between administered intravenous endotoxin and fatal Multi-organ Dysfunction Syndrome (MODS). (III) Inter-individual variation amongst rabbits receiving a lethal dose of endotoxin. *In-vitro studies:* Evaluation of the cellular and biochemical components of STIP in rabbits. (I) Development of rabbit ELISA. (II) Determination of the normal range, dose response and inter-individual variation. (III) Immunohistochemical evaluation of endotoxin induced lung injury. (IV) Dose related oxidative stress and DNA damage and (V) apoptosis in rabbit lung.

Results: There is a reciprocal relationship between graded SIRS and the outcome of ECMO. There is a linear relationship between the dose of endotoxin and the development of graded SIRS in rabbits. Significant DNA homology and cross-reactivity exists between humans and rabbits making this a useful model for immune experimentation.

Conclusions: ECMO is superior to conventional ICU management in selected groups of patients. New oxygenator technology has significantly reduced the haematic response to ECMO, however it has failed to influence survival. Cellular components such as neutrophils play a central role in SIRS activation, however thrombin appears to be the common biochemical component for feedback escalation and progression of severe SIRS to MODS.

ACKNOWLEDGEMENTS

My first thanks goes to my supervisors Giles Peek, Richard Firmin and Sir Peter Bell each of whom in their own way had great influence on making this work happen.

I am grateful to the following collaborators, Andrzej Sosnowski and Hillary Killer (ECMO), Richard Reeves (Perfusion), Tony Dux (Radiology), Tony Buckwell (Veterinary sciences), Roger James (Immunology), Kevin West (Histology and immunohistochemistry), Wilhelm Schwaeble (Complements), Jo Lunec (Oxidative stress) and Michael Festing and Nick Taub (Statistics).

The following scientists and technical specialists helped me inch forward with the lab work. Mike Jackson, Julie Chamberlain, Jan Harris, Peter Willan, Evelyn Topping, Debbie Bursnall, Jenny Nicholls, Colin Brooks, Peter Jubby, Terry McGee, Mark Esward, Joanne Jeory, Kay Holmes, Silke Roscher, Nick Lynch, Stephen Pool, Jason Wills, Christina Guillen, David Machin, Chris Thornburgh, Elizabeth Toon, Adel Hinnis and Nalini Foreman.

The following clinicians deserve to be mentioned for their contributions, Suneel Pooboni, Clair Westrope, Neil Roberts, Chris Harvey, Ravin Tiruvoipati, Senthil Balasubramanian and Inger Oye.

Thanks to all the nursing staff and ECMO specialists at Glenfield Hospital. Their dedication to the ECMO program and to saving lives inspired me to investigate this worthy cause.

My thanks to Rukhsana Tilly, Janice Garner, Linda Bradshaw, Rose Aldwinckle, Judith Amodio and Jane Allen for their guidance since the start of this project.

For provision of funding I would like to thank the Newman Foundation and my understanding bank manager Michelle Ward.

Last but certainly not least I'd like to thank Tracy who I married on the 29th of March 2003 during the period of this research. Her contribution to this thesis is immeasurable and I dedicate this thesis to her, my father Dr Ehsanollah Khoshbin who's footsteps I followed and my mother Zohreh who's determination I inherited but unfortunately not her good looks.

ABBREVIATIONS

Abbreviation Full Word

ACCP	American Collage of Chest Physicians
ACT	Activated Clotting Time
AECC	American-European Consensus Conference
AKP	Alkaline Phosphatase
ALI	Acute Lung Injury
APACHE II	Acute Physiology and Chronic Health Evaluation II
APC	Activated Protein C
A-PMP	Adult Poly-methyl Pentene
APTTR	Activated Partial Thromboplastin Time Ratio
ARDS	Acute Respiratory Distress Syndrome
ARK	Animal Research Kit
A-S	Adult Silicon
ATN	Acute Tubular Necrosis
ATS	American Thoracic Society
BAL	Broncho-alveolar Lavage
BALF	Broncho-alveolar Lavage Fluid
CARS	Counter Anti-inflammatory Response Syndrome
CD4 T cells	Helper T Cells
CDH	Congenital Diaphragmatic Hernia
CESAR	Conventional ventilation versus ECMO for Severe Adult Respiratory failure
Cm H ₂ O	Centimetre of Water
COPD	Chronic Obstructive Pulmonary Disease
COSHH	Control of Substances Hazardous to Health
CRP	C-reactive Protein
CV	Coefficient of Variation
CVVH	Continuous Veno-venous Haemofiltration
CXR	Chest X-rays
DAPI	4',6-diamidino-2-phenylindole
DIC	Disseminated Intravascular Coagulation
ECG	Electrocardiogram
ECLS	Extra-corporeal Life Support

ECMO	Extra-corporeal Membrane Oxygenation
ELISA	Enzyme-linked Immunosorbent Assay
ELSO	Extracorporeal Life Support Organisation
EPCR	Endothelial Protein C Receptor
ESICM	European Society of Intensive Care Medicine
EuroSCORE	European System for Cardiac Operative Risk Evaluation
FDPs	Fibrin Degradation Products
FFP	Fresh Frozen Plasma
FHF	Fulminant Hepatic Failure
FIO ₂	Fractional Inspired Oxygen
FITC	Fluorescein Isothiocyanate
g	Gram
g/dl	Gram Per Decilitre
GCS	Glasgow Coma Scale
H ₂ DCF-DA	Dichlorodihydrofluorescein Diacetate
H ₂ O ₂	Hydrogen Peroxide
HAS	Human Albumin Solutions
HFOV	High Frequency Oscillatory Ventilation
hpf	High-power Field
Hr	Hours
HRP	Horseradish Peroxidase
HSP	Heat Shock Proteins
I/R	Ischaemic Reperfusion
ICAM	Intracellular Adhesion Molecules
ICU	Intensive Care Unit
IFN	Interferon
IgE	Immunoglobulin E
IL	Interleukins
IMS	Industrial Methylated Sprit
iNO	Inhaled Nitric Oxide
INR	International Normalized Ratio
IPPV	Intermittent Positive Pressure Ventilation
ISF	International Sepsis Forum
ISS	Injury Severity Score

kg	Kilogram
L	Litres
LBP	LPS Binding Protein
LDH	Lactate Dehydrogenase
LIS	Lung Injury Score
LPS	Lipopolysaccharide
MAC	Membrane Attack Complexes
MARS	Molecular Adsorption Recycling System
MAS	Meconium Aspiration Syndrome
MCP	Monocyte Chemoattractant Protein
MDA	Malondialdehyde
mg	Milligram
Min	Minutes
MIP	Macrophage Inflammatory Protein
ml	Millilitre
mm Hg	Millimetre of mercury
MODS	Multi-Organ Dysfunction Syndrome
MODS	Multiple Organ Dysfunction Score
M _v SO ₂	Mixed Venous Saturation
ng	Nanogram
NGS	Normal Goat Serum
NK Cells	Natural Killer Cells
nm	Nanometer
N-PMP	Neonatal Poly-methyl Pentene
NRP	Normal Rabbit Plasma
N-S	Neonatal Silicon
OD	Optical Densities
OFR	Oxygen Free Radicals
PaCO ₂	Partial pressure of arterial carbon dioxide
PAI-1	Plasminogen Activation Inhibitor-1
PaO ₂	Partial Pressure of Arterial Oxygen
PaO ₂ /FiO ₂	Partial pressure of arterial oxygen / Fractional inspired oxygen
PAR	Pressure Adjusted Heart Rate
PBS	Phosphate Buffered Saline

PC	Protein C
PEEP	Positive End-expiratory Pressure
PEMOD	Paediatric Multiple Organ Dysfunction
PET	PBS/EDTA0.5M/Tween 20
pg	Pecogram
PIP	Peak Inspiratory Pressure
PMP	Poly-methyl Pentene
PPHN	Persistent Pulmonary Hypertension of the Neonates
PRISM	Paediatric RISK of Mortality
PTS	Paediatric Trauma Score
PVR	Pulmonary Vascular Resistance
RBC	Red Blood Cell
RDS	Respiratory Distress Syndrome
ROS	Reactive Oxygen Species
RT	Room Temperature
SCCM	Society of Critical Care Medicine
SD	Standard Deviation
Sec	Seconds
SIRS	Systemic Inflammatory Response Syndrome
SIS	Surgical Infection Society
SM	Silicon Membrane
SOFA	Sepsis Related Organ Failure Assessment
STIP	Systemic Thrombo-inflammatory Pathways
T	Thrombin
TAFI	Thrombin-activatable Fibrinolysis Inhibitor
TAPVD	Total Anomalous Pulmonary Venous Drainage
TAS	Total Antioxidant Status
TF	Tissue Factor
TGF β	Transforming Growth Factor Beta
TM	Thrombomodulin
TMB	3,3',5,5'-Tetramethylbenzidine
TNF α	Tissue Necrosis Factor
t-PA	tissue Plasminogen Activator
TT	Thrombin Time

u/kg/hr	Unit Per Kilogram Per Hour
VV-ECMO	Veno-venous ECMO
μg	Microgram
μl	Microlitre
μm	Micrometer

PUBLICATIONS & PRESENTATIONS

1) **Khoshbin E**, Dux A, Firmin RK, Peek GJ.

A comparison of radiographic signs of pulmonary inflammation during ECMO between silicon and poly-methyl pentane oxygenators.

Perfusion 2007; 22: 15-21.

2) **Khoshbin E**, Peek GJ, Sosnowski AW, and Firmin RK.

A potential propensity for failure secondary to clot embolism in neonatal ECMO:

Perfusion 2005; 20:177-181.

3) **Khoshbin E**, Machin D, Killer H, Peek GJ, Sosnowski AW, Firmin RK.

Poly-methyl pentene oxygenators have improved gas exchange capability and reduced transfusion requirements for adult extra-corporeal membrane oxygenation.

ASAIO Journal 2005; 51(3): 281-287.

4) **Khoshbin E**, Machin D, Killer H, Peek GJ, Sosnowski AW, Firmin RK.

Performance of poly-methyl pentene oxygenators for neonatal extra-corporeal membrane oxygenation: a comparison with silicon membrane oxygenators.

Perfusion 2005; 20:129-134.

5) **Khoshbin E**, Firmin RK, Bell P, Peek GJ.

Dose-response Relationship for the Development of ARDS and Multi-system Organ Failure by Administration of Intravenous Endotoxin L-2143 in Rabbits.

Presented at **The Society of Cardiothoracic Surgeons of Great Britain and Ireland research meeting**, Leicester and **The UK ECMO conference**, Glenfield Hospital, Leicester in Jun 2003.

6) **Khoshbin E**, Machin D, Killer H, Peek GJ, Sosnowski AW, Firmin RK. Poly-methyl Pentene Oxygenators for Extracorporeal Life Support – The Early Performance is Encouraging.

Presented at the **Perfusion congress**, Blackpool in Nov 2002.

7) **Khoshbin E**, Firmin RK, Bell P, Peek GJ.

Sepsis as an Indication for Extracorporeal Membrane Oxygenation.

Presented at the **UK ECMO conference**, Glenfield Hospital, Leicester. Jun 2002.

PREFACE

The Systemic Inflammatory Response Syndrome (SIRS), a whole body inflammatory activation {Goris et al. 1985} and ultimately Multi-Organ Dysfunction Syndrome (MODS) {Barie et al. 1995} are the major causes of death in the Intensive Care Units {Brun-Buisson et al. 2000}. They account for 50-80 percent of deaths in surgical intensive care units {Angus et al. 2001}. Acute Respiratory Distress Syndrome (ARDS) is one of the commonest manifestations of SIRS. ARDS is really the effects of this whole body disease seen in the lung. Many factors can stimulate SIRS, including systemic infection, burns, multiple trauma, pancreatitis and prolonged surgery with multiple blood transfusions. During this, the bodies natural defence mechanisms are switched on inappropriately leading to widespread inflammation which damages the cells lining the blood vessels (endothelium) throughout all the organ systems, a so called “pan-endothelial injury” {Moat et al. 1993}.

Attempts to modify the disease process and improve outcome using specific anti-mediators are usually effective in the laboratory {Leff et al. 1995} but ineffective in clinical practice {Clark et al. 1998}. We believe that this is because the inflammatory cascade is very complex, being composed of many branching and interweaved pathways, more like a network. Thus interrupting the pathway at one point has so far had little effect on the process as a whole. We know that this inflammatory cascade is important as high levels of inflammatory mediators correlate with a poor outcome {Meduri et al. 1995}.

A relatively new treatment for patients with ARDS and MODS who continue to deteriorate despite maximal conventional management is extra-corporeal membrane oxygenation (ECMO). This technique uses a modified heart-lung machine to provide prolonged cardiac or pulmonary support in the intensive care unit, thereby giving the patient a chance to recover. Unfortunately, however, ECMO itself causes activation of systemic inflammatory response {Plots et al. 1993}, which in some cases may hinder the resolution of the underlying disease.

We believe that ECMO is a useful treatment for severe SIRS however the outcome of using ECMO may still improve by manipulating the systemic inflammatory response at either a cellular or biochemical level. We believe that this systemic inflammatory response is largely mediated by neutrophils, which play a central role in SIRS. This systemic response may therefore be dampened by using specialised filters that remove neutrophils (Neutrophil depletion filter) or by removing effector molecules from the serum, for example by using Molecular Adsorption Recycling System

(MARS). Altering the components of the ECMO circuit for example the oxygenator may also influence the systemic inflammatory response, which may alter outcomes.

The understanding of the cellular, molecular and biochemical interactions that forms this systemic inflammatory response is essential and solid evidence is required to justify clinical trials. Certain aspects of this evidence can only be gathered by animal experimentation. We have therefore set out to review the use of ECMO for severe SIRS in our institution and to develop a reliable and reproducible animal model of SIRS that can assist in our ultimate challenge to improve the outcomes for patients with severe SIRS and MODS.

Chapter One

INTRODUCTION

INTRODUCTION

“ There is a circumstance attending accidental injury that does not belong to disease, namely that the injury done has in all cases the tendency to produce both the disposition and the means to cure”

John Hunter, London 1794

One of the greatest scientists of all time, surgeon John Hunter recognised that the host response to trauma was not only a means for recovery but also a clinical disposition to disease. This host response is the bodies natural defence mechanism whose function is to protect the host throughout its lifetime by seeking out and inactivating foreign antigens including those from microorganisms as well as mutant host cells with malignant potential and transplanted tissues {Radmond 1996}.

The term Systemic Inflammatory Response Syndrome (SIRS) denotes a clinical syndrome believed to be the result of an overly activated host response. This neologism embodied two evolving concepts in an understanding of the hosts response to an acute life threatening challenge. Firstly the responses of the host was similar to both infectious and non-infectious stimuli and secondly the severity of the response, rather than its trigger, was the critical determinant of patient survival {Okusawa et al. 1988, Parrillo et al. 1990 & Marshall et al. 1988}. This excessive hypercatabolic response that precipitates organ dysfunction has been termed malignant systemic inflammation {Moor et al. 1995}.

Many factors can stimulate SIRS, including systemic infection, burns, multiple trauma, pancreatitis and prolonged surgery with multiple blood transfusions. During SIRS, the bodies natural defence mechanisms are switched on inappropriately leading to widespread inflammation. This causes damage to the vascular endothelium throughout all the organ systems, a so-called “pan-endothelial injury” {Moat et al. 1993} and ultimately leads to Multiple Organ Dysfunction Syndrome (MODS). It is important that clinicians and researchers have the tools necessary to recognise SIRS and MODS in order to treat or to investigate novel treatment strategies against this deadly syndrome.

AIMS AND OBJECTIVES

This chapter will provide a conceptual review of the SIRS, MODS and Extracorporeal Membrane Oxygenation (ECMO). It examines the role of ECMO in severe SIRS, SIRS shock and MODS, and discusses the pathophysiology of the Systemic Thrombo-inflammatory Pathways (STIP) and “the Haematic response”. This will lay grounds, for the forthcoming chapters, using stratification and staging systems as a tool to discuss the outcomes of our clinical and animal experiments.

DEFINITIONS AND CLASSIFICATIONS

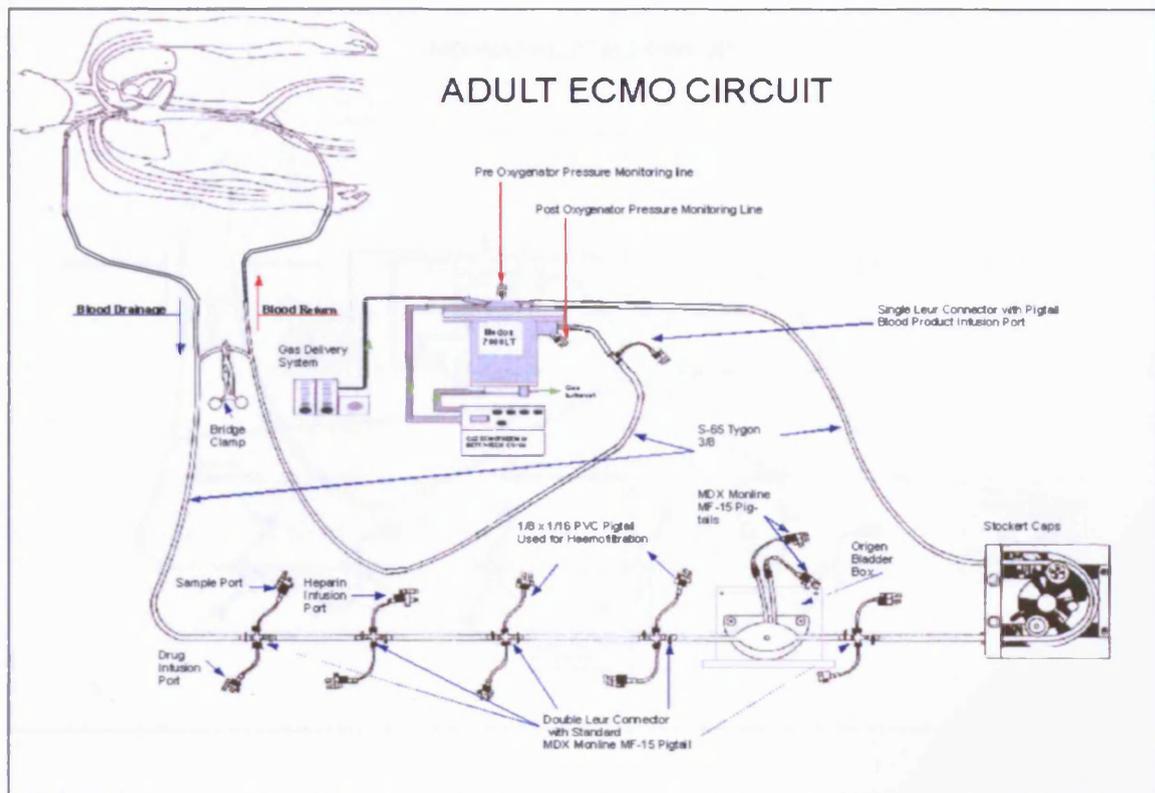
EXTRACORPOREAL MEMBRANE OXYGENATION (ECMO)

Extra-corporeal Membrane Oxygenation is an artificial heart-lung support system. It is a modified form of cardio-pulmonary bypass used in open-heart surgery, that enables prolonged period of cardiopulmonary support in an intensive care setting (Diagram 1.1).

The use of ECMO is currently restricted to only a few specialised units. Large cannulae are inserted via central blood vessels whilst anticoagulants prevent clot formation as blood comes into contact with the circuit. Patients are therefore at a high risk of haemorrhage not only from the cannulation / surgical sites, but also from bleeding into organs or body cavities. The use of ECMO is therefore restricted to occasions when conservative means of organ support fail. However as we become more experienced, and disease specific outcomes of ECMO become well recognised, the threshold for using ECMO will be lowered.

After two large clinical trials in the U.S.A. by Bartlett et al. 1985 and O'Rourke et al. 1989 and the collaborative randomised trial of neonates in the U.K. {Anonymous, 1996}, ECMO became an established therapy in neonates with severe but potentially reversible conditions, such as meconium aspiration syndrome (MAS) and persistent pulmonary hypertension of the neonates (PPHN). ECMO is however not as yet an established therapy for adult respiratory support.

Diagram 1.1: Illustrating the set up for adult ECMO circuit.

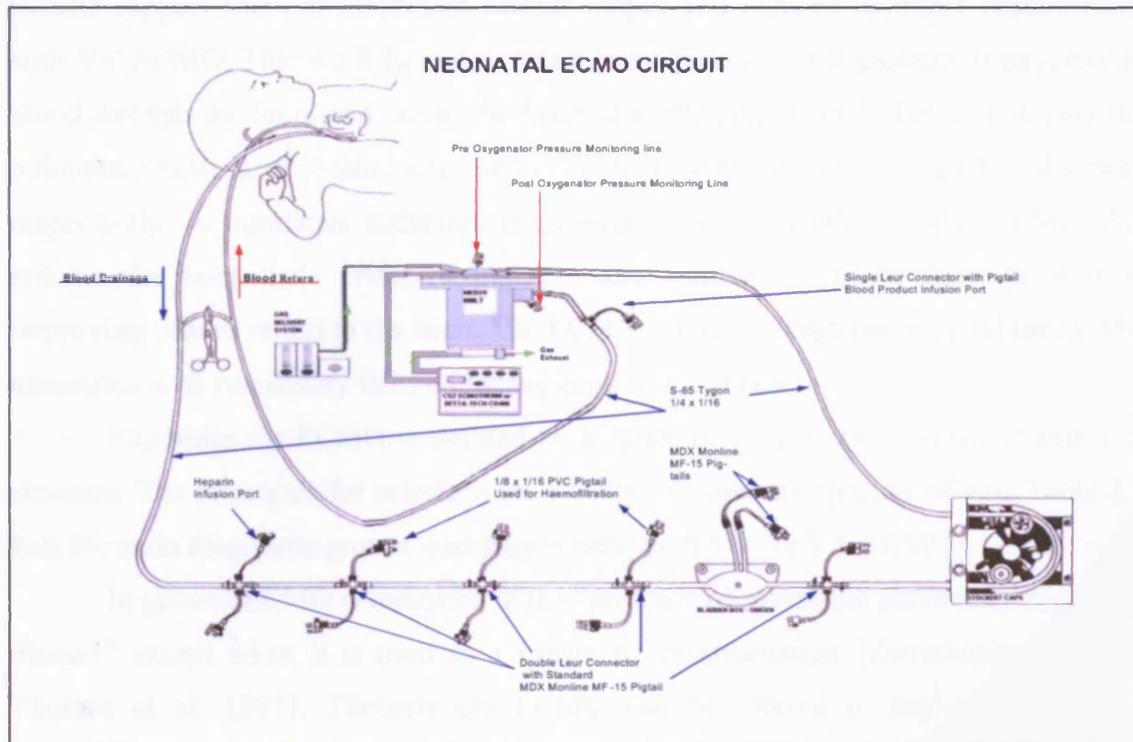


There are two types to ECMO, Veno-arterial ECMO (VA-ECMO) is preferred for patients requiring cardiac (Especially right heart) support and veno-venous (VV-ECMO) approach predominantly for isolated respiratory support {Klein et al. 1985 & Bartlett et al. 1995} with a degree of overlap. For example the use of veno-arterial ECMO in cases of severe Pertussis, where cardiac insufficiency is forecasted {Pooboni et al. 2004}.

In VA- ECMO blood is drained from a central vein (Jugular or femoral veins percutaneously or the right atrium during an open heart procedure), pumped through an oxygenator and returned to the patient via a central artery (i.e. The carotid or femoral arteries or the ascending aorta) bypassing the heart and lungs. This provides cardio-respiratory support and leads to instantaneous haemodynamic stability.

In contrast during VV-ECMO blood is returned back to a central vein or the right atrium without bypassing the heart or lungs. In the neonates cannulation for VV-ECMO is done through a single, double lumen cannula placed in the right internal jugular vein (Diagram 1. 2).

Diagram 1.2: Illustrating the set up for neonatal ECMO circuit.



It should be noted that VA-ECMO is particularly beneficial in right-sided cardiac support, for example post cardiac surgery for congenital heart diseases with secondary pulmonary hypertension in children or in massive pulmonary embolus in adults bypassing the lungs and hence reducing the right heart strain. It may also be used as a bridge to cardiac / cardiopulmonary transplantation or to support myocarditis and transplant rejection {Zwischenber 2000}. However during VA-ECMO an increase in aortic pressure increases the left ventricular afterload. Although this is not commonly a problem in neonates and children it may pose a threat to an adult with compromised left ventricular function by causing ventricular dilatation and myocardial stun {Holley et al. 1994 & Martin et al. 1991}. Allowing the left ventricle to eject by minimising the rate of blood flow into the aorta, reducing LV afterload (Either by drug therapy or by intra aortic balloon counter pulsation) or by ventricular venting may prevent this. Increasing the FiO_2 on the ventilator above the normal rest setting from 0.3 to 0.5 may also improve myocardial contractility by improving coronary oxygen supply without significant risk of oxygen toxicity.

Preliminary reports of cardiac function immediately before and after the start of ECMO support show an improved cardiac output and reduced inotropic requirements with VV-ECMO. That we believe is a direct consequence of, the passage of oxygenated blood through the lung, and out of the heart in to the sinuses of Valsalva, reducing the pulmonary vascular resistance and increasing the oxygenation of coronary blood supply respectively. A significant reduction in positive pressure ventilation after ECMO also reduces the tamponade effect of high-pressure ventilation on the heart as well as improving venous return to the heart. VV-ECMO is now the preferred method for ECMO associated with respiratory failure in many centres worldwide.

Eligibility for ECMO is defined by a series of general and specific (Table 1.1) criterion. The principals for selection are exactly the same irrespective of age. Table 1.2 lists the main diagnostic groups who may benefit from VV- or VA-ECMO.

In general ECMO is indicated in the “presence of severe but potentially reversible disease” except when it is used as a bridge to transplantation {Zwischenber 2000 & Thomas et al. 1997}. Theoretically ECMO can be offered to any patient with a potentially reversible cardiac, pulmonary or cardiopulmonary disease {Firmin et al. 1999}. However the outcome of some potentially reversible diseases is still poor and without novel therapeutic strategies any improvement seems unlikely.

The following generalised questions are used to justify selection versus exclusion from ECMO. a) Does the patient have a potentially reversible pulmonary, cardiac or cardiopulmonary disease? b) Are the neurological status and function of other organs consistent with a reasonable outcome? c) Is limited heparinisation contraindicated? d) Is there anything to be gained by further conventional treatment?

Specific criterion for cardiac support is common between all age groups. According to the University of Michigan they are; cardiac index $< 2 \text{ L/m}^2 / \text{min}$ for 3 hours, metabolic acidosis with a base deficit < -5 for 3 hours, hypotension (Mean arterial blood pressure < 40 in neonates < 50 in infants and < 60 in children or adults) with oligurea $< 0.5 \text{ ml/Kg/hr}$ for three hours or failure to wean from bypass in post operative patients.

The specific criterion for respiratory support in the presence of reversible lung disease varies with the age of the patient.

In neonates they are; gestation age > 34 weeks, birth weight $> 2 \text{ Kg}$ and an oxygenation index ($\text{OI} = \text{Mean airway pressure} \times \text{FiO}_2 / \text{Postductal PaO}_2$) > 40 for 3 hours which may be reduced to 25 when on treatment with inhaled nitric oxide (iNO)

{Belgium et al, 1999 & NINOS 2000}, or high frequency oscillatory ventilation (HFOV), and in the absence of contraindications to limited anticoagulation (For example > grade one intracranial haemorrhage, or coexisting severe congenital cardiac anomalies). Note that the period of intermittent positive pressure ventilation (IPPV) / High FiO₂ treatment should not exceed ten days in neonates as this predisposes them to lung fibrosis due to barotrauma and oxygen toxicity. Recent research suggests that novel therapies such as iNO and HFOV have decreased the use of ECMO in the neonates {Hintz et al. 2000 & Al-Alaiyan et al. 1999}. However this may lead to a delay in ECMO therapy of non-responders. This means referral of sicker neonates with poorer prognosis, but not necessarily with a higher OI ratio.

Eligibility criterion for paediatric ECMO are; aged between 28 days and 18 years, in the presence of severe but reversible respiratory disease, refractory to conventional treatment. This may manifest as a PEEP > 5 CmH₂O, Peak inflation pressure >30 CmH₂O, FiO₂ >0.8, PaO₂/FiO₂ < 150, Alveolar-arterial oxygen gradient > 450 mmHg [A-a O₂ in mmHg = FiO₂ x (P_{atm} - 47) - (PaCO₂ / 0.8) - PaO₂, where P_{atm} is 760 mmHg and normal gradient can be estimated as age (years)/4 plus 4], implying impaired gas diffusion or presence of uncompensated respiratory acidosis, PH < 7.2. The cut off for the duration of conventional ventilation may be dependent on the age of the child that is nine days for < 2 years old, eight days for 2-8 years old and seven days for >8 years old according to this institutions protocol.

Components:	Score Values				
	0	1	2	3	4
PaO ₂ / FiO ₂ (mmHg)	≥300	225-299	175-224	100-174	<100
CXR (Quadrants)	Normal	1	2	3	4
PEEP (CmH ₂ O)	≤5	6-8	9-11	12-14	≥15
Compliance (ml/CmH ₂ O)	≥80	60-79	40-59	20-39	≤19

Table 1.1: Illustrating the Murray scoring system. The lung compliance is calculated as the respiratory tidal volume in mls divided by the difference between peak inspiratory pressure and positive end expiratory pressure in Cm H₂O (TV/PIP-PEEP). The score is calculated by adding each individual scores from each component and dividing it by 4 to one decimal place. The total score may range between 0-4. Note that the FiO₂ should be 1 when calculating the Murray score.

Adults may be eligible if; aged between 18-65 years, on high-pressure (> 30 cm H₂O) and / or high FiO₂ (> 0.8), ventilation with a Murray score (Table 1.1) of > 3 {Peek

et al. 1998} or an uncompensated respiratory acidosis (PH < 7.2). Patients with intracranial bleeding or any uncontrollable bleed into an organ or cavity, which could get worse by anticoagulation, would not be eligible for ECMO. This would become less of a problem if ECMO circuits could run without anticoagulants.

Table 1.2: This table illustrates the main diagnostic groups of neonates, children and adults, which may be considered for cardiac, pulmonary or cardiopulmonary support by ECMO. Patients requiring cardiopulmonary support may receive VA-ECMO from the start or may have VV- to VA- conversion in cases of marked cardiac insufficiency with poor inotropic response.

Neonatal	Paediatric	Adult
MAS PPHN RDS CDH Sepsis Pneumonia Pulmonary haemorrhage Failure to wean from bypass Bridge to cardiac surgery Pulmonary atresia TAPVD	ARDS Pneumonia Sepsis Severe airway obstruction Aspiration Failure to wean from bypass Bridge to cardiac transplant Transplant rejection Viral myocarditis	ARDS Pneumonia Sepsis Pulmonary vasculitis Severe airway obstruction Aspiration Massive pulmonary emboli Failure to wean from bypass Bridge to cardiac transplant Transplant rejection

SYSTEMIC INFLAMMATORY RESPONSE SYNDROME (SIRS)

Roger C Bone in August 1991 {Bone et al. 1992} Chaired the American College of chest physicians / Society of Critical Care Medicine consensus conference committee held in Chicago. Their goal was to provide both a conceptual and a practical framework to define the systemic inflammatory response syndrome following a wide variety of causes including infection generally known as “Sepsis” and sepsis-associated organ dysfunction. The following definitions were derived from their recommendations in the hope to make early bedside detection of disease, thus allow early therapeutic interventions. They also aid standardization of research protocols to improve dissemination and application of information derived from clinical studies, for better understanding of cellular and immunologic mechanisms that lead to multiple organ dysfunction and death. SIRS is the systemic inflammatory response to a variety of severe clinical insults such as infection, trauma, burns, ischaemia, haemorrhagic shock, cardiac surgery, lung transplantation, pancreatitis and immune-mediated organ injury (Diagram 1.3). The response is manifested by two or more of the following conditions: (1) temperature >38 °C or <36°C; (2) heart rate > 90 beats per minute (or > 90th Centile for

age); (3) respiratory rate >20 breaths per minutes (or $> 90^{\text{th}}$ Centile for age) or $\text{PCO}_2 < 32$ mmHg; and (4) white blood cell count $> 12,000/\text{cubic mm}$, $<4,000/\text{cubic mm}$, or $>10\%$ immature neutrophils (“bands”) in absence of other causes of neutropenia and leucopenia. Muckart and Bhagwanjee {Muckart et al. 1997} used a similar approach to defining stages of SIRS as ACCP/SCCM. They described SIRS as a spectrum of conditions that may lead to severe SIRS (Patients with a non infective insult but pathophysiologic changes equivalent to the definitions of severe sepsis), sterile shock (Patients with a non infective insult but pathophysiologic changes equivalent to septic shock) and ultimately MODS. These critical stages in SIRS just like sepsis are likely to have independent prognostic implications {Sprung et al. 1990}. Table 1.4 jointly define each stage. The well-defined manifestations of severe SIRS / SIRS shock irrespective of cause is the development of organ dysfunction such as acute respiratory distress syndrome, acute cardiac failure / arrest, oligurea, electrolyte imbalance and acute tubular necrosis, acute hepatic dysfunction, GI bleed, leucopenia, thrombocytopenia and disseminated intravascular coagulation (Table 1.6). Classifying patients according to these critical stages as SIRS/Sepsis allows stratification of patients according to changes in metabolism that are characteristic of inflammatory disorders. This classification system has the advantage of simplicity because it is based on commonly obtained clinical data {Goldman et al. 1999}.

ACUTE RESPIRATORY DISTRESS SYNDROME (ARDS)

Acute respiratory distress syndrome is defined as a syndrome of inflammation and increased lung capillary permeability associated with a number of clinical, radiological and physiological abnormalities that cannot be explained by, but may coexist with heart failure (American-European Consensus Conference 1994). ARDS is one of the most common manifestations of SIRS. It is really the effects of this whole body disease seen in the lung.

The diagnosis of ARDS is essentially clinical. The two most common diagnostic tools available are: the ratio between the arterial blood oxygenation and the oxygen delivered by the ventilator, the $(\text{PaO}_2)/(\text{FiO}_2)$ ratio and the lung injury score (LIS) also known as the Murray score, illustrated above. ARDS may be diagnosed if the $(\text{PaO}_2)/(\text{FiO}_2)$ ratio is ≤ 200 mmHg (≤ 300 is classed as acute lung injury (ALI) which may lead to ARDS) or the $\text{LIS} \geq 3$.

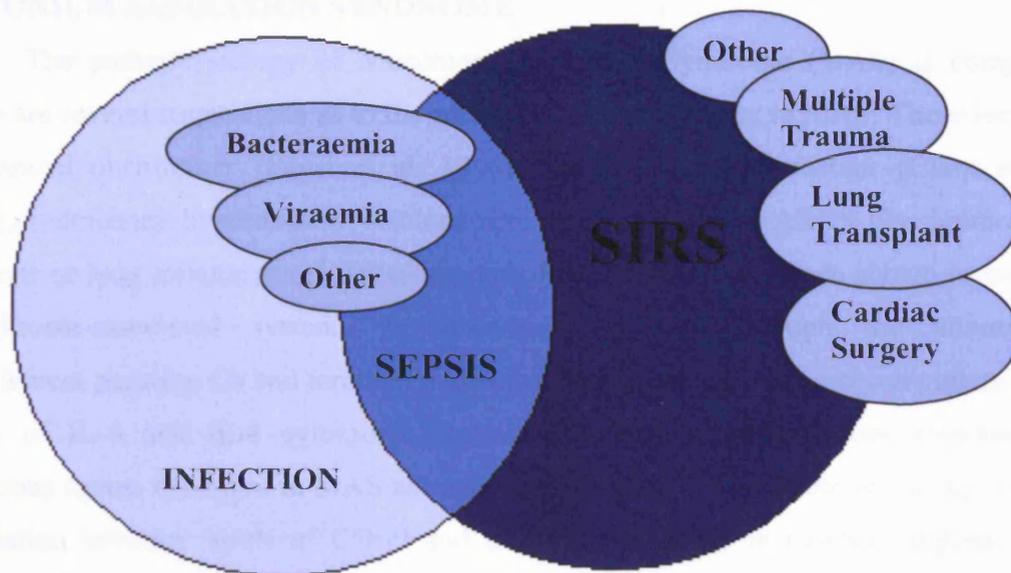


Diagram 1.3: Pie Chart illustrating SIRS and its common causes.

ARDS may be caused either by a direct or an indirect lung injury. Direct injury may be caused by pneumonia, aspiration or inhalation of toxic substances, pulmonary contusion and near drowning {Atabai et al. 2002}. Respiratory infection is considered as one of the most common aetiologies leading to direct ARDS {Baumann et al. 1986}. Multiple trauma, burns, Infection, fat emboli and haemorrhagic shock are all associated with a high risk of progression to ARDS. The incidence of ARDS due to infection is 40% {Pepe et al. 1982 & Hudson et al. 1995}.

The most important pathological finding in the lung during the early stages of ARDS is severe pulmonary oedema, due to increased permeability of the capillary endothelial and alveolar epithelial barriers (Exudative phase). Furthermore there is an increase in pulmonary vascular resistance (PVR) due to vasoconstriction and thromboembolism, which further increases the pulmonary oedema {ten Cate et al. 2001}. In an attempt to recover (Proliferative phase) alveolar type II cells proliferate and differentiate to type one cells {Bellingan et al. 2002}. Fibroblasts proliferate within the alveolar wall and migrate into airspaces where they organise the exudates and deposit collagen into fibrous tissue {Tomashefski et al. 2000}. As soon as 10 days after the first signs of respiratory failure, if the patient survives, intra-alveolar and interstitial fibrosis (Fibrotic phase) occurs {Bellingan et al. 2002}.

MECONIUM ASPIRATION SYNDROME

The pathophysiology of Meconium Aspiration Syndrome (MAS) is complex. There are several suggestions as to the mechanism of lung injury in MAS. These include mechanical obstruction {Foust et al. 1996}, inactivation of surfactant {Clark et al. 2000}, pulmonary hypertension {Holopainen et al. 1998} and ARDS-like pulmonary response or lung inflammation {Soukka et al. 1997}. MAS have been shown to induce complement-associated systemic inflammatory response through the alternative complement pathway C3 and terminal pathway C5b-9 complexes in newborn piglets. The levels of IL-6 and IL-8 cytokines, central mediators of inflammatory response to infectious agents increased in MAS animals compared to control. There was a significant correlation between levels of C5b-9 and these complements in newborn piglets. The concentrations of C5b-9 in these piglets correlated closely with their oxygenation index {Castellheim et al. 2005}. Therefore systemic inflammatory response is seen in experimental MAS. The mechanism of meconium aspiration leading to activation of inflammatory response however may be associated to the presence of a variety of toxins present in meconium.

SEPSIS

The systemic response to infection, manifested by two or more of the following conditions as a result of infection: (1) temperature $>38^{\circ}\text{C}$ or $<36^{\circ}\text{C}$; (2) heart rate > 90 beats per minute (or $> 90^{\text{th}}$ Centile for age); (3) respiratory rate >20 breaths per minutes (or $> 90^{\text{th}}$ Centile for age) or $\text{PCO}_2 < 32$ mmHg; and (4) white blood cell count $> 12,000/\text{cu mm}$, $<4,000/\text{cu mm}$, or $>10\%$ immature neutrophils (“bands”) in absence of other causes of neutropenia and leucopenia {Bone et al. 1992}. That is SIRS in the presence of an infection. This is the accepted definition by The International Sepsis Forum (ISF), according to Sibbald and Rackow {Sibbald et al. 1986}. Despite these consensus studies, the definition of sepsis and its clinical manifestations are still a source of controversy. No single physiological or laboratory parameter can universally identify sepsis. In many cases for example in the elderly, neonates or immuneosuppressed patients minimal or absent host response leads to colonisation, however colonisation itself may cause organ dysfunction. To confuse matters further, not all patients with sepsis demonstrate presence of infection ($<50\%$) {Brun-Buisson et al. 1995 & Rangel-Frausto et al. 1995}. Therefore the international sepsis conferences in 2001 {Levy et al. 2003}

introduced series of diagnostic criterion in addition to the definition criteria of SIRS plus infection, more in keeping with the reality of day to day clinical practice (Table 1.3).

It is of utmost importance to use correct terminology when speaking about sepsis. The word sepsis should only be used to describe the host's response to an infection. Sepsis begins with a simple infection and not all patients with sepsis are equally ill. The patients who develop sepsis (SIRS in the presence of an infection) may develop organ dysfunction (Severe sepsis) for example ARDS, circulatory collapse (Septic Shock) or progress to multiple organ dysfunctional syndrome (MODS). Table 1.4 illustrates the progress through each stage of this disease.

Based on a variation of TNM approach to tumour classification {Sobin et al. 1988}, in a special article in 2003, American Thoracic Society (ATS), Surgical Infection Society (SIS), European Society of Intensive Care Medicine (ESICM), Society of Critical Care Medicine (SCCM) and American Collage of Chest Physicians (ACCP) developed a classification scheme {Levy et al. 2003} which was later refined in 2005 {Steven et al. 2005}. In these reports sepsis was staged using a PIRO (Predisposition, Insult or infection, Response and Organ dysfunction) staging. The PIRO system was however proposed as a template for further investigation and not a model to be adopted.

Predisposition or pre-morbid factors, which may have substantial impact on the outcome of sepsis may be acquired or genetic. For example presence of a pre-morbid illness with reduced probability of short-term survival like severe trauma, burns, diseases predisposing to immunosuppression {Levy et al. 2003 & Dellinger et al. 2004} or requiring immunosuppressant therapy like in transplant patients and genetic polymorphisms in cellular or biochemical components of innate immune response. Genetic factors play a greater role in determining the risk of premature mortality in sepsis even more than other common conditions such as cardiovascular disease {Gospodarowicz et al. 1998}. Comparative studies indicate a strong predilection for severe infection and septic shock based on genetic background of individuals {Sorensen et al. 1988}. Defects in neutrophils {Platonov et al. 1998} complement {Merino et al. 1983} including the lectin pathway {Neth et al. 2001, Hibberd et al. 1999 & Westendorp et al. 1997}, signal transduction {Mira et al. 1999, Fang et al. 1999 & Stuber et al. 1996} and pattern recognition molecules {Gibot et al. 2002, LeVan et al. 2001 & Arbour et al. 2000}. The difference in cytokine responsiveness for example TNF α , IL-1 β {Levy et al. 2003, Mira et al. 1999 & Stuber et al. 1996} and genetic polymorphism of coagulative

and fibrinolytic response {Fang et al. 1999}. Another important predisposition factor in development of sepsis is age and sex of the patient {Levy et al. 2003}.

Insult or infection type, site and extent have a significant impact on prognosis. For example, the host response to gram-negative organisms is different to that of gram-positive organisms {Opal et al. 1999, Ziegler et al. 1991, Wortel et al. 1992 & McCloskey et al. 1994} the presence or absence of bacteraemia has been associated with a mixed outcome in sepsis {Opal et al. 2003 & Gluck et al. 2004}, however secondary nosocomial bacteraemia is associated with a higher risk of mortality than primary bacteraemia {Renaud et al. 2001} this may be a result of higher levels of pathogen being present in the blood or tissue of patients with secondary rather than primary bacteraemia. The quantity of microorganisms and their intrinsic virulence are important determinants of outcome in the presence of invasive microorganisms {Cross et al. 1993 & Opal et al. 1990}. However although the presence of a candida species in blood is particularly associated with poor prognosis the main prognosticator is the severity of disease process, which leads to immunosuppression and candida sepsis in the first place.

Response by the host is the key to understanding severe SIRS or SIRS shock. It depends on the physiological state of the host based on hyper/hypo-responsiveness to the insult. Putative biological markers of response severity include levels of non-specific markers of activated inflammation such as IL-6 {Damas et al. 1992} Procalcitonin or impaired host responsiveness HLA-DR {Harbarth et al. 2001 & Hausfater et al. 2002}. However, some non-specific markers of inflammation may be more valuable for making therapeutic decision than others. For example a marker of adrenal dysfunction may be more useful for making a therapeutic decision to treat with corticosteroids {Annane et al. 2002} whereas a coagulation marker may be more valuable in making a decision to treat with activated Protein C {Bernard et al. 2001}.

Organ dysfunction is the ultimate determinant of survival. It may be assessed simply by counting the number of dysfunctional organs or by a composite scoring systems such as multi organ dysfunction syndrome index (MODS index) {Leclerc et al. 2005}, sepsis related organ failure assessment (SOFA) {Vincent et al. 1998}, Logistic organ dysfunction system (LODS) {De Silva et al. 2001}, paediatric multiple organ dysfunction (PEMOD) {Leteurtre et al. 1999} or paediatric logistic organ dysfunction (PELOD) {Leteurtre et al. 2006}. Measuring cellular response to the insult for example oxidative stress and DNA damage or apoptosis may allow early prediction of organ

dysfunction and pre-emptive treatment. One attribute of PIRO is that its elements are readily testable in clinical research.

MULTIPLE ORGAN DYSFUNCTION SYNDROME (MODS)

The term MODS stems from the 1991-consensus conference. It is the progressive failure of two or more organ systems as a result of abnormal intravascular inflammation {Wilkinson et al. 1986 & Goh et al. 1999} fuelled by a number of cellular, humoral, and biochemical elements resulting in hypoperfusion, metabolic, hypoxic and oxidative stresses, which ultimately lead to cell death by a combination of necrosis and apoptosis. MODS describe a continuum of organ dysfunction, which is subject to modulation by numerous factors at varying time periods. The change in organ function over time may be an important element in prognostication. Therefore the major threat to life is less frequently the underlying illness but rather the progressive physiologic failure of several independent organ systems {Skillman et al. 1969, Tilney et al. 1973, Eiseman et al. 1977, Fry et al. 1980, Bell et al.1983 & Baue et al.1975}. Table 1.6 defines the criterion for major end organ failure.

MODS may be primary or secondary. Primary MODS is directly attributed to the insult itself for example in multiple traumas resulting in lung contusion, ATN caused by rhabdomyolysis or coagulopathy as a result of multiple blood transfusions. In this form of MODS the participation of the host inflammatory response is not as evident as in secondary MODS. However if the patients survive the immediate insult they may develop SIRS and associated secondary MODS. Hence secondary MODS is a direct consequence of SIRS.

It is unclear why some patients develop one type of organ dysfunction at early phases of their disease and others develop another despite similar stimuli. The sequence and pattern of organ dysfunction may provide some insight into the likelihood of survival. It is also interesting to know the likelihood of or the risk of developing organ dysfunction in the presence of pre-existing organ dysfunction during sepsis {Opal et al. 2005}.

According to Bone {Bone et al. 1992} “the increasing incidence of morbidity and mortality caused by multiple organ failure has paralleled improvement in the life support technology as technology is used in higher risk patients at later stages of illness”.

Table 1. 3: Diagnostic criteria for sepsis

Infection, documented or suspected, and some of the following:

General variables

- Fever (core temperature $>38.3^{\circ}\text{C}$)
- Hypothermia (core temperature $<36^{\circ}\text{C}$)
- Heart rate >90 bpm or >2 SD above the normal value for age
- Tachypnea
- Altered mental status
- Significant oedema or positive fluid balance (>20 mL/kg over 24 hrs)
- Hyperglycaemia (plasma glucose >120 mg/dL or 7.7 mmol/L) in the absence of diabetes

Inflammatory variables

- Leukocytosis (WBC count $>12,000$ /cu mm)
- Leukopenia (WBC count <4000 /cu mm)
- Normal WBC count but with $>10\%$ immature forms
- Plasma C-reactive protein >2 SD above the normal value
- Plasma procalcitonin >2 SD above the normal value

Hemodynamic variables

- Arterial hypotension (SBP <90 mm Hg, MAP <70 , or an SBP decrease >40 mm Hg in adults or >2 SD below normal for age)
- SvO₂ $>70\%$ (except in neonates or children)
- Cardiac index >3.5 (except in neonates or children)

Organ dysfunction variables

- Arterial hypoxemia (PaO₂/FIO₂ <300)
- Acute oligurea (urine output <0.5 mL/kg/hr or 45 mmol/L for at least 2 hrs)
- Creatinine increase >0.5 mg/dL
- Coagulation abnormalities (INR >1.5 or APTT >60 secs)
- Ileus (absent bowel sounds)
- Thrombocytopenia (platelet count $<100,000$ /cu mm)
- Hyperbilirubinaemia (plasma total bilirubin >4 mg/dL or 70 mmol/L)

Tissue perfusion variables

- Hyperlactatemia (1 mmol/L)
- Decreased capillary refill or mottling

Severity scoring systems will ensure that the increase complexity of disease is consistently represented in evaluation and therapy of patients and may be used to anticipate outcomes {Knaus et al. 1991}. The use of this approach is particularly important in clinical research. Rangel-Frousto & Pittet in 1995 {Rangel-Frousto et al. 1995 & Pittet et al. 1995} demonstrated the correlation between severity of MODS and mortality.

As well as generic scoring systems there are a variety of age specific and specialised scoring systems available for measurement of the extent of organ dysfunction or predicting mortality from organ dysfunction. For example MODS (Multiple Organ Dysfunction Score) and APACHE II (Acute Physiology and Chronic Health Evaluation II) are used mainly in adults, PRISM (Paediatric RISK of Mortality) in children,

EUROSCORE and Parsonnet score in cardiac surgery and ISS (Injury Severity Score) and Paediatric Trauma Score (PTS) in trauma.

Table 1.4: Defining classifications of SIRS and Sepsis derived from the work done by Bone in association with ACCP/SCCM, Muckart and Bhagwanjee.

Sepsis	Systemic Inflammatory Response Syndrome (SIRS), characterised by two or more abnormalities of heart rate, respiratory rate temperature and white cell count, in the presence of an infection.
Severe Sepsis / SIRS	Sepsis / SIRS associated with respiratory, renal, hepatic gastrointestinal, Haematologic or neurologic organ dysfunction, hypoperfusion, or hypotension which may include, but are not limited to lactic acidosis, oligurea, or an acute alteration in mental state.
Septic Shock/SIRS Shock	Severe sepsis / SIRS associated with hypotension (Systolic arterial pressure below 90 mmHg in adults (< 2 standard deviation below normal for their age in children), a MAP < 60 or a reduction in systolic pressure of > 40 mmHg from baseline despite adequate fluid resuscitation along with the presence of perfusion abnormalities may include, but are not limited to lactic acidosis, oligurea, or an acute alteration in mental state. Patients who are receiving inotropic or vasopressor agents may not be hypotensive at the time that perfusion abnormalities are measured. In children and neonates the shock state occurs long before hypotension that is a sign of late decompensated shock. Tachycardia with signs of decreased peripheral pulses compared with central pulses, altered alertness, flash capillary refill or capillary refill > 2 secs, mottled or cool extremities, or decreased urine output may be used as early signs of shock in children and neonates. SIRS shock is also known as sterile shock.
Multiple-organ dysfunction syndrome	Presence of altered two or more organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention.

A SIRS criterion is a simple abbreviated generic severity of illness measure. Muckart and Bhagwanjee {Muckart et al. 1997} showed that the incremental increase in severity of the inflammatory response embodied the definition of SIRS, Severe SIRS and Sterile (SIRS) shock are reflected by increases in mean APACHEII and ISS. In other words it is a variation in APACHE or Injury Severity Score System. Muckart {Muckart et al. 1997} also showed that mortality risk was independent of presence of infection in a cohort of patients with penetrating trauma whom developed SIRS and organ dysfunction.

Marshall {Marshall et al. 1995} developed an objective scale to measure the severity of the multiple organ dysfunction syndromes called MODS. This scoring system, constructed using simple physiologic measures of dysfunction in six organ systems, mirrors organ dysfunction as seen by clinicians and correlates strongly with the ultimate

risk of ICU mortality and hospital mortality (Table: 1.5). This system provides an objective measure of severity of organ dysfunction and correlates in a graded fashion with the ICU mortality rate, both when applied on the first day of ICU admission as a prognostic indicator and when calculated over the ICU stay as an outcome measure. For example in the latter ICU mortality was 25% at 9 to 12 points, 50% at 13 to 16 points, 75% at 17 to 20 points, and 100% at levels of more than 20 points.

Similarly MODS index is an age adjusted criteria for dysfunction of the cardiovascular, respiratory, neurologic, haematologic, renal, hepatic and gastrointestinal systems. It is based on clinical observation, laboratory measurements or a need for a specific therapy {Doughty et al. 1998 & Goh et al. 1999}.

Table 1.5: Multiple organ dysfunctional scoring system by Marshall et al. PAR is pressure adjusted heart rate and GCS, Glasgow coma scale.

Organ System	Score				
	0	1	2	3	4
Respiratory (PaO ₂ /FiO ₂ ratio)	>300	226-300	151-225	76-150	≤75
Cardiovascular (PAR)	≤10	10.1-15	15.1-20	20.1-30	>30
Renal (Serum creatinine)	≤100	101-200	201-350	351-500	>500
Hepatic (Serum bilirubin)	≤20	21-60	61-120	121-240	>240
Haematologic (Platelet count)	>120	81-120	51-80	21-50	≤20
Neurologic (GCS)	15	13-14	10-12	7-9	≤6

MODS Index is simpler, routinely and reproducibly measured and readily evaluable in a heterogeneous group of critically ill patients (Table 1.6). It measures the extent of organ system dysfunction using clinical as well as laboratory criteria based on objective evaluation of illness severity, hence making it an ideal scoring system for clinical research {Marshall et al. 1995}. It is defined, as the total number of organ system dysfunction divided by the maximum number of organ systems studied, expressed as a percentage, were any item within each category is considered diagnostic. Unlike MODS,

MODS Index does allow a degree of subjective clinical evaluation such as the use of therapeutic interventions to support organ function as well as physiologic measures.

The specific diagnostic criteria for organ dysfunction illustrated in Table 1.6, may be used to assess organ dysfunction prior to or during ECMO. ^IAcute respiratory distress syndrome (ARDS) is one of the commonest indications for ECMO. It is the commonest manifestation of SIRS and a clinical diagnosis. It is important not to confuse ARDS with Infant respiratory distress syndrome, which is caused by surfactant deficiency. ^{II} Circulatory collapse/shock is also a clinical diagnosis common in sepsis. It is an inability to maintain normal blood pressure and tissue perfusion despite adequate fluid resuscitation. However adequate fluid resuscitation is quite subjective and commonly patients present with gross peripheral oedema. ^{III} Acute tubular necrosis (ATN) may be a direct result of a primary insult, for example when associated with septicaemia or secondary to hypoperfusion caused by circulatory shock. ^{IV}Fulminant hepatic failure (FHF) is a clinical condition that results from acute, severe and extensive hepatocellular damage, which leads to Jaundice, coagulopathy and encephalopathy. In the context of SIRS, just like ATN, FHF may be caused by a primary hepatocellular insult like septicaemia or a result of poor perfusion and hepatic congestion caused by circulatory collapse. ^VMalabsorption is difficult to assess in a sedated patient and perhaps under diagnosed. ^{VI}Active GI bleed is a contra-indication to ECMO. Patients on ECMO are at an increase risk of noticeable GI haemorrhage. ^{VII} DIC is diagnosed when there is clinical signs of abnormal bleeding and microvascular thrombosis in the presence of a deranged intrinsic, extrinsic and fibrinolytic coagulation screen; ^{VIII}Diagnosis of brainstem death can only be confirmed after toxicological investigations for sedatives in order to eliminate the possibility of slow re-release of sedatives into the blood by the ECMO circuit. Plasma levels of Morphine and Midazolam may be raised days after stopping them.

To date there is neither an effective treatment for MODS or an effective means to prevent its onset. Undoubtedly, understanding the pathophysiologic mechanisms underlying the development of SIRS and MODS should provide important directions for development of successful treatments for these fatal syndromes.

Table 1.6: Criteria for specific organ dysfunction and MODS Index.

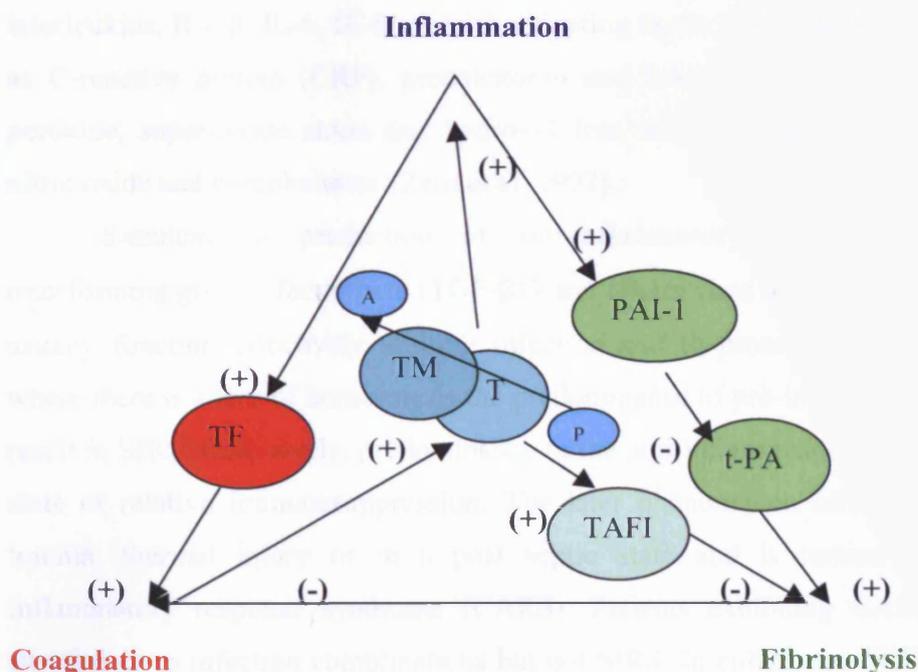
Organ	Criteria	MODS Score
Respiratory	Clinical diagnosis of acute respiratory distress syndrome ^I PaCO ₂ >65 mmHg, PaO ₂ < 40 mmHg, PaO ₂ /FiO ₂ ratio < 300 on mechanical ventilation (all types). ECMO for respiratory support.	1
Cardiovascular	Hypotension (age specific), requiring Dopamine > 5 mcg/kg/min (or adrenaline or nor-adrenaline). Bradycardia (age specific). Circulatory collapse / shock ^{II} Serum PH < 7.2 with normal PaCO ₂ . Cardiac arrest. ECMO for cardiac support.	1
Renal	Acute tubular necrosis ^{III} and raised serum creatinine > 300 mmol/l. Age specific oligurea despite adequate filling. Dialysis for acute renal failure.	1
Hepatic	Fulminant hepatic failure ^{IV} associated with conjugated hyperbilirubinaemia / Jaundice or other abnormally raised LFTs Use of Liver support systems (MARS)	1
Gastro-intestinal	Failure to absorb enteric nutrition (Malabsorption) ^V . Paralytic Ileus. Stress ulceration / GI haemorrhage ^{VI} .	1
Haematologic	Leukopenia (Leukocyte count < 1500 x 10 ⁶). Thrombocytopenia (Platelet count < 20000 x 10 ⁶). Diagnosis of DIC ^{VII} .	1
Neurologic	GCS ≤ 9. Fixed and dilated pupils. Stroke / Cerebral haemorrhage Clinical diagnosis of Brain stem death ^{VIII} .	1

PATHOPHYSIOLOGY OF SIRS

SYSTEMIC THROMBOINFLAMMATORY PATHWAYS

In his review of the pathogenesis of sepsis, Roger C. Bone wrote “... Damage in sepsis probably results from persistent and repetitive inflammatory insults. Eventually, these insults produce sufficient damage that down regulation can no longer occur; this leads to a state of metabolic anarchy in which the body can no longer control its own inflammatory response” (Bone, 1991). This is now better known as the loss of “Homeostasis” mechanism, which allows a cascade of inflammation, coagulation and loss of fibrinolysis to sweep through the bodies systems causing inflammatory infiltration, coagulopathy and microvascular thrombosis {Bone et al. 1991}. A clinical syndrome, that leads to organ dysfunction and ultimately death. This phenomenon can no longer be explained by its inflammatory component alone. Coagulative and anti-fibrinolytic pathways play an important role in the pathophysiology of SIRS. For simplicity I call these interlinked arrays of inflammatory, coagulative and anti-fibrinolysis the Systemic Thrombo-Inflammatory Pathways (STIP). This term has not been used in this context before. However to maintain continuity and to avoid confusion, we use SIRS to denote the clinical syndrome as it was defined by Bone in 1991.

Diagram 1.4: This diagram simplifies the interactions between the components of the Thrombo-Inflammatory Pathway. PAI-1 is plasminogen activation inhibitor 1; t-PA, tissue plasminogen activator; TF, tissue factor; TAFI, thrombin-activatable fibrinolysis inhibitor; TM, thrombomodulin; P, protein C; A, activated protein C and T, thrombin.



ANTIGEN CHALLENGE AND INNATE RESPONSE

The trigger to activation of STIP (Diagram 1.4) is antigen challenge. This will initiate a response to eradicate irritants or to potentiate tissue repair. This is the innate response. The innate response provides an immediate defence mechanism against antigens. It consists of a number of biochemical and cellular components (Table 1.7). The antigen is commonly an infective microorganism, an endotoxin for example the lipopolysaccharide (LPS) constituent of coliform organisms cell membrane, an exotoxin like Streptococcal exotoxin C or Staphylococcus aureus toxic shock syndrome toxin-1 {Sriskandan et al. 1999}. However non-infective stimuli can produce identical physiologic responses as seen in severe tissue injury or burns {Bone et al. 1991}. Therefore the final common pathway to a multitude of primary insults that can precipitate SIRS and MODS is independent of the presence of infection.

LOSS OF BALANCE BETWEEN PRO & ANTI-INFLAMMATION

The host immune response is functionally divided into innate and acquired immunities. The innate response is the first line of host defences. Breach of this component usually results in activation of the acquired or adaptive immune response {Redmond 1996}. However structurally the immune response may be divided into its cellular and biochemical components.

The presentation of antigen to the cellular components of the innate immune response such as the endothelial cells, neutrophils, macrophages and natural killer cells will trigger release of pro-inflammatory mediators; tissue necrosis factor (TNF α), interleukins, IL-1 β , IL-6, IL-8, platelet activating factor (PAF), acute phase proteins such as C-reactive protein (CRP), procalcitonin and heat shock proteins (HSP), hydrogen peroxide, super-oxide anion and hydroxyl free radicals, vasoactive mediators such as nitric oxide and complements {Zeni et al. 1997}.

Simultaneous production of anti-inflammatory cytokines such as IL-10, transforming growth factor beta (TGF- β 1) and others {Lee et al. 2003 & Ng et al. 2003} usually function effectively to limit infection and to promote tissue repair. However where there is a loss of homeostasis the predominance of pro-inflammatory response may result in SIRS. Conversely, predominance of the anti-inflammatory response may cause a state of relative immunosuppression. The later phenomenon often results after major trauma, thermal injury or in a post septic state and is termed the counter anti-inflammatory response syndrome (CARS). Patients exhibiting CARS may be more susceptible to infection complications but not SIRS. In either case MODS can occur but

in CARS this is due to overwhelming infection in one or more organ systems. It is therefore not unreasonable to presume a spectrum of mixed responses ranging from SIRS through to CARS {Jacobi et al. 2002}.

SYSTEMIC INFLAMMATORY ACTIVATION & ENDOTHELIAL INJURY

The cellular components of immune system, such as neutrophils and macrophages amplify the inflammatory response, associated with a further rise in pro-inflammatory cytokines. The release of cytokines causes an increased production of intracellular adhesion molecules ICAM-1 and ICAM-2 on the endothelial surfaces. The activation of selectins (L-Selectin and E-Selectin) on neutrophils facilitating their adhesion and the adhesion of platelet to endothelial surfaces result in an excessive production of complements and free radicals causing significant cellular and endothelial injury.

LOSS OF BALANCE BETWEEN COAGULATION AND FIBRINOLYSIS

The overwhelming release of inflammatory mediators and vasoactive substances result in wide spread endothelial dysfunction. A combination of vasodilatation and endothelial injury leads to endo-vascular leakage of fluids containing cellular and biochemical mediators of inflammation. This results in systemic hypotension, tissue oedema, organ hypoperfusion and dysfunction. Endothelial dysfunction is widely believed to be one of the key derangements underlying SIRS and its sequela. Damage to the endothelial surface results in the release of pro-coagulants and anti-fibrinolytic substances such as tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1). In the absence of further antigen challenge the inflammatory process may be fuelled by a lack of inhibition of coagulation, a relative lack of activated protein C (APC) or tissue factor pathway inhibitor (TFPI), or a lack of stimulation of the fibrinolytic pathways due to a lack of tissue plasminogen activator (t-PA). The ultimate imbalance between coagulation and fibrinolysis will lead to formation of thrombin clots, micro thrombosis and microvascular dysfunction.

ACTIVATION OF COAGULATION PATHWAYS

Clinically patients with SIRS exhibit low platelet count, elevated D-dimers and fibrinogen degradation products as well as other characteristics of low grade DIC {Jacobi et al. 2002}. Release of endothelial, neutrophil and macrophage derived tissue factor stimulates the coagulation cascade {Maier et al. 1996}. An uncontrolled systemic coagulation is prevented locally by the activation of natural anticoagulants such as APC and TFPI {Mann et al. 2002}, however, with a large inflammatory response these proteins are consumed and their effects overwhelmed by the intense pro-coagulation

response. In addition the inhibition of anticoagulants such as thrombomodulin (TM), alpha 1 anti-trypsin (α 1-AT) and heparin by the inflammatory process {Leviton et al. 2004} further stimulates STIP and endothelial injury acting as a vicious cycle of positive and negative feedback mechanisms.

INHIBITION OF FIBRINOLYTIC PATHWAY

Increased release of PAI-1 from the endothelial cells results in an inhibition of fibrinolysis. PAI-1 inhibits t-PA and deactivates fibrinolysis contributing further to the vicious cycle of STIP; increase production of thrombin, activation of platelet and microvascular thrombosis. Production of thrombin is the feedback mechanism linking coagulation and fibrinolysis to inflammation.

DEFICIENCY IN PROTEIN C ANTICOAGULANT PATHWAY

Protein C pathway is a mediator in preventing amplification of STIP. It blocks thrombosis, inhibits monocytes, TNF α formation and protects against endotoxin induced organ damage.

Inflammatory cytokines interact with endothelium to down regulate the protein C pathway by inhibiting TM and endothelial protein C receptor (EPCR) synthesis {Esmon et al. 1989}. Neutrophil activation particularly of endothelial attached neutrophils can decrease endothelial cell associated TM activity by release of oxidants that oxidise a sensitive Met residue on TM that is important in protein C activation {Glaser et al. 1992}, soluble TM levels rise in most inflammatory diseases {Takano et al. 1990}, This is probably as a result of neutrophil elastase release by adherent cells on the endothelial cell surface {Boehme et al. 1996}.

Inhibition of the protein C pathway increases the inflammatory cytokines such as TNF α in particular {Taylor et al. 1991}. In rats activated protein C infusion dampens the TNF α response to endotoxin {Hancock et al. 1992}. This appears to be due to a direct effect of APC on monocytes, since the enzyme and protein S function synergistically to block LPS induced cytokine production by more than 90% {Hancock et al. 1992}. APC did not block production of reactive oxygen intermediates, up-regulate intercellular adhesion molecule ICAM-1, major histocompatibility complex MHC class II molecule or IL-2 receptor or down-regulate CD59, suggesting that adhesion, phagocytosis and bacterial killing activities remain intact in presence of APC {Grey et al. 1994}. Alpha one anti-trypsin is an acute phase protein and a major APC inhibitor {Scully et al. 1993}.

Table 1.7: Cellular and Biochemical Components of The innate immune response.

Cellular components of innate response	
Endothelium	Damaged tissues Release Histamine, Bradykinin and Serotonin leading to innate immunity swelling and inflammation. Vasodilatation and Fever.
PMN	<p>Direct or indirect release of most mediators of inflammation.</p> <p>Release of free-oxygen species and lysosomal enzymes causing damage to the endothelium and vascular matrix.</p> <p>Aggregate to produce microemboli leading to tissue ischaemia.</p> <p>Adhere to endothelial surface and produces a variable effect on the tone of the vessel depending on its concentration.</p> <p>Releases neutrophil derived relaxing factor.</p> <p>Migrates through endothelium and accumulates in the sub-cellular space.</p> <p>Activated by extracorporeal circuit and accumulate in the lungs releasing vasoactive and cytotoxic substances.</p>
Monocytes	Precursor of tissue macrophages
Macrophages	<p>Remove damaged tissue and microorganisms from the tissue.</p> <p>Stimulated by antigen and performs chemotaxis.</p> <p>Gram positive organisms attached to CD14 of the Macrophages.</p> <p>Important source of inflammatory cytokines IL-1β, 6 and TNFα.</p>
Eosinophils	<p>Production of reactive oxygen species such as super oxide.</p> <p>Release of elastase and growth factors TGF-β1.</p> <p>Release of cytokines IL-1,2,4,5,6,8 and 13 and TNFα.</p>
NK cells	<p>Release of cytokines and chemokines.</p> <p>Trigger to apoptosis.</p>
Biochemical components of innate response to sepsis	
Acute phase proteins	
CRP	<p>Acute phase protein produced by liver during inflammation.</p> <p>Blood levels rise in response to tissue damage or infection within hours of insult and can reach 1000-folds within 24 to 48 hours and fall very rapidly once the stimulus is removed.</p>
Fibrinogen	<p>Fibrinogen (FGN) is an acute phase protein that is also a part of the coagulation cascade of STIP. Fibrinogen is converted to fibrin in the presence of thrombin.</p> <p>The amount of fibrinogen in the plasma can serve as a non-specific indicator of whether or not an inflammatory process is present. The fibrinogen levels commonly increase in the acute phase response.</p>

Heat shock proteins	
HSP 60	<p>Acute phase protein, prevent and repair cellular injury after hyperthermia and oxidative stress.</p> <p>HSP60 is identified as a danger signal for the innate immune system.</p> <p>Levels are associated with early atherosclerotic cardiovascular disease.</p> <p>There is a positive association between serum HSP60 and TNFα.</p>
Inflammatory mediators	
Cytokines and similar mediators	
TNF α	<p>Stimulates hypothalamus and cause fever.</p> <p>Stimulates production of polymorphonuclear cells.</p> <p>Increases the expression of adhesion molecules.</p> <p>Directly toxic to endothelial cells.</p> <p>Stimulates the release of other cytokines such as IL1,6,8 platelet activating factor, leukotrienes, Thromboxane A2.</p> <p>Activates the complement system.</p> <p>Activates common coagulation pathways and may inhibit thrombomodulin expression on endothelial surfaces.</p> <p>Direct myocardial depression.</p>
IL-1 β	<p>Main stimulant of fever through its action on hypothalamus.</p> <p>Act synergistic to TNFα.</p> <p>Stimulates release of TNFα and other cytokines.</p> <p>Increases the expression of adhesion molecules.</p> <p>Enhances tissue sensitivity to TNFα.</p> <p>Promotes polymorphonuclear cells activation and accumulation.</p> <p>Activates resting T Cells.</p> <p>Promotes release of adrenocorticotrophic hormone.</p>
IL-2	<p>Promote release of TNFα and IFγ.</p> <p>Required for T cell proliferation.</p>
IL-4	<p>Increase expression of antigen on endothelial cells in synergy to TNFα and IL-1β but antagonise expression of adhesion molecules against them.</p>
IL-6	<p>Acts as a T Helper Cell for T and B cell activation.</p> <p>Synergistically increases T cell proliferation by interacting IL-1β together with TNFα.</p> <p>Promotes polymorphonuclear cells activation and accumulation.</p>
IL-8	<p>Chemotactic to Neutrophils and Lymphocytes hence inducing tissue infiltration.</p> <p>Inhibits endothelial Neutrophil adhesion.</p>
IL-10	<p>Inhibits synthesis of proinflammatory cytokines.</p>
PAF	<p>Encourage platelet aggregation leading to thrombosis.</p> <p>Stimulates release of TNFα, Leukotrienes, Thromboxane A2.</p> <p>Promotes Leukocyte activation and free radical formation.</p> <p>Promotes endovascular cell retraction and leak due to loss of reciprocal contact.</p> <p>Negative inotropic effect on the Heart.</p> <p>May attenuate effect of endotoxin on hyperglycaemia.</p>

Leukotrienes	<p>Promote Neutrophil adhesion and chemotaxis. Weakly promote chemotaxis in Eosinophils. Promotes endovascular cell retraction and leak due to loss of reciprocal contact. Decrease coronary blood flow and myocardial contractility. Increase pulmonary vascular resistance. Decrease mesenteric blood flow but not renal blood flow Vasoconstrictor effect.</p>
Thromboxane A ₂	<p>Promotes release of Nitric oxide. Pulmonary vaso- and broncho-constriction. Causes platelet aggregation and Neutrophil accumulation. Increase vascular permeability.</p>
Prostaglandin E ₂	<p>Stimulates hypothalamus and cause fever. Inhibits endotoxin-induced hypotension. Increases the effects of serotonin and bradykinin on vascular permeability. Vasodilator and improves tissue perfusion. Inhibits IL-1β production. At low concentration stimulates TNFα. At High concentration suppress TNFα. Increases intracellular cyclic AMP levels.</p>
Prostacyclin	<p>Inhibits platelet aggregation and adhesion. Inhibits thrombus formation. Increases the effects of serotonin and bradykinin on vascular permeability. Produces smooth muscle relaxation.</p>
Interferon- γ	<p>Promotes release of TNFα IL1β and IL-6. Augments production of adhesion molecules. Synergistic to TNFα in cytotoxic effect. Synergistic to IL-2 in promotion of TNFα production. Helps to activate B cells for antibody production. May antagonise GMCSF production. Promotes Macrophage activation. Induces class I and II histocompatibility molecules.</p>
GMCSF	<p>Stimulates Polymorphonuclear cell phagocytosis, degranulation and cytotoxicity. Promotes Macrophage maturity, and enhances its activity.</p>
TGF- β 1	<p>Recruits Macrophages to sight of injury. Stimulates Release of IL-1β and more TGF-β1. Suppresses macrophages ability to release reactive oxygen species. Promotes leukocyte adhesion to endothelium. Suppresses TNFα induced inflammation. Promotes cell growth repair, metabolism. Increases production of extracellular matrix and collagen.</p>
Nitric Oxide	<p>Relaxes vascular smooth muscle. Inhibits platelet aggregation and adhesion. Inhibits mitogenesis in vascular smooth muscle.</p>
Endothelin 1	<p>Strongly promotes vasoconstriction. Increase glomerular resistance and renal hypoperfusion. Promote NO and prostacyclin release. Promotes mitogenesis of vascular smooth muscle.</p>

Adhesion molecules	Act as ligands for receptors on inflammatory cells. Attract and help inflammatory cells to migrate across endothelial monolayer.
Selectin	Stimulated by histamine and thrombin to facilitate leukocyte adhesion to the endothelial cells.
Lysosomal enzymes	Produced by PMN, Monocytes and Macrophages. Bactericidal lysosomal enzymes e.g. Myeloperoxidase.
Bradykinin	Promotes release of NO and prostacyclin.
Platelets	Promote release of Nitric oxide and prostacyclin. Produce TGF- β 1, thromboxane A2, serotonin. Release 12-hydroxy acids that are metabolised by neutrophils into leukotrienes B4. Induce vasoconstriction. Stimulate neutrophils.
Complements	
C3a	Causes mast cell degranulation and release of vasodilators (Alternative pathway).
C4a	Causes mast cell degranulation and release of vasodilators. It is less active than C3a.
C5a	Causes mast cell degranulation and release of vasodilators also enhances polymorphonuclear cell activation, migration adhesion and aggregation (Classical pathway). Induces capillary leak. Most active of the above anaphylatoxins.
Coagulative mediators	
Tissue Factor	Essential for thrombin production from prothrombin.
Protein C	Precursor of activated protein C produced by the liver.
Activated Prot C	Anti-thrombotic, pro-fibrinolytic and anti-inflammatory properties. Decrease synthesis and expression of Tissue Factor. Inactivates factor Va and VIIIa therefore limits production of thrombin. Limits activation of factor X. Increases fibrinolysis by decreasing PAI-1 and preventing inhibition of t-PA. Promotes fibrinolysis by inhibiting thrombin formation. Reduces inflammation by limited production of thrombin and monocytes chemoattractant protein-1. Inhibits TNF α production by Monocytes and endothelium.
Thrombomodulin	Thrombin induced activation of protein C.

Thrombin	Stimulates release of PAF from endothelial cells. Promotes release of NO and prostacyclin. Encourage Endothelin 1 release. Mediates the activation of IL-8 in pro-coagulant environment. Encourages fibrinogen consumption. Pulmonary vasoconstrictor. Activates Protein C Inactivates factors Va and VIIIa Important role in STIP.
Fibrinolytic mediators	
PAI-1	Inhibits t-PA
t-PA	Plays a role in cell migration. Stimulates hyper-fibrinolysis by converting plasminogen to plasmin. Plasmin degrades fibrin to fibrinogen.
TAFI	Inhibits fibrinolysis by inhibiting fibrin degradation.

MECHANISM OF ORGAN DYSFUNCTION AND MODS

The mechanism of host response leading to MODS is complex and some of its aspects still unclear. MODS is progressive failure of two or more organ systems as a result of abnormal intravascular inflammation {Wilkinson et al. 1986 & Goh et al. 1999} fuelled by a number of cellular, humoral, and biochemical elements resulting in hypoperfusion, metabolic, hypoxic and oxidative stresses, which ultimately lead to cell death by a combination of necrosis and apoptosis.

ENDOTHELIAL CELL DYSFUNCTION

An intact and correctly functioning microcirculation is essential for the efficiency of tissue perfusion and oxygenation. There are a considerable number of experimental data in animals showing reduced microcirculatory flow and increased heterogeneity of microvascular blood flow in SIRS and MODS {Cryer et al. 1987, Lam et al. 1994, Piper et al. 1996, Drazenovic et al. 1992, Farquhar et al. 1996, Madorin et al. 1999, Nakajima et al. 2001 & Walley et al. 1996}. The precise mechanisms involved in microcirculatory impairment are unclear {Vincent et al. 2005}. Altered vasomotor tone by vasomotor substances may increase endothelial permeability, release of neutrophil derived oxygen free radicals {Kirton et al. 1992}, endothelial apoptosis and fibrin deposition due to activation of thrombin and microvascular thrombosis {Vincent et al. 2005}. This may then lead to vascular congestion and result in a state of inadequate organ perfusion, organ

dysfunction and shock. Vasomotor substances such as platelet-derived growth factor, platelet activating factor, IL-1 β and adhesion molecules such as endothelial adhesion molecules and intracellular adhesion molecule-1, promote adhesion of neutrophils monocytes and lymphocytes to the endothelium via CD 11b and CD18 receptors {Dustin et al. 1986 & McMillen et al. 1993}. The response of the sympathetic nervous system during shock is to increase heart rate despite myocardial depression. Attempts to divert blood to vital organs by increasing peripheral vascular resistance often fail against this hyper-dynamic cardiovascular state {Kim et al. 2000}. At this stage without intervention organ hypoperfusion, hypoxia and acidosis, ultimately leads to a state, which is incompatible with life leading to cell necrosis.

OXIDATIVE STRESS AND DNA DAMAGE

There is convincing evidence of severe oxidative stress in patients with SIRS. In SIRS, Ischaemic reperfusion (I/R) injury may occur as a result of circulatory shock, tissue hypoperfusion attributable to altered vasodilatation patterns and pathologic supply dependent oxygen uptake. Ischaemia reperfusion injury is defined as the damage that occurs to an organ at the time of resumption of blood flow, after an episode of ischaemia. In SIRS all cells produce oxygen free radicals especially endothelial cells and neutrophils. Oxidative stress occurs when the homeostatic balance between formation of reactive oxidising oxygen species including superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^{\cdot}), and endogenous antioxidants is lost {Gutteridge et al. 1999}. These oxygen free radicals and other reactive oxygen species appear to be involved as messengers in cellular signal transduction and gene activation with implications for expression and control of thrombo inflammatory response (\cdot = unpaired electron of a free radical).

Reperfusion injury appears to be mediated by an initial production of super-oxide anion when oxygen is reintroduced into the system and the subsequent triggering of a free radical cascade {Abello et al. 1994}. Oxygen free radicals may act directly on organ cells or by activation of neutrophils. Accumulating evidence suggest that the cellular redox state and the equilibrium between oxygen free radicals (OFR) generation and detoxification influences the early stages of apoptosis which leads to MODS.

In sepsis several potential sources of reactive oxygen species, include the mitochondrial respiratory electron transport chain, xanthine oxidase activation as a result of ischaemia and reperfusion and the respiratory burst associated with neutrophil activation and arachidonic acid metabolism. Activated neutrophils produce superoxide as

a cytotoxic agent as part of the respiratory burst via the action of membrane bound NADH oxidase on molecular oxygen. Neutrophils also produce the free radical nitric oxide (NO.), which can react with superoxide to produce peroxynitrite, itself a powerful oxidant, which may decompose to form the hydroxyl radical. Under ischaemic conditions followed by reperfusion, the enzyme xanthine oxidase catalyses the formation of uric acid with coproduction of superoxide. Superoxide release results in the recruitment and activation of neutrophils and their adherence to endothelial cells, which stimulates the formation of xanthine oxidase in the endothelium, with further superoxide production.

During oxidative stress damage mediated by reactive oxygen species can occur. Oxidation of DNA and proteins may take place, along with membrane damage, because of lipid peroxidation, leading to alterations in membrane permeability modification of protein structure and function {Zimmerman et al. 1995}. Oxidative damage to the mitochondrial membrane can also occur, resulting in membrane depolarisation and uncoupling of oxidative phosphorylation, with altered cellular respiration {Nathan et al. 1999}. This can ultimately lead to mitochondrial damage, with release of cytochrome c, activation of caspases and apoptosis.

Many agents that induce apoptosis are either oxidants or stimulators of cellular oxidative metabolism. Conversely many inhibitors of apoptosis have anti-oxidant activities {Schumer et al. 1992}. Buchman {Buchman et al. 1993} reported that antioxidant factors inhibit LPS induced apoptosis. Interestingly bcl-2 a gene product inhibiting apoptosis has anti-oxidant properties.

Nitric oxide (NO) is a reactive radical that mediates a number of biological effects in relation to almost every organ system as well as immune, inflammatory and cytotoxic effects {Nathan et al. 1992}. Nitric oxide levels have been shown to be elevated in SIRS associated with trauma and sepsis {Ochoa et al. 1991}. Its production induced by LPS and implicated in the induction of apoptosis in a variety of cell types, including thymocytes neutrophils and endothelial cells {Fehsel et al. 1995}. The possible role of NO in apoptosis related to MODS has not been investigated. NO may mediate TNF α induced apoptosis {Larrick et al. 1990 & Wood et al. 1994}.

Antioxidants are central to the redox balance within the human body. They do not act in isolation, but synergistically. Primary antioxidants prevent oxygen radical formation, whether by removing free radical precursors or by inhibiting catalysts, e.g. glutathione peroxidase and catalase. Secondary antioxidants react with reactive oxygen species, which have already been formed, either to remove or inhibit them, e.g. vitamins

C and E. Endogenous antioxidant defences exist at a number of locations, namely intracellularly, on the cell membrane and extracellularly {Gutteridge et al. 1999}.

APOPTOSIS

Apoptosis or programmed cell death (PCD) is an active, gene-directed cellular self-destruction that may occur under physiologic or pathologic conditions. Mediators of SIRS and MODS such as IL-1 β , IL-6, TNF α reactive oxygen species, heat shock proteins and LPS have potential to enhance apoptosis in organ tissues and endothelial cells. Regulation of apoptosis is mediated by intracellular signalling after binding of a death specific receptor (fas) to its ligand (fasL), which forms an amino acid sequence necessary for apoptotic signal transduction. FasL belongs to the TNF family and has limited homology with TNF at what has been termed the “death domain”. {Itoh et al. 1993} through which both fas and TNF-RI (TNF receptor p55) may transduce apoptotic signals through other intracellular signalling proteins such as FADD (fas associated death domain protein), TRADD (TNF-RI associated death domain protein) and FLICE (FADD-like IL-1 β converting enzyme) which activates proteases (Caspase) which in turn executes the cell death program by activation of intracellular substrates essential for cell death and inactivation of structural proteins and substrates essential for cell survival {Wallach et al. 1996, Boldin et al. 1996, Miller et al. 1997 & Yuan et al. 1997}. Papathanassoglou {Papathanassoglou et al. 2000} explored the hypothesis that during SIRS the increase release of cytokines such as TNF α , IL-1 β , IL-6, IFN γ , oxidants such as H₂O₂, O₂[·], OH[·] and NO, heat shock proteins (HSP), glucocorticoids and LPS from bacteria in the serum results in the dysregulation of apoptotic homeostasis in the vascular endothelium, tissues and organs, resulting in accelerated apoptotic cell death and MODS. Abello and Buchman provided primary evidence by using endothelial cell culture experiments that at least four type of mediators in SIRS act sequentially to signal apoptosis: cytokines, endotoxins, heat-shock proteins, and reactive oxygen species. They produced consistent results on the role of oxygen free radicals in apoptosis and the finding that antioxidants in vivo may block apoptosis caused by inflammatory mediators {Buchman et al. 1993, Abello et al. 1994, Abello et al. 1994 & Abello et al. 1994}.

Neutrophils (Plymorphonuclear leucocytes) accumulate at the site of inflammation and promote opsinisation and antigen presentation as well as phagocytosis. Prolonged accumulation of neutrophils in inflamed tissue accounts for tissue injury because of their high oxidative properties and proteolytic activities. Non-stimulated neutrophils die by PCD. However in contrast to the finding that endotoxin LPS triggers

endothelial cell apoptosis it has been reported that LPS inhibits neutrophil apoptosis and prolongs its functional longevity in vitro {Lee et al. 1990}. However it has been shown that the degree of apoptosis in inflamed tissue depends on multiple counteracting signals {Watson et al. 1996}. It has been shown that TNF α and HSP induced apoptosis can override mechanism by which LPS delays PCD in neutrophils. However neutrophils of severely injured patients demonstrate a decreased spontaneous and fas induced apoptosis that appears to be mediated by proinflammatory cytokines {Ertel et al. 1997}.

T cell suppression and a decrease in total T lymphocyte numbers are characteristic derangement occurring in SIRS and MODS. T cells appear to be particularly sensitive to apoptotic signals. Wang demonstrated that apoptosis of thymocytes may account for thymic involution and T cell suppression in a mouse model of gram negative and positive sepsis and that thymocyte apoptosis may be mediated by TNF α {Wang et al. 1994}. Rat studies suggest thymocyte apoptosis and T cell depletion in sepsis may be related to IL-2 suppression {Teodorczyk-injeyan et al. 1992}.

In addition to neutrophils and thymocytes a mouse model of sepsis demonstrated parenchymal cells with increased apoptotic characteristics in lung, intestine and to a lesser degree in the kidney, however no apoptotic pattern was evident in the heart, Liver or brain cells {Hotchkiss et al. 1997}. Organ differences in the induction of apoptosis might account for the clinically apparent sequence of organ dysfunction in MODS that is most commonly initiated in the lungs followed by the liver, renal, intestine and myocardium. Bronchoalveolar lavage fluid obtained from ARDS patients demonstrated apoptotic characteristics in both endothelial cells and fibroblasts {Polunovsky et al. 1993}.

Endo {Endo et al. 1996} conducted an innovative study examining the concentration of the circulating apoptotic receptor sfas in MODS patients with or without infection. He recorded that sfas concentration were significantly elevated in all MODS patients and that the concentrations correlated with nitric oxide (NO) concentrations. Both sfas and NO rapidly decreased when MODS was relieved. In this study, sfas concentrations were conceptualised as a positive marker of apoptosis, and the correlation with NO was in accordance with previous findings on the role of NO and oxidative stress in apoptosis.

HEAT SHOCK RESPONSE

Heat shock response is a mode of cellular response to environmental stressors distinct from the acute phase response. Both responses may be induced during SIRS by inflammatory mediators and oxidative stress, respectively and they are both homeostasis-preserving mechanisms {Curtis et al. 1988}. The heat shock response is fundamentally different from the acute phase response that by being phylogenetically primitive, it is driven by the necessity to preserve intracellular homeostasis even at the expense of whole organism homeostasis. The heat shock response is triggered by stimuli that are potentially more threatening than those required for the acute phase response, including viral infection, abrupt cell temperature rise, glucose depletion, heavy metal ions, hydrogen peroxide, DNA damaging agents, anoxia, and recovery from anoxia {Jaattela et al. 1992}. An intriguing characteristic of this kind of cellular defence is that expression of its effector molecules, the HSP, overrides other programs of gene expression in the cell by inactivating key gene control loci {Westwood et al. 1991}. The implications of this transcriptional competition between HSP and other cellular programs may be that normal growth and development and constitutive peptide and hormone production by specific cells may be suspended as long as HSP proteins are expressed. Although favourable at the unicellular level, this kind of cellular “hibernation” may be detrimental for the homeostatic balance of the whole organism. This transition from organism defence to cellular self-defence, set in motion when the organism sustains an injury such as oxidative stress, may be clinically as organ specific dysfunction {Abello et al. 1994}. It appears that HSP can either protect a cell from apoptosis or stimulate apoptosis. It is probable that when moderate heat shock occurs, HSP might protect the cell from subsequent septic and oxidative insult or from cytokine transduced signals; however, in the presence of septic or inflammatory signals, subsequent HSP generation results in apoptosis. Migliorati {Migliorati et al. 1992} reported that heat shock (43 C, 20 min) induced DNA fragmentation and apoptotic cell death in mouse thymocytes.

HAEMATIC RESPONSE TO ECMO

The Oxford dictionary defines the word Haematic as “Of blood or containing blood”. In this thesis I will refer to activation of blood during extracorporeal life support as the haematic response to ECMO.

ECMO is used to treat patients with sepsis or systemic inflammatory response syndrome, associated with respiratory distress {Stewart et al. 1997}. ECMO itself

however causes a systemic response {Plotz et al. 1993}. This is a response to the circuit, including the oxygenator and is due to widespread activation of all defence mechanisms against non-self, which may lead to pan-endothelial injury {Moat et al. 1993}. I call it the haematic response because it is due to the contact activation of blood. This term has not been previously used to describe contact activation of blood.

The response caused by ECMO is clearly visible within the first 24 hours of cannulation. Pulmonary infiltrates diffuse to all areas of the lung even affecting areas previously spared by the primary disease. Therefore it is not unreasonable to assume that ECMO may hinder their recovery.

Almost all of the cellular and humoral elements of the blood are involved in the response to extracorporeal circuits {Peek et al. 1999}. The component of the extracorporeal circuit examined in this thesis is the oxygenators. Oxygenation requires a large surface area of blood contact. In the forthcoming chapters we will review the systemic response to ECMO and our experience with different types of oxygenators.

The haematic response to ECMO is similar in some respects to the systemic response observed in sepsis. It occurs as a result of cellular and humoral responses through the processes of inflammatory, coagulative and fibrinolysis against non-self and has a time dependent pattern. The first pattern emerges within 24 hours of cannulation and the second within 72 hours of ECMO {Plotz et al, 1993}. These patterns are easily recognisable by clinical observation.

The first response has two main simultaneous components, the activation of contact coagulation system and the complements cascades with some fibrinolytic component. The activation of the contact system results in an elevated factor XIIa, an increase in plasma Kallikrein {Edmunds et al. 1987}, thrombin-antithrombin III, and moderate generation of fibrinogen degradation products. Elevated C3a, decreased leukocyte count, elastase release, and TNF α production characterize the complement activation {Plotz et al. 1993 & Urlesberger et al. 1996}. The activation of the alternative pathway of complements leads to an activation of the lytic pathway (final common pathway) and formation of membrane attack complexes (MAC) or complement C5b-9 that cause haemolysis and organ dysfunction by a process of cell damage. Complements also increase vascular permeability and vasodilatation and activate leucocytes {Craddock et al. 1977}.

The second pattern of response is the further activation of coagulation and fibrinolytic pathways and the demise in complement activation. The circuit is then said to

have “passivated”. Little further inflammatory activation occurs from this point on {Plotz et al. 1993}, unless the circuit / oxygenator is changed, or as a result of secondary hospital acquired sepsis. The time course of this process coincides with the clinical behaviour of patients on ECMO. Within 24 hours of cannulation there is a marked worsening of the Chest X-ray {Maurer et al. 1998} caused by interstitial oedema and collapse. This observation is reiterated when replacing an old circuit.

Whilst the inflammatory activation weans within 24 hours from cannulation the coagulative cascades continue to be active. Activation of coagulation system, mostly by the intrinsic pathways, lead to formation of thrombin. Thrombin converts fibrinogen to fibrin {Edmunds et al. 1987}. This usually occurs at areas of turbulence for example at the connectors leading to a white (Fibrin) thrombus or in the areas of low shear stress, such as the oxygenator or the bladder, where red cells and platelets are trapped to form red (blood) thrombus. Fibrinogen also binds to the circuit and activates platelets, which activating other platelets as well as activating neutrophils and promote release of cytokines. This in turn activates more platelets {Edmunds et al. 1987 & Salzman et al. 1987}. Platelet consumption starts immediately after cannulation. Platelet production increases, but unless it exceed the amount consumed the platelet counts will continue to drop {Anderson et al. 1986}. A fall in platelet count during ECMO may be a result of several mechanisms, for example activation and increased destruction, platelet aggregation and adherence to the circuit / oxygenator membrane are only a few {Robinson et al. 1993}. Decreased productions, sequestration in the extra-vascular space or haemodilution are other possibilities. Activated neutrophils accumulate in the lung and may cause damage by releasing elastase and other enzymes {Edmunds et al. 1987}. The neutrophils also undergo an oxidative burst with generation of free radicals, resulting in oxidative stress and cell damage {Marcus et al. 1985}.

The fibrinolytic system is activated to remove accumulating thrombi. Plasminogen is converted to plasmin {Edmonds et al. 1987}. However, excessive activation of a fibrinolytic system like in sepsis may then lead to Disseminated Intravascular Coagulation (DIC) like state with consumption coagulopathy and uncontrollable thrombus formation in the circuit. Platelet consumption by the circuit escalates and the levels of fibrin (ogen) degradation products (FDPs) rise.

This aspect of the haematic response will be reviewed with reference to clinical practice in forthcoming chapters.

THE WAY FORWARD

The relationship between a SIRS inducing insult and the ultimate development of multi-organ dysfunction is complex. It consists of inflammatory, coagulative and anti-fibrinolytic pathways (STIP). During SIRS the body's homeostatic mechanism, regulating body's defences are lost. This leads to a whole body inflammatory activation. Irrespective of the precipitating condition widespread inflammatory activation results in auto-destructive inflammation {Bone et al. 1991} and pan-endothelial injury {Moat et al. 1993}. SIRS / sepsis progress to severe SIRS / sepsis, SIRS / septic shock when associated with organ dysfunction, and multi-organ dysfunction occurs when two or more organs fail simultaneously. This illustrates a graded progression of this disease process.

ECMO can be used to support patients with severe cardiac or respiratory failure associated with SIRS and MODS. Extracorporeal circuits such as cardiopulmonary bypass (CPB) {Moat et al. 1993}, haemodialysis circuits {Ing et al. 1995} and ECMO {Plotz et al. 1993} also activate the thrombo-inflammatory pathways. This activation is indistinguishable from SIRS and causes an ARDS-like picture. Cellular components of the innate response such as neutrophils and biochemical components such as cytokines play an active role in the balance of inflammatory homeostasis. Neutrophil depletion by in-line filtration is known to be effective in attenuating SIRS and lung injury caused by CPB {Al-Ebrahim et al. 1993 & Bando et al. 1990}. Molecular adsorption recirculating system (MARS) has also shown to measurably clear IL-6, IL-8, IL-10, TNF α and TNF α R1 in patients with acute on chronic liver failure {Stadlbauer et al. 2006}. However it has been unable to reduce serum levels of these cytokines presumably due to their continuous production {Stadlbauer et al. 2006}.

Survivals of 61% for paediatric cardiac support {Peek GJ et al. 1997}, 77% for paediatric respiratory support {Peek et al. 1997}, 66% for adult respiratory patients {Peek et al. 1997} and 75% for neonates {Peek et al. 1998} have been reported with ECMO in groups of patients who were essentially moribund and were not expected to survive. We examine the results of ECMO support for graded SIRS and MODS in the same group of patients, however since the current treatment including ECMO merely supports or replaces organ function until recovery occurs the survival of these patients are to a greater degree dependent on resolution of inflammatory response before irreversible organ injury occurs. There are many patients with fulminant ARDS/MODS who do not respond to supportive therapy. A disease modifying approach by interrupting the inflammatory process may be more effective than current supportive therapy by ECMO alone, for

example there is good theoretical basis to expect improvement in outcomes of these patients as a result of neutrophil depletion or MARS. However there is no data available concerning the safety or efficacy of their use in ECMO and in the absence of a reliable model of SIRS we have no means of assessing these approaches. For example longer-term neutrophil depletion (i.e. for several days) may reduce host defences to a level where nosocomial infection occurs. It is therefore essential, that these procedures must be investigated in an animal model before human use can be considered.

A reliable and reproducible model is essential when testing novel hypotheses. Models of sepsis and multi-organ dysfunction do exist in the published literature {Ito et al. 1978, Nishina et al. 1997 & Yokoi et al. 1997}, however we lack a sufficiently well characterized and reproducible model that fits our requirements. We believe that breaking the vicious cycles of STIP and haematic response is likely to result in improved outcomes.

Chapter Two

MATERIALS AND METHODS

INTRODUCTION

This chapter aims to provide clear descriptions of how this study was designed, the materials used, the way it was carried out and how the data was analysed. We hope that this will provide other researchers with a reliable and reproducible experimental methodology.

THESIS DESIGN

This thesis combines literature review with our institutional report in order to evaluate the role of ECMO in the management of neonates, children and adults with severe SIRS and MODS. We studied the prevalence of severe SIRS in our ECMO population and compared their outcomes with the available outcomes using conventional methods. In order to evaluate the haematic response to ECMO we designed a retrospective study comparing clinical and laboratory variation between two different oxygenator technologies. The pathophysiology of SIRS and MODS was reviewed in order to understand and design a reliable and reproducible animal model. We designed a rabbit model of SIRS and MODS and determined the dose response and inter-individual variation for the development of fatal SIRS caused by administration of intravenous lipopolysaccharide (LPS). We assessed the relationship between the dose of LPS and the development of MODS clinically and by using laboratory methods. A variety of other in-vitro experiments were designed and performed. We determined the interspecies cross reactivity for the serum/plasma proteins involved in STIP. Using ELISA method we quantitatively assessed the relationship between the severity of illness and expression of inflammatory, coagulative and anti-fibrinolytic components of STIP in rabbit serum/plasma. Semi-quantitative methods were used in order to assess end organ dysfunction, inflammatory infiltration, oxidative stress, DNA damage and apoptosis caused by SIRS/sepsis in rabbits.

LITERATURE REVIEW

This investigation involved a comprehensive literature review using Medline plus, in order to define and classify systemic inflammatory response and multiple organ dysfunction syndromes, and to explain the pathophysiology of these disease processes, their staging and management using ECMO support. Definitions and classifications were obtained from well-established peer reviewed sources. The use of abbreviations is kept to a minimum by using only the most commonly used terms, however we have introduced and defined two new terms STIP Systemic Thrombo Inflammatory Pathway to describe the inflammatory coagulative and anti-fibrinolytic path to SIRS and haematic response to

describe blood activation by ECMO, in order to avoid confusion between these similar entities.

CLINICAL EXPERIMENTS

There are two clinical components in this thesis. Chapter three; the institutional report of ECMO for severe systemic inflammatory response and multi organ dysfunction and chapter four, comparative review of ECMO oxygenator performance “The state of the art oxygenator”. These chapters assess the prevalence of SIRS and MODS in an ECMO population, and evaluate role of a novel oxygenator on haematic response to ECMO respectively. All patients were treated at an Extracorporeal Life Support Organisation (ELSO) registered institution (Glenfield Hospital, Leicester, United Kingdom). Patients were treated according to our standard institutional ECMO protocol. This was a retrospective study using the ECMO specialist charts, ELSO registry forms, perfusion charts and patient records. These charts were completed prospectively during the course of the patient’s admission. Chest x-rays were recovered from archives scored and reviewed by an independent expert radiologist. Information was stored on an excel spreadsheet, then converted to SPSS format prior to statistical analysis. Case reports were used when appropriate to emphasize points for discussion.

Institutional report of ECMO for severe systemic inflammatory response and multi organ dysfunction

Methodology: The diagnosis of SIRS is still clinical, based on Bone’s definition set in 1991. Unfortunately no single biomarker of SIRS exists. The SIRS criteria are met when there are alterations in any two of four parameters of temperature, heart rate, respiratory rate or white cell count (Table 2.1) Reports have shown that more than 2/3 of ICU patients and a substantial proportion of the ward patients meet SIRS criteria. This implies that SIRS criteria is very sensitive {Vincent et al. 1997} and probably a vast majority of patients referred for ECMO would satisfy its criteria.

In a special article, American Thoracic Society (ATS), Surgical Infection Society (SIS), European Society of Intensive Care Medicine (ESICM), Society of Critical Care Medicine (SCCM) and American Collage of Chest Physicians (ACCP) in 2001, agreed on a series of clinical findings to aid establishment of the diagnosis of systemic inflammatory response syndrome. These include haemodynamic instability, arterial hypoxemia, oligurea, coagulopathy and altered liver function. These clinical observations made diagnosis and classification of SIRS easier for clinical and research purposes. The 2001 criteria for the diagnosis of SIRS were used and patients that did not

satisfy any of these criteria were excluded (Table 1.3). All patients referred for ECMO had at least a single organ dysfunction. Therefore the definition classification of SIRS and sepsis were used to diagnose and differentiate clinical progression of systemic inflammatory response to an insult as severe SIRS / SIRS shock and MODS.

Based on a variation of TNM approach to tumour classification {Sobin et al. 1988}, a modified form of PIRO staging system for sepsis as defined in the introduction, was used as a template and a conceptual framework upon which our study was based. The attributes such as **predisposition, insult, response to treatment and organ dysfunction** were readily testable in this clinical research setting.

The **predisposing** factors included age, weight, severity of lung injury and presence of co-morbidities. In this thesis we selected those patients who according to the 2001 ACCP/SCCM consensus conference definitions {Levy et al. 2003} represented a spectrum of morbid conditions from severe SIRS / sepsis or SIRS / septic shock to MODS. Severe SIRS was defined as SIRS in the presence of organ dysfunction such as ARDS and severe sepsis as defined by the consensus conference (Table 1.3) as sepsis complicated by organ dysfunctions (Table 1.4). Patients were referred for ECMO with either a primary clinical diagnoses of an infection; commonly a pneumonia leading to severe sepsis or a non-infective **insult** that had provoked severe systemic inflammatory response manifested as acute respiratory distress syndrome. It is important to note that the mechanism of pneumonia leading to organ dysfunction is in itself a spectrum that can vary from CARS to SIRS. Therefore patient with severe sepsis may have respiratory failure due to overwhelming bacterial infiltration / consolidation of the lung, or develop ARDS secondary to bacteraemia caused by pneumonia (Intrinsic sepsis). Differentiation between these two in the absence of reliable inflammatory markers is quite difficult and in reality they both occur simultaneously but to varying degrees. The incidence and **response** of different categories of SIRS, adjustment for age according to reference levels as illustrated in the introductory chapter, was determined in each patient group. In order to minimise lead-time bias {Muckart et al. 1996}, the data for classification of severe SIRS / sepsis and MODS were determined using the worst physiology immediately before and during the course of ECMO treatment. When assessing disease progression, the use of inotropes / vasoactive substances prior to ECMO implied hypotension despite adequate fluid resuscitation and according to the consensus conference definition (Adjusted to include SIRS), this state of circulatory collapse is known as shock. The use of vasoactive drugs during ECMO, to the exclusion of Dopamine at < 5 mcg/Kg/min, use

of VA-ECMO / conversion from VV- to VA-ECMO, or requirement for additional cardiovascular support with intra-aortic balloon pump during ECMO also implied cardiovascular dysfunction.

The severity and outcome of **organ dysfunction** was determined by MODS index (Table 1.6). MODS was defined as the simultaneous dysfunction of two or more organ systems according to Wilkinson {Wilkinson et al. 1986}. Age adjusted criteria for dysfunction of respiratory, cardiovascular, renal, hepatic, neurologic, haematologic, and gastrointestinal systems were based on clinical and laboratory measurements or the need for specific therapies. Any item within each category was considered diagnostic.

In this study the patients who were labelled as no shock, either belong to the severe SIRS or severe sepsis groups. The patients labelled as in shock had cardiovascular dysfunction prior to the start of ECMO. In the presence of respiratory failure this may be classed as pre ECMO MODS.

Patients: Since the start of the ECMO programme in Aug 1989, 875 patients were treated with ECMO at Glenfield University Hospital, Heart link ECMO centre, Leicester, United Kingdom. There were 395 neonates, aged 28 days and below (Aug 1989- Feb 2005), 278 children aged above 28 days up to and including 17 years (Apr 1990- Feb 2005) and 202 adults from 18 to 65 years inclusive (Apr 1990- Jun 2001) with exclusion of those adults randomised into the CESAR trial (A multi-centred randomised controlled trial, comparing Conventional ventilation versus ECMO for Severe Adult Respiratory failure) since July 2001. Patients were referred from hospitals throughout the United Kingdom as well as Europe and were transferred to Leicester by land or air ambulance.

