A STUDY OF POTENTIAL SERUM MARKERS FOR A DIAGNOSIS OF GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIAL SEPSIS

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SUMMARY

Sepsis continues to be a major cause of morbidity and mortality as it can readily lead to severe sepsis, septic shock, multiple organ failure and death. The onset can be rapid and difficult to define clinically. Despite the numerous candidate markers proposed in the literature, to date a serum marker for sepsis has not been found.

The aim of this study was to assay the serum of clinically diagnosed patients with either a Gram-negative or Gram- positive bacterial sepsis for elevated levels of nine potential markers of sepsis, using commercially produced enzyme linked immunosorbent assays (ELISA). The purpose was to find a test marker for sepsis that would be helpful to clinicians in cases of uncertain sepsis and consequently expose false positive BC's caused by skin or environmental contaminants.

Nine test markers were assayed including IL-6, IL-10, IL12, TNF-α, lipopolysaccharide binding protein, procalcitonin, sE-selectin, sICAM-1 and a potential differential marker for Gram-positive sepsis- anti-lipid S antibody. A total of 445 patients were enrolled into this study from the Queen Elizabeth Hospital and Selly Oak Hospital (Birmingham). The results showed that all the markers were elevated in patients with sepsis and that patients with a Gram-negative sepsis consistently produced higher median/range serum levels than those with a Gram-positive sepsis. No single marker was able to identify all the septic patients. Combining two markers caused the sensitivities and specificities for a diagnosis of sepsis to increase to within a 90% to 100% range. By a process of elimination the markers that survived into the last phase were IL-6 with sICAM-1, and anti-lipid S IgG assays Defining cut-off levels for a diagnosis of sepsis became problematic and a semi-blind trial was devised to test the markers in the absence of both clinical details and positive blood cultures. Patients with pyrexia of unknown origin and negative BC were included in this phase (4). The results showed that IL-6 with sICAM-1 are authentic markers of sepsis. There was 82% agreement between the test marker diagnosis and the clinical diagnosis for sepsis in patients with a Gram-positive BC and 78% agreement in cases of Gram-negative BC. In the PUO group the test markers identified 12 cases of sepsis and the clinical diagnosis 15. The markers were shown to differentiate between early sepsis and sepsis, inflammatory responses and infection. Anti-lipid S with IL-6 proved be a sensitive marker for Gram-positive infections/sepsis.

Key words: sepsis; blood cultures; diagnostic; cytokines; ELISA.

To those I love

JESUS
RB
Philip
Hannah

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ABBREVIATIONS

α	alpha
ACCP/SCCM	American College of Chest Physicians and the Society of Critical Care Medicine
ADP	adenosine diphosphate
ALD	alcoholic liver disease
AML	acute myeloid leukaemia
APTT	activated partial thromboplastin time (ratio)
ARDS	adult respiratory distress syndrome
AT	antithrombin
β	beta
BMT	bone marrow transplant
BP	blood pressure.
C	Control patient code
C5a	complement-5 anaphylatoxin
Ca	carcinoma
CABG	coronary-artery by-pass graft
CAD	coronary artery
cAMP	cyclical adenosine monophosphate
CAPD	continuous ambulatory peritoneal dialysis
CD	cluster of differentiation
CDSC	Communicable Disease Surveillance Centre
cGMP	cyclic guanosine monophosphate
CLL	chronic leukocyte leukaemia
CLP	cecal ligation and puncture
CML	chronic myeloid leukaemia
CNS	coagulase-negative staphylococci
COAD	chronic obstructive airway disease
COPD	chronic obstructive pulmonary disease
COX	cyclooxygenase
CR	complement receptor
CRP	C-reactive protein

CRS	catheter related sepsis
CSF	colony stimulating factor
CV	coefficient of variation
CVC	central venous catheter
CVL	central venous line
cGMP	cyclic guanosine monophosphate
DIC	disseminated intravascular coagulation
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EC	endothelial cells
EIU	enzyme immunoassay units
ELISA	enzyme linked immunosorbent assay
ESR	erythrocyte sedimentation rate
ESRF	end stage renal failure
ET	endothelin
F	female
firbros.alv	fibrosing alveolitis
fMet	formylmethionine
FN	False negative
FP	False positive
g	gramme
GM-CSF	Granulocyte-macrophage colony stimulating factor
GNC	Gram-negative cocci
GNR	Gram-negative rod
GPC	Gram-positive cocci
HC	hypercholesterolaemia
HMGB-1	High mobility group -1
H_2O_2	hydrogen-peroxide
HP	hypertension;
HRP	horse radish peroxidase
hsp	heat shock proteins
ICU	intensive care units
IDDM	insulin dependent diabetes mellitus
IFN-γ	interferon-gamma

IL-1	Interleukin-1 (2,6,10 and 12)
ILra	interleukin receptor antagonist
ICNARC	Intensive Care National Audit and Research Centre
INR	international normalised ratio
L	litre
LBP	lipopolysaccharide binding protein
LFA-1	leucocyte functional antigen
LPS	lipopolysaccharide
L-selectin	leucocyte Selectin
LTA	lipoteichoic acid
LTB	leukotriens
M	molar
MAC	membrane attack complex
M/F	male/female
MI	myocardial infarction
MIF	macrophage inhibitory factor
μg	microgram
mg	milligram
ml	millilitre
MOD	multiple organ dysfunction
MOF	multiple organ failure.
MRSA	methicillin resistant *Staphylococcus aureus*
N	Negative sepsis patient code
(n)	number
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NCTC	National Collection of Type Cultures
nd	not done
ng	nanogram
NHL	Non Hodgkin's lymphoma
NIDDM	non-insulin dependant diabetes mellitus
NK	natural killer cells
nm	nanometre
NO	nitric oxide

NO	nitric oxide
NPV	negative predictive value
nr	no record
NSAIDS	non-steroidal anti-inflammatory drugs
osteo	osteoarthritis
p	pico
P	Positive (sepsis patient code)
PAF	platelet activating factor
PAI-1	plasminogen activator inhibitor
PAMPs	pathogen-associated molecular patterns
PARS	protease activated receptors
pbsct	peripheral-blood stem cell transplant
PCT	procalcitonin
PEM	protein energy malnutrition
PGI_2	prostaglandin 2 (is prostacyclin)
PHLS	Public Health Laboratory Service
PLA_2	phospholipase A2
PMN	polymorphonuclear (leucocyte)
PPV	positive predictive value
PRRs	pattern recognition receptors
P-selectin	platelet Selectin
PUO	pyrexia of unknown origin
RA	rheumatoid arthritis
R&D	research and development
RF	renal failure.
rhab	rhabdomyolysis
ROC	receiver operator curve
RPM	rate per minute
RTA	road traffic accident
SD	standard deviation
Sens	Sensitivity
sE-selectin	soluble endothelial -selectin
s-ICAM-1	Soluble intercellular adhesion molecule
SIRS	systemic inflammatory response syndrome

SLE	systemic lupus erythematosus
s-Le^x	sialyl-Lewis^x
SOP	standard operating procedure
Spec	Specificity
TAFI	thrombin activatable fibrinolysis inhibitor
TF	tissue factor
TFPI	tissue factor pathway inhibitor
Th2	T- helper 2 lymphocytes
TLR	toll-like receptor
TMB	tetramethyl benzidine
TN	true negative
TNF-α	tumour necrosis factor alpha
TP	True positive
TPN	total parenteral nutrition
t-PA	tissue type plasminogen activator
TSST-1	toxic shock syndrome toxin
TVCAD	triple vessel coronary artery disease
UTI	urinary tract infection
v	versus
vWF	von Willebrand factor
VAP	ventilator associated pneumonia
VDJ	variable diversity joining (region)
VRE	vancomycin resistant enterococci
WBC	white blood cell
WCC	White cell count
w/v	weight per volume
YPU	young person unit

CHAPTER 1: INTRODUCTION

1.1 DEFINING SEPSIS

Sepsis has been defined as a systemic inflammatory response in the presence of confirmed infection and is associated with a high mortality rate resulting primarily from shock (Bone, 1992). It is a frequent complication in patients who are critically ill with other diseases and it is reported to be the most common cause of death in non-coronary intensive care units (Giudici *et al.*, 1999). The current guidelines for a clinical diagnosis of sepsis were established in 1991 during a Consensus Conference sponsored by the American College of Chest Physicians and the Society of Critical Care Medicine (ACCP/SCCM) and are reproduced in table 1 (Bone, 1992).

A major problem with the definition of sepsis is the requirement for a confirmed infection to change the systemic inflammatory response. However many patients, especially in intensive care units do not have positive cultures, possibly due to prophylactic or on-going antibiotic treatment (Vincent, 2002). These types of cases have higher mortality rates thought to be due to delayed diagnosis (Reyes *et al.*, 1999).

The systemic inflammatory response syndrome (SIRS) criteria are reported as being too sensitive and aspecific for a bedside prediction of a possible microbial infection; 56 to 98 percent of hospitalised patients may have SIRS and therefore the predictive value of SIRS for infection and bacteraemia is poor. However patients with SIRS plus a positive blood culture are more likely to have sepsis than patients with a positive blood culture but without SIRS (Bossink *et al.*, 1998). Exacerbating the problems involved in defining this complex disease, SIRS criteria are known to include patients without a systemic inflammation (Takala *et al.*, 1999).

The onset of sepsis can be rapid and difficult to define clinically, as there are other non-infectious causes of a systemic inflammatory response such as acute pancreatitis, trauma, burns and post surgical inflammatory responses that are accompanied by classical signs of infection including fever, raised white cell count and hypotension (van Dissel, 2002). Therefore a serum marker or markers specifically reflecting the presence of an infection would be a valuable diagnostic test for patient management

Table 1.1 Guidelines for establishing the Clinical Diagnosis of Sepsis (ACCP/SCCM, 1991)

Clinical condition	Clinical Criteria
The **S**ystemic **I**nflammatory **R**esponse **S**yndrome (**SIRS**) At least two criteria from the list opposite.	Temperature < 36°.6C (96°F) or > 38°.3C (101°F) Heart Rate > 90 beats/minute Respiration >20/minute White cell count >12.0 x 10^9/l or < 4.0 x 10^9/l
Sepsis	SIRS plus a confirmed infection
Severe Sepsis: - Sepsis associated with manifestations of any organ dysfunction.	Hypotension – a systolic blood pressure of < 90 mm Hg Acute altered mental state –confusion Lactic acid acidosis –reflecting perfusion abnormalities Oliguria - < 400ml/day or 15ml/hour
Septic Shock: – When the organ dysfunction involved is the heart.	Sepsis plus a hypotension that does not respond to interventions such as fluid resuscitation or vasopressors.
Multiple Organ Dysfunction Syndrome (MODS) also called multiple organ failure (MOF)	Failure of more than one organ to maintain homeostasis in the acutely ill patient in the context of SIRS.

Primary multiple organ dysfunction (MOD) occurs as a result of a well defined insult such as renal failure due to diabetes. In secondary MOD the organ failure is secondary to a primary insult such as infection (Moreno *et al.*, 2002).

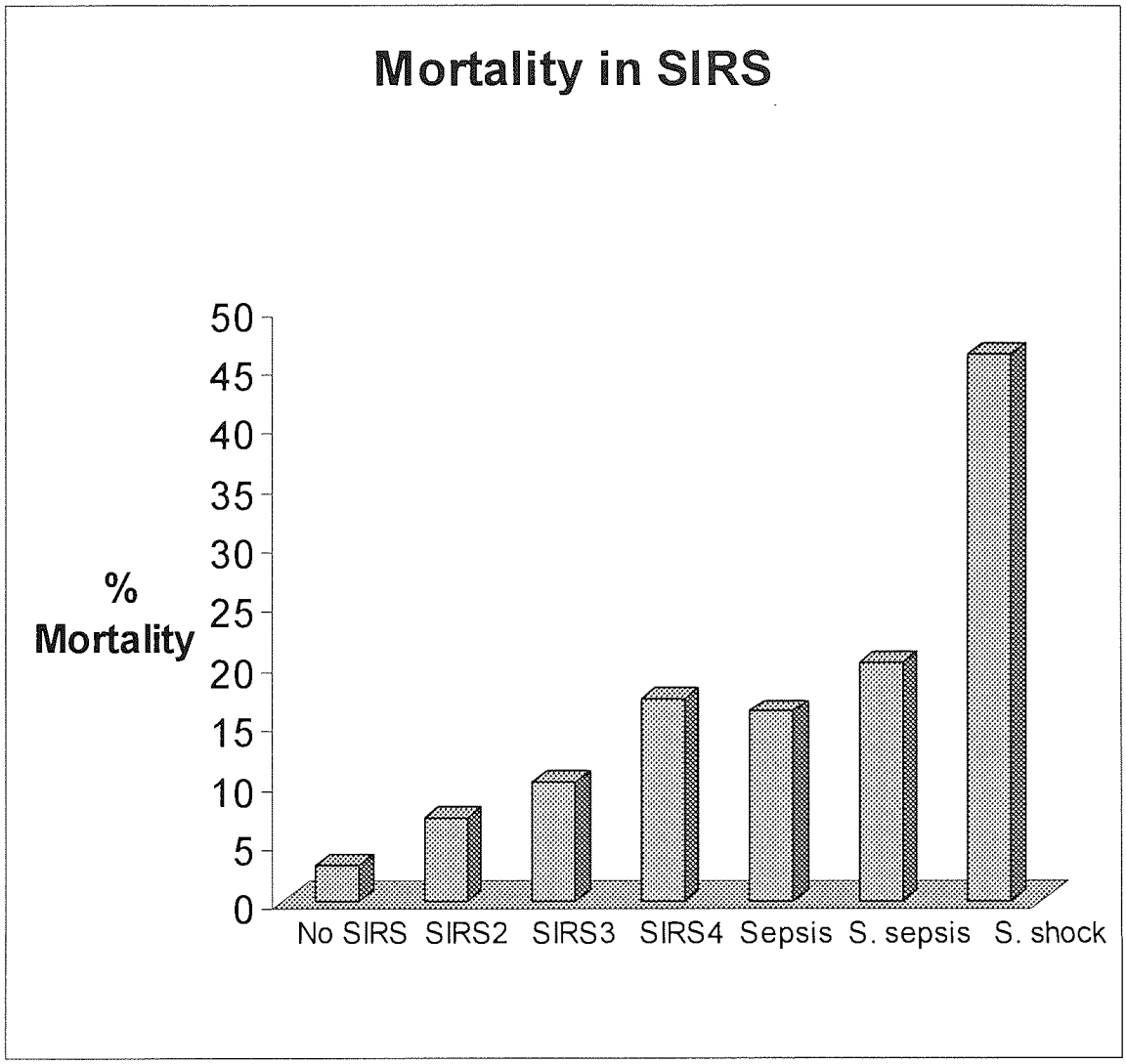
1.2 EPIDEMIOLOGY, INCIDENCE and MORTALITY RATES

For the past decade sepsis has been a major cause of death in intensive care units in both the United States and the United Kingdom. Sepsis was reported as being the 13th leading cause of death in America (Centers for Disease Control and Prevention, 1993). Two years later, a prospective study on patients with a systemic response syndrome (SIRS) demonstrated the clinical progression of SIRS to multiple organ failure (MOF) and death, with an incremental increase in the mortality rate from 7% to 46% (Rangel-Frausto *et al.,* 1995). This data is shown in figure 1.1. More recently the national incidence rate in the United States was reportedly 3.0 cases per 1000 population (2.26 cases/hospital discharge) and a hospital mortality rate of 28.6%. There was no gender difference in mortality rates but the rate increased with age, pre-existing disease and number of organ failures (Angus *et al.*, 2001).

The Intensive Care National Audit and Research Centre (ICNARC) based at Birmingham University reported the mortality rate for severe sepsis in the U.K. was 44.7%, and the incidence of severe sepsis during the first twenty-four hours in a total of 92 intensive Care Units (ICU) was 27.7%. This percentage represents about 21,000 cases per year in Britain (ICNARC 2001). However these figures do not include cases of severe sepsis that occur after the first 24 hours in the ICU or cases on other wards (NHSC, 2001).

More specifically the Public Health Laboratory Service (PHLS) reported 4,700 cases of meningococcal disease (caused by the Gram-negative microorganism *Neisseria meningitides*) in England and Wales during 1999, three quarters of these patients developed septicaemia (PHLS, 2001; Livermore *et al.,* 2001). Meningococcal disease is the most common infectious cause of death in people up to the age of 20 years, and the commonest cause of death in children aged 1-5 years with death occurring within twenty-four hours from the first symptom (PHLS, 2003).

A significant number of patients who survive a meningococcal septicaemia have long-term sequalae such as severe skin damage, amputations and neurological disorders (Levin *et al.*, 2000). Patients, who have survived a septic episode caused by other bacteria or have experienced a systemic inflammation, are said to be at greater risk of death for the next five years (Quartin *et al.*, 1997; Brod, 2000).



(Reproduced from Rangel-Frausto *et al.*, 1995)

Figure 1.1 Mortality in patients with a Systemic Inflammatory Response Syndrome

SIRS, systemic inflammatory response syndrome. SIRS with two criteria = SIRS2; SIRS with three criteria = SIRS3 etc., S. sepsis, is severe sepsis and S. shock is Septic shock. (The criteria are specified in table 1).

The graph in figure 1.1 represents SIRS as "a hierarchical continuum of an increased inflammatory response to infection" (Rangel-Frausto *et al.*, 1995). In the 'Italian Sepsis Study' mortality rates for patients in intensive care units with sepsis, severe sepsis and septic shock were 36, 52, and 82% respectively (Salvo *et al.*, 1995). A similar result for severe sepsis was found in a French study with a 58% mortality rate (Brun-Buissson *et al.*, 1995).

Other researchers argue that the death rate has decreased in some subgroups of patients with sepsis-induced organ failure, possibly due to early treatment of the infection together with improved supportive care in the intensive care units (Wheeler & Bernard, 1999). Alternatively the death rate only appears to have decreased because sepsis is not a notifiable disease whereas bacteraemias are. Bacteraemia is defined as the presence of bacteria in the blood. Bacteraemia and sepsis should not be considered as interchangeable terms for the same condition, as many patients with a bacteraemia never become septic and some septic patients may not be bacteraemic (McCabe *et al.*, 1983; Bone *et.al.*, 1989; Rangel-Frausto, 1999). However bacteraemia is reported to have a mortality rate of between 20 and 50 % (Wilson, 1994). Despite improvements in technology, therapy and patient care systems there has been little improvement in mortality from sepsis over time.

1.2.1 Mortality relating to blood culture isolates

Candida albicans is the most common cause of systemic fungal disease (Brown 2003). This yeast is considered to be a harmless commensal in the normal healthy population; nevertheless it is reported to have the highest mortality rate of 55% (Rangel-Frausto, 1999). In comparison 30% of patients with an *Enterococcal* sepsis die whereas systemic infections with coagulase negative *Staphylococcus epidermidis* (CNS) have the lowest mortality rates of approximately 15% (Rangel-Frausto, 1999).

These finding are confirmed by Jones (1998) who demonstrated that in 101 patients with systemic yeasts the mortality rate was 54 %. The second highest mortality rate was in 58 patients with a *Bacteroides* sp., 50% of whom died. In 152 patients with a *Pseudomonas* sp. in their blood culture, 46% died (Jones, 1998; Weinstein *et al.*, 1983; Roberts *et al.*, 1991). In a separate report *Candida* sp. was found to be independently associated with increased mortality, as well as pneumonia as a secondary infection and polymicrobial infections (Pittet, *et al.*, 1997).

1.2.2 Prognostic and risk of death scoring systems

Prognosis is defined as *an informed judgement of the course and probable outcome of a disease based on a knowledge of the facts of a particular case* (Koenigsberg, 1989). Prognostic scoring systems were first devised and published in the early 1970s. Their aim was to quantify the extent of injury in burns and trauma patients. An early system that remains in use today is the Glasgow Coma Score (GCS).

This evaluates the neurological status of a patient. It is a simple system that scores eye, verbal and motor responses.

Table 1.2 Glasgow Coma Score (GCS) Eye Responses

BEST EYE RESPONSE (4)	
1	No eye opening
2	Eye opening to pain
3	Eye opening to verbal command
4	Eyes open spontaneously

Table 1. 2 shows the scores for the eye. The cumulative scores should be represented for each parameter, for example a GCS of 11 should be reported as E3V3M5, that is eye score 3, verbal response 3 and motor 5. The potential total of 15 is the best score and 3 as a total score for all the parameters correlates with severe brain injury (Eidelman *et al.*,1996).

Other more complex physiological scoring systems have evolved to quantify the severity of illness and have been used to calculate a prediction of mortality. Examples include the acute physiology and chronic health evaluation (APACHE) scoring system and the sequential organ failure assessment (SOFA) system (Knause *et al.*, 1981; Vincent *et.al.*, 1996). APACHE III (an up-dated version), is used in intensive care units to evaluate risk of death. However they are not effective in determining an individual patient's prognosis and data obtained from such evaluations to withdraw intensive care is controversial (Sherck and Shatney, 1996). Probably the best use of these systems is in standardising patient groups for research and clinical trials; so that trial intervention therapies may be realistically evaluated in terms of outcome (Wood *et al.*, 1999).

1.3 PATHOGENISIS OF THE CLINICAL MANIFESTATIONS IN SEPSIS

Based on the American College of Chest Physicians and the Society of Critical Care Medicine (ACCP/SCCM) consensus conference definitions, patients with sepsis would be expected to have a temperature above 38°C or below 36°C, the latter case is less common and can occur late in the disease and is linked to a poor outcome particularly in Gram-negative septicaemia (Bryant and Hood, 1971). Human thermoregulation balances heat loss and heat production and is under the control of the autonomic nervous system and the hypothalamus (Noursadeghi and Cohen, 2000). Fever is caused by the production of endogenous pyrogens IL-1 and TNF-α released from mast cells, macrophages or neutrophils (Dinarello, 1999). They increase the synthesis of prostaglandin E (PGE) in the hypothalamus, which is the final chemical mediator of fever. Indeed hypothalamic injection of PGE cause fever in a murine model (Rang *et al.*, 1995).

A respiratory rate higher than the normal 20 breaths per minute (tachypnea) is another expected clinical manifestation, it can cause a partial pressure of carbon dioxide ($PaCO_2$) in arterial blood of less than 32 mm Hg (normal range 35-45) causing a respiratory alkalosis. A lower $PaCO_2$ could reflect a metabolic (lactate) systemic acidemia with increased blood [H+], which would also cause compensatory hyperventilation (Eccles, 1993).

Another clinical manifestation of sepsis is a heart rate greater than 90 beats per minute (tachycardia). Tachycardia increases cardiac output to compensate for systemic peripheral vasodilation which causes a reduction in the amount of blood returning to the heart. There are many vasodilators that are released by various cells during sepsis including nitric oxide (NO); bradykinin; complement anaphylotoxins (C3a and C5a); prostacycline; histamine and platelet-activating factor (PAF). They directly effect the smooth muscle cells in the microvascular circulation characterised by a decrease in vascular resistance and increase in cardiac output (Noursadeghi and Cohen, 2000). In severe sepsis cardiac output falls due to hypovolaemia from fluid loss and to increased vascular permeability

In one study with septic patients, the initial heart rate was greater than 120 beats per minute (bpm) and those patients whose heart rate dropped to less than 106 bpm within 24 hours had an improved survival rate (Parker *et al.*, 1987). Not all cases with

tachycardia have hypotension (Cruz and Dellinger, 2002). This may to due to a hypertensive baseline blood pressure prior to infection.

1.3.1 The Importance of Pyrexia in infectious diseases

Fever is described as the host's primary defence mechanism and a conserved response throughout evolution (Kluger, 1986). All host defence mechanisms are optimised during fever and work together to the detriment of the infective pathogen (Mackowiak, 1982; Graham *et al.*, 1990; Sawari and Mackowiak, 1996). Indeed a correlation has been observed between hyperthermia and cytoprotection and a failure to mount a fever response has been associated with an increase in mortality.

Given that an increase in temperature of 1°F (0.56°C) causes an increase in the pulse of 10 beats/minute, temperatures greater than 102°F (38.8°C) in patients with severe coronary disease are at risk of myocardial infarction; temperatures greater than 106°F (41°C) are detrimental to the host as there is a risk of damage to the central nervous system and protein denaturation of important enzymes resulting in death (Albrecht *et al.*, 1999). However there is no data either in the animal or human model to suggest that reducing a temperature has any benefit for septic patients. A temperature greater than 41°C does not occur in sepsis (Cunha, 2002).

Although aspirin is thought to reduce capsular expression in *Klebsiella* spp. and *Strep. pneumoniae* the use of antipyretics is said to adversely affect the action of antibiotics e.g. doxycycline so that there is no net gain for the host (Mackowiak, 1982; Plaisance and Mackowiak, 2000).

Fevers can be caused by many diseases apart from infections but a reduction in temperature in response to antimicrobial therapy is said to be the earliest and best indicator of successful treatment, an effect that is masked by antipyretic drugs (Shann, 1995). Therefore "reducing fever as part of therapy may be a mistake" (Villar and Slutsky, 2002).

1.4 THE PATHOPHYSIOLOGY OF SEPSIS

This account of the pathophysiology of sepsis will include the host's mediators of inflammation including the role of cytokines, macrophages; neutrophils and mast cells, followed by a description of microbial mediators of inflammation. Two examples of the interactions between these human and microbial mediators are then given to illustrate the mechanisms involved and the inflammation produced, firstly in a local tissue infection and secondly in a bacteraemia leading to sepsis.

The host's response to infection is normally a protective and co-ordinated one; resulting in the destruction and removal of microbial invaders, repairing damaged tissues and bringing about a return to a state of balance or homeostasis (Cannon, 1932; Edelman, 1984; Moreno *et al.*, 2002). This process involves the cells of the immune system and soluble protein mediators of inflammation called cytokines.

1.4.1 Cytokines

Four cytokines were investigated in this project, interleukin-6 (IL-6); tumour necrosis factor-alpha (TNF-α); IL-10 and IL-12, as potential markers of infection in patients with positive blood cultures and clinically diagnosed as septic. The data from this current study should provide valuable information towards an improved understanding of the occurrence and levels of these cytokines in a group of pyrexial patients who not only have sepsis but also other underlying diseases. Human data has been lacking as the majority of previous data on cytokine levels in sepsis has come from animal models and in vitro studies.

Cytokines are small protein molecules that facilitate communication between cells in an autocrine or paracrine way. Hundreds of cytokines have been identified and they include interleukins (ILs) and chemokines such as IL-8 secreted by endothelial cells; monocytes/macrophages and neutrophils (Gimbrone *et al.*, 1989). Other cytokines function as colony stimulating factors (CSFs) such as Granulocyte-macrophage –CSF (GM-CSF) (Rang *et al.*,1999). There are growth factor cytokines such as transforming growth factor-β (TGF-β) produced by lymphocytes and activated neutrophils. Recently identified as a cytokine is the high mobility group –1 protein (HMG-1), a gene transcription factor that exists in two forms; membrane bound and free in extra cellular spaces. It is produced by macrophages and pituitary cells and serum levels are higher in non-survivors of sepsis than survivors (Wang *et al.*, 1999).

Cytokines are important antigen non-specific effector molecules involved in cell growth and regulation of the immune response for instance, IL-2 secreted by activated T helper-1 lymphocytes promotes T-cell expansion. Interferon-gamma (IFN-γ) secreted by activated T cells and natural killers cells (NK), stimulates cell differentiation, and upregulates adhesion molecules on endothelium. Cytokines stimulate immune cell activation for example, IL-1 produced by monocytes (previously activated by bacterial products) acts on macrophages to produce IL-1 as well as TNF-a and IL-6 (Herbert *et al.*, 1995).

The specific cytokines produced during the early phase of an infection regulate the development of the type of T cell response produced, either in a T-helper1 (Th1) or T-helper 2 (Th2) direction (Kamogawa, *et al.*, 1993; Janeway & Travers 1997).

Table 1. 3 Cytokines in the Inflammatory Response

Cytokines Mediating the Inflammatory Response				
EFFECT	**IL-1**	**IL-6**	**TNF–α**	**Site of Action**
Fever	+	+	+	Hypothalamus
Leucocytosis	+	+		Bone marrow
Acute phase	+	+	+	Liver
Glucocorticoid-release	+	+		Pituitary and Adrenal glands

Modified from Reeves & Todd (2000). Cytokines mediating the initial inflammatory response are listed in table 1.3. IL-1 is an endogenous pyrogen secreted by monocytes and macrophages after stimulation by microbial products, when it activates other macrophages to produce more IL-1, tumour necrosis factor alpha (TNF–α) and IL-6 (Herbert *et al.*, 1995).

TNF-α is synthesised as a transmembrane protein and must be cleaved before it is released. It is an endogenous pyrogen secreted by activated mast cells, activated macrophages and NK cells and has multiple actions; it is preformed in the cytosol of mast cells and released rapidly upon cell activation (Herbert *et al.*, 1995). Interleukin-1 and TNF-α individually and synergistically orchestrate the inflammatory response (Cavaillon and Conquy, 2002). Interleukin 1 has two forms IL-1α and IL-1β. The former has never been reported in the serum of septic patients but the latter one has; although

the detection rate is variable across septic patients, it is always detected in the serum of patients with meningococcal sepsis (Van Deuren *et al.*, 1995).

IL-1α; TNF-α, IL-10 and IFN-γ as well as IL-15 are constitutive components on cell membranes. For example there is evidence that when serum levels of TNF-α could not be detected this cytokine was found as part of the cell membrane (Hauser *et al.*, 1997; Munoz *et al.*, 1991). TNF-α has been assayed on monocytes in a cell associated manner in patients with acute respiratory distress syndrome (ARDS) as has IL-8 on alveolar macrophages (Armstrong *et al.*, 2000; Donnerly *et al.*, 1993).

The vagus nerve is involved in the control of TNF-α release from macrophages (Wang *et al.*, 2003). Acetylcholine is released from the efferent vagus nerve after stimulation of the afferent vagus nerve by IL-1 and binds to specific receptors on macrophages which results in the suppression of TNF-α release, see figure 1.2

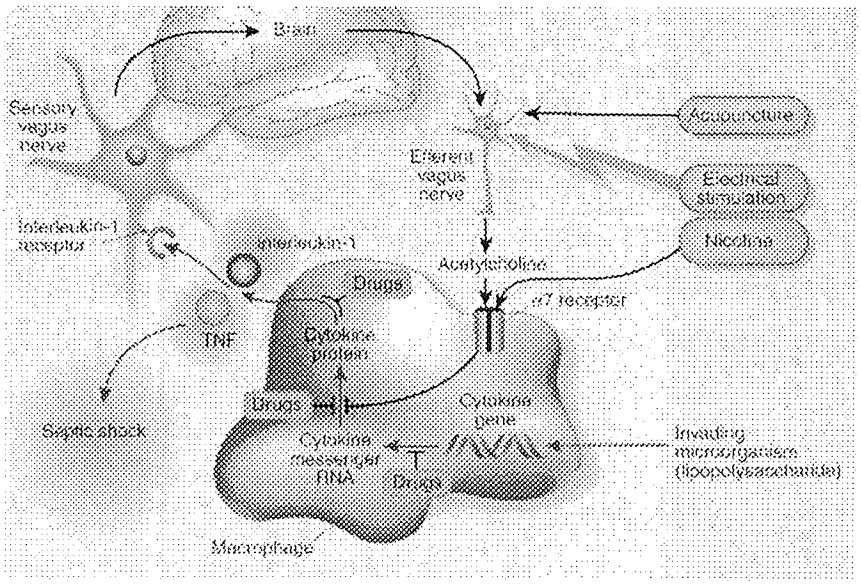


Figure reproduced from Libert, (2003).

Figure 1.2 Activation of the Nicotinic α7 receptor reduces TNF-α release from the macrophage

The α7 receptor recently characterised by Tracy and Cerami (1994), binds nicotine causing a systemic anti-inflammatory effect by down regulating TNF-α secretion, shown in Crohn's disease, a chronic inflammatory disease of the bowel

Cell associated IL-8 has also been detected on leucocytes in patients with sepsis (Marie *et al.*, 1997). The distinction to be made here is that secreted cytokines binding to cell surface receptors are usually internalised rapidly (Cavaillon and Conquy, 2002).

Inhibition of IL-8 caused a profound loss of neutrophil recruitment in a rabbit model of endotoxemia (Broaddus *et al.*, 1994). In human sepsis a high level of IL-8 is present in the serum as well as on cells, at the same time serum neutrophils appear to be immobilised. It has been postulated that as neutrophils physically encounter IL-8 they become desensitised to other IL-8 signals and this may be the mechanism that limits neutrophil extravasation and apparent immobilisation during intravascular inflammation (Marty *et al.*, 1994; Marie *et al.*, 1997; Gimbrone *et al.*, 1989; Cunha and Cunha, 1992). Normally IL-8 acts as a chemotatactic molecule such that neutrophils are drawn toward IL-8 via a chemical concentration gradient.

IL-6 is often called an inflammatory cytokine but others argue that its role in stimulating the liver to produce the acute phase proteins and the release of IL-1 receptor antagonist and a soluble TNF-α receptor puts this cytokine in an anti-inflammatory category (Tilg *et al.*, 1994).

Some researchers have found that pro-inflammatory cytokines such as TNF-α and IL-1 are significantly raised in the serum of patients with systemic inflammation and sepsis but other disagree (Gardlund *et al.*, 1995; Munoz, *et al.*, 1991). In many experimental models of septic shock, TNF-α is overproduced (Fabian *et al.*, 1995). Such data has resulted in the prevailing theory that sepsis represents an uncontrolled inflammatory response due to the abnormally high levels of pro-inflammatory cytokines (Fisher *et al.*, 1996). However in late sepsis immunodepression has been observed involving deactivated monocytes, a condition that was restored after treatment with interferon-gamma (Döcke *et al.*, 1997).

1.4. 2 Microbial mediators of inflammation and sepsis

Lipopolysaccharide (LPS) is considered to be the most potent stimulator of the host's immune response and has a central role in the pathogenesis of developing septic shock (Danner *et al.*, 1991). Picomolar concentrations of LPS are known to cause a massive upregulation of TNF-α from monocytes and neutrophils (Bone, 1991; Gazzano-Santoro *et al.* 1994; Foca *et al.*, 1998). TNF-α mediates many biological effects including vascular endothelial permeability and serum levels of this cytokine correlate with disease severity in septic patients (Nooteboom *et al.*, 2002; Casey *et al.*, 1993; Gardlund *et al.*, 1995).

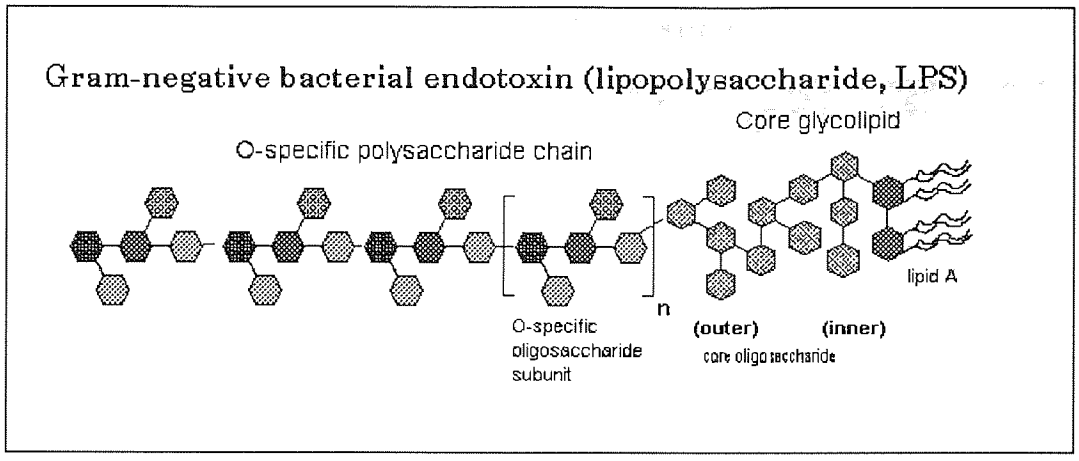


Figure 1.3 Schematic diagram of lipopolysaccharide (LPS)
Lipopolysaccharide and endotoxin have become interchangeable terms, however endotoxin refers to the lipid A moity and is considered to be the toxic part of the LPS molecule (Astiz, 2002). Lipid A anchors LPS into the bacterial cell wall. LPS is part of the outer wall of Gram-negative bacteria.

LPS actives the hosts defence system via specific receptors that recognise pathogen-associated molecular patterns (PAMPs) first expounded by Medzhitov and Janeway (1997). These microbial motifs are conserved molecules such as mannose and polysaccharides not found on human cells.

Table 1.4 lists microbial mediators of inflammation that have PAMPs and are recognised by the host's pattern recognition receptors (PRRs), with the exception of cytotoxins injected into host cells or extracellular spaces by bacterial type III secretion systems (that is, cytotoxins are mediators of inflammation without PAMPs). PRRs that recognise PAMPs include complement, mannose binding protein receptor and scavenger receptors on macrophages, monocytes and dendritic cells as well as toll-like receptors on most cell types including dendritic cells (Opal and Hubert, 2002).

Lipopolysaccharide–binding protein (LBP) an acute phase protein is constitutively expressed in the serum and opsonises LPS; this complex is recognised and binds to CD14 receptors on neutrophils, monocytes and macrophages (Wright *et al.*, 1990). CD14 lacks a cytoplasmic tail and cannot instigate a cellular response until it binds to Toll-like receptor 4 (TLR4) in conjunction with another protein MD2 illustrated in figure 1.4 (Takeuchi *et al.*, 1999). Activation of these cell receptors stimulates intracellular second messengers that ultimately cause the production and secretion of inflammatory cytokines.

Table 1. 4 Microbial mediators of inflammation

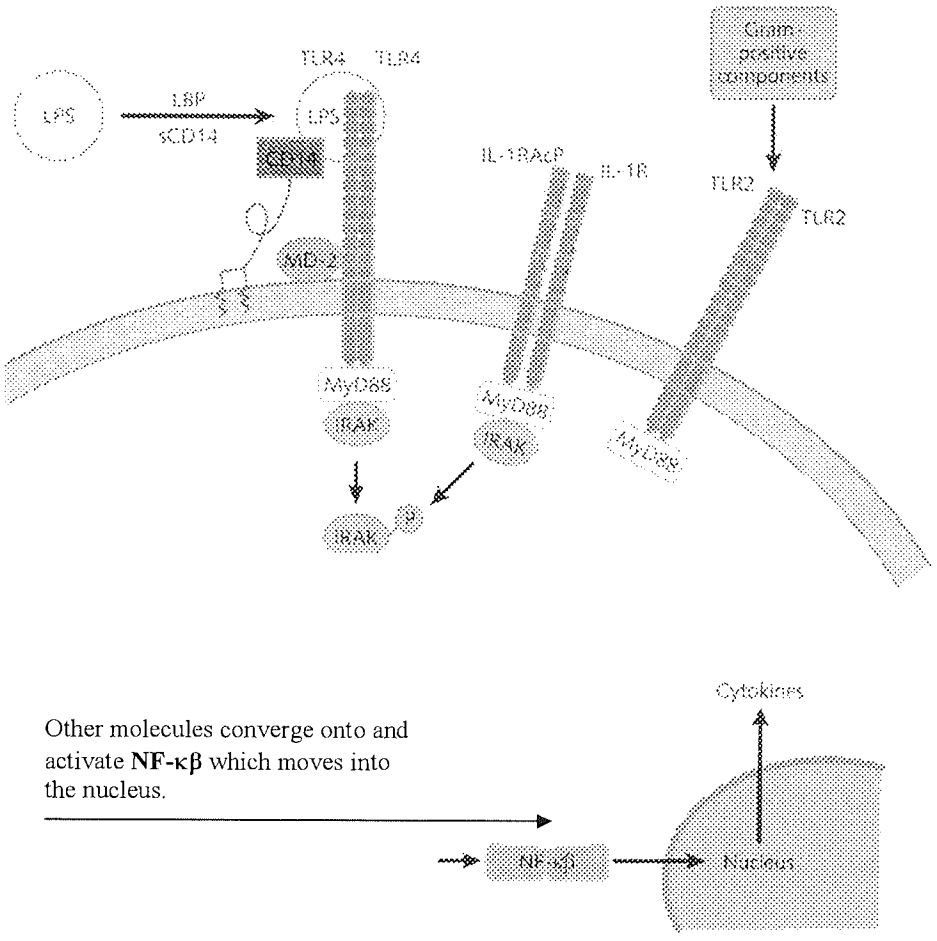
Mediator	Host receptor/action	Degree of activity	Reference
Lipopolysaccharide	LBP-CD14-TLR4-MD2	++++	1
Peptidoglycan	CD14-TLR2	++	1
Lipopeptides	CD14-TLR2	+++	1
Bacterial DNA	TLR9	++	2
Bacterial flagella	TLR5	+	3
Lipoteichoic acid	CD14-TLR2	++	4
Cytotoxins, type III Secretion systems	Disruption of cellular activity, apoptosis	+	5

Modified from Opal and Hubert (2002).

References: **1** – Takeuchi *et. al.*, 1999; **2** – Bauer *et al.*, 2001; **3**- Hayashi, 2001; **4**- Wang *et al.*, 2000; **5** – Galen and Collmer, 1999. TLR =Toll-like receptors

Gram-positive bacteria do not have endotoxin but their cell wall lipoteichoic acid (LTA) and peptidoglycan (PG) are known to be recognised by and bind to host receptors causing biological effects such as cytokine release causing activation of the innate immune by the host (Morath, *et al.*, 2001). Gram-positive cell wall components do not elicit the same potent inflammatory response as LPS, but they are known to produce exotoxins and superantigens that are implicated in toxic shock syndromes.

A novel antigen called lipid S has recently been described; it is a short form of LTA that is shed from viable coagulase negative staphylococci (Lambert *et al.*, 2000). Although it is not known as a mediator of inflammation it has been detected in patients with catheter-related sepsis caused by coagulase negative staphylococci (Elliott *et al.*, 2000). An assay for antibodies against lipid S has been developed and is included in this project to test if patients with Gram-positive sepsis can be differentiated from those with a Gram-negative sepsis. This molecule is described in chapter 2 (2.8).



MD-2 = necessary for the activation of TLR4 (Shimazu, *et al.*,1999). **MyD88** = Myeloid differentiation protein. **IRAK** = IL-1R-associated kinase. **P** =phosphate
NF-κβ =Nuclear factor –κappa B

Figure 1.4 Lipopolysaccharide-LBP-CD14 and TLR4 Complex

Proposed signalling pathway for Toll-like receptors (Le-Barillec *et al.*, 2000). Signal transduction within the cytosol leads to gene expression and the production of cytokines and other molecules that are the host's mediators of inflammation.

Soluble CD14 (sCD14) is found in normal serum, binding to LPS can activate cells that do not express CD14 (Fenton, 1998). Serum levels of sCD14 are elevated in sepsis and are associated with high mortality rates (Landmann, 1995).

1. 4. 2. 1 Microbial Virulence

An important mediator in the development of septic shock is the virulence of the invading bacteria, the size of the infective inoculum and the site of infection, for example a urinary tract infection is reported to be less life threatening than a lung infection (Plowman *et al.*, 1999). Virulence is a quantitative term that indicates the degree of pathogenicity of the microorganism and may be characterised as the ability of the

organism to attach to and colonise specific host sights and to form toxins, enzymes and other substances that will damage the host (Brock *et al.*, 1994). Table 1.5 lists some microbial virulence factors. An important example of a virulence factor is the superantigen toxic shock syndrome toxin (TSST-1).

Table 1. 5 Virulence strategies in bacterial and yeast pathogens

Virulence factors	Bacterial Pathogens	Yeasts
TOXINS		
entertoxin	*Vibrio cholerae*	*Aspergillus fumigatus*
diphtheria toxin	*Corynebacterium diphtheriae*	gliotoxins & ribotoxins
whooping cough toxin	*Bordetella pertussis*	
neurotoxin	*Clostridium tetani*	
neurotoxin	*Clostridium botulinum*	
α-Toxin	*Clostridium perfringens*	
TSST-1 and enterotoxins	*Staphylococcus aureus*	
exotoxins Streptolysin O & S	*Streptococcus pyogenes*	
ADHESION		
Fimbrial adhesions	*Haemophilus. Helicobacter,* *Escherichia* & *Salmonella* species	Candida, agglutin- like sequences *Candida albicans* transglutaminase targets
INVASION	*Mycoplasma, Yersinia, Salmonella* & *Shigella* species	*Candida albicans* using proteinases and lipases and by morphogenesis
EVASION		
By Capsules	*Streptococcal, Neisseria,*	
By Immunomodulation	*Mycoplasma* & *Escherichia* species. *Pseudomonas* & *Streptococcus* species.	*Candida albicans*
Complement inactivation	*Salmonella* & *Neisseria* species.	
By antigenic variation		*Candida albicans* by phenotypic switching

Modified from Brown, (2003).

The enzymes secreted by yeasts such as proteinases and lipases are included in table 1.5 whereas the enzymes secreted by bacteria are not included nevertheless they are important virulence factors.

Bacterial enzymes break down cells and tissues into digestible fragments. Enzymes such as heat stable DNAses from *S. aureus* destroy host cell DNA and hyaluronidase from streptococci, staphylococci and pneumococci promotes spreading through tissues (Brock *et al.*,1994).

A superantigen like the TSST-1 produced by some stains of *Staphylococcus aureus*, stimulate T lymphocytes with the same VDJ region on the T-cell's outer receptor edge illustrated in figure 1.5. Approximately 20% of all T cells will have this same VDJ specificity resulting in a massive increase in activated T-cells all secreting pro-inflammatory cytokines that are central to the lethality of this toxin (Lavoie *et al.*, 1999).

T-lymphocyte

Diagram taken from Kuby (1994).

Figure 1.5 Superantigen binding to T- lymphocytes

VDJ = variable, diversity and joining regions of both the alpha and beta chains of the T cell. One in a million T helper cells will have a receptor that is specific for the antigenic determinants of an antigen presented by the antigen presenting cell in the normal way. A superantigen overrides this specificity as 20% of T-cells will have the same outer VDJ regions causing all those T cells to become activated (Herman *et al.*, 1991).

1.4.3 Host mediators of inflammation.

The *rubor*, *calor*, *dolor* and *tumor* described by Cornelius Celsus a Roman physician two thousand years ago describes the classic signs of inflammation –redness, heat, pain and swelling. They are visible signs that the innate immunity is working, with mechanisms present and ready to deal with injury or infection. The components of the innate and adaptive immune systems will be described in terms of the following examples; a skin injury with infection and the host response to a bacteraemia. The first example is drawn from the work of Carl Nathan (2002), and describes the molecular events in mild tissue damage with infection.

Lining the perivascular tissue beneath the skin, in the alveoli and the mucosal surfaces of the gut, immune cells such as mast cells, dendritic cells and macrophages are stationed prior to an immune response recruiting other cells, in this example of tissue damage, the effects of an activated mast cell are described.

1.4.3.1 Tissue damage with infection

Tissue damage produces signals that activate the innate immune system; in response to pain, neurons release bioactive peptides such as substance P believed to be involved in pain transmission and a wide range of other responses including stimulating mast cells to release histamine (Maggi *et al.*, 1993; Steinhoff, 2000). Broken and dying cells release preformed molecules, such as heat shock proteins (hsp) and a transcription factor, high mobility group –1 (HMGB-1). When released into the extra-cellular space they promote cytokine production (Basu and Srivastava, 2000; Scaffidi *et al.*, 2002). Peptides from mitochondria are released with the fMet group characteristic of bacteria, this moiety is a potent chemoattractant for neutrophils (Carp, 1982). Finally, microbial surfaces and products bind to host cell PAMP receptors such as TLR2 and TLR4 causing intact mast cells to release *preformed* TNF-α, histamine and proteases (Nathan, 2002). TNF-α stimulates cytokine production in many cell types, including macrophages and it activates the endothelium to express adhesion molecules and synthesise prostacyclin, this molecule causes vasodilation (Rang *et al.*, 1995).

TNF-α from tissue mast cells and macrophages is protective in its effects; by increasing fluid entry to the site of injury the serum components including complement, acute phase proteins and phagocytic cells work together to clear the site of infection; these small blood vessels clot and so prevent bacteria from gaining access to the blood

stream. Fluid in the tissue drains into the lymphatic system where antigen from bacteria is taken to local lymph nodes and presented to B and T lymphocytes. B-cells then produce antibody (in the bone marrow) to the specific microbial invader; this process takes 4-5 days to complete, by which time the innate arm of the immune system may have resolved the infection (Janeway and Travers, 1997).

The TLRs on mast cells from mice have been shown to secrete different cytokine profiles depending on which receptor is stimulated. TLR2 receptors binding to peptidoglycan from *Staphylococcus aureus* (Gram-positive) produced TNF-α IL-4, IL-5, IL-6 and IL-13, whereas LPS from *Escherichia coli* (Gram-negative) binding to TLR4 produced TNF-α, IL-1β, IL-6 and IL-13. Interestingly binding TLR2 but not TLR4 caused mast cell degranulation. However TLR4 deficient mice had a higher mortality rate due to defective neutrophil recruitment (Supajatura *et al.*, 2002).

This work demonstrates that Gram-negative bacteria elicit a different host immune response than Gram-positive bacteria; this is an important factor when evaluating single serum markers for sepsis as in this current study.

Eicosanoids derived from arachidonic acid are not pre-formed, they are generated rapidly *de-novo* from cell membrane phospholipids (Rang *et al.*, 1995). Arachidonate metabolism differs in different cells, for instance in platelets the end product is thromboxane A_2 synthesis. Endothelial cells produce prostacyclin and in mast cells prostaglandin D_2 (PGD_2) is produced, the subscript indicates the number of double bonds (Rang *et al.*, 1995). Figure 1.6 illustrates these pathways. Binding to its receptor PGD_2 causes vasodilation and inhibits platelet aggregation, however when PDG_2 binds to a different receptor in the lungs it causes bronchioconstriction.

Leukotrienes and platelet activating factor (PAF) are also produced from phospholipids, PAF directly from the phospholipid and the leukotrienes from arachidonic acid but via a different enzyme to that of the prostanoids, and as the name suggests they are produced from white blood cells (Rang *et al.*, 1995). Leukotriene (LTB_4) from neutrophils is reportedly one of the most potent inducers of leucocyte chemotaxis, aggregation and degranulation (Samuelsson, 1981). Within minutes of exposure to LTB_4 (or C5a or histamine) endothelium expresses a stored pre-formed adhesion molecule called P-selectin; which begins the process of neutrophil and monocyte migration from the blood to sites of infection (Ebnet *et al.*, 1996).

The main action of non-steroidal anti-inflammatory drugs (NSAIDS) is the inhibition of the enzyme arachidonate cyclo-oxygenase and the production of prostaglandins in the hypothalamus. There are two known enzymes, cyclooxygenase-1 (COX 1) and 2 (COX 2); the former is constitutive in most cells; the latter is induced in cells by inflammatory mediators (Vane & Botting, 1996). However some NSAIDS *increase* inflammation and toxic oxygen radical production (Bahl *et al.*, 1994; Twomey & Dale, 1992). Aspirin is selective for COX-1 but paracetamol and ibuprofen are less selective for this enzyme (Rang *et al.*, 1995).

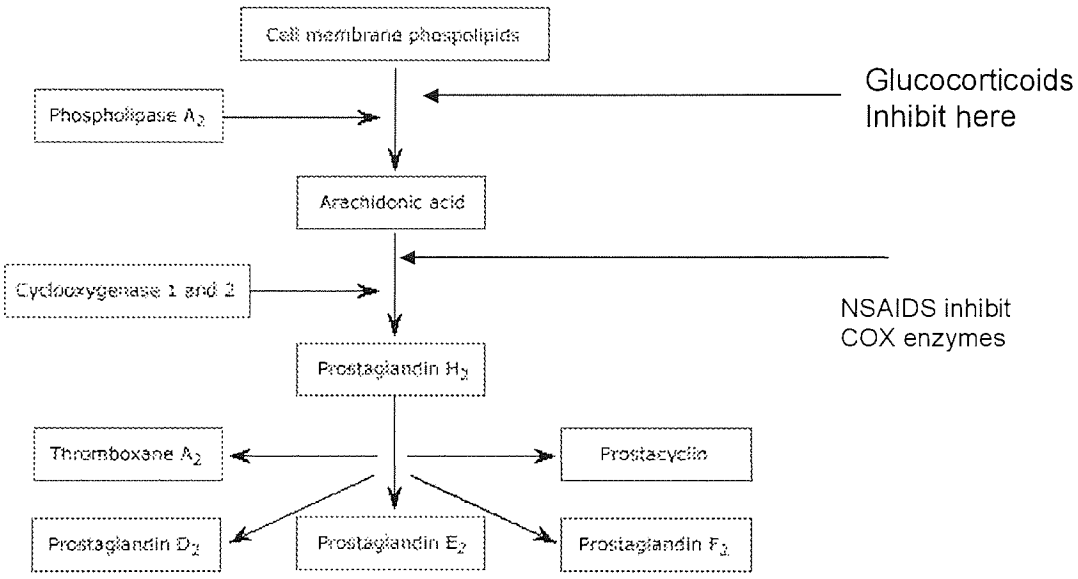


Figure 1.6 Products from the metabolism of Arachidonic acid
COX, cyclooxygenase. Anti-inflammatory inhibition of phospholipid inflammatory products with NSAIDS, non-steroid anti-inflammatory drugs.

Platelet activating factor (PAF) is a biologically active lipid, and a powerful mediator of inflammation. It is chemotactic to leucocytes and activates neutrophils and platelets, making it a pro-coagulant molecule; It is released from neutrophils, activated macrophages, eosinophils and basophils on interaction with antigen. Basophils are the serum version of tissue mast cells. Platelets also secret this molecule on stimulation with thrombin. All these cells have the enzymes to both synthesise and break down PAF. This molecule by its actions can produce two cardinal signs of inflammation; by producing local vasodilation, blood pools in the area causing redness (*rubor*) and increasing

vascular permeability allows cells and plasma exudate to leak into the tissues causing swelling (*tumor*).

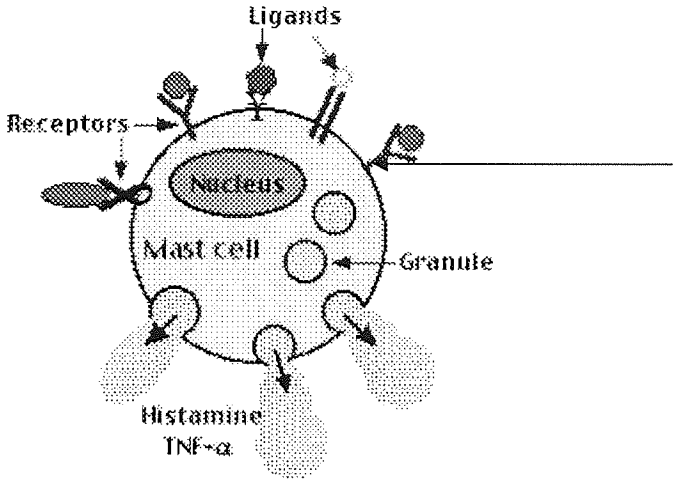


Figure 1.7 Schematic Mast cell as a first responder

The mast cell has receptors for complement components C3a and C5a, upon binding they can activate the cell to secrete the mediators of inflammation. CD88 is the receptor for C5a also found on neutrophils. All immune cells have receptors for TNF-α, binding TNF-α causes the cell to produce more TNF-α. This positive feedback system amplifies the response. Mast cells also have TLR 2 and TLR4 (Supajatura *et al.*, 2002).
Mast cells release heparin, leukotrienes, platelet activating factor (PAF) and interleukins (Nathan, 2002).

PAF can also stimulate the activation of phospholipase A_2, an enzyme that begins the metabolic pathway that can produce more PAF, a step that is inhibited by glucocorticoids (Rang *et al.*, 1995).

From a small area of tissue damage the histamine, eicosanoids, and proteases released into the tissues cause local microcirculatory vasodilation bringing more blood to the area (heat and redness), with immune cell recruitment and complement proteins bathed in plasma from the newly permeable vascular endothelium creating the swelling. Recruited neutrophils phagocytose bacteria and debris with a massive respiratory burst creating reactive oxygen species (ROS) such as hydrogen peroxide which is destructive to bacteria and host tissues. However some bacteria such as staphylococci produce catalase, an enzyme that breaks down hydrogen peroxide into harmless oxygen and water (Hart and Shears, 1996).

Only some of the players involved in the immune response to tissue injury with infection have been discussed, as even at this level of injury the immune system has many complex inter-relating stop-go signals that not only resolve the infection but repair and replace damaged tissue. The cumulative effects of the mast cell secretions, histamine, eicosanoids, newly synthesised cytokines and the proteases, produce vasodilation and extravasation of fluid from the local circulation, giving the classical signs of inflammation; redness, heat and swelling.

1.4.3.2 The vascular endothelium and bacteraemia

Vascular endothelium was formerly viewed as a passive lining a '"layer of nucleated cellophane"' with negative properties that merely separated the blood from underlying structures (Mantovani *et al.*, 1997; Volk and Kox, 2000). Normally blood cells are kept off endothelial cell (EC) surfaces, possibly by a negative net charge; by the mechanical characteristics of blood flow and constitutive nitric oxide secreted from ECs (Ziegelstein *et al.*, 1994).

Nitric oxide (NO) is a lipophilic free radical with a low reactivity relative to other free radicals. It is a key molecule in vascular regulation in health but is significantly increased in sepsis (Finney and Evans, 2002). NO has anti-aggregatory properties on platelets helping to preserve microvascular flow (Siess and Lapetina, 1989). This effect is mediated by increased levels of cyclic guanosine monophosphate [cGMP]). Inhibiting endothelial NO promotes P-selectin expression, so normal NO production may suppress the expression of P-selectin preventing immune cell rolling prior to binding and migration, reducing the efflux of macrophages and neutrophils; therefore normally produced NO actions are anti-inflammatory and anti-coagulatory (Finney and Evans, 2002).

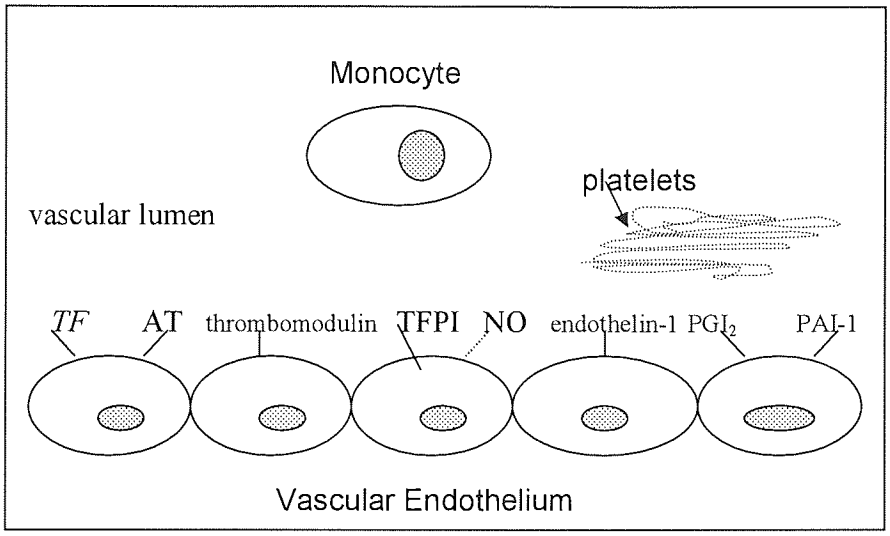
Erythrocytes are known to be scavengers of NO as the ligands for NO are metal ions in proteins such as the iron in oxyhaemoglobin leading to methaemoglobin and nitrate or a sulphur atom in proteins such as cysteine (Volk and Kox, 2000). NO has direct and indirect actions; the former effects dominate in health and the latter effects occur as a result of increased production of inducible NO resulting in cytotoxicity, altered gene expression and multiple signalling cascades (Liaudet *et al.*, 2000; Wink and Mitchell, 1998).

IL-1 and TNF-α act on endothelial cells (ECs) inducing them to secrete IL-8 and other chemokines, as well as colony-stimulating factors (CSFs), IL-6 and IL-1 (Mantovani *et al.*, 1991; Pober and Cotran, 1990). ECs express or secrete the molecules listed in figure 1.8 and TNF receptors p55 and p75, IL-1 receptor-1 (IL-1R1); CD40 receptor; chemokine receptors and many others including a putative receptor for lipopolysaccharide bound to sCD14 (Mantovani *et al.*, 1997). CD40 binds CD40 ligand on activated T cells and amplifies the induction of adhesion molecules, and it is essential for the proliferation of B-cells (Herbert *et al.*, 1995).

Infectious agents and their products and hypoxia can stimulate ECs to produce copious amounts of IL-6 (Yan *et al.*, 1995). *In vitro* and *in vivo* data suggests that IL-6 plays a role in amplifying leukocyte recruitment and activates EC functions in a unique way (Mantovani *et al.*, 1997). Indeed it has been shown that, once activated, ECs produce IL-1 and TNF-α as well as IL-6 and IL-8 which then act as autocoids to upregulate E-selectin and ICAM-1 adhesion molecules on the endothelium (Belmont and Abramson, 1999).

Complement components C3b and C5a are known to activate ECs in vitro and the assembly of the membrane attack complex (MAC, – the final pore-forming complex of the complement cascade) on the vascular endothelium participates in the upregulation of EC tissue factor promoting a procoagulatory state (Belmont and Abramson, 1999). The MAC should normally be prevented from forming on healthy ECs but not on infected cells and it has been demonstrated that *Staphylococcus aureus* can be internalised by human umbilical vein ECs (HUVEC) and induce IL-1 and IL-6 (Yao *et al.*, 1995; Lowy, 1998). *Pseudomonas aeruginosa* adheres to and enters ECs causing progressive damage, some persist in the endosomal membranes and Pseudomonal exotoxin A may directly injure ECs (Plotkowski *et al.*, 1994; Plotkowski and Meirelles, 1997).

In sepsis the hypotension and depression of myocardial contractibility together with coagulation abnormalities are due primarily to endothelial cell activation by LPS or by LPS induced IL-1 and TNF-α (Mantovani *et al.*, 1991; Pober and Cotran, 1990). Others say that even though LPS does stimulate these EC responses directly, the indirect route via LPS activated mononuclear phagocyte cytokines is more effective in activating ECs (Pugin *et al.*, 1993).



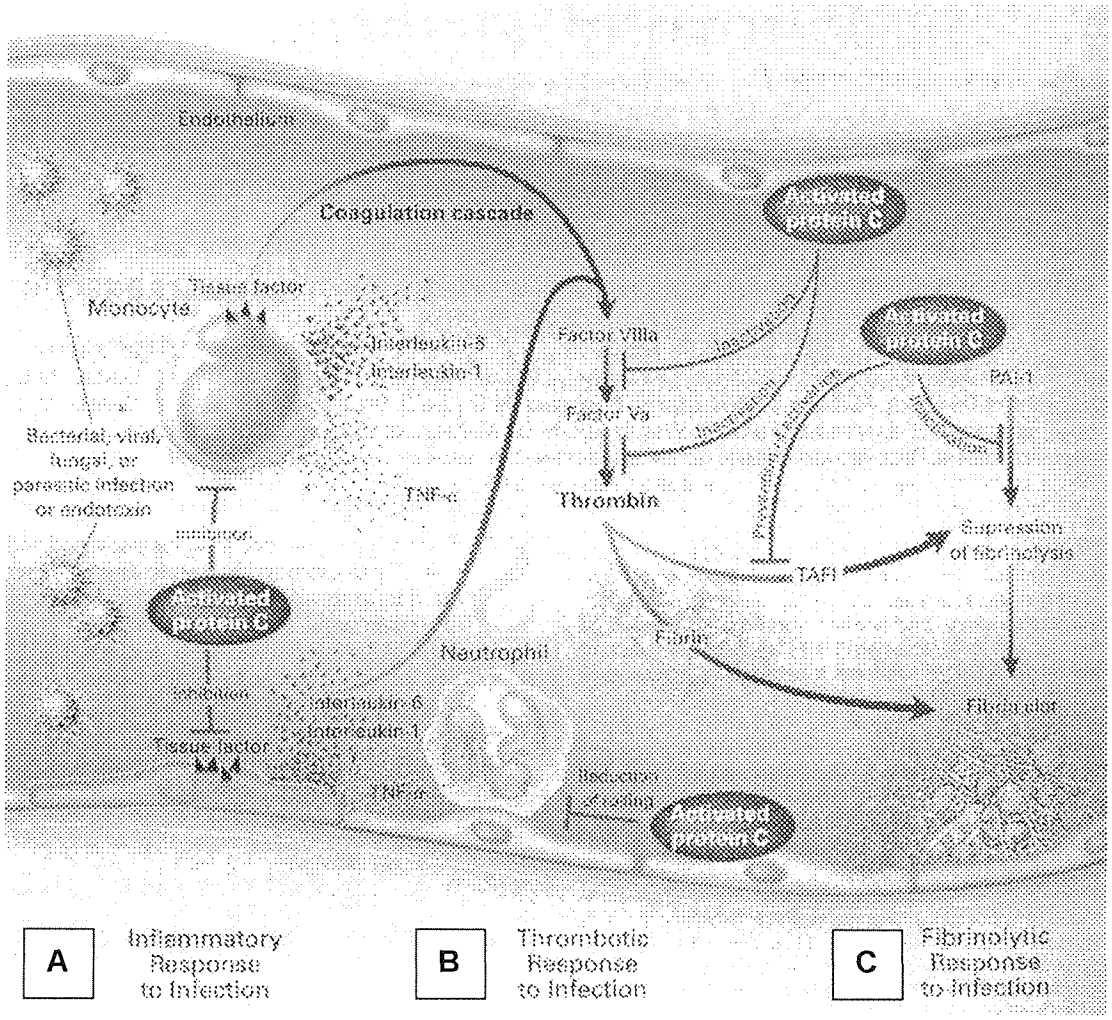
Modified from Vincent (2003).

Figure 1.8 Factors released or expressed by vascular endothelium

TF, tissue factor; AT, antithrombin; TFPI, TF pathway inhibitor; NO, nitric oxide; PGI_2, prostacyclin; PAI-1, plasminogen activator inhibitor.
Tissue factor, endothelin and PAI-1 promote coagulation; AT, thrombomodulin, NO and PGI_2 inhibit coagulation.

The vascular endothelium plays a vital role in preventing platelet adhesion and activation as well as being involved in haemostasis. An experimental model of sepsis in human volunteers given small doses of endotoxin or TNF-α demonstrated a procoagulant state with an initial profibrinolytic response followed by raised levels of plasminogen activator factor inhibitor –1 (PAI-1) expression; this molecule prevents the formation of plasmin, a molecule that slowly dissolves clots (Suffrenini *et al.*, 1989; Lorente *et al.*, 1993). However other human data supporting the endothelial procoagulatory state is lacking (Volk and Kox, 2000).

The evidence for a procoagulatory state in sepsis is deduced from clinical evidence demonstrated by laboratory measurements of platelets; prothrombin time; activated partial thromboplastin time ratio (APTT) and the products of fibrin breakdown (Eastham, 1984). Also sepsis is the most common cause of acute disseminated intravascular coagulation (DIC); large scale clinical trials revealed that DIC was present in 7-49% of patients with sepsis and was highest in patients with septic shock (Cohen and Carlet, 1996; Abraham *et al.*, 1998; Rangel-Frausto *et al.*, 1995).



Reproduced from Bernard *et al.*, (2001).

Figure 1.9 The lumen of the vasculature in microbially induced inflammation, with pro and anti coagulation responses, and the effects mediated by activated protein C.

TAFI, thrombin-activatable fibrinolysis inhibitor; PAI-1, plasminogen activator inhibitor-1. Factors Va and VIIIa are enzymes in the coagulation cascade.

