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The Role of Preoperative Antioxidant Status in the Development of the Systemic Inflammatory Response Syndrome in Elective Aortic Aneurysm Repair

by

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ABSTRACT

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THE ROLE OF PREOPERATIVE ANTIOXIDANT STATUS IN THE DEVELOPMENT OF THE SYSTEMIC INFLAMMATORY RESPONSE SYNDROME IN ELECTIVE AORTIC ANEURYSM REPAIR

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Reactive oxygen species (ROS) have been implicated in the development of the Systemic inflammatory response syndrome (SIRS). Antioxidants help prevent or limit the harmful effects of ROS.

The aim of this thesis was to investigate the role of the preoperative antioxidant status in this inflammatory response using elective abdominal aortic aneurysm patients as a model.

20 patients undergoing elective abdominal aortic aneurysm repair were enrolled following ethics committee approval and informed consent. Blood samples were collected pre-operatively, before and post clamp release and postoperatively to day 5. Glutathione(GSH), Vitamin E, β-carotene and α-carotene were determined using high-performance liquid chromatography. C-reactive protein (CRP) was also measured. Interleukin-6, 8 and 10 were analysed using an Enzyme Amplified Sensitivity Immunoassay (EASIA) The Acute Physiology Age and Chronic Health Evaluation (APACHE) II score at 24 hours and daily Sequential organ failure scores (SOFA) were calculated. Multi frequency bioelectric impedance was measured daily.

The patients had a median age of 71.8 (range 62-77), with a median APACHE II score of 16 (3.6), a predicted in hospital mortality of 10.6% (11) and an ICU stay of 25 (30.8) hours. 19 patients survived to hospital discharge. The only antioxidant measured that showed any effect was GSH. Pre-operative GSH is inversely related to the CRP at 72 hours ($r=0.515$, $P=0.02$) and also CRP area under the curve ($r=0.471$, $P=0.038$). Similarly, the SOFA score has a negative correlation at 24 hours ($r_s=0.453$, $P=0.045$) and SOFA area under the curve ($r_s=0.560$, $P=0.010$). Though APACHE II does not quite reach significance ($r_s=0.459$, $p=0.055$) it does show a similar negative trend as SOFA score. There was a significant difference between the mean GSH in patients with a phase angle $<$ or $> 5.3^{\circ}$ (2.90 mmol/l v 3.64 mmol/l $t=-3.791$ (df 18) $p=0.001$).

The results would suggest that GSH may be pivotal in modulating the inflammatory response. The enhancement of preoperative GSH may therefore ameliorate the adverse effects of inflammation and would be worth further study.

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Abbreviations

AA	Arachidonic Acid
AAA	Abdominal Aortic Aneurysm
AAPH	2-29-azobis(2-amidinopropane) hydrochloride
AF	Atrial Fibrillation
AFR	Ascorbyl Free Radical
AP-1	Activator Protein-1
APACHE II	Acute Physiology And Chronic Health Evaluation 2 Score
ARDS	Acute Respiratory Distress Syndrome
ARE	Antioxidant Response Element
BiPAP	Bilevel Positive Airway Pressure
cAMP	Cyclic Adenosine Monophosphate
cGMP	Cyclic Guanosine Monophosphate
COX	Cycloxygenase
CPAP	Continuous Positive Airway Pressure
CRP	C Reactive Protein
CT	Computer Tomography
DHA	Dehydroascorbic Acid
EC	Endothelial Cell
ELAM	Endothelial-Leucocyte Adhesion Molecules
ERK	Extracellular Signal Related Kinase
ESL-1	E-Selectin Ligand-1
FAD	Flavin Adenine Dinucleotide
GM-CSF	Granulocyte/Macrophage Colony Stimulating Factor
GPX	Glutathione Peroxidase
GSH	Glutathione
H_2O_2	Hydrogen Peroxide
HAL	Hypoxia Associated Ligand
HDU	High Dependancy Unit
HETE	Hydroxyeicosatetraenoic acids
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM	Intercellular Adhesion Molecule
ICE	Interleukin-1β converting enzyme
ICU	Intensive Care Unit
IKK	IkB kinase
IL-	Interleukin
IL-1R	Interleukin-1 Receptor
IL-1ra	Interleukin-1 receptor antagonist
IL-6Rα	Interleukin-6 receptor
JNK	c-jun N-Terminal Kinase
LFA-1	Lymphocyte Associated Function Antigen-1
LPS	Lipopolysaccharide
Mac-1	Macrophage antigen-1
MACR	Microalburnin Creatinine Ratio
MAP3K	Mitogen Activated Protein Kinase Kinase Kinase
MAPK	Mitogen Activated Protein Kinase
MDA	Malondialdehyde
MEK	Mitogen Activated Protein/Extracellular Signal Related Kinase
MKK	Mitogen Activated Protein Kinase kinase

MODS	Multiple Organ Dysfunction
NAC	N-Acetyl-L-Cysteine
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced form)
NFκB	Nuclear Factor-kappaB
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
$O_2^{\bullet}$	Superoxide
$ONOO^-$	Peroxynitrite
p38	p38 Mitogen Activated Protein Kinase
PAF	Platelet Activating Factor
PAI-1	Plasminogen Activator Inhibitor
PECAM	Platelet-Endothelial Cell Adhesion Molecule
PG	Prostaglandin
PLA_2	Phospholipase-A_2
PMN	Polymorphonuclear Cell or Neutrophil
PSGL-1	P-Selectin Gycoprotein Ligand-1
PUFA	Polyunsaturated Fatty Acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SAPK	Stress Activated Protein Kinase
SIRS	Systemic Inflammatory Response Syndrome
SOD	Superoxide Dismutase
SOFA	Sequential Organ Failure Score
SRF	Serum Resonse Factor
sTNF-Rs	Soluble Tumour Necrosis Factor Receptors
TAAA	Thoraco-Abdominal Aortic Aneurysm
TACE	Tumour Necrosis Factor-α Converting Enzyme
TBARS	Thiobarbituric Acid Reactive Substances
TBP	Tocopherol Binding Protein
TEM	Transendothelial Migration
TF	Tissue Factor
TH1	T helper subset 1
TH2	T helper subset 2
TNF	Tumour Necrosis Factor
tPA	Tissue Plasminogen Activator
TXB_2	Thromboxane B_2
uPA	Urinary Plasminogen Activator.
VCAM	Vascular Cell Adhesion Molecule
VSMC	Vascular Smooth Muscle Cells
vWF	Von Willbrand Factor
WBC	White Blood Count
XDH	Xanthine Dehydrogenase
XO	Xanthine Oxidase
XOR	Xanthine Oxidoreductase
αTTP	α Tocopherol Transfer Protein

Chapter 1 Systemic Inflammatory Response Syndrome

The introduction to this thesis is a literature review of the importance of the systemic inflammatory response syndrome, the influence that ischaemia and reperfusion have on inflammation together with the role that antioxidants play in protecting the body against these pathophysiological responses. The review will demonstrate the complex molecular and cellular interactions that occur in SIRS and ischaemia reperfusion injury demonstrating why antioxidants play only a part in modulating these effects.

Introduction

Following surgery or tissue injury there is a localised inflammatory response that is physiologically protective. This is tightly controlled and is aimed at initiating wound healing, controlling infection and returning the body to normal haemostasis. However if the homeostatic control of the inflammatory response is lost it can result in a massive systemic inflammatory reaction. Systemic inflammatory response syndrome (SIRS) is the clinical and haematological manifestation of this exaggerated inflammatory process.[1] This can in certain circumstances lead to organ failure or death. After developing SIRS there maybe a continuum through sepsis, septic shock to multiple organ dysfunction syndrome (MODS). These were defined in 1992 at a consensus conference of the American College of Chest Physicians and Society of Critical Care Medicine (ACCP/SCCM)[2] (table 1). Prior to these definitions the generalised term of sepsis covered this heterogeneous group of signs and symptoms. The lack uniformity in case definition had held back the understanding of the pathophysiology of SIRS as well as the assessment of the success of treatment.

Table 1 Criteria for systemic inflammatory response syndrome, sepsis and multiple organ dysfunction syndrome.[2]

SIRS	Temperature	>38°C or <36°C (rectal)
	Heart rate	>90 BPM
	Respiratory	>20 breaths per minute or Pa_{CO_2} < 4.3 kPa
	White cell count	>12,000 cells/mm^3 or <4,000 cells/mm^3 or 10% immature (band) forms
Sepsis	SIRS with documented infection.	
Severe Sepsis	Sepsis with hypotension, organ hypoperfusion or dysfunction.	
Septic shock	Severe sepsis with hypotension despite adequate fluid resuscitation.	
MODS	A state of physiological derangement in which organ function is not capable of maintaining homeostasis.	

Even so the ACCP/SCCM criteria have come under criticism as being too sensitive and unhelpful with the understanding of pathophysiology. The sensitive nature means that within the intensive care unit setting SIRS is common, hence of little clinical value. This has further implication in clinical trials as the sensitivity excludes its usefulness as entry criteria. The mixing of patients with a heterogeneous group of acute disease would also be possible within the SIRS criteria.[3,4]

However the ACCP/SCCM criteria does allow for a standardisation of research protocols and epidemiological studies. We must be mindful of the above criticisms but the ACCP/SCCM criteria have allowed for the comparison of clinical trials in the absence currently of a good biochemical marker of Sepsis.

Epidemiology

SIRS is important due to the associated morbidity and mortality. Due to problems in case definition an accurate estimate of incidence has been difficult. An epidemiological study by the Centers for Disease Control in 1990 showed the incidence of sepsis had increased from 73.6 to 175.9 per 100,000 from 1979 to 1987.[5]

Rangel-Frausto et al at the University of Iowa Hospitals and Clinics conducted a nine month prospective cohort study on three intensive care units and three wards with a 28 day follow up or until discharge.[6] There were 3708 patients admitted during the study of which 2527(68%) met at least two of the SIRS criteria. Of those patients with SIRS, 26% developed sepsis, 18% severe sepsis and 4% septic shock. The natural progression between the ACCP/SCCM definitions is confirmed with this study. Patients with two SIRS criteria developed sepsis in 32% of cases, with three criteria 36% developed sepsis and with four criteria 45% had sepsis by 14 days. Of those with sepsis 64% went on to severe sepsis within a median of 1 day but only 23% of those with severe sepsis developed septic shock. Mortality follows a stepwise progression from SIRS (7%), sepsis (16%), severe sepsis (20%) and septic shock (46%).

Sands et al at 8 academic tertiary referral centres prospectively surveyed a random sample of ICU patients and non-ICU patients who had blood cultures taken during a 15 month period.[7] They used 2 of 3 SIRS criteria, not prospectively surveying for white blood cell count (WBC). A total of 12,759 patients were monitored involving 15,515 surveillance episodes. SIRS was identified in 44% of these cases with 24.7% having sepsis. Of all admissions the extrapolated incidence of SIRS was 18%. These are probably

underestimates as they only used 3 of the 4 criteria for SIRS. The 28 day mortality for patients with severe sepsis and septic shock was 34%.

The Italian SEPSIS study preliminary results published in 1995 prospectively looked at the admission to 99 ICU's during a 12 month period.[8] On admission 52% of 1101 patients had SIRS, 4.5% sepsis, 2.1% severe sepsis and 3% septic shock. At any point during the study the incidence of SIRS for this population was 58% with 16.3% having sepsis, 5.5% severe sepsis and 6.1% septic shock. The mortality for SIRS was 26.5%, rising to 36%, 52% and 81.8% for sepsis, severe sepsis and septic shock respectively.

Brun-Buisson et al performed a prospective 2 month cohort study looking at 11828 admissions to 170 ICU's in France.[9] They identified patients with severe sepsis or septic shock using 2 out of 3 SIRS criteria (not including WBC) in addition to hypotension and at least one sign or symptom of acute organ dysfunction. Severe sepsis occurred in 6.3% of admission and mortality with severe sepsis and septic shock was 56%.

The above studies using ACCP/SCCM definitions have given wide range of incidence rates for patients admitted to intensive care units (ICU). These studies are from tertiary referral centres and therefore it is not possible to generalise the results to the wider hospital population. Importantly it can be seen that SIRS and associated sepsis is common particularly in ICU and has a significant mortality.

Pathophysiology of SIRS

Following tissue injury the body mounts a complex series of molecular and cellular events. Inflammatory cells such as macrophages, polymorphonuclear cells (PMN), and lymphocytes are recruited to the site of injury. These cells secrete inflammatory mediators. The coordination of this local inflammatory response requires the regulation of many different cell types. This is partly achieved by the paracrine effect of cytokines, which are soluble protein and lipid messengers. This inflammatory response is aimed at initiating wound healing, controlling infection and returning the body to normal haemostasis.

In 1996 Bone proposed that there were three stages in the development of SIRS.[1] The first stage is as described above and is a normal physiological response to injury. In stage 2 cytokines are released into the systemic circulation in small quantities enhancing the local response by recruiting macrophages, platelets stimulating growth factor synthesis and initiating the acute phase response. The synthesis of endogenous antagonists and decrease in the proinflammatory mediators regulates the cytokine effects and leads to establishment of normal homeostasis. Stage 3 occurs when homeostasis cannot be restored. This leads to a substantial systemic reaction where the previous protective role of cytokines becomes destructive. The circulation becomes inundated with inflammatory mediators leading to loss of microvascular integrity, sustained leukocyte activation, humoral cascade activation and end organ damage. If this stage cannot be controlled the result is MODS and possible death.

Mediators of SIRS

Cytokines are the principle mediators of SIRS, this is well established from three lines of evidence[10]:-

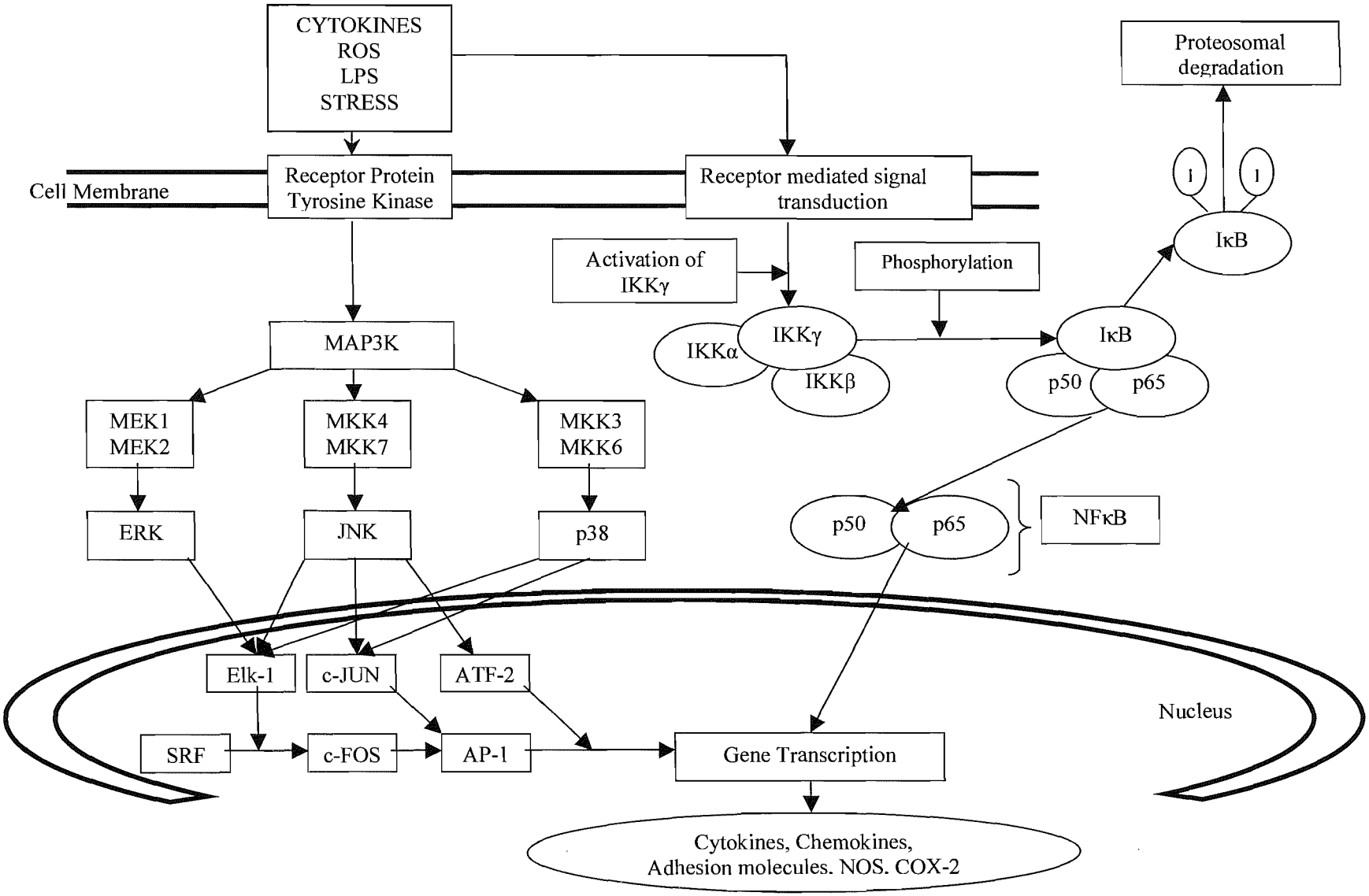
1. Humans and animals with sepsis have circulating levels of cytokines which correlate with outcome.[11,12]
2. Cytokines injected in humans or animals induce SIRS.[13]
3. Administration of anti-cytokines reduces the morbidity and mortality associated with sepsis.[14,15]

Though many mediators play a role in SIRS the most important pro-inflammatory cytokines are Tumour Necrosis Factor-α (TNF-α), Interleukin-1 (IL-1), Interleukin-6 (IL-6) and Interleukin-8 (IL-8). The chief anti-inflammatory mediators are IL-1 receptor antagonist (IL-1ra), Interleukin-10 (IL-10) and the soluble TNF-α receptors (sTNF-Rs). The biology and action of the above cytokines will be discussed individually with a brief review of those mediators with an increasing important role in SIRS.

Intracellular control of the inflammatory response

Cytokines are not stored intracellularly and are therefore newly synthesised following an inflammatory stimulus. This process is controlled within the cell by the interaction of a network of proteins that transmit extracellular signals to their intracellular target. This process of signal transduction results in the activation of effector proteins and the modification of protein expression and transcriptional activity. One such pathway that

Figure 2 MAPK and NFκB Intracellular Signalling Pathways.



has been identified as playing a key role in the inflammatory response is the mitogen activated protein kinase (MAPK) superfamily.This is a large family of protein kinases that are involved in intracellular signalling and are usually activated by extracellular signalling molecules binding to cell surface receptor associated tyrosine kinase.

Extracellular signal-related kinase (ERK), p38 MAPK (p38), and c-jun N-terminal kinases (JNK) also referred to as stress activated protein kinases (SAPK) have been shown to have an important function in the inflammatory response. They are usually activated by a classic MAPK three step pathway resulting in the dual phosphorylation of threonine and tyrosine separated by a single amino acid. These three MAPK's also have significant overlap in downstream substrate activation.

Lipopolysaccharide (LPS), cytokines and oxidant stress cause p38 activation in leukocytes, endothelial cells and monocytes/macrophage. Five different isoforms of p38 (α, β, $\beta 2$, γ, δ) have been identified which are selectively expressed in various tissue types. They also have differing upstream activators and substrate targets. One such upstream activator that has been shown to have proinflammatory role is mitogen activated protein kinase kinase 3 (MKK3). This is abundant in leukocytes and activates p38$\alpha/\gamma/\delta$ only. p38α is the isoform found in leukocytes and in neutrophils is selectively activated by lipopolysaccharide (LPS) via MKK3.[16] Several inflammatory responses in endothelial cells, macrophages and neutrophils have been shown to be p38 dependant. The expression of TNF-α, IL-1, IL-6, IL-8, cycloxygenase-2 (COX-2) and inhibition of apoptosis by monocytes/macrophages are dependant on p38.[17] In neutrophils the release of elastase, production of IL-8 and the oxidative burst similarly are p38 dependant[18]. Additional p38

dependant effects are the TNF-α and LPS induced generation of prostaglandins, COX-2 and E-selectin expression in endothelial cells.[19,20]

JNK is activated in a similar manner to p38. The major role of JNK is the activation of activator protein-1 (AP-1). AP-1 is a transcription factor complex composed of homodimers and heterodimers of the c-fos and c-jun proto-oncogenes.[21] JNK, p38 and ERK phosphorylates the transcription factor Elk-1 and c-jun. Elk-1 forms a complex with serum response factor, thus inducing c-fos by binding to the c-fos promoter. ATF-2 another transcription factor is also phosphorylated by the activated JNK. AP-1 has been shown to regulate TNF-α genes by complexing with other transcription factors such as ATF-2 and nuclear factor-κB (NFκB).[22] Il-8 may also be regulated by AP-1, an AP-1 like binding site has been identified in the IL-8 promoter in jurkat cells.[23] JNK probably through AP-1 activation is involved in stimulating cellular apoptosis.[24] E-selectin expression induced by TNF-α requires the activation of JNK.[25]

ERK is activated by a multitude of stimuli in addition to LPS. These include the following mitogens platelet derived growth factor, angiotensin-II transforming growth factor and insulin. Activation is also stimulated by adherance through integrin mediated activation of focal adhesion kinases in monocytes and endothelial cells.[26] This final mode of activation is important in priming the monocytes/macrophages for TNF-α production though other modes of activation may also be relevant.

The transcription factor, nuclear factor κB (NFκB) is rapidly activated and plays an important role in the regulation of several genes involved in immune and inflammatory responses. NFκB is localised in the cytoplasm in the majority of cell types. It is composed

of homo- or heterodimers of structurally related proteins. Currently, five subunits proteins have been identified that make up the NFκB. These are p50, p65, p52, c-Rel Rel-b with the predominant form within cells being the p50/p65 heterodimer. Other homo and heterodimer complexes of these subunits have alternate transcription factor activity. In unstimulated cells NFκB is bound non-covalently to an inhibitory protein called IκB. Seven IκB molecules have been identified to date with the principal forms being IκBα and IκBβ. The activation of NFκB requires the phosphorylation of IκB on two serine residues. This allows IκB to be targeted for ubiquination and proteosomal degradation.[27] The phosphorylation of IκB is via a complex of IκB kinases (IKK). Most evidence suggests that IKK is formed from a heterodimer of two catalytic subunits IKKα and IKKβ complexed with a homodimer of regulatory subunits IKKγ. Upstream activators interact with IKKγ resulting in the activation of the IKK complex and subsequent phosphorylation of IκB by the IKKβ subunit.The activation of NFκB mediated inflammatory response is via this pathway.[28] IKKα does not make a contribution to this pathway but is activated by an alternative route.This leads to the activation of p52 containing complexes by the processing of the precursor p100 subunit to p52. This pathway appears to have a role in b-cell mediated immunity.

The release of the NFκB subunits from IκB allows it to translocate into the nucleus and thus regulate a variety of genes. NFκB is involved in the induction of >150 target genes. Target genes include those that are involved in the positive and negative feedback mechanisms of NFκB control. Increased transcripton of IκB and p50 play a central role in negative feedback. The binding of IκB to NFκB within the nucleus promotes the resequestration of NFκB back to the cytoplasm. Increased production of p50 subunit

promotes the formation of a p50 homodimer. This binds to the promoter region of the target gene inhibiting gene activation. NFκB plays a fundamental role in stimulating the transcription of cytokines, chemokines, cell adhesion molecules and stress response genes (table 2).

Table 2 Small selection of target genes regulated by NFκB pathway

Cytokines	TNF-α IL-1β IL-6 IL-8
Cell adhesion molecules	Endothelial leucocyte adhesion molecule-1 Vascular cell adhesion molecule-1 Intracellular adhesion molecule-1
Acute phase proteins	Complement factors B, C3, C4 Serum amyloid A precursor
Enzymes	COX-2 iNOS

TNF-α and IL-1 play a role in positive feedback by stimulating the activation of NFκB and thus propagating the inflammatory response.[29] They also stimulate the transcription of IL-10 which plays a counter regulatory role by inhibiting NFκB activation.[30]

There has been an increasing interest in the role of apoptosis in the development of SIRS and MODS. Kerr et al used the term apoptosis in 1972 as a means of distinguishing a morphologically distinctive form of cell death which is associated with normal physiology.[31] Apoptosis was distinguished from necrosis, which is associated with acute injury to cells. Apoptosis is characterized by nuclear chromatin condensation, cytoplasmic shrinking, dilated endoplasmic reticulum, and membrane blebbing. Mitochondria remain unchanged morphologically.

In both trauma patients and those patients with SIRS following aortic surgery there is a decreased neutrophils apoptosis. The continued activity of these neutrophils may contribute to the progression of SIRS into MODS. In sepsis, apoptosis has been noted in parenchymal cells. This cell loss may have an important function in the development of MODS. NFκB activation in general is anti-apoptotic but in certain cell types it has a pro-apoptopic role.

In conclusion, the major function of NFκB is the coordination of gene transcription involved in the inflammatory response. It would appear that any imbalance in the activation of NFκB could result in either an amplified or suppressed activation of the NFκB target genes. Any resultant amplification of the proinflammatory target genes may lead to the development of SIRS. The evidence for NFκB activation having a significant influence in SIRS is limited but persuasive. The binding activity of NFκB in peripheral blood mononuclear cell was shown by Bohrer et al to be significantly elevated ($p<0.0001$) in non survivors of sepsis.[33] Similiarly in a small study of 10 patients who fulfilled the SIRS criteria the four non-surviving patients showed a marked increase in NFκB activation prior to death in mononuclear cell and neutrophils compared to the survivors ($p=0.0105$).[32] In a mouse model of endotoxaemia,the intravenous somatic gene transfer of IκBα given before LPS stimulation attenuated NFκB binding activity and increased survival.[33] Okada et al showed that the administration of 21-aminosteroid inhibited activation of NFκB in a mouse endotoxic shock model with improved survival rates.[34] Foulds et al in the neutrophils of patients undergoing thoracoabdominal aortic aneurysm repair demonstrated a significant difference between preoperative NFκB status in those patients who died or developed organ dysfunction and those who did not.[35]

Tumour Necrosis Factor-α and Interleukin-1

IL-1 acts synergistically with TNF-α and they have multiple overlapping functions. TNF-α is a 17 kDa protein the gene for which is located on chromosome 6. The release and biosynthesis of TNF-α is regulated at gene transcriptional (DNA to RNA) and translational (RNA to protein) level as well as post-translational protein processing.[36,37] It is initially expressed on the cell membrane as a 26kDa protein, which is cleaved into the 17 Kda soluble form by TNF-α converting enzyme (TACE), a metalloproteinase disintegrin.[38,39] Three of these soluble TNF monomers form a noncovalent polymer, and it this bell shaped structure that is the bioactive form.[40,41]

Cells of the Monocyte/Macrophages lineage are the principal source of TNF-α.[37] Other cell types that can secrete TNF-α, include lymphocytes, PMNs, keratinocytes, mast cells, eosinophils and glial cells.[42] The major stimulus for release of TNF-α is Lipopolysaccharide (LPS) also known as endotoxin.[37] This is part of the external gram negative bacteria cell wall and consists of three regions the O antigen polysaccharide, the core and the lipid A region. The latter is responsible for the majority of the LPS toxicity. Induction of TNF-α production can also occur by other factors including cytokines, activated complement products(C5a), C-reactive protein, granulocyte/macrophage colony stimulating factor(GM-CSF), antigen-antibody complexes and exotoxins.

The action of TNF-α is mediated by binding to specific cellular receptors that are present on all cell types except red blood cells. The two TNF- receptors (TNF-R) have homology in their extracellular domains the intracellular domains show no homology. The receptors are identified by their molecular weights, the 55 kDa TNF-R1 (p55) and 75 kDa TNF-R2

(p75).[43] Though the receptors are present on all cell types one may be dominant in its expression. Epithelial cells predominately have TNF-R1 and lymphoid cells TNF-R2.[44] In vitro and in vivo studies have suggested that TNF-R1 mediates the proinflammatory action of TNF-α[45] whereas TNF-R2 is associated with cellular proliferation.[46]

Soluble forms of the above TNF receptors (sTNF-Rs) are involved in the regulation of TNF-α activity. The receptors are cleaved from the cell surface probably by a metalloproteinase which may be similar to TACE.[47] Other proteases may also be involved in this cleavage process.[48] In vitro studies have suggested that p75 are released from mononuclear cells and p55 from endothelial cell.[49] The sTNF-Rs have a high affinity but reversible binding to TNF-α attenuating its action. Even with maximal concentrations of sTNF-Rs there still remains 10% of the TNF-α that is active. The soluble receptors will therefore not fully ameliorate the toxicity of TNF-α in SIRS but may go some way to modulating its biological activity.[50,51] The importance of the sTNF-Rs may be in its effect on the half life of TNF-α acting as a slow release biologically active pool rather than an antagonist to its action.[52]

The intravenous injection of endotoxin was first shown to induce the release of TNF-α in human volunteers by Michie et al in 1988.[53] It typically appears in the plasma after 45 to 60 minutes reaching a peak level at 90 minutes after the stimulus. This has been confirmed in several studies since. The haemodynamic and metabolic alterations seen in sepsis/SIRS can be induced by the infusion of recombinant TNF-α in animals.[54,55] The results of these studies as well as others suggest that TNF-α is the primary mediator of inflammation.

The three dimensional structure and receptors are clearly different for IL-1. Il-1 activity is mediated by two structurally related cytokines IL-1α and IL-1β which have only 26% homology. The genes are located on chromosome 2 and initially they are synthesised into a 31 kDa precursor. The precursors do not have a hydrophobic signal peptide which is required for post transcriptional modification in the golgi and subsequent secretion. The mature forms of the cytokines are 17 kDa in size. IL-1β unlike IL-1α requires cleavage of the precursor for optimal activity. A cysteine protease called IL-1β converting enzyme (ICE) is specific for IL-1β.[56,57] IL-1α appears to function predominately as an intracellular messenger but may be transported to the cell surface where it is a biologically active surface bound cytokine. IL-1β can be detected in the circulation and would appear to act as an extracellular mediator in its active form. It remains unclear how it is secreted. IL-1 is mainly produced by the monocytes/ macrophges but endothelial cells, keratinocytes, neutrophils and B lymphoctes also are able to synthesise IL-1. Though LPS is the main stimulus to its production other factors that induce its formation are TNF, GM-CSF and IL-1 itself.

Two types of IL-1 receptors (IL-1R) have been identified. The 80 kDa IL-1R type I which occurs on a variety of cell types including endothelial cells, T lymphocytes, fibroblast, keratinocytes and hepatocytes. The 68 kDa IL-1R type II have a more restricted cell expression on neutrophils, B lymphocytes and monocytes. Only the IL-1R type I is involved in signal transduction, binding of IL-1 with IL-1R type II inhibits its action. Soluble receptors (IL-1R type II) that bind and inhibit IL-1β, have been identified and are released from the surface of neutrophils in response to TNF or LPS.[58] A third member of the IL-1 family, IL-1 receptor antagonist (IL-1ra) competes with IL-1α and IL-1β for binding to the IL-1 receptors. It does not transduce signals and thus inhibits the action of

IL-1α and IL-1β. IL-1ra binds to both receptor types but with a 100% fold greater avidity for IL-1 type I.[59]

TNF-α and IL-1 have a multitude of effects but only those related to SIRS will be discussed here. They have a significant influence on endothelial cells. These include promoting procoagulant activity by enhanced expression of tissue factor, platelet activating factor (PAF), and von Willebrand factor.[60,61,62] TNF-α downregulates thrombomodulin expression. Thrombomodulin is required to accelerate the activation of protein c an anticoagulant.[63] Locally this procoagulant effect is useful to isolate infection but systemically promotes the development of disseminated intravascular coagulation. The expression of adhesion molecules on the endothelial cell surface is required to localise leucocytes at the site of infection or tissue injury. TNF-α and IL-1 enhance the de novo expression or upregulation of several adhesion molecules including Intracellular adhesion molecule-1 (ICAM-1), endothelial leucocyte adhesion molecule (ELAM) and vascular cell adhesion molecule (VCAM). Capillary leak is caused by structural changes within the endothelial cells and the loss of tight junction under the influence of TNF-α.[64,65,66] Complement production by the endothelial cells is upregulated by TNF-α and IL-1.[67] TNF-α also is involved in complement activation which may be one process in which TNF-α is involved in tissue injury.[68]

TNF-α and IL-1 stimulate the release of neutrophils from the bone marrow with a resultant leucocytosis. Neutrophil margination is initiated by TNF-α inducing the expression of cell surface adhesion molecules and therefore enhancing adhesion to the endothelium.[69,70] Transendothelial migration and augmented activation via increased superoxide production, phagocytosis, enzyme release and degranulation also occurs.[71]

Unlike TNF-α there is little evidence that IL-1 has an effect on neutrophil activity. The differentiation, maturation and activation of monocytes and macrophages is promoted by TNF-α and IL-1.[72,73] TNF-α and IL-1 induce cycloxygenase-2 (COX-2) and type-2 phospholipase-A_2 (PLA_2-II) gene expression which are important in the release and metabolism of eicosanoids.[74,75,76] The role of eicosanoids in the inflammatory response will be discussed later.

The mechanism of hypotension caused by TNF-α and Il-1 are not entirely clear but are probably due to the activation of nitric oxide (NO) and eicosanoids. Both TNF-α and IL-1 induce the expression of the nitric oxide synthase gene in vascular smooth muscle cells in rats and mice.[77,78]

Interleukin -6

IL-6 is a 26 kDa glycoprotein consisting of 212 amino acids and its gene has been mapped to the short arm of chromosome 7. The proposed tertiary structure is of 4 α-helices with interconnecting loops. IL-6 binds to a specific receptor to exert its function. This IL-6 receptor (IL-6Rα) is composed of a 80 kDa binding protein which has a short intracytoplasmic domain which does not transduce signal. This is associated with a 130 kDa glycoprotein signal transducer called gp130 which is also the signal transducer for other cytokines. This association forms a high affinity binding site for IL-6. Besides Monocytes/macrophages, most other cell types can synthesise IL-6 including granulocytes, T and B lymphocytes, endothelial cells, and smooth muscle cells. The main inducers of IL-6 production in SIRS are TNF-α, IL-1, and LPS, though other factors such as Prostaglandin-E_2 (PGE_2) and platelet derived growth factor may be involved. In

surgical patients IL-6 was detected in the circulation 2 to 4 hrs following the incision, peaking at 6 to 12 hrs.[79] Taniguchi et al measured the IL-6 levels in those patients who met the SIRS criteria over a four day period. They found that IL-6 levels remained elevated in non-survivors and gradually fell in survivors with the IL-6 being significantly higher in non-survivors after day 1. They found a strong correlation with poor outcome in those patients who had increased IL-6/IL-10 ratio.[80] Several previous and more recent studies have also shown that a poor prognosis in sepsis was related to elevated levels of IL-6.[81,82,83]

The exact role of IL-6 is difficult to determine as it has both pro-inflammatory and anti inflammatory properties. It appears relatively non-toxic as even high doses in non primates did not induce the development of shock, oedema or other toxic effects. A major role appears to be the stimulus of hepatocytes to produce acute phase proteins in conjunction with TNF-α and IL-1. Acute phase proteins are mostly anti-inflammatory in their effect. They scavenge free radicals and inhibit proteinases, however they do have some pro-inflammatory properties for example c-reactive protein activates complement. The enhanced synthesis of phospholiase A_2 is induced by IL-6.[84] This is involved in eicosanoid formation another of IL-6's pro-inflammatory properties. IL-6 alone does not cause neutrophilia or neutrophil activation except at doses >1000 ng/ml but may work synergistically with TNF-α in promoting phagocytosis and superoxide generation.[85,86] It stimulates differentiation of lymphocytes as well as antibody production and proliferation of B cells. The use of anti IL-6 monoclonal antibody attenuates the activation of coagulation in mice and chimpanzee's.[85,87] This suggests IL-6 may play a role in promoting coagulation by a currently unknown mechanism.

Interleukin -8

Il-8 is a member of structurally and functionally related cytokines called chemokines. There are two superfamilies of chemokines the so called C-C and C-X-C families. The C relates to the first two cysteine amino acid residues and the X to an intervening variable amino acid. IL-8 is a member of the C-X-C superfamily. It is initially synthesised as a 99 amino acid precursor protein with a 20 amino acid signalling sequence. The gene for C-X-C chemokines is on chromosome 4. The precursor protein is proteolytically cleaved into various N terminally processed forms which have minor biological property differences. The major form contains 72 amino acid residues. The effects of IL-8 are mediated by binding to specific receptors, CXCR1 and CXCR2 with resultant signal transduction. The predominant source of IL-8 are monocyte/macrophages, neutrophils and endothelial cells though a variety of other cells can also synthesise IL-8.