The patients included in this study had either severe sepsis as defined by consensus conference, or severe SIRS manifested as ARDS or SIRS in the presence of other organ dysfunction. Patients were categorised firstly according to their age. Each age group was then sub-divided into two main categories, severe sepsis and severe SIRS. Each sub category was further divided according to cause. For severe sepsis this was the causal organisms and in severe SIRS the insult. Patients were then categorised as no shock or in shock if they were on inotropes or constrictors in excess of low dose dopamine (≤ 5 mcg/kg/min) at the time of referral or immediately prior to ECMO.

Patients had MODS if they satisfied the criteria for two or more organ dysfunction. All patients in shock had suffered MODS prior to ECMO according to the

definition as having two or more organs failed. The number of organs failed on ECMO was determined for the calculation of MODS index. Chronic organ dysfunction for example chronic renal failure was not included in calculation of MODS index. The common organ dysfunction in patients of all age groups was the lungs. All patients satisfied the criteria for this specific organ dysfunction ($\text{PaO}_2/\text{FiO}_2 < 200$). Respiratory dysfunction in severe sepsis was either intrinsic, that is caused by pneumonia or extrinsic due to septicaemia. In adults and children clinical diagnosis of ARDS implied severe SIRS. In the introductory chapter we explained the controversy surrounding the pathophysiology of MAS. However increasingly evidence indicates that systemic inflammation induced by meconium aspiration is an essential part of the pathophysiology of this condition. Therefore we classed MAS as a cause of non-septic severe systemic inflammation and organ dysfunction in neonates.

Patients were excluded from this study if they died as a result of an iatrogenic cause or if they suffered from a predisposing lung disease like cystic fibrosis or chronic inflammatory diseases of the lung for example Wegener's granulomatosis. Patients with pneumocystis were excluded on the basis that they are more likely to represent a counter anti-inflammatory response syndrome (CARS) rather than severe systemic inflammatory syndrome (SIRS).

Outcome was measured in terms of recovery leading to ECMO decannulation and discharge alive (Survival). Patients may have received ECMO twice, however survival was only counted once upon discharge from the ECMO centre, as for calculations such as MODSI.

Comparative performance review of oxygenators: The state of the art in oxygenator technology

Methodology: ECMO is a relatively new supportive therapy for severe systemic inflammatory response and multiple organ dysfunction syndromes. However ECMO itself exacerbates inflammatory, coagulative and anti-fibrinolytic responses. One of the main components of the ECMO circuit, which is in constant contact with blood, is the oxygenator (Diagram 2.1). A novel type of oxygenator made from Poly-methyl pentene (Medos Hilite, Medizintechnik AG, Obere Steinfurt 8-10, D-52222 Stolberg, Germany) was launched in the new millennium. We began using Poly-methyl pentene (PMP) oxygenators in our institution in place of silicon membrane (SM) oxygenators (Medtronic, Medtronic Europe Sarl, Route du Molliau, Case Postale, CH-1131 Tolochenaz, Swetzerland) in March 2001. The Medos Hilite 7000LT was used in place of

the Medtronic 1-4500-2A for our adults and later we extended their use to neonates, using the Medos Hilite 800LT to replace the Medtronic 0800 (September 2001). The clinical impression was that patients with PMP oxygenators had reduced signs of blood activation on chest radiographs within the first 24 hours of cannulation and our experience with the first small group ($n = 6$) of adults {Peek et al. 2002}, prompted the investigation into the differences between these two oxygenator groups.

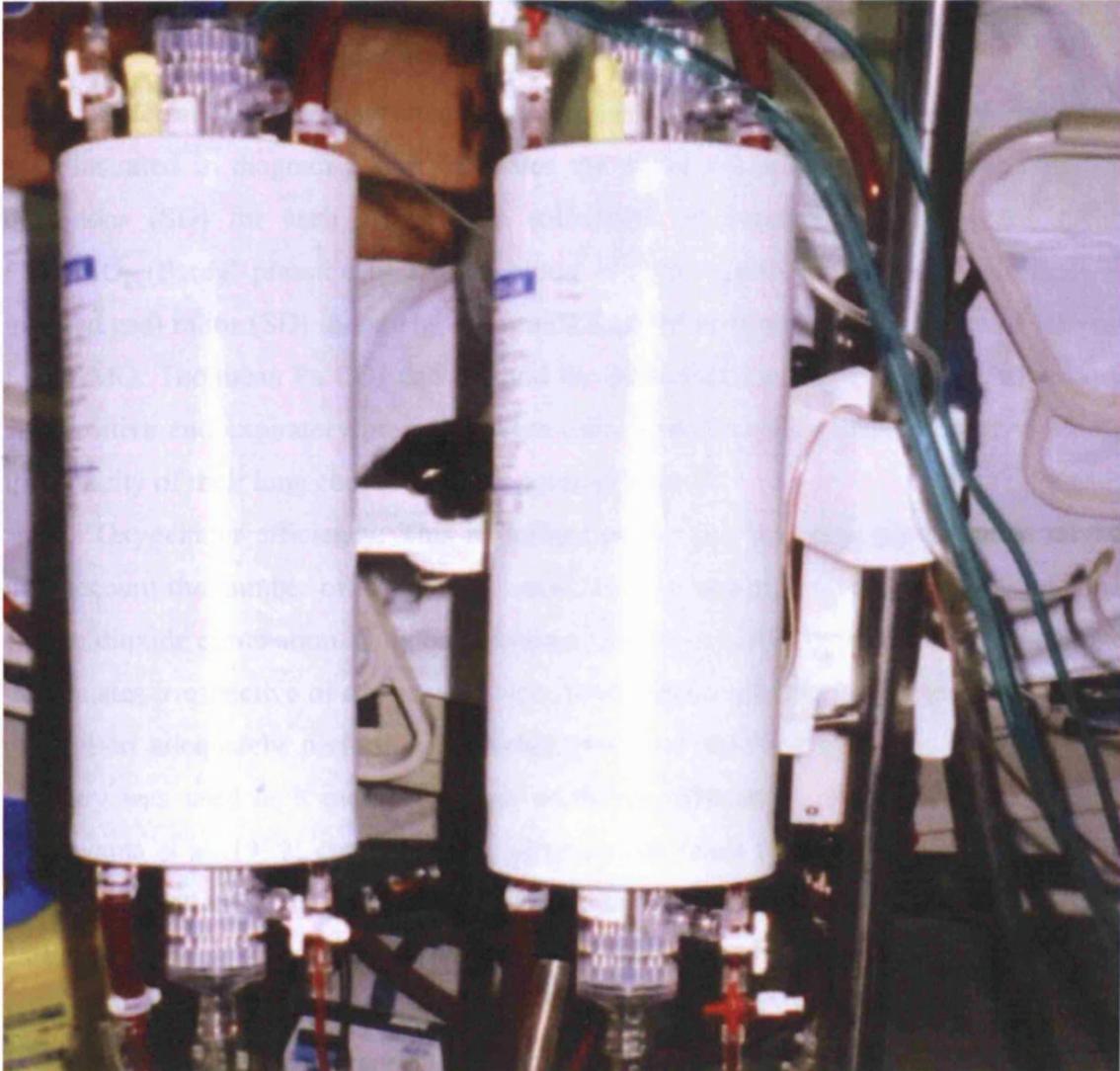
Diagram 2.1 Illustrating blood contact within the ECMO oxygenator.



Poly-methyl pentene oxygenators (Diagram 2.1) combined the solid membrane properties of silicon oxygenators (Diagram 2.2) mandatory for separation of blood and gas phases during gas exchange, with the hollow fibre design of polypropylene oxygenators (Used in cardio-pulmonary bypass) essential for lowering resistance and reducing the priming volume. In PMP oxygenators gas exchange occur through a plasma tight polymer matrix with hydrophobic properties to improve separation of blood and gas phases, hence preventing the plasma leakage, which occurs with micro-porous polypropylene hollow fibre devices {Thiara et al. 2007, Kind et al. 2006 & Keldenich et

al. 2000}. The physical properties and the structural design of these oxygenators had allowed them to be made smaller, with smaller surface area and lower priming volumes compared to Silicon oxygenators (Table 2.1).

Diagram 2.2: Adult silicon membrane oxygenators.



Eighty consecutive patients were selected retrospectively pre and post introduction of the new poly-methyl pentene oxygenators. They formed four equal groups of twenty, half adults and half neonates. They were selected according to the date they received ECMO for respiratory support and formed pairs of compatible groups with similar backgrounds.

Table 2.1: Comparing physical dimensions for each oxygenator. These measurements were obtained from the notes provided by the manufacturers.

Oxygenator	A-S (2 x Medtronic 1-4500-2A)	A-PMP (Medos 7000LT)	N-S (Medtronic 0800)	N-PMP (Medos 800LT)
Surface area (m ²)	9	1.9	0.8	0.31
Prime volume (ml)	1330	275	100	55

Referral details: Patients demographics were compared; the aetiological variations are illustrated in diagram 2.3. In neonates the mean oxygenation index and standard deviations (SD) for each group were calculated. In contrast for adults, the mean PaO₂/FiO₂ (Partial pressure of arterial blood oxygen / concentration of the oxygen in inspired gas) ratios (SD) in mmHg were calculated for each patient at the time of referral for ECMO. The mean Pa CO₂ and SD and the differences in the mean peak inspiratory and positive end expiratory pressures were calculated for each group in order to assess the severity of their lung condition at the point of referral.

Oxygenator efficiency: This is defined as the gas exchange performance taking into account the number of oxygenators used, oxygen transfer across the membrane and carbon dioxide elimination. Only one oxygenator was required for adequate gas exchange in neonates irrespective of oxygenator type, however the number of oxygenators used in parallel to adequately perform gas exchange varied between each adult group. This property was used as a measure of gas exchange efficiency. A study by Moriyama {Moriyama et al. 1999} complemented observations made by Cerra {Cerra et al. 1979} that inflammation increases the consumption of oxygen in critically ill patients. The partial pressure of oxygen in arterial blood (PaO₂) was used to assess tissue oxygenation in veno-venous patients and mixed venous saturation (MVSO₂) in the veno-arterial ECMO patient. The median partial pressure of arterial carbon dioxide (Pa CO₂) was used as a measure of CO₂ clearance. The bloods flow rate (Flow) and the gas flow rate through the oxygenator (Sweep) was taken into account when comparing gas exchange efficiency in each group. The median flow to sweep ratios for each patient were calculated and the mean values compared as an indicator of CO₂ exchange efficiency.

Coagulation data: The coagulation profiles including thrombin time (TT), activated clotting times (ACT), activated partial thromboplastin time ratios (APTTR), international normalized ratios (INR) and platelet counts were used to assess resistance to heparinisation and preservation of coagulation factors and platelets between groups.

Consumption of Blood products: Blood product consumption including coagulation factors and platelets during the course of ECMO in order to maintain a steady state may be indicative of the activation of STIP as a result of consumption given that the other component of the circuit mainly the raceway and the roller pump was unchanged. These were the differences in the mean red blood cell (RBC), fresh frozen plasma (FFP) cryoprecipitate and platelet transfusion requirements in ml/ecmo day. The values excluded the volume of RBCs used for priming the circuit.

Oxygenator pressure and resistance: The mean values of the median pre- and post-oxygenator pressures extracted for patient was used to calculate the resistance across the oxygenators. Resistance (Paul Wood units) = Pressure gradient / Flow, where pressure is measured in mmHg and flow in litres per minute. In the adult group the resistance between the sub-groups where some patients acquired two oxygenators in parallel were assessed using Kirchoff's law, $1/R = 1/R_1 + 1/R_2$ measuring the sum of oxygenator resistances.

Semi-quantitative methods have been used to accurately predict histopathological changes in ECMO patients with respiratory distress syndrome {Maurer et al. 1998}. We used this method to assess differences in the degree of inflammatory response that affects the lung during the early stages of ECMO between our groups. Chest x-rays (CXRs) were assessed at pre-cannulation, immediately post-cannulation, first day post-cannulation and from then on up to recovery or death. They were examined for the presence of alveolointerstitial lung shadowing at areas least affected by the primary disease on the immediate post-cannulation CXR and compared to subsequent chest radiographs. X-rays were scored by a single independent radiologist as 0 = No significant alveolointerstitial lung shadowing, 1 = mildly increased alveolointerstitial lung shadowing, 2 = moderately dense alveolointerstitial lung shadowing and presence of air bronchogram and 3 = severe (Total white out) with or without air bronchogram). The immediate post cannulation score for each patient was subtracted from that of the first day post cannulation score to give a final score. The number of days for the CXR appearance to return to the immediate post cannulation state was counted.

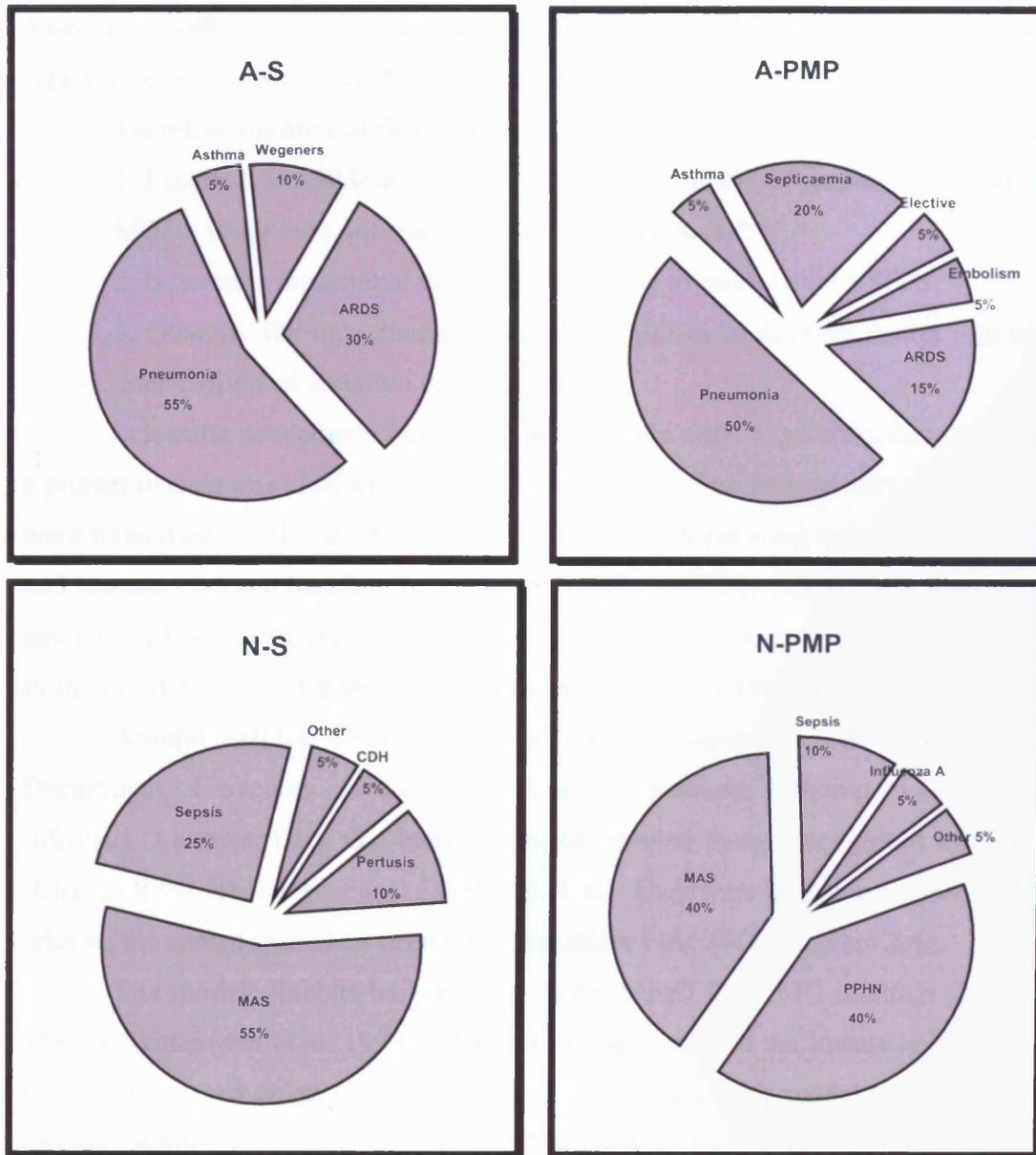
Currently all ECMO Centres in the United Kingdom have adapted to using PMP oxygenators, in patients of all age groups. However, concerns were raised regarding the tolerance of the smaller variety of oxygenators against clots.

Clots and oxygenator failure: We tackle the safety aspect of each oxygenator as oxygenator failure may occur suddenly with severe consequences. We use case reports in this section of the thesis to illustrate some important issues in the neonatal groups.

ECMO run and survival: These data reflect the use of resources in terms of length of treatment required and the primary outcome measure which is survival to discharge. These factors are important in terms of cost to the NHS and the patient.

Patients: They were patients in a single institution that received ECMO between May 2000 and June 2002. They formed four equal groups of twenty: Adult size PMP (A-PMP), adult size silicon (A-S), neonatal PMP (N-PMP) and neonatal silicon (N-S). The A-PMP patients were further sub-divided; according to the number of oxygenators they received simultaneously, to A-PMP I, for one oxygenator and A-PMP II, for two oxygenators placed in parallel. Adult size refers to patients with a body weight of 60 kg or above. The majority of patients received veno-venous ECMO (VV-ECMO). Seven patients required additional circulatory support with veno-arterial ECMO (VA-ECMO) from the start. One patient was converted from VV- to VA-ECMO shortly after the start of treatment and one patient with a previous pneumonectomy received elective ECMO prior to his lung surgery. Five patients in the N-PMP group and four in the N-S group had one or all of their CXRs missing. Two patients received a second run of ECMO, however for simplicity and to avoid bias by patient repetition, for each patient only the first run was included. Patient details are summarized in table 2.2.

Diagram 2.3: Pie charts comparing disease aetiology.



IN-VIVO-ANIMAL EXPERIMENTS

Methodology: This animal experiment was designed to characterize the graded clinical and pathophysiologic responses to the administration of intravenous endotoxin L-2143 and development of SIRS / sepsis and MODS in rabbits. Whilst similar models sepsis (SIRS) do exist in the published literature {Ito et al. 1978, Nishina et al. 1997 & Yokoi et al. 1997} they do not contain a sufficiently well characterized model that can be reproduced, nor illustrate any form of dose response and inter-individual variation.

Therefore the aims of this study is to,

1. Establish a reliable and reproducible Rabbit model of graded SIRS / sepsis and MODS using intra-venous Lipo-poly Saccharide (LPS).
2. Determine the optimal dose of LPS needed to cause lethal MODS.
3. Quantify the time course required for MODS to develop taking into account inter-individual variation in this species.

Scientific procedures (Animals) act 1986: In order to perform these experiments a project licence was obtained from The Home Office. The researchers and animal carers were trained by the Home Office and were issued with personal licences for appropriate and humane care and handling of the animals (PIL 80/8220). All animals were treated in strict accordance to the project license (PPL 80/1562), the department of Surgery Animal Protocol (SUR70) and the animal scientific procedures act 1986.

Animal welfare: Theatres and staff were provided by the Bio-medical Services Department, University of Leicester (Clinical Sciences Building, Leicester Royal Infirmary, Leicester UK). Out bred rabbits are ordered from a recognised establishment (Charles River laboratories; Inc. Edinburgh, UK). They were kept in isolation and looked after by the named animal carer and welfare officer NACWO (Diagram 2.4).

The model: Rabbits have previously been used in ECMO research {Kress et al. 1987 & Trittenwein et al. 1999}. They are the smallest and the lowest order of animals that can be placed on an existing human ECMO circuit. This model uses Female New-Zealand White {Granton et al. 1997 & Matsukawa et al. 1993} Rabbits > 3.5 Kg in Weight. This weight is similar to that of a neonate; therefore a neonatal ECMO circuit may be used on these animals, however large enough to allow adequate blood sampling throughout the experiment.

Diagram 2.4: Animal welfare.



This experiment was performed in two phases both using an identical procedure save for the dose of LPS administered (Table 2.2).

In this chapter we will concentrate on the practical aspects of the model such as the anaesthesia, surgical procedures, blood sampling and post-mortem examination.

In the first phase of this experiment we used 12 Rabbits. They were imported to our institute, acclimatised and observed for at least one week prior to experimentation. The animals were pre-medicated, anaesthetised and procedures performed in order to enable ventilation by surgical tracheostomy and monitoring by ECG, arterial and venous pressure lines and urinary catheter. The animals were stabilised and maintained at normal body temperature (37.5-38.5 °C) using a heated blanket / space blanket and hydrational state by continuous maintenance fluid at 4 ml/Kg/hr. Ventilation was adjusted to maintain normal blood gases. Fluid management was the only measure used to maintain blood pressure and organ perfusion. Except for the first three who received none (The controls) the remaining nine rabbits received incremental doses of Lipo-polysaccharide, L-2143 (Endotoxin from E-Coli K235, Sigma-Aldrich Company Ltd). The primary and secondary outcome measures were observed and the animals were followed to death or

sacrificed after 24 hours of reaching a stable state in controls or administration of LPS. Post-mortem examination was performed and tissue samples were taken from different organs. The animals were then disposed off in accordance with the departmental protocol.

We examined the dose response relationship for development of graded SIRS / sepsis, MODS and ultimately death. The following outcome measures were therefore observed.

Primary outcome: Death.

Secondary outcome: Development of graded SIRS and MODS.

Table 2.2: illustrating sequence of the experimental procedure.

Clock time	Experiment time	Event
0700	-3	Induction & tracheotomy
0800	-2	Monitoring lines & cystotomy
0900	-1	Stabilise for 1 hour & baseline blood samples
1000	0	LPS administration
1100	1	Sample, maintain anaesthesia & adjust life support measures
1200	2	Sample, maintain anaesthesia & adjust life support measures
1300	3	Sample, maintain anaesthesia & adjust life support measures
1400	4	Sample, maintain anaesthesia & adjust life support measures
1500	5	
1600	6	Sample, maintain anaesthesia & adjust life support measures
1700	7	
1800	8	Sample, maintain anaesthesia & adjust life support measures
1900	9	Deliver samples to Labs on ice, centrifuge samples and Freeze
2000	10	
2100	11	
2200	12	Sample, maintain anaesthesia & adjust life support measures
2300	13	
2400	14	
0100	15	
0200	16	Sample, maintain anaesthesia & adjust life support measures
0300	17	
0400	18	
0500	19	
0600	20	Sample, maintain anaesthesia & adjust life support measures
0700	21	
0800	22	
0900	23	
1000	24	Sample, schedule 1 euthanasia, post mortem examination
1100		Mop up
1200		Deliver over night samples to Labs on ice, centrifuge samples and Freeze

In the second phase all animals received the same dose of LPS in order to determine the inter-individual variation. In this phase five more rabbits from the same species were used. We used the dose response graph from the first phase to derive at our desired dose of LPS (Dose of LPS at T=5 was 0.018 mg/kg). This dose would allow MODS to occur without immediate death. It allows the desired amounts of time (Five hours), which would be needed, in future experiments to investigate possible treatments for MODS.

Rabbit anaesthesia

Rabbits are easily stressed by inexperienced pre-operative handling and during induction. The combined effect of stress and anaesthesia can result in cardiac and respiratory arrest. Care should be taken to prevent that by:

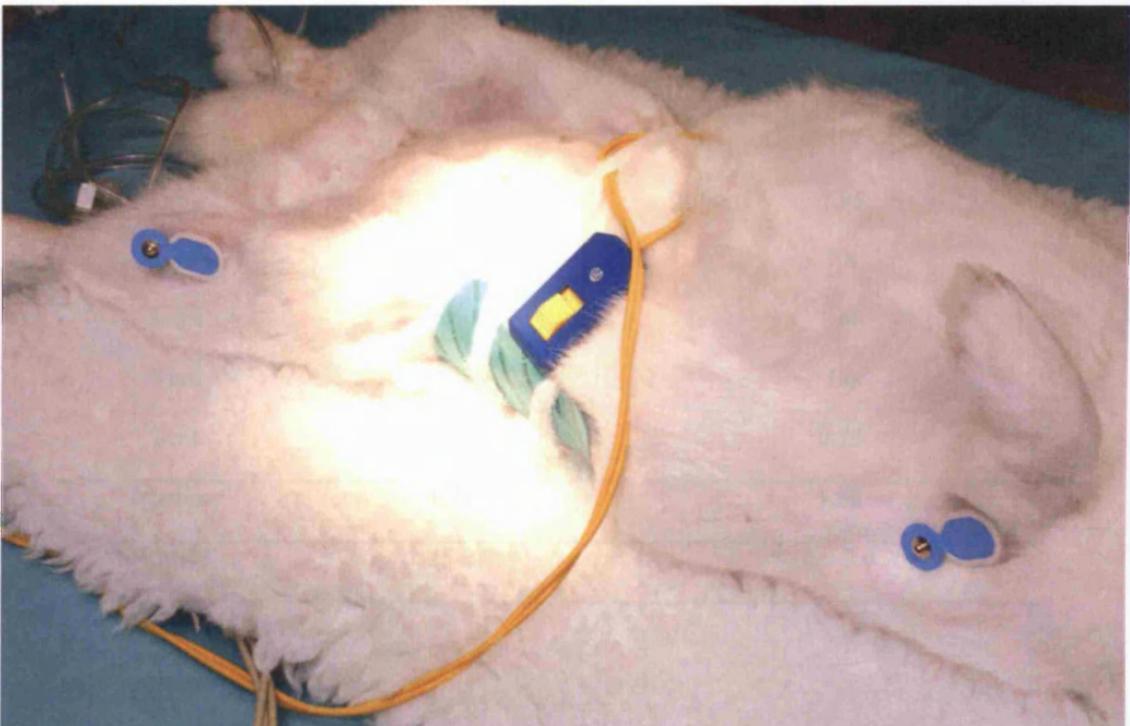
1. Choosing the rabbit: The ideal weight for the rabbit was between 3.5 and 5 kg. Rabbits larger than 5 kg have more problems with induction and maintenance of anaesthesia.
2. It is important to make sure that the rabbit is not suffering from any form of respiratory infection known as “the snuffles”. This typically presents as coryza.
3. Rabbit Pre-medication: This is performed by an intramuscular injection of a combination of Atropine (0.25 mg) and 1 in 10 Hypnorm 0.3 ml / kg, thirty minutes prior to induction.
4. Preparation of the skin: The rabbit coat is clipped at sites where surgical procedures or monitoring is to take place. These are:
 - a. The left ear
 - b. The ventral aspect of the neck for the tracheostomy.
 - c. Both groins for arterial and central lines.
 - d. Supra-pubic area for cystotomy.
 - e. Left and right elbow and left knee for ECG pads.
 - f. Lower back for diathermy pad.
5. Cannulate the dorsal vein on the rabbits left ear with a 20-24 G cannula and secure it with sleek tape (Diagram 2.5).
6. Place the ECG dots and the diathermy pad on the clipped areas.

NB: Cut the diathermy pad to the required size (7cm x 14 cm) and secure it with bandages (Diagram 2.6).

Diagram 2.5 (Below): Cannulation of the dorsal vein in rabbits ear. Note: the assistant is occluding the vein proximally make the cannulation easier.



Diagram 2.6 (Below): Position of the ECG and the diathermy pads.



7. Induce the anaesthesia by intravenous administration of a mixture of 1:10 Hypnorm 1 ml/Kg and Midazolam via the ear cannula.
8. Maintenance of anaesthesia: Intravenous Hypnorm and Midazolam were used. The initial dose was estimated by adding the consumption of anaesthetic agents during the initial surgical procedures. In order to maintain a satisfactory level of anaesthesia the maintenance was continuously titrated throughout the experiment by testing the level of consciousness in rabbit by their response to painful stimuli (Table 2.3).

Table 2.3: Illustrates the dose of anaesthetic used for maintenance in each named Rabbit.

Rabbit	Hypnorm 1 in 10 (ml/kg/hr)	Midazolam 2mg/ml (ml/kg/hr)
Gladys	3.3	0
Hermion	1.6	0
Ivy	1.25	1.25
Lidia	2.7	1.7
Mono	2.0	2.0
Nena	1.5	1.5
Orbit	2.1	1.3
Pady	1.95	1.95
Roman	1.8	1.8
Sama	1.68	1.68
Tina	1.6	1.6
Unit	1.25	0.75
Median	1.74	1.55
Range	1.25-2.7	0.0-2.0

Surgical procedure

It is essential to perform the surgical procedures with great care. Particular attention should be given to haemostasis as the rabbits have a small circulatory volume. Haemostasis is particularly important as this model is to be used for ECMO/ECLS therefore it is important to withstand anticoagulation. All surgical procedures should be performed under sterile conditions with sterile drapes, gowns and gloves.

1. Tracheotomy: Skin was prepared as per protocol with a 1 in 10 diluted solution of Betadine antiseptic scrub. It is important for this procedure to be done without delay as the rabbit has a tendency towards respiratory arrest so one must be prepared to perform the tracheotomy rapidly.
 - a. The rabbit is placed in a supine position.
 - b. A mix of air and oxygen is given to the rabbit during the procedure (Diagram 2.7).
 - c. The trachea is palpated gently in the neck.
 - d. The skin is incised in a median sagittal plane over the trachea.
 - e. Slings placed proximal and distal to the site of incision between the 3rd – 8th ring (Diagram 2.8).
 - f. The trachea incised and the Portex endo-tracheal tube (Size 3.5-4.5 mm) inserted to the 4 cm line.
 - g. Ventilation is maintained by bagging while the tracheotomy tube is secured in place by tying it with a 2-0 Silk tie.
 - h. The proximal stay is tied off.
 - i. The skin closed on top with 2-0 Nurolon Polyamide braided non-absorbable (Johnson & Johnson) continuous stitches.
 - j. The rabbit is attached to the ventilator (Sechrist, Baby log) pre set at Peak inspiratory pressure (PIP) of 25 cm H₂O, Positive end expiratory pressure (PEEP) of 5 cm H₂O, rate of 30 BPM, (Inspiratory time of 0.7 and Expiratory time of 1.3 seconds) and an FiO₂ of 0.6 (Diagram 2.9).



Diagram 2.7 (Above) and 2.8 (Below): Surgical tracheostomy.

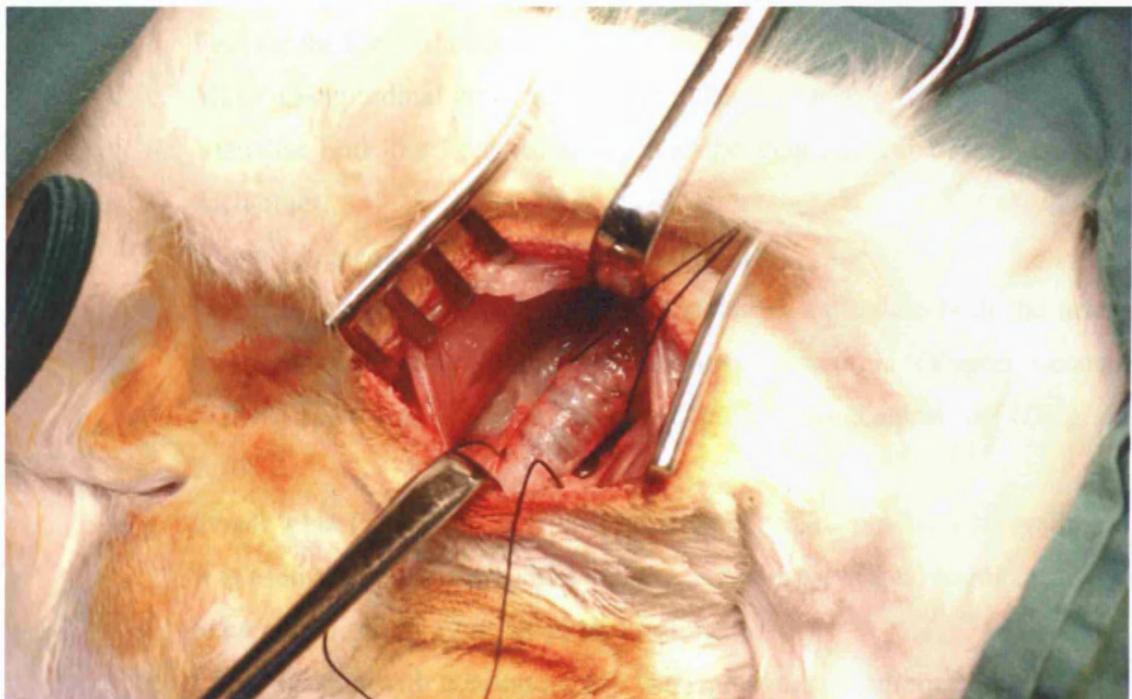


Diagram 2.9: Connection to positive pressure ventilation.



2. Femoral Cut down: This is performed for cannulation of the femoral vessels. It is important to prepare both femoral regions in case of a failed attempt on one side.
 - a. Prepare the area with 1 in 10 solution of Betadine.
 - b. Feel for the femoral pulse.
 - c. Make a longitudinal incision over the pulsatile artery.
 - d. Visualise both the Femoral artery and the vein and separate them from each other.
 - e. Apply slings to each both proximal and distal.
 - f. Using Seldinger technique under direct vision cannulate both the artery and vein using an 18-gauge teflon arterial cannula (Vygon Ecoen, France) and a size 7.5 F 10 cm triple lumen Vygon central line (Diagram 2.10).
 - g. Tissue glue (Tissumed II from Phoenix veterinary products laboratories) was used on the puncture sight and the ties to achieve total haemostasis.
 - h. Secure the lines by tying them to the slings.
 - i. Close the skin with continuous 2-0 Nurolon stitch (Diagram 2.12).

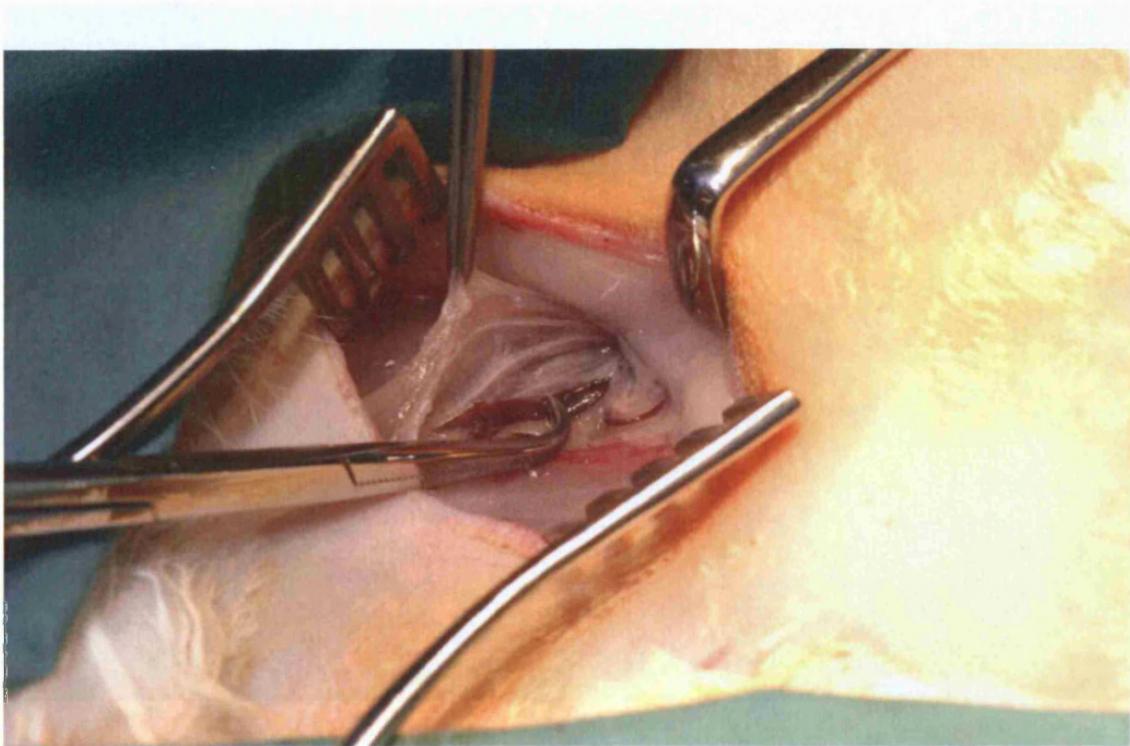


Diagram 2.10 (Above) and 2.11 (Below): Femoral cannulation.

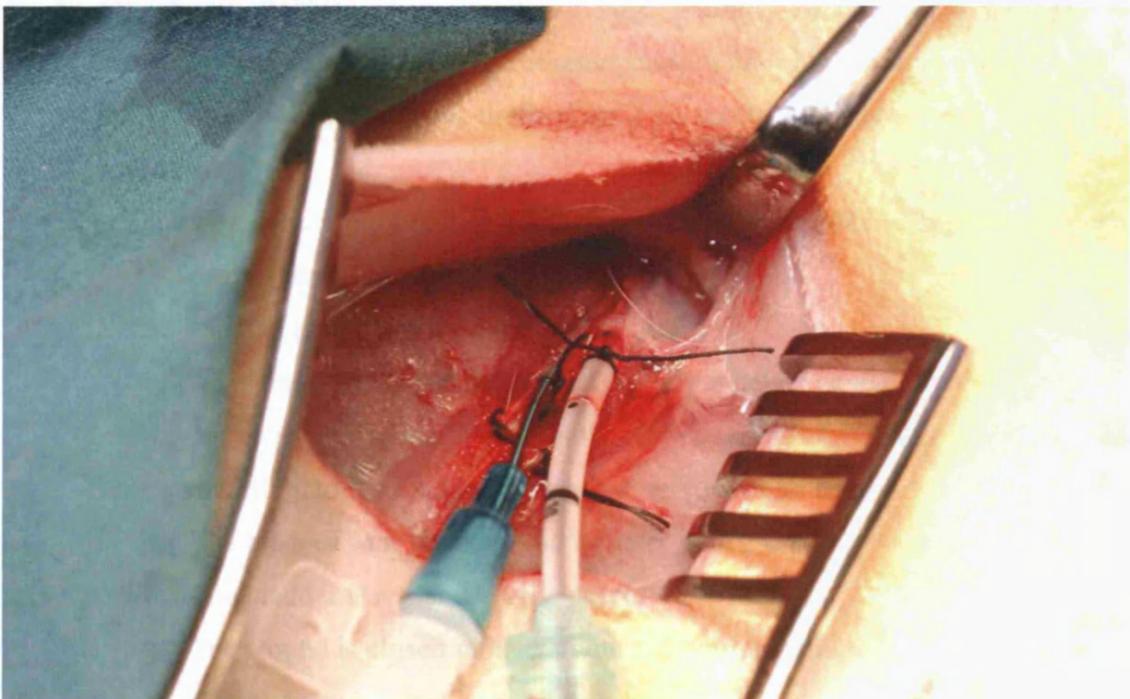
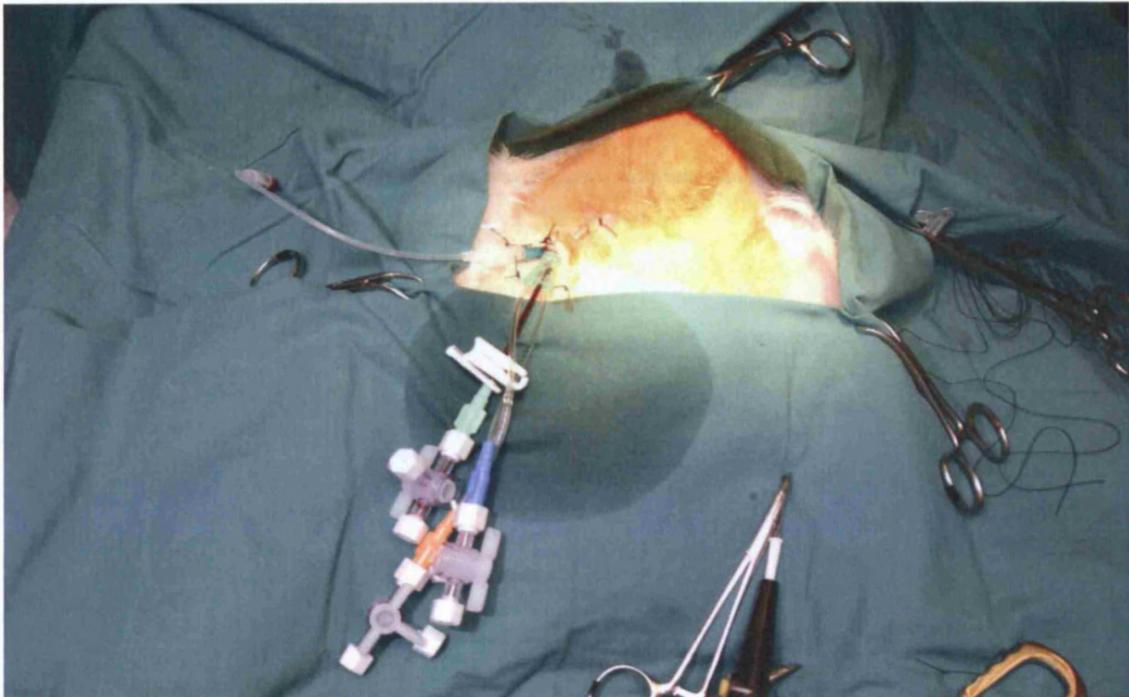
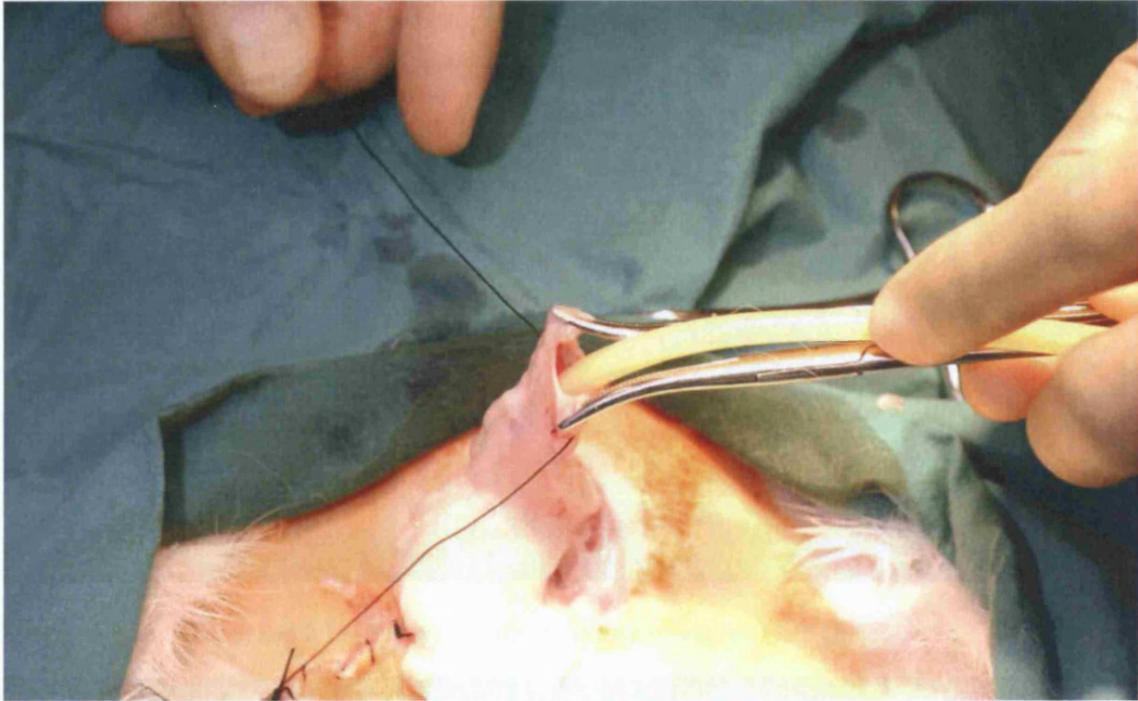


Diagram 2.12: Completion of the femoral cannulation.



1. Cystotomy: Rabbits urine contains a large quantity of residue so in order to measure urine output it requires catheterisation with a large bore catheter. This is too large to be inserted trans-urethral so it necessitates a supra-pubic cystotomy approach.
 - a. The area is prepped at the same time as the groin preparation for the central lines.
 - b. Median incision is made above the pubis.
 - c. The bladder is found and pulled out of the pelvis gently.
 - d. An incision is made by diathermy probe and catheter (Foley 12F) is placed directly into the bladder, which is a very vascular organ.
 - e. The bladder wall is tied to the catheter with 2-0 silk ties (Diagram 2.13).
 - f. The bladder is replaced in the pelvis.
 - g. The wound is closed in layers with 2-0 Nurolon suture.
 - h. The catheter is attached to an urometer.

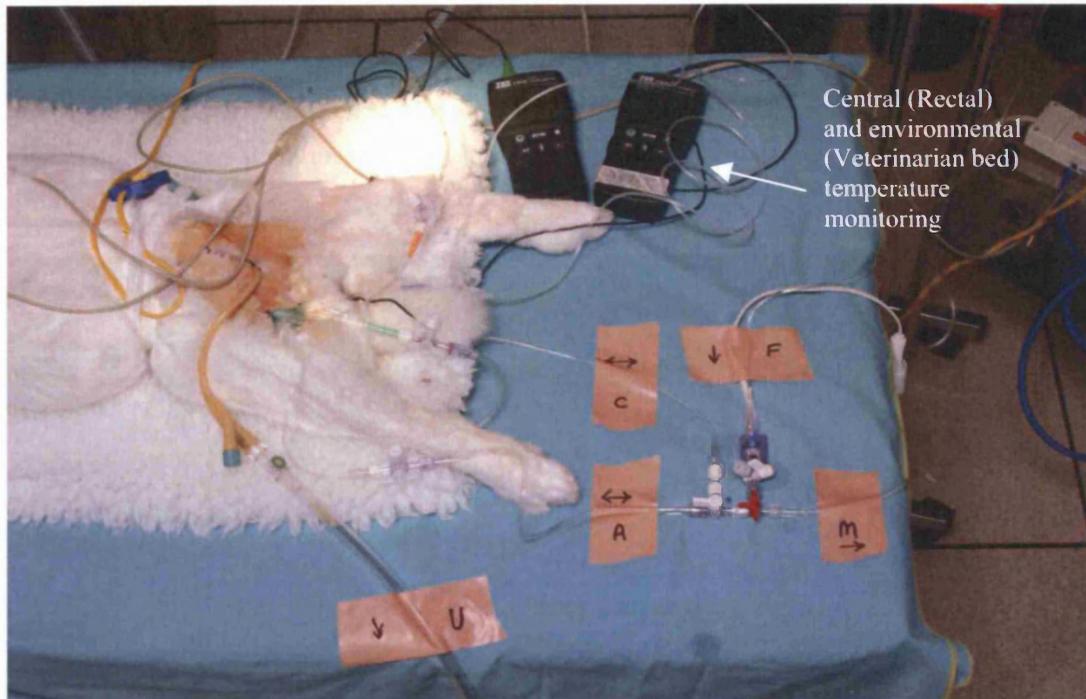
Diagram 2.13: Surgical cystostomy for supra-pubic catheterisation.



2. Connection to the monitors:

- a. 3 lead ECG connected to ECG monitor.
- b. Invasive blood pressure monitoring connected to pressure monitor.
- c. Central venous pressure monitoring connected to pressure monitor.
- d. Hourly urine output monitoring using an urometer.
- e. Temperature central (Rectal) and environmental (Veterinarian bed) monitoring (Diagrams 2.14 & 2.15).

It is important to use appropriate sterile instruments in each stage of the surgical procedure. Diagram 2.16 a, and b illustrate the instruments used for performing the tracheostomy and the vascular cut down.



Diagrams 2.14 (Above) and 2.15 (Below): Set up of the monitoring lines and devices.

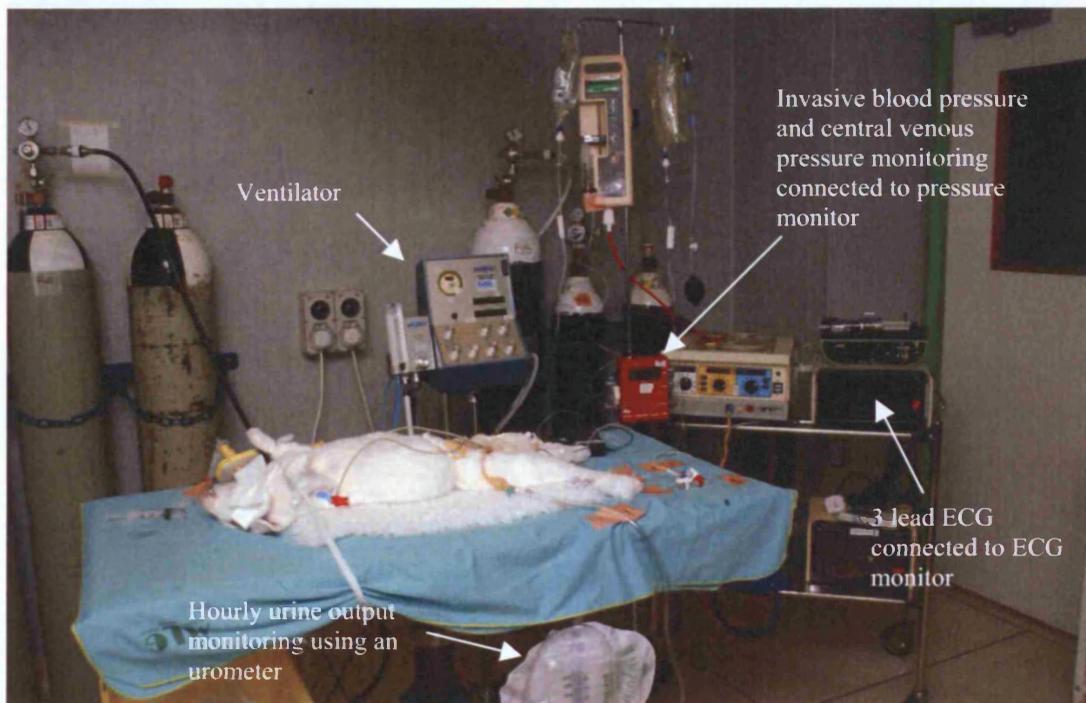


Diagram 2.16 a (Tracheostomy set) & b (Vascular down set): Illustrating the surgical instruments used in each experiment such as Langenbeck retractors, self-retaining retractors, scalpel, Debaquey forceps, Metsenbaum's scissors, towel clips, Mosquito and artery clips, O Shaughnessy clip, side biting vascular clamp, needle holders sterile drapes and diathermy.



Endotoxin preparation and administration

Lipo-polysaccharide L-2143, an endotoxin from E-Coli K235 (Sigma-Aldrich Company Ltd) is a dusty solid substance. This component of the bacterial outer membrane is the prime initiator of gram-negative sepsis {Morrison et al. 1987, Brade et al. 1988 & Glauser et al. 1991}. This product is harmful (Hazard category = medium) therefore precautions are necessary in handling and use to avoid contact with skin and mucus membranes. Protective gloves, goggles and facemask are necessary. A COSHH (Control of substances hazardous to health) assessment form was completed. The powder was mixed with sterile water at 50 mg in 50 ml to form a stock solution. Subsequent concentrations were obtained by further diluting the stock solution prior to the experiment. The stock solution was kept in a 50 ml syringe clearly marked as LPS at 4-8 °C (Diagram 2.17).

Management of anaesthetised rabbit

1. All surgical incisions were closed.
2. Monitoring lines were connected to heparin flush (To maintain their patency) and pressure transducer.
3. Animals received intravenous maintenance fluid (Dextrose 4%/Saline 0.18%, with potassium as required).
4. Anaesthesia was maintained throughout the experiment, as this was a terminal procedure.
5. Intermittent positive pressure ventilation (IPPV) was adjusted to maintain normal blood gas tensions.
6. Colloid (Gelofusin solution) was given in small boluses of up to 5 ml/kg to maintain cardiac filling pressures.
7. Arrhythmias and cardiogenic crises were treated on their merits as they arose, but infusions of inotropes and diuretics were not used, as they would confound the end points.
8. The animals were left to stabilise for 1 hour after surgery, then baseline blood samples and physiological observations were done. A total of up to 8.5 mls of blood was drawn from the arterial line and placed into containers for the following investigations:
 - Blood gases (Pre-heparinised mini syringe)

Diagram 2.17: Lipopolysaccharide L-2143 from E- Coli K-235 Lot number 81K4141.



- Liver function, urea, creatinine and electrolytes (Lithium-heparin paediatric tubes)
 - Full blood count (FBC) including the differential white cell count (EDTA paediatric tube).
 - Coagulation tests (Citratd paediatric tubes).
 - Serum and plasma samples for Plasma/serum protein levels by enzyme-linked immuno-sorbent assay (Plain tube and EDTA tube respectively).
9. Blood samples and physiological observations will be made at 1 hour after stabilization and repeated at 2,3,4,6,8,12,16,20 & 24 hours. This gives a total sample volume of approximately 90 ml, assuming a circulating volume of 80ml/kg, the worst-case scenario for a 3.5 kg rabbit, is that this amounts to 33% of the blood volume. Since the sampling will be spread over 24 hours and the filling pressure will be restored with colloid as indicated this should be well tolerated (Diagram 2.18).
10. Animals were observed continuously until they died, or 24 hours post stabilization.

Diagram 2.18: Sampling of blood and tissue.



11. If they survived the full 24-hour observation period they were euthanased by a Schedule-1 method (Intravenous injection of 20% Pentobarbital solution at 2 ml/Kg). A full post-mortem examination was performed on each rabbit. All the major organs were examined macroscopically and samples were taken for histology and wet to dry ratio measurements.
12. Lung function was assessed by calculation of $\text{PaO}_2/\text{FIO}_2$ ratios. This was calculated by using measured FIO_2 and the measured arterial oxygen tension; it gives a direct measure of the efficiency of the alveolar membrane, impaired in ARDS. Oxygenation index was calculated from the airway pressures and respiratory rate, thereby giving a measure of progression of ARDS. The wet to dry ratio was measured by weighing the tissue before (immediately after death) and after desiccation, this gave an estimate of the severity of tissue water content that is well known to be an important consequence of ARDS/MODS. semi-quantitative neutrophil immunohistochemistry, and histology were used to estimate the degree of neutrophil infiltration and the amount of lung damage caused.
13. Cardiovascular status was assessed via Mean arterial pressure, mixed venous O_2 saturation and myocardial wet to dry ratio.
14. Liver function (PT ratio and trans-aminase elevation were measured by routine haematological and biochemical techniques thereby giving an index of the livers synthetic function and the severity of any hepatocellular damage respectively. The wet to dry ratio was measured, this gave an estimate of the severity of tissue water content that is a well known consequence of MODS).
15. Renal failure was assessed by measuring the urine output, serum urea, electrolytes and creatinine and kidney wet to dry ratio.
16. Gut injury was assessed by wet to dry ratio.
17. Differential WBC was measured using routine haematological investigations. The serum $\text{TNF}\alpha$, interleukins, complements and other biological markers of STIP were measured by enzyme linked immuno-sorbent assay (ELISA) this data allowed us to quantify the inflammatory response provoked.
18. Oxidative stress and DNA damage to the lung tissue was assessed by lipid peroxidation giving an estimate of free radical activity, and by semi-quantitative immunofluorescence techniques.

Post-mortem Examination

Immediately after the death of the rabbit a post mortem examination was performed and tissue samples were taken for histology and wet dry ratio measurements.

1. This was performed shortly after death or euthanasia.
2. The instruments did not require sterility.
3. Involved examination of the internal organs.
4. Notes were taken of any technical faults.
5. Position of the lines and tubes were noted.
6. Any morphological abnormalities were noted.
7. All cavities except cranium were examined.
8. Individual organs within each cavity were examined.
9. Samples were taken and weighed from Lung, Heart, Liver, Kidney and Stomach for wet/dry ratio study.
10. A specimen of lung tissue was fixed in formalin and sent for histology.

Technical points

These technical points were learnt in previous rabbit experiments performed by our research group, which are not described in this thesis.

1. The tracheostomy tube may twist within the trachea as the animal's torso is twisted on the bead filled mattress. This may cause asphyxia.
2. Control the airway by tying the endo-tracheal tube to the trachea to avoid displacement.
3. Stress and blood loss from surgical sites may result in VF arrests.
4. Hypnorm and Midazolam provide stable anaesthesia during induction and maintenance. Inhalation anaesthesia does not provide smooth anaesthesia as rabbits may hold their breath, preventing administration of the agent.
5. Cannulation for central and arterial line needs to be done using magnifying instruments.
6. A Sechrist ventilator consumes high quantities of gas (3 x higher than measured flow) care should be taken by acquiring an adequate amount of oxygen and air for the duration of the study which takes >24 hours.
7. During Cystostomy care should be taken not to tie the ureters as they can be quite anterior to the bladder.

Wet to dry ratio

During the post-mortem examination representative tissue samples were obtained from the right lower lobe of the lung, the heart, a kidney, a piece of stomach and a section of the liver. These samples were immediately weighed with a sensitive scale to the closest milligram. Then they were frozen to minus seventy degrees Celsius until all samples could be processed together. The samples were then placed on pre-weighed baking paper, defrosted and dried with constant heat at 65°C in an oven (Pickstone equipment Ltd) for three days {Klinzing et al. 2000}. There was no change in the weight of the samples between day two and three. The weight of the baking paper was subtracted from the dry weight prior to calculation.

The wet-dry ratio W/D was calculated as:

$$\frac{(\text{Weight}_{\text{wet}} - \text{weight}_{\text{dry}})}{\text{weight}_{\text{wet}}} \times 100$$

This illustrates the percentage of water in each tissue sample, which can then be compared.

IN-VITRO-LABORATORY EXPERIMENTS

Rabbits are a good host for the production of polyclonal antibodies. These antibodies are frequently used to detect plasma and serum levels of a variety of proteins in other species including humans. However, there has been little effort to produce antibodies against rabbits. One reason being that there are not many other abundant sources of polyclonal antibodies and monoclonal antibodies although extremely specific, are not very sensitive making experimental methodology some what more difficult.

This part of the research project deals with the laboratory experiments that evaluated the biochemical, haematological, histological, immunological, immuno-histochemical and immuno-fluorescent techniques, which were employed to characterise the thrombo-inflammatory response in rabbits.

A simple ELISA method was designed to determine concentration of various inflammatory substances in rabbit serum or plasma. Immunohistochemical methods were

designed to determine infiltration of cellular components of innate response as well as to assess oxidative stress and DNA damage in the lung.

Standard Laboratory Investigations

The department of biochemistry at Leicester Royal Infirmary conducted standard biochemical investigations such as the urea, creatinine and electrolytes and liver function tests. The haematology department at Leicester Royal Infirmary conducted full blood count and coagulation tests.

Parafin blocks were made from the lung tissue samples in formalin and sections were cut and mounted on glass slides. Haematoxylin and Eosin (H&E) staining was performed on each block. The histology department at Leicester Royal Infirmary provided this service. These slides were also used for immunohistochemistry and immunofluorescent experiments.

In order to perform the in-vitro experiments a lab was set up at the department of cardiovascular sciences, Robert Kilpatrick Building (RKB) Leicester Royal Infirmary.

Enzyme Linked Immuno-sorbent Assay (ELISA)

A simple, cheap, reliable and reproducible method of quantitative measurement of serum / plasma levels of biochemical components of the innate immune system involved in SIRS / sepsis in rabbits was developed.

This method was used to determine concentrations of various thrombo-inflammatory proteins and other key elements of the innate response in rabbits. In a minority of cases where compatible kits were available they were used. In some cases where cross reactivity data was available or where protein gene sequence homology were available (ExpASY proteomics server), after careful consideration of the design of the kits and the substrates used, kits designed for other species were tried in rabbits. However in the majority of cases rabbit specific ELISA protocols were designed to quantify serum biomarkers (ELISA protocol 2.1). Wherever possible anti rabbit antibodies were especially produced otherwise antibodies (Mainly polyclonal) with highest % sequence homology was used in the protocol.

A non-competitive double antibody sandwich technique {Wreghitt et al. 1990} was used for quantitative measurement of the majority of serum/plasma markers of STIP. Highly-purified antibodies against specific surface antigens (Capture antibodies) are non-covalently adsorbed ("coated") as a result of hydrophobic interactions with the plastic micro well plates (Nunclon TM Surface CAT. NO.167008). After washing the plates in order to remove un-bound antibodies the immobilized antibodies on the plate serve to

specifically capture soluble proteins present in samples that were applied to the plate. The specificity of the solid phase antibody was improved further by blocking non-specific binding by adding a blocking buffer (For example: 5% BSA in PBS Tween). After washing away the block the sample was added to the wells and incubated. Unbound material was washed away and the captured serum/plasma proteins detected using biotin-conjugated antibodies against the particular antigen (detection antibodies). This was followed by an enzyme-labelled avidin or streptavidin stage and the addition of the chromogenic substrate. The level of coloured product generated by the bound, enzyme-linked detection reagents was measured spectrophotometrically using an ELISA-plate reader at an appropriate optical density (OD).

A standard curve was incorporated into the sandwich ELISA by making serial dilutions of a standard protein solution of known concentration. Standard curves (Aka “calibration curves”) were plotted using the standard protein concentrations in ng or pg/ml versus the corresponding mean OD value of the replicates. Each sample was at least in duplicate unless measurements were frequent and close to one another. The concentrations of the proteins captured from each sample were then interpolated from the standard curve.

Quality Control

Prior to performing the full experiment a pilot dilution experiment (Optimisation run) was performed on a random sample in order to assure that the OD would fall within the linear portion of the standard curve. Although opinions differ as to determining the ELISA sensitivity, we choose the lowest cytokine concentration that gives a signal, which were at least two or three standard deviations above the mean background signal value. The enzyme-mediated amplification of the detection antibody signal by sandwich ELISA can measure physiologically relevant (*i.e.*, > 5-10 pg/ml) concentrations of specific plasma/serum proteins in a mixed solution. Horseradish Peroxidase (HRP) and Alkaline Phosphatase (AKP) were the enzymes employed in these ELISA methods.

A random positive control sample was used at different intervals within and between plates to assess inter and intra plate variation. The absorbance values for these samples were used to calculate the mean, standard deviation and the coefficient of variation (CV). If the CV of < 10% was not achieved the assay system was investigated to ensure correct procedures are being followed and look for technical errors. All solutions were prepared in house and to the correct concentration and PH with purified water. For each specific protein care was taken to use the same batch of wells, solutions

and chemicals. This would reduce inter-plate variation. The procedures were recorded accurately with times for each step of the experiment. A stopwatch was used to aid time keeping. All calculations were documented in full.

Determination of plasma/serum concentrations

Upon measurement of the optical densities (OD), values were either manually transferred to an excel spreadsheet or a specific ELISA computer software program (Davies, C calibration 1994) used to calculate the protein concentrations (Complements only). The concentrations of the proteins were determined by comparing the ODs against the standard curve. The standard curve was the regression plot obtained from the serial dilutions. The best fitting plot provided the regression equation, which was then used to calculate the protein concentration. The concentrations obtained were then transferred to SPSS for graphical presentation.

Storage

Samples: After separation of the Plasma and Serum using a Centrifuge. The samples (up to a maximum of 500 μ l in volume) were placed in eppendorpf containers, re-labelled according to the blood sample they were derived from (e.g. AUD 004, 009, 014, 019 etc) and stored immediately at -75°C . These samples were thawed once and aliquoted into five 100 μ l samples (E.g. AUD 004 a,b,c,d and e), then re-frozen to -75°C immediately in especially designed and labelled racks to allow easy access.

Antibodies: Lyophilised proteins were stored for no more than twelve months at -20 to -75°C . Upon reconstitution, the antibodies were stored at $2-4^{\circ}\text{C}$ for no longer than one month this would ensure no detectable loss of activity as indicated by majority of the antibody suppliers. Reconstituted antibodies were divided into aliquots, each sufficient for one or more 96-well plates and stored at -75°C in a manual defrost freezer until ready for use (Maximum of six months). Repeated freeze-thaw cycles were avoided.

Rabbit ELISA protocol 2.1

Capture antibody (Solid Phase):

1. Dilute the purified capture antibody (Polyclonal Goat anti Rabbit / Human) in Binding Solution.
2. Add 100-200 μl of diluted antibody to each well of an enhanced protein-binding ELISA plate.
3. Seal plate to prevent evaporation.
4. Incubate.

Blocking:

5. Bring the plate to RT.
6. Remove the capture antibody solution (You may re-use it).
7. Wash wells three times with PBS.
8. Block non-specific binding by adding 200 μl of Blocking Buffer per well (BSA in PBS Tween).
9. Seal plate and incubate.
10. Wash wells three times with PBS/Tween.

Standards and Samples:

11. Add standards and samples (Diluted if necessary in Blocking Buffer/Tween at 100 μl per well).
12. Seal the plate and incubate it for 2-20 hrs at RT or overnight at 4°C (Overnight incubation of standards and samples is recommended).
13. Wash wells three times with PBS/Tween.

Detection antibody:

14. Biotinylate the primary Goat anti-Rabbit / Human antibody of interest using the Biotinylation Kit Sigma BK-200.
15. Dilute the biotinylated detection antibody in Blocking Buffer/Tween.
16. Add 100 μl of diluted antibody to each well.
17. Seal the plate and incubate for 1-3 hrs at RT.
18. Wash wells three times with PBS/Tween.

Conjugate:

19. Dilute the Av-HRP (Avidin-horseradish Peroxidase: Sigma A2528) conjugate to 1:100,000 (minimum for optimal concentration) in Blocking Buffer/Tween.
20. Add 100 μl per well.
21. Seal the plate and incubate it at RT for 30 min.
22. Wash wells three times with PBS/Tween.

Substrate:

23. Add 100 μl of TMB (Stabilised Hydrogen peroxide and Stabilised Chromogen, mixed in the dark, within 15 min of use, R&D895000&895001) to each well (Protect from light).
24. Incubate for up to 80 min in the dark at RT for colour development.
25. Add 50 μl of stop solution (R&D895032) to each well.
26. Read the optical density (OD) for each well with a microplate reader set to 450-570 nm.

Antibody purification

Some of the antibodies were unstable in purified form and required purification at the point of use. A MabTrap Kit (Amersham Biosciences) was therefore used. This kit is an affinity chromatography kit for fast and effective one step purification of monoclonal and polyclonal IgG antibodies.

Biotinylation of antibodies TPA

A biotinylation Kit from Sigma (Sigma-Aldrich Product Code BK-200) was used for the biotinylation of the detection antibody.

Optimisation

For each ELISA experiment immunoreagent concentrations and dilutions were optimised in order to achieve best signal and to develop a robust, reproducible assay for the sample over a biologically relevant range. This was achieved by adjustment of concentration of sample and reactants, incubation times and temperatures.

Determination of the dilution of the capture antigen and of the sample: This was done using a chessboard assay with high to low assay values and negative samples.

Optimum conjugate dilution: The recommended manufacturer dilution and several other dilutions on either side were used to determining the optimal conjugate dilution.

The optimum dilution for the capture layer and sample is the highest dilution of capture antigen which gives the highest positive to negative (P:N) ratio and a high positive sample control absorbance value of at least 0.8 {Wreghitt et al. 1990}.

Detection

A spectrophotometer was employed for quantitative evaluation of the 96-well microtitre plate-based system. In the majority of cases a dual beam reading was taken to minimise background noise from scratches or particulate matter on the wall. The spectrophotometer was regularly calibrated to ensure optimal performance.

Results

The results were expressed as absorbance values. The standards were incorporated with each assay and all other values were corrected with respect to the standard curve.

Standard curve

A linear cytokine ELISA standard curves was obtained in a series of eight two-fold dilutions of the pooled (Concentrated) normal rabbit serum/plasma. The range for

the standard immunoassay was from 2000 pg/ml to 15 pg/ml and 15 pg/ml to 0.5 pg/ml for highly sensitive immunoassay using an amplifier.

Calculation of antigen concentration

Linear regression was used to fit straight line to data and to compute a coefficient of linear correlation.

The following is the account of the variation in experimental methodology for each biomarker in each category.

Ready to use kits

CRP: Rabbit CRP Immuno-peroxidase assay for determination of C-reactive protein in rabbit sera based on double antibody sandwich ELISA principle (ELISA kit Manufactured by Immunology Consultants Laboratory).

Note: Prior to the experiment the optimal sample dilution was determined using serum specimens from random experimental animals. The optimal dilution was determined as 1:500. This was the same as the recommended dilution for the positive control. The concentrations were measured in duplicates. There were five standard duplicates and five positive control duplicates (Concentration = 40 ng/ml) and two negative control duplicates scattered at different points on the plates.

Nitric Oxide: Total rabbit serum nitric oxide levels were measured using a 96 well ELISA kit from R&D Systems (DE1600, R&D Systems Europe Ltd). This assay involves the conversion of nitrate to nitrite by the enzyme nitrate reductase. The detection of total nitrite is then determined as a coloured azo-dye product of the Griess reaction that adsorbs visible light at 540-570 nm. The samples yielded best results at a 1:3 dilution.

TGF- β 1: There were greater than 90% DNA homology between human and rabbit TGF- β 1. This kit utilised a monoclonal capture and a polyclonal detection antibody. This kit (mouse/rat/porcine TGF- β 1, Catalogue #: BMB100, R&D Systems Europe Ltd) had shown cross reactivity for mouse, rat and porcine species but there were no cross reactivity available with rabbits. Samples were diluted to 1:10 with the sample diluents provided by the kit.

Optimised rabbit ELISAs

TNF α : A purified Goat anti-rabbit TNF α Polyclonal antibody (Catalogue #: 551214, BD Biosciences) was used for the solid phase at an optimised concentration of 12ug/ml. The capture antibody was incubated at 37 °C for one hour. 2.5% BSA solution was used as block for one hour at 37 °C. Recombinant TNF α at a concentration of 1

$\mu\text{g/ml}$ was obtained from the National Institute for Biological Standards and Control (NIBSC). This was used as the standard x 8 dilutions from 1:40 in this experiment. The samples were diluted 1:10. Any lower dilutions resulted in signal inhibition. Standards and samples were incubated over night at 2-8 °C. Biotinylated Mouse anti-rabbit TNF Monoclonal antibody (Catalogue #: 552470, BD Biosciences) was used as the ELISA Detector antibody at a concentration of 2 $\mu\text{g/ml}$. Av-HRP was used as the conjugate at 1:10000 with 2.5% BSA for one hour at room temperature (RT). TMB substrate was left for 90 minutes at RT before being stopped by a one Molar solution of sulphuric acid and read at 450-630 nm.

IL-1 β : Rabbit serum containing customised goat anti-rabbit antibodies to IL-1 β were purchased from the National Institute for Biological Standards and Control (NIBSC). These antibodies were first purified according the above protocol and the purified antibody was used as the capture antibody in the concentration of 10 $\mu\text{g/ml}$ in PBS for 90 minutes at 37 °C. 5% BSA was used as the blocking agent for one hour at 37 °C. Recombinant Rabbit IL-1 β at a concentration of 1 $\mu\text{g/ml}$ was obtained from the National Institute for Biological Standards and Control (NIBSC). This was used as the standard x 8 dilutions from 1:20 in this experiment. Samples were diluted to 1:10 with PBS only and incubated overnight at 2-8 °C. The detection antibody was the biotinylated-purified goat anti rabbit IL-1 β antibody. The biotinylation protocol is described above. The concentration of the detection antibody was determined by optimisation experiments as 2 $\mu\text{g/ml}$. The optimal detection took two hours at RT with vibration at 120 RPM. Av-HRP at 1:10000 with 2.5% BSA was used as conjugate and was again left to vibrate at 120 RPM for one hour at RT. TMB substrate was left for 90 minutes at RT before being stopped by a Molar solution of sulphuric acid and read at 450-630 nm.

IL-5: We were unable to optimise a rabbit ELISA protocol for IL-5. Anti-human IL-5 antibody produced in goat immunised with purified recombinant human IL-5 (rhIL-5) was used as the solid phase capture antibody (Catalogue #: AB-205-NA, R&D systems, inc). The biotinylated antibody was used for detection and recombinant human Interlukin-5 (Catalogue #: PHC0055 from Biosource international) was used at different concentrations as the standard. Variations of every stage of the standard rabbit protocol were tried unsuccessfully, e.g. the concentrations of the antibodies the dilutions and the diluents used for the samples and the standards. The experiment was performed under variable PH, with different blocks (BSA vs. 3% Marvel) and conjugate (Avidin-

horseradish peroxidase vs. Avidin-alkaline phosphatase). In order to determine the cause of this failure we coated the plates with 1 µg/ml solution of rhIL-5 incubated it for one hour at 37 °C. After rinsing the plates 1% BSA block was applied for one hour at RT. The excess blocking solution was rinsed with PBS and the recombinant antigen was detected using variable concentrations of the biotinylated antibody between 0.2 to 2 µg/ml for one hour at RT. The excess antibody was washed away with PBS then the conjugate solution diluted to 1:10000 with 1% PBS was applied for 30 minutes followed by an other wash and neat TMB substrate. The reaction was stopped with strong acid. This experiment resulted in a graduated response according to the concentration of the biotinylated detection antibody, which implies that this assay works and the antibody and the recombinant antigen are both viable. The conclusion at this point was that the goat anti human antibody to IL-5 must have a single epitope which once bound to the solid phase will leave no other site for the attachment of the IL-5 and so the detection antibody. On the basis of this an inhibition assay was designed (Inhibition ELISA assay 2.2). In this assay free IL-5 in the sample and the standard compete with the bound IL-5 in the 96 well plates to bind with the detection antibody. This is called inhibition assay as the free IL-5 in the sample or the standard inhibits detection antibody from binding to the solid phase. Hence the lower the absorbance the higher the concentration of the IL-5 in the sample compared with the standards.

Competitive inhibition ELISA assay 2.2

Antigen binding (Solid Phase)

1. Dilute the recombinant antigen in binding solution.
2. Add 100-200 μl of diluted antigen to each well of an enhanced protein-binding ELISA plate.
3. Seal plate to prevent evaporation.
4. Incubate.

Blocking:

5. Bring the plate to RT.
6. Remove the recombinant antibody solution (You may re-use it).
7. Wash wells three times with PBS only.
8. Block non-specific binding by adding 200 μl of Blocking Buffer per well (BSA in PBS Tween).
9. Seal plate and incubate.
10. Wash wells three times with PBS/Tween.

Detection antibody, Standards and Samples:

11. Add 50 μl of the biotinylated detection antibody diluted in PBS only to 50 μl of standard or samples (Diluted if necessary in Blocking Buffer/Tween) making a 100 μl per well solution.
12. Seal the plate and incubate it for 90 minutes at RT.
13. Wash wells three times with PBS/Tween.

Conjugate:

14. Dilute the Av-HRP (Avidin-horseradish peroxidase: Sigma A2528 conjugate to 1:10,000 (minimum for optimal concentration) in Blocking Buffer/Tween
15. Add 100 μl per well.
16. Seal the plate and incubate it at RT for 30 min.
17. Wash wells three times with PBS/Tween.

Substrate:

18. Add 100 μl of TMB (Stabilised Hydrogen peroxide and Stabilised Chromogen, mixed in the dark, within 15 min of use, R&D895000&895001) to each well (Protect from light).
19. Incubate for up to 80 min in the dark at RT for colour development.
20. Add 50 μl of stop solution (R&D895032) to each well.
21. Read the optical density (OD) for each well with a microplate reader set to 450-570 nm.

IL-6: The DNA sequence homology between rabbits IL-6 and human or rats was very poor. It was not therefore worth testing cross reactivity between these species by using available human or rat IL-6 Kits on rabbit samples. Customised goat anti-rabbit IL-6 antibody and recombinant rabbit IL-6 were purchased from the National Institute for Biological Standards and Control (NIBSC). This antibody was used to coat the micro-wells after purification at a concentration of 10 µg/ml for one hour at 37 °C. Non-specific binding was minimised blocking with 2.5% BSA (Sigma-Aldrich, A7030) for one hour at 37 °C. Serum samples were diluted to 1:10 with PBS and standard x 8 dilutions from 1:20 using the 1 µg/ml recombinant solution. They were incubated for 12 hours at 2-8 °C. . The detection antibody was the biotinylated-purified goat anti rabbit IL-6 antibody. The biotinylation protocol is described above. The concentration of the detection antibody was determined by optimisation experiments as 2 µg/ml. The optimal detection took two hours at RT with vibration at 120 RPM. Av-HRP at 1:10000 with 2.5% BSA was used as conjugate and was again left to vibrate at 120 RPM for one hour at RT. TMB substrate was left for 90 minutes at RT before being stopped by 25 µl of one Molar Sulphuric acid and read at 450-630 nm.

IL-8: As always prior to the experiment the assay was optimised and reagents prepared. A solution of 2.5% BSA was prepared as the blocking solution. PBS and PBS Tween (0.5 %) were used for washing and rinsing the wells. Capture antibody, goat anti human IL-8 antibody (Catalogue #: AF-208-NA from R&D systems) was prepared by diluting it with PBS at 10 µg/ml and applied at 100 µl/well. The wells were incubated for 90 minutes at 37 °C while vibrating at 120 rpm. Block was applied for 60 minutes at RT and then 3 hours at 2-8 °C. Samples were diluted 1:10 in PBS. Standards human IL-8 at 1 µg/ml concentration was serially diluted from starting from 1/10 dilution. PBS was used as the negative control and 1/700 dilution for positive control. The samples and standards were incubated overnight at 2-8 °C on a vibrating plate at 120 rpm. The optimum concentration of detection antibody was 2 µg/ml in 2.5% BSA for two hours at RT vibrating at 120 rpm. The HRP conjugate was used at 1:3000 for 3 hours at RT in 1% BSA. TMB substrate was applied in the dark for 30 minutes prior to stopping the reaction with one Molar Sulphuric acid.

IL-10: The capture antibody, goat anti human IL-10 (Catalogue #: AF-217-NA from R&D systems) was prepared by diluting it with PBS at 10 µg/ml and applied at 100 µl/well. The wells were incubated for 90 minutes at 37 °C while vibrating at 120 rpm. Block (2.5% BSA) was applied for 60 minutes at RT and then 3 hours at 2-8 °C. Samples

were diluted 1:10 in PBS. Standards were serially diluted from 1/20 concentration of a 1 µg/ml solution. PBS was used as negative control and 1/700 dilution for positive control. The samples and standards were incubated overnight at 2-8 °C on a vibrating plate at 120 rpm. The optimum concentration of detection antibody was 2 µg/ml in 2.5% BSA for two hours at RT vibrating at 120 rpm. The HRP conjugate was used at 1:3000 for 3 hours at RT in 1% BSA. TMB substrate was applied in the dark for 30 minutes prior to stopping the reaction with one Molar Sulphuric acid.

Neutrophil elastase: Ten µg/ml of polyclonal sheep anti human Neutrophil Elastase capture antibody from Biodesign International (Catalogue #: K90052C, AMS Biotechnology Europe Ltd) formed the solid phase. The micro wells were incubated at 37 °C on a vibration plate at 120 rpm. After the rinse with PBS they were blocked with a filtered 5% BSA solution for one hour at 37 °C again on the vibrating plate at the same setting. After the wash and rinse step samples diluted to 1: 10 with PBS only and the standards Elastase from human leukocytes (Catalogue #: E8140, Sigma-Aldrich) were serially diluted starting from 800 ng/ml. The negative control was PBS and positive control a solution of 60 ng/ml concentration of human Elastase. The samples and the standards were incubated over night in the dark at 2-8 °C on a vibrating plate set at 120 rpm. They were detected using 2 µg/ml solution of the biotinylated sheep anti human antibody in 2.5% BSA, then incubated for one hour at RT and vibrated at 120 rpm. The concentration of the Av-HRP conjugate was 1:5000 and incubation for 30 minutes at RT without vibration. The plates were read at 450 nm after the substrate solution (Incubated for 90 minutes at RT and in a dark room), was stopped by a one Molar Sulphuric acid solution.

Tissue factor: Polyclonal Sheep anti rabbit tissue factor IgG (Product #: 4513, American diagnostica inc) was used at a concentration of 10 µg/ml. The Blocking solution was optimised as 2.5% BSA at 37 °C. The plasma samples were diluted in 1:10 with 10% normal rabbit plasma (NRP) for 24 hours at 2-8 °C in order to yield the best N: P ratio (8.5:1). The standards were prepared using recombinant rabbit tissue factor (Product #: 4520, non-lipidated) starting from 6400 ng/ml. The negative control was 10% NRP and positive control a solution of 300 ng/ml of the recombinant rabbit tissue factor. The captured rabbit tissue factor was detected using an in house biotinylated monoclonal antibody against rabbit tissue factor (Product #: 4511, American diagnostica). The incubation time for this stage was two hours at RT at a concentration of 1.5 µg/ml. The conjugate concentration was 1:1000 incubated in the dark for 30 minutes at RT.

PAI-1: Goat anti human PAI-1 IgG (Product #: 395G, American Diagnostica Inc.) was diluted with PBS to a concentration of 20 µg/ml. This constituted the solid phase antibody. The micro wells were incubated at 37 °C for one hour. After the wash with PBS the wells were blocked with 2.5 BSA for one hour at 37 °C on a vibrating plate at 600 rpm. The block was washed and rinsed with PBS Tween/PBS. The samples were diluted to 1:10 in PBS only, the standards serially diluted starting from 100 ng/ml and the positive control has a concentration of 18 ng/ml. PBS was used as negative control. Recombinant plasminogen activator inhibitor-one (rPAI-1) from Calbiochem (Catalogue #: 528205, Calbiochem) was used to constitute the standards. They were incubated for 90 minutes at RT on the vibrating plate set at 600 rpm. This time the standards and samples were washed with PBS/EDTA0.5M/Tween 20 (PET) only. The detection anti body was the biotinylated antibody used for the capture at the concentration of 2 µg/ml with 1% BSA in PET at RT. From now on all washes are performed with PET only. The conjugate was diluted 1:3000with PET/PBS 1% and incubated for 30 minutes at RT and vibration at 600 rpm. Vibration was also used during the substrate phase with TMB. The reaction was stopped using one Molar Sulphuric acid. The plates were read immediately at 450-570 nm.

Thrombomodulin: Goat anti-rabbit throrombomodulin IgG (Product #: 236, American diagnostica inc.) was diluted to 10 µl/ml with PBS and coated the plates at 37 °C for one hour. BSA at a concentration of 1% was used as the blocking agent, incubated for an hour at 37 °C. The standard (Rabbit lung Thrombomodulin, Product #: 237, American diagnostica) was diluted starting from 10 ng/ml and the sample at 1:10 with 1% BSA. The positive control was chosen as 2 ng/ml and the negative control consisted of 1% BSA. They were incubated over night at 2-8 °C. The detection antibody was the biotinylated goat anti rabbit Thrombomodulin IgG compound. It was diluted to 2 µg/ml and left to incubate at RT for 2 hours. Av-HRP conjugate was diluted to 1:3000 with 1% BSA and incubated for 30 minutes. TMB substrate was incubated for 30 minutes. The reaction was stopped using one Molar Sulphuric acid. The plates were read at 450-570 nm.

Fibrinogen: Fibrinogen concentration in rabbit plasma was detected using the in house ELISA protocol. Polyclonal goat anti-bovine fibrinogen (Product #: 3462 from American Diagnostica Inc,) was used to capture and the biotinylated compound to form the detection antibody. The plates were captured for one hour at 37 °C at a concentration of 20 µg/ml in PBS. Human albumin solutions (HAS) were used as a block at 2.25 %

concentration for one hour incubated at 37 °C. Samples were diluted to 1:10 in PBS. Fibrinogen from bovine plasma (Biochemika, >70% as protein, Sigma-Aldrich) was used for standard. The concentration of the standard ranged from 16 µg/ml to 15.6 ng/ml. PBS was used as the negative control and a sample solution of 300 ng/ml for positive control. Normal rabbit plasma was used at 1 in 10 dilution in PBS. Av-HRP (Sigma-Aldrich) in 1% HAS at 1 in 3000 dilution was used as conjugate and TMB (Biosource International) as substrate. 25ul of 1M H₂SO₄ was used as the stop solution and the plates were read at 450 nm.

Lectin pathway activation: This is a well-established functional assay to measure lectin pathway-dependent C4 activation in response to immobilised ligands. It was developed by Petersen in 2000. The sample dilution buffer contains 1 M NaCl, which permits high affinity binding of lectin pathway recognition components to their ligands, but prevents activation of endogenous C4 and excludes the participation of the classical pathway by dissociation of the C1 complex. The samples are added to the ligand-coated plate, followed by a constant amount of purified C4 in a buffer with a physiological concentration of salt. Bound recognition complexes contain MBL Serine Protease-2 (MASP-2) which cleave the C4, resulting in C4b deposition, which is detected with an antibody. During optimisation rabbit MBL (Mannan binding Lectin) did not bind to Mannan but to LPS, therefore LPS was used to coat the wells.

C3 activation assay: Activated rabbit C3 complement was detected by modifying an established human ELISA protocol where activated C3 molecules cleave to a particular ligand, in this case Lipo-polysaccharide.

C4 cleavage ELISA assay 2.3

Ligand binding (Solid Phase):

1. Dilute the Lipo-polysaccharide (LPS, L2143 from *Escherichia coli* K-235) in binding buffer (15mMNa₂CO₃ and 35 mM NaHCO₃ in ultra pure water, PH 9.6).
2. Add 100 µl of diluted antigen at 10 mg/ml concentration to each well of an enhanced protein-binding ELISA plate (Nunc Immuno plate, F96 Maxisorb, Catalogue #: 442404).
3. Seal plate to prevent evaporation.
4. Incubate over night at 2-8 °C.

Blocking:

5. Bring the plate to RT.
6. Remove the coating solution.
7. Block non-specific binding by adding 300 µl of Blocking Buffer per well (TBS in 0.1 % HAS) for 1-3 hours at RT.
8. Wash wells three times with TBS-Tween-CaCl₂ (10mM Tris, 140 mM NaCl, 0.05 % Tween and 5 mM Cal) PH 7.4, 200 µl/well.

Standards and Samples:

9. Dilute the standards and samples with the binding buffer as above. Start the standard dilutions by two folds from 1/10 and the samples at 1:20. Add 200 µl/well.
10. Seal the plate and incubate over night at 2-8 °C in a humidified chamber.
11. Wash three times with TBS-Tween-CaCl₂ (200 µl/well).
12. Add 100 µl/well of 1 µg/ml purified human C4 (From fresh human serum by iron exchange chromatography method developed by Dodds 1993) in Barbital-buffering-solution (BBS = 4 mM Barbital, 145 mM NaCl, 1 mM MgCl₂ and 2 mM CaCl₂, PH 7.4) incubate at 37 °C for 90 minutes.
13. Wash again three times with TBS-Tween-CaCl₂ (200 µl/well).

Conjugate:

14. Add alkaline phosphatase-conjugated chicken anti human C4c (Immunsystem AB, Uppsala, Sweden), diluted 1:1000 in TBS-Tween-CaCl₂. Incubate for 90 minutes at RT.
15. Wash plates three times with TBS-Tween-CaCl₂, 200 µl/well.

Substrate:

16. Add 100 µl of pNPP substrate solution (Sigma Fast p-Nitrophenyl phosphate tablet sets, Catalogue #: N-1891) to each well to detect alkaline phosphatase.
17. Incubate at RT until colour development.
18. Hydrolysis of the substrate is monitored quantitatively by measuring the absorption at 405 nm in a microtitre plate reader.

C3 activation ELISA assay 2.4

Ligand binding (Solid Phase):

1. Dilute the Lipo-polysaccharide (LPS, L2143 from *Escherichia coli* K-235) in binding buffer (15mMNa₂CO₃ and 35 mM NaHCO₃ in ultra pure water, PH 9.6).
2. Add 100 µl of diluted antigen at 10 mg/ml concentration to each well of an enhanced protein-binding ELISA plate (Nunc Immuno plate, F96 Maxisorb, Catalogue #: 442404).
3. Seal plate to prevent evaporation.
4. Incubate over night at 2-8 °C.

Blocking:

5. Bring the plate to RT.
6. Remove the coating solution.
7. Block non-specific binding by adding 300 µl of Blocking Buffer per well (TBS in 0.1 % HAS) for 1-3 hours at RT.
8. Wash wells three times with TBS-Tween-CaCl₂ (10mM Tris, 140 mM NaCl, 0.05 % Tween and 5 mM Cal) PH 7.4, 200 µl/well.

Standards and Samples:

9. Dilute the standards (1 µg/ml solution human complement C3, Department of immunology, University of Leicester) and samples with Barbitol-buffering-solution (BBS = 4 mM Barbitol, 145 mM NaCl, 1 mM MgCl₂ and 2 mM CaCl₂, PH 7.4). Start the standard dilutions by two folds from 1/10 and the samples at 1:50. Add 200 µl/well.
10. Seal the plate and incubate over night at 2-8 °C in a humidified chamber.

Detection:

11. Wash three times with 200 µl/well TBS-Tween-CaCl₂.
12. Add 100 µl/well of 1:2000 dilutions with TBS-Tween-CaCl₂, of a goat anti mouse antibody obtained locally (ARAS, Department of Immunology, Leicester University).
13. Incubate plate for 90 min at 37 °C.
14. Wash again three times with 200 µl/well TBS-Tween-CaCl₂.

Conjugate:

19. Add alkaline phosphatase-conjugated anti goat IgG diluted 1:10000 in TBS-Tween-CaCl₂. Incubate for 90 minutes at RT vibrating at 60 rpm.
20. Wash plates three times with TBS-Tween-CaCl₂, 200 µl/well.

Substrate:

21. Add 100 µl of pNPP substrate solution (Sigma Fast p-Nitrophenyl phosphate tablet sets, Catalogue #: N-1891) to each well to detect alkaline phosphatase.
22. Incubate at RT until colour development.
23. Hydrolysis of the substrate is monitored quantitatively by measuring the absorption at 405 nm in a microtitre plate reader.

Automated analysis

Total antioxidant status: Plasma samples were placed in an automated Olympus AU400 machine (Department of clinical biochemistry Leicester Royal Infirmary, Leicester U.K.). primed with colorimetric reagents (buffer, Chromogen and substrate) obtained from Randox Laboratories Ltd. (Catalogue #: NX2332, Randox). The machine was calibrated with total antioxidant status (TAS) standard solutions (Catalogue #: NX2615, Randox). Olympus AU400 worked on the basis of two-point standard curve. The positive controls (Catalogue #: NX2331, Lot 155NX Randox) yield highly accurate results.

Histology

Hematoxylin and eosin: Hematoxylin and eosin staining is often used to detect inflammation or to determine the integrity of a tissue. Haematoxylin and eosin staining is one of the most common method used in histology. Basophilic nuclei and calcium are stained blue with haematoxylin. Eosinophilic cytoplasm, connective, and all other tissues are counterstained red with eosin. Lung tissue specimens preserved in formaldehyde 10% V/V were paraffin fixed. Microtomes (4 μ m) were cut and mounted on to Vitrebond Adhesive Microscope Slides (Provided by histopathology department LRI). These were used for histological immunohistochemical and immunofluorescent experiments. The department of histopathology at Leicester Royal Infirmary performed the H&E staining. The rest of the blocks were stored in a cool place in the shade.

Immunohistochemistry

The cellular components of STIP were visualised by immunohistochemical methods either using an animal research kit (ARK) or an ABC protocol determined in the lab (ABC assay protocol 2.5). This experiment has to be performed in a fume cupboard (Diagram 2.19).

Animal Research Kit, ARK™: An ARK™ (Animal Research Kit, DAKOCytomation, Catalogue #: K3955) designed for use with primary antibodies from mice supplied by the user for the qualitative identification of antigens by light microscopy in formalin-fixed, paraffin-embedded tissue preparations from any animal species. The technique used in this system is based on the avidin-biotin and peroxidase methodologies. This system is formulated to minimize reactivity of secondary anti-mouse antibodies with endogenous immunoglobulin that may be present in the specimen. Prior to staining, tissue sections must be de-paraffinized.

Diagram 2.19: The set-up for the immunohistochemical experiments in the fume cupboard.



This was performed by firstly heating the slides to 50 °C for 15 minutes followed by dipping them in xylene (Catalogue #: X2377, Sigma-Aldrich) twice for 3 minutes each avoiding drying between dipping. This part of the experiment has to be performed in a fume cupboard. Xylene removes the embedding media. The slides are then re-hydrated with graded industrial methylated spirit (IMS) 99% followed by 95% IMS, 2 x one min dips in each before being placed in a bath of de-ionised water followed by a bath of wash buffer (PBS) for at least 5 minutes. Incomplete removal of paraffin will result in increased non-specific staining. Prior to application of the primary antibody to the specimen, the antibody is labelled using the biotinylation reagent, a modified biotinylated anti-mouse immunoglobulin. The primary antibody and the biotinylation reagent are mixed in solution, resulting in binding of biotinylated secondary antibody to the primary antibody. The blocking reagent, containing normal mouse serum, is then added to the mixture. The mouse immunoglobulin present in the blocking reagent binds residual biotinylation reagent not bound to the primary antibody, minimizing potential interaction with immunoglobulin endogenous in the specimen. The biotin-labelled primary antibody is then applied to the specimen. The specimen is next incubated with streptavidin-peroxidase, followed by reaction with diaminobenzidine / hydrogen peroxide as

substrate-chromogen. The slides were then counterstained with Hematoxylin, dehydrated (Reversal of rehydration steps) and mounted on a cover slip using Xam (Aqueous-based mounting medium donated by the department of Pathology, Leicester Royal Infirmary).

Quality control: A known positive control tissue was utilized for monitoring the correct performance of processed tissues and test reagents (Rabbit spleen / Human Tonsil). If the positive control tissues fail to demonstrate positive staining, results with the test specimens were be considered invalid. A negative control reagent (a mouse antibody of class/subclass identical to the primary antibody) was used in place of the primary antibody with a section of each test specimen to evaluate non-specific staining and allow better interpretation of specific staining at the antigen site. Biotinylation and subsequent blocking of the negative control antibody should be performed as for the primary antibody and the negative control antibody should be diluted to a final immunoglobulin concentration matching that of the primary antibody at its final dilution.

The methodology was tested using monoclonal anti-human CD45 (Catalogue #: V1162, Biomed) on human tonsil (Supplied by the department of immunology, LRI). This yielded excellent result. Prior to each rabbit experiment the best antigen retrieval method, optimum concentration of antibody and substrate solutions were determined. The exposure time to hematoxylin was also determined as its strength diminished with time.

Rabbit ABC Immunohistology: This method was used in addition to the ARK. This method allowed flexibility and ease of optimisation, however both methods worked perfectly well when used in association with a monoclonal antibodies. The ABC method had the advantage of enabling the use of polyclonal antibodies. This improved the chance of cross reactivity between rabbits and other species.

Cellular Components of STIP

Neutrophil: Monoclonal antibody to Rabbit Neutrophil Defensin 5 (NP-5 at 100 µg/ml, Catalogue #: HM4008, Hycult biotechnology) was used in association with ARK. After re-hydration the antibodies were retrieved by heating the slides for 20 minutes at 92 °C in citrate buffer 1:20 dilution. Then the slides were left to stand at RT for forty minutes. They were then washed with de-ionised water five times and set up in the humidity chamber. The concentration of the monoclonal antibody was optimised as 1:50.

Rabbit ABC Immunohistology Protocol 2.5

Antigen retrieval:

1. Heat slides to 50 °C for 15 minutes.
2. Removal of the embedding paraffin media using Xylene (Catalogue #: X2377, Sigma-Aldrich) twice for 3 minutes each avoiding drying between dipping.
3. Re-hydrating the slides by using graded alcohol solutions (99% IMS 2 x 1 minutes followed by 95% IMS 2 x 1 minutes and de-ionised water. industrial methylinated sprit (IMS) 99% followed by 95% IMS, 2 x one min dips in each before being placed in a bath of de-ionised water.
4. Antigen retrieval using one or more of Heating for 20 minutes at 92 °C in 1:20 dilution of citrated buffer solution, Pressure cooking in citrated buffer 1:20 dilution, High PH solution and microwave heat or Proteinase K (Catalogue #: S3020, DakoCytomation, www.dakocytomation.com).
5. Store the slides in de-ionised water until use.
6. Place slides in humidity chamber and cover the microtome with de-ionised water.
7. Remove excess water.

Block non-specific bindings:

8. Block non-specific staining with incubation buffer. That is normal goat serum (NGS) for CD4, CD57 rabbit N&T and Caspase or Bovine serum albumin (BSA) for CK.
9. Incubate for 30 minutes at RT.
10. Wash with PBS x 3.

Capture antigen:

11. Add 100 µl of Primary antibody diluted in incubation buffer at a predetermined dilution, for example 1:50-1:400 to each slide, making sure the microtome is covered.
12. Incubate in a humidity chamber over night at 2-8 °C.
13. Wash with wash buffer (PBS & 0.1% v/v Tween 20) x 2.

Detect the primary antibody:

14. Add 100 µl of detection antibody at predetermined dilutions for example 1:400 in incubation buffer. This antibody was a biotinylated goat anti mouse immunoglobulin (Catalogue #: E433, DAKO, www.dakocytomation.com).
15. Incubate for 30 minutes at RT.
16. Prepare ABC (ABCComplex Streptavidin biotin-peroxidase Complex, Catalogue #: BUF020, DAKO, www.dakocytomation.com) substrate solution now at 1:1000 dilutions with Tris Buffer Saline (TBS, 80 g NaCl + 6.05g Tris in 1 L de-ionised water. Adjust PH to 7.65 with HCl and make up to 10 L with de-ionised water).
17. Wash with wash buffer.

Substrate:

18. Add 100 µl of the prepared ABC substrate solution to each slide.
19. Incubate for 30 minutes at RT.
20. Wash with wash buffer.

Chromogen:

21. Add 100 µl of DAB (3,3'-Diaminobenzine Chromogen solution, Catalogue #: BUF021A, DAKO, www.dakocytomation.com) to each slide.
22. Incubate for 5 minutes at RT.
23. Wash under running tap water for five minutes.
24. Counterstain the slides with Mayers Hematoxylin.
25. Wash slides with tap water.

Mount:

26. Dehydrate the slide with graded IMS and Xylene (Reverse of the re-hydration step)
27. Mount cover slip with a drop of XAM.
28. View under light microscope.

Mononuclear Phagocytes: Mouse Monoclonal antibody (Ab-5) to Rabbit Macrophage cytoplasmic antigen (Clone RAM11, 50 µg/ml, Catalogue #: MS-1829-S0, Neo Markers, Labvision) was used at a 1 in 10 dilution. This assay only worked when antigen was retreated using Protease K (Catalogue #: S3020, DakoCytomation) for 30 minutes followed by Heat to 92 °C in citrate buffer 1:20 dilution for 20 minutes and RT for 40 minutes.

T lymphocytes: Mouse anti rabbit T cells and Neutrophils Monoclonal antibody (Catalogue #: MCA805, 10-50 µg/m, Serotec) was used in association with the ARK. After re-hydration the antibodies were retrieved by heating the slides for 20 minutes at 92 °C in citrate buffer 1:20 dilution. Then the slides were left to stand at RT for forty minutes. They were then washed with de-ionised water five times and set up in the humidity chamber. The concentration of the monoclonal antibody was optimised as 1:50.

T Helper Lymphocytes: Monoclonal mouse anti Rabbit CD4 (Catalogue #: MCA799, 10-50 µg/ml, Serotec) was optimised as 1:10 dilution and required Protease K antigen retrieval however this antibody failed to work with the ARK. This antibody was optimised after setting up a new rabbit immunohistology protocol (ABC method). This simple immunohistology protocol was utilized for all subsequent antibodies.

Histological and immunohistochemical scoring system: A semi-quantitative histopathological scoring system similar to one designed by Zeldin {Zeldin et al. 2001 & Gavett et al. 1999} was used in order to assess inflammatory response in rabbit lung. The scoring system was based on the presence or abundance of the following:

(a) Perivascular Oedema (0, absent; 1, mild to moderate, involving fewer than 25% of the perivascular spaces; 2, moderate to severe, involving more than 25% but less than 75% of perivascular spaces; or 3, severe, involving more than 75% of perivascular spaces); (b) Alveolar haemorrhage (0, absent; 1, mild to moderate, involving fewer than 25% of the alveolar spaces; 2, moderate to severe, involving more than 25% but less than 75% of alveolar spaces; or 3, severe, involving more than 75% of alveolar spaces); (c) Thrombosis in pulmonary vessels (0, absent; 1, mild to moderate, involving fewer than 25% of the pulmonary vessels; 2, moderate to severe, involving more than 25% but less than 75% of pulmonary vessels; or 3, severe, involving more than 75% of pulmonary vessels); (d) Alveolar consolidation with mononuclear and polymorphonuclear leukocytes or mixed inflammation (0, absent; 1, mild to moderate, involving fewer than 25% of the lung; 2, moderate to severe, involving more than 25% but less than 75% of the lung; or 3, severe, involving more than 75% of the lung); (e) Goblet-cell metaplasia of bronchioles

(0, absent; 1, few goblet cells present in one or two bronchiolar profiles; or 2, large numbers of goblet cells present abundant in the majority of airways); (f) Large macrophages in alveolar spaces (0, absent; 1, present in fewer than 25% of alveolar spaces; 2, present in 25–75% of alveolar spaces; 3, present in more than 75% of alveoli, often in tightly packed clusters); (g) Lymphoid aggregates (0, absent; 1, identifiable at low magnification around fewer than 50% of vessels and bronchi; 2, identifiable around more than 50% of vessels and bronchi; 3, identifiable around more than 50% of vessels and bronchi with acute inflammation and extension to involve small vessels); (h) Multinucleated giant cells (0, absent; 1, occasionally identified while examining slides at medium magnification [x10 objective] and not generally visible at low power [x4 objective]; 2, present and numerous, easily identified at lower magnification); (i) Eosinophils (0, absent; 0.5, a few identified within inflammatory exudates at high magnification; 1, present in multiple fields but never more than 10% of cells identified); (j) Large eosinophilic intracytoplasmic inclusions (crystals) in macrophages (0, absent; 1, identified in a few cells upon examination at high-power magnification [x40 objective]; 2, present and numerous, sometimes large and extracellular; 3, extremely numerous with many lying free within alveolar spaces); (k) Perivascular/peribronchial acute inflammation with neutrophil infiltration (0, absent; 1, mild acute inflammation in the perivascular oedematous space, with fewer than 5 neutrophils per high-power field (hpf); 2, moderate acute inflammation in the perivascular spaces, extending to involve the peribronchial spaces, with more than 5 neutrophils per hpf in these regions; or 3, severe, acute inflammation in the perivascular and peribronchial spaces with numerous neutrophils encircling most [$> 50\%$] of bronchioles).

Two observers, one an independent pathologist who trained the other, determined total histo-inflammatory scores taken as the sum of the individual scores. The independent pathologist who was blinded to the identity of the treatment/control groups. Intra-observer variability was determined by re-examination of 7 of the 11 parameters at a later time. The same value was assigned in 95% of the time. In the remaining 5%, the second score differed from the first by 1 grade.

CytoDEATH M30: Mouse monoclonal antibody IgG_{2b}, (Cytokeratin 18, Clone M30, Catalogue #: 2140322, 7 µg/ml, Roche Molecular Biochemicals) was incorporated in the Rabbit ABC Protocol (Above). During Apoptosis, vital intracellular proteins are cleaved. The proteases that mediate this process are called caspases (CysteinyI-aspartic acid proteases). Caspases are expressed as zymogens, which are activated by different

apoptosis inducers. Once activated, a single caspase activates a cascade of caspases. We were unable to optimise a monoclonal antibody to caspase 3 (CPP322, Catalogue #: M91210H, Biodesign) to detect early apoptosis in rabbit lungs. Until recently cytokeratins, in particular cytokeratin 18, was not known to be affected by early events of apoptosis. Recently, it has been shown, that the M30 antibody recognizes a specific caspase cleavage site within cytokeratin 18 that is not detectable in native CK18 of normal cells (Leers et al., in preparation). Consequently, the M30 CytoDEATH antibody is a unique tool for the easy and reliable determination of very early apoptotic events in single cells and tissue sections. This experiment was optimised and worked best when high PH and microwave heat was used for antigen retrieval and the antibodies were diluted to 1 in 400 for both the capture (Primary) and detection (Secondary) antibodies.

Immunofluorescence: These experiments were performed with the help of the department of clinical biochemistry at Leicester Royal Infirmary. They aimed to assess oxidative stress and DNA damage in paraffin fixed lung specimens obtained from our dose response experiments. This experiment has to be performed in a fume cupboard (Diagram 2.19).

Malondialdehyde (MDA) deoxyguanosine adduct: Monoclonal antibody M1G has been raised against Malondialdehyde deoxyguanosine adduct. The endogenous formation of MDA during intracellular oxidative stress and its reaction with biologically important macromolecules makes MDA–DNA adducts a suitable biomarker of endogenous DNA damage. This monoclonal antibody specific for MDA–DNA adducts, has been developed for the detection and quantification of DNA damage in cells. The concentration of the primary antibody was optimised as 1: 250 dilution with 2% normal goat serum (NGS). Sevilla {Sevilla et al. 1997}, outline characterisation and specificity of this antibody in the following manuscript. This antibody was a gift from Dr Sevilla. The secondary goat anti mouse IgG antibody was used at 1:1000 dilution with 2% NGS. FITC was diluted with phosphate buffered saline (PBS) to 1:50. Nuclear counterstaining with DAPI at 1 µg/ml generated a second fluorescent spectrum.

Rabbit immunofluorescence protocol 2.6

Antigen retrieval:

1. Heat slides to 50 °C for 15 minutes.
2. Removal of the embedded paraffin media using Xylene (Catalogue #: X2377, Sigma-Aldrich) twice for 3 minutes each avoiding drying between dipping.
3. Re-hydrating the slides by using graded alcohol solutions. Industrial methylinated sprit (IMS) 99%, 2 x 1 minutes followed by 95% industrial methylinated sprits (IMS), 2 x 1 minutes each before being placed in a bath of de-ionised water.
4. Antigen retrieval using Heating for 20 minutes at 92 °C in 1:20 dilution of citrated buffer and cooling for 40 minutes in that buffer at RT.
5. Wash and Store the slides in de-ionised water until use.
6. Place slides in a dark humidity chamber and cover the microtome with deionised water.
7. Remove excess water.