Figure 1.9 illustrates three sets of activation processes in the vasculature: **A** -systemic inflammatory response due to infection; **B** – coagulation; **C**- impaired fibrinolysis; representing the factors in septicaemia that can follow one after the other causing disseminated intravascular coagulation and organ failure, multiple organ failure and death (Offord, 2002).

1.4.3.3 Microbial systemic inflammation (section A)

The section of blood vessel illustrated in figure 1.9 represents a vessel in the microvasculature rather than an artery or vein. Post-capillary venules are the primary site of inflammatory cell trafficking (Lush and Kvietys, 2000). This is understandable considering that the speed of blood flow in the arteries is about 1000 micrometres per second, whereas the rolling velocity for immune cells preparing to leave the vasculature is 2-15 micrometres per second (Hammer and Apte, 1992).

Neutrophils and other leukocytes entering post-capillary venules are pushed to the walls allowing any initiation of rolling and adhesion to occur if the necessary adhesion molecules are expressed on the endothelium (Seilenkämper *et al.*, 2002). At these slower velocities microbial initiators of inflammation (LPS, peptidoglycan) can engage with the various PAMPS receptors on neutrophils and monocytes and initiate the alternative and mannose complement cascades resulting in the release of inflammatory cytokines such as TNF-α, IL-1 and IL-6. These cytokines then activate the coagulation system by stimulating tissue factor expression on monocytes and ECs (Bernard *et al.*, 2001). Phospholipid micoparticles shed from monocytes may also contain tissue factor and provide another intravascular source of this potent procoagulant (Satta *et al.*, 1994). Tissue factor has also been found on leucocytes infiltrating organs, and phospholipid microparticles expressing tissue factor may circulate in patients with meningococcal sepsis (Nieuwland *et al.*, 2000).

1.4.3.3.1 Tissue factor

Tissue factor (TF) is only constitutively expressed in extravascular tissues, and it usually initiates clotting of blood that has escaped into the tissues from damaged blood vessels. This is a very rapid response, a clot develops within 3-4 minutes following vascular injury (Sherwood, 1993). Like all cascades there is an amplification effect where one activated molecule actives hundreds of next molecule in a chain reaction.

In vitro studies show that activated protein C inhibits the production of cytokines by monocytes and indirectly the expression of tissue factor on monocytes and the endothelium, as illustrated in section A (Bernard *et al.*, 2001).

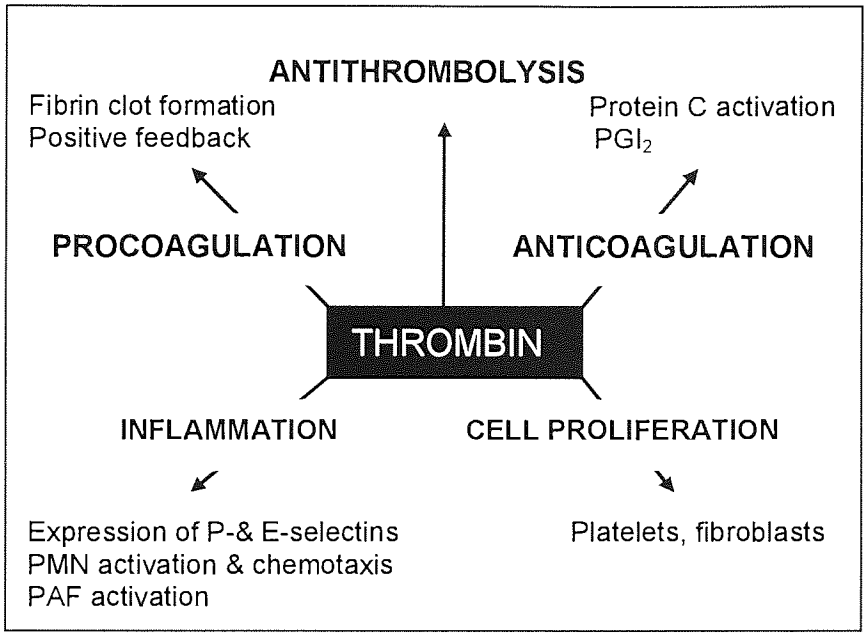
1.4.4 Microbially induced coagulation (section B)

Tissue factor together with factor VIIa in the presence of calcium ions and acidic phospholipid surfaces provided by platelets and damaged ECs leads to the formation of thrombin and thrombin is the central molecule in both pro and anticoagulant pathways (Coughlin, 2000).

1.4.4.1 Thrombin

Thrombin (IIa) is a multifunctional serine protease, but it acts like a hormone and elicits cellular responses. Signalling is mediated by a family of protease–activated receptors (PARs). They are seven membrane G-protein-coupled receptors and PAR1 is the prototype and is activated by thrombin (Vu *et al.*,1991). PARs carry their own ligands that are unmasked when the receptor is cleaved. PAR1 is like a two headed snake, thrombin cuts off one head leaving the other head attached to the receptor, this head is the ligand, it coils back and binds to the rest of the receptor intramolecularly causing transmembrane signalling (Coughlin, 2000). Activated PAR1 is internalised and degraded. New PAR1 appears on the surface from an internal pool on endothelial cells and fibroblasts but in platelets, they have to be synthesised (Coughlin, 2000).

PAR2, found on endothelial cells, is activated by trypsin and tryptase from mast cells (Nystedt *et al.*, 1994; Molino *et al.*, 1997). Tissue factor and factor X from the coagulation cascade are dependant on PAR2 activation by factor VIIa, an enzyme involved with tissue factor in the activation of factor X. This latter factor with factor Va (in the presence of calcium and a negatively charged phospholipid from activated platelets) activates prothrombin to give thrombin (Rang *et al.*, 1995). The suffix 'a' after a clotting factor indicates the active form of the molecule (Esmon, 1992). PAR2 is not activated by thrombin (Camerer *et al.*, 2000).



From Vincent, (2001).

Figure 1.10 Central role of thrombin in acute sepsis

PGI_2, prostaglandin 2, is prostacyclin; PMN, polymorphonuclear leucocytes; PAF, platelet activating factor.

Thrombin is a paradoxical molecule in that *different concentrations* elicit different biological responses; it catalyses the conversion of fibrinogen into fibrin demonstrating procoagulant activity, but smaller concentrations do not induce fibrin but activate protein C, which has anticoagulant effects, this is known as the thrombin paradox (Griffin, 1995). It also acts as an inflammatory mediator by causing endothelial cells to contract increasing their permeability. PARs also trigger mast-cell degranulation causing more inflammation (Cirino *et al.*, 1996).

Thrombin acting via PARs triggers shape change in platelets and the release of platelet activators; adenosine diphosphate (ADP), serotonin and thromboxane A2. It mobilises P-selectin and the CD40 ligand to the platelet surface and activates the integrin receptor that binds von Willebrand factor (vWF) and fibrinogen (Stenburg *et al.*, 1985; Henn *et al.*, 1998). Binding these factors causes platelet aggregation (Coleman, *et al.*, 1994). In cell cultures of fibroblasts and vascular smooth muscle cells thrombin regulates cytokine production and is mitogenic and causes calcium signalling in T-cells (Coughlin, 2000).

1.4.4.2 Protein C is a potent anticoagulant

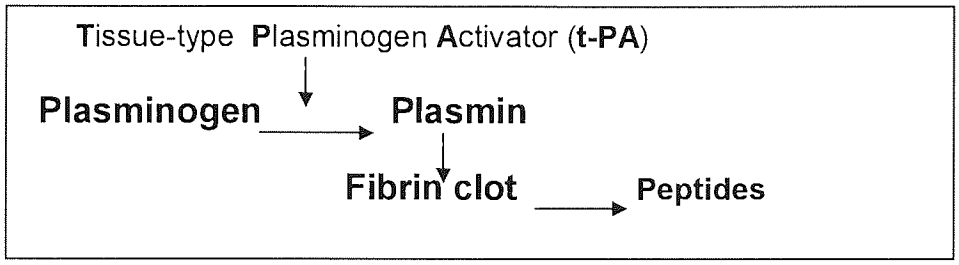
Protein C is a vitamin K dependent plasma glycoprotein and is a potent anticoagulant (Kisiel *et al.*, 1977). Although thrombin activates this enzyme, protein C inhibits further thrombin generation and thus its own activation (Esmon *et al.*, 1980; Marlar *et al.*, 1982; Gardener and Griffin, 1983). Human plasma-derived and human cell-produced protein C inhibits E-selectin mediated cell adhesion; this effect is not mediated through its serine protease activity but by binding its oligosaccharide moiety to endothelial cell E-selectin, it is a more potent ligand for this adhesion molecule than the sialyl-Lewis x ligand on leucocytes (Grinnell *et al.*, 1994).

Protein C is activated by a complex of thrombin and endothelial cell bound thrombomodulin. Thrombomodulin is expressed on vascular ECs and is an anti-inflammatory molecule that helps to keep blood fluid. Thrombomodulin binds to thrombin causing protein C to become activated, but simultaneously inactivates thrombin (Maruyama, 1998). Activated protein C (aPC) modulates the procoagulant activities of thrombin by binding to a co-factor protein S and this complex is a potent inhibitor of blood coagulation. Activated protein C (an enzyme), inactivates enzymes in the coagulation cascade, namely factors Va and VIIIa, both of these factors accelerate the formation of thrombin (Marlar *et al.*, 1982; Rang *et al.*, 1995). This part of the coagulation cascade is shown in section B in figure 1.9.

Recombinant human activated protein C has been made commercially, and the results of its administration to patients with sepsis have shown improved survival rates; it is a relatively cost effective product when used to treat patients with APACHE scores of 25 or more. This product is the first approved biological therapy for patients with severe sepsis (Manns *et al.*, 2002).

1.4.5 Host response to systemic coagulation induced by microbes (section C)

Normally there is a balance between pro- and anti-coagulant activities in the blood, anticoagulant factors include thrombomodulin expression on endothelial cells, antithrombin III and tissue -type plasminogen activator (t-PA).



From Stryer, (1988).

Figure 1.11 Fibrinolysis

Plasmin is formed from a precursor molecule plasminogen and breaks-down fibrin into peptides. Plasminogen activator-inhibitor-1 (PAI-1) prevents the formation of plasmin and can inactive formed plasmin.

The amount of thrombin and antithrombin in the blood are balanced, however as soon as coagulation occurs fibrinolytic mechanisms are set in motion. Antithrombin III is the most important inhibitor of the clotting factors, it inhibits the actions of thrombin irreversibly and other enzymes in the clotting cascade. The actions of antithrombin III are enhanced by heparin, a negatively charged polysaccharide on the surface of endothelial cells and in mast cells near the walls of blood vessels (Stryer, 1988).

Fibrin is split by plasmin (a serine protease) that acts locally within the clot. Fibrin is designed to dissolve and this process occurs slowly but readily in freshly formed clots, however two day old fibrin clots become resistant to lysis (West, 1989). If plasmin is detected free in the circulation, it is rapidly de-activated by plasmin inhibitors, including plasminogen activator inhibitor -1 (PAI-1) (Rang *et al.*, 1995).

1.4.5.1 Fibrinolysis is impaired in septic conditions

Fibrinolysis is known to be impaired in septic conditions and severe pneumonia (Vincent, 2001; Gunther *et al.*, 2000). Plasminogen activator inhibitor –1 (PAI-1) levels are raised in children with meningococcal septic shock and correlate with increased mortality (Kornelisse *et al.*, 1996). Others provide evidence that elevated PAI-1 in

patients with multiple organ failure (MOF) reflects the development of coagulation/fibrinolysis abnormalities (Mesters *et al.*, 1996).

Activated protein C directly inhibits the biological action of PAI-1, whereas thrombin-activatable fibrinolyis inhibitor (TAFI) is inhibited by thrombin (Bernard *et al.*, 2001). Studies have shown that protein C levels are reduced in patients with severe infection. Two possible reasons for this finding are: protein C is consumed in its anticoagulation role and secondly, the levels of thrombomodulin are significantly decreased due to disrupted vascular endothelial cells during systemic inflammation (Yan *et al.*, 2001; Vincent, 2001; Offord, 2002). As previously stated thrombomodulin is required to active protein C and is expressed on the surface of endothelial cells.

A rise in procoagulant PAI-1 due to the action of cytokines and low circulating levels of the potent anticoagulant factor, activated protein C, are thought to be the main contributing factors in impaired fibrinolysis and the coagulopathy seen in some patients with sepsis that result in a poor outcome (Mesters *et al.*, 1996; Offord, 2002).

1.4.5.2 Vitamin K and Warfarin

Vitamin K is essential for the synthesis of prothrombin as well as for factors VII, IX, and X, (procoagulants) and protein C (anticoagulant). The action of vitamin K allows prothrombin to bind calcium, this binding anchors prothrombin to phospholipid membranes derived from platelets after injury (Stryer, 1988; West, 1989).

Vitamin K antagonists such as wafarin, an oral anticoagulant, act by preventing the reduction of vitamin K. The physiological effect of warfarin takes several days to become established and its effect in the patient is measured by prothrombin time, expressed as an International Normalised Ratio (INR). Prothrombin time is a measure of the time taken for patient's blood to clot; prolonged clotting times are compatible with decreased hepatic synthesis of clotting factors (Mayne and Day, 1994).

Patients with liver diseases are at greater risk from the unwanted side effects of anticoagulants such as bowel or brain haemorrhage. Many drugs potentiate warfarin including antibiotics such as ciprofloxacin and metronidazole by inhibiting hepatic metabolism or by inhibiting the reduction of vitamin K such as the cephalosporins (Hirsh *et al.*, 1995; Rang *et al.*,1995).

1.4.5.3 Complement serum proteins

There are over twenty different serum proteins involved in the complement enzyme cascade and three possible activation pathways. Activating these processes results in the destruction of pathogens either directly, by membrane attack complex (MAC) forming pore holes in the bacteria causing them to lyse or indirectly by opsonising invaders for phagocytosis.

Opsonisation is the process whereby a complement component, C3b, coats the surface of pathogens. The C3b molecule then binds to specific receptors on phagocytes. In this way phagocytes can more easily engulf and destroy the bacteria (Reeves & Todd, 2000).

In the classical complement pathway factors C1 to C9 are activated sequentially by binding to antibody – antigen complexes (often IgM), resulting in the formation of the membrane attack complex (MAC), whereas spontaneous cleavage of C3 is the starting point for the alternative pathway (Frank and Fries 1991; Tomlinson, 1993). The lectin-binding pathway is initiated by serum mannose binding protein binding to foreign carbohydrates causing a serum protease to active C4 (Gerard, 2003).

The C3 component is spontaneously cleaved in the plasma into C3b and C3a. C3b is very reactive and binds to amino or hydroxyl groups. If binding does not occur within microseconds C3b is neutralised by binding to plasma water molecules, or it can be clipped by other proteins to form inactive 3Cb (*i*C3b).

C3b binding to host cell surfaces is inactivated by various means including a decay accelerating factor (DAF). Other controls include a membrane glycoprotein CD59, this molecule prevents the formation of the membrane-attack complex (MAC) on normal host cells (Morgan *et al.*, 1992).

The small peptides C4a, C3a and C5a bind to complement receptors on macrophages, neutrophils, mast cells and ECs causing cellular effects. C5a is a potent chemotactic factor for leucocytes. They are all anaphylatoxins that act directly on blood vessels causing vasodilation. C5a causes mast cell degranulation releasing histamine - a potent vasodilator (Drouin *et al.*, 2001).

Blocking the C5a receptors with antibody in an animal model of sepsis correlates with a decrease in serum levels of TNF-α and IL-6, suggesting that normal binding with C5a affects the production of cytokines. By contrast genetic deletion of C3a receptors in

mice increases their susceptibility to endotoxin shock as well as increasing levels of TNF-α and IL-1β (Riedemann *et al.*, 2002).

Pathogens have developed mechanisms for evading the effects of complement. Common pathogens such as *Staphylococcus aureus* and *Streptococcus pneumoniae* evade opsonisation by their cell surface capsular components which prevent C3b from binding. *Salmonella* species are covered in polysaccharide side chains which also prevent opsonisation by C3b (Reeves and Todd, 2000).

The complement cascade performs three major functions in the innate response to infection; they opsonise foreign bodies to enhance phagocytosis; they attract immune cells to the area under attack and they directly kill bacteria.

1.4.5.4 Summary of systemic infection with coagulopathy

The vascular endothelium it the largest organ in the body and yet it is never discussed in terms of an 'organ failure' when multiple organ failures are itemised and identified in severe sepsis; however it is now recognised that vascular endothelial cells play a key role in the pathogenesis of sepsis (Vincent, 2001).

The realisation that systemic inflammation triggers the coagulation cascade by various means and especially by the presence of infectious microorganisms or their products and that this cascade enhances inflammation, has led to a new understanding of the self-perpetuating mechanisms of SIRS. Repeating this cycle can eventually produce disseminated intravascular coagulation, leading to tissue hypoxia, organ failure, multiple organ failure and death.

A key cytokine in this process is TNF-α as it induces blood clotting. Secreted locally its effects are protective, however in a murine model when there is a systemic release of this cytokine the result can be fatal (Pfieffer *et al.*, 1993).

Nitric oxide is a key molecule in vascular regulation in health but is elevated in systemic sepsis and is heavily implicated in the pathogenisis of septic shock. Septic shock has been defined as a profound alteration in cellular oxygen metabolism in the presence of infection causing tissue hypoxia resulting in organ failure (Vincent, 1998). The cytotoxic effects of *inducible* nitric oxide has been shown to lead to a breakdown in mitochondrial function, as it inhibited oxidative phosphorylation in an animal model of cardiac myocytes (Oddis and Finkel, 1995).

The advent of the first biological therapy for sepsis in the form of activated protein C is welcomed as the first of many interventions that will reduce the high mortality rate associated with sepsis. However early detection of systemic infection with a highly sensitive and specific serum marker has yet to be identified; all the test markers in this project had the potential to fulfil this role and are discussed fully in chapter 2.

1.5 RISK FACTORS CONTRIBUTING TO THE INCREASING RATE OF SEPSIS

The steady increase in the mortality rate of patients with bacteraemia and sepsis is attributed to multiple risk factors. They can be divided into the following groups; *Patient; hospital*; *procedural* and *surgical* risk factors (Lynn, 2000). Microbial virulence and antibiotic resistance contributes to the risk of a poor out-come for the infected patient (Jones, 1998).

1.5.1 Patient's intrinsic risk factors

Sepsis has been described as a disease of the elderly, due in part to the concept of age-related immunodepression, and the recorded incidence rates (Linde-Zwirble, 1999). The nutritional status of many older patients on admission may be poor and a low protein intake will undermine the production of immuno-active molecules and cells causing an ineffective immune response to hospital acquired infections and clinical procedures (Reinhardt *et al.*, 1980). Protein energy malnutrition (PEM) causes gross reductions to occur in the synthesis of complement components and low secretory and mucosal IgA antibody; also it has been shown that PEM results in a reduction in the total number of T-cells but not of B-cells or other leucocytes (Mims *et al.*, 1993)

Hospitalised patients with serious underlying diseases other than infection are at particular risk from nosocomial and opportunistic infections as they are less likely to survive a septic episode (McCabe and Jackson, 1962b; Brod, 2000; NINSS, 2000). Patients who have organ transplants are immuno-suppressed to prevent organ rejection and they are particularly vulnerable to infections, as are patients undergoing chemotherapy for cancer treatment, causing them to become neutropenic and undermining their ability to mount an effective immune response to infections (Barriere and Lowry, 1995).

Similarly patients with the Human Immunodeficiency Virus (HIV) suffer from multiple infections due to the virus attaching to and invading CD4+ T-helper lymphocytes. These cells are a major component of the adaptive (cellular) immune response to infections (Janeway *et al.* 1996). Malnutrition is another cause of acquired immunodeficiency. The genetic makeup of an individual may contribute to a patient's innate ability to overcome infections; susceptibility genes have been described, this a new area of research and data remains to be evaluated (Stuber, 2002).

1.5.2 Hospital risk factors

The length of patient stay in hospital adds to the risk of an acquired infection. Hospital outbreaks and cross infections can occur and seriously undermine the patient's health. The practice of employing agency nurses is thought to increase infection risks for patients as these nurses move frequently from one ward to another and from hospital to hospital. They may act as carriers of infection and are often unaware of the hospitals infection control policies (House of Lords Select Committee, 1997). The hospital-specific presence of antibiotic resistant microorganisms generate difficult-to-treat infections that place patients at greater risk of developing sepsis. Indeed, patients with a hospital acquired infection are 7.1 times more likely to die than are uninfected patients (Plowman *et al.* 1999).

1.5.3 Procedural risk factors

Wound dressing and intravenous cannulae or urinary catheters as well as cannulae used for total parenteral nutrition (TPN) contribute to a higher risk for overwhelming sepsis (Johnson and Oppenheim, 1992; Raad and Bodey, 1992). TPN lines can become colonised and cause infections, using this method as opposed to enteral feeding is associated with a worse outcome (Hadfield *et al.*, 1995; Suchner *et al.*, 1996).

Vascular catheters and medical devices develop biofilms of bacteria that are resistant to many potent antimicrobial drugs and are a known cause of bacteraemia and sepsis in critical care units (DeKievit and Iglewski 2000).

1.5.3.1 Catheter-related infections (CRI) and ventilator associated pneumonia (VAP).

Eighty percent of hospital acquired infections in patients on intensive care units (ICUs) in the United States and Europe are due to ventilator associated pneumonia (VAP); followed by post surgical infections; or trauma, and catheter-related bacteraemias and sepsis (Maki, *et al.* 1998; NINSS, 2000). Bloodstream infections via central venous catheter (CVC) lines are reported to have higher rates of infection than those from peripheral lines and frequently cause life-threatening complications (Elliott and Faroqui 1992; Elliott, 1993; Elliott *et al.* 1998; Maki *et al.*, 1998).

Establishing an early diagnosis of a catheter-related infection is important for successful treatment (Elliott *et al.* 2000). However clinical diagnosis is difficult, as signs of inflammation may be absent or conversely present but due to a non bacterial thrombophlebitis (Eggimann and Pittet, 2000). The presence of common skin commensal coagulase negative staphylococci (CNS) in blood cultures or line tip cultures, adds to the diagnostic uncertainty of a CRI being present, as the isolates may simply be contaminants from the patient's own skin or the environment (Eggimann and Pittet, 2000).

Ventilator associated pneumonia (VAP) may lead to an increase in the rate of multiple organ failure and death as it can trigger cytokine release into the systemic circulation (Lynn, 2000; Heyland *et al.*, 1999). However others disagree (Petty, 1990).

1.5.4 Surgical risk factors

An increase in invasive surgical procedures, prosthetic implants and emergency versus elective surgery adds to the number of patients developing a hospital acquired infection that may lead to sepsis (Lynn, 2000).

A leading cause of death in surgical patients is infection-related organ failure (Carrico *et.al.*, 1986). Patients admitted to intensive care units following urgent surgery have a one in three chance of dying as a direct result of endotoxin, indeed some patients take years to recover from surgery as a direct result of the immune response to endotoxin (Mythen, 2003).

It is thought that there is a causal relationship between surgical procedures that cause haemorrhage and a severe paralysis of cell-mediated immunity to infections following an initial excessive immune response, predisposing these patients to

overwhelming sepsis (Faist *et al.*, 1992; Volk *et al.*, 1996). The pre-operative administration of 15 μg of granulocyte colony-stimulating factor (G-CSF) has been shown to prevent this immunosuppression (Angele and Faist, 2002).

1.5.5 Antibiotic resistant strains of bacteria

Antibiotic resistant strains of bacteria, for example, methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant enterococci (VRE) have the potential to cause serious life threatening infections. Treatment of MRSA with antibiotics such as vancomycin can have nephrotoxic side effects for the patient. Consequently hospital acquired infections with these microorganisms may add to the mortality rate. (PHLS. 2002).

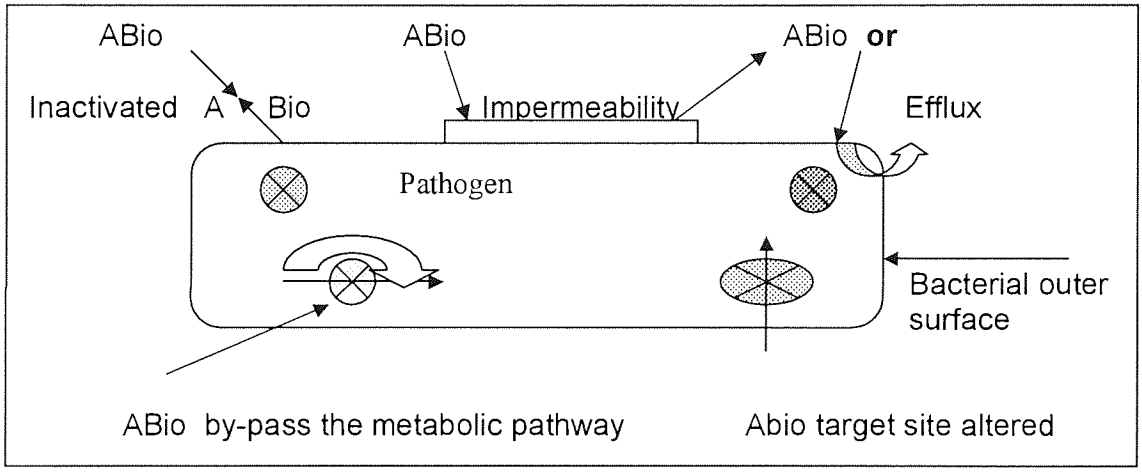


Diagram modified from Taylor *et al.*, (2002).

Figure 1. 12 Microbial mechanisms in acquired antibiotic resistance

Abio is antibiotic; ⊗ symbol represents bacterial resistance mechanisms.

Figure 1.12 shows the numerous ways in which bacteria have developed antibiotic resistance. These include (clockwise from the top left) antibiotics inactivated before reaching the target site; the outer bacterial wall becomes impermeable to the drug; antibiotics enter and are pumped out again; the bacterial target site is altered so that the drug becomes ineffective. When metabolic pathways are the targets for drug therapy, pathogens can develop alternative pathways to avoid this interference (Taylor *et al.*, 2002).

Antibiotic resistance can be transferred from one bacterium to another on plasmids. Plasmids are circular genetic elements that are separate from the bacterial chromosome. Bacteria replicate their plasmids and transfer them to other bacteria by cell to cell contact or by direct transfer of DNA by conjugation. Viruses are able to take host DNA and infect other cells by transfection.

The plasmids carrying genes for resistance to antibiotics are called R plasmids and their infectious nature allows them to spread antibiotic resistance through populations rapidly. A specific plasmid called R100 is able to transfer itself between enteric bacteria, *Escherichia*, *Klebsiella*, *Proteus*, *Salmonella* and *Shigella* but not to non-enteric bacteria such as *Pseudomonas* spp. (Brock *et al.*,1994). The global spread of antibiotic resistance in bacteria is a major health problem, turning once easily treated infections into statistics for mortality rates.

1.6 COMMON ISOLATES IN BACTERAEMIAS AND THE DISEASES THEY CAUSE

The Public Health Laboratory Service (PHLS) reported that bacteraemia caused by *Escherichia coli* had increased to over 12,000 cases in England and Wales during 2002. The highest number of reports came from the West Midlands, and was second only to *Staphylococcus aureus*, which was the leading isolate. Table 1.6 lists the most commonly reported bacteraemias in England and Wales in 2002.

In the United States (North America) *Staphylococcus aureus* was also the most prevalent pathogen isolated from patients with positive blood cultures (Pfaller *et al.*, 1998).

Bacteraemias with *E .coli* were highest in patients aged 65 years and over in 2002, with more cases in the male population (PHLS, 2003b). In the same year 49 % of the total number of surgical infections reported to the Communicable Disease Report centre (CDR) were caused by *S. aureus* and over half of these infections were antibiotic resistant MRSA strains (WHO, 2002).

Table 1.6 Bacteraemia in England and Wales: laboratory reports Jan-Dec 2002: Top-ten reported pathogens

ISOLATE	NUMBER OF REPORTS	OVERALL RANKING	G+VE	G-VE
S.aureus	12,285	1	1	
E.coli	12,185	2		1
CNS	6229	3	2	
Enterococcus spp*	4371	4	3	
S. pneumoniae	3951	5	4	
Klebsiella sp	3296	6		2
Pyogenic *Strep*	2541	7	5	
Pseudomonas spp.	2288	8		3
Enterobacter spp.	1814	9		4
Proteus spp.	1592	10		5

(Collated from the CDR weekly 16th Jan 2003).
G+ve = Gram-positive; G-ve =Gram-negative. CNS, coagulase negative staphylococcus; *Enterococcus* spp.* includes Group D *Streptococci;* Pyogenic streptococci, includes Lancefield grouping A,B, C and G. The total number of reported cases for this latter group in England, Wales and Northern Ireland for 2002 was 2698 cases (group B-991; A-921; G-580 and C-206).

The total number of Gram-negative isolates reported in 2002 from England and Wales was 25,326 and the total number of Gram-positive isolates was 33,055. Anaerobic bacteraemia totalled 1391 and other pathogenic isolates produced an overall total of 60,833 reported positive blood cultures in 2002. This was higher than the overall total for 2001 which was 59,053 (CDR weekly 16th Jan PHLS, 2003a).

The highest number of regional *E. coli* bacteraemia reported in 2002 came from the West midlands with 1679 cases (CDR weekly. 20th Feb. PHLS, 2003b).

1.6.1 The infections they cause

Infections due to bacteria can range from mild tonsillitis to life threatening septicaemias and from individual infections to epidemics of cholera or plague.

Disease occurs when micro-organisms gain access to normally sterile sites such as the blood and tissues, breeching the protective interface between the body and the environment. The potentially disease causing isolates listed in table 1.6 may be commensal bacteria that normally live in or on the human body and are often the source of the infection, particularly in surgical patients and patients with a urinary tract infection (UTI). Table 1.7 lists the types of infections usually caused by the isolates in table 1.6.

Table 1.7 Commonly reported pathogens and the infections they cause

Organism	Major-infection	Less commonly	Sites
S. aureus	Boils, impetigo, wounds, septicaemia osteomyelitis.	Pneumonia, endocarditis,toxic shock syndrome, food poisoning	Skin, blood, ears, chest, heart valves, gut.
E.coli	UTI, wounds, septicaemia, neonatal septicaemia	Some serotypes cause gasteroenteritis	urinary tract, skin, blood, intestines.
CNS	Bacteraemia in immunocompromised	Catheter – related septicaemia	blood
*Enterococcus sp**	UTI, wounds, intra-abdominal abscess	Bacteraemia, endocarditis	urinary tract, abdomen, blood, heart values
S. pneumoniae	Pneumonia, otitis media	Meningitis septicaemia	Chest, middle ear, meninges, blood
Klebsiella sp	UTI, chest	septicaemia	urinary tract, respiratory tract, blood
Pyogenic *Strep*	Tonsillitis, impetigo, cellulites, scarlet fever,	Puerperal sepsis erysipelas, septicaemia	Throat, skin, tissues, blood
Pseudomonas sp	Wounds, burns, UTI, septicaemia.	Pneumonia	Skin, urinary tract, blood, respiratory tract
Enterobacter sp	Wounds, UTI	septicaemia	Abdomen, urinary tract , blood
Proteus sp	UTI, wounds	septicaemia	urinary tract, blood

Collated from Hart and Shears (1996).

CNS, coagulase negative staphylococcus; *Enterococcus* spp.* includes *Enterococcus faecalis* and *E. faecium* (Lancefield group D, β haemolytic streptococci).

Staphylococcus aureus and *S. epidermidis* (a coagulase-negative staphylococcus), colonise the skin and *Streptococcus pneumoniae* together with *Haemophilus influenzae* and *Neisseria meningitidis* may be found as part of the normal flora of the throat (Hart and Shears 1996). The enterococci and enterobacter sp., with *E. coli*, *Klebsiella* sp., *Proteus* sp., are commensals in the large intestine. *Pseudomonas* sp. are widely distributed in the environment and are obligate aerobes that cause a range of infections including wound infections, UTI and septicaemia.

1.6.2 Blood Cultures in the diagnosis of septicaemia

Approximately ten percent of positive blood-cultures (BC) are due to contamination, this can result in patients receiving inappropriate antibiotic therapy (Washington, 1992). By definition 90% of positive blood-cultures represent a blood stream infection and are valuable in the diagnosis of sepsis. However it is not always possible to document an infection for the following reasons. Patients may already be receiving antibiotic therapy that interferes with microbiological results (Reyes *et al.*, 1999). If this is due to prophylactic therapy, treatment may be terminated and the infection would proceed unchecked.

Alternatively the causative organism in the blood culture may be a slow grower and the incubation time for a positive culture may exceed a laboratory's standard practice (Washington, 1992). For instance *Propionibacterium acnes,* a skin commensal was implicated in a case of possible post-surgical endocarditis. This organism takes on average 6-7 days to grow, but the range is 2-19 days and is often considered to be a contaminant (Jakab *et al.*, 1997). Generally automated blood cultures are incubated for up to 5 or 6 days. In this specific case the organism took 8 days to grow and a false negative would have been reported (Vanderbos *et al.*, 2001). Confounding the diagnosis of infective endocarditis in the latter case was the 20 month interval between surgery and the onset of fever. Indeed manifest symptoms may take several years to develop (Günthard *et al.*, 1994; Huynh *et al.*, 1995).

A diagnosis of bacterial endocarditis was made possible with the aid of gallium-67, an imaging technique that uses radioactive labelled molecules that bind to particular pre-specified cells and emit gamma rays. In this case the results showed hyperfixation of the isotope under the xyphoid process of the sternum. Consequently the index of suspicion for an infection was raised such that blood cultures were incubated for longer

than normal resulting in 8 out of 14 positive blood cultures with *P. acnes.* The source of the infection was thought to be a Teflon patch.

Gallium-67 has been used successfully to identify a focus of infection in patients with pyrexia of unknown origin (Vanderbos *et al.*, 2001; Mouratidis and Lomas, 1994).

Not all bacteria have the same growth requirements. *Heamophilus* spp., for instance will not grow unless factor X (haemin) and/or factor V (NADPH) a nucleic acid product is provided, *H. influenzae* requires both factors. *Mycobacteria* spp., are not detected by Gram's stain and an acid fast stain is required. These slow growing bacteria must have a chemically defined growth media that contains eggs and glycerol and take up to six weeks incubation to develop colonies. Whereas the commonly reported isolates listed in table 1.7 will form visible colonies within 24 hours. False negative cultures will occur if the exacting nutritional requirements are not supplied and/or incubation periods are too short.

M. tuberculosis was unknowingly transmitted to a transplant patient via a donor organ; symptoms of infection took twelve months to occur and treatment with the antibiotic flucloxicillen was ineffective. A differential diagnosis for a non-infective cause such as deep vein thrombosis was excluded by ultrasound but radioactive gallium-67 showed a significant uptake of the isotope around the area of pain and swelling. A biopsy was taken and cultured but it was not cultured for mycobacterium as the acid-fast stain was negative; the patient continued to have pain and discharge from the affected area (Graham *et al.*, 2001).

Four months later another similar case occurred and the clinicians noticed that this patient had also received a transplant organ from the same donor. The donor was investigated and a diagnosis of occult tuberculosis was considered. Consequently a further biopsy revealed an acid fast bacteria and tuberculosis was eventually diagnosed in both patients (Graham *et al.*, 2001). Gallium-67 is an invasive and potentially toxic procedure that is employed because other methods have failed to disclose an occult infection (Vandenbos *et al.*, 2001).

These case histories demonstrate an urgent need for diagnostic markers of infection in the absence of positive microbial cultures; markers that could become rapid, routine and non-invasive, a serum marker that is sensitive and specific for bacterial involvement.

Unusual bacteria not commonly isolated in Britain may be overlooked, for instance *Burkholderia pseudomalle* the causative organism of melioidosis, a febrile

illness that presents with no obvious infected wound or trauma and is characterised by abscess formation (Vatcharapreechaskul *et al.*, 1992; Wong *et al.*, 1995). However *B. pseudomallie* is an important source of sepsis in east Asia and northern Australia and accounts for 20% of all community acquired septicaemias in Thailand (Chaowagul *et al.*, 1993).

This Gram-negative bacterium is a soil organism transmitted by direct contact and is intrinsically resistant to many antibiotics (Dance *et al.*, 1989: Jenny *et al.*, 2001). Although effective antibiotic therapy is available, treatment involves a 20 weeks course with 10% of patients relapsing; adult patients require follow-up for life (White, 2003). The distribution and frequency of melioidosis is likely to be underestimated and is an emerging global problem (Dance, 2000).

A patient may be septic without being septicaemic. Based on the ACCP/ SCCM guidelines for the diagnosis of sepsis given in table 1.1 there has to be evidence of a systemic inflammatory response plus a confirmed infection (Bone *et al.*,1992). However, if insufficient volumes of blood are used in blood culture bottles the result may be a blood-stream infection going undetected (Mermel and Maki, 1993). It has been recommended that at least 10ml of blood is inoculated into each culture bottle to avoid these reported false negative results (Plorde *et al.*, 1985; Mermel and Maki, 1993).

Finally not all bacteria can be cultured, such as *Treponema pallidum;* obligate intracellular parasites such as *Coxiella burnetii* (Q fever) and *Chlamydia* spp. may be grown in cell cultures but are usually detected by serological tests including *T. pallidum* (Hart and Shears 1996).

Blood cultures are valuable in the diagnosis of sepsis, but for the reasons given many blood stream infections may be missed resulting in greater morbidity and mortality for the patient and extensive cost to the health services. There is clearly a need for fast and accurate serological tests to facilitate the diagnosis of bacterial infection as a contributory or underlying cause for the clinical presentation of symptoms.

1.7 MARKERS OF SEPSIS AND THERAPEUTIC INTERVENTIONS

Historically, fever with an elevated peripheral blood white cell count has been the gold standard indicating a probable infection. Within the last decade a large number of other markers have been suggested as more specific for bacterial infections leading to sepsis and some are listed in table 1.8.

Table 1.8 Experimental Markers of sepsis

MARKERS of SEPSIS	
Neopterin	Released from activated monocytes and macrophages
Procalcitonin	
LBP	Acute phase protein
IL-6; IL-10; IL-18; IL-8; TNF-α	Many cytokines
High mobility group-1	(HMG-1)
ICAM-1; sICAM-1; sE-selectin	Activated endothelial markers
C3b	Complement components
Protein C receptor	
thrombomodulin	Soluble fragments
HLA-DR	Soluble form

Neopterin and procalcitonin are surrogate markers of macrophage activation (Redl *et al.*, 2002).

Some of these markers of sepsis are currently being used to test patients for sepsis. Procalcitonin is a promising marker and there is a semi-quantitative cassette in use (Brahms, Germany).

Neopterin testing has been used routinely in an intensive care setting for over ten years in a European hospital. On the basis of the results, antibiotics have either been given or with-held to great effect together with bedside Gram staining smears (Strohmaier *et al.*, 1996). The background on this marker is so convincing it is difficult to know why this molecule is not more commonly utilised. Often the usefulness of a marker may depend on cost effectiveness and the time and effort required to produce a result. Or the rate at which a test marker is eliminated from the blood stream may make certain markers difficult to evaluate in sepsis.

1.7.1 Serum Kinetics of serum markers

In non-human primates with *E. coli*-induced sepsis, many of the plasma markers in table 1.8 had different serum kinetics; TNF-α or IL-10 followed short time kinetics and peak levels occurred within 10 hours. Procalcitonin peaked within 10 hours but

maintained this level for over twelve hours before gradually declining. Levels of LBP increased steadily over time and did not decline over the test period of 70 hours. C-reactive protein is a slow reacting marker but as it is elevated in all inflammatory conditions its value as a sepsis marker is considered to be limited (Redl *et al.*, 2002).

1.7.2 Therapeutic interventions

Many immunomodulatory therapies have been tested with disappointing results and others show some promise. Some strategies for these interventions are given in the following list:

- Blocking the action of microbial products, such as antibodies to LPS.
- Prevention of immune cell activation, for example by inhibiting transcription factors such as nuclear factor-kappa B.
- Inhibition of secondary mediators such as cytokines, nitric oxide, and lipid mediators for example platelet-activating factor antagonists.
- Blocking the activation of humoral pathways, including the coagulation and complement cascades.
- Neutralising target cells, such as the adhesion molecules.
- Immunostimulation, with interferon-gamma or colony stimulating factors. (Baumgarter and Calandra, 1999).

In large trials using anti-inflammatory corticosteroids there was no improvement in the survival rate of patients with sepsis or septic shock (Bone, 1996; Zeni *et al.*, 1997). Trials with monoclonal antibodies to endotoxin or anti-cytokine therapies have all failed to improve patient outcome (Bone, 1996).

Inhaled nitric oxide was given to patients with adult respiratory distress syndrome (ARDS) to increase oxygenation and flow of the blood. This procedure significantly improved oxygenation within 24 hours and caused a small decrease in pulmonary artery pressure. This trend was not maintained, nor was there any benefit on mortality. Prostacyclin, another endogenous vasodilator, was tested with similar results (Lynn, 2000).

A substance that inhibits macrophage migration called the macrophage inhibitory factor (MIF) is secreted by the pituitary gland in response to infection and stress

(Bernhagen *et al.*, 1993). MIF is also released by activated monocytes and macrophages and is pivotal to the innate immune response as it induces the expression of proinflammatory cytokines from macrophages and activates T cells (Calandra *et al.*, 1994; Calandra *et al.*, 1998). The median level of MIF in human controls was 3ng/ml; in patients with severe sepsis it was 12.2 ng/ml and in cases of septic shock 17.8ng/ml (Calandra *et al.*, 2000). This molecule may prove to be a useful indicator of infection in future studies. Anti-MIF-antibodies protected normal mice from lethal peritonitis and the only molecule (apart from stem cell factor) known to protect TNF-α knock-out mice from death induced by cecal ligation and puncture (CLP).

Like TNF-α, MIF appears to be both protective in small doses but lethal when over expressed. Antibodies developed against TNF-α are protective in animal models of endotoxic shock but not in CLP peritonitis or lethal doses of *E.coli* and are detrimental in sub lethal infections (Bagby *et al.*, 1991).

As stated earlier the only effective therapy to date in reducing MOF and death in patients with sepsis appears to be activated protein C (Bernard *et al.*, 2001).

Nevertheless all this research is productive as it demonstrates the effects of inflammatory mediators on other cells and identifies the order in which they appear during an infectious insult. In this current study the serum markers for sepsis were the nine listed in table 1.9.

Table 1.9 The project test markers for sepsis

PROJECT MARKERS for SEPSIS	
Interleukin-6	IL-6
Interleukin-10	IL-10
Interleukin-12	IL-12
Tumour necrosis factor-alpha	TNF-α
Soluble E-selectin	sE-selectin
Soluble intracellular adhesion molecule-1	sICAM-1
Procalcitonin	PCT
Lipopolysaccharide-binding protein	LBP
Anti-lipid S antibody	

Table 1.9 introduces a novel molecule called *anti-lipid S* antibody.

The lipid S molecule is elevated in patients with catheter related sepsis caused by coagulase negative staphylococci and may be a useful differential marker for patients with a Gram-positive bacteraemia (Elliott *et al.*, 2000). All the project test markers are discussed in detail in Chapter 2.

1.8 SUMMARY

There have been numerous studies to evaluate the plasma levels of potential markers of sepsis. Many have been alluded to in this introduction and more examples are given in chapter 2 where the project test markers are discussed in more detail. However many of these studies are based on the results from non-human primates with experimentally induced sepsis, and therefore are not always relevant to sepsis in humans.

Data collected from patients with sepsis is less common than data from animal models with sepsis. Although human studies have been conducted in the clinical setting particularly for serum levels of cytokines (especially IL-6 and TNF-α) there is no evidence that the nine test markers in this project have been collectively evaluated from the same serum sample in patients with sepsis.

In a recent book 'The Sepsis Text' there was a call for more data from the clinical setting, stating that "more prospective studies on sepsis markers are required to identify the most relevant and predictive markers" (Redl, *et al.*, 2002).

Data collected from patients in always unique and novel. They not only have unique finger-prints but their individual response to infection and sepsis differ based on multi-factorial causes and not least because of their genetic makeup (Stuber, 2002).

This project aims to find diagnostic common ground in patients with sepsis despite their individual differences, by evaluating the serum levels of the nine test markers in patients and controls with or without other underlying diseases.

1.9 AIMS AND OBJECTIVES OF THIS STUDY

Sepsis is a complex disorder of infection and inflammatory reactions that often manifests as a secondary complication to other underlying diseases, making diagnosis and subsequent treatment difficult, reflected in the high mortality rates (see 1.2). For sepsis to occur there has to be an underlying defect in the patient's immunity together with the presence of a potential pathogen. Patients with underlying diseases are less

likely to clinically present with the classical signs and symptoms of infection, or if they do these symptoms may be confused with their current health problem. It is important to find sensitive and specific markers for infection in these patients.

The number of cases of sepsis caused by Gram-positive bacteria now exceeds those caused by Gram-negative bacteria (table 1.6). Therefore a differential marker for Gram-positive sepsis would not only aid in the early detection and diagnosis of sepsis but would facilitate the start of appropriate antibiotic treatment, a practise that has been proven to be associated with a better outcome (Moreno *et al.*, 2002).

The aims of this current study were:

- To evaluate the level of the test markers in the serum of control patients and clinically defined septic patients that would be both sensitive and specific for a diagnosis of sepsis.

- To assess the validity of establishing a diagnostic cut-off value for the markers in relation to reported values found in other diseases.

- Ascertain whether the results from the above studies can be successfully applied to new groups of patients in a semi-blind trial. Group 1) known to have a positive blood culture but no other information supplied and Group 2) patients with a pyrexia and a negative blood culture but with no other patient information.

- To validate the results of the study by giving a 'blind' diagnosis based on the test markers alone and comparing these results with the actual clinical diagnosis of these cases as given by the participating Consultant Microbiologist.

- Evaluate Lipid S as a differential marker for Gram-positive sepsis.

Many of the proposed test markers can be found in other disease states, for example elevated serum levels of TNF-α in cases of trauma, burns and non-infectious inflammatory conditions. Therefore the value of the marker or markers may lie in the quantitative values found in sepsis in comparison with levels reported in published research literature for other diseases.

CHAPTER 2 THE PROJECT TEST MARKERS

2. INTRODUCTION

The test markers for sepsis selected for this study were, TNF-α; IL-6, IL-10 and IL-12, two leucocyte adhesion molecules; sE-selectin and sICAM-1, procalcitonin PCT, LBP. These markers were chosen because they have been reported to be elevated in the serum of patients with sepsis and are discussed in detail below. Anti-lipid S antibody was included as a differential marker for Gram-positive sepsis as it is elevated in central venous catheter-related sepsis caused by coagulase negative staphylococci (Elliott *et al.*, 2000).

2.1 TUMOUR NECROSIS FACTOR-α

Tumour necrosis factor- alpha (TNF-α) was the first cytokine to be described in patients with septicaemia (Waage *et al.*, 1986). TNF-α is a 12 kilodalton molecular weight cytokine composed of 157 amino-acids (Wilson *et al.*, 1998). TNF -α exists as a bell shaped trimer and is synthesised constitutively in limited amounts by unstimulated phagocytic macrophages and is stored pre-formed in mast cell granules (Nathen 2002). Following activation with lipopolysaccharide (LPS) there is a 10,000-fold increase in the rate of TNF synthesis and is a major secretory product of macrophages (Beutler *et al.*, 1985). TNF-α has a median half-life of 17 minutes and is degraded by the liver and other organs (Selby *et al.*, 1987).

TNF-α is also secreted by neutrophils, activated T lymphocytes, natural killer cells (NK), monocytes and endothelial cells (Ibelgauft 1999). Monocytes are macrophage precursors that are transient in the blood for 8 –24 hours before they leave the circulation and complete their maturation in the tissues.

The liver contains the largest number of resident macrophages known as Kupffer cells, which line the blood sinuses and remove foreign particles by phagocytosis. Kupffer cells are situated in a prime position to be activated by bacterial products such as LPS causing a massive systemic release of TNF-α, producing vasodilation, increased vascular permeability with loss of plasma volume leading to shock (Janeway *et al.*, 1998).

TNF-α is known to have a wide range of functions which include:

- Up-regulation of adhesion molecules, ICAM-1 and E-selectins (Nakae *et al.*, 1996).
- Increase of capillary permeability (Tracey *et al.*, 1986).
- Activation of neutrophils, causing a respiratory burst that generates oxygen radicals and nitric oxide, and the release of granule contents that contain proteolytic enzymes such as elastase and lysozyme plus bactericidal substances such as defensins (Murphy *et al.*, 1992).
- Acts synergistically with membrane attack complex (MAC) to further upregulate ICAM-1 (Kilgore *et al.*, 1995).
- Induces acute phase proteins and IL-8 from hepatocytes in the liver (Thornton *et al.*, 1990).
- Induces the secretion of IL-6 (Zilberstein *et al.*, 1986).

Cytokines are introduced in section 1.4.1 and it is clear that cytokines do not act in isolation but are involved in highly complex interactions with other cytokines. For instance, interferon-gamma (IFN-γ) (induced by IL-2) is produced mainly by T-cells, natural killer cells and B cells. IFN-γ acts synergistically with IL-1 and IL-2 and appears to be required for the expression of IL-2 receptors on the cell surface of T-lymphocytes. Co-expressed with TNF-α it acts synergistically to enhance inflammatory functions, by increasing the production of TNF and the expression of cell surface TNF receptors (Beutler and Cerami 1986; Old, 1985; Sheehan *et al.*, 1992). A. Billiau (1988), argues that IFN-γ may well be "The match that lights the fire" of shock and disseminated intravascular coagulation.

IFN-γ receptors are ubiquitous on all cell types except erythrocytes and they have a high affinity for IFN-γ (Anderson *et al.*, 1982; Van Loon *et al.*, 1991). For instance the number of receptors expressed on platelets is 300 per cell; given the large number of circulating platelets (3 x 10^8/ml) then there are ninety billion high affinity receptors for IFN-γ on platelets alone. This could explain why IFN-γ is not commonly found free in the circulation, making this molecule difficult to quantify in disease states (Molinas *et al.*, 1987).

Memelmans *et al.*, (1996) reported that TNF-α was seldom found in the circulation of septic patients. This could be due to an increase in high affinity TNF-α receptors, as is the case for IFN-γ. However other workers have reported that TNF-α is present in the serum of septic patients but the levels vary considerably (Cohen & Abraham, 1999).

Microorganisms such as *Pseudomonas aeruginosa* produce exotoxins known to diminish the production of TNF–α by inhibiting lymphocyte proliferation (Staugas *et al.*, 1992). This phenomenon may account for some of the reported variation and would adversely affect the veracity of TNF-α as a single marker for sepsis in patients with *P. aeruginosa.* Another possibility for diminished TNF-α levels in septic patients may be due to enhanced production of factors such as the anti-inflammatory cytokine IL-10, which inhibits the production of TNF-α (van der Poll *et al.*, 1997). Others have shown that an *in vitro* model of endotoxaemia using whole blood and LPS down regulated TNF-α secretion (Marchant, 1994; van der Poll *et al.*, 1995).

TNF-α has been shown to stimulate the release of endothelins from vascular endothelium in animal models (porcine) with Gram-negative induced septic shock (Han *et al.*, 1994). Endothelins (ET) are potent vasoconstrictors and induce the release of thromboxane A2 (a vasoconstrictor), prostacyclin and nitric oxide (vasodilators) (de Nucci *et al.*, 1988). ETs are known to be elevated in septic patients (Weitzberg *et al.*, 1991).

Two of the many interacting systems that are activated during sepsis are the coagulation and complement cascades. TNF-α and IL-1 causes tissue factor (TF) production on vascular endothelium and monocytes. Whenever TF comes into contact with the blood it initiates coagulation rapidly. Normally TF is only found constitutively expressed on fibroblasts and tissues in extravascular areas (Sam *et al.*, 1997). Experimental evidence suggests that in the absence of complement TNF-α ability to upregulate lung vascular ICAM is diminished, reducing inflammation because of the lack of leucocytes (Vaporciyan *et al.*, 1995).

Inflammation in the lungs is caused by activated macrophages. The possible mechanism of action of TNF-α on lung macrophages is to induce an oxidative burst which produces hydrogen-peroxide (H_2O_2), this in turn causes oxidative activation and cleavage of C5 (an inactive serum component of the complement system), resulting in the formation of the membrane attack complex (MAC) and releasing C5a; this molecule is an anaphylatoxin with chemotaxis properties that is thought to play an important role

in the further induction of TNF-α from other lung macrophages (Murphy *et al.*, 1992). Table 2.1 gives some TNF-α serum levels, which have been reported in septic patients.

Table 2.1 Cytokine serum concentration (range) in patients with and without bacteraemia; non-survivors and survivors

	Day	IL-6 pg/ml	TNF-α pg/ml	CRP mg/l
Bacteraemia	1	7 - 7637	0.35 - 49	90 - 570
	3	2 - 9999	4 - 64	80 - 420
Without	1	22 -1888	6 – 66	29 - 360
bacteraemia	3	0 - 500	3 - 48	100 -270
Non-	1	78 - 877	0 -203	93 – 368
survivors	3	0 – 448	0 - 23	83 - 398
Survivors	1	18 -7637	0 -124	29 – 570
	3	54 -9999 (range)	0 - 41	103 - 423

Data expressed as the range of values, from R&D Quantikine assays for IL-6 and TNF-α. (Preserl *et al.*, 1997).

2.2 INTERLEUKIN-6

Interleukin-6 (IL-6) is generally considered to be one of the three major pro-inflammatory cytokines produced during inflammation. The other two cytokines being TNF-α and interleukin –1 (IL-1). Interleukin–6 is raised in the serum of patients with sepsis, but is not specific for bacterial infections as levels of IL-6 are elevated after any form of physical trauma (Assicot *et al.*, 1993; Selburg *et al.*, 2000).

Many cell types secrete this cytokine including monocytes, macrophages, T-helper 2 lymphocytes (Th2) and fibroblasts. It is known to be induced by TNF-α (Zilberstein *et al.*, 1986).

A DNA binding protein (called nuclear factor-kappa B [NF-κβ]) is involved in the transcription of IL-6; this transcription factor is induced by IL-1 and TNF-α. As these two cytokines increase so does the level of IL-6 (Kuby, 1994). Increasing levels of intracellular nucleotides, cyclic adenosine monophosphate (cAMP), are thought to promote the synthesis of IL-6 but block the formation of TNF-α, demonstrating a regulatory mechanism in cytokine production (Zhang *et al.*, 1988).

IL-6 is also considered to be an anti-inflammatory cytokine because it down regulates the synthesis of TNF-α and IL-1 and promotes the synthesis of IL-1 receptor antagonist (ILra) (Xing, *et al.*, 1998) ILra competitively binds to IL-1 receptors inactivating them. IL-6 promotes the release of the anti-stress glucocorticoids via the hypothalamus pituitary adrenal axis in animal studies (Tilg *et al.*, 1994; Ruzek *et al.*, 1997; Opal and Depalo, 2000). Glucocorticoids effect a reduction in inflammation by suppressing cellular immune functions including the cytotoxic activity of natural killer cells (Zhou *et al.*, 1997).

IL-6 is an endogenous pyrogen and a major hepatocyte stimulating cytokine resulting in the production of the acute phase proteins such as C-reactive protein, fibrinogen, protease inhibitors and some complement components (Castell *et al.*, 1989; Herbert *et al.*, 1995).

IL-6 activates B-cells and T-cells and is also involved in platelet production and local fibroblast proliferation (Kuby, 1994). Newly created platelets are particularly responsive to IL-6 causing them to become more sensitive to thrombin activation with increased procoagulation activity (Esmon, 1999). Tissue hypoxia elicits IL-6 production adding to a damaging cycle of more platelets being produced allowing coagulation to occur leading to hypoxia and to the production of IL-6 (Yan *et al.*, 1995). IL-6 together with a soluble IL-6 receptor may be involved in chemokine production in endothelial cells, consequently attracting leucocyte adhesion and migration with an increase in the inflammatory response (Mantovani *et al.*, 1997).

There are a number of diseases attributed to over production of IL-6 these include rheumatoid arthritis, alcoholic liver cirrhosis, psoriasis and renal cell carcinoma. IL-6 is elevated in meningococcal septic shock and in animal studies there is a correlation between IL-6 levels and poor clinical outcome (Waage *et al.*, 1989).

The normal serum range of IL-6 in adults is approximately 0.2 –4.5 pg/ml (Nomura *et al.*, 1999). However comparing serum levels in various studies is problematic, as researchers have used different methods and expressed the results in a variety of ways. For instance in a study with 255 African neonates with probable and definite infections IL-6 levels ranged from 422 to 11,164 pg/ml respectively and the mean control level (without infection) was 58pg/ml (Magudumana *et al.*, 2000). The control is unexpectedly high given the normal adult range, but the 11,164 pg/ml upper range value concurs with Dollner *et al.*, (2001). In this latter study IL-6 was determined

by the IL-6 dependent mouse hybridoma cell line B 13.29 (clone B9), as described by Aarden *et al.*, (1987) together with a monoclonal antibody against IL-6 (Genzyme, Cambridge, MA, USA) to evaluate the specificity of measured IL-6 activity. These results cannot be realistically compared with serum levels determined by commercial ELISA. IL-6 and CRP values from Dollner and collegues (2001) are reproduced in table 2.2.

In a different study the median range of IL-6 in neonatal sepsis was 1620pg/ml and in non -septic cases the median level was 42pg/ml (Olander and Norman 2001).

Table 2.2 Serum concentrations of inflammatory mediators in infected, possibly infected, and non-infected neonates (Dollner *et al.*, 2001).

	Infected Median (range)	**Possibly infected Median (range)**	**Not infected Median (range)**
IL-6 pg/ml	164 (<10 – 12500)	17.6 (<10 – 3229)	<10 (<10 – 3028)
CRP mg/l	13 (< 5 – 105)	7.0 (< 5 – 55)	< 5 (<5 – 37)

IL-6 measurement using an IL-6 dependent mouse hybridoma cell line B 13.29 (clone B9), together with a monoclonal antibody against IL-6 (Genzyme, Cambridge, MA, USA).

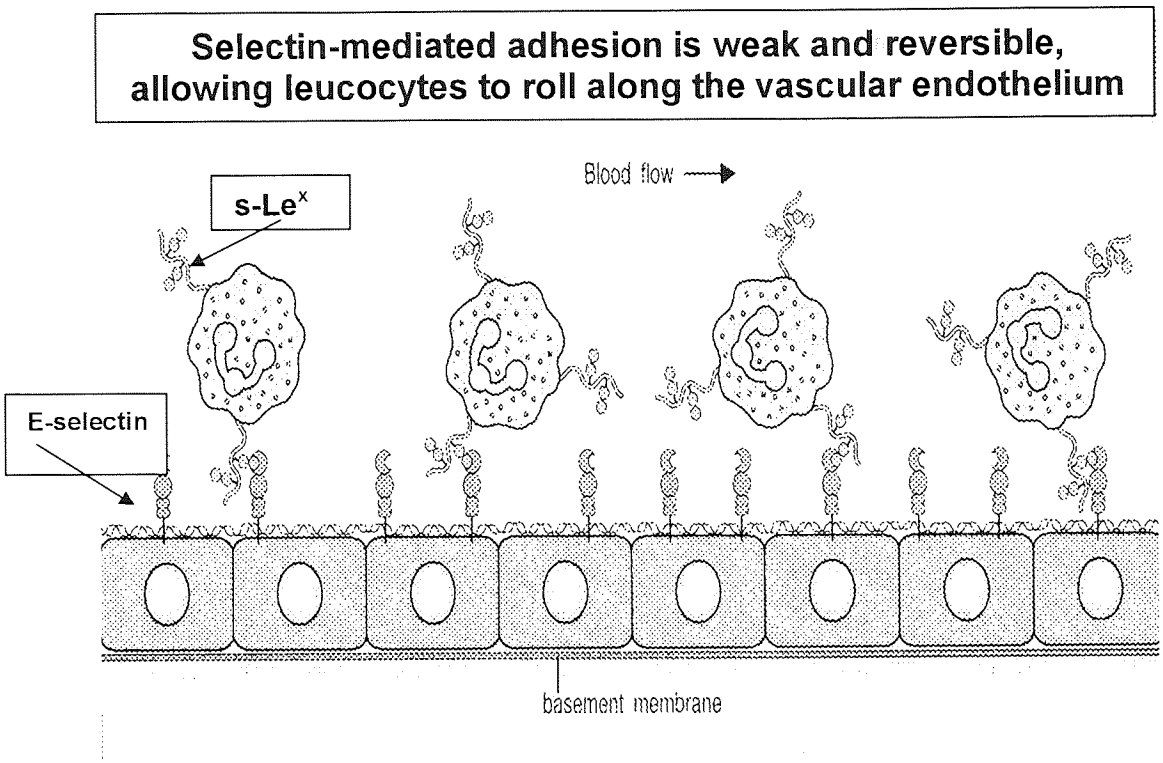
2.3 CELL ADHESION MOLECULES

Selectins are a family of carbohydrate binding transmembrane proteins that include P-selectin, E-selectin and L-selectin (Herbert *et al.*, 1995). Pre-formed P-selectin is stored in secretory granules called Weibel-Palade bodies inside vascular endothelial cells and platelets (Ebnet *et al.*, 1996). They are expressed within seconds after stimulation by histamine , thrombin, leukotriene B4 (LTB4) or by a breakdown product of complement C5a (Zimmermann *et al.*, 1985; Ebnet *et al.*, 1996). P-selectin allows transient attachment of neutrophils, monocytes and subsets of T lymphocytes to the vascular endothelium via intergrins on the surface of leucocytes (Jutila, *et al.*, 1994). P-selectin's adhesive role is taken over by E-selectin within minutes. Although soluble P selectin is found in serum, generally this molecule is internalised within the endothelium by endocytosis after E-selectin has been expressed on the endothelium; these two processes occur within a time frame of approximately thirty minutes (Herbert *et al.*, 1995; Janeway and Travers, 1997).

L-selectins are expressed on leukocytes and as with the other selectins binds to sialylated glycoproteins. Leucocytes also express sialylated glycoproteins recognised by E-selectins on vascular endothelium. L-selectin – E-selectin interactions allow neutrophil and monocyte binding to endothelium but not lymphocytes (Ebnet, *et al.*, 1996). L-selectin mediates binding of lymphocytes to high endothelial venules of peripheral lymph nodes by binding (possibly) GlyCAM-1. L-selectin is rapidly shed from the cell surface after activation – a process that may help leucocyte detachment and extravasation (Herbert *et al.*, 1995)

2.3.1 sE-selectin

E-Selectins are expressed on the surface of vascular endothelia but only after IL-1, TNF-α or LPS has activated the endothelium (McEver, 1994; Herbert *et al.*, 1995). They possess one carbohydrate binding domain that is dependent on the presence of calcium ions (McEver *et al.*, 1995). The carbohydrate ligand for E-selectins on leucocytes is a sialylated glycoprotein, sialyl-Lewis ^{x} (s-Le^{x}). Selectins cause leucocytes to slow down against the rate and flow of the blood stream by low affinity interaction with the s-Le^{x} moiety, see figure 2.1 (Janeway and Travers, 1997).



Reproduced from Janeway and Travers 1997

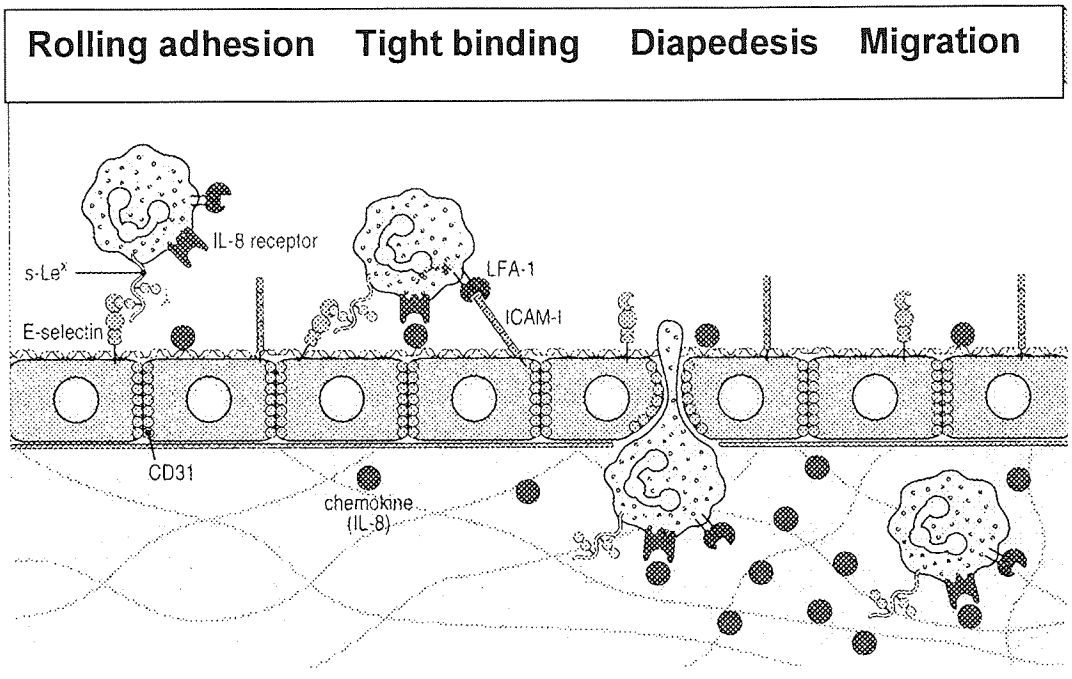
Figure 2.1 Adhesion molecules on vascular endothelium and leucocyte binding.

E-selectin on vascular endothelial cells binds reversibly to leucocytes via the sialyl Lewis^x (sLe^x) allowing the cell to roll along the vascular endothelium; followed by strong binding of integrins such as LFA-1 (also known as CD11a/CD18) to ICAM-1, see figure 2.2.

Soluble E-selectin (sE-selectin) is found in the blood of healthy individuals and it is thought that it is derived from proteolytic activity on the endothelial cell surface (Lobb, 1991). Other workers have shown that membrane E-selectins are removed from the cell surface by endocytosis (Subramanian *et al.*, 1993). It was thought that the soluble versions acted as anti-inflammatory regulating factors by preventing leucocytes from binding to the vascular endothelium; alternatively sE-selectins may activate neutrophils and therefore are proinflammatory agents (Cines *et al.*, 1998).

sE-selectin levels are raised in Kawasaki disease, a vasculitic syndrome of unknown aetiology and may be a marker for vascular endothelial damage (Takeshita *et al.*, 1997). Serum levels correlated with increased levels of CRP and was an early predictor of severe sepsis with MOF whereas sICAM-1 levels were a non-specific indicator of sepsis in a study by Egerer and collegues (1997), see table 2.3 for their

results. The normal range in serum for adults is 30 ± 7ng/ml (R&D Systems assay values). In a different research study the normal range value was given as a mean, 40.4 ± 4.5 ng/ml and the levels of sE-selectin in patients with sepsis were 231 ± 41.8 (Kayal *et al.*, 1998).



Reproduced from Janeway and Travers 1997

Figure 2.2 Neutrophil adhesion and extravasation from the vasculature

E-selectin on vascular endothelial cells binds reversibly to leucocytes via the sialyl Lewis^x moiety, allowing the cell to slow down and marginate; this weak binding is followed by strong binding of integrins to ICAM-1 causing the cell to stop, spread and migrate from the blood stream,

[LFA-1 (leucocyte functional antigen-1) an integrin- is also called CD11a/CD18; CD31- is an adhesion molecule; s-Le^x -sialyl Lewis^x the E-selectin ligand. Neutrophils are unique in that they express all three β_2 (CD18) integrin complexes, **CD11a**/CD18; **CD11b**/CD18 (or MAC-1, also called complement receptor3 [CR3]) and **CD11c**/CD18 also called complement receptor 4 [CR4] (Hynes and Lander 1992; Barnett *et al.*, 1996).