The major stimuli for IL-8 release in vitro are TNF-α, IL-1 and Endotoxin.[88] The use of NO synthase inhibitors has shown that TNF-α induced release of IL-8 from endothelial cells is NO dependant. Thrombin probably also promotes release of IL-8 from endothelial cells and monocytes it induces the release of other chemokines via activation of the thrombin receptor and indirectly by activation of platelets.[89] In addition the complement attack complex can stimulate endothelial cells to release IL-8. Reactive oxygen species are regulators of IL-8 gene expression and thus initiate its production in vitro.[90] The release of IL-8 has recently been observed to be biphasic in endotoxin stimulated human whole blood.[91] The initial phase is due to direct stimulation of the leukocytes and second phase due to stimulation from TNF and IL-1. This biphasic response has also been noted in vivo

with the second phase occurring 6 hours after a challenge with endotoxin or following administration of TNF.

IL-8 is a significant chemoattractant for neutrophils. It promotes neutrophil degranulation, respiratory burst, and arachidonate-5-lipoxygenase activation. It appears that IL-8 enhances neutrophil activation and transendothelial migration in vitro, as well as in certain circumstances inhibiting neutrophil adherance to activated endothelium.[92,93] This supports the concept that it plays a pivotal role in the regulation of neutrophil activation and migration.

Interleukin -10

IL-10 is an anti-inflammatory cytokine being a potent inhibitor of the synthesis and release of pro-inflammatory cytokines. It was originally characterised in T helper subset 2 (TH2) lymphocytes as a factor involved in the inhibition of T helper subset 1 (TH1) cytokine production. It subsequently has been shown to be produced by macrophages in response to LPS stimulation. Keratinocytes and associated cell lines also produce IL-10. Il-10s probable mode of action is the inhibition of the gene transcription of TNF-α, IL-1, Il-6, IL-8 and Interferonγ (IFNγ) in LPS stimulated neutrophils, macrophages or monocytes. This inhibition of gene transcription may in part due to IL-10s effect on NFκB, by inhibiting IKK activation and the DNA binding activity of NFκB. Tissue factor expression and the procoagulation activity of monocytes are inhibited by IL-10, as is the production of the eicosanoid, Prostaglandin E_2 (PGE_2)[94] via the down regulation of COX-2.[95]

Following experimental endotoxinaemia IL-10 reaches a peak 90-120 minutes after the stimulus. The administration of TNF-α to human volunteers induces a rise in IL-10 within 45 minutes and anti-TNF antibodies inhibit its release. It would appear that TNF is not the only stimulus for IL-10 as in a baboon sepsis model anti-TNF antibodies did not modulate IL-10 release.[96] Niho et al in human monocytes cultures found that PGE_2 significantly increased the expression of IL-10 mRNA.[97] Marchant et al found that IL-10 is elevated in patients who have either septic or a non-septic causes of circulatory shock. They also demonstrated a positive correlation with the intensity of the inflammatory response and the level of IL-10 in septic shock.[98] Patients with SIRS have also been shown to have elevated levels of IL-10 compared to controls. The level of IL-10 increased significantly in those patients with septic shock and is independently related to prognosis.[99,100]

The importance of IL-10 as an inhibitor of the pro-inflammatory cytokine response is demonstrated in a murine model of septic peritonitis. The pre-treatment with an anti-IL-10 monoclonal antibody resulted in increased circulating levels of plasma TNF-α and enhanced mortality.[101] The pre-treatment with low dose IL-10 in a murine model of endotoxinaemia showed a fall in LPS induced TNF-α release. In addition high dose IL-10 completely prevented the mortality associated with an LPS challenge.[102] The administration of IL-10 thirty minutes post LPS showed similar results.[103] Chernoff et al in a randomised controlled trial with varying dose of IL-10 given to healthy volunteers demonstrated that the ex-vivo production of TNF-α and IL-1β following endotoxin stimulation was inhibited (65%-95%) in a dose dependant manner.[104]

Acute phase proteins

C reactive protein (CRP) was the first acute phase protein to be described and was named following its ability to precipitate the somatic C-polysaccharide of *streptococcus pneumoniae*. CRP is a member of the ancient highly conserved pentraxin family of proteins and it is arranged in a cyclic homopentameric structure.

The acute phase response is a non-specific biochemical and physiological reaction to inflammation, infection, and tissue damage. They have been found to have an essential role in the inhibition of extracellular proteases, blood clotting, fibrinolysis, modulation of immune cell function, and the neutralization and clearance of harmful components from the circulation. Cytokines at the site of injury control the upregulation of acute phase protein synthesis with simultaneous suppression of normal export proteins chiefly in the hepatocytes. In addition to CRP other acute phase proteins include complement, transport proteins and proteinase inhibitors.[105] The predominant transcriptional control of CRP production by hepatocytes is by IL-6.[106] Other cytokines may also be involved in the control of the acute phase response for example IL-8.[107]

It has been proposed that CRP may be synthesised locally by lymphocytes at other sites.[108] The important role of CRP in innate immunity is largely due to its opsonizing abilities, its capability to activate human complement and to bind to immunoglobulin G receptors. CRP can bind phosphocholine largely present in bacterial membranes, cell membrane and lipoproteins, in addition CRP can recognize nuclear constituent in damaged cells. CRP can activate C3 convertase through the classical pathway but not C5 convertase resulting in generation of opsonic complement fragments. Interactions of CRP with Fc receptors leads

to the generation of proinflammatory cytokines by monocyte/macrophages while inhibiting neutrophil function.

Following stimulus of the hepatocyte the de novo synthesis of CRP is rapid with serum concentration reaching 5 mg/l by 6 hours and peaking by 48 to 72 hours. Scintigraphic studies of radio iodinated CRP have shown its half-life to be a constant 19 hours irrespective of health or disease.[109] The implication is that the only determinant of CRP plasma concentration is its synthesis rate and the intensity of the stimulus promoting this synthesis. It follows that the plasma CRP level falls quickly when the stimulus to synthesis is removed.

Lipid mediators in SIRS

Lipid mediators are lipid molecules that are formed from the phospholipid components of the cell membrane. The primary mediators are platelet-activating factor (PAF) and the eicosanoids, which are metabolised from arachidonic acid (AA). AA and PAF are released from the membrane by the enzyme PLA_2.

Phospholipase A_2 (PLA_2)

PLA_2 is found in both secretory and cystolic forms which are tightly regulated to prevent autodigestion of the cell. There are 2 forms of the secretory PLA_2 designated PLA_2-I and PLA_2-II. PLA_2-I is released from the pancreatic acinar cells and is involved in digestion and will not be discussed further. PLA_2-II is released from neutrophils, monocytes/macrophages and other inflammatory cells. Elevated levels of PLA_2-II have been demonstrated in sepsis, peritonitis, ischaemia reperfusion injury and trauma. In

addition increased serum levels of PLA_2-II have been identified as a marker of disease severity in patients with burns, SIRS, sepsis and MODS. [110,111,112,113] LPS and TNF-α stimulate the production of PLA_2-II in both animals and humans.[75,76,114]

The role of cytosolic PLA_2, a calcium dependant enzyme, in the inflammatory response is less clearly defined. PAF, LPS, TNF-α, and IL-1β stimulate its activity among other factors. Rodewald et al using human peripheral blood leucocytes stimulated with LPS ex-vivo showed an increase in $cPLA_2$ but not in PLA_2-II mRNA expression[115]. Levy et al using neutrophils from patients with SIRS and sepsis supported these results. They demonstrated an increase in $cPLA_2$ activity and protein expression. The level of expression was regulated at the mRNA level. These results suggest that $cPLA_2$ may play a major role in neutrophil function and thus in SIRS.[116]

The use of inhibitors of PLA_2 activity adds further weight to the role of this enzyme in the inflammatory response. A monoclonal antibody to PLA_2 has been shown to attenuate the pulmonary vascular leak in a rat model of gut ischaemia reperfusion. SB203347 a specific inhibitors of PLA_2-II, fully inhibits PLA_2-II activity in a human neutrophils homogenate and in addition the release of AA and PAF from intact human neutrophils was inhibited in a dose dependant manner. In a mouse model of endotoxin shock SB203347 prolonged their survival.[117]

Platelet Activating Factor (PAF)

Cells involved in the inflammatory response including, endothelial cells, macrophages, neutrophils and platelets, produce PAF rapidly. All these cells types also possess specific

membrane receptors for PAF, this allows the PAF released from the membrane to act in an autocrine and paracrine manner. The production of the inflammatory cytokines, TNF-α and IL-1β by monocytes/macrophages is enhanced by PAF. PAF also appears to have direct effects on the endothelium by stimulating increased neutrophil adherence and activation as well as potentiating the endothelial response to TNF-α. It may also play a role in increased vascular permeability as inhibition of PAF receptors in a human umbilical vein endothelial cells (HUVEC) culture reduces the permeability to albumin.[118]

The use of animal models has looked at the role of PAF in the inflammatory response. Following LPS infusion increased levels of PAF are demonstratable in serum as early as 1 minute and as late as 4 hours. The infusion of PAF in anaesthetised rats leads to a shock state that is similar to that seen in endotoxin-induced shock.[119] The use of a specific PAF antagonists though having variable results, generally show haemodynamic improvements and some survival benefit in endotoxic shock animal models.[120,121,122,123]

In humans, PAF levels have been found to be significantly elevated in septic shock and trauma patients compared to healthy controls.[111] A prospective randomised double blind placebo controlled trial with a PAF antagonist showed a reduction in 28-day mortality of 42% with the antagonist compared to 51% for the placebo group ($p=0.17$). This became significant when a subgroup with gram-negative sepsis was examined in comparison to the placebo group (57% v 33% $p=0.01$).[124] Thompson et al demonstrated that pre-treatment of PAF antagonist prior to LPS infusion in healthy volunteers showed significantly decreased symptoms in comparison to the control group.[125] These results show that PAF plays a role in the development of the inflammatory response especially to gram-negative sepsis.

Eicosanoids

Arachodonic Acid following its release from the membrane by PLA_2 is metabolised by the cyclooxygenase (COX) and lipooxygenase pathways.

LPS stimulated rat Kupffer cells have been shown to produce eicosanoids particularly in the first hour[126]. Slotman et al showed a rapid rise of thromboxane B_2 (TXB_2) with a slower rise in 6-keto-PGF_1 in a porcine model of graded bacteraemia.[127] Foex et al, in a porcine model of bacteraemic shock, showed significant increases in eicosanoid production but not in pigs undergoing 30 minutes of haemorrhagic shock.[128] Previous studies in animal models of sepsis have shown similar increase in eicosanoids. Multiply injured trauma patients who developed adult respiratory distress syndrome (ARDS) had significantly higher eicosanoid levels compared to those who developed sepsis or had an uncomplicated recovery.[129] Oettinger et al showed that increased levels of eicosanoids correlated with the severity of organ failure in patients with gram-negative sepsis. In humans with sepsis TXB_2 levels were found to be ten fold higher in non-survivors than in survivors. In contrast the use of PGE_2, PGI_2, and PGE_1 in animal models has been shown to improve survival in traumatic or hypovolaemic shock.

There are two isoforms of COX, COX-1 which is constituively expressed and COX-2 which is an inducible form. *In vitro*, COX-2 mRNA is induced by endotoxin and cytokines within endothelial cells, macrophages, pulmonary epithelial cells and fibroblasts. In rats exposed to LPS, COX-2 activity is up regulated with COX-1 activity being down regulated. In animal models the use of nonsteroidal anti-inflammatory drugs (NSAIDS), which are non-selective COX inhibitors have shown decreases in oxidant mediated tissue

injury, improved cardiovascular and pulmonary function with improved survival. A multicentre, randomised controlled trial in human sepsis patients has not shown the same improved survival except in a selected group of hypothermic septic patients.

The importance in SIRS of leukotrienes, Lipoxins and hydroxyeicosatetraenoic acids (HETEs), which are formed from AA via the lipoxygenase pathways, remains unclear. The use of 5' lipoxygenase and leukotriene receptor inhibitors in animal models of endotoxinaemia does suggest a possible role in SIRS for these lipoxygenase products. There is no evidence currently which shows a role for lipoxins and HETEs in SIRS.

Nitric Oxide (NO)

Nitric oxide is formed during the conversion of L-arginine to citrulline by nitric oxide synthase (NOS). There are 3 distinct isoforms of NOS that have been identified. nNOS predominately found in neuronal tissue and eNOS first identified in vascular endothelial cells. These two isoforms are also referred to collectively as cNOS as they are constitutively expressed. The third isoform is iNOS; this is inducible in several cell types, including macrophages, PMNs, kupffer cells, vascular endothelial and smooth muscle cells. The expression of iNOS is induced by endotoxin and proinflammatory cytokines including interferon-γ (IFNγ), TNF-α and IL-1. The anti-inflammatory cytokine IL-10 has been shown to inhibit iNOS mRNA expression.[130] Though eNOS is constitutively expressed it may be regulated in endothelial cells with an increase in mRNA transcription under the influence of shear stress and during hypoxia.[131]

Calmodulin and four cofactors (flavin adenine dinucleotide (FAD), flavin mononucleotide, tetrahydrobiopterin (BH_4), and heme) are necessary requirements for the activity of all NOS isoforms. nNOS and eNOS calmodulin binding is calcium dependant requiring high intracellular levels of calcium. Several receptor agonists cause a transient rise in intracellular calcium thus activating cNOS to produce NO, this response is short lived. The binding of calmodulin to iNOS is calcium independent requiring only basal levels of calcium. When iNOS is induced the only limiting factor to its production of NO is the availability of substrate and co-factors.

The major physiological role of NO is its activation of soluble guanylate cyclase resulting in the formation of cyclic guanosine monophosphate (cGMP) and the activation of a downstream signalling pathway. The resultant decrease in free intracellular calcium causes a multitude of effects, including relaxation of vascular and nonvascular smooth muscle, inhibition of both platelet aggregation and leucocyte adhesion to the endothelium.

NO has also been shown to regulate NFκB activity via concentration dependant effects. Low concentrations of NO enhance TNF-α induced NFκB DNA binding activity probably by increasing the activity of IKK[132]. The presence of NFκB binding sites in the iNOS gene promoter region suggests it is important in the transcription of iNOS. Inhibition of NFκB has been shown to reduce iNOS expression and thus NO production. High concentrations of NO inhibit NFκB DNA binding with a resultant decrease in iNOS expression[133] and the transcriptional activity of NFκBs target genes.[132,134] NO would appear to have a proinflammatory effect at low concentrations by stimulating NFκB activity. In contrast, NO opposes the expression of proinflammatory genes by inhibiting NFκB activation at higher concentrations.

The importance of NO in sepsis has not been fully determined. Small animal models of endotoxaemia have shown a significant rise in NO production and iNOS expression compared to controls.[135] The response in small animal models is less marked using caecal ligation and perforation (CLP), which induces peritonitis and probably more closely mimics clinical reality.[136] In humans, several small studies have demonstrated higher levels of NO in septic patients compared to non-septic patients.[135] Tissue debrided from areas of cellulitis in septic patients have greater iNOS mRNA expression than tissue taken away from the site of infection. It has also been shown in the neutrophils of septic patients that iNOS mRNA expression is increased.[137] The use of non-selective NOS inhibitors in endotoxic shock animal models though reversing hypotension had many adverse effects including increasing microvascular permeability, reduced cardiac output, decreased splanchnic blood flow and increased mortality. Bacteraemic animal models using NOS inhibitors have shown variable and opposing results which appear to be more dependant on experimental conditions. Non-selective inhibitors in human septic patients have shown a decrease in hypotension but again with a decreased cardiac output and increased pulmonary vascular resistance.[138]

The use of selective iNOS inhibitors in contrast have shown to have beneficial effects on the changes caused by LPS challenge in small animal models. These effects include preventing or reversing hypotension, cardiac output depression, microvascular permeability, endothelial dysfunction and end organ injury. They also showed either no change in or a decrease in mortality never an increase[139]. There are currently no published selective iNOS studies in humans.

It would appear from these report that NO produced by iNOS is an important factor in sepsis. eNOS inhibition on the other hand exacerbates the endothelial dysfunction caused by sepsis. The importance of eNOS has recently been demonstrated by Yamashita et al, using transgenic mice overexpressing eNOS there was a reduction in LPS induced hypotension, lung injury, adhesion molecule expression and increased survival.[140]

Conclusion

This chapter has reviewed the importance of SIRS in critical surgical illness. It has also looked at the role of intracellular signalling and activation of mediators in the development of this inflammatory response. The next chapter will look at the importance of oxidant stress in enhancing the inflammatory response.

Chapter 2 Ischaemia Reperfusion Injury

Introduction

The consequences of the loss of the blood supply to tissues (ischaemia) have long been recognised. Ischaemia can cause tissue necrosis and organ dysfunction due to the oxygen requirements of the tissue not being met (hypoxia). The restoration of the blood supply to prevent this sequela has long been the aim of physicians and surgeons. However, the reperfusion of tissue has also been acknowledged to cause further cellular injury, with resultant effects on organ function. This process is described as ischaemia and reperfusion injury (I/R). The microcirculation of organs in particular the endothelial cell lining seem most vulnerable to the I/R injury though parenchymal cells may also be affected. As such it is a potentially serious problem during thrombolytic therapy, coronary angioplasty, coronary artery bypass grafting, organ transplantation, following aortic cross clamping and limb revascularisation. The biochemical and molecular changes following I/R injury are consistent with an acute inflammatory response. If the response locally is severe then it may cause distant organ injury through a systemic inflammatory response.

Biochemistry and Pathophysiology of I/R injury

The role of ischaemia, hypoxia and reperfusion

The production of high-energy phosphate compounds by oxidative phosphorylation in the mitochondria is dependant on oxygen. Adenosine triphosphate (ATP) is required for the function of the membrane ionic pumps; in periods of ischaemia, ATP is depleted allowing intracellular accumulation of calcium (Ca^{2+}) [141]and sodium (Na^{2+}). The intracellular

accumulation of Ca^{2+} and Na^{2+} promote cellular swelling and this with intracellular acidosis secondary to lactic acid accumulation can lead to cell death if the period of ischaemia is not reversed. The period of ischaemia that leads to cell death is dependant on the cell type. Neurons, cardiomyoctes and renal tubular cells are sensitive to ischaemia and as such unable to survive prolonged hypoxia. However, endothelial cells(EC)[142]and skeletal muscle[143] are able to adapt to long-standing ischaemia.

Cyclic Nucleotides

Many cellular homeostatic functions are maintained by intracellular signalling pathways. Cell surface receptors when stimulated, activate adenyl cyclase in the cell membrane via a G-protein intermediate. The adenyl cyclase forms the cyclic nucleotides, 3',5'- adenosine monophosphate (cyclic AMP or cAMP) which is a secondary messanger. The function of these molecules include the phosphorylation of proteins via cAMP dependant protein kinases that change the functional properties of the proteins. The binding of cAMP to promotor regions of certain genes activates gene transcription. The secondary messenger 3',5'- guanosine monophosphate (cGMP) has many overlapping properties but is formed by the action of guanylate cyclase binding nitric oxide (NO) or carbon monoxide (CO).

These pathways are interrupted during ischaemic conditions. There is decreased synthesis of cAMP secondary to a decrease in adenyl cyclase activity in ECs. In vascular smooth muscle cells (VSMC) the adenyl cyclase activity is maintained but there is increased catabolism of cAMP. The increased catabolism is due to the amplified activity of type III and IV phosphodiesterase's . Under hypoxic conditions the constitutive NO production is suppressed with resultant decrease in cGMP. The decreased cellular concentration of the

cyclic nucleotides during hypoxia has multiple effects on the function of the cell. This is particularily relevant to the EC properties and its influence on vascular function. The effects on barrier function, procoagulation, leucocyte activation, cytokine and adhesion molecule expression and vasomotor function will be discussed in the following paragraphs.

Free Radicals

It is now well recognised that free radicals play an important role in I/R injury. Free radicals have one or more unpaired electrons in their atomic outer orbit. They are therefore highly reactive with other molecules in order to pair their electrons and thus gain a more stable state. They attack lipids most importantly, but also proteins and nucleic acids. The effect on lipids is to cause lipid peroxidation with a resultant alteration in the membranes permeability and fluidity, which may result in cell lysis. In addition, free radical production is propagated as the reactive electron passes from molecule to molecule causing further injury. Denaturation of structural and enzymatic proteins can be caused by free radical activity. Crosslinking, hydroxylation or scission (cutting) of the nucleic acids in deoxyribonucleic acid (DNA) may occur with possible lethal or mutagenic results.[144]

In biological systems, several free radicals are derived from molecular oxygen, which due to its two unpaired electrons is itself a free radical. Superoxide ($O_2^{\bullet-}$) and hydroxyl radical ($OH^{\bullet}$), are the most biologically relevant. Other molecules that are not free radicals but have oxidising effects are also present in biological systems these include hydrogen peroxide (H_2O_2). This group of molecules are also referred to as reactive oxygen species (ROS). Originally, it was believed that much of the toxic effects of $O_2^{\bullet-}$ were due to its

direct effects on cellular components. It has been increasingly noted that secondary formation of other more reactive free radicals from $O_2^{\bullet-}$ plays a major role in this toxicity. Dismutation of superoxide occurs spontaneously (rate constant of 8×10^4 M^{-1} s^{-1}) in an aqueous media forming H_2O_2 and oxygen, this reaction is further accelerated (rate constant of 2×10^9 M^{-1} s^-1) by superoxide dismutase (SOD). The H_2O_2 can then be enzymatically converted to water and oxygen by catalase in a two stage reaction. In the presence of transition metal ions, mainly the ferrous (Fe^{2+}) ion, H_2O_2 reacts with the Fe^{2+} in the iron catalysed Fenton reaction to form the hydroxyl radical (see table 3). The OH^- radical is highly reactive and is probably the major mediator of tissue injury. Unlike superoxide, H_2O_2 is a more stable molecule and is also able to diffuse freely across biological membranes, and thus may indirectly cause injury at a site distant from its production.

Superoxide has also been shown to react with nitric oxide ($NO^{\bullet}$), an endogenously produced free radical, which has an important role in maintaining vascular tone. The oxidant peroxynitrite ($ONOO^-$) is formed from this reaction with a rate constant of 6.7×10^9 M^{-1} s^{-1}. [145] Therefore from the rate constants it can be seen that if $NO^{\bullet}$ levels are increased and approach that of SOD it is able to compete for superoxide and so potentially $ONOO^-$ could be produced in biologically significant amounts. On protonation, peroxynitrite decomposes via peroxynitrous acid into OH^- and nitric dioxide ($NO_2^{\bullet}$)(see table 3).[146] Peroxynitrite is not a free radical but like nitric oxide is a member of a group of nitrogen containing oxidising molecules called reactive nitrogen species (RNS).

Table 3 Free radical formation reactions

$O_2 + e \rightarrow O_2^{\bullet-}$
$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$
$Fe^{3+} + O_2^{\bullet-} \longrightarrow O_2 + Fe^{2+}$
$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$
$NO^{\bullet} + O_2^{\bullet-} \rightarrow ONOO^- + H^+ \rightarrow ONOOH \rightarrow OH^{\bullet} + NO_2^{\bullet}$

Several free radicals have been detected during I/R injury. The use of paramagnetic resonance and spin trapping have confirmed the production of O_2^- and the OH^-, in reoxygenated endothelial cells.[147,148] Further evidence implicating free radicals in I/R injury is the identification of lipid peroxidation products following reperfusion. The use of antioxidants, such as superoxide dismutase has been shown to protect reperfused organs and cells. Wang et al using isolated rat heart model have demonstrated the increased production of O_2^- and $NO^{\cdot}$ following reperfusion. Chemilumenesce measurement showed a similar increase in $ONOO^-$ which was blocked by a nitric oxide synthase (NOS) inhibitor or SOD.[149]

There are several probable sources of free radical production during I/R injury. The enzyme Xanthine Oxidoreductase is the most extensively researched in its relationship to I/R injury. Increasingly more interest has been in examining the role of NADPH oxidase in the endothelium as well as its recognized role in activated leucocytes.

Xanthine Oxidoreductase (XOR)

The majority of XOR activity in humans is found in the liver and intestine with low levels of activity in other tissues. In addition, immunohistochemical staining has identified XOR in the capillary endothelium of human skeletal muscle. Xanthine dehydrogenase (XDH, D-form) comprises approximately 80% of the enzyme XOR in endothelial cells[150] and in most tissue types.[151] XOR metabolises the conversion of hypoxanthine to uric acid. XOR is composed of two identical subunits of 150 kDa each. Each subunit contain redox sites comprised of molybdopterin cofactor, flavin adenine dinucleotide (FAD) cofactor and two spectroscopically distinct iron-sulphur centres. Hypoxathine oxidation occurs at the molydopterin cofactor site with rapid electron transfer to the other centres.[152] NAD^+ is the electron acceptor for this reaction being reduced to NADH at the FAD cofactor site. Xanthine oxidase (XO, O-form) uses oxygen as the electron acceptor again at the FAD cofactor and comprises approximately 20% of the cellular XOR. In fresh rat heart, 15% is in an irreversible form and 5% is reversible to XDH.[153]

During ischaemia XDH is partially converted to XO. The mechanism is most likely the intracellular accumulation of Ca^{2+} promoting the conversion of XDH to XO by calcium activated calmodulin regulated proteases.[154] The inhibition of calmodulin by trifluoperazine prevents this irreversible proteolytic conversion of XDH to XO.[155,156] This proteolysis however has been shown to be slow and effects only a small proportion of the total enzyme.[157] In rat intestine the rate of conversion was shown to be 13% per hour of ischaemia.[158] *In vitro* studies of rat liver and kidney, as well as *ex vivo* perfused canine pancreas preparations suggest a role for the reversible oxidation of enzyme bound sulphydrl groups to disulphides in the transformation of XDH to XO.[159,160]

Crystallography of bovine milk XOR suggests that cleavage of a surface loop on XDH causes structural changes in a further loop which partially restricts access of NAD^+ to the FAD cofactor site during proteolytic conversion of XDH to XO.[152] XDH also has a lower reduction potential of the flavin FADH/$FADH_2$ compared to XO and such preferentially uses NAD^+ rather than oxygen.[161]

The catabolism of ATP through AMP to adenosine continues during hypoxia. Adenosine rapidly diffuses extracellularly where it's degraded to hypoxanthine. However the metabolism of hypoxanthine to uric acid by XDH is inhibited during ischaemia due to accumulation of NADH. Additionally the available XO requires O_2 to convert hypoxanthine, it therefore accumulates in the ischaemic tissue. This has little pathological significance during ischaemia.

In the prescence of oxygen XO metabolises hypoxanthine, the reaction producing O_2^- and hydrogen peroxide (H_2O_2). Due to the production of ROS, XO as been hypothesised as a major contributor to I/R injury following reoxygenation of the tissue.[162] This hypothesis was initially supported by many studies which demonstrated that administration of the xanthine oxidase inhibitors allopurinol, tungsten or oxypurinol reduced cell injury and infarct size.[163,164,165] The problem with this hypothesis is that the concentration of XO in the tissue is low and the rate of conversion during ischaemia has been shown to be slow. Nishino et al proposed that the accumulation of NADH in the ischaemic cell inhibits the activity of XDH. XO is then able to oxidise more hypoxanthine than normal on reperfusion.[166] Xia et al using perfused rat heart exposed to 30 minutes global ischaemia and variable periods of reperfusion showed an increase in XO activity from 7% to 11% of the total XOR activity. On reperfusion free radicals were generated only over the first five

minutes with the XO activity still being elevated at 45 minutes. The hypoxanthine levels prior to ischaemia were undetectable becoming elevated post ischaemia but rapidly falling over the next five minutes again becoming undetectable. These results suggest that high concentrations of substrate promote XO activity and associated ROS production.[167] XDH has also been shown to be able to use oxygen as an electron acceptor and produce O_2^- and H_2O_2. *In vitro,* the presence of NAD^+ inhibits this reaction and it only occurs with concentrations of NAD^+ below its K_m (the concentration of substrate that gives half-maximal activity) value (7μM)[161]. The relevance of this activity *in vivo* is unclear. XDH has been shown by Sanders et al to have intrinsic NADH oxidase activity, the oxidation of NADH with molecular oxygen results in O_2^- generation[168]. The maximal rate of O_2^- generation is 0.2 μmol/min/mg, which is four times greater than that for XO (0.05 μmol/min/mg) in the presence of hypoxanthine. This is consistent with an alternative mechanism for ROS production, as ischaemia leads to NADH accumulation in the cell.

XOR has also been found to be circulating at low levels in human plasma. Due to the presence of serum protease the circulating XOR is probably all in the oxidase form.[169] Tan et al in patients undergoing aortic cross clamping reported significantly increased levels of circulating XOR after seven minutes of reperfusion.[170] Similar results had previously been demonstrated in a rabbit model of aortic occlusion with subsequent reperfusion.[171] Ischaemia and reperfusion following the use of a tourniquet in humans have also shown significant increase in plasma XOR compared to the contralateral limb as a control.[172,173] This studies as well as others have led to the proposal that circulating XOR may be involved in remote organ injury. Yokoyama et al in a rat perfusate hepatic model of ischaemia and reperfusion showed a significant increase in XOR. This level of XOR in the presence of sufficient substrate caused severe injury to vascular endothelium *in vitro*[174].

Terada et al in rats treated with allopurinol or tungsten enriched diets showed a significantly decreased level of plasma XOR compared to sham controls after intestinal reperfusion. This resulted in lower pulmonary myeloperoxidase activity, neutrophil chemotaxis and decreased lung permeability in comparison to the controls.[175] The surface of vascular endothelial cells express surface glycoaminoglycans, which have been reported to bind circulating XOR.[176] Houston et al have demonstrated that XOR not only binds to endothelial cell it also retains its catalytic activity.[177] XOR could then be concentrated at sites distant to its production and thus be able to cause free radical mediated injury in organs with a low level of constitutive XOR.

NADPH oxidase

These are a group of plasma membrane associated enzymes found on leukocytes, endothelial cell, fibroblasts and vascular smooth muscle cells (VSMC). NADPH acts as the donor in the one electron reduction of oxygen to $O_2^{\cdot}$. In neutrophils the production of superoxide is used to produce other reactive oxidants including oxidised halogens. These are required by the neutrophils in killing of microrganisms. The reactive oxidants also can cause local tissue damage and the activation of NADPH oxidase has to be regulated so they are generated only when required.

Within vascular cells NADPH oxidase is a major source of superoxide but the estimated rate of production is one third of that found in neutrophils. In contrast to neutrophils there appears to be a degree of constitutive activity in vascular cells. Following stimulation the rate activation in EC, VSMC and fibroblast is the order of minutes to hours in comparison to the instantaneous release in neutrophils. In vascular cells NADPH oxidase is activated

by several factors including angiotensin II, thrombin, platlet derived growth factor, TNF-α and IL-1. Reoxygenation has also been demonstrated to activate NADPH oxidase.

Fisher et al in a perfused rat lung ischaemia model using PR-39 (proline /arginine rich 39 amino acid peptide that inhibits NADPH oxidase assembly) showed a concentration dependant inhibition of ROS. Pretreatment with allopurinol a XOR inhibitor had no effect on ROS generation. They also demonstrated no production of ROS with lung ischaemia in NADPH oxidase knock out mice. Wild type mice showed a similar ROS profile as rat lung. These results are consistent with the activation of NADPH oxidase being the source of ROS generation. Using fluorescence image the NADPH oxidase was localised to the endothelial cells.[178] Rac1 Guanidine triphosphatase (GTPase) regulates the production of ROS by NADPH oxidase in phagocytic and non phagocytic cells. Ozaki et al in an *in vivo* mouse model of ischaemia reperfusion completely suppressed the production of ROS, lipid peroxides, activation of NFκB and reduced liver necrosis using a dominant negative Rac1 (N17Rac1).[179] *In vitro* adenoviral gene transfer of N17Rac1 to VSMC, EC, fibroblast and ventricular myoctes inhibits the generation of ROS in a hypoxia reoxygenation model.[180] These reports show that Rac1 and its regulation of NADPH oxidase are probably a crucial step in the production of ROS in ischaemia reperfusion injury.

Oxidant stress and intracellular signalling

A large number of cellular processes are controlled by the interaction of a network of proteins that transmit extracellular signals to their intracellular target. There is increasing evidence that ROS and RNS may play an important physiological role in this intracellular

signalling. During I/R, the increased production of ROS/RNS may have pathophysiological consequences leading to cell injury in addition to the direct cytotoxic effects of these molecules. Several pathways have been identified that are redox sensitive and have a role in activation of transcription factors, which are involved in promoting apoptosis and cytokine generation.

The MAPK pathway has been identified as one such target of ROS, the mechanism and the precise molecular target are currently unclear. Oxidant stress activated MAPK enhances the phosphorylation and transcription of c-jun proto-oncogene. The c-jun N-terminal kinases (JNK) are also oxidant stress sensitive and are involved in the activation of c-jun. AP-1 has shown to be activated by H_2O_2 and superoxide in several cell lines.[181] Following reoxygenation, AP-1 has shown increased binding activity and subsequent induction of cellular apoptosis.[182] It is unclear whether this is secondary to MAPK and JNK activation or an independent mechanism. Oxidant stress stimulated MAPK pathway also may have a role in endothelial permeability and adhesion molecule regulation. This is discussed below in the relevant sections.

There has been increasing evidence that has suggested a role for ROS in the activation of NFκB. Schreck et al showed that H_2O_2 could induce the expression and replication of HIV-1 in a human T-cell line by a NFκB dependant mechanism. They proposed that ROS directly or indirectly caused the release of IκB from NFκB and therefore were acting as secondary messengers.[183] Schmidt et al using a cell line that overexpressed catalase inhibited NFκB activation. The inhibition of catalase by aminotriazol restored NFκB activity. In those cells that overexpressed SOD there was increased activation of NFκB due to in increased levels of H_2O_2 from superoxide.[184] However other cell lines have been

relatively insensitive to H_2O_2 such as endothelial[185], T lymphocte and monocytic cell lines.[186] Further evidence for the role of ROS has come from the use of antioxidants to inhibit NFκB activation. Schreck et al prevented NFκB activation by H_2O_2 by using N-acetylcysteine[183] an antioxidant and free radical scavenger.[187] Pyrolidine dithiocarbamate (PDTC) an antioxidant and metal chelator inhibits NFκB activation in several cell lines.[188,189,190] These studies were the strongest evidence to date that ROS played a central role in NFκB activation. However, Bowie et al using a transformed human endothelial cell line showed that other antioxidants failed to inhibit NFκB activation. However, other metal chelators showed a similar response to that of PDTC suggesting that metal chelation was the mode of action in its inhibition of NFκB activation. They further proposed that iron catalysed lipid peroxidation rather ROS generation was important in NFκB activation in these cells.[185] At the current time the evidence remains contradictory for the role of ROS in NFκB activation but if proven it would play a central position in the development of ischaemia reperfusion injury.