Block non-specific bindings:

8. Block non-specific staining with incubation with buffer (2% w/v Normal Goat Serum diluted in PBS).
9. Incubate for 30 minutes at RT.
10. Wash with PBS x 3.

Capture antigen:

11. Add 100 µl of Primary antibody diluted in incubation buffer at a predetermined dilution, for example 1:500 to each slide, making sure the microtome is covered.
12. Incubate in a humidity chamber for two hours at RT.
13. Wash with PBS x 3.

Detect the primary antibody:

14. Add 100 µl of detection antibody at predetermined dilutions for example 1:1000 in incubation buffer. This antibody was a biotinylated goat anti mouse immunoglobulin (Catalogue #: E433, DAKO, www.dacocytomation.com).
15. Incubate for 30 minutes at RT.
16. Wash with PBS x 3.

Substrate:

17. Add 100 µl of the prepared FITC-labelled Streptavidin (Catalogue #: SA-5001, Vector Labs) at appropriate dilution e.g. 1:50, to each slide.
18. Incubate for 30 minutes at RT. All procedures from this point must be carried out in the dark.
19. Wash with PBS x 3.

Counter staining of the nuclei:

20. Add 100 µl of 1 µg/ml DAPI (4,6-Diamino-2-phenylindole Chromogen solution, Sigma-Aldrich, Dorset) to each slide.
21. Incubate for 5 minutes at RT.
22. Rinse with PBS x 3.

Mount:

23. Dehydrate the slide with graded IMS and Xylene (Reverse of the re-hydration step).
24. Mount sections using vectashield fluorescence mounting media (H-1000, 10 ml, Vector Laboratories, Peterborough) and E-Z Mount (999430, 500 ml, Shandon, Pittsburgh).
25. Store in a light-tight container at 4°C.
26. View under Fluorescence microscope.

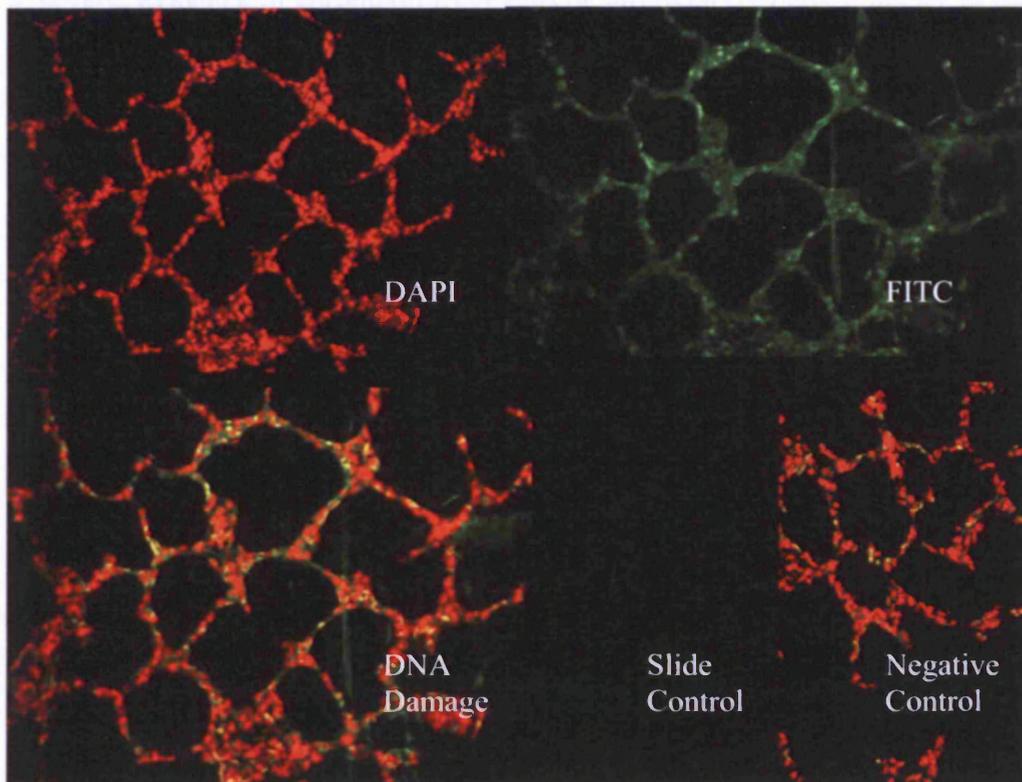
Anti 4-Hydroxy-2-nonenal monoclonal antibody: Oxidative damage of lipids caused by reactive oxygen species (ROS) play an important role in lesion of cell functions and ageing. Aldehydes such as Malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) have been reported as two of the advanced lipid peroxidation products. This monoclonal IgG antibody is manufactured in Japan by the Japan Institute for the Control of Ageing (JaiCA) and distributed outside Japan by Genox (Catalogue #: M10, Genox). The concentration of the primary antibody was optimised as 1: 250 dilution with 2% NGS. Toyokuni {Toyokuni et al. 1995} outlines characterisation and specificity of this antibody in his manuscript. The secondary goat anti mouse IgG antibody was used at 1:1000 dilution with 2% NGS. Fluorescein isothiocyanate (FITC) was diluted with PBS to 1:50. Nuclear counterstaining with 4',6-diamidino-2-phenylindole (DAPI) at 1 µg/ml generated a second fluorescent spectrum.

Data acquisition: Tissue sections were viewed using a Zeiss Axioskop Fluorescence microscope at x 40 magnifications. Images were taken with 0.1 µm Z-sten using a focus drive and were captured using a Hamamatsu 12-bit digital camera and FITC or DAPI filters on a 10- position filter wheel, which were controlled using Openlab 3.04 software (Improvision). Exposure was set at 240 ms for all tissue sections. Microscopy was then performed to visualize the cells by phase-contrast, plus the distribution of DAPI and FITC. The Z-stack of images was processed using Volocity software, which produced a 3D image.

Oxidative stress and DNA damage: Semi-quantitative assessment of oxidative stress and DNA damage was obtained from observation of any increase in the density seen in phase-contrast images performed by superimposing the fluorescent DAPI (Nuclear counter stain) and FITC images obtained by each marker of oxidative stress. Where the DAPI and FITC overlapped the yellow coloration denoted oxidative stress in the nuclear DNA (Diagram 2.20). Cells with two or more chromatin fragments or shrunken condensed nuclei were considered apoptotic. For each slide four different high power fields were assessed. These areas were the representative of perivascular / peribronchial region, consolidated region, a representative of lymphoid aggregates and normal looking lung tissue. The degree of oxidative stress in the nuclear DNA was categorized as: 0, no evidence of oxidative stress and DNA damage in the cell nuclei with non of the high power fields; 1, mild to moderate, evidence of nuclear oxidative stress

and DNA damage, in one of four high power fields; 2, moderate to severe, evidence of nuclear oxidative stress and DNA damage, in two of three high power fields; or 3, severe, evidence of nuclear oxidative stress and DNA damage, in three or four out of four high power fields. The examination was done for each of M1G and Genox assays separately and the mean value was considered.

Diagram 2.20: Illustrating oxidative stress and DNA damage by immunofluorescent technique.



Controls: Negative control slides were treated with 2% NGS/PBS instead of the primary antibody. There was one negative slide for every experimental/control slide ($n = 17$) and one de-waxed and mounted slide per each experimental slide (Slide control). The slide and negative controls are to exclude false positive auto-fluorescence and background staining respectively.

Apoptosis: A similar methodology was used to score the degree of apoptosis. This is as follows: Firstly for CytoDEATH: 0, absent of any Cytokeratin 18 expression in the lung; 1, mild expression, with fewer than 5 cells expressing Cytokeratin 18 per high-power field (hpf); 2, moderate expression, with more than 5 cells expressing Cytokeratin

18 per hpf; or 3, severe, with numerous cells expressing Cytokeratin 18. Secondly for each of the M1G and Genox immunofluorescent experiments: The degree of cells with two or more chromatin fragments or shrunken condensed nuclei were categorized as: 0, no evidence of chromatin fragments or shrunken condensed nuclei with non of the high power fields; 1, mild to moderate, evidence of chromatin fragments or shrunken condensed nuclei, in one of four high power fields; 2, moderate to severe, evidence of chromatin fragments or shrunken condensed nuclei, in two of three high power fields; or 3, severe, evidence of chromatin fragments or shrunken condensed nuclei, in three or four out of four high power fields. The examination was done for each of M1G and Genox assays separately and the mean value was considered.

The apoptosis score from CytoDEATH and the mean score for apoptosis determined by immunofluorescent techniques were used to assess the overall degree of apoptosis. This was then compared to the dose of LPS, the degree of oxidative stress and DNA damage and ultimately the survival of rabbits (Table 8.1).

Table 2.4: This is a proforma used for scoring histological, immunohistochemical and immunofluorescent slides.

Semi-Quantitative Analysis						Rabbits ID:
	Scoring System					
	0+	1+	2+	3+		Layout of slide
Perivascular Oedema						H&E
Alveolar Haemorrhage						
Vascular Thrombosis						
Alveolar Consolidation						
Goblet Cell Metaplasia						
Macrophages						
Lymphoid aggregates						
Multinucleated Giants						
Eosinophils						
Cytoplasmic Inclusions						
Neutrophils						
Histo-inflammatory Score					Total	
Oxidative stress M1G						
Oxidative stress Genox					Mean	
Apoptosis IF						
Apoptosis CytoD					Mean	
Vessel wall thickness						

STATISTICAL ANALYSIS

Comparing variables

Results were analysed using SPSS versions 11.5 and 14. Except for the continuous parametric variables, for example pre-ECMO positive end expiratory pressure and flow to sweep ratios during ECMO, where independent sample t-test was used, for other continuous non-parametric variables a Mann-Whitney test was employed to assess their variability. Chi-Square test was used to test the variability of all grouped variables, for example Clots in the oxygenator, Oxygenator failure and Haemorrhagic complications. They were corrected for ties and expressed in terms of minimum, maximum, median, first and third quartiles, mean and one standard deviation. A *p* value of <0.05 was considered to be significant. The confidence interval gave an indication of whether the power of the test was adequate and enabled us to assess clinical implications of results as a range of values. We interoperated the confidence interval as the range of values within which we were 95% confident that the true population mean lies.

Regression Plot

We employed a simple bivariate correlation and obtained a Pearson product-moment correlation coefficient in order to test the relationship between continuous variables for example % survival and MODSI.

A scatter diagram was used initially to check for outliers (Diagram 2.20). They were either very high or very low or away from the main cluster of points. The data was then double checked before excluding them from the database (Diagram 2.21). The direction of the relationship was determined for example a negative direction meant that the lowest value of one continuous variable corresponded to the highest value of the other variable (Diagram 2.22). The best fitting curve with the highest *r*-value was then selected. A Pearson *r* correlation coefficient was used to assess the strength of the relationship (Diagram 2.23). This can range between -1.00 to 1.00. A correlation of 0 indicates no relationship and a correlation of 1 indicates perfect correlation. The values between 0 and ± 1 were interoperated as follows. $r = \pm 0.1-0.29$ small correlation, $r = \pm 0.30-0.49$ medium correlation, $r = \pm 0.50-1.00$ large correlation.

A linear Pearson correlation coefficient was therefore calculated. This in diagram 2.23 illustrates a negative relationship. It also shows a strong relationship illustrated by the value of $r = 0.836$ and a correlation of determination or *r*-square of 0.699 (Variance = 69.9%). This shows almost 70% of the variance in MODSI on the perceived survival.

This is even better (77.2%) if a cubic plot is used. The significant level for the linear correlation is < 0.001 (Table 2.5).

Diagram 2.20: The Scatter diagram.

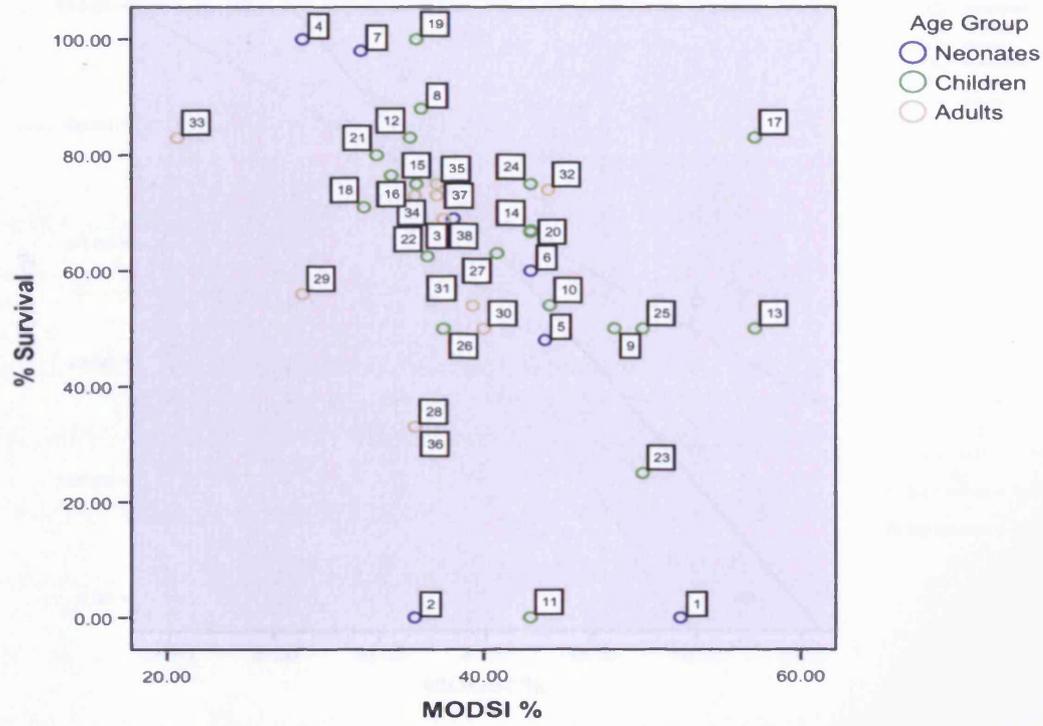


Diagram 2.21: Scatter plot excluding values considered to be outliers.

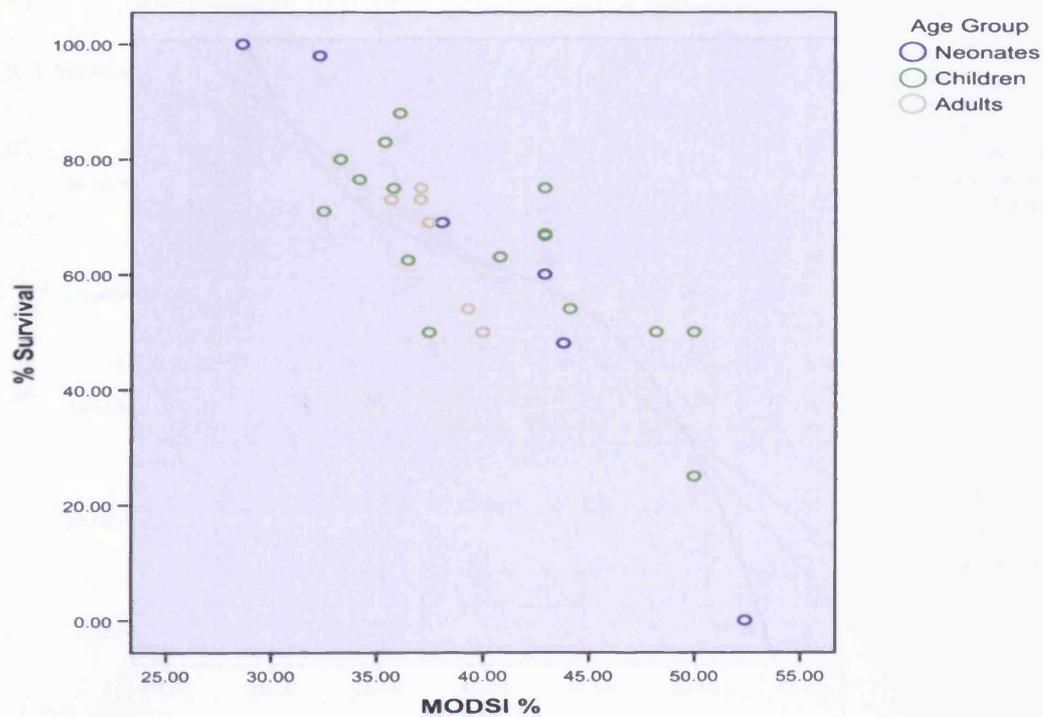


Diagram 2.22: Illustrating the direction and the strength of the correlation for each individual age group.

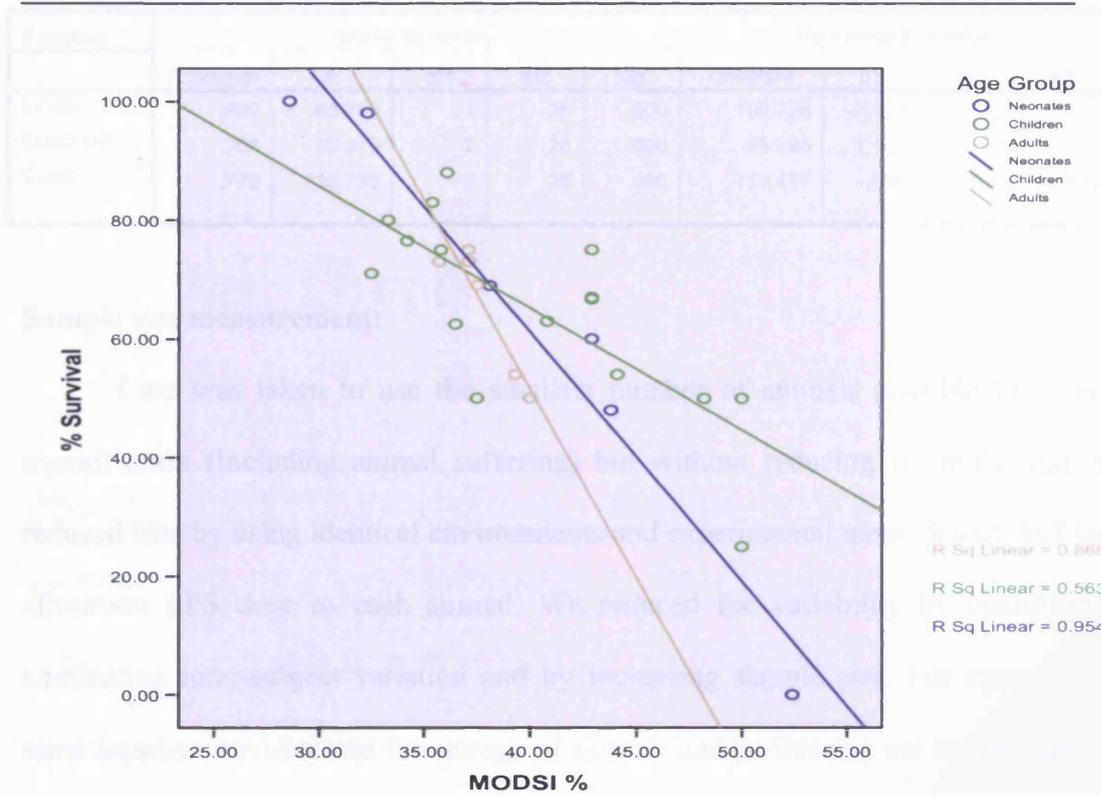


Diagram 2.23: This diagram illustrates the regression plots. Comparison between the cubic, quadratic and linear plots revealed no significant difference. However the cubic plot showed the highest coefficient of determination illustrated by R sq.

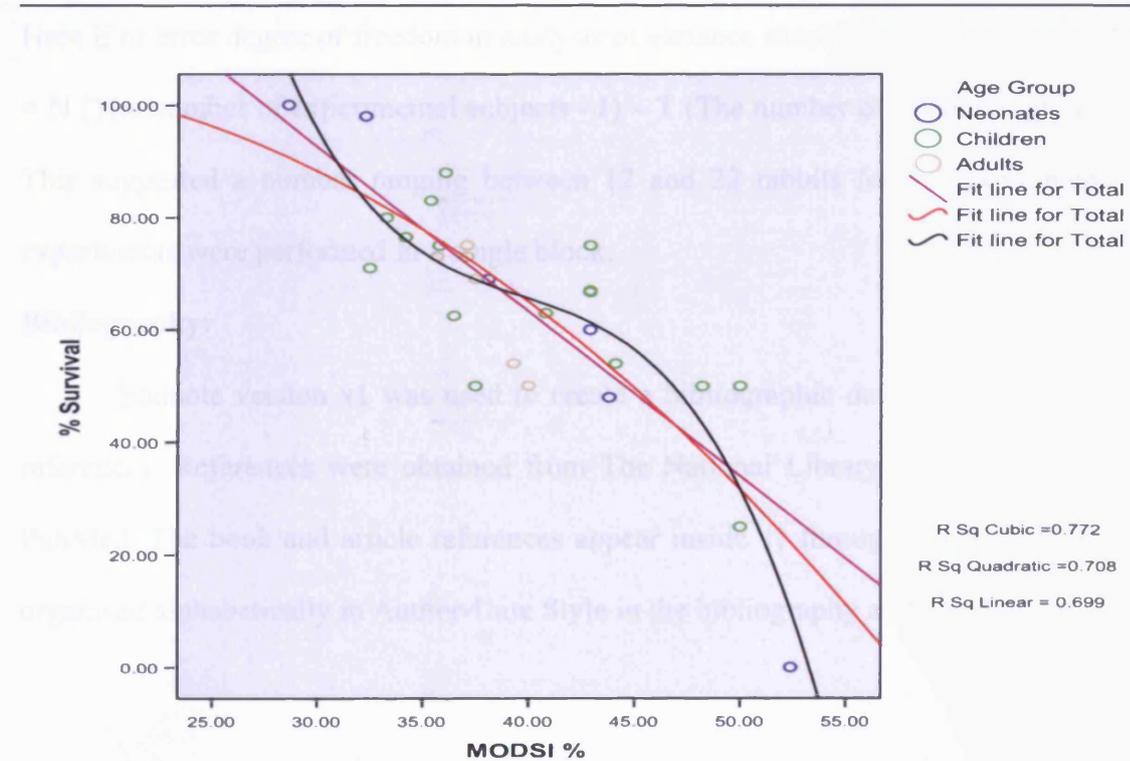


Table 2.5: SPSS output table shows how significantly each of the plots differs from zero.

Equation	Model Summary					Parameter Estimates			
	R Square	F	df1	df2	Sig.	Constant	b1	b2	b3
Linear	.699	60.253	1	26	.000	180.228	-2.925		
Quadratic	.708	30.310	2	25	.000	98.395	1.155	-.050	
Cubic	.772	30.736	2	25	.000	116.457	-.535	.000	.000

Sample size measurement:

Care was taken to use the smallest number of animals possible to reduce the overall costs (Including animal suffering) but without reducing scientific output. We reduced bias by using identical environments and experimental methodology and random allocation LPS dose to each animal. We reduced the variability by controlling and eliminating inter-subject variation and by increasing sample size. For example use of same supplier, environment for storage of animals and performing the experiments, same sex and weight within a narrow range. In the absence of reliable information to perform power analysis we used Mead's resource equation (RE) to calculate the sample size. Here E or error degree of freedom in analysis of variance should be between 10 and 20. $E = N - T$ (The number of experimental subjects - 1) - T (The number of treatment groups - 1). This suggested a number ranging between 12 and 22 rabbits for our experiment. All experiments were performed in a single block.

Bibliography:

Endnote version x1 was used to create a bibliographic database and to organise references. References were obtained from The National Library for Medicine (NLM) PubMed. The book and article references appear inside {} through out the text and are organised alphabetically in Author-Date Style in the bibliography at the end of the thesis.

Chapter Three

INSTITUTIONAL REPORT OF ECMO FOR SEVERE SYSTEMIC INFLAMMATORY RESPONSE AND MULTI ORGAN DYSFUNCTION:

EPIDEMIOLOGY

The prevalence of SIRS is very high. Brun-Buisson {Brun-Buisson et al. 2000} reviewed the published cohort studies of the epidemiology of SIRS, with an emphasis on intensive care unit (ICU) patients. In his report, SIRS affects one-third of all in-hospital patients, >50% of all ICU and >80% of surgical ICU patients. He described a graded severity from SIRS to sepsis, severe sepsis and septic shock, with an associated 28-day mortality of approximately 10%, 20%, 20%-40%, and 40%-60%, in adults respectively. Mortality rates were similar within each stage, whether infection is documented or not, and microbiological characteristics of infection do not substantially influence outcome, although the source of infection does.

Muckart {Muckart et al. 1997} documented trauma-associated mortality for SIRS to severe SIRS and SIRS (sterile) Shock as 9.2%, 15.8% and 66.7% respectively. The mortality rates in the same cohort of septic patients were similarly 9.2% to 13.1% and 63.7% for sepsis, severe sepsis and septic shock respectively. The graded mortality for paediatric sepsis was however 22.2%, 65.0% and 80.0% respectively {Goh et al. 1999}.

Factors like age, sex and geographical location will influence the incidence and outcome of SIRS. For example: Pre-term neonates are at a greater risk of developing sepsis than full term neonates. Bacteraemia is more often seen in male neonates {Remington 1989 & Davies 1989}. Trauma predominantly affects young previously healthy male. Infection is the commonest cause of SIRS in western countries {Salvo et al. 1995, Rangel-Fraustro et al. 1995, Brun-Buisson et al. 1995 & Pittet et al. 1995}, multiple trauma is commonest in African countries {Muckart et al. 1997}. Trauma patients are at particularly high risk of SIRS but sepsis increases the mortality {Tantaleán et al. 2003}.

The prognosis of SIRS is related to the underlying diseases and the severity of the inflammatory response and its sequela, reflected in shock and organ dysfunction. It has been estimated that sepsis accounts for approximately 15% of all neonatal mortality. Neonatal sepsis is one of the main reasons for mortality in the newborn and sepsis remains a leading cause of death in children > 4300 deaths annually in the U.S. {Goldstein et al. 2005}. Sepsis occurred in approximately 25% of adult ICU patients, depending on the case-mix, bacteraemic sepsis accounts for approximately 10%. In such patients, sepsis evolves to severe sepsis in the presence of organ failure, in >50% of cases.

Two-thirds of the overall ICU mortality can be attributed to severe sepsis {Brun-Buisson et al. 2000}. Approximately 150,000 people die annually in Europe and > 200,000 in United States {Angus et al. 2001} from severe sepsis.

A quarter of patients with severe sepsis have shock. Shock within the first 24 hours of ICU admission is the most important predictor of outcome in the severely injured and is an invaluable mean of stratifying patients. The presence of sterile or septic shock is a predictor of poor outcome, particularly when associated with MODS {Brun-Buisson et al. 2000}.

The transition to clinical MODS is a significant prognostic event because it heralds a change in mortality risk. In the western world MODS is the leading cause of mortality and morbidity in the intensive care {Baue et al. 1975 & Deitch et al. 1992}. It accounts for 91.5% of ICU mortality in children {Tantaleán et al. 2003}. MODS in children usually occur early, and sepsis increases their mortality {Tantaleán et al. 2003}. The mortality associated with adult trauma ranges from 60% to 98% in cases in which dysfunction involves three or more organs for ≥ 7 days {Barriere et al. 1995 & Marshall et al. 1995}. MODS is often irreversible {Knaus et al. 1985}.

Severe SIRS/sepsis and SIRS/septic shock especially when associated with MODS are conditions that deserve consideration for novel therapies. Severely ill patients are those most likely to benefit from novel therapies {Sibbald et al. 1995}. In patients with established MODS who frequently require massive doses of inotropic support the mortality rate is catastrophically high, in such patients relatively new therapies like ECMO may still have great benefits.

AIMS AND OBJECTIVES

To determine the association between disease aetiology, severity and outcome in a population of patients treated with ECMO for severe SIRS and its sequela compared to the outcomes available in the published literature.

PATIENTS AND METHODOLOGY

In brief from a total of 875 patients treated with ECMO since 1989 and 2005, n = 582, 66.5 % (220 neonates, 189 children and 173 adults), had severe SIRS/sepsis some of which were associated with shock and MODS. In this cohort the common organ dysfunction for patients of all age groups was the lungs. All patients satisfied the criteria

for ARDS ($\text{PaO}_2/\text{FiO}_2 < 200$). Respiratory dysfunction in severe sepsis could be intrinsic such as pneumonia or extrinsic such as peritonitis. In adults and children a clinical diagnosis of ARDS implied severe SIRS. In neonates the diagnosis of SIRS is more difficult. In the introductory chapter we explained the controversy surrounding the pathophysiology of MAS. Increasing evidence indicates that systemic inflammation induced by meconium aspiration is an essential part of the pathophysiology of this condition. Therefore we examined MAS as a cause of severe systemic inflammatory response and organ dysfunction in neonates.

VV- ECMO was the mode of choice in the treatment of all patients with primary respiratory failure in our institution. VA-ECMO was used for cardiac support or when cardiovascular support was necessary.

In this chapter we employ a modified form of the **PIRO** staging system for sepsis as defined in the methods chapter (Chapter 2) as a template and a conceptual framework upon which our study is based on. The attributes such as **predisposition** factors included age, weight, severity of lung injury and presence of shock, **insult** that had provoked severe systemic inflammatory response manifested as acute respiratory distress syndrome, **response to treatment** by ECMO in terms of survival and **organ dysfunction** were readily testable in this clinical research setting.

RESULTS

NEONATES: Table 3.1 illustrates the demographic data for each of the neonatal groups. Apart from one patient, who aspirated maternal blood during delivery, all other neonates in the other SIRS groups suffered from MAS.

There was a significant difference in admission weight between neonatal septic shock and SIRS shock groups. The mean admission weights were 3.2 (SD = 0.58) and 3.53 (SD = 0.56) respectively ($p = 0.02$). The analysis of variance revealed no significant difference between the $\text{PaO}_2/\text{FiO}_2$ ratios between groups ($p = 0.59$). All patients satisfied the criteria for acute respiratory failure as defined by MODSI Table 3.2. In fact in most cases this value was as much as a quarter of that defined as ARDS ($\text{PaO}_2/\text{FiO}_2 < 200$ mmHg).

Table 3.1: Illustrating patient demographics for the neonates. The significantly different groups are marked with an *.

Neonates				
	N (%)	Mean weight Kg (SD)	Median (range) age Hours	P/F mmHg Mean (SD)
Severe Sepsis	2(0.9)	3.6(0.1)	216(169)	39.8(27.6)
Septic Shock	44(20.0)	3.2(0.58)*	66(15-672)	53.3(32.8)
Severe SIRS	13(5.9)	3.4(0.51)	20(8-140)	49.1(22.9)
SIRS Shock	161(73.2)	3.53(0.56)*	23(6-192)	48.4(26.1)
Total	220	3.47(0.57)	24(6-672)	49.4(27.4)

Table 3.2 illustrates the sub-classifications according to their cause. Neonates were divided into septic and other SIRS (non-septic) and sub divided into their causal insult.

Circulatory shock was encountered in 93.2 % (n = 205) of neonates, characterised by inotropic/vasoactive drug therapy despite adequate fluid resuscitation immediately prior to ECMO. There were only two patients in the septic group not in circulatory shock prior to ECMO. There was a significant reduction in the use of inotropic/vasoactive drugs upon start of ECMO, from 93.2 to 21.4% ($p < 0.001$). In 14.1% of that this improvement could be attributed to the use of VA-ECMO. The rest occurred after the start of VV-ECMO ($p < 0.001$).

Septic shock was associated with the highest percentage of mortality of 50.0 %. The graded mortality for neonatal sepsis was therefore 0.0% and 50.0% for severe sepsis and septic shock, and the mortality rates in the same cohort of SIRS patients were 0.0 and 2.5% for severe SIRS and SIRS shock. In both cases of severe sepsis and severe SIRS the number of patients were perhaps too small to make a meaningful statistical comparison.

Table 3.2: Comparing groups in terms of severity of pre-ECMO disease, degree of MODS on ECMO and the primary outcome measure of survival.

Neonates	No Shock	Shock	MODS	MODSI Mean (SD)	Survival
<i>Septic</i>					
Pertussis	-	3	3	52.4 (16.5) %	0%
Pneumococcal	-	2	2	35.7 (10.1)%	0%
Streptococcal	-	13	9	38.1 (10.1) %	69%
Influenza A	-	1	1	28.6%	100%
Bacterial unspecified	1	20	15	43.8 (17.5)%	48%
RSV	1	4	4	42.9 (20.2)%	60%
Viral unspecified	-	1	0	-	100%
<i>Other SIRS</i>					
MAS	13	160	46	32.3 (8.2)%	98%
Aspiration	-	1	-	-	100%
Overall	15	205	80	36.1 (13.2)%	88%

Multiple organ dysfunctions was frequently encountered and managed on ECMO. MODS featured in 36.2% of neonatal ECMO patients (n = 80). Septic neonates had a significantly higher chance of developing MODS (73.9 %) compared to other SIRS (26.4%, $p < 0.001$). From the two patients in the severe sepsis group one developed MODS. The patients with septic shock had the highest incidence of MODS at 75.0%. Respiratory dysfunction was the common feature in all MODS. Cardiovascular dysfunction occurred in 23.2% of these neonates, renal dysfunction in 15%, neurologic dysfunction in 3.6%, hepatic dysfunction in 1.4%, GI dysfunction in 1.4% and haematologic dysfunction was recorded in 0.9% of our MODS population. Table 3.2 compares the frequency and the outcome of each neonatal group with reference to the development of MODS as well as the degree of organ dysfunction illustrated by MODSI, and percentage survival.

The percentage mortality from MODS caused by sepsis was 61.8 % compared with the mortality in the absence of MODS (8.3%, $p = 0.001$). The mortality associated with MODS by other causes of SIRS was 8.7 % vs. 0.0 % with out MODS ($p = 0.001$).

There was significant difference in mortalities between MODS caused by sepsis vs. other SIRS (61.8 vs. 8.7%, $p < 0.001$) or between them in the absence of MODS (8.3

vs. 0.0, $p = 0.001$). The combined mortality from MODS was 31.3 % compared to 0.7 % in cases not associated with MODS ($p < 0.001$).

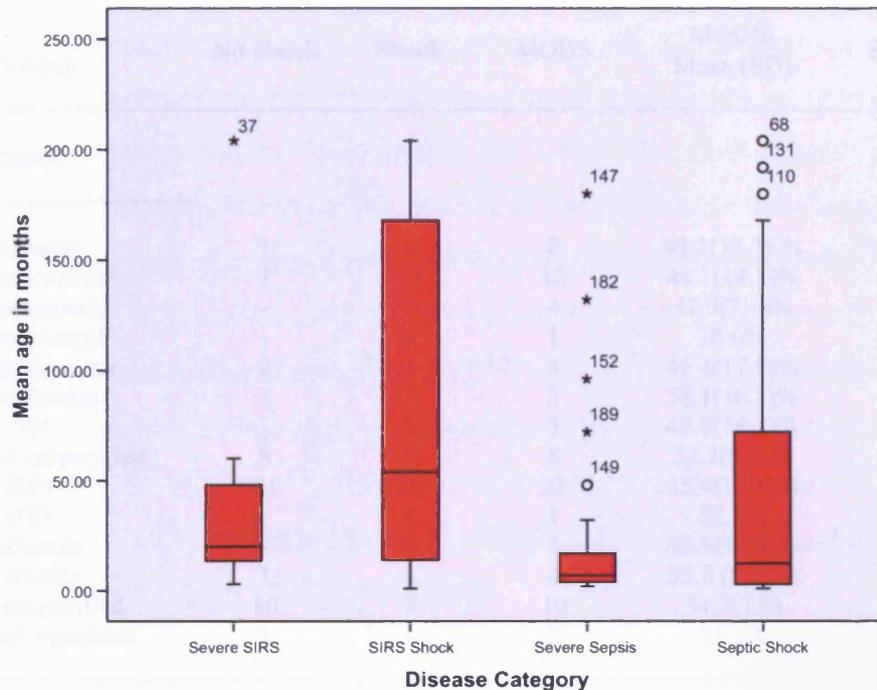
The mortality associated with presence of 2 ($n = 51$), 3 ($n = 19$), 4 ($n = 6$), 5 ($n = 3$) and 6 ($n = 1$) organ failures was 7.8, 63.2, 83.3, 100 and 100% respectively. The difference between percentage mortalities was statistically significant only between 2 and 3 organ dysfunction categories (2 vs. 3, 3 vs. 4 and 4 vs. 5 were < 0.001 , 0.356 and 0.453 respectively). Patients with 5 and 6 organ failures both had 100% mortalities. The number of patients with four, five and six organ failure were not however large enough to make a meaningful statistical comparison. Table 3.2 compares the frequency and the outcome of groups of neonates referring to the development of MODS as well as the degree of organ dysfunction illustrated by MODSI.

CHILDREN: Table 3.3 illustrates the patients demographics. There appeared to be a significant difference between the severe sepsis and SIRS shock groups with respect to age [Mean age of 23.7(SD=39.8) vs. 82.8(SD=79.2) months respectively, $p = 0.002$]. This is illustrated in the means plot (Diagram 3.1). All patients satisfied the criteria for respiratory failure as defined by MODSI (Table 1.6). In fact the mean values of $\text{PaO}_2/\text{FiO}_2$ ratios for each group were less than half that diagnostic for ARDS. The analysis of variance revealed no significant difference between the mean values of $\text{PaO}_2/\text{FiO}_2$ ratios between groups ($p = 0.10$).

Table 3.3. Illustrating patient demographics for the children. * Denotes the significantly different continuous variables.

Children			
	N (%)	Median (range) age Months	P/F mmHg Mean (SD)
Severe Sepsis	47(24.9)	7(2-180)*	56.8(3.1)
Septic Shock	97(51.3)	12.5(1-204)	61.7(32.5)
Severe SIRS	11(5.8)	20(3-204)	83.8(56.3)
SIRS Shock	34(17.9)	54 (1-204)*	67.4(42.7)
Total	189	13(1-204)	62.6(34.0)

Diagram 3.1: Box plot of mean age between disease categories in children. The values presented by a small circle are considered as outliers. Extreme points (Outliers by more than 3 box lengths from the edge of the box) are represented by an asterisk*.



Circulatory shock was encountered in 69.3% (n = 131) of children, characterised by the need for inotropic/vasoactive drug therapy despite adequate fluid resuscitation immediately prior to ECMO. There were twice as many patients in shock prior to ECMO than with no shock (n = 131 vs. 58). One patient had two runs of VV ECMO. Table 3.4 illustrates the sub-classifications according to the cause.

Children were divided into septic and other SIRS (non-septic) and sub-divided into their causal insult.

There was a significant reduction in the use of inotropic/vasoactive drugs upon start of VV-ECMO, from 66.1 to 44.4% (21.7%, $p < 0.001$). In 18.1% of above this improvement could be attributed to the use of VA-ECMO. The rate of conversion from VV to VA-ECMO was 7.8% (n = 10). VA was reserved for patients who required substantial circulatory support or those whom developed circulatory collapse requiring high doses of inotropes / vasoconstrictors on VV-ECMO. Occasionally in anticipation of

progression to cardiovascular failure, for example in some cases of Pertussis or RSV sepsis, VA-ECMO was used from the start.

Table 3.4: Illustrating the sub-classifications of Severe SIRS into one caused by Sepsis and that caused by other causes of SIRS in Children.

Children	No Shock	Shock	MODS	MODSI Mean (SD)	Survival
<i>Septic</i>					
Pertussis	1	9	8	48.2(13.1) %	50%
Meningococcal	1	13	12	44.1(14.2)%	54%
Pneumococcal	-	7	4	42.9(7.1)%	57%
Streptococcal	-	3	1	28.6%	100%
Staphylococcus	2	4	4	46.4(17.9)%	83%
H. Influenza	1	3	3	38.1(16.5)%	75%
TB	-	3	3	42.9(14.3)%	0%
Bacterial unspecified	8	9	8	32.1(6.6)%	88%
RSV	21	25	23	35.4(12.8)%	82%
HSV	1	1	1	57.1%	50%
Influenza	-	6	5	42.9(14.3)%	67%
Varicella	1	3	4	35.8 (8.3) %	75%
Viral unspecified	10	7	10	34.2(7.4)	77%
Atypical organisms	1	4	2	57.1%	83%
<i>Other SIRS</i>					
Aspiration	3	9	10	32.5 (18.2)%	71%
Acute Pancreatitis	-	2	2	35.8(10.1)%	100%
Burns / Smoke inh.	2	1	2	42.9(20.2)%	67%
Near drowning	1	4	3	33.3(8.3)%	80%
RTA / Post surgery	3	13	9	36.5(10.4)%	63%
Other	2	5	6	50.0(23.5)%	25%
Overall	58	131	120	38.8 (13.9)%	71%

It is not therefore a surprise to see a significantly worse outcome ($p < 0.001$) in patients whom received VA-ECMO (Survival 54.1%) compared with VV-ECMO (Survival 78.9%) in children. SIRS shock was associated with the highest percentage of mortality of 38.2%. The graded mortality for paediatric sepsis was therefore 10.6% and 34.0% ($p = 0.003$) for severe sepsis and septic shock, and the mortality rates in the same cohort of SIRS patients were 36.4% and 38.2% for severe SIRS and SIRS shock.

Multiple organ dysfunctions were encountered in 63.5% of children ($n = 120$) with a mean MODSI of 38.8%. The survival rate was variable however the mean survival was 71% across all groups Table 3.4.

Children with shock had the highest association with MODS on ECMO (74.0%). Only 39.7% of children with no shock developed MODS on ECMO ($p < 0.001$). Respiratory dysfunction was the common feature in all MODS. Cardiovascular dysfunction was a feature in 58.2% of children, renal dysfunction in 30.1%, neurologic dysfunction in 5.8%, hepatic dysfunction in 3.7%, GI dysfunction in 2.1% and haematologic dysfunction was recorded in 4.2% of the paediatric MODS population.

The percentage mortality from MODS caused by sepsis was 40.9 % compared with the mortality in the absence of MODS (3.6%, $p < 0.001$). The mortality associated with MODS by other causes of SIRS was 46.8 % vs. 15.3 % with out MODS ($p < 0.001$). There was no significant difference in mortalities between MODS caused by sepsis vs. other SIRS (40.9 vs. 46.8 %, $p = 0.599$) or between them in the absence of MODS (3.6 vs. 15.3, $p = 0.205$). The combined mortality from MODS was 42.5 % compared to 5.7 % in cases not associated with MODS ($p < 0.001$).

The mortality associated with presence of 2 ($n = 64$), 3 ($n = 30$), 4 ($n = 17$) and 5 ($n = 8$) organ failures was 20.3, 43.3, 94.1 and 100% respectively. The difference between percentage mortalities was statistically significant between 2 and 3, and 3 and 4 organ dysfunction categories (2 vs. 3, 3 vs. 4 and 4 vs. 5 were 0.020, 0.001 and 0.484 respectively). There were no patients with greater than five-organ dysfunction. The number of patients with five-organ dysfunction was not very large, however none of the eight patients survived this amount of organ dysfunction. Table 3.5. Compares the frequency and the outcome of groups of children referring to the development of MODS as well as the degree of organ dysfunction illustrated by MODSI.

ADULTS: The analysis of variance confirmed statistically significant differences in the age of patients between the two main groups (Sepsis and other SIRS). Septic patients were significantly older than the patients suffering from other types of SIRS (Diagram 3.2). There were no significant differences within the SIRS ($p = 0.968$) or Sepsis ($p = 0.932$) groups.

The patients demographics are illustrated by Table 3.5. All patients satisfied the criteria for respiratory failure ($\text{PaO}_2/\text{FiO}_2$ ratios < 200 mmHg) as defined by MODSI. In fact the mean values of $\text{PaO}_2/\text{FiO}_2$ ratios for each group were less than a third for ARDS.

The analysis of variance revealed no significant difference between the mean values of PaO₂/FiO₂ ratios between groups.

Table 3.5: Illustrating patient demographics for the adults. * Denotes the significantly different continuous variables.

Adults			
	N (%)	Median (Range) age years	P/F mmHg Mean (SD)
Severe Sepsis	35(20.2)	38.7(20-59)*	59.7(20.4)
Septic Shock	73(42.2)	40.1(18-65)**	58.8(18.9)
Severe SIRS	18(10.4)	29.7(18-65)*	51.2(15.0)
SIRS Shock	47(27.2)	31.2 (18-54)**	74.9(50.4)
Total	173	36.4(18-65)	63.3(33.0)

Circulatory shock was encountered in 69.4% (n = 120) of Adults, characterised by the need for inotropic/vasoactive drug therapy despite adequate fluid resuscitation immediately prior to ECMO. Table 3.6 illustrates the sub-classifications according to the cause. Prior to ECMO there were over twice as many patients in shock than not in shock (n = 120 vs. 53).

There was a significant reduction in the use of inotropic/vasoactive drugs upon start of ECMO, which is a 23.2% reduction ($p < 0.001$). In only 2.3% of cases this improvement could be attributed to the use of VA-ECMO (n = 4). The rate of conversion from VV to VA-ECMO was 1.7% (n = 3). VA was reserved for patients with considerable degree of cardiovascular instability not responding to inotropes or vasoconstrictors from the start or after a period of VV-ECMO. It is therefore disappointing but not surprising to see a mortality of 100% in adults who received VA-ECMO.

Septic shock was associated with the highest percentage of mortality of 41.1%. The graded mortality for adult sepsis was therefore 31.4% and 41.1% for severe sepsis

and septic shock, and the mortality rates in the same cohort of SIRS patients were 33.7% and 27.3% ($p = 0.653$) for severe SIRS and SIRS shock.

Multiple organ dysfunctions were encountered in 57.2% of adults ($n = 99$) with a mean MODSI of 39.0%. The survival rate was variable, however the mean survival was 65.3% across all groups table 3.6. Septic adults had a significantly higher chance of developing MODS (63.0 %) compared to other SIRS (47.7%, $p = 0.049$). The patients with septic shock had the highest incidence of MODS at 74%. Respiratory dysfunction was the common feature in all MODS. Cardiovascular dysfunction was a feature in 46.2% of adults, renal dysfunction in 41.0%, neurologic dysfunction in 3.5%, hepatic dysfunction in 5.2%, GI dysfunction in 6.4% and haematologic dysfunction was recorded in 1.1% of the adult population.

The percentage mortality from MODS caused by sepsis was 45.2 % compared with the mortality of 23.3% in the absence of MODS ($p = 0.018$). The mortality associated with MODS by other causes of SIRS was 50.0 % vs. 12.9 % with out MODS ($p = 0.002$).

Diagram 3.2: Box plot of mean age between disease categories in Adults.

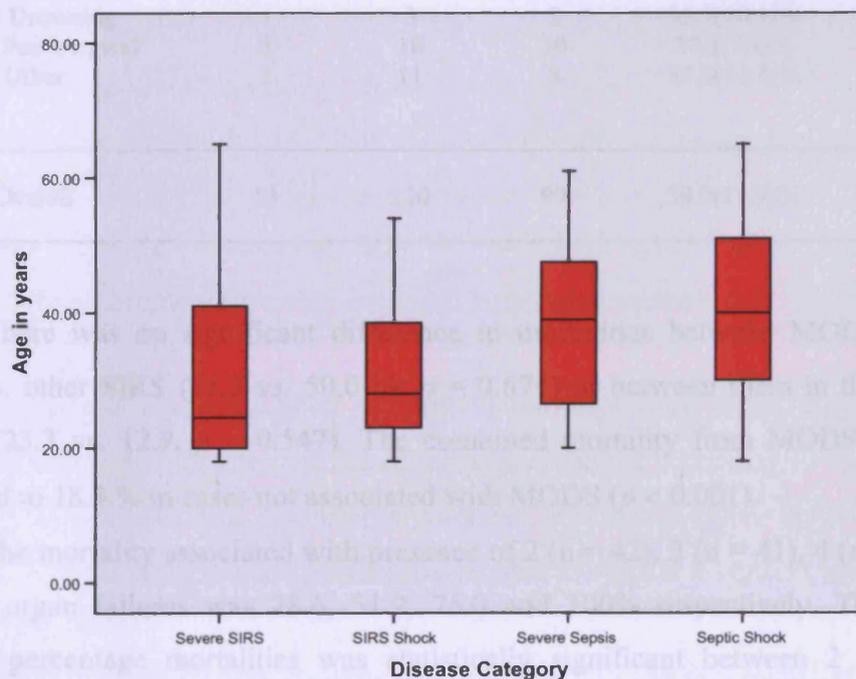


Table3.6: Illustrating the sub-classifications of Severe SIRS into one caused by Sepsis and that caused by other causes of SIRS in adults. Other causes included aplastic anaemia, Obstetric emergencies like ruptured ectopic pregnancy and pre-eclampsia, and drug tri-cyclic drug overdoses.

Adults	No Shock	Shock	MODS	MODSI Mean (SD)	Survival
<i>Septic</i>					
Meningococcal	1	3	1	42.9%	75%
Pneumococcal	1	11	9	50.0(15.3)%	50%
Streptococcal	4	6	8	37.5(7.4)	50%
Staphylococcus	1	7	7	40.8(9.9)%	63%
H. Influenza	-	3	2	35.7(10.1)%	33%
TB	-	1	1	28.6%	100%
Bacterial unspecified	1	3	1	42.9%	75%
Legionella	8	10	10	38.6(11.8)%	56%
Influenza	-	6	5	40.0 (15.6)%	50%
Varicella	9	4	4	39.3 (13.7) %	54%
Viral unspecified	8	14	14	44.0(10.9)	74%
Atypical organisms	1	5	6	20.7(21.9)%	83%
Parasitic	1	-	-	-	100%
<i>Other SIRS</i>					
Aspiration	6	9	6	35.7 (7.8)%	73%
Acute Pancreatitis	2	6	5	37.1(12.7)%	75%
Near Drowning	-	3	2	35.7(10.1)%	33%
RTA / Post surgical	8	18	10	37.1(7.4)%	73%
Other	2	11	8	37.5(13.1)%	69%
Overall	53	120	99	39.0(11.9)%	65%

There was no significant difference in mortalities between MODS caused by sepsis vs. other SIRS (45.2 vs. 50.0 %, $p = 0.674$) or between them in the absence of MODS (23.3 vs. 12.9, $p = 0.547$). The combined mortality from MODS was 46.5 % compared to 18.9 % in cases not associated with MODS ($p < 0.001$).

The mortality associated with presence of 2 ($n = 42$), 3 ($n = 41$), 4 ($n = 12$) and 5 ($n = 3$) organ failures was 28.6, 51.2, 75.0 and 100% respectively. The difference between percentage mortalities was statistically significant between 2 and 3 organ dysfunction categories only (2 vs. 3, 3 vs. 4 and 4 vs. 5 were 0.035, 0.144 and 0.333 respectively). There were no patients with greater than five-organ dysfunction. The numbers of patients with four- or five-organ dysfunctions were not large enough to make

a meaningful statistical comparison. Table 3.6 compares the frequency and the outcome of groups of adults referring to the development of MODS as well as the degree of organ dysfunction illustrated by MODSI.

DISCUSSION

More than two thirds of ITU patients have SIRS {Rangle-Frausto et al. 1995 & Salvo et al. 1995}. In this chapter we selected patients who represented a spectrum of morbid conditions that according to the ACCP/SCCM consensus conference definitions {Levy et al. 2003} closely resembled severe SIRS/sepsis and SIRS/septic shock. Many patients satisfied the criteria for SIRS as these criteria were quite sensitive {Vincent et al. 1997} therefore added care was taken when including patients in this study by particular attention to the clinical presentation and pattern recognition in order to improve this studies specificity.

Continuing on the common theme of PIRO staging system, **predisposition, insult, response to treatment and organ dysfunction**, we will now discuss our institutional outcome for ECMO support.

Predisposing factors were: neonatal weight, age of children and adults, severity of lung injury, assessed by calculation of $\text{PaO}_2/\text{FiO}_2$ ratios at the point of referral, and the presence of co-morbidities such as pre-ECMO shock represented by the use of inotropic / vasoconstrictor drugs despite adequate fluid resuscitation.

We know that pre-term neonates are at a greater risk of developing sepsis. Table 3.1 revealed that smaller neonates are predisposed to sepsis too. The two extremes of age, that is the youngest of children and the oldest of adults, also had the greatest predisposition to sepsis. There also exists an association between age groups and the type of insult.

All patient groups satisfied the criteria for respiratory organ failure. In fact all groups had $\text{PaO}_2/\text{FiO}_2$ ratios well below the specifications according to the criterion for respiratory failure. This was a quarter in neonates, less than a half in children and a third in adults, however there was no relationship or predisposition to a particular group based on $\text{PaO}_2/\text{FiO}_2$ ratios.

Circulatory shock was encountered in 93.2% of neonates, 69.3% of children and 69.4% of adults. The patients with shock prior to the start of ECMO satisfied the criterion

for cardiovascular dysfunction and as all patients had respiratory failure in common these patients may be considered as having pre-ECMO MODS.

The **insult** in severe Septic patients is the infective microorganism. Infection is the most common cause of severe inflammatory response referred for ECMO. In the majority of cases the source of infection was intrinsic to the lungs (Pneumonia). In severe SIRS, the insult is non-infective, even though the pathophysiology of the disease processes is similar the **response to treatment** in terms of survival however, was different.

Streptococcal severe sepsis (Excluding Pneumococcal) commonly occurred in all age groups. This type of severe sepsis was associated with a relatively low MODS index ranging from a mean of 28.6 in children to 38.1 in neonates and the survivals ranged from 50% in adults to 100% in children. Group B organisms were responsible for the vast majority (77%) of neonatal streptococcal sepsis. It is difficult to accurately compare the survival of these patients to that of the published literature. Many geographical factors may influence outcomes. Opal reported the neonatal survival in an army medical centre in Washington DC from Group B Streptococcal sepsis at 27% {Opal et al. 1988}, were as Nomura, reported the survival of neonates in a Brazilian private maternity hospital from Group B Streptococcal (GBS) sepsis to be 50% {Nomura, et al. 2004}. The mortality with ECMO in this group was 69%. It should also be remembered that these neonates were selected for ECMO as they were not responding to conventional treatment in the referring hospital and so their predicted survival was very low. All neonatal GBS patients required circulatory support prior to admission. Three of which required VA ECMO from the start. The survival rate for adults with Group B Streptococcal sepsis (50% of Streptococcal cases) treated by ECMO was 62% identical to that published by Opal {Opal et al. 1988}.

Pertusis (Whooping cough) and sepsis caused by RSV (Respiratory syncytial virus) infection affect both neonates and children. They both represented a significant treatment challenge as they were commonly associated with severe cardiovascular dysfunction and necessitated VA ECMO, either from the start or by subsequent conversion from VV-ECMO (100% of neonatal and 80% of paediatric Pertusis and 60% of neonatal and 37% of paediatric RSV severe sepsis). In some cases, cardiovascular dysfunction was pre-empted and patients were placed on VA-ECMO from the start, however even though this may have influenced the outcome in RSV severe sepsis, it has yet to prove a significant benefit in terms of survival in the Pertusis cases {Pooboni et al.

2003}, 42% according to Pooboni and 50% in this report. However according to the American Academy of Paediatrics, {Pertussis. In: Pickering LK, ed. Red Book: 2003 Report of the Committee of Infectious Disease. 2003: 472-86} of the 103 reported Pertussis-related fatalities in the United States, 99% were among infants. This data from 1990-1996 showed that the most severe morbidity occurred in infants younger than 1 year. This group accounted for 83% of hospitalised Pertussis patients and 66% of patients with Pertussis pneumonia. In other word a survival rate of 27-34%. According to the American Academy of Paediatrics Committee on Infectious Diseases published in Pediatrics {Anonymous, September 1993; 92(3):501-4} The use of ribavirin in the treatment of respiratory syncytial virus infection, RSV pneumonia is associated with a mortality rate ranging from 11-78%, depending on the severity of underlying immune suppression. This ranged from 18% (Children) to 40% (Neonates) in our ECMO population irrespective of immune status.

Commonly encountered bacterial sepsis in adults and paediatric ECMO such as meningococcal, pneumococcal and staphylococcal demonstrated similar degrees of organ dysfunction (Mean MODSIs ranging between 37.5 to 50.0). The survival from meningococcal severe sepsis ranged from 54% in children to 75% in adults. This is close to the survival range documented in the literature {Maat et al. 2007, Blanco et al. 2004 & Riordan et al. 1995} between 55% to 81% in children taking into account the presence or absence of shock but lower than that for adults at 91% {Cabellos et al. Abstract P716; 18th European Congress of Clinical Microbiology and Infectious Diseases, 2008}.

Two adults received ECMO therapy for respiratory failure caused by fulminant lung infection with *Pneumocystis Carinii*. These patients were excluded from the main study as they suffered from a variety of immune deficiency syndromes making them more likely to have had CARS than SIRS. Both patients died one from irreversible organ failure but the other developed MODS requiring cardiovascular and renal supports. This is a good reminder that inflammatory activation or the lack of is a spectrum, however in the absence of biochemical markers it is difficult to differentiate CARS from SIRS.

A considerable number of patents in each age group had developed clinical characteristics of either bacterial or viral severe sepsis. Despite the lack of knowledge of the organism involved for specific anti-microbial treatment, with the exception of the unspecified neonatal bacterial severe sepsis, all had survival rates in excess of 75%. The reason for this is not clear. However it could be that we are placing too much emphasis

on cultures results or that the presence of culture positive organisms indicated the presence of a more virulent strain.

All ECMO patients received broad-spectrum antibiotic therapy from the start. The institutional treatment strategy is to eradicate the culture proven primary insult by using the best first line anti-microbial treatment according to sensitivities or in culture negative cases by pattern recognition in close consultation with microbiologists. Prophylactic anti-microbial treatments were used during ECMO cannulation and decannulation or for likelihood of a secondary infection. In fact most patients on ECMO received some form of anti microbial treatment throughout ECMO and beyond.

ECMO was frequently used as a supportive therapy for aspiration. One of the common types of aspiration is MAS in neonates. The mechanism of MAS causing respiratory dysfunction is controversial. Several mechanisms have been attributed to the development of MAS, such as Mechanical obstruction, inactivation of surfactant {Clark et al. 1987}, pulmonary hypertension {Holopainen et al. 1998} and ARDS-like pulmonary response or lung inflammation {Soukka et al. 1997}. MAS has been shown to induce complement-associated systemic inflammatory response through the alternative complement pathway C3 and terminal pathway C5b-9 complexes in newborn piglets {Castellheim et al. 2005}. Assuming that SIRS is instrumental in the pathophysiology of MAS, ECMO is undoubtedly successful in the management of severe SIRS {Firmin et al. 1996}. However what is the difference between MAS and other insults which makes it such an ideal indication for ECMO. The survival of neonates with MAS despite a mean MODSI of 32.3% (SD = 8.2%) was 98%. Aspiration in children and adults had similarly good outcomes of 71% and 73% survival and similar mean MODSIs of 32.5% (SD = 18.2%) and 35.7% (SD = 7.8%) respectively. Similarly the outcome of therapy for severe SIRS associated with acute pancreatitis was excellent for children and adults, 100% and 75% respectively (MODSI = 37.1 and 35.8%). The percentage survivals for trauma / post surgical severe SIRS was also as good in children and adults (62.5 and 73.1% respectively, $p = 0.471$). It was difficult to comment on the response to treatment in burns, near drowning and other causes of severe SIRS as their incidence was low.

The overall **response to treatment** by ECMO in terms of survival fell from 88% in neonates to 71% in children and 65% in adults even though the MODSI was almost constant across groups (36.1, 38.8 and 39.0 respectively).

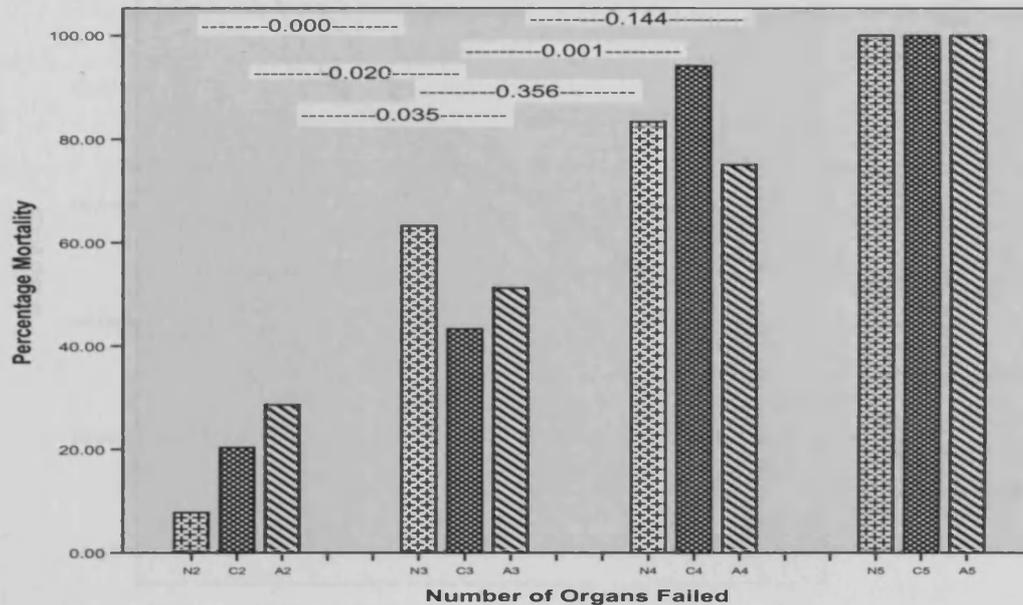
Organ dysfunction was commonly encountered in ECMO patients all patients had already satisfied criteria for single organ failure (Respiratory). A vast majority of

patients in each group 93, 63 and 69% of neonates, children and adults respectively had multiple organ dysfunction purely by satisfying the criteria for cardiovascular failure pre ECMO. This degree of organ dysfunction was reversed to 36.4% in neonates, remained the same 63.5% in children and reduced to 57.2% in adults on ECMO. The main reason for the reduction in the incidence of MODS post ECMO in neonates was a significant reduction ($p < 0.001$) in need for cardiovascular support after ECMO by 90.3%. Comparatively although still statistically significant ($p < 0.05$) this was only 21.7% for children and 23.2% for adults.

Now we will examine the relationship between the degree of MODS and the outcome of supportive therapy with ECMO. Diagram 3.3 illustrates and examines the relationship between the number of organs failed and the percentage mortality for this cohort of patients. The reason for the lack of significance between some of the groups with three and four organs or four and five organ failure was probably because as the number of organs failed increases the number of patients in that category reduces, i.e. the number of patients in each category was not large enough to draw statistical conclusion. For example only a handful of patients developed four or five organ failure in any age group and only one patient in the paediatric group had six organs failed and so, for simplicity that patient was not included in the diagram.

The relationship between the MODSI and percentage survival in ECMO patients caused by severe SIRS/severe sepsis was also examined the values of mean MODSI for each insult against the percentage survival from that particular insult were plotted. Diagram 3.4 tests the relationship between these two continuous variables for each age group. The coloured lines indicate a negative relationship between these variables for each age group. Note that the values of r-squared (R Sq) indicate strong linear relationship in all age groups.

Diagram 3.3: Illustrates the percentage mortality between MODS. In the category axis, N stands for neonate C for children and A for adult followed by the number of organs failed in that category. The p values between the first three categories are illustrated on the top left and centre. There was no significance between the last two categories so they were omitted.



The regression plot of the scatter diagram irrespective of age demonstrated a strong cubic relationship. This was determined by comparing the coefficient of determination (r-square) for each of the Linear (0.699), Quadratic (0.708) and Cubic (0.772) plots (Diagram 3.4), however there was no statistical significance between any of them (p for all three plots < 0.001).

The Pearson correlation coefficient r for the linear regression plot was -0.836 (Range = -1 to $+1$) showing a strong negative relationship. The coefficient of determination or r-squared was therefore 0.699 (Variance = 69.9%). This shows almost 70% of the variance in MODSI on the perceived survival ($p < 0.001$).

This plot may be used to assess the likely hood of survival in patients or groups of patients suffering from severe sepsis or ARDS on ECMO when associated with MODS. According to this graph more than 70% of patients with dysfunction of two or less organs are expected to survive with strong certainty (77.2% certain). $65-70\%$ of ECMO patients with dysfunction of three organs would survive were as there is a strong suggestion that no patients with four or more organs failed are expected to survive with ECMO alone.

Diagram 3.4: The regression plot between the mean MODS index and percentage survival for each insult presented for each age group and as a total of all groups.

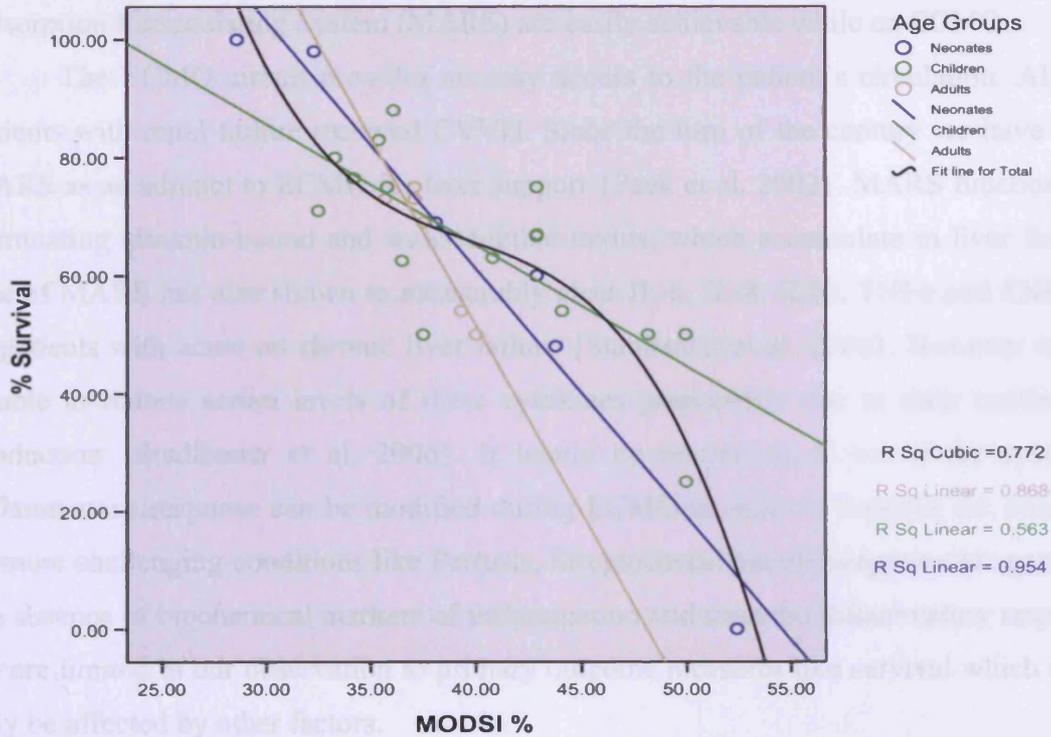
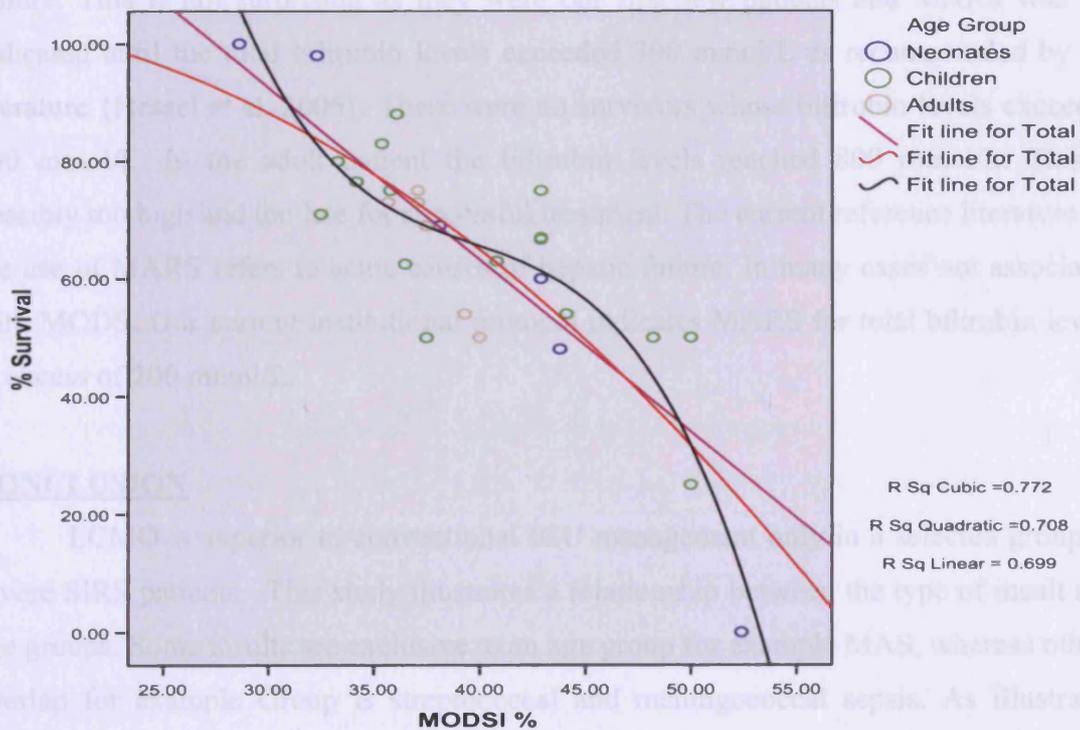


Diagram 3.5: Comparison between cubic, quadratic and linear regression lines irrespective of age. R Sq is the coefficient of determination or the variance when multiplied by 100.



In addition to the use of Venous-arterial (VA-) ECMO for circulatory support, use of adjuncts like Continuous Venous-venous Haemofiltration (CVVH) and Molecular Adsorption Recirculating System (MARS) are easily achievable while on ECMO.

The ECMO circuit provides an easy access to the patient's circulation. All our patients with renal failure received CVVH. Since the turn of the century we have used MARS as an adjunct to ECMO for liver support {Peek et al. 2002}. MARS functions by eliminating albumin-bound and water-soluble toxins, which accumulate in liver failure. Use of MARS has also shown to measurably clear IL-6, IL-8, IL10, TNF α and TNF α R1 in patients with acute on chronic liver failure {Stadlbauer et al. 2006}. However it was unable to reduce serum levels of these cytokines presumably due to their continuous production {Stadlbauer et al. 2006}. It would be interesting to see if the systemic inflammatory response can be modified during ECMO in order to improve the outcome of more challenging conditions like Pertussis, Streptococcal and Meningococcal sepsis. In the absence of biochemical markers of inflammation and thrombo inflammatory response we are limited in our observation to primary outcome measures like survival which itself may be affected by other factors.

In our study there was only one adult non survivor who received MARS (MODSI = 71.4), and three children, only one of whom survived, MODSI = 57.1% vs. 71.4 in the other two. The only survivor in these patients had the least degree of organ failure. This is not surprising as they were our first few patients and MARS was not indicated until the total bilirubin levels exceeded 300 mmol/L as recommended by the literature {Hessel et al. 2006}. There were no survivors whose bilirubin levels exceeded 400 mmol/L. In the adult patient the bilirubin levels reached 800 mmol/L. This is possibly too high and too late for successful treatment. The current reference literature for the use of MARS refers to acute causes of hepatic failure. In many cases not associated with MODS. Our current institutional protocol indicates MARS for total bilirubin levels in excess of 200 mmol/L.

CONCLUSION

ECMO is superior to conventional ICU management only in a selected group of severe SIRS patients. This study illustrates a relationship between the type of insult and age groups. Some insults are exclusive to an age group for example MAS, whereas others overlap for example Group B streptococcal and meningococcal sepsis. As illustrated compared to the conventional management reported in the literature our ECMO practice

has been a success, particularly in the management of RSV sepsis in children and neonates, acute pancreatitis and aspiration pneumonia in adults and children, as well as streptococcal sepsis and MAS in neonates. Although other causes of neonatal sepsis like Pertussis and Meningococcal septicaemia, particularly in children, remain a therapeutic challenge. For these and other indications ECMO offers an acceptably similar if not superior outcome to conventional methods.

The graded response to ECMO in terms of mortality from severe SIRS/sepsis, SIRS/septic shock to MODS is summarised in table 3.7. This table illustrates that in both neonates and adults the association between sepsis and shock and in most stages their staged progression from single organ respiratory failure to shock and MODS had negative influences on the outcome of treatment with ECMO. This outcome can be predicted in the form of percentage survival, by calculating the MODSI and using the regression plots (Diagrams 3.4 or 3.5), or simply by looking at the bar diagram 3.3. However in children the only significant negative influence came from the progression from severe sepsis to septic shock or from SIRS shock to MODS.

Table 3.7: Graduated percentage mortality in neonates, children and adults with Severe SIRS supported with ECMO.

Age Group	Severe SIRS	No Shock	Shock	MODS
Neonates	Septic	0.0%	50.0%	61.0%
	Sterile	0.0%	2.5%	8.7%
Children	Septic	10.6%	34.0%	40.9%
	Sterile	36.4%	38.2%	46.8%
Adults	Septic	31.4%	41.1%	45.2%
	Sterile	33.7%	27.3%	50.0%

Although it is evident that ECMO causes a haematic response, the highly significant reduction in the inotropic requirements immediately after the start of ECMO in all age groups may be evidence of down regulation of the thrombo-inflammatory response (From SIRS shock to severe SIRS). We have also shown a down regulation in the degree of MODS in specially neonates from a mean MODSI of 93% pre ECMO to 36% post ECMO.

Chapter Four

REDUCTION IN THE HAEMATIC RESPONSE TO ECMO: THE STATE OF THE ART IN OXYGENATOR TECHNOLOGY

INTRODUCTION

The haematic response to ECMO was discussed in the first chapter. In this chapter we evaluate two of the most common varieties of oxygenators used worldwide for Extracorporeal Membrane Oxygenation. Extra-corporeal Membrane Oxygenation works on the same principals as cardiopulmonary bypass. It does however allow much longer period of cardio-pulmonary support, required for recovery from a reversible disease. The transition from cardiopulmonary bypass to extracorporeal membrane oxygenation was made by the invention of silicon membrane (SM) oxygenators in the last century. Separation of blood and gas phase allowed prolonged perfusion that was not possible with the currently available screen and bubble oxygenators used for cardiopulmonary bypass.

A novel type of oxygenator, Poly-methyl pentene (PMP), was launched in the new millennium. We began using PMP oxygenators in March 2001. The change over from SM to PMP oxygenators in our institution presented an opportunity to investigate and to improve our understanding of oxygenators. The clinical impression was that PMP oxygenators had reduced chest radiographic signs of blood activation within the first 24 hours of cannulation, and that was highly suggestive of a reduction in the haematic response to these oxygenators.

MATERIALS AND METHODS

This is a retrospective single institutional study, comparing technical and physiological properties of two types of oxygenator commonly used for ECMO

Eighty consecutive patients were selected retrospectively pre and post introduction of the new poly-methyl pentene oxygenators (May 2000 and June 2002). They formed four equal groups of twenty, half adults and half neonates. Adult size PMP (A-PMP), adult size Silicon (A-S), neonatal PMP (N-PMP) and neonatal Silicon (N-S). The A-PMP patients were further sub-divided; according to the number of oxygenators they received simultaneously, to A-PMP I, for one oxygenator and A-PMP II, for two oxygenators placed in parallel.

The flow path resistance, gas and heat exchange efficiency, consumption of coagulation proteins and platelets, blood transfusion requirements, incidence of clots in the oxygenator and the appearance of chest x-rays were assessed for each group.

Data was collected from the ECMO specialist charts, ELSO registry forms, perfusion charts and patient records. These charts are filled in during the course of a

patient's admission. All patients were treated according to our standard institutional ECMO protocol.

Results were analysed using SPSS version 11.5. Except for the continuous parametric variables, pre-ecmo positive end expiratory pressure and flow to sweep ratios during ECMO, where independent sample t-test was used, all other continuous variables were non parametric and a Mann-Whitney test was employed to test their variability. Chi-Square test was used to test the variability of all grouped variables, Clots in the oxygenator, Oxygenator failure and Haemorrhagic complications. They were corrected for ties and expressed in terms of minimum, maximum, median, first and third quartiles, mean and one standard deviation. A p value of <0.05 was considered to be significant.

RESULTS

ADULTS

Referral details: The patients were demographically quite similar (Table 4.1). Aetiological variations are illustrated in diagram 2.3. $\text{PaO}_2/\text{FiO}_2$ ratios were calculated for each patient at the time of referral. The mean $\text{PaO}_2/\text{FiO}_2$ ratios and standard deviations (SD) for A-S and A-PMP were 73.4 (44.6) and 67.8 (45.2) mmHg respectively ($p = 0.73$). The mean Pa CO_2 and SD for each group were 73.1 (45.5) vs. 59.7 (16.8) mmHg respectively ($p = 0.55$). The differences in the mean peak inspiratory and positive end expiratory pressures were not statistically significant either (p values of 0.93 and 0.098 respectively).

Oxygenator efficiency: All A-S circuits had two oxygenators in parallel. Apart from the very first patient, all A-PMP patients received one oxygenator ($n=13$), except when patients weighed >100 Kg ($n=7$). Two oxygenators were then used in parallel.

The data in table 4.2 illustrates blood gas values for each group and sub-groups. The partial pressure of oxygen in arterial blood (PaO_2) was used to assess oxygenation in veno-venous patients and mixed venous saturation (MVSO_2) in the veno-arterial ECMO patient. Note that the only adult VA-ECMO run belonged to A-S group.

The median partial pressure of arterial carbon dioxide (Pa CO_2) was lower in patients with two oxygenators, irrespective of their type [Median Pa CO_2 for A-PMP II vs. A-PMP I was 39.0 vs. 43.5 mmHg respectively, $p = 0.05$ and for A-S vs. A-PMP I was 41.3 vs. 43.5 mmHg respectively, $p = 0.03$]. There was no statistically significant difference in patient oxygenation between any of the groups and sub-groups.

Table 4.1: Summary of patient's details, which includes patient demography, pre-ECMO assessment, ECMO run and immediate outcome.

Groups	A-S	A-PMP	N-S	N-PMP
Demographics				
Male	13	13	10	11
Female	7	7	10	9
Mean age (SD) Y=years, H=hours	36.7(16.2) Y	36.9(15.2) Y	94 (160) H	74 (88) H
Mean Weight (SD) Kg	87.0(12.9)	86.9 (21.4)	3.4 (0.6)	3.3 (0.7)
Pre-ECMO				
Mean PaO ₂ /FiO ₂ (SD) mmHg	73.4 (44.6)	67.8 (45.2)	-	-
Mean OI (SD)	-	-	50.3 (32.6)	35.1 (16.3)
Mean PaCO ₂ (SD) mmHg	73.1 (45.5)	59.7 (16.8)	59.9(32.9)	47.8 (18.7)
Mean PIP (SD) CmH ₂ O	38 (8.9)	37 (8.7)	35.3(7.4)	33.2 (7.0)
Mean PEEP (SD) CmH ₂ O	10.8 (6.0)	13.8 (4.0)	6.2(1.8)	5.4 (1.4)
Hand bagged prior to ECMO	4	3	4	1
ECMO				
VV-ECMO	19	20	16	17
VA-ECMO	1	0	4	2
VV to VA	0	0	0	1
Mean ECMO run (SD) Hours	228.2(130.5)	257.7(194.2)	141.4(117.3)	143.1(122.0)
CVVH	14	11	4	2
MARS	3	1	0	0
Haemorrhagic complications	2	6	2	1
Treatment with Aprotinin	1	6	2	1
Survived ECLS %	80	85	80	80
Survival to discharge %	80	80	75	70

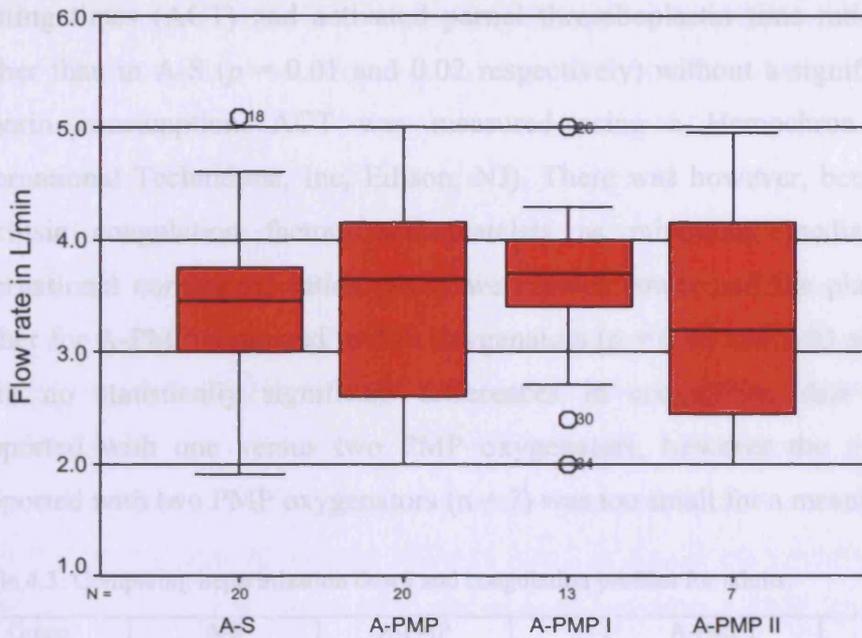
There was no statistically significant difference in the flow rates for the different types of oxygenator (Diagram 4.1). However there was a significant reduction in the sweep gas required (Median sweep; 5.5 from 8.4 L/min, $p = 0.03$) by A-PMP oxygenators compared to A-S devices (Diagram 4.2). There was no statistically significant difference in median sweep requirements ($p = 0.33$) between the sub-groups of A-PMP.

The median flow to sweep ratios for each patient was calculated and their mean values compared [1: 2.2 (SD=1.17) for A-S and 1: 1.6 (SD=0.37) for A-PMP, $p = 0.03$]. A-PMP oxygenators have a lower flow to sweep ratio, indicating more efficient CO₂ exchange.

Table 4.2: Gas exchange performance for adult ECMO. p values are included in the table.

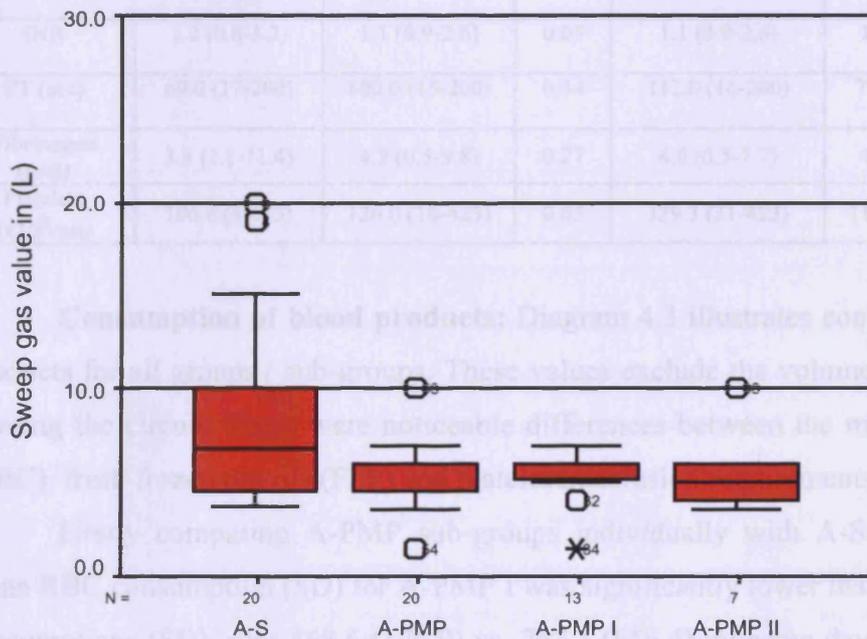
Groups	A-S	A-PMP		A-PMP I	A-PMP II	
Variable	Median (Range)	Median (Range)	p	Median (Range)	Median (Range)	p
PaO ₂ (mmHg)	57.8 (27.8-176.3)	54.8(33.8- 186.8)	0.69	54.0 (36.8-186.8)	55.5 (33.8-99.8)	0.97
MVSO ₂ (%)	71 (64-80)	-	-	-	-	-
PaCO ₂ (mmHg)	41.3 (25.5-72.0)	42.0 (23.3-91.5)	0.17	43.5 (24-91.5)	39.0 (23.3-57.8)	0.05

Diagram 4.1: Illustrating the box-and-whisker presentation of the flow rates for each of the oxygenator groups. $p = 0.62$ for A-S vs. A-PMP, $p = 0.61$ for A-PMP I vs. A-PMP II and $p = 0.31$ for A-S vs. A-PMP I. The values presented by a small circle are considered as outliers by the SPSS program.



Group	Median (Range)	Mean (Range)	Mean (Range)	p
A-S	3.5 (1.9-4.6)	3.5 (1.9-4.6)	3.5 (1.9-4.6)	0.62
A-PMP	3.7 (2.0-5.0)	3.7 (2.0-5.0)	3.7 (2.0-5.0)	0.61
A-PMP I	3.8 (2.7-4.3)	3.8 (2.7-4.3)	3.8 (2.7-4.3)	0.31
A-PMP II	3.2 (2.0-5.0)	3.2 (2.0-5.0)	3.2 (2.0-5.0)	

Diagram 4.2: Illustrating the box-and-whisker presentation of the sweep gas for each of the oxygenator groups. The values presented by a small circle are considered as outliers. Extreme points (Outliers by more than 3 box lengths from the edge of the box) are represented by an asterisk*.



Group	Median (Range)	Mean (Range)	Mean (Range)	p
A-S	7.0 (3.5-15.0)	7.0 (3.5-15.0)	7.0 (3.5-15.0)	0.62
A-PMP	5.5 (3.5-7.5)	5.5 (3.5-7.5)	5.5 (3.5-7.5)	0.61
A-PMP I	5.0 (4.0-7.0)	5.0 (4.0-7.0)	5.0 (4.0-7.0)	0.31
A-PMP II	4.5 (3.5-6.0)	4.5 (3.5-6.0)	4.5 (3.5-6.0)	

Coagulation data: The review of the coagulation profiles (Table 4.3) revealed significantly less resistance to heparinisation in the A-PMP group, as both activated clotting times (ACT) and activated partial thromboplastin time ratios (APTTR) were higher than in A-S ($p = 0.01$ and 0.02 respectively) without a significant difference in heparin consumption. ACT was measured using a Hemochron monitors (HCH; International Technidyne, Inc, Edison, NJ). There was however, better preservation of extrinsic coagulation factors and platelets as minimum, median and maximum international normalized ratios (INR) were much lower and the platelet counts much higher for A-PMP compared to A-S oxygenators ($p = 0.05$ and 0.05 respectively). There were no statistically significant differences in coagulation data between patients supported with one versus two PMP oxygenators, however the number of patients supported with two PMP oxygenators ($n = 7$) was too small for a meaningful comparison.

Table 4.3: Comparing heparinisation doses and coagulation profiles for adults.

Group	A-S	A-PMP		A-PMP I	APMP II	
Variable	Median (Range)	Median (Range)	<i>p</i>	Median (Range)	Median (Range)	<i>p</i>
Heparin (u/kg/hr)	18.0 (0-56.8)	20.4 (0-49.9)	0.10	20.8 (0-49.4)	19.8 (0-42.4)	0.66
ACT (sec)	191.0 (95-622)	200.2 (124-629)	0.01	201.5 (124-629)	197.9 (142-405)	0.48
APTTR	2.1 (0.9-6.6)	2.6 (0.9-6.6)	0.02	2.5 (0.9-6.6)	2.7 (1.3-6.6)	0.61
INR	1.2 (0.8-3.2)	1.1 (0.9-2.6)	0.05	1.1 (0.9-2.6)	1.1 (0.9-1.7)	0.56
TT (sec)	69.0 (17-200)	100.0 (15-200)	0.34	112.0 (16-200)	77.6 (15-200)	0.24
Fibrinogen (g/dl)	3.8 (1.1-11.4)	4.2 (0.5-9.8)	0.27	4.0 (0.5-7.7)	4.6 (1.3-9.8)	0.40
Platelets ($\times 10^9$ /ml)	106.6 (8-613)	126.0 (10-423)	0.05	129.3 (31-423)	119.8 (10-358)	0.32

Consumption of blood products: Diagram 4.3 illustrates consumption of blood products for all groups / sub-groups. These values exclude the volume of blood used for priming the circuit. There were noticeable differences between the mean red blood cell (RBC), fresh frozen plasma (FFP) and platelet transfusion requirements.

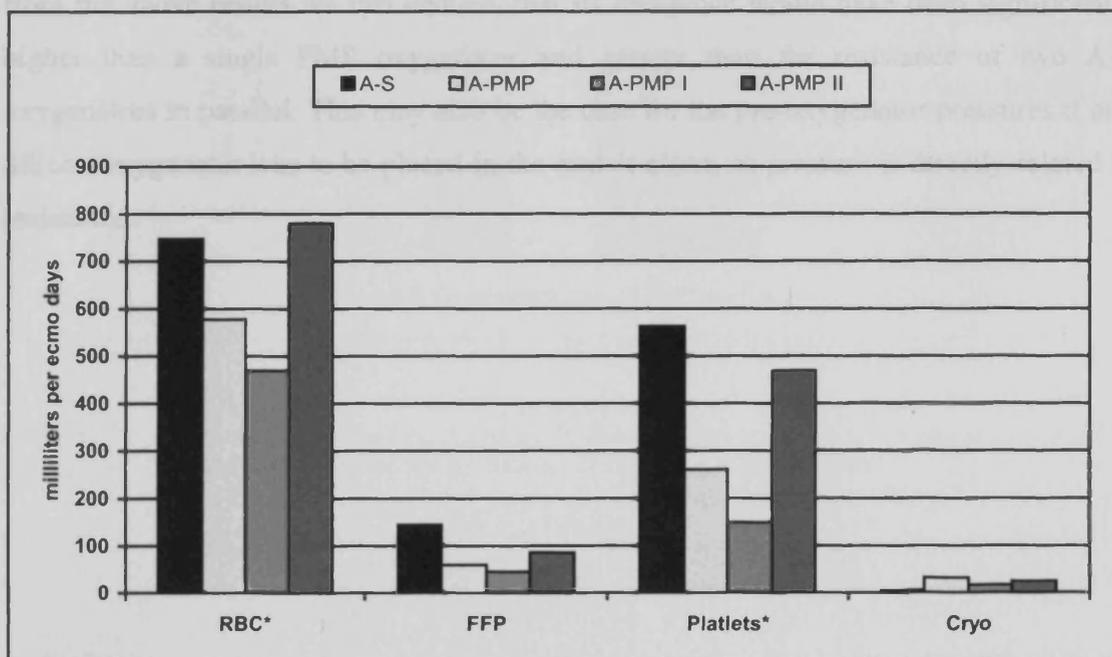
Firstly comparing A-PMP sub-groups individually with A-S revealed that the mean RBC consumption (SD) for A-PMP I was significantly lower than A-S [Mean RBC consumptions (SD) were 468.6 (174.9) vs. 747.1 (656.4) ml/ecmo day respectively, ($p = 0.005$)]. This is a 37.3% reduction in red blood cell usage per ecmo day. However there was no statistically significant difference in RBC consumption between the A-PMP II group, where two A-PMP oxygenators were used vs. two Silicon oxygenators in A-S

[Mean RBC consumptions (SD) were 780.0 (429.5) ml/ecmo day vs. 747.1 (656.4) ml/ecmo day respectively, ($p = 0.62$)]. Therefore it seems that the reduction in RBC consumption is the result of a lower surface area rather than the oxygenator type.

There were even bigger reductions in platelet consumption. The platelet consumption by A-PMP I was almost a quarter of that by A-S group [Mean platelet consumption (SD) of 148.7 (136.3) vs. 562.8 (388.7) ml/ecmo day respectively, $p = 0.03$]. The reduction in platelet consumption between A-PMP II and A-S groups, where both circuits utilized two oxygenators each, was not of statistical significance [Mean platelet consumption (SD) of 467.9(565.3) vs. 562.8 (388.7) ml/ecmo day respectively, $p = 0.35$], which reiterates the significance of a lower surface area for consumption of blood products.

Secondly, it is clear that continuing the current practice and using two A-PMP oxygenators for heavier patients will not result in significant overall reduction in RBC consumption. The red blood cell consumption between A-PMP group, 577.5 (317.5) ml/ecmo days vs. A-S group, 747.1 (656.4) ml/ecmo days, did not reach statistical significance ($p = 0.18$). This however was not the case for platelet consumption as statistically significant differences were still noted in the overall figures [Mean platelet consumption (SD) of 260.4 (370.2) vs. 562.8 (388.7) ml/ecmo day respectively, $p = 0.01$], in favour of A-PMP.

Diagram 4.3: Consumption of blood products. * Indicates the categories with statistically significant differences.



The requirements of FFP or Cryoprecipitate did not differ significantly between any of the groups / subgroups.

Oxygenator pressure and resistance: The Mean values of the median pre-oxygenator pressures for each group of patients are demonstrated in diagram 4.4. The pre-oxygenator pressure was significantly lower in the A-PMP group than the A-S [Mean of the median pre-oxygenator pressures (SD) were 187.7 (53.8) vs. 228.9 (46.8) mmHg respectively, $p = 0.03$]. The difference between A-PMP I and II however, did not reach statistical significance [Mean of the median pre-oxygenator pressures (SD) were 202.7(44.4) vs. 159.9 (62.0) mmHg respectively, $p = 0.13$].

The resistance across the oxygenators followed the same pattern. Resistance = Pressure gradient / Flow, where pressure is measured in mmHg and flow in litres per minute. Diagram 4.5; illustrates this relationship. The oxygenator resistance for the A-PMP group is found to be significantly lower than the A-S group [Mean of the median oxygenator resistance (SD) 19.6 (6.4) vs. 37.4 (9.1) Paul Wood Units or mmHg/(L/min) respectively, $p < 0.0001$]. The resistance between the sub-groups of A-PMP was also significantly different in favour of two oxygenators in parallel [The mean value of the median oxygenator resistance (SD) was 23.0 (4.1) vs. 13.5 (5.5) Paul Wood Units, $p = 0.001$]. This is explained by the way resistors behave when placed in parallel, $1/R = 1/R_1 + 1/R_2$ (Kirchoff's law).

There were no records of a single silicon oxygenator being used on an adult, but from the above results we can deduce, that its resistance would have been significantly higher than a single PMP oxygenator and greater than the resistance of two A-S oxygenators in parallel. This may also be the case for the pre-oxygenator pressures if one Silicon oxygenator was to be placed in the circuit alone, as pressure is directly related to resistance.

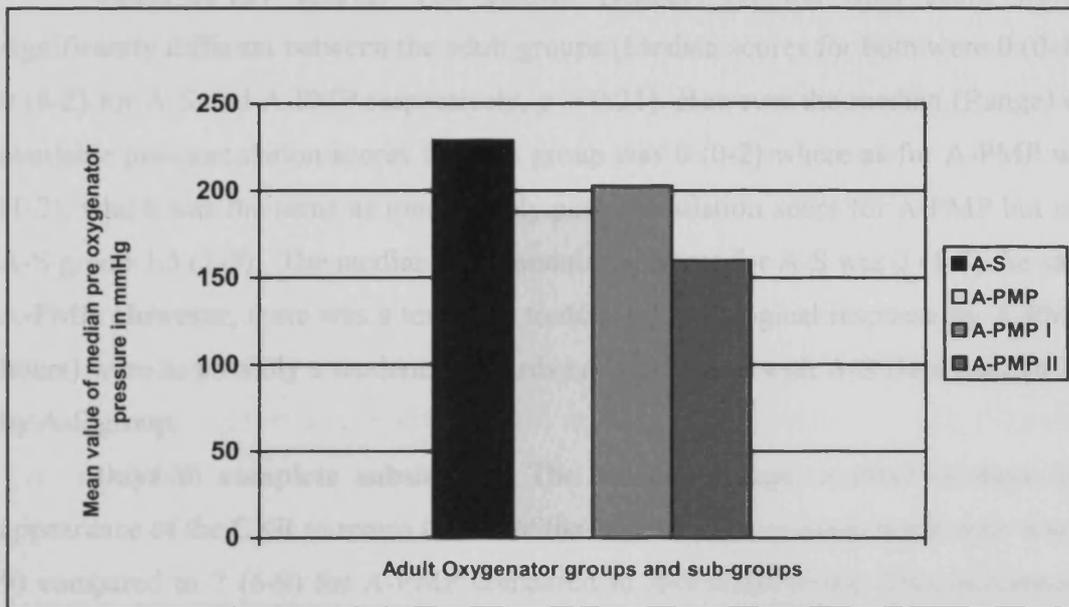


Diagram 4.4 (Above): Comparing the mean values of the median pre-oxygenator pressures for all adult groups and sub-groups.

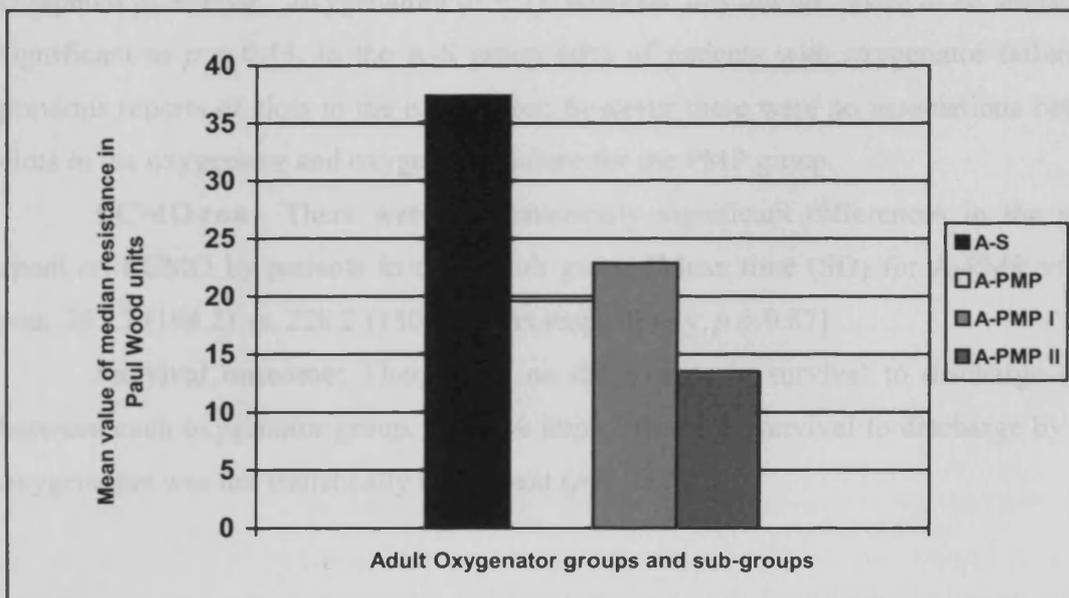


Diagram 4.5 (Above): Comparing the variation in mean values of median resistance across adult oxygenators according to their groups and the number of oxygenators in the circuit.

Chest X-ray scores: The median (Range) for the final score were not significantly different between the adult groups [Median scores for both were 0 (0-1) and 0 (0-2) for A-S and A-PMP respectively, $p = 0.21$]. However the median (Range) of the available pre-cannulation scores for A-S group was 0 (0-2) where as for A-PMP were 2 (1-2), which was the same as immediately post cannulation score for A-PMP but not for A-S group 1.5 (1-3). The median post cannulation score for A-S was 2 (1-3) the same as A-PMP. However, there was a tendency to delayed radiological response by A-PMP (48 hours) were as possibly a tendency towards early response with A-S (less than 24 hours) by A-S group.

Days to complete subsidence: The median (Range) number of days for the appearance of the CXR to return to that of the immediate post-cannulation state was 6 (2-9) compared to 7 (6-9) for A-PMP compared to A-S respectively. This difference was statistically insignificant ($p = 0.13$).

Clots and oxygenator failure: The reported number of patients to have clots in their oxygenators were four times higher in A-S ($n = 10$) compared to A-PMP ($n = 3$), $p = 0.006$. The incidence of oxygenator failure for A-S ($n = 6$) was three times higher compared to A-PMP oxygenators ($n = 2$), however this did not prove to be statistically significant as $p = 0.43$. In the A-S group 60% of patients with oxygenator failure had previous reports of clots in the oxygenator, however there were no associations between clots in the oxygenator and oxygenator failure for the PMP group.

ECMO run: There were no statistically significant differences in the period spent on ECMO by patients in each adult group [Mean time (SD) for A-PMP vs. A-S was, 257.7 (194.2) vs. 228.2 (130.5) hours respectively, $p = 0.87$]

Survival outcome: There were no differences in survival to discharge (80%) between each oxygenator group. The 5% improvement in survival to discharge by PMP oxygenators was not statistically significant ($p = 0.67$).

NEONATES

Referral details: The demographic details were quite similar between the two neonatal groups (Table 4.1). The aetiological indications are illustrated in diagram 2.3. The mean oxygenation index (SD) for N-S vs. N-PMP were calculated respectively as 50.3 (32.6) and 35.1 (16.3), but were not statistically significantly different $p = 0.15$.

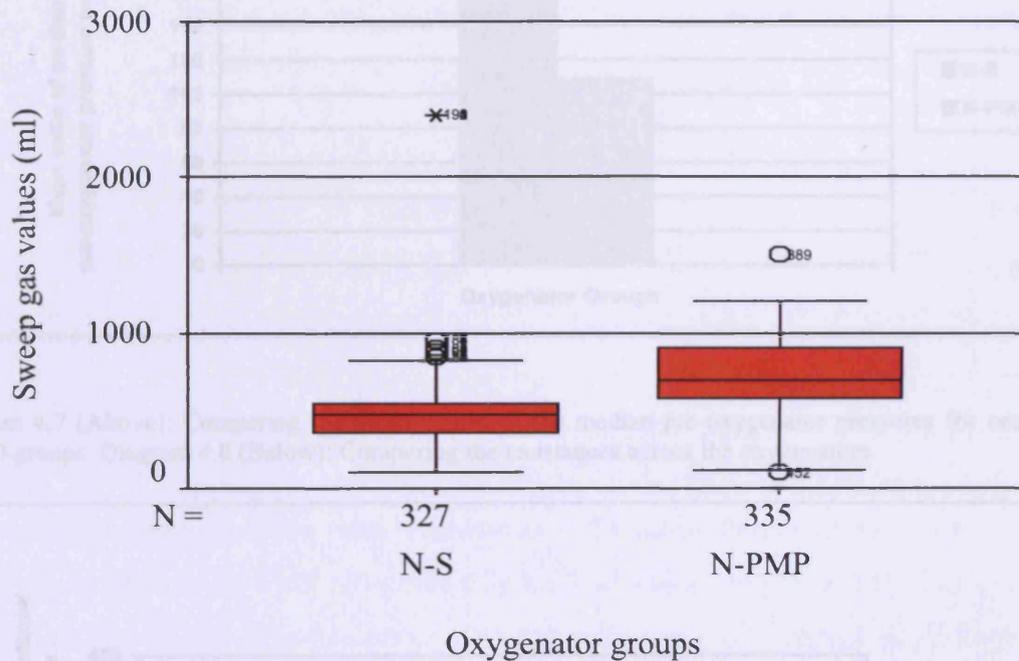
Oxygenator efficiency: This was defined as the gas exchange performance taking into account oxygen transfer across the membrane and carbon dioxide elimination. The mean values of median post oxygenator partial pressure of oxygen were compared between groups, [Mean values of PO_2 (SD) was 491.3 (43.5) and 393.8 (63.8) mmHg for N-S vs. N-PMP respectively, $p = 0.001$]. This shows a better oxygen transfer capability in favour of N-S. There were no statistically significant differences however, in the mean values of the median partial pressure of peripheral arterial oxygen (PaO_2), between veno-venous patients, [Mean values (SD) for N-S vs. N-PMP was 63.0 vs. 68.3 mmHg respectively, $p = 0.08$] on similar flow rates (Table 4.4). The mean flow to sweep ratios were, 1: 2.0 (SD = 0.80) for N-S and 1:3.0 (SD = 0.90) for N-PMP with a p value less than 0.001. Indicating that N-S oxygenators were also more efficient in terms of carbon dioxide transfer.

Table 4.4: Table comparing gas exchange performance for neonates.

Groups	N-S	N-PMP	
Variable	Median (Range)	Median (Range)	p
VV-ECMO			
PaO_2 (mmHg)	63 (26.3-252)	68.3 (30.8-229.5)	0.08
Flows (ml/min)	247.2 (130-410)	268.8 (80-500)	0.37
PCO_2 (mmHg)	44.3 (28.5-67.5)	45.8 (15.8-96)	0.39
Sweep (ml/min)	454.7(100-2400)	774.4 (100-1500)	<0.001
VA-ECMO			
$MVSO_2$ (%)	81.6 (46-93)	72.3 (55-93)	0.16
Flows (ml/min)	322.5 (130-600)	293.3 (140-400)	0.29
PCO_2 (mmHg)	53.3 (33.8-84.8)	52.5 (30.0-76.5)	1.00
Sweep (ml/min)	810 (100-4180)	1016.7 (100-2400)	0.72

There was however less variation in the sweep gas requirements for N-PMP group compared to N-S. The median sweeps were significantly higher for patients in the N-PMP group, who received VV-ECMO in order to maintain similar carbon dioxide levels as the N-S (Diagram 4.6). The difference between VV and VA Sweeps (on 100% Oxygen) were not statistically significant in either group ($p = 0.81$ and 0.26 respectively).

Diagram 4.6: Box-and-whisker presentation of sweep gas variations between the oxygenator groups. The values represented by a small circle are considered as outliers and the adjacent numbers are their identifiers. Extreme points (Outliers by more than 3 box lengths from the edge of the box) are represented by an asterisk*. N represents the number of measurements.