Normally the integrins (LFA-1, CR3 and CR4) adhere weakly but chemoattractants such as interleukin 8 (IL-8) released from cells at the site of infection trigger integrin conformational change (Ebnet, *et al.*, 1996).

The importance of the adhesion process is demonstrated in leucocyte-adhesion-deficiency (LAD) syndrome. LAD is a serious immunodeficiency characterised by recurrent bacterial infections and is caused by a genetic defect in the formation of the β_2

endothelium and consequently cannot move out into the tissues and attack the source of infection, pus fails to be produced and lesions fail to heal and tend to become necrotic (Herbert *et al*, 1995).

2.3.2 Intercellular adhesion molecule-1 (ICAM-1)

Cell membrane bound ICAM-1 is a 90kDa Type -1 transmembrane glycoprotein with five immunoglobulin (Ig) type domains. ICAM-1 mediates the adhesion of leucocytes to vascular endothelium. All type 1 transmembrane proteins have the N-terminal region of the amino-acid residue situated in the extracellular space.

Low levels of ICAM-1 are normally expressed on vascular endothelial cells and lymphocytes; but after these cells are exposed to IL-1, TNF-α or LPS there is an increase in ICAM-1 expression.

The ligands for ICAM-1 are integrins, LFA-1 and Mac-1/CR3. Integrins are Type 1 transmembrane heterodimer proteins with an α and β chain. LFA-1 has an alpha chain called CD11a and a beta chain CD18. Mac-1/CR3 has an alpha chain CD11b with CD18. The former molecule is located on most leucocytes and binds ICAM-1,2,or 3. Mac-1/CR3 (CD11b) is present on the myeloid cell series, neutrophils, macrophages and NK cells and binds to ICAM-1 and a complement protein iC3b (Herbert *et al.*, 1995). Integrins cause rolling leukocytes to stop against the shear flow, spread over and migrate between the endothelial cells.

Soluble ICAM-1 (sICAM-1) is thought to be produced by proteinase cleavage of membrane bound ICAM-1 and there is some evidence that IL-1α (from tumour cells) up-regulates the release of sICAM-1 (Fonsatti *et al.*, 1997). *In vitro* studies demonstrate that soluble ICAM-1 secretion is induced from endothelial cells and monocytes by the actions of IL-1 and TNF-α (Leeuwenberg *et al.*, 1992; Pigott *et al.*, 1992).

In healthy adults the reference range for serum sICAM-1 is 115-306 ng/ml (R&D Parameter assay) and is elevated in the serum of patients with systemic inflammation (Labarrere, 1997). Patients who did not survive sepsis with multiple organ failure had higher levels of sICAM-1 than those that survived (Egerer *et al.*, 1997). The results from this latter paper are given in table 2.3.

Table 2.3 sICAM-1 and sE-selectin in patients with infection and sepsis with multiple organ failure

(Extrapolated from Egerer *et al.*, 1997). S; survivor; NS; non-survivor. MOF-multiple organ failure.

	sICAM-1 ng/ml range (229 –410)	**sE-selectin ng/ml (9 – 42)**
Infection	300 - 440	30 - 38
Sepsis with MOF (S)	420 - 620	43 -130
Sepsis with MOF (NS)	600 - 1000	63 -110

The serum values in table 2.3 were detected with Parameter sE-selectin and sICAM-1 ELISA assays from R&D Systems Minneapolis, MN USA. Egerer and colleagues used a reference range for these molecules established by Gearing and Newman (1993). Where as the reference range used in this study for sICAM-1 was 115-306 ng/ml as given by the assay manufacturer R&D Systems. The R&D range for sE-selectin was 26 –63ng/ml.

Patients with sepsis in another study had sICAM-1 levels of 868 ± 131ng/ml (Kayal *et al.*, 1998). Only 60 % of the patients in this latter study had a bacteraemia and in those cases the mean sICAM-1 was 1039 ± 170 ng/ml and in patients with septic shock the mean levels were 1133 ± 200ng/ml.

sICAM-1 levels are elevated in other diseases such as in Graves disease (Ozata *et al.*, 1996), melanomas (Fonsatti *et al.*, 1999) and in necroinflammation of the liver due to Hepatitis C, (Lo Iacano *et al.*, 1998). In this latter paper it was suggested that soluble vascular cell adhesion molecule-1 (sVCAM-1) could be a marker for liver fibrosis. VCAM-1 and sICAM-1 differ in their cell binding properties, sVCAM-1 binds to VLA-4 (CD29/CD49d) on lymphocytes, monocytes, dendritic cells and interdigitating cells in the lymph nodes. In comparison ICAM-1 and sICAM-1 do not bind to lymphocytes (Herbert *et al.*, 1995).

sICAM-1 provokes *in vitro* neutrophils to release elastase by crosslinking two integrin receptors in a multivalent way. sICAM-1 engages both adhesion integrins LFA-1 (CD11a/CD18) and MAC-1 (CD11b/CD18) causing the neutrophil to release elastase (Barnett *et al.*,1996). Elastase is a proteolytic enzyme that can inactivate antithrombin, a

potent anticoagulant. Barnett and colleagues suggest that the release of elastase by sICAM-1 represents a mechanism provoking distal organ dysfunction.

It is hypothesised in this current study that sICAM-1 may be more elevated in septicaemia than in other diseases as a mechanism to prevent the migration of immune cells away from the focus of infection in the blood stream.

2.4 LIPOPOLYSACCHARIDE BINDING PROTEIN

Lipopolysaccharide binding protein (LBP) was first named by Tobias and colleagues (1986). It is an acute phase 60kDa plasma glycoprotein that binds to amphipathic molecules including microbial lipopolysaccharide (LPS). LBP is known to specifically bind bacterial LPS via lipid A (Ulevitch and Tobias 1999). A diagram of LPS is illustrated in chapter one figure 1.2. Lipid-binding proteins enable lipids to become transportable in aqueous environments such as the plasma (Schromm *et al.*,1997).

LBP is predominantly produced by the liver (Schumann *et al.*,1990). The serum levels of LBP in healthy adults are in the range of 12-22 μg/ml, and can increase up to 300μg/ml within 24 hours after the induction of an acute phase response in volunteers (Tobias & Ulevitch, 1993). Recently the serum reference range of LBP in healthy adults has been modified to 5-10 μg/ml and is adopted for this project (Kirschning *et al.*, 1997).

One of the functions of LBP is to transfer LPS in the plasma to soluble or membrane bound CD14 (Pugin *et al.*, 1993). CD14 is a GPI anchor-linked glycoprotein found on many cell types but mainly on monocytes, macrophages and neutrophils. Binding of this receptor appears to result in cell activation and the production of TNF-α and other cytokines (Kirchning *et al.*, 1997). However CD14 does not possess a cytoplasmic domain therefore it can not trigger cellular activation.

Toll-like receptors (TLR) appear to be the missing link in the LBP + LPS + CD14 complex (Medzhitov *et al.*, 1997). Another report infers that LPS binds directly to TLR4 without the help of LBP (Lien *et al.*, 2000), but when LBP is removed from the serum by immunoadsorption the serum loses its ability to active an LPS response (Schumann *et al.*, 1990).

In-vitro models show that LBP can detoxify LPS by transferring it to high-density lipoproteins and in the acute phase of inflammation LBP has a protective effect against LPS and bacterial infection (Hailman *et al.*, 1994; Ulevitch and Tobias 1994; Kirchning *et al.*, 1997).

LBP has been selected as a potential diagnostic marker for bacterial sepsis because of its apparent specificity for bacterial lipids, and because maximal serum levels peak earlier than either IL-6 or C-reactive protein (Opal *et al.*, 1998). Table 2.4 lists the levels of LBP in patients with early systemic inflammatory response syndrome (SIRS) and severe sepsis

Table 2.4 Lipopolysaccharide Binding Protein (LBP)

LBP **Reference range 5 - 10 μg/ml**	LBP μg/ml **mean (range)**	Reference
Controls	7.7 (not given)	Myc *et al.*, 1997
Early SIRS	36.6 (4.9 – 114)	Myc *et al.*, 1997
Severe sepsis	36 ±14 (2.1 – 162)	Opal *et al.*, 1998

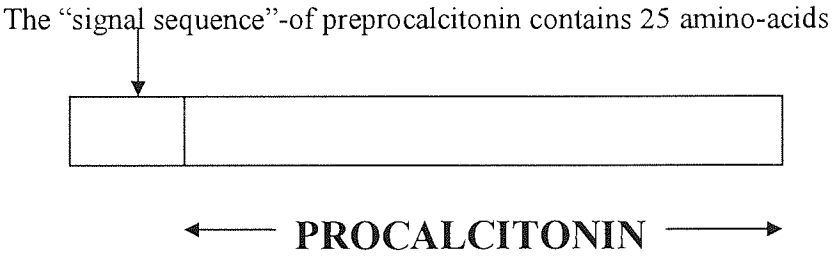
From Opal *et al.*, (1998), lower LBP levels were associated with lactic acidosis and in predicting a poor outcome and were routinely lower in older patients.

2. 5 PROCALCITONIN

Procalcitonin (PCT) is a 116 amino-acids polypeptide (13kDa) with a serum half-life of 25-30 hours (Assicot *et al.*, 1993). Figure 2.3 illustrates this molecule and the post translational cleavage products in figure 2.4 including calcitonin, a well described and understood hormone involved in calcium metabolism and known to be produced by the thyroid (Le Moullec *et al.*, 1984).

Animals with their thyroid glands removed still produced PCT so researchers studied other tissues and cells for this molecule. Oberhoffer *et al.*, (1999) found for the first time that peripheral blood mononuclear cells express this protein. *In vitro* hepatocytes produce large quantities of PCT after stimulation with TNF-α and IL-6, and as PCT behaves like a fast responding acute phase protein, the liver is probably a major source (Nijsten *et al.*, 2000).

Within the last seven years procalcitonin has been studied intensively and is significantly elevated in the serum of patients with Gram-negative sepsis (Assicot *et al.*, 1993; Whang *et al.*, 1998). Serum levels ranging from 0.6 to 34.7ng/ml were recorded in patients with SIRS to septic shock respectively; with a sensitivity of 60% and specificity of 79% (Al-Nawas *et al.*, 1996). Serum PCT levels in healthy adults are less than 0.1ng/ml (Brunkhorst *et al.*, 2002).



(Adapted from Carrol *et al.*, 2002).

Figure 2.3 A schematic diagram of preprocalcitonin

Preprocalcitonin is taken up into the endoplasmic reticulum with the help of the signal sequence, 25 amino acids in length; this portion is cleaved leaving **procalcitonin**.

Procalcitonin is cleaved again in the neuroendocrine cells of the thyroid (C-cells), lungs and pancreas producing three distinct molecules; aminoprocalcitonin (57 amino acids), calcitonin (32 amino acids) and katacalcin (21 amino acids) see figure 2.4 (Carrol *et al.*, 2002).

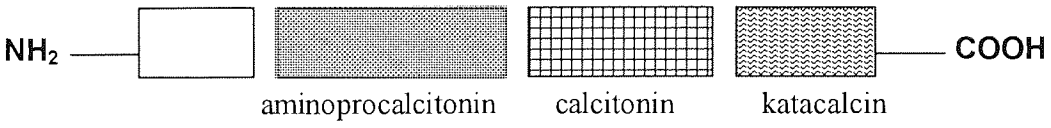


Figure 2.4 A schematic diagram of procalcitonin degradation products

During sepsis, the degradation of procalcitonin into aminoprocalcitonin, calcitonin and katacalcin is inhibited (Cusack *et al.*, 2000). However normal calcitonin levels were recorded in children with and without bacterial infections regardless of elevated PCT levels (Assicot *et al.*, 1993).

Procalcitonin may be specific for systemic bacterial infection but it is elevated in patients with pancreatitis, a disease not normally considered to be caused by microorganisms (Hughes *et al.*, 1995; Wilson, *et al.*, 1998; Kylänpää-Bäck *et al.*, 2001). However PCT may differentiate sterile from infected pancreatitis (Cusack *et al.*, 2000). Using a cut-off value of 1.8ng/ml over 2 days, PCT assays gave 94% sensitivity and 91% specificity for the diagnosis of infected necrosis of the pancreas (Rau *et al.*, 1997).

Other causes of raised PCT levels include severe heat stroke (Nylan *et al.*, 1998) and burns related inhalation injury (O'Neill *et al.*, 1992).

PCT does not appear to have the sensitivity to discern early bacterial infections in the new born; in these cases CRP and interleukin-8 together are more reliable indicators of bacterial infection (Franz *et al.*, 1999). Nor does it have a specific prognostic value in patients with community-acquired pneumonia as no correlation was found between high PCT serum levels and severity of disease (Raz *et. al.,* 2000). In a different study the mean PCT values in survivors was 4.4ng/ml whereas in non-survivors it was 15.2ng/ml suggesting a poor prognosis when serum levels of PCT are high (Al-Nawas and Shah 1998). Although PCT levels may not predict patient outcome, an increase in serum levels was related to infectious complications and acute septic episodes (Assicot *et al.*, 1993).

Serum levels of PCT decrease over time (Brunkhorst *et al.*, 2002). The potential prognostic value of this marker may reside in the relationship between the number of days PCT is detected in the serum during an infectious episode rather than the initial day one serum level. For example, a patient with a level of 25ng/ml PCT on day 1-2 of a septic episode may survive but a patient with < 8 ng/ml on day 12-14 is likely not to survive (Brunkhorst *et al.*, 2002). Although others state that PCT is higher on day one in non-survivors with sepsis (Oberhoffer *et al.*, 1996).

In cases of suspected meningitis where the question is 'is it viral or bacterial?' serum PCT values > 0.2ng/ml proved to have a sensitivity and specificity of one hundred percent in the diagnosis of bacterial meningitis (Viallon *et al.*, 1999). This finding suggests that procalcitonin may be an important serum marker of bacterial infection.

PCT has a serum half-life of 24 –30 hours and could be a useful marker for monitoring the effect of antibiotic treatment and may remove the need for inappropriate antibiotic therapy. In organ transplant recipients, increases in PCT reflect the severity of bacterial, fungal or protozoal infections (Hammer *et al.*, 1998). Patients with malaria had serum PCT levels that correlated positively with the density of *Plasmodium falciparum* infection (Hollenstein *et al.*, 1998).

Procalcitonin is a specific marker of infection in patients with neutropaenic fever, except in neutropenic patients with a Gram-positive infection, when PCT was reported to demonstrate poor sensitivity due to an absent or delayed response (Ruokonen *et al.*, 1999).

Table 2.4 Procalcitonin (PCT) serum levels in various studies

Disease		PCT ng/ml	Reference
Melioidosis	Mild infection	0.13 (0.02-0.46)	Smith *et al.*, 1995
Melioidosis	Controls	0.07 (0.03-0.15)	Smith *et al.*, 1995
Melioidosis	Severe infection (NS)	350 (63-3538)*	Smith *et al.*, 1995
Melioidosis	Severe infection (S)	19 (0.55-387)	Smith *et al.*, 1995
Septicaemia	With peritonitis	5420**	Gramm *et al.*, 1995
Meningitis	Bacterial	13.8 ±5 (0.22-101)	Viallon *et al.*, 1999
Meningitis	Viral	0.03 (0.01-0.1)	Viallon *et al.*, 1999

Meliodosis is caused by a Gram-negative bacillus *Malleomyces pseudomallei*
NS = non-survivors; S = survivors. * the initial PCT level was significantly higher in the patients that died. Smith *et al.*, (1995) show the median and range, whereas Viallon *et al.*, (1999) give the mean and range. **The highest reported level of PCT.

PCT is raised transiently in post surgical cases and burns without infection but the levels drop within 2 days. Severe viral infections, toxic acute respiratory distress syndrome (ARDS), and autoimmune disorders show little or no rise in serum PCT (Monneret *et al.*, 1998). In the latter study Monneret and colleagues were able to show that PCT assays identified covert infections. This characteristic is a vital attribute for a diagnostic marker, particularly in patients with complex underlying diseases that may mask the expected symptoms and clinical presentation of a serious infection, or in patients with a pyrexia of unknown origin. However no significant difference was found in the serum of children with bacterial as opposed to viral pneumonia, although very high levels of PCT, IL-6 and CRP probably indicated a bacterial pneumonia (Toikka *et al.*, 2000).

2.6 INTERLEUKIN -10

Interleukin-10 (IL-10) is a potent anti-inflammatory cytokine that prevents lethal experimental endotoxemia (Gerard *et al.*, 1993). IL-10 with IL-4 and transforming growth factor-beta (TGF-β), are physiological antagonists to the actions of other cytokines (Brandtzaeg *et al.*, 1996).

IL-10 is an acid sensitive homodimeric 18.5 kDa protein. Subunits are 160 amino-acids long. It has seventy-three percent homology with murine IL-10 (Ibelgaufts, 1999). It is mainly produced by helper-2 T-lymphocytes (Th2), but also by monocytes, activated macrophages and some activated B cells; non haematopoitic cells such as keratinocytes and cancerous cells in colon carcinoma and melanoma cells secret this cytokine (Delves *et al.*, 1998).

Interleukin-10 specifically inhibits macrophage activation by IFN-γ and cytokine production in activated monocytes and macrophages such as TNF-α; IL-1; IL-6; IL-8 and IL-12 (Gerard *et al.*, 1993; Yue Ho *et al.*, 1993; Delves *et al.*, 1998). Plasma IL-10 from patients with septic shock was found to deactivate monocytes (Brandtzaeg *et al.*, 1996).

IL-10 is known to inhibit T helper-1 (Th1) lymphocyte cytokine synthesis in the mouse. By down regulating the expression of MHC class II and co-stimulatory molecules on macrophages, T cell activation is inhibited (Fiorentino *et.al.*, 1989; Delves *et al.*, 1998; Ibelgauft, 1999). IL-10 also interrupts the action of neutrophils by suppressing the production of oxygen radicals and proteases. It controls tissue injury by downregulating the expression of integrins (CD11a and CD11b) and the TNF-α receptor; integrin receptors bind to ICAM-1 and complement protein *i*C3b (Kawai, 2000). *i*C3b is the inactivated form of C3b, but it is still able to activate leucocyte cell function and oposonise particles although it cannot form a membrane attack complex (Herbert *et al.*, 1995).

In a reported case study, a patient with *Crytococcus neoformans* meningitis had high levels of IL-10 in the cerebro-spinal fluid (146pg/ml) and less than normal values (< 8.9pg/ml) in serum; at the same time IL-12 was elevated in the serum and continued to rise over a four week period but was low in the cerebro-spinal fluid (Tascini, *et al.*, 2002). These results agree with others that IL-10 production is stimulated by the yeast and suppresses the inflammatory response (Vecchiarelli *et al.*, 1996).

IL-10 has been used to prevent the HIV virus from invading macrophages. CD4 (an accessory molecule to the MHC class II molecule) acts as a receptor for the human immunodeficient virus (HIV) and is mainly found on T-lymphocytes but human monocytes and macrophages also express this molecule and can become infected with the virus and transport it to lymph nodes where it has greater access to CD4+ T-lymphocytes (Weissman, 1996).

Virally induced TNF-α and IL-6 production is inhibited by IL-10 as well as causing a decrease in viral replication (presumably by preventing HIV access to the cells); clinical trials using infusions of IL-10 are being carried out on AIDS patients and cases with Crohns disease (an inflammatory bowel disease), both trials are producing remissions (Weissman, 1996; MAC, 1999; van Deventer *et al.*, 1999).

In a study of patients with SIRS it was found that after a multivariate analysis of heart-rate, mean arterial pressure (MAP), IL-6 and IL-10, the only relationship was between IL-6 and IL-10. In survivors the ratio of these cytokines remained stable, but in non-survivors the ratio changed with IL-6 increasing and IL-10 decreasing (Taniguchi *et al.*, 1999).

2.6.1 Drug effects on IL-10 production.

Research has demonstrated that immunosuppressive drugs such as cyclosporin A and rapamycin inhibit IL-10 production by human T cells (Cohen, 1997). This is a factor that needs to be taken into account when evaluating patient results in this project.

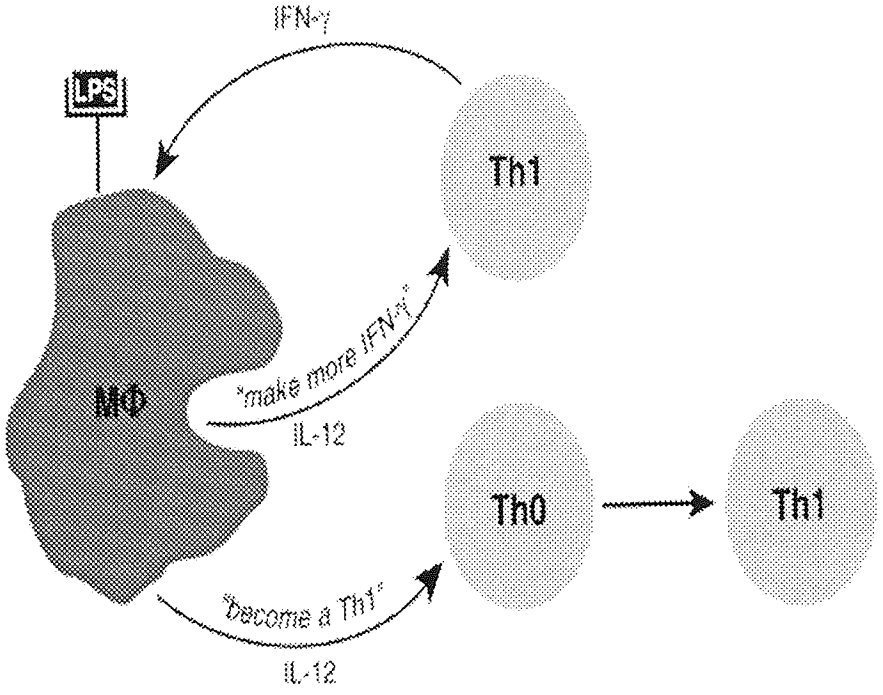
2.7 INTERLEUKIN -12

IL-12 is a 70kDa heterodimeric glycoprotein with a 40kDa (p40) and 35kDa (p35) subunit linked by disulphide bonds that are essential for its biological activity. The p70 heterodimer is the functional component of IL-12. There is a binding site for heparin on the p40 unit. The receptor for IL-12 is reported to be a single protein and between 1000 and 9000 high affinity receptors are expressed on activated peripheral blood mononuclear cells (Ibelgaufts, 1999).

IL-12 is a potent inducer of IFN-γ which is a major macrophage activating factor, resulting in enhanced killing of intracellular pathogens within macrophage (Hessle *et al.*, 2000).

IL-12 is secreted mainly by macrophages and other antigen presenting cells and induces the proliferation of natural killers (NK) cells and CD8+ cytotoxic T cells (Herbert *et al.*, 1995). IL-12 was formerly known as the natural killer (NK) cell activator. The most potent inducers of IL-12 are bacteria and parasites (Mountford *et al.*, 1998). The result of studies with IL-10 knock-out mice suggest high levels of IL-12 during microbial infection can be harmful, even lethal (Gately *et al.*, 1994). Conversely other

results suggest that IL-12 exerts a protective effect during experimental endotoxia by upregulating cellular immunity and phagocytic functions (Ertel *et al.*, 1997).



Reproduced from Sompayrac, (1999).

Figure 2.5 IL-12 from macrophages drives naïve T-helper 0 (Th0) cells towards a T-helper-1 (Th1) response.
IL-12 binding Th1 cells activates the T cell to make more IFN-γ, this in turn causes the macrophage to produce IL-12, this cytokine causes uncommitted T cells to become Th1 type helper cells. Consequently the number of Th2 cells diminishes. This is usually balanced by Th2 cells secreting IL-10. IL-10 then acts to decrease the rate of Th1 cell proliferation (Sompayrac, 1999).

Recent findings suggest that Gram-positive bacteria preferentially induce monocytes to express IL-12, whereas Gram-negative bacteria stimulate monocytes to express IL-10 (Hessle *et al.*, 1999). Other examples involving pneumococci versus *Haemophilus influenzae* (Arva & Andersson, 1999), *Streptococcus mutans* versus *Porphyromas endodontalis* (Jiang *et al.*, 1999), all concurred that Gram-negative bacteria resulted in the production of large amounts of IL-10 and limited IL-12 and the converse was true for the Gram-positive bacteria.

If these finding are confirmed then IL-10 or IL-12 could be potential differentiation markers between Gram-positive and Gram-negative sepsis.

2.8 LIPID S

Lipid S is a recently discovered moiety from *Staphylococcus epidermidis* and has been described as an exocellular short chain length glycerophosphoglycolipid (see figure 2.6) form of the cellular lipoteichoic acid (Lambert *et al.*, 2000).

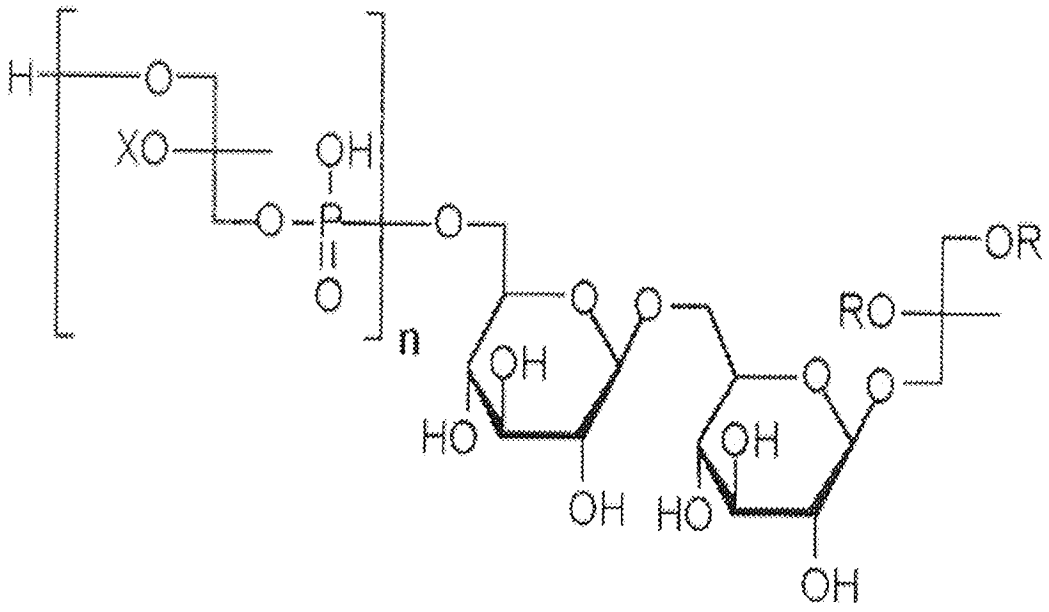


Figure 2.6 Schematic representation of the potential structure of lipid S.

R = fatty acid esters, X = alanyl esters or N-acetylglucosamine, n =6

(Copied with permission from Lambert *et al.* 2000). Lipoteichoic acid (LTA) is found in the outer cell wall of Gram-positive bacteria.

An indirect ELISA has been developed to detect serum antibody to lipid S (Lambert *et al.*, 2000). This test has been used successfully to detect infections in prosthetic joints caused by Gram-positive bacteria (Rafiq *et al.*, 2000).

More recently a rapid version of this ELISA has been developed for the diagnosis of endocarditis and intravascular catheter related sepsis caused by coagulase negative staphylococci (Connaughton *et al.* 2001; Worthington *et al.*, 2002). The sensitivity and specificity of this ELISA for the detection of anti-lipid S IgG antibody in patients with central venous catheter-related sepsis was found be seventy-five percent and ninety percent respectively (Elliott *et al.*, 2000).

This ELISA measures either preformed antibody to lipid S from a previous infection or antibody developed in response to a patient's current infection; in the latter case a negative lipid S titre may occur as antibody to specific antigen takes 5-7 days to develop.

The anti-lipid S assay was included in the project to ascertain its value as a differential marker between Gram-positive and Gram-negative sepsis. If this test was shown to be of value in this scenario it would facilitate the immediate application of the appropriate antibiotic therapy thereby improving patient outcome.

2.9 TEST MARKER SUMMARY

Many of the proposed test markers can be found in disease states other than infections, for example, elevated serum levels of TNF-α and IL-6 in cases of trauma, burns and non-infectious inflammatory conditions. Therefore the diagnostic value of the test marker or markers may lie in a higher cut-off value for sepsis as determined from this study in comparison with levels found in published research literature for other diseases.

Research into the pathophysiology of sepsis and septic shock is still a high priority as these conditions can readily progress into organ failure, multiple organ failure (MOF) and death. As the pathophysiology of sepsis and the mechanisms causing coagulopathies are unravelled new potential serum markers are discovered such as protein C, a potent anti-coagulant (Bernard *et al.*, 2001). Replacing serum levels of the activated form of this protein is a moderately effective therapy for patients with sepsis leading to organ failure (Bernard *et al.*, 2001). However the best defence against MOF and multiple organ dysfunction syndrome (MODS) is prevention and the best diagnostic tool for sepsis is said to be "suspicion" (Moreno, 2002; Cruz and Dellinger, 2002). Twenty years ago an early diagnosis of sepsis with treatment proved to reduce fatalities

by 50% (Kregar *et al.*, 1980), yet a reliable diagnostic serum marker for impending sepsis remains elusive.

2.9.1 Clinical Markers of infection

When a patient presents with a raised temperature, it is normal practice to measure the serum levels of the current markers of infection; which include the peripheral white cell count and serum C-reactive protein. These routine laboratory markers of infection are included in this project to compare the diagnostic information they present in comparison to the test markers.

Patient samples for microbial investigation are obtained from possible sites of infection. In this study all the patients had a blood culture taken to identify bacteraemia or septicaemia. Pathogens were cultured and their sensitivity to antibiotics ascertained. This process can commonly take greater than 48 hours to complete. Earlier evidence of infection may be obtained by measuring a patient's serum level of C-reactive protein and total peripheral white cell count as they are normally elevated during infections or inflammation. High levels of both markers may induce the clinician to start the patient on a broad spectrum antibiotic.

2.9.1.1 C-reactive protein

C-reactive protein (CRP) is an acute phase protein synthesised by hepatocytes in the liver after stimulation by IL-1 or IL-6 (Kolb-Bachofen, 1991; Jupe, 1996). CRP is constitutively expressed and is found in the serum of healthy adults at levels of less than 10mg/litre (Pepys, 1981; Clyne and Olshaker, 1999), or even less than 4mg/l (Spickett, 1999). A 100 fold increase can occur within hours of an inflammatory stimulus including trauma, burns or bacterial infection (Wolbink *et al.*, 1997). CRP has a circulating half-life of approximately eight hours and can be used to track the inflammatory response and to monitor the effect of antibiotic treatment in patients; as serum CRP levels decline rapidly when the inflammatory stimulus has been resolved (Young *et al.*, 1991; Clyne and Olshaker, 1999).

CRP is a pentameric molecule consisting of five identical subunits noncovalently bonded with a molecular weight of 120kD, smaller than antibody but larger than albumin (Clyne and Olshaker, 1999; Playfair and Lydyard 2000). It has a strong affinity for

phosphorylcholine, a component of some bacterial cell walls, for example *Streptococcus pneumoniae* and acts as a non-specific opsonin (Reeves & Todd 2000).

It is called C-reactive protein because of its reactivity with the C-substance polysaccharide of streptococci, a discovery first made in 1930 by Tillet and Frances. They identified a substance in the sera of patients with pneumococcal pneumonia that formed a precipitate when it was added to polysaccharide C of *Streptococcus pneumoniae* (Tillet and Frances, 1930; Kasper, 1990). CRP activates the classical pathway and binds to complement fragments on damaged tissues (Kushner and Kaplin, 1961; Wolbink *et al.*, 1997).

The established "normal" CRP serum levels have recently been challenged in a study that demonstrated that CRP levels fall into two distinct age groups of 20-50 years and 50 –80 years with mean values of 1.3 (+/- 1.27) mg/l and in the older group 0.43 (+/- 0.42) mg/l (Wu *et al.*, 2002). These findings are due in part to the development of new high sensitivity CRP assays with an analytical sensitivity of 0.04mg/ml (Jaye and Waites, 1997; Rifai and Ridker, 2001).

The main problem with CRP as a diagnostic marker for bacterial infection or sepsis is its lack of specificity. Levels of up to 100mg/l are seen in myocardial infarction, lymphomas, hypernephroma and some viral infections, especially from the Epstein Barr Virus (EBV) and the cytomegalavirus (CMV), although normal levels are seen in most other viral infections (Spickett, 1999). Serum levels greater than 100mg/l may be found in severe vasculitis and burns, however levels greater than 300mg/l are *only* found in cases of bacterial sepsis (Jupe, 1996: Spickett, 1999).

2.9.1.2 White Cell Count

The peripheral blood white cell count WCC) is an estimate of the number of white cells per millilitre. The normal range in adults is given in table 2.5. Deviations from these norms, such as a raised neutrophil count may be due to bacterial infection but can be elevated for other reasons such as severe exercise, inflammation, connective tissue disease corticosteroid therapy (due to a decreased rate of neutrophil egress from the circulation), diabetic ketoacidosis and Cushings syndrome (Karle and Hansen 1974; Eastham, 1984). Laboratory reports specify the differential white cell components of the peripheral blood. These include neutrophils, monocytes, basophils, eosinophils and

lymphocytes as shown in figure 2.7. The differential cell counts per litre are given in table 2.5.

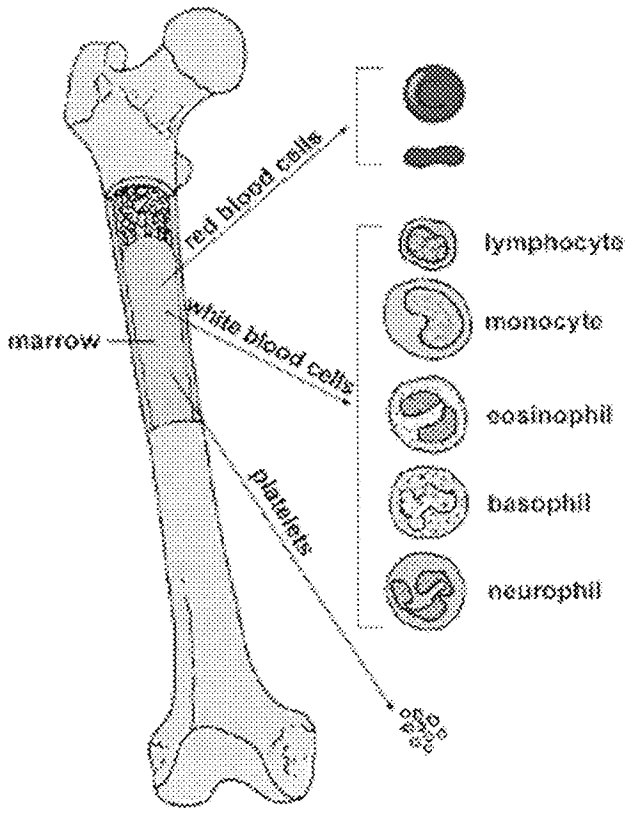


Figure 2.7 Cellular elements in the blood originate in the bone marrow

All blood cells differentiate from pluripotent haematopoietic stem cells in the bone marrow. These cells then divide into two other cells, the common lymphoid progenitor cell that produces the lymphocytes (T cells and B cells) and the myeloid stem cell from which all the other blood cells are derived (Goldie, 1991). The cells are carried in plasma, the fluid constituent of the blood. The packed cell volume of a given sample of peripheral blood is estimated using the haematocrit. A centrifuged sample of blood will separate into approximately 55- 58 percent plasma above a layer of white cells (also called the Buffy coat) and represent less than one percent of the total cells. Erythrocytes or red blood cells account for 42-45 percent of the haematocrit volume (Sherwood, 1993).

Whereas erythrocytes are involved amongst other things in transporting oxygen to and carbon dioxide from the tissues, white blood cells are involved exclusively in the defence of the host against infections from any exogenous source as well as disposing of normally occurring dead cells by phagocytosis. The structure and functions of white cells and their interactions in host defence encompasses a vast amount of information therefore each white blood cell is described here only in terms of it's major function see table 2.5.

Table 2.5 Total peripheral white cell count (WCC); differential cell count and major function.

Leucocytes	White cell count	Function
	4.0-11 x 10^9/l	
Neutrophils	2.0- 7.5 x 10^9/l	Phagocytosis
Lymphocytes (include B-lymphocytes T-lymphocytes)	1.0- 4.0 x 10^9/l	Anti-body production; destroy cells with intracellular pathogens, Produce cytokines
Monocytes	0.2- 0.8 x 10^9/l	Phagocytosis Produce cytokines
Eosinophils	0.0 – 0.4 x 10^9/l	Act against parasites
Basophils	0.0- 0.1 x 10^9/l	Not phagocytic release vasodilators when stimulated.

Table 2.5 lists the differential peripheral white cell count for adults, adapted from Eastham, (1984). T-lymphocytes include T-helper 1 and 2 cells as well as cytotoxic cells called natural killer cells (NK).

A characteristic neutrophil leucocytosis (neutrophils >11 x 10^9/l) occurs in the early stages of infection or tissue injury (Witko-Sarsat *et al.*, 2000). Activated macrophages release granulocyte colony stimulating factor (GCSF) and granulocyte

macrophage colony stimulating factor (GMCSF). These cytokines stimulate division of the myeloid progenitor cells in the bone marrow resulting in the release of millions of neutrophils into the circulation (Parker and Cohen, 2001). Conversely, neutropenia (circulating peripheral blood neutrophils < 2.0 x 10^9/l) can occurs in overwhelming infections such as sepsis and septicaemia (Huges-Jones and Wickramasinghe, 1991). However there are other causes of neutropenia such as systemic lupus erythematosus (SLE), hypothyroidism and certain drugs (Eastham, 1984).

The precise molecular mechanisms involved in leucocytosis and the opposite effect of sepsis-mediated bone marrow suppression creating a leucopenia are poorly understood (Cruz and Dellinger 2002).

The white cell count and the differential neutrophil count are useful markers of infection but relying on these markers of infection can be cause a delay in diagnosis in those patients with a WCC within the normal range (4.0 -11 x10^9/L).

A patient whose 'normal' WCC is in the lower end of the range could show an increase in WCC from 4 to 10 x10^9/L due to an infection but it would not be noticed. Also immunosuppressed patients such as those undergoing chemotherapy or organ transplant would be expected to have a drug induced lower white cell count. This effect would not only expose these subjects to a greater risk from infection but the effects of an infection in relation to an increase in peripheral neutrophils would be masked (Gates and Baehner, 1995; Fullwood *et al.*, 2001). Although this project does not include infants nevertheless a WCC is an inaccurate screen for bacteraemia in febrile infants (Bonsu and Harper 2003).

It is imperative to identify a serum marker indicating bacterial infection and sepsis for patients regardless of their WCC, underlying disease or drug regime, so that early appropriate treatment can be initiated and the infection controlled to prevent a possible progression towards sepsis.

CHAPTER 3 MATERIALS AND METHODS

3.0 CLINICAL SAMPLES

All subjects for this project were patients at the Queen Elizabeth and Selly-Oak Hospitals Birmingham. Sera from 92 patients were used in the first part of this study, termed Phase 1. Routine blood samples were collected aseptically by venepuncture and after blood cells were removed the sera was stored at minus 20°C. Clinical staff collected the samples for diagnostic tests such as serum C-reactive protein. Permission to use these routine laboratory samples for the project assays was approved by the hospitals Local Ethics Committee and Trial Research and Development Committee.

3.1 PATIENTS IN THE PRELIMININARY TRIAL (PHASE 1).

Twenty-three pre-operative patients admitted into hospital for coronary artery by-pass grafts (CABG) served as controls in the first part of this study. They had no clinical evidence of infection or sepsis twelve weeks prior to the test date.

Thirty-three cases of Gram-positive central venous catheter (CVC) sepsis and thirty-five patients with Gram-negative sepsis were enrolled on to this study. Sepsis was defined as the systemic inflammatory response syndrome (SIRS) in the presence of a confirmed infection. The criteria for SIRS was a temperature < 36.6°C (96°F) or ≥ 38°C (101°F) and a white cell count $< 4.0 \times 10^9$/l or $> 12.0 \times 10^9$/l as outlined by the American College of Chest Physicians and the Society of Critical Care Medicine (ACCP/SCCM) in Bone, (1992).

When a peripheral blood white cell count was missing from patient's records or not taken on the same date as the test serum or ambivalent in a clinically diagnosed septic patient (as in neutropenia) an elevated C-reactive protein was substituted as the second criteria for patient inclusion. Table 3.1 gives the patient's demographics

Bacterial sepsis (septicaemia) was defined as given above and bacteraemia was confirmed in all septic patients by positive blood cultures (BACTEC semi-automated blood culturing system; Becton Dickinson, Europe), Gram-staining and subculture onto the appropriate media. The isolate was identified by routine biochemical methods.

Table 3.1 PATIENT DEMOGRAPHICS

Group	Number	Gender M/F	Mean age
Controls	23	17 M 6 F	62 54.4
Gram-positive sepsis	33	21 M 12 F	53 49
Gram-negative sepsis	36	21 M 15 F	49.5 62.4

3.1.1 Inclusion Criteria

Patients with pyrexia and a Gram-negative or Gram-positive isolate in their blood culture together with a raised white cell count or C-reactive protein. Control patients without a recent (twelve weeks) documented infection. Patients meeting the sepsis criteria were included regardless of their underlying disease.

3.1.2 Exclusion criteria.

Patients were excluded if they had experienced a septic episode or infection just prior to the current infection that would otherwise have qualified them for the study. Patients with Gram-positive and Gram-negative bacteria in the same blood culture were excluded.

3. 2 PRINCIPLES OF THE ENZYME LINKED IMMUNOSORBENT ASSAYS (ELISA)

The assays for the test markers for sepsis (with the exception of the procalcitonin and Lipid S), employ a solid phase quantitative sandwich enzyme immunosorbent assay (ELISA) technique. Monoclonal antibodies specific for each marker are precoated onto a 96 well microplate by the manufacturer. Standards and patient samples are pipetted into the wells and any antigen (for example interleukin 6) is 'captured' by the bound antibody. After washing away any unbound substances an enzyme-linked second antibody is added making a sandwich of the test marker. Any unbound second enzyme-linked antibody is removed by washing and a substrate for the enzyme is added. For interleukin -6 the substrate is tetramethybenzidine (TMB- a chromogen) and hydrogen peroxide. The coloured product produced by the catalytic action of the enzyme is

proportional to the amount of the test marker bound in the initial step. Figure 3.1 illustrates the 'sandwich' principle of these assays.

3.2.1 Procalcitonin (PCT) LUMItest® (BRAHMS Diagnostica Gmbh, Germany) is a solid phase immunoluminometric (ILMA) assay that produces photons of light by an oxidative reaction with hydrogen peroxide. Measurement of the light given off from each test sample is recorded using a luminometer. Two antibodies are employed to form sandwich complexes with serum PCT. One antibody is bound to the sides of the assay tube and binds to the katacalcin portion of procalcitonin. A second antibody conjugated to a luminescent marker, binds to the calcitonin part of PCT. Following incubation and removal of unbound antibody the luminescent signal is measured with a luminometer. The intensity of the luminescence is directly proportional to the concentration of PCT in the sample.

3.2.2 The Lipid S assay is a quantitative indirect ELISA that measures serum immunoglobulin G (IgG) antibodies against lipid S. Lipid S is an exocellular short chain form of lipoteichoic acid (LTA). LTA is a part of the cell structure of Gram-positive bacteria. In this assay the antigen is bound to the microplate wells not the antibody. The patient's serum supplies the antibody not the antigen as is the case for all the other assays in this project. Bound antibody is them detected using an enzyme-linked animal anti-immunoglobulin raised against human IgG. The specificity is provided by the bound antigen. After the addition of the substrate (tetramethylbenzidine/hydrogen peroxide) the colour development is stopped with sulphuric acid. The lipid S antigen bound to the walls of the microplate is referred to as the BHI7 antigen. This nomenclature is based on the brain heart infusion (BHI) that grew the 7 strains of coagulase negative staphylococci isolated from patients with catheter related sepsis. Gel permeation chromatography (Superose 12) was used to recover the antigen (BHI7) from the culture medium (Lambert *et al.*, 1996; Lambert *et al.*, 2000; Elliott *et al.*, 2000; Worthington *et al.*, 2002).

3.2.3 IL-6 and TNF-α High Sensitivity assays (R&D Quantikine®, Oxen, UK) use alkaline phosphatase enzyme linked antibodies with nicotinamide adenine dinucleotide phosphate (NADPH). They incorporate an amplifier system that results in a maroon coloured product, read at wavelength 490nm. These assays are active in an alkaline pH

(optimum pH 8). In the amplification system, alkaline phosphatase dephosphorylates NADPH to NADH which acts as a specific co-factor that activates a redox cycle driven by alcohol dehydrogenase and diaphorase. NADH reduces iodonitrotetrazolium violet (INT –violet) to produce a coloured product and NAD^+. NAD^+ is reduced by ethanol, catalysed by alcohol dehydrogenase to NADH. The amount of coloured product is directly proportional to the amount of IL-6 or TNF-α.

3.2.4 Interleukin 10 and Interleukin 12p70 assays are quantitative ELISA and follow the sandwich principle illustrated in figure 3.1. and differ only in the second antibody. This is a biotin labelled antibody that binds streptavidin –horseradish peroxidase.

3.2.5 The lipopolysaccharide binding protein (LBP) assay kit is manufactured by HyCult biotechnology b.v. (HBT, Netherlands) and is a solid-phase sandwich ELISA assay that uses the same type of reagents as for IL-10 and IL-12p70, except the stopping agent is citric acid as opposed to sulphuric acid.

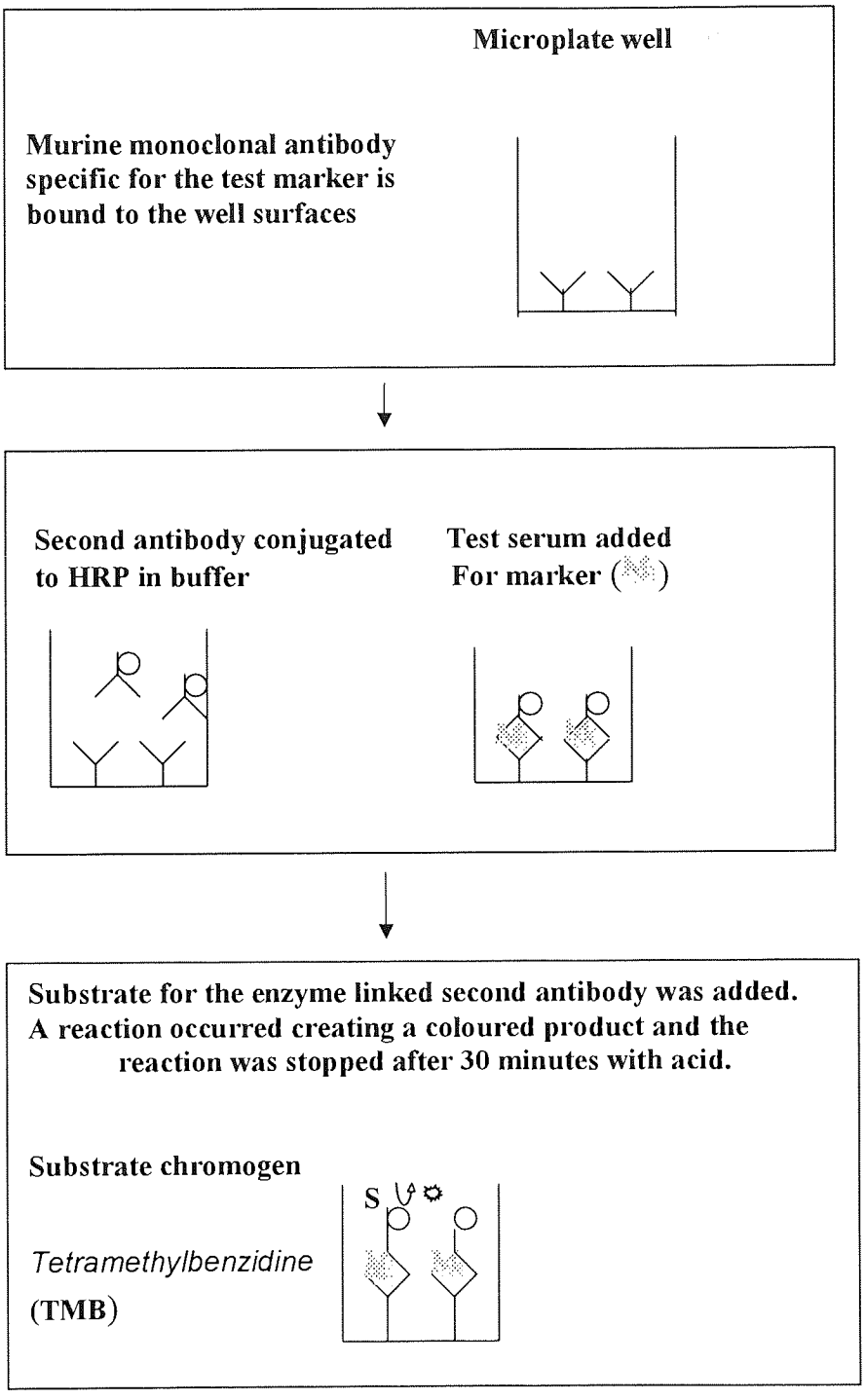


Figure 3.1 Quantitative sandwich ELISA using a Horseradish-peroxidase (HRP)/TMB-hydrogen peroxide system.

sE-selectin; sICAM-1; IL-6 and TNF-α Assays

The HRP/TMB-H_2O_2 system (see figure 3.1) is active over a broad pH range. Peroxide is reduced with hydrogen donors that can be measured after oxidation as a colour change. The TMB dye product is soluble and therefore detectable using a spectrophotometer at wavelength 450 – 620nm. The resulting blue colour turned yellow when the reaction was stopped by sulphuric acid.

3.3 METHODS COMMON TO ALL ASSAYS

To avoid repeating methods that are used in every assay such as the washing procedure, they are given here in full and subsequently abbreviated in the methods. A data collection sheet was prepared before proceeding with an assay. This was an invaluable tool ensuring that the correct sample was inoculated into the designated well.

IL-10 pg/ml	Standards	Standards	Tests			
	1	2	3	4	5	6
A	400	400	test samples	test samples	test samples	test samples
B	200	200	test samples	test samples	test samples	test samples
C	100	100	test samples	test samples	test samples	test samples
D	50	50	test samples	test samples	test samples	test samples
E	25	25	test samples	test samples	test samples	test samples
F	12.5	12.5	test samples	test samples	test samples	test samples
G	blank	blank	test samples	test samples	test samples	test samples
H	control	control	test samples	test samples	test samples	test samples

Figure 3.2 An example of a microplate template inoculation scheme

A microplate has 12 columns and 8 rows of wells, only 6 columns are shown here. All the wells are assigned a standard, a control or a coded patient's test sample on a data collection sheet. Spare wells were used to duplicate (approximately) 10% of the samples as an intra-assay control. Approximately 2% of the wells per plate were utilised for previously tested samples of the particular test marker and served as inter-assay controls.

3.3.1 Wash procedure (except for PCT and anti-lipid S assays).

An adhesive strip was stuck to the base of the microplate to hold the wells firmly in place when the plate was inverted during the washing procedure. The contents of the wells were removed by inverting the plate over a sink with running water. Excess liquid

clinging to the sides of the wells was removed by firmly tapping the inverted plate onto paper towels. Using an eight-channel pipette the wells were re-filled with 400µl of the prepared wash buffer. After approximately 30 seconds the buffer was decanted by inverting the plate over the sink. This procedure was repeated a further three times. After the fourth wash the plate was again tapped onto paper towels to remove any excess buffer. The anti-lipid S IgG antibody assay microplate was washed on an automated ELP-40 plate washer.

3.3.2 Microplate reader.

The assay absorbance was read using an Anthos 2001 microplate reader (Labtech, Ringmer, East Sussex, UK). This plate reader prints out the absorbance for each well and has a correction setting. The correction setting allows for the optical imperfections in the plate to be measured and subtracted from the final absorbance results. Half and hour before the end of each assay the plate reader was turned on to allow the machine to reach the optimum working temperature. Any bubbles in the wells would alter the accuracy of the assay and were removed prior to incubations and particularly before the plate was read. This machine was used to read the results from all the assays in this project with the exception of the procalcitonin assays and anti-lipid S assays. Procalcitonin assays were read with a luminometer Auto Clinilimat, (Berthold UK, Ltd. LB952T/16).

3.3.3 Inter-assay and Intra-assay precision tests.

All the assay kit manufacturers recommended that each standard and test sample should be performed in duplicate. Due to the high cost of the assay kits, for example a procalcitonin test cost £5 per patient, not all the patient samples could be performed twice. Therefore approximately 5% of patient samples where duplicated per assay. The reproducibility and precision of the results within an assay was evaluated from these duplicate samples using the mean, standard deviations and a coefficient of variance see section 3.5.6. for a fuller description. Inter-assay reproducibility (between plates) was tested using standards and patient samples with known levels of the test markers from previous assays. The results are given in Chapters 4.5 and 6.

3.3.4 Control of substances hazardous to health (COSHH).

Protective clothing and safe practices were followed in the laboratory. All reagents and serum was handled according to the regulations under the Health and Safety at Work Act 1974 governing the control of substances hazardous to health (COSHH).

The IL-6 and TNF-α high sensitivity assays contained mercury. Sodium azide was contained as a preservative in some of the reagents and was disposed of with plenty of water as it can react with lead or copper plumbing to form explosive metallic azides. All the assays contained acids.

3.4 THE ASSAYS; Preparations, Materials and Methods

3.4.1 INTERLEUKIN-6 HS ASSAY (detection limit < 0.09pg/ml)

Materials included in the Quantikine® HS (High Sensitivity) Assay Kit, R&D Systems Oxen, UK.

3.4.1.1 Materials IL-6 HS

96 well polystyrene microplate; Comprising of a frame that held 12 separate strips of 8 wells coated with murine monoclonal antibody against IL-6.

IL-6 Conjugate; 21ml of polyclonal antibody against IL-6 conjugated to alkaline phosphatase

IL-6 Standard for the calibration curve; 50 pg of recombinant human IL-6 in a buffered protein base.

Assay Diluent (HD1D); 6ml of buffered protein base for serum/plasma samples

Calibrator Diluent (HD6F); 21ml of animal serum for use with serum/plasma samples

Wash Buffer concentrate; 100ml of a 5- fold concentrated solution of buffered surfactant.

Substrate; lyophilized reduced nicotinamide adenine dinucleotide phosphate (NADPH) with stabilizers.

Substrate diluent; 7ml of buffered solution.

Amplifier; lyophilised amplifier enzymes (alcohol dehydrogenase and diaphorase).

Amplifier diluent; 7ml of a buffered solution containing ethanol and iodonitrotetrazolium violet (INT-violet).

Stop solution; 6ml of 2 N sulphuric acid.

Plate covers; 8 adhesive strips.

Additional materials (not included in the kit).

Gilson pipettes and sterilised disposable tips, multi-channel pipette; graduated measuring cylinders 100ml and 500ml, distilled water and a microplate reader Anthos 2001(Labtech, Ringmer, East Sussex UK), with a setting of 490nm a correction wavelength of 650 or 690nm.

3.4.1.2 Preparation IL-6 HS assay

The microplate and all the reagents where removed from the fridge and brought to room temperature before proceeding. Patient samples were thawed. The wash buffer was prepared by adding 400ml of distilled water to 100ml of wash buffer. The IL-6 HS standard was reconstituted with 5ml of calibrator diluent (HD6F) and gently agitated for 15 minutes to form a stock solution of 10 pg/ml. 500µl of calibrator diluent was added to 7 test tubes, labelled 1-7. 500µl of the 10pg/ml IL-6 standard was added to tube 1. Using a Gilson pipette the solution in tube 1 was mixed thoroughly. A fresh sterile pipette tip was used to remove 500 µl of this solution to tube 2. This process was repeated from tube 2 to tube 6 to obtain the standards for the calibration curve. Tube 7 was the zero standard (0 pg/ml) containing diluent. The dilutions halved the concentration of IL-6 per dilution with tube 6 having a concentration of 0.156pg/ml.

The Substrate and Amplifier were reconstituted with 6ml of substrate and amplifier diluent respectively and mixed thoroughly. These solutions were prepared during the assay 30 minutes before use.

The assay protocol states that 200µl of patient sample should be used, however patient samples were diluted 1:4 with 50µl serum to 150µl distilled water. This was done to avoid an "overflow" optical density effect caused by very high levels of IL-6 in the sample. This effect was anticipated from the serum of patients with sepsis.

3.4.1.3 Method IL-6 HS Assay.

50µl of assay diluent (HD1D) was added to each well using a Gilson pipette, then 200µl of each standard was consecutively placed into wells A1 to H1 using a sterile tip for each standard. The 10pg/ml standard was in A1 and the zero standard in well H1.

200µl of each patient's diluted serum was added to each well (as labelled on the template). The plate was covered with an adhesive strip and incubated overnight at room temperature. The adhesive strip was removed and a fresh strip was applied to the base of the wells to avoid the possibility of the wells becoming dislodged when the plate was inverted to remove the contents of the wells. The microplate was washed 4 times with the prepared wash buffer. 200µl of conjugate (antibody against IL-6 conjugated to an alkaline phosphatase) was added to each well. The plate was covered with an adhesive strip and incubated at room temperature for 6 hours. The conjugate was then discarded and the wells were washed four times as described above. 50µl of the prepared reconstituted substrate (NADPH) was added to each well and covered with adhesive

tape. The plate was incubated at room temperature for 1 hour. After substrate incubation the adhesive strip was removed and 50µl of amplifier solution was added to each well and incubated for a further 30 minutes at room temperature. The amplifier solution initiates the colour development. The reaction was stopped with 50µl of 2N sulphuric acid (stop solution). The optical density was measured using a microplate reader (Anthos 2001) set at 492 nanometres (nm) with a wavelength correction setting of 620nm. The correction setting subtracts any optical interference from the microplate. A print out of the absorbance from each well was used with Graph Pad Prism 3.0 data analysis computer package to produce the calibration curve from the standards. The unknown levels of IL-6 in patient samples were computer generated from the data. The results obtained were multipled by the dilution factor (x4). The results from this assay are given in Chapter 4.

3.4.2 TUMOUR NECROSIS FACTOR – alpha (TNF-α) HS Assay (detection limit < 0.18 pg/ml)

Materials included in the TNF-α Quantikine® HS (High Sensitivity) Assay Kit, R&D Systems Oxen, UK.

3.4.2.1 Materials TNF-α HS

96 well polystyrene microplate; Comprising of a frame that held 12 separate strips of 8 wells coated with murine monoclonal antibody against TNF-α.

TNF-α Conjugate; 21ml of polyclonal antibody against TNF-α conjugated to alkaline phosphatase

TNF-α Standard for the calibration curve; 3 vials (32 pg/ml) of recombinant human TNF-α in a buffered protein base.

Assay Diluent (HD1-11); 6ml of buffered protein base for serum/plasma samples.

Calibrator Diluent (HD65); 21ml of animal serum for use with serum/plasma samples

Wash Buffer concentrate; 100ml of a 5- fold concentrated solution of buffered surfactant.

Substrate; lyophilized reduced nicotinamide adenine dinucleotide phosphate (NADPH) with stabilizers.

Substrate diluent; 7ml of an undefined buffered solution.

Amplifier; lyophilised amplifier enzymes (alcohol dehydrogenase and diaphorase).

Amplifier Diluent; 7ml of a buffered solution containing ethanol and iodonitrotetrazolium violet (INT-violet).

Stop solution; 6ml of 2 N sulphuric acid.

Plate covers; 8 adhesive strips.

Other materials;

Gilson pipettes (50µl and 200µl) and sterilised disposable tips, a plate reader (ref), multi-channel pipette; graduated measuring cylinders 100ml and 500ml.

3.4.2.2 Preparation TNF-α HS assay

The microplate and all the reagents where removed from the fridge and brought to room temperature. Patient samples were thawed. The wash buffer was prepared by adding 400ml of distilled water to 100ml of wash buffer. The TNF-α HS standard was reconstituted with 1ml of calibrator diluent (HD6J) and gently agitated for 15 minutes to form a stock solution of 32 pg/ml. 500µl of calibrator diluent was added to 7 test tubes, labelled 1-7. 500µl of the 32 pg/ml reconstituted TNF-α standard was added to tube 1. Using a Gilson pipette the solution in tube 1 was mixed thoroughly. A fresh sterile pipette tip was used to remove 500 µl of this solution to tube 2. This process was repeated from tube 2 to tube 6 to obtain the standards for the calibration curve. Tube 7 was the zero standard (0 pg/ml) containing diluent. The dilutions halved the concentration of TNF-α per dilution; tube 6 had a concentration of 0.5 pg/ml.

The Substrate and Amplifier were reconstituted with 6ml of substrate and amplifier diluent respectively and mixed thoroughly. These solutions were prepared during the assay 30 minutes before use.

3.4.2.3 Method TNF-α HS Assay

50µl of assay diluent (HD1D) was added to each well using a Gilson pipette, then 200µl of each standard was consecutively placed into wells A1 to H1 using a sterile tip for each standard. The 10pg/ml standard was in A1 and the zero standard in well H1.

200µl of each patient's diluted serum (100µl serum plus 100µl distilled water) was added to each well (as labelled on the template). The plate was covered with an adhesive strip and incubated overnight (14-20 hours) in a refrigerator (2-8°C). The adhesive strip was removed and a fresh strip was applied to the base of the wells prior to washing. The microplate was washed 4 times with the prepared wash buffer. 200µl of conjugate (antibody against TNF-α conjugated to an alkaline phosphatase) was added to each well. The plate was covered with an adhesive strip and incubated at room temperature for 3

hours. The conjugate was then discarded and the wells were washed four times. 50µl of the prepared reconstituted substrate (NADPH) solution was added to each well and covered with adhesive tape and incubated at room temperature for 1 hour. After substrate incubation the adhesive strip was removed and 50µl of amplifier solution was added to each well and incubated for a further 30 minutes at room temperature. The amplifier solution initiates the colour development. The reaction was stopped with 50µl of 2N sulphuric acid (stop solution). The optical density was measured using a plate reader set at 492 nanometres (nm) with a wavelength correction setting of 620nm. The correction setting subtracts any optical interference from the microplate. Computer generated results from this assay are given in the results in Chapter 4.

3.4.3 sE-SELECTIN-ASSAY (minimum detectable < 0.1ng/ml)
A quantitative solid phase sandwich enzyme-linked immunosorbent assay (ELISA).
Materials that were included in the Parameter® sE-selectin assay kit (R&D Systems Oxen, UK).

3.4.3.1 **Materials (sE-selectin)**

A 96 well microplate (12 strips each with 8 wells) coated with a murine mononuclear antibody to human sE-selectin

sE-selectin standards. 6 vials of recombinant human sE-selectin ng/ml containing a blue dye.

Sample diluent. 2 x 21ml bottles of buffered protein base with a blue dye and preservative.

sE-selectin Conjugate concentrate. 0.3ml of antibody to human sE-selectin conjugated to horseradish peroxidase (HRP) in a buffer with preservative.

Conjugate diluent. 11 ml of diluent for HRP-conjugate concentrate.

sE-selectin Control. 1 vial of lyophilized human serum containing sE-selectin (ng/ml). With the reconstituted specific concentration of sE-selectin printed on the vial.

Wash buffer concentrate. 20ml of a 25-fold concentrated buffered surfactant with preservative.

Substrate. 11 ml of stabilized tetramethylbenzidine (TMB).

Stop solution. 11 ml of acid (type not specified).

Additional materials (not supplied in the test kit).

A multi-channel pipette, 25µl, 100µl and 1000µl Gilson pipettes with sterile disposable tips. Distilled water, a 500 ml graduated cylinder and a automated microplate reader capable of measuring an absorbance set at 450nm and a correction wavelength set at 620 or 650nm. Eppendorfs for patent's diluted samples.

3.4.3.2 Preparations for the sE-selectin Assay.

All the reagents where brought to room temperature before use and the patient samples were thawed. Eppendorfs were labelled with the patient's code name and 25µl of patients serum was added to their eppendorf and mixed with 475µl of sample diluent giving a 20-fold dilution. The wash buffer concentrate was diluted with 480ml of distilled water to make a 500ml solution. The sE-selectin standards were reconstituted with 800µl of distilled water and mixed by gentle swirling and inversion. The Control was reconstituted with 500µl of distilled water. 25µl of the reconstituted control was added to a clean test tube (labelled) and mixed with 475µl of sample diluent to give a 20-fold dilution. 250µl of the concentrated conjugate was withdrawn from the stock bottle and added to the conjugate diluent bottle and mixed gently by swirling. A data collection sheet was labelled with the respective well number and row for each of the following, the standards, control and specific patient samples.

3.4.3.3 Method sE-selectin

100µl of diluted sE-selectin conjugate was added to all the wells. 100µl of the appropriate standard, control or patient sample was added to the conjugate in their pre-designated wells. Fresh sterile pipette tips were used for each sample. The plate was covered with an adhesive plate cover and incubated at room temperature for 1.5 hours.

The plate was then washed 6 times using the method previously described. 100µl of substrate was added to all the wells and covered with a fresh plate sealer. The plate was incubated for 30 minutes at room temperature and the reaction (colour development) was stopped with 100µl of an acid solution. The plate was read on an Anthos plate reader set at 450nm with a 620nm correction setting. The results from this assay are given in Chapter 4.

3.4.4 SOLUBLE INTERCELLULAR ADHESION MOLECULE-1 ASSAY

(minimum detection < 0.35 ng/ml).

A quantitative solid phase sandwich enzyme-linked immunosorbent assay (ELISA).

Materials included in the Parameter® human soluble ICAM-1 assay kit (R&D Systems Oxen, UK).

3.4.4.1 Materials sICAM-1

A 96 well microplate (12 strips each with 8 wells) coated with a murine monoclonal antibody to human sICAM-1.

sICAM-1 standards. 6 vials of recombinant human sICAM-1 ng/ml containing a blue dye.

Sample diluent. 2 x 21ml bottles of buffered protein base with a blue dye and preservative.

sICAM-1 Conjugate concentrate. 0.3ml of antibody to recombinant human sICAM-1 conjugated to horseradish peroxidase (HRP) in a buffer with preservative.

Conjugate diluent. 11 ml of diluent for HRP-conjugate concentrate

sICAM-1 Control. 1 vial of lyophilized human serum containing recombinant sICAM-1. The reconstituted specific concentration of sICAM-1 was printed on the vial (191-289ng/ml)

Wash buffer concentrate. 20ml of a 25-fold concentrated buffered surfactant with preservative.

Substrate. 11 ml of stabilized tetramethylbenzidine (TMB).

Stop solution. 11 ml of acid (type not specified).

Additional materials (not supplied in the test kit).

A multi-channel pipette, 25µl, 100µl and 1000µl Gilson pipettes with sterile disposable tips. Distilled water, a 500 ml graduated cylinder and a automated microplate reader capable of measuring an absorbance set at 450nm and a correction wavelength set at 620 or 650 (nm). Eppendorfs for patients diluted samples.

3.4.4.2 **Preparations and method** (sICAM-1).

The preparations and methods for this assay were the same as those given for the sE-selectin assay, with one exception. The 20-fold dilution of patient samples and the sICAM-1 control was 15µl of sample (or control) into 285 µl of sample diluent. Every preparation before and during the assay and all procedures, including the preparation of patient samples and the construction of a data collection template were identical. The results from this assay are given in Chapter 4.

3.4.5 INTERLEUKIN -10 ASSAY (minimum detectable dose < 5 pg/ml)

A quantitative solid phase sandwich enzyme linked immunosorbent assay (ELISA) kit from DIACLONE Research (France).