Barrier Function

ECs form a selectively permeable barrier which allows solutes to pass through depending on their size. Large molecules however require a transport mechanism to pass through the endothelium. Leucocytes are able to transmigrate through the tight junctions with no apparent change to endothelial permeability. Hypoxia however causes changes to the permeability of the endothelium with it no longer being selective. In vitro the EC actin based cytoskeleton on exposure to hypoxia shows structural changes which causes the appearance of intercellular gaps (0.5 to 1 micron) between the ECs.[191] This allows a leakage of solutes, proteins and leucocytes into the extracellular tissue. This is clinically

recognised as tissue oedema. The use of a cAMP analogue prevents these changes in the endothelium during hypoxia. Inauen et al using cultured bovine pulmonary artery endothelial cells exposed to hypoxia/reoxygenation demonstrated an increase in permeability over that found with hypoxia. This increased permeability was prevented by oxypurinol and superoxide dismutase suggesting that the changes are due to XOR free radical induced endothelial cell dysfunction.[192]

Procoagulation

The increased permeability of the endothelium allows exposure of subendothelial tissue factor and collagen which promotes the activation of the coagulation system. Tissue factor(TF) promotes fibrin deposition. Mice exposed to hypoxia showed increased TF expression compared to mice in a normoxic enviroment. Mononuclear phagocytes(MP) recruited into hypoxic mice lung tissue showed increased TF expression compared to mice deficient in MP. The blocking of TF with goat anti-rabbit TF IgG showed a decrease in pulmonary fibrin deposition and cross linking during hypoxia. It appears therefore that hypoxia activated MP promote thrombosis by increased TF expression.[193]

The exposure of human umbilical vein ECs (HUVEC) in vitro to hypoxic conditions stimulated the release of von Willebrand factor (vWF) by exocytosis of the intracellular Weibel-Palade bodies.[194] vWF factor promotes the activation and adhesion of platelet to the endothelium. Prolonged hypoxia downregulates thrombomodulin expression, which is required to accelerate the activation of protein c an anticoagulant.[191] EC cultures under hypoxic conditions directly activated Factor X but not other coagulation factors. This may

be by a membrane associated Factor X activator requiring calcium to convert Factor X to Xa.[191]

Plasminogen-activator inhibitor N1 (PAI-1) is a serine protease inhibitor that prevents the conversion of plasminogen to plasmin by plasminogen activators. Plasmin is a prime mediator in fibrinolysis. In a murine model[195] and human hepatoma cell line[196], hypoxia promotes gene transcription of PAI-1. The major source of the PAI-1 were mononuclear phagocytes as identified by immunohistochemical staining[195], though it is also found in other cells including endothelial, liver, hepatocytes, platelets and smooth muscle cells.[197] Hypoxia suppresses gene expression for tissue plasminogen activator (tPA) and urokinase type plasminogen activator (uPA) which also inhibit fibrinolysis. In a rat model of acute peripheral ischaemia by infrarenal aortic cross clamping there was an initial transient rise in tPA with a fall in PAI-1. However in well perfused tissue sites mRNA expression for PAI-1 and also tPA were elevated between 60 and 120 minutes suggesting a humoral mediator.[198]

Leucocyte activation and cellular adhesion molecules

The accumulation of leucocytes especially polymorphonuclear neutrophils (PMNs) within the tissue plays an important role in the host defence mechanism and is a major contributor to inflammatory tissue injury. The passage of PMNs into tissue follows a three-step pathway, which are rolling, adherence and transendothelial migration (TEM).
The reversible binding of PMNs via transmembrane glycoprotein adhesive molecules called selectins allows for the capture of PMNs from the blood flow and subsequent

rolling along the endothelial surface. Selectins are found on both the PMNs (L-selectin) and EC (E-selectin and P-selectin).

L-selectin is expressed with the highest concentration on newly released PMNs from the bone marrow with shedding of L-selectin over time in part by metalloproteinase activity, proteolysis and the effect of rolling. There currently is no evidence that following shedding of L-selectin that it can be replaced. Under normal conditions L-selectin will intermittently form a very loose association with the endothelium via a endothelial expressed ligand which is constitutively expressed.

The Weibel-Palade bodies within the EC are a rich source of preformed P-selectin. P-selectin is rapidly mobilised to the cell membrane following exposure to inflammatory mediators (complement, cytokines, reactive oxygen species (ROS)). Pinsky et al using cultured HUVECs showed increased expression of P-selectin by hypoxia alone.[194] The binding of P-selectin to the PMN is via P-selectin glycoprotein ligand-1 (PSGL-1). PSGL-1 is also expressed on monocytes and platelets and are able to bind to activated ECs. The PSGL-1/ P-selectin interaction in comparison to L-selectin is much longer lived, causes slowing of PMN rolling and finally tethering of the PMN to the EC. This process continues to be reversible if no other adhesive interactions come into play. E-selectin is not constitutively expressed or stored and therefore needs gene transcription for it to be expressed. Following exposure to inflammatory mediators peak activity and expression occurs in vitro at 4 to 6 hours and thus may maintain PMN rolling following down regulation of P-selectin. E-selectin binds to E-selectin ligand-1 (ESL-1) on the PMN membrane.

Adherence of the PMN to the EC is via interaction of integrins and intercellular adhesion molecules. Integrins, transmembrane glycoproteins are found on PMN and other haemopoetic cells that are involved in cell-cell and cell-extracellular matrix adhesion. Integrins are composed of two subunits one α (16 known subunits) and one β (8 known subunits). Two integrins are involved in adherence of PMNs to the ECs these are CD11b/CD18 ($\alpha_M\beta_2$ or macrophage antigen-1(Mac-1)) and CD11a/CD18 ($\alpha_L\beta_2$ or lymphocyte associated function antigen-1(LFA-1)). CD11b/CD18 is stored preformed within the PMN and can be mobilised rapidly to the cell surface following stimulation of the PMN by lipopolysaccharide or TNF-α. Baudry et al studied leucocyte rolling and adherence in the venules of rat cremaster under systemic and local hypoxic conditions. Antibody to CD11/CD18 prevented hypoxia induced leucocyte adhesion.[199] The effects were seen within one hour of hypoxia. In vitro incubation of PMNs in hypoxic conditions showed an increase in CD11b/CD18 expression.[200]

Intracellular adhesion molecule-1 (ICAM-1) is an endothelial cell expressed ligand for CD11b/CD18. It has a low constitutive expression but following stimulation by inflammatory cytokines, expression is markedly upregulated. CD11a/CD18 can bind to ICAM-1 but has increased affinity for Intracellular adhesion molecule-2 (ICAM-2), which also can bind CD11b/CD18 with low affinity. Arnould et al demonstrated that in the presence of anti-ICAM-1 antibodies using HUVEC (*in vitro*) and human umbilical vein (*ex vivo*) the hypoxia induced adherence of PMNs was inhibited by 61% and 77% respectively.[201] Zund et al demonstrated that ICAM-1 expression was not increased by hypoxia however in the presence of LPS, hypoxia increases ICAM-1 expression. This is in line with previous studies that have shown that hypoxia alone does not induce ICAM-1.[202] It remains unclear how adherence to the ECs is mediated under hypoxic conditions as

there is no upregulation of ICAM-1 or ₪2. It may be due to an increased affinity of the PMN integrins to the constitutively expressed ICAM-1 and -2. A further possibiltity is a hypoxia activated adhesion molecule (hypoxia associated ligand (HAL) 1/13), which in vitro significantly increases PMN adhesion following hypoxia. The increased adhesion being blocked by a monoclonal antibody (HAL 1/13 mAb).[203,204] The mechanism of the HAL 1/13 ligand binding to the PMN appears to be integrin independent as the use of anti-leucocyte integrin mAb is additive to that of HAL 1/13 mAb[203].

Ichikawa et al in a HUVEC model of anoxia and reoxygenation demonstrated two phases of neutrophils adhesion. The first phase peaking at 30 minutes of reoxygenation, was inhibited by the use of monoclonal anti ICAM-1 and anti P-selectin antibodies. Oxypurinol and Catalase also attenuated this first phase of adhesion suggesting a role for XOR and ROS. The second phase of neutrophils adhesion peaking at 240 minutes of reoxygenation was inhibited by E-Selectin antibodies. ds-oligonucleotides contain multiple copies of the DNA binding sites for NFκB and AP-1 there presence in the cell prevent the binding of NFκB and AP-1 to the promoter region of there target genes. The pre-treatment of the cells with ds-oligonucleotides significantly attenuated the phase 2 adhesion, suggesting a transcription dependant expression of adhesion molecules. The use of WEB2086 a specific antagonist of PAF prevented neutrophils adherence in both phases.[205] Yoshida et al had also demonstrated that catalase and WEB2086 had inhibited neutrophil adhesion to HUVECs.[206] Further evidence for the role of ROS in recruitment of neutrophils has been reported by Gasic et al using a cultured venous endothelium model as well as perfused canine artery and vein. Those vessels and cell cultures exposed to H_2O_2 showed a significant concentration dependant transient increase in neutrophil adherence to and migration through the vascular endothelium. This adherence was inhibited by catalase,

dimethylurea, a hydroxyl radical scavenger and WEB2086. These results suggest that the neutrophil adherence induced by H_2O_2 is PAF dependent. The use of ICAM-1 and CD18 monoclonal antibodies similarly prevented neutrophil adherence.[207]

The passage of the PMNs through the endothelium occurs at the junctions of the ECs. Platelet-endothelial cell adhesion molecule-1 (PECAM-1) appears to have an important role in localising the PMNs at the sites for TEM as it is concentrated at the EC intracellular junction. PECAM-1 is also expressed uniformly on the surface of PMNs (and platelets) and as such it acts has its own ligand. In a HUVEC model, antibodies to PECAM-1 do not prevent adhesion of PMNs but significantly inhibit TEM by up to 70%. Unlike TNF-α, hypoxia doesn't increase EC expression of PECAM-1 or increase its localisation at the intracellular junction.[208]

The *in vitro* incubation of PMNs with hypoxic HUVEC results in the activation of the PMNs characterised by a rise in intracellular calcium, release of superoxide, and leukotriene B4 production. The activation appears to be a result of adhesion to the HUVEC, as the medium from hypoxic HUVEC did not activate PMNs. In addition blocking of adhesion by anti-ICAM and oleic acid protected the HUVEC from cytotoxic damage. The use of superoxide dismutase and catalase significantly reduced cytotoxic damage to the hypoxic HUVEC (94% inhibition) unlike the use of anti-proteases. These results suggest that free radicals produced by PMNs are important in cell injury.[209]

Cytokines and other mediators

The effects of ischaemia and reperfusion in many ways are similar to the host response to inflammation that is leucocyte adherence and activation, procoagulation and increased vascular permeability. These similarities have resulted in the focusing of research on cytokine production by hypoxic cells.

Ertel et al using a mouse hypoxaemic model showed a significant increase in the plasma level of TNF-α compared to normoxic controls. They also demonstrated that in macrophages harvested from the mice and incubated *ex vivo* with LPS had significantly elevated TNF-α production in the hypoxic model compared to the normoxic control. These results would suggest that hypoxia can induce cytokine release in addition to priming macrophages. Similar results were obtained for IL-6[210]. Colleti et al in a rat model of hepatic I/R demonstrated TNF-α release following 45 to 90 minutes ischaemia and reperfusion. They also showed in the same model an increase in pulmonary capillary permeability, which was attenuated by pretreating with anti-TNF[211]. Ischaemia and reperfusion of a rat hind limb resulted in significantly increased levels of TNF-α, IL-1 and IL-6 in the plasma. There was also an increase in vascular permeability both locally and within the lung. The use of polyclonal TNF-α and IL-1 antibodies inhibited this increase in vascular permeability.[212] Wakai et al in patients undergoing arthroscopy of the knee demonstrated significantly increased plasma levels of TNF-α and IL-1β following the use of a tourniquet with subsequent reperfusion compared to those without.[213]

Endothelial cells exposed to hypoxic condition in vitro showed increased activity of IL-1α with elevated levels of IL-1α mRNA. There was also an associated expression of

Endothelial leucocyte adhesion molecule-1 (ELAM-1) following the increased IL-1 activity. On reoxygenation of the cells, ICAM-1 expression was upregulated.[214] Hypoxia has also been shown to stimulate the release of IL-8 from endothelial cells in vitro. There is increased expression of IL-8 mRNA within the hypoxic endothelial cells with resultant increased rate of transcription. PMNs showed increased chemotatic activity, which was blocked by IL-8 antibodies. Similar results were shown with human umbilical vein segments in vitro.[215] Blood taken from the coronary sinus in patients undergoing cardiac transplant in comparison to patients having elective cardiac surgery showed elevated levels of IL-8 suggesting the longer ischaemic time may play a role.[216] De Perrot et al in 18 human patients undergoing lung transplant demonstrated that the levels of IL-8 were predominantly raised following reperfusion in comparison to TNF-α and IL-10 which were raised during the period of ischaemia.[217]

Yan et al demonstrated increased transcription of IL-6 in cultured endothelial cells with associated increased IL-6 mRNA expression. Mice exposed to hypoxia showed increased IL-6 activity in the lung and immuno-staining of the lung tissue showed increased expression of IL-6 antigen.[218] Kukeikla et al in an awake canine model of myocardial ischaemia and reperfusion established that IL-6 mRNA expression was induced following reperfusion. This was in contrast to the minimal IL-6 mRNA expression in myocardium exposed to ischaemia for 4 hours without reperfusion. Twenty fours of ischaemia did produce levels of IL-6 mRNA expression similar to that of the I/R model.[219]

Hypoxia also induces the production of PAF in HUVEC, becoming maximal at 90 minutes of hypoxia.[220] This confirmed the earlier work by Caplan et al who also demonstrated an upregulation of PLA_2 in hypoxic HUVEC.[221] Michiels et al showed both an activation of

PLA_2 and a five to ninefold increase in PGE_2, PGD_2, $PGF_{2\alpha}$, and PGI_2 in HUVEC exposed to hypoxia compared to normoxic controls[222]. Following I/R in the small intestine of the rat the level of circulating PLA_2 was significantly increased.[223] The injury caused by I/R in the intestinal mucosa was inhibited by quinacrine, a PLA_2 inhibitor.[224] The use of a PLA_2 inhibitor in a canine liver model of ischaemia and reperfusion attenuated hepatic I/R injury.[225] The inhibition of PAF by WEB 2086 in sprague dawley rats subjected to intestinal I/R significantly reduced the pulmonary vascular leak compared to sham operated rats.[226]

Another class of prostaglandins called isoprostanes are produced by a non cyclo-oxygenase mechanism involving free radical catalysed peroxidation of arachidonic acid. They were originally described over 20 years ago in vitro but only identified in humans by Morrow et al in 1990.[227] The isoprostanes most extensively studied are isomers of the COX derived $PGF_{2\alpha}$ and as such are called F_2-isoprostanes (F_2-iPs). Isoprostanes have been used as markers of lipid peroxidation but also have been shown to have biological activity and may thus be mediators of oxidant stress. The evidence for this is currently limited and mostly derived from the use of 8-iso-$PGF_{2\alpha}$. In several animal models it has been shown to cause vasoconstriction in pulmonary, renal and coronary arteries.[228,229,230] It has also been reported to cause bronchoconstriction in rats and guinea pigs.[228,231] Its action may be via the thromboxane receptor as a specific thromboxane receptor antagonist prevents these effects. Funkunaga et al demonstrated that 8-iso-$PGF_{2\alpha}$ actions on vascular smooth muscle were only partly inhibited by a thromboxane receptor antagonist suggesting that there may be a specific 8-iso-$PGF_{2\alpha}$ receptor related to but distinct from the thromboxane receptor.[232] 8-iso-$PGF_{2\alpha}$ has also been shown to have role in promoting platelet aggregation by increasing release of intracellular calcium, shape change and in

inositol phosphate concentration.[233,234] It has been reported by Minuz et al that platelet adhesion is also promoted by 8-iso-$PGF_{2\alpha}$ via a dose dependant increase in adhesion to fibrinogen and expression of the adhesion molecule glycoprotein IIb/IIIa. The anti-aggregatory effects of NO were also inhibited by 8-iso-$PGF_{2\alpha}$.[235] Fontana et al in vitro have shown that 8-iso-$PGF_{2\alpha}$ cause rapid adhesion of neutrophils in a dose dependant manner. It did not however cause neutrophil chemotaxis or activation of NADPH oxidase.[236]

Conclusion

This chapter has reviewed the role of ischaemia and reperfusion in the development of the inflammatory response. In particular looking at the cellular and molecular responses to periods of ischaemia followed by reperfusion and how these may result in tissue injury. Many of these responses are secondary to the production of reactive oxygen and nitrogen species. In the next chapter, the role of antioxidants in attenuating the effects of ROS/RNS will be reviewed.

Chapter 3 Role of Antioxidants

Introduction

The production of reactive oxygen and nitrogen species occurs to a lesser extent in healthy cells where they can have direct deleterious effects. It has been demonstrated in the previous chapter that following ischaemia reperfusion injury that there increased production has a potentially significant role in promoting SIRS. To help prevent or limit these effects several defensive mechanisms exist. These defences are generally referred to as antioxidants.

Antioxidants can be divided into two systems, those that have an enzymatic role in detoxifying reactive species and those that sequester reactive species. These enzymatic and endogenous antioxidant systems will be discussed below. The main thrust of the chapter will be to concentrate on the role of those antioxidants that can be potentially manipulated nutritionally. These are glutathione, α-tocopherol (vitamin E), the carotenes, and ascorbate (vitamin C).

Enzymatic antioxidants

The most important components of the enzymatic system are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GR).

Superoxide dismutase

The ROS, superoxide reacts with itself to form the less reactive hydrogen peroxide and molecular oxygen, this is described as dismutation. This reaction occurs at a rate constant of 8×10^4 mol^{-1} l s^{-1} but this is accelerated by SOD with a rate of 2.9×10^9 mol^{-1} l s^{-1}. There are three forms of SOD in mammalian tissue. Manganese-SOD (SOD2) is found within the mitrochondria. Copper/Zinc-SOD is localised to the cytosol (SOD1) and a secretory form of Copper/Zinc-SOD is found in the extracellular space (SOD3).

The importance of SOD as an antioxidants is demonstrated from several lines of evidence. SOD2 would appear to be the most important form of SOD. This is best highlighted by the fact that mice with a SOD2 knockout do not survive past 3 months whereas SOD1/SOD3 knockouts only show any difference following traumatic injury. The expression of SOD preserves myocardial function after ischaemia and reperfusion.[237,238] In rats, SOD has been shown to improve survival in a model of kidney I/R injury as well as preserve renal function.[239] The use of an adenoviral vector encoding for SOD in donor rat liver showed improved survival and decreased hepatic injury following reperfusion after transplantation.[240] In isolated rat pancreatic acinar cells the use of SOD and other antioxidants reduced oxidant mediated NFκB activity and inhibited production of TNF-α and IL-6.[241] This has been further supported by the adenoviral transfection of kupffer cells with the SOD gene. This suppressed SOD production and decreased LPS NFκB activation and TNF-α production though not IL-10.[242]

Catalase

Hydrogen peroxide is in itself a ROS and can cross cellular membranes easily. Catalase enzymatically converts H_2O_2 to water and molecular oxygen protecting the cell from H_2O_2. Catalase is a 240 kDa tetrameric enzyme composed of 4 ferriprotoporphyrin containing subunits.

The importance H_2O_2 as a ROS and the role of catalase in ameliorating its potentially harmful activity in promoting I/R injury is suggested in the evidence below. Li et al in a isolated heart model of ischaemia reperfusion showed that there was a significantly increased cardiprotective effect in transgenic mice with enhanced levels of catalase compared to non transgenic mice.[243] The immunotargeting of catalase to pulmonary endothelium in a donor rat model of transplantation demonstrated an improved graft function, decreased oxidative stress and reduction in I/R injury.[244]

Glutathione and Glutathione peroxidase

The tripeptide, glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) is the predominant low molecular weight thiol in mammalian cells. It was originally discovered in 1888 by de Rey-Paihade and called philothion but in 1921 Hopkins renamed it glutathione after elucidating it was a sulphur containing compound. It wasn't until 1931 that its tripeptide structure was fully demonstrated. It has been the focus of much research since.
The major site of *de novo* synthesis of GSH is the liver, supplying 90% of the circulating GSH under normal conditions. The turnover is rapid with a half life of 2-3 hours in rat liver. GSH is present in all cell types mainly concentrated in the cytosol with

approximately 10% in the mitochondria and a small percentage in the endoplasmic reticulum. The cellular concentration of GSH varies from organ to organ. The GSH concentration in the liver, duodenal and gastric mucosa is 2 to 3 times that of muscle (3.69, 4.47, 2.49 mmol v 1.34 mmol/Kg of wet weight).The majority of GSH in whole blood (0.57 mmol/L) is concentrated in erythrocytes as the plasma concentration is only 4.9μmol/L.[245] Whole blood GSH is thought to reflect the synthetic capacity of the liver.

Glutathione is synthesised via two ATP dependant enzymatic pathways. Initially the formation of the dipeptide, γ-glutamylcysteine is catalysed by γ-glutamylcysteine synthetase(γ-GCS also referred to as glutamate-cysteine ligase) using L-glutamate and L-cysteine as substrate. γ-GCS is a heterodimeric protein composed of a catalytic heavy and a regulatory light subunit. GSH synthetase, a homodimeric protein completes the process with the addition of glycine to γ-glutamylcysteine.

The synthesis of GSH has been shown to be regulated by three major factors these are:-

1. Feedback inhibition of GSH on γ-glutamylcysteine synthetase.[246]
2. Substrate availability. Predominately this is due to L-cysteine levels being consistently lower than glycine and L-glutamate. Other factors influencing hepatocellular levels of cysteine availability is membrane transport of cysteine, cystine (cysteine is oxidised to the predominant disulphide cystine extracellularily) and methionine. Methionine in the liver can be converted to cysteine via a transulphuration pathway.
3. Regulation of cellular γ-glutamylcysteine synthetase levels. Up regulation of γGCS has been demonstrated to occur through antioxidant response elements(AREs), AP-1 and NFκB binding sites located in the genes promoter

region[247]. TNF-α has been shown to upregulate γGCS transcription though it is unclear whether this through NFκB[248] or AP-1.[249] Oxidant stress is well recognised to activate NFκB and AP-1. Glucocorticoids and insulin also stimulate γGCS transcription.[250]

Intracellular degradation is resisted by the amino terminal peptide bond of cysteine being through the γ carboxyl group of glutamate rather than the usual α-carboxyl group. The bond can only be hydrolysed by the γ-glutamyltranspeptidase (GGT), which is located on the extracellular cell surface. In addition the terminal carboxyl group on glycine prevents cleavage by intracellular γ-glutamyltransferase. GSH is transported from the cell into the plasma, bile and mitochondria by distinct GSH transporters. The mitochondrial pool can only maintained by this transport process as it is not able to synthesize GSH. Under normal physiological conditions, the majority of cellular turnover of GSH is due to export of intact GSH from the cell only a small amount of GSH being lost by the formation of GSH conjugates. Plasma GSH levels are dependant on export of GSH into the hepatic sinusoids. This demonstrates the importance of the liver in interorgan GSH homeostasis.[251] However, the difference in concentration gradient between intracellular and extracellular GSH makes transport of intact GSH into the cell thermodynamically unfavourable. A process called the γ-glutamyl cycle overcomes this by cleaving GSH. γ-glutamyltranspeptidase transfers the γ-glutamyl moiety to an amino acid leaving cysteinylglycine. The γ-glutamyl amino acid is transported into the cell where glutamate is resynthesised. A dipeptidase acting on cysteinylglycine generates cysteine and glycine. These are also transported into the cell where GSH is resynthesised. Intact GSH absorption from the intestine occurs via Na^+ independent transport protein in the brush border membrane with Na^+ dependant GSH transporter in the basolateral membrane.[252]

Research has also focused on the possibility of GSH transport protein on other membranes, which will import intact GSH into the cell. Kannan et al *in vitro* have demonstrated such a transport protein. This is a Na^+ dependant GSH transport protein located in human cerebrovascular endothelial cells.[253]

GSH plays a major role in a cells defence against oxidative stress as well as other cellular functions. These roles include:-

1. A co-substrate for the enzymatic reduction of H_2O_2 and lipid peroxides by glutathione peroxidase (GPX).
2. Scavenging free radicals
3. Maintaining the sulphydrl status of proteins and enzymes in a reduced state.
4. Store and transporting cysteine in a non toxic state.
5. Modulating DNA synthesis.

Though GSH has many roles we will only discuss those in relation to its antioxidant function.

GPX is a member of a group of selenium containing proteins. Four distinct isoenzymes of GPX have been identified, cytosolic GPX (cGPX), plasma GPX, gastrointestinal GPX (giGPX) and phospholipid hydroperoxide GPX (phGPX). The most ubiquitous is cGPX, which in the presence of sufficient selenium is expressed in all cell types. It is at its highest levels in those tissues with high peroxide production such as liver, erythrocytes, lung and kidney. pGPX is found in extracellular fluid the main source being the kidney but is also present in the lung, liver, muscle, pancreas, and heart . In humans giGPX is localised to the epithelium of the gastrointestinal tract and the liver. phGPX is at low levels in all other organs except for the testes. GPX has a major role as a antioxidant

enzyme in protecting cells from hydroperoxides. However, hydroperoxides have been identified as having a role in modulating cellular signalling. There is increasing evidence that GPX as a modulator of hydroperoxide activity acts as a highly specialised redox regulator of cellular processes.[254]

In the presence of GPX, GSH reduces endogenously produced hydrogen peroxide to H_2O and is itself oxidised to GSSG. It also reduces lipid hydroperoxide to its corresponding alcohol. GSSG is reduced back to GSH by NADPH (from the pentose phosphate pathway) thus forming a redox cycle. In conditions of severe oxidative stress the ability to reduce GSSG may be overcome with a resultant increase in intracellular GSSG. This would shift the redox balance, but to compensate for this GSSG is actively transported from cell by an ATP dependant mechanism. GSSG can also react with protein sulphydrl groups to form protein-glutathione mixed disulphides these have a longer half life than GSSG. The result is cellular depletion of GSH.

Thioredoxin

Thioredoxin reductase (TrxR) is a selenium containing oxidoreductase, which reduces thioredoxin (Trx) by a NADH dependant mechanism. TrxR and Trx are ubiquitous in mammalian cells with multiple biological functions. Trx plays an important role in regulating cell growth and inhibiting apoptosis. In addition, Trx on translocation to the nucleus enhances the nuclear binding of the transcription factors AP-1 and NF-κβ. Trx has also been demonstrated to regulate the synthesis of several cytokines (TNF-α, IL-1, IL-6 and IL-8). Extracellularly, Trx also plays a role in inducing chemotaxis of PMN and mononuclear cells.

The synthesis of Trx is induced by hydrogen peroxide and ischaemia reperfusion. Trx acts as an electron donor for periredoxins or thioredoxin peroxidase, which catalyse the reduction of hydrogen peroxide as well as having endogenous antioxidant activity. TrxR has been shown to be able to reduce lipid hydroperoxide and hydrogen peroxides. A further demonstrated antioxidant role for TrxR is in the recycling of ascorbate from both the ascorbyl free radical and dehydroascorbic acid. *In vivo,* it remains unclear how important TrxR is in this recycling role.

Vitamin E

Vitamin E was originally discovered in 1922 by Evans and Bishop who found it to be essential for reproduction in rats.[255] It wasn't until the 1950s when Schwarz recognised its role in the cellular antioxidant system.[256] Vitamin E acts as a lipid soluble chain breaking antioxidant that prevents propagation of free radical reactions. The term vitamin E actually covers a group of molecules, these are four tocopherols (α,β,γ,δ) and four tocotrienols (α,β,γ,δ). α-tocopherol is the most abundant and biologically active form.

Dietary vitamin E is absorbed in the intestine, entering the circulation via the lymphatic system. It is absorbed with lipids and transported in chylomicrons to the liver. The majority of vitamin E in plasma is α-tocopherol with around 10% coming from γ-tocopherol.[257] Even with elevated levels of dietary γ-tocopherol, α-tocopherol still has the highest concentration in plasma. This is explained by the liver containing a 32 kDa transfer protein that selectively recognizes α-tocopherol called α-tocopherol transfer protein (α-TTP). Gene mutation of α-TTP result in low plasma vitamin E concentration.

Supplementation with vitamin E can return plasma levels back to normal but they fall rapidly if supplements are discontinued.[258] Transport of vitamin E was found to be normal in chylomicrons but defective in VLDL.[259] It would therefore appear that α-TTP is essential in maintaining plasma and cellular levels of vitamin E.

α-Tocopherol is incorporated into very low density lipoprotein (VLDL) exported from the liver and subsequently taken up into cell membranes. The transfer and regulation of tocopherols in peripheral cells is currently poorly understood. α-TTP has been found to be expressed in other tissues including the brain, spleen, lung and kidney.[260] In addition, a widely expressed binding protein for tocopherols has been identified. Tocopherol associated protein is 46 kDa in size and may be involved in intracellular tocopherol transport.[261] The preferential binding of α-tocopherol by a 15kDa tocopherol binding protein (TBP) may be influential in intracellular distribution of α-tocopherol. The tissue levels of tocopherols may be regulated by a membrane TBP.[262] The majority of β, δ and γ tocopherols are secreted into the bile and excreted in the faeces.

Lipid peroxidation occurs by a free radical removing a hydrogen atom from a methylene carbon on a polyunsaturated fatty acid (PUFA). The resultant carbon centred radical following rearrangement will react with oxygen forming a peroxyl radical. This radical can remove a hydrogen atom from an adjacent PUFA side chain resulting in propagation of oxidative injury.[263] α-Tocopherol within the lipid membrane is initially separated from the carbon radical and so is unable to interact. Once the peroxyl radical is formed it moves to the water/membrane interface. This brings it close to the chromonal nucleus of α-tocopherol and its reactive hydroxyl group. The peroxyl radical is reduced to a lipid hydroperoxide forming a α- tocopheroxyl radical thus preventing chain propagation. The

lipid hydroperoxide is cleaved by PLA_2 and is reduced to an alcohol by GSH and GPX. Even though α-tocopheroxyl is a free radical, it is neither strongly oxidising nor reducing. It also reacts poorly with oxygen so it does not produce any superoxide or form a peroxyl radical. To become an effective antioxidant the α-tocopheroxyl radical needs to be reduced back to α-tocopherol. Constantinescu et al described a network of redox reactions, which results in the recycling of α-tocopherol. As the hydroxyl group on the chromanol nucleus of α-tocopherol is at the water/membrane interface ascorbate (vitamin C) a water soluble antioxidant vitamin is able to reduce the α-tocopheroxyl back to α-tocopherol.[264]

Vitamin C

Vitamin C or ascorbic acid (AA) is one of the most important water soluble antioxidants with a role as free radical scavenger and also in redox reactions with other antioxidants. It is also an important cofactor in the enzymatic biosynthesis of collagen, carnitine, catecholamines and peptide neurohormones.

In most adult mammals, vitamin C can be synthesised *de novo* in the liver from glucose. This is not the case in humans and thus they require vitamin C from dietary sources. When AA becomes oxidised it is converted via a short lived ascorbyl free radical(AFR) to dehydroascorbic acid (DHA). Both DHA and AA are abdundant within the diet. The ingestion of DHA raises the AA level to similar extent as that of AA ingestion alone. This is important as the processing of food as a tendency to oxidised AA.

Both AA and DHA are absorbed from the gastrointestinal tract with further DHA being produced by the presence of oxidants in the gut lumen oxidising AA. AA is absorbed via a

Na^+-ascorbate cotransporter into the enterocyte all along the small intestine. DHA is taken up into the cell by facilitated diffusion by Na+ independent mechanism. The enterocytes maintain a gradient for DHA facilitating continued DHA uptake by converting DHA to AA via DHA reductase. It remains unclear how AA is transported from the enterocytes into the blood. There is some evidence to suggest that AA enters the extracellular fluid through volume sensitive anion channels and then enters the blood through gaps in capillary endothelium.[265] AA circulates in the blood as an ascorbate anion. An intracellular transmembrane concentration gradient is formed within the cells due to the active uptake of ascorbate by Na^+-ascorbate cotransporters. In erythrocytes there is no active uptake mechanism and the concentration in plasma and the erythrocytes are similar.[266] Within the cell the DHA produced when AA is utilised either in its enzymatic cofactor role or as an antioxidants can be transported from the cell via facilitative glucose transporters. AA is not a substrate for glucose transporters and thus accumulates in the cell. DHA at physiological pH is labile with a half life of approximately 6 minutes. It undergoes an irreversible ring opening forming 2,3-diketo-1-gulonate finally being metabolised via the pentose phosphate pathway. This is prevented by reducing DHA to AA non enzymatically by GSH, thioredoxin reductase and glutaredoxin. Erythrocytes, neutrophils, smooth muscle cells, and hepatocytes are among the many cell types that take up DHA from the extracellular fluid and reduce it to AA. In addition, if the plasma DHA level increases it is filtered in the kidney, reabsorbed, and reduced in the renal tubules. AA is also reabsorbed in the renal tubules by the Na^+-ascorbate cotransporter.[267]

In redox reactions ascorbate can donate one or two electrons. The single loss of an electron results in the formation of the AFR. This is stabilised by resonance on the three adjacent carbonyl groups and thus is less reactive than ascorbate. The loss of a second

electron results in the formation of DHA. AFR can be reduced back to ascorbate by AFR reductase activity within the cell. This would be advantageous in maintaining ascorbate concentrations due to the labile nature of DHA at physiological pH.[268]

Vitamin C has a wide scope in its action of scavenging physiologically relevant ROS and RNS. These include hydroxyl, alkoxyl, peroxyl radicals, superoxide and peroxynitrite. As previously, mentioned vitamin C is instrumental in the regeneration of the α-tocopheroxyl radical. It also can regenerate other antioxidants such as GSH, urate, and β-carotene from their radical species.[269]

Carotenoids

Carotenoids are lipid soluble plant pigments. Multiple caretenoids have been identified within human tissue and extracellular fluid. These can be derived from dietary sources and include the cyclic carotenes (β-carotene, α-carotene), acyclic carotenes (lycopene, phyotene) and the xanophylls (lutein, β-cryptoxanthin and zeaxanthin).[270]

Carotenoids are released from their food source, incorporated into micelles and absorbed by the enterocytes of the small intestine via passive diffusion. No active or facilitated transports mechanisms have currently been identified for carotenoids. Carotenoids are secreted into the lymph following incorporation into chylomicrons. β-carotene, α-carotene and cryptoxanthin may be partially converted to rentinyl esters in the enterocytes. Chylomicrons are hydrolysed by lipoprotein lipase and converted into chylomicron remnants. These remnants are rapidly taken by the hepatocytes. The carotenoids are

incorporated into VLDL except in the fasting state when the majority of carotenoids are associated with LDL and HDL.

In vitro, three possible mechanism have been demonstrated by which carotenoids can react with free radicals:-

1. Electron transfer resulting in the production of a cation, anion or alkyl radical.
2. Hydrogen abstraction
3. Radical species addition

The nature of the radical species influences both the rate and mechanism of scavenging of these species by the carotenoids. β-carotene has been shown to be more reactive with peroxyl radicals than superoxide and hydroxyl radicals.

In vivo it is difficult to demonstrate the above reactions. This partly due to the heterogenous nature of carotenoids in tissue but also the lower tissue concentrations compared to those used *in vitro.* Another confounding factor is that carotenoids are poorly soluble in solution and are found concentrated in the hydrophobic regions of biological membranes usually tightly associated with proteins and lipoproteins. The different structure of the carotenoids influences the orientation of the molecule within the membrane. β-carotene lies deep in the hydrophobic core of the membrane but is orientated parallel to the membrane surface. This is strongly contrasted by zeaxanthin, which spans the whole membrane. Woodall et al using liposomal membranes showed that β-cryptoxanthin and zeaxanthin had a greater protective effect than β-carotene or lycopene against peroxyl radicals induced by 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) oxidation. In solution, both β-carotene and zeaxanthin had similar response to

peroxyl radicals.[271,272] The different position and orientation in the membrane of the carotenoids would influence the collision rate of the peroxyl radicals and thus the difference in antioxidant behaviour.

Carotenoid radicals have been shown to be stable in aqueous solution. They are however long lived decaying by second order reactions and as such are potentially capable of acting in a pro-oxidant manner. Truscott proposed a mechanism of interaction between β-carotene, vitamin C and vitamin E which results in the recycling of vitamin E and β-carotene from their respective radicals.[273] Vitamin C has been shown to convert the carotenoid radical to the parent carotenoid. This occurs both in methanol and in unilamellar liposomes of dipalmitoyl phosphatidyl choline.[274] This result with the liposome may appear to be suprising as the carotenoids are found entirely within the hydrophobic core of lipid membranes unlike vitamin C which is in the aqueous phase. The properties of the carotenoid radicals are different to its parent species and may reorientate to come in to contact with the aqueous phase. Truscott proposed that the carotenoids would recycle the α-tocopheroxyl radical back to α-tocopherol. Mortensen et al have shown the converse to be true with α-tocopherol being able to reduce all the carotenoid radicals back to their parent molecules.[275,276]

Due to the reason outlined above it's difficult to demonstrate these antioxidant reactions *in vivo*. There are several studies, which suggest that carotenoids do have antioxidant properties *in vivo*. Winklhofer-Roob et al gave β-carotene supplements to children with cystic fibrosis and demonstrated a fall in malondialdehyde (MDA) levels and increase in the lag time of LDL oxidation.[277] Rust et al showed similar results.[278] Dixon et al placed women on a carotenoid deficient diet with a subsequent increase in MDA levels, which

were reversed with supplements of carotenoids.[279] Agrawal et al supplemented 19 healthy subjects with tomato juice and showed a two fold increase in plasma lycopene and a decrease in MDA and conjugated dienes.[280] Allard et al in a randomised double blind controlled trial in smokers and non smokers showed that breath pentane levels in smokers were significantly elevated compared to non smokers. β-carotene supplementation for four weeks decreased breath pentane levels in smokers but had no effect in non smokers or both placebo groups.[281]

Urate, Bilirubin and Albumin

There is increasing evidence that albumin acts as a free radical scavenger in blood and extracellular fluids though the precise mechanism of its antioxidant activity is unclear.[282] Bovine serum albumin has been shown to prolong the lag phase in copper mediated LDL oxidation as well as significantly reduce thiobarbituric acid reactive substances (TBARS).[283] TBARS are end products of fatty acid oxidation.