Coagulation data: The Fibrinogen levels were better preserved in the N-PMP group with a median of 2.2 (g/dl), ranging between 0.6 to 7.8 (g/dl) compared to N-S with a median of 1.8 (g/dl) and a range of 0.5 to 5.2 (g/dl), $p = 0.048$. Thrombin time was significantly prolonged in the N-PMP group, 106 (sec) ranging between 18-200 (sec) compared to N-S with a thrombin time of 51.6 (sec) and a range of 15-200 (sec), $p = 0.004$. The other parameters, such as the ACT, APTTR, INR and the platelet counts were almost identical for both groups (Table 4.5).

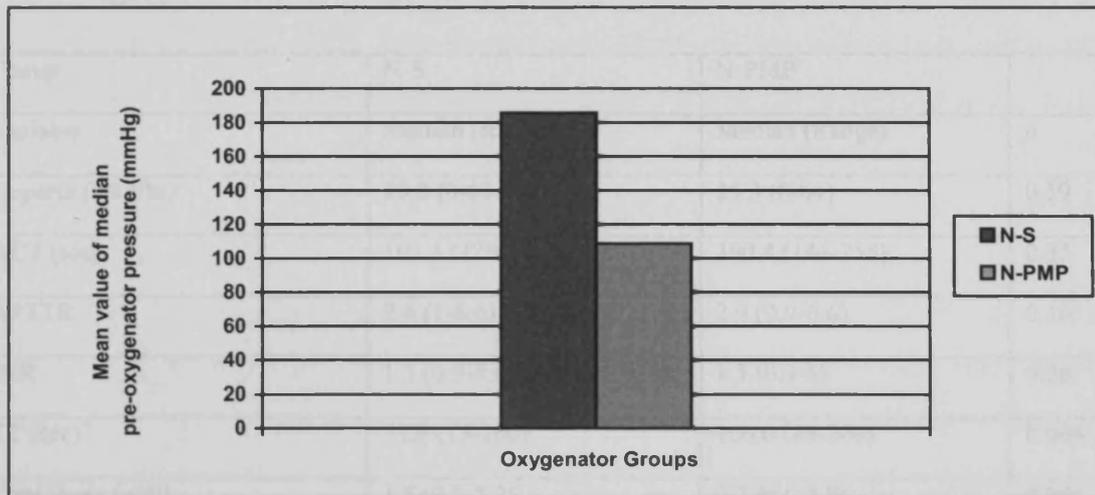
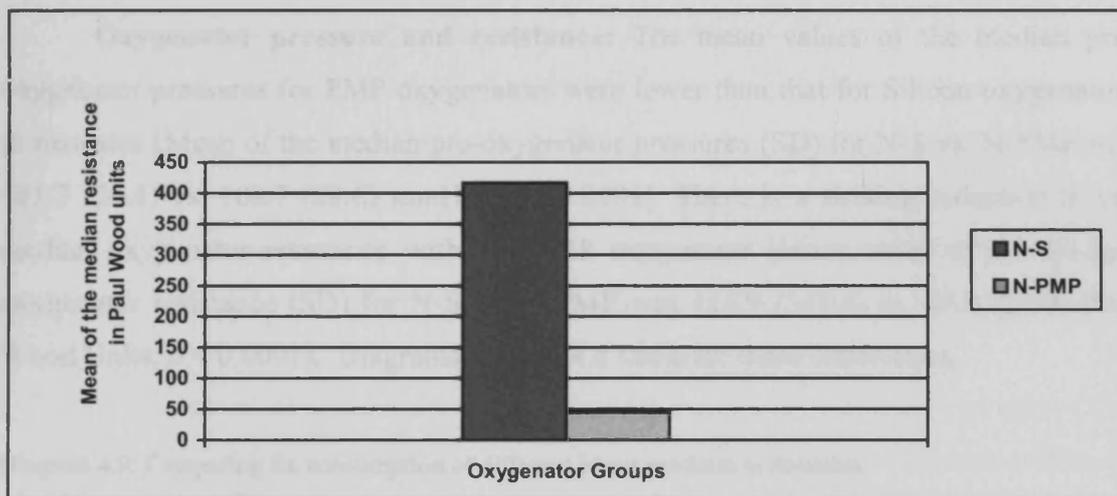


Diagram 4.7 (Above): Comparing the mean values of the median pre-oxygenator pressures for neonatal ECMO groups. Diagram 4.8 (Below): Comparing the resistances across the oxygenators.



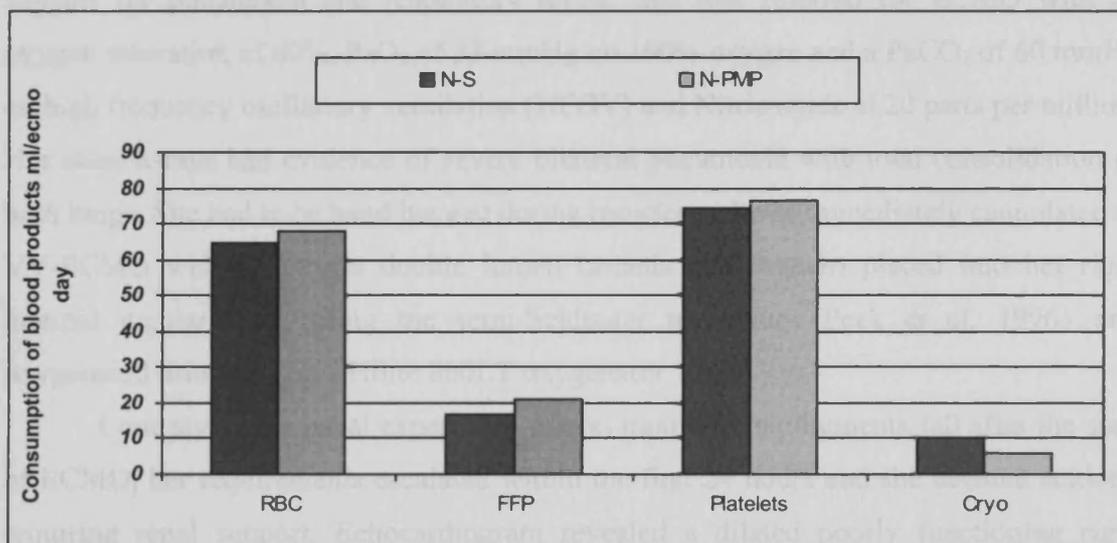
Consumption of blood products: There was no statistically significant difference in transfusion requirements between the two groups. The mean RBC consumption (SD) in the N-PMP group was 67.9 (90.2) ml/ecmo day compared to N-S at 64.7 (37.2) ml/ecmo day, $p = 0.53$. The mean FFP consumption (SD) was 20.8 (42.0) ml/ecmo day for N-PMP compared to 16.5 (33.0) ml/ecmo day for N-S, $p = 0.47$. Platelet consumption (SD) was 76.6 (89.9) ml/ecmo day for N-PMP compared to N-S at 74.2 (42.9) ml/ecmo day, $p = 0.40$ and a mean of 5.7 (14.1) ml/ecmo day of Cryoprecipitate (SD) was consumed in N-PMP compared to 10.3 (25.4) ml/ecmo day in N-S, $p = 0.17$ (Diagram 4.9).

Table 4.5: Heparinisation dose and coagulation profiles for neonates.

Group	N-S	N-PMP	
Variable	Median (Range)	Median (Range)	<i>p</i>
Heparin (u/kg/hr)	23.0 (0-61.5)	25.3 (0-64)	0.59
ACT (sec)	191.4 (129-598)	190.4 (144-755)	0.85
APTTR	2.4 (1-6.6)	2.9 (0.9-6.6)	0.16
INR	1.2 (0.9-4.6)	1.3 (0.9-5)	0.38
TT (sec)	51.6 (15-200)	106.0 (18-200)	0.004
Fibrinogen (g/dl)	1.8 (0.5-5.2)	2.2 (0.6-7.8)	0.048
Platelets ($\times 10^9$ /ml)	103.7 (10-217)	106.3 (17-436)	0.76

Oxygenator pressure and resistance: The mean values of the median pre-oxygenator pressures for PMP oxygenators were lower than that for Silicon oxygenators in neonates [Mean of the median pre-oxygenator pressures (SD) for N-S vs. N-PMP was 185.7 (34.1) vs. 108.7 (28.6) mmHg, $p < 0.0001$]. There is a striking reduction in the median oxygenator resistance with the PMP oxygenator [Mean value of the median oxygenator resistance (SD) for N-S vs. N-PMP was 416.9 (145.4) vs. 45.0 (27.4) Paul Wood Units, $p < 0.0001$]. Diagrams 4.7 and 4.8 illustrate these differences.

Diagram 4.9: Comparing the consumption of different blood products in neonates.



Chest x-ray scores: The median (Range) pre-cannulation scores were 1 (0-2) for both oxygenator groups and were similar to that of the immediate post-cannulation scores, 1 (0-3). However the median (Range) score for first day post-cannulation was 1 (1-3) for N-PMP versus 3 (1-3) for N-S. The median (Range) final score for N-PMP was 0 (0-3) versus 1 (0-3) for N-S. The final scores were significantly lower for N-PMP than for N-S ($p = 0.023$).

Days to Complete Subsidence: The median number of days for the appearance of the CXR to return to that of the immediate post-cannulation state was 2 (2-3) compared to 5 (2-11) for N-PMP compared to N-S respectively. This was statistically significant in favour of N-PMP with a mean number of days of 2.2 versus 5.3 for N-S ($p < 0.0001$).

Clots and oxygenator failure: There were no reports of clots in the oxygenators for N-PMP group, compared to N-S group, where clots were subjectively reported in as many as eleven patients. However there were two incidences of oxygenator failure in the N-PMP group compared to one in N-S group and more incidences of sudden oxygenator failure since this study. There was no association between clots in the oxygenator and oxygenator failure for any of the neonatal oxygenator groups in this series.

ECMO run: There was no statistically significant difference between the mean numbers of days each group spent on ECMO. The mean (SD) run was 143.1 (122.0) and 141.4 (117.3) hours respectively, ($p = 0.84$).

Case report of a fatal neonatal oxygenator failure: A 2.5 Kg neonate with a corrected gestational age of three weeks had been treated with antibiotics and ventilatory support for pneumonia and respiratory arrest. She was referred for ECMO with an oxygen saturation of 60%, PaO₂ of 33 mmHg on 100% oxygen and a PaCO₂ of 60 mmHg on high frequency oscillatory ventilation (HFOV) and Nitric oxide at 20 parts per million. Her chest x-rays had evidence of severe bilateral pneumonia with total consolidation of both lungs. She had to be hand bagged during transfer and was immediately cannulated to VV-ECMO with an Origin double lumen cannula (12 French) placed into her right internal jugular vein, using the semi-Seldinger technique {Peek et al. 1996} and oxygenated using a Medos Hilite 800LT oxygenator.

Contrary to our usual experience where, inotropic requirements fall after the start of ECMO, her requirements escalated within the first 24 hours and she became acidotic requiring renal support. Echocardiogram revealed a dilated poorly functioning right ventricle and pulmonary arteries. There was minimal flow through her pulmonary

arteries. A transformation from VV- to VA-ECMO allowed extra cardiac support by surgical cannulation of the right internal carotid artery using a 10 French cannula (Biomedicus). The inner lumen of the existing Origin cannula was used to drain and with the outer lumen (The y-end) connected to the venous side of the bridge.

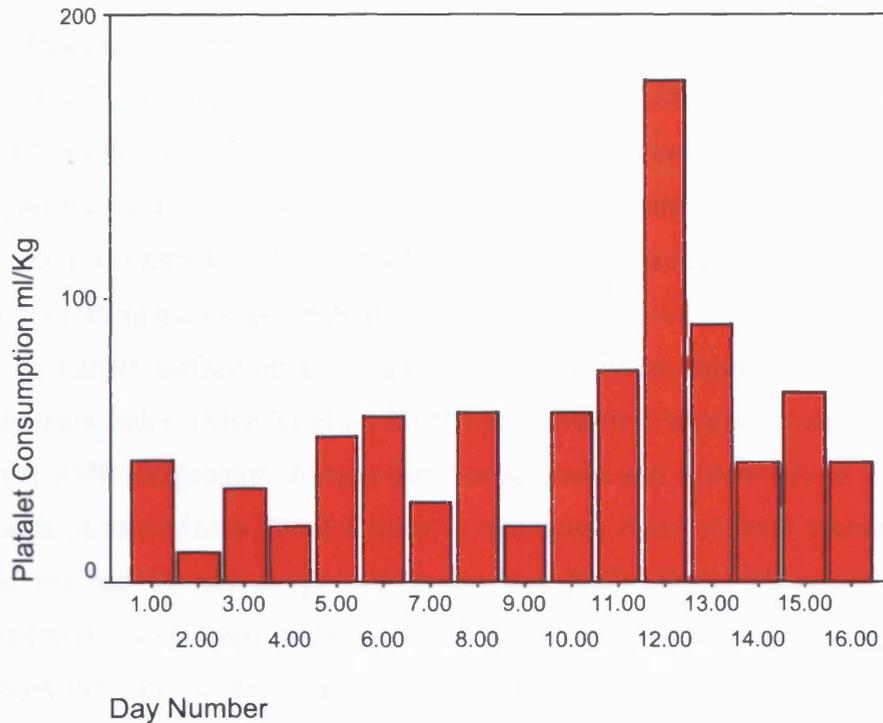
During the course of ECMO treatment it was clear that she had severe lung infection, which was proved to be Influenza A. She had also developed secondary bacterial sepsis and was treated with a number of antibiotics.

In the second half of her treatment with ECMO, she had a circuit change as numerous clots were noted throughout the circuit and platelet consumption had risen (Diagram 4.10). She did not tolerate this very well with oxygen saturations dropping to 30% during the change. However both blood pressure and saturations returned to range rapidly on going back on to the new circuit, and remained stable. Her median platelet count was $120 \times 10^9/\text{ml}$, ranging from 23 to $436 \times 10^9/\text{ml}$. The median ACTs were 180 sec, with a range of 148-296 sec on heparin infusion directly into the circuit. Her median Fibrinogen level, INR and APTTRs were 3.2 g/dl, 1.0 and 1.3 respectively. The increase in fibrinogen levels (from 1.3 to above 5 g/dl) followed an increase in thrombin time from 30 up to 200 (s) by day seven and back down again. However her fibrinogen levels continued to stay high. She had minimal requirements for Fresh frozen plasma or Cryoprecipitate throughout her treatment with ECMO.

On the morning of ECMO day seventeen, the circuit suddenly stopped. This was due to a clot moving from the bladder, occluding the oxygenator. There was a sudden rise in trans oxygenator pressure from about 20 to 200 mmHg. As she was totally dependent on the circuit she lost cardiac output and suffered a cardiac arrest. Despite a new circuit, after 20 minutes, resuscitation was abandoned, as there was no possibility of a successful outcome.

Survival outcome: There was no difference in percentage of survival to decannulation, or survival to discharge between groups. Survival of ECMO was 80% for both groups, whereas survival to discharge was 75 vs. 70 % in favour of N-S but that was not statistically significant ($p = 0.72$).

Diagram 4.10: Representing the daily consumption of platelets in ml/Kg. The histogram peaks at day 12. The first oxygenator change occurred at day 13 and the terminal event occurred on the morning of day 17.



DISCUSSION

Galletti {Galletti et al. 1962} described the design objectives of an “ideal“ oxygenator as “efficient gas exchange, minimal blood trauma, small priming volume and safety”. Bramson {Bramson et al. 1965} added two other basic design desiderata of low haemodynamic resistance and adequate temperature control. The same ideals still hold true, as we strive to improve clinical outcomes. They will now be discussed in relation to these oxygenators. However since the advent of membrane oxygenators the reduction in surface area has been one of the main aims in design of newer oxygenators.

Mueller {Mueller et al. 2000} discussed the impact of low surface area by reducing gas exchange capability, increasing the blood path resistance as well as blood trauma. However we will demonstrate how the material property and design characteristics together with heparin bounding allow considerable reduction in the oxygenator surface area before any of these adverse effects arise.

Surface area of oxygenators and gas exchange efficiency: The material properties of PMP oxygenators and their structural design have made these oxygenators significantly better at gas exchange. In adults despite a reduction in surface area, as much

as five times (Table 2.1) by substitution of A-S (Medtronic 1-4500-2A) oxygenators by A-PMP I (Medos Hilite 7000LT), there was no significant reduction in patient oxygenation. The median oxygenation was similar for all adult groups and sub-groups (Table 4.2), and were achieved using similar flow rates (Diagram 4.2). Also similar PaCO₂ levels were achieved with significantly lower sweep gases in A-PMP group even though the number of oxygenators and the surface area of gas exchange were greatly reduced (Diagram 4.3). The A-PMP oxygenators had a lower flow to sweep ratio to A-S, also indicating their superiority in CO₂ exchange.

Larger surface areas for gas exchange may be required for patients with larger body mass index {Mueller et al. 2000}. However it is almost always unnecessary to use two A-PMP oxygenators for gas transfer purposes and is detrimental for haematological considerations. Medos 7000LT allow a maximum Flow of 7000 ml/min. The maximum flow reached by any oxygenator group was 5000 ml/min (Diagram 4.1). If the post oxygenator blood were well saturated, using two oxygenators would not change the oxygen delivery to the patient. The only reason for using two oxygenators in heavier patients would be to achieve higher sweeps. Patients with a body weight above 100 kg automatically received two PMP oxygenators in this study. These patients had significantly better CO₂ removal ($p = 0.05$) compared to lighter patients with one oxygenator (Table 4.1). However the maximum sweep required by any adult PMP patient in this study was 10 litres (Diagram 4.2) and that is manageable through a single oxygenator. Thus one may use a single PMP oxygenator for all patients irrespective of their weight. This may however leave little margin for safety.

However the results in neonates confirmed Mueller's concern. Neonatal poly-methyl pentene oxygenators (Medos Hilite 800 LT) were less than half the size of Silicon membrane (Medtronic 0800) oxygenators. They were also more than six times smaller than the A-PMP oxygenators (Table 2.1). Therefore it was not surprising to find that N-S oxygenators were significantly more efficient in gas transfer across their much larger membrane. However, despite higher post oxygenator PO₂ there was no significant difference in PaO₂ in the peripheral blood. This may have been a result of re-circulation or caused by a difference in cardiac output between these patient groups. Other factors that may affect peripheral PaO₂ are post oxygenator PO₂ and difference in the severity of disease between the groups. The values of post oxygenator PO₂ were in fact higher in N-S group. Both groups were similar in terms of demographical and pre-ECMO conditions (table 4.1).

Contrary to the experience in the adults, significantly higher sweeps had to be employed to achieve similar levels of PaCO₂ for N-PMP compared to N-S. This observation together with the fact that, the median arterial carbon dioxide levels were lower in adult patients with two oxygenators irrespective of their type, illustrates how lowering surface area affects carbon dioxide excretion even more than it affects oxygen transfer and points to a threshold below which reduction in surface area may no longer have desirable effects.

Although Medos 800LT appear not to be as efficient in gas exchange as Medtronic 0800, they seem to be perfectly adequate for the need of our neonates. In this study the maximum pump flow never exceeded 500 ml/min (Maximum flow capability of Medos 800LT = 800 ml/min) and sweep never rose above 2400 ml/min (Maximum sweep capability = 10000 ml/min).

Surface area of oxygenators and blood path resistance: The damage to the blood cells may be a consequence of interaction with the surface membrane and shear stress within the oxygenator {Beckley 1995}. A high-pressure drop across the oxygenator is associated with high resistance and high shearing forces {Bramson et al. 1965}. Significance of lowering oxygenator resistance is in reducing shearing stress and hence reducing blood trauma {Beckley et al. 1995}. Therefore a reduction in the surface area by PMP oxygenators should reduce blood path resistance and blood trauma. But in this study higher resistance did not correlate to high blood trauma.

The resistance to blood flow was significantly lower for both adult and neonatal PMP oxygenators. The greatest difference in resistance was noted when we compared similar arrangements of oxygenators, for example: A-S and A-PMP II (Both with two oxygenators in parallel) or N-S and N-PMP (one oxygenator per circuit), as illustrated in diagrams 4.5 and 4.8.

In adults although we found significantly lower resistance in A-PMP II group than A-S (More than halved), the consumption of RBCs between them were not significantly different [Mean RBC transfusion (SD), 774.8 (410.8) vs. 747.1 (656.4) ml/ecmo day respectively, $p = 0.62$]. The surface area in A-PMP II was also over half that of A-S (Table 2.1). However the consumption of RBCs was significantly less in favour of A-PMP I compared with A-S [Mean RBC transfusion (SD), 468.6 (174.9) vs. 747.1 (656.4) ml/ecmo day respectively, $p = 0.005$], where the surface area of A-PMP I was smaller by more than four times, but its resistance not even as much as half that of A-S. Similarly the resistance across the A-PMP II was significantly less than A-PMP I [The mean value

of the median oxygenator resistance (SD) was 13.5 (5.5) vs. 23.0 (4.1) mmHg/(L/min) respectively, $p = 0.001$], but the consumption of RBCs was significantly higher for A-PMP II than I [Mean RBC transfusion (SD), 774.8 (410.8) vs. 471.3 (200.7) ml/ecmo day respectively, $p = 0.03$].

The same applied to the neonates; even though there was an eight times reduction in resistance from silicon to PMP oxygenators (Diagram 4.8) the reduction of surface area was not relatively as large (Halved). This may explain why the consumption of blood products was not significantly different between the two neonatal groups. A significant reduction in RBC consumption in adults occurred only when the surface area was reduced by a factor of four.

These results suggest that the consumption of RBC is related to surface area of the oxygenator rather than to oxygenator resistance. However, as long as the resistance caused by the oxygenators necessitates the use of a roller pump {Motomura et al. 2002}, blood trauma and hemolysis caused by the pump may be a factor in RBC consumption. We used roller pumps for all our circuits.

Surface area of oxygenators and haematic response: The physiological advantage of an oxygenator with a small surface area of blood-gas interface is in reducing blood contact with foreign surfaces {Mueller et al. 2000 & Bramson et al. 1965}, Hence a reduction in contact activation of blood. Thus, one would expect a reduction in the activation of contact coagulation proteins, inflammatory products, fibrinolytic pathways and platelet consumption, the major components of the response to extracorporeal circuits {Plotz et al. 1993}.

This investigation demonstrated a significant reduction in resistance to heparinisation and better preservation of extrinsic coagulation factors. It also revealed a statistically significant reduction in platelet consumption with adult PMP oxygenators. The reduction in platelet requirements could not be explained just by a reduction in number of oxygenators, as there was no statistical difference between the platelet requirements for A-PMP II vs. I. Poly-methyl pentene oxygenators were however heparin (Rheoparin) coated (Medos Medizintechnik AG). There is much published literature in support of heparin coating and its role in enhancing biocompatibility of oxygenators {Ovrum et al. 2003, Tong et al. 1990, Pradhan et al. 1991, Ranucci et al. 2002, Svenmarker et al. 2001, Beckley et al. 1996 & Videm et al. 1991}. Studies of heparin coated bypass circuits have shown a reduction in complement activation {Videm et al. 1991, Pekna et al. 1994, Ovrum et al. 1995, Plotz et al. 1992 & te Velthuis et al. 1996},

granulocyte activation {Fosse et al. 1994 & Borowiec et al. 1992}, reduction in platelet adhesion, improvement in platelet function {Gorman et al. 1996 & Hatori et al. 1994} and inhibiting the release of pro-inflammatory cytokines, including TNF α {Plotz et al. 1992, Gu et al. 1993 & Redmond et al. 1993} and soluble TNF receptors {Weerwind et al. 1995}. In vitro models of extracorporeal circuits suggest reduction in leukocyte activation and adhesion {Hogevold et al. 1997} an important effector in the inflammatory response process {Peek et al. 1999}.

Maintenance of an adequate platelet count is the most important factor in prevention of haemorrhage {Stallion et al. 1994}, a real threat to any ECMO patient. Experimental and clinical studies also suggest that heparin coated circuits exert a protective effect on pulmonary function {Redmond et al. 1993 & Ranucci et al. 1996}. Heparin activates ATIII, inhibits factor Xa {Samama et al. 1996}, inhibit compliment activation {Nojiri et al. 1995} and activates platelets {Edmunds et al. 1987}. However prolonged exposure to heparin causes platelet dysfunction {Robinson et al. 1993}. Therefore it is possible that coating the entire circuit, including the cannula, with heparin would further reduce the inflammatory and coagulative responses to ECMO. However this may not be justified in neonates, as although heparin coated oxygenators have visibly reduced inflammatory and coagulative response to ECMO, they have not led to an improved outcome nor reduced consumption of blood products and platelets.

A-PMP oxygenators significantly preserve platelet counts in comparison to A-S [Mean value of median platelet count (SD) was 1293 (10-423) vs. 1066(8-613) respectively, $p = 0.05$]. However despite that, there was a non-significant increase in the incidence of haemorrhagic complications in A-PMP compared to A-S [3 vs. 1, $p = 0.11$]. Comparing the platelet counts and the incidence of bleeding in neonates however, showed no significant difference in the mean value of the median platelet counts (SD) between PMP and Silicon oxygenator groups [103.7 (19.3) vs. 106.3 (16.1) respectively, $p = 0.76$] and consequently no significant difference in the incidence of haemorrhagic complications between the two groups [One vs. none, $p = 0.31$].

The improved preservation of coagulation proteins and platelets observed in this study strongly suggest a reduction in activation of the contact coagulation system. However, the main inflammatory component of the response to extracorporeal circuits in the first twenty four hours is the activation of complements, which are impossible to quantify in retrospect. Activation of complements occurs within the first 24 hours of cannulation {Plotz et al. 1993}. The complement activation and leukocyte inflammatory

reaction during the initial period cause a capillary leak syndrome and might therefore explain the frequently observed temporary compromised lung function in extracorporeal life support. This inflammatory response to ECMO is, clearly visible a day after cannulation. Therefore semi-quantitative analysis of their CXRs was used to compare the degree of inflammatory response, caused by different oxygenators.

The CXRs taken immediately post-cannulation to check the position of the cannulae were used as a baseline to compare subsequent X-rays. In almost two out of three patients there were no comparable pre-cannulation CXRs as almost all patients were transported from other hospitals and cannulated immediately on arrival.

Once ECMO was established after cannulation the ventilation was weaned to the institutional rest settings in order to protect the lungs from further barotrauma and oxygen toxicity. These settings were: PIP of 20 Cm H₂O, PEEP of 10 CmH₂O and FiO₂ of 30 at a rate of 10 breathes per minutes.

However, there were no statistically significant differences between the CXR scores pre- and post-cannulation ($p = 0.27$ for N-PMP and 0.10 for N-S). The median pre- and immediate post-cannulation scores were the same for both groups (Median Scores = 1).

The primary disease affected each patient differently. The scores do not represent the difference in severity between the primary disease processes. They reflect the change in the radiographic appearance of the region least affected, on the immediate post-cannulation CXR.

Even though there was a statistically significant reduction in the intensity of alveolointerstitial infiltrates caused by inflammatory response to the circuit and quicker radiological recovery by N-PMP group, there were no statistically significant reductions in the length of ECMO runs or any survival advantages by using N-PMP oxygenators, as predicted by the oxygenation index. The intensity of alveolointerstitial infiltrates did not appear different between the immediate post cannulation CXR and 24 hour post cannulation in adults. This was contrary to the findings in the neonates. However, the tendency toward early radiological response, which in some cases may be almost instantaneous, in the A-S group may have masked any radiographic changes in the first 24 hours of cannulation. Unfortunately in many cases a pre-cannulation x-ray was not available making interpretation difficult. However, the median pre-cannulation scores for A-S group was 0 compared to 2 for A-PMP. The post cannulation score for A-PMP group stayed the same as pre cannulation score at 2, hence followed the same trend as in the

neonatal groups. However, there was an increase in the median post cannulation CXR score from 0 to 1.5 indicating a sudden rise in the x-ray score soon after cannulation.

It is not unreasonable to predict that coating not only the oxygenator but also the entire circuit with heparin would further reduce the inflammatory and coagulative responses to ECMO. Coating the entire circuit including the cannula may be difficult and costly. The ease of heparinisation depends on the surface, whether it forms ionic or covalent bonds and whether it is needed as a temporary or permanent measure. However, even in neonates where there was a clear reduction in the radiological signs of inflammatory response to ECMO by using heparin coated oxygenators, there were no improvement in outcomes in terms of survival to discharge, the length of ECMO runs or a reduction in the consumption of blood products and platelets

Safety of oxygenators: The material properties and structural design of PMP oxygenators have allowed adequate gas exchange to occur through much smaller surface area. This has made PMP a more ideal oxygenator for ECMO. One oxygenator may be used in A-PMP instead of two in A-S. The SM oxygenators were noticeably bulky and took too much room. However, they seem to have tolerated showers of clots from the circuit quite well even in the neonates. The N-PMP (Medos Hilite 800LT) on the other hand has proved to have a lower reserve for clots. There were two incidences of oxygenator failure in N-PMP compared to one for N-S. Since the completion of this study there have been two more incidences of oxygenator failures in neonates with PMP oxygenators. They were all unexpected and were accompanied by a sudden rise in pre-oxygenator pressures. Unfortunately one incidence proved fatal. The earlier report was therefore not our only incident of oxygenator failure, but the only fatal incident in the recent years. The severity of the patient's condition may be a crucial factor in surviving these events. This case unfortunately illustrates how dangerous a common disease like influenza A can be in a very small neonate, who inevitably has little immunity at this stage of life. It also brings about questions about the tolerance of small oxygenators towards clot emboli. As a result we changed our practice by lowering the tolerance of clots in the circuit changed our institutional parameters to reduce incidence of clots in the circuit and stopped using Hilite LT800 oxygenators. The parameters for activated clotting time (ACT) during ECMO were increased from 180-200 (s), to 200-220 (s) for Hemocron ACT monitors, using glass activator ACT tubes (International Technidyne, New Jersey, and USA); and 160-180 (s) for Actalyte ACT monitors, using the MAX-ACT tubes (GTA Ltd, Nottingham, UK). Currently all our ACT measurements are made

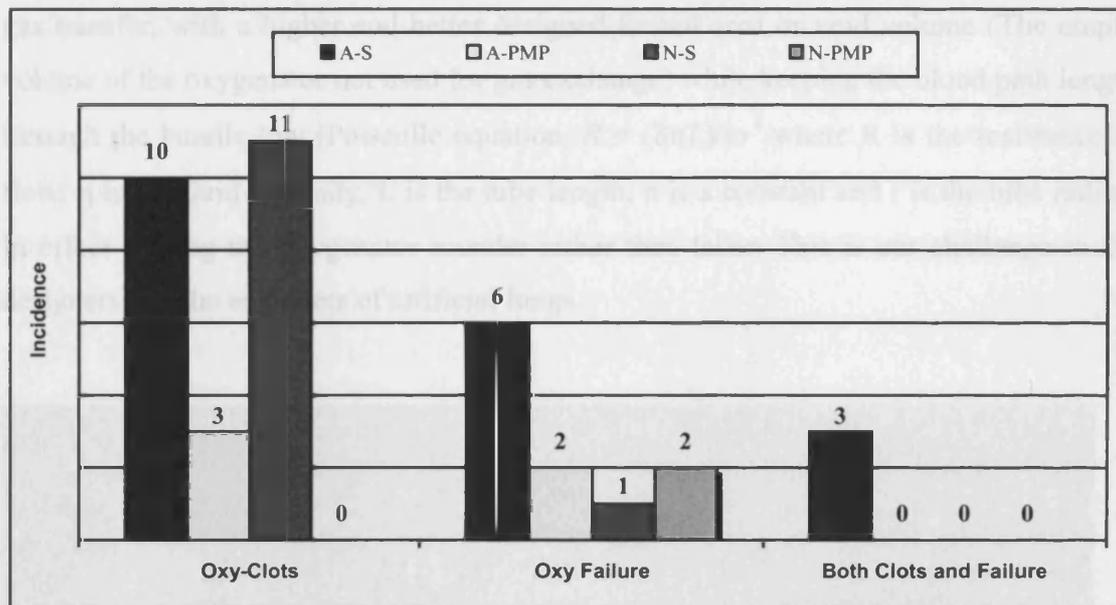
using the MAX-ACT device. We also lowered our platelet transfusion parameters to maintain platelet counts above 75 ($\times 10^9/\text{ml}$) rather than above 100 ($\times 10^9/\text{ml}$). There have been no further incidences of sudden unexpected oxygenator failure after these changes.

Raising the ACTs and lowering the platelet transfusion parameters did not significantly change the incidence of neurologic haemorrhage by Pearson Chi-square test. Extra efforts to monitor these patients with regular cranial ultrasound revealed no significant change in the incidences of post ECMO neurological haemorrhage, since the change in our practice. According to the institutional database, there were seven incidences of neurological haemorrhage compared to nine ($p = 0.37$) and one incidence of neurological infarct compared to four ($p = 0.12$) in patients treated with Medos oxygenators on the modified protocol (Between Dec 2001- Dec 2004, $n = 98$) compared with patients on either oxygenator treated with the old protocol (Between Dec 1998- Dec 2001, $n = 82$). The failure to increase the incidence of haemorrhage may also be related to a better preservation of coagulation proteins by Medos oxygenators.

There was no correlation between the presence of clots in the oxygenator and oxygenator failure for any group (Diagram 4.11).

Diagram 4.12 demonstrates a cross-sectional photograph of a Medos Hilite 800LT oxygenator. The arrows point at a narrow port where blood enters the cylindrical heat-exchange column. This narrow port represents the only reserve these oxygenators have against external clots. A relatively large clot for example from the bladder can instantly block this port resulting in sudden oxygenator failure. The diameter, size and the shape of the bladder may also be partly to blame. A Silicon bladder is the widest section of the circuit. It is soft therefore easily distorted; which may cause further upset to the flow dynamics causing more turbulence. The volume of a Silicon bladder is approximately 30 mls depending on how filled it is, therefore large clots may form within it. A better-constructed bladder {Tamari et al. 1999} may reduce turbulence and hence formation of clots in this part of the circuit.

Diagram 4.11: illustrating the number of patients reported as having clots in their oxygenator, the incidence of oxygenator failure and the number of patients who had reports of clots in the oxygenator followed by oxygenator failure for each group.



A primed circuit may be kept as a backup in case of an emergency {Kawahito et al. 2000}, for oxygenator failure in a very sick patient or even as a resuscitation tool for rapid response ECMO or E-CPR {Carmichael et al. 2002 & Younger et al. 1999}. In this incidence having a wet circuit as a backup may have changed the course of events, however the overall outcome was quite possibly the same.

Medos oxygenators are relatively quick to prime. CO₂ flushing in these oxygenators is unnecessary, saving on crucial time and blood needed for priming. However prevention is better than cure. The contact activation that affects platelets might explain the continuous platelet consumption observed during extracorporeal life support. Therefore, aprotinin given in the pump prime might be effective to prevent platelet consumption and impairment of haemostasis, however recent comparative studies of Aprotinin vs. other anti-fibrinolytic agents used in cardiac surgery {Fergusson et al. 2008} have shown an increase mortality in post cardiac surgery patients with only modest reduction in the risk of bleeding. Aprotinin is now used cautiously only in severe cases of bleeding on ECMO. Increasing the size of the oxygenator for example by using a Medos Hilite 2400 LT instead of 800 LT may allow a 'residual' of gas exchange to occur in the event of a relatively large clot being dislodged into the oxygenator, hence it may buy valuable time for urgent oxygenator change. An increase in the size of the oxygenator and its tolerance to withstand larger clots would necessitate an increase in the surface area of blood and

gas phase {Wickramasinghe et al. 2002}, and that would not be desirable {Mueller et al. 2000}. However, It is possible to make a low-resistance device of same surface area of gas transfer, with a higher and better designed frontal area or void volume (The empty volume of the oxygenator not used for gas exchange) while keeping the blood path length through the bundle low (Poiseuille equation, $R = (8\eta L)/\pi r^4$ where R is the resistance to flow, η is the fluid viscosity, L is the tube length, π is a constant and r is the tube radius. In effect making the oxygenator rounder rather than taller. This is our challenge to the designers and the engineers of artificial lungs.

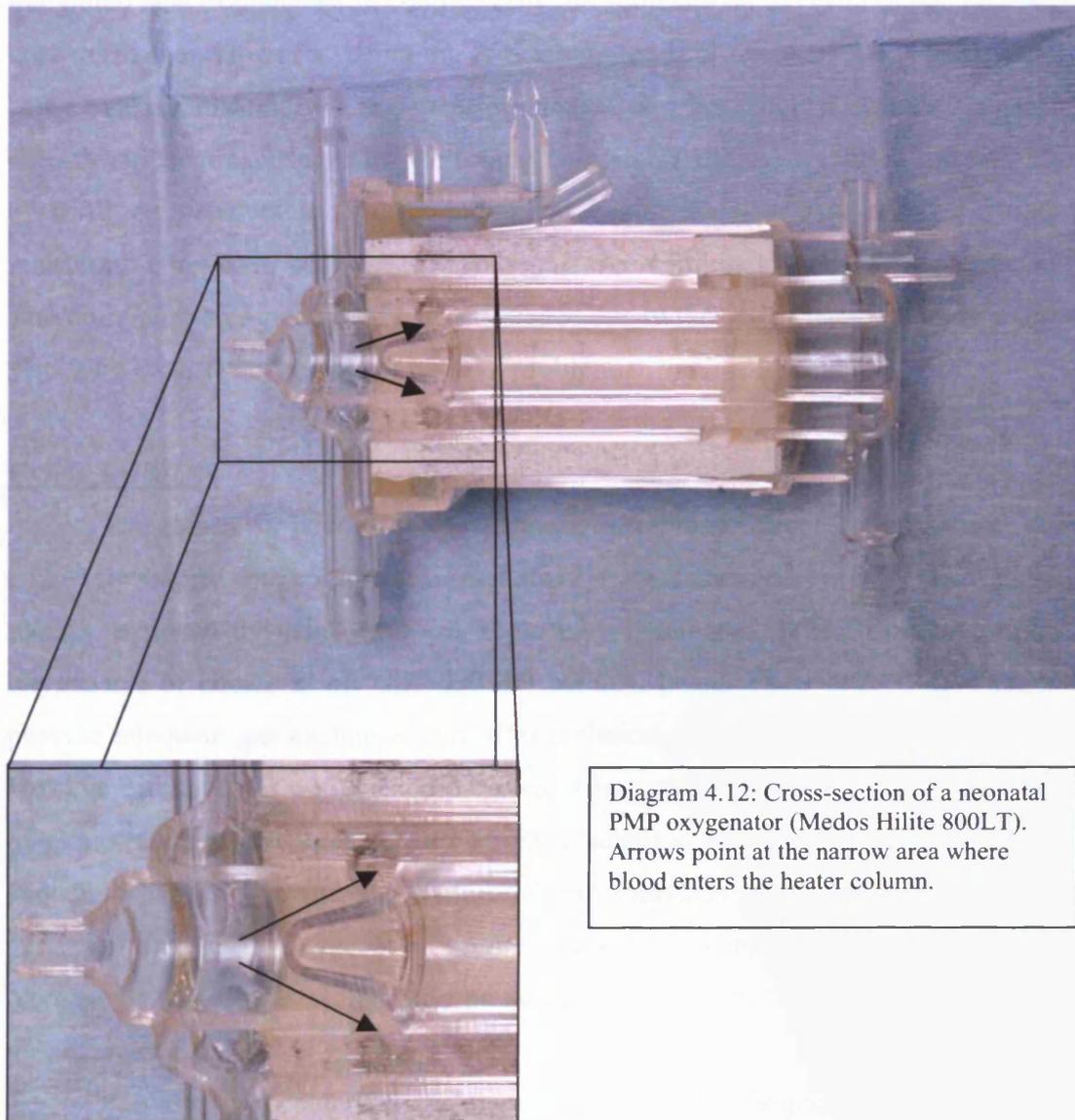


Diagram 4.12: Cross-section of a neonatal PMP oxygenator (Medos Hilite 800LT). Arrows point at the narrow area where blood enters the heater column.

Prime volume: Poly-methyl pentene (Medos) oxygenators are noticeably smaller than silicon (Medtronic) devices. The priming volume of a Medos 7000LT used in adults is almost five times less than that of two Medtronic 1-4500-2A oxygenators. The neonatal Medos Hilite 800 LT is almost half the size of Medtronic 0800 (Table 2.1). The importance of smaller priming volume is in reducing the amount of blood transfusion needed to prime these oxygenators. It may also save some time in priming. However, better designs of oxygenators with better tolerance for clots but smaller contact surface area may necessitate a larger void volume.

Heat exchange: Both neonatal and adult PMP oxygenators have functioning integrated heat exchangers. In contrast only the adult silicon oxygenator had an integrated heat exchanger. However, using the A-S integrated heat exchanger was thought to have cause extensive haemolysis and therefore not utilised. An external heat exchanger device (Bio-therm Heat exchanger by Medtronic perfusion systems) was used instead. The use of PMP oxygenators with their integrated heat exchanger had reduced blood path resistance compared with the SM oxygenators with their external heat exchangers. Therefore an increase in blood path resistance due to the use of integrated heat exchanger {Beckley et al. 1996} no longer posed a problem.

CONCLUSION

The search for an ideal extra-corporeal membrane oxygenator continues. There is a need for improvement in the design of small oxygenators and the ECMO circuits. Poly-methyl pentene oxygenators have replaced Silicon membrane oxygenators as the oxygenator of choice in all U.K. ECMO centres. In our experience PMP oxygenators provide adequate gas exchange and offer technical advantages to Silicon oxygenators. However, in neonates we adjusted our coagulation parameters and lowered our tolerance of clots (reducing our threshold for circuit change) in order to reduce the likelihood of sudden unexpected oxygenator failure. These measures may reduce the incidence of sudden unexpected oxygenator failure. However using a slightly larger Medos oxygenator in neonates would buy the valuable time needed to arrange an oxygenator / circuit change.

Poly-methyl pentene oxygenators have reduced radiographic signs of the host's response to ECMO in neonates and delayed that in adults. However, they have not significantly changed outcomes in terms of the duration of ECMO runs and survival to discharge for neonates or adults.

Adult PMP oxygenators showed lower blood path resistance than SM oxygenators. However, lower consumption of blood products in these oxygenators was a direct result of their smaller surface area and heparin-coated design, reducing contact activation of coagulation factors. These oxygenators are noticeably smaller; require lower priming volumes and have better gas exchange capability. They showed greater stability and preservation of coagulation factors and platelets compared to SM oxygenators. They also had the advantage of a functioning integrated heat exchanger. Using a single PMP oxygenator in the first instance may be adequate for the majority of adult patients and would significantly reduce red blood cell consumption during ECMO.

Chapter Five

THE RABBIT MODEL OF GRADED SIRS/SEPSIS AND MODS

AIMS AND OBJECTIVES

A disease modifying approach by interrupting the inflammatory cascades could be more effective than current supportive treatments such as ECMO alone. There is good theoretical basis to expect an improvement in outcomes by using a neutrophil depletion filter or MARS during ECMO, however there is currently no data available concerning the safety or efficacy of their prolonged use. For example the longer-term effect of neutrophil depletion (i.e. for several days) may reduce the hosts defences to a level where nosocomial infection occurs.

In the absence of a reliable model we have no means of assessing new approaches to treatment of severe SIRS / sepsis. Before any clinical trials can be performed the efficacy and safety of that treatment must be investigated in an animal model. There are models of sepsis and multi-organ dysfunction in the published literature {Ito et al. 1978, Nishina et al. 1997 & Yokoi et al. 1997}, however they lack sufficiently well characterized and reproducible properties that we require.

Rabbits have previously been used in ECMO research {Kress et al. 1987 & Trittenwein et al. 1999}. They are the smallest and the lowest order of animals that can be placed on an existing human ECMO circuit (chapter 2). We aim to develop a reliable and reproducible rabbit model of severe SIRS / sepsis, SIRS / septic shock and MODS. This model may then be used to assess the efficacy and safety of neutrophil depletion or MARS as an adjunct to ECMO. These investigations are essential in order to strengthen our case to continue our research in this field. A successful animal study would furnish sufficient information to support a Phase-I clinical trial in patients.

MATERIALS AND METHODS

Detailed descriptions regarding methods used in this thesis are included in Chapter 2. In this section, only a brief summary of the materials and methodological principles are addressed. Briefly seventeen female New-Zealand white rabbits (> 3.5 Kg in weight) were anaesthetised, surgically intubated, ventilated and monitored using surgically established arterial and venous accesses. Endotoxin from E-Coli K235 (LPS, L-2143) was administrated intravenously in order to trigger severe sepsis (SIRS), MODS and ultimately death. Ventilation was adjusted to maintain normal blood gases. Fluid management was the only measure used to maintain blood pressure and organ perfusion. Primary and secondary outcome measures were assessed for each animal up to death or until they were euthanased, and post-mortem samples were taken. Blood samples were

taken hourly for the first 4 hours, then every 2 hours for 4 hours, and then every 4 hours for the remainder of the experiment. The animals were kept anaesthetised and ventilated for the entire duration of the experiment.

The experiment was performed in two phases both using an identical procedure save for the dose of LPS administered. In the first phase of the experiment a total of twelve animals were used. This phase determined the optimum dose of endotoxin, and the amount of time that was required to allow development of fatal MODS. This is illustrated by the dose response curve. The rabbits received incremental doses of endotoxin, the prime initiator of gram-negative sepsis, starting from zero in three control animals. In the second phase in order to determine reproducibility the same lethal dose of LPS, determined from the first phase, was used on five more experimental animals. This established the inter-individual variation amongst these rabbits. This data will be essential in sample size selection for subsequent experiments.

RESULTS

Phase one experiment: Dose response relationship between administration of LPS and the development of MODS

Primary outcome

The primary outcome measure was death. All the controls ($n = 3$) survived the full 24 hours. They received no LPS. The first three high dose experimental rabbits died rapidly within the first two hours. The dose of LPS was halved after each of the above starting with 1 mg/Kg then 0.5 mg/Kg and 0.25 mg/kg. The subsequent six, who received lower doses, < 0.025 mg/Kg, survived to develop clinical signs of severe sepsis, MODS and died at different times (Table 5.1). One rabbit died secondary to surgical complication and was removed from the experiment.

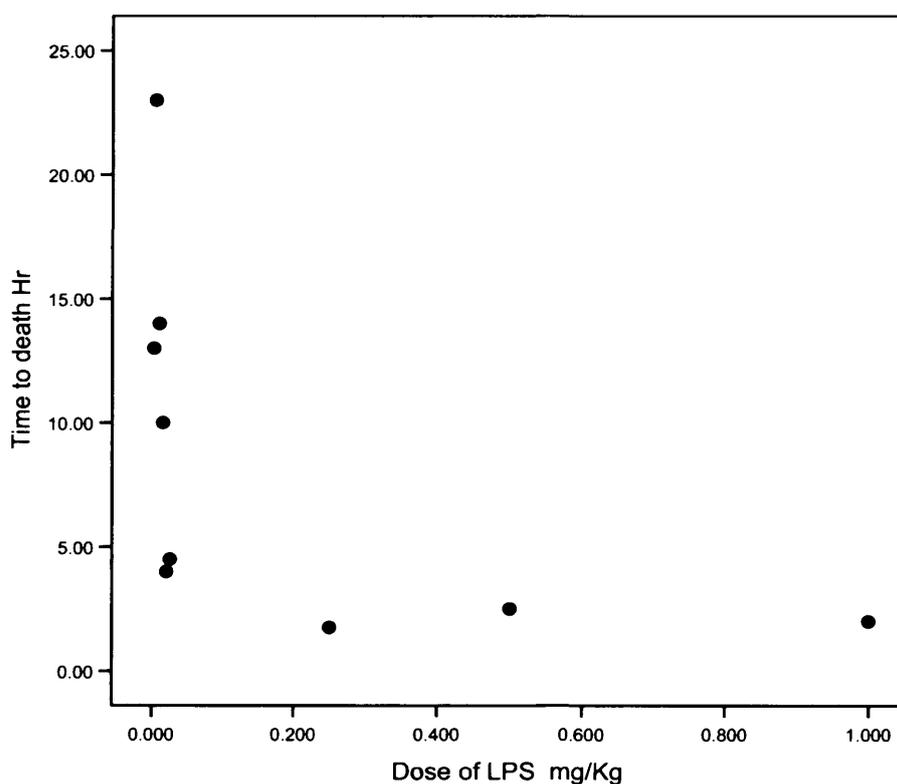
Dose response curve: The first graph illustrates the time to death (Vertical axis) for a given dose of LPS (Horizontal axis) for all the experimental animals ($n = 9$). Note that the first three highest doses resulted in rapid deterioration and death. The fast demise of these animals did not allow determination of a dose response relationship.

Diagram 5.1 is the scatter plot that illustrates the correlation between the dose of LPS and time to death. The graph (Diagram 5.2) illustrates the dose response relationship for development of severe sepsis and MODS resulting in death within 4 – 23 hours of administration of the LPS ($n= 6$). This illustrates a negative relationship.

Table 5.1 illustrates the dose of LPS administered in the order that they were given and the survival time in hours.

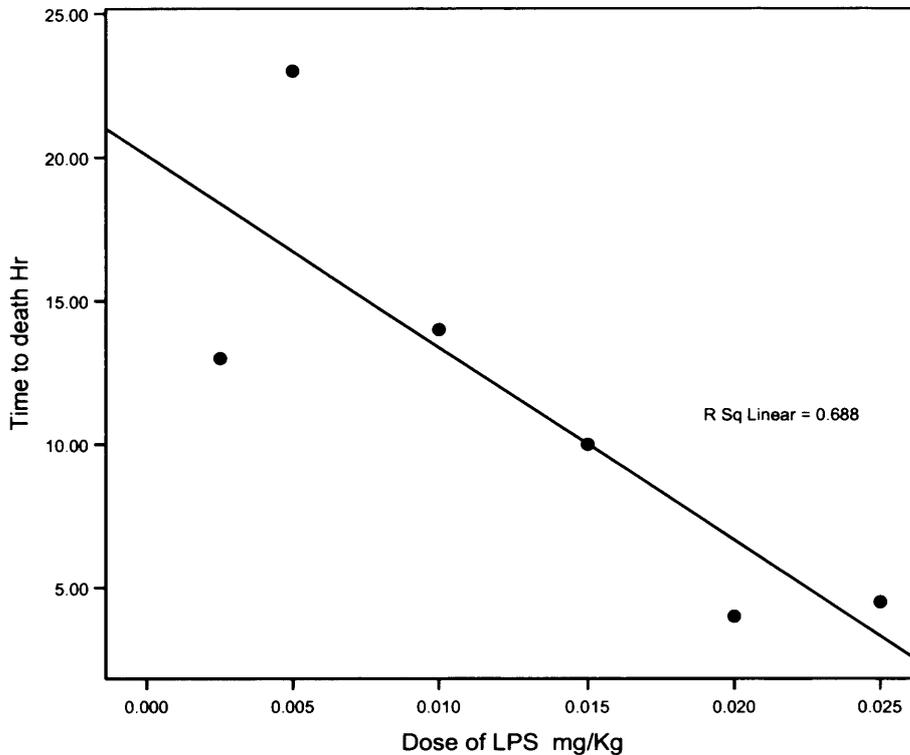
Rabbit	Control / LPS	Survival to 24 hours	Dose of LPS mg/Kg	Hours to death
GLA	C	Yes	0	24
HER	C	Yes	0	24
IVY	C	Yes	0	24
KAT	L	No	1.0	2.00
LID	L	No	0.5	2.50
MON	L	No	0.25	1.75
NEN	L	No	0.025	4.5
ORB	L	No	0.01	14
ROM	L	No	0.02	4
SAM	L	No	0.005	23
TIN	L	No	0.015	10
UNI	L	No	0.0025	13

Diagram 5.1: Scatter plot illustrating the dose response relation between the dose of intravenous LPS and primary outcome of death in all experimental animals.



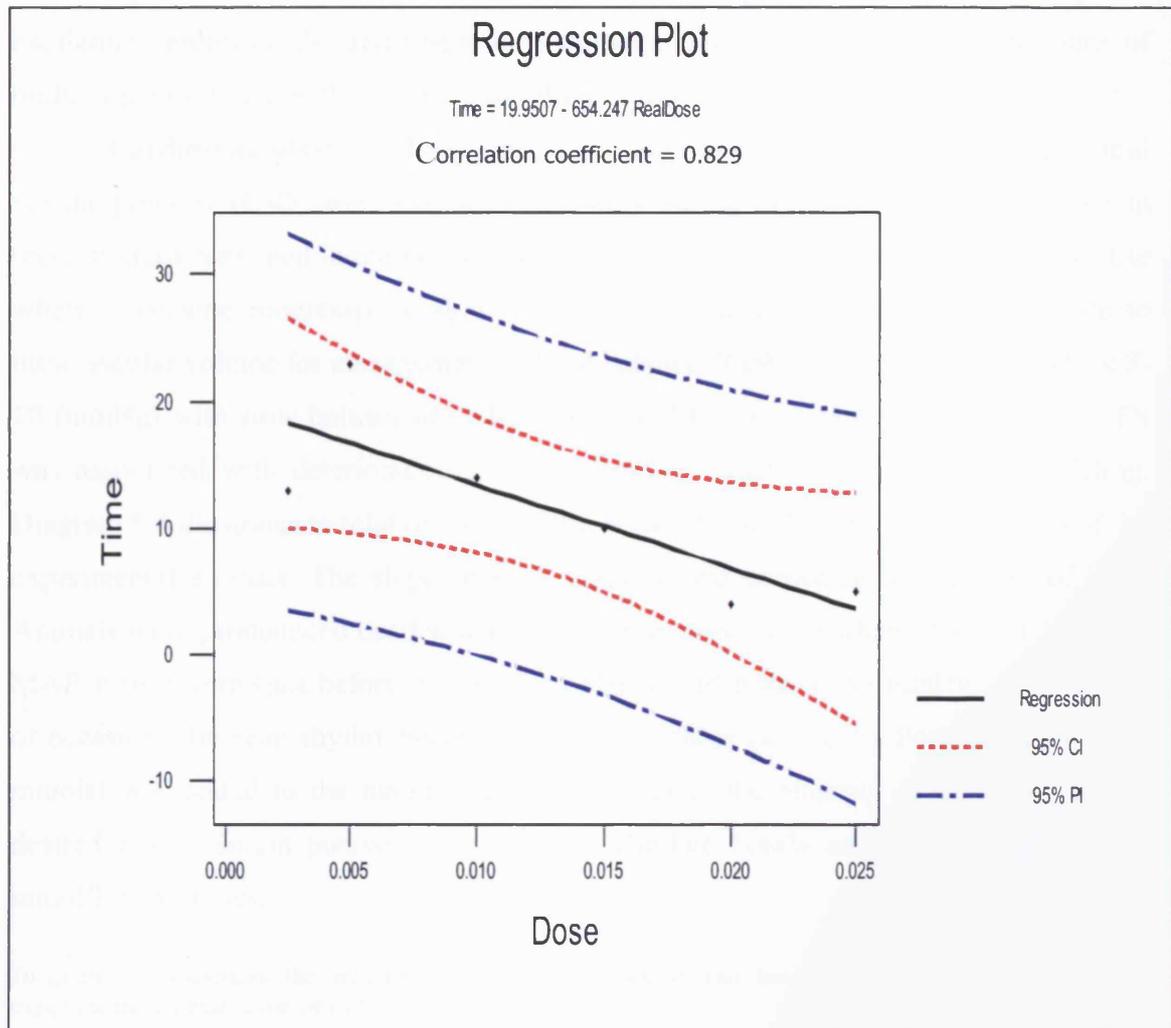
A 2-tailed Pearson correlation test showed a statistically significant ($p = 0.041$) and strong ($r = 0.829$) correlation between the dose of LPS and the response time to death. The regression plot demonstrates this linear relationship for these selected doses, which is statistically significantly different from zero.

Diagram 5.2: The Dose response graph between intravenous administration of LPS and response time to develop fatal MODS.



The coefficient of determination or $R^2 = 0.688$, therefore the % variance is 68.8% which means a reasonable degree of overlap between the two variables (Diagram 5.3). The values of R^2 were not significantly different between linear, cubic and quadratic (0.688, 0.694 and 0.865 respectively). The regression plot (Diagram 5.3) demonstrates a strong negative linear relationship between the given points. The regression equation is demonstrated at the top of the chart. The graph illustrates the 95% confidence interval (CI) that is the area between the red dotted lines where with 95% confidence the distribution of sample means lies. In other words the area where the true line lies with 95% confidence. The wider region between the blue lines illustrates the Predictive Interval (PI) where we expect 95% of the observations to lie.

Diagram 5.3: The regression plot illustrating the confidence interval and the prediction interval.

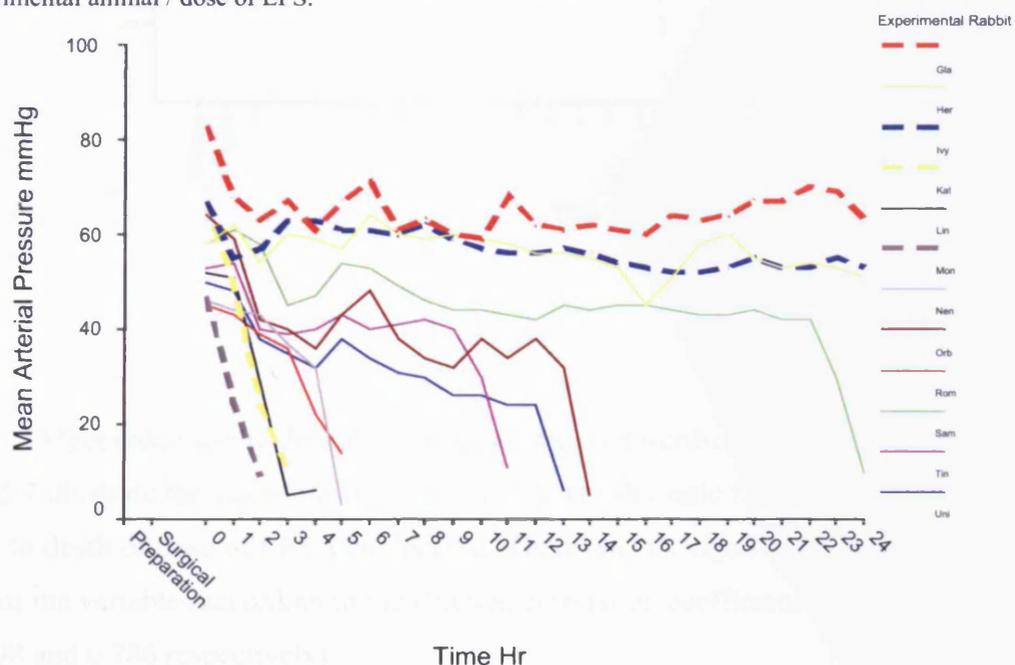


Secondary outcomes

Upon administration of LPS the animals became profoundly unwell and required escalating cardiovascular and respiratory supports. The rabbits had developed signs of multi organ dysfunction these were quantified as shown below.

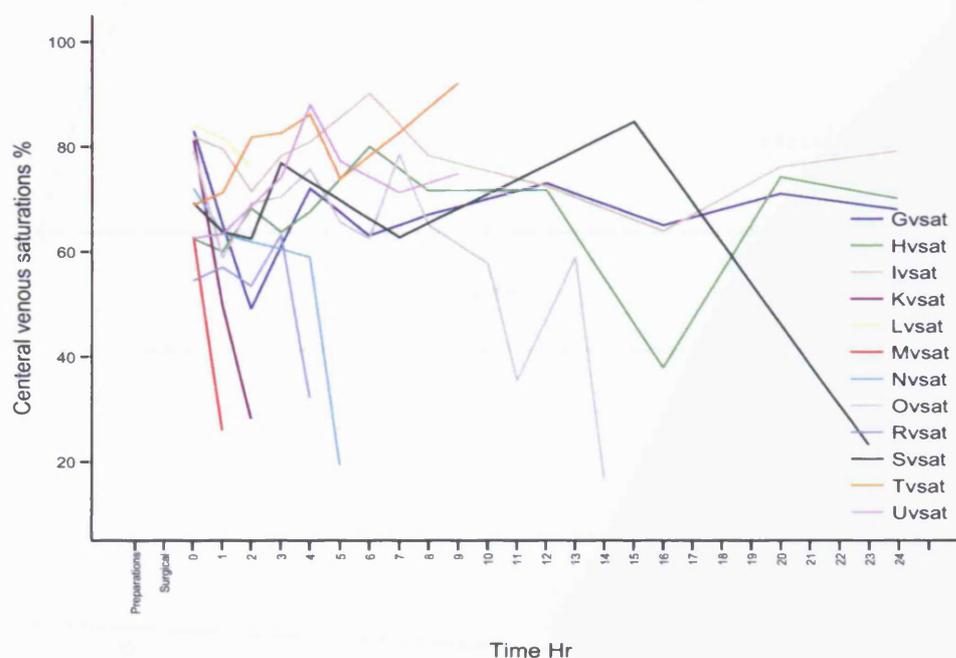
Cardio-vascular: The heart rate, mean arterial pressure (MAP) and the central venous pressure (CVP) were documented hourly during experiments. The heart rate in these animals remained in the normal range (~ 220 BPM) except for the last hour of life where it became progressively slow and stopped. The CVP was used as a guide to intravascular volume for management of fluid balance. Rabbits were filled to a CVP of 5-10 (mmHg) with slow boluses of Gelofusin up to 20 ml each time. Infusion of the LPS was associated with deterioration in cardiovascular function despite adequate filling. Diagram 5.4 illustrates in relation to time, the mean arterial blood pressure for each of the experimental animals. The slope of each graph varied according to the dose of LPS. Animals were pronounced dead when they became asystolic or when MAP = CVP. The MAP at time zero (just before infusion of LPS) ranged from 45-85 mmHg. In a number of occasions the heart rhythm became irregular. In these cases extra Potassium (KCl, 10 mmols) was added to the maintenance fluid. This in the majority of animals had the desired effect. Serum potassium levels were checked hourly and maintained above 4 mmol/L at all times.

Diagram 5.4 illustrates the relation ship between mean arterial blood pressure and time for each experimental animal / dose of LPS.



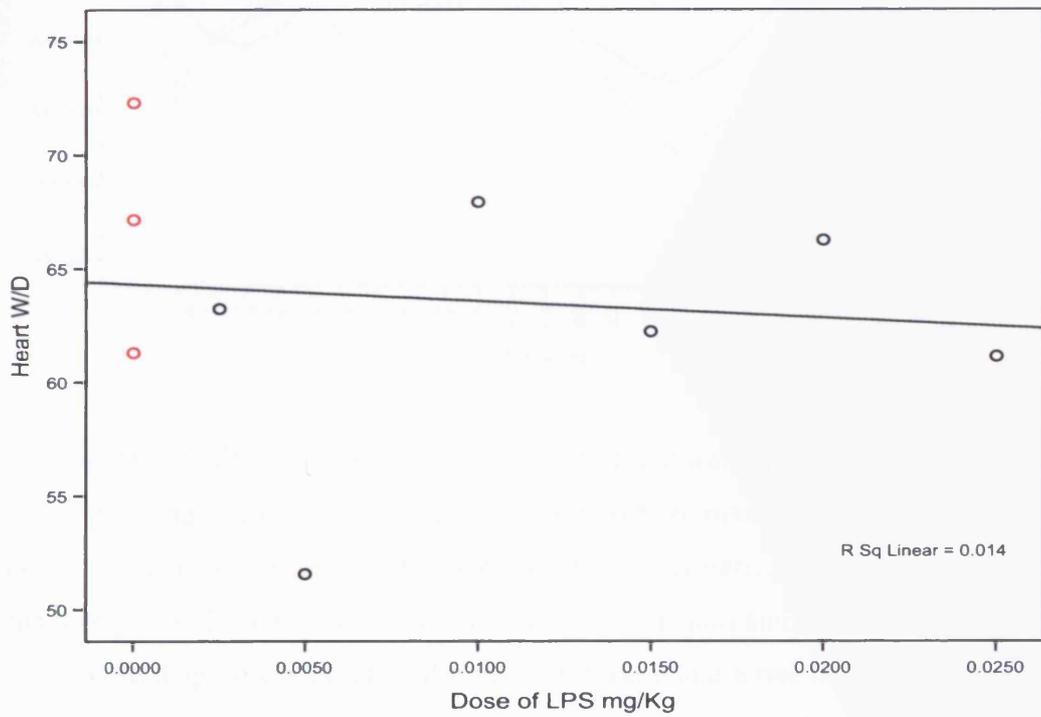
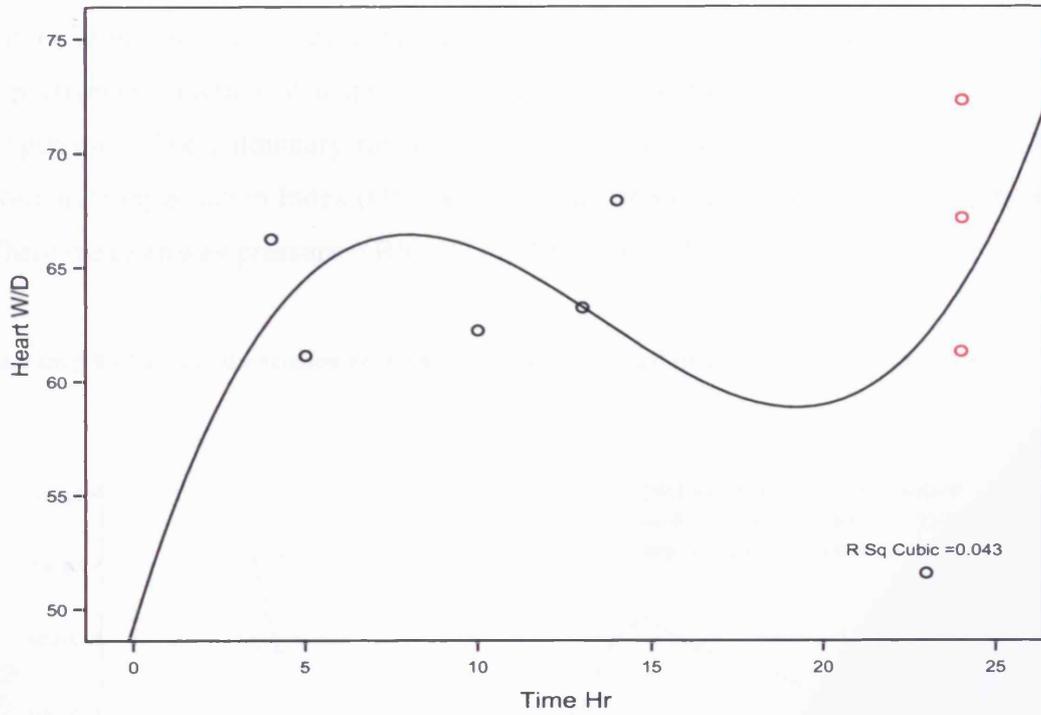
The central venous saturation (ScvO₂) was monitored as a measure of early haemodynamic and circulatory disturbance {Scalea et al. 1988}. The ScvO₂ of below 65% has been demonstrated in trauma {Scalea et al. 1988}, severe sepsis {Rady et al. 1992}, myocardial infarction and cardiac failure {Hutter et al. 1970 & Ander et al. 1998}. Diagram 5.5 illustrates the trend in ScvO₂ for each experimental animal. The value of ScvO₂ dipped to less than 65% immediately after the LPS administration. This in some cases may have been exacerbated by a fall in arterial saturation (SaO₂) in a number of experimental animals however although SaO₂ returned to normal immediately after ventilatory adjustment the venous saturation continued to fall or rose and fell again as the animals condition deteriorated. This suggests myocardial suppression.

Diagram 5.5: Trend of ScvO₂ (%) with time post LPS administration (Hours).



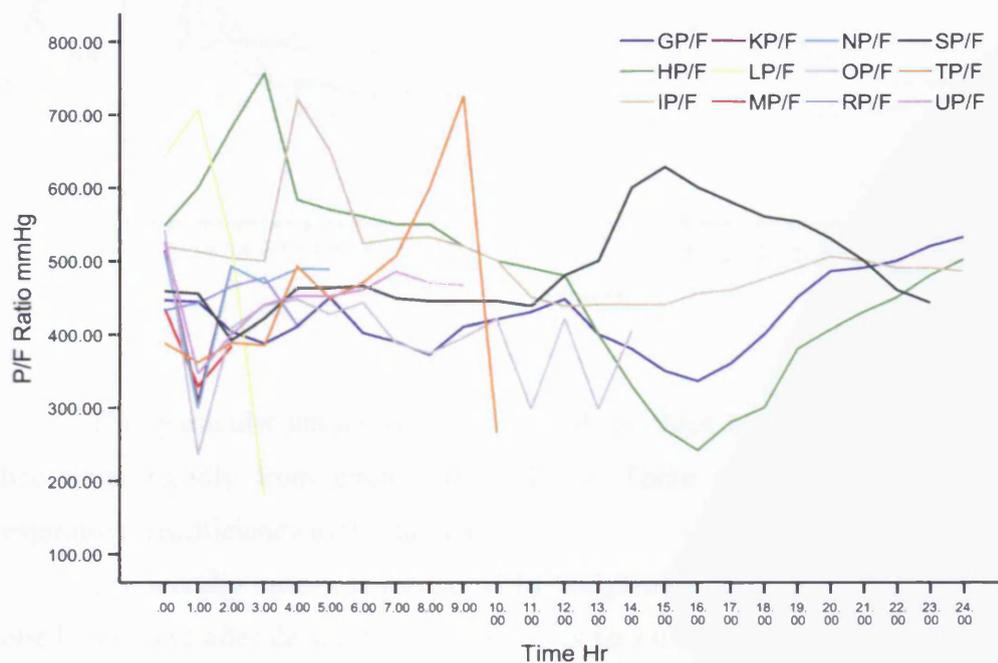
Myocardial wet to dry ratio was measured as described in chapter 2. Diagrams 5.6 and 5.7 illustrate the variations in myocardial wet to dry ratio for each experiment against time to death or dose of LPS administered. There was no significant correlation between any of the variables according to the Pearson correlation coefficient ($r = 0.2$ and 0.1 and $p = 0.98$ and 0.786 respectively).

Diagrams 5.6 (Top) and 5.7 (Bottom) show the relationship between myocardial oedema and time to death or the dose of LPS administered in mg/Kg. Red circles represent the control group.



Respiratory: The arterial blood gases (ABGs) were measured hourly and the ventilation was adjusted to maintain normal blood gases and to treat respiratory or mixed acidosis. The ventilator settings such as respiratory rate were manipulated by adjusting the inspiratory time (Ti) and expiratory time (Te) to between 25-90 breaths per minutes (Physiological = 50 BrPM). The tidal volume (Physiological = 20 ml) was adjusted by manipulating the peak inspiratory and positive end expiratory pressures (PIP and PEEP respectively). Fraction of inspired oxygen (FiO₂) and PEEP were adjusted to maintain oxygenation. The pulmonary functional state was calculated as PaO₂ (mmHg)/FiO₂ (P/F ratio) and Oxygenation Index (OI = Mean airway pressure (cmH₂O) x FiO₂ x 100/ PaO₂). Where mean airway pressure = RR x (Ti x PIP + Te x PEEP)/ 60.

Diagram 5.8 illustrates the relation between PaO₂/FiO₂ ratios and time for each experimental animal.

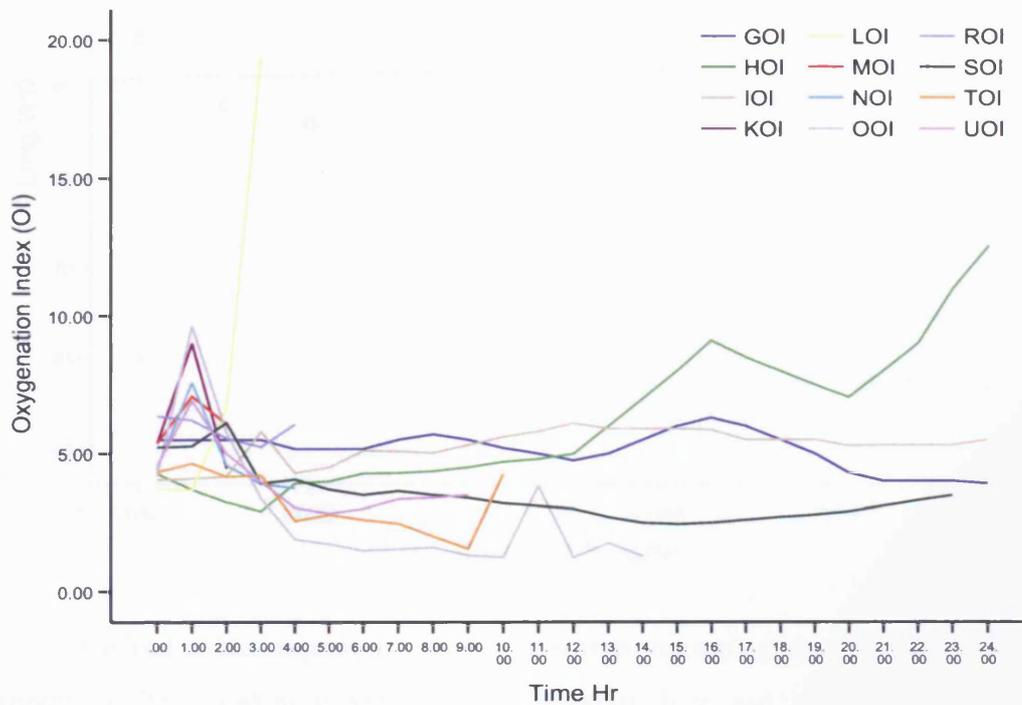


Within the first hour after infusion of LPS there was a severe deterioration in lung function needing escalation in respiratory support to maintain ABGs and acid base balance. Diagrams 5.8 and 5.9 illustrate the deterioration in respiratory function immediately after LPS administration for each experimental animal.

These diagrams illustrate a dip in the P/F ratio and a rise in OI immediately after administration of LPS except for the control animals. They clearly developed respiratory dysfunction. The respiratory dysfunction was immediately treated with heightened

ventilatory support. Significant high pressure / FiO₂ ventilatory support was required to maintain life in these animals. One of the experimental animals (LID) showed a severe deterioration in respiratory function, which was unresponsive to high ventilatory support.

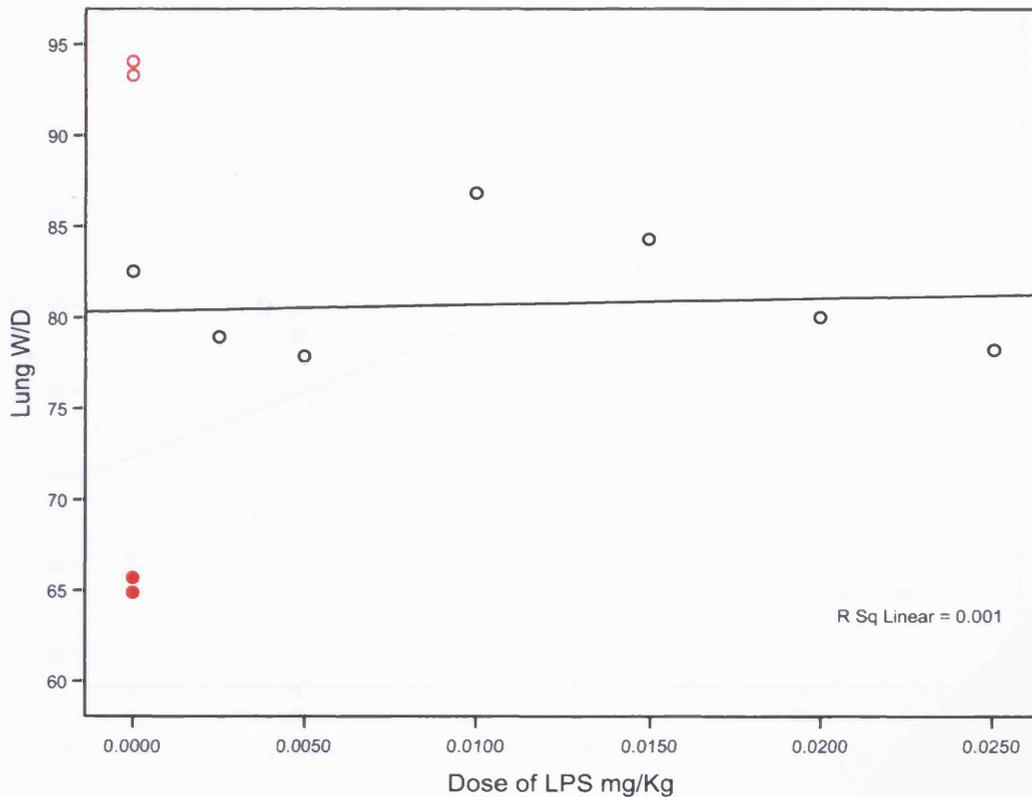
Diagram 5.9 illustrates the relation between Oxygenation Index and time for each experimental animal.



This particular animal received one of the three highest doses of LPS. They all died quite rapidly from circulatory collapse. There were no signs of predisposing respiratory insufficiency in this animal.

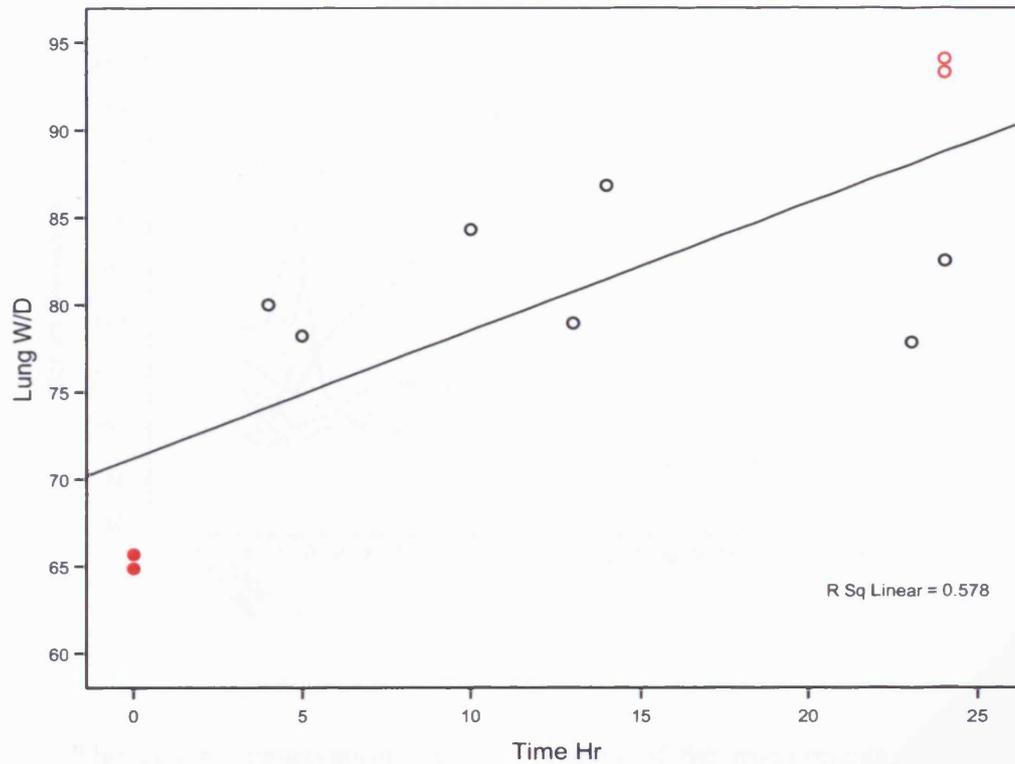
The wet/dry ratio was measured by weighing a piece of each rabbits right lower lobe before and after desiccation, this gave us an estimate of the severity of tissue water content that is well known to be an important consequence of ARDS the respiratory manifestation of severe SIRS / sepsis. Diagrams 5.10, demonstrates the scatter diagram of the relationship between the lung wet/dry ratio and the dose of LPS used in each experimental animal. There is no significant correlation ($r = 0.1$ and $p > 0.05$).

Diagram 5.10: Relationship between lung wet-dry weight ratio (%) and the dose of LPS mg/Kg used on each experimental animal.



The red circles represent tissues taken from normal animals not taken part in the experiment. They had no procedures performed on them and were not ventilated. Their life was terminated by a schedule one method and the only organ removed for that experiment was the heart (Hence no normal values for the cardiovascular graphs). The red ring represents two of the control animals. The value for the third was missing.

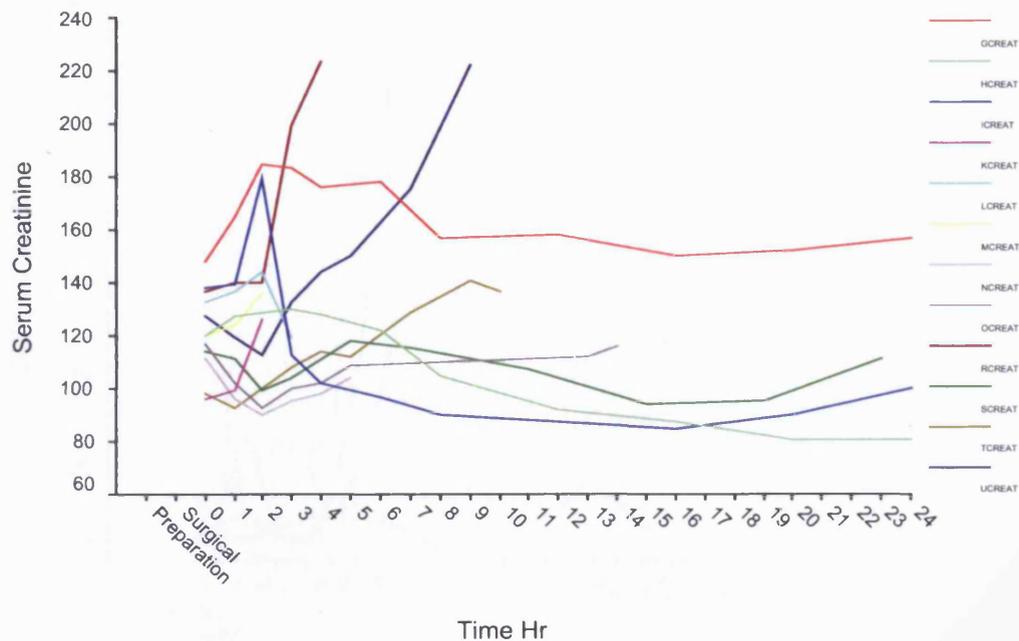
Diagram 5.11: The correlation between the wet-dry weight ratios (%) vs. the time from administration of LPS in hours.



The Pearson 2-tailed correlation test revealed a strong positive relationship between the percentage tissue water in the lungs and the time (Diagram 5.11) since administration of the LPS ($r = 0.768$, $n = 11$ and $p = 0.006$). From these last two plots we suspect that this is a relationship between severity of tissue oedema and the length of positive pressure ventilation rather than the dose of LPS.

Renal: This outcome measure was assessed by continuous measurement of urine output and serum creatinine levels. Diagrams 5.12 and 5.13 illustrate that as renal function deteriorated and serum creatinine rose the urine output fell. The slope of each individual graph varied according to the dose of LPS. In the high dose experiments rabbits developed rapid renal impairment and became anuric prior to death. The desired effect was noted in the experiments where the rabbit developed gradual renal impairment (Rom and Uni). In these experiments renal dysfunction accompanied respiratory and cardiac dysfunctions in a desired period between four to eight hours. The majority of animals became anuric in the last hour(s) of their life.

Diagram 5.12 illustrating the relationship between serum creatinine in mmol/L and time in hours from LPS administration.



The general observation towards the end of the experiments was that a large quantity of water had to be aspirated from the ET tubes and post-mortem examination revealed severe tissue oedema especially around the head and back. Also pleural and peritoneal effusions were found.

None of the animals lived to reach levels of plasma creatinine considered in humans as renal failure, that is a creatinine of above 300 mmol/L. However in an experiment where the primary outcome is prolonged these levels would probably reach that level as the animal becomes anuric.

Please note that in some lower LPS dose experiments within a few hours of the insult the rabbit became polyuric in some instances this followed a short oliguric phase. This is interesting, as the rabbit has developed polyuric renal failure, which may have prevented fluid overload.

Measurement of wet-dry weight ratio from the post mortem specimens of the right kidney is illustrated in diagrams 5.14 5.15 and 5.16.

Diagram 5.13 illustrating the relationship between urine output and time in hours from LPS administration.

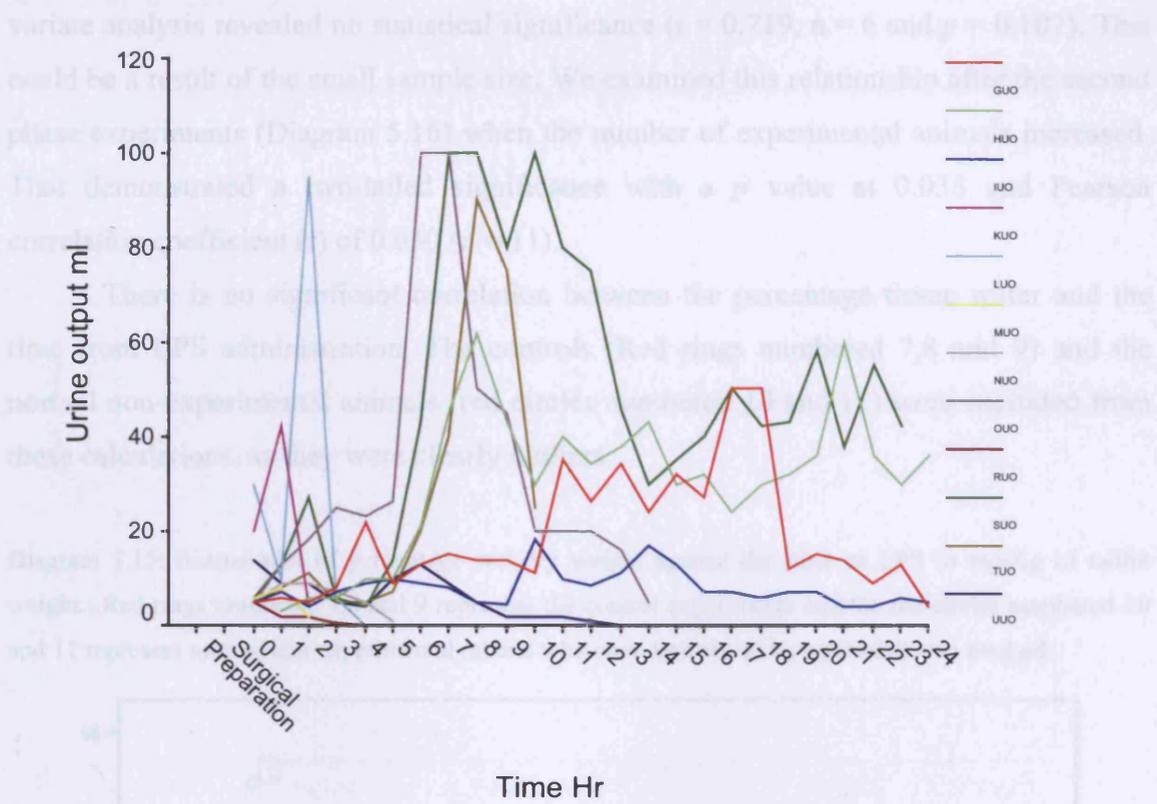
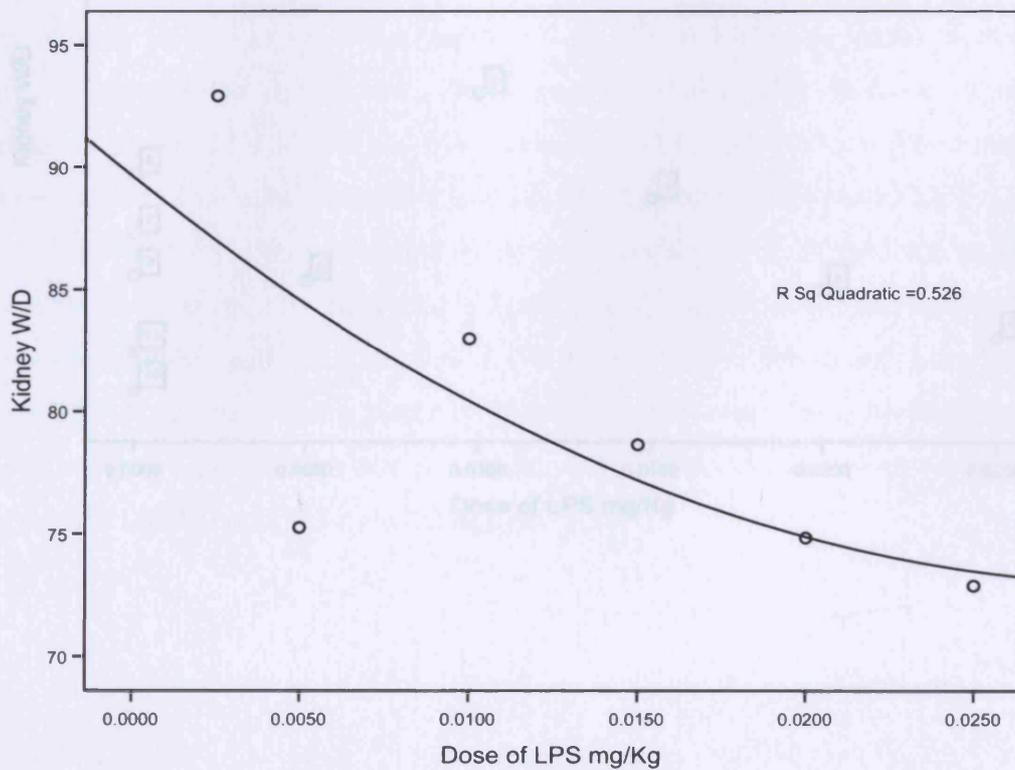


Diagram 5.14: The correlation between the dose of LPS (mg/Kg) on the X-axis and the percentage kidney tissue water on the Y-axis.



This plot demonstrates a strong negative correlation, however the Pearson bi-variate analysis revealed no statistical significance ($r = 0.719$, $n = 6$ and $p = 0.107$). This could be a result of the small sample size. We examined this relationship after the second phase experiments (Diagram 5.16) when the number of experimental animals increased. That demonstrated a two-tailed significance with a p value at 0.038 and Pearson correlation coefficient (r) of 0.630 ($n = 11$).

There is no significant correlation between the percentage tissue water and the time from LPS administration. The controls (Red rings numbered 7,8 and 9) and the normal non-experimental animals (red circles numbered 10 and 11) were excluded from these calculations, as they were clearly outliers.

Diagram 5.15: Scatter plot of percentage wet-dry weight against the dose of LPS in mg/Kg of rabbit weight. Red rings numbered 7,8 and 9 represent the control experiments and the red circles numbered 10 and 11 represent normal non experimental rabbits who were terminated by a schedule one method.

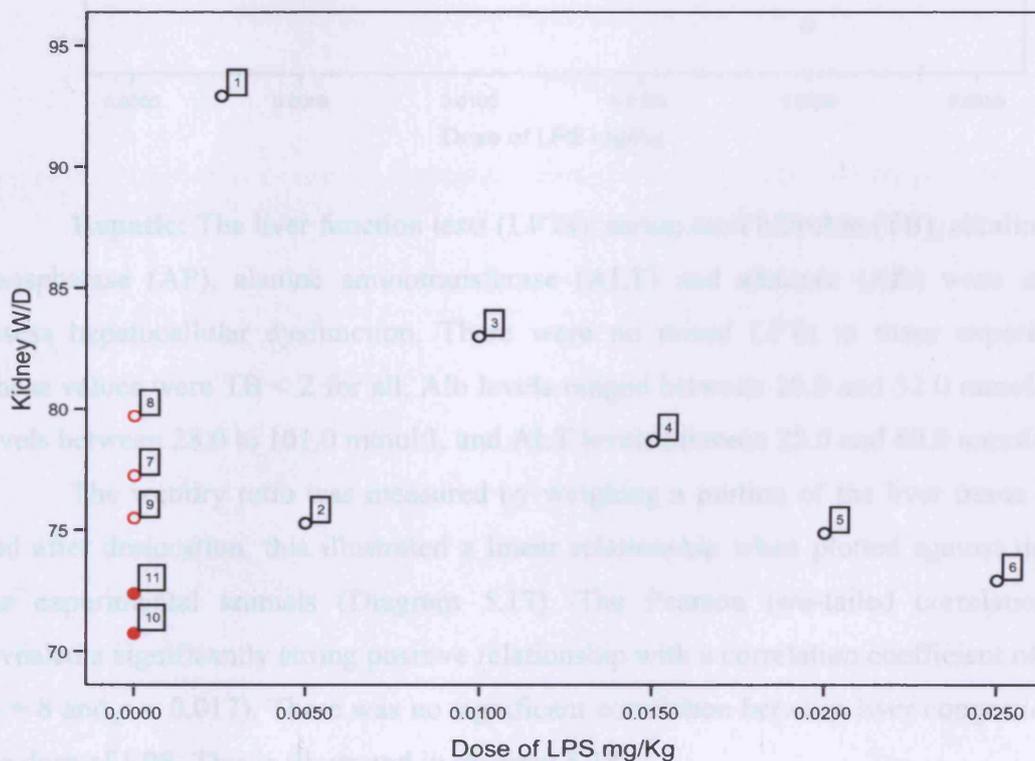
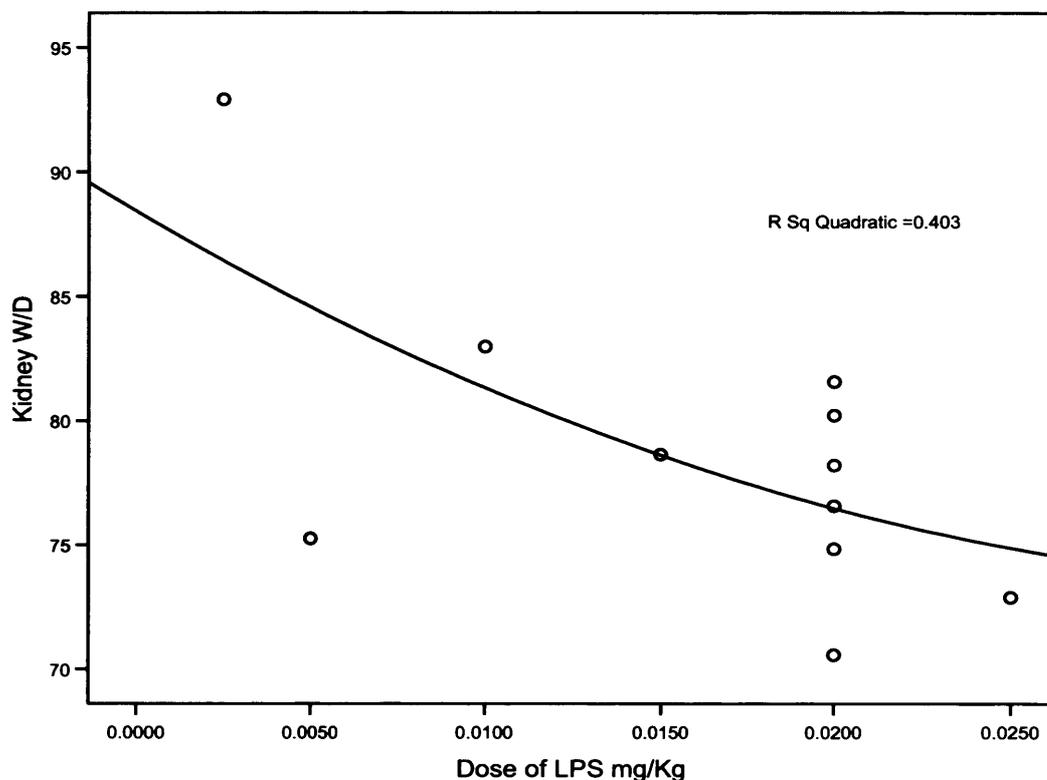


Diagram 5.16: The correlation between the dose of LPS (mg/Kg) on the X-axis and the percentage kidney tissue water on the Y-axis.



Hepatic: The liver function tests (LFTs); serum total bilirubin (TB), alkaline phosphatase (AP), alanine aminotransferase (ALT) and albumin (Alb) were used to assess hepatocellular dysfunction. There were no raised LFTs in these experiments. These values were TB < 2 for all, Alb levels ranged between 20.0 and 32.0 mmol/L, AP levels between 28.0 to 101.0 mmol/L and ALT levels between 25.0 and 80.0 mmol/L.

The wet/dry ratio was measured by weighing a portion of the liver tissue before and after desiccation, this illustrated a linear relationship when plotted against time for the experimental animals (Diagram 5.17). The Pearson two-tailed correlation test revealed a significantly strong positive relationship with a correlation coefficient of 0.802 ($n = 8$ and $p = 0.017$). There was no significant correlation between liver congestion and the dose of LPS. This is illustrated in diagram 5.18.

Diagram 5.17: Scatter diagram assessing liver congestion versus time. The red rings are the control experiments.

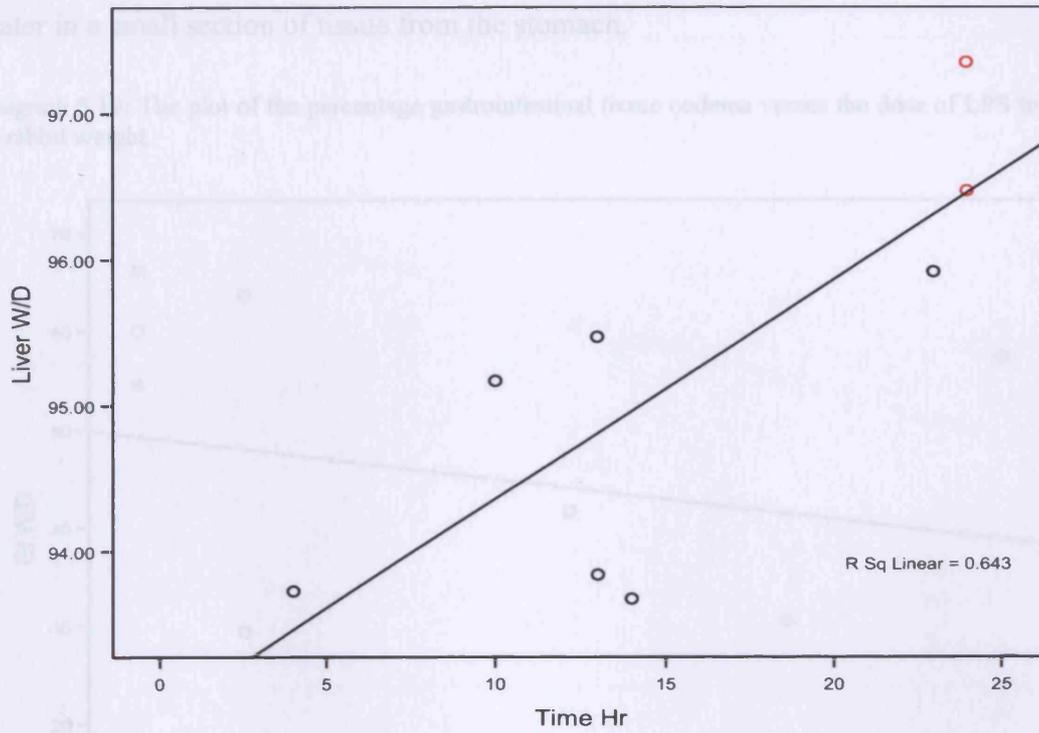
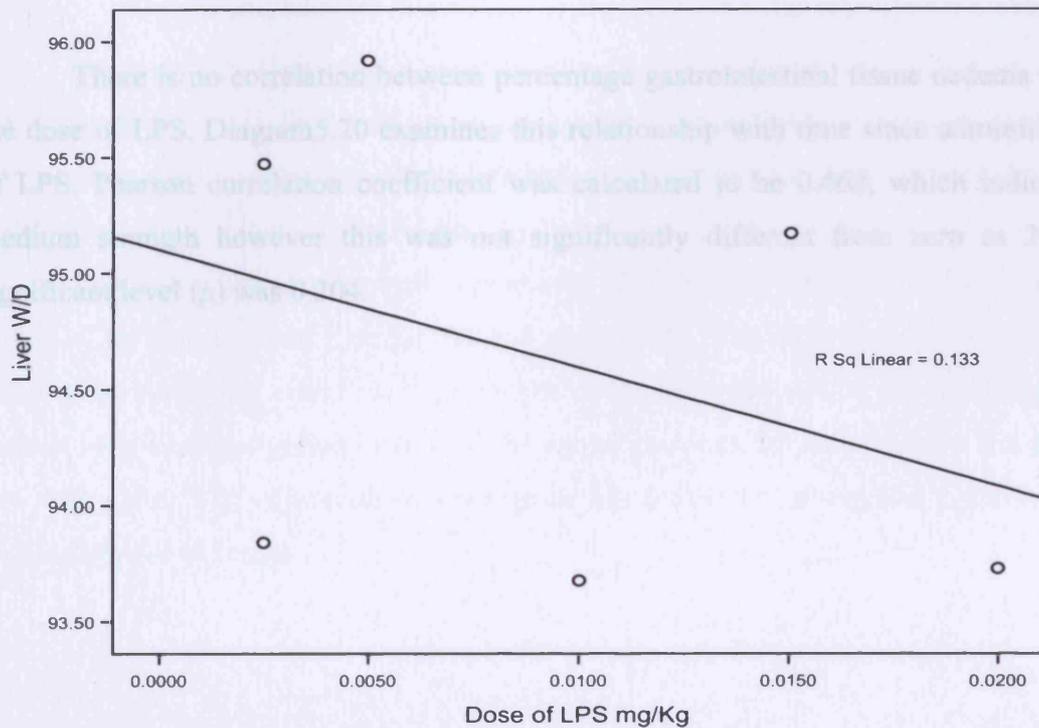
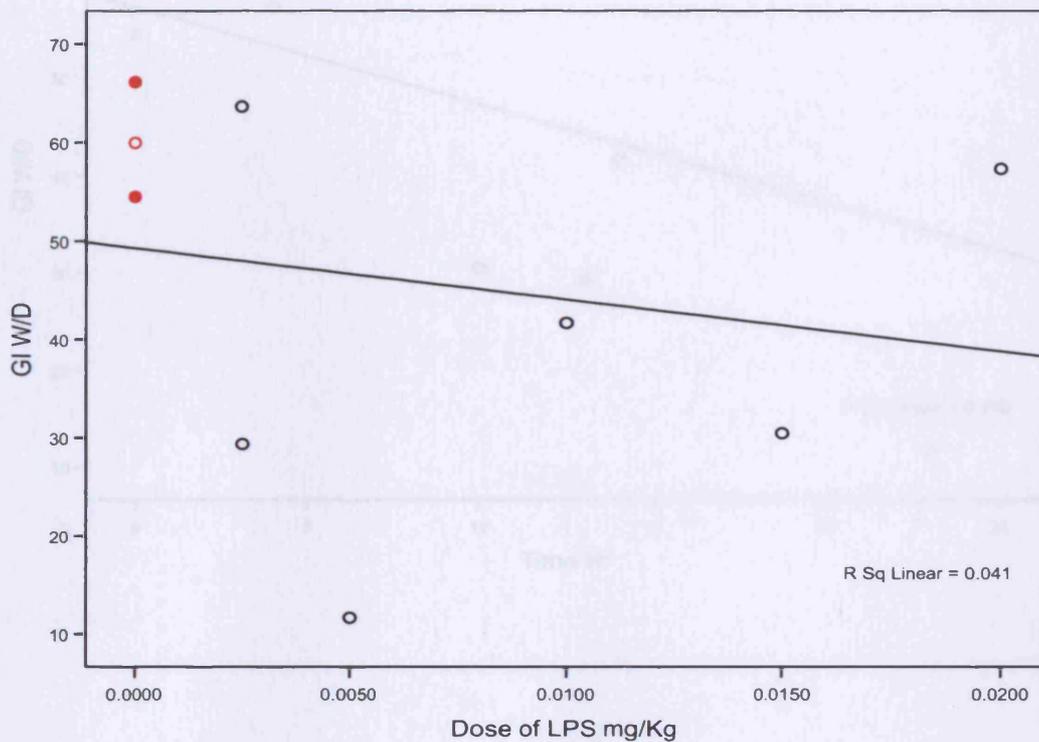


Diagram 5.18: The scatter plot and the regression plot showing no significant relationship between the dose of LPS and development of liver congestion.