Materials included in the kit.

3.4.5.1 **Materials IL-10**

A 96 well microplate. Pre-coated with monoclonal antibody specific for IL-10.

Plastic cover.

Standard. 2 vials each containing 400 pg/ml IL-10 when reconstituted.

2 Control vials. The concentration of reconstituted IL-10 was 226 ± 57 pg/ml.

Standard diluent buffer, 25ml of x 10 concentrate.

Standard Diluent: human serum, 7ml ready to use.

0.4 ml Biotinylated anti-IL-10.

13ml Biotinylated antibody diluent.

Streptavidin-horse radish peroxidase (HRP), 5µl.

HRP diluent, 23 ml.

Washing buffer. 10ml of concentrated wash buffer (200x)

Chromogen Tetramethybenzidine (TMB), 24 ml.

Sulphuric acid (1.8N H_2SO_4), 11 ml of stopping reagent.

Additional materials (not included in the kit).

Distilled water, pipettes with sterile disposable tips; 10ul; 50µl; 100ul; 200µl and 1000µl. A magnetic stirrer, and a microplate reader with a 450nm setting and a correction setting optimally 620nm (but 610 to 650nm was acceptable).

3.4.5.2 **Preparations** for the IL-10 Assay.

All the reagents where brought to room temperature before proceeding. Patient sera was thawed. Patients expected to have high levels of IL-10 in their sera (Gram-negative bacterial sepsis) were tested twice, once with 100µl of undiluted sera and again with a diluted serum sample. 10µl sample to 90µl Standard diluent: human serum. 25ml of Standard diluent buffer was diluted with 250ml of distilled water in a clean container and mixed using a magnetic stirrer.

The Standard was reconstituted with 1.19ml of ready to use Standard Diluent:human serum, this solution was then diluted with one part of the reconstituted Standard to nine parts of diluent giving a stock of 400pg/ml of IL-10. The Control was reconstituted with 0.5 ml of Standard Diluent:human serum, giving a concentration of 226 ± 57 pg/ml IL-10. 5ml of concentrated wash buffer was diluted with 1000 ml of distilled water and mixed using a magnetic stirrer.

3.4.5.3 **Prepared immediately prior to use.**

240µl of Biotinylated antibody against IL-10 was mixed with 6360µl (6.36ml) in a clean glass container with a wide opening to allow access to a multi-channel pipette. 0.5ml of Horseradish peroxidase (HRP) diluent was added to a 5µl vial of Streptavidin-HRP and mixed together by gentle swirling. 150µl of this solution was removed to a clean container holding 10ml of Streptavidin-HRP diluent and mixed.

3.4.5.3 **Method IL-10**

100µl of Standard diluent: human serum, was added to wells B1, B2, C1,C2 ,D1,D2, E1, E2, F1,F2, G1 and G2. 200µl of prepared Standard (400pg/ml) was added into wells A1 and A2 as shown in table 3.1. 100µl of this was transferred from A1 to B1 and a 100µl was transferred from A2 to B2. The contents of wells B1 and B2 were mixed by repeated aspirations and ejections using a fresh disposable tip for each transfer and subsequent mixing. This procedure was repeated from wells B1 and B2 to wells C1 and C2 up to wells F1 and F2. After the final mixing in wells F1 and F2 100µl was withdrawn and discarded. Wells G1 and G2 served as the IL-10 zero pg/ml control. 100µl of the reconstituted control was added to wells H1 and H2. Following the prepared inoculation scheme 100µl of each patient sample and diluted patient sample was added to their respective wells. 50µl of prepared (extemporarily) diluted biotinylated anti-IL-10 was added to all the wells using a multi-channel pipette. The plate was covered with a plastic

lid and incubated at room temperature (18°C -25°C) for 2 hours. The cover was removed and the plate washed 3 times as described previously. The streptavidin-HRP solution was prepared just prior to the washing procedure. 100µl of this solution was added to each well. The plate was covered and incubated at room temperature for 30 minutes. The wells were emptied and washed 3 times using the wash buffer as described previously. 100µl of ready to use Substrate (tetramethybenzidine –TMB) solution was added to all wells and incubated for 15 minutes at room temperature protected from the light with aluminium foil. The colour development reaction was stopped with 100µl of acid solution (H_2SO_4). The absorbance of each well was read on the Anthos spectrophotometer using 450nm as the primary wavelength and 620nm as the correction wavelength. The resulting absorbance was entered into a computer data analysis programme (Prism) that generated a standard curve from which levels of IL-10 in patient samples were extrapolated. The results from this assay are recorded in Chapter 4.

3.4.6 INTERLEUKIN-12 ASSAY (minimum detection dose < 3 pg/ml)

A quantitative solid phase sandwich enzyme linked immunosorbent assay (ELISA) kit from DIACLONE Research (France). This assay is specific for antibodies against IL-12p70 heterodimer in human serum.

Materials included in the kit.

3.4.6.1 **Materials IL-12**

A 96 well microplate. Pre-coated with monoclonal antibody specific for IL-12.

Plastic cover.

Standard. 2 vials, each containing 200 pg/ml IL-12 when reconstituted.

Standard diluent buffer, 25ml of x 10 concentrate.

Standard Diluent: human serum, 7ml ready to use.

0.4 ml Biotinylated anti-IL-12.

13 ml Biotinylated antibody diluent.

Streptavidin-horse radish peroxidase (HRP). 5µl.

HRP diluent, 23 ml.

Washing buffer. 10ml of concentrated wash buffer (200x)

Chromogen Tetramethybenzidine (TMB), 24 ml.

Sulphuric acid (1M H_2SO_4), 11 ml of stopping reagent.

Additional materials for IL-12 (not included in the kit).

Distilled water, pipettes with sterile disposable tips; 10ul; 50µl; 100ul; 200µl and 1000µl. A magnetic stirrer, and a microplate reader with a 450nm setting and a correction setting optimally 620nm (but 610 to 650nm was acceptable).

3.4.6.1 Preparations and Method for the IL-12 Assay.

The preparations and methods for this assay were the same as IL-10, with two exceptions; the initial incubation time was 3 hours instead of 2 hours and the kit did not include an assay validating control. Every preparation before and during the assay and all procedures, including the preparation of patient samples and their position on the template were identical. The results from this assay are given in Chapter 4.

3.4.7 PROCALCITONIN ASSAY (minimum detection limit 0.15 ng/ml).

A quantitative solid phase immunoluminometric assay (ILMA). Materials included in the LUMItest® PCT Assay kit (BRAHMS Diagnostica GmbH. Berlin).

3.4.7.1 **Materials** Procalcitonin (PCT).

100 Tubes (A); coated with murine anti-PCT antibody.

Tracer (B); a luminescence labelled (an acridinium derivative) anti-PCT antibody.

29 ml Buffer (C) ; to reconstitute the tracer

4 ml of Zero serum (G); human serum to reconstitute the standards, calibrators and controls.

6 Standards; vials containing PCT standards labelled as S1,S2,S3,S4,S5,S6.

2 vials PCT Controls; labelled Ko1 and Ko2.

Additional materials (not included in the kit).

LUMItest ® Basiskit containing reagents for the generation of the luminescence signal.

BR1; 3 x 105ml bottles containing 0.5% hydrogen peroxide in 0.1M nitric acid (HNO_3)

BR2; 3 x 105ml bottles containing 0.25M sodium hydroxide (NaOH)

BK1; 2 vials of Basiskit control. 2ml each when reconstituted with distilled water

BK2; 2 vials of Basiskit control. 2ml when reconstituted with distilled water.

LUMItest® Waschkit. 6 x 40ml wash buffer concentrate.

Pipettes with disposable plastic tips 20µl and 250µl, a horizontal rotator, bench vortex, distilled water, luminometer with two injectors (Auto Clinilumat, Berthold UK Ltd. LB952T/16). Spare uncoated test tubes to prime luminometer injection tubes (wash or flush with BR1 and BR2).

3.4.7.2 **Preparations** for the PCT assay.

All the reagents and patient samples were brought to room temperature (18°C-25°C).

The luminometer (Berthold UK Ltd., Auto Clinilumat) was prepared by flushing the injections system with 4.5ml of the Basiskit reagents BR1 and BR2. Following the manufactures instructions a protocol was programmed into the machine that included the number of controls used (2), the number of standards (6) with their known concentrations in ng/ml and the number of samples to be tested. The amount of luminescence reagents (BR1 and BR2) automatically injected into each tube was set at 300µl, with the recommended measuring time of 1 second per tube.

Each PCT standard was reconstituted with 0.25ml zero serum (G) which gave the following concentrations;

Standard	S1	S2/K1	S3	S4/K2	S5	S6
PCT ng/ml	0.08	0.45	1.7	18	185	474

The controls (Ko1 and Ko2) were reconstituted with 0·25 ml of zero serum (G), to give a concentration of 1.61 ± 0.40 ng/ml PCT in Ko1 and 44.5 ± 9.0 ng/ml in Ko2.

The standards labelled S2/K1 and S4/K4 represent the two standards to be used if the samples are run against a PCT master curve supplied by the manufactures. This method is recommended for the daily follow-up determination of serum PCT concentrations and was not used in this study, as the manufactures report that the results can deviate by 20±%.

The Tracer (B) was reconstituted with 29ml of buffer (C). 40ml wash buffer concentrate was diluted with 1960ml of distilled water. Tubes for patient samples were labelled a, b etcetera and put into test tube racks. A data collection sheet was used to record patients name and code letter.

3.4.7.3 **Method** PCT assay.

20µl of each reconstituted standard was added to coated tubes S1 to S6 using a new pipette for each transfer. 20µl of reconstituted control was added to the tubes labelled Ko1 and Ko2 and 20µl of each patient's serum was added to their pre-labelled coated tube. 250µl of reconstituted tracer was then added to all the tubes and mixed for a few seconds using a bench vortex. The tubes were protected from the light with aluminium foil and incubated at room temperature for 2 hours on a horizontal rotator. Before decanting the contents 1 ml of diluted wash buffer was added to all the tubes. The liquid was decanted into a plastic disposable container for autoclaving prior to disposal. 1ml of wash buffer was added and swirled around the length of the tubes to remove any residual tracer. This washing process was repeated two times. The tubes were inverted on disposable paper for 5 minutes and the tops blotted dry. All the tubes were loaded into the luminometer in the order defined by the protocol and data collection sheet. The luminometer automatically injected 300µl of reagents BR1 and BR2 into each tube and recorded the relative light units (RLU) emitted. The machine prints out a standard curve and the results in RLU with the corresponding PCT levels in ng/ml. The results from this assay are given in Chapter 4.

3.4.8 LIPOPOLYSACCHARIDE BINDING PROTEIN ASSAY (minimum detection 0.4ng/ml)

A solid phase enzyme linked immunosorbent assay (ELISA) kit for the quantitative measurement of human lipopolysaccharide binding protein (LBP) in human plasma or serum. Materials included in the HyCult biotechnology b.v. (Uden, Netherlands) assay kit.

3.4.8.1 **Materials** LBP

2 x 96 wells microplates (12 strips per plate each with 8 wells) coated with anti-human LBP antibody.

2 x 20 ml of concentrated (20x) wash/dilution buffer (A) containing protein stabilized phosphate buffered saline and Tween 20 the preservative is 2-chloracetamide.

(Tween 20 is polyoxyethylene sorbitan monolaurate)

20ml of concentrated (40x) wash/dilution buffer (B) containing magnesium chloride (MgCl) with a preservative.

Concentrated human LBP Standard (2 vials labelled 3A/B).

Tracer (2 vials 4A/B) a biotinylated antibody to human LBP.

Conjugate (vial 5) concentrated streptavidin peroxidase.

6ml Substrate (6) tetramethybenzidine-(TMB).

6ml Substrate buffer (7).

12ml Substrate dilution buffer (8).

22ml Stopping (blocking) solution containing 2M citric acid.

4 Adhesive covers for microplates and a batch control with the exact concentration of the LBP Standard.

Additional materials, not included in the kit.

Micropipettes and disposable tips; a multi-channel pipette; distilled water; measuring cylinders; polypropylene tubes and a spectrophotometer (450nm setting) for reading the absorbance from the microplate.

3.4.8.2 **Preparations** LBP.

The reagents, microplate and patient samples were brought to room temperature. Freeze dried reagents (the standard 3A; tracer 4A and the peroxidase conjugate 5) were reconstituted with 1 ml of distilled water injected into each rubber topped vial and mixed. The standard concentration is given on the batch-control sheet and was 370ng/ml. 20 ml of the concentrated wash/dilution buffer (A) was diluted with 180ml of distilled water and 10ml of concentrated wash/dilution buffer (B) was added to 190ml of distilled water, both solutions A and B were mixed together 1:1, this was sufficient for a 96 well plate. 11ml of this diluted wash/buffer was added to the reconstituted tracer (4A). Eight test tubes were labelled 1 to 8 for the standard dilutions. Tube 8 was the zero LBP ng/ml containing 500µl of prepared wash/dilution buffer. The amount of wash/dilution buffer added to tube 1 differs with each assay kit and is specified in the batch-control. Specifically, 405µl of wash/dilution buffer was added to tube 1. 225µl of wash/dilution buffer was added to tubes 2 to 7. 150µl of the reconstituted standard was transferred to tube 1 and mixed thoroughly by aspiration and ejection. Using a clean pipette tip 150µl from tube one was transferred to tube 2. This process was repeated until tube 7. After mixing tube 7, 150µl of the solution was discarded. The concentration of LBP in each tube was;

Tubes 1	2	3	4	5	6	7	8
LBP-100	40	16	6.4	2.5	1.02	0.4	0.00 ng/ml

Patient samples were diluted 2000 times. 2.5µl of patient's serum was added to 5ml of wash/dilution buffer in prepared plastic screw top jars, labelled with the patient's name and mixed by gently inverting.

3.4.8.3 **Method** LBP assay.

The microplate was washed (as previously described) 3 times prior to inoculation with standards and patient samples, a process that was unique to this assay. 100µl of standard and patients serum was transferred to their assigned wells (following the scheme on the prepared data sheet). An adhesive cover was applied over the wells and the plate was incubated for 60 minutes at room temperature. The plate was inverted and emptied over the sink with running water and washed three times and tapped dry on disposable paper. 100µl of diluted tracer was added to each well, the plate was covered and incubated for 60 minutes at room temperature. The plate was emptied and washed. 100µl of diluted streptavidin-peroxidase conjugate was added to each well, the plate was covered and incubated again for one hour at room temperature. During this incubation period the substrate was prepared. In a clean plastic container in a ratio of 1:1:2 3ml of TMB (6) was added to 3ml of substrate buffer (7) and 6ml of substrate dilution buffer (8). The solution was protected from the light with aluminium foil. The plate was emptied and washed as previously described then100µl of the TMB solution was added to the wells. The plate was covered with aluminium foil and incubated at room temp for 20 minutes. 100ml of stop solution (9) was added to the wells to stop the reaction. The plate was read at 450nm on the spectrophotometer. The data was entered into Prism computer package for data analysis and the results are given in Chapter 4.

3.4.9. ANTI-LIPID S ANTIBODY ASSAY (minimum detection > 0.00 EIU/L)

3.4.9.1 **MATERIALS** (anti-Lipid S IgG assay)

A 96 well microplate (Immulon 2HB, Dynex Technologies, Chantilly, USA).

Antigen dilution buffer; sodium carbonate/bicarbonate (0.05 M. pH 9.6).

Microplate blocking solution and wash/buffer; TBS-tween (containing 0.01 M Tris-HCL. pH 7.4, NaCl 0.9% w/v, Tween-20 0.3%v/v).

Antihuman IgG conjugate (Sigma, Poole Dorset, UK) stored at -20°C.

Substrate; 3,3',5,5'-tetramethybenzidine (TMB-Sigma).

Substrate solvent; Dimethyl sulfoxide (DMSO- C_2H_6SO).

Substrate diluent/buffer; containing sodium acetate/citrate buffer (0.1 M, pH 6.0)

Hydrogen peroxide (H_2O_2 -6% v/v).

BHI7 antigen was supplied by Dr. Sue Lang (Aston Uni. Birmingham, UK).

Anti-Lipid S antibody controls; There were four controls, high, medium, low and negative. The high positive control serum came from a patient with catheter related sepsis and the medium and low controls from other patients with medium and low levels of anti-lipid S IgG serum antibodies. The negative control was normal human serum (Bradsure Biologicals, Loughborough, UK).

HCL acid 1M

Sulphuric acid 1 N (Stock 98%: N= 35.9 and 1N is equivalent to 18M. 1ml of H_2SO_4 98% to 35ml distilled water is equivalent to 1M)

80 test tubes; for serum dilutions.

ELP-40 plate washer.

3.4.9.2 **Preparations** (anti-Lipid S)

All the reagents were brought to room temperature.

3.4.9.2.1 **Preparation of wash/dilution-buffer,** concentrated stock solution; TBS-Tween 0.1M (10x) dilution buffer pH 7.4

12.1g of Tris-HCl (Sigma) and 90g of sodium chloride (Fisher chemicals) with 30mls of Tween 20 was added to 970ml of distilled water in a flask. This was mixed using a magnetic stirrer and the pH was adjusted to pH 7.4 using a pH meter and several ml of hydrochloric acid (HCL 1M). 100ml of this solution was added to 900ml of distilled water to make 1L of 0.01M TBS-Tween 20 wash/buffer.

3.4.9.2.2 **Preparation of stock carbonate buffer 0.5M** (x10) antigen dilution buffer pH 9.6.

A quantity of stock sodium carbonate/bicarbonate buffer was made up by mixing sodium carbonate (0.5M) pH 13.45 with sodium bicarbonate (0.5M) pH 8.00 until the solution had a pH of 9.6. This was achieved using a magnetic stirrer and a calibrated pH meter.

This solution was diluted 1:10 with distilled water to produce sodium carbonate/bicarbonate buffer (0.05M pH 9.6).

3.4.9.2.3 **Preparation of substrate dilution buffer**; concentrated stock solution of sodium acetate/citrate buffer (1M pH 6.0).

Sodium acetate (1M) pH 8.3 was added to a quantity of citric acid (1M) pH 2.29 until the pH of the solution was pH 6. A magnetic stirrer and a pH meter were used to establish the pH of the solution. 10ml of the concentrated sodium acetate/citrate buffer was diluted with 90 ml of distilled water to provide 100ml of 0.1M sodium acetate/citrate buffer.

3.4.9.2.4 **Preparation of the chromogenic peroxidase substrate solution**.

(This solution was prepared immediately before use). 0.01g of tetramethybenzidine (TMB) was dissolved in 1ml of dimethyl sulphoxide (DMSO). 50µl of hydrogen peroxide (H_2O_2) was added and mixed. 100ml of sodium acetate/citrate buffer (0.1 M) was added to the solution and protected from the light with aluminium foil until used.

3.4.9.2.5 **Microplate preparation; method.**

500µl of thawed antigen BHI7 (see 3.2.2) was diluted with 200ml of sodium carbonate/bicarbonate buffer (0.05M, pH 9.6), 100µl of this solution, containing 0.125µg/ml of antigen, was applied to each well. The plates were incubated overnight at 4°C. The antigen solution was discarded and the plate was washed in TBS/Tween buffer. The wells were filled with TBS/Tween to block unbound sites and incubated for 1 hour at 4°C. The plate was washed as described previously and tapped dry. The plate was stored at -20°C before use and then brought to room temperature prior to performing the assay.

3.4.9.2.6 **Dilution of patient samples and controls.**

All the serum and reagents were brought to room temperature before proceeding.

Test tubes were labelled for the controls and patients. The controls were **H**- for high anti-lipid S IgG; **M**-medium; **L**-low and **N** –negative for anti-lipid S IgG antibodies. Control and patient serum was diluted 1:6400 in TSB-tween buffer. 5µl of control or patient serum was added to 2mls of buffer in a labelled test tube and mixed by withdrawing and expelling the solution six times using a Gilson pipette. 100µl of this diluent was added to 1.5ml of TBS-tween buffer in a separate test tube and mixed thoroughly.

3.4.9.3 Method (anti-Lipid S IgG assay).

A paper template of the microplate was labelled. Column 1 (rows A-H) blank, column 2 and 12 (A to H) with the high, medium, low and negative controls in duplicate. Columns 3 to 11 (A to H) were labelled with the patients names in duplicate.

100µl of TBS-tween was added to each well in column 1. 100µl of each control was added in duplicate to their designated wells in column 2, wells A to H and repeated in column 12. Finally 100µl of patients diluted serum was added to their designated wells in duplicate. The plate was covered with an adhesive plate cover and incubated at 37°C for 2 hours. An automatic plate washer was used in this assay. Prior to use the plate-washer was flushed with a rinse solution and then primed with the TBS-tween wash/buffer and programmed to automatically decant and wash the plate three times. The plate was removed from the plate washer and tapped dry as described previously. 50µl of anti-IgG conjugate (stored in aliquots of 50µl) was dissolved in 50ml of TBS-tween and mixed. 100µl of this diluted antihuman IgG (Sigma, Poole Dorset, UK) conjugate was added to all the wells; the plate was covered with an adhesive strip and incubated at 37°C for 1hour. The plate was automatically emptied and washed 3 times by the plate washer. The substrate was prepared. 0.01g of TMB was dissolved in 1ml of dimethyl sulfoxide (DMSO) and 50µl of hydrogen peroxide (6% v/v) was added. Finally 100ml of citrate acetate buffer 0.1M was added and mixed. 100µl of this solution was added to all the wells and the plate was incubated at 37°C for 25 minutes. The reaction was stopped by addition of 100µl of sulphuric acid (1M). The optical density was read at 450nm. The optical density of each row was given after the machine automatically subtracted the optical density from the first well in each row from all the other wells in that same row.

The anti-lipid S titres were calculated from the mean absorbance for each control and patient sample using the following formula.

Calculating the IgG titres:

$$\frac{Ab_{450}\text{ test sample} - Ab_{450}\text{ negative control}}{Ab_{450}\text{ positive control} - Ab_{450}\text{ negative control}} \times 100{,}000$$

Interpretation	**Titre**
Borderline positive	up to 1,000 (repeat suggested)
Low positive	1,000 – 5,000
Moderate positive	5,000 – 20,000
High positive	20,000 +

3.5 STATISTICAL ANALYSIS

The statistics used in this study are largely directed towards testing the discrimination of the test marker or markers for a diagnosis of sepsis, therefore the sensitivity and specificity values for these test markers are ascertained. High sensitivity and specificity values are a pre-requisite for any marker to become valuable in the routine clinical setting.

The median values are useful for comparing differences (if any) between Gram-positive and Gram-negative cases, and between the test groups and controls; including inter-assay and operator consistency.

3.5.1 Median and significance values

Comparative continuous descriptive statistics in a computer software programme called Analyse-it for Microsoft Excel (Leeds, UK), was used on the results. This programme determines visually as well as statistically whether the data to be analysed should be treated as parametric (normally distributed) or non-parametric, by constructing a frequency histogram of the dataset from each patient group overlaid with a normal distribution curve.

Apart from LBP, all the test markers exhibited distribution free characteristics, therefore the following non-parametric statistics were applied to the results; the median, range, confidence interval (CI) and the confidence level (CL) as a percent. A significant difference (if any) of the control median in relation to the test groups (Gram-positive and Gram-negative sepsis) was tested using the Analyse-it for Microsoft Excel Mann-Whitney test. The smaller the resulting p-value (set at a significance level of 0.05) the more untenable the null-hypothesis (that there is no significant difference between the serum levels of the test markers in the septic group as opposed to the controls).

The CIs are computed around the median indicating the range of values likely to contain the true population or median, this statistic provides more information than the p-value in a non-parametric test. The confidence level is given as a percentage, usually 95%. This statistic indicates how certain we want to be that the interval includes the population statistic (Analyse-it for Microsoft Excel Leeds, UK).

The 'Range' is the difference between the largest and smallest serum level in a dataset, this statistic is sensitive to outliners (Everitt, 1995). This statistic is not the same as the 'range of values' given for the confidence interval.

3.5.2 Box and Whisker plots

This method displays the important characteristics of a set of results. The "box" shows the inter-quartile range of data, the "whiskers" indicate the range of observations and the line through the box indicates the median, the 50th percentile. The median is the value that divides the data into two parts of equal size, half above the median value and half below this value (Everitt, 1995).

3.5.3 Sensitivity and Specificity

The definition of sensitivity, specificity, a positive predictive value (PPV) and a negative predictive value (NPV) are given here with a method for translating these indices of test performance into percentages.

3.5.3.1 Sensitivity defined:

Sensitivity is an index of the performance of a diagnostic test, calculated as the performance of individuals *with a disease* who are correctly classified as having the disease. A test is sensitive to the disease if is positive for most individuals having a disease (Everitt, 1995).

3.5.3.2 Specificity defined:

Specificity is an index of the performance of a diagnostic test, calculated as the performance of individuals *without the disease* who are classified as not having the disease, that is the conditional probability of a negative test result given that the disease is absent. A test is specific if it is positive for a small percentage of those without the disease (Everitt, 1995).

It follows from these definitions that the positive predictive value (PPV) is a measure of the probability that a person (who has a positive result) has the disease. Conversely the negative predictive value (NPV) is the probability that a person with a negative result on the diagnostic test does not have the disease.

Sensitivity (%) True positive ÷ (True positives + False negatives) x 100

Specificity (%) True negative ÷ (True negatives + False positives) x 100

Positive predictive value (PPV) (%) TP÷ (TP + FP) x 100

Negative predictive value (NPV) (%) TN÷ (TN + FN) x 100

False positive fraction for **ROC** FP÷ (TN+FP) (1- specificity)

Where: - TP is True positive

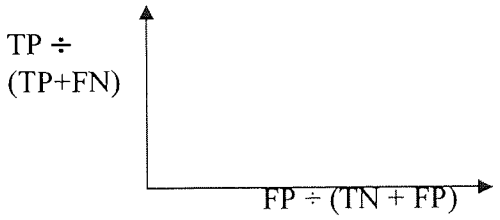
FP is False positive

TN is True negative

FN is False negative

3.5.4 Receiver –Operating Characteristic (ROC)

ROC curves plot the sensitivity or True positive fraction on the Y-axis and 1-specificity or the False positive fraction on the X-axis.



ROC plots give an index of test performance accuracy; qualitatively the nearer the plot is to the upper left hand corner (1.0 or 100% perfect sensitivity and 0 on the X-axis, giving perfect specificity) the higher the accuracy of the test (Zweig and Cambell, 1993).

3.5.5 Intra-assay precision (precision within an assay).

The quantitative values of duplicate samples were used to calculate the average, plus or minus (+/-) the difference between the results. Any difference is presented as a percentage of the average. A percentage difference less than or equal to 5% is preferred but less than 10% was considered acceptable. .

3.5.6 Inter-assay Precision (precision between assays).

Patient samples or standards with known levels of a test antigen from one assay were used in subsequent assays to test the reproducibility of results between assays. The same calculations were performed on these results as given for the intra-assay measures of reproducibility in 3.5.5.

The results from the nine assay types described in this chapter are given in Chapter 4, together with the results of data analysis. Chapter four includes a discussion on the results and an introduction to the next research phase of this project.

CHAPTER 4 - PHASE 1 RESULTS

This chapter contains the results from the assays described in chapter 3. Other trials are contained within one chapter (Phase 2, in chapter 5; Phase 3, in chapter 6; and finally Phase 4 in chapter 7)

Serum samples were obtained from patients with Gram-positive bacterial or Gram-negative bacterial associated sepsis and from a control group without infection. The blood samples were separated and serum obtained. The nine assays as described in Chapter 3 were carried out on these samples. The results and statistical analysis are presented in this chapter

4.1 CONTROL GROUP RESULTS

A blood sample from each patient was taken on admission for elective surgery for coronary artery by-pass grafts (CABG). Clinical details and any underlying diseases are given in table 4.1. None of the patients had any clinical evidence of infection or sepsis during the previous three months.

Patient codes were assigned for data/patient protection. AC1 represents; 'A' for patients in this preliminary trial (Phase 1) of test markers. 'C' represents a control patient followed by a number; N and P represent patients in the Gram-negative and Gram-positive groups respectively. All the control patients had body temperatures within the normal oral range which can vary throughout the day from 36.5°C to 37.5°C (West, 1990).

4.2 GRAM-NEGATIVE BACTERIAL SEPSIS GROUP

These patients were primarily selected for inclusion in this project based on their positive blood culture results. Test serum samples were collected within 2 days of blood culture being taken that subsequently grew a Gram-negative aerobic bacillus. The majority were same day samples. Table 4.2 lists the inclusion criteria; the isolate; the white cell count (WCC) and C-reactive protein (CRP).

All the patients in the sepsis groups had a raised or a sub-normal temperature. This was often indicated as a 'fever' or 'pyrexia' by the clinicians or nursing staff, as opposed to a quantitative temperature reading.

Patients with sepsis were drawn from medical and surgical wards; renal, cancer and intensive care units in the participating hospitals. There were thirty-six patients in

the Gram-negative sepsis group, twenty-one males with a mean age of 49.5 years and fifteen females with a mean age of 62.4 years.

Table 4.1 CONTROL PATIENT CHARACTERISTICS: Including underlying disease (if any); white cell count (WCC); blood pressure (BP) and pulse on the day the test sample was obtained.

PATIENT CODE	AGE YEARS	M/F	UNDERLYING DISEASE (IF ANY)	WCC $1X10^9/L$	BLOOD PRESSURE	PULSE RPM
AC1	55	F	Hypertension	4.9	120/80	78
AC2	29	M	ESRF-gout	no	available	notes
AC3	**75**	**F**	MI 2 yrs ago	8.0	110/70	70
AC4	59	M	HT and HC	13.8	155/85	62
AC5	62	F	P-X elasticum	13.2	176/103	105
AC6	69	M	Diabetic	4.9	140/70	78
AC7	80	M	HT and HC	5.4	160/80	70
AC8	64	F	Hypothyr. anaemia	5.8	152/79	110
AC9	70	M	none	11.1	140/90	66
AC10	77	M	Arthritis, HT/HC	8.7	145/76	54
AC11	70	M	IDDM and HC	4.86	155/95	62
AC12	60	M	HC, reflux-oes.	4.5	137/80	70
AC13	49	M	MI 1 year ago	5.8	124/74	84
AC14	79	M	None	6.3	100/50	64
AC15	53	M	HC /dysponea	6.0	143/87	62
AC16	66	F	HC and diabetic	7.3	130/80	52
AC17	53	M	IDDM and HC	11.1	165/75	66
AC18	43	M	Diabetic, nec-toe	no	available	notes
AC19	69	M	HC, IDDM	6.9	120/69	102
AC20	45	M	Recent MI -RA	6.2	122/62	74
AC21	71	M	HC and HT	5.9	160/90	94
AC22	60	F	Emergency admis.	6.4	110/65	60
AC23	59	M	Allergy to Cipro.	9.9	140/80	64

M/F; male /female. WCC; white cell count (normal range is 4.0-11 x 10^9/L). BP; blood pressure. RPM; rate per minute. ESRF; end stage renal failure. HT; hypertension; HC; hypercholesterolaemia. MI; myocardial infarction. P-X elasticum (rare); Peudo-xanthoma elasticum. Hypothyr; Hypothyroidism and chronic anaemia. Reflux-oes; reflux-oesophogitis. Nec-toe; necrotic toes. RA; rheumatoid arthritis. Cipro; Ciprofloxacin. Patient AC3 died post-operatively (6 weeks).

Table 4.1 shows that ten of the control patients had an underlying disease apart from their coronary artery disease; six patients had diabetes; another patient had end stage

renal failure; one suffered from ulcerative colitis and another patient was being treated for hypothyroidism.

Table 4.2 GRAM-NEGATIVE AEROBIC BACTERIAL SEPSIS GROUP:

Clinical data

WCC normal range 4-11 $x10^9$/L C- reactive protein (CRP) normal range < 10mg/L

PATIENT	WCC	CRP	ISOLATE	UNDERLYING DISEASE
AN1	7	37	*Citrobacter freundi*	Diabetic ESRF
AN2	**13**	**nd**	*Citrobacter freundi*	Bowel cancer (post resect)
AN3	nk	nk	*Proteus mirabilis*	nk
AN4	**24**	**237**	*Enterobacter cloacae*	Chronic renal failure
AN5	**135**	**15**	*Citrobacter freundi*	CML-AML, NIDDM
AN6	5.6	154	Coliform	Peritonitis -CAPD
AN7	**6.5**	**112**	*Pseudomonas aeruginosa*	Post-op CABG
AN8	nk	nk	*Klebsiella chinos*	Over-dose, liver damage.
AN9	nk	nk	*P. aeruginosa*	Renal failure
AN10	nk	nk	*Klebsiella pnuemonia*	nk
AN11	**33.6**	**nd**	*Enterobacter cloacae*	Pancreatic cancer, diabetic
AN12	21	nd	*Acinetorbacter* spp. x 2	Renal carcinoma
AN13	nk	nk	*Xanth. maltophilia*	Cancer
AN14	**9.4**	**nd**	*Enterobacter gergoriae*	Cirrhosis/oeso.varices
AN15	**17**	**nd**	*Enterobacter cloacae*	Alcoholic liver disease
AN16	nk	nk	Klebsiella pneumonia	Cancer
AN17	nk	nk	*Proteus mirabilis*	Bone marrow transplant
AN18	nk	nk	Coliform	nk
AN19	nk	nk	Coliform	nk
AN20	nk	nk	*Campylobacter* spp.**	ITU
AN21	nk	nk	*Acinetobacter spp.*	MOF
AN22	**18**	**287**	*Escherichia coli*	Pylonephritis
AN23	23	nd	*Escherichia coli*	Haematemesis
AN24	**11**	**11**	*Serratia* spp.	Myasthenia gravis
AN25	17	140	*P. aeruginosa*	Diabetic
AN26	4.9	126	*K. oxy* & *Pseu.* spp.	Surgical-(short bowel syn).
AN27	nk	nk	*Escherichia coli*	nk
AN28	nk	nk	*Enterob. aerogenes**	nk
AN29	nk	nk	*Enterobacter cloacae*	Diabetic
AN30	16.7	nd	*Serratia* spp.	Perforated bowel
AN31	nk	nk	Coliform	nk
AN32	nk	nk	Coliform	nk
AN33	nk	nk	Coliform	nk
AN34	nk	nk	Coliform	nk
AN35	7.3	nd	*Acinetobacter spp.*	Subdural bleed/liver disease
AN36	nk	nk	*E.coli*	nk

WCC; White cell count. nd; not done. nk; not known. ESRF; end stage renal failure. Bowel resect; bowel resection. CAPD; Continuous ambulatory-peritoneal dialysis. CML-

AML; Chronic myeloid leukaemia, acute myeloid leukaemia. NIDDM; non-insulin dependant diabetes mellitus. syn; syndrome. inf; infection. ITU; intensive care unit. The blood culture from patient AN20 was shown to have an additional Gram-negative isolate *Ent.cloacae* (post inclusion into this study) and patient AN28 was polymicrobial with a Gram –positive *Enterococcus* spp and the Gram-negative isolate (*Enterobacter aerogenes*). Patients highlighted in bold in table 4.2 did not survive their septic episode or underlying disease.

4.3 GRAM-POSITIVE BACTERIAL SEPSIS GROUP

The original research aim of this project was to find a serum marker or combination of markers for patients with Gram-negative sepsis. The patients with Gram-positive sepsis were collected as a secondary control. All these patients had central venous line (CVL) related sepsis caused by coagulase negative staphylococci (CNS). There were thirty-three patients in this group twenty-one males and twelve females with an age range of eighteen to eighty-two years.

4.4. SCATTER GRAPHS; nine serum markers

The quantitative values for each patient's serum markers are given as scatter graphs in the following figures. The reference ranges for the serum markers are taken from the manufacturers assay kits, except TNF-α, this reference range is from Nomura *et al* (1999). The reference range relates to those values found in a finite sample of healthy adults therefore the term 'normal' range is avoided.

The reproducibility of these results was calculated from paired serum samples within an assay (intra-assay precision) and between assays (inter-assay precision). The results are tabulated in section 4.8 showing the sample average, plus or minus the sample difference from the average. This value is recorded as a percent.

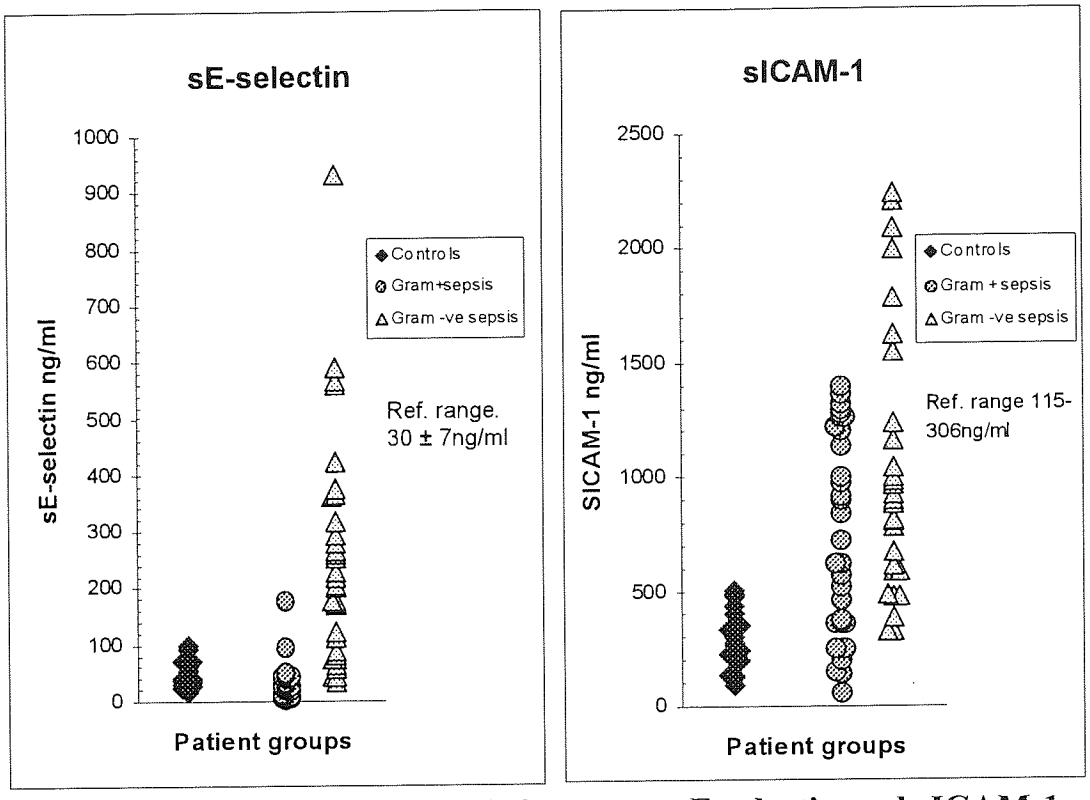


Figure 4.1 SCATTER GRAPHS; for serum sE-selectin and sICAM-1

The graphs show the concentration (ng/ml) assayed from each patient's serum for the adhesion molecules, sE-selectin and sICAM-1. The normal reference range for each marker is given in the graphs.

Table 4.3.1 Median and confidence interval (CI) for sE-selectin ng/ml; all patient groups

sE-selectin	Controls	Gram-positive sepsis	Gram-negative sepsis
Median	42	26	201
CL% (CI)	96.5 (36 to 70)	96.5 (12 to 33)	95.9 (111 to 267)
Range	96	173	897

Table 4.3.1 The CI is a range of values and the confidence level (CL) states the percentage likelihood that the CI contains the population statistic for sE-selectin (ng/ml).

Table 4.3.2 Median and CI for sICAM-1 ng/ml; all patient groups

sICAM-1	Controls	Gram-positive sepsis	Gram-negative sepsis
Median	270	620	891
CL% (CI)	96.5 (210 to350)	96.5 (370 to1000)	97.1 (675 to1040)
Range	410	1340	1913

Table 4.3.2 gives the median, CL/CI values. The 'Range' is the difference between the largest and smallest observations for sICAM-1 (ng/ml).

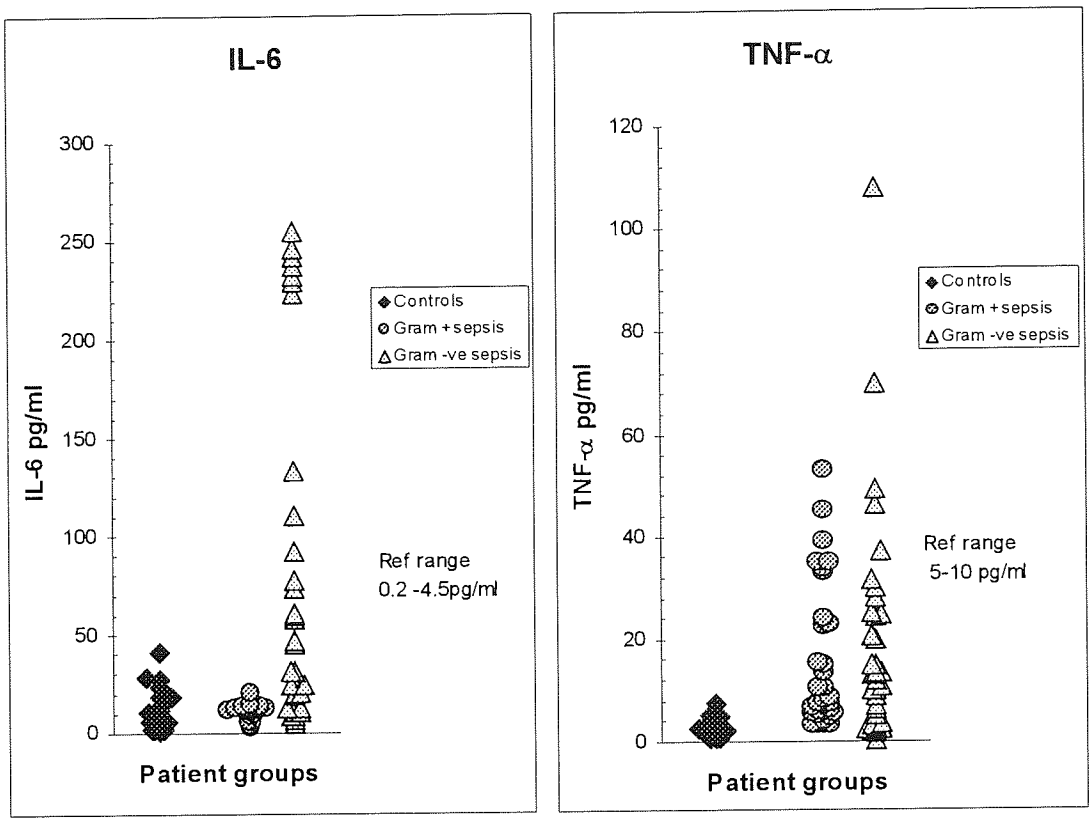


Figure 4.2 SCATTER GRAPHS; for serum IL-6 and TNF-a

The graphs in figure 4.2 show the concentration (pg/ml) assayed from each patients serum for IL-6 and TNF-α. The reference range given for each marker equates to the range expected in healthy adults

Table 4.3.3 Median and CI for IL-6 pg/ml; all patient groups

IL-6	Controls	Gram-positive sepsis	Gram-negative sepsis
Median	6.8	12.6	28.2
CL% (CI)	96.5 (4.6 to 14)	96.5 (12.1 to 13)	95.6 (20.5 to 57.9)
Range	40.72	18.5	250

Table 4.3.3 Indicates that the median, CL % and CI serum levels of IL-6 are greater in patients with a sepsis caused by a Gram-negative bacillus.

Table 4.3.4 Median and CI for TNF-α pg/ml; all patient groups

TNF-α	Controls	Gram-positive sepsis	Gram-negative sepsis
Median	2.1	8.3	13.19
CL% (CI)	96.5 (1.28 to 3.1)	96.5 (6.3 to 15.5)	95.9 (9.01 to 20.55)
Range	6.92	49.8	107

Table 4.3.4 gives the median, confidence level and CI for TNF-α (pg/ml). TNF-α is clearly elevated in patients with sepsis as opposed to the controls. The confidence level (CL) states the percentage likelihood that the CI contains the population statistic.

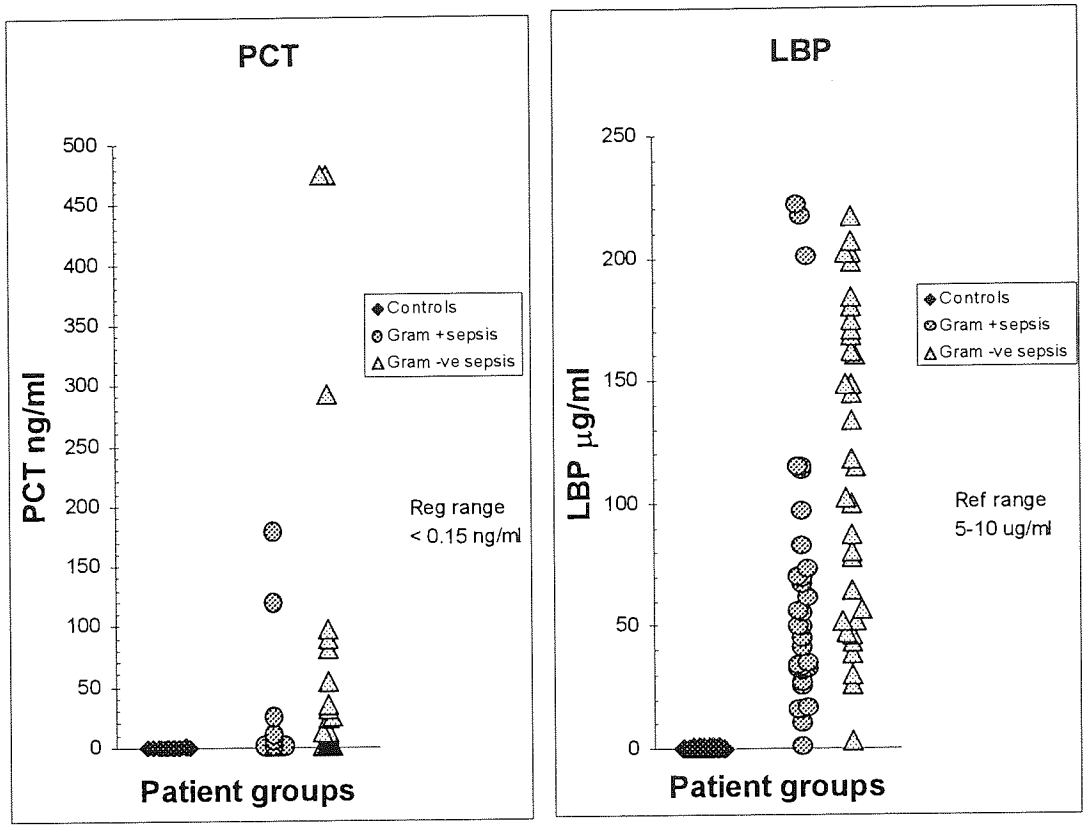


Figure 4.3 SCATTER GRAPHS; for serum Procalcitonin and LBP

The graphs show the concentration (ng/ml and µg/ml) assayed from each patient's serum for the proteins procalcitonin (PCT) and lipopolysaccharide binding protein (LBP). The normal reference range for each marker is given in the graphs

Table 4.3.5 Median and CI for PCT ng/ml; all patient groups

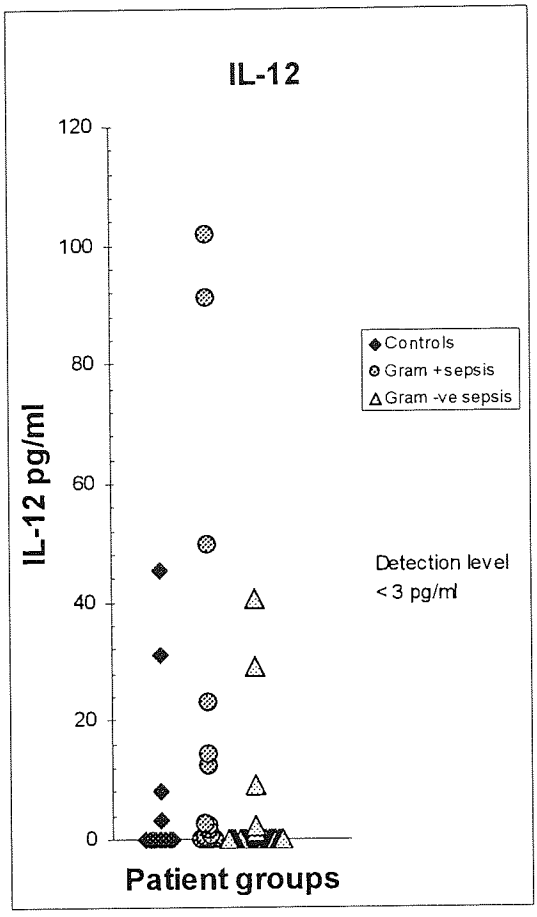
PCT	Controls	Gram-positive sepsis	Gram-negative sepsis
Median	0.21	0.92	8.86
CL% (CI)	98.3 (0.09 to 0.4)	97.1 (0.54 to 1.20)	95.7 (3.89 to 24.96)
Range	0.83	178.9	473

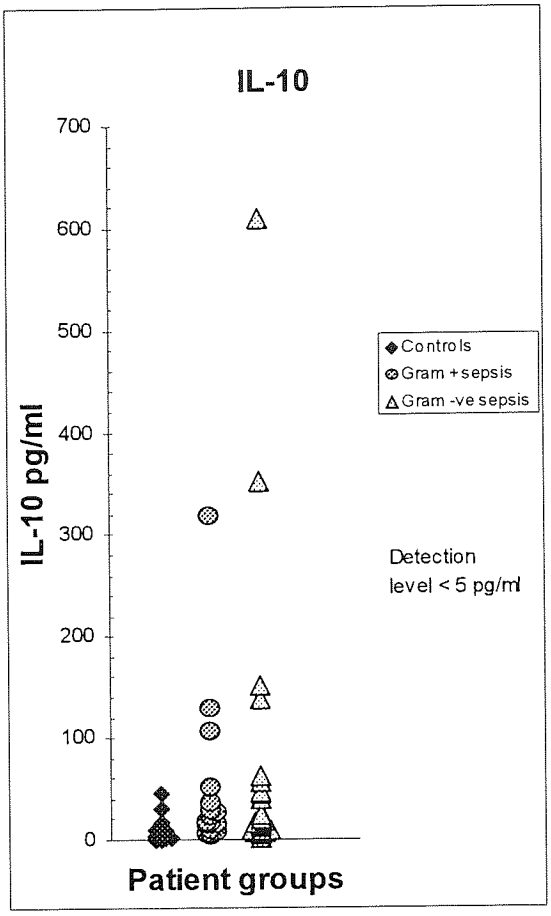
Table 4.3.5 the range values are much higher in the patients with sepsis as opposed to the controls. Less than 0.09 for PCT indicates that the serum level (if any) was below the detection limit for the assay.

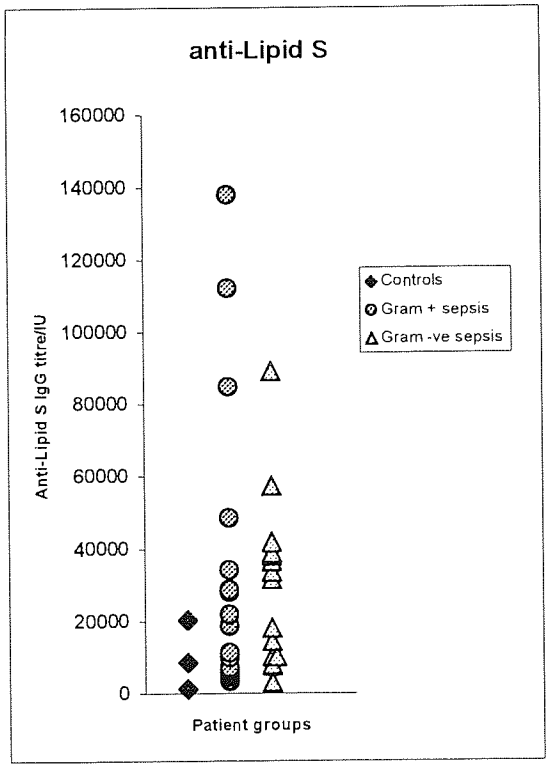
Table 4.3.6 Median and CI for LBP µg/ml; all patient groups

LBP	Controls	Gram-positive sepsis	Gram-negative sepsis
Median	16.53	49.74	125.97
CL% (CI)	96.5 (12.7 to 24.7)	96.5 (33.06 to 69.5)	97.1 (77.88 to 168.2)
Range	47.6	220	213

Table 4.3.6 LBP (µg/ml) CI for the septic groups are clearly elevated as opposed to the controls.







These graphs in figure 4.4 show the serum concentration (pg/ml) obtained from each patient for IL-12 and IL-10. The anti-lipid S IgG assay was the only one that measured patients' antibodies. 18 of the 21 control patients were negative for lipid S antibodies and are not shown on the graph.

Figure 4.4 SCATTER GRAPHS; for serum IL-10 and IL-12 and anti -Lipid S IgG

Table 4.3.7 Median and CI values for IL-10 pg/ml; all patient groups

IL-10	Controls (number 17)	Gram-positive sepsis (17)	**Gram-negative sepsis** (30)
Median	5.20	17.35	14.11
CL% (CI)	95.1 (1.14 to 11.68)	98.1 (6.82 to 35.58)	95.7 (8.84 to 24.64)
Range	46	315	606

Table 4.3.7 lists the median, CL%, CI and the range of values for IL-10 (pg/ml) assayed from the sera of patients in all groups; the number of patients tested is in brackets.

According to the Hessle hypothesis (Hessle, 1999) **Gram-negative bacteria** induce the expression of **IL-10** more than they induce the expression of IL-12. Patient's sera was limited, therefore not all the patients could be tested for IL-10 and IL-12.

Table 4.3.8 Median and CI values for IL-12 pg/ml; all patient groups

IL-12	Controls (number 16)	**Gram-positive sepsis** (17)	Gram-negative sepsis (30)
Median	0.00	0.72	0.00
CL% (CI)	97.9 (0.00 to 3.20)	98.1 (0.00-14.18)	95.7 (0.00 to 0)
Range	45	101	40.4

Table 4.3.8. **Gram-positive bacteria** are thought to preferentially induce the expression of **IL-12**, a theory that is supported from these results. The 'Range' shows the outlines.

Table 4.3.9 Median and CI for anti-lipid S IgG EIU/L; all patient groups

Anti-lipid S anti-bodies	Controls (21)	Gram-positive sepsis (21)	Gram-negative sepsis (34)
Median	0.00	11313.0	0.00
CL% (CI)	97.3 (0.00 to 0)	97.3 (4664 to 28740)	97.6 (0.00 to 10173)
Range	20287	138000.0	89118.0

Table 4.3.9 anti-lipid S was included as a potential differential marker for Gram-positive sepsis and the medians show that this marker is a strong candidate. However the range shows that there are positive outlines for both the controls and Gram-negative sepsis.

The significance of the difference (if any) between the median values for each marker in the septic patients and the control median value was evaluated using a computer generated Mann-Whitney test (Analysis-it, Software Ltd. Leeds, U.K). The results are given in table 4.4

Table 4.4 Mann-Whitney test; *p*-values (normal approximation, with correction for ties).

2-tailed *p*-value	(normal approximation,	correction for ties).
Test markers	Controls/Gram +ve sepsis	Controls/Gram-ve sepsis
sE-selectin	<0.0002	<0.0001
sICAM-1	<0.0001	<0. 0001
IL-6	0.08	<0. 0001
TNF-α	<0.001	<0. 0001
PCT	<0. 0001	<0. 0001
LBP	<0.0001	<0. 0001
IL-10	0.005	0.004
IL-12	0.06	0.73
Anti-lipid S IgG	<0. 0001	0.0134

Table 4.4 Lists the significance of difference between the median control values for a marker with respect to the median values for the sepsis patients.

A 2-tailed *p*-value <0.05 was considered to be significant. IL-6 serum levels in patients with a Gram-positive sepsis was not significant and IL-12 in both groups was also not significant.

4.5 BOX and WHISKER GRAPHS SHOW QUARTILES

Displaying the results in box and whisker form, illustrates the data more fully when there is a large data base. The box extends from the 25th percentile to the 75th percentile. The line in the box is the median, the 50th percentile. One quarter of the values lie between the top whisker and the top of the box, and one quarter of the values lie between the bottom of the box and the bottom whisker. These graphs were generated using GraphPad Prism ® 3.0.

Six box and whisker graphs are shown in figure 4.5. IL-12, IL-10 and Lipid S assays contained fewer patients from each group as test serum was limited and many negative (not detected) results which cannot be displayed in this form of graph.

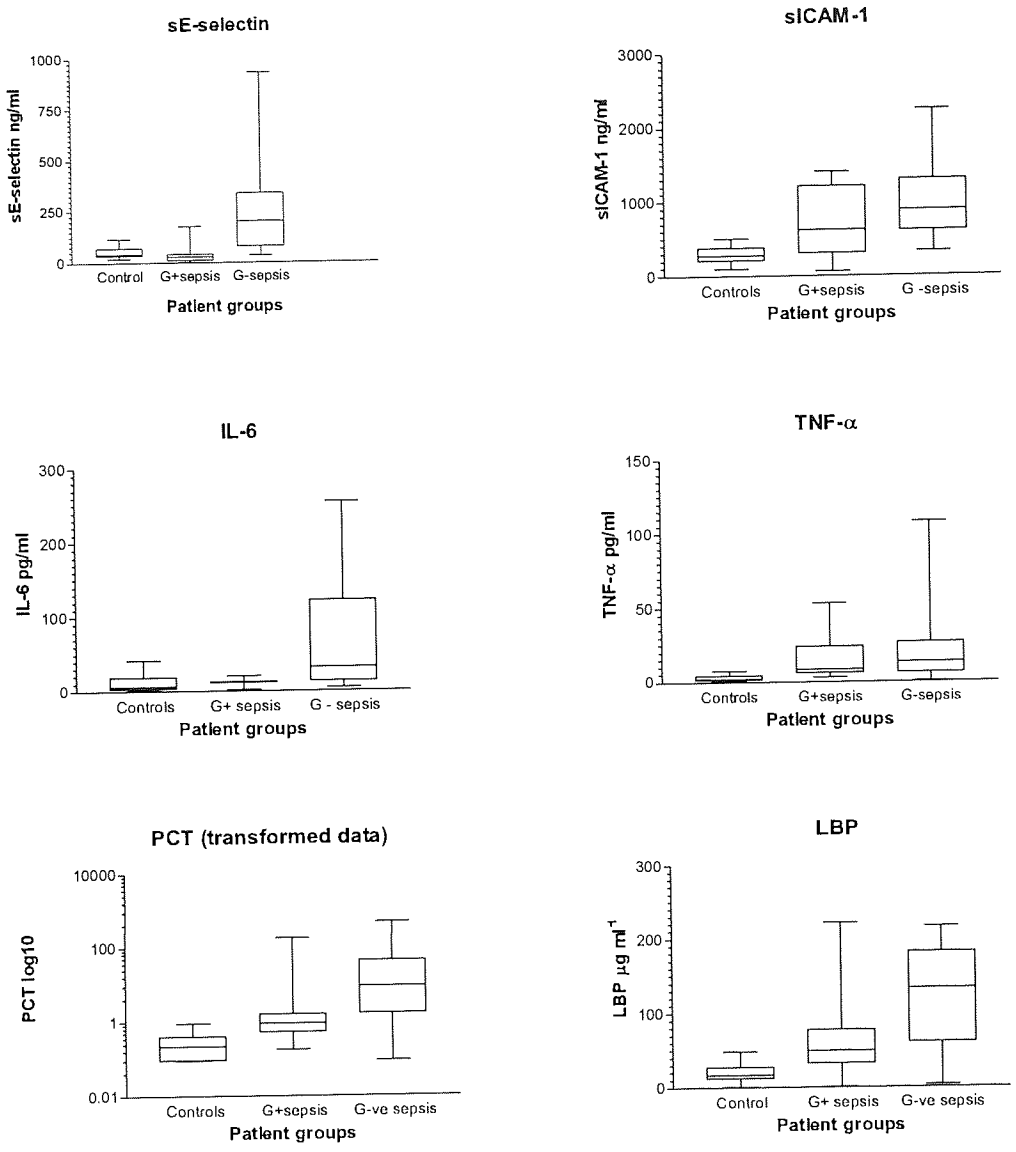


Figure 4.5 BOX and WHISKERS: sE-selectin; sICAM-1; IL-6; TNF-α; PCT and LBP

The line through the box represents the median (the value that divides the data equally in size, see section 3.5.2). The serum values for the test markers in figure 4.5 were higher in the Gram-negative group than the Gram-positive group, a finding that was consistent throughout this project.

4.6 RECEIVER-OPERATOR CHARACTERISTIC (ROC)

The value of an individual marker for discriminating patients with sepsis was calculated using a receiver-operator characteristic (ROC) curve analysis. The sensitivity and specificity for a given marker was established at various arbitory cut-off levels and plotted, using the formulae given in chapter three, sections 3.5.3.2 and 3.5.4.

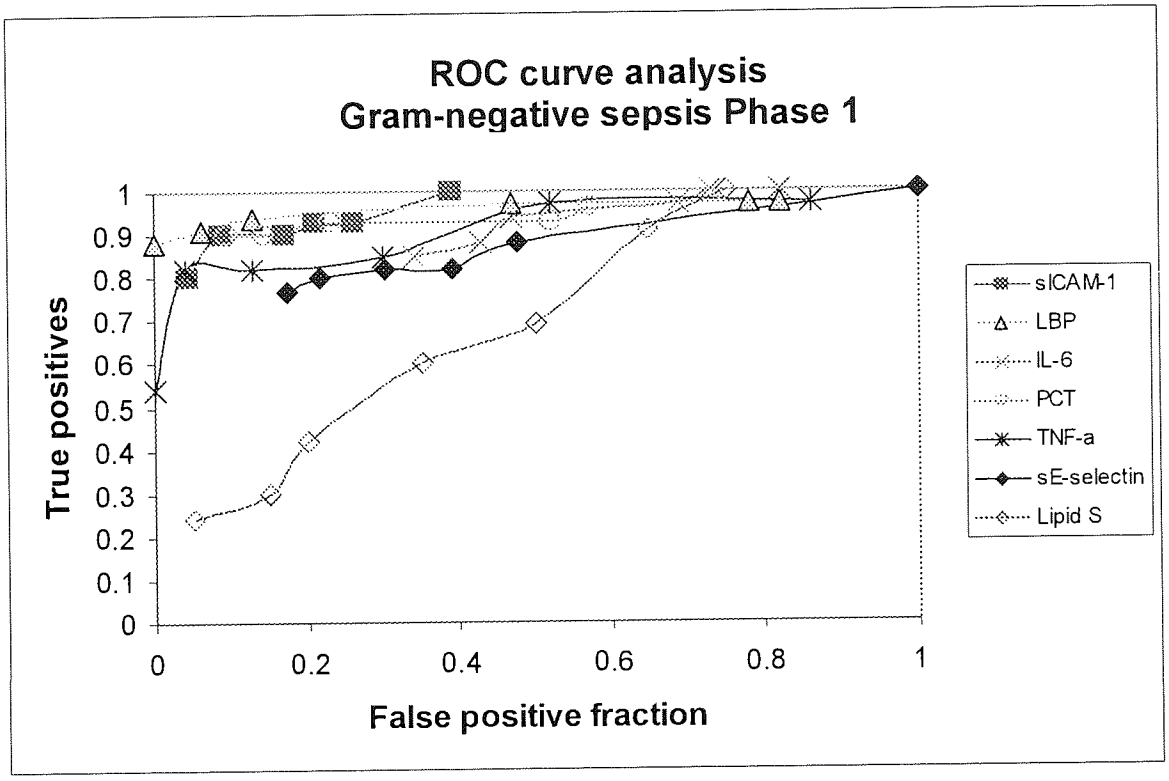


Figure 4.6.1 ROC curves for Gram-negative sepsis. The points on a curve are arbitrary cut –off values used to calculate the false positive and true positive fractions.

A diagnostic marker for sepsis must have a sensitivity and specificity as close to 100% as possible for it to have a realistic practical value, therefore only those markers that have a value of 90% and above are given in table 4.5.1.

Table 4.5.1 SENSITIVITY AND SPECIFICITY: Gram-negative sepsis

Best Markers	Cut-off values	Sensitivity (%)	Specificity (%)
LBP	40 µg/ml	91	93
sICAM-1	450 ng/ml	90	91
PCT	0.5 ng/ml	90	90

Table 4.5.1 lists the cut-off levels (ng/ml and µg/ml) for each test marker to achieve the given sensitivities and specificities.

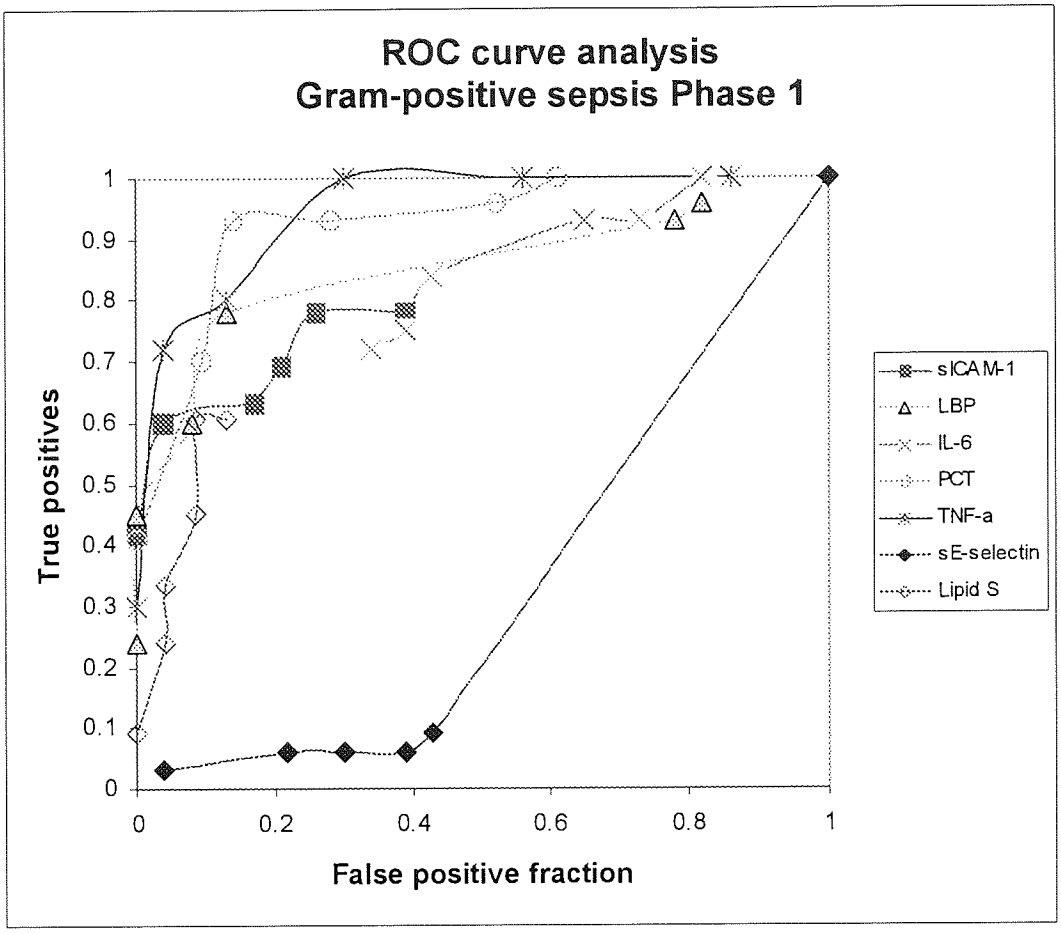


Figure 4.6.2 ROC curves for Gram-positive sepsis

The closer the curve to the top left of the graph (1, on the y axis and 0 on the x axis) the more discriminating the test marker for the test. 1 is equivalent to 100% sensitivity and 0 to 100% specificity. From this graph sE-selectin is the least discriminating marker for sepsis in Gram-positive patients.