Bilirubin is an end product of the catabolism of haem and can have cytotoxic effects if not excreted from the body. Bilirubin has been demonstrated to be an effective scavenger of peroxyl radicals *in vitro*. It also is more effective in liposomes of preventing lipid peroxidation than α-tocopherol.[284] Bilirubin is a lipid soluble molecule, the majority in plasma is transported bound to albumin. Neuzil et al have also demonstrated the free radical scavenging activity of albumin bound bilirubin for the hydroxyl radical and superoxide. They also showed that bilirubin protected albumin from hydroxyl oxidation.[285]

Urate as previously mentioned is an end product of purine metabolism catalysed by xanthine oxidoreductase. Though long considered to have no physiological value it has been demonstrated to have antioxidant activity. It is active in free radical scavenging of superoxide, hydroxyl and peroxynitrite radicals.

It is still to be determined how important these three molecules are as antioxidants *in vivo.* However, in measurements of the total antioxidant capacity of plasma they make up a considerable proportion of this activity.

Antioxidants, SIRS and clinical implications

It has been demonstrated that the markers of oxidative injury are elevated in SIRS, sepsis and IR injury. Several studies have also shown a fall in the total antioxidant capacity of plasma in the same groups of patients. Galley et al in patients on the ICU who fitted the criteria for SIRS had significantly lower total vitamin C levels compared to normal controls. Hammarqvist et al demonstrated in eleven critically ill patients in the ICU that the reduced and total glutathione was 57% and 62% of the levels in the eleven healthy controls that had undergone elective surgery. Patients undergoing abdominal aortic aneurysm repair with up to 90 minutes of leg ischaemia showed falls in muscle GSH of up to 40% similar to that in patients undergoing abdominal surgery. The glutathione levels following reperfusion were only marginally affected. Goode et al in 16 patients with septic shock had significantly lower vitamin E, β-carotene and lycopene levels when compared to healthy controls. 8 of the patients had undetectable β-carotene and lycopene and the rest were below their reference range. Takeda et al have also shown that vitamin E levels fall in critically ill patients. This has also been confirmed in patients that have acute

respiratory distress syndrome the commonest form of organ dysfunction following SIRS. Basu et al in a porcine model of septic shock showed a steady decrease in vitamin E in survivors and non-survivors. Curran et al measured the antioxidant levels of 13 patients admitted with acute pancreatitis. In comparison to controls they found that the concentration of α-tocopherol, α-carotene, β-carotene, lutein and lycopene were reduced on admission and remained low throughout the seven days. A previous study by Scott et al had demonstrated that the admission concentration for vitamin C in 29 patients with acute pancreatitis was low compared to controls. They also looked at patients with other acute abdominal conditions who also had reduced levels of vitamin C but not as low as in the pancreatitis group. Goode et al showed in 12 patients undergoing orthotopic liver transplant for chronic liver disease that they had significantly lower vitamin E, β-carotene and lycopene in comparison with healthy controls. On reperfusion of the graft liver the vitamin E levels fell further.

With this data in mind, several studies in both human, cell and animal models have looked at the effect of endogenous antioxidant supplementation. Mendez et al treated rabbit alveolar macrophages with the antioxidants vitamin E and N-acetylcysteine (NAC). On stimulation of the macrophages with LPS they showed that TNF mRNA expression and TNF synthesis were reduced in pretreated cells.[286] Fox et al showed similar results in rat kuppfer cells. Kuppfer cells stimulated with LPS showed that TNF-α, NFκB activation, and cytokine mRNA expression was inhibited by the addition of vitamin E and NAC following activation.[287] Bulger et al enterally supplemented Sprague dawley rats for 6 days with vitamin E. In comparison to the control groups they showed a two fold increase in serum vitamin E levels. After 6 days supplementation peritoneal and blood macrophages were harvested. In vitro following LPS stimulation of the macrophages the vitamin E

group showed suppression of the TNF response.[288] These results suggest that at the cellular level that antioxidants may be able to suppress the inflammatory response.

Several studies have looked at the effect of vitamin E supplementation in animal models of sepsis. These studies showed an improved survival with vitamin E.[289,290] The administration of vitamin E for seven days in a rat model of warm ischaemia increased the survival rate from 0 to 46.7%. Following four hours reperfusion the ATP levels in the vitamin E group was increased and the creatinine levels were lower than the control group after 2 days.[291] Marubayashi et al pretreated rats with vitamin E for three days prior to subjecting them to 90 minutes of liver ischaemia. Those rats that were pretreated showed an increased survival from 0% to 45.5%. The period of ischaemia caused a fall in the endogenous vitamin E, total glutathione, hepatic ATP and lipid peroxides. Reperfusion in the control group resulted in a continued decrease except for lipid peroxides, which increased. The reperfused vitamin E group showed no change in vitamin E and glutathione compared to that following ischaemia. There was an increase in ATP synthesis and suppression of lipid peroxide formation.[292] Kearns et al looked at the effect of vitamin C supplementation in a Sprague-Dawley rat model of ischaemia reperfusion. They randomised 18 rats into either a control group with normal diet, ischaemia reperfusion with normal diet and ischaemia reperfusion with vitamin C supplemented normal diet. They measured the myeloperoxidase activity (a marker of tissue neutrophil sequestration) and neutrophil concentration in bronchoalveolar lavage(BAL). The myeloperoxidase activity was significantly increased in the I/R group compared to the controls. The vitamin C group showed a significant reduction in myeloperoxidase activity compared to the I/R group. The neutrophil concentration was also significantly reduced in the vitamin C pretreated group. BAL protein concentration (a marker of increased pulmonary

microvascular permeability) was shown to be reduced in the pretreated animals in comparison to the I/R group. These results would suggest a protective effect of vitamin E and C in ischaemia reperfusion models.

Human studies with antioxidant supplements have also shown some promising results. Devaraj et al studied the monocytes function of 21 healthy volunteers at baseline, following 8 weeks of vitamin E supplementation, and after a 6 week washout period. They showed a significant increase in plasma and monocytes vitamin E levels following supplementation compared to the baseline and following the washout period. There was a significant decrease in superoxide release, TBARS activity and IL-1β release following vitamin E supplementation in resting monocytes. Though, there was an increase in these three parameters following LPS stimulation the difference with vitamin E supplementation remained. They also suggested that the effects of vitamin E may not entirely be due to its antioxidant role. It may partially be due to the inhibition of protein kinase c as similar effects were seen with calphostin c a PKC inhibitor which has no antioxidant activity.[293]

A further study by Devaraj et al looked at the effect of vitamin E supplementation in type 2 diabetics in comparison with a control group. They measured serum hs-CRP and IL-6 production by LPS stimulated monocytes at baseline, following 3 months vitamin E supplement and after a 2 month washout period. After vitamin E supplementation in controls and diabetics there was a significant decrease in serum hs-CRP and monocytes IL-6 production compared to washout and baseline.[294] Nathens et al in a large study of 595 patients admitted to ICU randomised them to antioxidant supplementation with vitamin E and vitamin C or standard treatment without antioxidant supplements. The supplemented group showed increased plasma levels of both vitamins. The risk of developing pneumonia

or ARDS was decreased in the supplemented group (relative risk 0.81, 95% CI 0.6-1.1) similarly the risk of developing multi organ dysfunction was also reduced (relative risk 0.43 95% CI 0.19-0.96). The supplemented patients also had a shorter ICU stay and period of mechanical ventilation.[295] Novelli et al randomised 12 patients undergoing abdominal aortic aneurysm repair to either preoperative vitamin E supplementation for 8 days prior to surgery or no supplements. In the control group, they showed that the MDA content of the quadriceps leg muscle was not increased during ischaemia but was significantly elevated during reperfusion. Neutrophils were recruited into the muscle during ischaemia in the control group. The rise in MDA during reperfusion was ameliorated in the treated group with decreased ischaemia related neutrophil infiltration. The ultrastructural changes in muscle fibres following reperfusion were also significantly less in the treated compared to the untreated group.[296]

Ortolani et al randomised 30 patients with septic shock into one of three groups. All patients received standard therapy but one group was supplemented with intravenous GSH and a further group with intravenous GSH and N-acetylcysteine (NAC) for five days. . The plasma levels of GSH was significantly increased in the supplemented groups. The GSH and GSH/NAC groups showed a significant decrease in the markers of lipid peroxidation (MDA and Breath ethane) at day 5 compared to baseline and the control group. There was no difference in mortality between the groups at day five but the control group became twice that of the supplemented group by day 10. The APACHE II score and logistic organ dysfunction scores showed no significant difference between the groups by day 5. By day 10, the treatment groups showed a significant difference in scores in comparison with the basal values and the control group.[297] These results show some

probable benefits for clinical outcome with GSH and NAC supplements in septic shock patients but the sample size is small.

Animal and human studies have shown that antioxidant supplements can decrease the levels of lipid peroxidation markers, reduce organ dysfunction, improve survival and modulate the inflammatory response.

Conclusion

In this chapter, we have looked at the metabolism and the evidence for the antioxidant activity of the endogenous antioxidants as well as a brief review of those enzymatic antioxidants. Finally, reviewing the current literature on the effects of antioxidants in ameliorating the effects of SIRS and ischaemia reperfusion. The next chapter will review the current literature on SIRS and ischaemia reperfusion in elective abdominal aortic aneurysms.

Chapter 4 Ischaemia Reperfusion Injury in Elective AAA Repair

An abdominal aortic aneurysm (AAA) is present when the infrarenal aortic diameter exceeds 3 cm. Rupture of abdominal aortic aneurysms caused about 6800 deaths in England and Wales in the year 2000. In those older than 65 years, ruptured abdominal aortic aneurysms are responsible for 2·1% of all deaths in men and 0.75% of all deaths in women[298]. The Multicentre Aneurysm Screening Study (MASS) found the prevalence of AAA in men between 65 and 74 to be 4.9% of those who attended for ultrasound screening. 12% of these were greater than 5.5cm the size usually considered for repair. [299]

Abdominal Aortic Aneurysm repair

There are two possible techniques for the repair of an AAA the more traditional open surgical repair and more recently the endovascular approach though there is some interest in laparoscopic repair of AAAs. The aorta lies behind the peritoneum in the abdomen and so can be approached transperitonealy or extraperitonealy. Either way the aorta and the iliac arteries have to be cross clamped while a tube graft is sewn in situ. If the iliac arteries are aneurysmal then a bifurcated graft is used. This leads to a period of relative ischaemia in the lower torso and potentially the left side of the colon as the inferior mesenteric artery arises from the aneurysm sac. Left colon ischaemia arises if the collateral flow from the internal iliac artery or superior mesenteric artery is inhibited in anyway. Following clamp release the lower torso is reperfused with a potential ischaemia reperfusion injury which will be discussed below.

The endovascular approach involves the deployment of a stent under radiological guidance into the aneurysm sac via the femoral artery. Several studies have compared endovascular

to open aneurysm repair particularly looking at the inflammatory response in the following review only the open repair data is reported.

Morbidity and Mortality

In the MASS study those patients undergoing elective repair of their AAA the reported 30 day mortality was 6%.[299] A literature review by Hallin et al showed a mean mortality of 5% in those papers that had data on 30 day mortality for elective AAA repair. The proportion of elective patients having a myocardial infarction was 3.3% (range 0-19.6%) (25 studies, 5487 patients). The need for dialysis for postoperative renal failure was 0.9% (range 0-34%) (8 studies, 1293 patients).[300] Blankensteijn et al in a literature review reported mortality and morbidity broken down into five levels of evidence. In those that were prospective population or hospital based studies the cardiac morbidity was 10.6% (range 8.5-13.2%) and 12.0% (range 10.5-13.9%), pulmonary morbidity was 5.3% (range 3.8-7.3%) and 9.8% (range 8.3-11.6%) and renal morbidity 7.0% (range 5.3-9.2%) and 4.8% (range 3.8-6.2%) respectively.[301] Elkouri et al in 261 patients having an elective open AAA repair showed a thirty day cardiac morbidity rate of 22%, pulmonary morbidity of 16% with renal morbidity being 4.2%. [302] Akkersdijk et al reported both pulmonary and cardiac morbidity as 10% in 291 patients having an elective open AAA repair. The overall reported complication rate of varying severity was 26%.[303]

There is significant variation in the recorded morbidities for elective AAA repair. This is likely to be due differences in definition of cardiac or pulmonary morbidity between studies. The strict criteria used by Elkouri et al probably more closely reflects the true morbidity following open AAA repair.

Cytokines

In 1990 Cruickshank et al demonstrated a rise in IL-6 in patients undergoing aortic surgery which was significantly greater than that in patients having intermediate or minor surgery.[304] Baigrie et al showed a similar increase in IL-6 which peaked at a median of 8 hours in 25 patients undergoing elective AAA repair. They also demonstrated a rise in IL-1β which preceded that of IL-6 by several hours. There was a subsequent increase in CRP which was maximal at 72 hours.[305] Holmberg et al compared 23 patients undergoing AAA repair with 11 patients having spinal surgery. In the AAA repair group IL-6 and IL-10 both rose significantly intraoperatively. They did not demonstrate any significant difference between samples taken from the femoral vein or veins in the arm. Both IL-6 and IL-10 remained significantly elevated compared to baseline at one week. They showed no significant changes in IL-6 or IL-10 in the control group.[306] Oldenburg et al showed a peak rise in IL-10 in patients undergoing AAA repair at 30 minutes after clamp release falling to undetectable levels by 24 hours. This is in comparison to patients who had a thoraco-abdominal aortic aneurysm (TAAA) repair where the peak was at 4 hours following clamp release and only becoming undetectable after 48 hours.[307] Welborn et al compared IL-8 in patients undergoing TAAA repair with those having an infrarenal AAA repair. IL-8 peaked earlier with the plasma concentration being significantly lower in AAA repair than that in TAAA repair.[308] Rowlands et al showed that IL-8 peaked 4 hours following clamp release before showing a gradual decline towards baseline by 144 hours.[309] Cabič et al detected TNF-α in the systemic and portal blood in 14 patients undergoing AAA repair. This was increased following release of the aortic clamp and was significantly higher in portal blood compared to the systemic blood. They also demonstrated endotoxin in the portal blood in 71% of cases following clamp release.[310]

Holzheimer et al also detected TNF-α which was maximal 120 minutes following clamping but this rise was not significant.[311] This is consistent with the majority of studies that have looked at TNF-α in AAA repair where TNF-α is either not detected or shows a minimal elevation in concentration. This is probably due to the cyclical nature of TNF-α release and as such more intense sampling is required to detect any changes.

Reactive oxygen species

Thompson et al demonstrated the production of ROS in blood taken from the femoral vein in patients undergoing open or endovascular AAA repair. They used the oxidation of IgG as a quantifiable marker of ROS. They found that ROS production was maximal following 5 minutes of reperfusion declining by 30 minutes of reperfusion.[312] Lindsay et al in 22 patients undergoing elective AAA repair measured acyloins and F_2-isoprostanes both markers of oxidative stress. No significant changes were seen in F_2-isoprostanes in comparison to pre-induction concentrations. However there was a gradual increase in total acyloins from 15 minutes post clamp release which only became significant at 24 hours. The acyloin, 3-hydroxynonan-2-one showed a similar rise but became significant at 1 hour post clamp release.[313] Kretzschmar et al studied 3 groups of patients undergoing elective AAA repair. They gave N-acetylcysteine to one group just before clamping, mannitol to the second group prior to clamp release and the third control group had no additional treatment. They found a significant rise in oxidised GSH and lipid peroxides in the mannitol and control group following clamp release but not in the N-acetylcysteine group.[314] Kazui et al used ethane in exhaled breath as a biomarker of lipid peroxidation. They found a transient 2 fold increase in ethane at 15 minutes post clamp release.[315]

Lower limbs and Neutrophils

Lau et al using neutrophil elastase/α_1-antitrypsin complexes as a marker of neutrophil activation showed a significant increase in concentration following release of the aortic cross clamp, peaking 6 hours postoperatively.[316] Barry et al demonstrated a significant increase in the neutrophil expression of the integrin CD11b following release of the aortic cross clamp. This change was noted in pre-pulmonary venous blood but not in post pulmonary arterial blood. The increase in expression of CD11b was only transient. The neutrophil respiratory burst activity was also shown to increase following clamp release.[317] Norwood et al have also shown an increase in expression of CD11b in neutrophils from blood sampled from the femoral vein in comparison with portal or systemic blood. Interestingly this increased expression started prior to application of the clamp.[318] Swartbol et al incubated donor leucocytes with the plasma of patients undergoing endovascular and open AAA repair. They showed an increase in CD11b expression in the donor neutrophils prior to clamping reaching a peak 60 minutes post operative. However this expression was significantly greater in those undergoing the endovascular procedure compared to an open repair.[319]

Formigili et al took leg muscle biopsies from the quadriceps prior to clamping, just prior to clamp removal and 30 minutes post clamp. They showed histological neutrophil infiltration of the muscle during ischaemia and reperfusion. Blood sampled from a superficial leg vein showed these changes paralleled complement activation and increased circulating neutrophils.[320] In another study they looked at E-selectin expression (a marker of endothelial activation) in quadriceps muscle biopsies at the same time points using preoperative samples as controls. There was increased expression of E-Selectin on the

venular endothelium during ischaemia and reperfusion. This seemed to match the neutrophil accumulation in the biopsy.[321] Novelli et al again using quadriceps muscle biopsies showed neutrophil infiltration during ischaemia and reperfusion with ultrastructural muscle changes. They also measured the malondialdehyde (MDA) content of the biopsies as a marker of lipid peroxidation. This only increased during reperfusion but not during ischaemia. They treated a second group with vitamin E which showed a significant decrease in MDA, neutrophil infiltration and ultrastructural changes during reperfusion.[296]

Lung Injury

Paterson et al demonstrated pulmonary interstitial oedema with pulmonary wedge pressures that excluded left ventricular failure in the chest x-rays of 20 patients undergoing open AAA repair. They also showed a rise in TxB2 which started during aortic cross clamping rising further on cross clamp release which was related to pulmonary dysfunction.[322] Smith et al demonstrated pulmonary dysfunction as assessed by chest x-ray and PaO_2:FiO_2 ratio in 10 out of 40 patients undergoing AAA repair. At 4hrs these patients in comparison to those who recovered uneventfully had a significantly higher microalbumin/creatinine ratio (MACR) (a renal marker of systemic vascular permeability).[323] Barry et al showed a significant increase in pulmonary vascular resistance and mean pulmonary artery pressure after clamp release. These pulmonary changes correlated with an increase in plasma TxB_2 following clamp release. Clinical evidence of pulmonary oedema was seen in six patients however this was transient. [317]

Gastrointestinal tract

Soong et al measured the intramucosal pH of the sigmoid colon in 21 patients undergoing AAA repair. 10 patients whose sigmoid pH was less than 7 had a significant increase in plasma concentrations of endotoxin, TNF-α and IL-6.[324] Roumen et al using a dual absorption sugar test showed that the lactulose/mannitol ratio was significantly greater in those patients undergoing either elective or emergency AAA repair compared to the controls. This indicates a significant increase in intestinal permeability following AAA repair. Lau et al compared patients undergoing either a transperitoneal or extraperitoneal AAA repair. Those patients having a transperitoneal repair had a significantly increased intestinal permeability at day 1 as measured by the lactulose/mannitol ratio. Endotoxin measured in the systemic and portal circulation was also significantly elevated in the transperitoneal group.[325]

Conclusion

It can be seen that patients undergoing elective open AAA repair do have a significant inflammatory response as demonstrated by the elevated cytokines particularly IL-6. It would appear that this response is systemic as femoral vein sampling showed no difference in cytokine concentration compared to systemic blood. Several biomarkers of lipid peroxidation have been measured which suggest an increase in reactive oxygen species following clamp release. The increased MDA content in leg muscle biopsies would suggest that the legs are the source of the ROS. In elective AAA repair the exact role that ROS plays in promoting the inflammatory response currently remains unclear.

Neutrophils have been shown to be activated in AAA repair both *in vivo* and *ex-vivo* though this starts before aortic cross clamping. This would suggest that surgery itself causes activation of neutrophils rather than the result of an ischaemia reperfusion injury. A further theoretical explanation could be that there is an increased expression of endothelial adhesion molecules which binds the activated neutrophils and thus they are removed from the circulation and so masking a post reperfusion. Norwood et al also proposed that there may be allosteric hinderance secondary to soluble ICAM-1 in plasma binding to the CD11b and preventing binding of the labelled monoclonal anti- CD11b prior to flow cytometry.[318] Neutrophil activation appears to be initiated in the lower limbs with further evidence suggesting that neutrophils may be trapped in the pulmonary circulation causing pulmonary injury.

The left colon can be prone to ischaemia as reflected by a low sigmoid pH. This does not occur in all cases and though an increase in cytokine and endotoxin is demonstrated in these patients, other studies have shown an increased intestinal permeability with elevated portal endotoxin. The increased intestinal permeability is unlikely to be due to direct ischaemia reperfusion injury as aortic cross clamping only effects the left colon and therefore may be secondary to bowel manipulation. It may also be due to distant inflammatory mediator release and neutrophil activation. This increase in permeability allows translocation of endotoxin which has been identified as one of the drivers of the inflammatory response.

These studies suggest that patients undergoing elective open AAA repair would be an excellent human model for investigating factors involved in modulating the inflammatory response and ischaemia reperfusion injury for instance antioxidants or diet.

Chapter 5 Outcome Measures:- The evidence

Introduction

Many scoring systems and biochemical markers have been studied to try and determine a method for predicting outcome in those patients admitted for surgery or to the intensive care unit. Unfortunately none of these are 100% specific or sensitive in determing outcome. However several methods have been well validated and therefore have been used as markers of outcome in this study in addition to morbidity and mortality.

Sequential Organ Failure Assessment (SOFA) Score

This scoring system was developed during a consensus conference of the European Society of Intensive Care and Emergency Medicine, it was initially called the sepsis related organ failure score though it can be applied to all groups of patients and so was renamed. An initial retrospective study of the score at 24 hours in 1643 patients admitted to ITU showed a good correlation to mortality.[326] A subsequent prospective multicenter study of 1449 critically ill medical and surgical patients admitted to ITU showed that the SOFA score was predictive of patient prognosis and in identifying the development and monitoring of organ dysfunction.[327] Further studies have confirmed the usefulness of SOFA as an outcome predictor.[328,329,330]

The SOFA scoring system allows the calculation of the level of dysfunction for 6 organ system (respiratory, coagulation, hepatic, cardiovascular, neurological and renal). Each organ system has up to four levels of dysfunction (see table 4).

Table 4 SOFA Scores[326]

Variables	0	1	2	3	4
Respiratory PaO_2/FiO_2 (kPa)	>53.3	<53.3	<40	<26.7	<13.3
Coagulation Platelets	>150	<150	<100	<50	<20
Hepatic Bilirubin(μmol/l)	<20	20-32	33-101	102-199	>200
Cardiovascular Hypotension	MAP*>70	MAP<70	Dopamine** <5 or Dobutamine any dose	Dopamine>5 or Epinephrine<0.1 or Norepinephrine<0.1	Dopamine>15 or Epinephrine>0.1 or Norepinephrine>0.1
Neurological Glasgow coma scale	15	13-14	10-12	6-9	<6
Renal Creatinine(μmol/l) or urine output	<110	110-170	171-299	300-440 or <500 ml/day	>440 or <200 ml/day

***Mean arterial pressure**
****doses are in μg/Kg per minute administered for at least I hour**

Acute Physiology And Chronic Health Evaluation II (APACHE II)

In 1985 Knaus et al proposed the APACHE II scoring system. This was a modification of the original APACHE score. It uses a point scoring system for 12 physiological variable, age and previous health status giving a range from 0 to 71 (see appendix A). The score is calculated using the worst values in the first 24 hours in ITU. The APACHE II also includes an equation to estimate prognosis based on the information collected in the first 24 hours in ITU.

The original validation was on 5815 patients admitted to 13 intensive care units. They found that increasing score was predictive of mortality and could prognostically stratify

critically ill patients.[331] The APACHE II score has been further validated in several studies in surgical patients.[332,333,334]

Microalbumin Creatinine Ratio (MACR)

Proteinuria occurs secondary to two possible mechanisms, these being an increase in glomerular permeability and impairment of tubular reabsorption. It is generally accepted in normal physiological conditions that proteins such as IgG with a radius of 55 A are not filtered by the glomerulus. 55 A is larger than the restrictive pore size (37 to 48 A) in the glomerulus. However, the low glomerular permeability of albumin with a radius of 36 A cannot be explained by the restriction of movement by pore size. The movement of albumin is probably restricted by its negative charge with resulting electro repulsion by the negatively charged glomerulus.[335] It has been shown that inhibition of the negative charge by polycations increases the permeability to albumin. Albumin in normal human subjects is restricted by a factor of 100 compared to that of a 36 A uncharged probe.[336] The role of charge in determining selectivity of filtration has been questioned. The charge on the glomerulus has been calculated to be less than that required to effect the filtration of anionic proteins.[337] A recent study however by Ohlson et al developing a gel membrane model has shown strong support for the permselective nature of the glomerular membrane with charge selectivity.[338] Other proteins with a radius lower than 30 A are freely permeable to the glomerular membrane but only appear in the urine in negligible amounts. These proteins are practically, totally reabsorbed by the proximal tubule.

Microalbuminuria is defined as persistent elevation of albumin in the urine, of 30-200 mg/day (20-200 μg/min). These values are less than the values detected by routine urine

dipstick testing, which does not become positive until protein excretion exceeds 300-500 mg/day. As previously discussed in the introduction one of the effects of the inflammatory response is an increase in capillary permeability. Fleck et al measuring transcapillary escape rate of albumin demonstrated that in septic shock the rate of loss was increased by 300% and a 100% following cardiac surgery.[339] Jensen et al in a group of clinically healthy human subjects with persistent microalbuminuria compared to normoalbuminuric individuals showed a significantly increased systemic transcapillary leak using ^{125}I-labelled albumin as a marker of fractional clearance from the plasma.[340] It has become well recognised that the rate of microalbuminuria is elevated in multiple pathological conditions including diabetes[341], hypertension[342], acute myocardial infarction[343], trauma[344], sepsis[345] and major surgery[346] and it probably reflects a generalised increased vascular permeability. There is an increasing body of evidence that has shown that an elevated microalbuminuria is predictive of outcome and the development of MODS. Smith et al measured the MACR in 40 patients undergoing infrarenal aortic aneurysm repair.[323] They showed that the degree of increase in MACR appeared to predict the development of pulmonary dysfunction. Shearman et al in patients with pancreatitis demonstrated that those patients with the highest levels of microalbuminuria were more likely to develop complications.[347] More recently, Abid et al measuring the trend in microalbuminuria in 40 medical patients admitted to an ICU showed that increasing microalbuminuria over 48 hours had good sensitivity and specificity to predict the development of acute respiratory failure and MODS.[348] Several recent studies have shown increased levels of MACR on admission to the ICU were predictive of mortality and have also demonstrated an association with physiological scoring systems.[349,350,351]

Bioelectric Impedance Analysis (BIA)

BIA is a well recognised method for estimating body composition in both clinical and research settings. If a radiofrequency electrical current is applied to the body it will either pass through the extracellular or extracellular and intracellular fluid compartments. BIAs ability to measure body composition is related to the principal of a conductive body's volume being proportional to its length squared and inversely proportional to its resistance.

$$V \propto \frac{L^2}{R}$$

The human body is represented as a series of interconnected cylinders. The electrical resistance of a cylinder is dependant on both its physical dimensions and its resistivity(ρ). The relationship can be written as:-

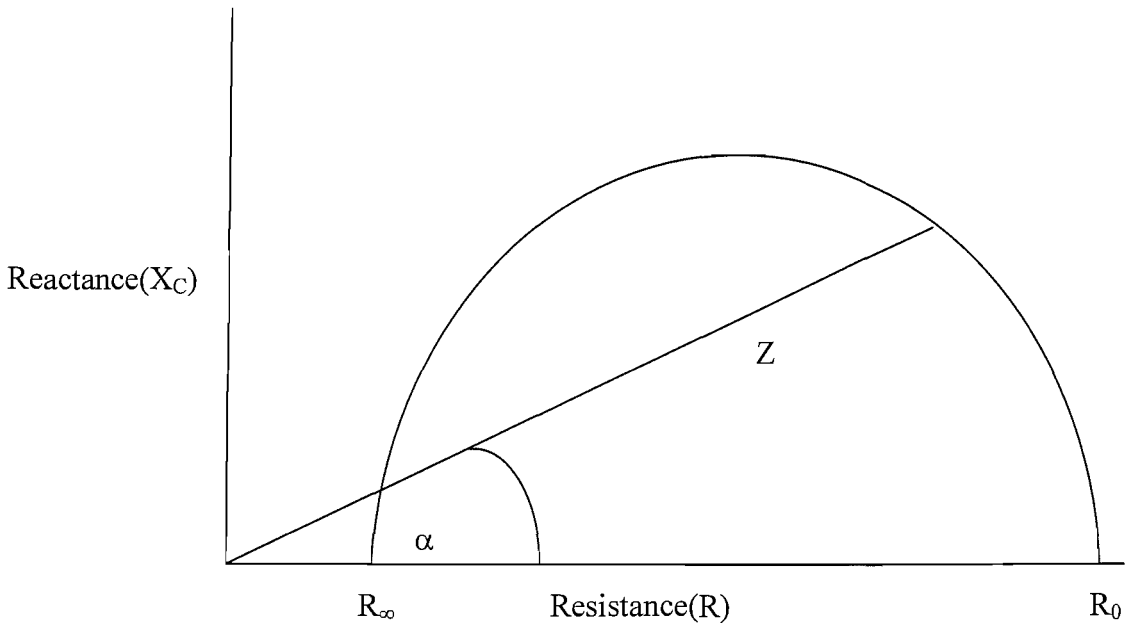
$$V = \rho \frac{L^2}{R}$$

Cell membranes when exposed to alternating current act as capacitors. A capacitor stores electric charge for a period of time. Capacitance opposes the instantaneous flow of electric current and this effect is called reactance (X_c). If current is applied at zero frequency then the cell membrane capacitors act as an insulator and the current passes through the extracellular water. The resistance at zero frequency (R_0) is representative of the ECW. In contrast if an infinite frequency current is applied then the cell membrane act as a perfect conductor and the current passes through all fluid compartments and the resistance (R_∞) is

representative of TBW. These values can be predicted from a cole-cole plot by plotting X_c against R for a spectrum of measured frequencies (fig 2).

Impedance (Z) is the frequency-dependent opposition of a conductor to the flow of an alternating electric current, and is composed of two vectors, resistance (R) and reactance (Xc). Phase angle reflects the relative contributions of fluid (resistance, or R) and cell membranes (reactance, or Xc) to the observed impedance in the human body. Mathematically, phase angle equals the arc tangent of Xc/R.[352] Any alteration in cell membrane function can affect its capacitance and thus the reactance. Similarly changes in fluid balance will be reflected in the resistance and thus the phase angle.

Figure 2 Cole-Cole Plot



Gudivaka et al reviewed 7 previously published BIA models in normal volunteers receiving Ringers Lactate or the diuretic bumetanide. Their ability to estimate changes in TBW and ECW was compared with deuterium and bromide dilution techniques. They found that the only method that predicted changes accurately was the multi frequency

Cole-Cole plot model.[353] The comparison of multifrequency BIA with bromide dilution in 37 critically ill patients showed an underestimation by BIA of the ECW. The changes however in ECW between the two methods agreed closely (4.42 +/- 4.25 (s.d.) vs 4.43 +/- 4.84 1).[354] Segal et al had previously shown that multiple frequency BIA was reproducible in 36 healthy volunteers for estimation of TBW and ECW in comparison to isotopic dilution techniques.[355] Marx et al measured ECW by inulin distribution method which showed a good relationship with BIA (ht^2/R_{5khz}).[356]

Cheng et al showed in comparison with young patients that elderly patients had a prolonged expansion of ECW and this was associated with a poorer clinical outcome.[357] Schwenk et al in 78 patients using multifrequency bioelectrical impedance analysis to estimate the ECW/TBW ratio showed in patients with significant bacteremia that there was an early shift from intracellular to extracellular water. This was associated with an unfavourable prognosis.[358] Shime et al in paediatric heart patients using BIA to reflect changes in body composition showed that a sustained decrease in the ratio of preoperative to postoperative values was associated with a higher probability of non survival.[359] Patients with SIRS using BIA to measure the phase angle were more likely to survive if the initial phase angle was > 4^o.[360] Schwenk et al in patients with human immunodefiency virus showed that those patients in lower quartile of phase angle (<5.3^o) had a shorter estimated survival.[361] Other authors have shown similar results with phase angle measurements.[362]

In summary BIA measurement is a useful tool to measure changes in the distribution and volume of body water. There is increasing evidence that these measurements also have a role in predicting outcome.

Chapter 6 Methods:-Clinical outcome measures

Sequential Organ Failure Assessment (SOFA) Score

The worst score for each organ system was taken for each 24 hour period. Following discharge from the intensive care unit or from the surgical high dependency unit it was assumed for the respiratory system that the PaO_2 was 13.3 kPa if the oxygen saturations were >95%. The FiO_2 continued to be recorded.

Acute Physiology And Chronic Health Evaluation II (APACHE II)

The APACHE II score was calculated based on the worst scores in the first 24 hours for each of the 12 variables.

Systemic inflammatory response score

The criteria used were those defined in 1992 at a consensus conference of the American College of Chest Physicians and Society of Critical Care Medicine (ACCP/SCCM)(table 1).[2] A patient was determined as having SIRS if they scored in two or more of the four criteria. The worst values in the 24 hour period were used to calculate the SIRS score. A patient was determined to have SIRS if they met two or more criteria.

Biochemical parameter used in scoring systems

The creatinine was measured using a modified Jaffe reaction. Due to the negative interferance from bilirubin this is oxidised with potassium ferricyanide prior to the reaction. Creatinine reacts with alkaline picrate forming a coloured complex. The rate of complex formation is measured at 505nm and is proportional to creatinine concentration. The method for measurement of bilirubin is described in chapter 7.

Sodium and Potassium measurements are based on an indirect potentiometric procedure using ion selective electrodes which selectively respond to Na^+ and K^+ according to the nernst equation. Electrolyte ion concentration is therefore determined by measuring the electrolyte activity in solution. All assays were performed on a Bayer Advia 1650 autoanalyser by the Chemical Pathology Department, Southampton General Hospital, UK.

Microalbumin Creatinine Ratio(MACR)

In addition to measuring MACR as marker of outcome, IgG creatinine ratio has also been measured to confirm that changes in excretion are due to an increased permeability of the glomerulus to proteins. The low molecular weight protein α_1-microglobulin has also been measured. This has been found to be a marker of proximal tubular dysfunction as normally it is reabsorbed in the tubule. It has the advantage over $ß_2$-microglobulin, another tubular marker, in that it is stable at a low pH.[363] Results are expressed as a urinary creatinine ratio to compensate for errors due to changes in urinary flow. The other advantage is that it avoids the need for timed urine samples.

Urine albumin IgG and alpha 1 microglobulin were measured in urine by automated polyethylene glycol enhanced automated immunoturbidimetry. Antibodies, buffers and calibrants were purchased from DakoCytomation (Ely Cambridgshire UK) and measurements were made using a Cobas Mira S (Roche Diagnostics Lewes Sussex UK). Urine creatinine was measured by a modification of the Jaffe reaction on an Ilab 900 automatic analyser.