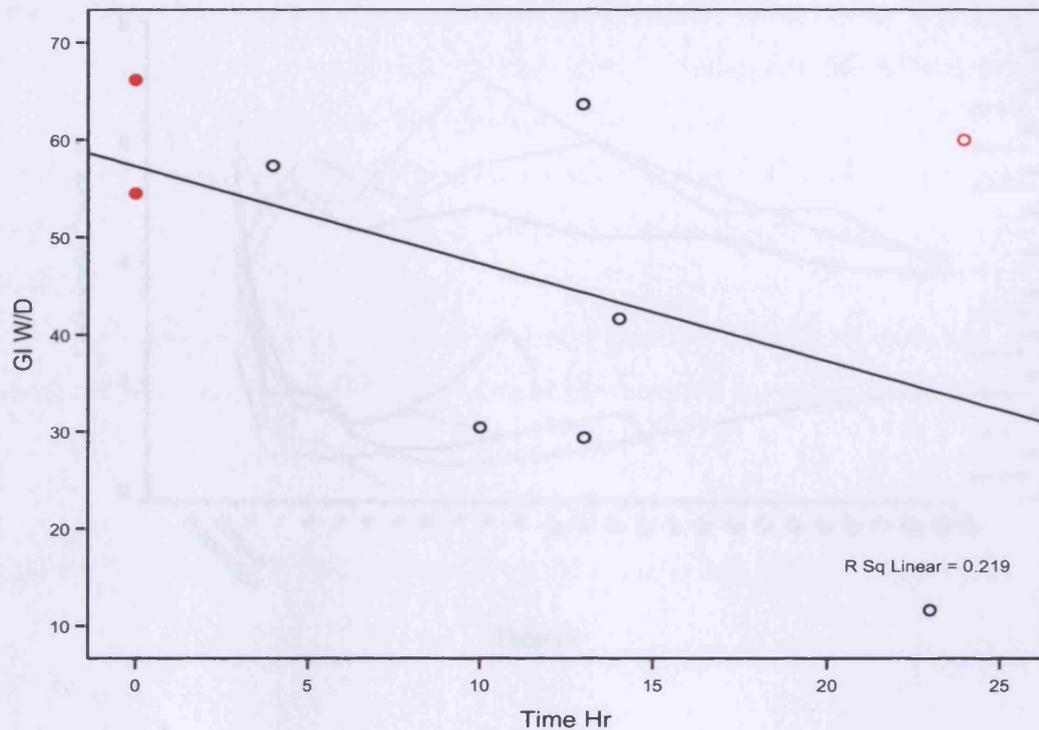
Gastrointestinal: The only means of assessing gastrointestinal dysfunction was by assessing the wet-dry weight ratios. Diagram 5.19 illustrates the percentage tissue water in a small section of tissue from the stomach.

Diagram 5.19: The plot of the percentage gastrointestinal tissue oedema versus the dose of LPS in mg/Kg of rabbit weight.



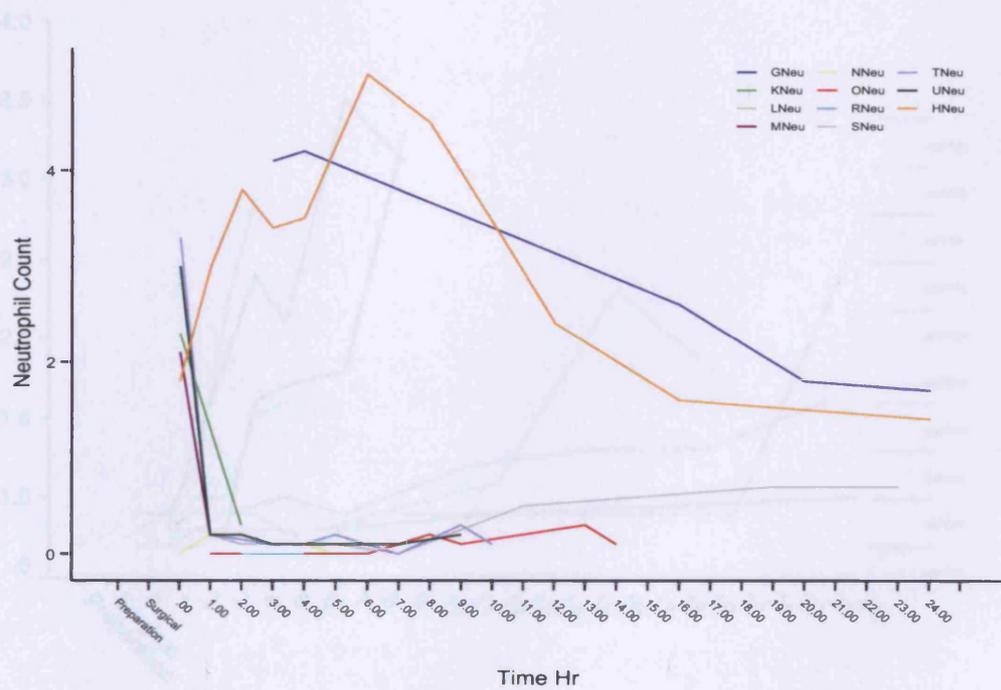
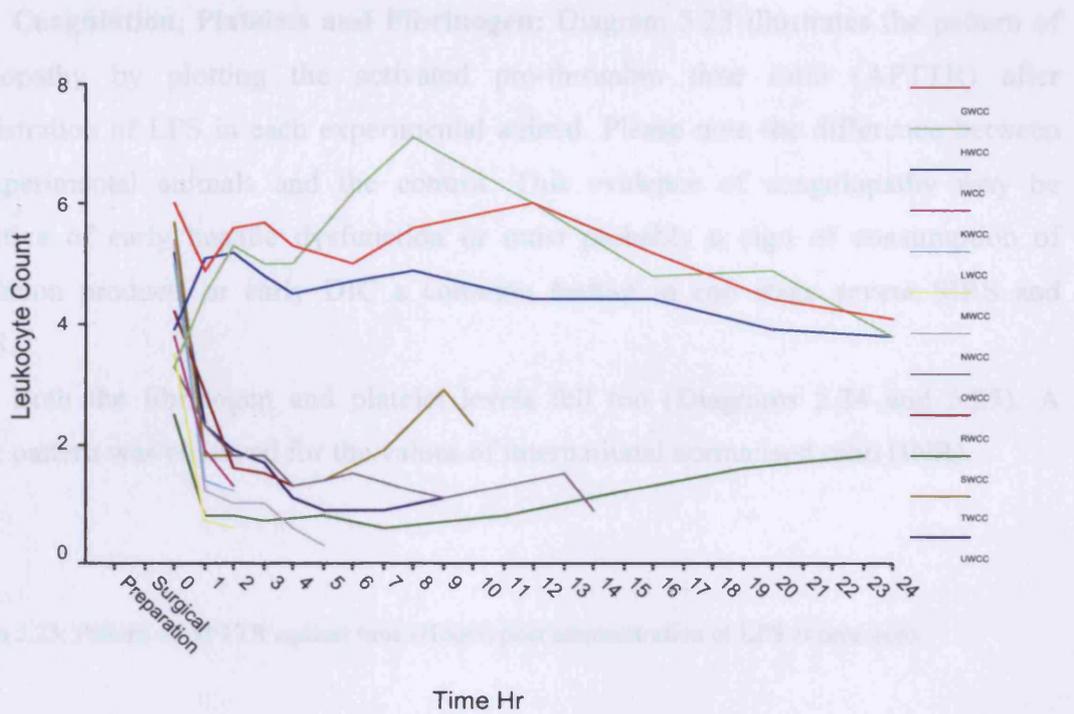
There is no correlation between percentage gastrointestinal tissue oedema versus the dose of LPS. Diagram 5.20 examines this relationship with time since administration of LPS. Pearson correlation coefficient was calculated to be 0.468, which indicates a medium strength however this was not significantly different from zero as 2-tailed significant level (p) was 0.204.

Diagram 5.20: Regression plot comparing percentage wet-dry weight of gastrointestinal tissues vs. the time since the administration of LPS in hours.



Leukocytes: There was a sudden sharp fall in leukocyte count just after LPS administration. This is illustrated by our next graph. This fall is as a result of adhesion of these cells to the vessel wall and their infiltration into the tissues. Diagram 5.21 illustrates this for each experimental dose of LPS.

The fall in leukocyte count was mostly attributed to neutrophils. This was in a region of ten times greater than the fall in lymphocytes. The diagram 5.22 illustrates the differential neutrophil count. It is important to note that the neutrophil count starts to recover after an initial period of drop. If the animal survives this may return to baseline or rise above that. The activation of neutrophils has a key role in cellular component of innate response to sepsis.

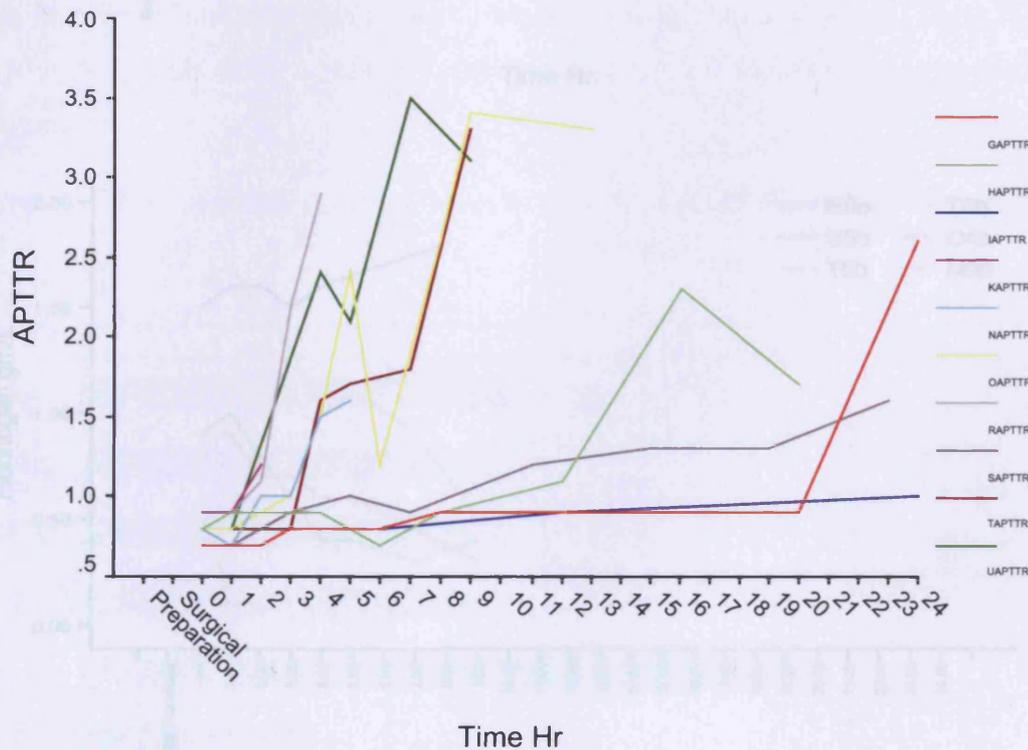


Diagrams 5.21 (Top) and 5.22 (Bottom): Graphs of leukocyte and neutrophil count ($\times 10^9/L$) on the top and bottom verses time (Hours) respectively post LPS administration.

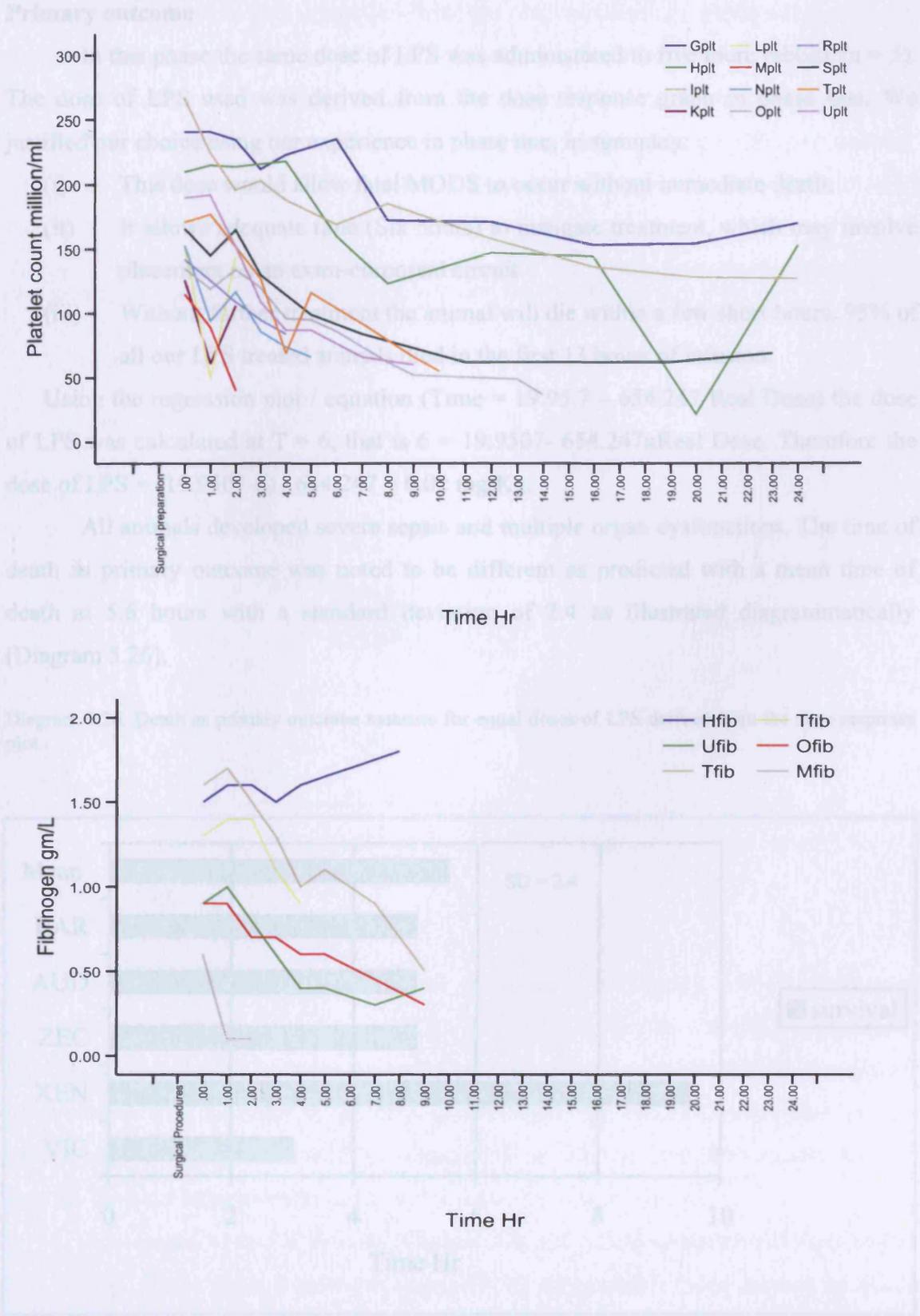
Coagulation, Platelets and Fibrinogen: Diagram 5.23 illustrates the pattern of coagulopathy by plotting the activated pro-thrombin time ratio (APTTR) after administration of LPS in each experimental animal. Please note the difference between the experimental animals and the control. This evidence of coagulopathy may be suggestive of early hepatic dysfunction or most probably a sign of consumption of coagulation products or early DIC a common feature in end stage severe SIRS and MODS.

Both the fibrinogen and platelet levels fell too (Diagrams 5.24 and 5.25). A similar pattern was observed for the values of international normalised ratio (INR).

Diagram 5.23: Pattern of APTTR against time (Hours) post administration of LPS at time zero.



Diagrams 5.24 (Top) and 5.25 (Bottom): Illustrate the consumption of platelets and fibrinogen during severe sepsis in experimental animals. Please note there were missing values in the fibrinogen graph. They were due to technical failure, therefore only six experiments could be included.



Phase two experiments: Inter-individual variation in rabbits injected with lethal dose of endotoxin L-2143

Primary outcome

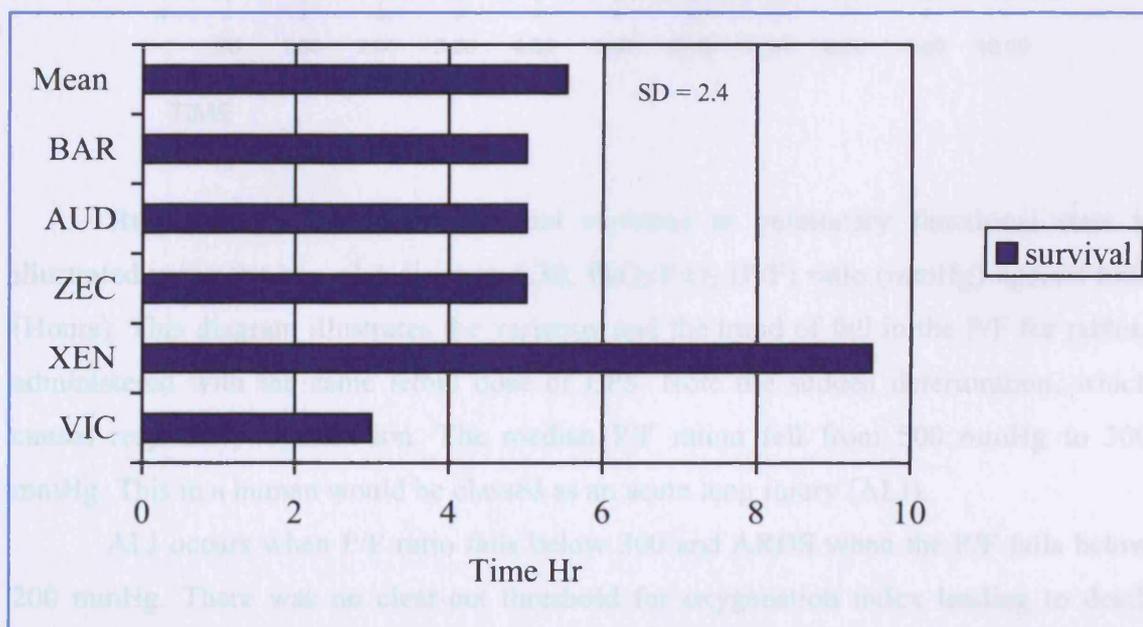
In this phase the same dose of LPS was administered to five more rabbits (n = 5). The dose of LPS used was derived from the dose response graph in phase one. We justified our choice using our experience in phase one, in summary:

- (i) This dose would allow fatal MODS to occur without immediate death.
- (ii) It allows adequate time (Six hours) to instigate treatment, which may involve placement on an extra-corporeal circuit.
- (iii) Without further treatment the animal will die within a few short hours. 95% of all our LPS treated animals died in the first 13 hours of infusion.

Using the regression plot / equation (Time = 19.95.7 – 654.247 Real Dose) the dose of LPS was calculated at T = 6, that is $6 = 19.9507 - 654.247 \times \text{Real Dose}$. Therefore the dose of LPS = $(19.9507 - 6) / 654.247 = 0.02 \text{ mg/Kg}$.

All animals developed severe sepsis and multiple organ dysfunctions. The time of death as primary outcome was noted to be different as predicted with a mean time of death at 5.6 hours with a standard deviation of 2.4 as illustrated diagrammatically (Diagram 5.26).

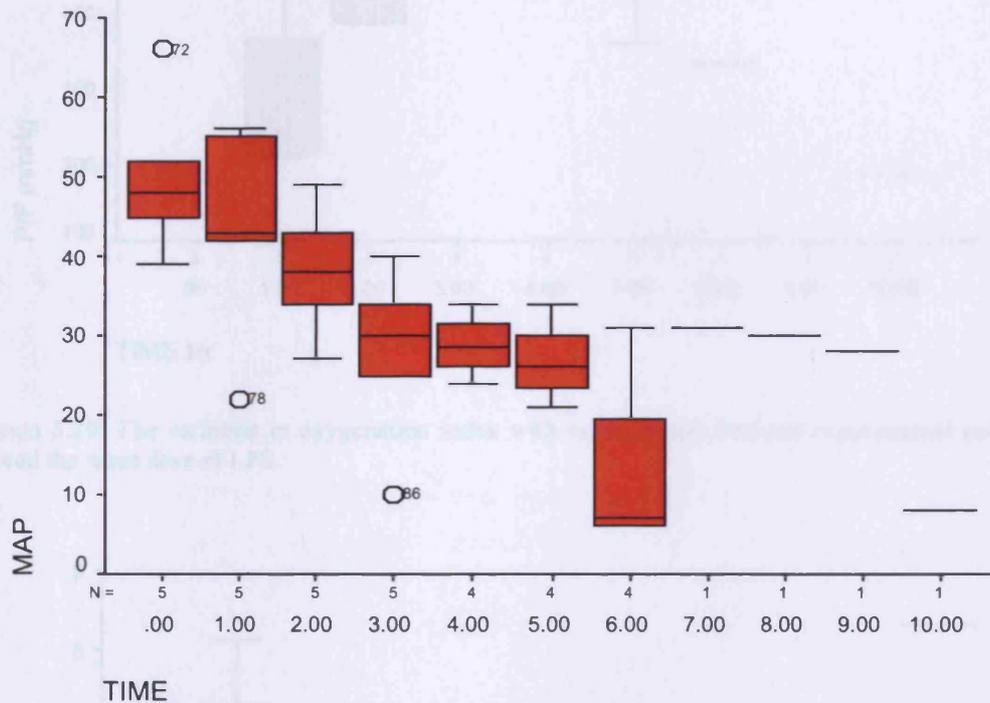
Diagram 5.26: Death as primary outcome measure for equal doses of LPS derived from the dose response plot.



Secondary outcomes

Cardiovascular: The haemodynamic variations are demonstrated using a box plot (Diagram 5.27). This diagram illustrates the variation in mean arterial blood pressures between the phase two animal experiments at different times up to death. It shows the median (central line), Inter-quartile range (Edges of the box) and the range (arms of the diagram). Outliers are demonstrated as circles and an identification number. The mean arterial pressure fell consistently amongst experimental rabbits that received the same dose of LPS.

Diagram 5.27 illustrating the inter-individual variation in mean arterial blood pressures (mmHg) or MAP at each given time (Hours) during the experiment. N is the number of values at each given time.



Respiratory: The inter-individual variation in pulmonary functional state is illustrated using the box-plot diagram 5.30, $\text{PaO}_2/\text{FiO}_2$ (P/F) ratio (mmHg) against time (Hours). This diagram illustrates the variation and the trend of fall in the P/F for rabbits administered with the same lethal dose of LPS. Note the sudden deterioration, which causes respiratory dysfunction. The median P/F ration fell from 500 mmHg to 300 mmHg. This in a human would be classed as an acute lung injury (ALI).

ALI occurs when P/F ratio falls below 300 and ARDS when the P/F falls below 200 mmHg. There was no clear-cut threshold for oxygenation index leading to death from the phase one experiments, however the oxygenation index rose from a median of 2

to 3.5 within the first hour of LPS administration or immediately after ALI. Diagram 5.29 illustrates the inter-individual variation between experimental animals as OI against time (Hours).

Diagram 5.28: Box plot diagram of PaO₂/FiO₂ (P/F) ratios (mmHg) against time (Hours).

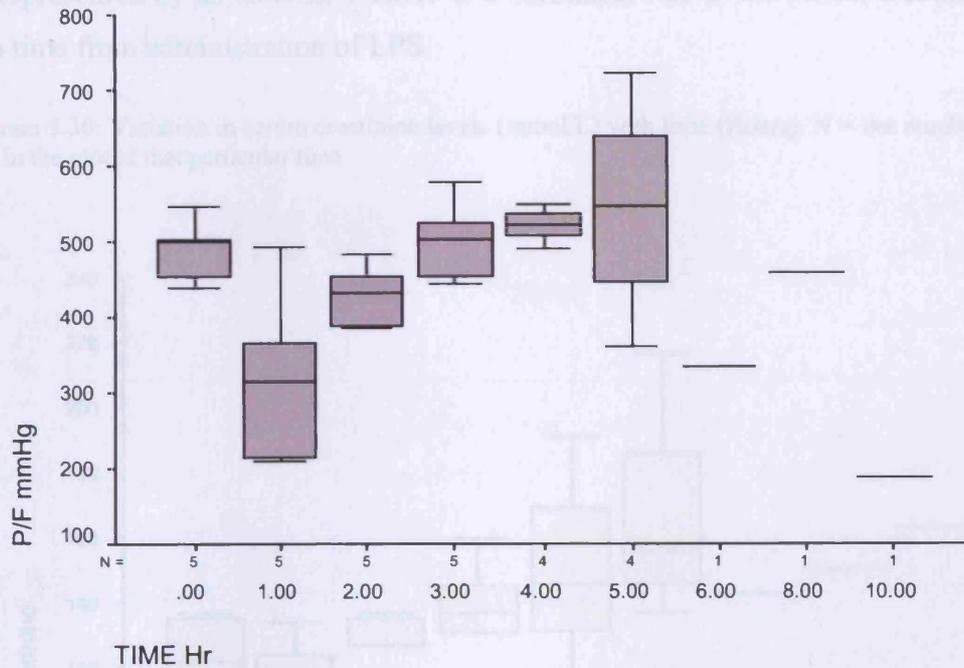
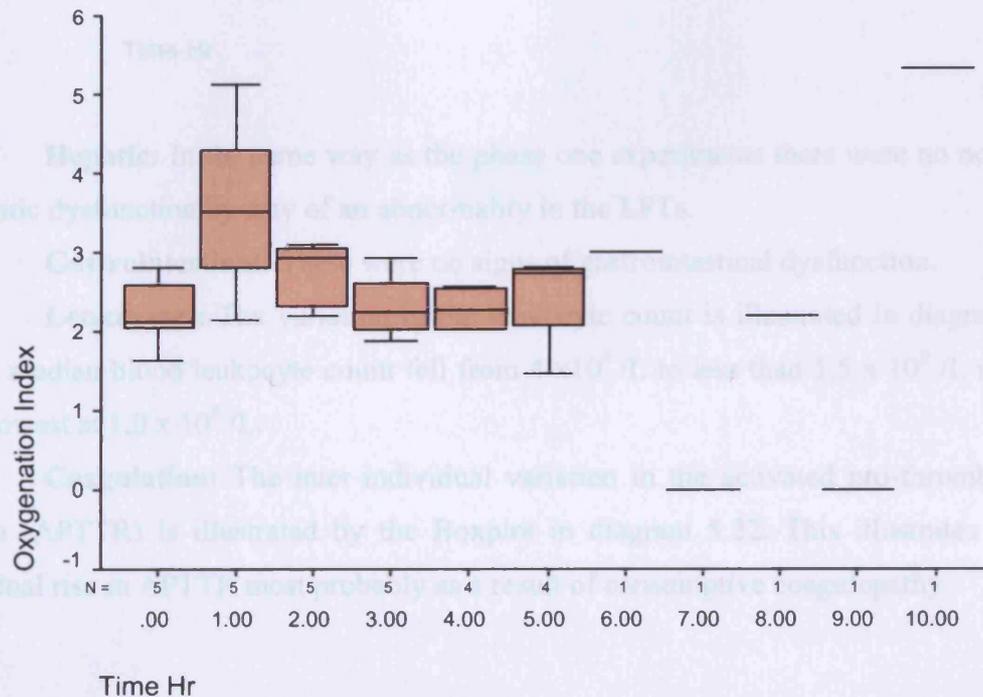
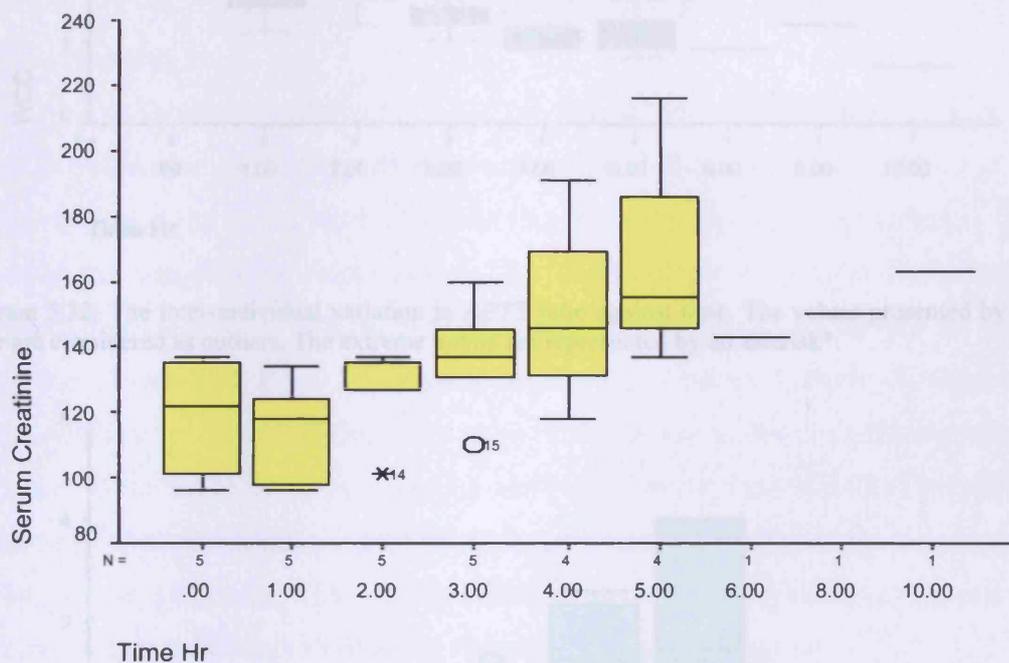


Diagram 5.29: The variation in oxygenation index with time (Hours) between experimental animals that received the same dose of LPS.



Renal: The graph below illustrates the variation in creatinine levels in the phase two experiments. The median is plotted as a horizontal line across the box. The values presented by a small circle are considered as outliers (1.5 box-length from the edge of the box). The extreme points (Outliers by more than 3 box lengths from the edge of the box) are represented by an asterisk*. There is a consistent rise in the serum creatinine levels with time from administration of LPS.

Diagram 5.30: Variation in serum creatinine levels (mmol/L) with time (Hours). N = the number of values used in the plot at that particular time.



Hepatic: In the same way as the phase one experiments there were no noticeable hepatic dysfunction by way of an abnormality in the LFTs.

Gastrointestinal: There were no signs of gastrointestinal dysfunction.

Leukocytes: The variation in the leukocyte count is illustrated in diagram 5.32. The median blood leukocyte count fell from $4 \times 10^9 /L$ to less than $1.5 \times 10^9 /L$ reaching its lowest at $1.0 \times 10^9 /L$.

Coagulation: The inter-individual variation in the activated pro-thrombin time ratio (APTTR) is illustrated by the Boxplot in diagram 5.22. This illustrates a more gradual rise in APTTR most probably as a result of consumptive coagulopathy.

Diagram 5.31: Box plot diagram illustrating the inter-individual variation in blood leukocyte count vs. time after administration of the same dose of LPS.

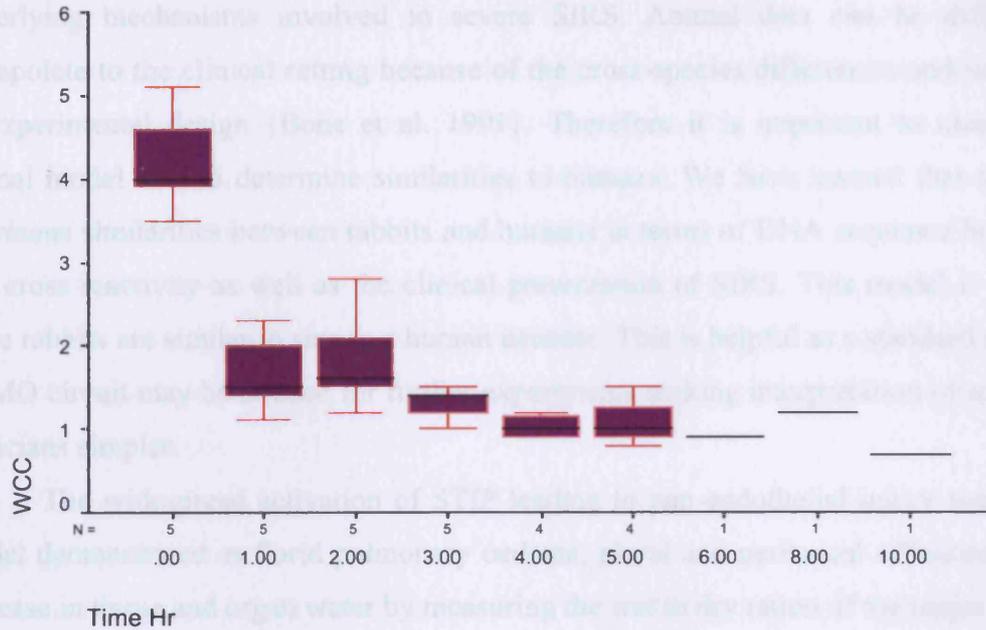
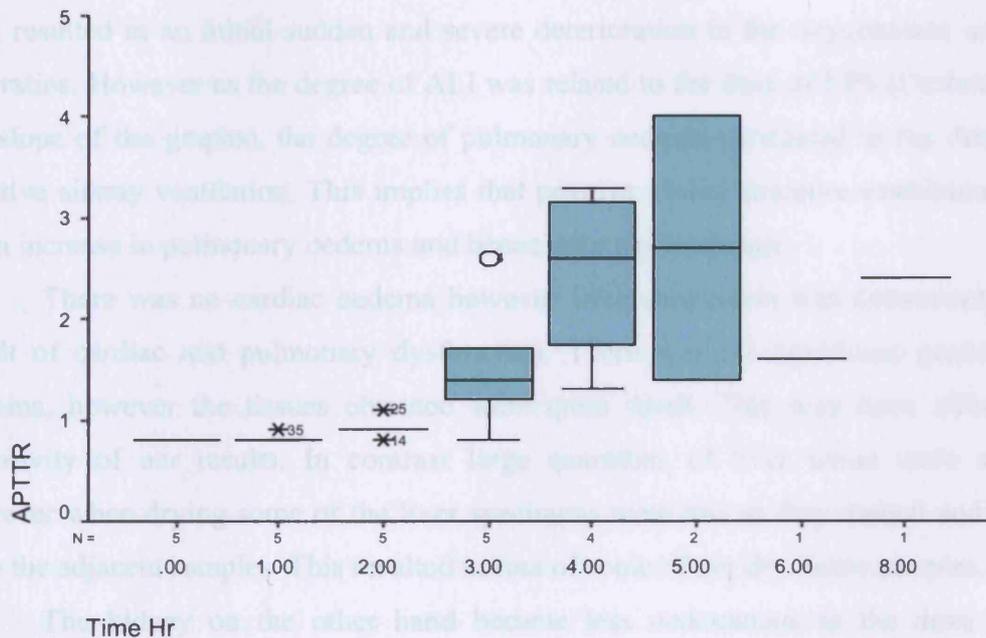


Diagram 5.32: The inter-individual variation in APTT ratio against time. The values presented by a small circle are considered as outliers. The extreme points are represented by an asterisk*.



DISCUSSION

Experimental animal models are useful tools for the better understanding of the underlying mechanisms involved in severe SIRS. Animal data can be difficult to extrapolate to the clinical setting because of the cross-species differences and variations in experimental design {Bone et al. 1991}. Therefore it is important to examine an animal model first to determine similarities to humans. We have learned that there are enormous similarities between rabbits and humans in terms of DNA sequence homology and cross reactivity as well as the clinical presentation of SIRS. This model is ideal as these rabbits are similar in size to a human neonate. This is helpful as a standard neonatal ECMO circuit may be utilised for further experiments making interpretation of results by clinicians simpler.

The widespread activation of STIP leading to pan-endothelial injury was in this model demonstrated as florid pulmonary oedema, plural and peritoneal effusions and an increase in tissue and organ water by measuring the wet to dry ration of the major internal organs at the post-mortem examination. The degree of tissue oedema in the lung was irrespective of the dose of LPS but strongly related to the time spent on positive pressure ventilation. Acute lung injury occurred in all the experimental animals. The infusion of LPS resulted in an initial sudden and severe deterioration in the oxygenation index and P/F ratios. However as the degree of ALI was related to the dose of LPS (Determined by the slope of the graphs), the degree of pulmonary oedema correlated to the duration of positive airway ventilation. This implies that positive airway pressure ventilation results in an increase in pulmonary oedema and hence poor gas exchange.

There was no cardiac oedema however liver congestion was demonstrated as a result of cardiac and pulmonary dysfunction. There was no significant gastric tissue oedema, however the tissues obtained were quite small. This may have affected the sensitivity of our results. In contrast large quantities of liver tissue were sampled, however when drying some of the liver specimens were lost as they melted and merged with the adjacent samples. This resulted in loss of some of our dry tissue samples.

The kidney on the other hand became less oedematous as the dose of LPS increased. This did not show significance in the first phase of our experiments due to the small sample size however it reached significance as the numbers of animals increased from 6 to 11 in the second phase.

We also demonstrated varying degrees of organ dysfunction by simple biochemical and haematological investigations. The liver function however appeared to

be unaffected by LPS. But the time course of this experiment may not have been enough to develop liver dysfunction. The abnormality in the coagulation profile could either be attributed to STIP or demonstrative of an early hepatic dysfunction.

This model is the first step in the evaluation of treatments that could have important survival benefits for a large number of patients in intensive care units.

The epidemiological review in chapter 3 illustrates a clear need for improvement in the outcome of therapy with both conventional means as well as ECMO for severe SIRS. Following these experiments treatment modalities may be evaluated to determine their effects such as neutrophil filter or MARS. The characterisation of the model is essential to allow rational design of the second wave of experiments, particularly with respect to sample size.

CONCLUSION

In this chapter we reached our objectives by developing a simple, reliable and reproducible model, which uses the lowest order of animal suitable for ECMO research. We demonstrated the dose response relationship between administration of intravenous LPS, development of severe SIRS/sepsis and lethal MODS. There is no such model, which exists, in the published literature.

We demonstrated a strong negative linear relationship between the dose of LPS administered and the survival of rabbits. We used this relationship to test inter-individual variation amongst rabbits given the same dose of desired LPS that would result in death if left untreated, however allowing enough time to intervene using an extracorporeal strategy. That dose was 0.02 mg/kg rabbit body weight. This allowed a mean (SD) time to death of 5.6 (2.4) hours derived from the study of inter-individual variations. The inter-individual variation was again tested for each of the physiological and biochemical secondary outcome measures.

This animal model will enable us to assess adjunctive therapies prior to their use in humans for their safety and efficacy, and to improve our current outcomes with ECMO by changing the course of progression from SIRS to severe SIRS and MODS using disease-modifying strategies.

Chapter Six

BIOMARKERS OF THE INNATE IMMUNE RESPONSE IN RABBITS

INTRODUCTION

The innate immune system provides an immediate defence mechanism against a variety of insults including infection. It consists of a number of biochemical and cellular components. In this chapter we will examine some of the important biochemical components of the innate immunity in rabbits. We will describe the quantitative variation in these components in relation to the dose of LPS administered / severity of disease, for the first time in rabbits.

MATERIALS AND METHODS

We have already described the laboratory setting, the experimental methodology, optimisation and quality control used in determining serum / plasma concentration of biomarkers of the rabbits innate immunity in Chapter 2.

RESULTS

ACUTE PHASE PROTEINS

C-Reactive Protein: The intra assay variation was assessed using coefficient of variation. This was acceptable (CV = 7.3%). The value of R^2 was > 0.99 for all standard curves. The median (Range) for CRP prior to administration of LPS is illustrated in table 6.1. The CRP levels fell in all animals within the first few hours of the experiment. This occurred faster in experimental animals that received high dose LPS to almost undetectable in the last hour of their life (Diagram 6.1). The CRP levels however started to rise after a short fall and continued to rise in rabbits that survived close to 24 hours. The rise in CRP was not restricted to the experimental animals and the control also illustrated an equally as high rise in CRP at 24 hours. The inter-individual variation in CRP was assessed at 3 and 5 hours post LPS administration. This is illustrated in diagram 6.2. Only one animal in the constant dose group (XEN) survived more than five hours. Therefore the serum CRP level there after would not illustrate a variation and was therefore excluded.

Diagram 6.1: Trend of CRP in ng/ml against time in hours. Note that in the interest of lowering the experimental costs only one control rabbit (IVY) was included in this assay.

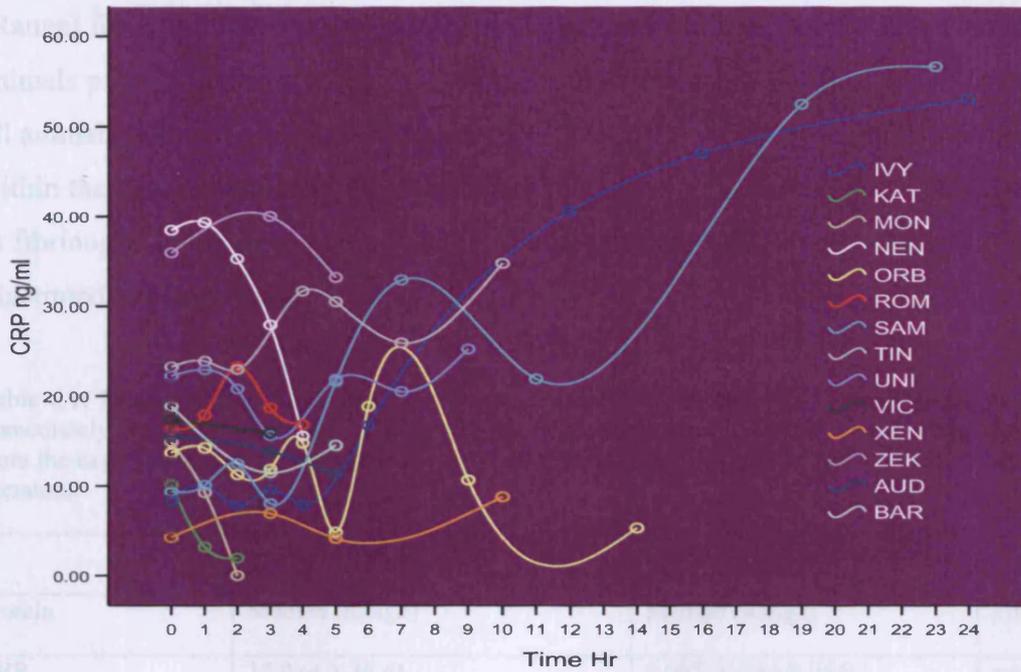
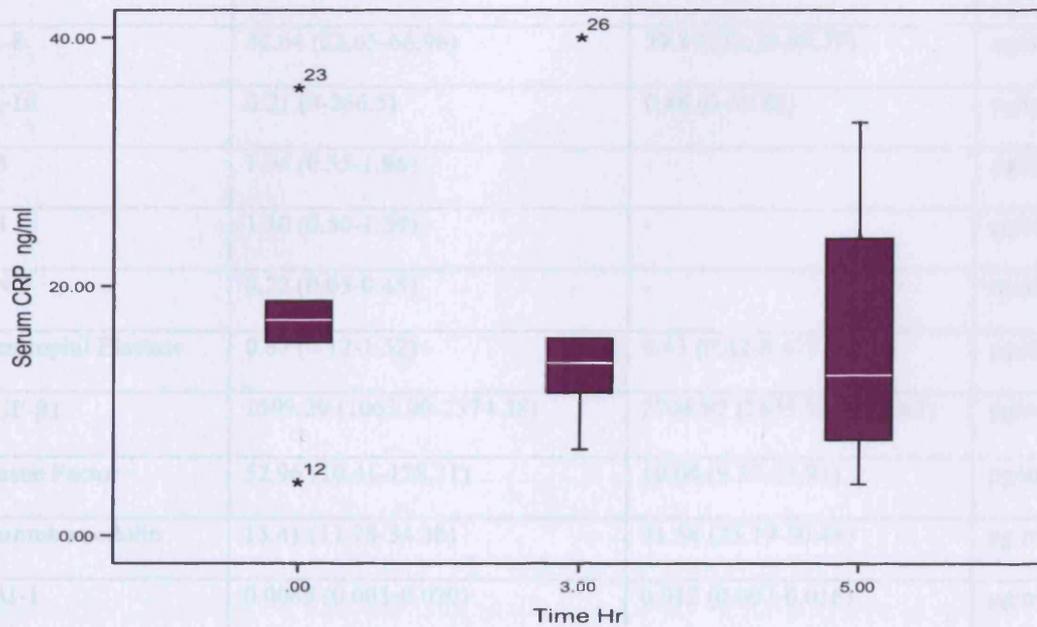


Diagram 6.2: Illustrating the inter-individual variation in serum CRP in ng/ml at 0, 3 and 5 hours amongst experimental animals that received the same dose of LPS. * Represents the extreme points these are outliers by more than 3 box lengths from the edge of the corresponding box. The number represents the ID number on the SPSS spread sheet.



Fibrinogen: The inter assay variations ranged from 3.06 to 7.2 % and the intra assay variation was 9.9%. The value of R^2 was > 0.96 for all standard curves. The median (Range) for rabbit Fibrinogen (FGN) levels and the fibrinogen levels in our experimental animals prior to administration of LPS is illustrated in table 6.1. The FGN levels rise in all animals both experimental and control. However the levels rose quicker in the former within the first few hours of the experiment (Diagram 6.3). The inter-individual variation in fibrinogen levels post administration of a constant pre-calculated dose of LPS. This is illustrated in diagram 6.4.

Table 6.1: Illustrating the variations in the concentration of Thrombo-inflammatory mediators in rabbits immediately before administration of LPS and in normal rabbit serum/plasma. These values were derived from the experiments performed in this thesis. These values are otherwise not established in the published literature.

	Experimental and Control rabbits	Normal rabbit serum/plasma	
Protein	Median (Range)	Median (Range)	Units
CRP	15.9 (4.3-38.4)	0.055 (0.056-0.054)	ng/ml
Fibrinogen	0.014 (0.0014-0.027)	0 (0-.0007)	µg/ml
TNF α	2.68 (1.24-35.04)	3.161 (2.38-4.33)	ng/ml
IL-1 β	1.07 (0.99-3.02)	1.25 (1.21-1.48)	ng/ml
IL-6	11.89 (9.10-19.70)	14.69 (12.45-16.18)	ng/ml
IL-8	32.64 (23.65-66.96)	39.87 (32.10-50.79)	ng/ml
IL-10	0.21 (0-266.5)	0.88 (0-19.86)	ng/ml
C3	1.04 (0.35-1.96)	-	µg/ml
C4	1.10 (0.50-1.39)	-	µg/ml
TNO	0.22 (0.05-0.45)	-	mmol/L
Neutrophil Elastase	0.67 (0.12-1.52)	0.43 (0.42-0.47)	µg/ml
TGF- β 1	1509.29 (1063.99-2374.28)	2706.97 (2635.31-2778.63)	pg/ml
Tissue Factor	52.96 (10.41-128.31)	10.64 (9.37-11.91)	pg/ml
Thrombomodulin	15.41 (11.78-34.38)	31.54 (25.19-50.48)	ng/ml
PAI-1	0.0089 (0.003-0.020)	0.012 (0.007-0.016)	µg/ml
TAS	1.26 (0.94-1.77)	0.69 (0.77-1.77)	mmol/L

Diagram 6.3: Trend of fibrinogen in $\mu\text{g/ml}$ against time in hours in all experimental and control rabbits.

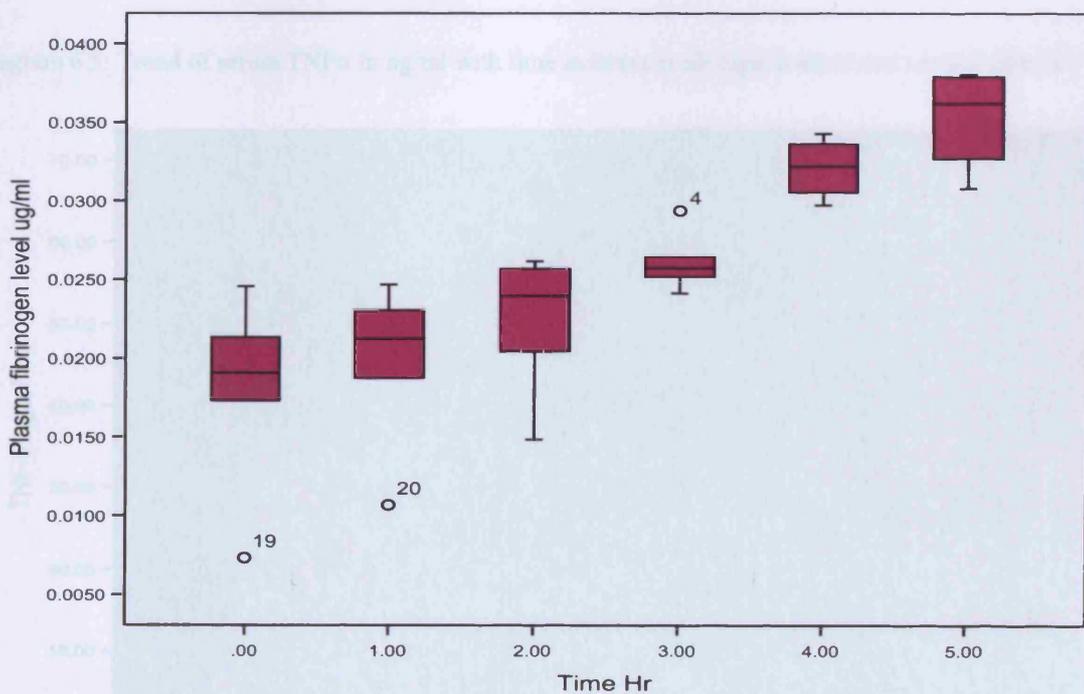
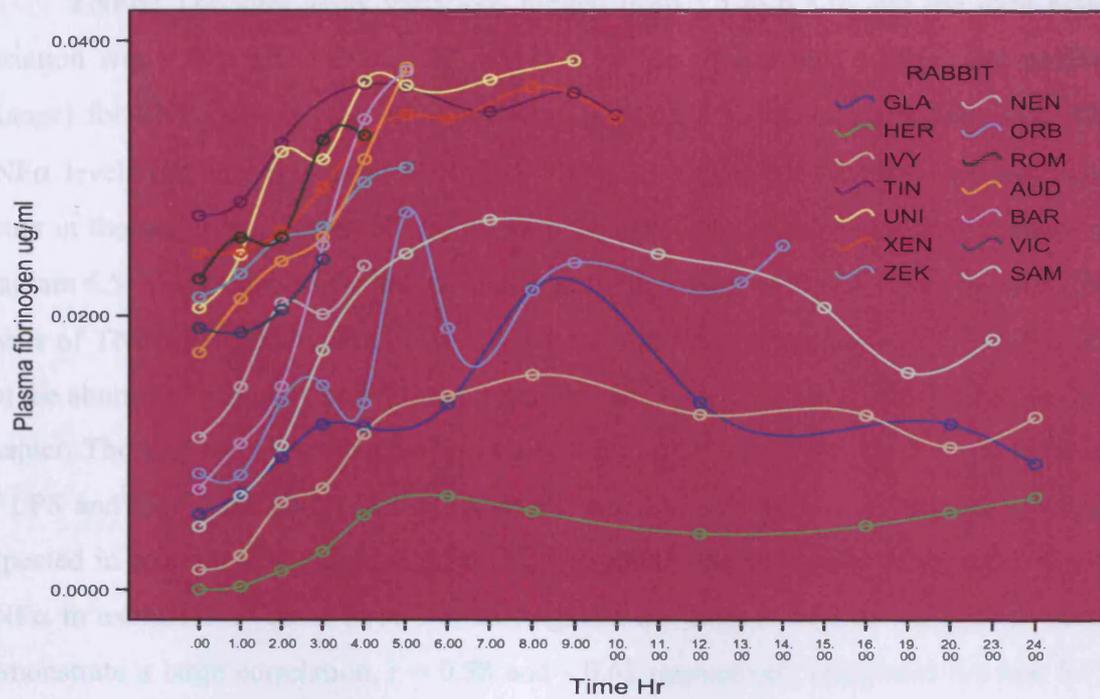


Diagram 6.4: Illustrating the inter-individual variation in plasma Fibrinogen levels in $\mu\text{g/ml}$ up to and including five hours post administration of the same dose of LPS. ° Represents outliers and the number represents the ID on the SPSS spread sheet.

CYTOKINES

TNF α : The inter assay variations ranged from 3.5 to 6.3 % and the intra assay variation was 9.8%. The value of R^2 was > 0.98 for all standard curves. The median (Range) for TNF α levels prior to administration of LPS is illustrated in table 6.1. The TNF α levels rise in response to LPS. The TNF α levels peaked between one and three hours in the majority of cases at two hours post LPS administration as demonstrated in diagram 6.5. The animal ROM had an abnormally high level of TNF α from the start. The levels of TNF α reached maximum at T = 1 hour and fell appropriately. This animal did not die abnormally quickly and followed the dose response pattern as described in the last chapter. The first three experimental animals (KAT, LID and MON) received high doses of LPS and died quite early. In these animals the serum TNF α did not rise as much as expected in relation to the dose of LPS. The graphical representation of the peak serum TNF α in exclusion of these three animals, against the dose of LPS or the time to death demonstrate a large correlation, $r = 0.58$ and $- 0.62$ respectively (diagrams 6.6 and 6.7). In these diagrams the curved lines on either side of the linear plot is the confidence interval where 95% of the sample means distribution would lie.

Diagram 6.5: Trend of serum TNF α in ng/ml with time in hours in all experimental and control rabbits.

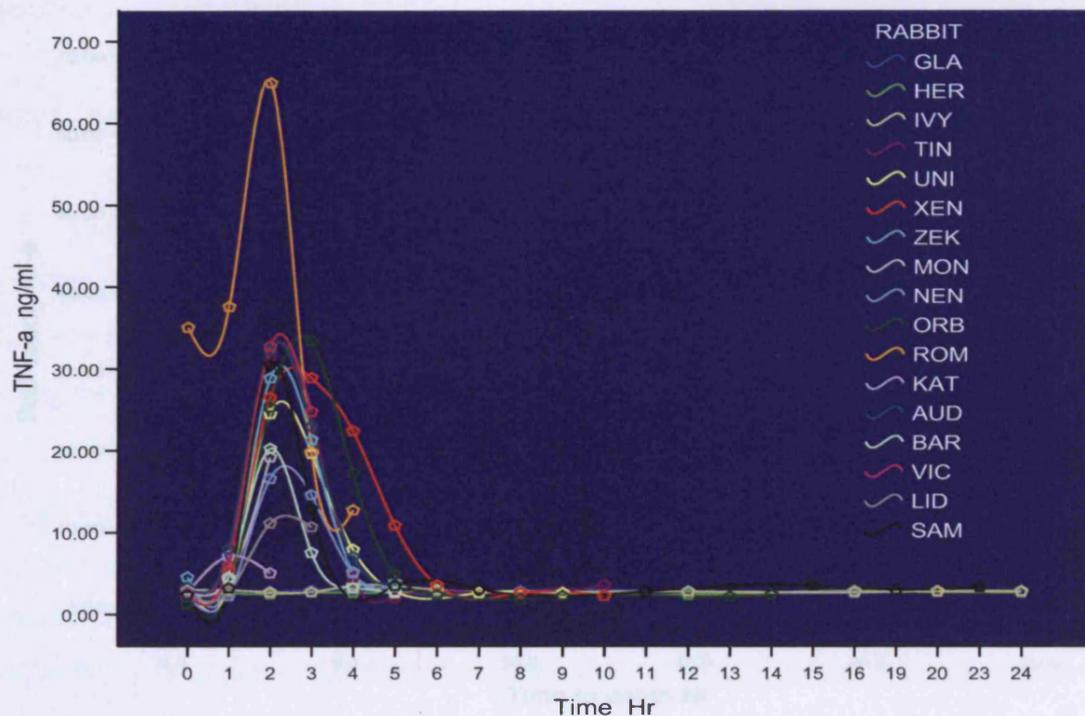


Diagram 6.6: The relation ship between the dose of LPS administered and the peak serum levels of TNF α in experimental and control rabbits.

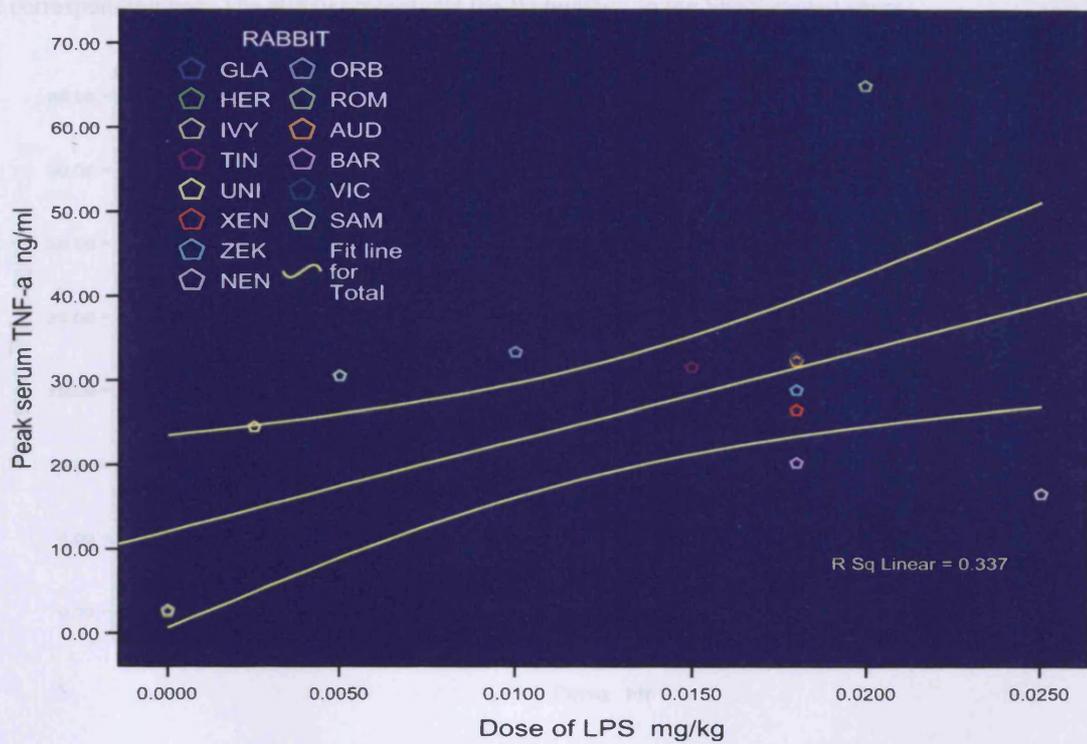


Diagram 6.7: The relation ship between the peak serum levels of TNF α and time to death in experimental and control rabbits. The curved lines represent the 95% confidence interval.

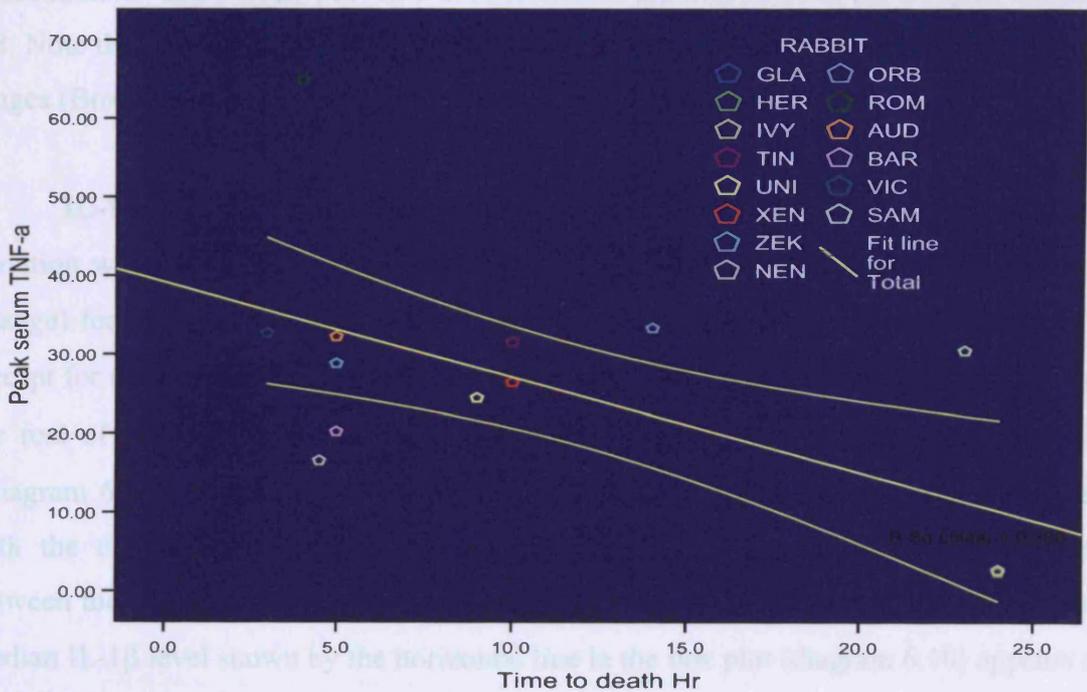


Diagram 6.8: Illustrating the inter-individual variation in serum TNF α in ng/ml at 0, 2 and 5 hours amongst experimental animals who received the same dose of LPS. Note that only one experimental animal in this group survived past 5 hours. Therefore the serum TNF α level there after would not illustrate a variation and hence excluded from this graph. ° Represents the outliers by less than 3 box lengths from the edge of the corresponding box. The number represents the ID number on the SPSS spread sheet.

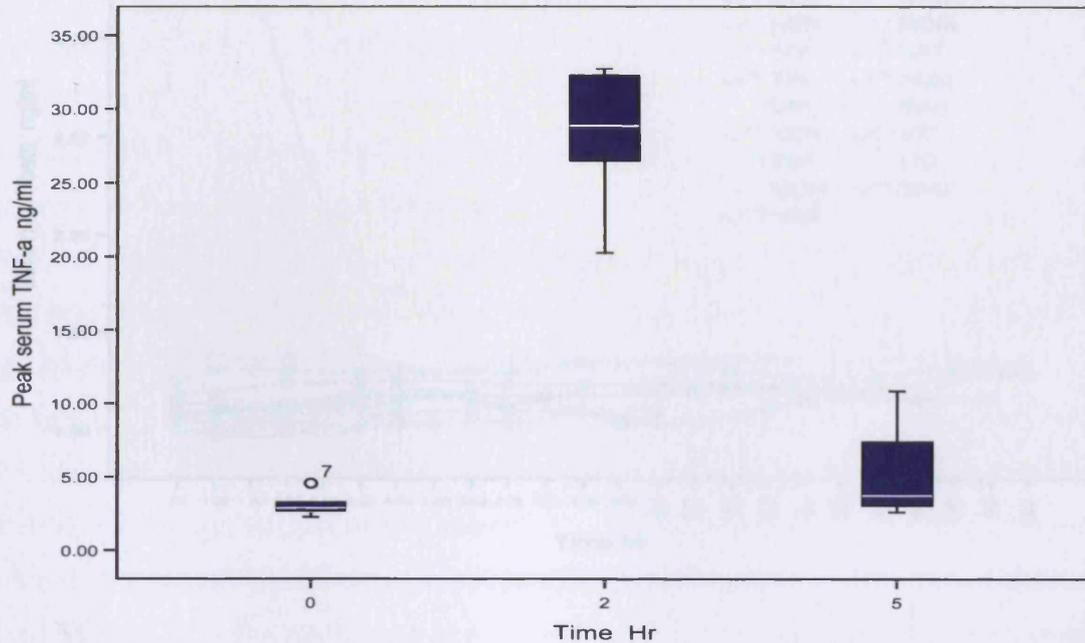


Diagram 6.8: Illustrating the inter-individual variation in serum TNF α in ng/ml at 0, 2 and 5 hours amongst experimental animals who received the same dose of LPS. Note that only one experimental animal in this group survived past 5 hours. Therefore the serum TNF α level there after would not illustrate a variation and hence excluded from this graph. ° Represents the outliers by less than 3 box lengths from the edge of the corresponding box. The number represents the ID number on the SPSS spread sheet.

The inter-individual variation in serum levels of TNF α at T = 0, 2 (Peak concentration) and 5 hours post LPS administration are illustrated in the Boxplot diagram 6.8. Note that there is only one outlier and that the range (Whiskers) and the inter quartile ranges (Box) containing 50% of the values are relatively small.

IL-1 β : The inter assay variations ranged from 2.05 to 10.7 % and the intra assay variation was 7.8 %. The value of R^2 was > 0.94 for all standard curves. The median (Range) for serum IL-1 β levels prior to administration of LPS is illustrated in table 6.1. Except for the experimental animal ROM who had an IL-1 β level three times higher than the rest of the animals to start with the level of IL-1 β did not change significantly (Diagram 6.9). The IL-1 β levels were in fact higher in the control group as compared with the experimental group. Diagram 6.10 illustrates the inter-individual variation between the experimental animals receiving same dose of LPS at T = 0 to 5 hours. The median IL-1 β level shown by the horizontal line in the box plot (diagram 6.10) appears to be steadily rising.

Diagram 6.9: Trend of serum IL-1 β in ng/ml with time in hours in all experimental and control rabbits.

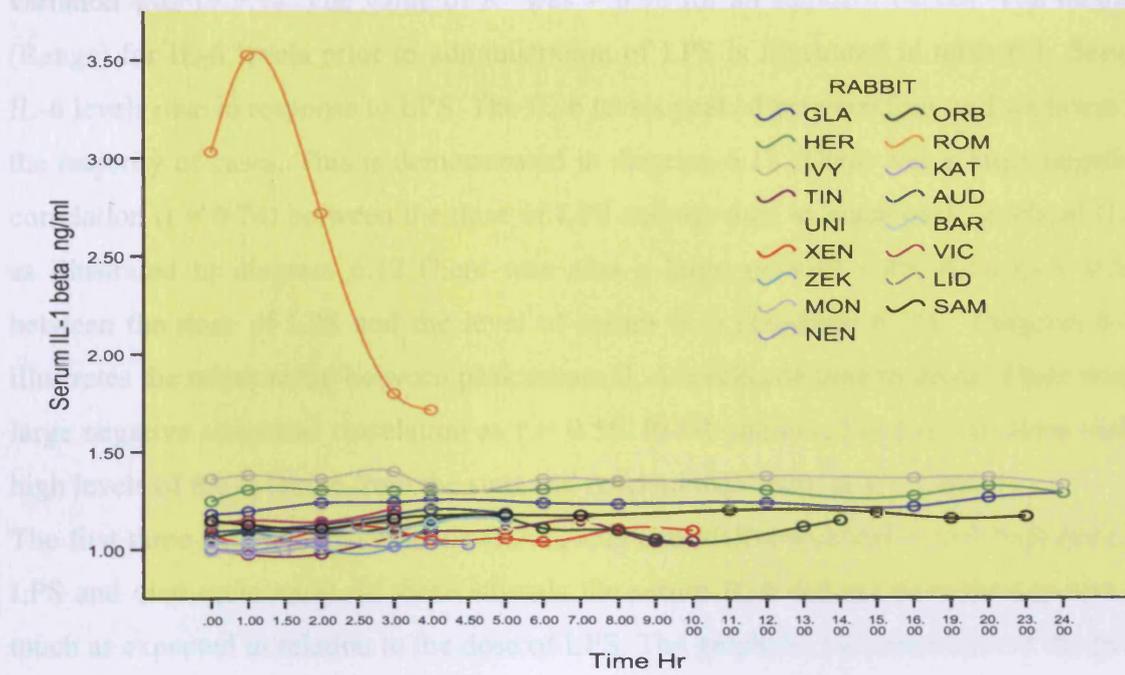
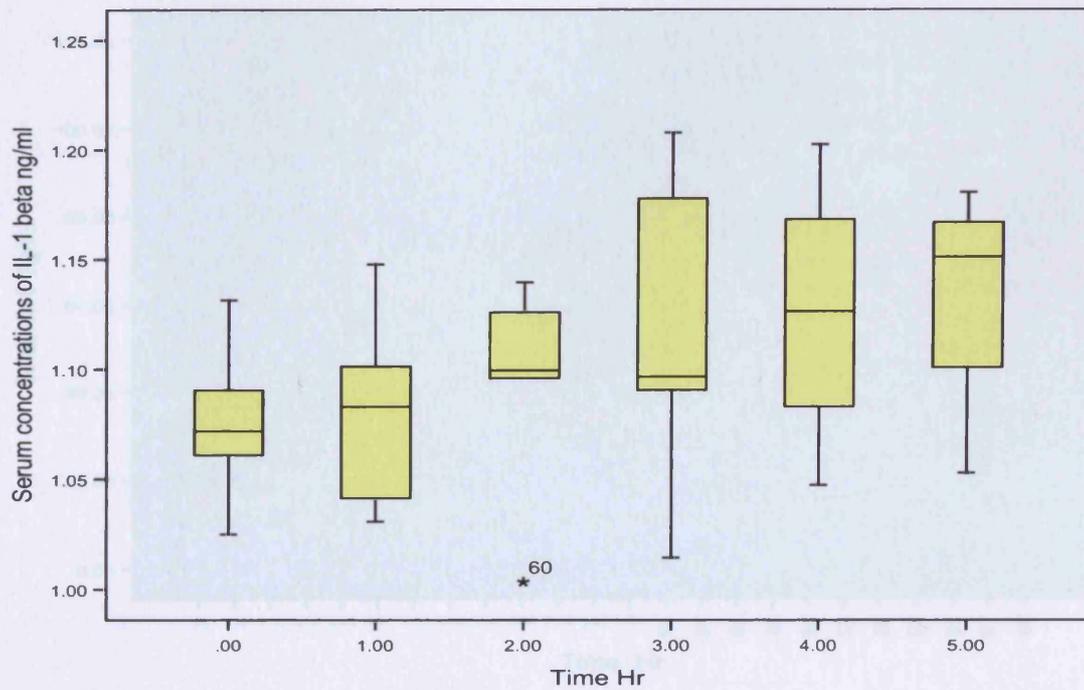


Diagram 6.10: Illustrating the inter-individual variation in serum IL-1 β in ng/ml at different times. Note that only one experimental animal in this group survived past 5 hours. Therefore the serum IL-1 β level there after would not illustrate a variation and hence excluded. * Represents an extreme point. The number represents the ID number on the SPSS spread sheet.



IL-6: The inter assay variations ranged from 5.8 to 8.8 % and the intra assay variation was 17.3 %. The value of R^2 was > 0.98 for all standard curves. The median (Range) for IL-6 levels prior to administration of LPS is illustrated in table 6.1. Serum IL-6 levels rose in response to LPS. The IL-6 levels peaked between four and six hours in the majority of cases. This is demonstrated in diagram 6.11. There was a large negative correlation ($r = 0.74$) between the dose of LPS and the time to reach peak levels of IL-6 as illustrated in diagram 6.12. There was also a large positive correlation ($r = 0.53$) between the dose of LPS and the level of serum IL-6 (Diagram 6.13). Diagram 6.14 illustrates the relationship between peak serum IL-6 levels and time to death. There was a large negative statistical correlation as $r = 0.56$. ROM continued to have an abnormally high levels of the cytokine from the start and reached maximum at $T = 1$ hour.

The first three experimental animals (KAT, LID and MON) received a very high dose of LPS and died quite early. In these animals the serum IL-6 did not have time to rise as much as expected in relation to the dose of LPS. The graphical representation of the peak serum IL-6 excludes these three animals. In these diagrams the curved lines on either side of the linear plot is the confidence interval where 95% of the sample means distribution would lie. The inter-individual variation in serum levels of IL-6 up to and including $T = 5$ hours post LPS administration are illustrated in the Box plot diagram 6.15.

Diagram 6.11: Trend of serum IL-6 in ng/ml with time in hours in all experimental and control rabbits.

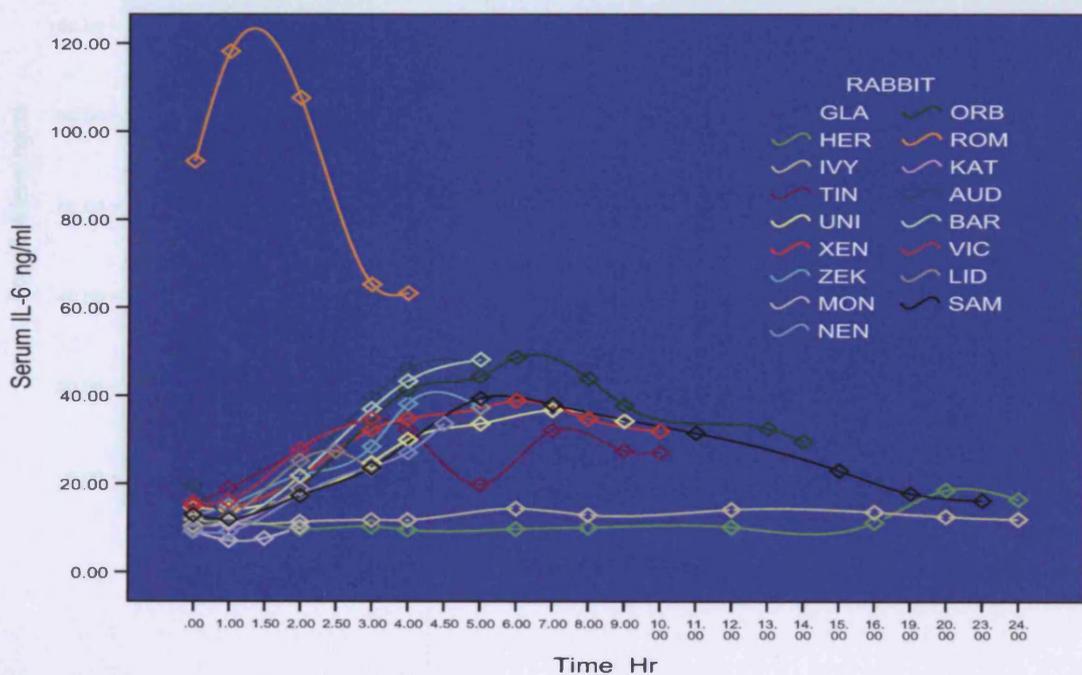


Diagram 6.14: The relationship between the peak serum levels of IL-6 and the time to reach the peak levels.

Diagram 6.12: The relation ship between the peak serum levels of IL-6 and the time to reach the peak levels.

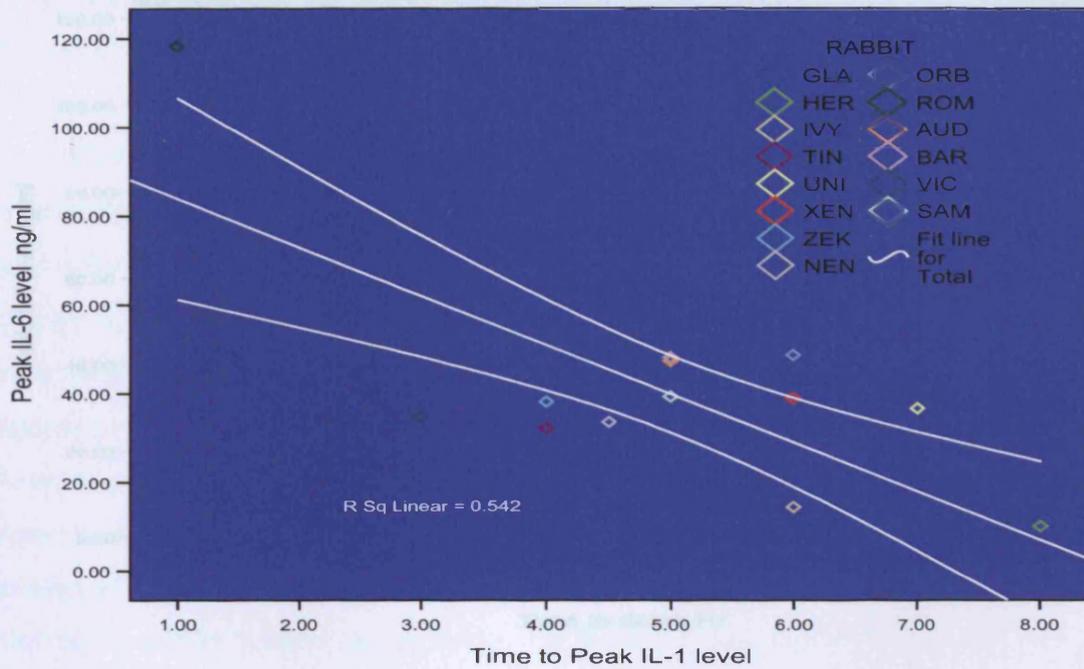


Diagram 6.13: The relation ship between the peak serum levels of IL-6 and the dose of LPS administered.

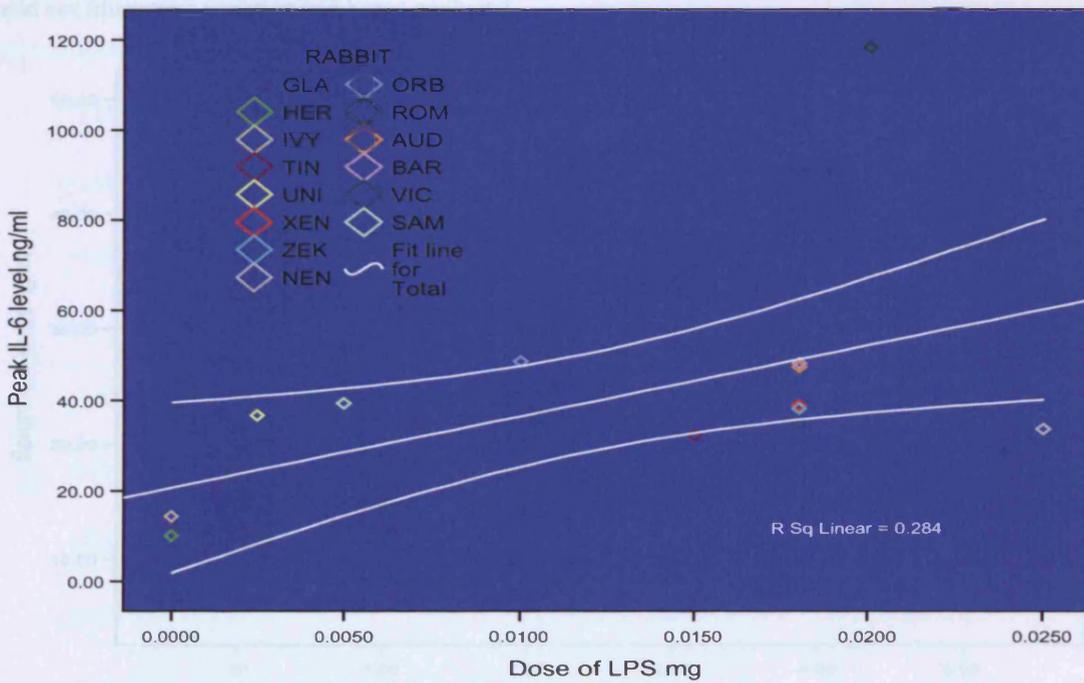


Diagram 6.14: The relationship between the peak serum levels of IL-6 and the time to death.

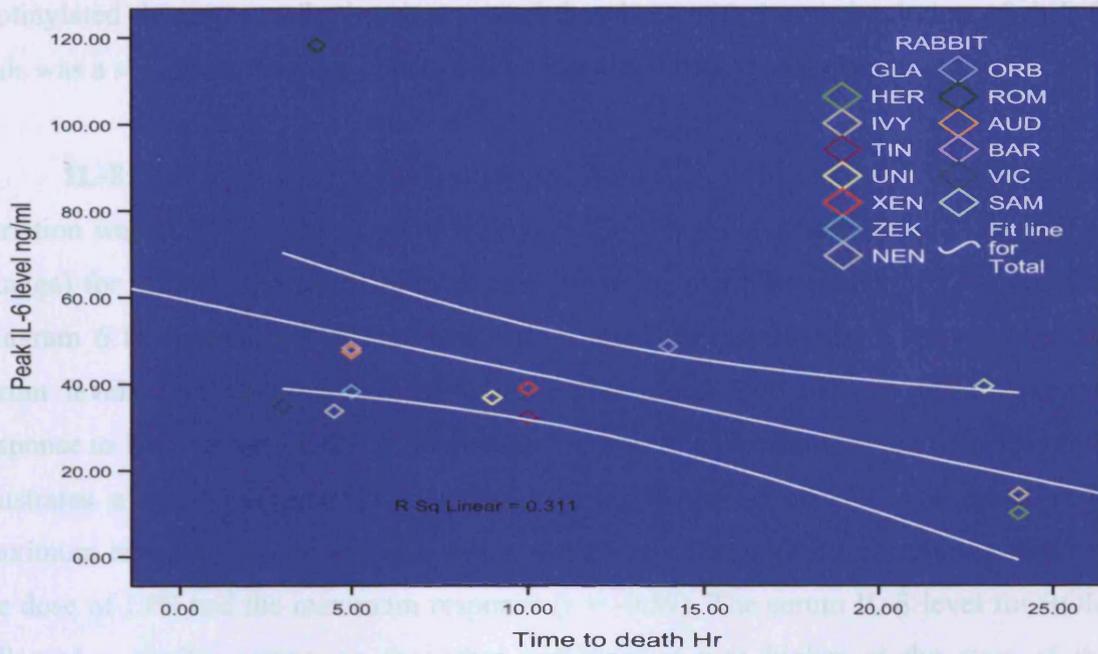
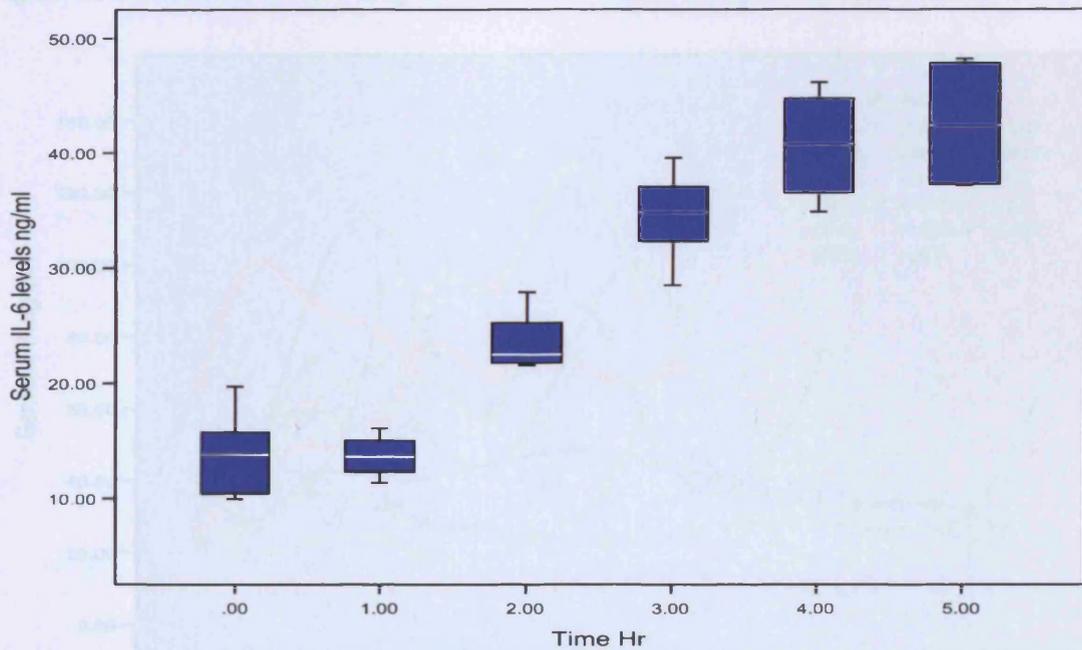


Diagram 6.15: Illustrating the inter-individual variation in serum IL-6 in ng/ml at different times. Note that only one experimental animal in this group survived past 5 hours. Therefore the serum IL-6 level there after would not illustrate a variation and hence excluded.



IL-5: We were unable to optimise a standard rabbit ELISA protocol for IL-5. However we were able to show a graduated response according to the concentration of the biotinylated detection antibody when coated the plates with 1 µg/ml solution of rhIL-5. This was a significant finding, which will be discussed later in this chapter.

IL-8: The inter assay variations ranged from 2.28 to 10.86 % and the intra assay variation was 19.80 %. The value of R^2 was > 0.97 for all standard curves. The median (Range) for normal IL-8 levels prior to administration of LPS is illustrated in table 6.1. Diagram 6.16 illustrates a rise in serum IL-8 levels in response to LPS, however the serum levels fluctuate and may peak more than once. The time to reach maximal response to LPS varied greatly in relation to the dose of LPS administered. Diagram 6.17 illustrates a strong negative relationship between the dose of LPS and the time to maximum response. However there was a statistically insignificant correlation between the dose of LPS and the maximum response ($r = -0.59$). The serum IL-8 level for ROM followed a similar pattern as the other cytokines it was higher at the start of the experiment and peaked in the first hour post LPS administration. The inter-individual variation in serum levels of IL-8 up to and including T = 5 hours post LPS administration are illustrated in the Box plot diagram 6.18.

Diagram 6.16: Trend of serum IL-8 in ng/ml with time in hours in all experimental and control rabbits.

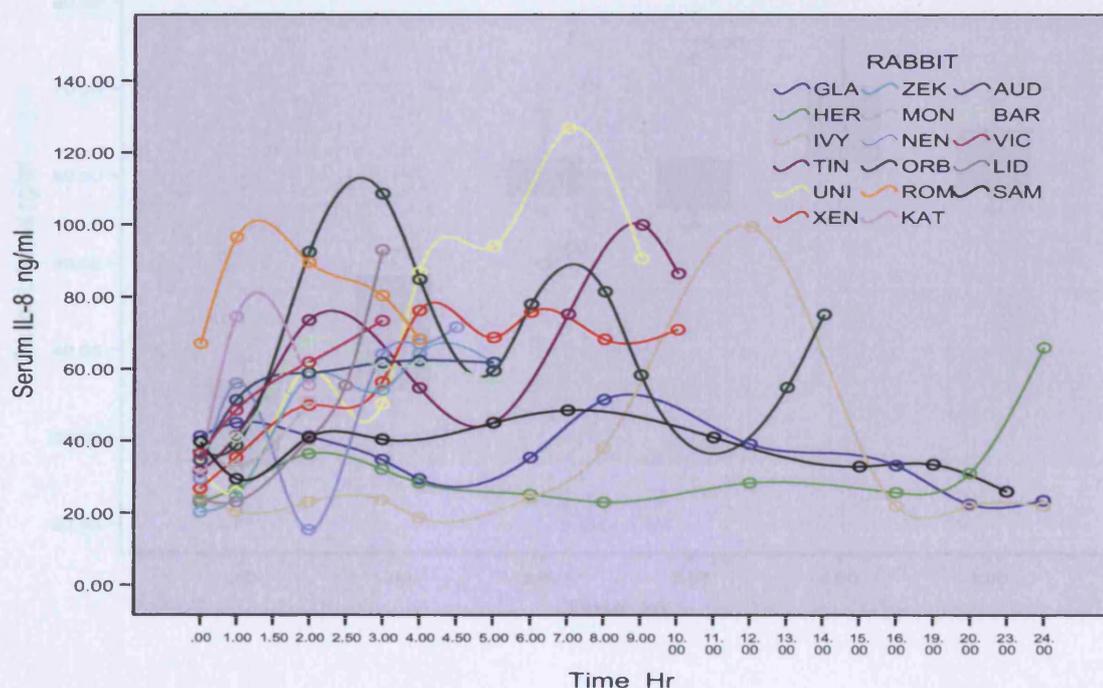


Diagram 6.17: The relationship between the dose of LPS administered and the time to reach peak serum levels of IL-8. In this diagram the curved lines on either side of the linear plot is the confidence interval where 95% of the sample means distribution would lie.

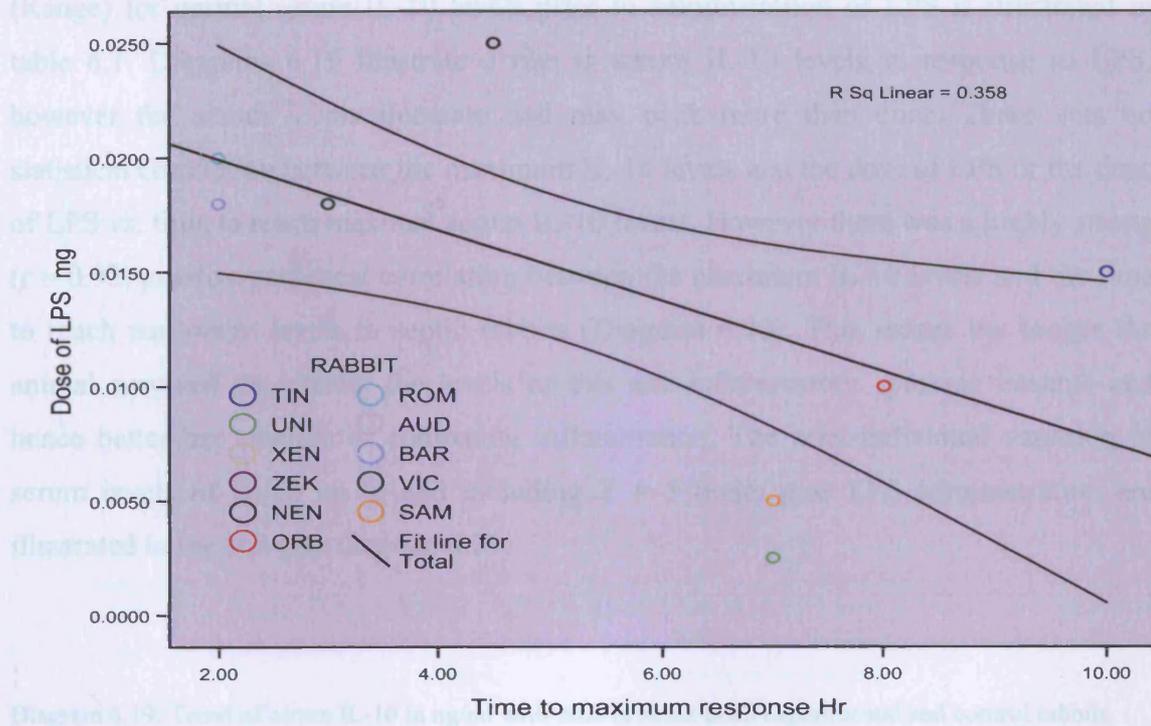
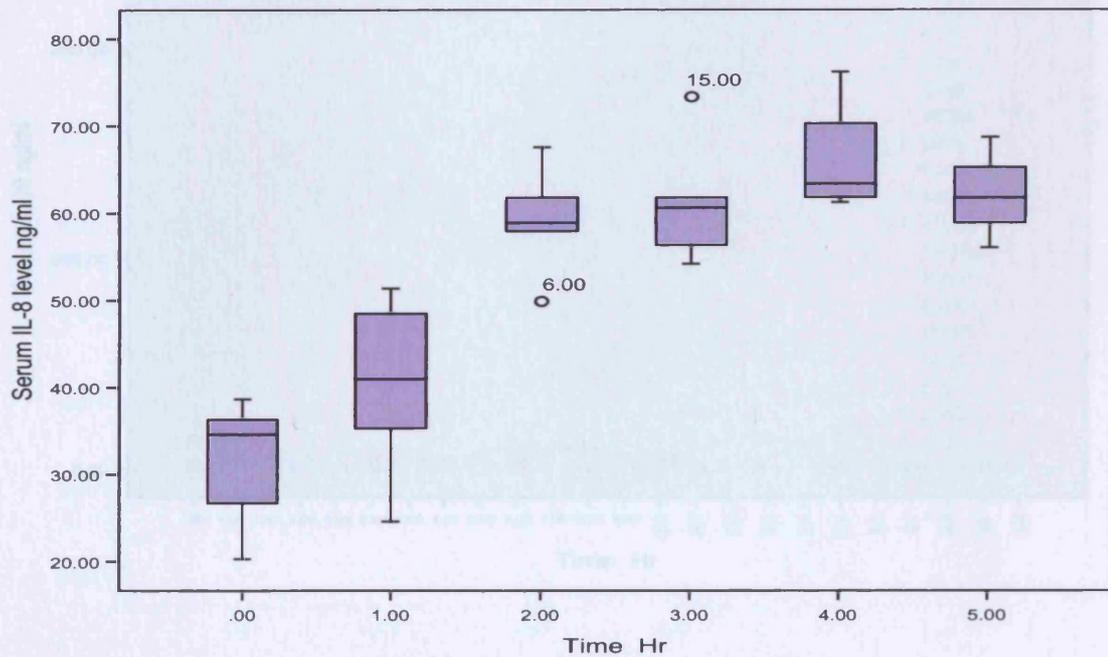


Diagram 6.18: Illustrating the inter-individual variation in serum IL-8 in ng/ml at different times. Note that only one experimental animal in this group survived past 5 hours. Therefore the serum IL-8 level there after would not illustrate a variation and hence excluded. ° Represent the outliers. The numbers represent the ID number on the SPSS spread sheet.



IL-10: The inter assay variations ranged from 7.3 to 14.3 % and the intra assay variation was 9.6 %. The value of R^2 was > 0.99 for all standard curves. The median (Range) for normal serum IL-10 levels prior to administration of LPS is illustrated in table 6.1. Diagrams 6.19 illustrate a rise in serum IL-10 levels in response to LPS, however the serum levels fluctuate and may peak more than once. There was no statistical correlation between the maximum IL-10 levels and the dose of LPS or the dose of LPS vs. time to reach maximal serum IL-10 levels. However there was a highly strong ($r = 0.92$) positive statistical correlation between the maximum IL-10 levels and the time to reach maximum levels in septic rabbits (Diagram 6.20). This means the longer the animal survived the greater the levels of this anti-inflammatory cytokine became and hence better her chances of combating inflammation. The inter-individual variation in serum levels of IL-10 up to and including $T = 5$ hours post LPS administration are illustrated in the box plot diagram 6.21.

Diagram 6.19: Trend of serum IL-10 in ng/ml with time in hours in all experimental and control rabbits.

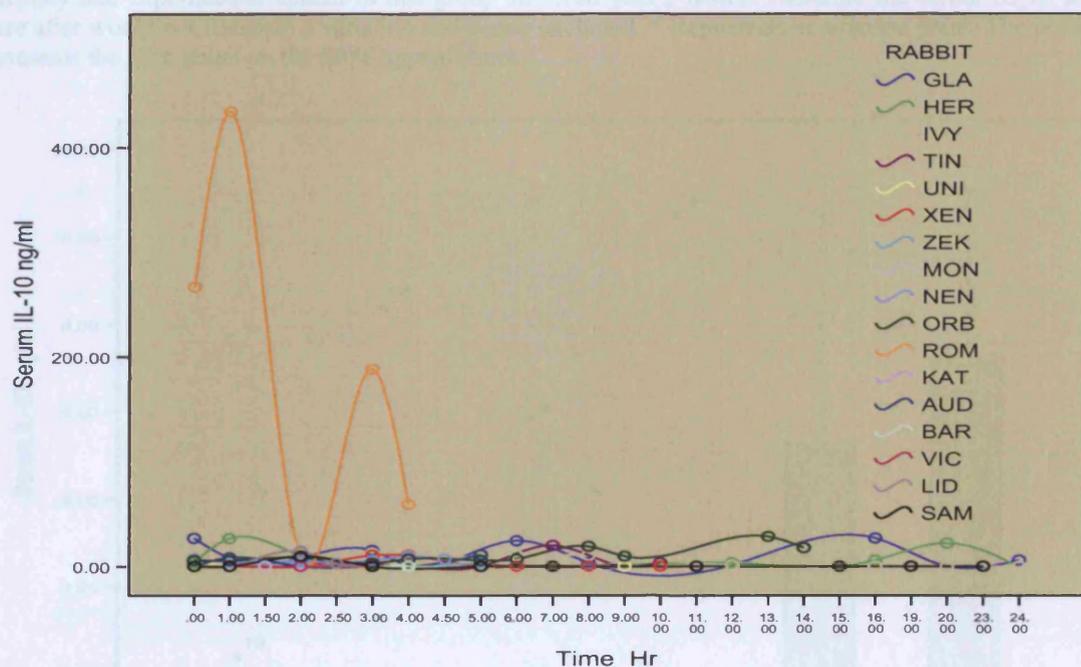


Diagram 6.20: Illustrates the correlation between the maximum level of IL-10 in ng/ml reached and the time to reach it in hours. This plot excludes the value for ROM. Its value would be an extreme point.

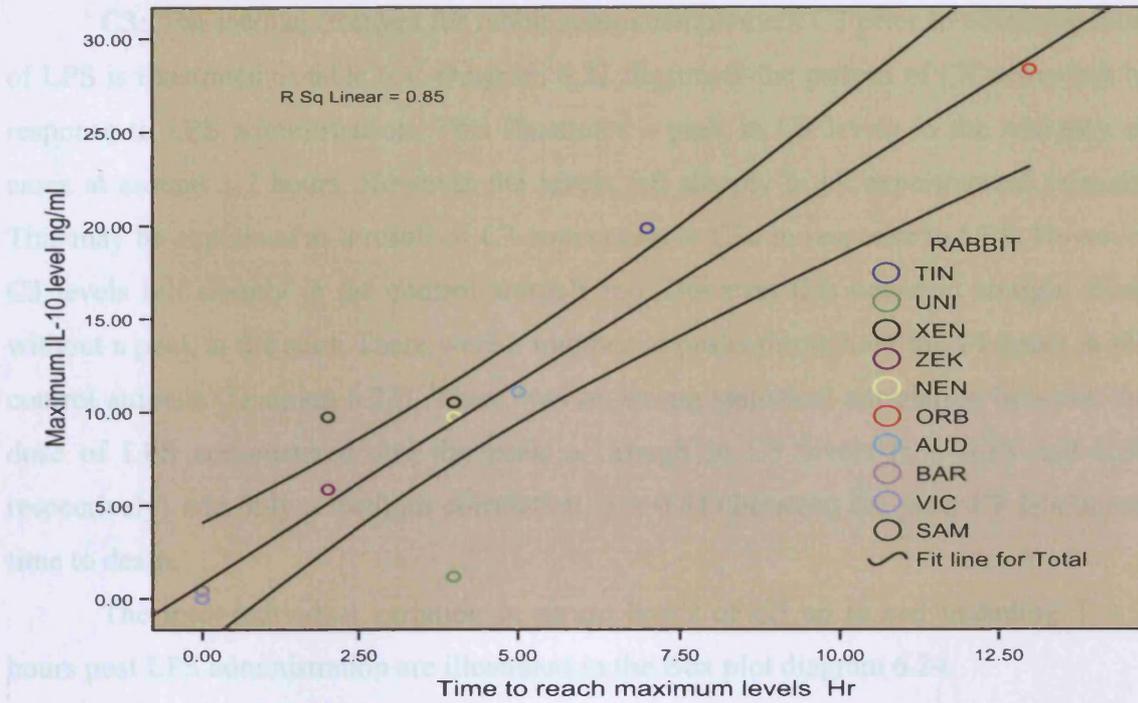
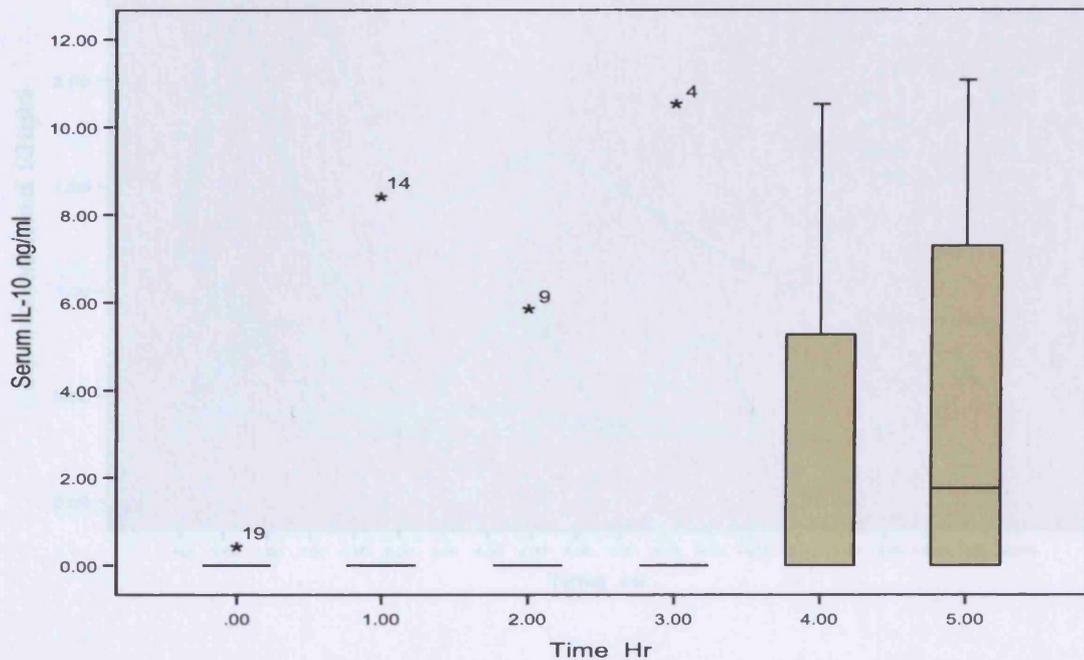


Diagram 6.21: Illustrating the inter-individual variation in serum IL-10 in ng/ml at different times. Note that only one experimental animal in this group survived past 5 hours. Therefore the serum IL-10 level there after would not illustrate a variation and hence excluded. * Represents an extreme point. The number represents the ID number on the SPSS spread sheet.



COMPLEMENT

C3: The median (Range) for rabbit serum complement C3 prior to administration of LPS is illustrated in table 6.1. Diagram 6.22 illustrates the pattern of C3 activation in response to LPS administration. This illustrates a peak in C3 levels in the majority of cases at around 1-2 hours. However the levels fell sharply in all experimental animals. This may be explained as a result of C3 conversion to C3a in response to LPS. However C3 levels fell sharply in the control animals too. However this occurred straight away without a peak at the start. There were a number of peaks throughout the 24 hours in the control animals (Diagram 6.23). There was no strong statistical correlation between the dose of LPS administered and the peak or trough in C3 levels ($r = 0.18$ and 0.28 respectively) and only a medium correlation ($r = 0.41$) between the peak C3 levels and time to death.

The inter-individual variation in serum levels of C3 up to and including $T = 5$ hours post LPS administration are illustrated in the Box plot diagram 6.24.

Diagram 6.22: Trend of serum C3 in $\mu\text{g/ml}$ with time in hours in all experimental rabbits.

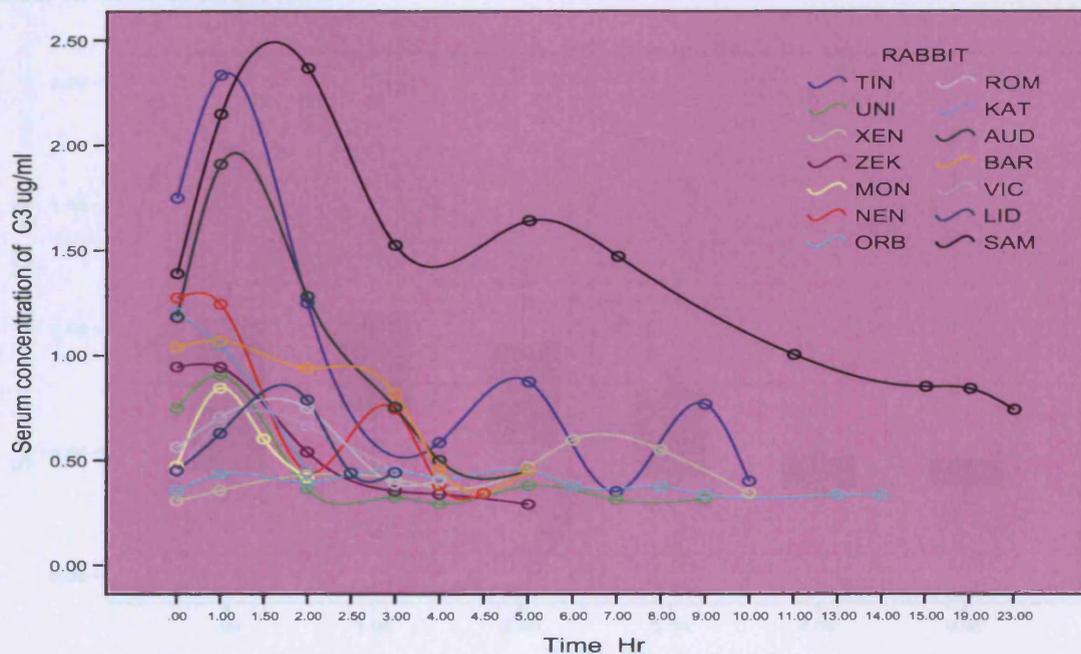


Diagram 6.23: Trend of serum C3 in $\mu\text{g/ml}$ with time in hours in all the control rabbits.

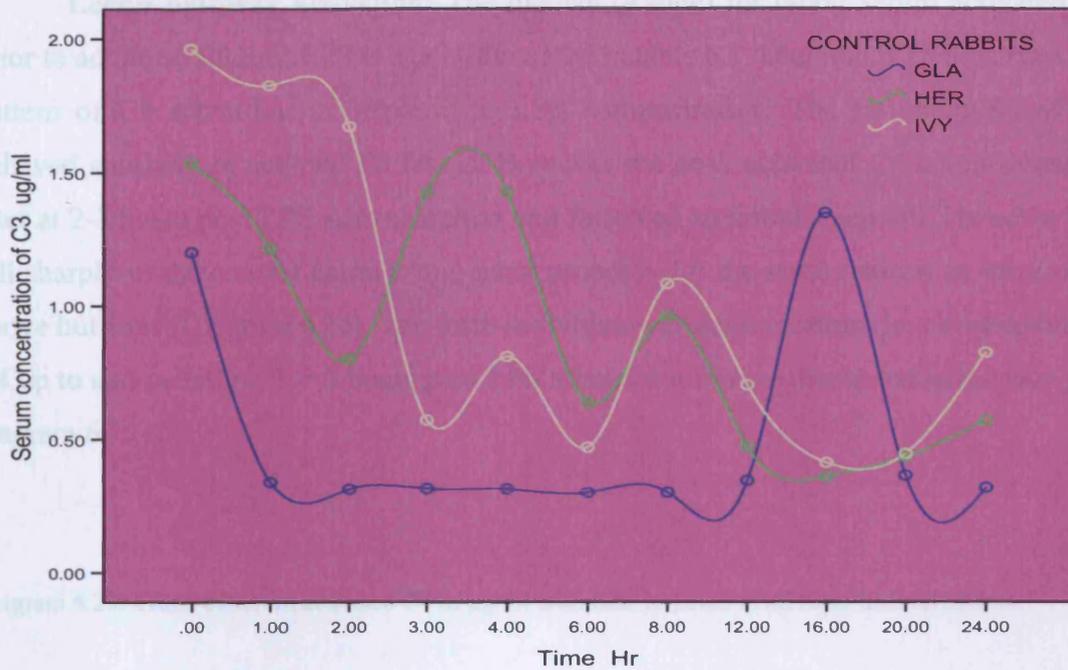


Diagram 6.24: Illustrating the inter-individual variation in serum C3 in $\mu\text{g/ml}$ at different times. Note that only one experimental animal in this group survived past 5 hours. Therefore the serum C3 level there after would not illustrate a variation and hence excluded. ° Represent the outliers. The numbers represent the ID number on the SPSS spread sheet.