Table 4.5.2 SENSITIVITY AND SPECIFICITY: Gram-positive sepsis

Best Markers	Cut-off values	Sensitivity (%)	Specificity (%)
PCT	0.4 ng/ml	93	85
TNF-α	3.0 pg/ml	100	69
IL-6	5 pg/ml	93	34

Table 4.5.2 lists the quantitative cut-off levels for each test marker to achieve the given sensitivities and specificities.

PCT, TNF-α and IL-6 gave high sensitivity values in the Gram-positive group but their specificities were low. The specificity increased as the sensitivity decreased at different

cut-off levels for instance; there was a 100% specificity for sICAM-1 (cut-off level 900 ng/ml), LBP (cut-off level 50μg/ml) and PCT (cut-off 0.15ng/ml) but their corresponding sensitivities were 42%, 45% and 41% respectively.

The cut-off value for TNF-α (3.0 pg/ml) in table 4.5.2 that resulted in a sensitivity of 100% for a diagnosis of sepsis in the Gram-positive group, was below the reported "normal" range 5-10 pg/ml, (Nomura, 1999). However the control group's mean value for serum TNF-α was 2.61 pg/ml (or median 2.1) and agrees with the manufacturers (R&D) who state that TNF-α (high sensitivity assay) is either not detectable in the serum of healthy adults or has a mean 2.7pg/ml. The mean (and median) values for TNF-α in the Gram-positive group were 11.6 (12.6) and 77 (28.2) pg/ml for the Gram-negative patients.

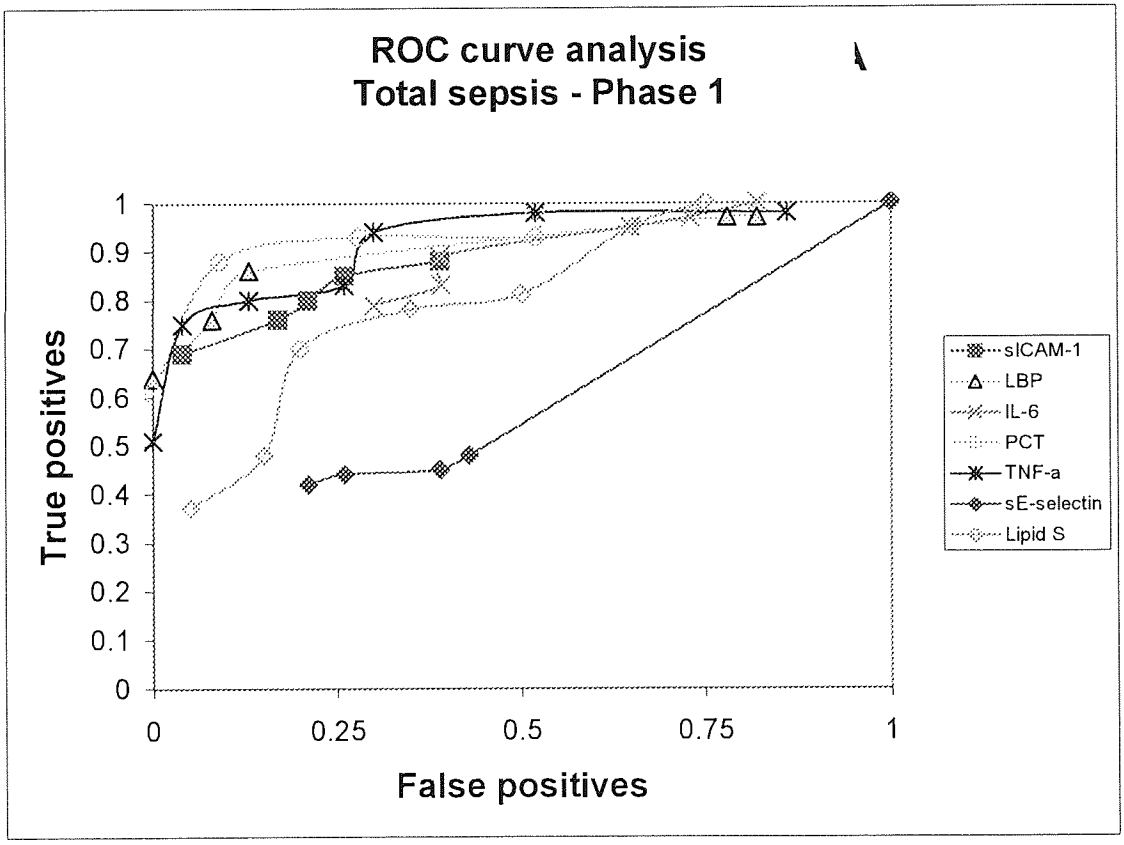


Figure 4.6.3 ROC curves for both Gram –positive and Gram-negative patients combined (total sepsis).

The ROC curves in figure 4.6.3 combine the values for Gram-negative and Gram-positive cases of sepsis (total) versus the controls. Procalcitonin gave the highest sensitivity (93%) and specificity (71%) in total sepsis.

Table 4.5.3 SENSITIVITY AND SPECIFICITY: Gram-positive plus Gram-negative (total) sepsis versus the controls

Best Markers	Cut-off values	Sensitivity (%)	Specificity (%)
PCT	0.35 ng/ml	93	71
LBP	40 μg/ml	76	91
TNF-α	5pg/ml	80	86

Table 4.5.3 lists the quantitative cut-off levels for each test marker to achieve the given sensitivities and specificities.

Procalcitonin had a consistently high sensitivity and specificity in both Gram-negative and Gram-positive patients and was the most sensitive marker in the combined total sepsis and in the Gram-positive group. However LBP was more specific (93%) and maintained a high sensitivity (91%) in patients with sepsis due to an infection with Gram-negative bacteria.

4.6.1 Area under the curve (AUC)

Results obtained from Receiver Operating characteristic (ROC) curves are often expressed in terms of the area under the curve (AUC). This latter method was not adopted in this report as the sensitivities (%) and specificities (%) extrapolated from the graphs gave the quantitative values required to evaluate the test markers.

4.7 TWO-WAY SCATTER GRAPHS

Two markers from each patient were combined in scatter graphs to find a combination of markers that may give higher sensitivity and specificity for sepsis than one marker alone. Only those two-way scatter graphs with the highest values are shown. Tables are included to illustrate the various cut-off levels for each pair of markers that produced the best sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV).

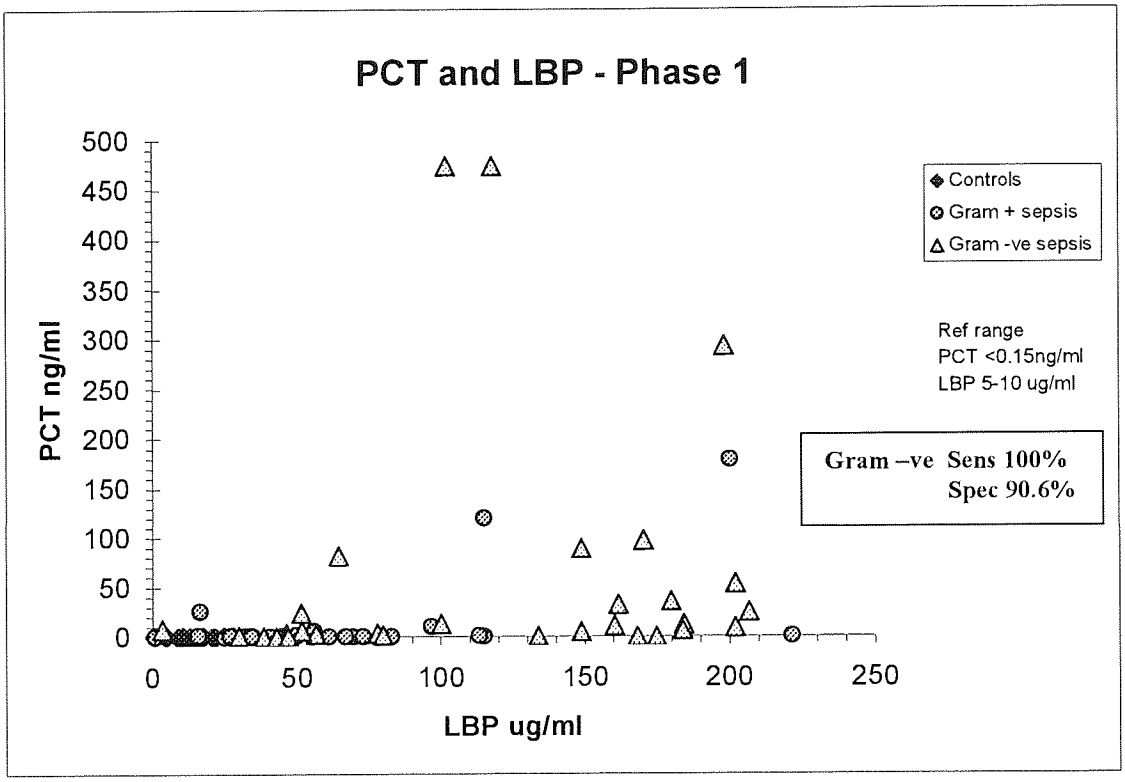


Figure 4.7.1 Two-Way Scatter Graph for PCT and LBP combined

Figure 4.7.1 illustrates that both PCT and LBP are elevated in patients with sepsis. The PCT cut-off level for a diagnosis of sepsis is below 10 ng/ml and is obscured in this graph. Figure 4.7.2 below illustrates the results more clearly.

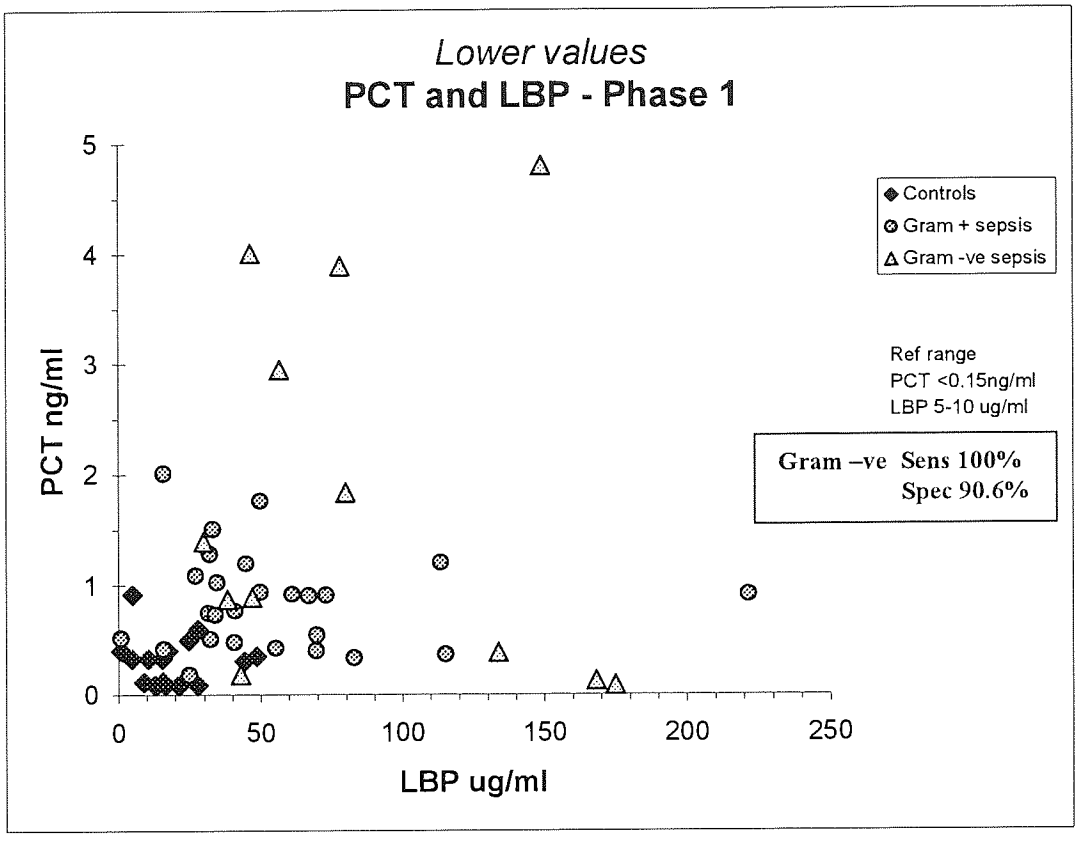


Figure 4.7.2 Magnified area from the PCT/LBP scatter graph in figure 4.7.1

Each symbol on the graph in figure 4.7.2 represents one patient's serum PCT and LBP levels. A cut-off value of 1.0ng/ml PCT and 30 mg/ml LBP gave the best sensitivity and specificity for patients with Gram-negative sepsis see table 4.5.4. A number of controls had elevated LBP levels but none of their corresponding PCT levels were above 1ng/ml.

Table 4.5.4 Sensitivity and Specificity for PCT and LPB in Gram-negative sepsis

PCT ng/ml /LBPμg/ml Cut-off for sepsis	Sensitivity (%)	Specificity (%)	PPV	NPV
2/40	93	90	93	90
1/30	**100**	**90.9**	**93**	**100**

Two serum markers (PCT/LBP) from a patient's sample improved the sensitivity of the test from 91% for LBP and 90% for PCT, to 100% sensitivity, although the specificity was reduced for LBP from 93% to 90.9%. See table 4.5.1

Table 4.5.5 Sensitivity and Specificity for PCT and LPB Combined in Gram-positive sepsis and total sepsis.

Patient group	PCTng/ml/LBPμg/ml Cut-off for sepsis	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
G +ve	1/30	90	90	93	86.9
Total sepsis	1/30	95	90	96	86.9

The positive predictive value (PPV) indicates the probability that a person with a positive result on a diagnostic test actually does have the disease and a negative predictive value (NPV) reflects the probability that a person having a negative result on a diagnostic test does not have the disease (Everitt, 1995). These values are not as high as the Gram-negative patients.

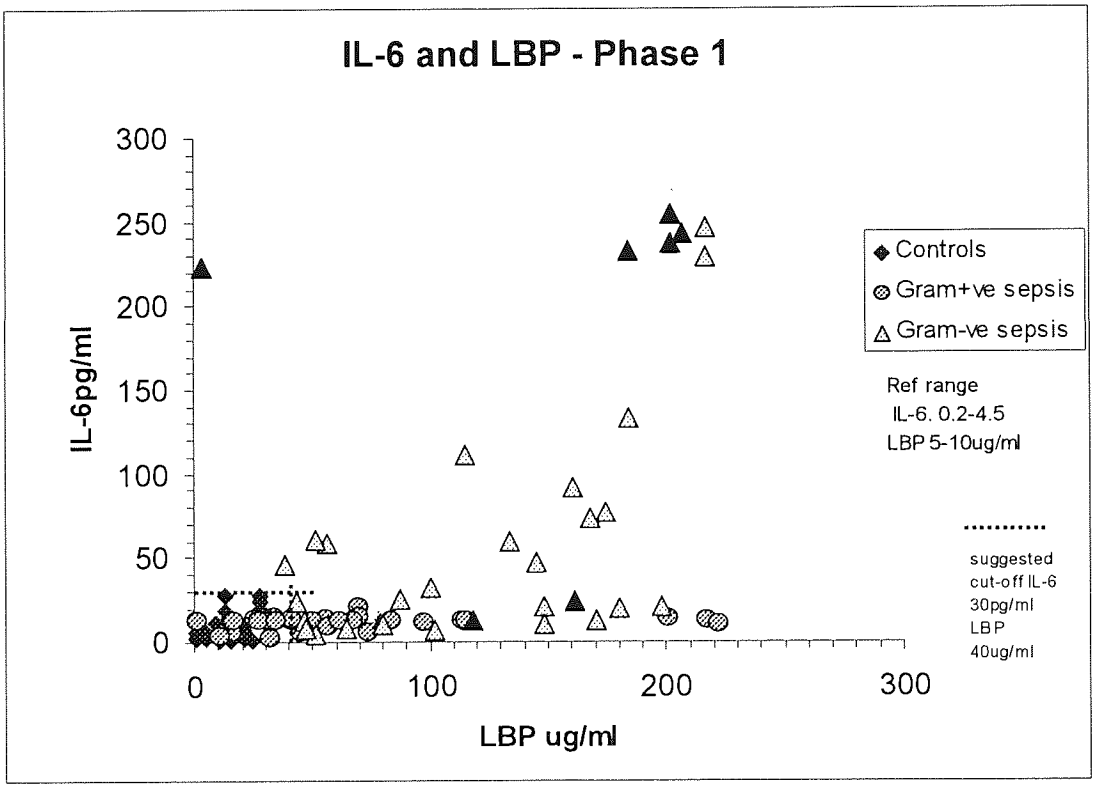


Figure 4.7.3 Two-way scatter graph for IL-6 and LBP

Figure 4.7.3 combines the results of two markers in a patient's serum. The triangles in black (G-ve sepsis), represent patients who did not survive their septic episode.

Table 4.5.6 Sensitivity and Specificity for IL-6 and LPB combined; Gram-negative group

Patient group	IL-6 pg/ml and LBPμg/ml Cut-off for sepsis	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
G -ve	30/40	100	91	94	100

The best sensitivities and specificities for LBP when paired with IL-6, TNF-α or sICAM-1 was achieved with a cut-off value of 40 μg/ml; for the best sensitivity and specificity with PCT however only 30 μg/ml was required.

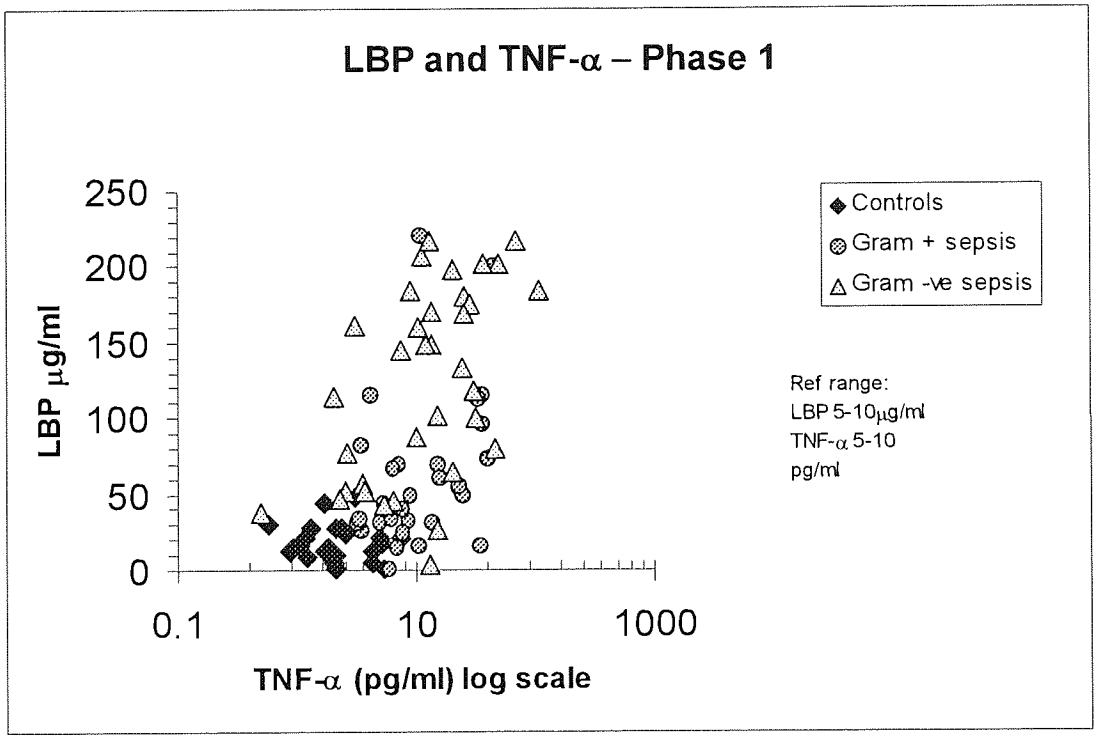


Figure 4.7.4 Two-way scatter graph for LBP and TNF-α

The Gram-negative patient (AN13) with an LBP of 38.4 μg/ml and TNF-α 0.49 pg/ml was a young female (25 yrs) from oncology with a Hickman line in situ. Her diagnosis of sepsis was overturned when her case was reviewed and this is reflected in the position of the marker on the graph.

Table 4.5.7 Sensitivity and Specificity for LPB and TNF-α Combined

Patient group	LBPμg/ml and TNF- pg/ml Cut-off for sepsis	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Gram -ve	**40/10**	**97**	**91**	**94.4**	**95.5**
Gram +ve	40/10	69	91	92	67.7
Total sepsis	40/10	82.6	91	96	65.6

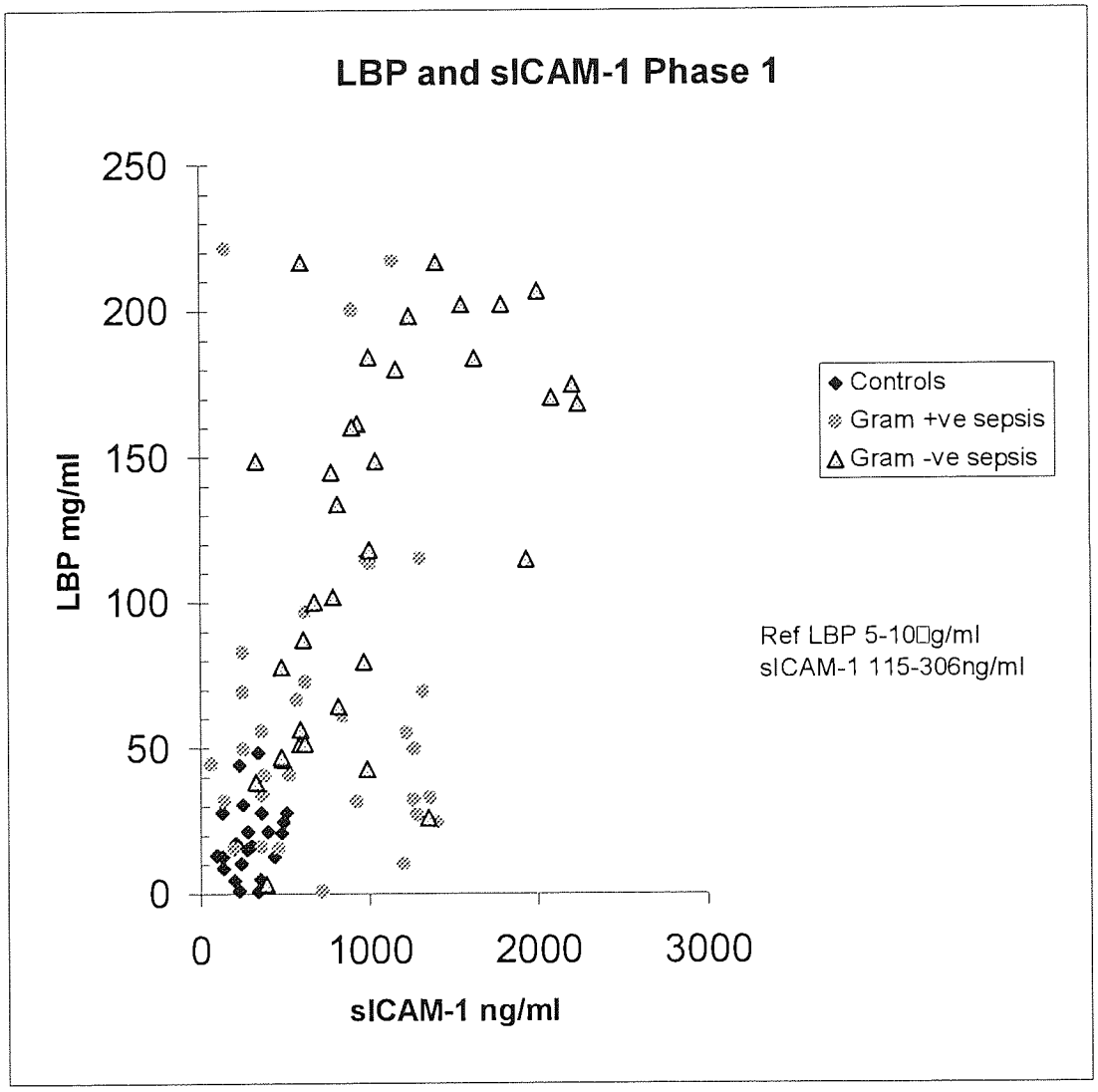


Figure 4.7.5 Two-way scatter graph for LBP and sICAM-1
Each symbol represents an individual patient's serum LBP and sICAM-1 level.

Table 4.5.8 Sensitivity and Specificity for LPB and sICAM-1 Combined

Patient group	LBPμg/ml and sICAM-1ng/ml Cut-off for sepsis	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Gram -ve	**40/500**	**94**	**86.9**	**91.8**	**90.9**
Gram +ve	40/500	78	86.9	89.6	76.9

4.8 SUMMARY OF RESULTS AND THE REPRODUCIBILITY OF ASSAYS

The preliminary assays clearly show that there is a significant difference between the median serum levels of the test markers in the controls (without infection) and those with a positive bacteraemia (with infection/sepsis). The probability that these results are not by chance is represented by the probability factor p-value <0.05; The p-value for each test marker is given in table 4.4. There were two exceptions sE-selectin in Gram-positive patients and IL-12 in Gram-negative patients.

The reproducibility of the assay results were evaluated by comparing the results of duplicate samples within a plate (intra-assay precision) and by comparing the results of the same sample between plates (inter-assay precision). The results are given in tables 4.6.1 to 4.6.7.9.

4.8.1 INTER-ASSAY AND INTRA-ASSAY PRECISION RESULTS.

The following tables show the average of the sum of each evaluation, plus or minus the difference between the average and this difference is also expressed as a percentage. A low percentage difference reflects good reproducibility between the sample pairs and the higher the percentage the larger the difference between the samples.

Table 4.6.1 sICAM-1 inter-assay and intra-assay precision

INTRA-ASSAY PRECISION (precision within the assay)		**sICAM-1** ng/ml			INTER-ASSAY PRECISION (precision between assays)				
sICAM-1	**n**	**average**	**+/-**	**%**	**sICAM-1**	**n**	**average**	**+/-**	**%**
1	2	1042	75.4	7.2	1	2	1468	228	15.5
2	2	400	31	7.7	2	2	955	105	10.9
3	2	155	14	9.0	3	2	1201	201	16.73
					4	2	938	8	0.86

Table 4.6.2 IL-6 inter-assay and intra-assay precision

INTRA-ASSAY PRECISION		**IL-6 HS** pg/ml			INTER-ASSAY PRECISION				
IL-6 HS	**n**	**average**	**+/-**	**%**	**IL-6**	**n**	**average**	**+/-**	**%**
1	2	37.76	6.2	16.4	1	2	16.65	1.8	17.3
2	2	6.7	1.5	22	2	2	44.2	2.6	5.8
					3	2	28.5	2.5	8.8

HS; high sensitivity assay

Table 4.6.3 TNF-α inter-assay and intra-assay precision

INTRA-ASSAY PRECISION		**TNF-a HS** pg/ml			INTER-ASSAY PRECISION				
TNF-α HS	**n**	**average**	**+/-**	**%**	**Sample**	**n**	**average**	**+/-**	**%**
1	2	29.44	7.6	25.6	1	2	5.8	0.47	8.1
2	2	47.0	6.6	14.0	2	2	41.5	11.5	27.7
					3	2	47	7.0	14.8

HS; high sensitivity assay

Table 4.6.4 sE-selectin inter-assay and intra-assay precision

INTRA-ASSAY PRECISION		**sE-selectin ng/ml**			INTER-ASSAY PRECISION				
sE-selectin	**n**	**average**	**+/-**	**%**	**Sample**	**n**	**average**	**+/-**	**%**
1	2	0.52	0.0	0.0					
2	2	4.65	0.019	0.07	**Discontinued assay**				
3	2	9.7	0.05	0.6					

Table 4.6.5 LBP inter-assay and intra-assay precision

INTRA-ASSAY PRECISION **LBP μg/ml** INTER-ASSAY PRECISION

(precision within the assay) (precision between assays)

LBP	**n**	**average**	**+/-**	**%**	**LBP**	**n**	**average**	**+/-**	**%**
1	2	15.4	0.56	3.6	1	2	122.5	10.5	8.57
2	2	96.86	3.14	3.24	2	2	11.84	2.3	19.5
3	2	1.95	0.93	47	3	2	19.2	2.6	13.7

The intra-assays appear to be more reliable than the inter-assay results.

Table 4.6.6 IL-10 inter-assay and intra-assay precision

INTRA-ASSAY PRECISION **IL-10 pg/ml** INTER-ASSAY PRECISION									
IL-10	**n**	**average**	**+/-**	**%**	**IL-10**	**n**	**average**	**+/-**	**%**
1	2	13.8	1.7	12.3	1	2	48	8.6	18
2	2	56.85	0.2	0.36	2	2	36.7	2.12	5.79
3	2	22	1.0	4.5	3	2	156	5	3.2
4	2	8.3	0.36	4.4					

Table 4.6.7 IL-12 inter-assay and intra-assay precision

INTRA-ASSAY PRECISION **IL-12 pg/ml** INTER-ASSAY PRECISION									
IL-12	**n**	**average**	**+/-**	**%**	**IL-12**	**n**	**average**	**+/-**	**%**
1	2	14.5	0.1	0.68	1	2	22.6	6.3	28.2
2	2	77.8	13.2	16.9	2	2	38.5	6.5	16.8
3	2	14.5	0.139	0.95					

Table 4.6.8 PCT inter-assay and intra-assay precision

INTRA-ASSAY PRECISION **PCT ng/ml** INTER-ASSAY PRECISION									
PCT	**n**	**average**	**+/-**	**%**	**PCT**	**n**	**average**	**+/-**	**%**
Not done	-	-	-	-	1	2	17	16.48	96.9

The higher the percentage the poorer the reliability and reproducibility; more test samples are required to give a more reliable view of the reproducibility of this assay.

Table 4.6.9 anti-lipid S IgG titre inter-assay and intra-assay precision

INTRA-ASSAY PRECISION **anti-lipid S IgG titres** INTER-ASSAY PRECISION									
Anti-lipid S	**n**	**average**	**+/-**	**%**	**sample**	**n**	**average**	**+/-**	**%**
1	2	23,697	1149	4.4	1	4	99,055.9	1050	1.06
2	2	6684	214	3.2	2	4	25,891.5	9.5	0.03
3	2	3208	374	11.6	3	4	10,835.9	475	4.3

Tables 4.6.1 to 4.6.9 show that 17 paired tests from a total of 44 exceeded the acceptable 10% difference. There are many possible reasons for these results and they will discussed in the major section 4.9.

4.8.2 Gram-negative sepsis group

With the exception of anti-Lipid S and IL-12 all the test markers were significantly elevated in patients with sepsis induced by Gram-negative bacteria (*p*-value 0.05; see table 4.4) The most discriminating (sensitive and specific) markers for Gram-negative sepsis were LBP, sICAM-1 and procalcitonin see table 4.5.1.

4.8.3 Gram-positive sepsis group

A significant difference between the medians in the control group assays and the Gram-positive patients was established (p-value 0.05) for all the test markers with the exception of IL-6 and IL-12 see table 4.4. Sixty percent of the patients in the Gram-positive group were positive for anti-Lipid S antibodies as opposed to forty-one percent in the Gram-negative group and fourteen percent in the control group. TNF-α and PCT gave the best sensitivities but the specificity for TNF-α was low at 69%.

4.8.4 ROC Analysis

The diagnostic value of the test markers are reflected in the ROC curves. The nearer the curve is to the top left-hand side of the graph (Y = 1 and X = 0) the more discriminating the test. A curve that bisects the graph or falls below the 45° angle such as sE-selectin (see figure 4.6.2) is equivalent to demonstrating that there is no diagnostic value in the marker.

The most discriminating test for sepsis regardless of the causative organism was procalcitonin (PCT) and then lipopolysaccharide binding protein (LBP) see figure 4.6.3. In Gram-negative patients, lipopolysaccharide (LBP) was more discriminating and PCT was more sensitive (93%) in Gram-positive patients. Anti Lipid-S antibody titres were more specific for patients with Gram-positive sepsis.

4.8.5 Combining two markers for Sensitivity, Specificity, PPV and NPV

In the Gram-negative patients the best over-all values came from was combining IL-6 with LBP. This was calculated from a serum level of not less than 30pg/ml IL-6 and not less than 40μg/ml LBP in the same patient. These markers gave 100% sensitivity and 100% negative predictive values (NPV) with a positive predictive value (PPV) of 94% and a specificity of 91% (table 4.5.6). Similar results were found for PCT with LBP in the Gram-negative group (table 4.5.4).

The values for sensitivity, specificity, PPV and NPV in patients with Gram-positive sepsis did not reach the same high percentages as those for Gram-negative sepsis, nevertheless values reaching 90% were attained when PCT and LBP were combined (table 4.5.5).

The most discriminating pair of markers for all the septic patients combined (Gram-negative and the Gram-positive patients as one group) in total sepsis was PCT

and LBP; patients with a serum level of not less than 1.0 ng/ml PCT and not less than 30μg/ml LBP (table 4.5.5), produced a test sensitivity of 95% and specificity of 90%.

4. 9 DISCUSSION; PHASE 1

A blood culture is not routinely performed except in certain patient groups such as those undergoing renal dialysis. Often it is the onset of pyrexia that results in a request for a blood culture, white cell count and possibly a serum C-reactive protein. Therefore in this study the onset of a potential sepsis has been assumed to coincide with the day a blood culture was taken and subsequently grew a Gram-negative or Gram-positive microorganism. Consequently the patient's test serum sample date was selected to match the day blood was taken for culture. The discussion will show that variations in the test markers in patients diagnosed with the same disease vary and this may be due in part to the relationship between the test sample date and the onset of the sepsis.

4.9.1 The Markers

The patients in the control group for the preliminary trial had heart disease and some had underlying conditions such as diabetes or renal disorders, therefore their test marker serum levels may not represent the 'normal' ranges expected in a healthy population. However none of the control group exhibited any current symptoms indicative of an infection or sepsis.

The project was not designed to evaluate the levels of the test markers in patients with underlying disease such as liver or kidney disorders. However some patients in the septic groups had similar underlying diseases as the control group, such as diabetes. If any of the test markers are in fact affected by such underlying diseases (as opposed to the effects of sepsis) this may be reflected in the serum levels of the control patients with the same underlying disease. For example, if LBP (an acute phase protein produced by the liver) is shown to be elevated in septic patients but is lower than expected in septic patients with liver disease, and was found to be lower than expected in the control patients with the same disease, then it may be possible to speculate that the liver disease could be a reason for a down regulation of LBP. Therefore LBP as a universal marker for sepsis would be ineffective in such patients and would always produce false negative results. Any such findings or hypotheses would have to be verified with appropriate research; however at this early stage such speculations may suggest reasons for result anomalies.

An effective test marker for sepsis must be diagnostic for the condition regardless of the patients other diseases (if any). Therefore the control group may have served two purposes; the initial purpose (as a group without sepsis) and to infer (tentatively) that a given underlying disease does or does not appear to effect the serum levels of the test markers.

All the selected test markers were significantly elevated in the patient groups with sepsis as opposed to the controls, although not all septic patients had elevated levels of all the test markers, these findings are discussed under the specific test marker headings.

The test markers in patients with Gram-positive isolates in their blood cultures were invariably lower than those in the Gram-negative group confirming the findings of others, that Gram-negative and Gram-positive bacteria illicit different immune responses (Opal and Cohen, 1999).

4.9.2 TNF-α and IL-6

Serum levels of TNF-α in patients with sepsis varied considerably, showing a range of values from 0.49 pg/ml to 107 pg/ml. These values agree with the findings of Cohen & Abraham (1999). TNF-α was not sensitive or specific enough to be considered as a diagnostic marker for sepsis. However when TNF-α was combined with LBP as a two marker test for sepsis, the sensitivity and specificity reached 97% and 91% respectively but only in patients with Gram-negative sepsis.

High serum levels of TNF-α in this trial became synonymous with potential non-survivors; four of the nine non-survivors in the Gram-negative group had the highest levels of TNF-α see table 4.7.1. This finding agrees with Nooteboom *et al.*, (2002), high serum levels of this cytokine correlate with disease severity. Others argue that it is rarely found in the serum of septic patients (Memelmans *et al.*, 1996). The latter finding was not confirmed in Phase 1, as all the patients including the controls had detectable levels of TNF-α. This cytokine may not be a candidate diagnostic marker for sepsis but it could be a useful marker of disease progression and prognosis.

Table 4.7.1 Serum markers in non-survivors with Gram-negative sepsis.

Patient G -ve	TNF-a pg/ml	IL-6 pg/ml	Number of days	LBP µg/ml	Gram-negative bacillus
AN11	10.98	242	8	206	*Citrobacter freunii*
AN14	13.06	223	20	3.3	*Enterobacter* spp.
AN15	49.26	236	7	202	*Enter. cloacae*
AN16	37.11	254	4	202	*Kleb. pneumonia*
AN24	107.91	232	4	184	*Serratia* spp.
AN2	13.5	**20.5**	**98**	148	*Citrobacter freunii*
AN4	30	**12.7**	**10 months**	118	*Enter. cloacae*
AN5	2.6	**12.5**	**110**	77.8	*Citrobacter freunii*
AN7	3	**24.06**	**33**	161	*P. aeruginosa*

The 'number of days' indicates the length of time between the test serum date and patient outcome.

There appears to be two distinct groups in the patients with Gram-negative sepsis see figure 4.7.3 (triangles) and table 4.7.1; patients with high levels of IL-6 and those with lower values but equally high levels of LBP. Five of the seven patients with IL-6 above 200pg/ml died and four of these patients died within a week of the test sample date. There may be a correlation between disease severity and equally elevated serum levels of IL-6 and LBP.

A patient in the Gram-negative group (AN7, see table 4.7.1) with a low level of TNF-α (3pg/ml) had a *Pseudomonas aeruginosa* in her blood culture. This organism is reported to interfere with the host's production of TNF-α (Staugas *et al.*, 1992). Conversely patient (AN25) with the same microorganism had a high serum TNF-α and survived even though she was an elderly (80yrs) diabetic with chronic renal failure (see table 4.7.2).

Table 4.7.2 Patient AN25

Patient G -ve	TNF-α pg/ml	IL-6 pg/ml	Discharged days	LBP μg/ml	Isolate
AN25	46.44	10.63	30	79.9	*P. aeruginosa*

The lowest level of TNF-α from patients in this study (including the controls), was 0.49 pg/ml. This patient (AN13) had *Stenotrophomonas maltophilia* (previously known as *Pseudomonas maltophilia)* in his blood culture and within several weeks of this septic episode produced a positive blood culture with three species of *Pseudomonas.*

The high sensitivity (HS) assays for TNF-α and IL-6 produced a number of 'overflows' when the absorbance was read. Overflows occur when the concentration of the test antigen exceeds the absorbance parameters. Overflows were diluted to obtain a reading and the results multiplied by the dilution factor. This can lead to inaccuracies. Therefore this assay type was changed to a non-high sensitivity product from R&D systems. These assays kits had a higher pg/ml detection range for IL-6 and TNF-α but were less sensitive to very low serum levels of the test antigen.

Recently a novel rapid immunostrip test for IL-6 called SEPTEST was evaluated for a bedside diagnosis of sepsis. This immunostrip registers a positive result only at 1000pg/ml (Panacek *et al.* 2000). As a diagnostic marker for sepsis this strip would render all but one of the septic patients in this project non-septic.

4.9.3 The Adhesion molecules.

ICAM-1 and sE-selectin are elevated in vascular diseases, the latter is thought to be a good marker for vascular endothelial damage as it only appears on activated endothelial cells (Takeshita *et al.* 1997). The range of serum levels for these molecules in this study for Gram-negative sepsis cases were 325 –1932ng/ml for sICAM-1 and 32-930 ng/ml for sE-selectin, see tables 4.3.1 and 4.3.2. These values are generally higher than those reported in the literature for other diseases. For instance in episodes of relapsing–remitting-progressive multiple sclerosis the levels of sICAM-1 were raised from 154ng/ml to 222 +/- 58ng/ml (Khoury *et al.*1999). "Raised" levels in this case

represent a slight increase above their study's normal reference range, but are within the reference range expected in healthy adults in this study.

The working hypothesis in this project has been that all the tested markers are significantly elevated above those reported for other diseases, and could be diagnostic for sepsis at a given cut-off point. In the literature sICAM-1 was shown to be higher in septic patients (1266 ± 261 ng/ml) than trauma patients and all the non-survivors had values greater than 800ng/ml (Boldt *et al.*, 1996). The latter study reported that the trauma patients had elevated sICAM-1 but these values did not increase over time whereas the septic patient's sICAM-1 increased to 2022 ± 609ng/ml (5 days post diagnosis). In this current study, seven of the nine non-survivors in the Gram-negative septic group had sICAM-1 levels above 800ng/ml but so did ten patients from the surviving group. Serum levels of sICAM-1 reached 2238 ng/ml in one patient in this study although not all the septic patients had high levels they were all above the reference range (115-306 ng/ml).

sE-selectin was less discriminating as a marker for sepsis in this study as reflected by the ROC curve analysis see figures 4.6.1 and 4.6.2. Arbitrary cut-off values for sE-selectin from 54 to 75 ng/ml (reference range 30 ± 7 ng/ml), produced sensitivities from 88% to 77% with their respective specificities ranging from 52% to 82%. A cut-off level that produced 100% sensitivity had a corresponding specificity of 0%.

4.9.4 Procalcitonin and Lipopolysaccharide binding protein

LBP was shown by ROC curve analysis (figure 4.6.1) to be the most sensitive and specific marker for sepsis in patients with Gram-negative sepsis, whereas PCT appeared to be the marker of choice for patients with Gram-positive sepsis (sensitivity 93% and specificity 85%; calculated from a cut-off level of 0.4 ng/ml. Table 4.5.2).

However PCT was still the most discriminating test for bacterial sepsis regardless of the causative organism in the total sepsis ROC curve analysis (figure 4.6.3) and PCT gave the most discriminating results for Gram-negative sepsis when combined with LBP as a two-marker test (table 4.5.4).

Serum levels of PCT above 0.5ng/ml are pathological (Cusack *et al.*, 2000); this statement is only partially true according to this study, as seven Gram-positive patients and two Gram-negative cases had serum levels below this and two of the control patients had PCT levels above 0.5 ng/ml (0.6 and 0.92 ng/ml). Nevertheless the median PCT

levels in the Gram-positive and Gram-negative septic patients were 0.92 and 8.86 respectively see table 4.3.5.

Serum levels of PCT ranged from 9-474 ng/ml in both the non-surviving and surviving patients with Gram-negative sepsis therefore PCT does not appear to be an indicator of prognosis.

The patent holders of this molecule (BRAHMS Diagnostika, Berlin) are in the process of developing a more sensitive assay for PCT as levels are moderately elevated in patients with fungal and parasitic infections (Al-Nawas and Shah, 1997). Although this statement is open to debate as other researchers disagree with the latter findings (Gerard *et al.* 1997; Huber *et al.*, 1997).

In every case of Gram-negative sepsis in this preliminary trial, all the patients bar one (AN14) had significantly higher LBP levels than those in the control group. Seventeen of the twenty-three control patients had LBP levels above the reported normal reference range (5-10μg/ml) with a mean level of 18μg/ml but the mean value for Gram-negative sepsis was 120 μg/ml, and in the patients with a central venous catheter (CVC) related sepsis the mean was 66.91 μg/ml see table 4.3.6.

Patient-AN14 was in her mid forties and had cirrhosis of the liver. This fact may explain her low level of LBP (3.3μg/ml), as the liver synthesises LBP (Schumann *et al.* 1990). However patients with alcoholic liver disease in the Gram-negative septic group had high levels of LBP. A post-mortem was performed on patient AN14. Her liver had such an unusual appearance that the examiners suspected that the liver damage might have been related in part to self-administered homeopathic medicines. This lady was allergic to many foods including eggs, milk, cashew nuts, sweet corn and caffeine. She was also allergic to penicillin and erythromycin. The patient was diagnosed with Sjogren's syndrome (an immunologic disorder characterised by deficient moisture production) and hypothyroidism several years before inclusion into this study.

It is important to investigate patient history when evaluating the diagnostic potential of a serum marker because underlying conditions can affect the host's immune response to infection. This project aims to find a marker or markers for sepsis that would not be adversely affected by the pathology of underlying diseases. This is particularly relevant for patients who are immunosuppressed or immunodeficient.

4.9.5 IL-10 and IL-12

Due to limited amounts of test sera only 16 controls, 17 patients from the Gram-positive group and 29 patients from the Gram-negative group could be tested. The results from these assays were disappointing because of the high number of negative results. As there were so few results they are all given in table 4. 8.

Table 4.8 IL-10 and IL-12 in all patient groups

Patient group	IL- 10 normal levels < 5 pg/ml	IL-12 normal levels < 3 pg/ml
Contols (16)		
AC7	nd	45.3
AC9	nd	31.04
AC14	19.5	nd
AC17	nd	3.2
Gram-positive sepsis (17)		
AP2	nd	**49.75**
AP6	26.2	nd
AP7	96	1.65
AP8	nd	2.73
AP10	nd	2.42
AP14	nd	**23.15**
AP16	nd	**14.18**
AP22	nd	0.41
AP24	364.4	0.72
AP27	nd	0.108
AP30	nd	**91.04**
AP31	6.18	**12.17**
AP33	125.25	**101.9**
Gram-negative sepsis (29)		
AN3	**10.28**	1.03
AN11	**33.38**	29.03
AN12	**21.065**	nd
AN15	**407**	nd
AN16	**152.9**	40.47
AN20	nd	2.11
AN24	**732.455**	nd
AN29	nd	1.34
AN32	**19.52**	nd
AN34	**40.05**	nd
AN35	nd	9.078
AN36	**137**	0.727

nd-not detected

Patients with negative (not detected) results for both IL-10 and IL-12 are not listed.

According to the Hessle hypothesis (Hessle *et al.*, 1999) Gram positive bacteria preferentially induce monocytes to express cytokine IL-12 (in bold) and Gram-negative bacteria preferentially induce monocytes to secrete IL-10 (in bold).

The serum kinetics of IL-10 are known to be short term (Oberhoffer *et al.*, 1999); serum levels peak within 2 hours after surgical trauma and disappear from the serum within 24 hours; this probably accounts for the high number of negative results in this study.

IL-10 was detected in more patients with a Gram-negative sepsis than in Gram-positive patients and the converse was true for the Gram-positive group and IL-12. These findings partially support the Hessle hypothesis.

When patients in the Gram-positive group did have an elevated serum IL-10 their corresponding serum IL-12 was lower. Three Gram-negative patients negative for IL-10 had normal levels of IL-12.

The results from the control group were surprising. Two patients, AC7 and AC9 had high levels of IL-12 (45 and 31 pg/ml respectively), and patient AC14 had an elevated level of IL-10. The best inducers of IL-12 are some bacteria and intracellular parasites (Mountford *et al.*, 1998). By definition a successful parasite would not want to illicit an overt immune response and be expelled from the host. In the absence of clinical signs of infection and in the presence of normal levels of inflammatory markers such as IL-6 and the adhesion molecules in the control group, IL-12 may be a beacon marker for occult intracellular infections and a project investing this proposal would be interesting and possibly valuable.

IL-12 is crucial for the activation of macrophages and is known to drive naive T helper (Th-0) cells towards the proliferation of a sub-set of T cells known as T helper-1 (Th-1) cells (Croft *et al*, 1995). In comparison, IL-10 is mainly secreted from T-helper-2 (Th-2) cells. Th-2 cells are the most effective activators of B-cells and the antibodies they produce. IL-10 inhibits many cell types including macrophages and is an important anti-inflammatory cytokine (Fiorentino *et al.*, 1989).

Van-Dissel and colleagues (1998) demonstrated an increase in mortality in patients with a high ratio of IL-10 to TNF-α, a finding that is consistent with the fact that immunosuppression increases the risk of death from infection (Vincent, 1998). The highest levels of TNF-α in this preliminary trial were recorded from non-survivors AN24 (107 pg/ml) and AN15 (49 pg/ml). However AN24 also had the highest level of IL-10.

The relationship between increasing levels of IL-6 and declining levels of IL-10 in non-survivors shown by Taniguchi and colleagues (1999) cannot be confirmed or denied from this report as it would require serial measurements of both cytokines.

However several of the non-survivors with Gram-negative sepsis had very high serum levels of both cytokines; patient AN24 serum IL-10 reached 608pg/ml with IL-6 232 pg/ml, and patient AN15, with IL-10 352 and IL-6 151 pg/ml.

4.9.6 Anti-lipid S antibody as a differential marker for Gram-positive sepsis

Patients with a Gram-positive microorganism in their blood culture can be difficult to diagnose, as these isolates may be skin or environmental contaminants (Siegman-Igra *et al.*, 1997). This is particularly true for patients with indwelling catheters when positive blood cultures may be due to catheter contamination, colonisation or sepsis (Elliott *et al.*, 2000). Infections associated with central venous catheters in hospitalised patients are known to cause high morbidity and mortality (Elliott and Faroqui 1992; Elliott and Tebbs 1998). Therefore it is important to find a marker for Gram-positive sepsis that would indicate categorically the difference between a true positive blood-culture and a false- positive one.

Lipid S is an extracellular moiety recently isolated from *Staphylococcus epidermidis* (Lambert *et al.*, 2000) see chapter 2 (2.8). Therefore Lipid S could be an exciting new molecule specific for Gram-positive organisms and may be a marker for Gram-positive sepsis and a differential marker between Gram-negative and Gram-positive sepsis.

66% of the Gram-positive group were positive for anti-lipid S antibodies, however fourteen patients (14/33) in the Gram-negative group and three controls were also positive. Patient notes were able to suggest some reasons for these results. For example patient AN35 had previously suffered from a chest infection (*S. aureus*-methicillin resistant) but this was only apparent from his notes and after he had been enrolled onto this trial and tested. One patient proved to be polymicrobial with a Gram-positive and a Gram-negative isolate in the same blood culture, this patient had a positive anti-lipid S titre. Patient history (AN14, mentioned earlier with low levels of LBP) proved to be a very complex case that revealed many opportunities for this patient to develop antibodies to a Gram-positive isolate. One of the controls positive for lipid S was a diabetic and had been on insulin for 24 years; his notes mentioned eschar on leg wounds and it is speculation that these infections where caused by Gram-positive skin commensals.

When a patient develops antibodies against lipid S it is not known how long they remain detectable in the serum, although there is some evidence that it may be many

months as a patient strongly positive for lipid S became negative for this antibody at some point within six months. Residual antibodies to a previous infection could account for the unexpected results in the Gram-negative group and controls.

Patients in the Gram-positive group with negative anti-lipid S titres could be in the process of developing antibodies to the current infection that would not be detectable for days. An assay that detects the lipid S molecule in serum samples would answer many of these questions.

4.9.7 Potential sources of error

Recent papers highlight the need to standardise cytokine assays because they are being used with increasing frequency and the values found can vary dramatically (Ledur *et al.*, 1995; Banks, 2000). The latter paper points out the variability in the literature of 'normal' reference ranges for these serum molecules. Therefore reliable, normal healthy population levels need to be firmly established for serum proteins, taking into consideration age, sex, ethnic background and diurnal rhythmicity (Banks, 2000).

The published reference levels served as a rough background guide in this study because the diseased but non-infected controls served as the major reference range for establishing cut-off levels for a diagnosis of sepsis. The levels of sE-selectin, LBP and to some extent IL-6 in the control group did not conform to the normal reference ranges given in the literature or by the assay manufactures.

The high sensitivity assays (R&D Systems, Minneapolis, USA), are particularly vulnerable to cross contamination from laboratory glass wear that may have contained phosphate buffered saline (PBS) as this buffer is a strong competitive inhibitor of alkaline phosphatase a reagent in the IL-6 (HS) assay. As mentioned previously some of the test samples contained such high levels of the test antigen that an 'overflow' occurred when the plate was read with the automatic plate reader. Diluting the sample at this point is less accurate than diluting the sample at the start of the assay and is another potential source of error.

Errors can occur when measurements are extrapolated beyond the standard curves formed with the standards provided by the manufacturer. Beyond this value the dose response curve may be non-linear. This was true for the IL-10, IL-12, PCT and sICAM-1 assays therefore computer-generated values for high unknowns may be inaccurate (Ledur *et al.*, 1995).

Another source of error and not previously described is the fact that the various computer analysis tools generate different values from the same data. A comparison between Graph Pad Prism® and Excel standard curve values and the computer generated values for the unknowns (test samples) was conducted for the serum levels of LBP, some results are given in table 4.9.

Table 4.9 A comparison between Prism and Excel data analysis for serum LBP

Patient	Prism values μg/ml	Excel values μg/ml
AC17	16.53	12.72
AC21	13.23	9.92
AP3	0.9	2.32
AP14	96.8	121.42
AP15	**41**	**41.52**
AP20	113.46	143.02
AP29	56.01	62.92
AP30	221.35	263.62

The values differ and are greater in Excel after a mid point of 40μg/ml. When the serum levels are very high the difference between the two estimated values increases and this can have an important bearing on establishing cut-off levels. For consistency in this project the calibration curves were generated using GraphPad Prism 3.0.

Operator error can occur at various points in an assay and may include incomplete washing of the micro-titre plate between procedures that would allow non-specific binding to the conjugate. Other factors include incorrect dilutions and calculations. Assays kits such as sICAM-1 and LBP contained prepared controls with known amounts of the test antigen. The assay is validated when the control results fall within the control range of the antigen.

4.9.7 Discussion Summary

An aim of this study was to evaluate the levels of the selected test markers in patients with Gram-negative and Gram-positive sepsis and compare the findings with a control group without infection. The preliminary trial shows that all the markers are generally elevated in septic patients as opposed to the controls. However there are exceptions. These exceptions raise questions that need to be answered for a sepsis marker to be valid. For instance; why are serum markers raised in one patient but not

another with apparently the same condition? Obviously there are going to be individual differences based on age, underlying conditions and their genetic makeup. Also the time the test serum was taken and tested in relation to the progress of the septic episode will make a difference to the level of the test markers based on the serum kinetics of the various test markers.

Polymicrobial infections should have been excluded, as different Gram staining bacteria may give different cytokine profiles, as has been suggested in the literature. Unexpected results were often clarified when patient's notes were studied. This was particularly true for the Lipid S assays, when previously unknown infections revealed potent stimuli for the production of anti-lipid S antibodies. Some of the anti-lipid S IgG positive results in patients with Gram-negative sepsis were shown to have had or currently had mouth ulcers, leg ulcers or a post operative Gram-positive infection. One such patient had breast carcinoma with an abscess caused by a Gram-positive microorganism. A mastectomy was performed six months before this patient was enrolled onto this trial, a photograph of the patient's wounds 2 months later revealed the infection still persisted.

There were many such examples. Patient notes are often unavailable to clinicians when a febrile patient is admitted and a diagnosis is required. The need for a stringent rapid serological test marker for sepsis is therefore all the more imperative.

Another aim of this study was to establish cut-off levels for the markers that would be diagnostic for sepsis. The possible values are; sICAM-1 500 ng/ml, 30μg/ml for LBP, and greater than 1ng/ml for PCT.

If sICAM-1 were the sepsis marker of choice then 6 known cases of Gram-negative sepsis would have been missed; 2 cases if LBP was chosen; 6 cases if PCT was the marker of choice.

On the other hand if IL-6 was selected and based on the normal reference range (0.2-4.5pg/ml) seventeen (from twenty-three) of the control patients would be diagnosed as septic. Consequently it became apparent as the project progressed that two markers would be required to confirm a diagnosis of sepsis and that this second marker should preferably be one associated with infection as opposed to inflammation, such as LBP or PCT.

Results from this preliminary trial (phase 1) demonstrate that procalcitonin (PCT) together with lipopolysaccharide binding protein (LBP) had the highest sensitivity

(100%); specificity (90%); PPV (93%) and NPV (100%) for Gram-negative sepsis (see table 4.5.4). These figures were calculated from a cut-off for PCT greater than 1.0 ng/ml and 30μg/ml for LBP. The results for total sepsis were almost as high (table 4.5.5).

As previously mentioned a septic episode, even when successfully treated, reduces the life expectancy of the patient by as much as 5 years (Brod, 2000). So it is important to detect and diagnose sepsis early, and at the same time eliminate a potential miss-diagnosis that may occur from false-positive blood cultures.

The best systemic marker or markers would be one that is specific for bacterial infection as PCT and LBP appear to be, but with the sensitivity to detect the early phase of sepsis and the specificity to confirm the clinical diagnosis.

4.9.9 Further research

The next phase (2) of this study aims to enrol 75 new patients; 25 controls, 25 patients with a Gram-positive sepsis and 25 patients with Gram-negative sepsis. In this phase patients with a Gram-positive sepsis will include positive blood cultures with coagulase negative staphylococcal (CNS) as before but will include other species such as *S. aureus*, and other genus such as streptococci.

The cut-off values for a particular marker diagnostic for sepsis have been suggested in the first part of this study, but they need to be tested against a new cohort of patients.

Chapter 5 will contain a short introduction, with the methods and materials for the selected assays not described in chapter 3; the results and discussion.

CHAPTER 5 INTRODUCTION PHASE 2

Following the results from phase 1 (chapters 3 and 4) it became evident that some of the patients with sepsis had underlying confounding factors. Indeed a few patients had both possible Gram-positive and Gram-negative sepsis. It was therefore decided to select patients into the Gram-negative and Gram-positive sepsis groups initially based on both clinical and microbiological findings. The hypothesis to be tested was that investigations of patients who had no underlying confounding factors should facilitate more accurate determinations of the sensitivities and specificities of the test markers.

The results from the phase 1 showed that two markers assayed from the same serum sample produced a more discriminating test for sepsis in terms of higher sensitivities and specificities than any single marker. The markers that produced the best results were included in this phase of the project, and they are the assays for TNF-α, IL-6, sICAM-1, LBP and anti-lipid S IgG antibody.

Although the procalcitonin assay gave good results this test was not brought forward because it is currently being researched intensively by many other research centres; whereas there is little research being conducted using assays for all the other test markers; individually these markers are researched but rarely collectively on the same serum sample from patients with sepsis.

Soluble endothelial selectin (sE-selectin) deserves more attention as do interleukin -10 (IL-10) and interleukin -12 (IL-12) but based on the results from chapter 4 these assays were discontinued.

This chapter includes the methods and materials as well as the results from a new group of patients together with a discussion of the results. The main aim was to confirm that the serum levels of the test markers established in phase 1 were reproducible in a new group of patients with and without sepsis. Another aim was to establish cut-off levels for these markers in serum for a diagnosis of sepsis.

5.1 CLINICAL SAMPLES

The Queen Elizabeth Hospital's Ethics committee approved this research. The patients were enrolled into this part of the study after being verified by a Consultant Microbiologist based at Queen Elizabeth Hospital. A strict criterion for the diagnosis of sepsis was applied and only patients with either Gram-positive or Gram-negative sepsis were entered.

Blood samples were obtained from the control patients and those with either a Gram-positive bacterial or Gram-negative bacterial associated sepsis. The samples were separated and the serum obtained was stored at minus 20°C until assayed.

Patient's blood cultures were processed by the microbiology staff at the Queen Elizabeth Hospital.

5.1.1 Control patients

Twenty six pre-operative in-patients waiting for a coronary artery by-pass graft (CABG) at the Queen Elizabeth Hospital (Birmingham, UK) were enrolled into this study. They were a heterogeneous group with no recent or current clinical evidence of infection or sepsis. As described previously a blood sample from each patient was taken on admission for elective surgery for coronary artery by-pass grafts (CABG). There were 21 males with an average age of 60 years (age range 43 to 74), and 5 females with an average age of 68.8 years (age range 53 to 78).

5.1.2 Gram-negative sepsis, patients defined

Twenty-four patients from the Queen Elizabeth and Selly Oak hospitals (Birmingham, UK) were enrolled into this study. They were a heterogeneous group that were acutely unwell for an average of two days prior to producing a positive blood culture. The patients were enrolled after being verified by a Consultant Microbiologist.

Sepsis was defined as the presence of two or more clinical signs of a systemic inflammatory response syndrome (SIRS) in the presence of documented systemic infection (Bone *et al.*, 1992).

Table 1.1 in chapter 1 has a complete list of the clinical signs of SIRS. In this study the clinical signs indicating systemic inflammation were; a temperature < 36°.6C (96°F) or > 38°.3C (101°F) and a white cell count >12.0 x 10^9/l or < 4.0 x 10^9/l. If the former parameter was not present then other parameters of SIRS would be considered. An elevated C-reactive protein was accepted as a substitute for the white cell count if the latter was not available. The patients had a positive blood culture containing Gram-negative bacillus, after subculture these isolates were identified by routine microbiological methods. There were 10 males with an average age of 60.1 years (age range 43 to 74), and 14 females with an average age of 62.8 years (age range 41-92).

5.1.3 Gram-positive bacterial sepsis, patients defined

Twenty patients from the Queen Elizabeth and Selly Oak hospitals (Birmingham, UK) were enrolled onto this study. They were a heterogeneous group that were acutely unwell for an average of two days prior to producing a positive blood culture. Sepsis was defined as given in 5.1.2 and the diagnosis was verified by a Consultant Microbiologist. There were 12 males with an average age of 55.3 years (age range 29 to 82) and 8 females with an average age 46.6 (age range 28 to 68).

5.1.4 Inclusion criteria.

Patients with pyrexia and a Gram-negative or Gram-positive isolate in their blood culture together with a raised white cell count or C-reactive protein. Control patients without a recent (twelve weeks) documented infection. Patients meeting the sepsis criteria were included regardless of their underlying disease.

5.1.5 Exclusion criteria

Patients were excluded if they had recently experienced an infection. The laboratory database Telepath was checked for this information. Patients were excluded if they had both a Gram-positive and a Gram-negative microorganism in their blood culture.

5.1.6 Patient codes

Patients have been assigned a code for data/patient protection. In this current phase the control patients are C and a number; the Gram-positive patients are P and a number and the Gram-negative group N and a number. This system is used to differentiate the patients in this chapter from those in phase1.

5.2 METHODS and MATERIALS Phase 2

The assays for the selected test markers sICAM-1; LBP; and anti-lipid S antibodies, were conducted as given in chapter three sections 3.4.4, 3.4.8 and 3.4.9 respectively. The non-high sensitivity IL-6 and TNF-α (Quantikine® R&D) assays were performed for the first time here on patient's serum samples from this current phase (2). The methods and materials for these assays are given in 5.2.1 and 5.2.2

5.2.1 INTERLEUKIN-6 ASSAY (minimum detection dose < 0.7pg/ml)

Materials included in the Quantikine® Assay Kit, R&D Systems Oxen, UK.

5.2.1.1 Materials IL-6

96 well polystyrene microplate; Comprising of a frame that held 12 separate strips of 8 wells coated with murine monoclonal antibody against IL-6. The plate is labelled down the length with letters A to H and across the top with numbers1 to12.

IL-6 Conjugate; 21ml of polyclonal antibody against IL-6 conjugated to horseradish peroxidase

IL-6 Standard for the calibration curve; 1.5ng of recombinant human IL-6 in a buffered protein base.

Assay Diluent (RD1A); 11ml of buffered protein base.

Calibrator diluent (RD6F); 21ml of animal serum for use with serum/plasma samples.

Wash Buffer concentrate; 21ml of a 25-fold concentrated solution of buffered surfactant.

Colour Reagent A; 12.5ml of stabilised hydrogen peroxide

Colour Reagent B; 12.5ml of stabilised chromogen (tetramethybenzidine-TMB).

Stop solution; 6ml of 2 N sulphuric acid.

Plate covers; 4 adhesive strips.

Additional materials (not included in the kit).

Gilson pipettes and sterilised disposable tips, multi-channel pipette; graduated measuring cylinders 100ml and 500ml, distilled water, Anthos 2001 plate reader (Labtech, Ringmer, East Sussex UK), with a 450nm setting and a correction setting of either 540 or 570nm.

5.2.1.2 PreparationsIL-6 assay

The microplate and all the reagents where removed from the fridge and brought to room temperature before proceeding. Patient samples were thawed. The wash buffer was prepared by adding 480ml of distilled water to 20ml of the concentrated wash buffer. The IL-6 standard was reconstituted with 5ml of calibrator diluent (RD6F) and gently

agitated for 15 minutes to form a stock solution of 300 pg/ml. Seven test tubes were labelled 1 to 7. 667µl of calibrator diluent was added to tube 1 and 500µl to tubes 2 to 7. 333µl of the reconstituted standard (300pg/ml) was transferred to tube 1 and thoroughly mixed by aspiration and ejection using a Gilson pipette. 500 µl of the diluted standard in tube 1 was transferred to tube 2 with a new disposable pipette tip This process was repeated from tube 2 to tube 6 to obtain the standards for the calibration curve. Tube 7 was the zero standard (0 pg/ml) containing calibrator diluent and the undiluted standard was the high standard (300pg/ml). The dilutions from tube 1 halved the concentration of IL-6 per dilution; tube 1 (100pg/ml) to tube 6 (3.12pg/ml). The substrate was prepared during the assay 15 minutes before use and protected from the light.

5.2.1.3 Method IL-6 Assay.

100µl of assay diluent (RD1A) was added to each well using a Gilson multi-channel pipette. 100µl of each standard was consecutively placed into wells A1 to H1 using a sterile tip for each standard. The 300pg/ml standard was in A1 and the zero standard in well H1. 100µl of each patient's sample was added to their pre-designated well. The plate was covered with an adhesive strip and incubated at room temperature for 2 hours. The adhesive strip was removed and a fresh strip was applied to the base of the wells to avoid the wells becoming dislodged when the plate was inverted. The microplate was washed 4 times with the prepared wash buffer as described previously in 'methods common to all assays' chapter three, section 3.3.1. 200µl of conjugate (antibody against IL-6 conjugated to horseradish peroxidase) was added to each well. The plate was covered with an adhesive strip and incubated at room temperature for 2 hours. The conjugate was then discarded and the wells were washed four times. (During the last incubation period the substrate was prepared. 10ml of Reagent A was added to 10mls of Reagent B in a clean container and protected from the light with aluminium foil). Directly following the last wash, 200µl of substrate was added to each well using a multi-channel pipette; the microplate was covered with foil and incubated for 20 minutes at room temperature. 50µl of stop solution was added (2N sulphuric acid). The optical density was measured using a microplate reader (Anthos, 2001) set at 450 nanometres (nm) with a wavelength correction setting of 570nm. The correction setting subtracts any optical interference from the microplate.

A print out of the absorbance from each well was used with a data analysis computer package to produce the calibration curve from the standards. The unknown

levels of IL-6 in patient samples were computer generated from the data. The results from this assay are given in the form of scatter graphs.

5.2.2 TUMOUR NECROSIS FACTOR – alpha (minimum detection dose < 4.4 pg/ml)
Materials included in the TNF-α Quantikine® Assay Kit, R&D Systems Oxen, UK.

5.2.2.1 **Materials TNF-α**

96 well polystyrene microplate; Comprising of a frame that held 12 separate strips of 8 wells coated with murine monoclonal antibody against TNF-α.

TNF-α Conjugate; 21ml of polyclonal antibody against TNF-α conjugated to horse-radish peroxidase.

TNF-α Standard for the calibration curve; 3 vials (1 ng/vial) of recombinant human TNF-α in a buffered protein base.

Assay Diluent (RD1F); 6ml of buffered protein base for serum/plasma samples.

Calibrator Diluent (RD6); 21ml of animal serum for use with serum/plasma samples

Wash Buffer concentrate; 21ml of a 25- fold concentrated solution of buffered surfactant.