Bioelectric Impedance Analysis (BIA)

A SEAC SF3.1 bioelectric impedance machine was used to measure multiple frequency impedance. Following cleaning of the skin with alcohol wipes four electrodes were placed on the wrist/hand and ankle/foot on the same side and connected to the impedance monitor. The same side was used for all measurements on any particular patient. Three sequential measurements were taken for every patient preoperatively and daily until day 5. Resistance and reactance were measured at 124 frequencies between 4 KHz and 1024 KHz. The data was analysed using a Cole-Cole plot of measured reactance and resistance at these frequencies allowing extrapolation to estimate resistance at zero frequency (R_0) and at infinite frequency (R_{inf}). R_0 corresponds to the resistance of extracellular water (ECW) and R_{inf} represents the resistance of total body water (TBW). It follows that a falling resistance represents an increase in extra cellular or total body water. The volume of ECW and TBW are estimated by the calculated ratio of height(ht)$^2/R_0$ and ht^2/R_{inf} respectively. The phase angle was also recorded for individual frequencies of 5, 50 and 100 KHz..

Chapter 6 Methods:-Inflammatory response

Introduction

It has been previously discussed that cytokines are a significant driving force behind the development of the inflammatory response. It is well recognised that patients undergoing AAA repair have a major inflammatory response in part promoted by ischaemia reperfusion injury. The aim was to clearly define the inflammatory response in elective AAA patients.

Cytokines

Cytokines were assayed at all time points throughout the study period. The cytokines assayed were selected due to their recognised role in the systemic inflammatory response as previously described in the introduction.

All the assays were solid phase Enzyme Amplified Sensitivity Immunoassay (EASIA) performed in duplicate on microtitre plates with controls and appropriate standards to determine the standard curve. (Biosource Europe, Nivelles Belgium). The assay is based on an oligoclonal system in which a blend of monoclonal antibodies are directed against distinct epitopes (a portion of an antigenic macromolecule recognized and bound by a specific antibody) of the relevant cytokines. The use of a number of distinct monoclonal antibodies gives a highly sensitive assay with extended standard range and short incubation time. The microtitre plates are coated with capture monoclonal antibodies the sample or standards/controls are added to the plate and then incubated with a second

monoclonal antibody conjugated with horseradish peroxidase (HRP). Following washing to remove unbound enzyme labelled antibodies the bound enzyme labelled antibodies are measured with a chromogenic reaction using Tetrametylbenzidine and hydrogen peroxide. After a predetermined time the reaction is stopped with stop solution (H_2SO_4). The plates were read on a Titertekplus plate reader with dual wavelength mode. The wavelength of the test filter was 450nm and the reference filter 650nm. Standard curve was plotted and fitted by linear spline fit. (see appendix F for summary of assay procedures)

C-Reactive Protein (CRP)

Unlike other laboratory test of the acute phase response such as plasma viscosity the plasma levels of CRP mirror ongoing inflammation and tissue injury more precisely. CRP in addition does not show any diurnal variations and though liver failure may impair synthesis no other pathologies do. Only drugs that influence the pathology that is providing the stimulus for CRP production have any effect on its plasma circulating levels. These factors therefore make CRP a very useful albeit non-specific biochemical marker of inflammation.

CRP was measured preoperatively and on days 1, 3 and 5.The principle of the method is the CRP in the samples combines specifically with anti-human CRP antibodies in the buffer-antibody reagent mix forming an aggregate. The aggregate is insoluble and the increased turbidity is measured optically with the degree of turbidity being proportional to CRP concentration. Measurements were performed on a Bayer Advia 1650 autoanalyser in the department of Chemical Pathology, Southampton General Hospital, UK.

Chapter 7 Methods:-Antioxidants

Introduction

The role of antioxidants in ameliorating the effects of free radicals has been discussed in chapter 3. The aim of this part of the study was to look at the effect of surgery on antioxidant status intraoperatively and postoperatively. In addition to defining the consequence that preoperative antioxidant may have on outcome and cytokine response.

Glutathione

Whole blood for glutathione measurement was taken at the time points as previously described and stored in monobromobimane (see appendix B). A freshly prepared solution of ~62 mg Glutathione dissolved in a 100ml distilled water is made up. The molarity is calculated using a molecular weight of 307. Serial dilutions of the glutathione solution are made up in distilled water(0:5, 1:4, 2:3, 3:2, and 4:1). 10µl of each of these concentrations are diluted in 300µl monobromobimane and analysed to obtain a standard calibration curve.

The samples were defrosted and protein was precipitated using 50µl working perchloric acid which is also added to the standards(dilute stock 70% perchloric acid with distilled water 1:4 ratio. Store at 4°C). The samples were then centrifuged in a Beckman Microfuge for 5 minutes at 10,000 rpm. Aliquots of 200µl supernatant are added to an amber vial and a further 250µl of buffer A(see Appendix B) is added. The vials are capped and stored in the dark at 4°C until analysed by high performance liquid chromatography (HPLC).

Samples and standards were analysed in duplicate using a Beckman HPLC with a C18 column and fluorimeter set with an excitation wavelength 390nm with an emission wavelength of 480nm. The running buffer is composed of buffer A (Methanol 50ml, 1.0ml glacial acetic acid made up to 500ml with distilled water. The pH is adjusted to 4.0 with 1M NaOH) and buffer B (methanol 450ml and 50ml distilled water) with an initial composition of 25:75. A preset programme controls the run.

The area of the peaks for glutathione and the internal standard penicilliname are calculated and the ratio of glutathione peak to penicillinamine peak is used to calculate the glutathione concentration from the standard curve. To correct for changes in red blood cell concentration the haematocrit was measured using a Coulter Counter autoanalyser and used to express the glutathione as mmol per litre of red blood cells.

Vitamin E, Triglycerides and Cholesterol

These were measured concurrently with HPLC using a method modified from Thurman et al.[364] Vitamin E, with Tocopherol Acetate as an internal standard, is extracted from plasma with Hexane prior to reverse phase chromatography with UV detection @ 324 nm and 292 nm. (see appendix C). As Vitamin E is transported in an array of lipoproteins it is expressed as a ratio of Vitamin E:total lipid.[365]

The triglycerides are converted to glycerol and free fatty acids by lipoprotein lipase. The glycerol is then converted to glycerol-3-phosphate by glycerol kinase in the presence of glycerol-3-phosphate-oxidase to form hydrogen peroxide. A coloured complex is formed from hydrogen peroxide, 4-aminophenazone and 4-chlorophenol

under the catalytic influence of peroxidase. The absorbance of the complex is measured as an endpoint reaction at 505 nm.

The cholesterol esters are hydrolyzed by cholesterol esterase to cholesterol and free fatty acids. The cholesterol is converted to cholesterol-3-one by cholesterol oxidase in the presence of oxygen to form hydrogen peroxide. A colored complex is formed from hydrogen peroxide, 4-aminoantipyrine and phenol under the catalytic influence of peroxidase. The absorbance of the complex is measured as an endpoint reaction at 505 nm. Both assays were performed on a Bayer Advia 1650 autoanalyser by the Chemical Pathology Department, Southampton General Hospital, UK.

Carotenoids

Carotenoids are extracted from serum with Hexane after deproteinisation with Methanol. The artificial carotenoid , Ethyl B-apo-8'-carotenate(trans) is used as an internal standard. After reverse phase chromatography, the carotenoids are quantified by spectrophotometric detection @ 453nm (see appendix D). Only β-carotene can be measured with any accuracy as this the only one of the measured carotenoids with a calibration curve standard. However α-Carotene, lycopene and β- cryptoxanthin absorb @ 453nm the same as β-carotene and thus their value can be estimated from the chromatogram. Though this is not an accurate value it is likely to be close to the true value and it allows the monitoring of the trend in their values post operatively.

Albumin, Bilirubin and Urate

Albumin is known to bind to certain dyes. The peak absorption wavelength of bromocresol purple shifts when its bound to albumin. Plasma is diluted with buffered bromocresol purple at pH 5.2 and the increase in absorbance is measured at 596nm.

The measurment of bilirubin is based on the technique described by Van den Bergh and Muller in 1916. Caffeine releases any protein bound unconjugated bilirubin making it soluble in solution. This allows for the rapid reaction of both conjugated and unconjugated bilirubin with diazo sulphanilic acid at low pH to produce azobilirubin. The absorbance of azobilirubin is measured as an endpoint reaction at 545nm.

Urate measurement is based on the methods suggested by Trinder[366] and Fossati.[367] Urate is oxidised by uricase to allantoin and hydrogen peroxide. A coloured complex is formed from hydrogen peroxide, 4-aminphenazone and N-ethyl-N-(2hydroxy-3-sulphopropyl)-3-methylaniline under the catalysis of peroxidase. This is an endpoint reaction with the absorbance being measured at 545nm.

All three assays were performed on a Bayer Advia 1650 autoanalyser by the Chemical Pathology Department, Southampton General Hospital, UK.

Chapter 8 Study Design

Aims

The primary aim of this study was to determine whether preoperative antioxidant status has a role in the development of the systemic inflammatory response syndrome in patients undergoing elective abdominal aortic aneurysm repair. The secondary aim of the study was to measure the longitudinal postoperative physiological, biochemical and haematological changes that occur in patients following elective AAA surgery.

Hypothesis

Currently, there is no evidence to demonstrate that baseline preoperative antioxidants have an influence on the inflammatory response. Therefore, the null hypothesis for this study is that individual baseline antioxidant concentrations do not affect the inflammatory response or outcome in postoperative elective abdominal aortic aneurysm patients.

Study Design

Twenty patients with abdominal aortic aneurysm requiring elective open surgical repair were recruited. The study design was approved by Southampton University Hospitals Local Research Ethics Committee and all patients were enrolled following fully informed written consent.

Patients were recruited when all inclusion and no exclusion criteria were present.

Inclusion critera

1. Adult male or female patients undergoing elective open infrarenal abdominal aortic aneurysm repair.

Exclusion Criteria

1. Diseases requiring high dose corticosteroid i.e 20mg Prednisilone or equivalent.
2. Regular non steroidal inflammatory drug use in two weeks prior to abdominal aortic aneurysm repair.
3. On any investigative drug.
4. Concurrent Malignancy.

All patients were investigated preoperatively and until five days postoperatively.

All patients had their operations under a standardised protocol for general anaesthesia. An epidural was inserted for post operative analgaesia with bupivicaine and fentanyl. Intraoperative and post operative monitoring was via an arterial line inserted in the right or left radial artery for blood pressure and central venous pressure(CVP) via a catheter placed in the internal jugular vein. A further one or two intravenous catheters were placed in the right or left arm for intravenous fluids. A urinary catheter was inserted following anaesthesia to monitor urine output. At induction of anaesthesia all patients received intravenous antibiotic prophylaxis with metronidazole, gentamicin and fluclocloxacillin or cefuroxime (if patient penicillin sensitive). Induction of anaesthesia was with propofol, fentanyl and midazolam. Intraoperative muscle relaxant was with the non depolarising neuromuscular blockers vecuronium or rocuronium. Maintenance of anaesthesia throughout the operation was with sevoflurane.

Abdominal wall incisions were either transverse or midline. Following exposure of the aneurysm at the top end and the bottom end 5000 units of heparin was administered two minutes prior to crossclamping of the aorta. In this series the clamps were all placed infra renal. In all cases a Dacron tube or bifurcated graft was used to repair the aneurysm. Any intraoperative fluid given was either crystalloid (Hartmans or 0.9% saline) or colloid (gelofusin only). Blood loss was replaced with autologous blood, which was collected via a cell saver and returned to the patient as red blood cells following washing with 0.9% saline. Additional blood, if required was transfused following crossmatching for recipient compatibility. Coagulation factors in the form of group specific fresh frozen plasma and cryopreciptate were given to those patients with a significant blood loss and therefore potentially at risk of coagulopathy. All drugs given intraoperatively usually to maintain cardiovascular function were recorded. To standardise the protocol Mannitol was to be given to all patients at a dose of 5mls/Kg, in some cases this did not occur but this has been recorded.

All patients were admitted to the intensive therapy unit (ITU) post operatively. Patients remained in ITU until they were fit enough to be discharged to the surgical high dependency unit or back to the ward. All patients remained nil by mouth until the presence of bowel sounds or passage of flatus. Fluids only were given initially but diet was introduced quickly if fluids were tolerated usually by day 3.

Pre-operatively demographic data was documented. Risk factors and comorbidity were also documented from the patient or patient's notes. Size and type of aneurysm was determined from a preoperative Computed Tomography (CT) angiogram. Bioelectric impedance (BIA) measurements were taken daily. Blood and urine samples were taken at

the following time intervals, preoperatively, prior to removal of clamp, twenty minutes post clamp, 6 hours postoperatively and then daily to day five (see Appendix E). The time point for clamp release was just prior to reperfusion of the legs. In the case of bifurcated graft insertion it was just prior to reperfusion of the first leg. The post clamp time was 20 minutes following reperfusion or reperfusion of the first leg. Samples were prepared and stored in 2.0 millilitre Eppendorfs. Further data was recorded intraoperatively and postoperatively (see table 5).

Preoperative data	**Intraoperative data**	**Postoperative data**
Demographics	*Length of operation*	*SIRS criteria*
Age	*Anaesthetic agent*	*APACHE score (ITU)*
Weight	*Drugs used*	*SOFA score*
Height	*Clamp time*	*Microbiology*
Smoking history	*Blood loss*	*Clinical outcome*
Risk Factors and Comorbididity	*Blood transfused*	*Fluid balance*
Diabetes	*Cell saved blood used*	*Length of stay (ITU/HDU)*
Ischaemic heart disease	*Colloid used*	*Length of stay*
Cerebrovascular disease	*Crystalloid used*	
Hypertension	*Mannitol*	
Hypercholesterolaemia		
Other medical history		
Drug history		
Aneurysm (type and size)		

Table 5 Data recorded during the study

Sample Preparation

Blood samples were taken from either the arterial line or central venous line preoperatively and at the other time points until they were removed. Blood samples were then taken from a vein in the ante-cubital fossa until day 5. The samples were collected into bottles containing ethylene diamine tetra-acetic Acid (EDTA) as an anticoagulant and immediately placed on ice until sample preparation, this was within 45 minutes (See Appendix E).

Chapter 9 Statistical Analysis

All measurements were assessed for normal distribution using the Shapiro-Wilks test. Normally distributed values were expressed as means with standard error. An attempt was made to transform data that was not normally distributed with natural log or square root transformation. Normally distributed longitudinal data was analysed using repeated measures analysis of variance with post hoc pairwise comparisons using least square difference and bonferroni correction. For the comparison of two sets of continuous data Pearson's correlation were performed. One way ANOVA was used to examine the difference between means for three or more grouped variables with the independent t-test used to determine a difference in means for two grouped variables and for pairwise comparison of three or more grouped variables. Levene's test was used to show whether there was equal variance between groups and results expressed appropriately.

Data that is not normally distributed is expressed as median with minimum and maximum values to show the range. Longitudinal data was analysed using Friedman's test to see if there is a difference in mean ranks. The relationship between two sets of continuous data was examined with the non parametric Spearman Rho correlation. The difference between the mean ranks for three or more grouped variables was examined using Kruskal-Wallis test. Pairwise comparisons of two or more grouped variables were analysed using the Mann-Whitney U test with and without Dunns correction of the alpha value.

Data is also represented graphically either as line plots with the data points being plotted with standard error bars or boxplots which show the median, interquartile range, outliers,

and extreme cases of individual variables. Scatterplots are used to show the association between two variables.

Other statistical test performed that are different from the above are described in the results. The level of significance for rejection of the null hypothesis was set at $p<0.05$. All statistical analysis were performed using SPSS 12.

Chapter 10 Results

Demographics

Twenty patients, nineteen males and one female with asymptomatic infrarenal abdominal aortic aneurysms requiring elective surgery were recruited between August 2001 and March 2002. Baseline clinical characteristics and demographic data are tabulated below.

Table 6 Baseline Demographic and Clinical data

Variable	Patients (n=20)
Age*	71.5(62-77)
BMI*	26.9(20-32)
Aneurysm size (mm.)*	60.5(49-105)
Current smoker	5 (25%)
Ex-smoker	13 (65%)
Diabetic	1 (5.0%)
Hypercholesterolaemia	6 (30%)
Hypertension	12 (60%)
Ischaemic Heart disease	7 (35%)
Cerebrovascular disease	3 (15%)
Pulmonary disease	2 (10%)
Chronic renal failure (creatinine >120 μmol/l)	3 (15.0%)
Peripheral vascular disease	3 (15%)

* Median with range

Intraoperative data

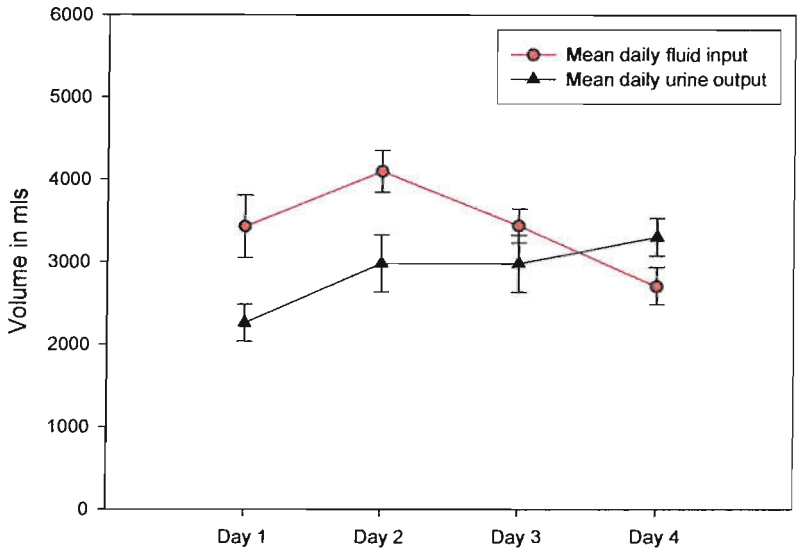
6 patients with an aortoiliac aneurysm required repair with a Dacron trouser graft the remaining fourteen patients were repaired with a Dacron tube graft. The mean time for induction of anaesthesia to starting surgery was 79 minutes (se 4.36). The median operative time was 177.5 minutes (range 140-460) with a median clamp time of 72.5

minutes (range 35-210). The median blood loss was 2100 mls (range 457-15712); a cell saver was used in 16 patients with a median autologous blood return to the patient of 466.5 mls (range 0-2736). Thirteen patients received cross-matched blood with a median transfusion of 2 units. All patients were given the colloid gelofusin with a mean infused volume of 3.285 litres (se 2.506). All patients had crystalloid with a mean infused volume of 1.495 litres (se 0.162). Mannitol was given to 13 patients prior to clamp release with a median volume of 355mls (range 0-465).

Postoperative fluid balance

The postoperative daily fluid balance was recorded. The cumulative mean fluid input up to day 4 postoperatively was 14737.43mls (se=822.81). This compared with a total mean urine output of 11759.43 mls (se= 1168.07) over the same period. The cumulative fluid balance showed a positive mean of 2628.93 mls (se= 1770.05). Using a paired samples t test there was no significant between the cumulative mean input and output t=1.477 (df 13) (95% CI -1208.58 to 6436.58) p=0.163. The mean daily fluid input and output was 3468.52 (se=148.09) v 3089.68 (se=175.64) with no significant difference between the mean input and output (p=0.146). However, using a paired samples t test to compare the individual daily means there is a significant difference between input and output on day 1 (t=2.776 (df 18) (95% CI 285.5 to 2061.7) p=0.012) and day 2 (t=2.186 (df 19)(95% CI 247.6 to 2189.9) p=0.042). The daily values for mean input and output are demonstrated graphically (see Graph 1)

Graph 1:-Plot of the mean daily fluid input and output with standard error



Haemoglobin

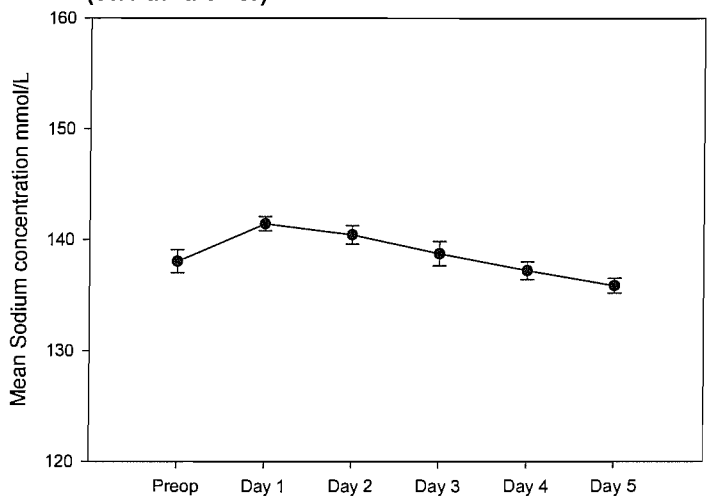
The mean preoperative haemoglobin was 139.21 g/l (se 2.75) which postoperatively on day 1 had fallen to a mean of 104.15 g/l (se 3.23). This fall persisted to day 5 with a mean Hb of 108.50 g/l (se 3.55). Using a paired samples t test with Bonferroni correction there is a significant difference between preoperative and day 1 to day 5 haemoglobin concentration $p<0.0005$.

Electrolytes

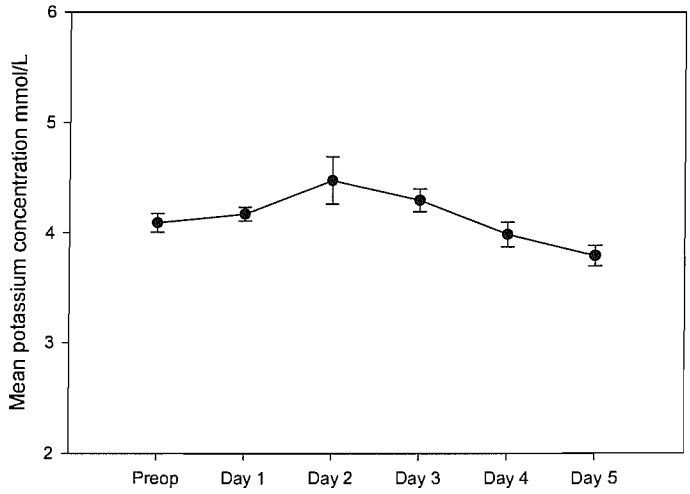
Postoperatively on day 1 the mean Sodium had risen to 141.6 mmol/l (se 0.65) from a preoperative mean of 138.0mmol/l (se 1.04) this difference was significant with the paired t test $p<0.0005$. Over the next four days the Sodium fell gradually to a mean of 135.9mmol/l (se 0.658), this again was significantly different from the preoperative value $p=0.044$ with Bonferroni correction (see Graph 2). Potassium showed a similar pattern to

Sodium rising postoperatively peaking at a mean of 4.47 mmol/l (se 0.21) on day 2 from a preoperative mean of 4.09 mmol/l (se 0.09) before falling to mean of 3.78mmol/l (se 0.09) on day 5. There was no significant difference between the preoperative and other daily values. (see Graph 3)

Graph 2:-Daily Mean Sodium Concentration (standard error)



Graph 3:- Daily mean potassium concentration (standard error)

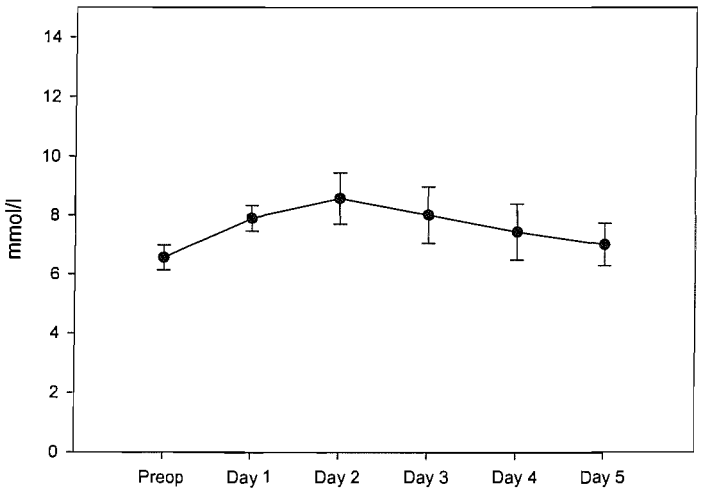


Renal Function

Renal function was monitored on a daily basis with the measurement of urea, creatinine

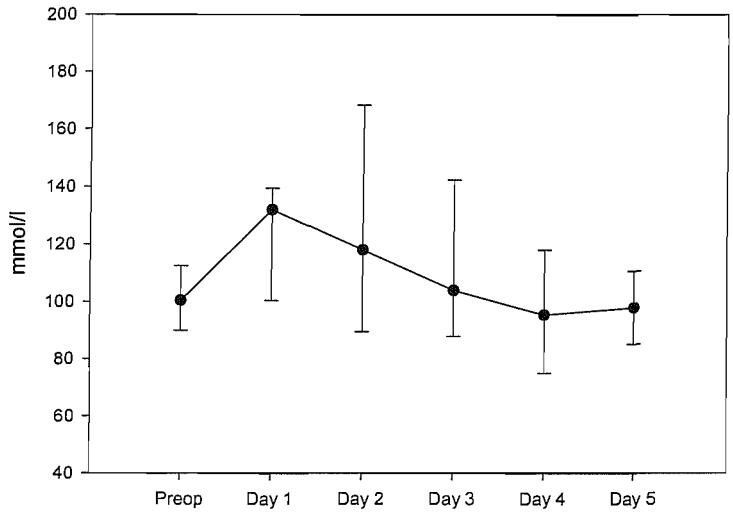
and α1-microglobulin creatinine ratio. The 3 patients with chronic renal failure were included as there was no significant difference in the results if they were excluded. Both urea and creatinine concentrations increased initially postoperatively before falling. Mean preoperative urea was 6.57 mmol/L (se 0.42) which rose postoperatively to a peak mean of 8.58 mmol/L (se 0.87) on day 2. The mean urea concentration then fell to a mean of 7.02 mmol/L (se 0.72) by day 5 (Graph 4). Repeated measures analysis of variance suggest there is no significant difference in the daily urea concentrations within subjects (F=3.043 (df 2.1 27.2) p=0.062 with Huyn-Feldt correction). However, pairwise comparisons show that the mean difference is significant between preoperative and day 1 urea concentration p=0.038 with Bonferroni correction.

Graph 4:-Daily mean urea concentration (standard error)



The median preoperative creatinine was 100.5 μmol/L (range 78-166) rising to a median creatinine of 132.0 μmol/L (range 88-225) by day 1 falling to a median of 98.0 μmol/L (range 62-214) by day 5 (Graph 5). Friedman test showed that there is a significant difference in the mean ranking of the daily creatinine concentration (χ^2=38.67 df 5 p<0.0005).

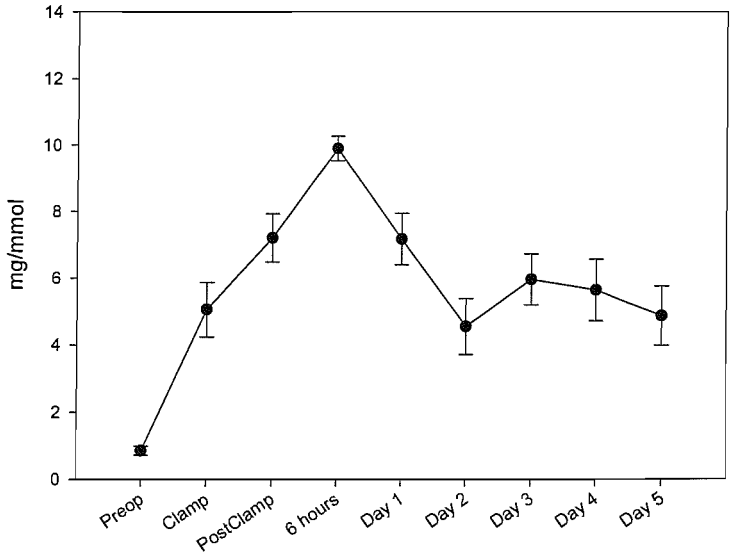
Graph 5:- Daily median creatinine concentration (interquartile range)



Renal Function:-Proximal Tubule

The urinary α1- microglobulin creatinine ratio preoperative mean was 0.86 mg/mmol (se 0.13) with a peak mean of 9.9 mg/mmol (se 0.37) at 6 hours. There is a subsequent fall to a mean of 4.56 mg/mmol (se 0.83) by day 2, rising again on day 3 (mean 5.96 mg/mmol (se 0.76)) then gradually falling to a mean of 4.86 mg/mmol (se 0.88) on day 5 (Graph 6). Repeated measures shows that there is a significant difference in the daily ratios $F=12.9$ (df 4.26 59.6) $p<0.0005$ with Huyn-Feldt correction. Pairwise comparison demonstrates that this significant difference between the preoperative mean ratio and the other time points is maintained throughout the five days postoperatively using Bonferroni adjustment ($p<0.0005$ to $p=0.008$) except for day 2 ($p=0.092$).

Graph 6:-Mean α1-microglobulin creatinine ratio (standard error)



Proximal tubule function and aneurysm type

Spearman rho correlation demonstrated a moderate correlation between α1- microglobulin creatinine ratio and clamp time at the end of clamping (r_s=0.555 p=0.014) and post clamp (r_s=0.744 p<0.0005). Aneurysm's were divided into two types by CT angiography, juxtarenal where the aneurysm is closely apposed to the renal arteries and infrarenal where there is a length of non aneurysmal aorta before the renal arteries. In juxtarenal aneurysm's the clamp is likely to be placed close to the renal arteries if not above them. To examine the effect of aneurysm type an independent t-test was used to compare juxtarenal and infrarenal with α1- microglobulin creatinine ratio post clamp, 6 hours, day 1 and day 2. There was no significant difference in mean α1- microglobulin creatinine ratio between the groups postclamp, at 6 hours and at day 2. On day 1 the mean juxtarenal α1- microglobulin creatinine ratio was 10.04 mg/mmol (se 1.35) v the infrarenal mean of 6.21 mg/mmol (se 0.81) which was significant t=2.393 (df 18) p=0.028. The interaction between aneurysm type and clamp time was performed using multivariate ANOVA with

aneurysm as a fixed factor and clamp time as a covariate this showed no significant interaction effect (Wilks lambda F=1.196 (df 10 ,22) p=0.345).

Proximal tubule function and Mannitol

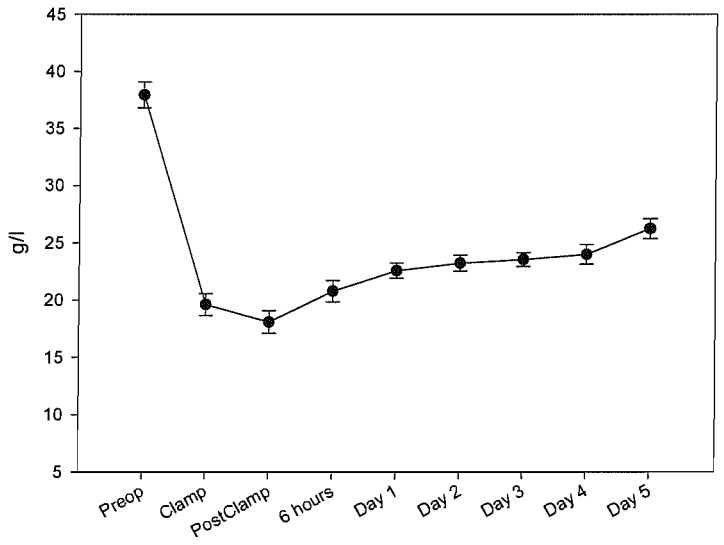
An independent t-test was used to determine if there was a significant mean difference in the α1- microglobulin creatinine ratio in those receiving mannitol and those not. The Levenes test for equality of variance was not significant at the post clamp (p=0.252) and 6 hour (p=0.342) time points and so equal variance was assumed. Both the post clamp and 6 hour α1- microglobulin creatinine ratio demonstrated a significant difference between the two groups. The mean in those not receiving mannitol post clamp was 14.81 mg/mmol (se 2.74) compared to a mean of 8.62 mg/mmol (se 1.16) in the recipients of mannitol (t=2.44 (df 18) p=0.025. At 6 hours the mean for the non recipients of mannitol was 60.18 mg/ml (se 6.9) v 29.48 mg/ml (se 3.12) for the mannitol group (t=4.68 (df 16) p<0.0005).

Albumin

Plasma albumin was measured at all time points with a mean preoperative concentration of 37.95 g/l (se 1.13). Intraoperatively albumin fell, this was maximal post clamp (mean 18.10 g/l (se 0.992)) with a subsequent gradual increase in mean value over the following time points reaching a mean of 26.25 g/l (se 0.864) by day 5 (Graph 7). Repeated measures analysis of variance with Huyn-Feldt correction demonstrates that there is a significant difference in the daily concentrations F=58.58 (df 5.0, 90.04) p<0.0005. Pairwise comparison with Bonferroni correction shows that preoperative albumin is significantly different to all time points (p<0.0005).

The association of intraoperative blood loss with albumin and albumin change post clamp, 6 hours and day 1 was examined with Spearman rho as blood loss is not normally distributed. The association of albumin change showed a significant negative correlation from post clamp to day 1 but decreasing in the strength of correlation. (r_s =-0.757 $p<0.0005$, r_s =-0.657 p=0.002 and r=-0.467 p=0.044). The actual values of albumin showed only a significant correlation post clamp r_s=0.720 p=0.001 and at 6 hours r_s=-0.608 p=0.006 but not for day 1 albumin r_s=-0.253 p=0.296.

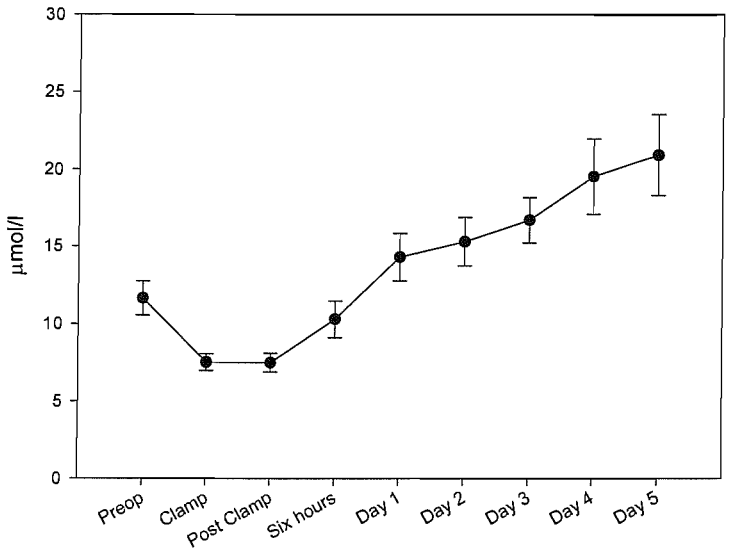
Graph 7:-Mean plasma albumin concentration (standard error)



Bilirubin

The mean preoperative bilirubin was 11.65 μmol/l (se 1.10) and as with albumin, bilirubin fell to a mean of 7.50 μmol/l (se 0.61) post clamp. It gradually increased postoperatively reaching a mean of 20.90 μmol/l (se 2.63) by day 5 (Graph 8). The sphericity assumption was not met so with Huyn-Feldt correction there is a significant difference in the daily concentrations F=14.34 (df 4.07, 73.30) $p<0.0005$. In post hoc analysis using pairwise comparison with Bonferroni correction preoperative bilirubin was only significantly different to day 3 (p=0.041).

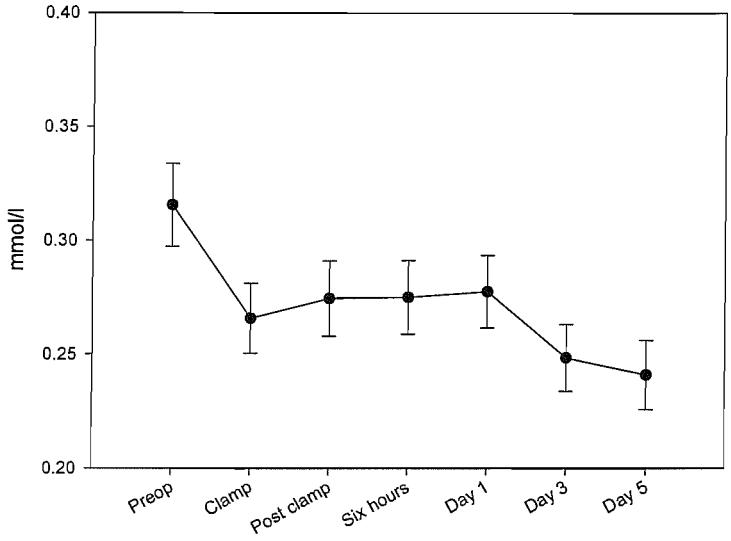
Graph 8:- Mean plasma bilirubin concentration (standard error)



Urate

Urate had a preoperative mean of 0.315 mmol/l (se 0.018) falling to a mean of 0.266 mmol/l (se 0.015) prior to clamp release with a further fall on day 3 to 0.249 mmol/l (se 0.015)(Graph 9).Repeated measures ANOVA with Huyn-Feldt correction shows a significant difference in the means $F=9.32$ (df 2.84 45.44) $p<0.0005$. Post hoc test using pairwise comparisons with Bonferroni correction demonstrate that the significant difference is between preoperative urate and all other time points ($p<0.0005$ to $p=0.042$). No other time points show any significant mean differences.