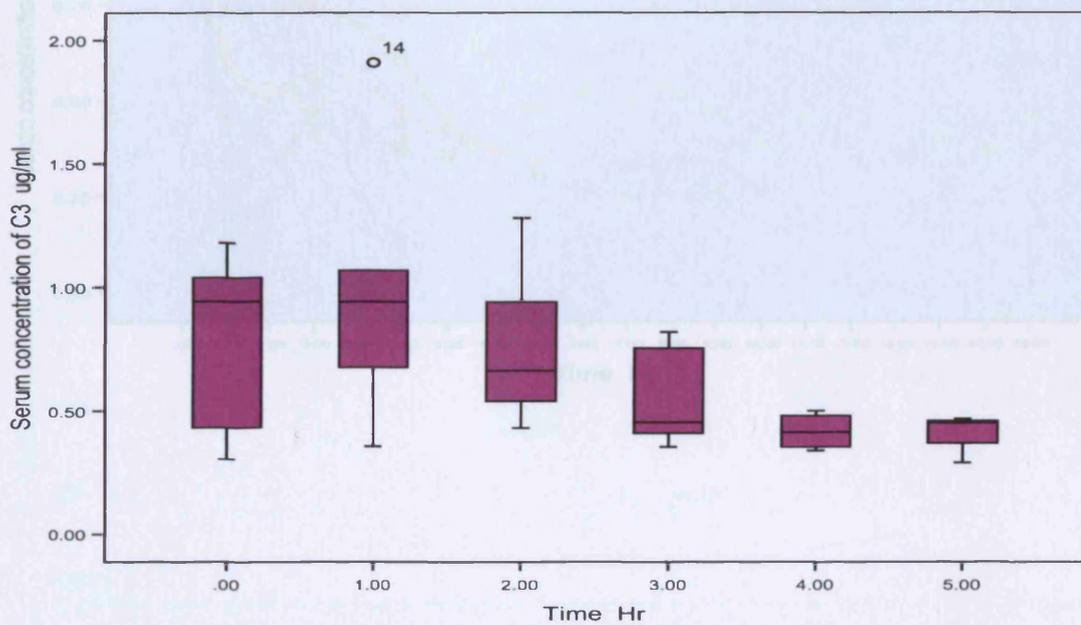


Diagram 6.25: Trend of serum activated C4 in $\mu\text{g/ml}$ with time in hours in all experimental rabbits

Lectin pathway activation: The median (Range) for rabbit serum activated C4 prior to administration of LPS is again illustrated in table 6.1. Diagram 6.25 illustrates the pattern of C4 activation in response to LPS administration. The serum levels of C4 behaved similarly to activate C3 levels. However the peak activated C4 levels occurred later at 2-3 hours post LPS administration and followed an initial steep fall. However this fell sharply in the control animals too, most probably for the same reasons as mentioned above but later (Diagram 6.26). The inter-individual variation in serum levels of activated C4 up to and including T = 5 hours post LPS administration are illustrated in the Box plot diagram 6.27.

Diagram 6.25: Trend of serum activated C4 in $\mu\text{g/ml}$ with time in hours in all experimental rabbits.

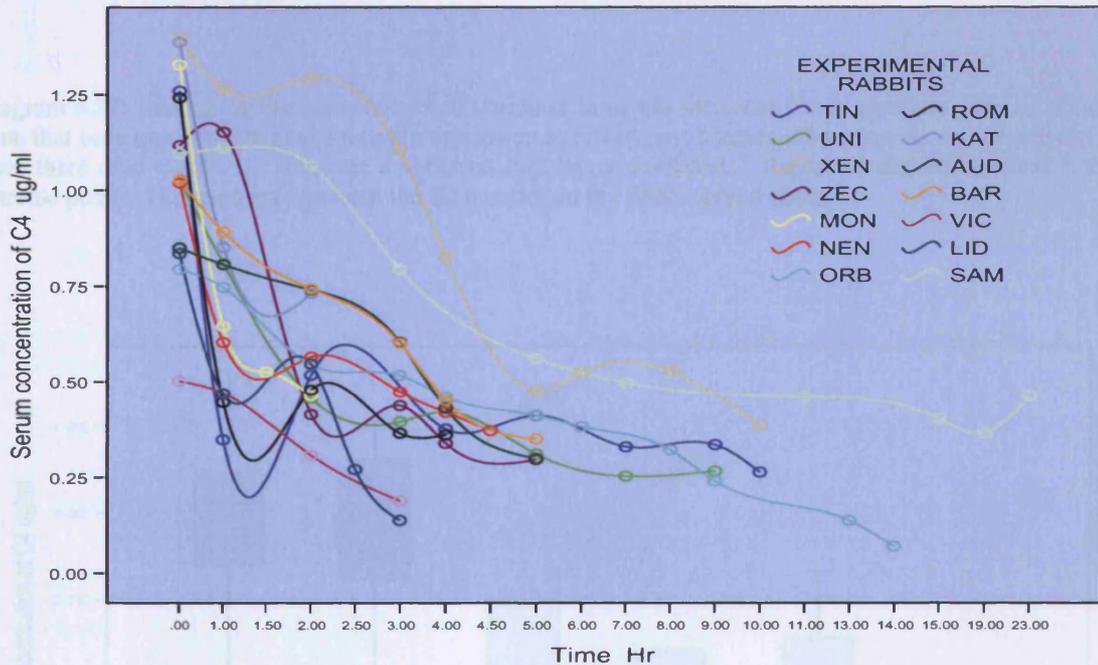


Diagram 6.26: Trend of serum activated C4 in $\mu\text{g/ml}$ with time in hours in all the control rabbits.

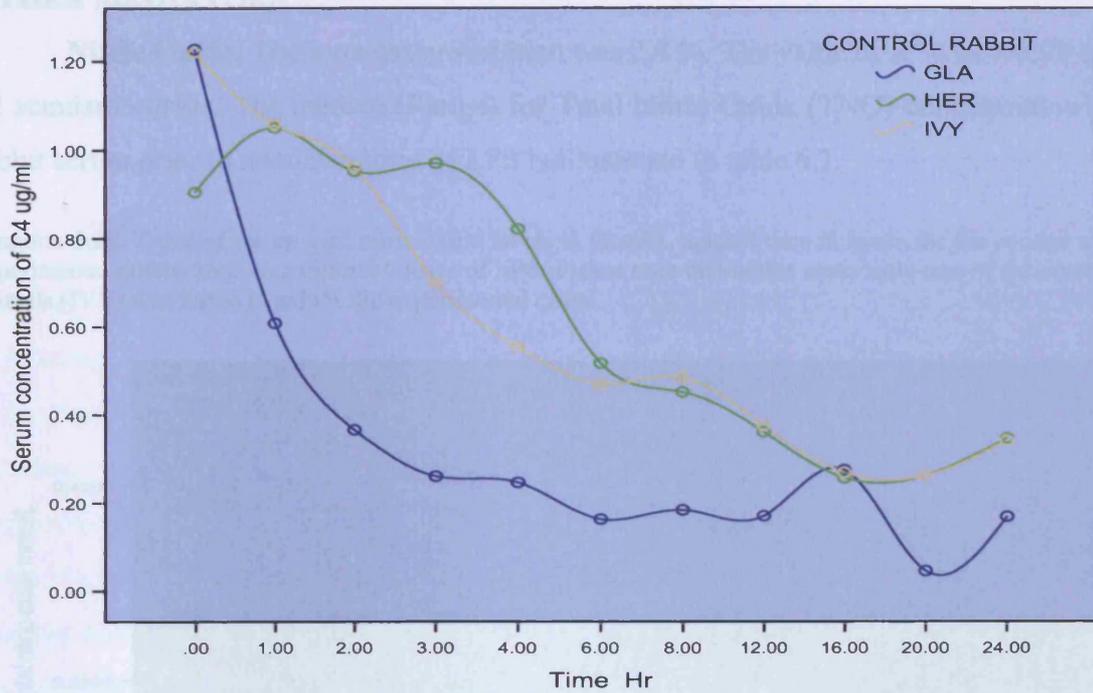
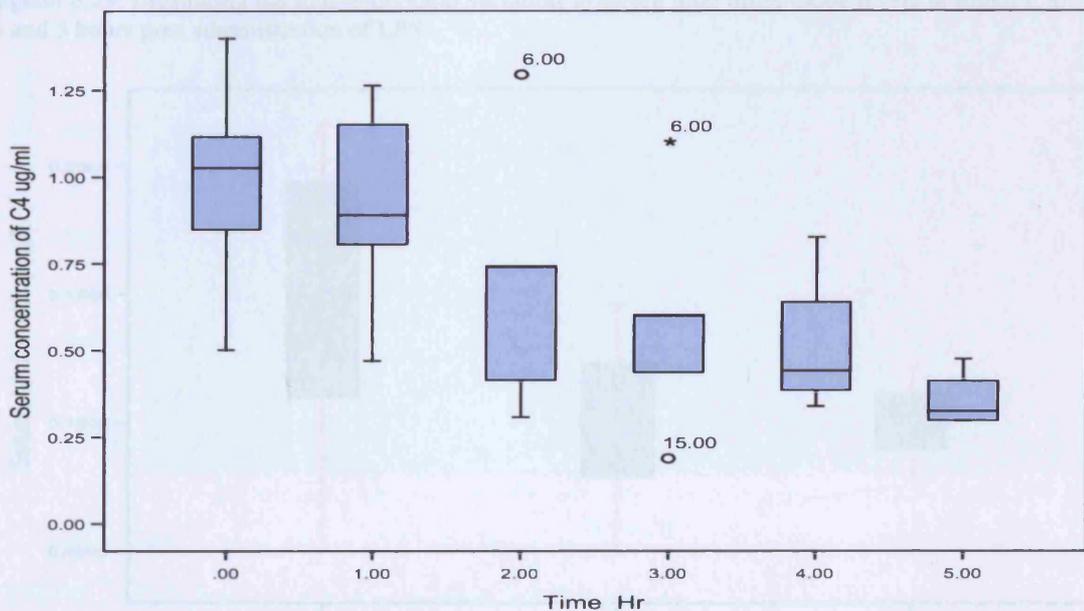


Diagram 6.27: Illustrating the inter-individual variation in serum activated C4 in $\mu\text{g/ml}$ at different times. Note that only one experimental animal in this group survived past 5 hours. Therefore the serum active C4 level there after would not illustrate a variation and hence excluded. ° Represent the outliers and * the extreme points. The numbers represent the ID number on the SPSS spread sheet.



OTHER MEDIATORS

Nitric Oxide: The intra assay variation was 2.4 %. The value of R^2 was > 0.99 for all standard curves. The median (Range) for Total Nitric Oxide (TNO) concentration in rabbit serum prior to administration of LPS is illustrated in table 6.1.

Diagram 6.28: Trend of serum total nitric oxide levels in mmol/L against time in hours for the control and experimental rabbits receiving different doses of LPS. Please note that in this assay only one of the control animals (IVY) was tested to reduce the experimental costs.

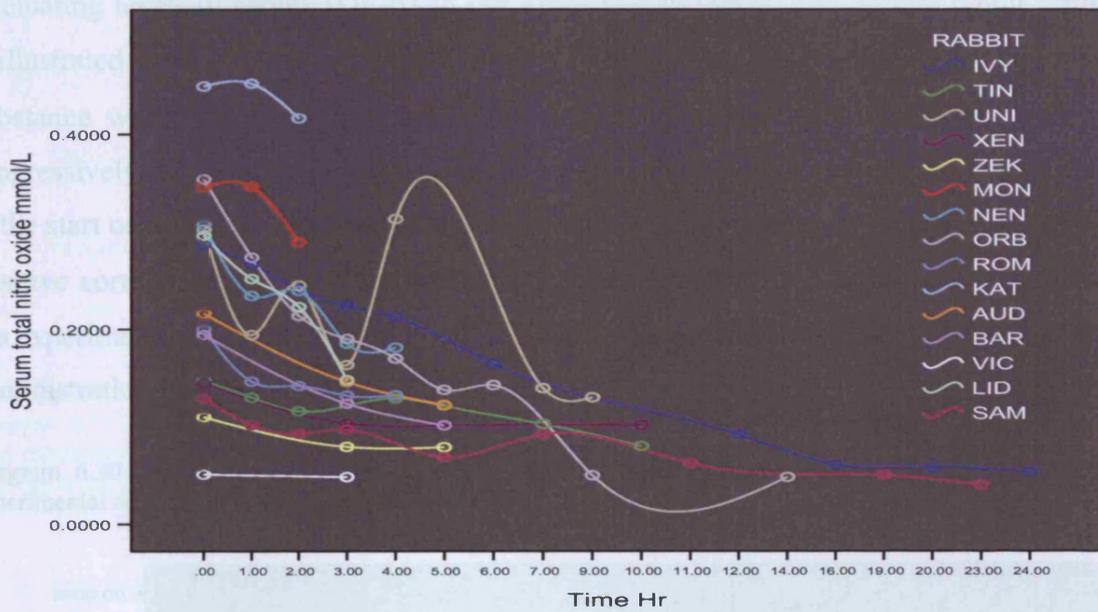


Diagram 6.29: Illustrating the inter-individual variation in serum total nitric oxide levels in mmol/L at T = 0,3 and 5 hours post administration of LPS.

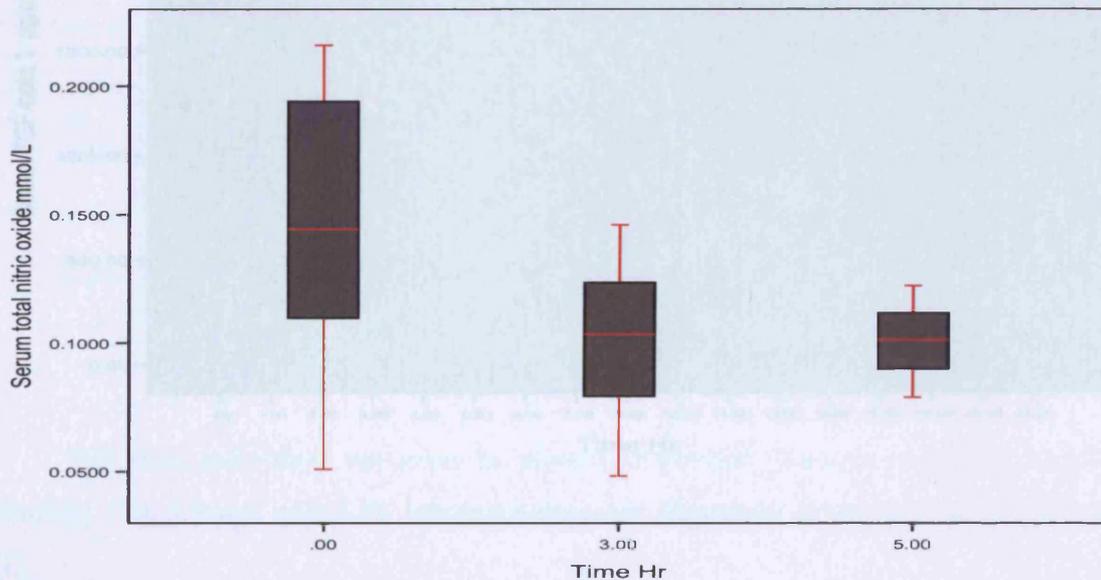
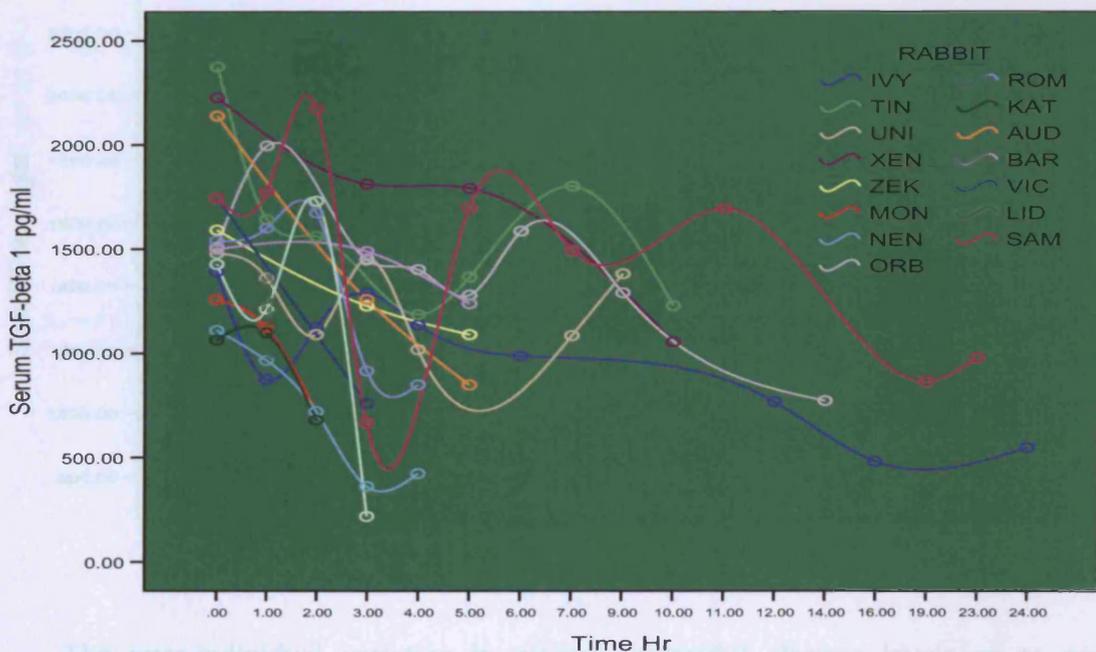


Diagram 6.28 illustrates the variation in TNO with time after administration of LPS. The TNO fell in all experimental animals. This diagram illustrates a large variation in TNO levels in serum from different rabbits. The inter-individual variation in serum TNO up to and including T = 5 hours post LPS administration are illustrated in the Box plot diagram 6.29.

TGF- β 1: The intra assay variation for transforming growth factor beta one assay was 3.0 %. The value of R^2 was > 0.99 for all standard curves. The median (Range) for circulating levels of serum TGF- β 1 in our experimental rabbits and normal rabbit serum is illustrated in table 6.1. Diagram 6.30 illustrates the variation in circulating levels of this substance with time from the start of the experiment. The serum levels of TGF- β 1 progressively fell in all rabbits including the control. The highest serum levels were either at the start or within the first two hours of the experiment. Diagram 6.31 illustrates strong positive correlation between the maximum TGF- β 1 levels within the first two hours of the experiment and survival time ($r = 0.61$). Same strong relation existed for the pre LPS administration levels ($r = 0.62$).

Diagram 6.30: Trend of serum TGF- β 1 levels in pg/ml against time in hours for the control and experimental rabbits receiving different doses of LPS.



The inter-individual variation in pre-LPS circulating TGF- β 1 levels up to and including T = 5 hours post LPS administration are illustrated in the Box plot diagram 6.32.

Diagram 6.31: illustrates the correlation between the maximum levels of TGF-β1 in pg/ml in the first 2 hours of LPS administration and survival time in hours.

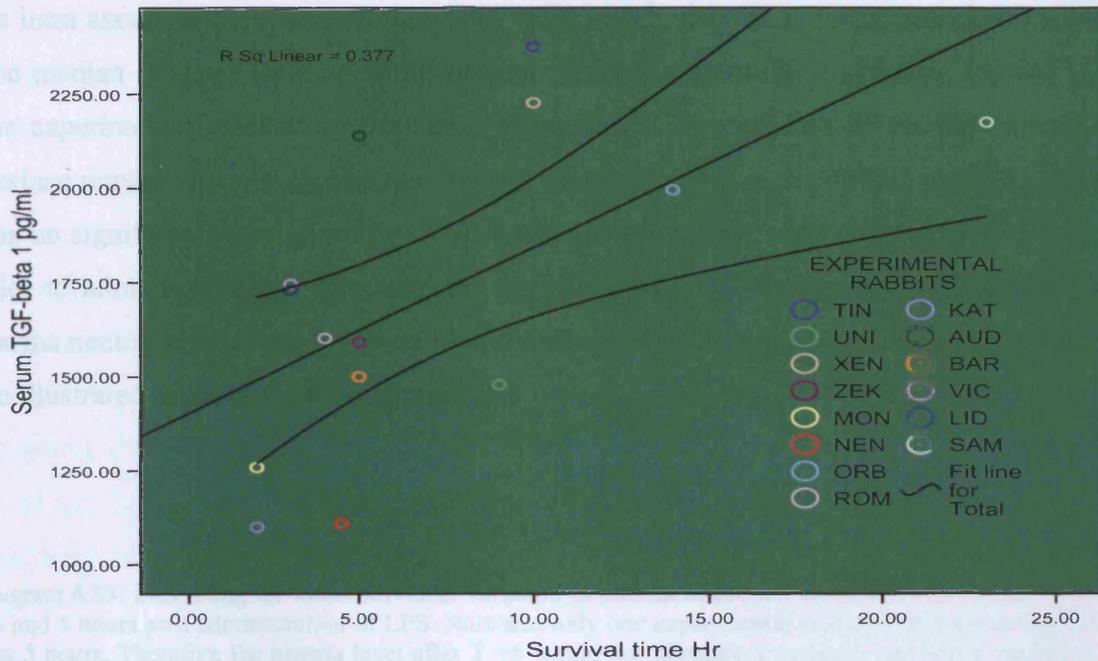
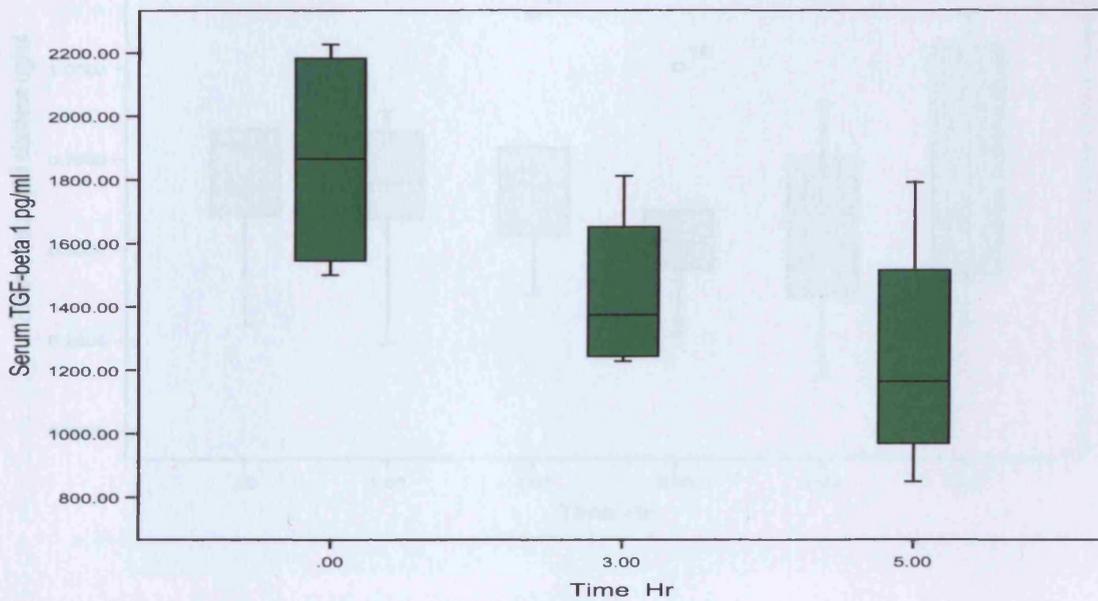


Diagram 6.32: illustrating the inter-individual variation in TGF-β1 levels in pg/ml at T = 0,3 and 5 hours post administration of LPS. Note that only one experimental animal in this group survived past 5 hours. Therefore the plasma level after T =5 would not illustrate a variation and therefore excluded.



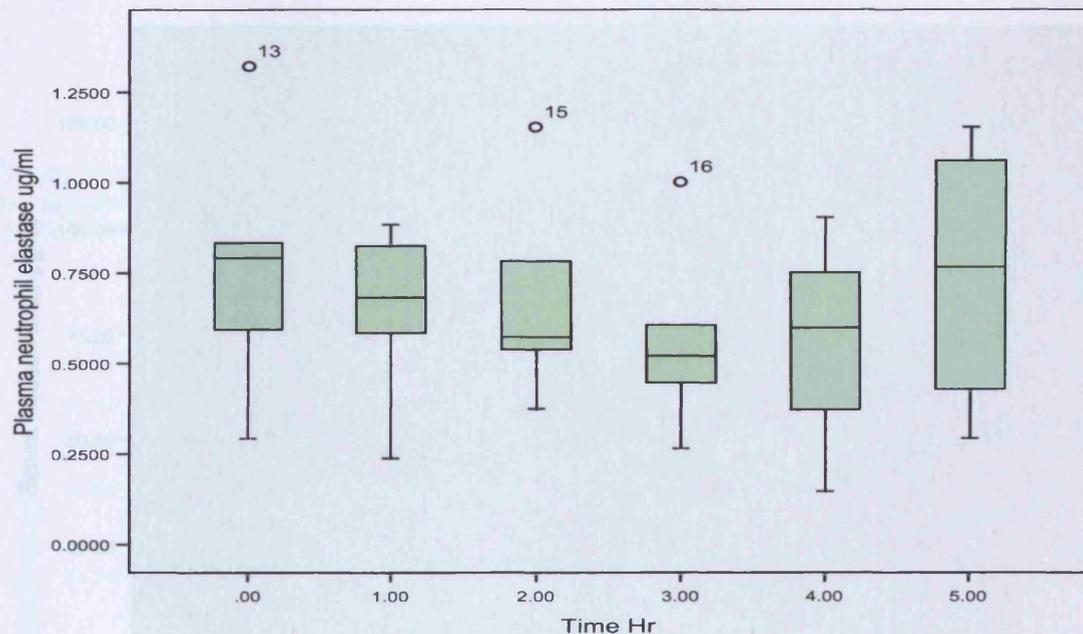
The inter-individual variation in plasma neutrophil elastase levels up to and including T = 5 hours post LPS administration are illustrated in the Box plot diagram 6.32.

COAGULATIVE MEDIATORS

Neutrophil Elastase: The inter assay variations ranged from 3.3 to 19.3 % and the intra assay variation was 30.6 %. The value of R^2 was > 0.95 for all standard curves. The median (Range) for circulating plasma levels of neutrophil elastase in normal and our experimental rabbits is illustrated in table 6.1. The plasma levels of neutrophil elastase remained stable for the majority of the control and experimental animals. There was no significant correlation ($r = 0.04$) between the plasma levels of neutrophil elastase prior to administration of LPS and the time to death. The inter-individual variation in plasma neutrophil elastase levels up to and including $T = 5$ hours post LPS administration are illustrated in the Box plot diagram 6.33.

TF levels (Diagram 6.34). The inter-individual variation in plasma TF levels up to and including $T = 5$ hours post LPS administration are illustrated in the Box plot diagram 6.35.

Diagram 6.33: illustrating the inter-individual variation in plasma neutrophil elastase levels in $\mu\text{g/L}$ at $T = 0, 3$ and 5 hours post administration of LPS. Note that only one experimental animal in this group survived past 5 hours. Therefore the plasma level after $T = 5$ would not illustrate a variation and hence excluded. $^{\circ}$ Represent the outliers. The numbers represent the ID number on the SPSS spread sheet.



COAGULATIVE MEDIATORS

Tissue Factor: The inter assay variations ranged from 6.6 to 16.1 % and the intra assay variation was 31.3 %. The R^2 was > 0.95 for all standard curves. The median (Range) for circulating levels of plasma TF in a normal and our experimental rabbits is illustrated in table 6.1. Diagram 6.34 illustrates the variation in circulating levels of Plasma Tissue Factor with time for the experimental and control rabbits. The plasma TF rose in all experimental rabbits compared with the control. This occurred within the first three hours and in the majority of cases in the first hour after the LPS administration. There was a medium positive correlation ($r = 0.48$) between the dose of LPS and the peak TF levels (Diagram 6.35). The inter-individual variation in plasma Tissue Factor levels up to and including $T = 5$ hours post LPS administration are illustrated in the Box plot diagram 6.36.

Diagram 6.34: Trend of plasma TF levels in pg/ml against time in hours for the control and experimental rabbits pre and post administration of a varying dose of LPS in mg/Kg.

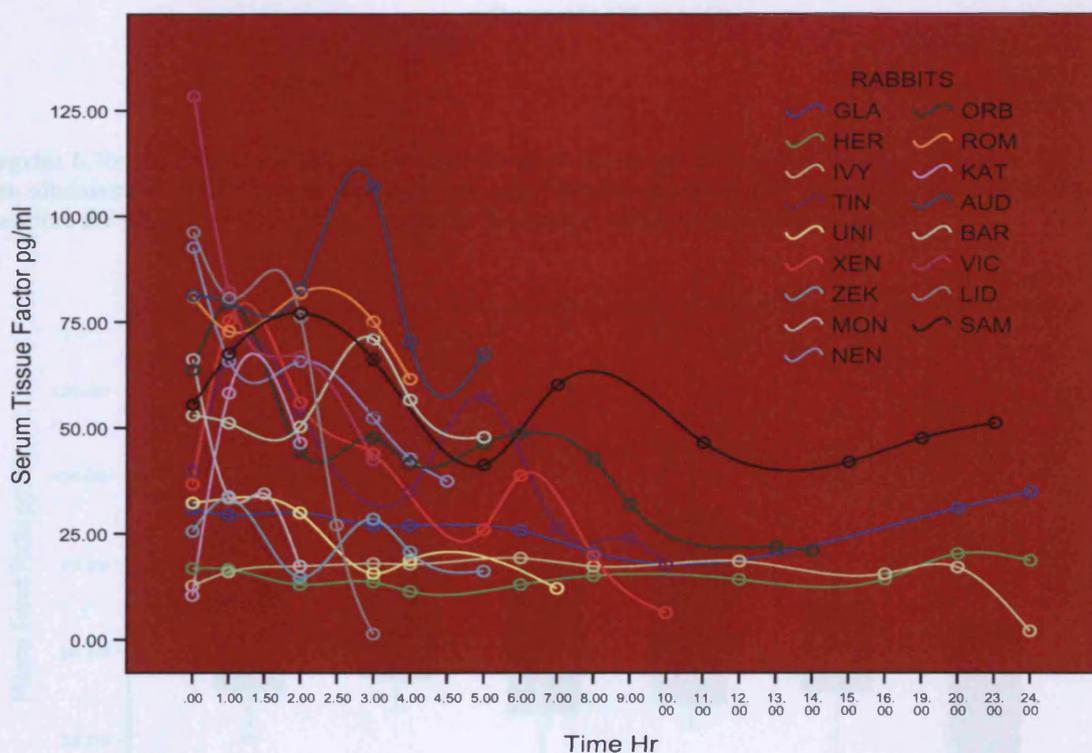


Diagram 6.35: Illustrates the correlation between the peak plasma levels of Tissue Factor in pg/ml and the dose of administered LPS in mg/kg body weight.

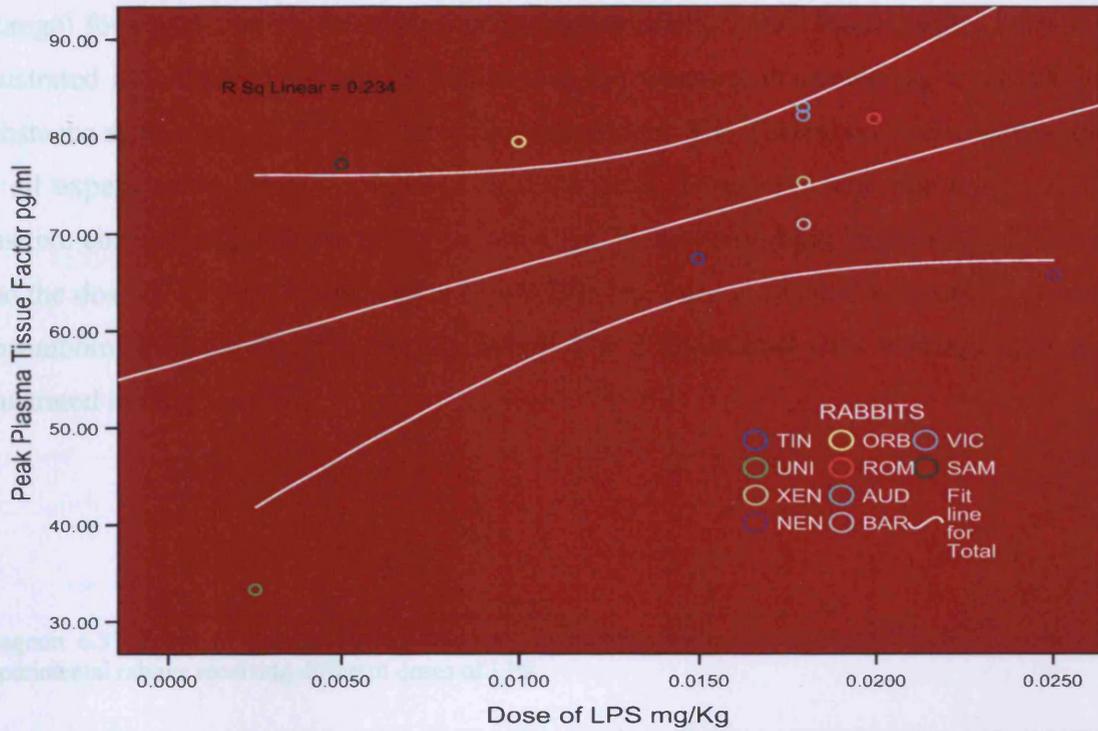
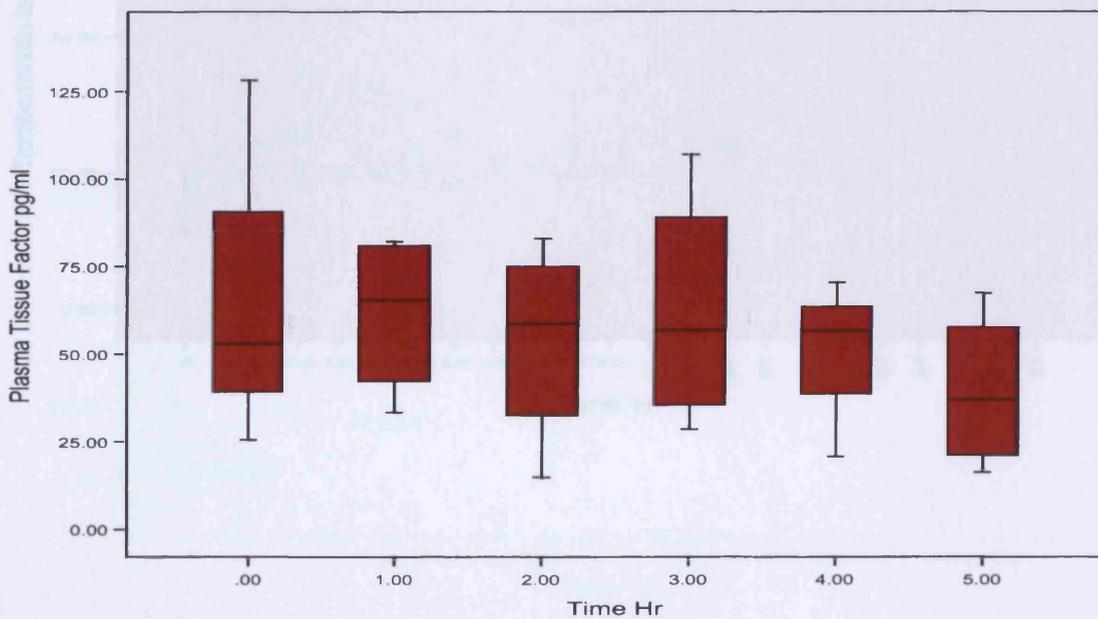


Diagram 6.36: Illustrating the inter-individual variation in plasma TF levels in pg/ml at T = 0 to 5 hours post administration of LPS. Note that only one experimental animal in this group survived past 5 hours. Therefore the plasma level after T =5 would not illustrate a variation and hence excluded.



Thrombomodulin: The inter assay variations ranged from 1.9 to 6.1 % and the intra assay variation was 12.68 %. The R^2 was > 0.98 for all standard curves. The median (Range) for circulating levels of plasma Thrombomodulin in our experimental rabbits is illustrated in table 6.1. Diagram 6.37 illustrates the variation in circulating levels of this substance against time from the start of the experiment. The Thrombomodulin levels rose in all experimental animals compared to the control. However there was only a small positive correlation (Diagram 6.38), between the Thrombomodulin levels at T = 3 hours and the dose of LPS per rabbit weight ($r = 0.27$). The inter-individual variation in plasma Thrombomodulin levels up to and including T = 5 hours post LPS administration are illustrated in diagram 6.39.

Diagram 6.37 Trend of plasma Thrombomodulin in ng/ml against time in hours for the control and experimental rabbits receiving different doses of LPS.

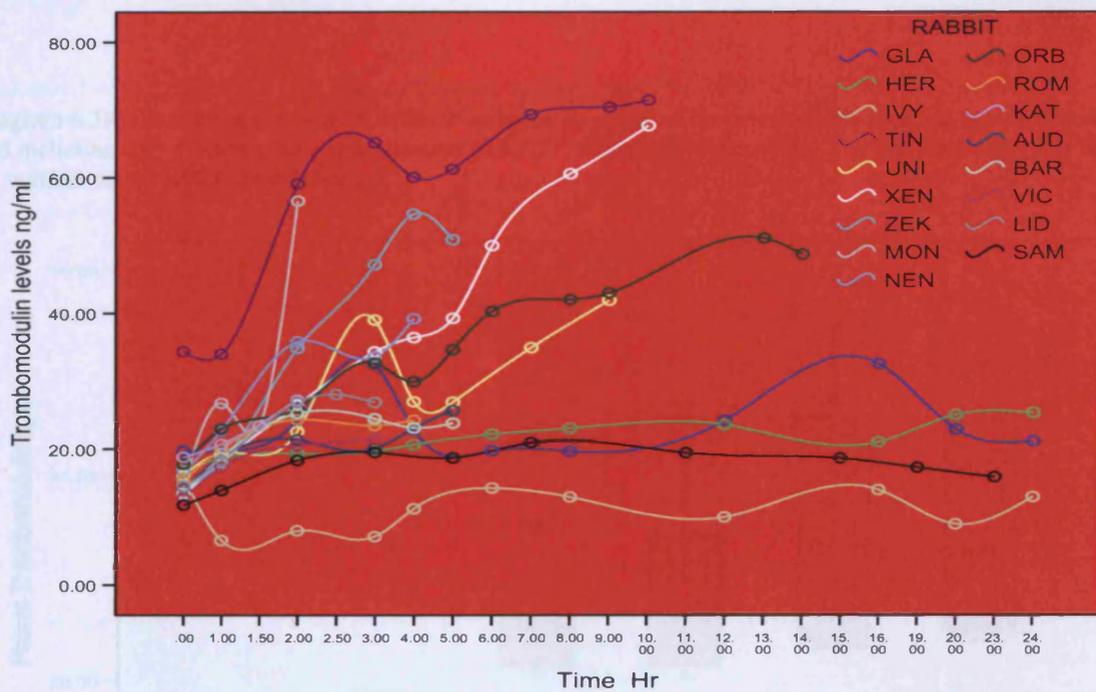


Diagram 6.38: Illustrates the correlation between the plasma levels of Thrombomodulin in ng/ml at T = 3 hours after administration of LPS and the dose of LPS in mg/kg body weight.

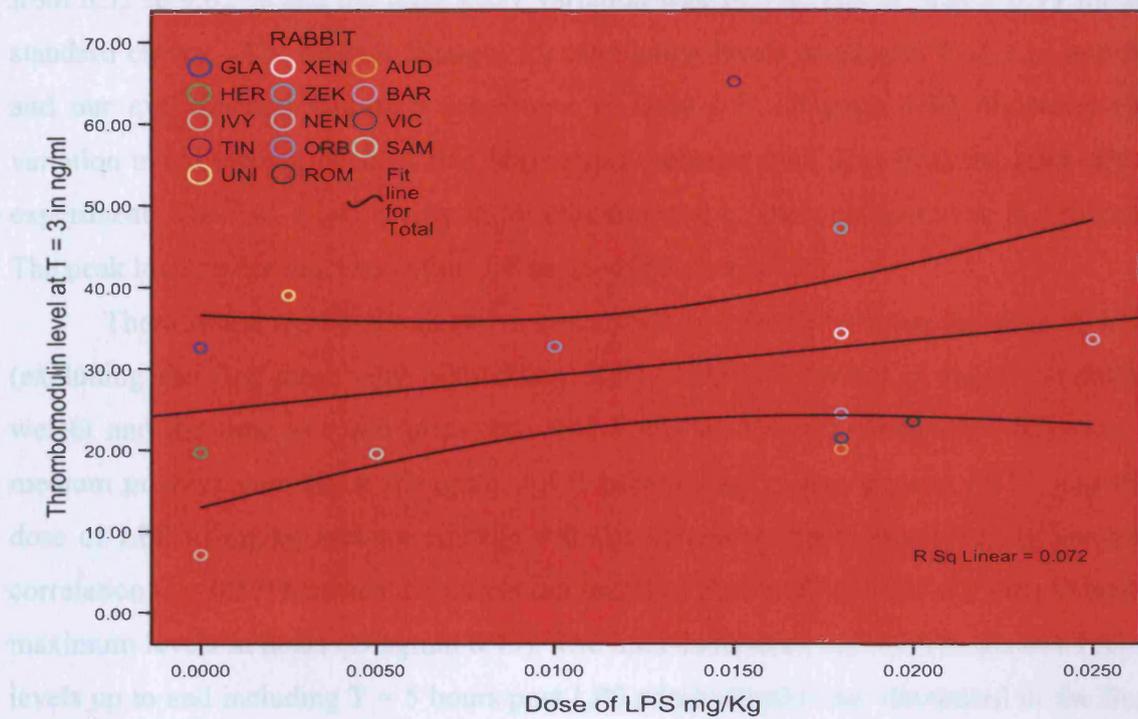
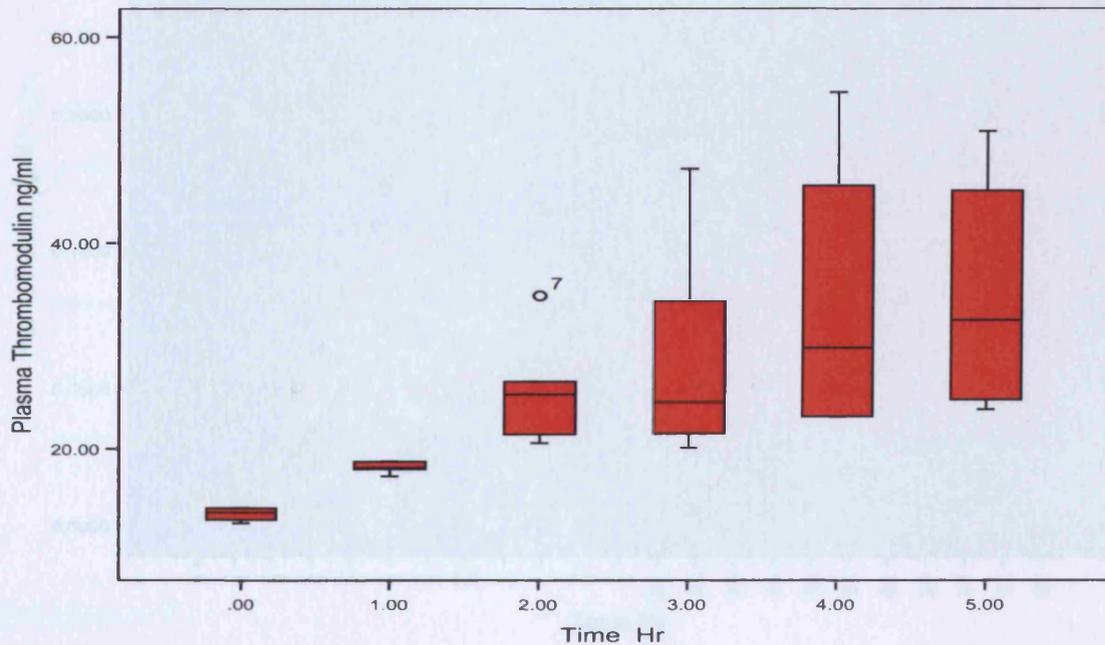


Diagram 6.39: Illustrating the inter-individual variation in plasma Thrombomodulin levels in ng/ml at up to and including T = 5 hours post administration of LPS. ° Represent the outliers. The numbers represent the ID number on the SPSS spread sheet.



FIBRINOLYTIC MEDIATORS

Plasminogen activator Inhibitor-1 (PAI-1): The inter assay variations ranged from 6.31 to 9.02 % and the intra assay variation was 14.7%. The R^2 was > 0.99 for all standard curves. The median (Range) for circulating levels of plasma PAI-1 in normal and our experimental rabbits is illustrated in table 6.1. Diagram 6.40 illustrates the variation in circulating levels of this fibrinolytic mediator with time from the start of the experiment. The PAI-1 levels rose in all experimental animals compared to the control. The peak levels were reached within 3-8 hours of receiving LPS.

There was a reasonable negative correlation ($r = 0.45$) between the dose of LPS (excluding the first three very high doses, KAT, LID and MON) in mg/ml of rabbit weight and the time to reach maximum PAI-1 levels in $\mu\text{g/ml}$ (Diagram 6.41), also a medium positive correlation (Diagram 6.42) between maximum plasma PAI-1 and the dose of LPS in mg/kg rabbit weight ($r = 0.32$). However there was a strong positive correlation ($r = 0.57$) between the maximum levels of plasma PAI-1 and the time to reach maximum levels in hours (Diagram 6.43). The inter-individual variation in plasma PAI-1 levels up to and including $T = 5$ hours post LPS administration are illustrated in the Box plot diagram 6.44.

Diagram 6.40: Trend of plasma PAI-1 in $\mu\text{g/ml}$ against time in hours for the control and experimental rabbits receiving different doses of LPS.

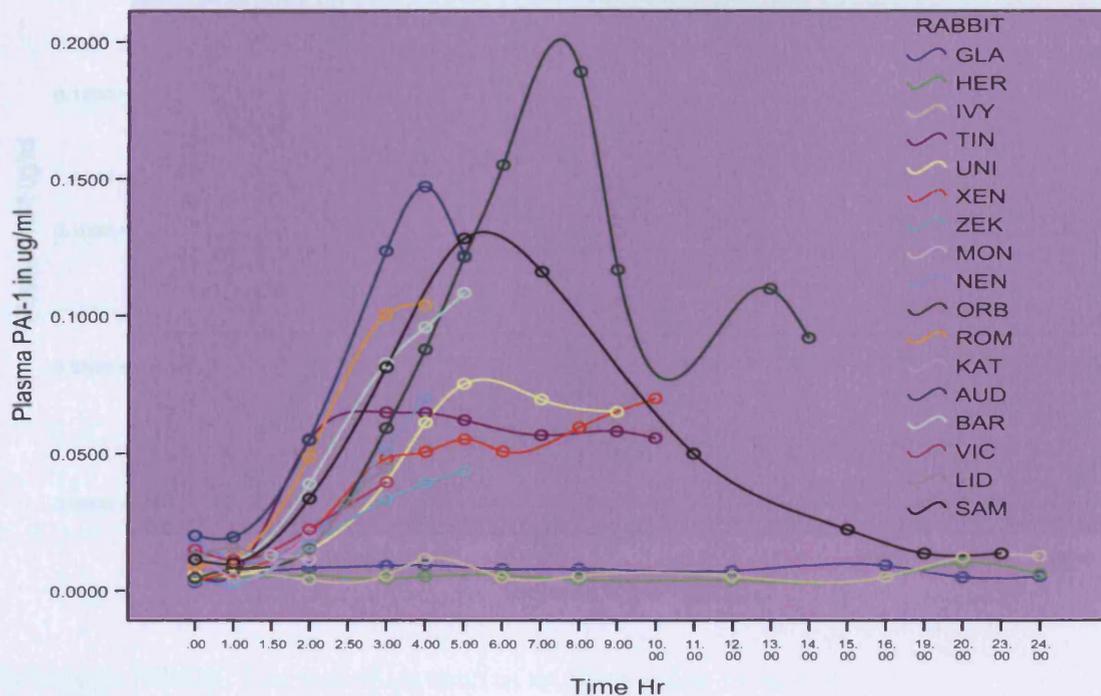


Diagram 6.41: Illustrates the correlation between the maximum plasma levels of PAI-1 in $\mu\text{g/ml}$ and the time to reach maximum levels in hours.

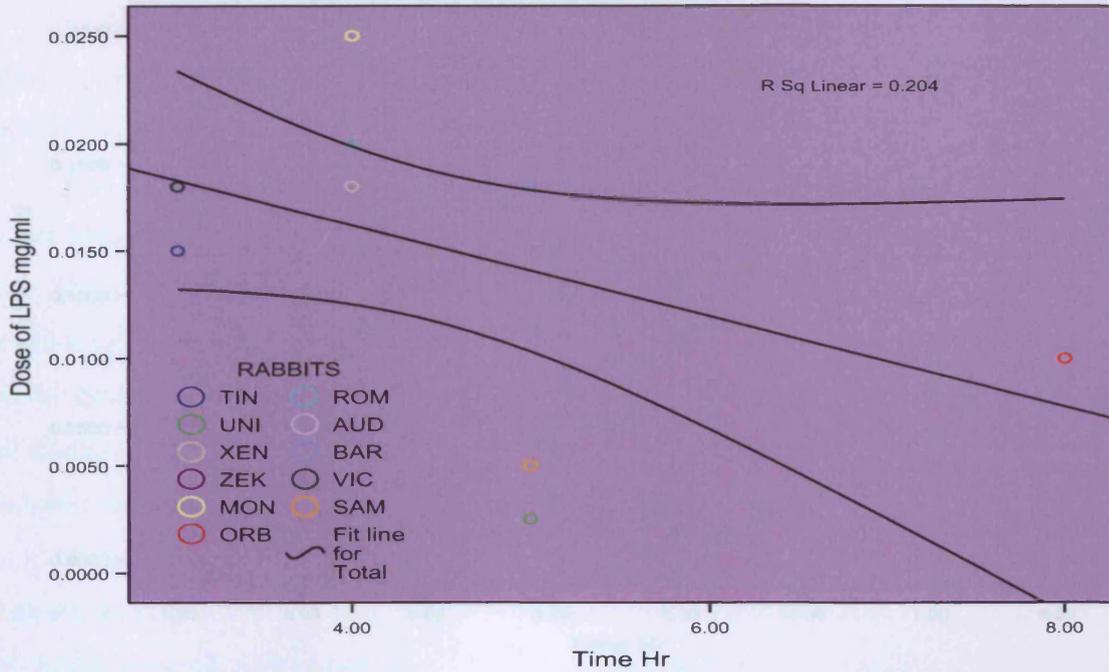
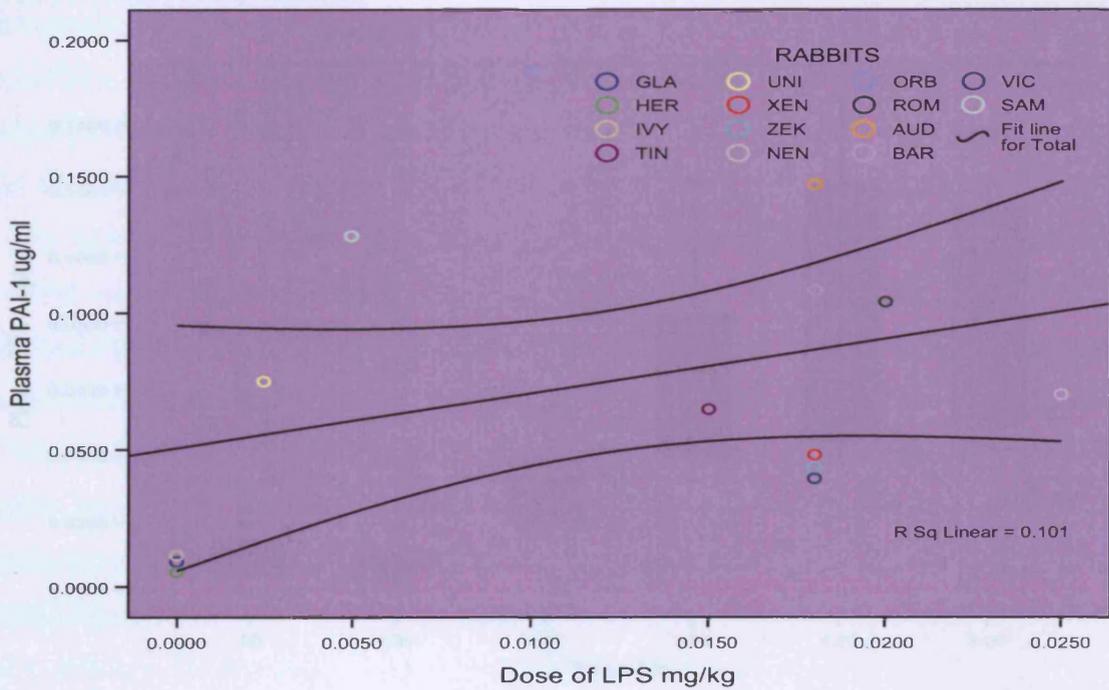


Diagram 6.42: Illustrates the correlation between the maximum plasma levels of PAI-1 in $\mu\text{g/ml}$ and the dose of LPS per rabbit body weight in mg/kg .



Fibrinogen (FNG): This was illustrated in the same plots above.

Diagram 6.43: Illustrates the correlation between the maximum plasma levels of PAI-1 in $\mu\text{g/ml}$ and the time to reach that maximum level in hours.

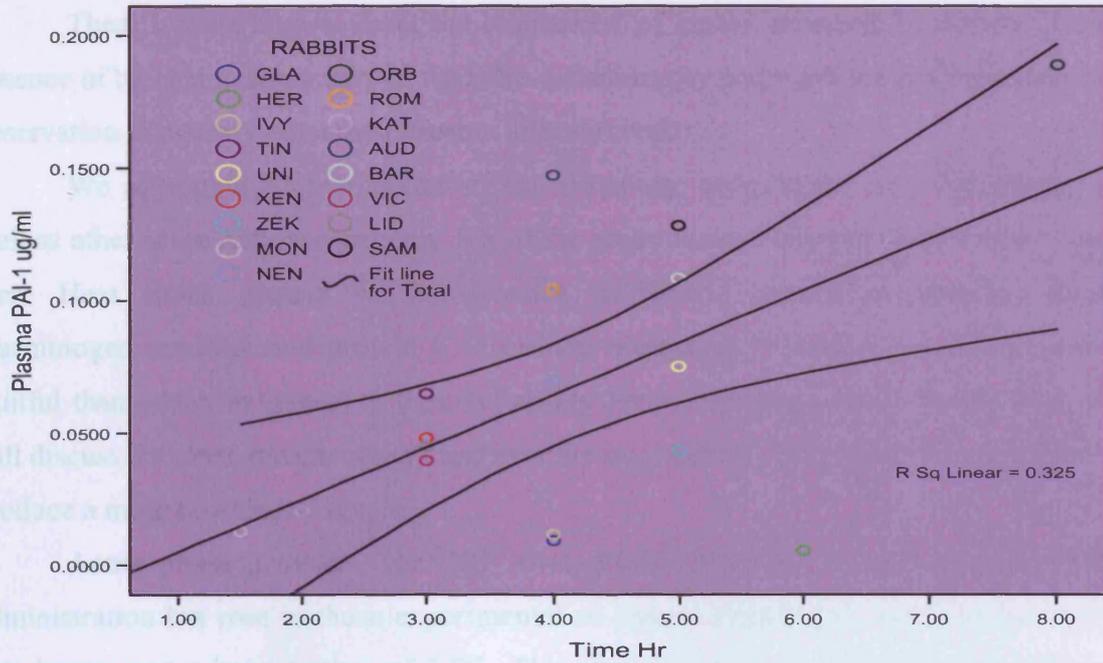
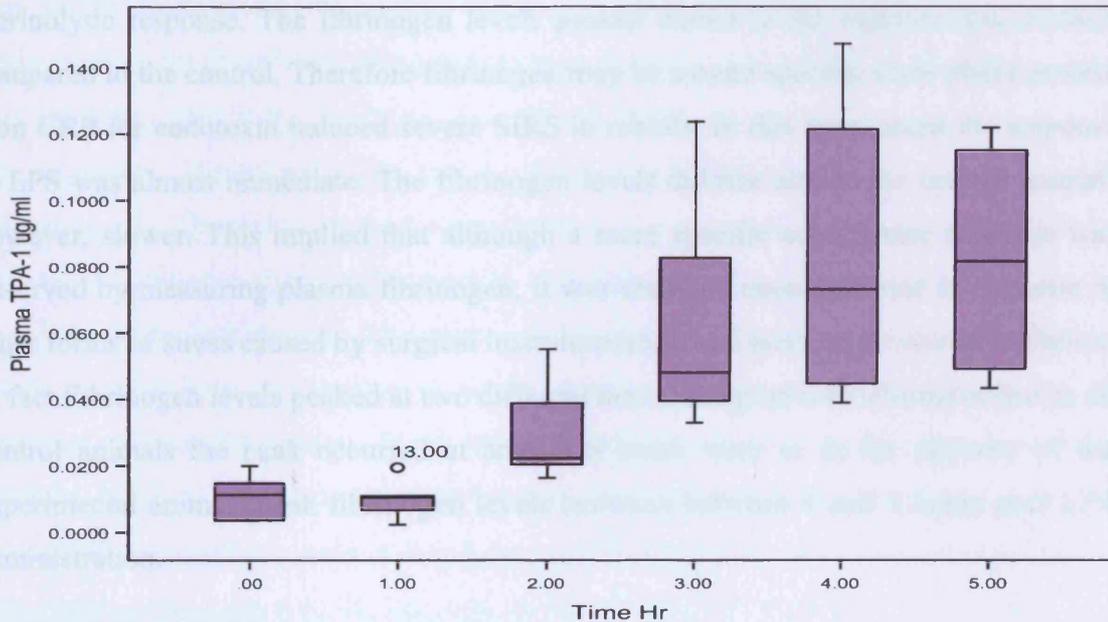


Diagram 6.44 illustrating the inter-individual variation in plasma PAI-1 levels in $\mu\text{g/ml}$ up to and including T = 5 hours post administration of LPS. Only one experimental animal in this group survived past 5 hours, therefore the plasma levels after T = 5 were excluded. $^{\circ}$ Represent the outliers. The numbers represent the ID number on the SPSS spread sheet.



Fibrinogen (FNG): This was illustrated as an acute phase protein above.

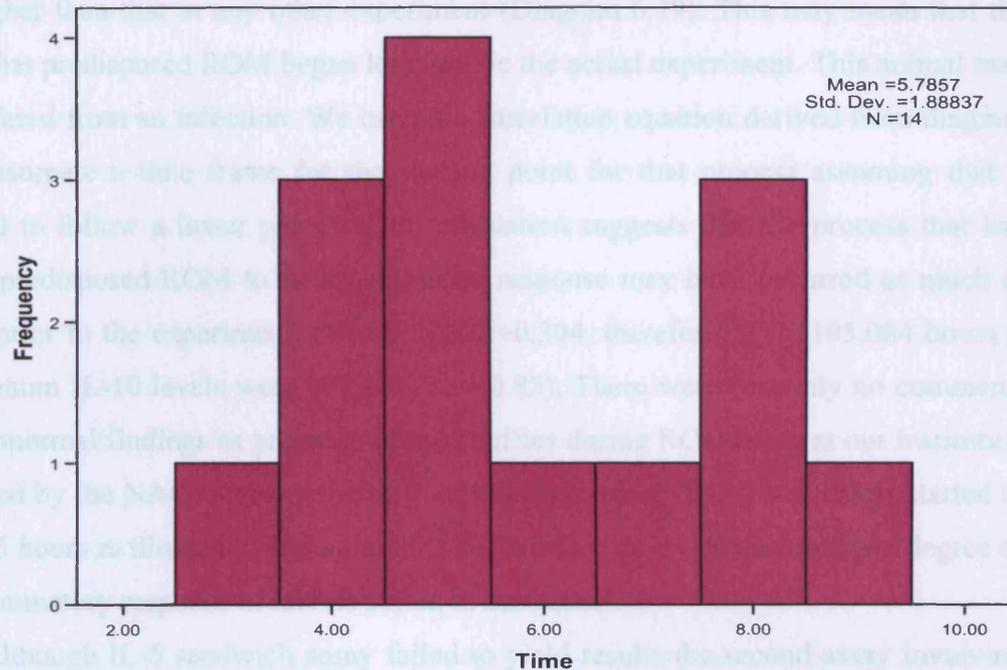
DISCUSSION

There is little known about the biomarkers of innate immunity in rabbits. In the absence of biochemical markers of thrombo-inflammatory pathways we are limited in our observation to primary outcome measures like survival.

We attempted 22 biomarkers of inflammatory, coagulative, and fibrinolytic as well as other acute response proteins. Six of the above assays failed to yield results. They were Heat shock protein 60, sL-Selectin, E-Selectin, Interferon gamma, tissue plasminogen activator and protein C. From the remaining 16 assays, some were more fruitful than others in respect to their suitability for our desired animal model. Here we will discuss the most suitable assays and how we may modify the other assays in future to produce a more meaningful result.

Acute phase proteins: The CRP levels fell in all rabbits immediately post LPS administration but rose in those experimental or control rabbits that survived the initial few hours post administration of LPS. This response was not significantly different between the control and the experimental groups. This may be related to the effect of instrumentation and ventilation as well as the LPS. The CRP rise may take as much as 12-15 hours. This makes CRP unsuitable for our model. In contrast Fibrinogen levels were assessed for their role as an acute phase protein as well as a component of the anti-fibrinolytic response. The fibrinogen levels peaked earlier in the experimental animals compared to the control. Therefore fibrinogen may be a more specific acute phase protein than CRP for endotoxin induced severe SIRS in rabbits. In this experiment the response to LPS was almost immediate. The fibrinogen levels did rise also in the control animals however, slower. This implied that although a more specific acute phase response was observed by measuring plasma fibrinogen, it was sensitive enough to rise in response to other forms of stress caused by surgical instrumentation and positive pressure ventilation. In fact Fibrinogen levels peaked at two different times. Diagram 6.45 illustrates that in all control animals the peak occurred at around 8 hours were as in the majority of the experimental animals peak fibrinogen levels occurred between 4 and 5 hours post LPS administration.

Diagram 6.45: Plot of the frequency to peak levels of plasma Fibrinogen versus time to peak levels in hours. This demonstrates a bimodal distribution. The first peak corresponds to the experimental animals and the second peak the controls.



Cytokines: Cytokines IL-6 was the most suitable marker. Its levels rose slowly and peaked within five hours of LPS administration. TNF α was the second best. Its levels rose within two hours of LPS administration. Therefore using TNF α alone may not allow adequate time for installation of the extracorporeal circuit and a treatment strategy. TNF α may be best suited if the treatment was started before or within the first hour of LPS administration. The animal ROM had an abnormally high level of TNF α from the start (Diagram 6.5). The levels reached a maximum at T = 1 hour and fell appropriately. This animal did not die abnormally quickly and followed the dose response pattern as described in the last chapter. This may indicate that the time to death is not dependent solely on peak level or the time to reach peak levels. However conclusion cannot be based on a single observation. ROM continued to have an abnormally high levels of the cytokine IL-1 β , IL-6 and IL-10 from the start which also peaked T = 1 hour. This was particularly interesting in case of IL-1 β , as no other rabbit had shown a significant rise in this cytokine (Diagram 6.9). IL-1 β levels in ROM who had levels three times higher than the rest of the animals to start with sharply fell after an initial peak at T = 1. This may illustrate that the levels of IL-1 β only rise in certain predisposed rabbits.

The serum level of the anti-inflammatory cytokine IL-10 for ROM followed a similar pattern as the other cytokines. However the serum IL-10 concentration for ROM at the start of the experiment was massively raised with levels in excess of 400 ng/ml, much higher than that in any other experiment (Diagram 6.19). This may mean that the process that predisposed ROM began long before the actual experiment. This animal may have suffered from an infection. We used the correlation equation derived from diagram 6.20 to estimate a time frame for the starting point for that process assuming that it continued to follow a linear pattern. Our calculation suggests that the process that had possibly predisposed ROM to such heightened response may have occurred as much as 46 days prior to the experiment. ($Y = 0.3926X + 0.304$, therefore $X = 1105.084$ hours if the maximum IL-10 levels were 434.161, $r^2 = 0.85$). There were certainly no comments on any abnormal findings or presence of the snuffles during ROMs stay at our institution as reported by the NACWO or at the time of the experiment. The IL-10 levels started to rise at 4-5 hours as illustrated in diagram 6.21. Therefore its levels may indicate degree of anti-inflammatory response in rabbits suited to our model.

Although IL-5 sandwich assay failed to yield results the second assay involving the antigen (rhIL-5) as a capture antibody (Solid phase) tested the goat antibody and concluded that this anti human antibody must have only a single epitope for human IL-5, which once bound to the solid phase will leave no other site for the attachment of the IL-5 and so ability to bind to the detection antibody. We followed this experiment by designing a competitive inhibition assay, however due to time constraints we were unable to optimise. IL-5 is an important mediator of eosinophil activation, one of the important features of the acute lung injury in rabbits as will be examined in the next chapter.

Chemokines: The assay of chemokine IL-8 showed favourable results. The levels of this pro-inflammatory protein rose in response to LPS at a desired time frame. It reached peak levels at four hours as illustrated by diagram 6.18.

Complements: The levels of activated C3 and C4 were almost a mirror image of each other. As activated C3 levels peaked activated C4 levels fell. These levels in ROM did not behave differently to other experimental animals in response to LPS administration. This suggests that the path of complement activation in rabbits is via the alternative pathway and not via the lectin pathway in rabbits treated with LPS. However the time course of activation was almost immediate therefore as for TNF α this assay may

be best utilised if the treatment was started before or within the first hour of LPS administration.

Other inflammatory mediators: The total nitric oxide levels fell to its lowest within three hours of LPS administration. Nitric oxide is a major secretory product of mammalian cells that initiates host defence. Its function is both as an extra-cellular affecter and an intra-cellular signal. The total nitric oxide levels fell in both experimental and control animals. This implies no specific extra-cellular secretion in response to LPS in rabbits. A fall in exhaled nitric oxide levels in absence of inflammatory response is known to be due to epithelial damage caused by abnormal stresses related to opening and closing of small airways in a rabbit model {D'Angelo et al. 2007}. This may also imply heightened intra-cellular consumption of nitric oxide in response to positive pressure ventilation hence contribute to the mechanisms leading to lung injury by apoptosis. This therefore raises a question, does administration of nitric oxide in ventilated patients fuels apoptosis during positive pressure ventilation?

TGF- β 1 is known as an "Immunosuppressive cytokine" {McDonanell et al. 1999}. TGF- β 1 is a useful assay to assess susceptibility of rabbits to develop SIRS/sepsis. Diagram 6.31 illustrates that the higher the pre LPS levels of TGF- β 1 the better the outcome in terms of survival. This implies that TGF- β 1 is a good predictor of survival in septic rabbits. For this reason TGF- β 1 is a very useful marker for our model.

The plasma levels of neutrophil elastase remained stable after LPS administration and were similar to that of the control. It is highly unlikely therefore that this assay would yield a useful result in response to LPS. However although highly unlikely the variability in the plasma levels may be a reflection of the relatively high inter and intra plate coefficient of variation rather than true serum variability. Therefore if this assay were to be repeated attention would be required to reduce the intra and inter assay variability. The current result shows a small trough at T = 3 hours (Diagram 6.33).

Central and Coagulative mediators: Thrombomodulin appears to be a specific marker of innate response to LPS in rabbits. The persistently high levels of Thrombomodulin compared to other biomarkers, which declined within a short few hours of LPS administration are significant. Its levels continue to rise and may be measurable hours post insult (Diagram 6.37). Thrombomodulin is the only one of the biomarkers in this extensive experiment that behaves in this manner. Therefore Thrombomodulin is a useful thrombo-inflammatory marker especially in this model. Activated partial

thromboplastin time discussed in the last chapter is the best coagulative mediator in contrast to Tissue factor that failed to produce a visible response even though highly specific rabbit antibodies were used. It is therefore not recommended for use in this model. However if this assay were to be repeated attention would be required to reducing the intra and inter assay variability.

Fibrinolytic mediators: PAI-1 proved to be a useful biomarker of thrombo-inflammatory pathway activation by LPS in rabbits. However although the peak plasma levels correlated with the dose of LPS administered, the rate of its production was not dependent on the severity of the insult. All this however occurred within the first five hours especially in the inter-individual variation experiment (Diagram 6.43). Therefore both PAI-1 and Fibrinogen are good markers of thrombo-inflammatory pathways leading to SIRS in this rabbit model.

CONCLUSION:

Of the sixteen assays that produced meaningful results we have chosen a handful, which would be useful in our specific model of severe SIRS/sepsis in rabbits. After administration of LPS the rabbit is placed on an extracorporeal system in order to test various new strategies to brake the vicious cycle of STIP and reduce haematic response. The critical factor in this design is the time course of change in the plasma/serum protein expression that can be detected reliably and reproducibly with small inter-individual variability. Fibrinogen is ideal as an acute phase reactant and an anti-fibrinolytic marker. IL-6 as a pro-inflammatory, IL-10 as an anti-inflammatory cytokine and IL-8, the only chemokine studied, all proved to be useful markers in this rabbit model. The “Immunosuppressive cytokine” TGF- β 1 levels pre administration of LPS was a useful indicator of predisposition and so a predictor of outcome of endotoxic challenge. Thrombomodulin was a useful central marker of STIP and may be used in association with APTT a coagulative or PAI-1 an anti-fibrinolytic biomarker of the STIP in this model. In cases where the treatment strategy is already or readily installed within the first hour of LPS administration, TNF α and C3 complement activation assays may be used. C3 activation assay is a useful assay for measuring changes in the first stage of haematic response. What is certain is that more than one marker should be tested in future experiments to improve our perception of any significant alteration to the progression of SIRS and deactivation of STIP or the haematic response in rabbits.

Chapter Seven

CELLULAR COMPONENTS OF THE INNATE RESPONSE IN RABBITS

INTRODUCTION

Acute Respiratory Distress Syndrome (ARDS) is one of the commonest manifestations of Severe SIRS. It is really the effects of this whole body disease seen in the lung. The most important pathological finding in the lung during the early stages of ARDS is severe pulmonary oedema, due to increased permeability of the capillary endothelial and alveolar epithelial barriers (Exudative phase). Furthermore there is an increase in pulmonary vascular resistance (PVR) due to vasoconstriction and thromboembolism, which further increases the pulmonary oedema {ten Cate et al. 2001}, also bronchoconstriction, and bronchial hyper-responsiveness. The severe lung inflammatory response is characterized by activation of the cellular components of the innate response such as alveolar macrophages, lymphocytes, eosinophils and recruitment of polymorphonuclear leukocytes (PMNs) into the airways. This results in the release of proinflammatory cytokines (e.g., tumour necrosis factor TNF α , interleukin IL-1 β and IL-6), chemokines (e.g. IL-8, macrophage inflammatory protein MIP-2), eicosanoids (e.g., leukotriene LTB $_4$), reactive oxygen species, cytolytic proteases and lysosomal enzymes {Michel et al. 1997, Wohlford-lenane et al. 1999 & Lefort et al. 1998}. In this chapter we concentrate on the cellular components of inflammatory response responsible for acute lung injury leading to acute respiratory distress syndrome in rabbits.

MATERIALS AND METHODS

We have already described the laboratory setting, the experimental methodology, optimisation and quality control used in determining the inflammatory response in a rabbits lung (Chapter 2). A semi-quantitative histopathological scoring system is used in order to assess the inflammatory response in a rabbits lung. This scoring system is based on the presence or absence of perivascular oedema, alveolar haemorrhage, thrombosis in pulmonary vessels, alveolar consolidation with perivascular/peribronchial acute inflammation, goblet-cell hyperplasia / metaplasia of bronchioles, presence of large macrophages in alveolar spaces, lymphoid aggregates, multinucleated giant cells, eosinophils, large eosinophilic intracytoplasmic inclusions (crystals) in macrophages and mononuclear and polymorphonuclear leukocytes.

RESULTS

PERIVASCULAR OEDEMA: Perivascular oedema was scored using the following; 0, absence of oedema in the low power fields; 1, mild to moderate, involving fewer than 25% of the perivascular spaces; 2, moderate to severe, involving more than 25% but less than 75% of perivascular spaces; or 3, severe, involving more than 75% of perivascular spaces. Diagram 7.1 illustrates severe perivascular oedema in one of the control animals.

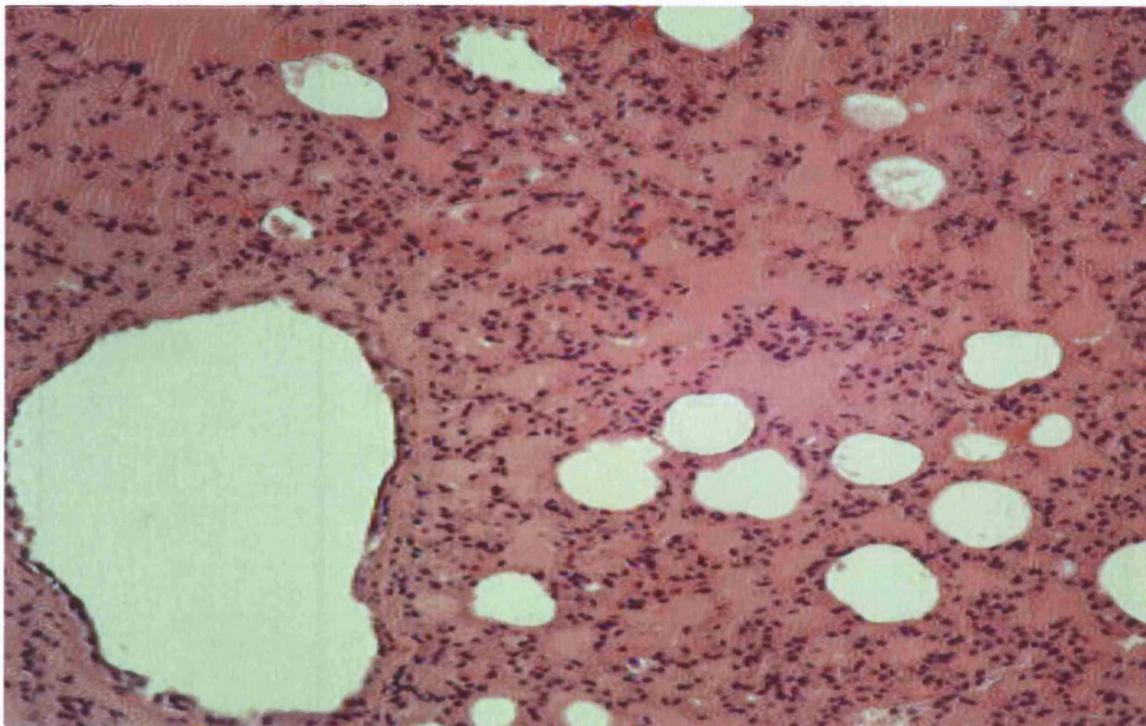


Diagram 7.1: Illustrating severe pulmonary oedema in a control rabbit. Low power field (X4 objective).

The three control rabbits had the highest score for pulmonary oedema. They were ventilated for 24 hours. The experimental animals that were ventilated the longest also had higher perivascular oedema. This was confirmed also by measurement of their wet/dry ratio. The control animals lung had significantly higher wet dry ratio and hence tissue water than the experimental animals ($p = 0.001$).

Table 7.1: Rabbit lung wet to dry ratios.

	Rabbit Identity code	Dose of LPS (mg/Kg)	Survival time (Hr)	Wet/Dry Ratio (%)	Control vs. Experimental Groups	
1	UNI	.0025	13.00	81.40	E	
2	SAM	.0050	23.00	79.82	E	
3	ORB	.0100	14.00	Missing	E	
4	TIN	.0150	10.00	86.04	E	
5	ROM	.0200	4.00	82.61	E	
6	NEN	.0250	5.00	80.87	E	
7	MON	.2500	2.00	83.16	E	
8	LID	.5000	3.00	78.92	E	
9	NEN	1.0000	2.00	83.45	E	
10	VIC	.0200	3.00	84.59	E	
11	ROM	.0200	10.00	80.48	E	
12	KAT	.0200	5.00	79.28	E	
13	AUD	.0200	5.00	83.28	E	
14	BAR	.0200	5.00	86.51	E	
15	GLA	.0000	24.00	94.78	C	
16	HER	.0000	24.00	94.09	C	
17	IVY	.0000	24.00	83.79	C	
N	17	17	17	16	17	
Total	Mean	9.0000	.113382	10.3529	83.9404	1.1765
	Std. Deviation	5.04975	.2618846	8.44794	4.66790	.39295

ALVEOLAR HAEMORRHAGE: Alveolar haemorrhage was scored using the H&E slides: 0, absent haemorrhage from all low power fields; 1, mild to moderate, involving fewer than 25% of the alveolar spaces; 2, moderate to severe, involving more than 25% but less than 75% of alveolar spaces; or 3, severe, involving more than 75% of alveolar spaces. Diagram 7.2 illustrates severe pulmonary haemorrhage. This was not a feature in the control and normal negative control rabbits.

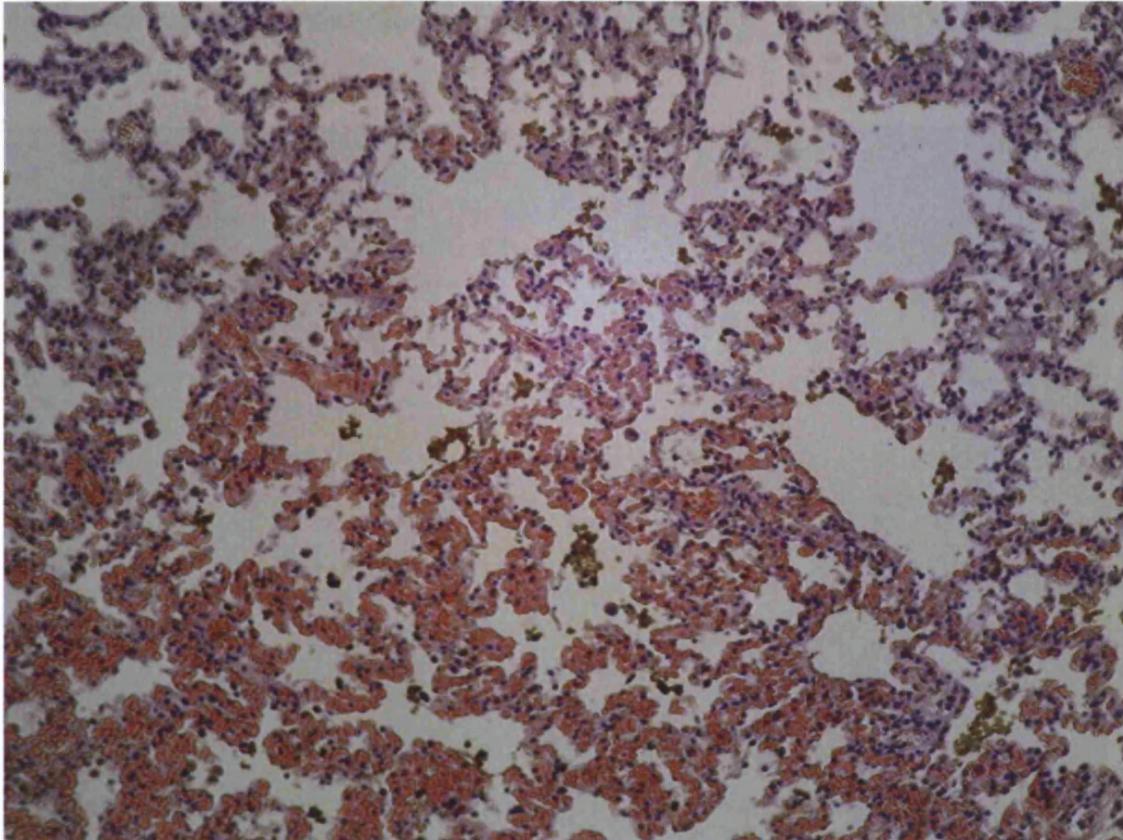


Diagram 7.2: Severe pulmonary haemorrhage in an experimental animal. Low power field (X 4 objective).

THROMBOSIS IN PULMONARY VESSELS: Thrombosis in pulmonary vessels was differentiated from the presence of blood in the vessels by the homogeneity of the thrombus with no clear red cell morphology (Diagram 7.3). H&E slides was scored as; 0, absence of pulmonary thrombus; 1, mild to moderate, involving fewer than 25% of the pulmonary vessels; 2, moderate to severe, involving more than 25% but less than 75% of pulmonary vessels; or 3, severe, involving more than 75% of pulmonary vessels. Mild pulmonary thrombosis was observed in three of the experimental rabbits however the only severe thrombosis was observed in a control animal.

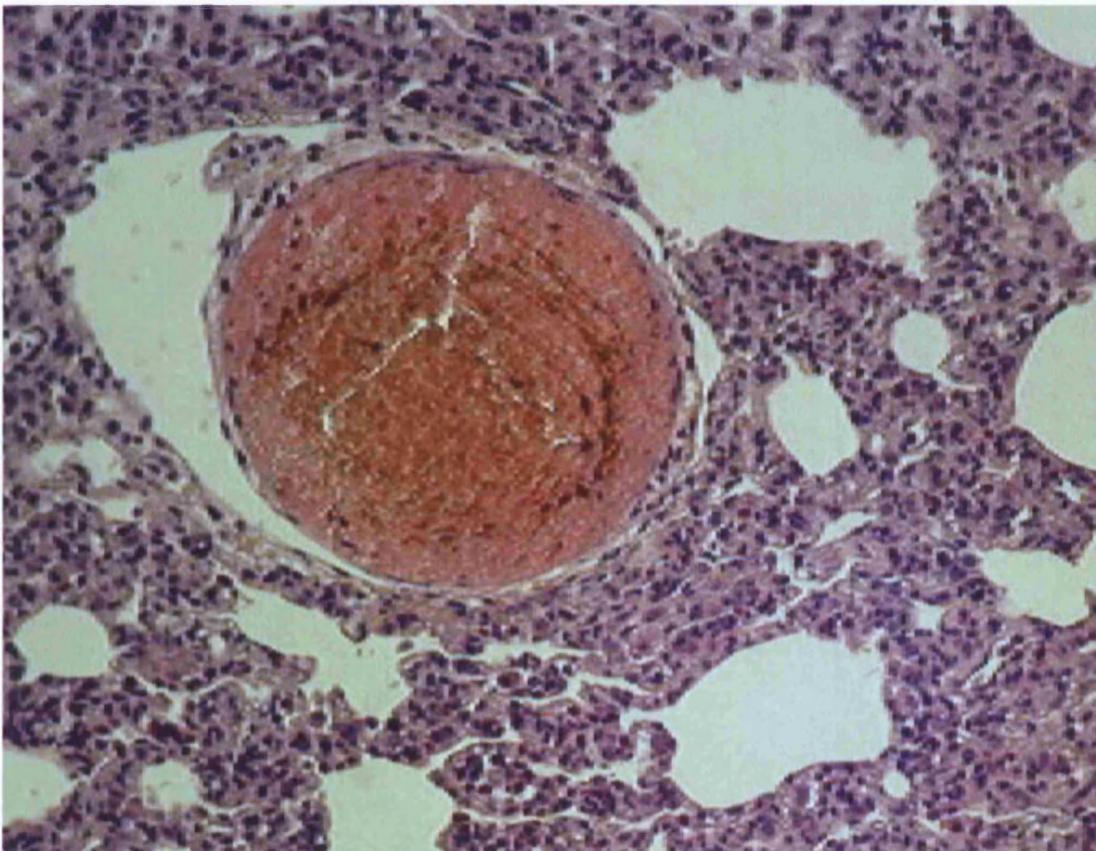


Diagram 7.3: A pulmonary thrombus in an area of collapsed consolidated rabbit lung. Medium power magnification (x 10 objective).

ALVEOLAR CONSOLIDATION: Alveolar consolidation with mononuclear and polymorphonuclear leukocytes or mixed inflammation was reviewed using the H&E slides in low power magnification (x4 objective). The following is a guide for scoring this component of lung injury scoring system. 0, absent areas of consolidation in any of the low power fields; 1, mild to moderate, involving fewer than 25% of the lung; 2, moderate to severe, involving more than 25% but less than 75% of the lung; or 3, severe, involving more than 75% of the lung. Diagram 7.4 illustrates the histological appearance of the lung in one of the normal negative control rabbits.

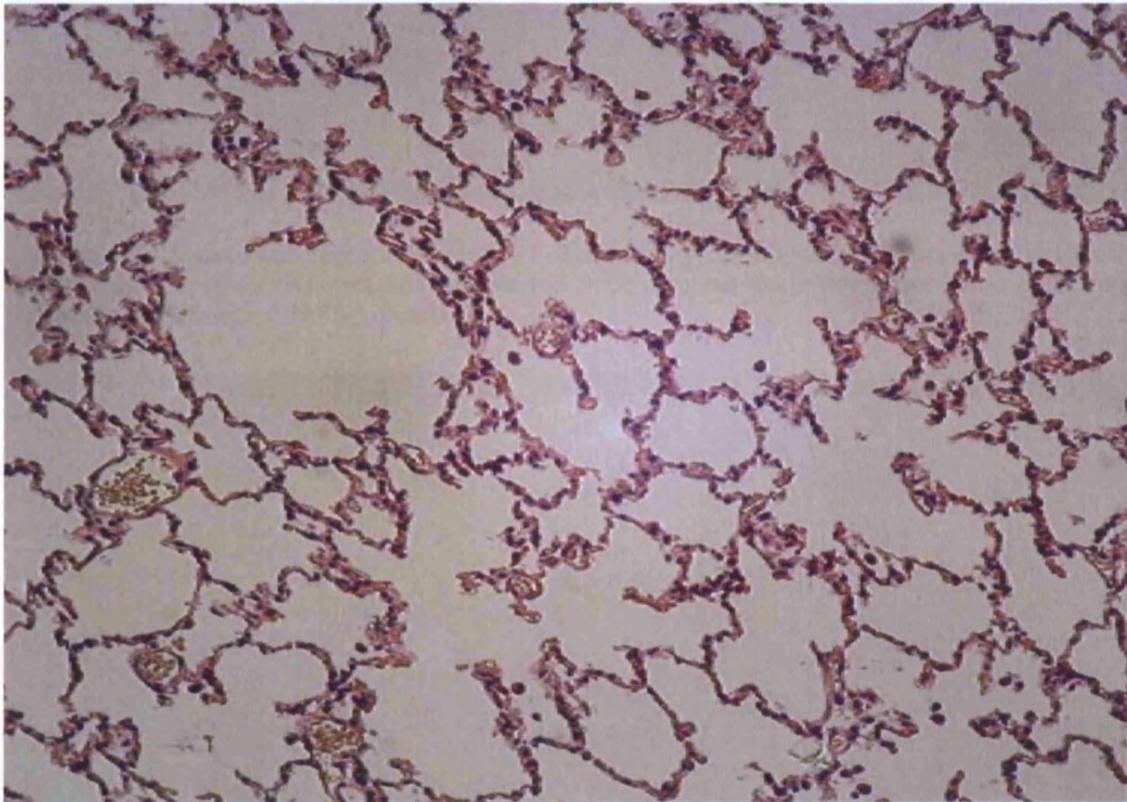


Diagram 7.4: Normal rabbit lung. Low power field (X4 objective)

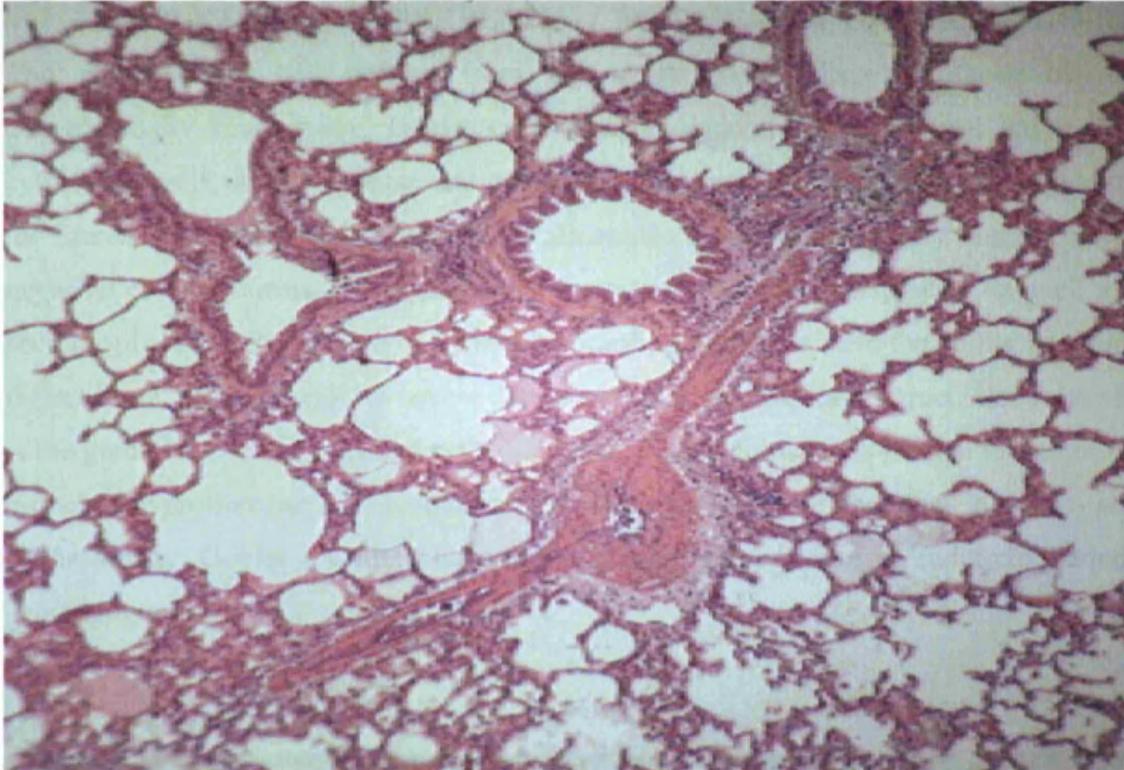
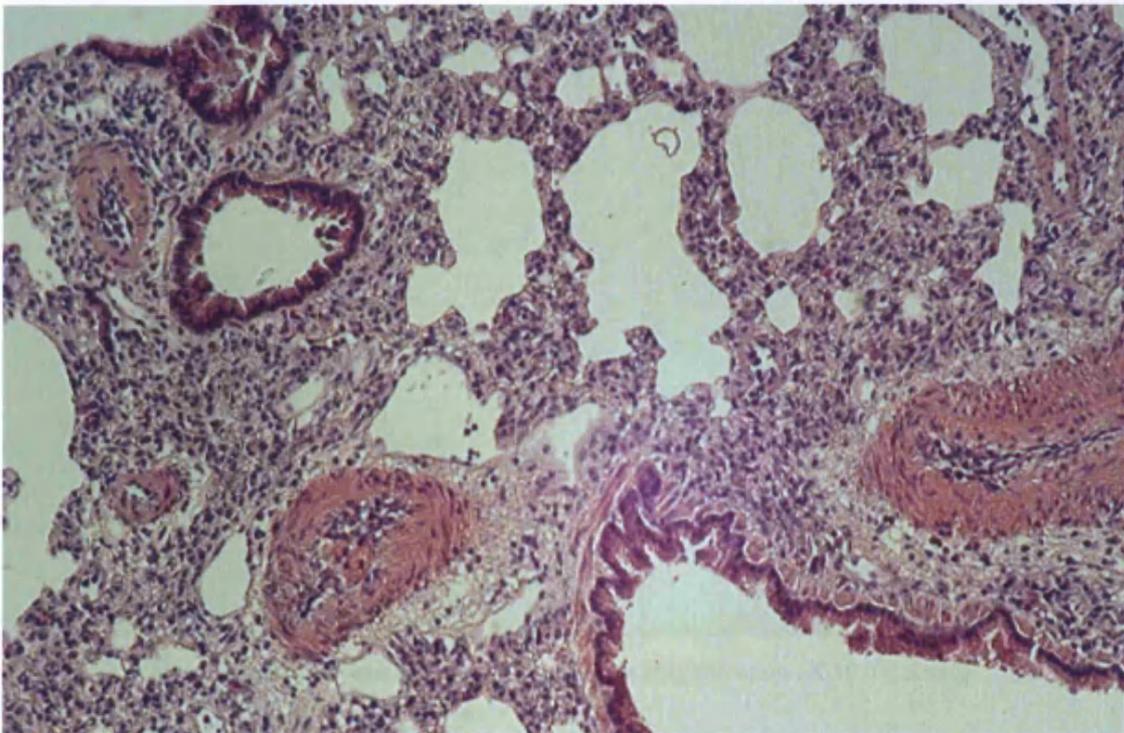


Diagram 7.5 (Above) and diagram 7.6 (Below): Respectively show mild to moderate and moderate to severe alveolar consolidation with mononuclear and polymorphonuclear leukocytes or mixed inflammatory infiltration. Low power field (X 4 objective).



GOBLET-CELL HYPERPLASIA / METAPLASIA OF BRONCHIOLES:

The name goblet cell derives from the characteristic shape of these cells in conventionally fixed tissue. Goblet cells have a distinctive polarity and are seen as cylindrical cells. A narrow base and expanded apical portion that sometimes extends in the lumen of the bronchus. Diagram 7.7 illustrates a normal respiratory mucosa from a negative control normal rabbit and 7.8 illustrate goblet cell metaplasia. As well as a secretory function in response to irritating stimuli, goblet cells have the ability to rapidly differentiate into other cells in severe disease states in the respiratory tract. The following is the guide to scoring for goblet cells. 0, absent; 1, few goblet cells present in one or two bronchiolar profiles; or 2, large numbers of goblet cells present abundant in the majority of the airway. Goblet cell metaplasia was not a common feature of endotoxin induced acute lung injury.



Diagram 7.7: Normal respiratory mucosa in medium power magnification (X 10 objective).

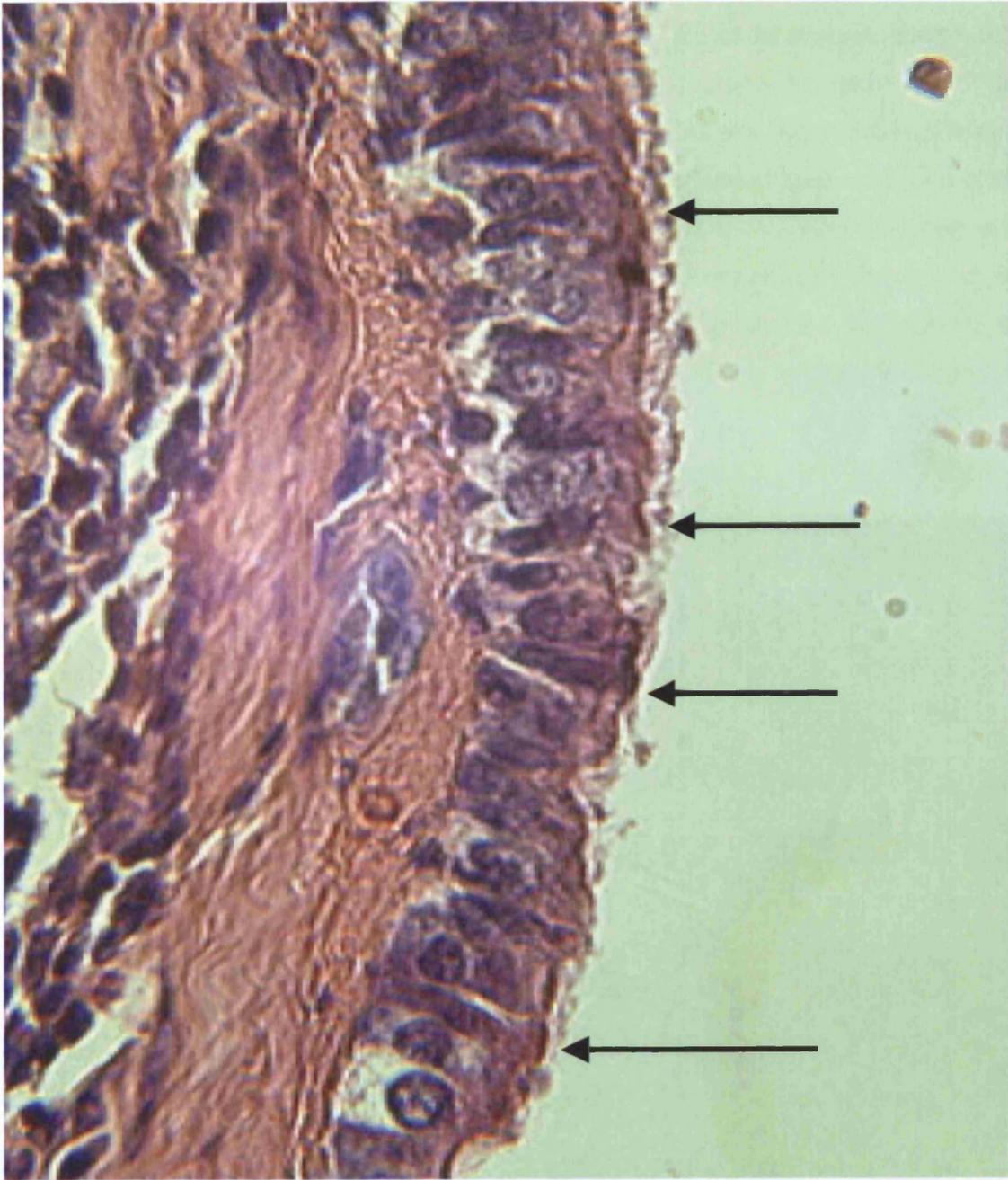


Diagram 7.8: The arrows point at the metaplastic goblet cells. High power magnification (X 40 objective).

MACROPHAGES IN ALVEOLAR SPACES: Macrophages were stained with the immunohistochemical technique described in chapter 2. Their involvement was measured semi quantitatively by using the following; 0, absent of alveolar spaces from any macrophages; 1, present in fewer than 25% of alveolar spaces; 2, present in 25–75% of alveolar spaces; 3, present in more than 75% of alveoli, often in tightly packed clusters. Diagram 7.9 illustrates the stained macrophages against the background of haematoxylin. Diagrams 7.10 and 7.11 illustrate presence of macrophages in less than 25% and more than 75% of the alveolar spaces respectively. The experimental animals showed varying degrees of macrophage infiltrations. Two of the control animals had no visible macrophages however the third expressed a high degree of macrophage presence per alveolar spaces.

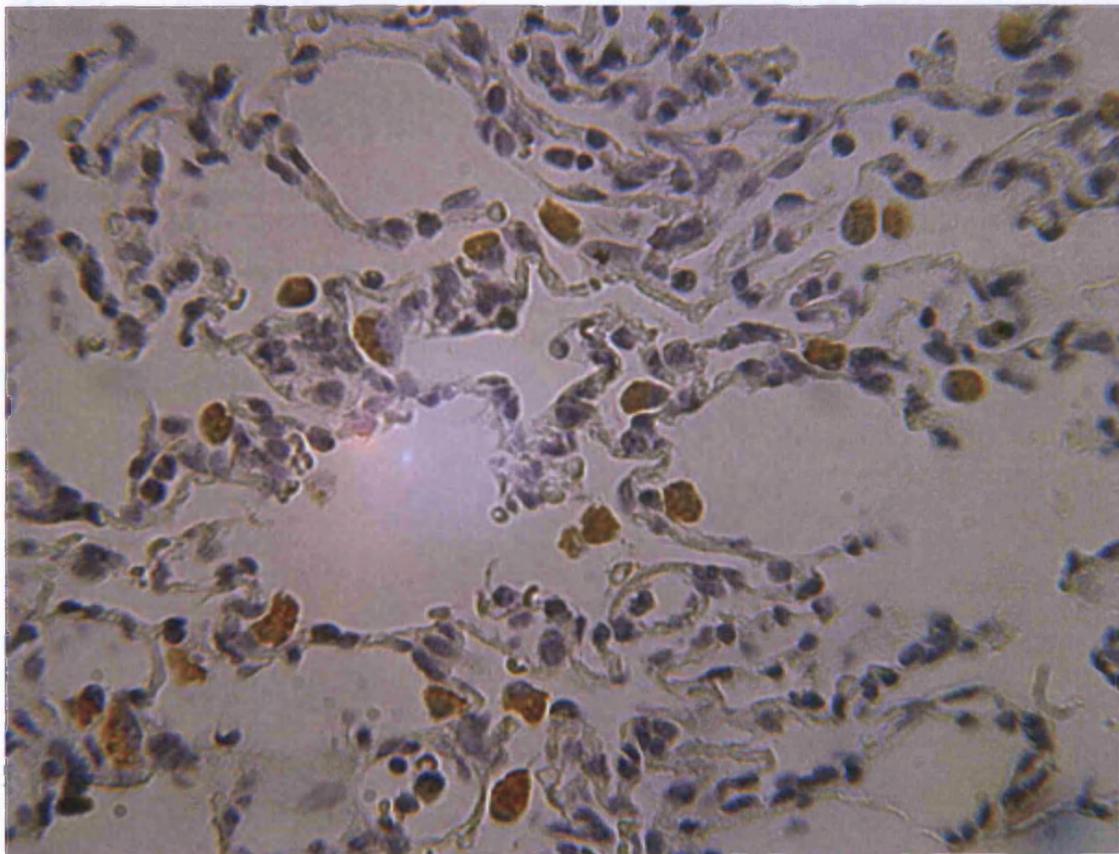
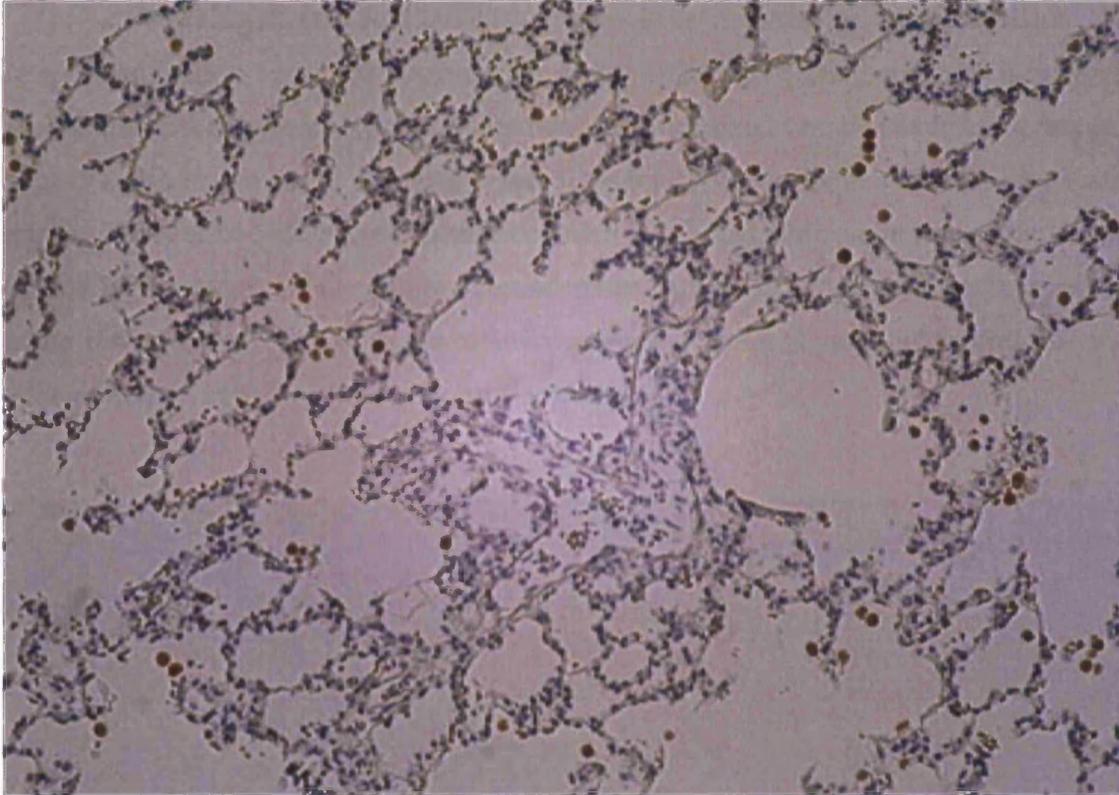
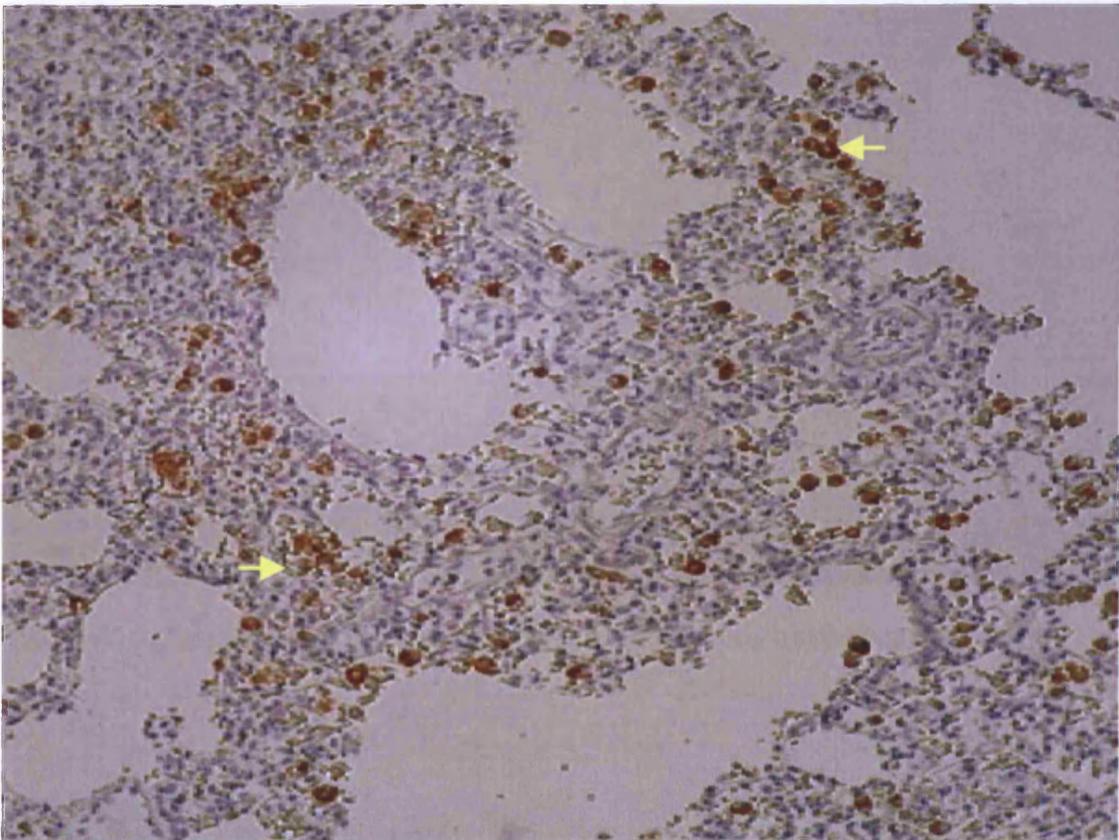


Diagram 7.9: Macrophages seen in a medium power field (X10 objective).