Colour Reagent A; 12.5ml of stabilised hydrogen peroxide.

Colour Reagent B; 12.5ml of stabilised chromogen (tetramethybenzidine-TMB).

Stop solution; 6ml of 2 N sulphuric acid.

Plate covers; 8 adhesive strips.

Additional materials (not included in the kit);

Gilson pipettes (50µl and 200µl) and sterilised disposable tips, Anthos 2001 plate reader (Labtech, Ringmer, East Sussex UK), multi-channel pipette; graduated measuring cylinders 100ml and 500ml.

Incubation refrigerator 2-8°C.

5.2.2.2 **Preparations TNF-α assay**

The microplate and all the reagents where removed from the fridge and brought to room temperature. Patient samples were thawed. The wash buffer was prepared by adding 480ml of distilled water to 20ml of concentrated wash buffer. The TNF-α standard was reconstituted with 1ml of calibrator diluent (RD6) and gently agitated for 15 minutes to form a stock solution of 1000 pg/ml. 500µl of calibrator diluent was added to 7 test tubes, labelled 1-7. 500µl of the reconstituted TNF-α standard was added to tube 1.

Using a Gilson pipette the solution in tube 1 was mixed thoroughly. A fresh sterile pipette tip was used to remove 500 µl of this solution to tube 2 and mixed. This process was repeated from tube 2 to tube 6 to obtain the standards for the calibration curve. Tube 7 was the zero standard (0 pg/ml) containing assay diluent. The reconstituted standard was the high standard -1000pg/ml. The dilution series halved the concentration of TNF-α per dilution; tube 6 had a concentration of 1000pg/ml to 15.6pg/ml. The substrate was prepared during the assay 15 minutes before use and protected from the light with aluminium foil.

5.2.2.3 Method TNF-α assay

50µl of assay diluent (RD1F) was added to each well using a Gilson pipette, then 200µl of each standard was consecutively placed into wells A1 to H1 using a sterile tip for each transfer. The 1000pg/ml standard was in A1 and the zero standard was in well H1. 200µl of each patient's sample was added to their designated well according to the data sheet protocol. The plate was covered with an adhesive strip and incubated for 2 hours at room temperature. The adhesive strip was removed and a fresh strip was applied to the base of the wells prior to washing. The microplate was washed 3 times with the prepared wash buffer as described previously in chapter three section 3.3.1.

200µl of conjugate (antibody against TNF-α conjugated to horseradish peroxidase) was added to each well. The plate was covered with an adhesive strip and incubated at room temperature for 2 hours. The substrate was prepared by adding 10ml of Colour Reagent A to 10ml of Colour Reagent B in a clean plastic container and protected from the light with foil. After incubation the plate was emptied and washed 3 times. 200µl of the prepared substrate solution was added to each well and covered with aluminium foil and incubated at room temperature for 20 minutes. The reaction was stopped with 50ml of 2N sulphuric acid (stop solution). The optical density was measured using a plate reader (Anthos 2001) set at 450 nanometres (nm) with a wavelength correction setting of 570nm. The correction setting subtracts any optical interference from the microplate. Computer generated results from this assay are given in the results in the form of scatter graphs.

5.3 RESULTS PHASE 2

The new assay types described in this chapter for TNF-α and IL-6 and the assays described in chapter 3 for sICAM-1, LBP and anti-lipid S IgG antibodies were carried out with the phase 2 patients; the results and statistical analysis are presented here. Tables 5.1, 5.2 and 5.3 below give the patient characteristics for each patient group. The assay results are presented graphically as scatter graphs in figures 5.1-5.6.8

Table 5.1 CONTROL PATIENT CHARACTERISTICS: Underlying disease (if any); white cell count (WCC); temperature (°C) blood pressure (BP) and pulse in rates /minute (RPM)

PATIENT CODE	UNDERLYING DISEASE	WCC 1×10^9/L	TEMP °C	BP	PULSE (RPM)
C1	TVCAD/HC	8.6	36.5	129/87	56
C2	COAD/HC/HT	6.3	apyrexial	146/58	55
C3	TVCAD/arthritis	9.6	36.4	110/54	52
C4	HC	11.2	37	nk	nk
C5	CAD/HT/HC	13.1	nr	129/75	96
C6	CAD/HT/HC	12.8*	nr	121/63	80
C7	TVCAD	9.7	nr	nk	nk
C8	CAD/HT	8.7	35.9	160/91	51
C9	TVCAD/HC/HT	5.1	nr	116/68	70
C10*	TVCAD	10.2	nr	110/70	70
C11*	COPD/HT/HC	15.5	39	121/71	66
C12*	TVD	9.7	37	97/67	58
C13	Psoriasis-osteo.	nd	nr	141/82	90
C14	Severe TVD	4.5	35.8	167/85	60
C15	TVD	6.7	37.2	140/70	68
C16	CAD/HT/HC	10.0	nr	107/86	80
C17	CAD/HT/HC	nd	nr	146/88	90
C18	CAD/HT/HC	9.2	36.5	147/111	74
C19	CAD/HT/HC	nd	37.5	139/70	60
C20	Chronic RF	8.5	nr	127/71	85
C21*	CAD/lung ca.	18.3*	nr	128/75	70
C22	Spondylosis TVD	8.8	nr	114/80	91
C23	CAD	13.6	36.4	180/100	60
C24*	TVD/HT/firbros.alv	4.8	nr	130/65	65
C25	TVD/HC	11.5	nr	110/67	67
C26	TVD/Ca bowel/liv	7.0	nr	180/92	65

TVCAD-triple vessel coronary artery disease. HT-hypotension. HC-hypercholesterol. COAD- chronic obstructive airway disease. CAD- coronary artery disease. COPD – chronic obstructive pulmonary disease. osteo- osteoarthritis. RF-renal failure. Ca-carcinoma. firbros.alv-fibrosing alveolitis. Ca bowel/liv-cancer bowel/liver. nr-no record.

The control patients admitted for coronary artery by-pass graphs (CABG) have blood samples taken pre-operatively for routine haematology. However on investigation the

patients marked with an asterisk in table 5.1 had their sample taken post operatively and this may account for the raised white cell count in some of these patients. All the patients had coronary artery disease but a few had other underlying diseases such as spondylosis (C12), arthritis (C3) renal failure (20) and two had cancer, C21 (lung) and C26 (liver).

Table 5.2 GRAM-POSITIVE PATIENTS CHARACTERISTICS: Underlying disease; white cell count (WCC); C-reactive protein (CRP) and Gram-positive microorganism in blood-culture

PATIENT Code	BC date Days	UNDERLYING DISEASE	ISOLATE IN Blood culture	WCC 4-11x10^9/L	CRP < 10 mg/L
P1	0	Renal dis.	MRSA	11.4	35
P2	0	50% burns	MRSA	6	133
P3	0	New Diabetic	CNS	11.8	nd
P4	0	pyrexia-loin pain	CNS	14.1	32
P5	0	Breast cancer	*Coryne*.spp.	4.4	59
P6	0	Non HLymphoma	CNS	0	119
P7	2*	pulmonary oedema	*Staph. aureus*	10.5	58
P8	0	Hepatic cancer	*Strep. pneumonia*	6.3	137
P9	0	Cancer -tongue	Strep Group G	16.8	11
P10	1	AML	CNS	0.3	241
P11	1	Stroke/ diabetic	*Staph. aureus*	11.5	9
P12	1	Head injury/haem	*Staph. aureus*	9.4	80
P13	1	IDDM/ abscesses	*Staph. aureus*	27.3	106
P14	1	Epilep /neuroma	CNS	2.1	119
P15	2	AML	*Staph. epidermidis*	0.7	50
P16	2	Chronic Renal F	*Staph. aureus*	16.9	182
P17	2	Crohn's disease	CNS	2.8	89
P18	2	Isch bowel	MRSA	16.1	89
P19	3	Lewy body dem.	MRSA	6.8 (25.9)	nd
P20	9	fever inf joint	*Staph. aureus*	18.3	63

BC- blood culture. dis –disease. CNS-coagulase negative staphylococcus. – Coryne-spp-*Corynebacterium* species (a Gram positive rod). Non H Lymphoma-non Hodgkin's lymphoma. AML-acute myoloid leukaemia. Haem-haematoma. Isch bowel-Ischemic bowel. IDDM –insulin dependent diabetes mellitus. Epilep-epilepsy. Renal F-renal failure; dem-dementia. inf-infection.

'BC date days' this column in table 5.2 represents the number of days between the positive blood-culture day and the test sample date. 0, means that the test sample date was the same day as the blood culture date. Patient P5 had chemotherapy three days prior to the positive blood culture. Patient P9 had just completed a course of radiotherapy that produced chronic inflammation of the mastoid air cells. Patient P10 and P15 had neutropenia post chemotherapy and both had acute myeloid leukaemia (AML). Patient P14 and was on intravenous steroids. Steroids are anti-inflammatory drugs that effect the production and activity of white blood cells (see table 5.9.4.1) to produce antibodies and

the cytokines they secrete, including TNF-α and IL-6 (Rang *et al.*, 1995). Patient P19 – died 3 months later (dementia) his WCC on the day before his blood culture was taken was 25.9 (x 10^9/l), but was 6.8 on the day of the test sample. All the patients with a positive blood culture had a raised body temperature.

Table 5.3 GRAM-NEGATIVE PATIENTS CHARACTERISTICS: Underlying disease; white cell count (WCC); C-reactive protein (CRP) and Gram-positive microorganism in blood-culture

PATIENT	BC date	UNDERLYING	ISOLATE IN	WCC	CRP
Code	Days	DISEASE	Blood culture	4-11x10^9/L	< 10 mg/L
N1	0	Non HL –post BMT	*E. coli*	0.8	403
N2	0	CLL	*Citrob. freundii*	39	422
N3	0	Burns 80%/amp.	*P. aeruginosa*	16.4	245
N4	0		*S. paratyphi*	4.2	150
N5	0	AML	*Enter.cloacae*	0.2	229
N6	0	Post pbsct	Coliform	0.9	261
N7	1	Pancreatic cancer	*E. coli*	14.4	130
N8	1	ALD	*E. coli*	nd	147
N9	1	RF 2° rhab.	*E. coli*	20	nd
N10	1	Cancer (YPU)	*Sten. maltophilia*	9.0	107
N11	1	cholecystitis/NIDDM	*Enterobacter* spp.	12.1	321
N12	1	ESRF	*P.mirabalis*	11.7	196
N13	1	Diverticulitis	*Acin, baumanni*	7.3	96
N14	1	Carcinoma	*K.pneumonia*	0.9	344
N15	1	(on liver unit)	*Citrob. freundii*	12.7	122
N16	1	Pyelonephritis	*E. coli*	26.7	150
N17	1	RTA	*Citrob. freundii*	10.3	94
N18	1	(92yrs)	*E. coli*	17.1	98
N19	2	Pace-maker wire inf.	*Proteus* spp	13.4	181
N20	3	Liver ca/liver resect	*Serratia* spp	0.5	nk
N21	3	ALD	*E. coli*	15.7	nk
N22	3	Cholangitis	*K.pneumonia*	9.8	91
N23	3	Recurrent pneumonia	*E. coli*	11.6	154
N24	3	Renal failure.	*E. coli*	17.3	177

Non HL Non HL Non Hodgkin's lymphoma. BMT- bone marrow transplant. CLL-chronic leukocyte leukaemia. *Citrob-citrobacter. Enter.*spp.-*Enterobacter* species. pbsct-peripheral-blood stem cell transplant. AML-acute myeloid leukaemia. RF 2° rhab- renal failure secondary to rhabdomyolysis. YPU-young person unit. nd.-not done. NIDDM-non insulin dependent diabetes mellitus. ESRF-end stage renal failure. RTA-road traffic accident. Resect-resection. ALD –alcoholic liver disease. nk-not known.

The majority of the WCC and the CRP levels recorded in table 5.3 are the levels in a patient's blood on the day the blood culture was taken, with a few exceptions.

5.4 SCATTER GRAPHS; illustrating the assay results from patients in Phase 2

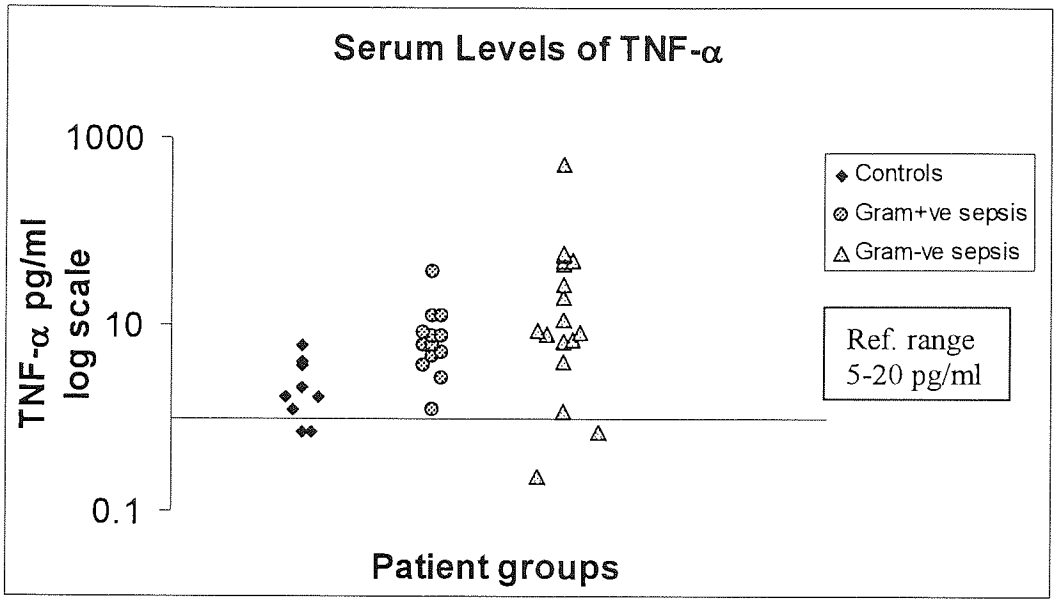


Figure 5.1 SCATTER GRAPH showing serum levels of TNF-α in controls, Gram-positive and Gram-negative sepsis patients.
Scatter graphs with a log scale cannot present zero values. Seventeen patients in the control group, six Gram-negative patients and seven Gram-positive patients had no detectable TNF-α in their serum sample.

Table 5.4 Median and confidence interval (CI) for TNF-a pg/ml; all patient groups

TNF-α	Controls	Gram-positive sepsis	Gram-negative sepsis
Median	0.00	4.10	7.48
CL% (CI)	97.1 (0.00 to 0.72)	95.9 (0.00 to 7.48)	97.7 (0.241 to 26.8)
Range	6.03	37.9	525* (or 60.)

As described previously the confidence level (CL, a percentage, usually 95%) is a measure of statistical confidence that the confidence interval (CI) contains the median value, and the median is the value that divides the data into two equal halves. The range is the difference between the largest and smallest serum sample in the assay. This statistic is sensitive to the outliers whereas the median is not.

*Patient N10 was a juvenile with cancer, his serum TNF-α level was 525 pg/ml. This extraordinarily high level of TNF-α is probably due to his cancer and not a function of his sepsis. The next highest level of this cytokine was 60.1 pg/ml and is less than the comparable value of 107pg/ml from phase 1 patients with Gram-negative sepsis given in chapter 4 table 4.3.4

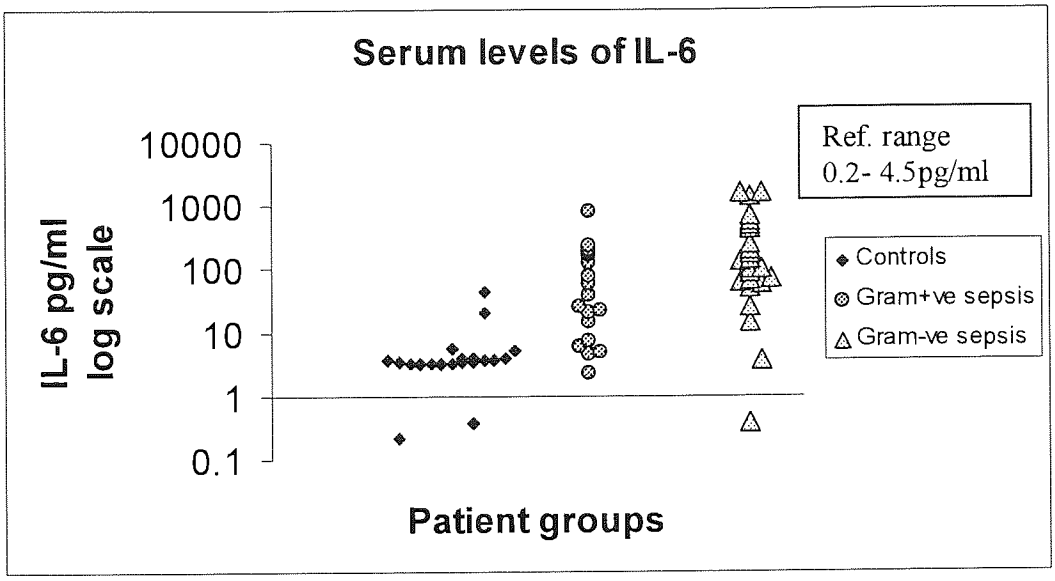


Figure 5.2 SCATTER GRAPH showing serum levels of IL-6 in controls, Gram-positive and Gram-negative sepsis patients.

The straight line cluster of IL-6 in the control group shown in figure 5.2 reflects the expected reference range for this marker in patients without an inflammatory immune response.

Table 5.4.1 Median and CI for IL-6 pg/ml; all patient groups

IL-6 pg/ml	Controls	Gram-positive sepsis	Gram-negative sepsis
Median	3.31	49.11	120
CL% (CI)	97.1 (0.37 to 3.8)	95.9 (15.14 to 174.93)	97.7 (61.90 to 453)
Range	45.6*	821	1699

*Patient (C13, a control) had an unexpectedly high level of IL-6 (45.6 pg/ml). The range for the controls excluding this result was 20.2 pg/ml (the reference range in healthy adults 0.2-4.5pg/ml) and is comparable to the control range from the phase1 see table 4.3.3. Further investigation showed that patient C13 had on-going psoriasis and osteoarthritis.

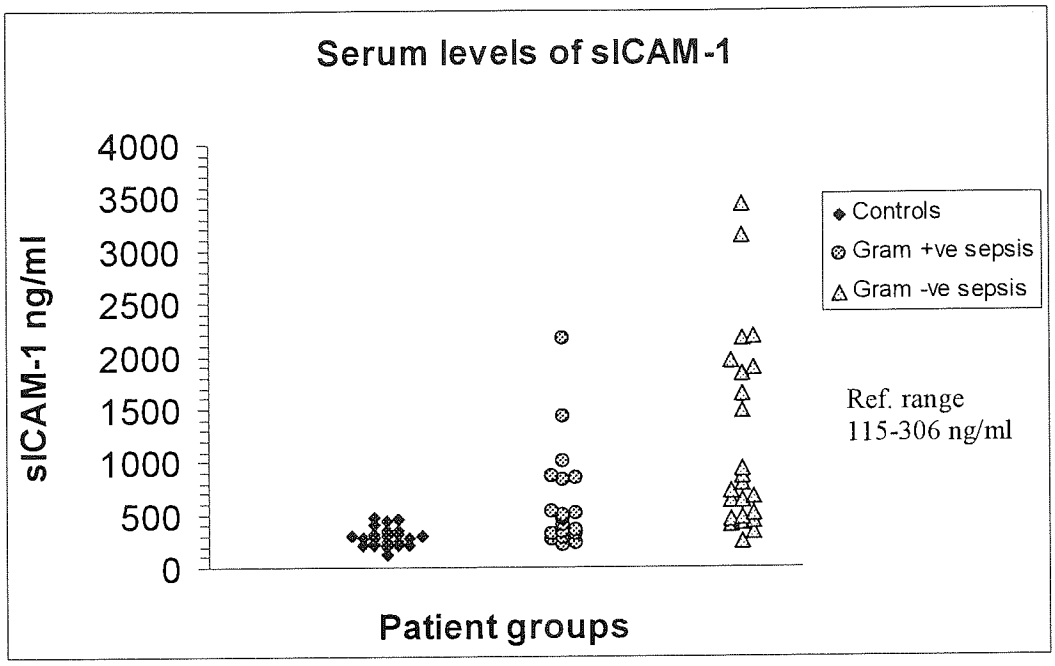


Figure 5.3 SCATTER GRAPH showing serum levels of sICAM-1 in controls, Gram-positive and Gram-negative sepsis patients.

This graph shows that the control group had serum levels of sICAM-1 below 500 ng/ml. However eleven of the Gram-positive and seven of the Gram-negative patients also had levels of sICAM-1 below 500ng/ml see figure 5.3.1

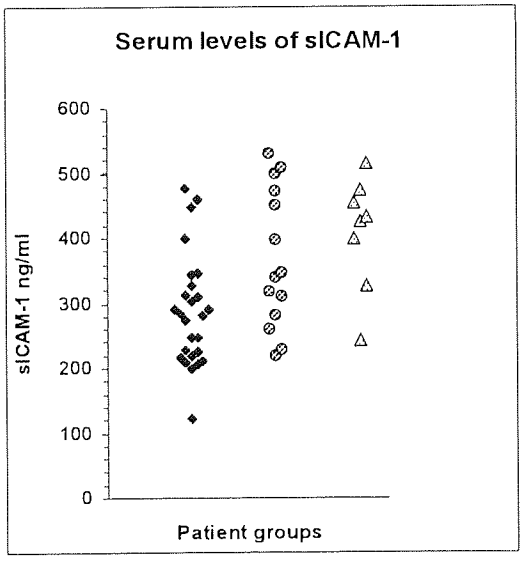


Figure 5.3.1 shows the lower range of serum sICAM-1 levels (0.0 to 500 ng/ml) from figure 5.3 for all the patient groups

If a cut-off level of 500ng/ml sICAM-1 in a patient's serum was used for a diagnosis of sepsis there would be a total of eighteen false negatives. However table 5.3 shows that

serum levels of sICAM-1 are highly elevated in some patients with sepsis. For this reason two markers would be required to demonstrate septic serum.

Table 5.4.2 Median and CI for sICAM-1 ng/ml; all patient groups

sICAM-1	Controls	Gram-positive sepsis	Gram-negative sepsis
Median	282	461	717
CL% (CI)	97.1 (222 to 314)	95.9 (317 to 832)	95.7 (515 to 1632)
Range	356	1936	3197

Table 5.4.2 gives the medians and confidence level (CL) as a percentage and the confidence level as a range around the median value. The range is the difference between the largest recorded sample and the lowest.

There is a clear distinction between the median level of serum sICAM-1 in the controls and the median levels in patients with sepsis.

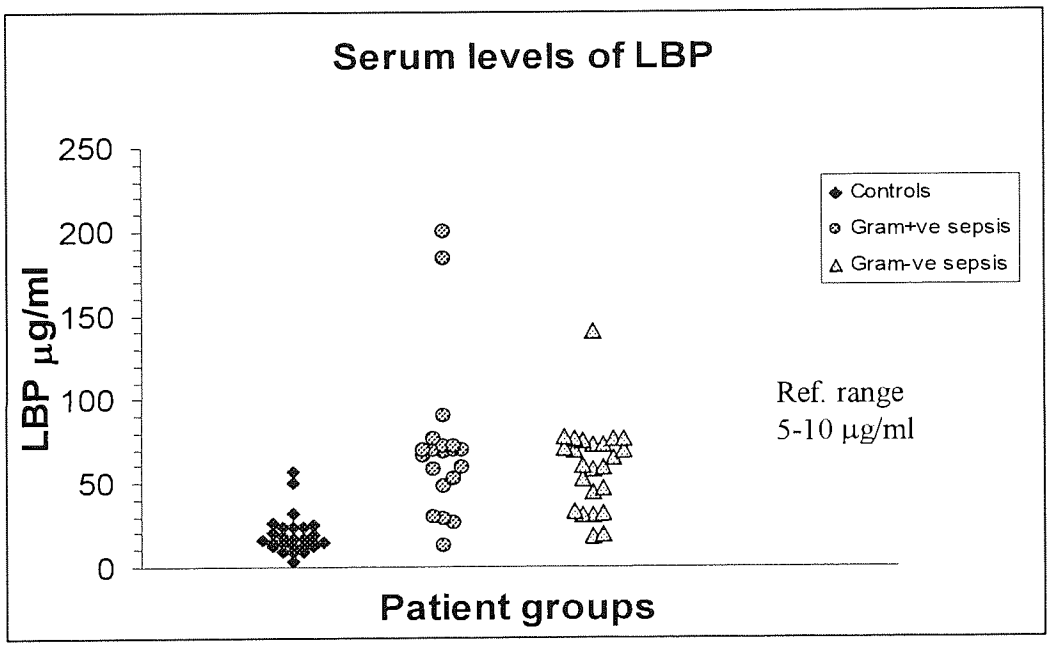


Figure 5.4 SCATTER GRAPH showing serum levels of LBP in controls, Gram-positive and Gram-negative septicaemia patients.

Figure 5.4 shows that a cut-off value in excess of 30µg/ml of LBP in serum would be required for a diagnosis of sepsis. In this case there would be 2 false positive controls and 4 false negative Gram-positive cases and 2 false negative results in the Gram-negative group.

Table 5.4.3 Median and CI for LBP µg/ml all patient groups

LBP µg/ml	Controls	Gram-positive sepsis	Gram-negative sepsis
Medians	16.45	68.7	62.5
CL% (CI)	97.1(13.0 to 23.5)	95.9(52.0 to72.0)	97.7(44.6 to 73.6)
Range	54	187	121

*Patient C13 (a control), had an elevated level of serum LBP as well as high IL-6 see table 5.4.1 This patient had on-going psoriasis and osteoarthritis.

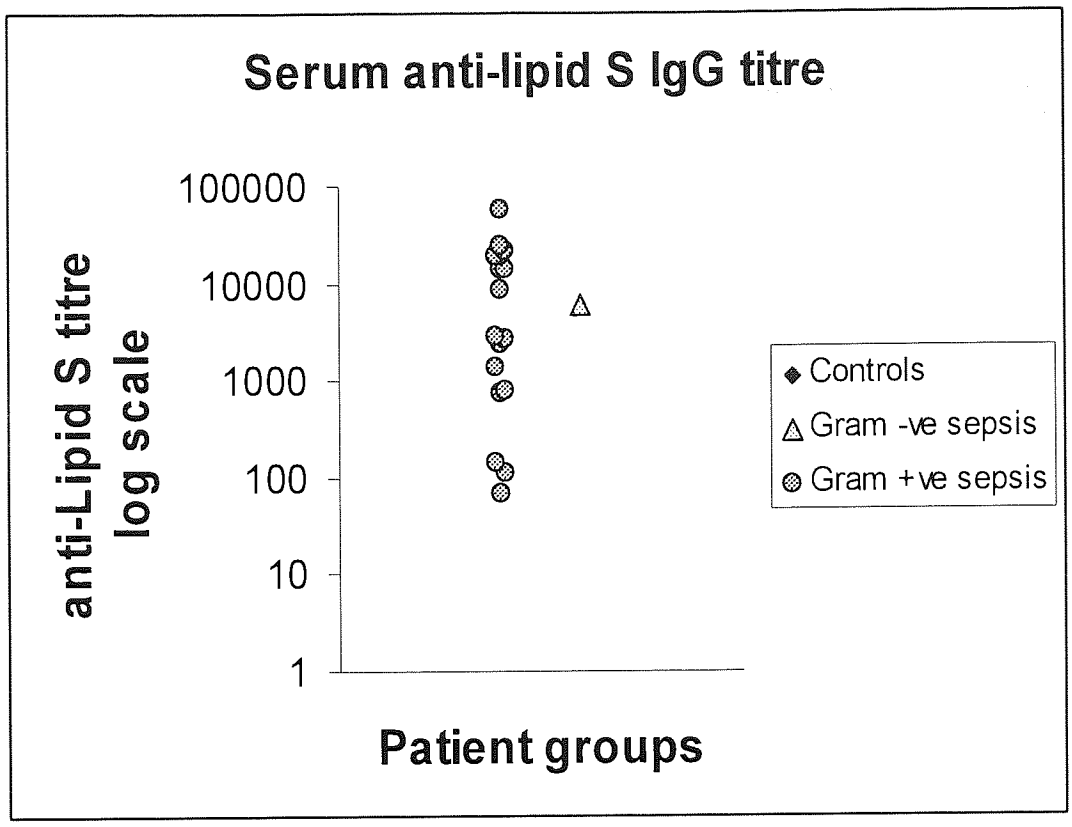


Figure 5.5 SCATTER GRAPH showing serum levels of anti-lipid S immunoglobulin (IgG) titres in Gram-positive and Gram-negative sepsis patients.

All the controls were negative for anti-lipid S immunoglobulin as were 23/24 patients in the Gram-negative group; negatives values cannot be plotted correctly on a log scale graph.

Table 5.4.4 Median and CI for anti-lipid S IgG EIU all patient groups

Lipid S	Controls	Gram-positive sepsis	Gram-negative sepsis
Median	0	2560	0
CL% (CI)	-	95.9 (140 to14227.0)	97.7 (0.000 to 0)
Range	0.0	58389	5930

EIU Enzyme immunoassay units.

Table 5.4.4 gives the levels of serum antibody to lipid S and exhibits excellent differential marker characteristics by being positive in the Gram-positive sepsis group as opposed to the controls or patients with sepsis due to a Gram-negative microorganism.

Table 5.5 Mann-Whitney test (Phase 2) for non-parametric *p*-values

2-tailed *p*-value					
	TNF-α	IL-6	sICAM-1	LBP	Lipid S
Controls with G-positive sepsis	0.0034	<0.0001	<0.0003	<0.0001	<0.0001
Controls with G-negative sepsis	0.0002	<0.0001	<0.0001	<0.0001	0.3074
Controls with Total sepsis*	0.0002	<0.0001	<0.0001	<0.0001	<0.003

2-tailed *p*-values (normal approximation, with correction for ties). The significance level was set at 0.05.
* Total sepsis combines both the Gram-positive and Gram-negative groups as one group.

Table 5.5 lists the statistical significance of the difference between the median values for all the markers from the patients in this current phase and for comparison, the *p*-values for the same markers from the patients in phase 1 are given in table 5.5.1. All the *p*-values are significant except for anti-lipid S in the Gram-negative sepsis group.

Most noteworthy is the difference between IL-6 in the Gram-positive group in this phase (*p*-<0.0001) and the non-significant result for the same group (different patients) from phase 1 (*p*-0.08).

Table 5.5.1 Mann-Whitney test (Phase 1) for non-parametric *p*-values

2-tailed *p*-value					
	TNF-α	IL-6	sICAM-1	LBP	Lipid S
Controls versus G-positive sepsis	<0.001	0.08	<0.0001	<0.0001	<0.0001
Controls versus G-negative sepsis	<0.0001	<0.0001	<0.0001	<0.0001	0.013
Controls with Total sepsis	nd	nd	nd	nd	nd

nd –not done.

High sensitivity assays where used in phase 1 for TNF-α and IL-6 which may account for the higher number of patients with detectable levels of TNF-α in phase 1 patients as opposed to the patients tested in phase 2. It is not clear at this point why the IL-6 levels in the Gram-positive group (phase 1) differ so markedly. Stringent inclusion criteria for patients in phase 2 may account for this difference.

5.5 TWO -WAY SCATTER GRAPHS

The scatter graphs in 5.4 show that although the test markers are elevated in patients with sepsis as opposed to the controls there are many false negatives. Therefore as previously described in chapter 4, two markers combined for a diagnosis of sepsis may eliminate the false negatives. The two-way scatter graphs with the best sensitivities and specificities are presented here. The sensitivities and specificities for all the possible combinations of the test markers are given in tables 5.6; 5.7 and 5.8.

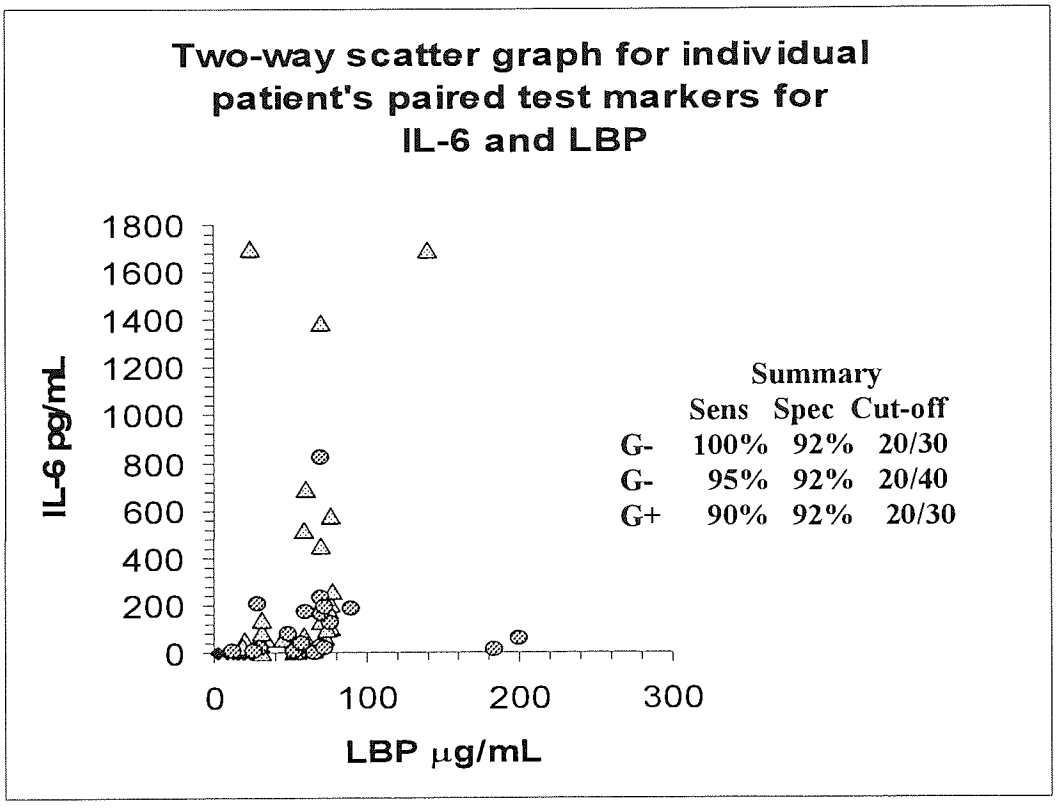


Figure 5.6 TWO-WAY SCATTER GRAPH for IL-6 and LBP
The cut-off levels for these markers in figure 5.6. are for IL-6 first then LBP.

This combination of test markers gave some of the highest sensitivities and specificities for a diagnosis of sepsis from patients in the Gram-negative and total sepsis group. The total sepsis group combined the Gram-positive and Gram-negative groups. The lower range of values for IL-6 and LBP are obscured in figure 5. 6 therefore an expanded version is given in figure 5.6.1.

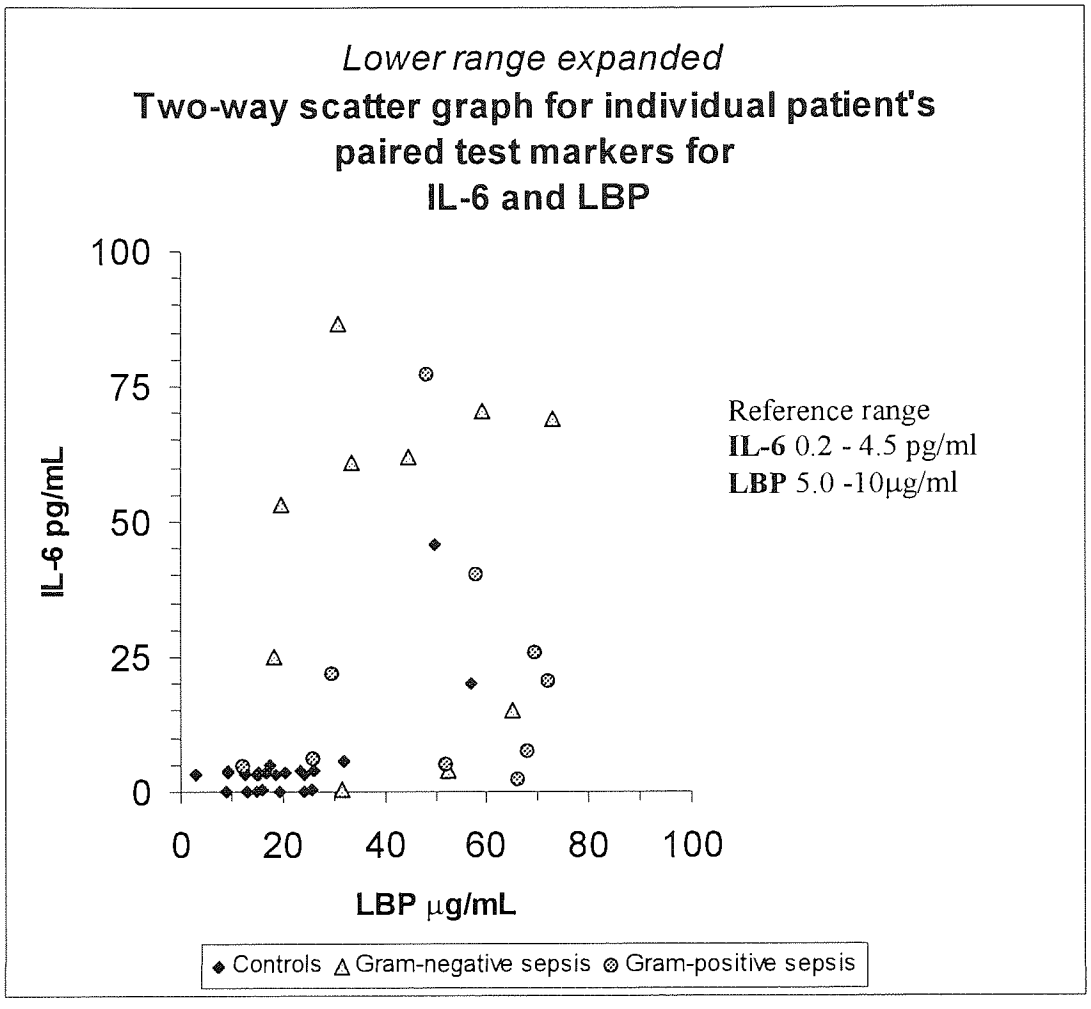


Figure 5.6.1 Lower range of values from figure 5.6.1 for IL-6 and LBP
This graph shows the number and group of patients within the cut-off area 20pg/ml IL-6 and 30µg/ml (or 40µg/ml) for LBP.

If a patient has a minimum 20pg/ml of serum IL-6 with 30µg/ml of LBP for a diagnosis of sepsis then 2 patients from the Gram-positive group would be missed as well as one patient from the Gram-negative group. With these two makers set at this level then 2 controls patients would qualify (on the markers alone) as septic. If these two markers can be elevated in patients without a known infection or sepsis then the value of these two markers for a diagnosis of sepsis is undermined. Other patients known to be septic with only one marker below the cut-off value were pulled into a septic 'diagnosis' by the level of the other marker. Every test marker has been shown to be elevated above the normal level in one or other of the control patients. Therefore it is still important to establish a cut-off level for individual markers such that the level reflects a host's response to infection as opposed to an inflammatory response from any other insult.

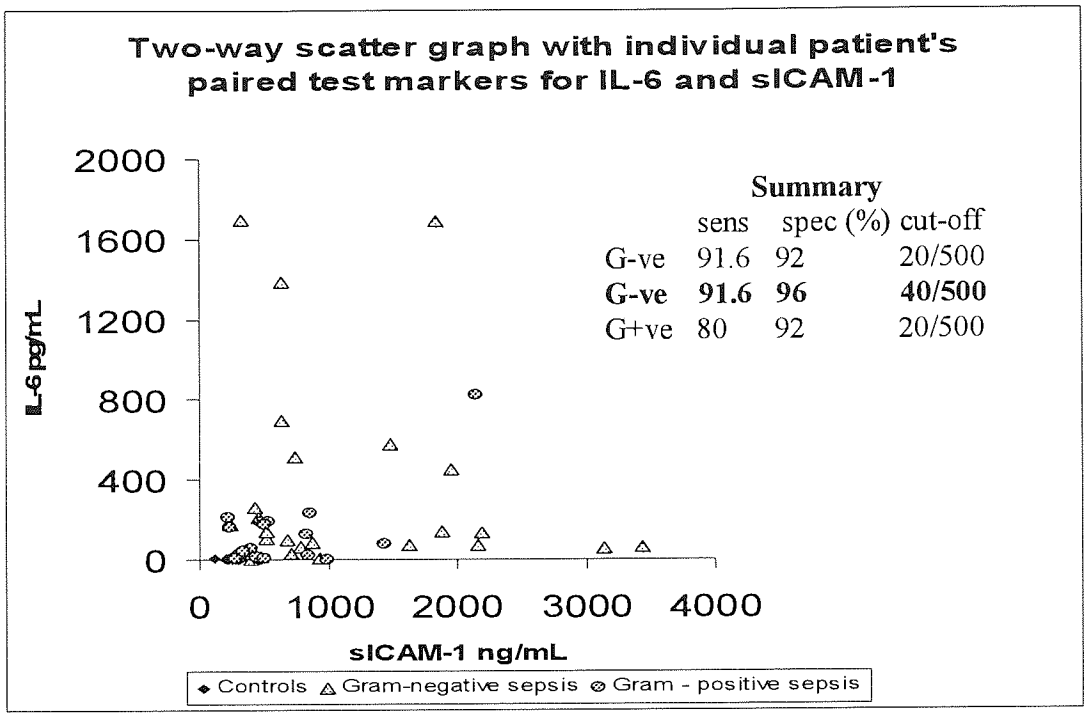


Figure 5.6.2 TWO-WAY SCATTER GRAPH for IL-6 and sICAM-1
The cut-off values used to establish the sensitivities and specificities were 20 and 40pg/ml IL-6 and 500ng/ml for sICAM-1

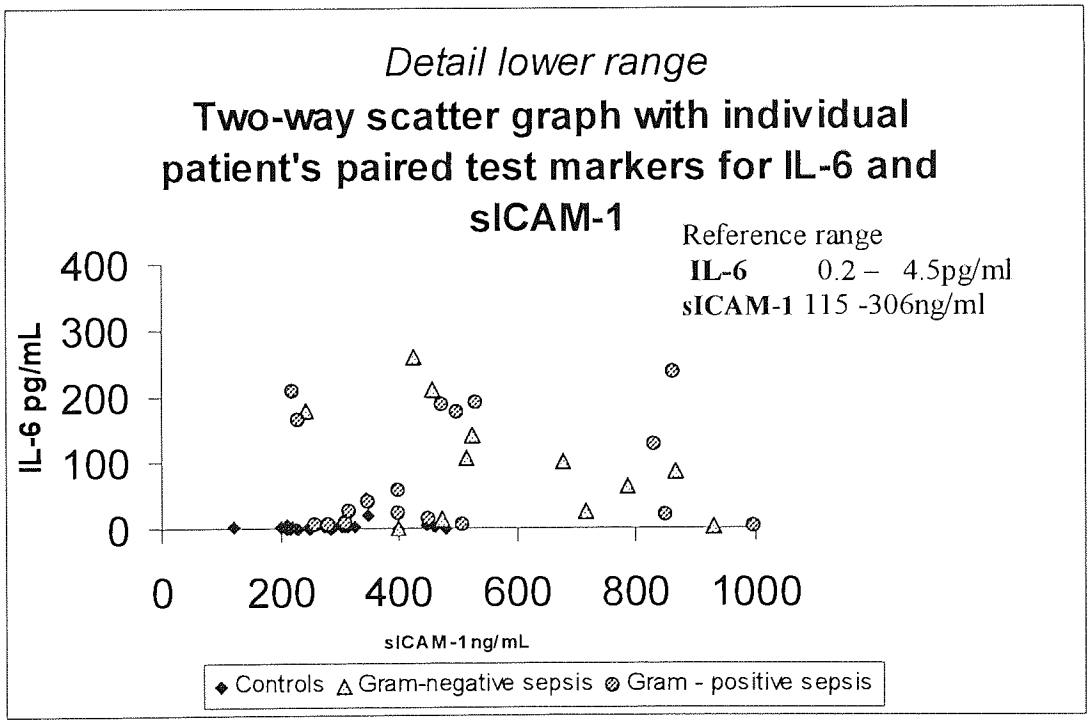


Figure 5.6.3 Lower range of values from figure 5.6.2 IL-6 and sICAM-1. This figure shows the cut-off area for a possible diagnosis of sepsis; 40pg/ml (or 20) of IL-6 with 500ng/ml sICAM-1.

Two patients (P17 and P18) with low levels of IL-6 had test serum taken two and three days post their positive blood culture. The serum kinetics for IL-6 are short term, that is serum levels can peak within 2 hours and then rapidly drop (Meisner, 2000). Another patient (P7) proved not to be septic by definition. The reasons why this patient is still included in this trail will be discussed in section 5.8. sICAM-1 was consistently elevated in patients with a Gram-negative sepsis although there were exceptions and these will be discussed in section 5.8.

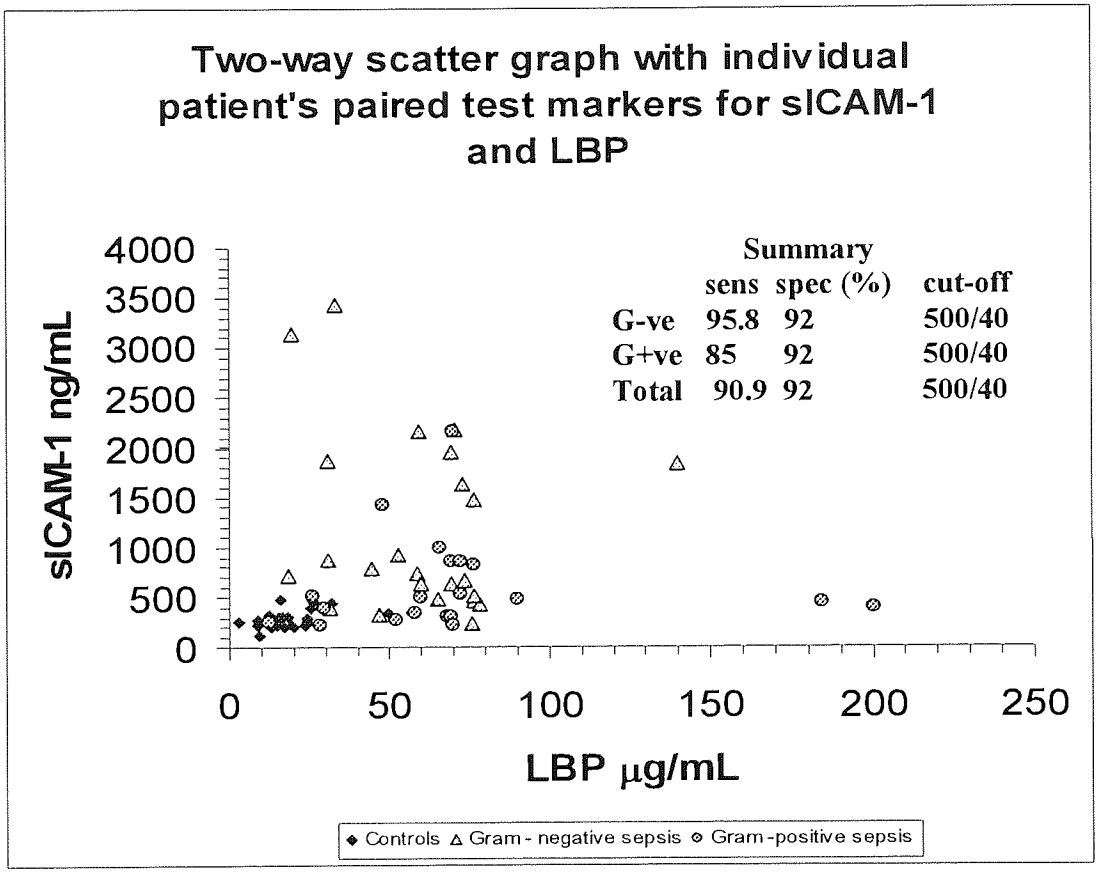


Figure 5.6.4 TWO-WAY SCATTER GRAPH for sICAM-1 and LBP
The cut-off values are for sICAM-1ng/ml and then LBPµg/ml.

sICAM-1 with LBP has a higher sensitivity (95.8%) than sICAM-1 with IL-6 (91.6%) but the specificity is higher with sICAM-1 and IL-6 at 96.1% as opposed to 92.3%; these results relate to patients with Gram-negative sepsis. The best combined marker for patients with a Gram-positive sepsis was IL-6 and anti-lipid S IgG (see figure 5.6.7). The lower cut-off range is obscured in figure 5.6.4 and an expanded version of this range is given in figure 5.6.5.

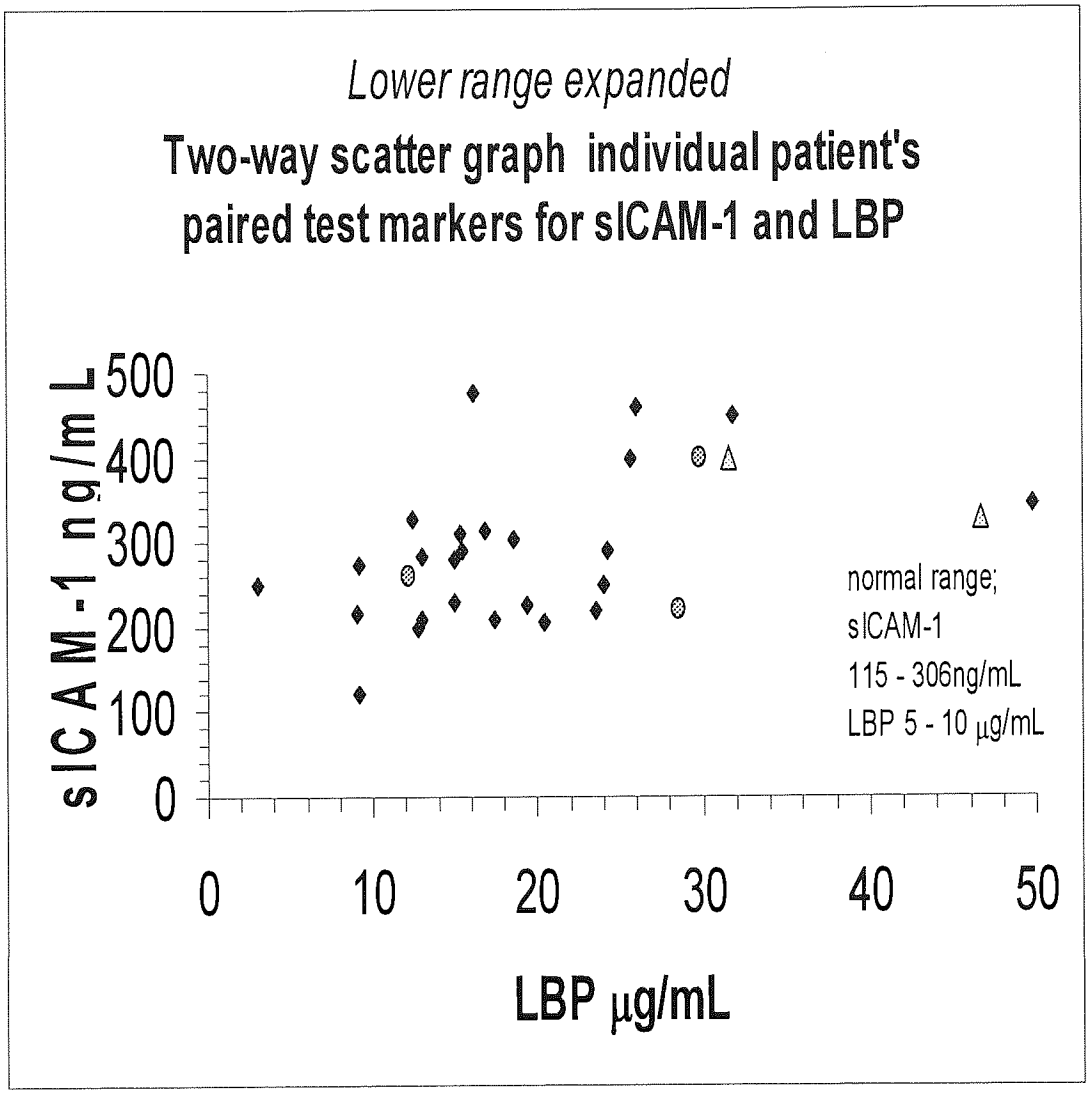


Figure 5.6.5 Lower range from figure 5.6.4 illustrating the proposed cut-off range for sepsis; 500 ng/ml sICAM-1 with 40µg/ml LBP

Figure 5.6.5 clearly shows that 3 Gram-positive patients (circles) and 1 Gram-negative patient (triangle) would be missed as false negatives. There are two controls that are false positives. One (patient C2) had an LBP 57 µg/ml and is off the scale in figure 5.6.5. The other control was patient C13.

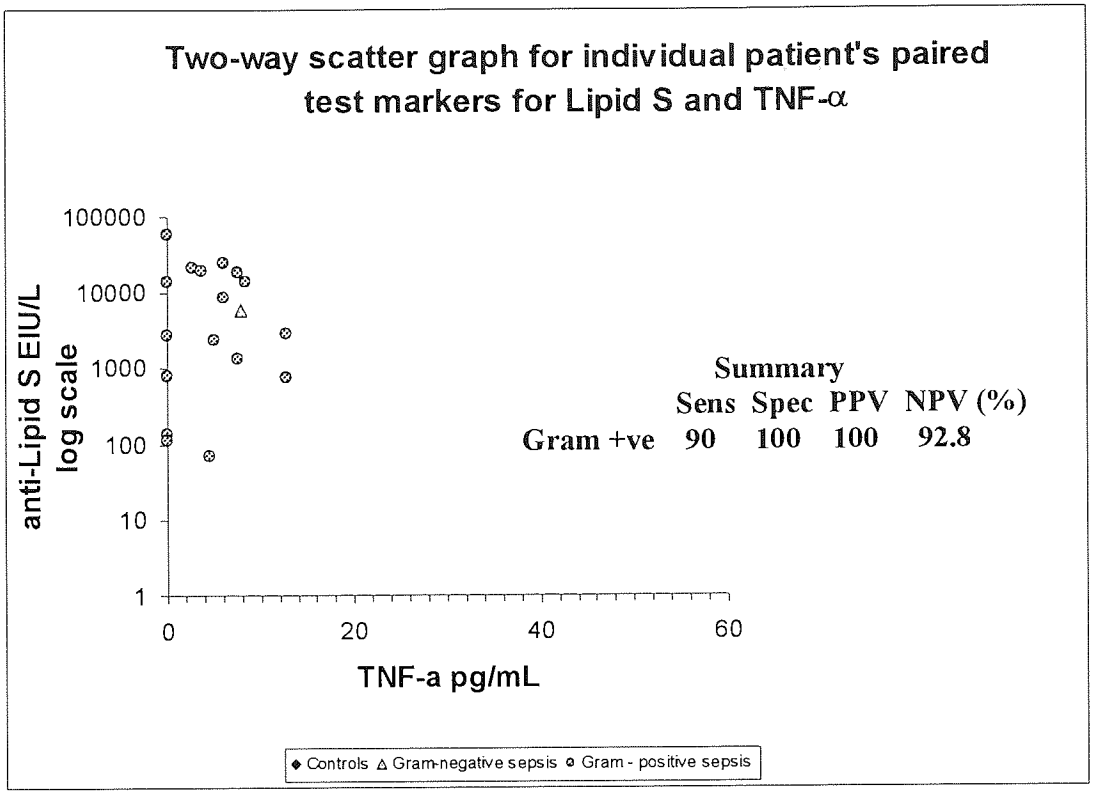


Figure 5.6.6 TWO-WAY SCATTER GRAPH for anti-lipid S and TNF-α. The cut-off levels for the sensitivity, specificity, positive predictive value and the negative predictive values given in figure 5.6.6 are TNF-α 30 (or 20) pg/ml and anti-lipid S greater than 0.

Apart from the combined markers IL-6 and LBP (figures 5.6 and 5.6.1) the best sensitivities and specificities for distinguishing patients with sepsis in the Gram-positive group are those that combine anti-lipid S with a single other test marker. These include TNF-α as shown in figure 5.6.6 and IL-6 shown in figure 5.6.7 and with LBP figure 5.6.8.

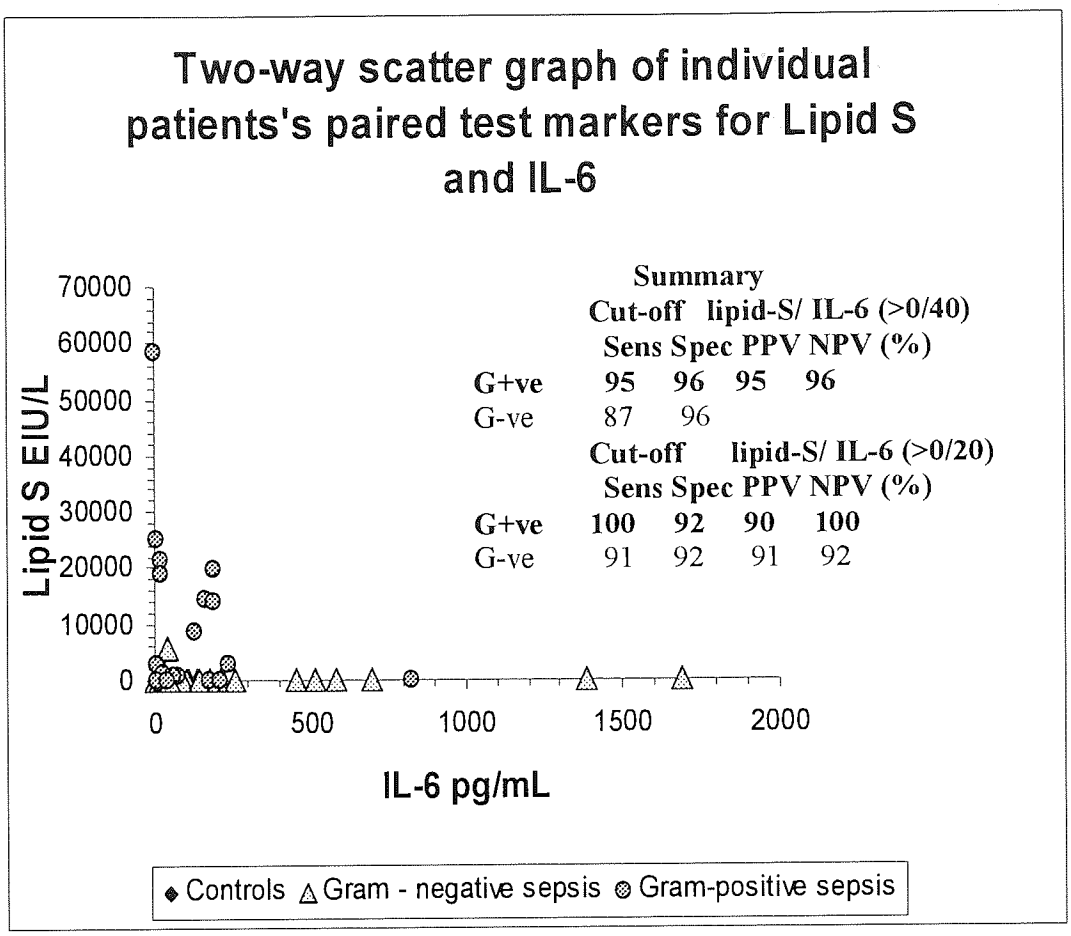


Figure 5.6.7 TWO-WAY SCATTER GRAPH for anti-lipid S and IL-6
The controls were all negative for anti-lipid S and are obscured by the Gram-positive markers (circles).

The high sensitivity (91%) and specificity (92%) for the Gram-positive patients shown in figure 5.6. 7 are due to the sensitivity and specificity of IL-6 in sepsis. However these high values in figure 5.6.7 are calculated from a cut-off level of 20pg/ml IL-6 and there are many disease states and conditions apart from sepsis that could induce this moderate rise in serum IL-6. At a cut-off level of 40pg/ml the sensitivities and specificities remain elevated in the Gram-positive patients but the sensitivity drops to 87% in patients with a Gram-negative sepsis, this value represents four patients with a serum IL-6 below 40pg/ml.

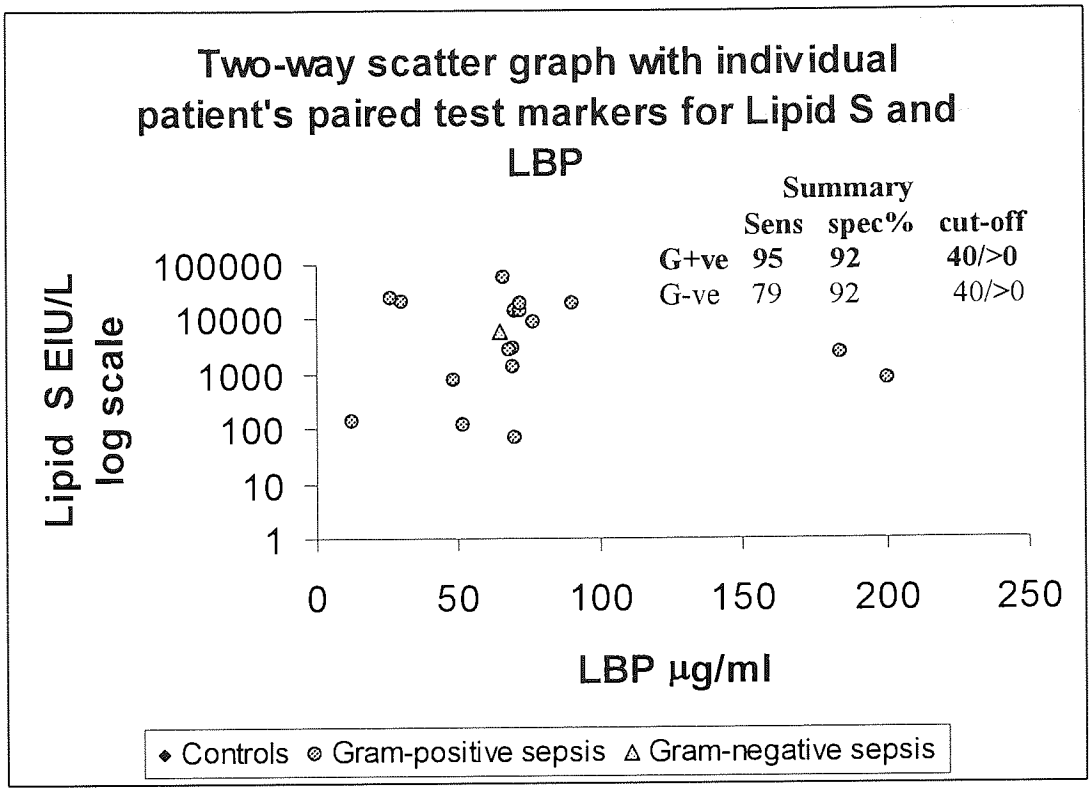


Figure 5.6.8 TWO-WAY SCATTER GRAPH for anti-lipid S and LBP
The normal reference serum level for lipopolysaccharide binding protein (LBP) is 5-10µg/ml. The single Gram-negative case positive for anti-lipid S had on-going psoriasis and this may account for his positive result for antibodies against lipid S.

When the cut-off level for LBP was 30µg/ml the sensitivity remained the same but the specificity was lower (88%) in the Gram-positive patients see table 5.7. The single Gram-negative patient with anti-lipid S antibodies will be discussed in section 5.7. Sensitivities and specificities for lipid S with sICAM-1 are given in table 5.7. The sensitivities and specificities calculated for all the patient groups and combined serum markers are given in the tables that follow (tables 5.6, 5.6.1 and 5.6.2). In these tables the positive predictive values (PPV) and the negative predictive valves (NPV) are calculated for those combined test markers with a sensitivity and specificity above 90% and are highlighted in bold. The PPV is a measure of probability that the patient has the disease and the NPV is the probability that the patient does not have the disease. For a fuller explanation of all these terms and the methods for calculating their values see section 3.5.3.

Table 5.6 SENSITIVITY (%) and SPECIFICITY (%) for Gram-negative patients paired test markers

Cut-off values	Sensitivity	Specificity	PPV	NPV
TNF-α with IL-6	%	%	%	%
20/20	87.5	92.3		
20/40	83.3	96.1		
30/20	87.5	92.3		
30/ 40	83.3	96.		
TNF-α with sICAM-1				
20/350	95.8	84.6		
20/500	75	100		
30/350	95.8	84.6		
30/500	75	100		
TNF-α with LBP				
20/30	91.6	88.4		
20/40	83.3	92.3		
30/30	91.6	88.4		
30/40	79.1	92.3		
TNF-α with Lipid S				
20/>0	33	100		
30/>0	29	100		
IL-6 with sICAM-1				
20/350	100	80.76		
20/500	**91.6**	**92.3**	**91.6**	**92.3**
40/350	100	76.92		
40/500	**91.6**	**96.1**	**91.6**	**92.3**
IL-6 with LBP				
20/30	**100**	**92.3**	88.8	100
20/40	**95.8**	**92.3**	88.8	100
40/30	**95.8**	**92.3**	88.4	100
40/40	**91.6**	**92.3**	**92.3**	**92.3**
IL-6 with Lipid S				
20/>0	**91.6**	**92.3**	**91.6**	**92.3**
40/>0	87.5	96.1		
sICAM-1 with LBP				
350/30	100	76.9		
350/40	100	76.9		
500/30	100	88.4		
500/40	**95.8**	**92.3**	**92**	**96**
sICAM-1with Lipid S				
350/>0	91.6	84.6	91.6	84.6
350/>0	75.0	100	75	100
LBP with Lipid S				
30/>0	91.6	88.4		
40/>0	79.0	92		

Table 5.6 highlights the best sensitivities in bold and in these cases the positive predictive value (PPV) and negative predictive values (NPV) have been calculated.

Table 5.6.1 SENSITIVITY (%) and SPECIFICITY (%) for Gram-positive patients paired test markers

Cut-off values	Sensitivity	Specificity	PPV	NPV
TNF-α with IL-6	%	%	%	%
20/20	70	92		
20/40	50	96.1		
30/20	70	92.3		
30/ 40	50	96.1		
TNF-α with sICAM-1				
20/350	60	84.6		
20/500	45	100		
30/350	60	84.6		
30/500	45	100		
TNF-α with LBP				
20/30	80	88.4		
20/40	80	92.3		
30/30	-	-		
30/40	-	-		
TNF-α with Lipid S				
20/>0	**90**	**100**	**100**	**92.8**
30/>0	**90**	**100**	**100**	**92.8**
IL-6 with sICAM-1				
20/350	85	86.7		
20/500	80	92		
40/350	70	76.9		
40/500	65	96		
IL-6 with LBP				
20/30	**90**	**92.3**	85.7	92
20/40	85	92.3		
40/30	85	92.3		
40/40	85	92.3		
IL-6 with Lipid S				
20/>0	**100**	**92.3**	**90.9**	**100**
40/>0	**95**	**96.1**	**95**	**96.1**
sICAM-1 with LBP				
350/40	85	76.9		
500/30	85	88.4		
500/40	85	92.3		
sICAM-1 with Lipid S				
350/>0	90	84.6		
350/>0	85	100		
LBP with Lipid S				
30/>0	95	88		
40/>0	**95**	**92**	**90.4**	**96**

Table 5.6.2 SENSITIVITY (%) and SPECIFICITY (%) for TOTAL Sepsis with patients paired test markers

Cut-off values	Sensitivity	Specificity	PPV	NPV
TNF-α with IL-6	%	%	%	%
20/20	79.5	92.3		
20/40	68	96		
30/20	-	-		
30/ 40	-	-		
TNF-α with sICAM-1				
20/350	79.5	84.6		
20/500	61.3	100		
30/350	-	-		
30/500	-	-		
TNF-α with LBP				
20/30	90	88.4		
20/40	81.8	92.3		
30/30	-	-		
30/40	-	-		
TNF-α with Lipid S				
20/>0	59	100		
30/>0	56.8	100		
IL-6 with sICAM-1				
20/350	**93**	**80.76**		
20/500	86	92.3		
40/350	86.3	76.2		
40/500	79.5	96.1		
IL-6 with LBP				
20/30	**95.4**	**92.3**	**93.3**	**92**
20/40	**93.1**	**92.3**	**95.3**	**88**
40/30	**90.9**	**92.3**	**93**	**85**
40/40	88.6	92.3		
IL-6 with Lipid S				
20/>0	**95.4**	**92.3**	**95.6**	**92.3**
40/>0	**93.1**	**96.1**	**97.5**	**86.2**
sICAM-1 with LBP				
350/30	93.1	76.9		
350/40	93	76.9		
500/30	93	88.4		
500/40	**90.9**	**92.3**	90	85.7
sICAM-1 with Lipid S				
350/>0	90.9	84.6		
350/>0	79.5	100		
LBP with Lipid S				
30/>0	93.1	88		
40/>0	84	92		

Total sepsis combines the two septic groups as one group versus the controls.