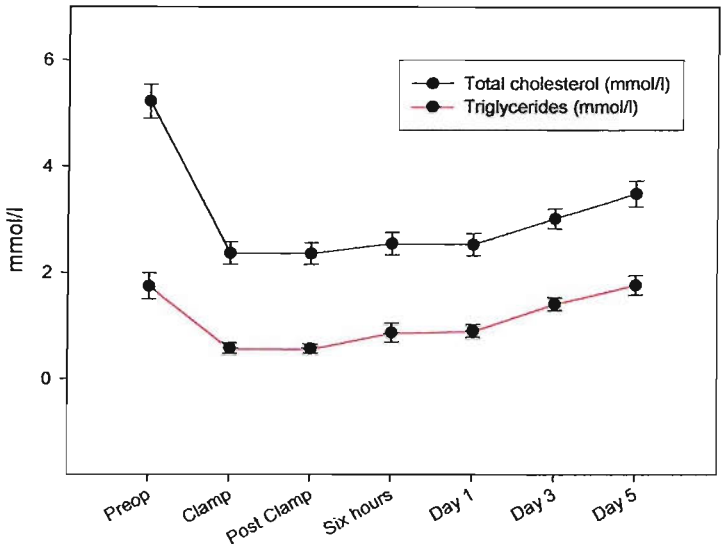
Graph 9:- Mean plasma urate concentration (standard error)



Cholesterol and Triglycerides

The mean preoperative total cholesterol was 5.22 (se 0.32) mmol/l with a rapid fall prior to clamp release reaching a trough mean of 2.35 (se 0.20) mmol/l following release of the clamp. There was trend back to baseline from day 3 with a mean of 3.02 (se 0.19) mmol/l rising to mean of 3.49 (se 0.24) mmol/l on day 5. Triglycerides showed the same pattern as total cholesterol reaching a minimum median value of 0.465 (range 0.29 to 2.10) mmol/l from preoperative median of 1.40 (range 0.87 to 5.89) mmol/l. The median value rose to 1.24 (range 0.75 to 2.67) mmol/l by day 3 and reached baseline with a median of 1.57 (range 0.86 to 3.93) mmol/l by day 5.

Graph 10:- Mean concentration of total cholesterol and triglycerides (standard error)



Repeated measures ANOVA demonstrates a significant difference between the means for total cholesterol F=49.12 (df 3.6, 57.1) p<0.0005. Post hoc pairwise comparison with Bonferroni correction shows a significant mean difference between the preoperative value and all other time points (p<0.0005). There is no significant difference in the means between clamp, post clamp, 6 hours and day 1. Day 3 shows a significant mean difference with day 5 (p<0.0005) but not with day 1 (p=0.458) and 6 hours (p=0.093). Friedmans test showed a significant difference in the mean ranks for triglyceride at all measured time points χ^2=78.45 (df 6) p<0.0005. Post hoc pairwise comparison was performed using Wilcoxon signed ranks which showed difference in mean ranks between the preoperative and all other time points (p<0.0005 to p=0.010) except day 5 P=0.794).

Outcome and response to surgery

Nineteen patients survived to discharge with a single patient dying 19 days postoperatively of MODS. The median length of stay was 12 days (range 8-26) with a median intensive therapy unit (ITU) stay of 25 hours (range 4-103). Fourteen patients required additional

care on the surgical high dependency unit before being discharged to the ward with a median stay of 1 day (range 0.5-10). Six patients developed postoperative complications. One patient developed bi-basal pneumonia confirmed on chest x-ray requiring respiratory support with Bi-level Positive Airway Pressure (BiPAP) eventually growing a gram negative bacillus from his sputum. He required 7 days on the high dependency unit (HDU) before being discharged home 12 days after admission. A second patient also developed bi-basal pneumonia confirmed on chest x-ray no causative organism was grown. He required respiratory support with Continuous Positive Airway Pressure (CPAP), remaining on HDU for 10 days with a total length of stay of 20 days. A further patient developed a urinary tract infection with coliforms being grown in a mid stream urine specimen. He was treated with oral antibiotics (trimethoprim).

Myocardial infarction was diagnosed in 2 patients. The first developed left ventricular failure 3 days post operatively with ischaemic changes on an electrocardiogram (ECG) with subsequent confirmation with an elevated Troponin I (a specific marker of cardiac muscle injury). He was discharged on day 11. The second patient developed atrial fibrillation (AF) 2 days postoperatively which was rate controlled with digoxin. A subsequent Troponin I confirmed a probable myocardial infarction being the cause of the AF. His total length of stay was 11 days.

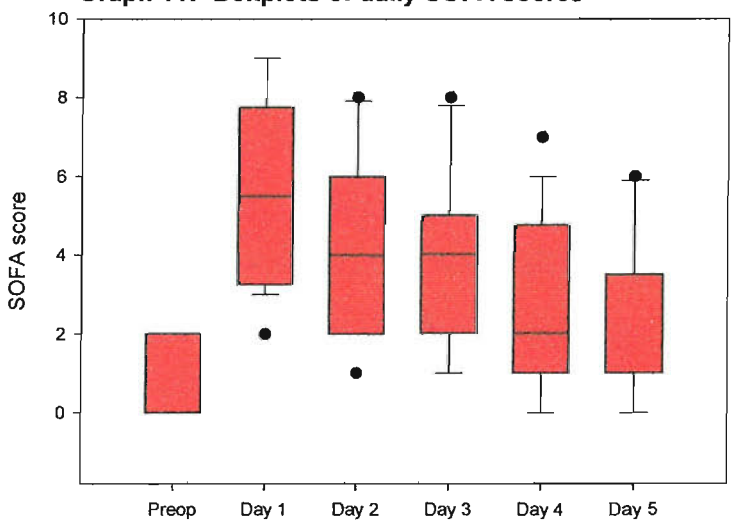
The patient who died remained on the HDU postoperatively for four days before returning to the ward. 6 days later he developed AF, hypotension, increasing oxygen requirements, pulmonary oedema and falling urine output with deteriorating renal function. He was readmitted to HDU where he improved before developing acute renal failure and acute respiratory distress syndrome (ARDS), dying 7 days after is readmission to HDU. The

cause of death at post mortem was MODS with evidence of a spontaneous subdural haematoma.

Physiological scores

The mean APACHE II score at 24 hours was 16.00 (range 11-27) with a median predicted in hospital mortality of 10.6% (range 5.4-37). The median SOFA score at twenty-four hours was 5.50 (range 2 to 9). Over the study period there was a downward trend in the median SOFA score but it did not reach the preoperative score, the median score at day 5 was still 2.0 (range 0-6)(see graph 11).

Graph 11:- Boxplots of daily SOFA scores

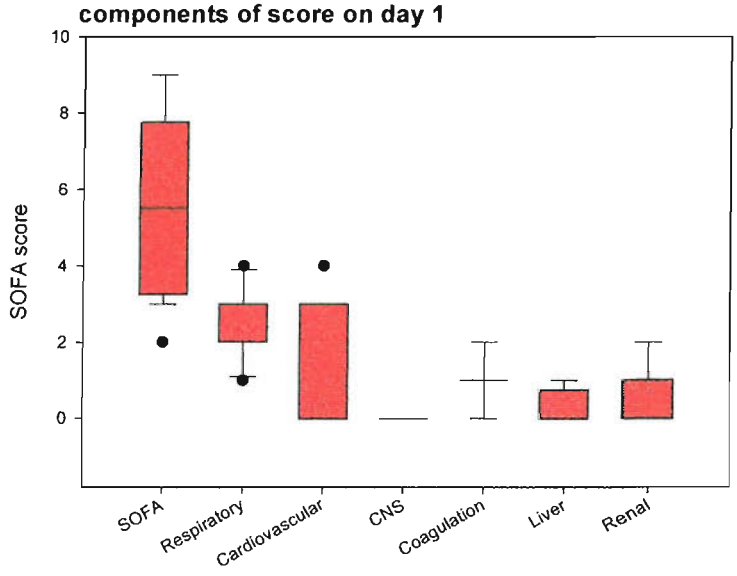


Friedman test confirms that there is a significant difference in the mean ranks χ^2=64.79 (df 5) p<0.0005. Using wilcoxon signed rank test for pairwise comparison of preoperative SOFA score to the other daily SOFA scores there is a significant difference to day 4 (p<0.0005 to P=0.001 exact). Preoperative to day 5 pairwise comparison using exact significance p=0.011 with Dunns correction for multiple comparisons the α value is 0.003 and therefore the comparison is not significant. There is a positive moderate correlation

between SOFA score at 24 hours and APACHE II score (r_s=0.489 p=0.035) SOFA and APACHE II scores were examined with the Mann-Whitney test to see if there were any difference in the mean ranks between those patients who had septic complication and those who had none. APACHE II showed no significant difference between the mean ranks(U=11.0 p=0.296). The mean ranks for the sepsis group were significantly higher than the no complications group for SOFA AUC (U=8.5 p=0.045), SOFA 24 hours (U=8.5 p=0.045), SOFA day 4 (U=6.0 p=0.023) and SOFA day 5 (U=7.5 p=0.032). Though the trend was still there for day 2 and day 3 the difference in mean ranks were not significant (U=18.0 p=0.412 and U=11.5 p=0.102).

Respiratory SOFA score makes the most significant contribution to the total SOFA score (see graph 12). The median respiratory SOFA score was 2.0 (range1-4) on day 1 postoperatively and remained at this level until day 4 when the trend was downward towards 0 on day 5. The median SIRS score on day 1 and day 2 was 1.0 (range 0-3) and 0.5 (Range 0-2) on day 3 and 4 returning to a 0 on day 5. Only 5% of patients had a SIRS score of 3 on day 1 and 2. On day 1, 5% of patients scored 2 increasing to 25% by day 2 and decreasing to 20%, 10% and 0% on day 3, 4 and 5 respectively.

Graph 12:- Boxplot of SOFA score and individual components of score on day 1



Bioelectric impedance

The coefficient of variation between the 3 sequential measurements at each time point for R_0 and R_{inf} was a mean of 0.54%. Using BIA, R_0 which is representative of extracellular water was measured on a daily basis. The mean daily R_0 values are tabulated below in table 9.

Table 7 Daily mean Ro values with standard error

	Mean(Ω)	Std. error
Preoperative	565.9	15.6
Day 1	496.1	18.4
Day 2	457.0	18.1
Day 3	427.6	18.1
Day 4	440.5	23.7
Day 5	458.2	20.7

The repeated measures analysis of variance shows that there is a significant difference in the daily R_0 BIA measurement $F=16.063$ (df 2.88, 43.13) $p<.0005$ with Huyn-Feldt correction. Using post-hoc testing with Bonferroni correction there is a significant mean difference between preoperative R_0 and day 1 R_0 $p=0.047$. This significant difference persist between the preoperative R_0 value and all subsequent time points ($p<0.0005$ to $p=0.014$). Day 1 R_0 also has a significant mean difference in comparison to day 2 and day 3 R0 ($p=0.015$ and $p=0.001$) with no significant mean difference in contrast to day 4 and day 5 ($p=0.177$ and $p=1.00$) (see graph 13)

The ratio of height^2(ht^2)/R_0 is also representative of ECW and was calculated at all daily time points(see graph 14). The mean daily value rose to a mean of 70.97 cm^2/Ω (se 2.97) from a preoperative mean of 52.59 cm^2/Ω (se 1.45) then falling to mean of 66.62 cm^2/Ω (se 3.10) by day 5. Repeated measures ANOVA with no assumption of sphericity shows

that there is a significant differences in the daily means F=16.43 (df 2.90, 43.33) p<0.0005 with Huyn-Feldt correction. Post hoc testing with Bonferroni correction using pairwise comparisons shows a significant difference between the preoperative mean and all other daily means (p<0.0005 to p=0.47). The day 1 mean value also shows a significant difference in comparison to day 2 and day 3 mean values (p=0.006 and p=.002) but not day 4 and day 5 (p=0.078 and p=0.867). There is no significant difference in comparison of day 3, day 4 and day 5.

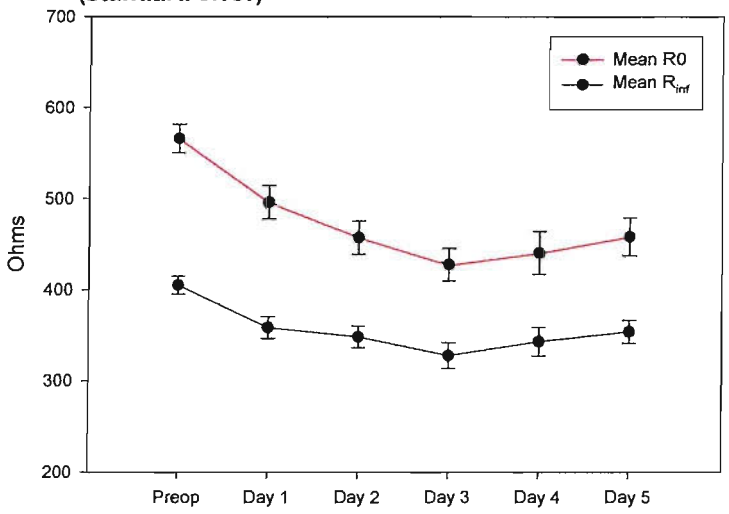
Total body water is represented by the measurement of R_{inf}, the mean daily values are tabulated below in table 10.

Table 8 Daily mean R_{inf} values with standard error

	Mean(Ω)	Std. error
Preoperative	404.9	9.9
Day 1	358.3	12.0
Day 2	348.1	11.7
Day 3	327.5	14.0
Day 4	343.0	15.7
Day 5	353.9	12.6

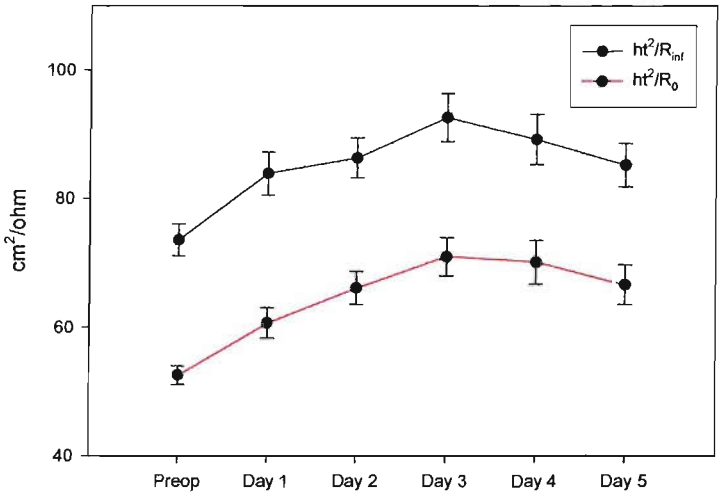
Sphericity is not assumed but with Huyn-Feldt correction there is a significant difference in the daily R_{inf} measurements F=9.691 (df 3.2, 48.67) p<.0005. The trend in the mean R_{inf} is downward until day 3. Using pairwise comparisons with Bonferroni correction as post hoc tests, there is a significant mean difference between preoperative R_{inf} and day 1 (p=0.022) which remains across all time points (p=0.003 to p=0.047). There are no significant mean differences between the other time points (see graph 13)

Graph 13:- Daily mean R_0 (ECW) and R_{inf} (TBW) (standard error)



A further estimate of total body water was calculated using ht^2/R_{inf} over the measured time points(see graph 14). The mean preoperative ht^2/R_{inf} was 73.58 cm^2/Ω (se 2.47), this increased to a mean value of 92.61 cm^2/Ω (se 3.74) by day 3 subsequently falling to a mean of 85.19 cm^2/Ω (se 3.39) by day 5. With Huyn-Feldt correction there is significant differences in the daily mean values $F=9.32$ (df 3.23, 48.48) $p<0.0005$. Using pairwise comparisons with Bonferroni correction the mean difference between the preoperative mean and the day1 to day 4 mean values are significant ($p=0.005$ to $p=0.042$). There is no significant difference between the preoperative and day 5 mean values ($p=0.057$). There is no significant difference between the mean values at any of the other time points.

Graph 14:- Daily means of ht^2/R_{inf} (TBW) and ht^2/R_0 (ECW) (standard error)



The ratio of ECW/TBW is represented by the calculated ratio of R_0/R_{inf} the median values in this case are tabulated (table 11) below due to a significant Shapiro-Wilk test of normality.

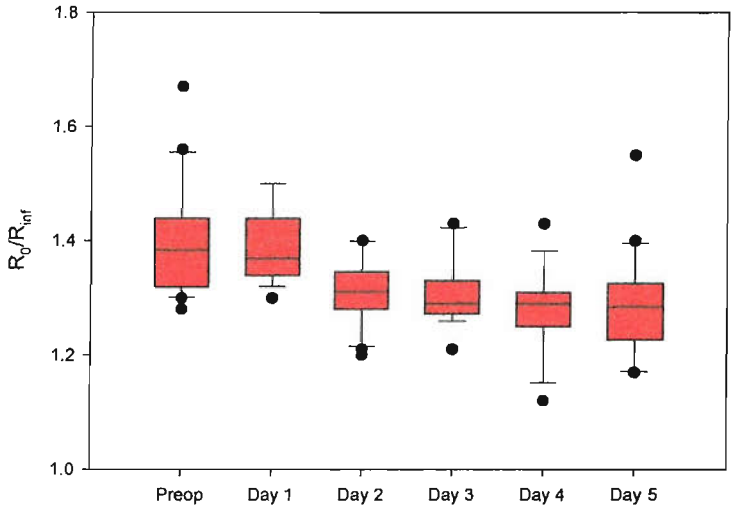
Table 9 The median daily Ro/R_{inf} ratio with range

	Median	range
Preoperative	1.399	1.28 to 1.67
Day 1	1.372	1.30 to 1.50
Day 2	1.312	1.20 to 1.40
Day 3	1.293	1.21 to 1.43
Day 4	1.287	1.12 to 1.43
Day 5	1.286	1.17 to 1.55

Following natural log transformation one way repeated measures ANOVA with the assumption of sphericity demonstrated that there was a significant difference in the daily measurement of the R_0/R_{inf} ratio $F=8.36$ (df 5, 75) $p<0.0005$. However post hoc testing showed that there was no significant difference between preoperative R_0/R_{inf} and day 1 R_0/R_{inf} ($p=1.00$) but the mean difference became significant between the preoperative R_0/R_{inf} and day 2 and day 4 R_0/R_{inf} ($p=0.035$ and $p=0.021$)) with Bonferroni correction.

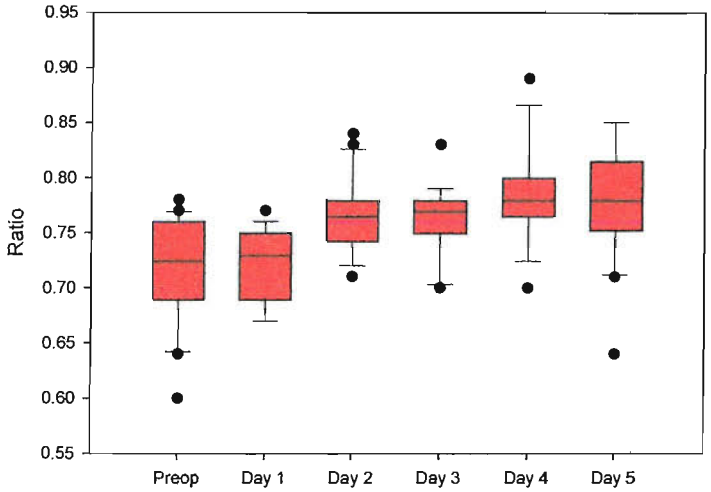
Day 1 R_0/R_{inf} had a significant mean difference between day 2 and all subsequent time points (p=0.002 to p=0.044). There was no significant mean difference in comparison of day 3, day 4 and day 5 (p=1.00).

Graph 15:- Boxplot of daily R_o/R_{inf} ratio



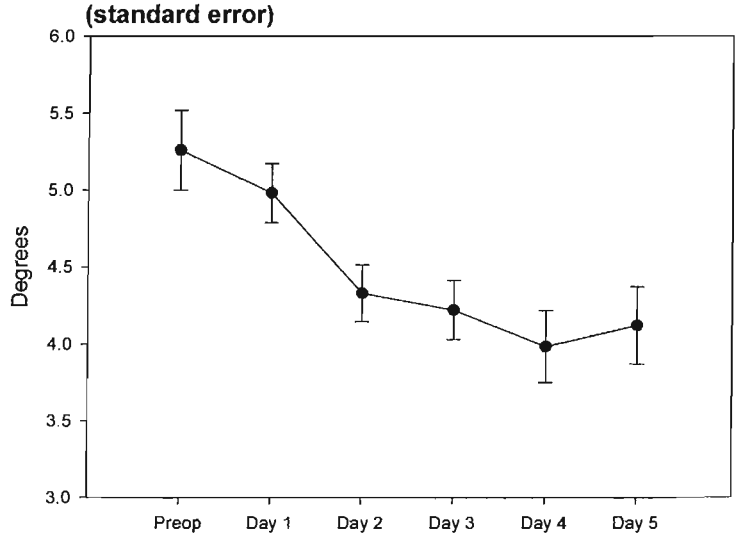
The ratio of $(ht^2/R_0)/ (ht^2/R_{inf})$ is a further representation of ECW/TBW and was calculated at all daily time points. The mean preoperative ratio was 0.718 (se 0.01) rising to mean ratio of 0.784 (se 0.01) by day 4 before falling to a mean ratio 0.779 (se 0.01) on day 5. Repeated measures with an assumption of sphericity shows a significant difference in the mean daily ratios F=8.493 (df 5, 75) p<0.0005). The mean difference between the preoperative mean daily ratio only becomes significant between day 2 and day 4 (p=0.030 and p=0.019) with Bonferroni correction. The day 1 mean ratio is significantly different to all the following days mean ratios (p=0.002 to p=0.040). There is no significant difference in the mean daily ratio for the day 2 to day 5 time points (see graph 16)

Graph 16:- Boxplot of daily $(ht^2/R_0)/(ht^2/R_{inf})$ ratio



The phase angle measured at 50 kHz had a preoperative mean of 5.25° (se 0.26) with a continued fall to a mean value of 3.98° (se 0.23) on day 4. The phase angle increased on day 5 to a mean of 4.12° (se 0.25) (see graph 17). The repeated measures ANOVA demonstrates a significant difference in the daily mean values F=6.36 (df 3.92, 58.83) p<0.0005) with Huyn-Feldt correction. Pairwise comparisons with Bonferroni correction is only significant between the preoperative and day 4 mean difference (p=0.042). Day 1 has a significant mean difference between day 2 and day 3 (p=0.041 to p<0.0005). No other time points demonstrated a significant mean difference.

Graph 17:- Mean daily phase angle measured at 50Khz (standard error)



Age was introduced into the one way ANOVA as a fixed factor in comparison with preoperative BIA measurements as dependant variables. Age with an assumption of homogeneity did demonstrate a significant difference between the groups in preoperative R_0 and R_{inf} ($F=4.175$ (df 2 18) $p=0.035$ and $F=3.674$ (df 2 18) $p=0.049$). Pairwise comparisons with Bonferroni correction showed that the mean difference between the younger (mean 506 .6 Ω (se 11.98) and older (mean 605.9 Ω (se 29.9) age range for R_0 was significant $p=0.032$ but not the middle age range (mean 558.8 Ω (se 29.9) ($p=0.381$ and 0.431).

An independent t- test was used to analyse BMI and preoperative BIA parameters BMI was coded into two groups (BMI < and > 27.5). R_0 did not show equality of variance with Levene's test $F=24.19$ $P<0.0005$. The mean R_0 in the non obese group was 599.3 Ω (se 21.03) v 516.2 Ω (se 9.1) which was still significant without the assumption of equality $t=3.629$ (df 12.2) $p=0.003$. For R_{inf} the assumption of equality was met with the mean for the non obese patients being 428.4 Ω (se 12.3) v 373.7 Ω (se 10.1) for obese patients with a significant mean difference $t=3.398$ (df 17) $p=0.003$. The mean ht^2/R_0 for non obese patients was 50.1 cm^2/Ω (se 1.92) in comparison to a mean of 55.6 cm^2/Ω (se 1.84) in obese patients this did not quite give a significant mean difference $t=-2.05$ (df 18) $p=0.055$ with an assumption of equality. With a change in the alpha value for Bonferroni correction to $\alpha=0.05/7=0.007$ R_0 an R_{inf} remain significant.

The daily cumulative fluid balance was calculated until day 3 and compared with the appropriate daily BIA parameters e.g day 2 R_0 to day 2 cumulative fluid balance. Due to the normal distribution and continuous nature of all variables the association with fluid balance and BIA was examined with Pearson correlation. The only correlation was with day 3 ht^2/R_{inf} and day 3 cumulative fluid balance $r=0.513$ $p=0.030$. The cumulative fluid

balance on day 3 when correlated with the day 4 and day 5 ht^2/R_{inf} demonstrates the correlation remains r=0.535 p=0.040 and r=0.649 p=0.004 respectively. In addition ht^2/R_0 and R_0 also show a moderate significant correlation to day 3 cumulative fluid balance on day 4 (r=0.594 p=0.020 and r=0.580 p=0.023) and day 5 (r=0.703 p=0.001 and r=0.652 p=0.003).

The association between changes from preoperative values in markers of ECW (R_0 and ht^2/R_0) and changes from preoperative concentration in albumin were examined with Pearson's correlation. There is a negative correlation for changes in albumin and changes in ht^2/R_0 when matched day for day(r=-0.688 p=0.001 day 1, r=-0.520 p=0.029 day 2, r=-0.515 p=0.020 day 3, r=-0.718 p=0.001 day 4 and r=-0.745 p<0.0005 day 5). This is mirrored in the positive association with changes in R_0 and in albumin on matched days (r=0.694 p=0.001 day 1, r=0.549 p=0.012 day 3 and r=0.528 p=0.017). The change in values of albumin, R_0 and ht^2/R_0 from day 1 to day 5 were also analysed to see if the association between ECW and albumin still stood. There was a significant negative correlation for ht^2/R_0 r=-0.678 p=0.001 and significant positive correlation for R_0 r=0.631 p=0.004.

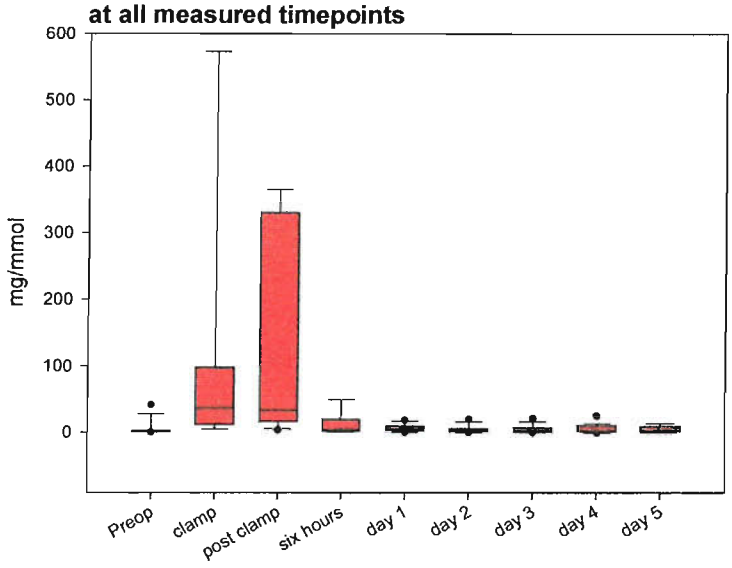
Phase angle on day 1 was recoded into a grouping variable with a cut off <5.3° and using a Mann-Whitney test, the means were compared for APACHE II, SOFA 24 hours and SOFA AUC. The difference in mean ranks for APACHE II score did not quite reach significance with the mean rank in the lower group 11 v 5.6 for the higher group (U=13.0 p=0.052). A similar trend was seen for SOFA 24 hours with a mean rank in the lower group of 11.93 v 6.20 in the higher group (U=16.0 p=0.058). There was no significant mean difference in ranks between the lower and higher groups for SOFA AUC (mean ranks 11.67 v 7.0 U=20 p=0.126). The means were compared for CRP 24 hours and CRP

AUC using an independent t-test with no significant difference between the groups (p=0.619 v p=0.768). The phase angle at 24 hours as a continuous variable was also correlated with the above variables using Spearman rho. Only SOFA 24 hours showed a significant negative correlation r_s=-0.539 p=0.017 but APACHE II score did show a trend towards a significant negative correlation r_s=-0.475 p=0.054.

Microalbumin Creatinine Ratio

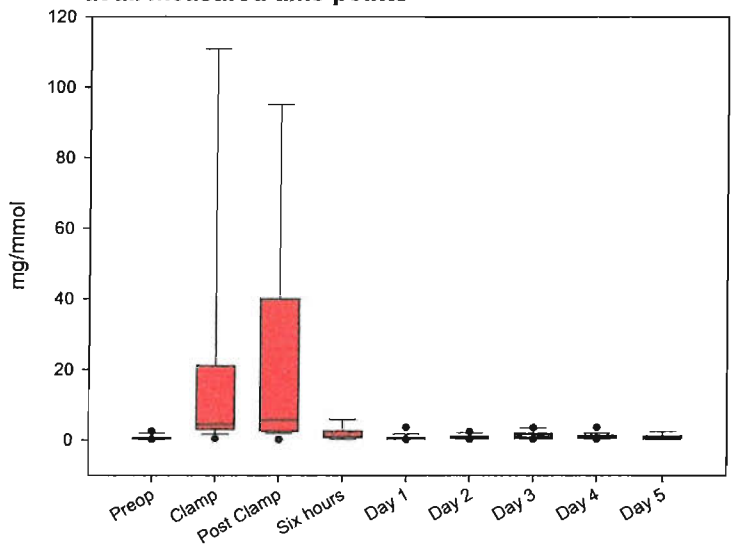
The preoperative median MACR was 1.41 mg/mmol (range 0.34 to 41.88) which intraoperatively reached a peak median value of 32.71 mg/mmol (range 1.7 to 970) post clamp removal. 6 hours postoperatively the median value had fallen to 5.17 mg/mmol (range 0.37 to 117.9) with a continued gradual fall to 3.93 mg/mmol (range 0.19 to 29.38) (see graph 18). Following natural log transformation the repeated measures ANOVA was significant using Huyn-Feldt correction F=22.11 (df 6.79, 101.82) p<0.0005. Post hoc testing with Bonferroni correction showed no significant difference between the clamp and post clamp means (p=1.000) but these two time points were significantly different to all other time points (p<0.0005). No other time points showed any significant mean differences. No association was identified between the maximal change in MACR and change in albumin post clamp to day 1 using Pearson correlation.

Graph 18:- Boxplot of Microalbumin creatinine ratio at all measured timepoints



The immunoglobulin G (IgG) creatinine ratio was also measured with a preoperative median of 0.322 mg/mmol (range 0.16 to 2.47). It also showed a similar pattern to MACR rising to a peak of 5.904 mg/mmol (range 0.27 to 221.78) post clamp and falling to a median of 1.115 mg/mmol (range 0.43 to 13.08) at 6 hours. The trend continued downwards reaching a median of 0.685 mg/mmol (rang 0.22 to 4.44) at day 5 (see graph 19). The Shapiro-Wilks test of normality as with MACR showed that the IgG creatinine ratio is not normally distributed and thus required natural log transformation. Using Huyn-Feldt correction as sphericity is not assumed there is a significant difference in the daily means $F=26.23$ (df 4.62 69.23) $p<0.0005$. Post hoc testing using pairwise comparison show that there is a significant mean difference between clamp, post clamp and 6 hours ($p<0.0005$, $p<0.0005$ and $p=0.013$) with Bonferroni correction. Clamp and postclamp comparisons demonstrates a significant mean difference at all time points except between themselves. No other time points show any significant mean differences.

Graph 19:- Boxplot of immunoglobulin G creatinine ratio at all measured time points



The Mann-Whitney U test was used to determine if the use of mannitol made any significant difference to the MACR or IgGCR over the individual measured time points as well as area under the curve (AUC). At 6 hours a significant difference was demonstrated in IgGCR in those receiving mannitol (mean rank 12.0) compared to those not receiving mannitol (mean rank 3.0) U=0.0 p=0.001. A further significant difference was shown for the IgGCR AUC in those receiving mannitol (mean rank 12.6 v 6.57) U=18 p=0.029. No significant difference were shown for MACR though at 6 hours the trends is towards significance U=13 p=0.055. There was no significant correlation using Spearman rho between APACHE II, SOFA score and MACR.

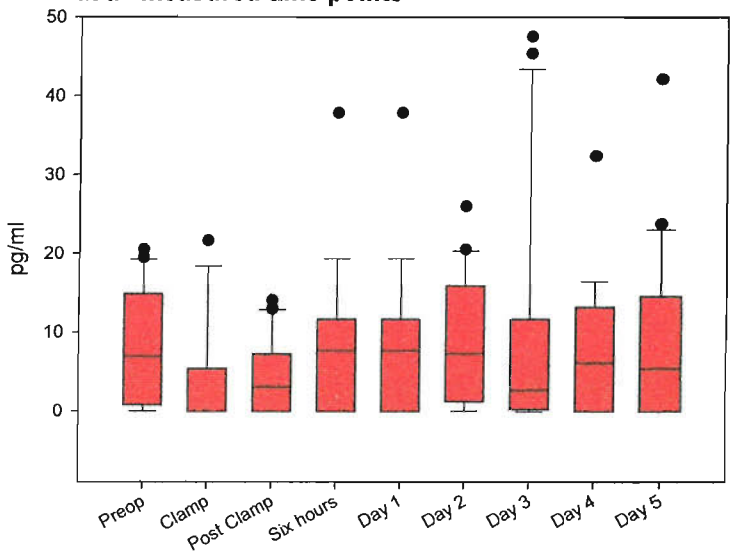
Cytokines

All the cytokines measured IL-1β, IL-6, IL-8 and IL-10 on testing for normality with the Shapiro-Wilk test were found not to be normally distributed.

Interleukin 1β

The median preoperative plasma concentration was 6.87 pg/ml (range 0 to 20.52) with a peak median concentration of 7.65 pg/ml at 6 hours postoperatively (see graph 20). Friedmans test shows there is no significant difference between the mean ranks χ^2=11.75 (df 8) p=0.163.

Graph 20:- Boxplot of interleukin 1β concentrations at all measured time points

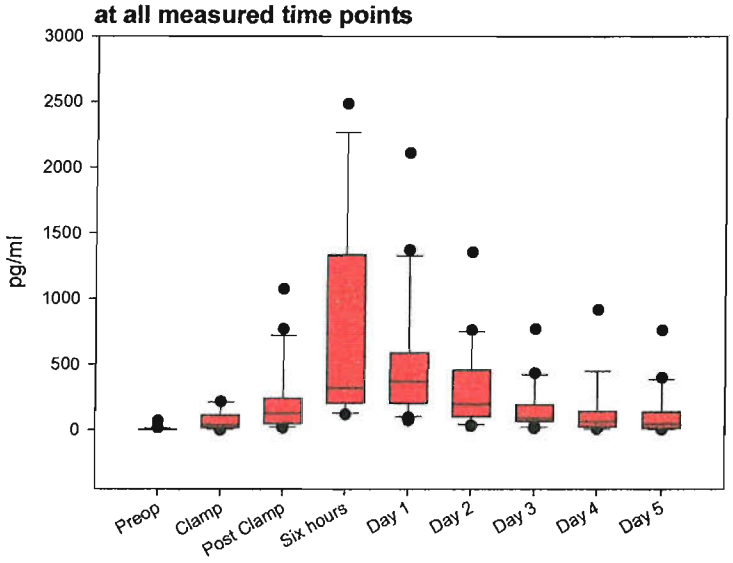


Interleukin 6

Preoperatively the median IL-6 concentration was 0.0 pg/ml (range 0 to 69.7) rising intraoperatively and postoperatively to a median of 365.51 pg/ml by day 1. The median concentration fell over the subsequent days from 198.79 pg.ml (range 32.2 to 1353.58) on day 2 to 50.27 pg/ml (range 7.98 to 756.77) by day 5.(see graph 21). Log and square root transformation did not normalise the IL-6 data. Friedmans test demonstrated a significant difference in the mean ranks χ^2=96.53 (df 8) p<0.0005. Using the wilcoxon signed ranks

test the paired differences between preoperative IL-6 and all other time points is significant P<0.0005.