Diagrams 7.10 (Above) and 7.11 (Below): Illustrating the presence of macrophages in less than 25% and more than 75% of the alveolar spaces respectively. Note the presence of macrophage clusters (Arrows).



LYMPHOID AGGREGATES: Lymphoid aggregates are easily visualised with low magnification some are even visible to the naked eye. Diagram 7.12 illustrates a moderate sized lymphoid aggregate close to a medium sized vessel. The H&E slides were scored 0, when lymphoid aggregates were absent; 1, identifiable at low magnification around fewer than 50% of vessels and bronchi; 2, identifiable around more than 50% of vessels and bronchi; 3, identifiable around more than 50% of vessels and bronchi with acute inflammation and extension to involve small vessels. Lymphoid aggregates were present in both experimental and control rabbits.

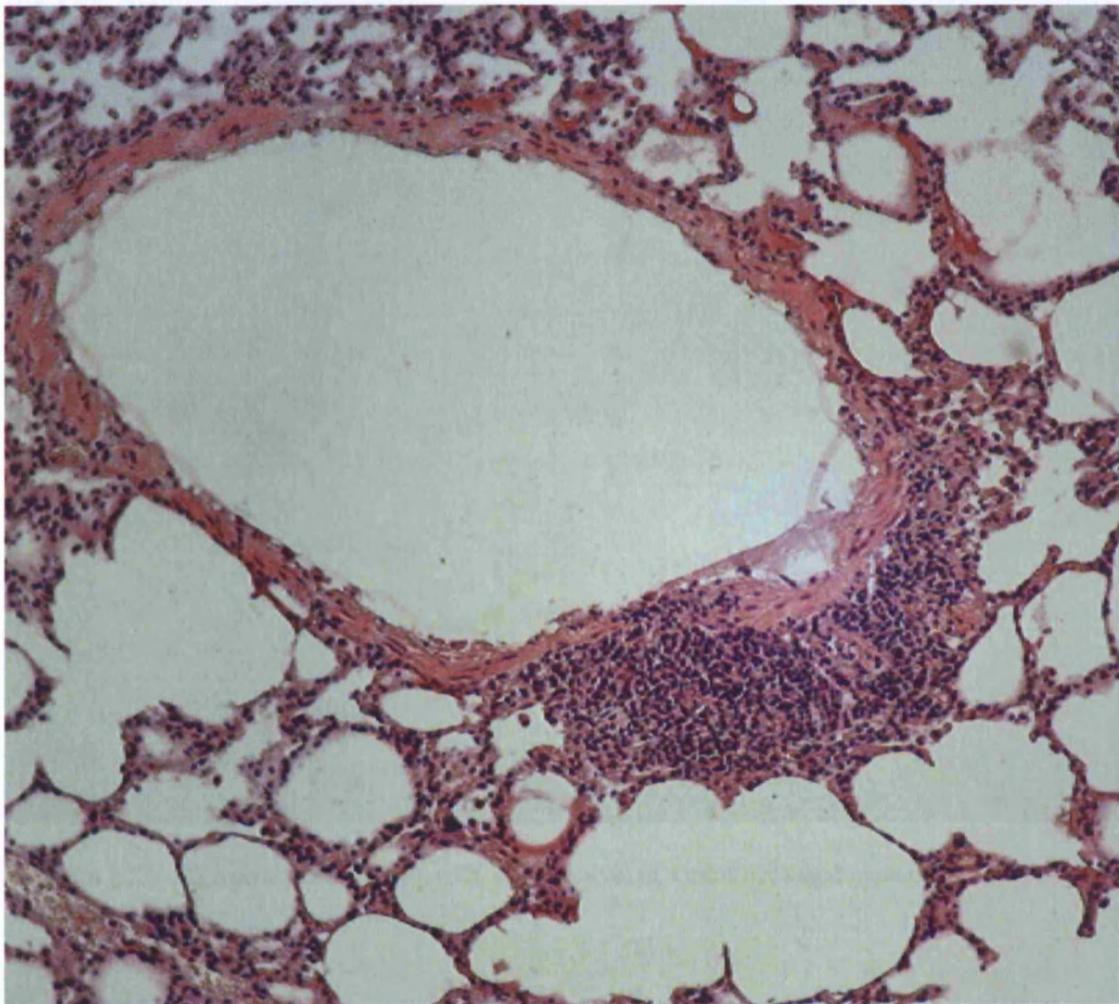


Diagram 7.12: Low power field (x 4 objective) diagram of a pulmonary lymphoid aggregate.

MULTINUCLEATED GIANT CELLS: Multinucleated giant cells are an integral part of the host immune response. Lymphocytic stimulation by specific antigens of T cell mitogens produce a soluble factor that causes peripheral blood monocytes to fuse and form multinucleated giant cells in vitro {Postlethwaite et al. 1982}. Production of the giant cell factor by antigen-stimulated peripheral blood lymphocytes correlates with the existence of cell-mediated immune to specific antigen. Diagram 7.13 illustrates a high power light microscope image of a multinucleated giant cell.

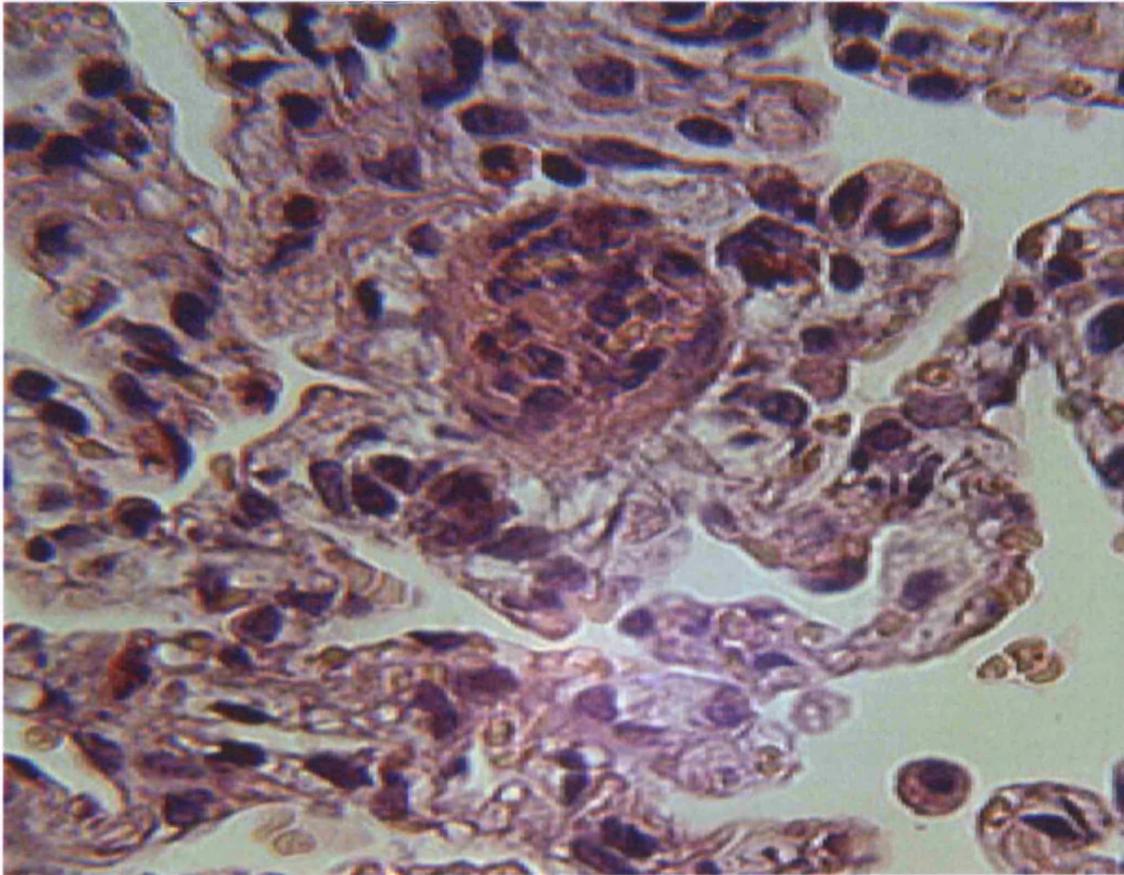


Diagram 7.13: High power magnification (X 40 objective) of a multinucleated giant cell.

We scored 0, in the absence of visible giant cells; 1, occasionally identified while examining slides at medium magnification [x10 objective] and not generally visible at low power (x4 objective); 2, present and numerous, easily identified at lower magnification. They were present at different degrees in almost all of the experimental and control animals but absent from the normal rabbits.

EOSINOPHILS: Diagram 7.14 shows a high power field examination of an experimental rabbits lung. Eosinophils were in abundance in rabbit lungs. They were therefore scored 0, for absent; 0.5, for a few identified within inflammatory exudates at high magnification and 1, present in multiple fields but never more than 10% of cells identified.

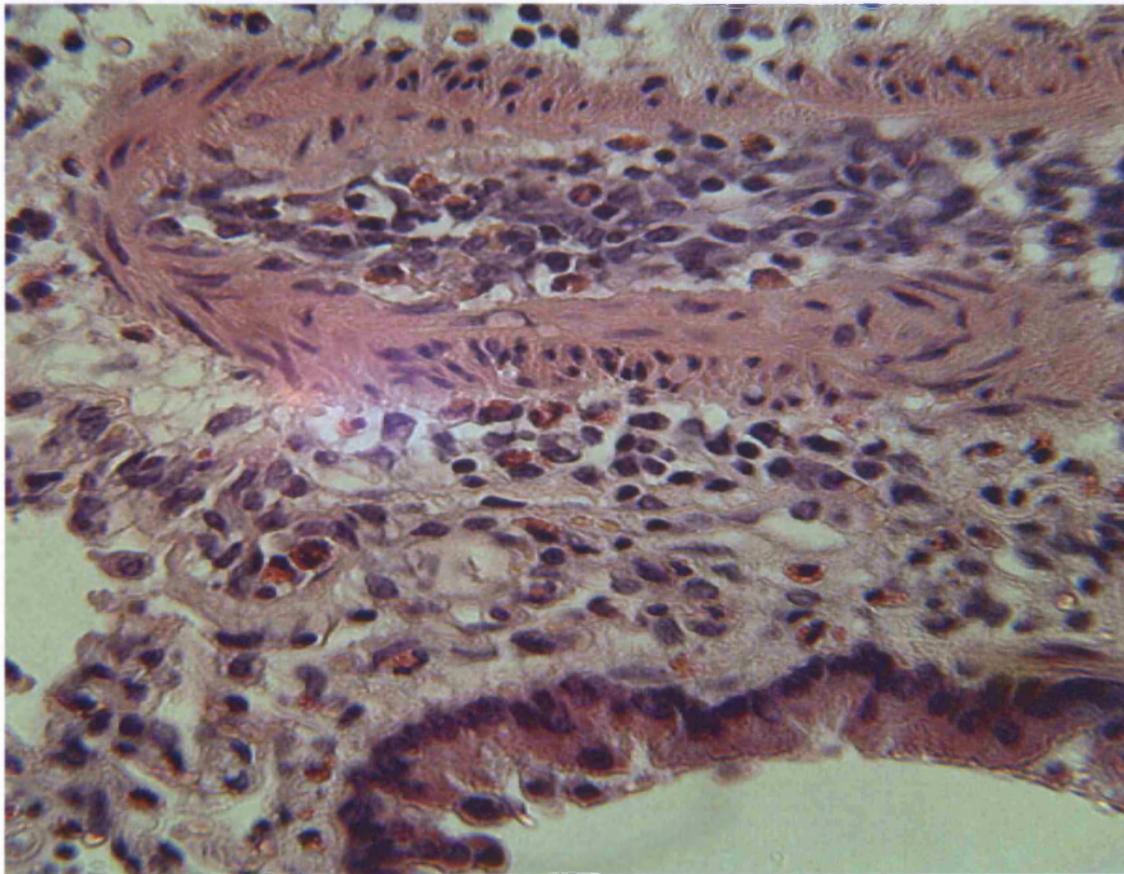


Diagram 7.14: High magnification of eosinophils mixed with other inflammatory cells.

EOSINOPHILIC INTRACYTOPLASMIC INCLUSIONS: Large eosinophilic intracytoplasmic inclusions (crystals) in macrophages are structurally distinct, inducible, non-nuclear sites for enhanced synthesis of paracrine eicosanoid mediators of inflammation {Bozza et al. 1997}. Diagram 7.15 illustrates an extracellular eosinophilic crystal. After examination of at least 10 random high power fields a score of 0 denoted absent of intracytoplasmic inclusions; 1, when identified in a few cells upon examination at high-power magnification ($\times 40$ objective); 2, present and numerous, sometimes large and extracellular; 3, extremely numerous with many lying free within alveolar spaces.

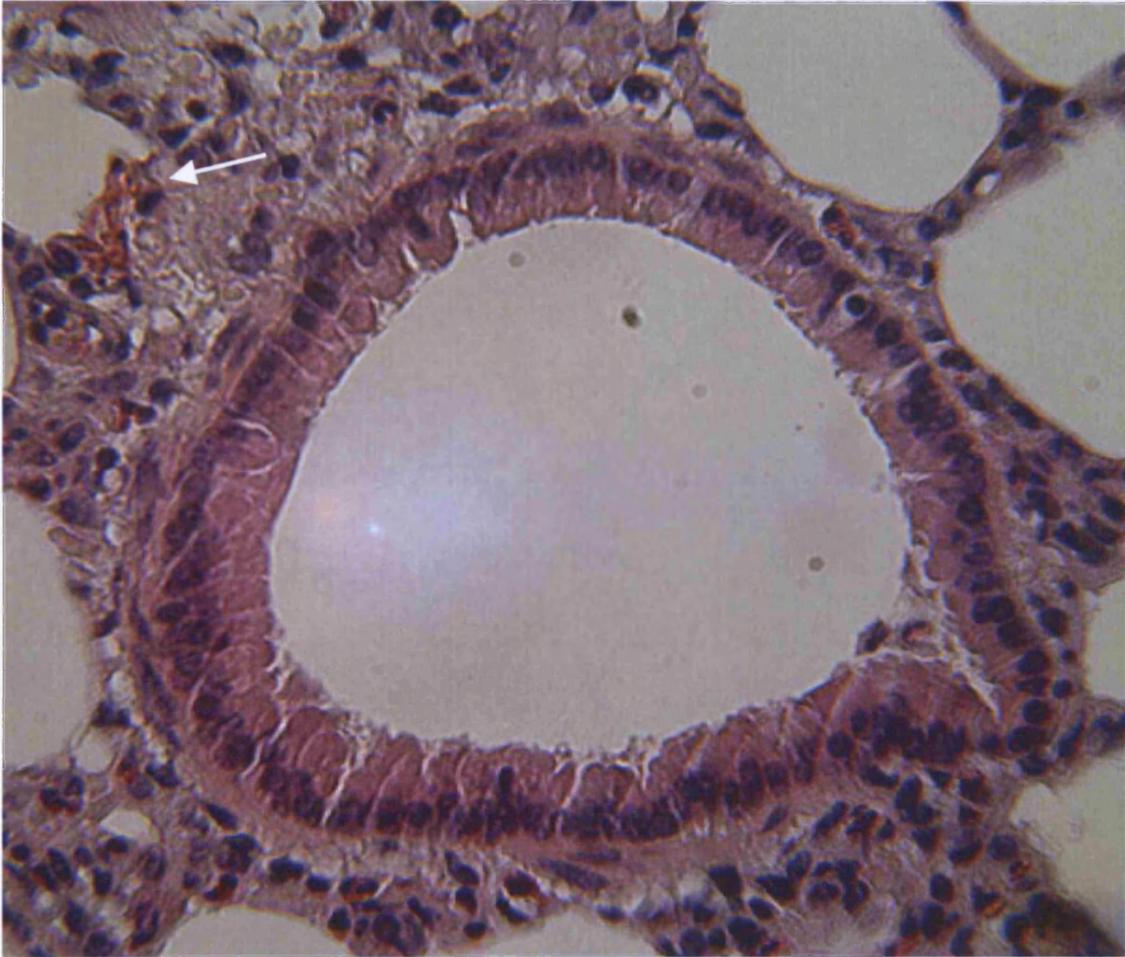


Diagram 7.15: Illustrates medium power magnification (X 10 objective) of a large extracellular eosinophilic crystal (Arrow) from macrophages.

MONONUCLEAR AND POLYMORPHONUCLEAR LEUKOCYTES:

Perivascular/peribronchial acute inflammation with neutrophil infiltration was scored as follows: 0, absent; 1, mild acute inflammation in the perivascular oedematous space, with fewer than 5 neutrophils per high-power field (hpf); 2, moderate acute inflammation in the perivascular spaces, extending to involve the peribronchial spaces, with more than 5 neutrophils per hpf in these regions; or 3, severe, acute inflammation in the perivascular and peribronchial spaces with numerous neutrophils encircling most [$> 50\%$] of bronchioles.

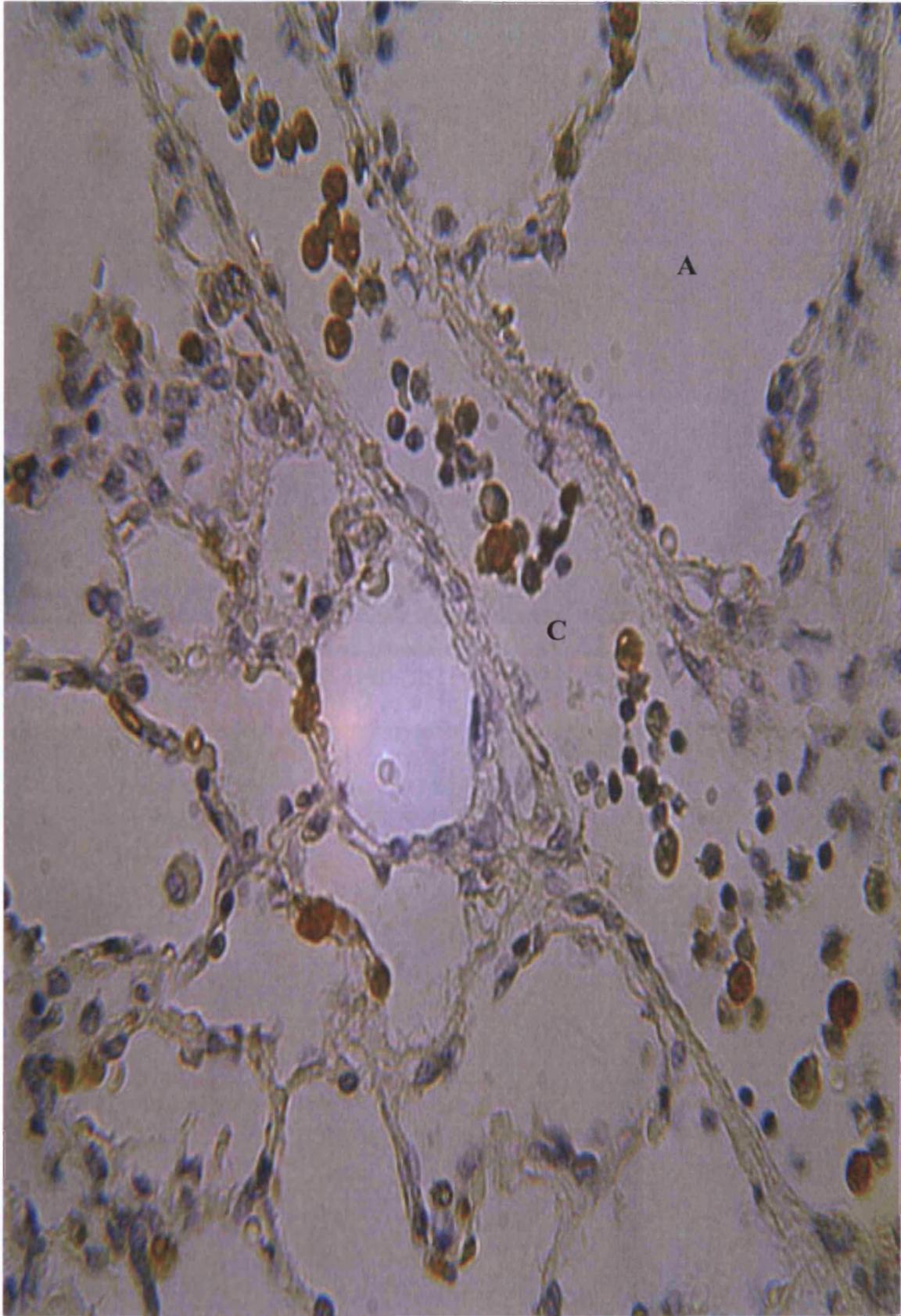


Diagram 7.16: Illustrates the increased number of neutrophils (Immuno-stained brown) in experimental rabbits lung in response to LPS. This diagram shows a cross-section of a pulmonary capillary (C) supplying a number of alveoli (A).

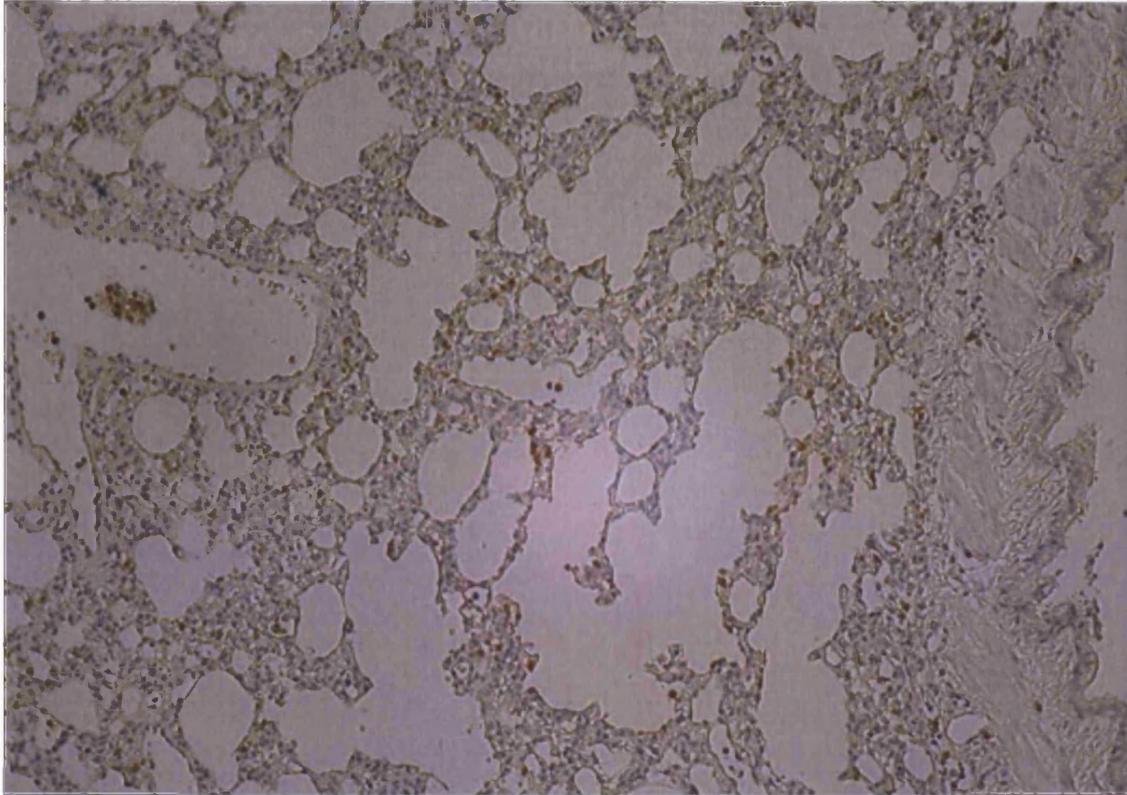
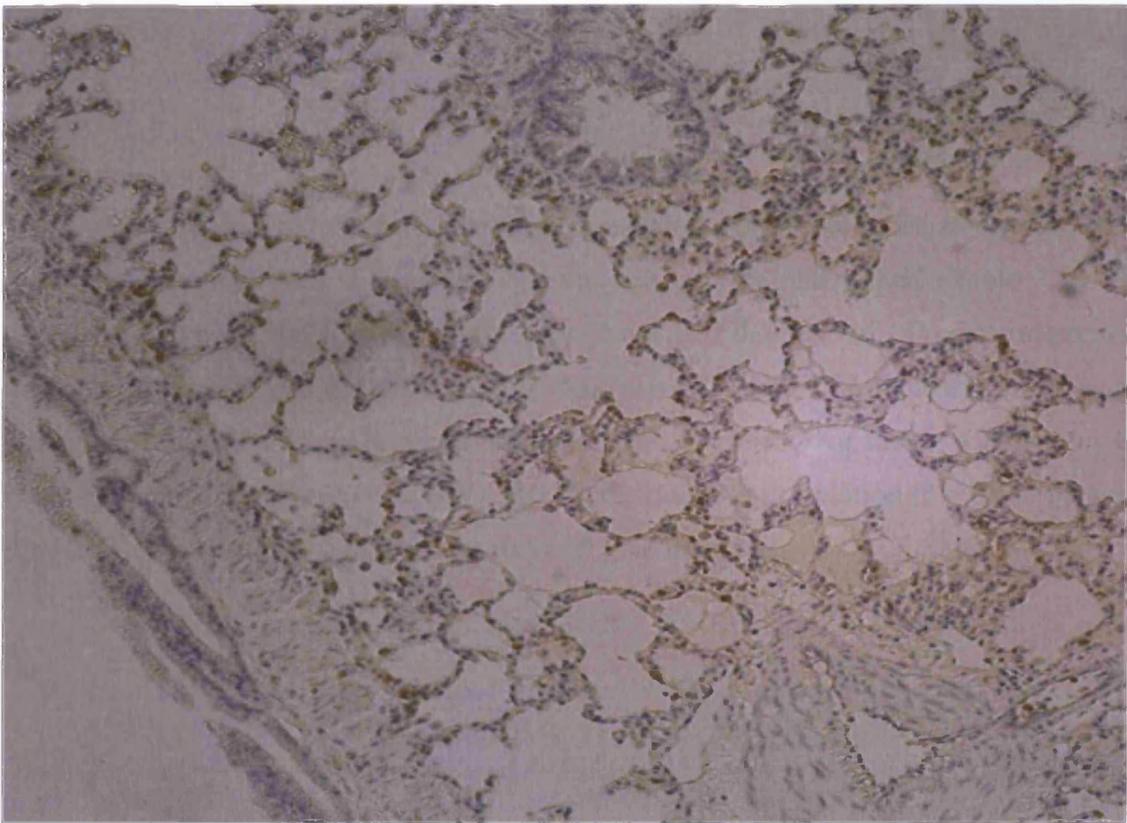


Diagram 7.17 (Above) and 7.18 (Below): Illustrating mild and moderate neutrophil infiltration. Note that these pictures are at low power field (X 4 objective) for illustration purposes.



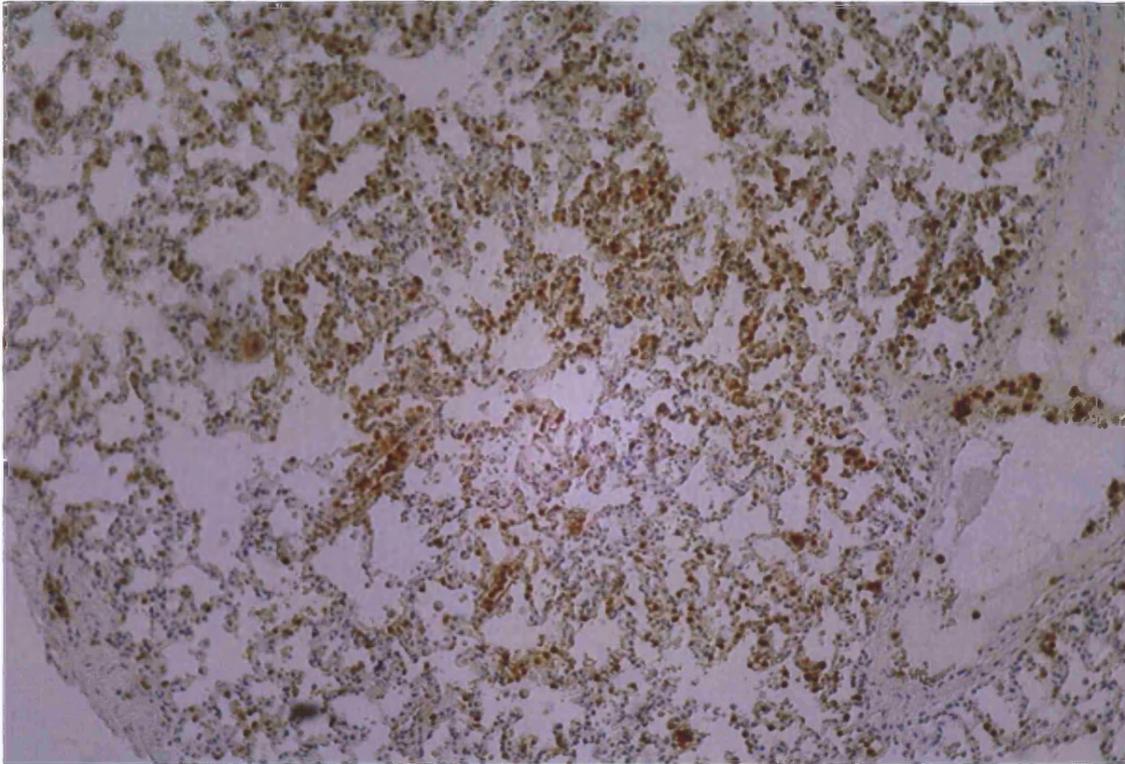


Diagram 7.19: Illustrating severe neutrophil infiltration at low power field (X 4 objective).

TOTAL SCORE: A total histological inflammatory score taken as the sum of the individual histology scores from each of the above, were determined (Table 7.2). The correlation between the Histo-inflammatory scores and the dose of LPS administered as well as the survival are illustrated in the diagrams 7.20 and 7.21 (below). There was a strong positive correlation between the Dose of LPS in mg/Kg rabbit weight and the Histo-inflammatory score ($r = 0.66$) and a strong negative correlation ($r = 0.61$) between the Histo-inflammatory score and the survival time in hours.

Table 7.2: Histo-inflammatory score summary. Sc1 animal underwent a schedule one procedure. That is no instrumentation as for the control or experiments were performed on that rabbit.

Number	LPS dose	HI Score	Survival Time
1	.0025	13.00	13.00
2	.0050	11.50	23.00
3	.0100	12.00	14.00
4	.0150	13.00	10.00
5	.0200	13.00	4.00
6	.0250	13.00	5.00
7	.0200	13.00	3.00
8	.0200	18.00	10.00
9	.0200	16.00	5.00
10	.0200	16.00	5.00
11	.0200	14.00	5.00
12	.0000	9.50	24.00
13	.0000	7.00	24.00
14	.0000	14.00	24.00
15	.0000	2.00	Sc1
Total	N	15	15
	Mean	.011833	12.3333
	Grouped Median	.015000	13.0000
	Minimum	.0000	2.00
	Maximum	.0250	18.00
	Range	.0250	16.00

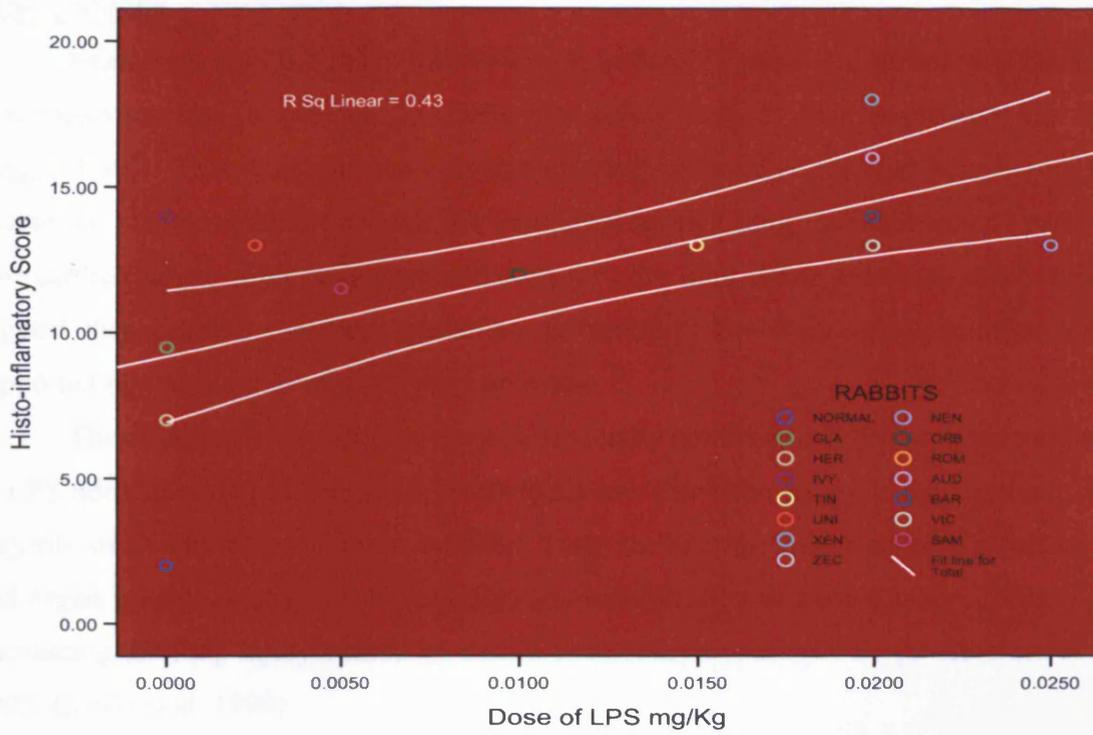
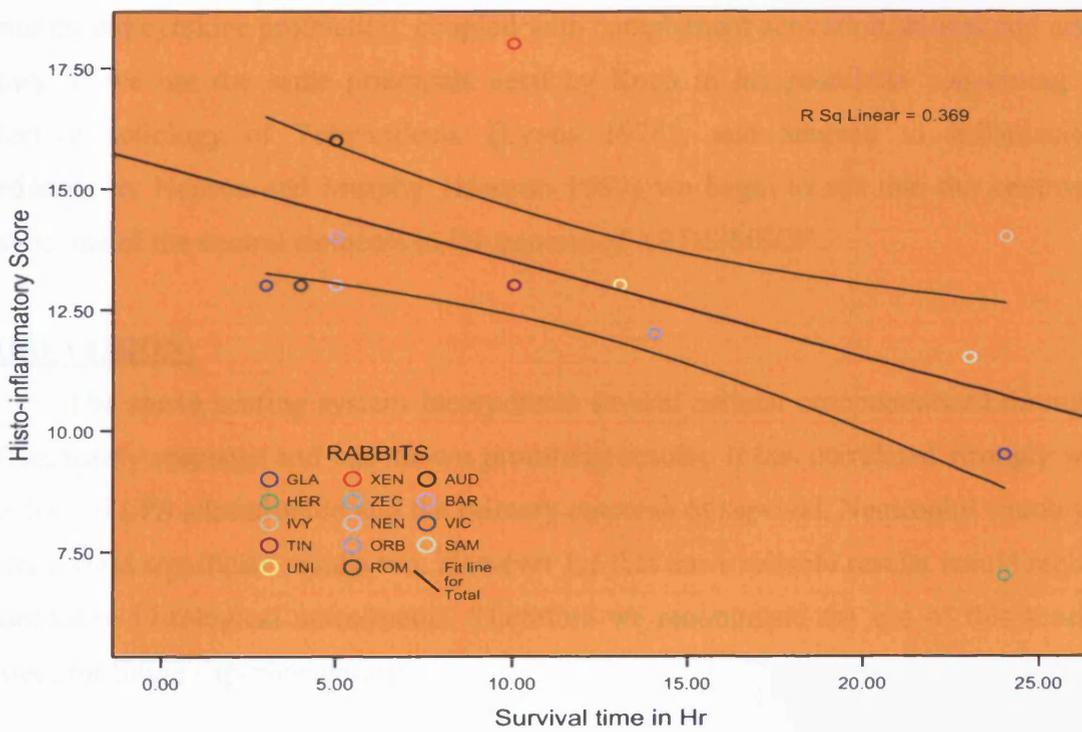


Diagram 7.20 (Above) and 7.21 (Below): Illustrate the significant correlations between the dose of LPS administered the histo-inflammatory score and the survival.



DISCUSSION

Examination of the H&E slides showed noticeably increased pulmonary oedema in rabbits ventilated for prolonged periods even at low levels of positive pressure and low oxygen levels. This illustrates the cumulative effect of positive pressure ventilation. Of course not many normal individuals are ventilated for that long. The majority of patients post cardiac surgery don't require ventilation for very long either. However these results suggest that positive pressure ventilation is harmful. The histological findings were supported by the findings of the Wet / Dry ratios.

The circulatory levels of leukocytes especially neutrophils fell sharply in response to LPS administration (Chapter 5). Neutrophils are either the target or the source of the majority of cytokines involved in ARDS. They are also the major cellular effectors of end organ injury. Neutrophil depletion by in-line filtration is known to be effective in attenuating the lung injury caused by cardio-pulmonary bypass (CPB) {al-Ebrahim et al. 1993, Bando et al. 1990}.

Neutrophils occupy a unique central position in the cascade, as they are the source of numerous mediators, and the target of many others. They are also the most important effectors of inflammatory tissue injury, as once stimulated they undergo degranulation and release cytotoxic and stromolytic enzymes. The combined effect of these granule contents and cytokine production, coupled with complement activation, causes end organ injury. If we use the same principals used by Koch in his postulates concerning the infective aetiology of Tuberculosis {Lyons 1978}, and adapted to inflammatory mediators by Henson and Murphy {Henson 1989} we begin to see that the neutrophil maybe one of the central elements in the genesis of ARDS/MSOF.

CONCLUSION:

The above scoring system incorporates several cellular components of thrombo-inflammatory response and has shown promising results. It has correlated strongly with the dose of LPS administered and the primary outcome of survival. Neutrophil counts *per se* may yield significant results too. However for that more reliable results would require quantitative histological assessments. Therefore we recommend the use of this scoring system for future experimentations.

Chapter Eight

OXIDATIVE STRESS, DNA DAMAGE AND APOPTOSIS IN A RABBIT MODEL OF SEVERE SIRS

INTRODUCTION

Oxidative stress occurs when the homeostatic balance between formation of reactive oxidising oxygen species and endogenous antioxidants is lost {Gutteridge et al. 1999}. These oxygen free radicals and other reactive oxygen species appear to be involved as messengers in cellular signal transduction and gene activation with implications for expression and control of thrombo inflammatory response.

Oxygen free radicals may act directly on organ cells or by activation of neutrophils. Accumulating evidence suggests that the cellular redox state and the equilibrium between oxygen free radicals (OFR) generation and detoxification by antioxidants influences the early stages of apoptosis, which leads to MODS.

In sepsis there are several potential sources of oxidative stress. These have been discussed in chapter one. Oxidative stress causes damage mediated by reactive oxygen species. Oxidation of DNA and proteins may take place, along with membrane damage, because of lipid peroxidation, leading to alterations in membrane permeability modification of protein structure and function {Zimmerman et al. 1995}. Oxidative damage to the mitochondrial membrane can also occur, resulting in membrane depolarisation and uncoupling of oxidative phosphorylation, with altered cellular respiration {Nathan et al. 1999}. This can ultimately lead to mitochondrial damage, with release of cytochrome c, activation of caspases and apoptosis.

In this chapter we will assess oxidative stress in our experimental and control rabbits by comparing plasma levels of total antioxidants or total antioxidant state (TAS), with immunofluorescent determination of DNA damage in rabbit lungs obtained after post-mortem examination of those rabbits.

In order to assess apoptosis in rabbits suffering from severe SIRS, we used Cytokeratin 18 (CytoDEATH) and Caspase 3 expression in lung specimens. Unfortunately we were unable to optimise our parafin embedded slides to determine the degree of apoptosis by Caspase 3.

RESULTS

TOTAL ANTIOXIDANT STATUS (TAS): The median (Range) for TAS concentration in rabbit plasma prior to administration of LPS is illustrated in table 6.1. Diagrams 8.1 and 8.2 illustrate the variation in TAS with time after administration of LPS. The TAS fell in all experimental animals.

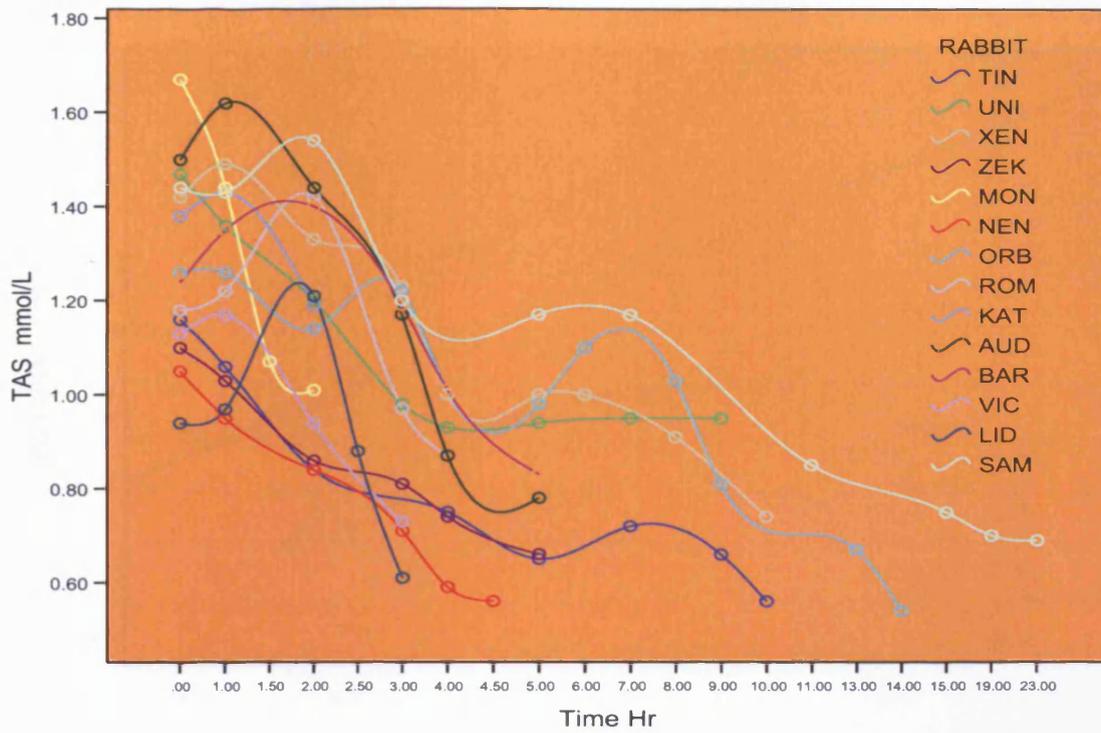


Diagram 8.1: Trend of TAS in mmol/L with time in hours in all experimental rabbits.

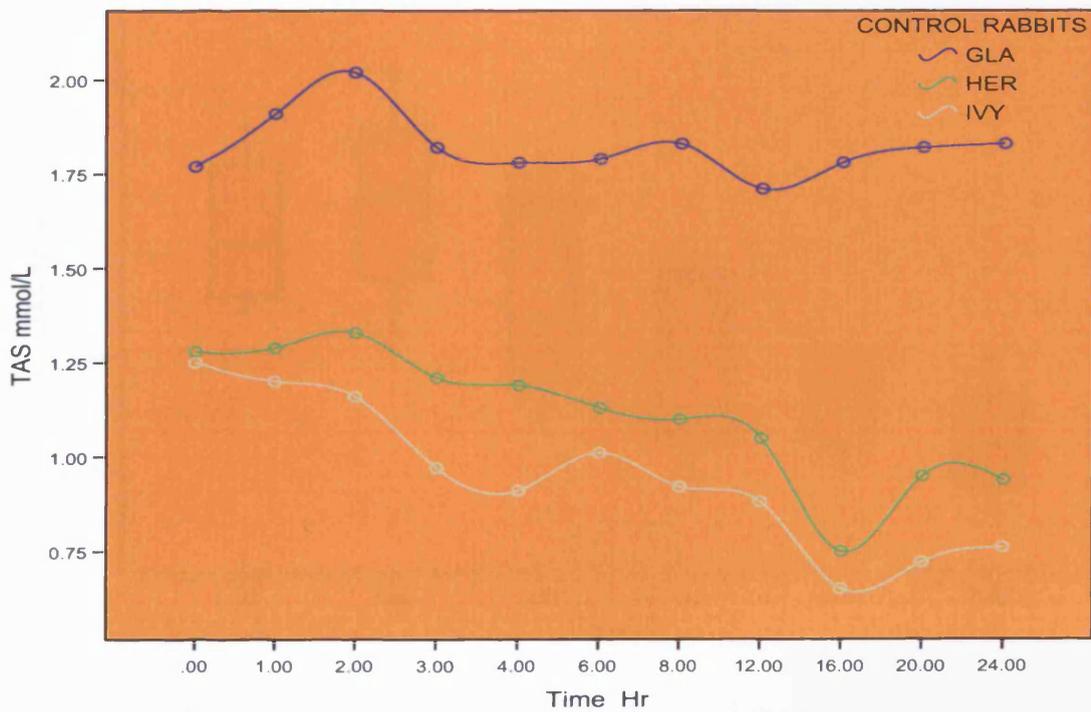


Diagram 8.2: Trend of TAS in mmol/L against time in hours in all the control rabbits.

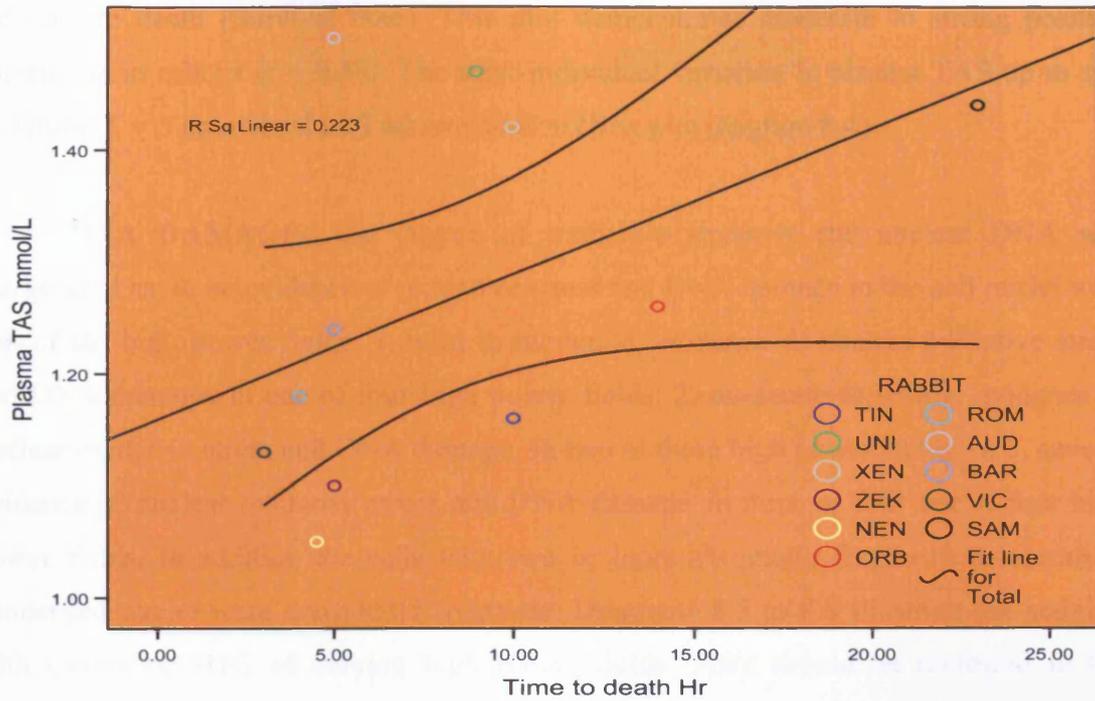


Diagram 8.3: The relationship between TAS and time to death in hours and the dose of LPS administered.

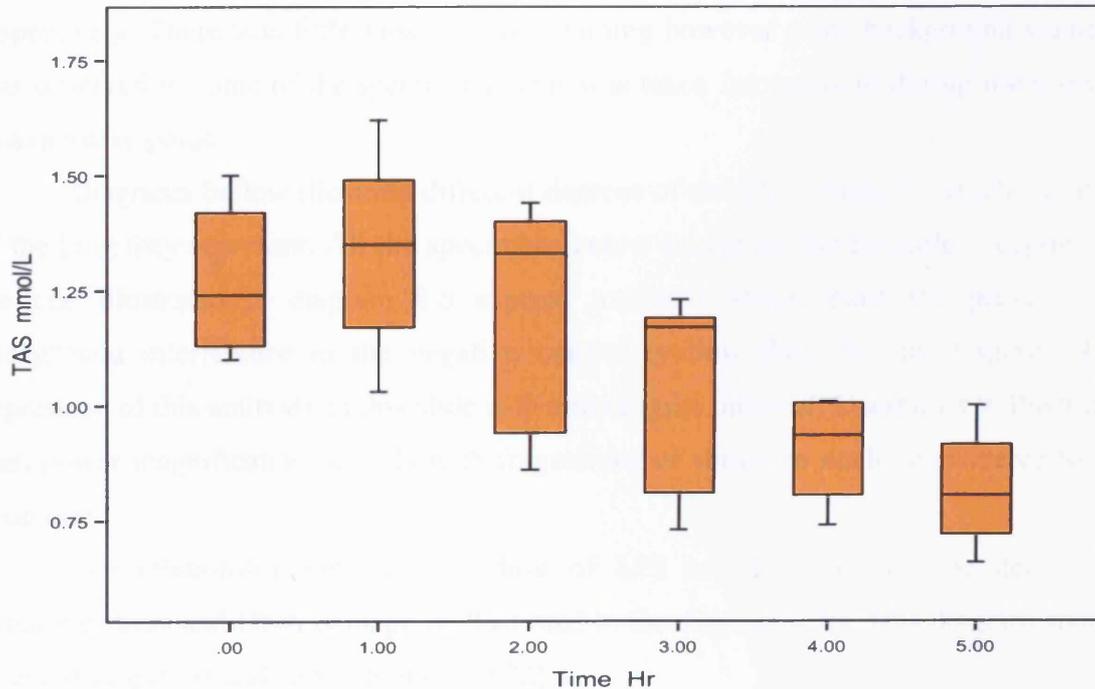


Diagram 8.4: Illustrating the inter-individual variation in plasma TAS in mmol/L at different times during the experiment when all animals received the same dose of LPS. Note that only one experimental animal in this group survived past 5 hours. Therefore the plasma TAS there after would not illustrate a variation and hence was excluded.

Diagram 8.3 illustrates the correlation between the Total Antioxidant Status and the time to death (Survival time). This plot demonstrates moderate to strong positive correlation in rabbits ($r = 0.48$). The inter-individual variation in plasma TAS up to and including T = 5 hours post LPS administration (Box plot diagram 8.4).

DNA DAMAGE: The degree of oxidative stress in the nuclear DNA was categorized as: 0, no evidence of oxidative stress and DNA damage in the cell nuclei with non of the high power fields; 1, mild to moderate, evidence of nuclear oxidative stress and DNA damage, in one of four high power fields; 2, moderate to severe, evidence of nuclear oxidative stress and DNA damage, in two of three high power fields; or 3, severe, evidence of nuclear oxidative stress and DNA damage, in three or four out of four high power fields. In addition the cells with two or more chromatin fragments or shrunken condensed nuclei were considered apoptotic. Diagrams 8.5 to 8.8 illustrate the staining with Genox or MIG of varying high power fields. They should be reviewed in the sequence of DAPI, FITC and overlap illustrated by the slide demonstrating the nuclear DNA oxidative stress and damage, which fluoresce in yellow. The slide and negative controls are to exclude false positive auto-fluorescence and background staining respectively. There was little false positive staining however some background staining was observed in some of the specimens. This was taken into account during the scoring as a negative point.

Diagrams bellow illustrate different degrees of oxidative stress in whichever part of the lung they represent. All the specimens below except for the possible exception of the one illustrated in diagram 8.6 express oxidative stress. Note the presence of background interference in the negative control (yellow dots) in this diagram. The expression of this antibody in this slide is therefore quite minimal. Diagram 8.9 illustrates high power magnification of cells with fragmented or shrunken nuclei considered to be apoptotic.

The relationship between the dose of LPS administered and the degree of oxidative stress and DNA damage is illustrated in the diagram 8.10. This diagram shows a very strong statistical correlation ($r = 0.72$).

Diagram 8.5: Illustrating oxidative stress and DNA damage in the peribronchial region. This immunofluorescent technique involved Genox antibody.

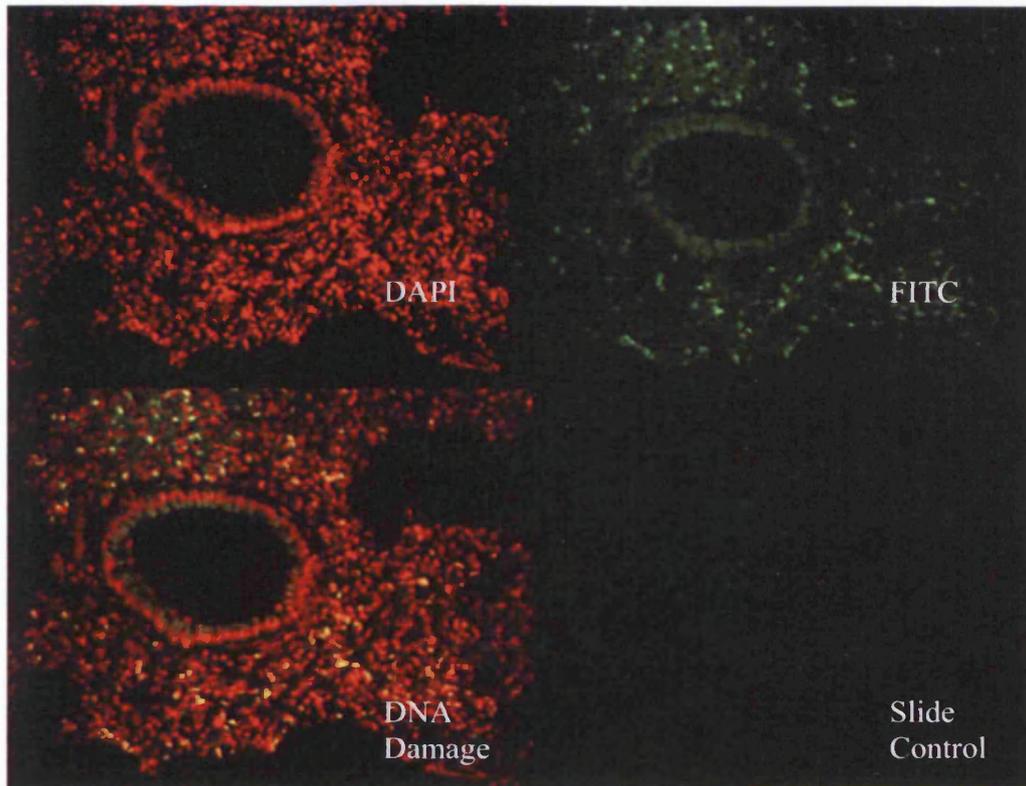


Diagram 8.6: Illustrating oxidative stress and DNA damage by immunofluorescent technique utilizing M1G. This represents an area of well-ventilated alveoli.

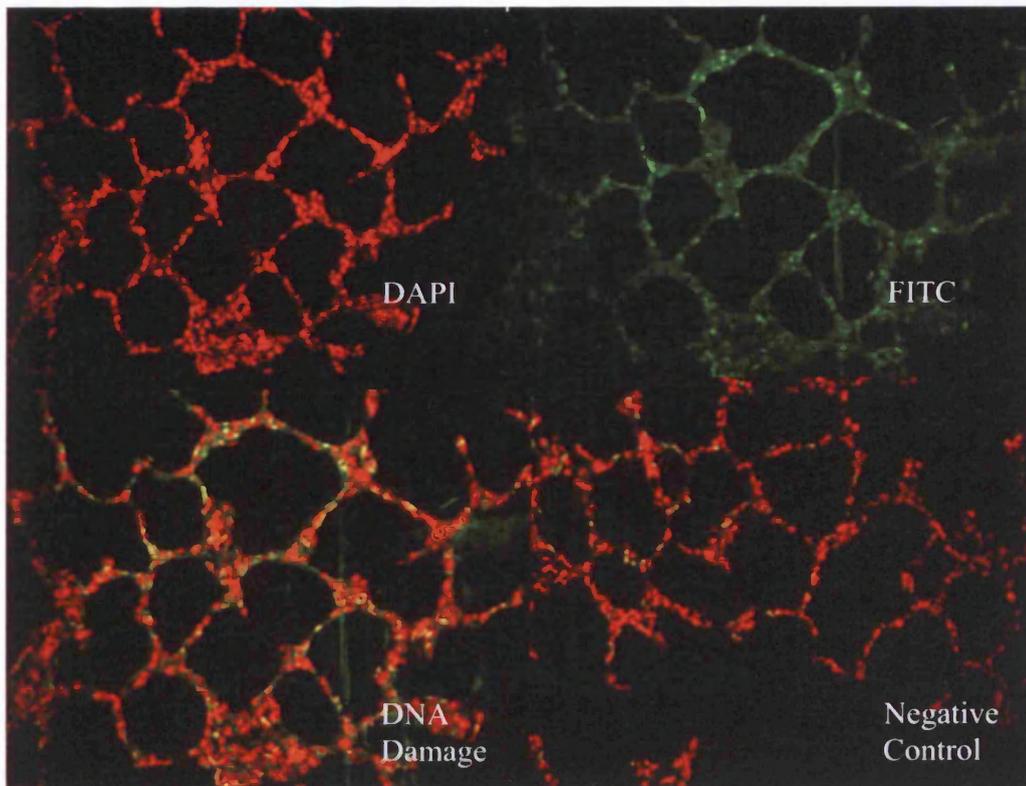


Diagram 8.7: Illustrating oxidative stress and DNA damage by immunofluorescent technique using MIG antibody. This is a high power field of an area of lymphoid aggregate.

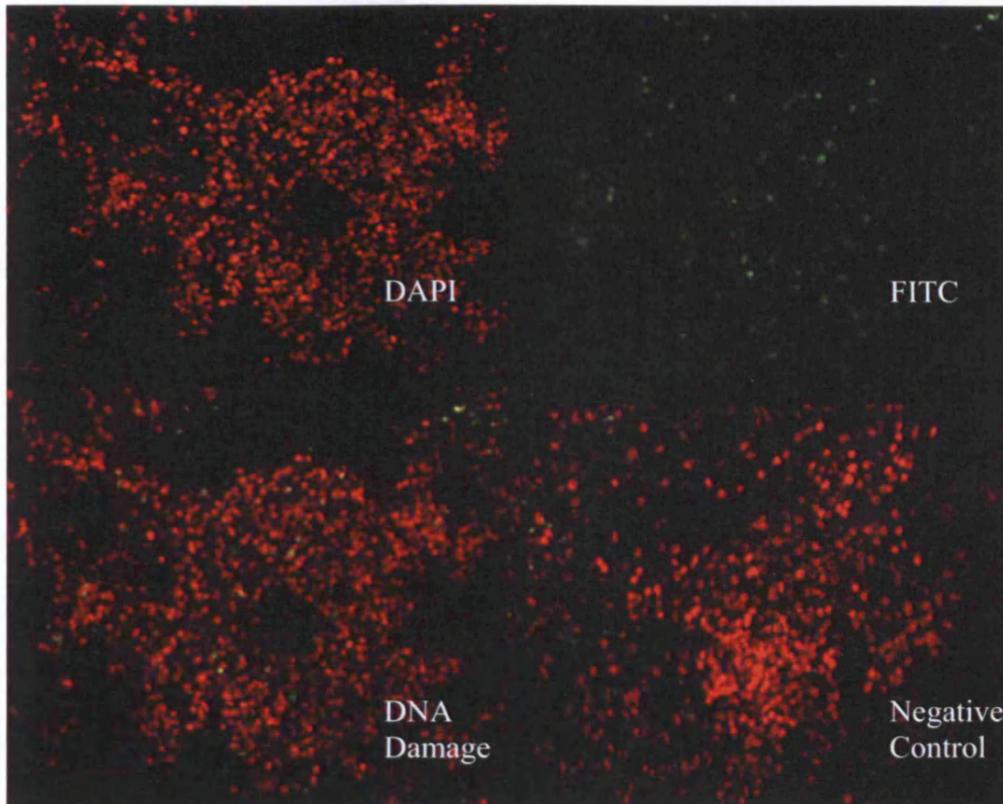
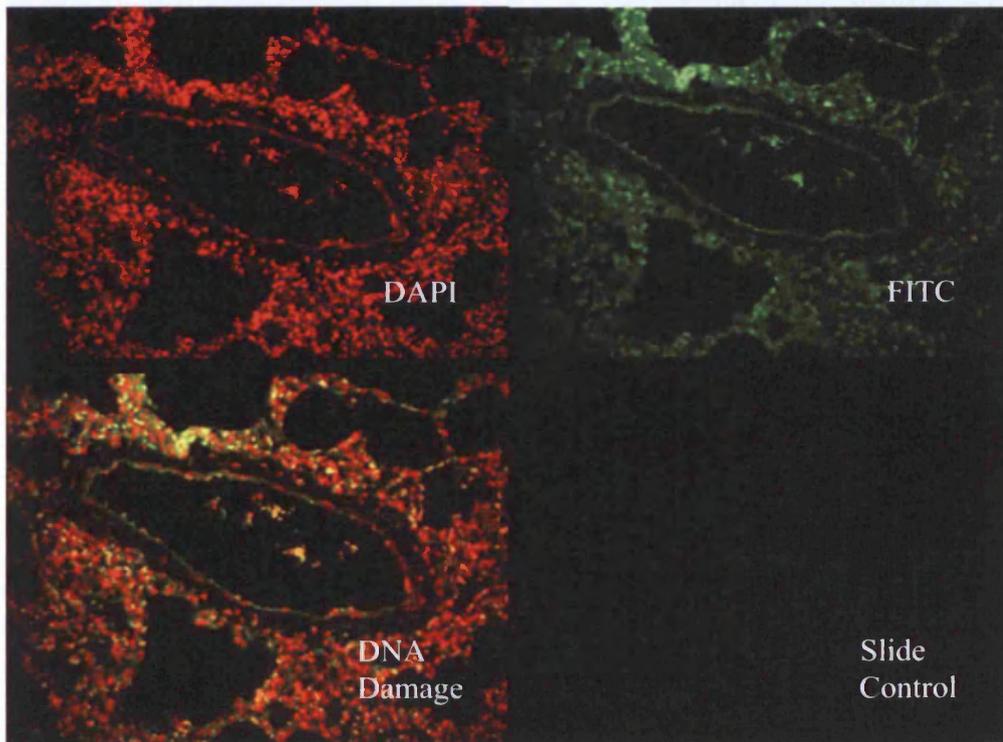


Diagram 8.8: Illustrating oxidative stress and DNA damage by immunofluorescent technique using Genox antibody. This slide represents an area of perivascular consolidation in an experimental rabbits lung tissue.



Rabbits that had lower oxidative stress survived longer as illustrated by diagram 8.11. This also showed a strong correlation with a coefficient of 0.59. Expression of DNA fragmentation was not surprisingly correlated with signs of apoptosis in experimental rabbits. This however only expressed a small negative statistical correlation ($r = 0.28$).

Diagram 8.9 illustrates cells with fragmented or shrunken nuclei considered to be apoptotic.



APOPTOSIS: The degree of apoptosis was scored as follows: Firstly for CytoDEATH: 0, absent of any Cytokeratin 18 expression in the lung; 1, mild expression, with fewer than 5 cells expressing Cytokeratin 18 per high-power field (hpf); 2, moderate expression, with more than 5 cells expressing Cytokeratin 18 per hpf; or 3, severe, with numerous cells expressing Cytokeratin 18.

Diagram 8.10: Relationship between the dose of LPS and the degree of oxidative stress and DNA damage.

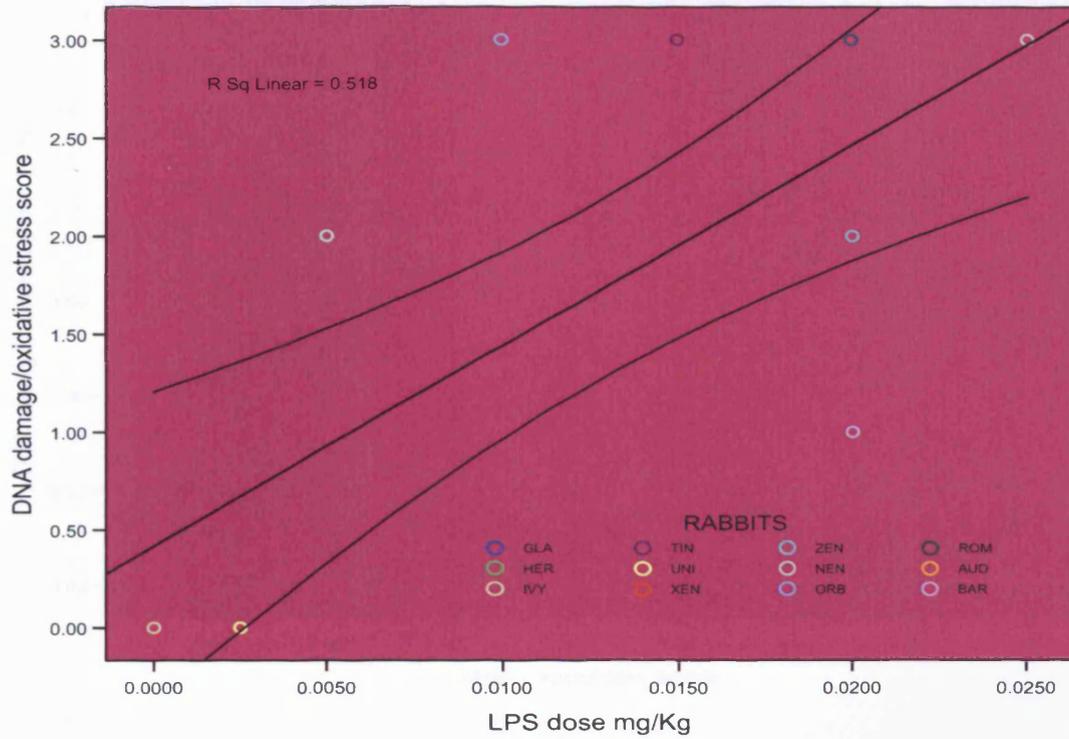


Diagram 8.11: The relationship between oxidative stress and survival in experimental and control rabbits.

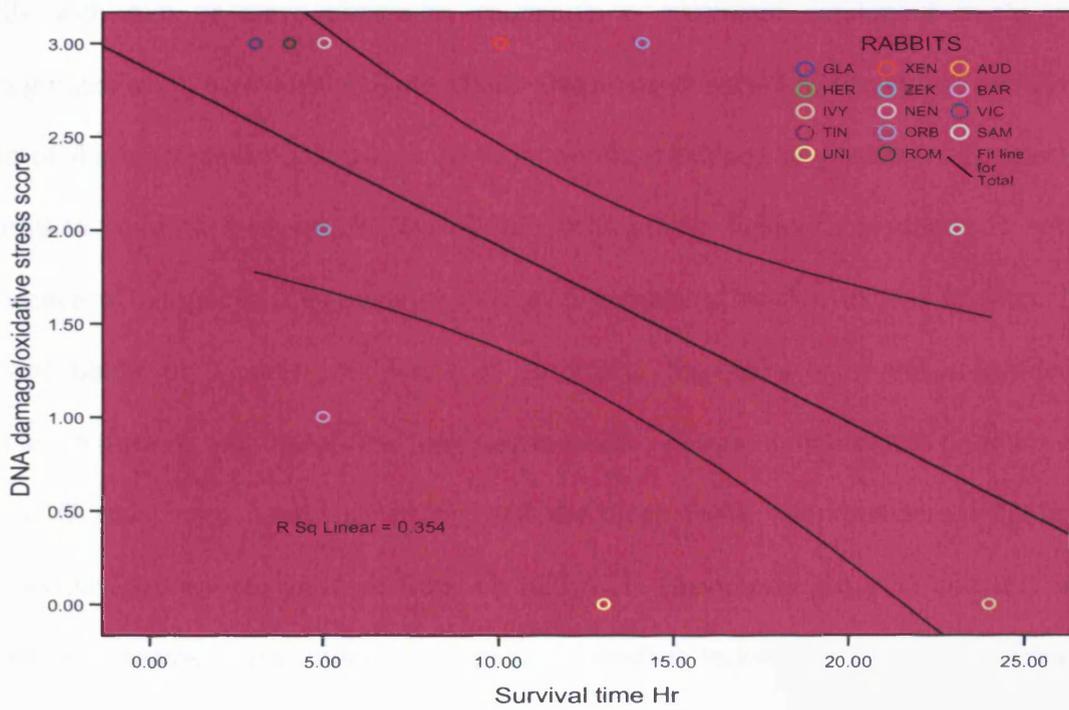
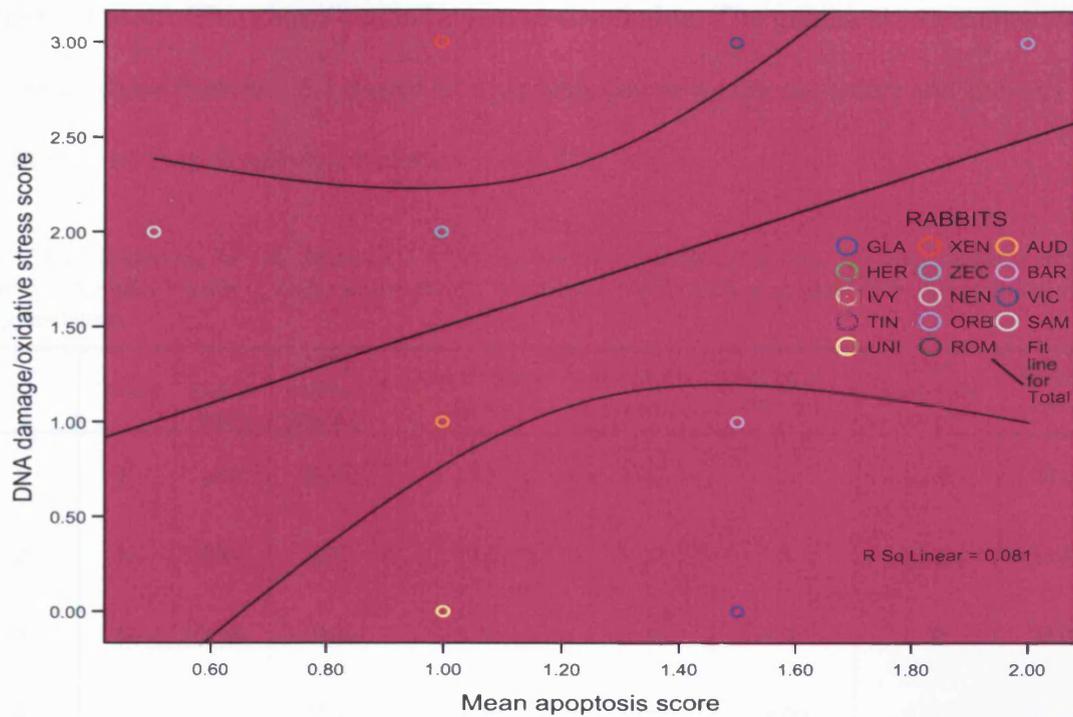


Diagram 8.12: The relationship between oxidative stress and apoptosis in experimental and control rabbits.



Secondly for each of the MIG and Genox immunofluorescent experiments: The degree of cells with two or more chromatin fragments or shrunken condensed nuclei were categorized as: 0, no evidence of chromatin fragments or shrunken condensed nuclei with non of the high power fields; 1, mild to moderate, evidence of chromatin fragments or shrunken condensed nuclei, in one of four high power fields; 2, moderate to severe, evidence of chromatin fragments or shrunken condensed nuclei, in two of three high power fields; or 3, severe, evidence of chromatin fragments or shrunken condensed nuclei, in three or four out of four high power fields. The examination was done for each of MIG and Genox assays separately and the mean value was considered. Table 8.1 shows how the apoptosis score from CytoDEATH (Apoptosis score 1) and the mean score for apoptosis determined by immunofluorescent techniques (Apoptosis score 2) were used to assess the overall degree of apoptosis (Mean Apoptosis Score). This was then compared to the dose of LPS, the degree of oxidative stress. Diagram 8.13 illustrates

the relationship between the dose of LPS and signs of apoptosis as described by the mean apoptosis score. This shows a small positive correlation. The graphical representation of the relationship between the degree of apoptosis (mean apoptosis score) and the survival showed a medium negative correlation ($r = 0.32$).

Table 8.1: Illustrating the raw data for DNA damage score and apoptosis scores. E = Experimental and C = Control. Apoptosis score 1 refers to the results from the CytoDEATH and score 2 from combined DNA fragmentation.

	Group	Rabbit code	LPS dose mg/Kg	DNA damage score	Apoptosis score 1	Apoptosis score 2	Mean apoptosis score	Survival time Hr
1	E	UNI	.0025	.00	2.00	.00	1.00	13.00
2	E	SAM	.0050	2.00	1.00	.00	.50	23.00
3	E	ORB	.0100	3.00	3.00	1.00	2.00	14.00
4	E	TIN	.0150	3.00	1.00	2.00	1.00	10.00
5	E	ROM	.0200	3.00	1.00	2.00	1.50	4.00
6	E	NEN	.0250	3.00	1.00	2.00	1.50	5.00
7	E	VIC	.0200	3.00	2.00	1.00	1.50	3.00
8	E	XEN	.0200	3.00	1.00	2.00	1.00	10.00
9	E	ZEK	.0200	2.00	2.00	1.00	1.00	5.00
10	E	AUD	.0200	1.00	2.00	.00	1.00	5.00
11	E	BAR	.0200	1.00	2.00	1.00	1.50	5.00
12	C	GLA	.0000	.00	3.00	.00	1.50	24.00
13	C	HER	.0000	.00	2.00	.00	1.00	24.00
14	C	IVY	.0000	.00	2.00	.00	1.00	24.00
N	14	14	14	14	14	14	14	14
Median	1.00	8.0000	.017500	2.0000	2.0000	1.0000	1.0000	10.0000
Range	1	16.00	.0250	3.00	2.00	2.00	1.50	21.00

Diagram 8.13: The relationship between the dose of LPS in mg/Kg rabbit weight and the mean apoptosis score.

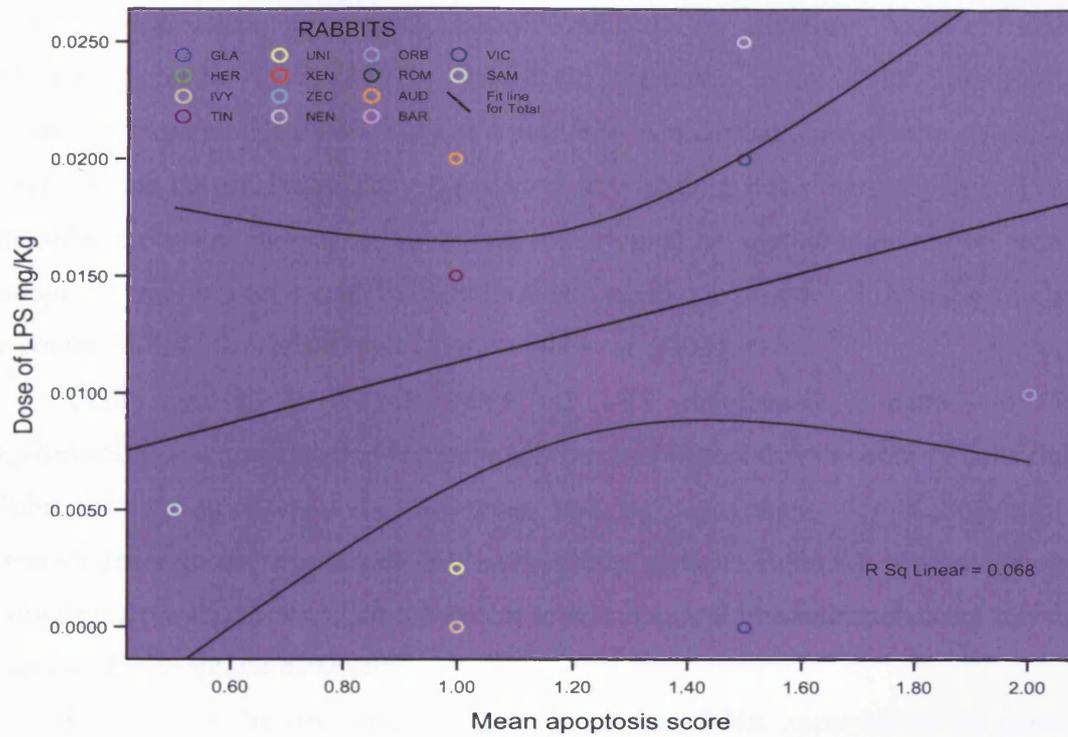
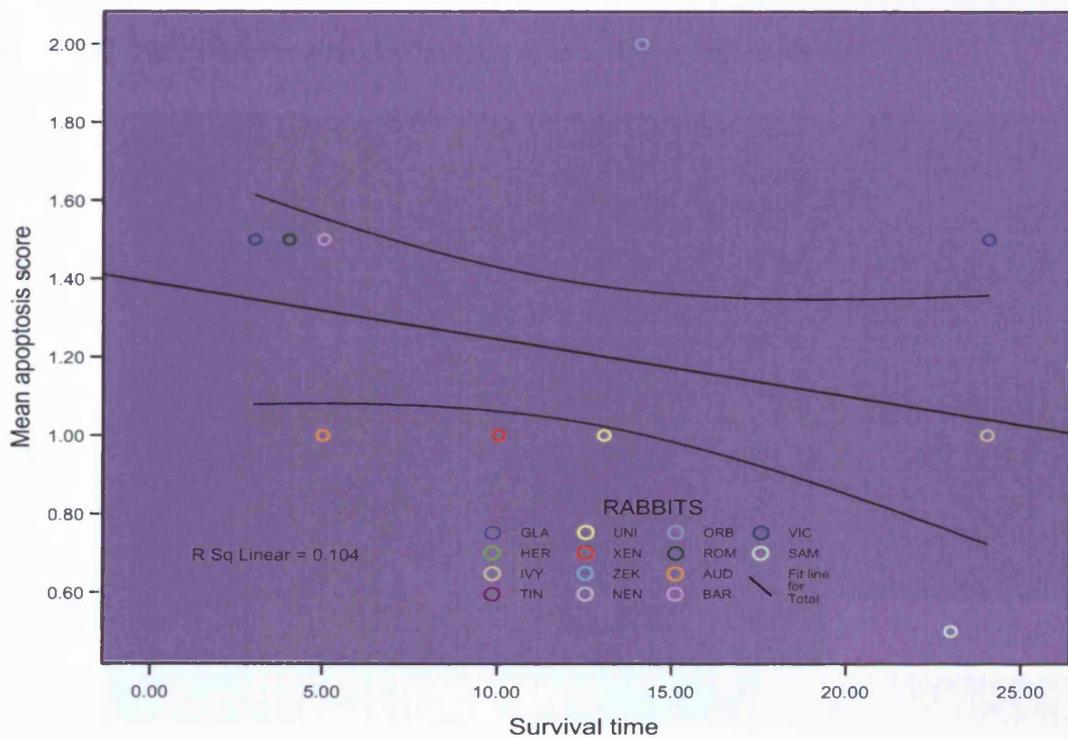


Diagram 8.14: The relationship between the mean apoptosis score and survival in septic rabbits.



DISCUSSION

Many agents that induce apoptosis are either oxidants or stimulators of cellular oxidative metabolism. Conversely many inhibitors of apoptosis have anti-oxidant activities {Schumer et al. 1992}. Buchman {Buchman et al. 1993} reported that antioxidant factors inhibit LPS induced apoptosis. Antioxidants are central to the redox balance in the human body. They do not act in isolation, but synergistically. Primary antioxidants prevent oxygen radical formation. Hence therapeutic intervention with anti oxidant therapy to alter signal transduction and mediator production helping to change the course of SIRS is a possibility {Macdonald et al. 2003}.

Cells exposed to concentrations of LPS manifested a pattern of DNA fragmentation characteristic of apoptosis. One would expect that the control cells did not exhibit an apoptotic pattern; however staining specimens for Cytokeratin 18 demonstrated a greater expression of this apoptotic marker. Table 8.1 illustrates how the scoring between the immunohistochemical expression and immunofluorescent technique of scoring for apoptosis differed.

In some of the immunofluorescent slides the DNA appeared to be generally spread throughout the cell, in some occasions the DNA fragments were concentrated in extra nuclear compartments and the FITC staining was at background levels.

Diagram 8.15: The relationship between Oxidative stress and programmed cell death.

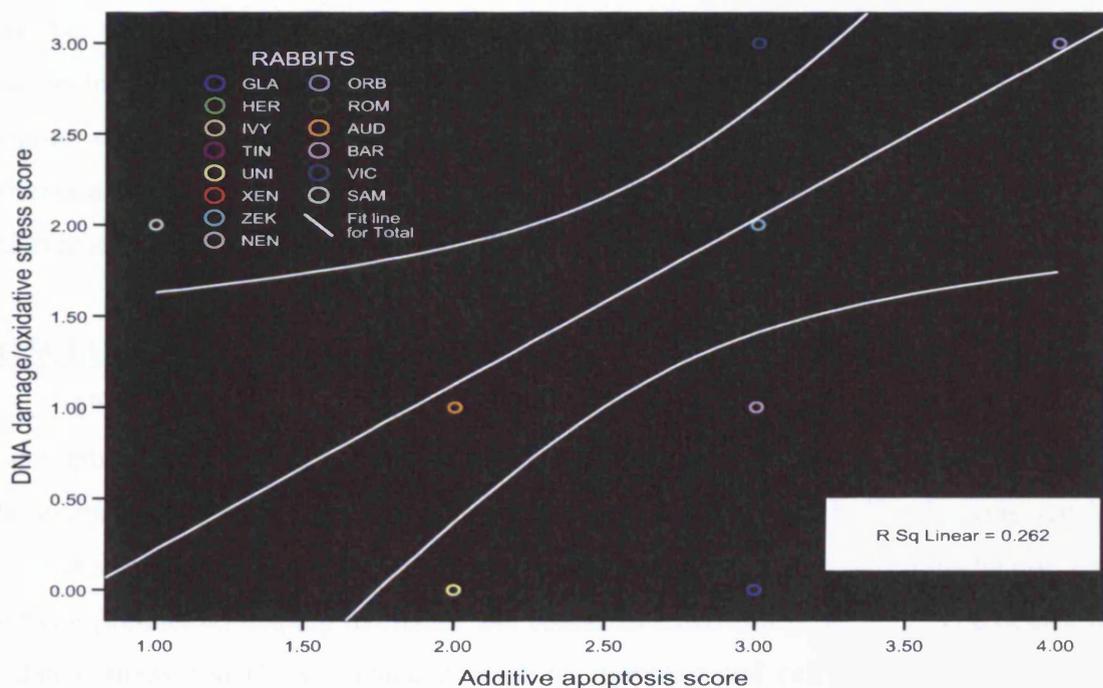
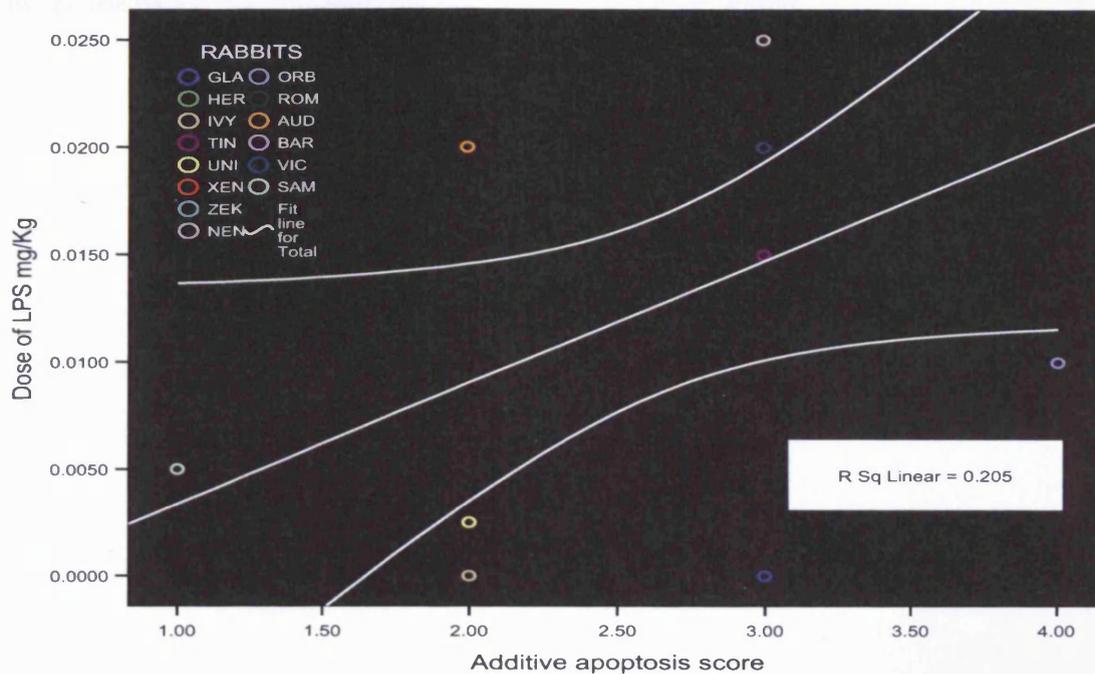


Diagram 8.16: The relationship between the dose of LPS and apoptosis induced in rabbits lung.



This implies that the DNA has escaped the nucleus and has compartmentalised itself in the cytoplasm. The immunochemical method relies on cytoplasmic expression of Cytokeratin 18 as opposed to the immunofluorescent method that relies on the morphological appearance of the nucleus. This explains the inconsistency in the results between the apoptosis score 1 and 2. Therefore adding the two apoptotic scores 1 and 2 may be the more appropriate choice. Diagram 8.15 illustrates a much stronger relationship (positive statistical correlation, $r = 0.51$) between oxidative-stress versus program cell death assessed by using the additive apoptosis score. Diagram 8.16 also presents a stronger positive correlation ($r = 0.50$) between the dose of administered LPS and extent of programmed cell death in these experimental and control rabbits.

CONCLUSION

There was a strong positive correlation between the plasma TAS and survival. This implies that TAS is a valuable predictor of survival in septic rabbits. Its levels pre-administration of LPS is also a predictor of response to endotoxin induced severe sepsis. Hence therapeutic intervention with anti oxidant therapy to alter signal transduction and mediator production helping to change the course of SIRS is a possibility. The degree of oxidative stress and DNA damage through to apoptosis and cell death can be reliably assessed by using a combination of immunofluorescence with MIG and Genox and

immunohistochemistry by Cytokeratine 18 (CytoDEATH) and additive apoptosis score between the two.

Chapter Nine

CONCLUSION

ECMO IS SUPERIOR TO CONVENTIONAL ICU MANAGEMENT ONLY IN A SELECTED GROUP OF SEVERE SIRS PATIENTS

ECMO may have provided us with a cohort of patients which otherwise had no chance of survival. Patients referred to this institution were amongst the sickest in the United Kingdom. They were referred for ECMO at a time when conventional therapy had already failed. They suffered from severe SIRS/sepsis, SIRS/septic shock and MODS. All patients included in this study satisfied the criterion set by the 2002 ACCP/SCCM consensus conference definitions.

This study illustrates a relationship between the type of insult and age groups. Some insults are exclusive to an age group for example MAS, whereas others overlap for example streptococcal (Group B Strep) and meningococcal sepsis. As illustrated in chapter three compared to the conventional management reported in the literature, our ECMO practice has been a success particularly in the management of RSV sepsis in children and neonates, acute pancreatitis and aspiration pneumonia in adults and children, streptococcal sepsis and MAS in neonates. Although other causes of neonatal sepsis like Pertussis and meningococcal septicaemia, particularly in children remain a therapeutic challenge. For these and other indications ECMO offers an acceptably similar if not superior outcome to conventional methods.

The graded response to ECMO in terms of mortality from severe SIRS/sepsis SIRS/septic shock to MODS is summarised in table 9.1. This table illustrates that in both neonates and adults the association between sepsis and shock and in most stages their staged progression from single organ respiratory failure to shock and MODS had negative influences on the outcome of treatment with ECMO. This outcome can be predicted in the form of percentage survival, by calculating the MODSI and using the regression plots (Diagrams 3.4 or 3.5), or simply by looking at the bar diagram 3.3. However in children the only significant negative influence came from the progression from severe sepsis to septic shock or from SIRS shock to MODS.

Geographic variation is one of the particular predisposing factors that make epidemiological comparison difficult. However despite that the outcome of ECMO for supportive management of severe systemic inflammatory response syndrome and multiple organ dysfunction seems acceptable.

Table 9.1: Graduated percentage mortality in neonates, children and adults with Severe SIRS supported with ECMO.

Age Group	Severe SIRS	No Shock	Shock	MODS
Neonates	Septic	0.0%	50.0%	61.0%
	Sterile	0.0%	2.5%	8.7%
Children	Septic	10.6%	34.0%	40.9%
	Sterile	36.4%	38.2%	46.8%
Adults	Septic	31.4%	41.1%	45.2%
	Sterile	33.7%	27.3%	50.0%

There was a significant reduction in inotropic requirements after the start of the VV-ECMO. This may be a consequence of the improved myocardial oxygenation and reduced uncompensated metabolic acidosis by VV-ECMO. In some cases improved oxygenation alone was not enough to support circulatory failure. In these cases VA-ECMO remained the preferred mode for circulatory support in the presence of respiratory compromise. The other analogy is that SIRS is down regulated (From SIRS shock to severe SIRS); however although the new oxygenator technology has significantly reduced the haematic response to ECMO, it has failed to influence the survival. We have also shown a down regulation in the degree of MODS in all patients from a MODSI of 93,63 and 69 percent in neonates, children and adults respectively pre ECMO to 36,63 and 57 percent post ECMO.

LIMITATIONS TO THE MANAGEMENT OF SEVERE SIRS ON ECMO

An attempt to successfully treat severe SIRS, would involve a treatment strategy directed to eliminate the ongoing insult, supportive therapy of the organ dysfunction and an attempt to alter the systemic thrombo-inflammatory response. As well as being a useful supportive therapy for patients with severe cardiac or respiratory failure, ECMO can accommodate renal or hepatic support by haemofiltration or molecular adsorption recirculating system respectively. This makes ECMO an ideal supportive platform for MODS. However ECMO is not a treatment against systemic inflammation, in fact it will exacerbate haematic response, which is indistinguishable from SIRS (Chapter 1).

Two of the most common varieties of artificial lung worldwide were evaluated in chapter four. The novel PMP oxygenator have reduced haematic response and improved efficiency during ECMO. It is smaller but more efficient in terms of gas exchange, hence reducing the number of oxygenators required per adult circuit. Better biocompatibility of these heparin treated oxygenators improved coagulation stability, reduced activation and hence consumption of coagulation factors which lead to less transfusion requirements, especially of platelets. Using a single PMP oxygenator for all adults would also significantly reduce transfusion of red blood cells.

The smaller variety of PMP oxygenators (Medos Hilite 800 LT) in neonates provided adequate gas exchange and offered technical advantages in terms of more efficient priming, reduced hemodynamic resistance and better control and preservation of coagulation proteins than Silicon oxygenators. However there is a need for improvement in the design of smaller oxygenators. Adjustment of the coagulation parameters, and lowering the tolerance towards clots in the circuit by electively changing them, may reduce the incidence of sudden unexpected oxygenator failure, however using a slightly larger design (Medos Hilite 2400LT) may buy valuable time to arrange oxygenator change in an emergency. Ideally an oxygenator with the smallest surface area but efficient enough gas exchange, low enough resistance not to require a roller pump, coated with strongly bound heparin and designed with safety features to prevent sudden unexpected oxygenator failure will have optimum efficiency and further reduce haematic response to ECMO. PMP oxygenators reduced radiographic signs of the hosts response to ECMO in neonates and delayed that response in adults. Coating oxygenators with heparin may have contributed to the reduced inflammatory and coagulative response to ECMO.

Cellular components of the innate response such as neutrophils and biochemical components such as cytokines and chemokines play an active role in the balance of inflammatory homeostasis. An attempt at alteration of the systemic thrombo-inflammatory response by neutrophil depletion using in-line filtration is known to be effective in attenuating SIRS and lung injury caused by cardio-pulmonary bypass (CPB) {Al-Ebrahim et al. 1993 & Bando et al. 1990}. Molecular adsorption recirculating system (MARS) has also shown to measurably clear cytokines in patients with acute on chronic liver failure {Stadlbauer et al. 2006}. However it has been unable to reduce serum levels of these cytokines presumably due to their continuous production {Stadlbauer et al. 2006}, as once activated the inflammatory process is propagated through feedback pathways. Since the current treatment including ECMO, CVVH and MARS merely

supports or replaces organ functions until recovery occurs, there are still many patients with fulminant MODS who do not respond to these supportive therapies per se.

A disease modifying approach to treatment of severe SIRS while supporting the organ dysfunction by interrupting the thrombo-inflammatory cascades of feedback mechanisms may be the solution and more effective than supportive treatments alone. Our study of the oxygenators in chapter four showed a significant reduction in the haematic response illustrated by haematologic and radiological signs but did not significantly alter the outcomes in terms of ECMO survival, duration of treatment and length of stay in hospital in either group. This raises the question would a reduction in haematic response to ECMO alone be as beneficial as we think or a much greater manipulation of the STIP is required?

THE RABBIT MODEL

In the absence of a reliable model we have no means of assessing new approaches to treatment of severe SIRS / sepsis. Before any clinical trials can be performed the efficacy and safety of that treatment must be investigated in an animal model.

In this thesis we developed a reliable and reproducible rabbit model of sepsis/SIRS by administration of intravenous LPS and determined the dose response relationship for both the primary outcome measure of death and a number of secondary outcome measures such as physiological, haematological, chemical and immune responses.

We proved that there is a linear relationship between the dose of LPS and time to death according to the dose response curve and that rabbits behave very similarly to humans with respect to graded response to sepsis. The dose response curve enabled determination of the dose that would result in death if left untreated however allowing enough time to intervene using an extracorporeal strategy. We chose that dose to be 0.02 mg/kg rabbit body weight. This allowed a mean (SD) time to death of 5.6 (2.4) hours derived from the study of inter-individual variations. The inter-individual variations were again tested for each of the physiological and biochemical outcome measures. This proved consistent results. However not all the cellular, biochemical and thrombo-inflammatory markers yielded desired outcomes. We therefore recommend the following secondary outcome measures in any follow-up experiments.

- 1) Physiological: Measurement of the temperature, heart rate, mean arterial pressure, the central venous pressure and urine output.

- 2) **Biochemical:** The arterial blood gases, the central venous saturation, serum urea and electrolytes.
- 3) **Haematological:** The differential leukocyte count, the full coagulation screen.
- 4) **Thrombo-inflammatory markers of STIP:** The markers of the immune response in rabbits best suited to this experiment were; Fibrinogen as a sensitive acute phase protein with a response time within the first five hours of LPS administration. The best pro-inflammatory cytokine was IL-6 with a peak levels at four to five hours and a small inter-individual variation. The IL-8 chemokine demonstrated peak concentration at four hours post LPS. The “Immunosuppressive cytokine” TGF- β 1 levels pre administration of LPS was a useful indicator of predisposition and so a predictor of out comes of endotoxic challenge. Thrombomodulin is a useful central component of STIP that would suit this model well and APTT a sensitive coagulative marker that rises within the first five hours of LPS administration. PAI-1 is the perfect marker of anti-fibrinolytic response for this model with peak levels within the first five hours. In cases where the treatment strategy is already in place or readily installed within the first hour of LPS administration, TNF α and C3 complement activation assays may be used. C3 activation assay as well as fibrinogen are useful assays for measuring changes in the first stage of haematic response as compared to coagulation marker such as APTT or central markers such as thrombomodulin. In cases where the assay did not give fruitful results we suggest further optimisation to improving the coefficient of variation prior to performing the experiment.
- 5) **Histological and immunohistochemical markers of STIP:** The H&E slides were greatly helpful in assessing signs of thrombo-inflammation in rabbits. Neutrophil and macrophage counts were important parts of the semi-quantitative analysis and histo-inflammatory scoring system, which proved to be a success as its results correlated strongly with the dose of LPS and the survival time.
- 6) **Oxidative stress, DNA damage and apoptosis:** Total anti-oxidant levels fell in all experimental animals compared with the control. This test together with the immunofluorescent techniques of oxidative stress (M1G and Genox) show reciprocal relationships which also can be correlated with apoptosis using either the Cytokeratine 18 or a combination of that and degree of DNA fragmentation in the nucleus (additive apoptosis score).

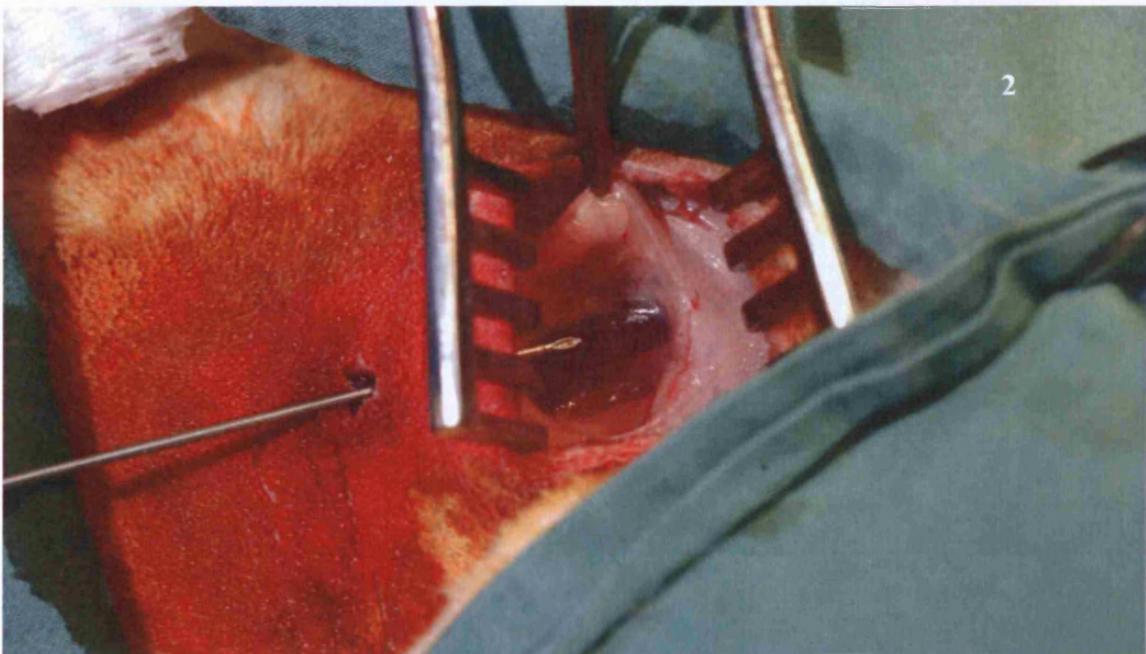
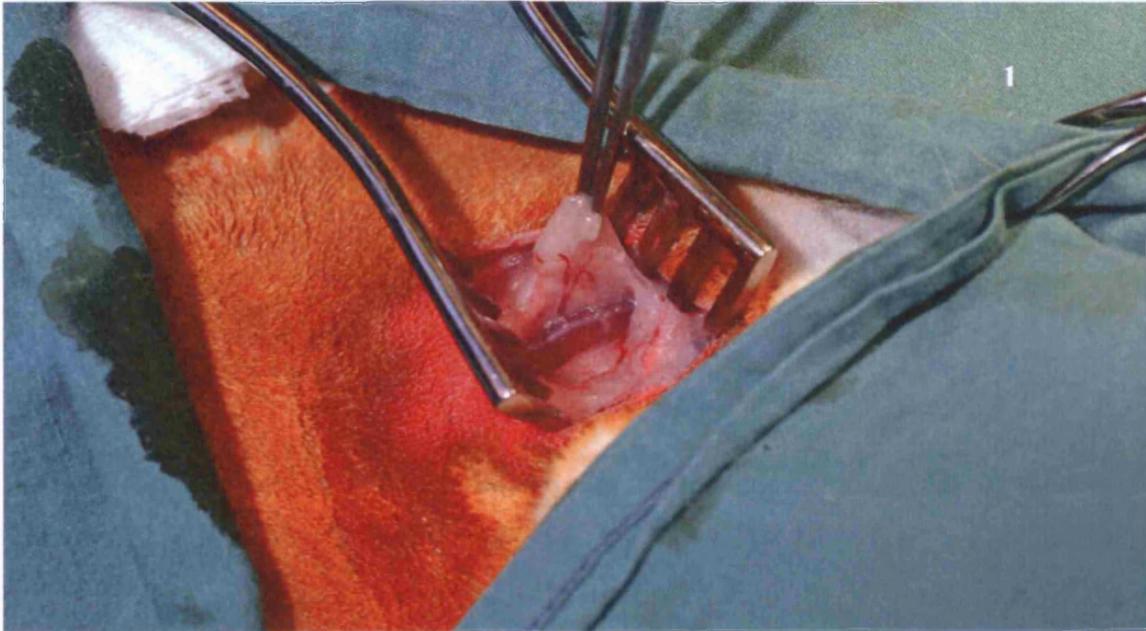
This model clearly demonstrates a reliable and reproducible rabbit model of graded sepsis / SIRS and multi organ dysfunction. Activation of the STIP which leads to pan endothelial injury further fuels this vicious cycle of inflammation, pro-coagulation and anti-fibrinolysis leading to micro-thrombosis as well as further activation of platelets and neutrophils. This is exacerbated by sympathetic response resulting in hypo-perfusion, hypoxia, metabolic acidosis oxidative stress which leads to DNA damage. In the absence of antioxidants this process is amplified and triggers apoptosis or programmed cell death. Then multi organ dysfunction is inevitable unless this process is interrupted. We believe this model would provide a simple solution to our experimental needs in the study of SIRS, MODS and ECMO.