5.6 INTER-ASSAY and INTRA-ASSAY PRECISION RESULTS.

The following tables show the average of the duplicate samples and the plus or minus difference between the individual sample pair. The difference is also expressed as a percentage. It is preferred that the difference is < 0.05 but given the low number of repeat samples 10% was considered to be acceptable.

Table 5.7
INTRA-ASSAY PRECISION **IL-6** pg/ml INTER-ASSAY PRECISION

IL-6 HS	n	average	±	%	IL-6	n	average	±	%
1	2	neg	-	-	1	2	30.7	1.09	3.5
2	2	neg	-	-	2	2	113	6.8	6.0

neg-negative. The duplicated patients samples tested for reproducibility within a plate were negative for IL-6. Three other patients had two samples assayed, one was from two different samples taken on the same day, and two other patients had consecutive day samples tested.

Table 5.7.1
INTRA-ASSAY PRECISION **TNF-a** pg/ml INTER-ASSAY PRECISION

TNF-α HS	n	average	±	%	Sample	n	average	±	%
1	2	1.44	0.24	16.6	1	2	27.79	3.9	14.0
2	2	5.30	0.72	13.5	2	2	32.2	2.24	6.9
					3	2			

The intra and inter-assay precision tests given here and those for TNF-α from phase 1 (table 4.6.3) are generally above the acceptable level of reproducibility (10%). Assay reproducibility is discussed in section 5.7.1.

Table 5.7.2
INTRA-ASSAY PRECISION **sICAM-1** ng/ml INTER-ASSAY PRECISION
(precision within the assay) (precision between assays)

sICAM-1	n	average	±	%	sICAM-1	n	average	±	%
1	2	393.5	3.87	0.98	1	2	722	47.3	6.5
2	2	278	0.78	0.27	2	2	937.9	8.03	0.85

Table 5.7.2 shows that sICAM-1 assays had a high degree of reproducibility and within a plate and between assay plates.

Table 5.7.3

INTRA-ASSAY PRECISION **LBP μg/ml** INTER-ASSAY PRECISION
(precision within the assay) (precision between assays)

LBP	n	average	±	%	LBP	n	average	±	%
1	2	26.4	0.36	1.3	1	2	70	30	42
2	2	122.9	10	8.1	2	2	71	46.5	65
3	2	176.6	21.39	12	3	2			

The reproducibility of results for serum LBP between assays was poor.

Table 5.7.4
INTRA-ASSAY PRECISION **anti-lipid S IgG EIU/L** INTER-ASSAY PRECISION

Anti-lipid S	n	average	±	%	sample	n	average	±	%
1	2	11,871	49	0.4	1	4	96,687	1318	1.3
2	2	2635	369	14	2	4	26,965	1064	3.9
3	2	7610	74	0.9	3	4	11,148	163	1.4

Anti-lipid S showed a high degree of reproducibility within and between plates

5.7 RESULTS SUMMARY Phase 2

The median values for the test markers from this current study vary with those from the preliminary trial reported in chapter 4, particularly in the Gram-positive group median values for interleukin-6 (IL-6) and anti-lipid S (all groups). Whereas the median values for sICAM-1 are consistent in both trials and in all groups. Table 5.8 compares the median values for all the test markers in this phase with those of the same markers from phase 1 in chapter 4.

As established in phase 1, the serum markers are significantly higher in patients with sepsis as opposed to the controls. Table 5.5 reports the *p*-values for the patient groups in phase 2 and for comparison phase 1 results are given in table 5.5.1.

It is interesting to note that *p*-values calculated as being not significant can never the less be valuable results. For example, anti-lipid S in the Gram-negative patients versus the controls has a *p*-value of 0.3; there is no significant difference between anti-lipid S levels in the Gram-negative patients and the controls. This makes anti-lipid S a prime candidate marker for the differential diagnosis of patients with sepsis caused by a Gram-positive microorganism. All the markers (except anti-lipid S) are higher in patients with Gram-negative sepsis. LBP in the current phase (2) had equivalent values in both

septic groups; whereas the median value (in Phase 1) for LBP in the Gram negative group was higher, 125 μg/ml versus 62.5μg/ml (table 5.8).

Table 5. 8 A comparison of the median values between the test markers from the first and second trials (Phase 1 and Phase 2)

MEDIAN Normal range	IL-6 pg/ml (0.2-4.5)	TNF-α pg/ml (5-20)	sICAM-1 ng/ml (115-306)	LBP μg/ml (5-10)	anti-LIPID S EIU/L (>0)
CONTROLS					
Phase 1	6.8	2.1	270	16.53	0.00
Phase 2	3.31	0.00	282	16.45	0.00
GRAM +VE					
Phase 1	12.6	8.3	620	49.74	11313.0
Phase 2	49.11	4.10	461	68.70	2560
GRAM-VE					
Phase 1	28.2	13.9	891	126	0.00
Phase 2	120	7.48	717	63	0.00

Table 5.8 summarises the medians from both trials and are given as a reference for the discussion.

The confidence intervals (CI) for each test marker from the two trials are given in table 5.8.1. The confidence levels for all the calculated CIs are omitted as they were >95% (95.6 to 97.7%).

Table 5.8.1 A comparison of the confidence intervals for the test markers from the first and second trials (Phase 1 and Phase 2)

Confidence Intervals	IL-6 pg/ml (0.2-4.5)	TNF-α pg/ml (5-20)	sICAM-1 ng/ml (115-306)	LBP μg/ml (5-10)	anti-LIPID S EIU/L >0
CONTROLS					
Phase 1	4.6-14.0	1.28-3.10	210-350	12.7-24.7	0.00-0
Phase 2	0.36-3.8	0.00-0.72	227-314	13.0-23.5	0.00-0
GRAM +VE					
Phase 1	12.1-13.0	6.2-15.5	370-1000	33.0-69.5	4664-28740
Phase 2	15.14-174	0.00-7.48	317-832	52.0-72.0	140-14227
GRAM-VE					
Phase 1	20.5-57.9	9.01-20.55	675-1040	77.8-168.2	0.00-10173
Phase 2	61.9-453	0.24-26.8	515-1633	44.6-73.6	0.00-5930

Table 5.8.1 compares the serum range for each marker from both trials.

The CI is insensitive to outliers such as the one for TNF-α (525pg/ml) in the current phase from a young man with cancer (N10). This single result caused to range to change from 60 to 525pg/ml. The equivalent result from phase 1 was 107pg/ml.

5.7.1 Assay reproducibility

A major drawback to a realistic evaluation of intra-assay precision tests was the limited number of possible duplicate samples per plate. The inter-assay reproducibility for LBP was imprecise, although the intra-assay precision was acceptable; there are several possible causes that include poor recovery from stored serum samples; unequal antigen binding to the plate, or operator error.

The manufacturers (Hycult biotechnology b.v, France) preferred and recommended a -70°C storage system for serum samples and state that "storage at -20°C can affect recovery of LBP". However all the test serum for this project was routinely stored at -20°C and before this assay type was originally included in the trial. It was considered preferable to keep all the protocols exactly the same for phase 2. Samples for inter-assay precision tests were stored for longer periods between assays and trials. Therefore it is possible that the recovery of LBP from previously tested samples was partially lost. Indeed this is bourn out by the fact that the duplicated samples gave higher LBP values in their first assay than in the second.

The intra-assay values for the LBP assays are acceptable but the reproducibility of the results between assays was poor with percent differences of 42 and 60%.

Intra and inter-assay precision results showed good reproducibility for the anti-lipid S and sICAM-1 assays.

TNF-α had differences between the paired test samples greater than the acceptable 10%, however the plus/minus difference was small (0.24pg/ml) and acceptable with so few samples.

Inter-assay evaluation for IL-6 suffered from a paucity of duplicated samples. The selected patients tested in duplicate had no detectable serum levels of IL-6. It was an error to choose patients from the control group for these reproducibility tests. However there were no negative results for IL-6 in the control group in phase 1 using the high sensitivity (HS) version IL-6 assay. The *non* -high sensitivity Quantikine® IL-6 assay (R&D systems, Oxen) was used in the current phase and is by definition less sensitive to

lower amounts of serum IL-6 and may account for the negatives in some controls in this phase.

5.7.2 The Sensitivity and Specificity of the test markers for sepsis.

It was established from phase 1 that two markers would be needed for a more accurate diagnosis of sepsis as calculated from the sensitivities and specificities of the test-markers. In the current phase the best combined markers for sepsis are given in table 5.9.

Table 5.9 High value sensitivities and specificities for the combined test markers

Patient groups	Cut-off values	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Gram-negative					
sICAM-1& LBP	500/40	**95.8**	**92**	**92**	**96**
IL-6 & sICAM-1	40/500	91	96	91	92
Gram-positive					
IL-6 & lipid S	40/>0	**95**	**96**	**95**	**96**
TNF-α & lipid S	20 or 30/>0	90	100	100	92
Total sepsis					
IL-6 & lipid S	20/>0	**95**	**92**	**95**	**92**

Total sepsis combines both septic groups and is compared as one group versus the controls. All the sensitivities and specificities are given in tables 5.6; 5.6.1 and 5.6.2 for all the possible combinations and for each patient group.

5.8 DISCUSSION

The median values between the two trials probably reflect the difference between the stringency of selection for patient enrolment into the phase 2 study. In the phase 1 trials patients were primarily selected based on positive blood culture results (as well as pyrexia and a raised white cell count). In this current study the controls and the patients in the Gram-negative group were more carefully selected in terms of their possible previous exposure to Gram-positive microorganisms which may give rise to an anti-lipid S IgG. Indeed previously undisclosed ulcers and various skin lesions were thought to be

the cause of the anti-lipid S IgG positive results in the controls and Gram-negative patients from the preliminary trial.

Serum marker anomalies will be discussed; the effect of anti inflammatory and immunosuppressive drugs such as steroids and chemotherapy will be considered in terms of their possible effect on the levels of the test markers in serum. The effect, if any of underlying diseases such as acute myeloid leukaemia (AML) on the level of serum markers will be explored.

5.8.1 Test marker anomalies, patient P7

Even after great care was taken to enrol the definitive septic patients into this current phase (2) patients were included who proved not to be septic for example; patient P7 was waiting for a mitral value replacement and developed a temperature, she was admitted as an emergency with shortness of breath and left ventricular fibrillation (LVF). A blood culture was taken which subsequently grew a methicillin sensitive *Staphylococcus aureus* from the aerobic bottle. She was eventually diagnosed with a joint infection. Her markers are given in table 5.9.1

Table 5.9.1 Patient P7; serum markers and white cell count and C-reactive protein

TNF-α	IL-6	sICAM-1	LBP	anti-lipid S	CRP	WCC*
pg/ml	pg/ml	ng/ml	μg/ml	EIU/L	mg/l	BC
5-20	0.2-4.5	115-306	5-10	> 0	< 10	day
0.0	**4.98**	**282**	**52**	**115**	**58**	**16.2**
-	**-**	**-**	**+**	**+/-**	**+**	**+**

WCC- white cell count. The normal range for white blood cells is between 4-11 $x10^9$/l. This patient is listed in table 5.2 in the wrong place as her test sample was 2 days post her positive blood culture. The CRP and WCC values relate to the date of the blood culture unless otherwise specified.

Table 5.9.1 relates to the serum levels of the test markers in the patient's blood 2 days after she was admitted and before intravenous antibiotics. An extra row has been added with negative and positive signs. As other patients are discussed it will become clear that septic patients have a distinctly different pattern of plus and minuses. The WCC and CRP results show that the patient has an inflammatory response, and the elevated LBP suggest an infection as there can be a 10 fold increase in the level of LBP during the acute stage response (Wilde *et al.*, 1994)

5.8.2 Test markers anomalies, patient P11

5.8.2 Test markers anomalies, patient P11

Another example of a patient being included in this trial who eventually proved not to be septic by definition was P11. This 67 year old gentleman was admitted with right hemi-paresis and was newly diagnosed as a diabetic. He developed a temperature 3 days later and this increased as did his blood pressure (190/ 95) and pulse rate (110 rpm). Brain abscesses were suspected. A blood culture was taken and after one day the aerobic bottle grew a bacterial microorganism -*S. aureus*. The test serum was a day older than the blood culture sample and the results are shown in table 5.9.2 normal reference ranges are given in table 5.9.1.

Table 5.9.2 Patient P11; serum markers and white cell count and C-reactive protein

TNF-α	IL-6	sICAM-1	LBP	Anti-lipid S	CRP	WCC
0	**4.45**	**260**	**12.2**	**140**	**9**	**11.5**
-	**-**	**-**	**+/-**	**+/-**	**-**	**+/-**

None of the test markers were elevated in this patient except for a small rise is LPB.

Patient P11 is a good example of a patient who clinically presents with many of the signs of an overwhelming infection indicative of sepsis; the WCC was going up, his temperature was spiking, his respiration increased and he had positive blood culture. Yet clearly from the markers this patient was not septic. He was diagnosed as having a bacteraemia from an unknown site was given antipyretics and antibiotics and was eventually discharged as having experienced a right side stroke.

It is in the very nature of this project to address the problems caused by conflicting clinical signs and symptoms of apparent infection and to establish a marker or markers of infection that are trustworthy in the face of confounding clinical signs caused not by infection but by the patients underlying condition or disease. Further more, this putative marker must be robust enough to allow a diagnosis of sepsis regardless of any concurrent symptoms from the underlying condition, as well as being able to show that evidence of infection such as a positive blood culture is either truly positive and real evidence of a systemic infection or a false positive culture caused by contamination.

5.8.3 Test marker anomalies, patient N17 (N29 in phase 3)

(patient N17 is re-labelled in phase 3 as patient N29)

Patient N17 was known to be an "uncertain septic" patient on the day of her blood culture. This lady (51years) had been involved in a road traffic accident six days previously and was in the critical care unit with lung contusions and fractures to both femurs. She was already on antibiotics, with an endo- tracheal tube in situ. A blood culture was taken because she became hypotensive. After one days incubation an unknown Gram-negative rod was isolated from the aerobic bottle and subsequently identified as *Citrobacter freundii*. This ladies test markers are from a serum sample taken one day after her blood culture sample; the results are shown in table 5.9.3

Table 5.9.3 Patient N17; serum markers and white cell count and C-reactive protein

TNF-α	IL-6	sICAM-1	LBP	Anti-lipid S	CRP	WCC
0	86.6	867	30.8	0	94	7.8
-	+	+	+	-	+	-

Patient N17; with early sepsis. She had four positive markers for sepsis in her serum and were elevated before the conventional marker of infection-a raised WCC. The test marker serum was taken one day after the blood culture sample. Due to the coding system used this patient becomes (N29) in phase 3.

This lady was considered to be septic 4 days after her positive blood culture (BC); 2 days after that (a total of 6 days post BC) her CRP level was 134 mg/ml and her WCC was 21 (x 10^9/l). Although her WCC started to increase to above normal levels two days after the blood culture was taken (14.0 x10^9/l).

It is her high serum sICAM-1 levels that signal a serious warning together with an elevated IL-6 (and /or LBP). Later work will show that other conditions such as drug over-dose induced liver damage may contribute to seriously high levels of sICAM-1, however the other markers in this case suggested that she was not septic (described in section 7.4.5).

5.8.4 Patients on Steroids (P14) and Chemotherapy (P5)

A brief overview of the known effects of glucocortiocoids (steroids) is included here in order to evaluate the test marker results against the background of steroid therapy. Figure 1.6 in chapter 1, illustrates one of the actions of glucocorticoids in

relation to the inhibition of phospholipase A_2 (PLA_2) and the generation of the lipid mediators of inflammation.

5.8.4.1 Potential effects of anti-inflammatory and immunosuppressive Steroids.

Glucocorticoids are endogenous steroid hormones. The major one is cortisol (or hydrocortisone). It is synthesised in response to any form of stress. At physiological levels cortisol increases the level of blood glucose by stimulating glycogenesis and inhibiting glucose uptake. It causes an increase in amino-acids in the blood by stimulating protein breakdown and blood levels of fatty acids increase. When exogenous glucocorticoids are administered (such as prednisolone and dexamethasone) resulting in higher than physiological levels, new effects are produced with anti-inflammatory and immunosuppressive effects, due in part to the induction of lipocortins (Sherwood, 1993). Lipocortins are anti-inflammatory proteins. Table 5.9.4. lists some of the known effects of glucocorticoids. It is estimated that 1% of the genome may be steroid regulated therefore their potential influence on the body is far reaching (Peers, 1994).

Table 5.9.4. Glucocorticoids; Prednisolone and Dexamethasone (Steroids)

Mechanisms	Inhibits	Reference
By direct effect upon endothelial cells	Vasopermeability	Yarwood *et al.*, 1993
	Neutrophil accumulation	
By preventing gene translation	Induction and secretion of PLA_2	Pruzanski & Vadas, 1991
	PG synthesis	Rang *et al.*, 1995
By gene transcription	Cytokine synthesis TNF-α	Beutler and Cerami 1986
	IL-1, IFN	Tobler *et al.*, 1992
	IL-8	Waage *et al.*, 1988
	IL-2;IL-3;IL-4;IL-6	Rang *et al.*, 1995
	GM-CSF	Lieschke and Burges, 1992
	Decreased clonal expansion of T and B cells	Rang *et al.*, 1995
	Reduction in the concentration of complement components in the plasma	Rang *et al.*, 1995
	Decreased histamine release from basophils	Rang *et al.*, 1995

GM-CSF- Granulocyte-macrophage colony stimulating factor. This cytokine stimulates the development of neutrophils, monocytes, eosinophils. The normal functional activity of these cells is enhanced by the action of GM-CSF (Lieschke and Burges, 1992). PLA_2, Phospholipase A_2.

Prednisolone is four times more anti-inflammatory and immunosuppressive in its effects than endogenous hydrocortisone; whereas dexamethasone is 25-30 times more anti-inflammatory and immunosuppressive (Jasani, 1979). The anti-inflammatory responses relate to the relative affinity of these substances for the glucocorticoid receptors in human foetal lung cells in comparison with the endogenous hydrocortisone (Baxter and Rousseau, 1979). Dexamethasone is also the drug of choice for the suppression of the adrenocorticotrophic hormone (ACTH) or corticotrophin. ACTH stimulates the synthesis and release of the glucocorticoids as they are not pre-formed or stored but are generated *de novo* (Rang *et al.*, 1995).

Considering all these possible effects on the host's immune response to infection caused by the action of steroids, it is surprising to see that patient P14 serum test markers for sepsis are all positive except for her negative anti-lipid S antibody level. This patient was given intravenous steroids (dexamethasone) 4 days before her positive blood culture. However her IL-6 and sICAM-1 levels are moderate in comparison with other patients in this trial with sepsis and are given in table 5.9.4.1.

Table 5.9.4.1 Patient P14; test marker results with WCC and C-reactive protein

TNF-α	IL-6	sICAM-1	LBP	Anti-lipid S	CRP	WCC
1.21	39.9	348	58	0	96	5.0*
-	+	+	+	-	+	±

WCC- white cell count. The test markers and CRP results are from a serum sample taken I day after the blood culture (BC) sample, whereas the WCC levels relates to the BC date. *Her WCC on the test sample date had dropped to 2.1 ($x10^9$/l), whereas her CRP had increased from 96 to 140 mg/l. (This patient's code became P32 in phase 3)

This patient's infectious episode developed 13 days after her admission with a 5 month history of step-wise visual loss, learning difficulties and seizures. She was discharged 4 months later to a nursing home. This lady was aged 50, blind with two inter-cranial pathologies; hydrocephalus associated with a right acoustic neuroma and an epidermoid cyst. Apart from other medication, this lady was given intravenous phenytoin for her epilepsy and intravenous steroids. Dexamethasone was administered for a total of 22 days, starting 4 days before her positive BC with a high dose (4mg twice a day for four days) and ending with 2mg per day.

The blood culture for P14 was requested because she developed a temperature (38.9°C) with rigors and her pulse rate was 120 beats /minute. The blood culture became positive for bacterial growth 2 days after incubation (37°C) in the anaerobic culture medium but not in the aerobic bottle. The isolate was later identified as a coagulase negative staphylococcus. Apart from this patient's zero antibody level against lipid S, steroid therapy has not completely suppressed serum elevations of the test markers and importantly does not appear to have affected a normal CRP response.

5.8.4.2 Patient P 5 and Chemotherapy

A course of antibiotics is chemotherapy, however the word is associated more often with substances that kill cancerous cells (or rapidly dividing cells). Patient P5 had just

finished her second cycle of Epirubicin (an cytotoxic antibiotic) that binds to DNA and RNA inhibiting their synthesis. Cytotoxic antibiotics cause bone marrow suppression and cardiotoxicity although Epirubicin is less cardiotoxic than other drugs in this class (Rang *et al.*, 1995) table 5.9.4.2 gives P5 test marker results.

Table 5.9.4.2 Patient P5 post chemotherapy

TNF-α	IL-6	sICAM-1	LBP	Anti-lipid S	CRP	WCC
0	7.38	310	69	2733	59	4.4
-	-	-	+	+	+	+/-

This patient's WCC was 1.6 x 10^9/l the day before the BC but granulocyte-colony stimulating factor (G-CSF) was administered and 3 days later she became neutrophilic with a WCC of 24.2 x 10^9/l.

Patient P5 had a mastectomy due to breast cancer 6-8 weeks before she was admitted with a temperature (39.5°), nausea, vomiting and diarrhoea. She had consumed an 'herbal remedy' for her chemotherapy induced nausea that made her condition worse. Her blood culture became positive in one bottle after 3 days incubation, the microorganism was identified as a *Corynebacterium* spp. She had an on going urinary tract infection. Her diagnosis was neutropenic sepsis and she was discharged home after treatment 16 days later. Lipopolysaccharide binding protein (LBP), C-reactive protein and anti-lipid S antibodies were the only markers in this patient's serum to suggest an infection.

Chemotherapy is known to induce bone marrow suppression but can it be assumed that her low WCC was the reason why the IL-6 was so low? Three patients with acute myeloid leukaemia and very low WCC are compared.

5.8.4.3 Markers in Patients with Acute myeloid leukaemia (AML)

Tables 5.9.4.3 and 4 and 5 give the test markers for three patients with acute myeloid leukaemia (AML). Two patients have a Gram-positive sepsis and one has a Gram-negative sepsis but all have neutropenia.

Table 5.9.4.3 Patient N5 Underlying AML with neutropenic sepsis (G-ve)

TNF-α	IL-6	sICAM-1	LBP	Anti-lipid S	CRP	WCC
8.44	106	515	76.4	0	229	0.2
-	+	+	+	-	+	-

All the test markers are positive for a diagnosis of sepsis in patient N5. This patient has a low WCC and a high level of IL-6.

Table 5.9.4.4 Patient P10 Underlying AML with neutropenic sepsis (G+ve)

TNF-α	IL-6	sICAM-1	LBP	Anti-lipid S	CRP	WCC
3.618	186	472	90	19,662	241	0.3
-	+	+	+	+	+	-

In this case, the patient had a high positive anti-lipid S result, in addition to all the positive test markers. The fact that the patient had few white blood cells shows that antibodies must have been produced prior to his neutropenia. The remaining white blood cells in this patient were all lymphocytes (normal range, 1.0-4.0 $x10^9$/l)

Table 5.9.4.5 Patient P15 Underlying AML with neutropenic sepsis (G+ve)

TNF-α	IL-6	sICAM-1	LBP	Anti-lipid S	CRP	WCC
2.65	21.9	340	29.8	21,558	50	0.7
-	+	+/-	+	+	+	-

This patients test markers are not as elevated as the other two cases, but they are all positive for sepsis.

These patients provide a small sample so it cannot be said that AML does not interfere with the test markers ability to diagnose sepsis, but it is possible to suggest that this underlying disease with its accompanying low WCC does not alter the effective use of these test markers for sepsis in patients with this specific cancer.

5.8.5 Liver pathologies and high serum sICAM-1 in sepsis

It is interesting to note that patients with any underlying liver pathologies such as alcoholic liver disease or liver carcinoma commonly have some of the highest levels of sICAM-1 in their serum see table 5.9.5 for a list of these patients.

Table 5.9.5 An association between high serum sICAM-1 in patients with sepsis and underlying liver disease.

Patients with sepsis	Underlying disease	sICAM-1 (ref. 115-306 ng/ml)
N7	Jaundiced-pancreatic ca.	3440
N8	Oesop. cancer. ALD	2190
N11	Cholecystitis alcohol excess	1630
N20	Liver cancer.	3140
N21	Alcoholic liver disease (ALD)	2160
P8	Liver carcinoma	2155

Patients with a Gram-negative sepsis and liver pathologies appear to have very high serum levels of sICAM-1.

There were other patients in this phase with high levels of sICAM-1 and a Gram-negative sepsis listed in table 5.9.6. In these cases there is either no known liver connection or no apparent liver connection.

Changes in serum sICAM-1 is implicated in a number of diseases as an immunological sign of disease pathology; these include Graves disease (hyperthyroidism); multiple sclerosis; Hepatitis C viral induced liver damage; melanomas; radiation pneumonitis; after neuroleptic treatment for schizophrenia and it is strongly implicated in the mechanism of progressive liver fibrosis in patients with biliary atresia (a congenital absence or underdevelopment of a biliary structure; (Anderson *et al.*, 1994; Sonnet *et al.*, 1999; Mysliwiec *et al.*, 1999; Trojano *et al.*, 1999; Fonsetti *et al.*, 1999; Ishii and Kitamura, 1999; Schwarz *et al.*, 1998; Kobayashi *et al.*, 2001). The serum levels of sICAM-1 in these diseases did not reach the high levels found in septic patients and septic patients with underlying liver disease except in children with biliary atresia.

Table 5.9.6 High serum levels of sICAM-1

in patients who may or may not have any liver disorder- it is not know.

Patients with sepsis	Underlying disease	sICAM-1 (ref. 115-306 ng/ml)
N3	80% burns	1950
N10	Carcinoma site unknown	1630
N24	Chronic renal failure	1880
P1	Not known	1430

Patients with Gram-negative sepsis and exceptionally high serum levels of sICAM-1 with no known liver pathology.

sICAM-1 is implicated in the pathogenesis of type 1 diabetes and serum levels may be a marker of the preclinical stage of this autoimmune disease (Mysliwiec *et al.*, 1999). It has been proposed that sICAM-1 is immunosuppressive as it binds to circulating leucocytes (via LFA-1 receptors also called CD11a/CD18) making these leucocytes unable to bind to cell surface ICAM-1 on target cells, consequently suppressing their specific activity. For instance, the activity of natural killers cells (NK) that bind and kill host cells infected with intracellular pathogens (Kaihara *et al.*, 1998). NK cells are referred to in section 2.9.1.2 and table 2.5. Interleuken-1 beta (IL-1β) increased the production of sICAM-1 from human endothelial cells in cell culture studies (Kaihara *et al.*, 1998). As mentioned previously the destructive aspect of increased serum sICAM-1 may be due to elastase released from polymorphonuclear leucocytes via sICAM-1 binding CD18 (Barnett *et al.*, 1996).

5.8.6 Levels of TNF-α, LBP sICAM-1 and IL-6 in serial samples

The short-term pharmacokinetics of IL-6 reported in Miesener (2000) and mentioned in this project under the two-way scatter graph for IL-6 and sICAM-1 (figure 5.6.3), refers to the serum levels of IL-6 in a surgical patient who was given an infusion accidentally contaminated with bacteria (Brunkhorst *et al.*, 1998). In this example IL-6 serum levels peaked after 2 hours then gradually disappeared from the blood over the next 72 hours. It infers a small window of opportunity to detect IL-6 in the serum.

To test these finding (if only tentatively) patients serum was sampled on two consecutive days to see if their serum markers had changed over time, table 5.9.7 displays these results.

Table 5.9.7 A comparison of the test markers in serum samples from the same patient over time

PATIENT	DAY	TNF-α pg/ml	IL-6pg/ml	sICAM-1 ng/ml	LBP μg/ml
		5-20	0.2-4.5	115-306	5-10
P4	1	ND	58.2	397	31.96
	3	ND	349	390	>200
P10	a.m	ND	186	472	183
	p.m	ND	320	723	90
N6	1	-	177	243	75
	2	-	3.2	191	45
N7	1	-	60	1718	33.4
	2	-	82	1350	53

ND-not detected, P10 same day samples. A dash line, not tested for this marker
Table 5.9.7 shows that IL-6 increases in the serum of patients with sepsis over time in three out of the four samples.

There is an increase in IL-6 and sICAM-1 in P10 but not a corresponding increase in LPB. Patient N6 most closely resembles the finding in Meisener (2000) that IL-6 disappears quickly from the serum by virtue of its short-term pharmacokinetics.

5.8.7 LBP and the Control patients

Lipopolysaccharide binding protein (LBP) is a promising marker for sepsis. Indeed LBP is said to have a better predictive value for sepsis than other acute phase markers, such as CRP, IL-6 or PCT (Schumann and Zweigner, 1999). The level expected in healthy adults is between 5-10 μg/ml (Wilde *et al.*, 1994) All the septic patients were positive for this protein, however all but three of the controls are also positive for LBP.

LBP, like procalcitonin may be elevated in circulatory dysfunction and cardiogenic shock without bacterial involvement (Al-Nawas *et al.*, 1996; Meisner 2000). Procalcitonin reaches very high concentrations in the plasma of patients with prolonged cardiogenic shock, similar to the levels found in septic shock (Brunkhorst, *et al.*, 1995). Yet low levels of LBP are found in septic patients and in such cases the out come is poor (Wilde *et al.*, 1994).

All the controls have serious cardio-vascular disease and their elevated LBP levels may be a normal non-bacterial response to their coronary artery disease. Alternatively the LBP levels in the controls may reflect more accurately the 'normal' levels in the adult population. A significant cut-off level for sepsis would be required to distinguish between non-bacterial (an acute phase response), local bacterial infections and systemic bacterial infections, requiring an extensive research programme.

Two control patients had levels of LBP that were so high as to indicate an infection they were C2 and C13; in addition their IL-6 levels were high. Patient C2 had a serum LBP level of 57μg/ml and an elevated IL-6 (20.2pg/ml). C2 had a high though normal level of TNF–α (6.03 pg/ml). The patient was aged 77 years with chronic obstructive airways disease (COAD) and because her LBP and IL-6 levels indicated an infection, her serum C-reactive protein (CRP) level was tested (for research only), and found to be 122 mg/l. This CRP level was from a serum sample taken on the day of her operation and it is not clear if the sample was taken pre-or post operationally.

Due to this highly unexpected result all the other control patients test serum was assayed for CRP using the hospital facilities paid for from the research fund. The results showed that all the control patients had CRP levels within the normal range < 10mg/l, and 17 of these were < 3.0 mg/l. All that is except for three patients C2, C13 and C24, the latter patient also had and elevated LBP but a normal IL-6 reading.

The elevated LBP level with corresponding normal level of CRP in the serum of patient C24 may be empirical evidence for suggesting that LBP appears before CRP in the serum of patients with an acute phase response to trauma or infection.

Normally CRP levels are not required from patients waiting for coronary artery by-pass grafts (CABG) surgery. C2 was admitted fourteen days *before* her operation. This is unusual as CABG patients are routinely admitted the day before their operation. An infection was suspected (despite negative microbiology) as her CRP levels were requested and she was started on augmentin five days after surgery. Interestingly, the CRP levels on post-operation days 7 and 8 were 56 and 43 mg/l respectively (normal levels < 10mg/l) indicating that the stimulus for CRP production occurred on or before the operation day, (see figure 7.5) illustrating the serum pattern of CRP in a patient with sepsis.

The white cell count (WCC) on the day of patient C2's operation was within the normal range (normal range 4-11 $x10^9$/l) but her differential WCC was not, the

lymphocyte count was below normal 0.8 x 10^9/l (normal range 1.0-4.0 x 10^9/l), the monocyte count and platelet count were also below normal. The day following surgery her neutrophil count had increased but the lymphocyte count was lower at 0.3 x10^9/l. This suggests that the test sample was taken post operatively, as lymphopenia is seen after surgery, however it is also seen after trauma, administration of corticosteroids, acute infections, uraemia, systemic lupus erythematosus (SLE) and human immunodeficiency virus (HIV) infection (Hughes-Jones and Wickramasinghe, 1991).

Control patient C13 had an even higher serum level of IL-6 and a high LBP. His CRP was 34mg/l; he had ongoing osteoarthritis and psoriasis. Interestingly this patient was also admitted six day before his operation and was discharged seven days after his CABG.

The only other control patient with an elevated CRP (27mg/ml) and an elevated LBP (25.6 μg/ml) was patient C24. His notes show categorically that the test serum sample was taken post operatively therefore this rise in acute phase proteins is understandable. His WCC was normal but his lymphocytes were not (0.4 x 10^9/l), a post surgical phenomemon

Some 'septic' patients who had sICAM-1 within the normal range proved not to be septic (P7; P11) however there is no explanation at this time for a septic patient with low levels of sICAM -1 (N6).

Patients with a Gram-positive sepsis often had lower levels of the test markers than patients in the Gram-negative group and if an individual patient's pre-septic base line level of sICAM-1 is in the lower end of the 'normal' range (115 ng/ml) then an elevation of an extra 200ng/ml would not be seen as elevated but more or less the normal range (115-306ng/ml) Alternatively and presumptively a low serum level may reflect a non-septic condition.

TNF-α assays from this phase were disappointing because of the number of negative results using the Quantikine ® non-high sensitivity assay kit. 17/26 controls, 7/20 Gram-positives and 6/24 Gram-negatives were negative for TNF-α. In addition the positive results in the septic patients varied over a wide range of serum levels. Neverthe less the appearance of elevated serum levels of this molecule will be seen (in time) as important evidence of a patients immune response to trauma and infection and as a prognostic serum marker, helping to complete the picture of the human response to sepsis and other disease states.

5.9 Conclusion

The test markers that yielded the best sensitivities and specificities in patients with a Gram-negative bacterial sepsis in this current phase 2 were LPB, IL-6 and sICAM-1; for patients with a Gram-positive sepsis IL-6, anti-lipid S and LBP gave better results. When all the septic patients were combined to produce a total group IL-6, LBP and anti-lipid S were the best markers. All the sensitivities and specificities from this current phase are given in tables 5.6; 5.6.1 and 5.6.2.

One of the aims for this phase of the research was to establish cut-off levels for a diagnosis of sepsis for each test marker. In the first trial this was 30pg/ml for IL-6 and 40μg/ml for LBP. In this phase 40pg/ml (IL-6) gave better results with 30μg/ml for LBP. sICAM-1 gave consistent results at a cut-off for sepsis of 500ng/ml in both trials, particularly in patients with Gram-negative sepsis. However eleven patients in the Gram-positive group had sICAM-1 levels below this cut-off level but we have established that two markers combined are required for a diagnosis of sepsis, and for the patients with a Gram-positive sepsis anti-lipid S was the best second marker.

In this current phase of the project there were 20 patients in the Gram-positive group and several of these patients were not septic; 24 in the Gram-negative group and 26 controls. It was proposed that a total of 50 patients in each group would provide a better estimation of the test markers reliability to diagnose sepsis and that IL-6, sICAM-1 and anti-lipid S should be carried forward to the next phase.

5.9.1 The next phase

It was the purpose of the next phase to establish IL-6 and sICAM-1 as markers for sepsis and anti-lipid S as a differential marker for Gram-positive sepsis and to confirm cut-off levels for these markers from the collective data from this current phase 2 and the data from the next phase 3, reported in chapter 6.

Finally the markers were tested in a semi-blind trial (phase 4 in chapter 7), based on the initial blood culture results supplied as either a named isolate or simply as a Gram-negative rod (GNR) or a Gram-positive cocci (GPC). A diagnosis was predicted from the serum test markers alone, then again with the added information of the patients CRP and WCC values and finally these results were compared with the previously unknown clinical diagnosis.

CHAPTER 6 INTRODUCTION (PHASE 3)

This section of the sepsis screen study continues to evaluate three test markers from chapter 5 they are IL-6, sICAM-1 and anti-lipid S antibodies with a new group of patients. A main aim is to find serum markers that would aid clinicians in the diagnosis of sepsis in patients with positive blood cultures and uncertain sepsis. LBP and TNF-α from phase 2 (chapter 5) were not brought forward into this phase for the reasons given in section 5.8.7.

Two of the control patients from the previous phase (2) had elevated IL-6 and LBP, this was an unexpected finding. Therefore serum level of CRP, (the traditional marker of inflammation or infection) was measured in all the control patients and was found to be above the normal range (< 10mg/l) in three patients, C2 and C13 the patients with elevated IL-6 and LBP and patient C24. The latter patient had a normal IL-6 but an elevated LBP. Therefore CRP levels were obtained for the control patients in the current phase (3) and in patients from the two sepsis groups who did not have a CRP result on the day of their blood culture sample.

In the previous phase the test markers were evaluated in a total of 70 patients from the Queen Elizabeth Hospital (Birmingham). Twenty patients with a Gram-positive sepsis and twenty four with a Gram-negative bacterial sepsis and twenty-six control patients without sepsis or infection. The results showed that IL-6 and sICAM-1 combined were sensitive (91%) and specific (96%) markers of sepsis in patients with a Gram-negative sepsis, when serum cut-off levels were 40pg/ml for IL-6 and 500ng/ml for sICAM-1. Whereas the Gram-positive group IL-6 with anti-lipid S gave a 95% sensitivity and 92.35% specificity, at a cut-off for IL-6 of 20pg/ml and > 0 for anti-lipid S. When the cut-off level in the Gram-positive group was 40pg/ml for IL-6 the sensitivity remained high at 95% but the specificity increased to 96%.

In order to consolidate these results 25 controls patients, 30 patients with a Gram-positive sepsis and 28 with a Gram-negative sepsis were enrolled onto this current phase-phase 3. Serum samples from these new patients were assayed for three serum markers IL-6, sICAM-1 and anti-lipid S antibodies. The results from the current phase (3) are presented in combination with the results for the same three markers from the previous trial (phase 2) in chapter 5.

6.1 Clinical samples

As previously described in chapter 5.1, the Queen Elizabeth Hospital's Ethics committee approved this continuing study. Serum samples were obtained from the control patients and those with either a Gram-positive bacterial or Gram-negative bacterial associated sepsis. Blood samples were separated and the serum obtained was stored at minus 20°C until assayed.

Sepsis was defined as the presence of two or more clinical signs of a systemic inflammatory response syndrome (SIRS) in the presence of documented systemic infection (Bone et al., 1992). The clinical signs indicating systemic inflammation were; a temperature < 36°.6C (96°F) or > 38°.3C (101°F) and a white cell count >12.0 x 10^9/l or < 4.0 x 10^9/l. If the former parameter was not present due to the administration of antipyretics such as paracetamol then other parameters of SIRS would be considered. An elevated C-reactive protein was accepted as a substitute for the white cell count if the latter had not been performed, or if the patient was leucopenic due to steroid therapy or immunosuppression. The patients had a positive blood culture containing either a Gram-negative bacillus or Gram-positive coccus; after subculture these isolates were identified by routine microbiological methods.

6.1.1 Control patients

25 pre-operative in-patients waiting for elective coronary artery by-pass graft (CABG) at the Queen Elizabeth Hospital (Birmingham, UK) were enrolled into this study. They were a heterogeneous group with no recent or current clinical evidence of infection or sepsis. As described previously a blood sample from each patient was taken on admission for elective surgery for coronary artery by-pass grafts (CABG). There were 18 males with an average age of 61 years (age range 40 to 78), and 7 females with an average age of 56 years (age range 54 to 78).

6.1.2 Gram-negative sepsis. Patients defined

Twenty-eight from the Queen Elizabeth and Selly Oak hospitals (Birmingham, UK) were enrolled into this study. They were a heterogeneous group that were acutely unwell for an average of two days prior to producing a positive blood culture. The diagnosis of sepsis was verified by a Consultant microbiologist. There were 11 males with an average age of 52 years (age range 21 to 79), and 17 females with an average age of 56 years (age range 21 to 94).

6.1.3 Gram-positive sepsis. Patients defined

Thirty patients from the Queen Elizabeth and Selly Oak hospitals (Birmingham, UK) were enrolled into this study. They were a heterogeneous group that were acutely unwell for an average of two days prior to producing a positive blood culture. Sepsis was defined as given in 6.1 and the diagnosis of sepsis was confirmed by the Consultant microbiologist. There were 20 males with an average age of 57 years (age range 21 to 85) and 10 females with an average age 58 years (age range 43 to 82).

6.1.4 Inclusion and Exclusion criteria

The inclusion and exclusion criteria for patients in this phase (3) are identical to those given in chapter 5.1.4 and 5.1.5.

6.1.5 Patient codes

Patients have been assigned a code for data/patient protection. In this current phase (as in the previous phase 2), the control patients are C and a number; the Gram-positive patients are P and a number and the Gram-negative group N and a number.

In the results section of chapter 6 patients from the last phase (2) were combined with the patients from the current phase (3). Patients are numbered down the page according to the number of days on or after their positive blood culture (BC) date. For example, serum test samples taken on the same day as the blood culture sample were designated as zero days and listed first. Patients with test samples collected 1 day after the BC sample were listed next as "1". This affects some of the patient numbers from the last phase such that patient P10 from the previous phase (2) became patient P28 in this phase (3). To minimise any possible confusion, a patient from phase 2 discussed in this phase will have their new number in brackets.

6.2 METHODS and MATERIALS Phase 3

The assays for the selected test markers sICAM-1; anti-lipid S antibodies were conducted as given in chapter three, 3.4.4 and 3.4.9. The non-high sensitivity IL-6 assay was performed as given in the materials and methods section 5.3.1 chapter 5.

6.2 RESULTS

Unless otherwise stated the results from the current phase (3) are presented together with the IL-6, sICAM-1 and anti-lipid S results from phase 2 (chapter 5).

The median, confidence interval (set at a confidence level of 95%) and the significance of the difference between the medians in the septic groups and the controls is given by a 2-tailed *p*-value calculated from the Mann-Whitney test. This test is the non-parametric equivalent to the students *t*-test used for parametric data. These statistics were computed using the software programme Analyse-it for Microsoft Excel, Leeds UK. The Range is given as the sum of the difference between the largest serum value of the marker and the smallest value (Everitt, 1995).

6.3.1 SERUM LEVELS OF THE TEST MARKERS AND CRP AND WCC

The serum levels of IL-6, sICAM-1 and anti-lipid S are presented in figures 6.1.1 to 6.1.3. In addition, the CRP and white cell count (WCC) for each patient is given in figures 6.2.1 to 6.2.3.

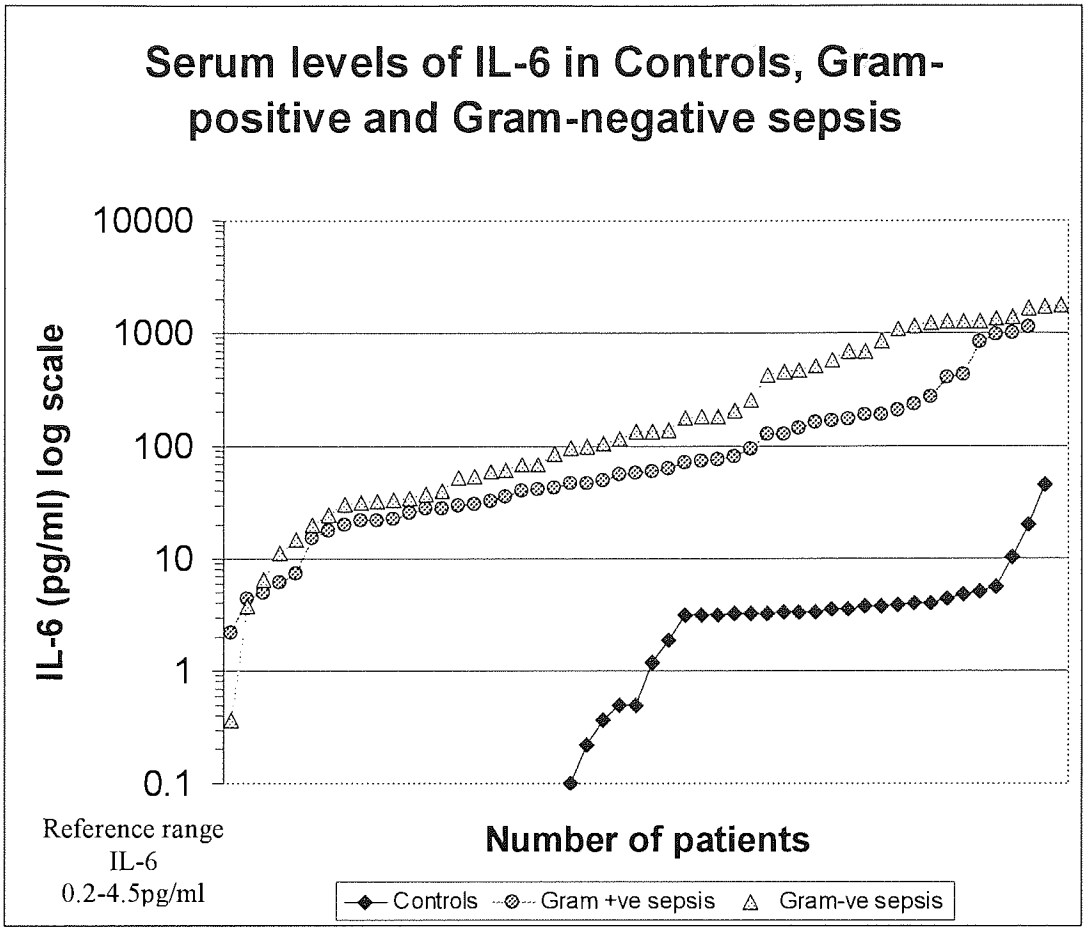


Figure 6.1.1. Illustrating the serum levels of IL-6 (pg/ml) for each patient.

Zero levels are not charted on a logarithmic scale. 21 controls had no detectable serum IL-6.

Figure 6.1.1 clearly demonstrates that patients with a Gram-negative sepsis tend to have higher serum levels of IL-6 than those with a Gram-positive sepsis.

Table 6.1.1 Median, confidence interval (CI) and *p*-value forIL-6 pg/ml; all patient groups

IL-6 pg/ml	Controls (51)	Gram-positive sepsis (50)	Gram-negative sepsis (52)
Median	**0.5**	**57.30**	**134**
CL% (CI)	**95.1 (0.00-3.28)**	**96.7 (35.4-94.2)**	**96.4 (69.1-433)**
***p* 0.05**		**0.0001**	**0.0001**
Range	**45.6**	**1109**	**1799**

(n) number of patients tested. Results are said to be significant when p-value is = or<0.05.

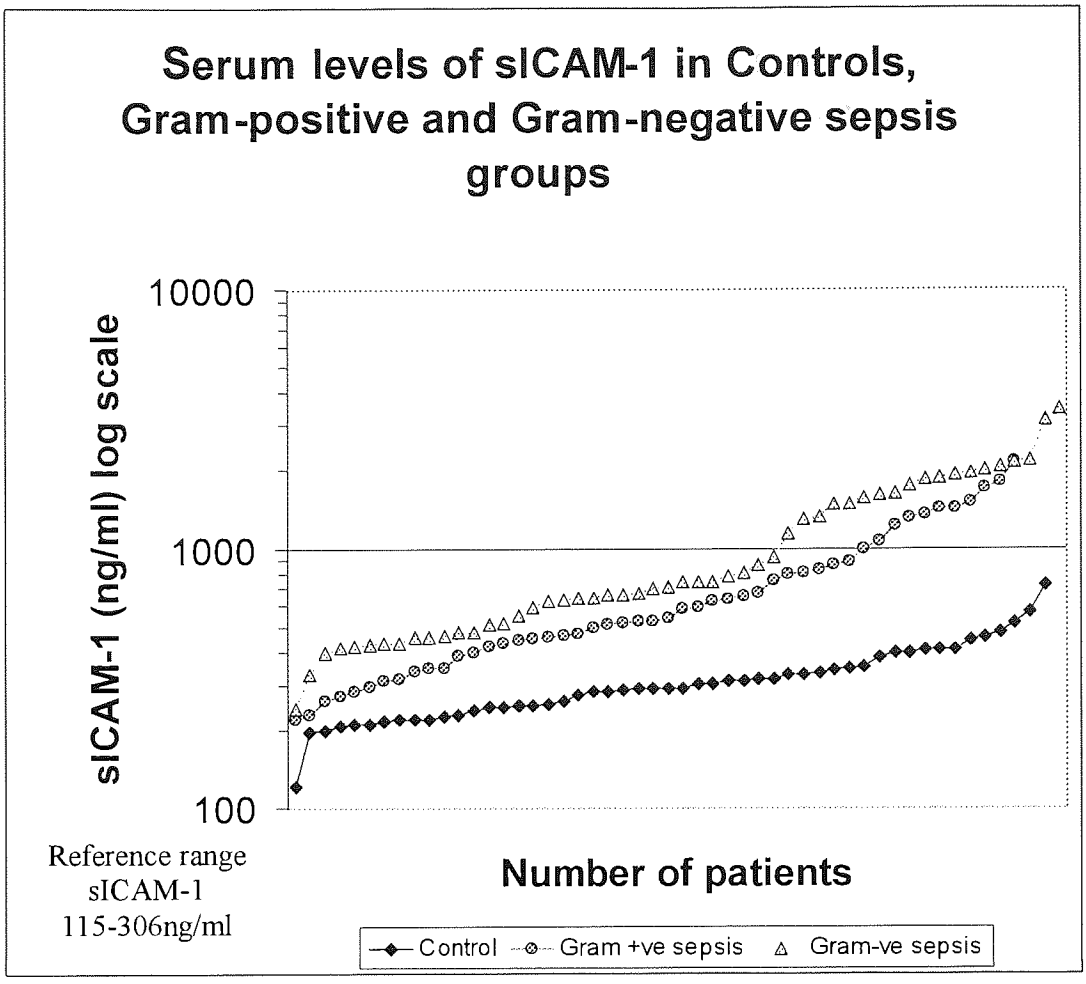


Figure 6.1.2 Individual serum levels of sICAM-1 for each patient
Patients with a Gram-negative sepsis (triangles) tend to have higher concentrations of serum sICAM-1 than patients with sepsis caused by Gram-positive bacteria.

Table 6.1.2 Median, CI and *p*-value for sICAM-1ng/ml; all patient groups

sICAM-1	Controls (51)	Gram-positive sepsis (50)	Gram-negative sepsis (52)
Median	291	536	728
CL% (CI)	95.1 (258-317)	96.7 (460-755)	96.4 (641-1139)
p 0.05		> 0.0001	> 0.0001
Range	600	1941	3197

CL%, is the confidence level set at 95% for the confidence interval.

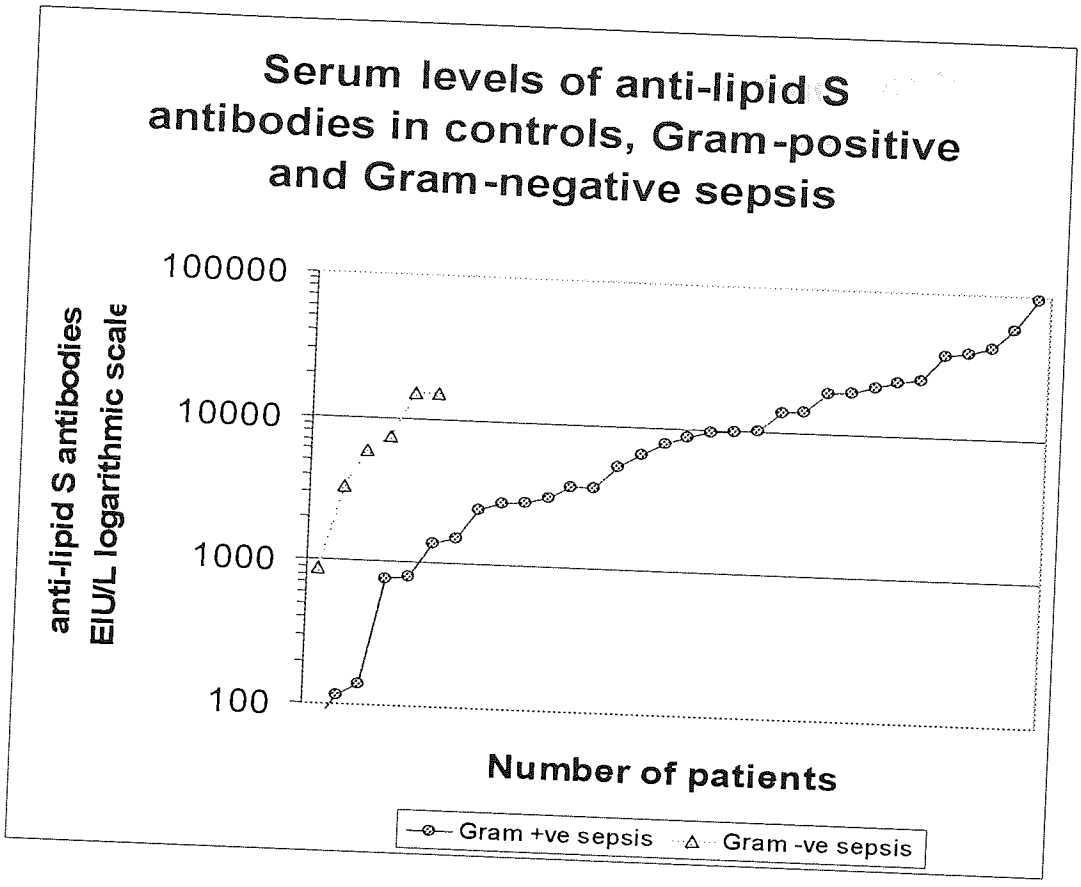


Figure 6.1.3 Individual serum levels of anti-lipid S antibody
Only positive results are shown in figure 6.1.3. The control patients were negative for anti-lipid S antibodies as were 47/52 patients from the Gram-negative group.

32/50 of the patients with a Gram-positive sepsis were positive for anti-lipid S antibodies. As this marker is an antibody there is a time delay between the appearance of the antigen (lipid S) and the development of antibodies. Six patients from the Gram-negative group were positive for anti-lipid S antibodies. As mentioned previously, it is not yet known how long anti-lipid S antibodies persist in the serum after an infection with a Gram-positive microorganism.

Table 6.1.3 Median, CI and *p*-value for anti-lipid S antibodies; all patient groups

Lipid S	Controls (51)	Gram-positive sepsis (50)	Gram-negative sepsis (52)
Median	0.00	1949	0.00
CL% (CI)	(0.00-0)	96.7 (0.00-6477)	96.4 (0.00-0)
p-0.05		0.0001	0.012
Range	0.00	95317	15107

(n) number of patients tested. Titres are in enzyme immunoassay units (EIU).

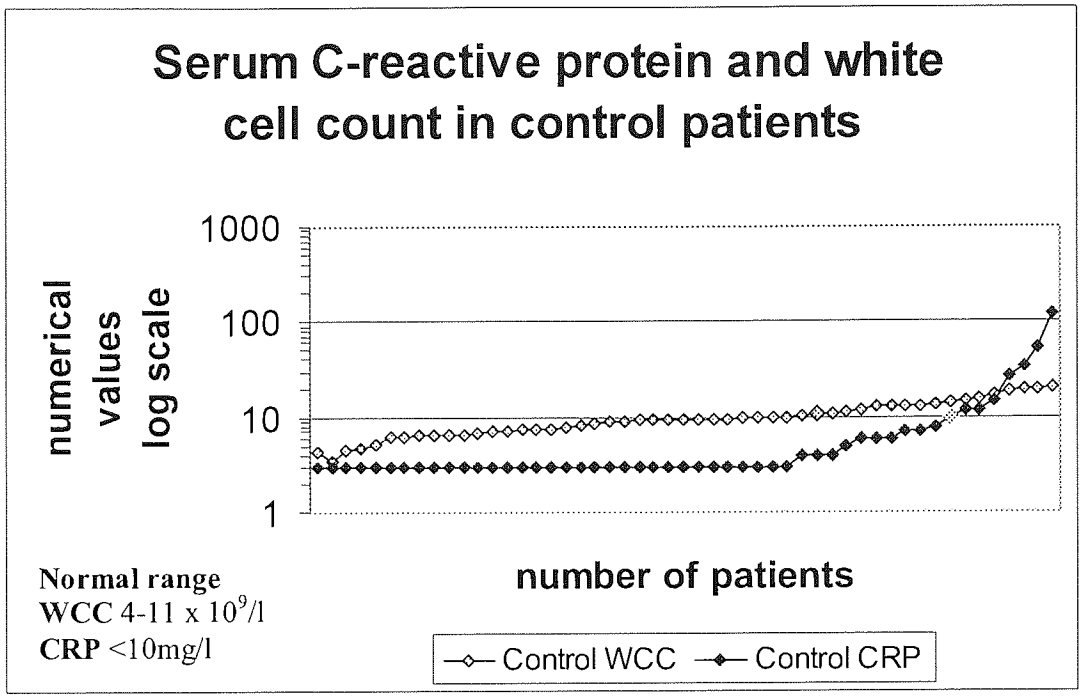


Figure 6.2.1 CRP and WCC in the serum of the control patients
The larger orange diamond represents the upper limit of the normal range for WCC and CRP. Serum CRP levels are not normally obtained from patients waiting for CABG.

The additional information given by the WCC and CRP in the control patients was useful extra information that helped to interpret unexpected elevations in the test markers.

Table 6.1.4 Median, *p*-value and range for serum WCC/l; all patient groups

WCC	Controls (51)	Gram-positive sepsis (50)	Gram-negative sepsis (52)
Median	9.4	11.5	12.0
p-value	-	0.149	0.103
Range	17.1	29.3	63

(n) number of patients. A WCC* of 63 x 10^9/l was obtained for a patient with Gram-negative sepsis, this was an unusual result, the next highest level from this group was 22.3 x 10^9/l

Table 6.1.5 Median, *p*-value and range for serum CRPmg/l; all patient groups

CRP	Controls (51)	Gram-positive sepsis (50)	Gram-negative sepsis (52)
Median	8.6	123	133
p-value	-	> 0.0001	>0.0001
Range	119	349	440

(n) number of patients. Control patients with elevated CRP levels are C2 (CRP 122); C13 (CRP 34) and C24 (CRP 27) from phase 2 and patient C48 (CRP 54) is new to phase 3.

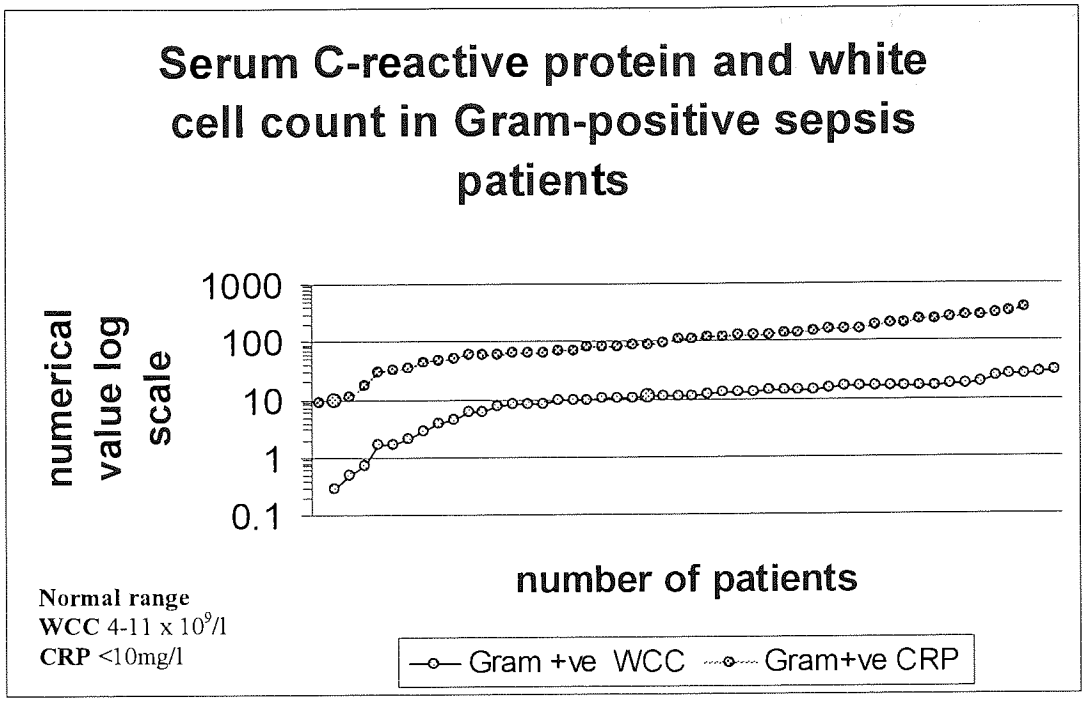


Figure 6.2.2 CRP and WCC in the serum of Gram-positive patients
The larger orange circles represent the upper limit of the normal range for WCC and CRP.

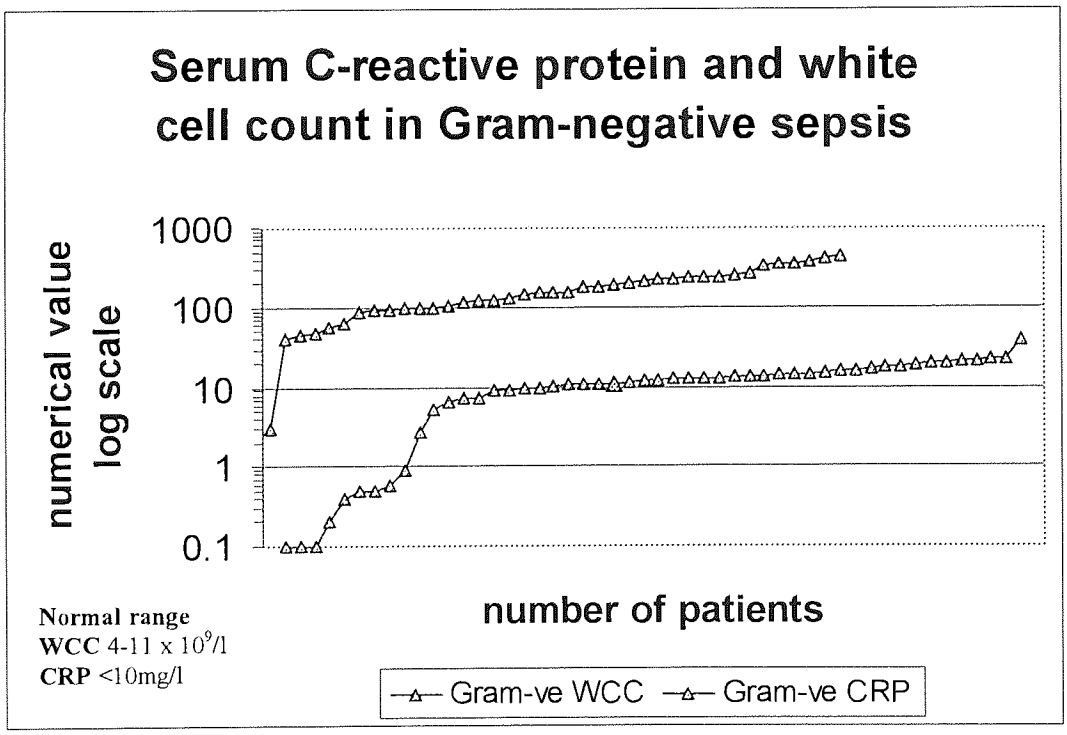


Figure 6.2.3 CRP and WCC in the serum of Gram-negative patients
The larger orange triangle represents the upper limit of the normal range for WCC.

The median, *p*-value and range for WCC and CRP in the Gram-positive and Gram-negative groups are give in tables 6.1.4 and 6.1.5. The sensitivities and specificities for IL-6, sICAM-1 anti-lipid S and CRP are given in tables 6.2.1 and 6.2.2

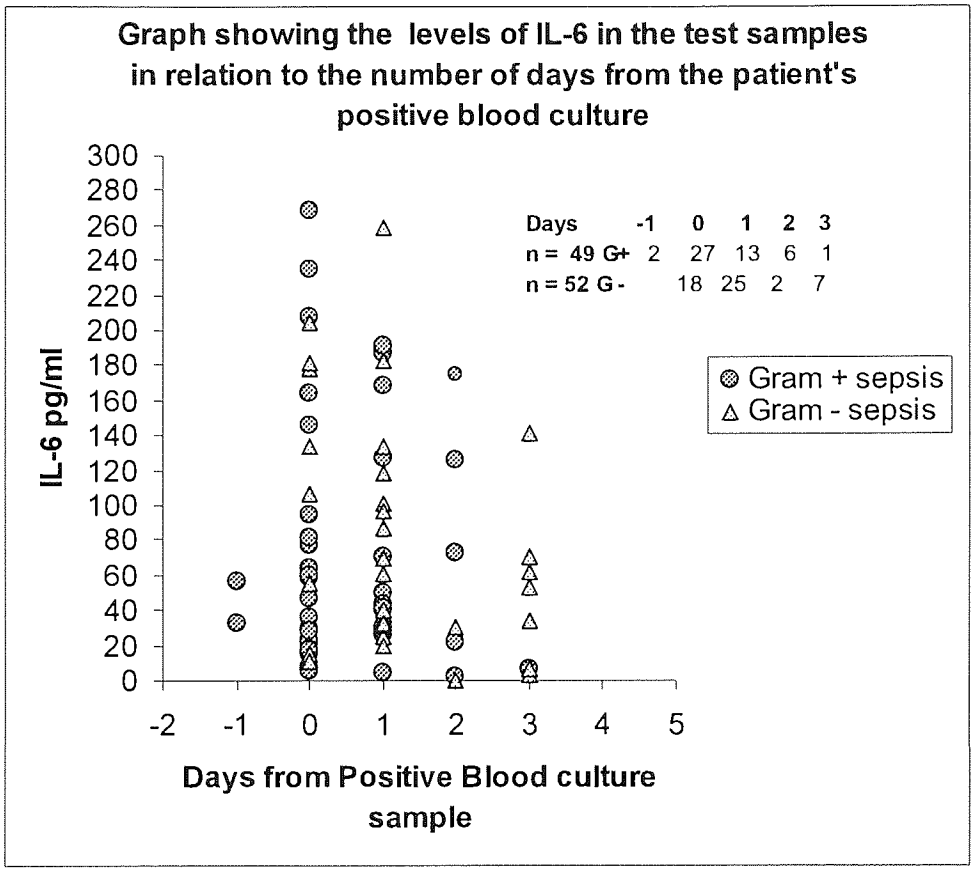


Figure 6.3 The concentration of IL-6 pg/ml in the serum of septic patients before and after BC day.