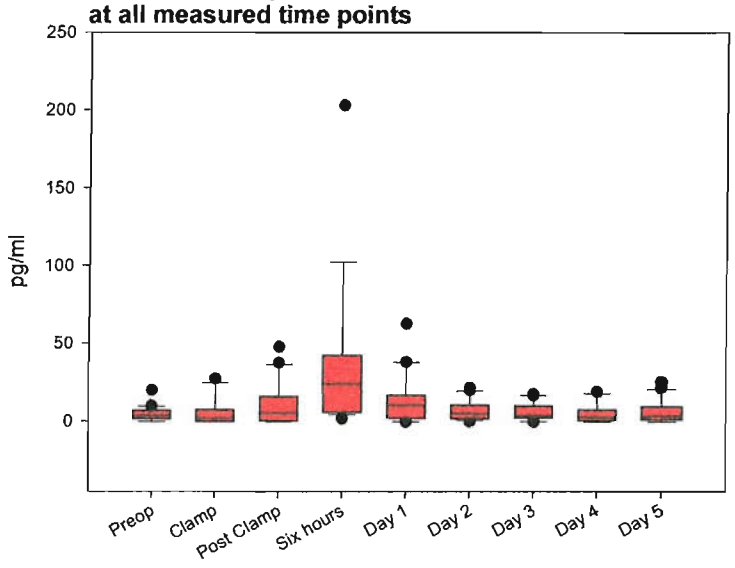
Graph 21:- Boxplot of Interleukin 6 concentration at all measured time points



Interleukin 8

The median IL-8 concentration rose to 24.69 pg/ml (range 2.01 to 203.6) at 6 hours from a preoperative median of 3.62 pg/ml (range 0 to 19.79). This fell to a median 10.30 pg/ml (range 0.00 to 62.69) by day 1 and 3.47 pg/ml (range 0 to 25.33) on day 5(see graph). Square root transformation normalised the data and therefore repeated measures with pairwise comparison was used to determine any significant mean differences. There was no assumption of sphericity but with Huyn-Feldt correction there is a significant difference in the mean values F=6.91 (df 6.79, 101.77) p<0.0005. With Bonferroni correction there was a significant mean difference between preoperative and the 6 hour IL-8 means (p=0.004) but at no other time points. The 6 hour mean difference was also significant at the clamp, day 3 and day 5 time points (p=0.029, p=0.013, p=0.006) and nearly reached significance between day 2 and day 4 (p=0.051).

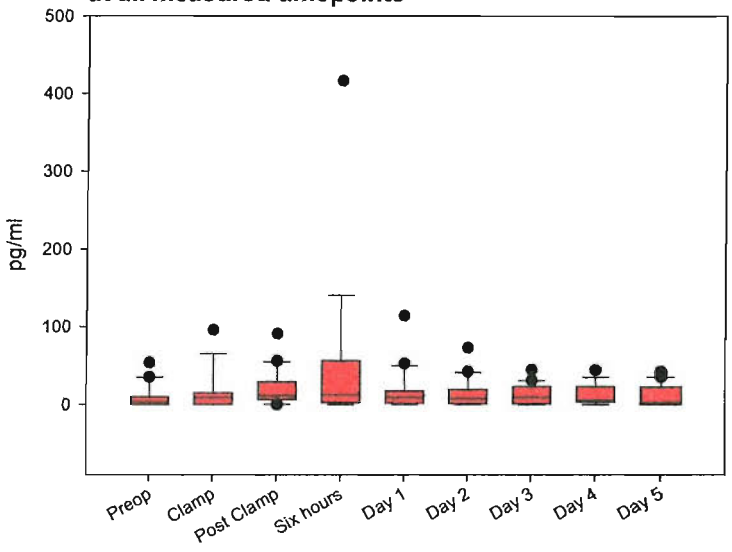
Graph 22:- Boxplot Interleukin 8 concentration at all measured time points



Interleukin 10

Changes in IL-10 showed a similar pattern to IL-6 and IL-8 with a preoperative median of 3.62 pg/ml (range 0 to 53.96) rising to a median12.02 pg/ml (range 0 to 415) at 6 hours. The general trend was downwards from a median 9.36 pg/ml (range 0 to 114.5) on day 1 to 3.19 pg/ml on day 5 (see graph). Normalisation of the data with square root transformation allowed the use of repeated measures which demonstrated no significant difference in the mean values F=0.857 (df 5.03 75.43) p=0.515 with Huyn-Feldt correction. This was confirmed with the Friedman test on the untransformed data χ^2=3.84 (df 8) p=0.871.

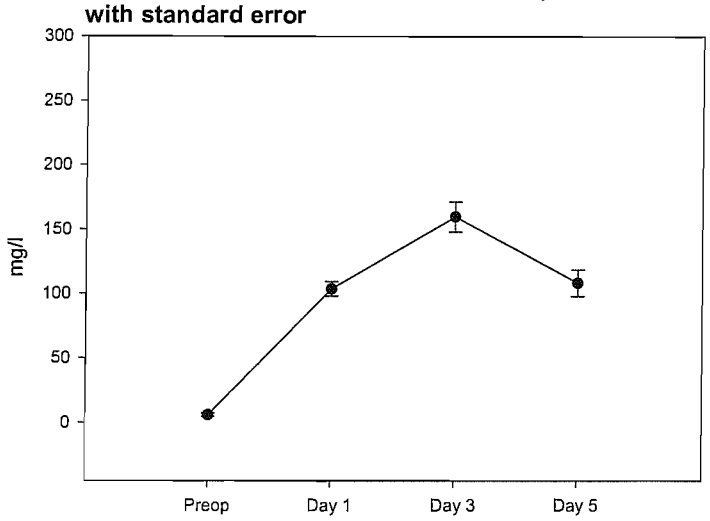
Graph 23:- Boxplot of interleukin 10 concentration at all measured timepoints



C-reactive protein

CRP was normally distributed with a mean preoperative plasma concentration of 5.94 mg/l (se 1.27). The peak CRP concentration was 159.6 mg/l (se 11.63) on day 3 before falling to a mean of 108.25 mg/l (se 10.23) by day 5 (see graph 24). Repeated measures ANOVA confirms that there is a significant difference in the mean daily CRP measurements $F=83.03$ (df 2.28, 43.32) $p<0.0005$ with Huyn-Feldt correction. Post hoc pairwise comparison demonstrates that there is a significant mean difference ($p<0.0005$) between all time points except day 2 and day 4 $p=1.000$. The association between CRP and IL-6 was examined with Spearman rho correlation. Day 1, 2 and day 3 IL-6 showed a significant correlation to peak CRP on day 3 ($r_s=0.546$ $p=0.013$, $r_s= 0.725$ $p<0.0005$ and $r_s= 0.632$ $p=0.003$) and CRP AUC ($r_s=0.547$ $p=0.012$, $r_s=0.698$ $p=0.001$ and $r_s=0.605$ $p=0.005$). There was no correlation between CRP and the summary statistics IL-6 max ($r_s= 0.247$ $p=0.259$) and IL-6 AUC ($r_s= 0.414$ $p=0.070$).

Graph 24:- Mean CRP at measured timepoints with standard error



Effect of intraoperative factors

Using the Mann-Whitney U test no significant difference was demonstrated for mannitol on cytokine or CRP response over the individual measured time points as well as area under the curve.

Spearman rho correlations were performed to determine whether there was any association with clamp time, operation length and blood loss on the cytokine and CRP response. Following clamp release there is a significant moderate correlation between IL-6, clamp time (r_s=0.570 p=0.009) and blood loss (r_s=0.586 p=0.008). The correlation between IL-6 and operation length was significant at 6 hour (r_s=0.562 p<0.015) and remained significant until day 3 (r_s=0.535 p=0.015) and was also born out with the correlation between the operation length and IL-6 AUC (r_s=0.603 p=0.005).

IL-8 showed a significant correlation with the operation length at 6 hours (r_s=0.712 p=0.001), day 1 (r_s=0.604 p=0.005) and IL-8 AUC (r_s=0.544 p=0.013).There was no

significant correlation with clamp time but blood loss showed a moderate correlation post clamp (r_s=0.631 p=0.004) but not at 6 hours. IL-10 showed no significant correlation with clamp time, operation length or blood loss. CRP shows a significant moderate correlation with clamp time on day 1 (r_s=0.479 p=0.033), day 3 (r_s=0.509 p=0.022) and AUC (r_s=0.463 p=0.040). There were no significant correlations with blood loss or operation length.

Further Spearman rho correlations were performed to identify any association between cytokines and the physiological scoring systems SOFA and APACHE II. SOFA AUC was analysed in association to IL-6 AUC, IL-8 AUC and IL-10 AUC with a positive correlation for IL-6 AUC r_s=0.676 p=0.001 and IL-8 AUC r_s=0.804 p<0.0005 but no correlation with IL-10 AUC r_s=-0.138 p=0.560. IL-6 max and Il-6 24 hours were correlated with SOFA 24 hours with a positive association r_s =0.505 p=0.023 and r_s=0.648 p=0.002 respectively. Similar positive correlation were found with IL-6 max and IL-6 24 hours and APACHE II score r_s=0.547 p=0.019 and r_s=0.572 p=0.013. A different pattern was seen with IL-8 max with no significant correlation found with SOFA score 24 hours r_s=0.324 p=0.163 and APACHE II score r_s=0.421 p=0.082. For IL-8 at 24 hours the converse was true with a strong correlation with APACHE score r_s=0.778 p=<0.0005 and SOFA score 24 hours r_s=0.708 p<0.0005. No significant correlations were identified for IL-10 at 24 hours and SOFA or APACHE II score. There was no correlation between CRP 24 hours and SOFA or APACHE II score but SOFA AUC and CRP AUC showed a moderately significant association r_s=0.489 p=0.029.

Four patients developed postoperative complications related to the SIRS Continuum these patients were compared with those without complication to day 4 and day 5 IL-6, IL-8 and IL-10 using Mann-Whitney. The mean rank on day 4 for the IL-6 sepsis group was 12.0 v

5.7 for the no complication group this was significant (U=2.00 p=0.008). IL-8 showed similar results with sepsis mean rank of 13.13 v 6.14 for the no complication group again this was significant U=1.5 p=0.003. Though the sepsis mean rank on day 5 was 10.5 for IL-6 and 11.5 for IL-8 compared to a mean rank of 7.83 for IL-6 and 8.12 for IL-8 in the no complication group these were not significant U=16 p=0.379 and U=14.5 p=0.202 respectively. There were no significant differences between the mean ranks on day 4 and 5 for IL-10. For CRP an independent T-test was used to determine whether there is a difference in the means between the sepsis and no complication group. There was no significant mean difference between the two groups for CRP day 3 and CRP AUC. On day 5 the mean for the sepsis group was 140 mg/l (se 12.89) v 95.9 mg/l (se 11.85) for the no complications group this was not significant t=-1.93 (df 15) p=0.073 with equal variance assumed.

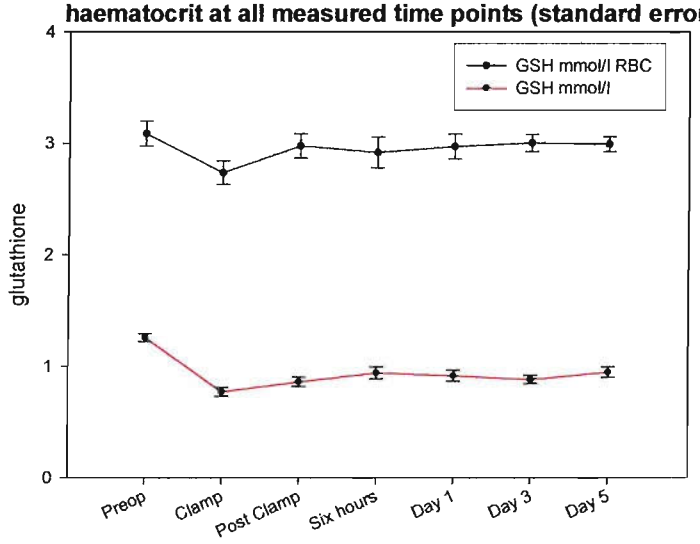
Glutathione

Prior to correction for haematocrit the GSH had a preoperative mean of 1.257 mmol/l (se 0.035) falling prior to clamp release to 0.771 mmol/l (se 0.038) then rising post clamp to mean of 0.861 mmol/l (se 0.041) and 0.940 mmol/l (se 0.053) at 6 hours (see graph 25). Repeated measures ANOVA shows that there is a significant difference between the means f=17.69 (df 3.4 47.61) p<0.0005 with Huyn-Feldt correction. Post hoc testing with pairwise comparisons using Bonferroni correction demonstrates that this significant difference is between preoperative GSH and all other time points (p<0.0005). No other time point showed any significant mean difference.

Following correction the mean preoperative GSH was 3.087 mmol/l RBC (se 0.111) falling prior to clamp release to a mean of 2.737 mmol/l RBC (se 0.107). The corrected

GSH then rose to mean of 2.977 mmol/l RBC post clamp. (see graph 25). Sphericity was not assumed and so with Huyn-Feldt correction no significant difference was shown between the means F=1.39 (df 4.54 72.64) p=0.242. The least significant difference pairwise comparison test suggested that there was a significant mean difference between preoperative and clamp GSH p=0.006 with the use of Bonferroni correction in pairwise comparison there is no significant difference p=0.111.

Graph 25:- Mean Glutathione corrected and uncorrected for haematocrit at all measured time points (standard error)



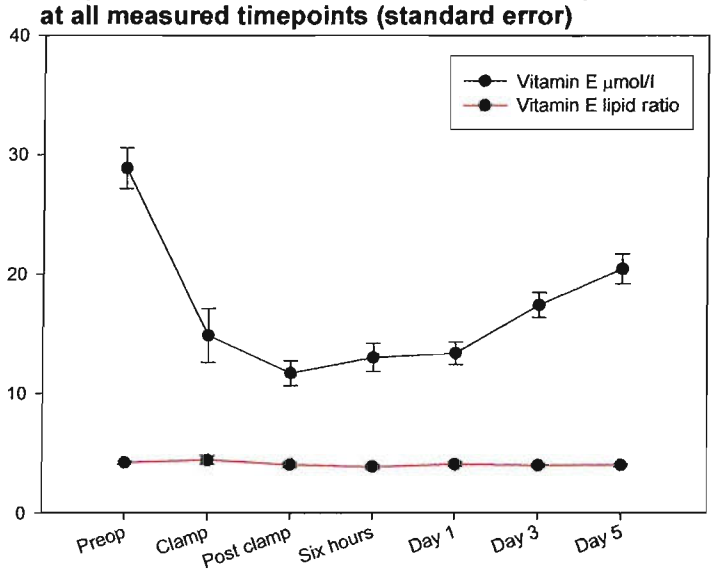
Vitamin E

The mean preoperative uncorrected vitamin E concentration was 28.87 µmol/l (se 1.72) falling to a mean of 11.7 µmol/l (se 1.19) post clamp. The uncorrected vitamin E rose gradually from a mean of 13.02 µmol/l (se 1.19) at 6 hours to 20.45 µmol/l (se 1.23) by day 5 (see graph 26). A significant mean difference was demonstrated between the time points using repeated measures ANOVA F=45.43 (df 4.18, 62.64) p<0.0005 with Huyn Feldt correction. Pairwise comparisons using Bonferroni correction demonstrated that there was a significant mean difference between preoperative vitamin E and all other time points (p<0.0005). The only pairwise comparisons that did not show any significance were

between clamp, post clamp and 6 hour time points (p=1.000) and in addition clamp and day 3 (P=0.356).

The vitamin E lipid ratio preoperatively had a mean of 4.206 (se 0.132) which fell slightly to a mean of 3.863 (se 0.154) at 6 hours with a mean of 4.01 (se 0.138) on day 5(see graph 26). No significant difference was seen between the time points with repeated measures ANOVA F=1.66 (df 2.00, 30.04) p=0.207.

Graph 26:- Mean Vitamin E and Vitamin E Lipid ratio at all measured timepoints (standard error)



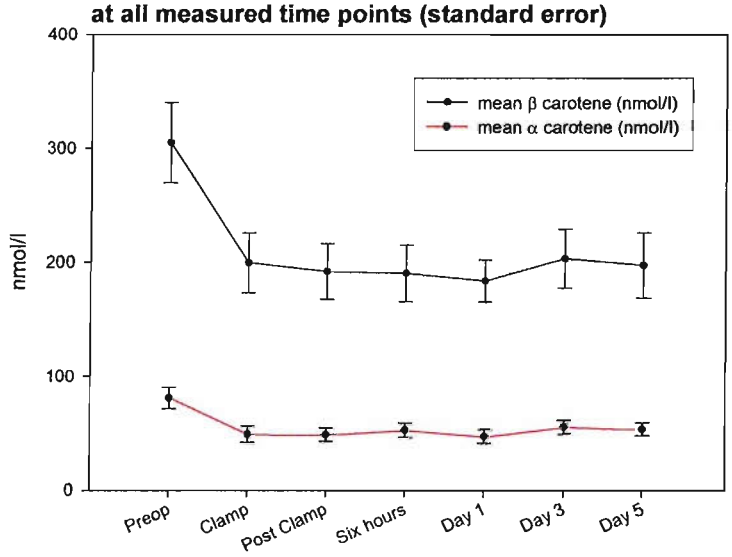
β carotene and α carotene

During clamp β carotene fell from a preoperative mean of 304 nmol/l (se 35.34) to 199.63 nmol/l (se 26.18) further falling until day 1 reaching a trough mean of 183.75 nmol/l (se 18.34). β carotene subsequently rose to a mean of 203nmol/l (se 25.71) on day 3 (see graph 27). Repeated measures without the assumption of sphericity showed a significant mean difference between the time points F=14.33 (df 2.87, 40.15) p<0.0005. The only

significant mean difference was between preoperative β carotene and all other time points ($p<0.0005$ to $p=0.003$) using pairwise comparisons with Bonferroni correction.

The mean preoperative α carotene was 81.15 nmol/l (se 9.35) which fell to a mean of 49.37 nmol/l (se 7.01) prior to clamp release with a mean of 53.90 nmol/l (se 5.66) by day 5 (see graph 27). As was found with β carotene there is a significant mean difference of α carotene over the time points $F=18.53$ (df 3.72 52.04) $p<0.0005$ with Huyn-Feldt correction. This significant mean difference was between preoperative α carotene and α carotene at all other time points ($p<0.0005$ to $p=0.003$) using pairwise comparison with Bonferroni correction. No other time points showed any significant mean difference.

Graph 27:- Mean β carotene and α carotene at all measured time points (standard error)



Preoperative Antioxidants

An independent t-test was used to examine the effect of smoking and BMI on the preoperative antioxidant concentrations with an assumption of equal variances as Levene's test was not significant. The effect of smoking was only significant with the preoperative vitamin E lipid ratio. The mean for non smokers being 4.36 (se 0.134) v 3.75 (se 0.270)

for smokers t=-2.18 (df 18) p=0.043. The mean preoperative GSH concentration in obese patients was 2.813 mmol/l RBC (se 0.150) v 3.311 mmol/l RBC (se 0.115) in the non obese patients the mean difference was significantly different t=2.529 (df 18) p=0.021. The effect of age on preoperative antioxidant status was examined using one way ANOVA with age as the grouping variable and an assumption of homogeneity of variance. There was no significant effect of age on preoperative antioxidant concentration.

Preoperative antioxidant status and outcome

The analysis of the preoperative antioxidants was performed using the continuous variables but also re-categorising the antioxidants into grouped variables in tertiles.

Bioelectric impedance

The BIA parameters examined for any effect of preoperative antioxidant status were phase angle 50Khz, R_0/R_{inf}, and $(ht^2/ R_0)/ (ht^2/ R_{inf})$ these had previously been identified in the literature to have been associated with outcome.

As all continuous variables were normally distributed correlation were performed using Pearson's correlation and showed no significant associations between preoperative antioxidants and preoperative BIA parameters. One way ANOVA was used to compare the preoperative BIA parameters as dependant variables to the individual preoperative antioxidants as factors. With the assumption of homogeneity there was no significant difference in the means for any of the groups analysed. The GLM univariate analysis was used to compare all possible interactions of the 4 preoperative antioxidants with the individual preoperative BIA parameters with no significant interaction effect being found.

R_0/R_{inf}, and $(ht^2/ R_0)/ (ht^2/ R_{inf})$ summary statistics were calculated of the maximum change and the change between preoperative and day 1 values. Pearson's correlation showed no significant association between preoperative antioxidants and BIA parameters. The one way ANOVA showed no significant mean difference between the groups and there was no significant interaction of the four antioxidant using GLM univariate analysis. Phase angle 50 Khz on day 1 was grouped into values < and > 5.3° and then compared to the preoperative antioxidants using an independent t-test. The mean GSH in the <5.3° group was 2.90 mmol/l v 3.64 mmol/l in the >5.3° group which was a mean significant difference t=-3.791 (df 18) p=0.001 equal variance assumed. There was no significant difference for any of the other preoperative antioxidants.

MACR and IgG creatinine ratio

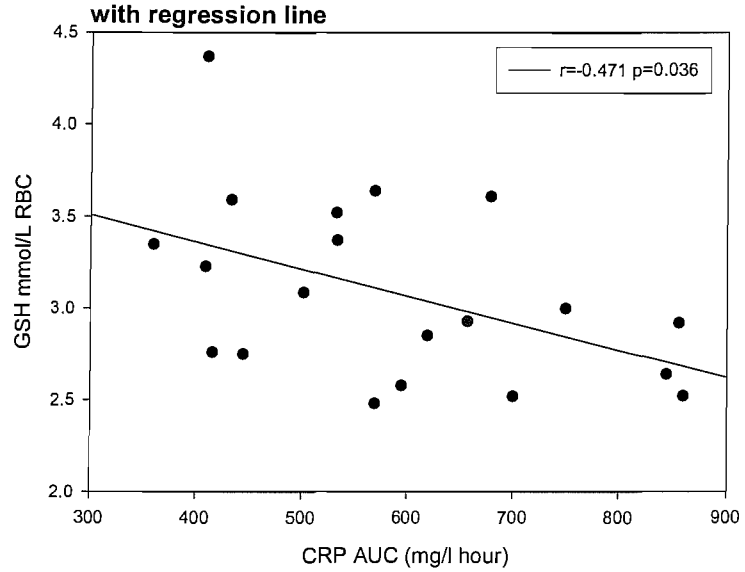
The maximum change in MACR and IgGCR from there preoperative values and area under the curve over the whole five days were used as summary statistics. The MACR and IgGCR variables are not normally distributed so Spearman rho was used for correlation with the continuous preoperative antioxidant variables. No significant correlations were found. The Kruskall-Wallis test was used to compare the mean ranks of the grouped individual antioxidants but no significant difference was shown for the mean ranks.

Cytokines and CRP

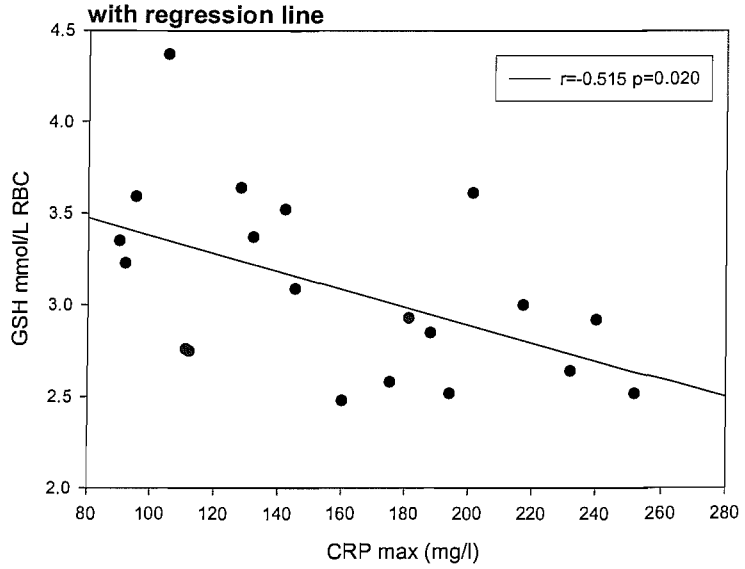
The summary statistics used were area under curve over the five days and maximum change in concentration for IL-6, IL-8 and CRP. IL-6 and IL-8 are not normally distributed and were analysed using non parametric methods.

Spearman rho correlation showed no significant association with the IL-6 and IL-8 summary statistics and any individual antioxidants. IL-10 AUC also showed no significant association with Pearson's correlation. Preoperative GSH did show a moderate negative correlation with CRP AUC r=-0.471 p=0.036 and CRP max r=-0.515 p=0.020.

Graph 28:- Scatterplot of preoperative GSH v CRP AUC with regression line



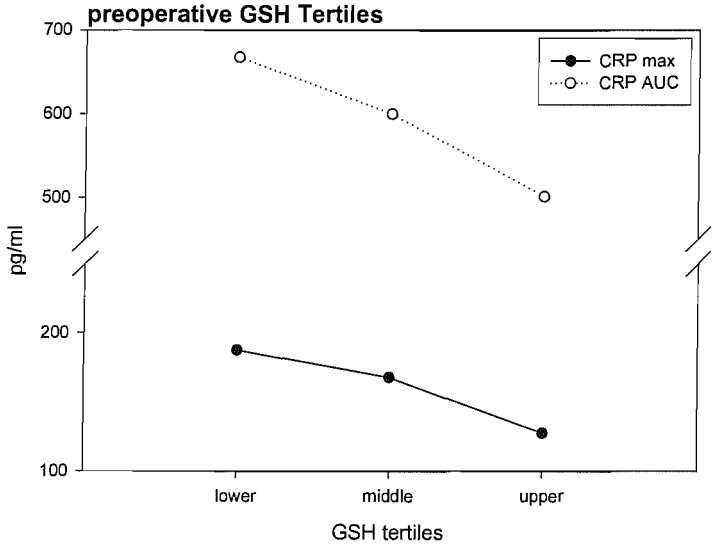
Graph 29:- Scatterplot of preoperative GSH v CRP max with regression line



Similar negative correlation was also seen with α-carotene and CRP AUC r=-0.488 p=0.029 and CRP max r=-0.482 p=0.032. CRP showed no significant association with β-carotene or vitamin E lipid ratio.

One way ANOVA with homogeneity assumed showed no significant difference between the groups for GSH and CRP max (F=2.676 p=0.098) and CRP AUC (F=2.067 p=0.157). Visual examination of the mean plots suggested a relationship between the means (see graph 30).

Graph 30:- Means plot of CRP AUC and CRP max for preoperative GSH Tertiles



The mean CRP AUC for the low GSH group was 667.8 mg/l hour(se 66.63) v 501.8 mg/l hour (se 41.11) for the higher GSH group and the middle GSH group mean was 600.3 mg/l hour (se 63.8). Post hoc pairwise comparison showed no significant difference in the means high v low group p=0.061 with no correction and p=0.182 with Bonferroni correction. For CRP max the means low to high GSH were 187.5 mg/l (se 20.65) v 167.7 (se 20.53) v 127.6 mg/l (se 14.3) with pairwise comparison the mean difference between

the low and high GSH groups was significant p=0.038 but with Bonferroni correction it was no longer significant p=0.182.

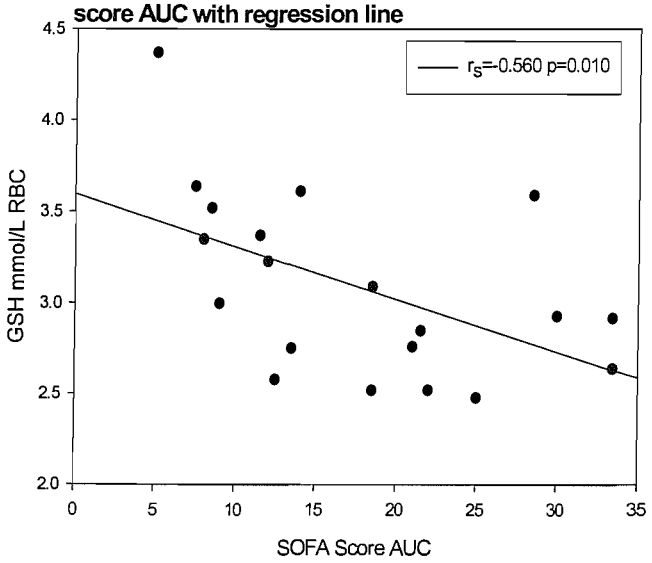
The one way ANOVA with an assumption of homogeneity showed a significant mean difference between the α-carotene groups for CRP AUC (F=4.30 p=0.027) and CRP max (F=4.53 p=0.031). CRP AUC means for low to high α-carotene were 705.4 mg/l hour (se 68.3) v 539.1 mg/l hour (se 107.7) v 507.0 mg/l hour (se 36.4) which post hoc pairwise comparison showed a significant mean difference between the low to high group p=0.041 with Bonferroni correction. The mean values for CRP max high to low were 200.0 mg/l (se 20.8) v 143.0 mg/l (se 17.1) v 133.4 (se 12.4), the mean difference between high and low mean values was significant p=0.037 with Bonferroni correction. There are no significant mean differences between CRP AUC and CRP max with vitamin E lipid ratio and β-carotene. There was no significant mean difference demonstrated with IL-10 and the individual preoperative antioxidants using one way ANOVA. GLM univariate analysis showed no interaction effect of the four antioxidants with CRP AUC, CRP max and IL-10 as dependant variables.

For IL-6 AUC, IL-6 max, IL-8 AUC and IL-8 max the Kruskal-Wallis test was used to see if there was any difference in the rank means of the groups for each of independent preoperative antioxidants. The only significant result was for IL-8 AUC and vitamin E lipid ratio χ^2=6.250 (df 2) p=0.044 all the other groups were not significant. The Mann-Whitney test was used for pairwise comparison of IL-8 AUC and vitamin E lipid ratio. The mean rank in the lowest group was 4.00 compared to 9.57 in the highest vitamin E lipid ratio group this was significant (U=3.0 p=0.008). Using Dunns correction for multiple comparisons the α=0.002 and so the result becomes not significant.

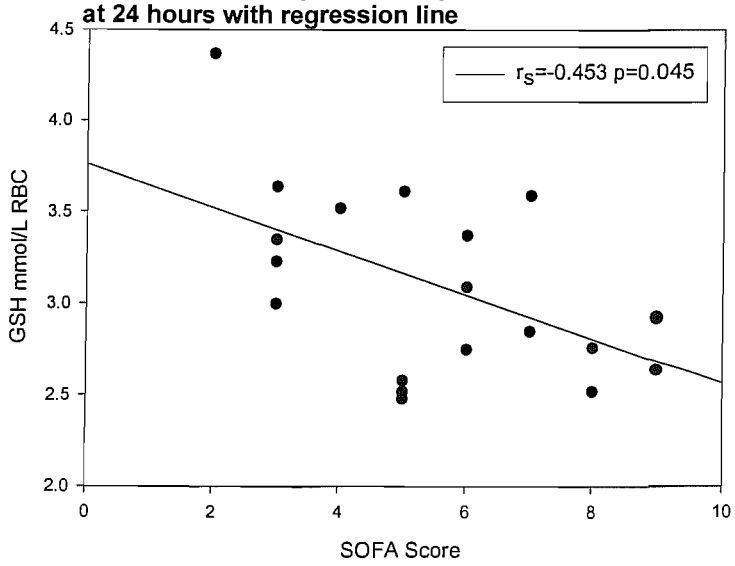
Physiological scores

Summary statistics for SOFA score were AUC for the five day period and SOFA score at 24 hours. There was only one time point at 24 hours for APACHE II score. Correlation using Spearman rho demonstrates a significant negative association of preoperative GSH with SOFA AUC and SOFA 24 hours (r_s=-0.560 p=0.010 and r_s=-0.453 p=0.045) (see graphs 31 and 32).

Graph 31:- Scatter plot of Preoperative GSH V SOFA score AUC with regression line

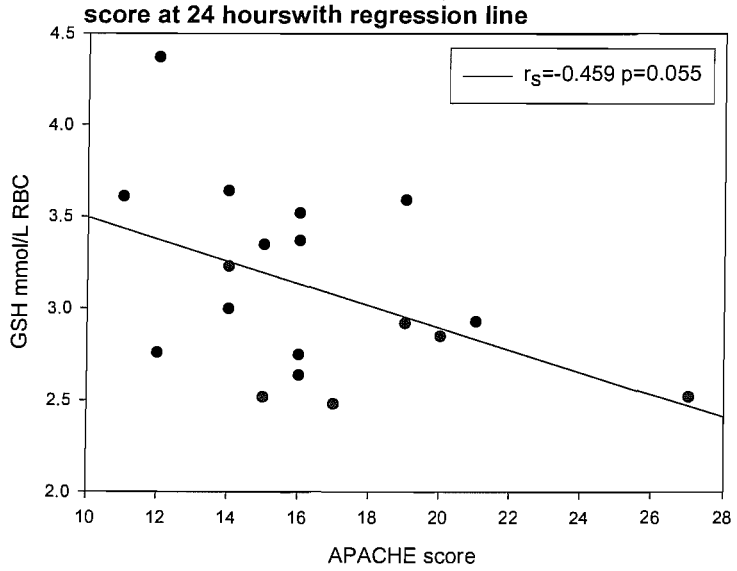


Graph 32:- Scatterplot of preoperative GSH v SOFA score at 24 hours with regression line



The APACHE II score at 24 hours showed a negative correlation for GSH but did not quite reach significance r_s=0.459 p=0.055 (see graph 33).

Graph 33:- Scatterplot of Preoperative GSH v APACHE II score at 24 hourswith regression line



Kruskal-Wallis test was used to compare the preoperative antioxidants as groups with the physiological scores. APACHE II and SOFA score showed no significant difference between the mean ranks for any of the antioxidants. However SOFA AUC tended towards significance with preoperative GSH (χ^2=5.88 (df 2) p=0.053). A Mann-Whitney test for pairwise comparison showed the mean rank for lowest GSH group to be 9.33 compared to the mean rank in the highest group of 5.00 u=7.00 p=0.051 so not quite significant. The use of Dunns correction makes this even less significant.

Length of stay

The use of correlation with Spearman rho showed no significant association with preoperative antioxidants compared with total hospital stay, ITU stay or ITU and high

dependency stay combined. Similarly one way ANOVA showed no significant mean difference for the above parameters and preoperative antioxidants as a grouped variable.

Chapter 11 Discussion

The Response to surgery

Fluid balance .

Bioelectric impedance measurements were performed to estimate the changes in ECW and TBW over the five day study period. The R_{inf} and ht^2/R_{inf} both demonstrated that the TBW had increased from preoperative levels by day 1. This increase continued until day 3 with R_{inf} being 81% of its preoperative value and ht^2/R_{inf} suggesting a 25% increase in TBW. Even by day 5, the R_{inf} was still 87% of the preoperative value with ht^2/R_{inf} showing a 16% increase in TBW.

Estimates of ECW (R_0 and ht^2/R_0) showed the same pattern as TBW with the ECW increasing from day 1 with a maximal fall by day 3. By day 3 R_0 was 75% of its preoperative value with ht^2/R_0 values suggesting a 35% increase in ECW. R_0 by day 5 was 80% of the preoperative value with an estimated increase in ECW of 26.6% from the change in ht^2/R_0. These results would suggest that the majority of the increase in TBW is secondary to extracellular water expansion. This is further confirmed by the increase in the ht^2/R_0 / ht^2/R_{inf} ratio and fall in the R_0/ R_{inf} ratio. The ratios would suggest that the relative expansion of the ECW in comparison to the TBW is more prolonged with little change in the ratios up to day 5.