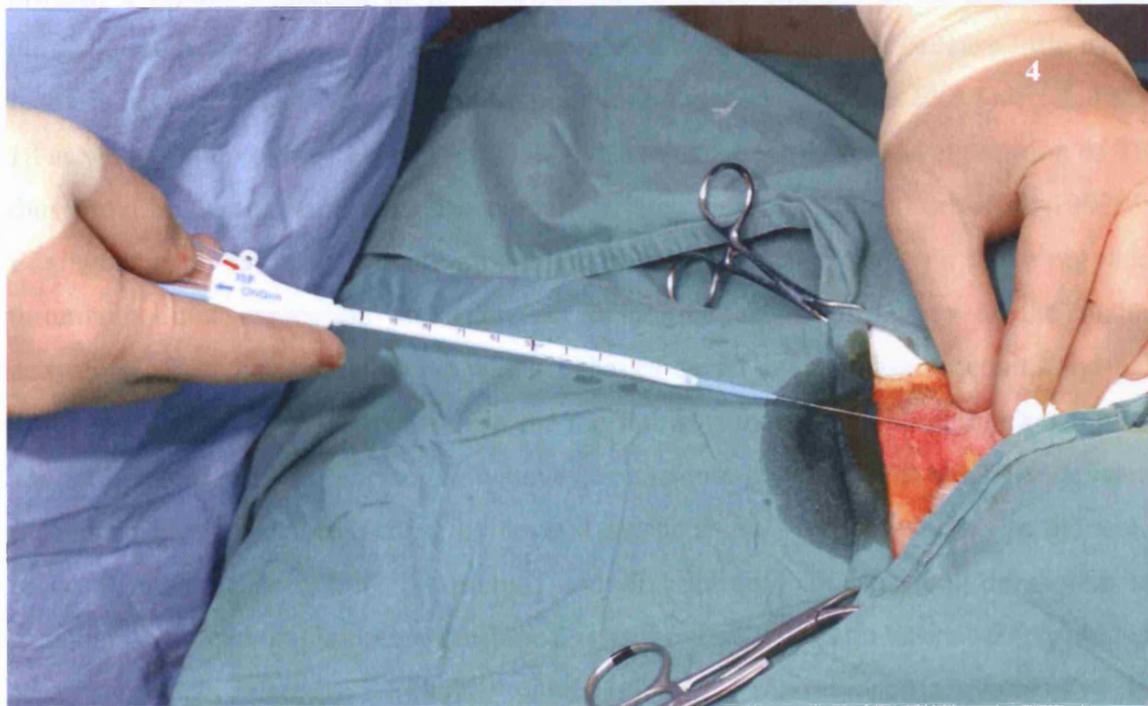
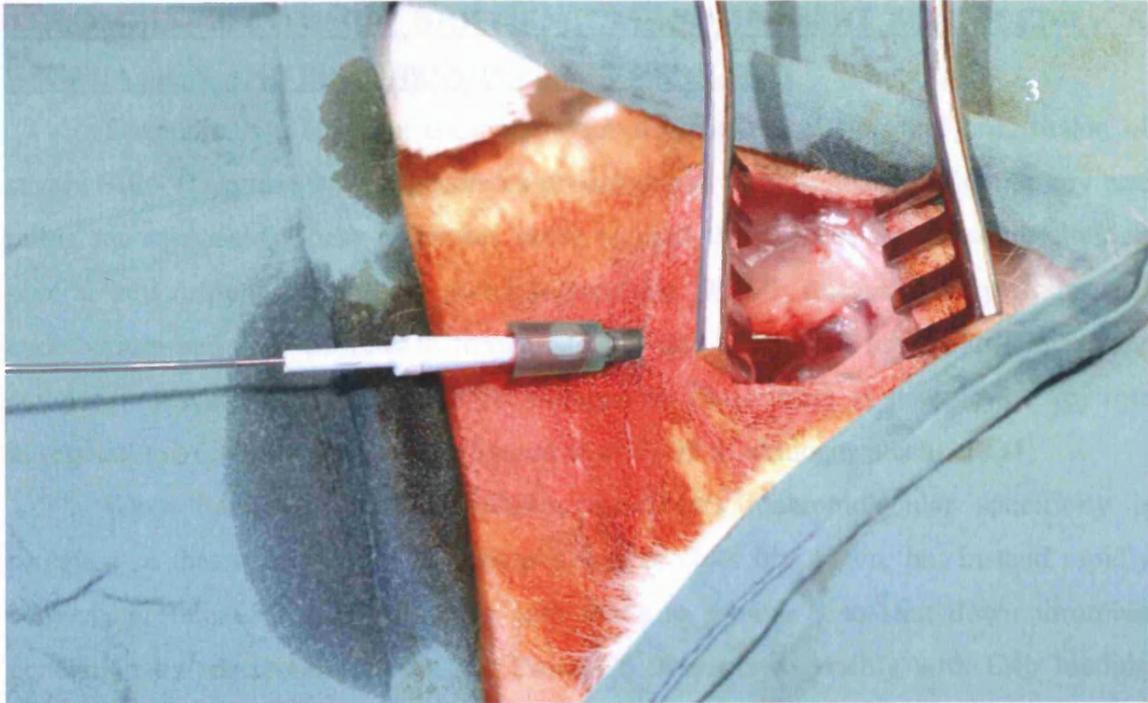
THE NEXT PHASE OF ANIMAL EXPERIMENTS

Now that we have a reliable and reproducible model of severe SIRS/sepsis, and have a number of physiological, biochemical, haematological thrombo-inflammatory and histochemical tools to assess the outcome of novel treatment strategies, we need to move to the next phase of the animal experiments which involves placing the rabbit on an extracorporeal circuit. This new phase of rabbit experiments would involve development of a protocol for setting up the rabbit extracorporeal circuit. Rabbits have previously been used in ECMO research {Kress et al. 1987 & Trittenwein et al. 1999}. They are the smallest and the lowest order of animals that can be placed on an existing human ECMO circuit. This would be very similar to that of a neonate. We would then be able to evaluate the safety and efficacy of extracorporeal therapeutic adjuncts proposed in this thesis such as specialised filters that remove neutrophils (Neutrophil depletion filter) or by removing effector molecules from the serum, by using Molecular Adsorption Recycling System (MARS). A randomised controlled rabbit experiment may be necessary before any clinical trials can begin.

In the next phase the animals would be anaesthetised, intubated and surgically instrumented for measurement of physiological outcomes as before. Same protocol for LPS administration and sampling is followed with the dose of 0.02 mg/kg of LPS as determined in chapter five. Diagram 9.5.1, .2, .3 and .4 illustrate the approach to cannulation of a rabbit for extracorporeal experiments. This approach is also the same as that of cannulating a neonate and has also been used in children and adults. The set-up of the rabbit extracorporeal circuit would be the same as that described by Kress {Kress et al. 1987 & Trittenwein et al. 1999}.

Diagram 9.5.1, .2, .3 and .4: Illustrates the semi-seldinger technique of cannulating the right internal jugular vein in a schedule one animal. The numbers on the top left corner illustrates the technical sequence of cannulation. 1. Incision exposing the right internal jugular, 2. Insertion of the introducer needle under direct vision, 3. Introducing the Seldinger wire and 4. Cannulation with a 15 F OriGen double lumen neonatal ECMO cannula.



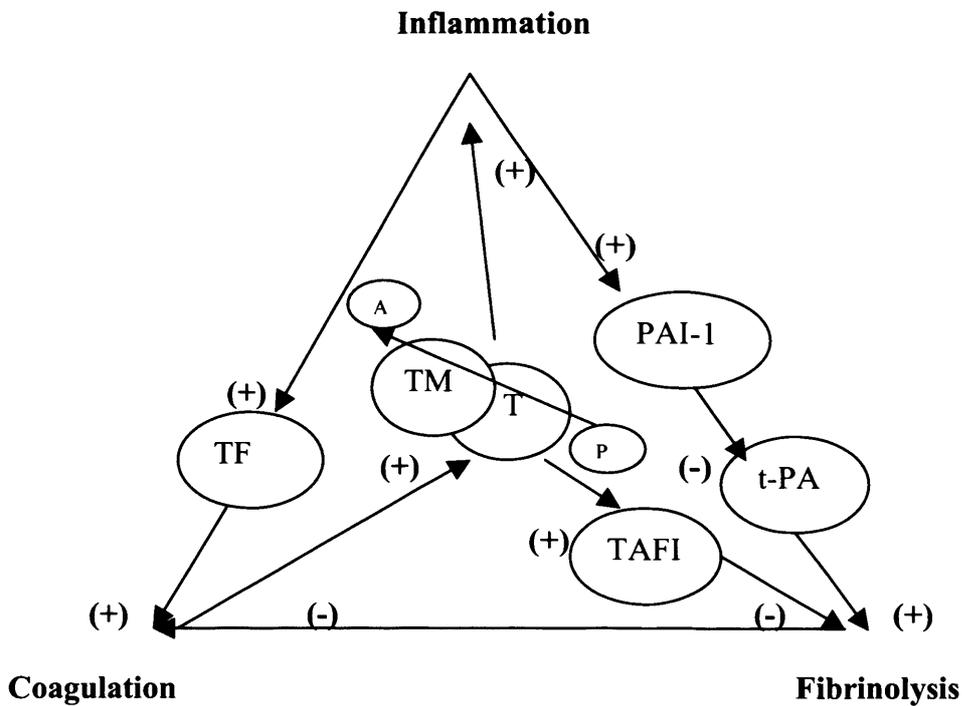


THROMBIN; A COMMON BIOCHEMICAL COMPONENT FOR FEEDBACK ESCALATION AND PROGRESSION OF SEVERE SIRS

Thrombin is a common component for feedback escalation and progression of severe SIRS (Diagram 9.1). It generates procoagulant, anticoagulant, inflammatory and mitogenic responses. These responses serve to shift the homeostatic balance, eliciting several key responses including platelet activation (PDGF release from platelets and endothelium and TGF- β 1 release from platelets) and fibrin clot formation (Encourage cellular proliferation for example direct mitogen for fibroblasts). It also plays a key role in regulation of its own production {Esmon et al. 2005 & Shinozawa et al. 2004}.

When bound to thrombomodulin, thrombin's macromolecular specificity is switched so that it no longer activates platelets or clots fibrinogen, but instead rapidly converts protein C to APC which in turn binds to protein S to shut down thrombin generation by inactivating factor Va. Protein S interacts reversibly with C4b binding protein, a regulator protein of the complement system {Esmon et al. 2006}. Under normal circumstances there is little thrombin generation, in large part because little tissue factor is in contact with blood. The rise in the inflammatory cytokines like TNF α and IL-1 β as well as endotoxins interact with monocytes to cause synthesis and expression of TF thus providing a trigger to innate coagulation {Beutler et al. 2008, Port et al. 1992 & Edgington et al. 1991}. The acute inflammatory mediators such as TNF α down regulate thrombomodulin expression and increase alpha one anti-trypsin, one of the major activators of protein C inhibitors, which behaves as an acute phase reactant. The net influence of these events is to diminish the mechanisms that control thrombin generation and a raise in thrombin. Fibrinogen behaves as an acute phase protein too. The elevated fibrinogen levels promote clotting by several mechanisms. Clot deposition is a dynamic process involving both fibrin formation and dissolution. The fibrinolytic system is responsible for clearing intravascular blood clots, primarily through tissue t-PA mediated activation of plasminogen. PAI-1 is a rapid inhibitor of plasminogen activator. Rise in PAI-1 induced by cytokines impairs clot lysis by preventing plasminogen activation. These events all lead to an increased thrombin formation {Esmon et al. 2004}.

Diagram 9.1: Diagrammatic illustration of the Systemic Thrombo-Inflammatory Pathway (STIP). PAI-1 is plasminogen activation inhibitor 1; t-PA, tissue plasminogen activator; TF, tissue factor; TAFI, thrombin-activatable fibrinolysis inhibitor; TM, thrombomodulin; P, protein C; A, activated protein C and T, thrombin.



The link between the coagulation and inflammatory response is accentuated when one examines the potential responses mediated by thrombin. Thrombin not only plays a role in clot formation and anticoagulant response, but also mediates cellular inflammation {Coughlin et al. 2000}. For example thrombin causes the expression of platelet activating factor (A potent neutrophil agonist) and the expression of Selectin on the endothelial cell surface {Lorant et al. 1993}, probably facilitating the proteolytic release of TM from the endothelium mediated by neutrophil elastase {Takano et al. 1990}. Since soluble TM has lower activity than cell surface TM, It is likely that this proteolytic attack on the TM creates a net shift in the haemostatic balance favouring the clotting process. The loss of thrombo-resistance from the endothelium would likely result in propagation of the coagulation response, thus generating additional thrombin. Thrombin mediated vascular injury is neutrophil dependent, probably a manifestation of thrombin induced up regulation of adhesion molecules and selectins {Sugama et al. 1992}. Since thrombin is chemotactic for neutrophils, thrombin generation at the sight of injury would also facilitate additional neutrophil recruitment to the injury sight, further amplifying the process. This thrombin signalling is probably achieved by activating the PAR-1-type thrombin receptor {Cirino et al. 2000}. This in turn stimulates endothelial cell activation

resulting in the availability of adhesion molecules {Levi et al. 2002}, which facilitates the transendothelial migration of leukocytes into the surrounding parenchyma {Harlan et al. 2002}. Endothelial cell activation also promotes the synthesis of pro-inflammatory cytokines {Krishnaswamy et al. 1999}. Some of the protective effects of activated protein C are undoubtedly due to its ability to diminish thrombin generation at or near the vessel surface, and hence to minimise the thrombin dependant amplification of coagulation and inflammatory responses. In support of this concept other anticoagulants can protect baboons challenged with lethal levels of E coli. These anticoagulants include monoclonal antibodies to tissue factor {Taylor et al. 1991}, tissue factor pathway inhibitor {Creasey et al. 1993}, active site blocking factor VIIa {Taylor et al. 1991}, and anti thrombin a relatively non-specific protease inhibitor that is only effective at extremely high levels {Emerson et al. 1989}. In contrast Heparin and active site blocking factor Xa a competitive inhibitor of pro-thrombin activation do not prevent death but do block the coagulation response at least as effectively as agents that prevent the lethal response {Coalson et al. 1978 & Taylor et al. 1991}. Therefore Thrombin is capable of stimulating multiple inflammatory pathways {Stouthard et al. 1996, Esmon et al. 1991, Conkling et al. 1988, Bevilacqua et al. 1986}. It also function by triggering cell activation. For example its effects on endothelium are procoagulant, proinflammatory and impair vascular integrity, increase capillary permeability and capillary leak syndrome {Garcia et al. 1995}.

Even though thrombin generation is responsible for DIC. Thrombin infusion has shown to lead to formation of a net anticoagulant response due to the formation of activated protein C {Comp et al. 1982}. Anti-thrombin (anti-thrombin III) is a cofactor for heparin anticoagulation. Deficiency of anti-thrombin III results in heparin resistance and a hyper-coagulable state. Low anti-thrombin levels in DIC are associated with increased mortality {Wilson et al. 1996} the anti-inflammatory effect of anti-thrombin may be related to endothelial binding and promotion of prostacyclin release. However anti-thrombin replacement has not been shown to improve outcome of sepsis {Wilson et al. 1996}. Protein C is a natural anti-thrombotic substance produced by the liver. Activated by the thrombin-thrombomodulin complex APC inhibits thrombin production (anti-thrombin effect) hence; exhibits anticoagulant, anti-inflammatory and fibrinolytic properties. The concentration of APC is low in patients with sepsis {Fisher et al. 2000} this may be due to a relative lack of production of its precursor by the Liver predisposing patients to sepsis. In a study of sepsis {Mesters et al. 2000} patients with sepsis had

significantly lower concentration of protein C and the patients with septic shock had the lowest concentration of protein C. Activated protein C has Anti-thrombotic, pro-fibrinolytic and anti-inflammatory properties {Joyce et al. 2002}. It modulates the coagulation system by a reduction in the synthesis and expression of Tissue Factor {Shua et al. 2000}. It inactivates factor Va and VIIIa therefore limits production of thrombin and limits activation of factor X {Mosnier et al. 2007}.

Thrombin induced activation of endothelium platelets and vascular smooth muscle directly damage vascular endothelium and neutrophil cell adhesion which further exacerbates inflammation. Thrombin production leads to enhanced microvascular coagulation endothelial cell dysfunction and apoptosis. Ultimately microvascular function is compromised which results in DIC and microvascular thrombosis, decreased tissue perfusion and hypoxaemia and organ dysfunction.

Control of Thrombin therefore would be instrumental in breaking the vicious cycle between Inflammation, Coagulation and Fibrinolysis. Diagrams 9.2, 9.3 and 9.4 illustrate that inhibition of any of the three components of STIP alone would still allow positive feedback mechanism that fuels this process. Diagram 9.5 illustrates the central role of thrombin in the feedback mechanism that keeps STIP spiralling out of control. In this diagram controlling plasma levels of thrombin may brake the vicious cycle of proinflammatory, pro-coagulative and anti fibrinolytic feedback. It is therefore not surprising how treatment strategies that influence thrombin production such as activated protein C has influenced the outcome of severe SIRS and MODS in clinical practice {Riedemann et al. 2003}.

Complete inhibition of thrombin however is not desirable either. Low levels of systemic thrombin would result in protein C activation. Activated protein C formation is critical for survival. Pittsburgh mutant is a potent inhibitor of both thrombin and APC {Heeb et al. 1990} Infusion of this inhibitor into baboons challenged with E Coli blocked DIC response but exacerbated the shock response resulting in decreased survival time {Harper et al. 1998}. Both endotoxin and proinflammatory cytokines promote thrombin generation hence impair anticoagulation and diminish fibrinolysis {ten Cate et al. 1997}.

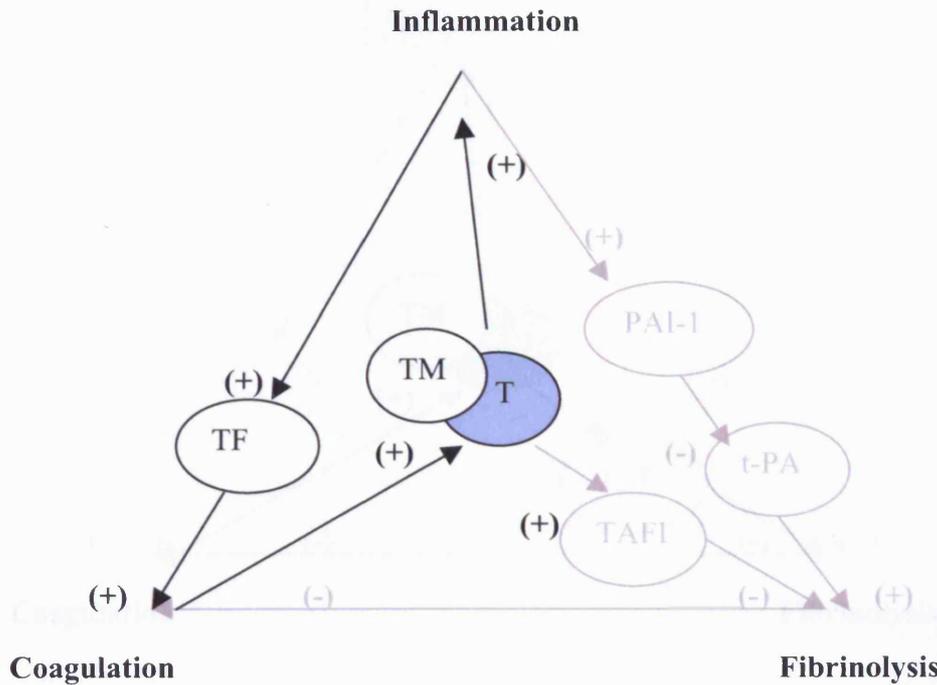
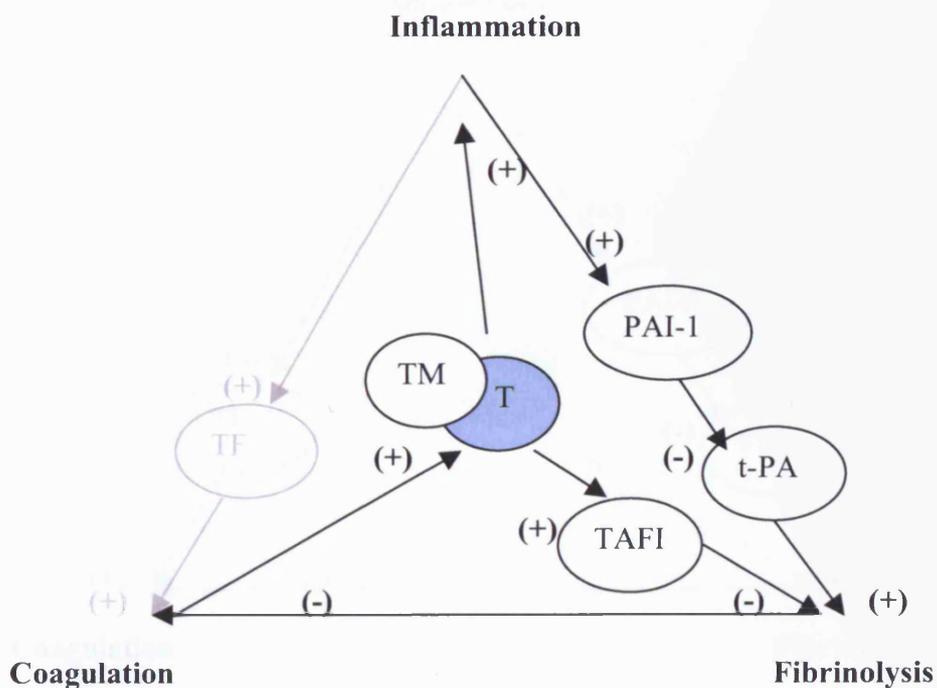


Diagram 9.2 (Above): Illustration of the Systemic Thrombo-Inflammatory Pathway (STIP) when interrupted by eliminating the positive feedback mechanisms that connect fibrinolysis to the other components of STIP. Note that the feed back mechanism still persists (Direction of the darker arrows) between the inflammatory and the coagulative components. Diagram 9.3 (Below): Illustrates persistence of feedback mechanism between the inflammatory, coagulation and fibrinolysis component up on elimination of the direct feedback between inflammatory and coagulative components.



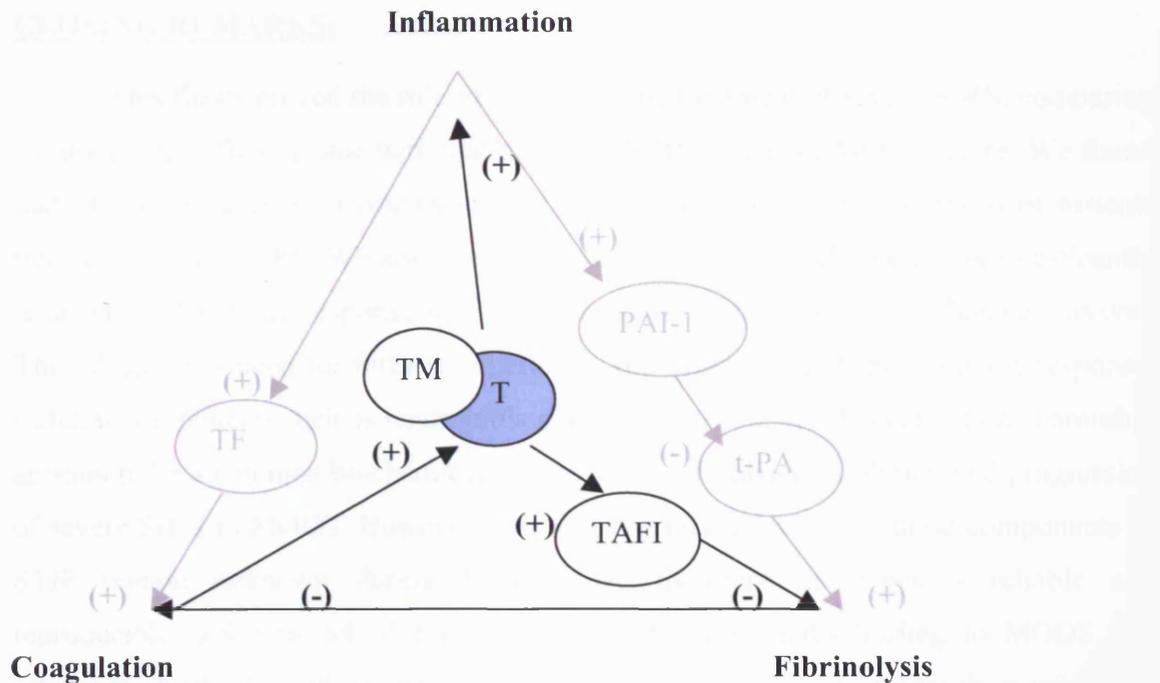
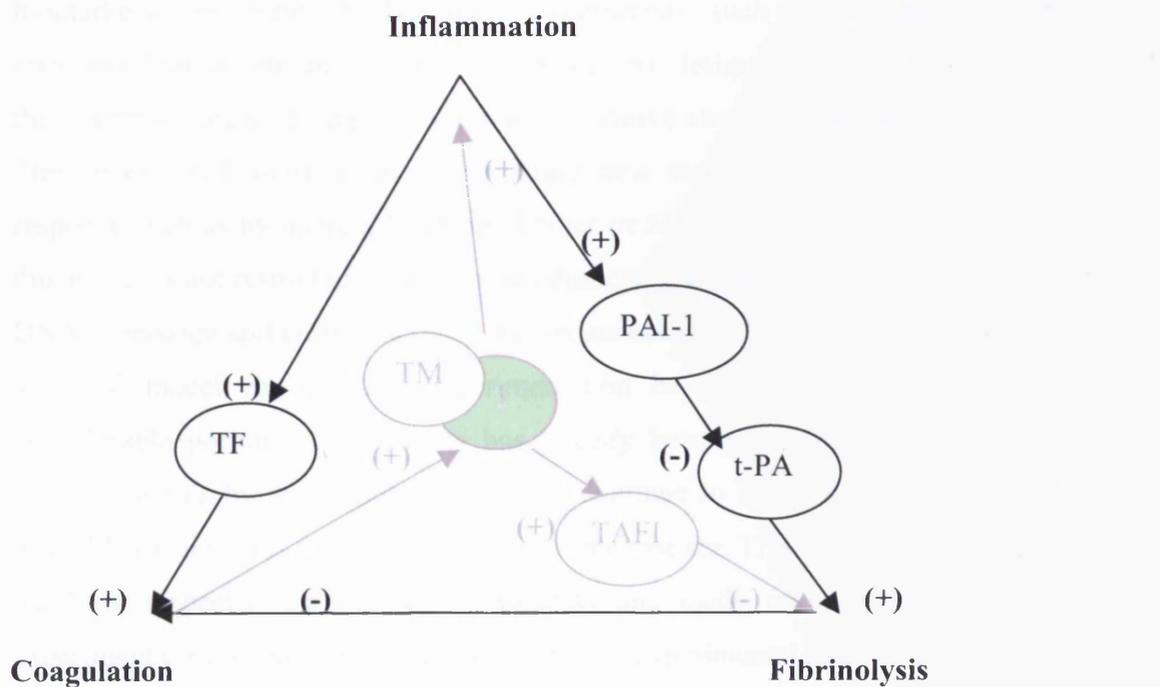


Diagram 9.4 (Above): Illustration of the Systemic Thrombo-Inflammatory Pathway (STIP) when interrupted by eliminating the feedback mechanism between the inflammatory and with coagulative and fibrinolytic components. Note that the feed back mechanism still persists (Direction of the darker arrows) between the inflammatory and the coagulative components. Diagram 9.5: Illustrates the components of the Systemic Thrombo-Inflammatory Pathway (STIP) without circular feedback mechanisms that propagates the vicious cycle between the pro-inflammatory, pro-coagulant and anti-fibrinolytic components. Note that the feed back mechanism has ceased to persist (Direction of the darker arrows no longer form a complete circle).



CLOSING REMARKS:

This thesis proved the role of ECMO in the treatment of severe SIRS, comparing its disease specific outcome with that of severe SIRS in non-ECMO literature. We found that ECMO is superior to conventional ICU management in selected groups of patients treated for severe SIRS. We also found that new oxygenator technology has significantly reduced the haematic response to ECMO, however it has failed to influence survival. This illustrates a need for further reduction in the cycle of STIP and haematic response. Cellular components such as neutrophils play a central role in SIRS activation. Thrombin appears to be a common biochemical component for feedback escalation and progression of severe SIRS to MODS. However the clinical effects of reducing these components of STIP remain unknown during ECMO. We therefore developed a reliable and reproducible rabbit model of endotoxin induced severe SIRS leading to MODS and ultimately death. We will use this model to test novel strategies to ensure their safety and efficacy prior to their trial in human subjects. We have determined the dose response and inter-individual variation for development of primary outcome measure of death and many secondary outcomes. We defined the pathophysiology of STIP and the haematic response to ECMO and tested their components in our rabbit model. We have developed a physiological, biochemical, haematological, immunological, histological and immunohistochemical profile of rabbits in endotoxic shock. These cellular and biomarkers were tested by laboratory experiments, such as quantitative ELISAs and semi-quantitative immunocytochemistry which we designed and optimised. We assessed the degree of organ damage by studying oxidative stress, DNA damage and apoptosis. This model will assist us in investigating new strategies for reducing the haematic response such as by using a Neutrophil filter or MARS on ECMO. However the use of this model is not restricted only to these adjuncts. We have found that there is significant DNA homology and cross-reactivity that exists between humans and rabbits making this a useful model for immune experimentation beyond the scope of this thesis. A considerable portion of this thesis has already been presented and published in peer review journals by the author and we will continue to make the findings of this thesis available to clinicians and scientists in the same manner. Throughout this research project we have respected the privacy of patients and made maximal use of any animal experiment conducted by keeping the number of experimental animals to a minimum.

Appendix One

SUPPLIERS

Alphabetical list and address of surgical, veterinary and biochemical suppliers mentioned in this thesis.

American Diagnostica Inc,
222 Railroad ave, P.O.Box 1165, Greenwich, CT 06836-1165
United Kingdom

Amersham Biosciences UK Ltd,
Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA
United Kingdom

AMS Biotechnology Europe Ltd,
63B Milton Park, Milton, Abingdon, Oxon, OX14 4RX
United Kingdom

BD Biosciences,
BD Biosciences- Customer Service, The Danby Building, Edmund Halley Road, Oxford
Science Park, Oxford, OX4 4DQ
United Kingdom

Biodesign International,
60 Industrial Park Road, Saco, ME 04072
United States

Biomedica,
1191 Chess Drive, Suite F, Foster City, CA 94404
United States

Biosource International,
BioSource Europe S.A., rue de l'Industrie, 8- B 1400
Belgium

Calbiochem,
Merck Chemicals Ltd,
Padge Road, Beeston, Nottingham, NG9 2JR
United Kingdom

Cambridge BioScience Ltd,
24-25 Signet Court, Newmarket Road, Cambridge, CB5 8LA
United Kingdom

Charles River Laboratories Inc,
Edinburgh,
United Kingdom

Dako Uk Ltd,
Cambridge House, St Thomas Place, Ely, Cambridgeshire, CB7 4EX
United Kingdom

Genox Corporation,
1414 Key Highway, Suite 300, Baltimore, MD 21230
United States

Hycut Biotechnology,
Linda Keizer, Frontstraat 2A, 5405 PB Uden
The Netherlands

Immunology Consultants Laboratory Inc,
141 N. Elliott Rd, Newberg, OR 97132,
United States
(Provided by Dunn Labortechnik GmbH, www.dunnlab.de)

Johnson & Johnson Medical Ltd,
Coronation Road, Ascot, Berkshire, SL5 9EY
United Kingdom

Lab Vision Products,
Thermo Fisher Scientific, 93-96 Chadwick Road, Runcorn, Cheshire, WA 71PR
United Kingdom

Medos Medizintechnik AG,
Obere Steinfurt 8-10, D-52222 Stolberg
Germany

Medtronic Europe Sarl,
Route du Molliau, Case Postale, CH-1131 Tolochenaz,
Switzerland

NIBSC (National Institute for Biological Standards and Control),
Blache Lane, South Mimms, Potters Bar, Hertfordshire, EN6 3QG
United Kingdom

Randox Laboratories Ltd,
Diamond Road, Crumlin. Co. Antrim, BT29 4QY
United Kingdom

R&D Systems Europe Ltd,
19 Barton Lane, Abingdon Science Park, Abingdon, Oxon OX14 3NB
United Kingdom

Roche Molecular Biochemicals,
Charles Avenue, Burgess Hill, RH15 9RY
United Kingdom
Sigma-Aldrich Company Ltd,
Fancy Road, Poole, Dorset, BH12 4QH
United Kingdom

Serotec MorphoSys UK Ltd,
Endeavour House, Langford Business Park, Kidlington, Oxford, OX5 1GE
United Kingdom

Vector Laboratories Ltd,
3 Accent Park, Bakewell Road, Orton Southgate, Peterborough, PE2 6XS, United
Kingdom

Appendix Two

BUFFERS & SOLUTIONS

Buffers & Solutions:

The following are the constituents of commonly prepared stock solutions and Buffers used in this thesis.

PBS: Phosphate Buffer Saline (1L solution of Phosphate Buffered Saline (PBS) contains 137mM Sodium Chloride, 2.7mM Potassium Chloride and 10mM Phosphate Buffer).

PBS / Tween 20: Phosphate Buffered Saline (PBS) solution with the detergent Tween 20 at 0.1% v/v at pH of 7.4 for use as a wash buffer and diluent for ELISA.

PET (PBS/EDTA0.5M/Tween 20): 0.5 molar solution of EDTA with PBS / Tween 20.

BSA (Bovine Serum Albumin): Bovine Serum Albumin powder made to 1, 2.5 & 5% w/v solutions with PBS.

Blocking buffer: 1, 2.5& 5% w/v solutions of BSA in PBS / Tween 20.

Stop Solution: One molar solution of Sulphuric acid (1M H₂SO₄).

NRP: Normal Rabbit Plasma. Diluted w/v in PBS.

NGS: Normal Goat Serum. Diluted w/v in PBS.

HAS: Human Albumin Solutions. Diluted v/v from salt poor albumin solution at 20% concentration.

LPS binding buffer: 15mMNa₂CO₃ and 35 mM NaHCO₃ in ultra pure water, pH 9.6.

TBS: Tris Buffer Saline, 80 g NaCl + 6.05g Tris in 1 L de-ionised water. Adjust pH to 7.65 with HCl and make up to 10 L with de-ionised water.

TBS in 0.1 % HAS: HAS diluted to 0.1% v/v with TBS.

TBS-Tween-CaCl₂: 10mM Tris, 140 mM NaCl, 0.05 % Tween and 5 mM CaCl₂, pH 7.4.

BBS: Barbital-buffering-solution = 4 mM Barbital, 145 mM NaCl, 1 mM MgCl₂ and 2 mM CaCl₂, pH 7.4.

BIBLIOGRAPHY

Bibliography

- Abello, P. A. and T. G. Buchman (1994). "Heat shock-induced cell death in murine microvascular endothelial cells depends on priming with tumor necrosis factor-alpha or interferon-gamma." Shock **2**(5): 320-3.
- Abello, P. A., T. G. Buchman, et al. (1994). "Shock and multiple organ failure." Adv Exp Med Biol **366**: 253-68.
- Abello, P. A., S. A. Fidler, et al. (1994). "Thiol reducing agents modulate induced apoptosis in porcine endothelial cells." Shock **2**(2): 79-83.
- Abello, P. A., S. A. Fidler, et al. (1994). "Antioxidants modulate induction of programmed endothelial cell death (apoptosis) by endotoxin." Arch Surg **129**(2): 134-40; discussion 140-1.
- Abraham, E. (2000). "NF-kappaB activation." Crit Care Med **28**(4 Suppl): N100-4.
- Al-Alaiyan, S. and E. Neiley (1999). "Inhaled nitric oxide in persistent pulmonary hypertension of the newborn refractory to high-frequency ventilation." Crit Care **3**(1): 7-10.
- al-Ebrahim, K. and H. Shafei (1993). "Our experience with using the Pall LG6 leukocyte depleting filter during cardiopulmonary bypass." Ann Thorac Surg **56**(5): 1216-7.
- Ander, D. S., M. Jaggi, et al. (1998). "Undetected cardiogenic shock in patients with congestive heart failure presenting to the emergency department." Am J Cardiol **82**(7): 888-91.
- Anderson, H. L., 3rd, R. E. Cilley, et al. (1986). "Thrombocytopenia in neonates after extracorporeal membrane oxygenation." ASAIO Trans **32**(1): 534-7.
- Angus, D. C., W. T. Linde-Zwirble, et al. (2001). "Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care." Crit Care Med **29**(7): 1303-10.
- Angus, D. C., W. T. Linde-Zwirble, et al. (2001). "Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care." Crit Care Med **29**(7): 1303-10.
- Annane, D., V. Sebille, et al. (2002). "Effect of treatment with low doses of hydrocortisone and fludrocortisone on mortality in patients with septic shock." JAMA **288**(7): 862-71.
- Arbour, N. C., E. Lorenz, et al. (2000). "TLR4 mutations are associated with endotoxin hyporesponsiveness in humans." Nat Genet **25**(2): 187-91.

Bibliography

- Atabai, K. and M. A. Matthay (2002). "The pulmonary physician in critical care. 5: Acute lung injury and the acute respiratory distress syndrome: definitions and epidemiology." Thorax **57**(5): 452-8.
- Bach, F. H., W. W. Hancock, et al. (1997). "Protective genes expressed in endothelial cells: a regulatory response to injury." Immunol Today **18**(10): 483-6.
- Bajzar, L., R. Manuel, et al. (1995). "Purification and characterization of TAFI, a thrombin-activable fibrinolysis inhibitor." J Biol Chem **270**(24): 14477-84.
- Bando, K., R. Pillai, et al. (1990). "Leukocyte depletion ameliorates free radical-mediated lung injury after cardiopulmonary bypass." J Thorac Cardiovasc Surg **99**(5): 873-7.
- Barie, P. S. (1995). "Organ-specific support in multiple organ failure: pulmonary support." World J Surg **19**(4): 581-91.
- Barnes, P. J. and M. Karin (1997). "Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases." N Engl J Med **336**(15): 1066-71.
- Barriere, S. L. and S. F. Lowry (1995). "An overview of mortality risk prediction in sepsis." Crit Care Med **23**(2): 376-93.
- Baue, A. E. (1975). "Multiple, progressive, or sequential systems failure. A syndrome of the 1970s." Arch Surg **110**(7): 779-81.
- Baumann, W. R., R. C. Jung, et al. (1986). "Incidence and mortality of adult respiratory distress syndrome: a prospective analysis from a large metropolitan hospital." Crit Care Med **14**(1): 1-4.
- Beckley, P. (1995). Oxygenator for extracorporeal circulation. Cardiopulmonary Bypass, Principals and Techniques of Extracorporeal Circulation. New York, Springer Verlag: 199-219.
- Beckley, P. D., S. M. Morris, et al. (1996). "Comparison of the performance characteristics of three generations of membrane oxygenators: Univoxregistered, Univoxregistered Goldtrademark and SpiralGoldtrademark." Perfusion **11**(1): 61-70.
- Bell, R. C., J. J. Coalson, et al. (1983). "Multiple organ system failure and infection in adult respiratory distress syndrome." Ann Intern Med **99**(3): 293-8.
- Bellingan, G. J. (2002). "The pulmonary physician in critical care * 6: The pathogenesis of ALI/ARDS." Thorax **57**(6): 540-6.

Bibliography

- Bellingan, G. J. (2002). "The pulmonary physician in critical care * 6: The pathogenesis of ALI/ARDS." Thorax **57**(6): 540-6.
- Bernard, G. R., E. W. Ely, et al. (2001). "Safety and dose relationship of recombinant human activated protein C for coagulopathy in severe sepsis." Crit Care Med **29**(11): 2051-9.
- Bernard, G. R., J. L. Vincent, et al. (2001). "Efficacy and safety of recombinant human activated protein C for severe sepsis." N Engl J Med **344**(10): 699-709.
- Beutler, B., I. W. Milsark, et al. (1985). "Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin." Science **229**(4716): 869-71.
- Beutler, B., I. W. Milsark, et al. (2008). "Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. Science, 1985, 229(4716):869-871. Classical article." J Immunol **181**(1): 7-9.
- Bevilacqua, M. P. and R. M. Nelson (1993). "Selectins." J Clin Invest **91**(2): 379-87.
- Bevilacqua, M. P., J. S. Pober, et al. (1986). "Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular endothelium: characterization and comparison with the actions of interleukin 1." Proc Natl Acad Sci U S A **83**(12): 4533-7.
- Blanco Quiros, A., J. Casado Flores, et al. (2004). "[Meningococcal sepsis in pediatrics. Parameters associated with poor outcome]." An Pediatr (Barc) **61**(4): 305-13.
- Boehme, M. W., Y. Deng, et al. (1996). "Release of thrombomodulin from endothelial cells by concerted action of TNF-alpha and neutrophils: in vivo and in vitro studies." Immunology **87**(1): 134-40.
- Boehme, M. W., Y. Deng, et al. (1996). "Release of thrombomodulin from endothelial cells by concerted action of TNF-alpha and neutrophils: in vivo and in vitro studies." Immunology **87**(1): 134-40.
- Boldin, M. P., T. M. Goncharov, et al. (1996). "Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death." Cell **85**(6): 803-15.

Bibliography

- Bombeli, T., A. Karsan, et al. (1997). "Apoptotic vascular endothelial cells become procoagulant." Blood **89**(7): 2429-42.
- Bone, R. C. (1991). "The pathogenesis of sepsis." Ann Intern Med **115**(6): 457-69.
- Bone, R. C., R. A. Balk, et al. (1992). "Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine." Chest **101**(6): 1644-55.
- Borowiec, J., S. Thelin, et al. (1992). "Heparin-coated circuits reduce activation of granulocytes during cardiopulmonary bypass. A clinical study." J Thorac Cardiovasc Surg **104**(3): 642-7.
- Bozza, P. T., W. Yu, et al. (1997). "Eosinophil lipid bodies: specific, inducible intracellular sites for enhanced eicosanoid formation." J Exp Med **186**(6): 909-20.
- Brade, H., L. Brade, et al. (1988). "Structure, endotoxicity, immunogenicity and antigenicity of bacterial lipopolysaccharides (endotoxins, O-antigens)." Prog Clin Biol Res **272**: 17-45.
- Bramson, M. L., J. J. Osborn, et al. (1965). "A New Disposable Membrane Oxygenator with Integral Heat Exchange." J Thorac Cardiovasc Surg **50**: 391-400.
- Brun-Buisson, C. (2000). "The epidemiology of the systemic inflammatory response." Intensive Care Med **26 Suppl 1**: S64-74.
- Brun-Buisson, C., F. Doyon, et al. (1995). "Incidence, risk factors, and outcome of severe sepsis and septic shock in adults. A multicenter prospective study in intensive care units. French ICU Group for Severe Sepsis." JAMA **274**(12): 968-74.
- Buchman, T. G., P. A. Abello, et al. (1993). "Induction of heat shock response leads to apoptosis in endothelial cells previously exposed to endotoxin." Am J Physiol **265**(1 Pt 2): H165-70.
- Carmichael, T. B., E. P. Walsh, et al. (2002). "Anticipatory use of venoarterial extracorporeal membrane oxygenation for a high-risk interventional cardiac procedure." Respir Care **47**(9): 1002-6.
- Castellheim, A., P. H. Lindenskov, et al. (2005). "Meconium aspiration syndrome induces complement-associated systemic inflammatory response in newborn piglets." Scand J Immunol **61**(3): 217-25.

Bibliography

- Castellheim, A., P. H. Lindenskov, et al. (2005). "Meconium aspiration syndrome induces complement-associated systemic inflammatory response in newborn piglets." Scand J Immunol **61**(3): 217-25.
- Cerra, F. B., J. H. Siegel, et al. (1979). "Correlations between metabolic and cardiopulmonary measurements in patients after trauma, general surgery, and sepsis." J Trauma **19**(8): 621-9.
- Cervellera, M., G. Raschella, et al. (2000). "Direct transactivation of the anti-apoptotic gene apolipoprotein J (clusterin) by B-MYB." J Biol Chem **275**(28): 21055-60.
- Chen, W., U. Syldath, et al. (1999). "Human 60-kDa heat-shock protein: a danger signal to the innate immune system." J Immunol **162**(6): 3212-9.
- Cirino, G., C. Napoli, et al. (2000). "Inflammation-coagulation network: are serine protease receptors the knot?" Trends Pharmacol Sci **21**(5): 170-2.
- Clark, D. A., G. F. Nieman, et al. (1987). "Surfactant displacement by meconium free fatty acids: an alternative explanation for atelectasis in meconium aspiration syndrome." J Pediatr **110**(5): 765-70.
- Clark, M. A., L. D. Plank, et al. (1998). "Effect of a chimeric antibody to tumor necrosis factor-alpha on cytokine and physiologic responses in patients with severe sepsis--a randomized, clinical trial." Crit Care Med **26**(10): 1650-9.
- Coalson, J. J., B. Benjamin, et al. (1978). "Prolonged shock in the baboon subjected to infusion of E. coli endotoxin." Circ Shock **5**(4): 423-37.
- Comp, P. C., R. M. Jacocks, et al. (1982). "Activation of protein C in vivo." J Clin Invest **70**(1): 127-34.
- Comp, P. C., R. R. Nixon, et al. (1984). "Familial protein S deficiency is associated with recurrent thrombosis." J Clin Invest **74**(6): 2082-8.
- Conkling, P. R., C. S. Greenberg, et al. (1988). "Tumor necrosis factor induces tissue factor-like activity in human leukemia cell line U937 and peripheral blood monocytes." Blood **72**(1): 128-33.
- Coughlin, S. R. (2000). "Thrombin signalling and protease-activated receptors." Nature **407**(6801): 258-64.
- Craddock, P. R., J. Fehr, et al. (1977). "Complement and leukocyte-mediated pulmonary dysfunction in hemodialysis." N Engl J Med **296**(14): 769-74.
- Creasey, A. A., A. C. Chang, et al. (1993). "Tissue factor pathway inhibitor reduces mortality from Escherichia coli septic shock." J Clin Invest **91**(6): 2850-60.

Bibliography

- Cross, A. S., S. M. Opal, et al. (1993). "Choice of bacteria in animal models of sepsis." Infect Immun **61**(7): 2741-7.
- Cryer, H. M., R. N. Garrison, et al. (1987). "Skeletal microcirculatory responses to hyperdynamic Escherichia coli sepsis in unanesthetized rats." Arch Surg **122**(1): 86-92.
- Curtis, W. E., M. E. Muldrow, et al. (1988). "N,N'-dimethylthiourea dioxide formation from N,N'-dimethylthiourea reflects hydrogen peroxide concentrations in simple biological systems." Proc Natl Acad Sci U S A **85**(10): 3422-5.
- Dahlback, B. (1991). "Protein S and C4b-binding protein: components involved in the regulation of the protein C anticoagulant system." Thromb Haemost **66**(1): 49-61.
- Damas, P., D. Ledoux, et al. (1992). "Cytokine serum level during severe sepsis in human IL-6 as a marker of severity." Ann Surg **215**(4): 356-62.
- Davies, P. (1989). Bacterial infections in the foetus and newborn Philadelphia, WB Saunders.
- De Silva, R. J., A. Anderson, et al. (2001). "Sequential organ scoring as a measure of effectiveness of care in the high-dependency unit." Anaesthesia **56**(9): 850-4.
- Deitch, E. A. (1992). "Multiple organ failure. Pathophysiology and potential future therapy." Ann Surg **216**(2): 117-34.
- Dellinger, R. P., J. M. Carlet, et al. (2004). "Surviving Sepsis Campaign guidelines for management of severe sepsis and septic shock." Crit Care Med **32**(3): 858-73.
- Deveraux, Q. L. and J. C. Reed (1999). "IAP family proteins--suppressors of apoptosis." Genes Dev **13**(3): 239-52.
- Dimmeler, S. and A. M. Zeiher (1999). "Nitric oxide-an endothelial cell survival factor." Cell Death Differ **6**(10): 964-8.
- Donnelly, S. C., C. Haslett, et al. (1994). "Role of selectins in development of adult respiratory distress syndrome." Lancet **344**(8917): 215-9.
- Doughty, L., J. A. Carcillo, et al. (1998). "Plasma nitrite and nitrate concentrations and multiple organ failure in pediatric sepsis." Crit Care Med **26**(1): 157-62.

Bibliography

- Drazenovic, R., R. W. Samsel, et al. (1992). "Regulation of perfused capillary density in canine intestinal mucosa during endotoxemia." J Appl Physiol **72**(1): 259-65.
- Dreyfus, M., M. Masterson, et al. (1995). "Replacement therapy with a monoclonal antibody purified protein C concentrate in newborns with severe congenital protein C deficiency." Semin Thromb Hemost **21**(4): 371-81.
- Dustin, M. L., R. Rothlein, et al. (1986). "Induction by IL 1 and interferon-gamma: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1)." J Immunol **137**(1): 245-54.
- Edgington, T. S., N. Mackman, et al. (1991). "The structural biology of expression and function of tissue factor." Thromb Haemost **66**(1): 67-79.
- Edmunds, L. H., Jr. (1987). "Thrombotic and bleeding complications of prosthetic heart valves." Ann Thorac Surg **44**(4): 430-45.
- Eiseman, B., R. Beart, et al. (1977). "Multiple organ failure." Surg Gynecol Obstet **144**(3): 323-6.
- Emerson, T. E., Jr., M. A. Fournel, et al. (1989). "Efficacy of antithrombin III supplementation in animal models of fulminant Escherichia coli endotoxemia or bacteremia." Am J Med **87**(3B): 27S-33S.
- Endo, S., K. Inada, et al. (1996). "Nitrite/nitrate (NOx) and sFas antigen levels in patients with multiple organ failure." Res Commun Mol Pathol Pharmacol **92**(2): 253-6.
- Ertel, W., M. Keel, et al. (1997). "Detectable concentrations of Fas ligand in cerebrospinal fluid after severe head injury." J Neuroimmunol **80**(1-2): 93-6.
- Esmon, C. (2000). "The protein C pathway." Crit Care Med **28**(9 Suppl): S44-8.
- Esmon, C. T. (1989). "The roles of protein C and thrombomodulin in the regulation of blood coagulation." J Biol Chem **264**(9): 4743-6.
- Esmon, C. T. (1992). "The protein C anticoagulant pathway." Arterioscler Thromb **12**(2): 135-45.
- Esmon, C. T. (1993). "Molecular events that control the protein C anticoagulant pathway." Thromb Haemost **70**(1): 29-35.
- Esmon, C. T. (2001). "The normal role of Activated Protein C in maintaining homeostasis and its relevance to critical illness." Crit Care **5**(2): S7-12.
- Esmon, C. T. (2001). "The normal role of Activated Protein C in maintaining homeostasis and its relevance to critical illness." Crit Care **5**(2): S7-12.

Bibliography

- Esmon, C. T. (2001). "Plasma glycolipids levels: new factors regulating the protein C anticoagulant pathway and determining thrombotic risk." Blood **97**(7): 1905.
- Esmon, C. T. (2001). "Protein C anticoagulant pathway and its role in controlling microvascular thrombosis and inflammation." Crit Care Med **29**(7 Suppl): S48-51; discussion 51-2.
- Esmon, C. T. (2001). "Protein C anticoagulant pathway and its role in controlling microvascular thrombosis and inflammation." Crit Care Med **29**(7 Suppl): S48-51; discussion 51-2.
- Esmon, C. T. (2001). "Role of coagulation inhibitors in inflammation." Thromb Haemost **86**(1): 51-6.
- Esmon, C. T. (2001). "Sepsis. A myriad of responses." Lancet **358** Suppl: S61.
- Esmon, C. T. (2002). "New mechanisms for vascular control of inflammation mediated by natural anticoagulant proteins." J Exp Med **196**(5): 561-4.
- Esmon, C. T. (2002). "Protein C pathway in sepsis." Ann Med **34**(7-8): 598-605.
- Esmon, C. T. (2002). "Protein C pathway in sepsis." Ann Med **34**(7-8): 598-605.
- Esmon, C. T. (2003). "Coagulation and inflammation." J Endotoxin Res **9**(3): 192-8.
- Esmon, C. T. (2003). "Inflammation and thrombosis." J Thromb Haemost **1**(7): 1343-8.
- Esmon, C. T. (2003). "The protein C pathway." Chest **124**(3 Suppl): 26S-32S.
- Esmon, C. T. (2003). "The protein C pathway." Chest **124**(3 Suppl): 26S-32S.
- Esmon, C. T. (2004). "The impact of the inflammatory response on coagulation." Thromb Res **114**(5-6): 321-7.
- Esmon, C. T. (2004). "Interactions between the innate immune and blood coagulation systems." Trends Immunol **25**(10): 536-42.
- Esmon, C. T. (2004). "Structure and functions of the endothelial cell protein C receptor." Crit Care Med **32**(5 Suppl): S298-301.
- Esmon, C. T. (2004). "Why do animal models (sometimes) fail to mimic human sepsis?" Crit Care Med **32**(5 Suppl): S219-22.
- Esmon, C. T. (2004). "Why do animal models (sometimes) fail to mimic human sepsis?" Crit Care Med **32**(5 Suppl): S219-22.
- Esmon, C. T. (2005). "Coagulation inhibitors in inflammation." Biochem Soc Trans **33**(Pt 2): 401-5.

Bibliography

- Esmon, C. T. (2005). "The interactions between inflammation and coagulation." Br J Haematol **131**(4): 417-30.
- Esmon, C. T. (2005). "Is APC activation of endothelial cell PAR1 important in severe sepsis?: No." J Thromb Haemost **3**(9): 1910-1.
- Esmon, C. T. (2006). "The endothelial protein C receptor." Curr Opin Hematol **13**(5): 382-5.
- Esmon, C. T. (2006). "Inflammation and the activated protein C anticoagulant pathway." Semin Thromb Hemost **32 Suppl 1**: 49-60.
- Esmon, C. T., W. Ding, et al. (1997). "The protein C pathway: new insights." Thromb Haemost **78**(1): 70-4.
- Esmon, C. T., K. Fukudome, et al. (1999). "Inflammation, sepsis, and coagulation." Haematologica **84**(3): 254-9.
- Esmon, C. T., J. M. Gu, et al. (1999). "Regulation and functions of the protein C anticoagulant pathway." Haematologica **84**(4): 363-8.
- Esmon, C. T., F. B. Taylor, Jr., et al. (1991). "Inflammation and coagulation: linked processes potentially regulated through a common pathway mediated by protein C." Thromb Haemost **66**(1): 160-5.
- Fang, X. M., S. Schroder, et al. (1999). "Comparison of two polymorphisms of the interleukin-1 gene family: interleukin-1 receptor antagonist polymorphism contributes to susceptibility to severe sepsis." Crit Care Med **27**(7): 1330-4.
- Farquhar, I., C. M. Martin, et al. (1996). "Decreased capillary density in vivo in bowel mucosa of rats with normotensive sepsis." J Surg Res **61**(1): 190-6.
- Fehsel, K., K. D. Kroncke, et al. (1995). "Nitric oxide induces apoptosis in mouse thymocytes." J Immunol **155**(6): 2858-65.
- Fijnvandraat, K., B. Derkx, et al. (1995). "Coagulation activation and tissue necrosis in meningococcal septic shock: severely reduced protein C levels predict a high mortality." Thromb Haemost **73**(1): 15-20.
- Firmin, R. K. and H. M. Killer (1999). "Extracorporeal membrane oxygenation." Perfusion **14**(4): 291-7.
- Firmin, R. K., G. J. Peek, et al. (1996). "Role of extracorporeal membrane oxygenation." Lancet **348**(9030): 824.
- Fisher, C. J., Jr. and S. B. Yan (2000). "Protein C levels as a prognostic indicator of outcome in sepsis and related diseases." Crit Care Med **28**(9 Suppl): S49-56.

Bibliography

- Fosse, E., O. Moen, et al. (1994). "Reduced complement and granulocyte activation with heparin-coated cardiopulmonary bypass." Ann Thorac Surg **58**(2): 472-7.
- Fourrier, F., C. Chopin, et al. (1992). "Septic shock, multiple organ failure, and disseminated intravascular coagulation. Compared patterns of antithrombin III, protein C, and protein S deficiencies." Chest **101**(3): 816-23.
- Foust, R., 3rd, N. N. Tran, et al. (1996). "Liquid assisted ventilation: an alternative ventilatory strategy for acute meconium aspiration injury." Pediatr Pulmonol **21**(5): 316-22.
- Fry, D. E., L. Pearlstein, et al. (1980). "Multiple system organ failure. The role of uncontrolled infection." Arch Surg **115**(2): 136-40.
- Fukudome, K. and C. T. Esmon (1994). "Identification, cloning, and regulation of a novel endothelial cell protein C/activated protein C receptor." J Biol Chem **269**(42): 26486-91.
- Fukudome, K. and C. T. Esmon (1995). "Molecular cloning and expression of murine and bovine endothelial cell protein C/activated protein C receptor (EPCR). The structural and functional conservation in human, bovine, and murine EPCR." J Biol Chem **270**(10): 5571-7.
- Galletti, P. M. (1970). "Artificial blood-gas interfaces and their application to "membrane oxygenators." Author's oxygenator module represents closest approximation to natural lung yet devised." R I Med J **53**(9): 503-5 passim.
- Gando, S., S. Nanzaki, et al. (1998). "Activation of the extrinsic coagulation pathway in patients with severe sepsis and septic shock." Crit Care Med **26**(12): 2005-9.
- Garcia, J. G., F. M. Pavalko, et al. (1995). "Vascular endothelial cell activation and permeability responses to thrombin." Blood Coagul Fibrinolysis **6**(7): 609-26.
- Gavett, S. H., S. L. Madison, et al. (1999). "Allergic lung responses are increased in prostaglandin H synthase-deficient mice." J Clin Invest **104**(6): 721-32.
- Gibot, S., A. Cariou, et al. (2002). "Association between a genomic polymorphism within the CD14 locus and septic shock susceptibility and mortality rate." Crit Care Med **30**(5): 969-73.
- Glaser, C. B., J. Morser, et al. (1992). "Oxidation of a specific methionine in thrombomodulin by activated neutrophil products blocks cofactor activity. A

Bibliography

- potential rapid mechanism for modulation of coagulation." J Clin Invest **90**(6): 2565-73.
- Glauser, M. P., G. Zanetti, et al. (1991). "Septic shock: pathogenesis." Lancet **338**(8769): 732-6.
- Gluck, T. and S. M. Opal (2004). "Advances in sepsis therapy." Drugs **64**(8): 837-59.
- Goh, A. and L. Lum (1999). "Sepsis, severe sepsis and septic shock in paediatric multiple organ dysfunction syndrome." J Paediatr Child Health **35**(5): 488-92.
- Goldman, R. K. and M. T. Haupt (1999). "Oxygen consumption: a marker of the systemic inflammatory response?" Crit Care Med **27**(10): 2293-4.
- Goldstein, B., B. Giroir, et al. (2005). "International pediatric sepsis consensus conference: definitions for sepsis and organ dysfunction in pediatrics." Pediatr Crit Care Med **6**(1): 2-8.
- Goldstein, B., S. Nadel, et al. (2006). "ENHANCE: results of a global open-label trial of drotrecogin alfa (activated) in children with severe sepsis." Pediatr Crit Care Med **7**(3): 200-11.
- Goris, R. J., T. P. te Boekhorst, et al. (1985). "Multiple-organ failure. Generalized autodestructive inflammation?" Arch Surg **120**(10): 1109-15.
- Gorman, R. C., N. Ziats, et al. (1996). "Surface-bound heparin fails to reduce thrombin formation during clinical cardiopulmonary bypass." J Thorac Cardiovasc Surg **111**(1): 1-11; discussion 11-2.
- Gospodarowicz, M., L. Benedet, et al. (1998). "History and international developments in cancer staging." Cancer Prev Control **2**(6): 262-8.
- Granton, J. T., C. M. Goddard, et al. (1997). "Leukocytes and decreased left-ventricular contractility during endotoxemia in rabbits." Am J Respir Crit Care Med **155**(6): 1977-83.
- Grey, S. T., A. Tsuchida, et al. (1994). "Selective inhibitory effects of the anticoagulant activated protein C on the responses of human mononuclear phagocytes to LPS, IFN-gamma, or phorbol ester." J Immunol **153**(8): 3664-72.
- Griffin, J. D., O. Spertini, et al. (1990). "Granulocyte-macrophage colony-stimulating factor and other cytokines regulate surface expression of the

Bibliography

- leukocyte adhesion molecule-1 on human neutrophils, monocytes, and their precursors." J Immunol **145**(2): 576-84.
- Grinnell, B. W., R. B. Hermann, et al. (1994). "Human protein C inhibits selectin-mediated cell adhesion: role of unique fucosylated oligosaccharide." Glycobiology **4**(2): 221-5.
- Grinnell, B. W., R. B. Hermann, et al. (1994). "Human protein C inhibits selectin-mediated cell adhesion: role of unique fucosylated oligosaccharide." Glycobiology **4**(2): 221-5.
- Grinnell, B. W. and S. B. Yan (1998). "Novel antithrombotics based on modulation of the protein C pathway." Coron Artery Dis **9**(2-3): 89-97.
- Gruber, A., J. H. Griffin, et al. (1989). "Inhibition of platelet-dependent thrombus formation by human activated protein C in a primate model." Blood **73**(3): 639-42.
- Gruber, A., S. R. Hanson, et al. (1990). "Inhibition of thrombus formation by activated recombinant protein C in a primate model of arterial thrombosis." Circulation **82**(2): 578-85.
- Gu, Y. J., W. van Oeveren, et al. (1993). "Heparin-coated circuits reduce the inflammatory response to cardiopulmonary bypass." Ann Thorac Surg **55**(4): 917-22.
- Gutteridge, J. M. and J. Mitchell (1999). "Redox imbalance in the critically ill." Br Med Bull **55**(1): 49-75.
- Hancock, W. W., A. Tsuchida, et al. (1992). "The anticoagulants protein C and protein S display potent antiinflammatory and immunosuppressive effects relevant to transplant biology and therapy." Transplant Proc **24**(5): 2302-3.
- Hanson, S. R., J. H. Griffin, et al. (1993). "Antithrombotic effects of thrombin-induced activation of endogenous protein C in primates." J Clin Invest **92**(4): 2003-12.
- Harbarth, S., K. Holeckova, et al. (2001). "Diagnostic value of procalcitonin, interleukin-6, and interleukin-8 in critically ill patients admitted with suspected sepsis." Am J Respir Crit Care Med **164**(3): 396-402.
- Harlan, J. M. and R. K. Winn (2002). "Leukocyte-endothelial interactions: clinical trials of anti-adhesion therapy." Crit Care Med **30**(5 Suppl): S214-9.
- Harper, P. L., F. B. Taylor, et al. (1998). "Recombinant antitrypsin Pittsburgh undergoes proteolytic cleavage during E. coli sepsis and fails to prevent the

Bibliography

- associated coagulopathy in a primate model." Thromb Haemost **80**(5): 816-21.
- Hartman, D. (1998). "A dose dependent reductions in the plasma levels of markers of coagulation and inflammation." Intensive Care Medicine **24**: S77.
- Hatori, N., H. Yoshizu, et al. (1994). "Biocompatibility of heparin-coated membrane oxygenator during cardiopulmonary bypass." Artif Organs **18**(12): 904-10.
- Hausfater, P., S. Garric, et al. (2002). "Usefulness of procalcitonin as a marker of systemic infection in emergency department patients: a prospective study." Clin Infect Dis **34**(7): 895-901.
- Heeb, M. J., R. Bischoff, et al. (1990). "Inhibition of activated protein C by recombinant alpha 1-antitrypsin variants with substitution of arginine or leucine for methionine358." J Biol Chem **265**(4): 2365-9.
- Henson, D. (1989). Mediators of the Inflammatory Response. Amsterdam, Elsevier.
- Hessel, F. P. (2006). "Economic evaluation of the artificial liver support system MARS in patients with acute-on-chronic liver failure." Cost Eff Resour Alloc **4**: 16.
- Hibberd, M. L., M. Sumiya, et al. (1999). "Association of variants of the gene for mannose-binding lectin with susceptibility to meningococcal disease. Meningococcal Research Group." Lancet **353**(9158): 1049-53.
- Hintz, S. R., D. M. Suttner, et al. (2000). "Decreased use of neonatal extracorporeal membrane oxygenation (ECMO): how new treatment modalities have affected ECMO utilization." Pediatrics **106**(6): 1339-43.
- Hogevold, H. E., O. Moen, et al. (1997). "Effects of heparin coating on the expression of CD11b, CD11c and CD62L by leucocytes in extracorporeal circulation in vitro." Perfusion **12**(1): 9-20.
- Holley, D. G., B. L. Short, et al. (1994). "Mechanisms of change in cardiac performance in infants undergoing extracorporeal membrane oxygenation." Crit Care Med **22**(11): 1865-70.
- Holopainen, R., H. Soukka, et al. (1998). "Meconium aspiration induces a concentration-dependent pulmonary hypertensive response in newborn piglets." Pediatr Pulmonol **25**(2): 107-13.
- Holopainen, R., H. Soukka, et al. (1998). "Meconium aspiration induces a concentration-dependent pulmonary hypertensive response in newborn piglets." Pediatr Pulmonol **25**(2): 107-13.

Bibliography

- Hotchkiss, R. S., P. E. Swanson, et al. (1997). "Apoptosis in lymphoid and parenchymal cells during sepsis: findings in normal and T- and B-cell-deficient mice." Crit Care Med **25**(8): 1298-307.
- Hutter, A. M., Jr. and A. J. Moss (1970). "Central venous oxygen saturations. Value of serial determinations in patients with acute myocardial infarction." JAMA **212**(2): 299-303.
- Ing, T. S., F. K. Wong, et al. (1995). "The 'first-use syndrome' revisited: a dialysis centre's perspective." Nephrol Dial Transplant **10 Suppl 10**: 39-44.
- Ito, Y., A. Wakabayashi, et al. (1978). "Temporary cardiopulmonary bypass for the treatment of endotoxic shock." Am J Surg **136**(1): 80-4.
- Itoh, N. and S. Nagata (1993). "A novel protein domain required for apoptosis. Mutational analysis of human Fas antigen." J Biol Chem **268**(15): 10932-7.
- Jaattela, M. and D. Wissing (1992). "Emerging role of heat shock proteins in biology and medicine." Ann Med **24**(4): 249-58.
- Jackson, C. V., B. D. Bailey, et al. (2000). "Pharmacological profile of recombinant, human activated protein C (LY203638) in a canine model of coronary artery thrombosis." J Pharmacol Exp Ther **295**(3): 967-71.
- Jacobi, J. (2002). "Pathophysiology of sepsis." Am J Health Syst Pharm **59 Suppl 1**: S3-8.
- Joyce, D. E., L. Gelbert, et al. (2001). "Gene expression profile of antithrombotic protein c defines new mechanisms modulating inflammation and apoptosis." J Biol Chem **276**(14): 11199-203.
- Joyce, D. E. and B. W. Grinnell (2002). "Recombinant human activated protein C attenuates the inflammatory response in endothelium and monocytes by modulating nuclear factor-kappaB." Crit Care Med **30**(5 Suppl): S288-93.
- Jung, T. M. and M. O. Dailey (1990). "Rapid modulation of homing receptors (gp90MEL-14) induced by activators of protein kinase C. Receptor shedding due to accelerated proteolytic cleavage at the cell surface." J Immunol **144**(8): 3130-6.
- Jutila, M. A., L. Rott, et al. (1989). "Function and regulation of the neutrophil MEL-14 antigen in vivo: comparison with LFA-1 and MAC-1." J Immunol **143**(10): 3318-24.
- Kansas, G. S. (1996). "Selectins and their ligands: current concepts and controversies." Blood **88**(9): 3259-87.

Bibliography

- Karsan, A., E. Yee, et al. (1996). "Cloning of human Bcl-2 homologue: inflammatory cytokines induce human A1 in cultured endothelial cells." Blood **87**(8): 3089-96.
- Kawahito, K., S. Murata, et al. (2000). "Resuscitation and circulatory support using extracorporeal membrane oxygenation for fulminant pulmonary embolism." Artif Organs **24**(6): 427-30.
- Keldenich, S., R. Kopp, et al. (2000). "Application of a new dynamic flow model for investigating the biocompatibility of modified surfaces." ASAIO J **46**(1): 134-41.
- Kelly, K. A., J. R. Allport, et al. (2005). "Detection of vascular adhesion molecule-1 expression using a novel multimodal nanoparticle." Circ Res **96**(3): 327-36.
- Kelly, K. J., W. W. Williams, Jr., et al. (1996). "Intercellular adhesion molecule-1-deficient mice are protected against ischemic renal injury." J Clin Invest **97**(4): 1056-63.
- Kim, P. K. and C. S. Deutschman (2000). "Inflammatory responses and mediators." Surg Clin North Am **80**(3): 885-94.
- Kind, K., N. Reiss, et al. (2006). "Introduction of a new oxygenator including a tight fiber for long-term ECMO in infants." ASAIO J **52**(2): 217-8.
- Kirton, O. C., R. G. Gore, et al. (1992). "Recurrent episodes of gram-negative bacteremia or endotoxemia change reactivity of pre- and post-capillary pulmonary segments to angiotensin or free radicals." Intensive Care Med **18**(5): 293-8.
- Klinzing, S., T. Lesser, et al. (2000). "Wet-to-dry ratio of lung tissue and surfactant outwash after one-lung flooding." Res Exp Med (Berl) **200**(1): 27-33.
- Knaus, W. A., E. A. Draper, et al. (1985). "Prognosis in acute organ-system failure." Ann Surg **202**(6): 685-93.
- Knaus, W. A., D. P. Wagner, et al. (1991). "Short-term mortality predictions for critically ill hospitalized adults: science and ethics." Science **254**(5030): 389-94.
- Knowlton, A. A., P. Brecher, et al. (1991). "Rapid expression of heat shock protein in the rabbit after brief cardiac ischemia." J Clin Invest **87**(1): 139-47.
- Kress, D. C., D. J. Cohen, et al. (1987). "Pump-induced hemolysis in a rabbit model of neonatal ECMO." ASAIO Trans **33**(3): 446-52.

Bibliography

- Krishnaswamy, G., J. Kelley, et al. (1999). "Human endothelium as a source of multifunctional cytokines: molecular regulation and possible role in human disease." J Interferon Cytokine Res **19**(2): 91-104.
- Kubo, H., N. A. Doyle, et al. (1999). "L- and P-selectin and CD11/CD18 in intracapillary neutrophil sequestration in rabbit lungs." Am J Respir Crit Care Med **159**(1): 267-74.
- Kurosawa, S., D. J. Stearns-Kurosawa, et al. (1997). "Identification of functional endothelial protein C receptor in human plasma." J Clin Invest **100**(2): 411-8.
- Lam, C., K. Tynl, et al. (1994). "Microvascular perfusion is impaired in a rat model of normotensive sepsis." J Clin Invest **94**(5): 2077-83.
- Lander, E. S. (1996). "The new genomics: global views of biology." Science **274**(5287): 536-9.
- Larrick, J. W. and S. C. Wright (1990). "Cytotoxic mechanism of tumor necrosis factor-alpha." FASEB J **4**(14): 3215-23.
- Laterre, P. F. (2007). "Clinical trials in severe sepsis with drotrecogin alfa (activated)." Crit Care **11 Suppl 5**: S5.
- Leclerc, F., S. Leteurtre, et al. (2005). "Cumulative influence of organ dysfunctions and septic state on mortality of critically ill children." Am J Respir Crit Care Med **171**(4): 348-53.
- Lee, A., M. K. Whyte, et al. (1993). "Inhibition of apoptosis and prolongation of neutrophil functional longevity by inflammatory mediators." J Leukoc Biol **54**(4): 283-8.
- Lee, E. G., D. L. Boone, et al. (2000). "Failure to regulate TNF-induced NF-kappaB and cell death responses in A20-deficient mice." Science **289**(5488): 2350-4.
- Lee, Y. C., K. B. Lane, et al. (2003). "Transforming growth factor-beta induces collagen synthesis without inducing IL-8 production in mesothelial cells." Eur Respir J **22**(2): 197-202.
- Leff, J. A., C. P. Wilke, et al. (1995). "Interleukin-1 pretreatment prevents interleukin-1-induced lung leak in rats." Am J Physiol **268**(1 Pt 1): L12-6.
- Lefort, J., M. Singer, et al. (1998). "Systemic administration of endotoxin induces bronchopulmonary hyperreactivity dissociated from TNF-alpha formation and neutrophil sequestration into the murine lungs." J Immunol **161**(1): 474-80.

Bibliography

- Leteurtre, S., A. Duhamel, et al. (2006). "Paediatric logistic organ dysfunction (PELOD) score." Lancet **367**(9514): 897; author reply 900-2.
- Leteurtre, S., A. Martinot, et al. (1999). "Development of a pediatric multiple organ dysfunction score: use of two strategies." Med Decis Making **19**(4): 399-410.
- LeVan, T. D., J. W. Bloom, et al. (2001). "A common single nucleotide polymorphism in the CD14 promoter decreases the affinity of Sp protein binding and enhances transcriptional activity." J Immunol **167**(10): 5838-44.
- Levi, M., H. ten Cate, et al. (2002). "Endothelium: interface between coagulation and inflammation." Crit Care Med **30**(5 Suppl): S220-4.
- Leviton, A. and O. Dammann (2004). "Coagulation, inflammation, and the risk of neonatal white matter damage." Pediatr Res **55**(4): 541-5.
- Levy, M. M., M. P. Fink, et al. (2003). "2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference." Crit Care Med **31**(4): 1250-6.
- Lorant, D. E., M. K. Topham, et al. (1993). "Inflammatory roles of P-selectin." J Clin Invest **92**(2): 559-70.
- Lyons, A. (1978). Medicine, an illustrated history. . New York, Abradale Press.
- Maat, M., C. M. Buijsse, et al. (2007). "Improved survival of children with sepsis and purpura: effects of age, gender, and era." Crit Care **11**(5): R112.
- Macdonald, J., H. F. Galley, et al. (2003). "Oxidative stress and gene expression in sepsis." Br J Anaesth **90**(2): 221-32.
- Madorin, W. S., C. M. Martin, et al. (1999). "Dopexamine attenuates flow motion in ileal mucosal arterioles in normotensive sepsis." Crit Care Med **27**(2): 394-400.
- Mahidhara, R. and T. R. Billiar (2000). "Apoptosis in sepsis." Crit Care Med **28**(4 Suppl): N105-13.
- Maier, R. V. and E. M. Bulger (1996). "Endothelial changes after shock and injury." New Horiz **4**(2): 211-23.
- Mann, H. J. (2002). "Recombinant human activated protein C in severe sepsis." Am J Health Syst Pharm **59** Suppl 1: S19-23.
- Marcus, A. J., L. B. Safier, et al. (1985). "Inhibition of platelet function in thrombosis." Circulation **72**(4): 698-701.
- Marshall, J. C., N. V. Christou, et al. (1988). "Immunomodulation by altered gastrointestinal tract flora. The effects of orally administered, killed

Bibliography

- Staphylococcus epidermidis, Candida, and Pseudomonas on systemic immune responses." Arch Surg **123**(12): 1465-9.
- Marshall, J. C., D. J. Cook, et al. (1995). "Multiple organ dysfunction score: a reliable descriptor of a complex clinical outcome." Crit Care Med **23**(10): 1638-52.
- Martin, G. R., B. L. Short, et al. (1991). "Cardiac stun in infants undergoing extracorporeal membrane oxygenation." J Thorac Cardiovasc Surg **101**(4): 607-11.
- Marton, M. J., J. L. DeRisi, et al. (1998). "Drug target validation and identification of secondary drug target effects using DNA microarrays." Nat Med **4**(11): 1293-301.
- Matsukawa, A., S. Ohkawara, et al. (1993). "Production of IL-1 and IL-1 receptor antagonist and the pathological significance in lipopolysaccharide-induced arthritis in rabbits." Clin Exp Immunol **93**(2): 206-11.
- Maurer, J., A. Kendzia, et al. (1998). "Morphological changes in chest radiographs of patients with acute respiratory distress syndrome (ARDS)." Intensive Care Med **24**(11): 1152-6.
- McCloskey, R. V., R. C. Straube, et al. (1994). "Treatment of septic shock with human monoclonal antibody HA-1A. A randomized, double-blind, placebo-controlled trial. CHES Trial Study Group." Ann Intern Med **121**(1): 1-5.
- McEver, R. P. (1994). "Selectins." Curr Opin Immunol **6**(1): 75-84.
- McGill, S. N., N. A. Ahmed, et al. (1998). "Endothelial cells: role in infection and inflammation." World J Surg **22**(2): 171-8.
- McMillen, M. A., M. Huribal, et al. (1993). "Common pathway of endothelial-leukocyte interaction in shock, ischemia, and reperfusion." Am J Surg **166**(5): 557-62.
- Meduri, G. U., S. Headley, et al. (1995). "Persistent elevation of inflammatory cytokines predicts a poor outcome in ARDS. Plasma IL-1 beta and IL-6 levels are consistent and efficient predictors of outcome over time." Chest **107**(4): 1062-73.
- Merino, J., V. Rodriguez-Valverde, et al. (1983). "Prevalence of deficits of complement components in patients with recurrent meningococcal infections." J Infect Dis **148**(2): 331.

Bibliography

- Mesters, R. M., J. Helterbrand, et al. (2000). "Prognostic value of protein C concentrations in neutropenic patients at high risk of severe septic complications." Crit Care Med **28**(7): 2209-16.
- Michel, O., A. M. Nagy, et al. (1997). "Dose-response relationship to inhaled endotoxin in normal subjects." Am J Respir Crit Care Med **156**(4 Pt 1): 1157-64.
- Migliorati, G., I. Nicoletti, et al. (1992). "Heat shock induces apoptosis in mouse thymocytes and protects them from glucocorticoid-induced cell death." Cell Immunol **143**(2): 348-56.
- Miller, D. K. (1997). "The role of the Caspase family of cysteine proteases in apoptosis." Semin Immunol **9**(1): 35-49.
- Mira, J. P., A. Cariou, et al. (1999). "Association of TNF2, a TNF-alpha promoter polymorphism, with septic shock susceptibility and mortality: a multicenter study." JAMA **282**(6): 561-8.
- Moat, N. E., D. F. Shore, et al. (1993). "Organ dysfunction and cardiopulmonary bypass: the role of complement and complement regulatory proteins." Eur J Cardiothorac Surg **7**(11): 563-73.
- Moore, F. A. and E. E. Moore (1995). "Evolving concepts in the pathogenesis of postinjury multiple organ failure." Surg Clin North Am **75**(2): 257-77.
- Moriyama, S., K. Okamoto, et al. (1999). "Evaluation of oxygen consumption and resting energy expenditure in critically ill patients with systemic inflammatory response syndrome." Crit Care Med **27**(10): 2133-6.
- Morrison, D. C. and J. L. Ryan (1987). "Endotoxins and disease mechanisms." Annu Rev Med **38**: 417-32.
- Mosnier, L. O., X. V. Yang, et al. (2007). "Activated protein C mutant with minimal anticoagulant activity, normal cytoprotective activity, and preservation of thrombin activable fibrinolysis inhibitor-dependent cytoprotective functions." J Biol Chem **282**(45): 33022-33.
- Motomura, T., T. Maeda, et al. (2002). "Extracorporeal membrane oxygenator compatible with centrifugal blood pumps." Artif Organs **26**(11): 952-8.
- Muckart, D. J. and S. Bhagwanjee (1997). "American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference definitions of the systemic inflammatory response syndrome and allied

Bibliography

- disorders in relation to critically injured patients." Crit Care Med **25**(11): 1789-95.
- Muckart, D. J., S. Bhagwanjee, et al. (1996). "Prediction of the risk of death by APACHE II scoring in critically ill trauma patients without head injury." Br J Surg **83**(8): 1123-7.
- Mueller, X. M., H. T. Tevæarai, et al. (2000). "Impact of hollow-fiber membrane surface area on oxygenator performance: Dideco D903 Avant versus a prototype with larger surface area." J Extra Corpor Technol **32**(3): 152-7.
- Murakami, K., K. Okajima, et al. (1996). "Activated protein C attenuates endotoxin-induced pulmonary vascular injury by inhibiting activated leukocytes in rats." Blood **87**(2): 642-7.
- Murakami, K., K. Okajima, et al. (1997). "Activated protein C prevents LPS-induced pulmonary vascular injury by inhibiting cytokine production." Am J Physiol **272**(2 Pt 1): L197-202.
- Nadel, S. (1995). Current management of meningococcal septicaemia and meningitis. In Meningococcal Disease., John Wiley and Sons 207-243.
- Nadel, S., B. Goldstein, et al. (2007). "Drotrecogin alfa (activated) in children with severe sepsis: a multicentre phase III randomised controlled trial." Lancet **369**(9564): 836-43.
- Nakajima, Y., N. Baudry, et al. (2001). "Microcirculation in intestinal villi: a comparison between hemorrhagic and endotoxin shock." Am J Respir Crit Care Med **164**(8 Pt 1): 1526-30.
- Nakamura, K., E. Bossy-Wetzel, et al. (2000). "Changes in endoplasmic reticulum luminal environment affect cell sensitivity to apoptosis." J Cell Biol **150**(4): 731-40.
- Nathan, A. T. and M. Singer (1999). "The oxygen trail: tissue oxygenation." Br Med Bull **55**(1): 96-108.
- Nathan, C. (1992). "Nitric oxide as a secretory product of mammalian cells." FASEB J **6**(12): 3051-64.
- Neth, O., I. Hann, et al. (2001). "Deficiency of mannose-binding lectin and burden of infection in children with malignancy: a prospective study." Lancet **358**(9282): 614-8.

Bibliography

- Newman, W., L. D. Beall, et al. (1993). "Soluble E-selectin is found in supernatants of activated endothelial cells and is elevated in the serum of patients with septic shock." J Immunol **150**(2): 644-54.
- Ng, P. C., K. Li, et al. (2003). "Proinflammatory and anti-inflammatory cytokine responses in preterm infants with systemic infections." Arch Dis Child Fetal Neonatal Ed **88**(3): F209-13.
- Nishina, K., K. Mikawa, et al. (1997). "Inhaled nitric oxide does not prevent endotoxin-induced lung injury in rabbits." Acta Anaesthesiol Scand **41**(3): 399-407.
- Nojiri, C., K. Hagiwara, et al. (1995). "Evaluation of a new heparin bonding process in prolonged extracorporeal membrane oxygenation." ASAIO J **41**(3): M561-7.
- Nomura, M. (2004). "Colonização materna e neonatal por estreptococo do grupo B em gestantes com trabalho de parto prematuro e/ou ruptura prematura pré-termo de membranas " Medicina.
- Ochoa, J. B., A. O. Udekwu, et al. (1991). "Nitrogen oxide levels in patients after trauma and during sepsis." Ann Surg **214**(5): 621-6.
- Okusawa, S., J. A. Gelfand, et al. (1988). "Interleukin 1 induces a shock-like state in rabbits. Synergism with tumor necrosis factor and the effect of cyclooxygenase inhibition." J Clin Invest **81**(4): 1162-72.
- Opal, S. M. (2005). "Concept of PIRO as a new conceptual framework to understand sepsis." Pediatr Crit Care Med **6**(3 Suppl): S55-60.
- Opal, S. M. and J. Cohen (1999). "Clinical gram-positive sepsis: does it fundamentally differ from gram-negative bacterial sepsis?" Crit Care Med **27**(8): 1608-16.
- Opal, S. M., A. Cross, et al. (1988). "Group B streptococcal sepsis in adults and infants. Contrasts and comparisons." Arch Intern Med **148**(3): 641-5.
- Opal, S. M., G. E. Garber, et al. (2003). "Systemic host responses in severe sepsis analyzed by causative microorganism and treatment effects of drotrecogin alfa (activated)." Clin Infect Dis **37**(1): 50-8.
- Ovrum, E., G. Tangen, et al. (2003). "Heparin-coated circuits (Duraflo II) with reduced versus full anticoagulation during coronary artery bypass surgery." J Card Surg **18**(2): 140-6.

Bibliography

- Papathanassoglou, E. D., J. A. Moynihan, et al. (2000). "Does programmed cell death (apoptosis) play a role in the development of multiple organ dysfunction in critically ill patients? a review and a theoretical framework." Crit Care Med **28**(2): 537-49.
- Parent, C. and P. Q. Eichacker (1999). "Neutrophil and endothelial cell interactions in sepsis. The role of adhesion molecules." Infect Dis Clin North Am **13**(2): 427-47, x.
- Parrillo, J. E., M. M. Parker, et al. (1990). "Septic shock in humans. Advances in the understanding of pathogenesis, cardiovascular dysfunction, and therapy." Ann Intern Med **113**(3): 227-42.
- Peek, G. J. and R. K. Firmin (1997). "Extracorporeal membrane oxygenation for cardiac support." Coron Artery Dis **8**(6): 371-88.
- Peek, G. J. and R. K. Firmin (1999). "The inflammatory and coagulative response to prolonged extracorporeal membrane oxygenation." ASAIO J **45**(4): 250-63.
- Peek, G. J., R. K. Firmin, et al. (1996). "Cannulation of neonates for venovenous extracorporeal life support." Ann Thorac Surg **61**(6): 1851-2.
- Peek, G. J., H. M. Killer, et al. (2002). "Early experience with a polymethyl pentene oxygenator for adult extracorporeal life support." ASAIO J **48**(5): 480-2.
- Peek, G. J., H. M. Killer, et al. (1998). "Extracorporeal membrane oxygenation: potential for adults and children?" Hosp Med **59**(4): 304-8.
- Peek, G. J., H. M. Killer, et al. (2002). "Modular extracorporeal life support for multiorgan failure patients." Liver **22 Suppl 2**: 69-71.
- Peek, G. J., H. M. Moore, et al. (1997). "Extracorporeal membrane oxygenation for adult respiratory failure." Chest **112**(3): 759-64.
- Peek, G. J. and A. W. Sosnowski (1997). "Extra-corporeal membrane oxygenation for paediatric respiratory failure." Br Med Bull **53**(4): 745-56.
- Peek, G. J., S. White, et al. (1998). "Severe acute respiratory distress syndrome secondary to acute pancreatitis successfully treated with extracorporeal membrane oxygenation in three patients." Ann Surg **227**(4): 572-4.
- Pekna, M., L. Hagman, et al. (1994). "Complement activation during cardiopulmonary bypass: effects of immobilized heparin." Ann Thorac Surg **58**(2): 421-4.

Bibliography

- Pigott, R. and A. M. Davies (1987). "The monoclonal antibody 69A1 recognizes an epitope found on neurones with axons that fasciculate but not on those with non-fasciculating processes." Development **100**(3): 489-500.
- Piper, R. D., M. Pitt-Hyde, et al. (1996). "Microcirculatory changes in rat skeletal muscle in sepsis." Am J Respir Crit Care Med **154**(4 Pt 1): 931-7.
- Pittet, D., S. Rangel-Frausto, et al. (1995). "Systemic inflammatory response syndrome, sepsis, severe sepsis and septic shock: incidence, morbidities and outcomes in surgical ICU patients." Intensive Care Med **21**(4): 302-9.
- Platonov, A. E., G. A. Shipulin, et al. (1998). "Association of human Fc gamma RIIa (CD32) polymorphism with susceptibility to and severity of meningococcal disease." Clin Infect Dis **27**(4): 746-50.
- Plotz, F. B., W. van Oeveren, et al. (1993). "Blood activation during neonatal extracorporeal life support." J Thorac Cardiovasc Surg **105**(5): 823-32.
- Plotz, F. B., W. van Oeveren, et al. (1992). "A heparin-coated circuit reduces complement activation and the release of leukocyte inflammatory mediators during extracorporeal circulation in a rabbit." Artif Organs **16**(4): 366-70.
- Polunovsky, V. A., B. Chen, et al. (1993). "Role of mesenchymal cell death in lung remodeling after injury." J Clin Invest **92**(1): 388-97.
- Pooboni, S., N. Roberts, et al. (2003). "Extracorporeal life support in pertussis." Pediatr Pulmonol **36**(4): 310-5.
- Porat, R., D. D. Poutsiaika, et al. (1992). "Interleukin-1 (IL-1) receptor blockade reduces endotoxin and *Borrelia burgdorferi*-stimulated IL-8 synthesis in human mononuclear cells." FASEB J **6**(7): 2482-6.
- Postlethwaite, A. E., B. K. Jackson, et al. (1982). "Formation of multinucleated giant cells from human monocyte precursors. Mediation by a soluble protein from antigen-and mitogen-stimulated lymphocytes." J Exp Med **155**(1): 168-78.
- Powars, D. R., Z. R. Rogers, et al. (1987). "Purpura fulminans in meningococemia: association with acquired deficiencies of proteins C and S." N Engl J Med **317**(9): 571-2.
- Pradhan, M. J., J. S. Fleming, et al. (1991). "Clinical experience with heparin-coated cardiopulmonary bypass circuits." Perfusion **6**(3): 235-42.

Bibliography

- Rady, M. Y., E. P. Rivers, et al. (1992). "Continuous central venous oximetry and shock index in the emergency department: use in the evaluation of clinical shock." Am J Emerg Med **10**(6): 538-41.
- Rangel-Frausto, M. S., D. Pittet, et al. (1995). "The natural history of the systemic inflammatory response syndrome (SIRS). A prospective study." JAMA **273**(2): 117-23.
- Ranucci, M., A. Cazzaniga, et al. (2002). "The antithrombin III-saving effect of reduced systemic heparinization and heparin-coated circuits." J Cardiothorac Vasc Anesth **16**(3): 316-20.
- Ranucci, M., A. Cazzaniga, et al. (2002). "The antithrombin III-saving effect of reduced systemic heparinization and heparin-coated circuits." J Cardiothorac Vasc Anesth **16**(3): 316-20.
- Redl, H., H. P. Dinges, et al. (1991). "Expression of endothelial leukocyte adhesion molecule-1 in septic but not traumatic/hypovolemic shock in the baboon." Am J Pathol **139**(2): 461-6.
- Redmond, H. P. (1996). Essential Surgical Immunology, Butterworth Heinemann.
- Redmond, J. M., A. M. Gillinov, et al. (1993). "Heparin-coated bypass circuits reduce pulmonary injury." Ann Thorac Surg **56**(3): 474-8; discussion 479.
- Remington, J. (1989). Infectious disease of the foetus and the newborn Philadelphia.
- Renaud, B. and C. Brun-Buisson (2001). "Outcomes of primary and catheter-related bacteremia. A cohort and case-control study in critically ill patients." Am J Respir Crit Care Med **163**(7): 1584-90.
- Riedemann, N. C. and P. A. Ward (2003). "Anti-inflammatory strategies for the treatment of sepsis." Expert Opin Biol Ther **3**(2): 339-50.
- Riordan, F. A., O. Marzouk, et al. (1995). "The changing presentations of meningococcal disease." Eur J Pediatr **154**(6): 472-4.
- Robinson, T. M., T. S. Kickler, et al. (1993). "Effect of extracorporeal membrane oxygenation on platelets in newborns." Crit Care Med **21**(7): 1029-34.
- Sakata, Y., D. J. Loskutoff, et al. (1986). "Mechanism of protein C-dependent clot lysis: role of plasminogen activator inhibitor." Blood **68**(6): 1218-23.
- Salvo, I., W. de Cian, et al. (1995). "The Italian SEPSIS study: preliminary results on the incidence and evolution of SIRS, sepsis, severe sepsis and septic shock." Intensive Care Med **21 Suppl 2**: S244-9.

Bibliography

- Salzman, E. W., J. Lindon, et al. (1987). "Role of fibrinogen in activation of platelets by artificial surfaces." Ann N Y Acad Sci **516**: 184-95.
- Samama, M. M., L. Bara, et al. (1996). "New data on the pharmacology of heparin and low molecular weight heparins." Drugs **52 Suppl 7**: 8-14; discussion 14-5.
- Scalea, T. M., M. Holman, et al. (1988). "Central venous blood oxygen saturation: an early, accurate measurement of volume during hemorrhage." J Trauma **28(6)**: 725-32.
- Schumer, M., M. C. Colombel, et al. (1992). "Morphologic, biochemical, and molecular evidence of apoptosis during the reperfusion phase after brief periods of renal ischemia." Am J Pathol **140(4)**: 831-8.
- Scully, M. F., C. H. Toh, et al. (1993). "Activation of protein C and its distribution between its inhibitors, protein C inhibitor, alpha 1-antitrypsin and alpha 2-macroglobulin, in patients with disseminated intravascular coagulation." Thromb Haemost **69(5)**: 448-53.
- Sevilla, C. L., N. H. Mahle, et al. (1997). "Development of monoclonal antibodies to the malondialdehyde-deoxyguanosine adduct, pyrimidopurinone." Chem Res Toxicol **10(2)**: 172-80.
- Shinozawa, Y., X. Q. Xie, et al. (2004). "[Correlation between intravascular coagulation/fibrinolysis system and cytokines]." Nippon Rinsho **62(12)**: 2253-61.
- Shua, F., H. Kobayashia, et al. (2000). "Activated protein C suppresses tissue factor expression on U937 cells in the endothelial protein C receptor-dependent manner." FEBS Lett **477(3)**: 208-12.
- Sibbald, W. J. and J. L. Vincent (1995). "Round table conference on clinical trials for the treatment of sepsis. Brussels, March 12-14, 1994." Intensive Care Med **21(2)**: 184-9.
- Sibbald WJ, S. C. (1986). Perspectives of Sepsis and Septic Shock, Fullerton.
- Skillman, J. J., L. S. Bushnell, et al. (1969). "Respiratory failure, hypotension, sepsis, and jaundice. A clinical syndrome associated with lethal hemorrhage from acute stress ulceration of the stomach." Am J Surg **117(4)**: 523-30.
- Sobin, L. H., P. Hermanek, et al. (1988). "TNM classification of malignant tumors. A comparison between the new (1987) and the old editions." Cancer **61(11)**: 2310-4.

Bibliography

- Sorensen, T. I., G. G. Nielsen, et al. (1988). "Genetic and environmental influences on premature death in adult adoptees." N Engl J Med **318**(12): 727-32.
- Soukka, H., M. Rautanen, et al. (1997). "Meconium aspiration induces ARDS-like pulmonary response in lungs of ten-week-old pigs." Pediatr Pulmonol **23**(3): 205-11.
- Soukka, H., M. Rautanen, et al. (1997). "Meconium aspiration induces ARDS-like pulmonary response in lungs of ten-week-old pigs." Pediatr Pulmonol **23**(3): 205-11.
- Spertini, O., P. Callegari, et al. (1994). "High levels of the shed form of L-selectin are present in patients with acute leukemia and inhibit blast cell adhesion to activated endothelium." Blood **84**(4): 1249-56.
- Sprung, C. L., P. N. Peduzzi, et al. (1990). "Impact of encephalopathy on mortality in the sepsis syndrome. The Veterans Administration Systemic Sepsis Cooperative Study Group." Crit Care Med **18**(8): 801-6.
- Sriskandan, S. and J. Cohen (1999). "Gram-positive sepsis. Mechanisms and differences from gram-negative sepsis." Infect Dis Clin North Am **13**(2): 397-412.
- Stadlbauer, V., P. Krisper, et al. (2006). "Effect of extracorporeal liver support by MARS and Prometheus on serum cytokines in acute-on-chronic liver failure." Crit Care **10**(6): R169.
- Stallion, A., B. R. Cofer, et al. (1994). "The significant relationship between platelet count and haemorrhagic complications on ECMO." Perfusion **9**(4): 265-9.
- Stefanec, T. (2000). "Endothelial apoptosis: could it have a role in the pathogenesis and treatment of disease?" Chest **117**(3): 841-54.
- Stefanec, T. (2000). "Endothelial apoptosis: could it have a role in the pathogenesis and treatment of disease?" Chest **117**(3): 841-54.
- Stewart, D. L., T. V. Dela Cruz, et al. (1997). "The use of extracorporeal membrane oxygenation in patients with gram-negative or viral sepsis." Perfusion **12**(1): 3-8.
- Stouthard, J. M., M. Levi, et al. (1996). "Interleukin-6 stimulates coagulation, not fibrinolysis, in humans." Thromb Haemost **76**(5): 738-42.
- Stuber, F., M. Petersen, et al. (1996). "A genomic polymorphism within the tumor necrosis factor locus influences plasma tumor necrosis factor-alpha

Bibliography

concentrations and outcome of patients with severe sepsis." Crit Care Med **24**(3): 381-4.

Sugama, Y., C. Tirupathi, et al. (1992). "Thrombin-induced expression of endothelial P-selectin and intercellular adhesion molecule-1: a mechanism for stabilizing neutrophil adhesion." J Cell Biol **119**(4): 935-44.

Svenmarker, S., E. Sandstrom, et al. (2001). "Neurological and general outcome in low-risk coronary artery bypass patients using heparin coated circuits." Eur J Cardiothorac Surg **19**(1): 47-53.

Takano, S., S. Kimura, et al. (1990). "Plasma thrombomodulin in health and diseases." Blood **76**(10): 2024-9.

Takano, S., S. Kimura, et al. (1990). "Plasma thrombomodulin in health and diseases." Blood **76**(10): 2024-9.

Tamari, Y., K. Lee-Sensiba, et al. (1999). "An improved bladder for pump control during ECMO procedures." J Extra Corpor Technol **31**(2): 84-90.

Tantalean, J. A., R. J. Leon, et al. (2003). "Multiple organ dysfunction syndrome in children." Pediatr Crit Care Med **4**(2): 181-5.

Taylor, F., A. Chang, et al. (1991). "C4b-binding protein exacerbates the host response to Escherichia coli." Blood **78**(2): 357-63.

Taylor, F. B., Jr., A. Chang, et al. (1987). "Protein C prevents the coagulopathic and lethal effects of Escherichia coli infusion in the baboon." J Clin Invest **79**(3): 918-25.

Taylor, F. B., Jr., A. Chang, et al. (1991). "Lethal E. coli septic shock is prevented by blocking tissue factor with monoclonal antibody." Circ Shock **33**(3): 127-34.

Taylor, F. B., Jr., A. C. Chang, et al. (1991). "DEGR-factor Xa blocks disseminated intravascular coagulation initiated by Escherichia coli without preventing shock or organ damage." Blood **78**(2): 364-8.

te Velthuis, H., P. G. Jansen, et al. (1996). "Specific complement inhibition with heparin-coated extracorporeal circuits." Ann Thorac Surg **61**(4): 1153-7.

Tedder, T. F., A. C. Penta, et al. (1990). "Expression of the human leukocyte adhesion molecule, LAM1. Identity with the TQ1 and Leu-8 differentiation antigens." J Immunol **144**(2): 532-40.

Bibliography

- ten Cate, H., S. H. Schoenmakers, et al. (2001). "Microvascular coagulopathy and disseminated intravascular coagulation." Crit Care Med **29**(7 Suppl): S95-7; discussion S97-8.
- ten Cate, J. W., T. van der Poll, et al. (1997). "Cytokines: triggers of clinical thrombotic disease." Thromb Haemost **78**(1): 415-9.
- Teodorczyk-Injeyan, J. A., B. G. Sparkes, et al. (1992). "IL-2 regulation of soluble IL-2 receptor levels following thermal injury." Clin Exp Immunol **90**(1): 36-42.
- Thiara, A. P., T. N. Hoel, et al. (2007). "Evaluation of oxygenators and centrifugal pumps for long-term pediatric extracorporeal membrane oxygenation." Perfusion **22**(5): 323-6.
- Thomas, B. A. (1927). "Vital Factors in the Management of Prostatic Obstruction." Ann Surg **86**(4): 563-76.
- Tilney, N. L., G. L. Bailey, et al. (1973). "Sequential system failure after rupture of abdominal aortic aneurysms: an unsolved problem in postoperative care." Ann Surg **178**(2): 117-22.
- Tomashefski, J. F., Jr. (2000). "Pulmonary pathology of acute respiratory distress syndrome." Clin Chest Med **21**(3): 435-66.
- Tong, S. D., M. R. Rolfs, et al. (1990). "Evaluation of Duraflo II heparin immobilized cardiopulmonary bypass circuits." ASAIO Trans **36**(3): M654-6.
- Totan, M. (1999). "Protein C, Protein S and Antithrombin III Levels in A Rabbit Sepsis Model." Tr. J. of Medical Sciences **29**: 389-391.
- Toyokuni, S., N. Miyake, et al. (1995). "The monoclonal antibody specific for the 4-hydroxy-2-nonenal histidine adduct." FEBS Lett **359**(2-3): 189-91.
- Trittenwein, G., A. T. Rotta, et al. (1999). "Lipid peroxidation during initiation of extracorporeal membrane oxygenation after hypoxia in endotoxemic rabbits." Perfusion **14**(1): 49-57.
- Urlesberger, B., G. Zobel, et al. (1996). "Activation of the clotting system during extracorporeal membrane oxygenation in term newborn infants." J Pediatr **129**(2): 264-8.
- Videm, V., T. E. Mollnes, et al. (1991). "Biocompatibility of extracorporeal circulation. In vitro comparison of heparin-coated and uncoated oxygenator circuits." J Thorac Cardiovasc Surg **101**(4): 654-60.

Bibliography

- Vincent, J. L. (1997). "Dear SIRS, I'm sorry to say that I don't like you." Crit Care Med **25**(2): 372-4.
- Vincent, J. L., G. R. Bernard, et al. (2005). "Drotrecogin alfa (activated) treatment in severe sepsis from the global open-label trial ENHANCE: further evidence for survival and safety and implications for early treatment." Crit Care Med **33**(10): 2266-77.
- Vincent, J. L. and D. De Backer (2005). "Microvascular dysfunction as a cause of organ dysfunction in severe sepsis." Crit Care **9 Suppl 4**: S9-12.
- Vincent, J. L., A. de Mendonca, et al. (1998). "Use of the SOFA score to assess the incidence of organ dysfunction/failure in intensive care units: results of a multicenter, prospective study. Working group on "sepsis-related problems" of the European Society of Intensive Care Medicine." Crit Care Med **26**(11): 1793-800.
- Wallach, D. (1996). "Suicide by order: some open questions about the cell-killing activities of the TNF ligand and receptor families." Cytokine Growth Factor Rev **7**(3): 211-21.
- Walley, K. R. (1996). "Heterogeneity of oxygen delivery impairs oxygen extraction by peripheral tissues: theory." J Appl Physiol **81**(2): 885-94.
- Wang, S. D., K. J. Huang, et al. (1994). "Sepsis-induced apoptosis of the thymocytes in mice." J Immunol **152**(10): 5014-21.
- Watson, R. W., H. P. Redmond, et al. (1996). "Bacterial ingestion, tumor necrosis factor-alpha, and heat induce programmed cell death in activated neutrophils." Shock **5**(1): 47-51.
- Weerwind, P. W., J. G. Maessen, et al. (1995). "Influence of Duraflo II heparin-treated extracorporeal circuits on the systemic inflammatory response in patients having coronary bypass." J Thorac Cardiovasc Surg **110**(6): 1633-41.
- Westendorp, R. G., J. A. Langermans, et al. (1997). "Genetic influence on cytokine production and fatal meningococcal disease." Lancet **349**(9046): 170-3.
- Westwood, J. T., J. Clos, et al. (1991). "Stress-induced oligomerization and chromosomal relocalization of heat-shock factor." Nature **353**(6347): 822-7.
- Wheeler, A., J. Steingrub, et al. (2008). "A retrospective observational study of drotrecogin alfa (activated) in adults with severe sepsis: comparison with a controlled clinical trial." Crit Care Med **36**(1): 14-23.

Bibliography

- White, B., M. Schmidt, et al. (2000). "Activated protein C inhibits lipopolysaccharide-induced nuclear translocation of nuclear factor kappaB (NF-kappaB) and tumour necrosis factor alpha (TNF-alpha) production in the THP-1 monocytic cell line." Br J Haematol **110**(1): 130-4.
- Wickramasinghe, S. R., C. M. Kahr, et al. (2002). "Mass transfer in blood oxygenators using blood analogue fluids." Biotechnol Prog **18**(4): 867-73.
- Wilkinson, J. D., M. M. Pollack, et al. (1986). "Outcome of pediatric patients with multiple organ system failure." Crit Care Med **14**(4): 271-4.
- Wilson, R. F., E. F. Mammen, et al. (1996). "Antithrombin levels related to infections and outcome." J Trauma **40**(3): 384-7.
- Wohlford-Lenane, C. L., D. C. Deetz, et al. (1999). "Cytokine gene expression after inhalation of corn dust." Am J Physiol **276**(5 Pt 1): L736-43.
- Wood, K. A. and R. J. Youle (1994). "Apoptosis and free radicals." Ann N Y Acad Sci **738**: 400-7.
- Wortel, C. H., M. A. von der Mohlen, et al. (1992). "Effectiveness of a human monoclonal anti-endotoxin antibody (HA-1A) in gram-negative sepsis: relationship to endotoxin and cytokine levels." J Infect Dis **166**(6): 1367-74.
- Wreghitt, T. (1990). ELISA In The Clinical Microbiology Laboratory, Public Health Laboratory Service.
- Xu, H., J. A. Gonzalo, et al. (1994). "Leukocytosis and resistance to septic shock in intercellular adhesion molecule 1-deficient mice." J Exp Med **180**(1): 95-109.
- Yan, S. (1999). "Contribution of late professor T. C. Tung to the experimental embryology of Amphioxus. In memory of the 20th anniversary of Professor T. C. Tung's death." Dev Growth Differ **41**(5): 503-22.
- Yokoi, K., N. Mukaida, et al. (1997). "Prevention of endotoxemia-induced acute respiratory distress syndrome-like lung injury in rabbits by a monoclonal antibody to IL-8." Lab Invest **76**(3): 375-84.
- Younger, J. G., R. J. Schreiner, et al. (1999). "Extracorporeal resuscitation of cardiac arrest." Acad Emerg Med **6**(7): 700-7.
- Yuan, J. (1997). "Transducing signals of life and death." Curr Opin Cell Biol **9**(2): 247-51.

Bibliography

- Zeldin, D. C., C. Wohlford-Lenane, et al. (2001). "Airway inflammation and responsiveness in prostaglandin H synthase-deficient mice exposed to bacterial lipopolysaccharide." Am J Respir Cell Mol Biol **25**(4): 457-65.
- Zeni, F., B. Freeman, et al. (1997). "Anti-inflammatory therapies to treat sepsis and septic shock: a reassessment." Crit Care Med **25**(7): 1095-100.
- Ziegler, E. J., C. J. Fisher, Jr., et al. (1991). "Treatment of gram-negative bacteremia and septic shock with HA-1A human monoclonal antibody against endotoxin. A randomized, double-blind, placebo-controlled trial. The HA-1A Sepsis Study Group." N Engl J Med **324**(7): 429-36.
- Zimmerman, J. J. (1995). "Defining the role of oxyradicals in the pathogenesis of sepsis." Crit Care Med **23**(4): 616-7.
- Zwischenberger, J. B. (2000). Extracorporeal Cardiopulmonary Support in Critical Care, Extracorporeal Life Support Organization.