The above changes in ECW and TBW are consistent with previous studies using different methods. Plank et al in 23 septic patients with peritonitis who underwent urgent surgery demonstrated an increase in the TBW mainly accounted for by an increase in ECW. They

used tritium and bromide method to measure TBW and ECW. They studied the patients over 21 days showing a gradual fall in TBW towards estimated pre illness levels. At day 21 there was still a relative expansion of the TBW accounted for by ECW. Monk et al part of the same group showed similar results in trauma patients but with an increased rate of return of TBW towards normal. Cheng et al found that in patients >60 years old compared to younger patients that there was a prolonged period of ECW expansion with no significant change up to 10 days[357].

The cumulative fluid balance by day 3 was positive by 2.2 litres this is slightly at odds with the estimated increase in TBW of 25%. The mean weight of the subjects was 79 kg with an estimate of 60% of weight being TBW the 25% change would suggest an increase in TBW of nearly 12 litres. The 35% estimated increase in ECW with assumption of 20% body weight being ECW would give an increase in ECW of 5.5 litres. Part of this discrepancy is probably due to the inherent inaccuracy in the keeping of fluid balance charts. This was demonstrated in a survey by Stoneham et al which showed charts were incomplete in 42% of patients[368]. Plank et al in a comparison of methods to estimate TBW in intensive care patients showed that changes shown by BIA were not significantly different to those from tritium dilution but BIA was a poor predictor of TBW volume[369].

The correlation of cumulative fluid balance with ht^2/R_0 and ht^2/R_{inf} on day 4 and 5 would suggest that BIA is useful in monitoring changes in fluid balance. The serial measurement of daily weights a good indicator of fluid balance could have helped reflect the value of BIA in monitoring these changes in fluid balance. This is difficult in the critically ill patient unless they are on a bed that allows weight measurement.

Albumin falls rapidly intraoperatively before recovering but remains significantly below preoperative values by day 5. The fall in albumin is probably multifactorial and is explained by a combination of capillary leak, fluid resuscitation, intraoperative blood loss and synthesis.

The presence of increased capillary permeability in this study was assessed by the use of MACR and IgGCR. These proteins are not usually present in urine in any significant quantity and microalbuminuria reflects increased systemic capillary leak[370] The MACR and IgGCR rose rapidly peaking after the release of the clamp before falling at 6 hours towards normal levels by day one. Systemic capillary leak allows the rapid transit of albumin into the extracellular space. The decrease in capillary leak postoperatively as demonstrated by the fall in MACR would trap albumin in the extracellular space. The removal of this excess albumin is then dependant on effective lymphatic drainage. The change in the colloid osmotic pressure (COP) gradient following this phase would suggest this may promote extracellular water expansion. The COP in healthy subjects is dependant on serum albumin but this is not the case in critical illness. In critically ill patients albumin contributes little to the COP[371] and it is partly compensated for by acute phase proteins[372]. This study suggests that albumin in the latter phases may have an increasing effect on COP as there is a significant negative correlation showing that as serum albumin increases the value of ht^2/R_0 decreases.

Fluid resuscitation can result in the fall of serum albumin by up to 20% as demonstrated by Lobo et al. They compared two groups of normal volunteers who were given either 2 litres of 0.9% saline or 5% dextrose. Both groups showed a rapid fall in serum albumin but the albumin had returned to normal within one hour of the infusion of 5% dextrose. The 20 % fall in albumin with the 0.9% saline group was greater than that of the packed cell

volume and was sustained beyond 6 hours[373]. It was suggested that this difference may be explained by convection with the crystalloid pulling the albumin into the extracellular space during redistribution.

Blood loss intraoperatively represents a direct loss of both red blood cells and plasma and therefore would be expected to have a direct influence on the intraoperative and initial postoperative level of albumin. The effect of fluid resuscitation in this scenario would have a more pronounced effect in increasing the percentage fall in albumin. The effect of blood loss is confirmed with the significant negative correlation between intraoperative and postoperative albumin.

This reduced albumin state in the early postoperative stages is further enhanced by the increased breakdown of albumin as part of general protein catabolism seen in the post injury response. Synthesis of albumin has been shown to be reduced in animal and human *in vivo* studies by the action of TNF-α and IL-6 reducing albumin mRNA expression[374,375,376].

Electrolytes and Renal function

Both sodium and potassium are seen to rise initially postoperatively with sodium peaking on day one and potassium being maximal on day two before falling to concentrations below preoperative levels. The mean results over the whole study period did not fall out of the normal range for sodium (135-145 mmol/l) or potassium (3.5 - 5.0 mmol/l).

The retention of sodium in the first 24 hours following surgery is well recognised and is secondary to the activation of the renin-angiotensin-aldosterone system(RAAS) with a

decreased urinary sodium excretion[377]. Lobo et al in volunteers receiving 0.9% saline demonstrated the suppression of RAAS allowing the increased excretion of sodium. Drummer et al previously in normal volunteers had shown that RAAS was suppressed and there is an increased urinary excretion of sodium for 2 days following a 2 litre 0.9% saline infusion. A further study by Lobo et al in patients undergoing colonic resection showed increased urinary sodium from day 2 postoperatively which was still present at day 4. Though it's not possible to directly correlate the events in normal individuals to those in the postoperative patient it would appear following the initial postoperative sodium retention that there would be a suppression of RAAS and thus prolonged excretion of urinary sodium.

We also have to take into account the results of the BIA measurements which showed an increase in ECW up to day 3 before it falls. It has been suggested that changes in plasma sodium are reflection of changes in water rather than sodium balance with a 1 mmol rise in sodium in a 70kg man requiring 280ml more water. This would suggest that the initial postoperative rise in sodium is secondary to fluid deficit of about one litre. The BIA measurements contradict this as ECW is already rising by day 1 and would suggest the sodium retention is due to RAAS activation.

Lobo et al had also shown that urinary potassium excretion is increased from day 1 postoperatively and continues through the postoperative period. This is not reflected in the plasma potassium results in this study until day 4. This is partly to do with intravenous potassium replacement and the increased potassium release secondary to the catabolism of protein and glycogen postoperatively. Following the reintroduction of diet there is an increased intracellular uptake of potassium. If there is a postoperative deficit of

intracellular and extracellular potassium this would be reflected by a subsequent fall in plasma potassium concentration.

α1-microglobulin remained elevated from preoperative levels throughout the study period. This would suggest that there is a sustained period of proximal tubular dysfunction. This dysfunction is further indicated by the elevation in postoperative urea and creatinine. The longer the clamp time the more likely that there will be proximal tubular dysfunction as indicated by the positive correlation of clamp time with α1-microglobulin creatinine ratio. Mannitol would appear to have a protective effect towards proximal tubule function as the mean α1- microglobulin creatinine ratio is significantly less in those receiving mannitol.

Inflammatory response

There were no significant detectable changes in the median IL-1β throughout the study period. Visual examination of the raw data shows that over the study period within subjects that the IL-1β is undetectable at several time points. This variability in results is secondary to the episodic release of IL-1β, the local nature of its action and the peak release occurring 90 to 180 minutes post injury. Baigrie et al have detected IL-1β in open AAA repair but only with more intensive sampling peaking at 2 hours post surgery[305]. IL-10 in contrast did show a peak rise at 6 hours with prolonged trend towards preoperative values by day 5. The mean concentrations of IL-10 at the different time points were not significantly different.

Median IL-6 started rising rapidly just prior to clamp release peaking by day 1 before falling steeply towards baseline by day 3. There continued to be a significant mean difference between preoperative and day 5 IL-6 though the trend was gradually down.

Results in previous studies have shown that the peak rise in IL-6 occurs around 6 hrs [308,379]. Though this has not been consistently the case as other studies have shown a peak at 24 hours [309,378]. The correlation between operation time and IL-6 would suggest that the IL-6 response is more dependant on length of surgical insult rather than length of peripheral ischaemia[304]. This is borne out by the clamp time only showing an association with IL-6 at 6 hours.

The two physiological scoring systems used in this study SOFA and APACHE score correlated with plasma IL-6 concentration. As previously discussed in the introduction all three of these have been show to be predictors of prognosis and these relationships help strength that argument. There does seem to be a difference in the level of IL-6 in those with septic complication compared to those without though this was only significant on day 4. The one patient who died had persistently elevated IL-6 throughout the study period.

IL-8 started rising rapidly post clamp peaking at 6 hours before falling towards preoperative levels by day 2. Other studies in aneurysm repair have shown a peak in IL-8 between 3 to 4 hours post surgery before returning toward preoperative values by 24 to 48 hours[379, 377]. Il-8 as with IL-6 showed that the length of operation but not clamp time influenced the level of IL-8. It also showed a similar pattern of correlation with APACHE and SOFA score and the same mean rank score difference on day 4 with septic complications.

The role of IL-6 in stimulating the production of the acute phase protein CRP seems to be confirmed in this study with the correlation between IL-6 and CRP. As would be expected it reaches a peak at 72 hours before falling towards but not returning to base line levels by

day 5. There is no association between CRP and operation length but there is some correlation with clamp time which seem a little at odds with the IL-6 results. CRP synthesis is also regulated by IL-1β and TNF-α and so it's feasible that clamp time has an effect on their local production but due to sampling we are unable to determine this here. There is a moderate association between SOFA AUC and CRP AUC but no significant mean differences in CRP day 3, day 5 and CRP AUC in those developing septic complications. Several previous studies have assessed CRP in its ability to determine severity of inflammation with highest mean values being higher in septic shock patients and decreasing towards those patients with SIRS[380,381]. In this study there does not appear to be that association with septic complication but there does appear to be a relationship with an increased organ dysfunction score overall as represented by SOFA AUC.

Physiological scores

The use of the SIRS criteria in determining whether patients were undergoing a systemic response proved to be poor in this study as only two patients met two or more criteria on day 1. There is some correlation between APACHE II and SOFA score as would be expected. This correlation is not particularly strong and this is probably secondary to the differences in calculating the score. The single non survivor had an APACHE II Score of 21 predicted mortality 19.7% with one of the highest SOFA scores of 9 at 24 hours reflected in a high SOFA AUC. With the added evidence of significantly higher SOFA mean ranks in patients with septic complication it would appear that this study shows that SOFA is a good outcome marker. The major determinant of the total SOFA score is the respiratory score. This would fit with the findings that pulmonary dysfunction is common following AAA repair[323,382]. A lower phase angle has previously been identified as being

associated with outcome. This study certainly seems to show an association with phase angle and the physiological scores at 24 hours in particular with SOFA score.

Antioxidants

The normal values for GSH show great variability from laboratory to laboratory the reasons for this are probably multifactorial in nature and include different methodology, sample processing and subject selection. One large scale study by Richie et al in 715 free living adults demonstrated a mean GSH of 1.020 (sd 0.167) mmol/l with a threefold range of GSH concentrations. The mean GSH without correction was in this study 1.257 mmol /l this is higher than the mean 0.561 (sd 0.09) mmol/l as determined by Luo et al using a similar method in 8 preoperative patients[383]. It is difficult with such variability to determine an inadequate status but it is unlikely any of the subjects in this study achieved this with the minimum GSH value being 0.975 mmol/l.

Intraoperatively, GSH fell significantly just prior to clamp release with some recovery but not to preoperative values by 6 hours where it stayed until day 5. These results would suggest a significant loss in GSH in the intraoperative period which does not recover significantly in the postoperative period. The greater fall during the clamp period may be secondary to free radical activity during this period. However, sustained loss of GSH would only be possible if the enzymatic conversion of GSSG to GSH was overwhelmed. Surplus GSSG would then be transported out of the erythrocytes or bound to protein sulphydryl groups resulting in a net loss of GSH. It would be expected that GSH levels would rise towards normal in the postoperative period secondary to *de novo* synthesis by the liver this is not reflected in these GSH results. The most likely reason for this persistent fall is that measurement of whole blood GSH is not a true reflection of its status

as 99% of GSH is found in the red blood cells. Expressing GSH as a litre of red blood cells better reflects its true status and corrects for changes in haematocrit.

The correction of GSH changed the profile of GSH concentration during the study period. The mean preoperative GSH only showed a significant difference to the GSH just prior to clamp release and had returned to baseline value post clamp. This would suggest that there is significant oxidative stress occurring during clamping but it resolves rapidly following clamp release. The return of GSH to baseline post clamp would be consistent with previous work by Mathru et al. In ten patients undergoing knee replacement under tourniquet they showed an increase in local but not systemic H_2O_2 production following reperfusion. This had peaked at 5 minutes of reperfusion but had returned to pre tourniquet levels by 10 minutes of reperfusion[173].

Vitamin E showed a rapid fall from its from preoperative concentration just prior to clamp release with a smaller fall post clamp before showing a slow gradual recovery. As for GSH these results are misleading as they do not represent the true status of vitamin E which is best represented as a ratio with lipid[384]. The profile for vitamin E lipid ratio over the study period showed no significant change in concentration at any time point. A large British dietary and nutritional survey in 2000 and 2001 showed a mean vitamin E of 23.3 μmol/l in the 50 to 64 age group with a inadequate status being defined as < 11.6 μmol/l[385]. For vitamin E lipid ratio the mean was 4.17 μmol/mmol with the cut off for inadequate status being 2.2μmol/l[257]. Though one patient preoperatively had a low vitamin E of 14.3 μmol/l, the vitamin E lipid ratio showed this as with all subjects to be well above the level of inadequate status.

The preoperative mean value for β-carotene in this study was 304 nmol/l (range 93 to 762 nmol/l) and for α-carotene was 81.1 nmol/l range(10 to 171 nmol/l). The values for β-carotene are higher than those seen in the British nutritional and dietary survey where the mean value for men aged 50 to 64 was 248 nmol/l and 384 nmol/l in women. The survey demonstrated a mean α-carotene for men of 74 nmol/l and for women 101 nmol/l in the 50 to 64 age group.

The wide range seen with the carotenoids is due to the multifactorial nature of carotenoid bioavailability. Some major factors limiting the availability of carotenoids include physical disposition in food sources (food matrix), chemical structure, interaction of carotenoids with other nutrients (mainly dietary fat) and nutritional status[386].

β-carotene and α-carotene both show the same pattern with a rapid fall in concentration prior to clamp release and subsequently staying low with little recovery in concentration towards base line. As for vitamin E the carotenoids are transported in lipoproteins the plasma values are probably not a true reflection of their status. The lipid profile over the study period shows an acute fall prior to clamp release with a gradual sustained rise postoperatively but not reaching baseline this is a similar pattern to that of the carotenoids. This would suggest that the changes seen in the carotenoids status would not be as pronounced if any actual change occurs at all. Its difficult to recalculate the values of the carotenoids in line with lipid profile as in fasting blood 75% of carotenoids are associated with low density lipoprotein (LDL) and the remaining with high density lipoprotein (HDL) and very low density lipoprotein (VLDL). The more pronounced rise in the lipids from day 3 is secondary to the reintroduction of diet with the carotenoids slower recovery still reflecting the period of postoperative fasting.

Several studies have shown the level of total antioxidant capacity (TAC) to fall following vascular surgery. Sparks et al in patients undergoing femorodistal bypass demonstrated a fall in TAC following reperfusion. This fall in TAC was greater in those patients who had two or more of the SIRS criteria. The TAC remained below preoperative levels for seven days postoperatively[387]. Khaira et al in patients undergoing AAA repair showed that TAC fell during clamping and following reperfusion but returned to preoperative levels by twenty four hours[388]. This is in contrast to Cornu-Labat et al who showed that the TAC remained below preoperative levels at 24 hours postoperatively in patients having surgery for AAA and Aorto-occlusive disease[389]. This study would suggest that there is no significant change in the four antioxidants measured over the study period. Urate which has powerful antioxidant activity fell significantly during clamping and remained significantly below its preoperative concentration throughout the study period. Urate accounts for 76.4% of the TAC using the chemiluminesence method (Sparks and Khaira) and 21% of the Fe/metmyoglobin method (Cornu-labat). The falls in TAC seen in these previous studies could probably be largely accounted for by changes in urate. As none of these studies measured urate it's difficult to determine if this is the case. Proteins including albumin can also have a confounding effect accounting for 45% of the TAC but only in the Fe/metmyoglobin method. The chemiluminesence method only measures the chain breaking antioxidants including vitamin E, vitamin C and urate[390]. Bilirubin also has an antioxidant role normally determining 1% of the TAC but obviously in jaundiced patients this contribution may be significantly increased.

Preoperative Antioxidant status and Outcome

Outcome in this study was determined by several surrogate markers that in previous studies had shown some evidence of predicting complications or survival.

The major intracellular antioxidant GSH was the only antioxidants to be associated with several of the measured outcome measures.

The relationship of GSH with phase angle is probably related to phase angle being representative of cell membrane integrity rather than its reflection of ECW/ICW ratio. This raises the possibility that lower intracellular GSH leaves the cell membrane prone to free radical induced lipid peroxidation at times of increased oxidative stress. The same situation was not reflected in the preoperative phase angle suggesting that during periods of low oxidative stress that intracellular GSH is able to cope with the free radicals produced. The vitamin E and the carotenoids have an important role in protecting the cell membrane from lipid peroxidation but no relationship is seen with phase angle. To maintain its chain breaking antioxidant activity vitamin E requires to be recycled from the α- tocopheroxyl radical. In addition to ascorbate Reddy et al have proposed that there is a GSH dependant regeneration of α-tocopherol catalysed by a heat labile factor[391]. Scholz et al have demonstrated in rat hepatic liposomes that there is a GSH dependant inhibition of lipid peroxidation. This inhibition was more pronounced in those liposomes that contained vitamin E than in those where vitamin E was absent[392].Its reasonable to suppose that with an adequate vitamin E status that inhibition of lipid peroxidation would be dependant on glutathione status. The lack of relationship with the other BIA parameters and MACR suggest that within the range of antioxidants measured there is no influence on endothelial permeability.

The negative correlation of preoperative GSH with the summary statistics for CRP suggests that it may have a role in modulating the inflammatory response. There was no demonstrable relationship between GSH and IL-6, the major stimulus for CRP synthesis. The other stimuli of CRP transcription IL-1β and TNF-α may be modulated by GSH but

have not been assessed in this study. It's possible that GSH has a direct effect on synthesis or release of CRP by a currently undetermined mechanism. The only other antioxidant measured that showed any correlation with CRP was α-carotene.

Potential clinical outcome was determined by the use of two validated physiological scoring systems APACHE II and SOFA score. In both of these there was a negative correlation with preoperative GSH. This was only significant with SOFA score but the trend was towards significance with APACHE II score. These results would certainly suggest that increased GSH levels could potentially influence clinical outcome.

The study was designed to look at the time base of the longitudinal changes that occur in elective AAA repair and therefore allow in depth comparisons of those factor that may effect these changes. This was a small pilot study and it can be argued that it statistical power is therefore likely to be low especially in a group of patients with a mortality and morbidity rate of 5.0% (1 patient) and 30% (6 patients) respectively. The morbidity is across the whole spectrum with a smaller percentage (20%, 4 patients) of these being related to the SIRS continuum. One of the aims of the study was to look at the longitudinal changes of physiological and biochemical parameters that have previously been recognised to be influenced by or are part of the inflammatory response. It follows that any modulation of the inflammatory response is likely to effect these longitudinal changes. The data in this study does suggest a relationship between GSH and some of the physiological and biochemical parameters. The small sample size may have introduced a type I error in these results but it's not just a single parameter that is significant. It can also be argued that a type II error may have occurred with the other measured antioxidants. The data from this small study does justify the development of a larger study particularly

looking at the role of GSH and gives enough information to calculate appropriate sample size.

If looking for a 25% (assumed from correlation coefficients for SOFA score) reduction in patients developing complications related to the SIRS continuum between high and low GSH groups with 80% power and significance of 0.05 would require 356 patients. Using SOFA score this is reduced to a sample size of 60 using the calculated means between the GSH groups from this study. For CRP a sample size of 9 achieves 84% power to detect a difference of 50.0 mg/l as found in this data.

The study design for this study was standardised as much as possible. Certain factors that have been recognised to modulate the inflammatory response such as non steroidal anti-inflammatory drugs and steroids have been excluded. The problem is that this is a human clinical model and as such there is heterogeneity in the intraoperative and postoperative management of the patients. Factors such as clamp time, length of operation, blood loss, fluid and blood replacement are dependant on each individual patient. Preoperative antioxidant status is independent of these factors but they may affect the magnitude of the inflammatory response as was shown by the relationship with clamp time and IL-6. It should follow that if there is a relationship between inflammatory response and preoperative antioxidant status it should not be affected by these factors. Mannitol, a recognised hydroxyl scavenger was supposed to have been received by all the patients but this did not occur. It did not have any effect on the inflammatory response but does remain as a potential confounding factor.

Conclusion

It's been well documented that patients with abdominal aortic aneurysm undergoing repair have an associated ischaemia reperfusion injury. The relationship with this response and the development of the systemic inflammatory response syndrome has been examined in depth in the introduction of this thesis. The modulation of the I/R and SIRS is likely to require a multifactorial approach. The majority of studies examining those factors that may influence outcome are done in critically ill patients who already have a significant inflammatory response. Elective AAA repair patients have no significant preoperative inflammation with a subsequent demonstratable intraoperative and postoperative systemic inflammatory response. There is an early rise in cytokines, systemic capillary leak, prolonged expansion of ECW, elevation of acute phase proteins, evidence of multiple organ dysfunction mainly pulmonary and renal and slow resolution to homeostasis. Although these responses have been shown individually in other studies it's the first time that the longitudinal changes have been studied in AAA patients. The use of patients undergoing AAA repair as a model to look at factors that effect or may modulate the inflammatory response is demonstrated in this thesis.

This was a small study and it maybe that the relationships seen with GSH are artefactual. However it does seem that there is a consistent association with several outcome measures. In addition the small sample size may have missed significant association with other antioxidants.

The primary aim of this thesis was to look at the role preoperative antioxidants status may play in modulating the inflammatory response secondary to ischaemia reperfusion. The

only antioxidant that consistently demonstrated an effect with any of the markers measured was GSH. It would appear that there are no significant changes in the concentration of the intracellular GSH and fat soluble antioxidants within blood following surgery. This would suggest that enhancing preoperative GSH may be pivotal in modulating the inflammatory response as long as the other dietary acquired antioxidants are not of inadequate status.

Appendix A

APACHE II

Acute Physiology And Chronic Health Evaluation II Scoring System

Physiological variable	High abnormal range							Low abnormal range		
	4	**3**	**2**	**1**	**0**	**1**	**2**	**3**	**4**	**Points**
Temperature Rectal ^0C	>41	39 to 40.9		38.5 to 38.9	36 to 38.4	34 to 35.9	32 to 33.9	30 to 31.9	<29.9	
Mean Arterial Pressure mmHg	>160	130 to 159	110 to 129		70 to 109		50 to 69		<49	
Respiratory rate	>180	140 to 179	110 to 139		70 to 109		55 to 69	40 to 54	<39	
Oxygenation A-aDO_2 or PaO_2 a FI02 > 0.5 record A-$aD0_2$	>66.4	46.5 to 66.3	26.6 to 46.4		<26.6					
b FI02 <0.5 record PaO_2					>9.3	8.1 to 9.3		7.3 to 8.0	<7.3	
Arterial pH (preferred)	>7.7	7.6 to 7.69		7.5 to 7.59	7.33 to 7.49		7.25 to 7.32	7.15 to 7.24	<7.15	
Serum HCO_3 (venous mEq/l)	>52	41 to 51.9		32 to 40.9	22 to 31.9		18 to 21.9	15 to 17.9	<15	
Serum Sodium	>180	160 to 179	155 to 159	150 to 154	130 to 149		120 to 129	111 to 119	<110	
Serum Potassium	>7	6 to 6.9		5.5 to 5.9	3.5 to 5.4	3 to 3.4	2.5 to 2.9		<2.5	
Serum creatinine (double point score for acute renal failure)	>305	170 to 304	130 to 169		54 to 129		<54			
Haematocrit %	>60		50 to 59.9	46 to 49.9	30 to 45.9		20 to 29.9		<20	
White Blood Count	>40		20 to 39.9	15 to 19.9	3 to 14.9		1 to 2.9		<1	
Glasgow Coma Score (GCS) Score=15 minus GCS										
A. Total physiology Score (sum of 12 above points)										
B. Age points (years) <44=0 45 to 54=2 55 to 64=3 65 to 74=5 >75=6										
C. Chronic Health Points (see next page)										
Total APACHE II Score (A+B+C)										

Appendix A:-Acute Physiology And Chronic Health Evaluation II (APACHE II) Scoring System

Chronic Health Points (CHP)

1. Assigned if the patient has a history of severe organ system insufficiency or is immunocompromised.
2. For non-operative or emergency post-operative patients, **5 points**.

   For elective post-operative patients, **2 points**.
3. Organ insufficiency or an imunocompromised state must have been evident before hospital admission and must conform to the following criteria:

   Liver: biopsy proven cirrhosis and documented portal hypertension, episodes of past upper gastrointestinal bleeding attributed to portal hypertension, or prior episodes of hepatic failure/encephalopathy/coma.

   Cardiovascular: New York Heart Association Class IV (i.e. symptoms of angina or cardiac insufficiency at rest or during minimal exertion).

   Respiratory: chronic restrictive, obstructive, or vascular disease resulting in severe exercise restriction, i.e. Unable to climb stairs or perform household duties, or documented chronic hypoxia, hypercapnia, secondary polycythemia, severe pulmonary hypertension (>40 mmHg), or respirator dependency.

   Renal: receiving chronic dialysis.

   Immunocompromised: the patient has received therapy that suppress resistance to infection, e.g. immunosuppression, chemotherapy, radiation, long term or recent high dose steroids, or has a disease that is sufficiently advanced to suppress resistance to infection, e.g. Leukemia lymphoma, AIDS.

Appendix B

Whole Blood Glutathione (GSH) Assay

Previously prepared 300µl aliquots of working monobromobimane (MBB) reagent were defrosted and kept in the dark. The blood was well mixed and 10µl was pipetted into the monobromobimane and well mixed. The sample was stood in the dark for 20 minutes before being refrozen at -20°c until sample analysis within one month. Samples were stored in triplicate.

Working Monobromobimane Buffer

Reagent list

Monobromobimane (Fluka, Cat no. 69898 MW 271,25 mg, 100mg)

Acetonitrile (HPLC grade)

Dulbeccos PBS (Sigma Dulbeccos PBS 0.137M NaCl, 1 mM KH_2PO_4, 3mM KCl, 8mM NaH_2PO_4, pH 7.5)

Serine (Sigma #S4500, MW 105.1)

Boric acid (Sigma #B0252, 100g)

Stock Monobromobimane 100 mMol/l

MBB is extremely light sensitive work in subdued light

Dilute 100mg MBB with 3.69ml acetonitrile or in proportion to give a 100mM solution.

Store at N20°C in the dark until ready for use.

PBS-serine-boric acid buffer PAS/S/B

Add 1 vial of Dulbeccos PBS to 1 L deionised water.

Add 5.26g/L serine and 3.09g/L boric acid and adjust pH to 7.4-7.5 Store at 4°G in fridge.

Shelf life is around one month.

(For analysis of whole blood reduced glutathione a 10 mmoL/l phosphate buffer may be used.)

Penicillamine stock internal standard 5mmol/L

Prepare freshly when preparing working MBB buffer as below. Ca 7.5 mg penicillamine in 10 ml water

Working monobromobimane buffer with penicillamine internal standard

Prepare in subdued light

To 15ml PBS/S/B buffer add 0.1 ml stock MBB and 0.1 ml penicillamine standard

Mix well, stand in dark for 10 min and dispense 0.3 ml aliquots into 1.5 ml microtubes. 1 aliquot will be used for each analysis.

Store frozen at -20C until use

Appendix C

Vitamin E Methodology

Reagents

BDH Chemicals Ltd

Ammonium Acetate	10013
Glacial Acetic Acid	10001CU

FISHER Chemicals

Methanol	M/4056/17
Dichloromethane	D/1852/17
Hexane	H/0406/17
Acetonitrile	A/0626/17
Ethanol	E/0665DF/17

SIGMA Chemical Corporation

Triethylamine	T-0886
Butylated hydroxytoluene	B-1378
Tocopherol acetate	T-3001

Mobile phase

Methanol	450 ml
Acetonitrile	450 ml
Dichloromethane	100 ml

B.H.T.	100 mg
Ammonium Acetate	100 mg
Glacial Acetic Acid	1 ml
Triethylamine	200 µl

Equipment

9095 Autosampler	Varian
9010 HPLC Pump	Varian
9050 UV-Vis detector	Varian
Apex II 3µ 250 X 4.6 mm HPLC column	Kinesis

Stock internal standard

(Tocopherol Acetate 2.75 mmol/L) At room temperature Tocopherol Acetate will melt to form oil. Add 130 mg to 100 ml Ethanol. Store at 4°C.

Working internal standard

Dilute 0.5 ml of this Stock with 25 ml Ethanol (1 in 50). (Tocopherol Acetate 55 µmol/L). Freshly prepare for each batch.

Calibration Standard

ChromSystems Product no 34.004.Prepare according to makers insert; store in 100 µL aliquots @ -20°C. Stable for 6 months. Check batch number for assigned values.

Controls

ChromSystems Controls Levels 1 and 2. Product no 0032 Prepare according to makers insert; store in 100 µL aliquots @ -20°C. Stable for 6 months. Check batch number for assigned values.

Method

1. Pipette 100 µL of standard, sample or control into appropriately labelled vials

2. Add 500 µL working internal standard.

3. Vortex for 15 seconds setting 6.

4. Add 500 µL Hexane; cap and vortex for 1 minute setting 6. Thorough mixing is vital.

5. Leave @ room temperature in the dark for 10 minutes then briefly vortex at setting 6.

6. Centrifuge @ 2000 rpm for 10 minutes.

7. Carefully pipette 250 µL of the upper organic phase into labelled 5 ml tubes.

8. Evaporate under air @ room temperature.

9. Reconstitute with 100 µL mobile phase, stand for 5 minutes then very briefly vortex at setting 6. Transfer to sample vials; crimp cap and place onto the autosampler and run according to preset protocol.

Typical Elution times

Vitamin E	5.0 min

Tocopherol acetate	5.5 min

Reference ranges

Plasma Vitamin E		11.6 - 37.1	µmol/L
Conversion factors	Tocopherol	mg/L x 2.32 = µmol/L	

Appendix D

Caretonoid Methodology

Reagents

BDH Chemicals Ltd

Ammonium Acetate	10013
Glacial Acetic Acid	10001CU

FISHER Chemicals

Methanol	M/4056/17
Dichloromethane	D/1852/17
Hexane	H/0406/17
Acetonitrile	A/0626/17

SIGMA Chemical Corporation

Triethylamine	T-0886
Butylated hydroxytoluene	B-1378

FLUKA

Ethyl-B-apo-8'-carotenoate(trans)	10815

Mobile phase

Methanol	450 ml
Acetonitrile	450 ml
Dichloromethane	100 ml
B.H.T.	100 mg

Ammonium Acetate 100 mg

Glacial Acetic Acid 1 ml

Triethylamine 200 µl

Stock Internal standard (100mg/L)

Dissolve 5 mg Ethyl-B-apo-8'-carotenoate in 50 ml Methanol. Store @ -20°C

Working internal standard (0.5mg/L)

Dilute 500 µL stock solution to 100 ml with Methanol. Freshly prepare for each batch.

Calibration Standard (B-Carotene)

Freshly prepare for each batch ChromSystems Product no 32003. Lot Number 302

Calibration level 1041 nMol/L

Controls

Freshly prepare for each batch.

ChromSystems Levels 1 and 2. Product no 0025. Lot Number 481.

Quoted value β-Carotene Level 1 743 nMol/L

Quoted value β-Carotene Level 2 1438 nMol/L

Method

1. Pipette 200µL of standard, sample or control into appropriately labelled vials

2. Add 500µL working internal standard.

3. Vortex mix for 15 seconds setting 6.

4. Add 500µL Hexane, cap and vortex mix for 1 minute setting 6.

5. Leave @ 4°C for 10 minutes vortex 5 seconds setting 6.

6. Centrifuge @ 2000rpm for 10 minutes.

7. Carefully pipette 250 µL of the upper organic phase into labelled 5 ml tubes.

8. Evaporate under air @ room temperature.

9. Reconstitute with 100 µL mobile phase, stand for 5 minutes then briefly vortex mix. Transfer to sample vials; crimp cap and place onto the autosampler using the preset routine.

Typical Elution times mins

I.S.	4.0
B-crypto-xanthine	4.6
Lycopene	7.4
α-Carotene	10.9
β-carotene	11.6

Reference ranges

Plasma β-carotene	180 – 1580 nmol/L

Appendix E

Blood and Urine sampling times for each test

	pre-op	clamp	post clamp	6 hours	24 hours	day 2	day 3	day 4	day 5
Blood samples									
Haemoglobin	x				x	x	x	x	x
Haematocrit	x	x	x	x	x	x	x	x	x
White blood cells	x				x	x	x	x	x
Platelets	x				x	x	x	x	x
Sodium	x				x	x	x	x	x
Potassium	x				x	x	x	x	x
Urea	x				x	x	x	x	x
Creatinine	x				x	x	x	x	x
Bilirubin	x				x	x	x	x	x
Albumin	x				x	x	x	x	x
Urate	x				x	x	x	x	x
Interleukin 1b	x	x	x	x	x	x	x	x	x
Interleukin 6	x	x	x	x	x	x	x	x	x
Interleukin 8	x	x	x	x	x	x	x	x	x
Interleukin 10	x	x	x	x	x	x	x	x	x
C reactive protein	x				x		x		x
Glutathione reduced	x	x	x	x	x		x		x
Vitamin E	x	x	x	x	x		x		x
Caretenoids	x	x	x	x	x		x		x
Lipid Peroxidation	x	x	x	x	x	x	x	x	x
Urine Samples									
Microalbumin	x	x	x	x	x	x	x	x	x
IgG	x	x	x	x	x	x	x	x	x
α1-microglobulin	x	x	x	x	x	x	x	x	x
Creatinine	x	x	x	x	x	x	x	x	x

Appendix F

Summary of cytokine assay procedures

IL-1β

Summary of assay procedure

	Standards/controls (ul)	Plasma samples (ul)
Standards/controls	200	-
Plasma samples	-	200
Anti Il-1β HRP conjugate	50	50
Incubate	2 hours room temperature with continuous shaking (700 rpm)	
Aspirate	the contents of each well	
Wash	3 times with 0.4ml of wash solution and aspirate	
Chromogenic solution	200	200
Incubate	15 min at room temperature with continuous shaking (700 rpm)	
Stop Solution	50	50

IL-6

Summary of assay procedure

	Standards/controls (ul)	Plasma samples (ul)
Solution B (Buffer)	50	50
Standards/controls	100	-
Plasma samples	-	100
Incubate	1 hours room temperature with continuous shaking (700 rpm)	
Aspirate	the contents of each well	
Wash	3 times with 0.4ml of wash solution and aspirate	
Anti Il-6HRP conjugate	100	100

Solution A	50	50
Incubate	1 hours room temperature with continuous shaking (700 rpm)	
Aspirate	the contents of each well	
Wash	3 times with 0.4ml of wash solution and aspirate	
Chromogenic solution	200	200
Incubate	15 min at room temperature with continuous shaking (700 rpm)	
Stop Solution	100	100

IL-8

Summary of assay procedure

	Standards/controls (ul)	Plasma samples (ul)
Solution B (buffer)	100	100
Standards/controls	100	-
Plasma samples	-	100
Anti Il-8 HRP conjugate	50	50
Incubate	2 hours room temperature with continuous shaking (700 rpm)	
Aspirate	the contents of each well	
Wash	3 times with 0.4ml of wash solution and aspirate	
Chromogenic solution	200	200
Incubate	30 min at room temperature with continuous shaking (700 rpm)	
Stop Solution	50	50

IL-10

Summary of assay procedure

	Standards/controls (ul)	Plasma samples (ul)
Solution B (Buffer)	100	100
Standards/controls	100	-
Plasma samples	-	100
Incubate	2 hours room temperature with continuous shaking (700 rpm)	
Aspirate	the contents of each well	
Wash	3 times with 0.4ml of wash solution and aspirate	
Anti Il-10 HRP conjugate	50	50
Solution A	100	100
Incubate	2 hours room temperature with continuous shaking (700 rpm)	
Aspirate	the contents of each well	
Wash	3 times with 0.4ml of wash solution and aspirate	
Chromogenic solution	200	200
Incubate	30 min at room temperature with continuous shaking (700 rpm)	
Stop Solution	50	50

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