Serum concentrations of IL-6 greater than 300pg/ml are not shown as the majority of results fall within the given range. Not shown; 3 Gram-positive patients (range 422-1112 pg/ml) and 12 patients from the Gram-negative group (range 433-1690pg/ml). The graph shows that more test samples were obtained on the BC day rather than saying that the zero BC date is equivalent to the highest serum levels of IL-6.

Assuming that IL-6 is reaching its peak serum level at the point when a clinician suspects a serious systemic infection and requests a BC, the graph shows IL-6 is still detectable in patients serum after three days, and at concentrations on or above a proposed cut-off level for sepsis of 40pg/ml (when judged in conjunction with another marker such as sICAM-1).

6.3.2 TWO-WAY SCATTER GRAPHS

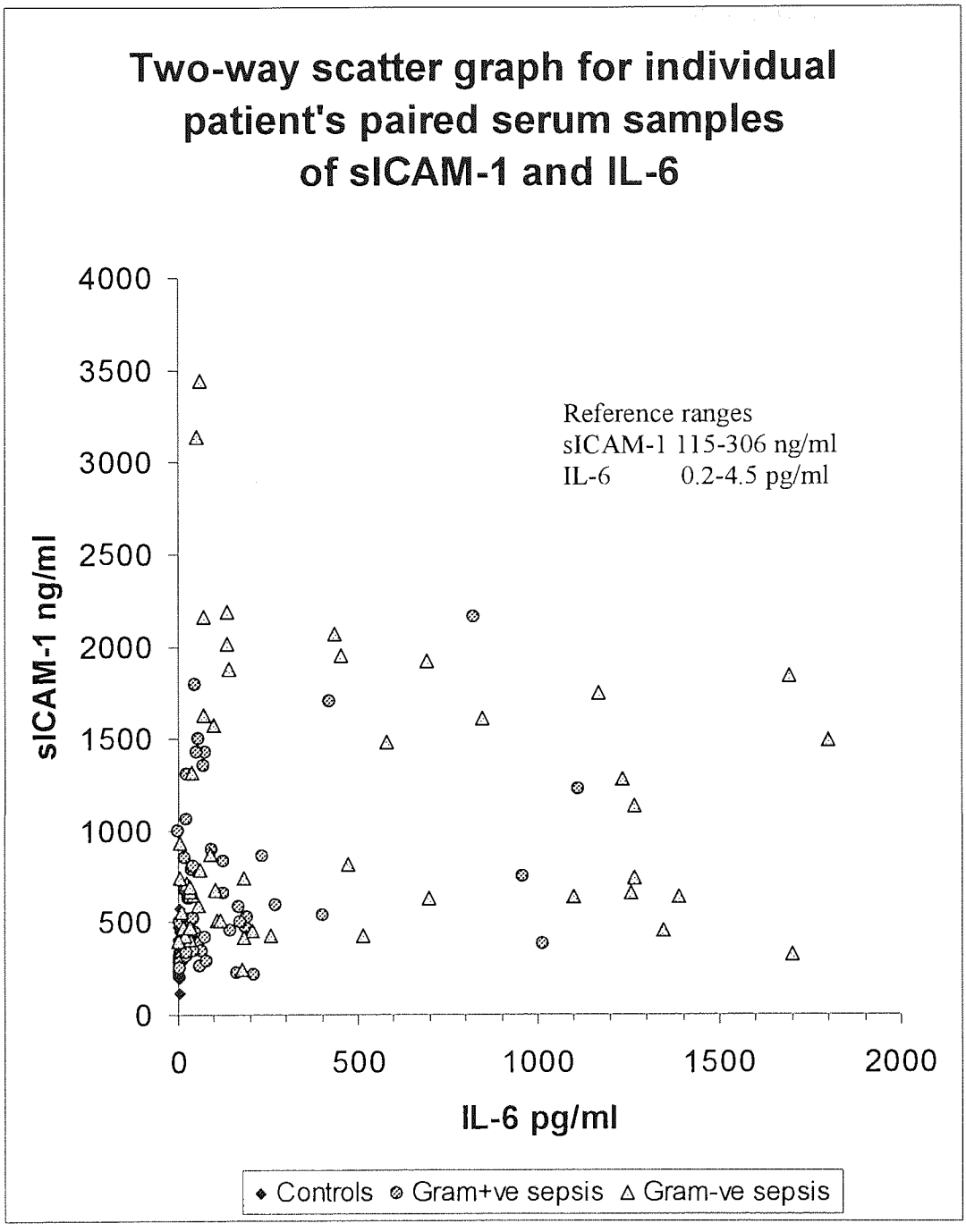


Figure 6.4.1 Two-way scatter graph showing individual patient's paired serum levels of sICAM-1 and IL-6

Figure 6.4.1 shows a cluster of results with IL-6 levels under 100pg/ml and the control patients results are obscured. Figure 6.4.1.1 below gives an expanded view of this important area of the graph.

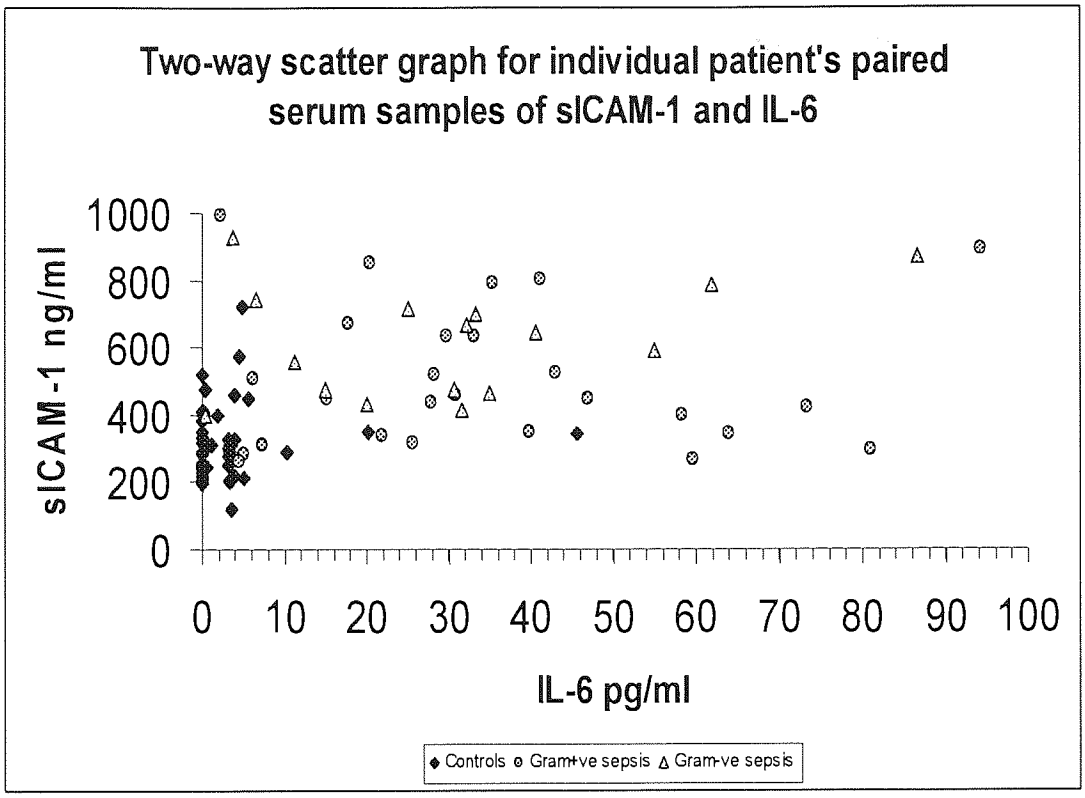


Figure 6.4.1.1 an expanded view of figure 6.4.1 highlighting the control patient's combined serum levels of sICAM-1 and IL-6.

The control patients with 45pg/ml and 20pg/ml concentrations of serum IL-6 were discussed in the previous chapter. The highest serum concentration of IL-6 in the current group of control patients was 10.3pg/ml.

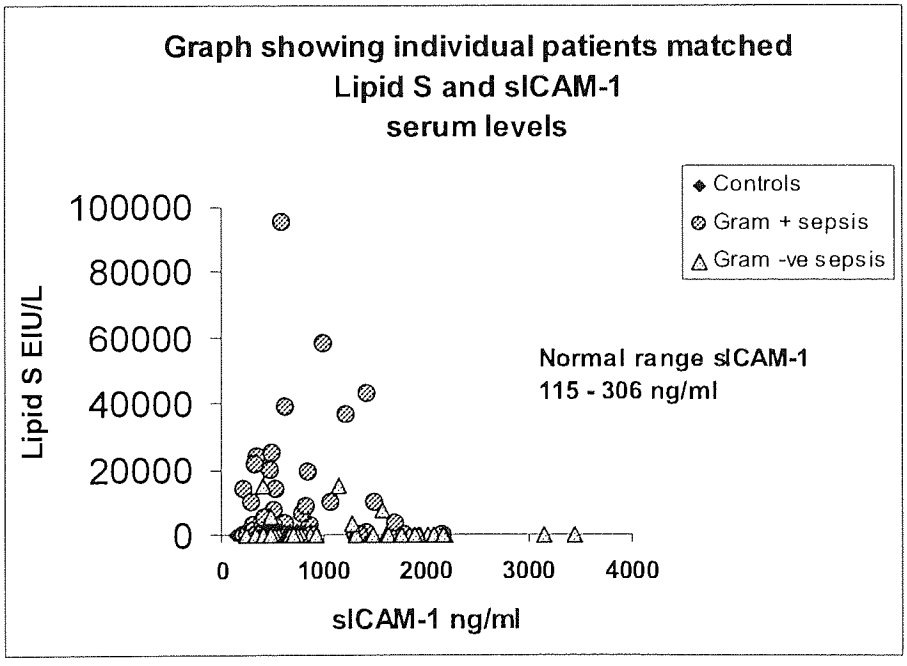


Figure 6.4.2 Two-way scatter graph for anti-lipid S and sICAM-1

All the controls were negative for lipid S.

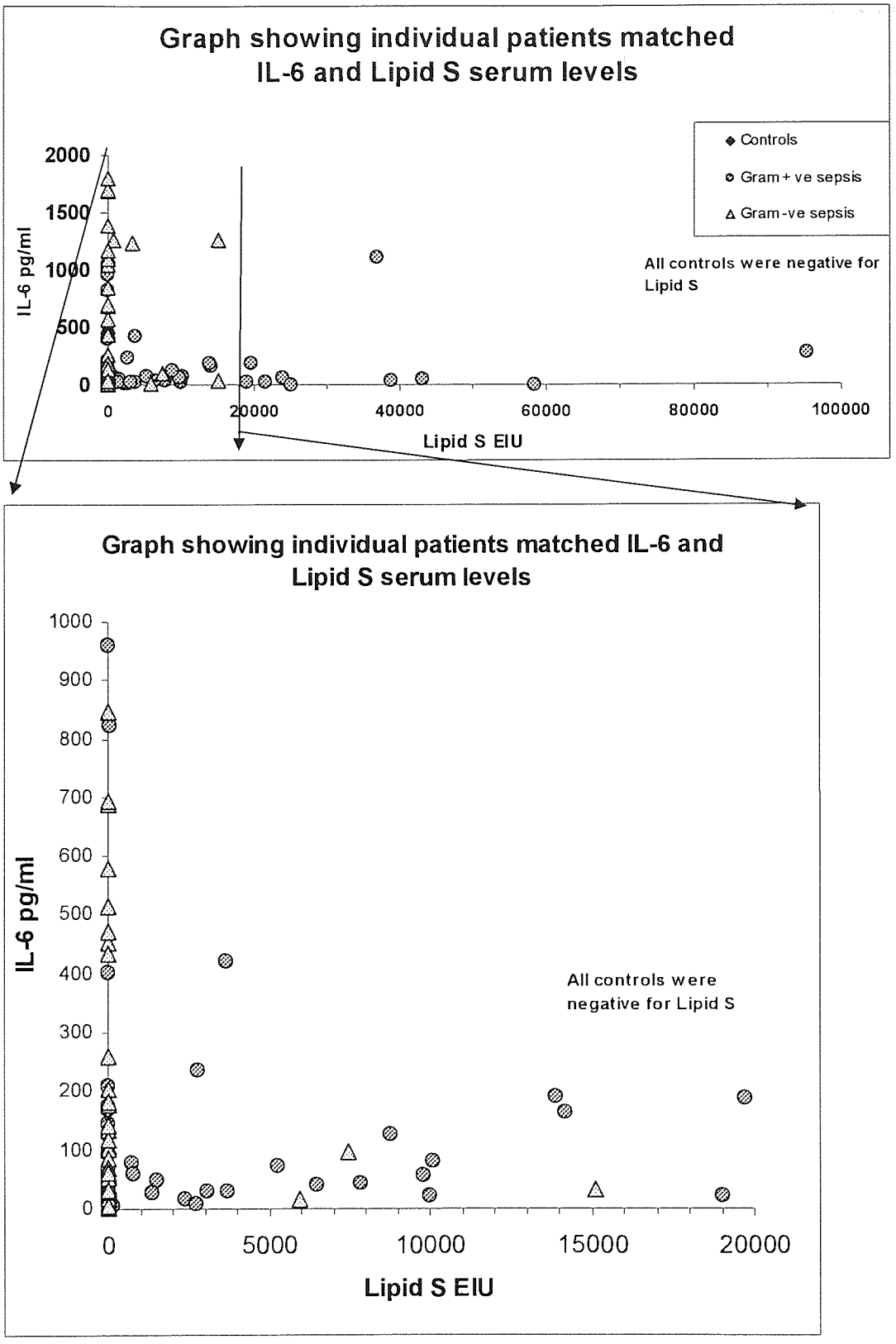


Figure 6.4.2.1 Two-way scatter for IL-6 and anti-lipid S antibody titres

In the Gram-positive group 18/50 patients were negative for Lipid S; 10/18 of these were taken on the same day as the positive BC, however 16 of the lipid S positive results came from sera taken on the same day as the positive BC.

6.3.3 TWO-WAY SCATTER GRAPHS FOR IL-6 AND CRP; CRP AND WCC; IL-6 AND WCC

The serum levels of CRP and the WCC given in figures 6.2.1, 6.2.2 and 6.2.3 showed that there is a clear elevation of both these traditional markers of infection in the septic groups as opposed to the controls. The median, *p*-value and range of CRP and WCC in all patient groups are given in tables 6.1.4 and 6.1.5.

Two-way scatter graphs were created to see if IL-6 with CRP or IL-6 and the WCC could give a better indication of sepsis than IL-6 with sICAM-1 or IL-6 with anti lipid S.

Each graph relates exclusively to one of the three patient groups, and has three colour codes with the appropriate patient group symbol; diamonds for controls, circles for the Gram-positive patients and triangles for the Gram-negative group. The colour codes relate to the same paired combinations for example IL-6 and CRP are orange in all the patient groups.

The figures 6.5.2 and 6.5.3 below, illustrate that serum levels of IL-6 and CRP combined in two-way scatter graphs produce a distinctly different picture in the patients with sepsis as opposed to the controls shown in figure 6.5.1.

The sensitivities and specificities for IL-6 and CRP combined were calculated for patients with a Gram-positive and Gram-negative sepsis and compared with those of IL-6 combined with sICAM-1 and anti-lipid S antibodies, the results are given in tables 6.3.1 to 6.3.4.

Three graphs were presented on a single page to allow for easier comparison between the control values and the septic groups and between the two septic groups.

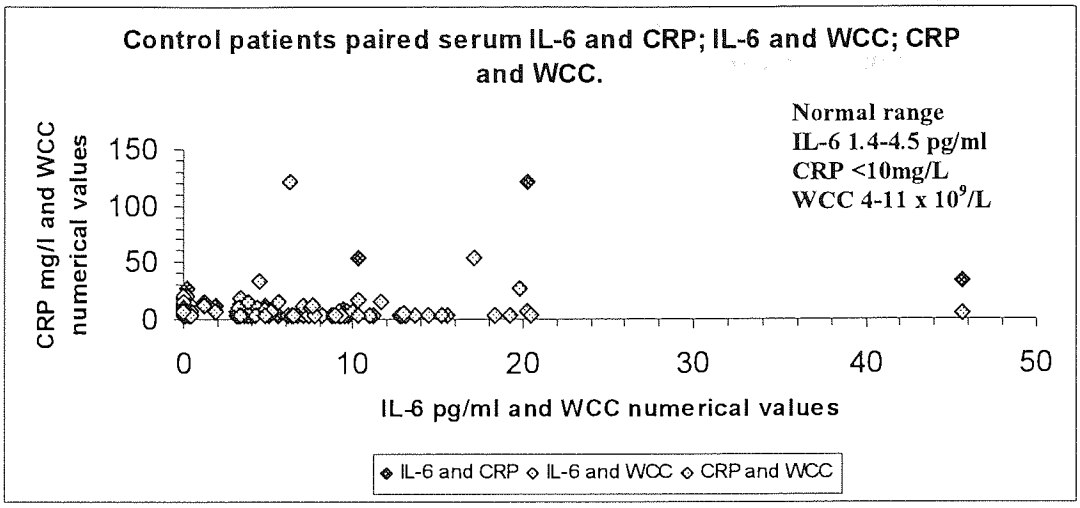


Figure 6.5.1 two-way scatter graph for control patients paired IL-6 and CRP.

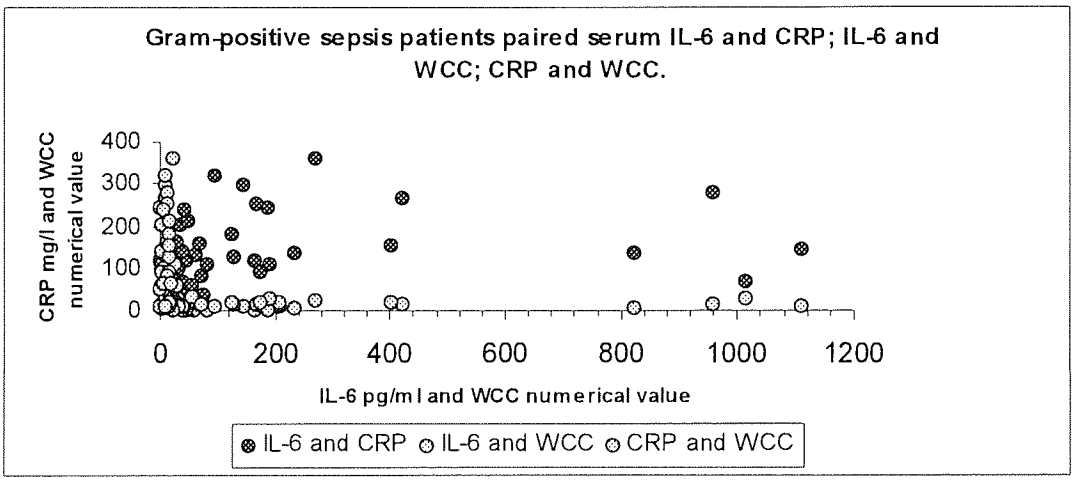


Figure 6.5.2 two-way scatter graph for Gram-positive patients IL-6 with CRP.

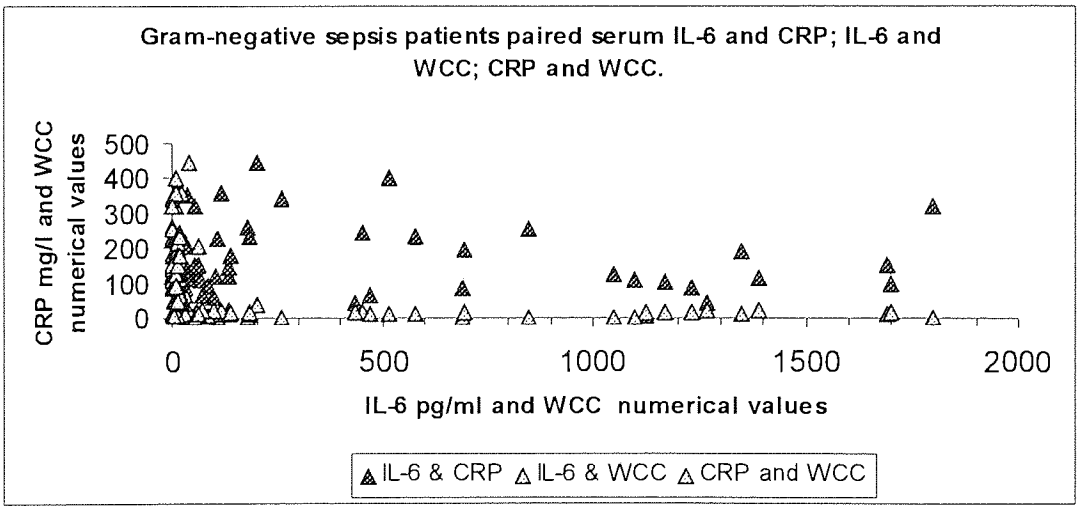


Figure 6.5.3 two-way scatter graph showing Gram-negative patients paired IL-6 with CRP; IL-6 with WCC and CRP and WCC.

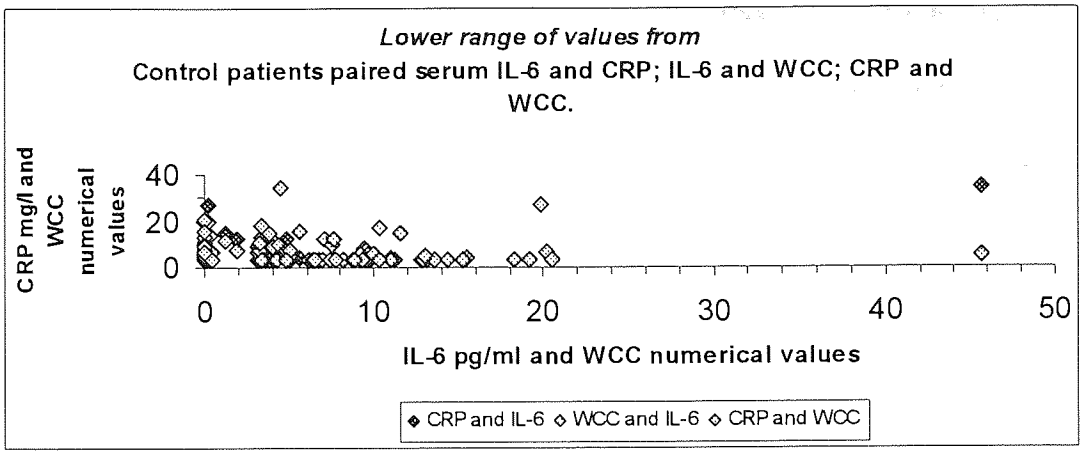


Figure 6.5.1.1 expanded version of graph 6.5.1. Control group

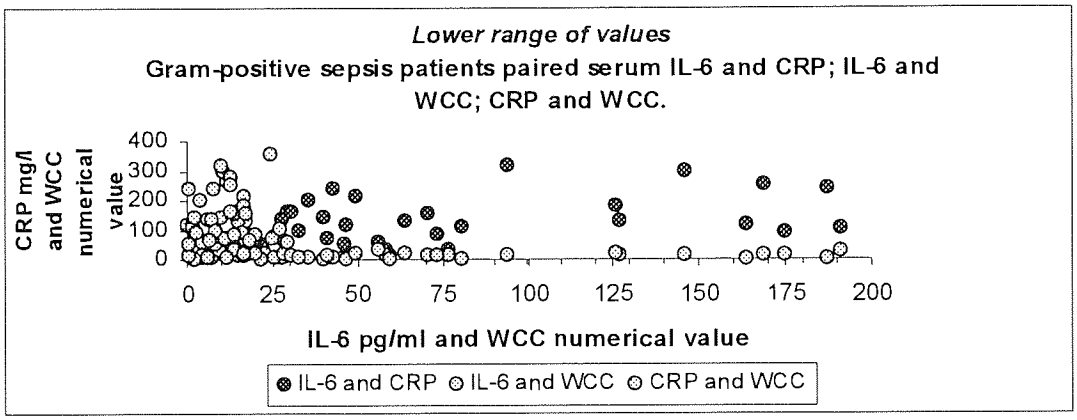


Figure 6.5.2.1 expanded version of figure 6.5.2. Gram-positive group

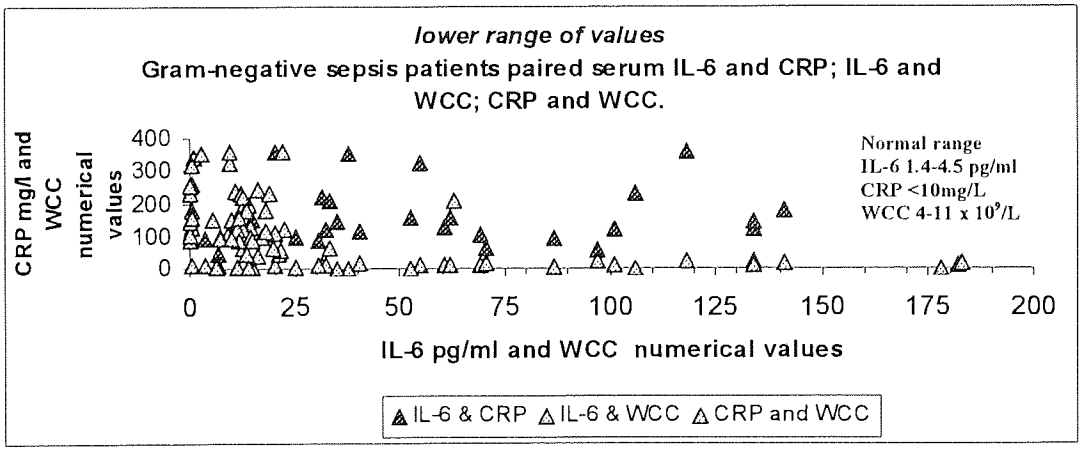


Figure 6.5.3.1 expanded version of graph 6.5.3 Gram-negative group

6. 3.4 SENSITIVITIES AND SPECIFICITIES: SINGLE MARKERS

Table 6.2.1 GRAM-NEGATIVE SENSITIVITY (%) and SPECIFICITY (%)(Gram-negative sepsis n = 52)

Cut-off values	Sensitivity %	Specificity %	PPV %	NPV %
IL-6	**G -ve**			
4.5	98	88	89	95.7
10	**98**	**94**	**94**	**94**
20	**92**	**96**	**95.9**	**90**
40	78	98		
50	74	100		
sICAM-1	**G -ve**			
306	**98**	56		
350	**96**	74		
400	**96**	80		
450	84	90		
Lipid S	**G -ve**			
> 0	11	100		
500	11	100		
1000	9.6	100		
2000	9.6	100		
CRP				
12	**98**	**90**		
20	**98**	**92**		
40	**98**	**96**		
60	**94**	**98**		

CRP the traditional marker of inflammation and infection produced the best sensitivities and specificities in the project so far. However four control patients (C2, C13, C24 and C48) had elevated CRP levels. Two of these patients had elevated IL-6 and LBP pre-surgery and both were pyrexial post-surgery for coronary artery bypass graphs. Therefore they may have been incubating an infection prior to their operation. In which case, CRP, IL-6 and LBP may be important indicators of an early infection.

It is becoming clearer as the project advances that single markers reflect a single aspect of the human response to physical trauma or infection and that two markers naturally reflect a larger part of the story. Incorporating CRP and the WCC serum levels with the test markers produces a more convincing framework for diagnosing sepsis. What is not yet apparent is how to read these combined markers when they are not always as elevated as expected in patients with sepsis, or are elevated in a few controls.

Table 6.2.2 GRAM-POSITIVE SENSITIVITY (%) and SPECIFICITY (%)
(Gram-positive sepsis n = 49)

Cut-off value	Sensitivity %	Specificity %	PPV %	NPV %
IL-6	**G +ve**			
4.5	96	88		
10	**91**	**94**	**93.7**	**90**
20	89	96		
40	67	98		
50	51	100		
sICAM-1	**G +ve**			
306	88	56		
350	78	74		
400	74	80		
450	66	90		
Lipid S	**G +ve**			
> 0	64	100		
500	58	100		
1000	54	100		
2000	48	100		
CRP				
12	**95**	**90**		
20	**93**	**92**		
40	87	96		
60	77	98		

Patient P29 (previously P11) was not included in the calculations for sensitivities and specificities in the Gram-positive group as he proved not to be septic. Therefore the total number of Gram-positive patients was 49.

The sensitivities and specificities for IL-6 at a serum cut-off level of 5 and 10pg/ml are higher than previously found in the Gram-positive group given in table 4.5.2. The 100% specificities for anti-lipid S are due to the fact that all the controls were negative for anti-lipid S. A cut-off level for IL-6 of 10 or 20 pg/ml for a diagnosis of sepsis is unrealistic as many other conditions (without infections) can cause an elevation of IL-6 within this range.

Table 6.2.3 GRAM-POSITIVE AND GRAM-NEGATIVE SENSITIVITY (%) and SPECIFICITY (%)
(=TOTAL-Sepsis n = 101)

Cut-off values	Sensitivity %	Specificity %	PPV %	NPV %
IL-6	**G +ve/G-ve**			
4.5	**96**	**88**	**94**	**93.7**
10	**92**	**94**	**96.9**	**85**
20	88	96		
40	69	98		
sICAM-1	**G +ve/G-ve**			
306	**93**	56		
350	87	74		
400	86	80		
450	76	92		
Lipid S	**G +ve/G-ve**			
>0	37	100		
>1000	31	100		

Results showing high sensitivities and specificities are highlighted in bold and their respective positive predictive values (PPV) and negative predictive values (NPV) were calculated as given in section 3.5.3.2

6.3.5 SENSITIVITIES AND SPECIFICITIES: TWO MARKERS

A single marker for sepsis has not been found from the nine test markers assayed at the beginning of the project, however two markers combined have previously been shown to increase the sensitivity and specificity of the markers for a diagnosis of sepsis.

Table 6.3.1 TWO-MARKERS COMBINED (Gram-negative sepsis)

Cut –off values	Sensitivity %	Specificity %
IL-6 with sICAM-1	**G -ve**	
20/350	100	70
40/350	100	72
20/500	**96**	**90**
40/500	**92**	**92**
IL-6 with Lipid S	**G -ve**	
10/>0	**94**	**93**
20/>0	88	95
40/>0	80	97
sICAM-1 with Lipid S	**G-ve**	
350/>0	96	74
500/ >0	76	94

Gram-negative patients individually paired test markers, n =52.

Table 6.3.2 Two markers combined (Gram-positive sepsis)

Cut –off values	Sensitivity %	Specificity %
IL-6 with sICAM-1	**G +ve**	
20/350	95	70
40/350	91	72
20/500	**93**	**90**
40/500	83	92
IL-6 with Lipid S	**G +ve**	
10/>0	**100**	**93**
20/>0	**97**	**95**
40/>0	**91**	**97**
sICAM-1 with Lipid S	**G+ve**	
350/>0	93	74
500/ >0	83	94

Gram-positive patient's individually paired test markers, n = 49

Table 6.3.3 Sensitivity and specificity of IL-6 with CRP in Gram-negative sepsis

Cut –off values	Sensitivity %	Specificity %
IL-6 with CRP		
10/12	100	86
20/20	**98**	**92**
20/40	**98**	**96**
40/40	**98**	**96**
50/60	**96**	**98**

The specificity for IL-6 with CRP increased as the cut-off level for CRP increased, this is shown by the 20/20 - 20/40 cut-off values. However CRP as a single marker loses sensitivity as the cut-off value increases; see tables 6.2.1 and 6.2.2

This is an exciting finding as the combination of IL-6 with CRP makes these markers both sensitive and specific for sepsis. However a note of caution, one of the controls had a CRP level of 122mg/l and a concurrent 20.0 pg/ml of IL-6 and although this patient (C2) was subsequently given antibiotics and therefore probably infected he was not septic. Therefore establishing the serum cut-off levels for sepsis remains to be established

Table 6.3.4 Sensitivity and specificity of IL-6 with CRP in Gram-positive sepsis

Cut –off values	Sensitivity %	Specificity %
IL-6 with CRP	**Gram-positive sepsis**	
20/20	**100**	**92**
20/40	**100**	**96**
40/40	**95.9**	**96**
50/50	**93**	**96**
50/60	**87**	**98**

Table 6.3.4 The sensitivity drops for Gram-positive cases when CRP > 50mg/l

6.4 DATA ANALYSIS

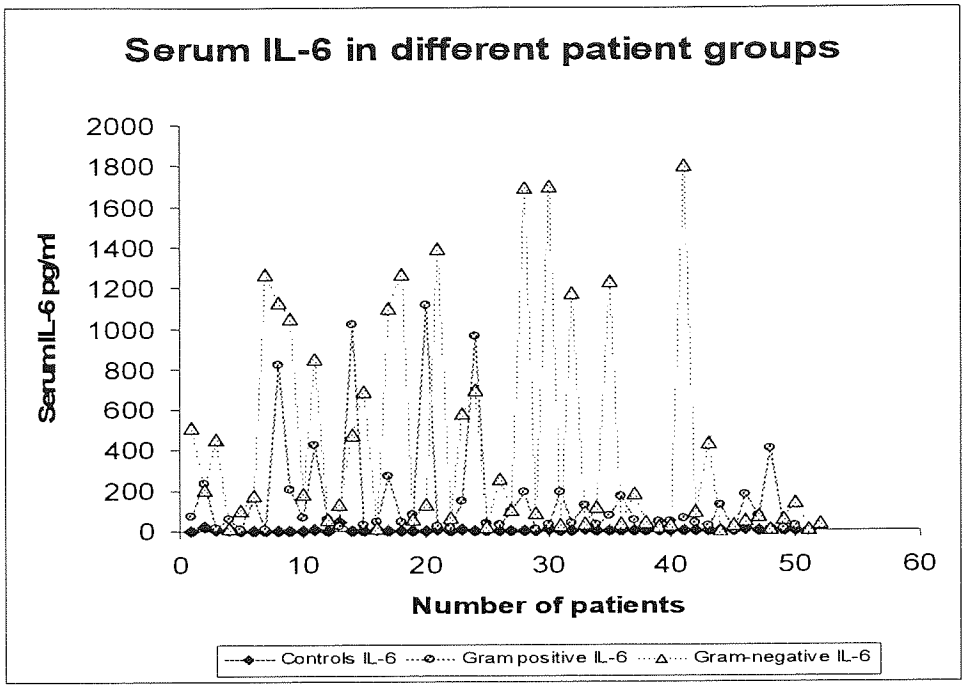


Figure 6.6. IL-6 in all the patient groups.
The joining lines merely connect one patient's serum IL-6 level to the next patient in line. The lines illustrate an increase in a patients marker above the control line, illustrated more clearly in figure 6.6.1

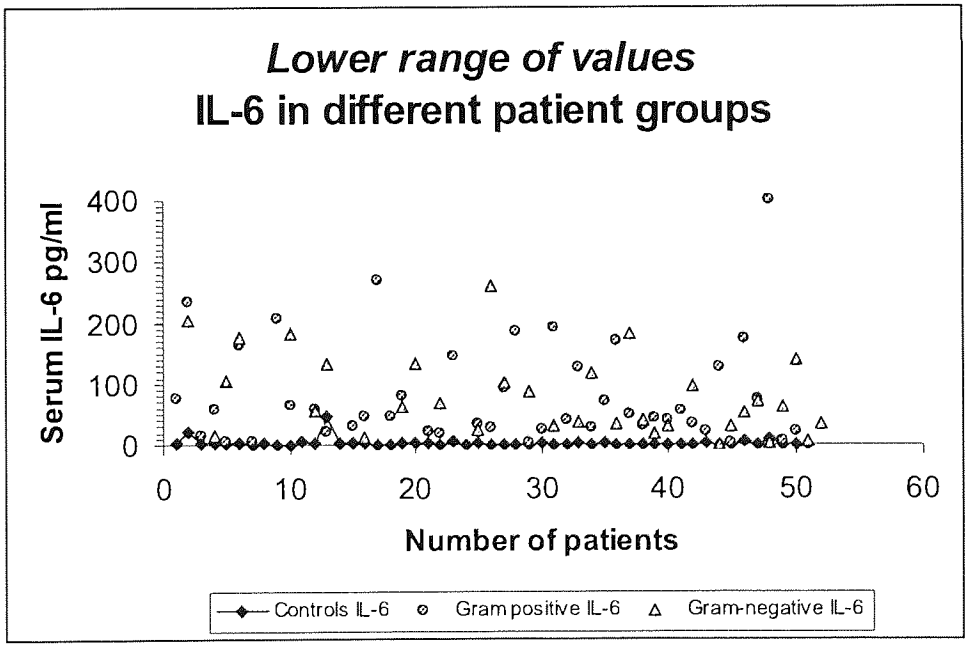


Figure 6.6.1 Lower range serum IL-6 values in all patient groups
The controls form a chain of connected results, only interrupted by three results with elevated serum IL-6. Whereas the Gram-positive and Gram-negative results vary extensively and may reflect degrees of sepsis such as early sepsis, severe sepsis and approaching septic shock.

Table 6.4 Analysis of the different serum levels of the test markers in patients with Gram-negative sepsis

Number of patients (%)	Group division	sICAM-1 ng/ml	CRP mg/ml (range)	WCC 1 x 10^9/L	IL-6 pg/ml (range)
11 (21%)	All IL-6 >1000 pg/ml (1098-1800)	All above normal; 5 > 1000ng/ml (328-1840)	All (bar 1) >45 (46-318)	All >11 (bar 1*) (12.6-21) E for L	
15 (28.8%)	sICAM-1 > 1000 ng/ml (1321-3440)		All > 40 (42-355)	All > 11 (bar 3*) E for L	All > 37 (bar 1) (37-690)
14 (26.9%)	Intermediate sICAM-1 > 500 ng/ml (515-867)		All > 45 10 > 110 (118-323)	All > 11 (bar 3*) (11.1-18.7) E for L	All (bar 2) (25-694)
11 (20%)	Low sICAM-1 (243-514)		All > 89 (89-433)	4 > 11 4* < 11 E for L (3)	All > 20 (bar 1**) (20-515)

E for L - except for leukaemias. Table 6.4 divides the patients into 4 groups. The first group includes all the patients with IL-6 serum levels greater than 1000pg/ml. The three other groups are divided on the basis of their serum sICAM-1 levels, high, medium and low.

The asterisks in table 6.4 are next to WCC counts that were within the normal range, but in every case their corresponding CRP levels were highly elevated. The patient with a low IL-6 ** (in the low sICAM-1 group) had a WCC of 13.4 and CRP-181mg/ml.

There was only one patient (new N16; not the phase 2 N16) in the Gram-negative group that stood alone and is not included in the table as every marker was normal except for sICAM-1 which was 555ng/ml. This patient is discussed in section 6.6 and table 6.8

It is interesting to note that patients with the highest levels of CRP had the lowest levels of sICAM-1. This finding could possibly reflect that their septic episode is in the early phase.

6.4.1 Patients with a Gram-positive BC and their serum IL-6, CRP and WCC.

Patients with a Gram-positive BC and a WCC above the normal range (4.0-11.0 x 10^9/l) were put together in group 1 (n=26). Group 2 contained patients with a WCC within the normal range (n=14) and group 3, patients with a WCC below the normal range (n=10).

All the patients in the first group had elevated WCC and all had raised CRP levels except patient P11 (from phase 2, discussed in chapter 5 and not septic). Patients in this group had elevated IL-6 (except P11) and elevated sICAM-1 except P11 and P9.

Although the second group had WCC within the normal range all their CRP levels were elevated (range 31- 317mg/l). They had elevated levels of IL-6 (bar 2 and range 21-1112pg/ml). Twelve patients (12/14) had raised sICAM-1 (range 435-2160ng/ml) and 10/14 were positive for anti-lipid S antibodies.

The third group of patients in the Gram-positive group with WCC below the normal range, contained cancer patients (7/10) many of whom had just completed a course of chemotherapy for example P5 (discussed in chapter 5). Two other patients were on steroids and one had chronic renal failure. They all had elevated levels of CRP (range 50-241mg/l), elevated levels of IL-6 (bar 2) ranging from 21 to 187pg/ml and 6/10 had moderately elevated sICAM-1. Three of these patients with acute myeloid leukaemia (AML) were discussed in chapter 5 (tables 5.9.4.3/4/5).

The anti-lipid S assays reflected the involvement of a Gram-positive microorganism in the disease process for the majority of patients with a Gram-positive sepsis. This assay not only served to differentiate between a Gram-positive sepsis and a Gram negative sepsis but may indicate the degree of infection and/or the length of time a patient's immune system had been exposed to the infection. Five of the Gram-negative septic patients (5/52) had anti-lipid S antibodies and in the absence of microbiology may reflect an occult involvement of a Gram-positive infection, or a recent previously unknown infection with Gram-positive bacteria.

6.5 PHASE 3 SUMMARY

A total of 83 new patients from the Queen Elizabeth and Selly Oak hospitals (Birmingham, UK) were enrolled onto this current phase of the sepsis screen study. There were 25 control patients and 30 patients with a Gram-positive sepsis and 28 with a Gram-negative sepsis. The controls, in keeping with all the other controls were hospital patients waiting for CABG surgery. Patients with sepsis had similar underlying conditions as those in the previous phase (2) and the first phase (1).

Serum samples were assayed for IL-6, sICAM-1 and anti-lipid S antibodies. WCC for all the patients and CRP values for the septic patients came from patient's records. CRP serum levels for the control patients and those septic patients without a CRP record for the day of their BC were tested for this study by hospital personnel funded by the research grant.

Assay results from this new group of patients were presented in this chapter in combination with the results from the previous phase (2) in chapter 5, making a total of 153 patients (51 controls, 50 Gram-positive and 52 Gram-negative).

The results show that the mean values for the test markers and CRP are significantly higher in the septic patients than the controls with p-values < 0.05. These results are listed in tables 6.1.1 (IL-6); 6.1.2 (sICAM-1); 6.1.3 (anti-lipid S) and 6.1.5 (CRP). The white cell counts in both septic groups did not show a significant difference as shown in table 6.1.4.

The most discriminating markers for sepsis defined by sensitivities (%) and specificities (%) above 90% for both septic groups was IL-6 in combination with CRP (tables 6.3.3 and 6.3.4). Next best for Gram-negative patients was IL-6 with sICAM-1 at a cut-off 20pg/nl for IL-6 and 500ng/ml -sICAM-1 (sensitivity 96% specificity 90%). For the patients with a Gram-positive sepsis the next best markers were IL-6 (20pg/ml) combined with anti-lipid S antibodies > 0 (sensitivity 97% and specificity 95%).

6.6 DISCUSSION

The main aim for this phase of the project was to define serum cut-off levels for a diagnosis of sepsis. The sensitivities and specificities suggest serum values such as 40pg/ml for IL-6 and 500ng/ml for sICAM-1 and CRP values of 40mg/l. Anti-lipid S titres greater than zero indicated the involvement of a Gram-positive microorganism, except in patients that were immunosuppressed since the onset of the current infection.

However there is a problem with this simplistic view. According to these cut-off levels not all the patients in the septic groups were septic. 14 patients in the Gram-positive group had less than 500ng/ml of sICAM-1, and 17 patients had less than 40pg/ml of IL-6. Seven of these latter patients were counted in the group with less than 500ng/ml of sICAM-1 listed in table 6.4.

Each patient had a range of results that included the test markers and their WCC and CRP results and 20/50 patients had LBP results from the previous phase. Shapes or patterns emerged from these collective results suggesting degrees of infection and sepsis and in some cases possibly not septic at all, such as patient P11 discussed in chapter 5 (table 5.9.2).

Alternatively the range of serum levels in the test markers could relate to a stage in sepsis development such as early sepsis, fully developed sepsis, severe sepsis with organ failures, or resolving sepsis due to antibiotic treatment.

Probably the most surprising results were the unexpectedly high sensitivities and specificities achieved by combining IL-6 with CRP. They did not always correlate but supported each other; when one serum level was lower than expected the other was high and taken together with other markers they helped to define patients as infected/septic when definitive cut-off levels for the test markers appeared to fail.

Patient data can be analysed in terms of age, gender and underlying disease or grouped together by the microorganism isolated from their BC. Another grouping for analysis could be the length of time a BC was incubated before it became positive – hours, a day, several days and by the number of positive BC bottles 1 or 2. The particular relevance of the length of time it takes for a BC to become positive relates to the assumption that a fast positive BC has more microorganisms/ml in the inoculating sample than BC's requiring longer incubation times.

The Gram-positive group of patients were analysed by their WCC (section 6.4.1) with more surprising results. They were divided into three groups, those with elevated WCC, those with normal WCC and patients with lower than normal WCC. The results showed that all the patients (26/50) with an elevated WCC had elevated levels of CRP. All except one patient (P11 discussed in chapter 5, table 5.9.2) and he proved not to be septic. Therefore these patients were already positive for the two gold standard markers of infection, an elevated WCC and raised CRP. Added to this they all presented with a pyrexia and a positive blood culture. The test markers would appear to be irrelevant in

these cases. Nevertheless the extra information about the serum levels of IL-6, anti-lipid S antibodies or sICAM-1 may add to the clinical picture, help in patient management and possibly improve the outcome as six (6/50) patients subsequently died. In addition 48% of the remaining Gram-positive patients did not have elevated WCC. These patients in particular need extra diagnostic tests to improve their management.

It was stated earlier that shapes and patterns emerge when all the markers are viewed together and that these patterns make the shapes that suggest an infection not sepsis, or an inflammatory response not an infection.

To draw an analogy from music where the notes are the markers; the first few notes of a melody can inform you about the whole song. This involves the individual notes, their values and the position they take on the scale and crucially their *relationship to each other*. In the same way the combination of markers and the degree to which they are elevated in relation to each other may help the clinician to differentiate between inflammation/infection, infection/sepsis and sepsis/severe-sepsis. Examples are given in tables 6.5 to 6.8 to illustrate these points.

In terms of the tune analogy a false note rings when a patient's sICAM-1 levels are normal even though others markers are elevated. Two patients are compared to illustrate this point.

Table 6.5 Patient P9 with normal serum sICAM-1

IL-6	sICAM-1	LBP	Anti-lipid S	CRP	WCC
0.2-4.5	115-306	5-10 μg/ml	> 0	<	4-11
pg/ml	ng/ml			10mg/l	$x10^9$/l
208	**219**	**70**	**0**	**11**	**16.8**
+	**-**	**+**	**-**	**+/-**	**+**

P9 eventually proved to have a mixed blood culture, and by definition - septic (elevated WCC, pyrexia and positive BC), but his test markers do not conform to the 'septic tune'. The aerobic BC was positive after I days incubation.

The test serum was taken the same day as the BC sample. The aerobic BC bottle was positive with Gram-positive short-chain cocci, which was identified as a beta (β) haemolytic streptococci (group G, Lancefield's typing) a common cause of throat, respiratory tract and skin infections (Banerjee, 1985). Five days later the anaerobic bottle became positive with a Gram-positive rod, identified as a *Propionibacterium* spp.

P9 was admitted with epiglottitis and a temperature of 38.3 °C that increased to 39.9°C. A BC was taken and he was given antibiotics (cefuroxime and metronidazole). The patient had a history of cancer at the base of the tongue (15 months previously) and a special note was made 8 days after his positive BC that chronic inflammatory changes had been observed in the mastoid air cells caused by radiotherapy. It was not clear if the radiotherapy was pre or post his epiglottitis and positive BC. Nevertheless the test marker (LBP) and WCC suggest an infection and an inflammatory response (the latter because of the high IL-6 in relation to a low CRP), rather than sepsis.

It is difficult to make precise comparisons between patients unless the differences between them can be minimised, however the following patient (P37) had the same WCC as patient P9 and he was a similar in age (47 years and P9 was 50).

Table 6.6 Patient P37 a more convincing septic profile

IL-6	sICAM-1	LBP	Anti-lipid S	CRP	WCC
0.2-4.5	115-306	5-10 μg/ml	> 0	<	4-11
pg/ml	ng/ml			10mg/l	$x10^9/l$
49.5	**1425**	**nd**	**43,054**	**210**	**16.8**
+	**+**		**+**	**+**	**+**

nd-not done. The BC grew an MRSA from both bottles in 1 day.

Does the balance of markers in this patient reflect sepsis, or severe sepsis? Alternatively do these results indicate the type of septic response elicited specifically by MRSA? Even though his IL-6 is quite low in comparison with other patients with sepsis, his CRP level is convincing elevated for a bacterial infection. The high level of anti-lipid S antibodies suggests that the original focus of infection had probably been developing over time. The very high sICAM-1 is reminiscent of patients with a liver disease. This patient was diagnosed with severe necrotising pancreatitis.

Another patient with a normal sICAM-1 but with an elevated CRP and LBP was P7 (already cited in chapter 5) this patient was not septic but did have an infected joint. Three patients with AML have already been described in chapter 5 however there is one other patient with AML patient P12, whose markers (listed in table 6.7) are quite different from the other three cases (N5, P10 and P15, tables 5.9.4.3/4/5).

Table 6.7 Patient P12, a patient with AML

IL-6	sICAM-1	LBP	Anti-lipid S	CRP	WCC
0.2-4.5	115-306	5-10 µg/ml	> 0	<	4-11
pg/ml	ng/ml			10mg/l	$x10^9/l$
59.6	**270**	**nd**	**0**	**10**	**0.5**
+	**-**		**-**	**-**	**-**

nd-not done. This patient had a fever and a positive BC with a CNS in both bottles after incubation for a day. (This patient is not the P12 from Phase 2)

The problem with these results is the lack of evidence of sepsis from his serological markers (apart from an elevated IL-6) unless they reflect an example of an early onset sepsis in patients with AML. Patients such as this are very vulnerable to infections because of their low white cell count and any suspected infection has to be dealt with quickly. However, if the positive BC was shown to be due to contaminants (if indeed it was) and the elevated IL-6 was known to be induced by the chemotherapy (again, that is making an assumption) then clinicians in this field (knowing the serum values in table 6.7) would have the confidence to with-hold unnecessary antibiotic treatment. For this to become a reality in practice the test markers would have to become routinely available.

Patients with a Gram-negative sepsis are known to evoke a stronger immunological response than patients with a Gram-positive sepsis. This is also evident from the higher median concentrations and range values for all the test markers in this project, except for anti-lipid S antibodies a differential marker for Gram-positive infections. However one patient with a Gram-negative sepsis (N16) had unusual results shown in table 6.8.

Table 6.8 Patient N16 and unusual results.

IL-6	sICAM-1	LBP	Anti-lipid S	CRP	WCC
0.2-4.5	115-306	5-10 µg/ml	> 0	<	4-11
pg/ml	ng/ml			10mg/l	$x10^9/l$
11.3	**555**	**nd**	**0**	**<3**	**6.5**
+/-	**+**		**-**	**-**	**-**

nd-not done. Could the results in Table 6.8 be evidence for the test markers becoming positive before the traditional markers of infection, CRP and WCC? Or is transplant immunosupression the reason for these non-septic results (N16 is a phase 3 patient),

This 51 year old patient was immunosuppressed due to a previous liver transplant, he became pyrexial (39°C) and blood cultures grew a *Klebsiella pneumoniae* (both bottles within 12 hours). His CRP level 4 days after the test marker date had increased moderately to 26 mg/l. He was positive for hepatitis C antibodies and was due for a renal biopsy two weeks later. He was placed on antibiotics. Nothing more was known about this case but his serum markers are worth recording given that he was diagnosed as septic. It is possible that patients with hepatitis C cannot produce the acute phase liver proteins such as IL-6, LBP and CRP. Another marker would be needed (possibly procalcitonin) to indicate sepsis in such patients.

6.6.1 The next phase

The trials so far have been concerned with identifying markers for sepsis from nine candidate markers and then to establish serum cut-off levels for these markers that would be diagnostic for sepsis.

IL-6, sICAM-1 and anti-lipid S antibodies became the markers of choice and at cut-off levels of 40pg/ml for IL-6 and 500ng/ml for sICAM-1. High titres of anti-lipid S reflected a Gram-positive sepsis and when a patient had a Gram-negative sepsis and anti-lipid S antibodies, an occult Gram-positive infection was indicated.

It was shown that definitive serum cut-off levels for the test markers fail to identify and diagnose all the septic patients, particularly in the patients with a Gram-positive sepsis and in patients with certain underlying conditions such ongoing immunosuppression due to an organ transplant.

What emerged from these trials was something different and unexpected. It was not necessarily the serum levels of these markers on there own that signalled a septic state but the levels of the markers in relation to each other that reflected sepsis.

Defining exactly what these relational serum levels may be was put to the test in a semi blind trial. Phase 4 proposes to determine if the serum levels of IL-6, sICAM-1 and anti-lipid S IgG facilitate the diagnosis of sepsis in the absence of conventional methods such as blood cultures.

CHAPTER 7 SEPSIS SCREEN STUDY

7.1 INDRODUCTION

The serum markers for sepsis have been evaluated in patients with and without sepsis and found to be significantly elevated in septic patients as opposed to the controls. However do the test markers add significantly to the diagnosis of sepsis in the clinical setting? Would it be helpful to the clinician to know the serum levels of these markers when confronted with patients presenting with uncertain sepsis, or when a positive BC contains a microorganism of low virulence that may be a skin or environmental contaminant?

A protocol was devised to test the markers in a semi-blind trial. Random consecutive BC results from 100 patients showing either a Gram-negative rod or Gram-positive coccus were selected for this phase of the study. Nothing else was known about these patients. Fifty new CABG patients with no known infection served as controls but in this phase BC were performed to demonstrate that they did not have bacteria in their blood.

A diagnosis for each patient was made based on the results from the test marker assays. Later referral to the patient's notes and the clinical diagnosis given by a Consultant Microbiologist in the normal way would confirm or refute the test markers ability to diagnose the degree and extent of septicaemia, from early infection to sepsis; severe sepsis; multiple organ failure and the likelihood of a poor outcome

To ascertain if the test markers facilitate the diagnosis of infection/sepsis in the absence of conventional methods such as positive blood cultures, patients with a pyrexia of unknown origin (PUO) and negative BC were enrolled into this phase.

As this phase (4) relied largely on the results of the tests markers to make a diagnosis (as opposed to knowing the diagnosis before hand) all the patients serum was tested for antimicrobial activity. This information was obtained primarily to help in the interpretation of the PUO results, as only 24% of PUO are caused by infections and another 25% remain undiagnosed.

7.2. Clinical samples

After the Queen Elizabeth's ethics committee approved this study, serum samples were obtained from the control patients and those with either Gram-positive coccus (GPC) or

Gram-negative rod (GNR) in their BC. Blood samples were separated and the serum obtained was stored at minus 20°C until assayed.

In collaboration with doctors in the cardiology department and the surgical team involved in performing coronary artery by-pass grafts for patients at the Queen Elizabeth Hospital (Birmingham), a signed consent form was obtained for blood to be taken from CABG patients for this study.

7.2.1 Controls patients

Fifty CABG patients with no known infection that produced a clinically negative blood culture acted as the controls in this part of the study. After obtaining the patient's signed consent, theatre staff removed 15ml of blood from the distal central venous catheter (CVC) line and aseptically injected 5ml into each of the 2 blood culture bottles provided. The remaining blood was injected into a sterile vial. The BC's were incubated at 37°C and the blood samples were separated by centrifugation and the serum obtained was stored at minus 20°C.

7.2.2 Patients with Positive Gram-stains

A daily print-out listing all the patients with a positive Gram-stain from their BC's was available in the microbiology department and this was used to take the first 50 consecutive patients with a GNR and the first 50 patients with GPC. Serum from these randomly collected patients was obtained from routine laboratory samples and stored at minus 20°C until assayed.

7.2.3 PUO's

Traditionally a patient is said to have a PUO when they have presented with a fever for more than three months. However this term has become corrupted, and now tends to mean that initial and obvious causes of fever have been eliminated and the PUO remains undiagnosed. In this study PUO will be defined as a patient presenting with pyrexia lasting >1 day, with negative initial investigations (microbiological and serological) including a BC that remained negative for 5 days. 56 patients that met these criteria were enrolled into this study.

7.2.4 Patient codes

Codes were used to protect patient/data. In phase 4 the controls, GNR and GPC patients were represented by a lower case *c*; *n* and *p* respectively and italicised. Patients with a PUO –are ascribed - *u*

7. 3 METHODS AND MATERIALS

The method and materials for the sICAM-1 assay and anti-lipid S antibody assay were as given in Chapter 3 sections 3.4.4 and 3.4.9. The method and materials for IL-6 are given in chapter 5 section 5.2.

7.3.1 Control Blood Cultures

The blood cultures from the CABG patient's were tested for bacterial growth after two and five days incubation at 37^0C. A sample from the BC was removed aseptically and sub-cultures onto 2 blood agar (BA) plates and a McConkey's agar plate. Plates were labelled and incubated overnight at 37^0C in air/carbon-dioxide and one BA was incubated in the anaerobic cabinet. Any subsequent growth would be Gram stained and identified using routine microbiological procedures.

7.3.2. CRP levels

The CRP levels for the controls were established from their test serum. Other patient's in this phase without a CRP result for the day of their positive BC were also tested. These tests were performed by the hospital biochemistry personnel, funded by a research grant.

7.3.3 Antimicrobial testing

Possible antimicrobial activity in a patient's serum was tested using a confluent growth of *Bacillus subtilis* grown on iso-sens agar and the addition of 5µl of patient's test serum, (6 patient samples per plate). The antimicrobial activity in a patient's serum was measured by the zone of no growth around the sample in millimetres (mm).

7.4 RESULTS

When all the assays were completed and with no other patient information (CRP and WCC were included later) the first 'diagnosis' of patients with a GNR or a GPC was made, based initially on the serum levels of the test markers.

7.4.1 The Controls BC results

The results from the CABG control BC's were expected to be negative. However this was not the case. Thirty-six of the blood cultures were positive for Gram-positive microorganisms and 14 were negative. These results are a significant finding as it suggests that three quarters of all BC positive with a Gram-positive microorganism are probably false positives, the implications of these findings will be discussed in 7.9.

Table 7.1 CABG Control BC results Phase 4

CABG	BC-SUB-O_2	ANO$_2$ BOTTLE	ISOLATES
c1	CNS	NG	Skin contaminants
c2	CNS	NG	Skin contaminants
c3	CNS	NG	Skin contaminants
c4	CNS/GPC	NG	Skin contaminants
c5	NG	NG	NOS
c6	GPC	NG	Contaminant
c7	NG	CNS	Contaminant
c8	CNS/Dipthroids	GPC	Skin contaminants
c9	CNS	CNS/GPR	Skin+enviro contam
c10	NG	CNS x 2	Contaminants
c11	CNS	CNS	Skin contaminants
c12	GPC x3 types	CNS	β-haem/Proprion
c13	NG	NG	NOS
c14	CNS	CNS	Skin contaminants
c15	GPC	GPC	Skin contaminants
c16	GPC	GPC	Skin contaminants
c17	NG	NG	NOS
c18	GPC	CNS	Mixed skin contaminants
c19	CNS	CNS/GPR	Skin contaminants
c20	CNS	CNS	Skin contaminants
c21	NG	NG	NOS
c22	CNS	GPC	Skin contaminants
c23	CNS	β- haem cols	Skin contaminants
c24	CNS	CNS/GPC	Skin contaminants/H_2S
c25	NG	NG	NOS
c26	NG	NG	NOS
c27	CNS	CNS/GPC-	Skin& environ contam
c28	CNS/GPR	CNS	Mixed skin contaminants
c29	GPR	NG	Environ ?Bacillus sp.
c30	NG	NG	NOS
c31	NG	NG	NOS
c32	NG	NG	NOS
c33	NG	NG	NOS
c34	CNS	NG	Skin contaminants
c35	CNS/GPR	NG	Skin contaminants
c36	NG	NG	NOS
c37	NG	NG	NOS
c38	CNS	NG	Skin contaminants
c39	CNS	NG	Skin contaminants
c40	NG	NG	NOS
c41	NG	CNS	Skin contaminants
c42	GPC x2+ mixed	NG	Skin contaminants/env
c43	α-haem Strep	NG	Gross contaminant
c44	CNS	GPR	Skin contaminants
c45	NG	NG	NOS
c46	NG	GPR	Skin contaminants
c47	CNS	CNS x 2	Skin contaminants
c48	CNS	NG	Skin contaminants
c49	CNS	NG	Skin contaminants
c50	CNS	NG	Skin contaminants

Patients highlighted in bold are all positive BC's. BC-SUB- subculture. CNS-coagulase negative staphylococcus. NG-no growth. NOS- no organisms seen.env-environmental. H_2S – hydrogen sulphide.

The control blood culture results are given in full so that comparisons may be made between table 7.1 and 7.2. Approximately half of the patients with elevated markers in their serum had BC that were apparently positive and half showed no growth.

Table 7.2 Control patients with elevated markers.

Patient	Lipid	IL-6	sICAM-1	CRP	WCC x 10^9/L
	EIU/L	**0.2 -4.5 pg/ml**	**115-306 ng/ml**	**< 10mg/L**	**4.0-11**
***c*11**	0	1.23	228	**18**	11.0
***c*14**	0	1.29	**467**	1.9	**12.9**
***c*15**	0	1.45	222	2	**17.8**
***c*18**	0	**145***	144	2	6.2
***c*21**	0	3.12	285	**18**	6.6
***c*23**	0	**14.08**	203	2	**11.2**
***c*24**	0	**0**	172	10	**21**
***c*30**	0	**6.48**	**402**	**28**	**18.1**
***c*32**	0	0	273	**15**	**26.7**
***c*33**	0	0.31	223	1.9	**13.1**
c37	0	0	284	6	**17.8**
***c*41**	0	2.75	236	**11**	9.4
***c*44**	**15,350**	0	186	2	9.5
***c*45**	0	0	272	4	**16.2**
***c*49**	**86,900**	0	217	3	7.5

Patients notes were consulted (post test-marker assays and diagnosis) when possible, to find answers to the questions that were raised by the results in table 7.2. These will be discussed in 7.9. The notes for *c*49 were unavailable and this patient had no microbiological history on Telepath, but it interesting that the high titre for anti-lipid S antibodies does not appear to have affected the test markers, suggesting a recent infection with a Gram-positive organism.

None of the positive blood cultures from the controls were considered to reflect a bacteraemia. One clue is in the nature of the isolates (skin commensals and environmental microorganisms) that is not to say that these microorganisms would be insignificant if they were isolated from the blood.

Another indication that these isolates were contaminants is that only one bottle was positive in many cases (bearing in mind that some microorganisms can be defined by the fact that they only grow in one bottle, for example *Pseudomonas* spp. are obligate

aerobes); thirdly the results of elevated markers in table 7.2 show that patient *c*18 and *c* 30 are the most likely candidates for a positive blood culture reflecting an infection but *c*30 BC had no growth and *c*18 had experienced a myocardial infarction (MI) just prior to his operation (strongly indicated by the massive IL-6 level) and confirmed by an elevated troponin level 2 days after the operation (troponin 3.1 –reference < 0.6 ng/ml). Troponin is a protein specifically found in myocardial cells and increased levels indicate a recent MI.

By following up the control patients notes and microbiological records it was possible to check if any of these patients have been incubating an infection at the time of their BC prior to surgery. Several patients had infections 5 to 8 days post-operatively, which may have accounted for their raised WCC and CRP. Patient *c*15 had a sputum sample taken the day before the operation which subsequently grew a *Candida* spp., (WCC-17.8). Patient *c*24 was known to have cellulitis (WCC-21) but his CABG surgery was urgent.

7.4.2 Test-marker diagnosis, patients with GPC

Establishing cut-off levels for the test markers for a diagnosis of sepsis was said to be too simplistic (phase 3) and that the test markers with CRP and the WCC had a septic tune or shape and it was their relationship to each other that gave a diagnosis of sepsis. To clarify this relationship and establish exactly what this relationship may be in a quantifiable way three graphs were constructed, one based on the test markers IL-6, and sICAM-1 results, for patients in the Gram-positive group. A 'diagnosis' was made for each patient and recorded without knowing the patients CRP and WCC levels. The results are given in figure 7.1. Then a second 'diagnosis' was made with the added information given by the patient's CRP and WCC. These results are recorded in figure 7.2.

Based on the levels of the test-markers in relation to each other, definite groups were formed and they were; 'not infected', 'an infection but not sepsis', 'early sepsis or resolving sepsis', 'sepsis' and (arbitrarily) a liver disease group. The range of markers in each group is given in table 7.3.

Finally a clinical diagnosis was made by a Consultant Microbiologist, and the results are given in figure 7.3. The test marker diagnosis was then compared with the clinical diagnosis.

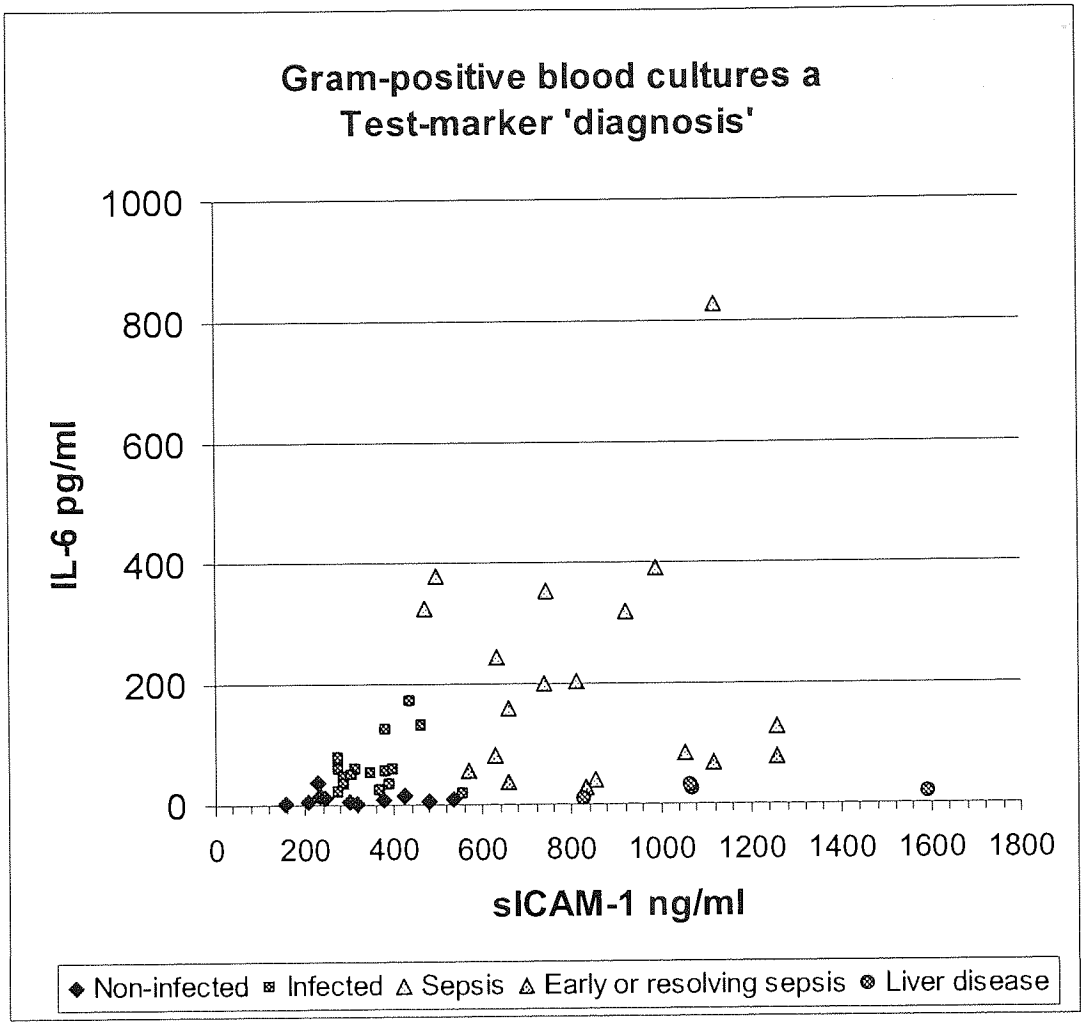


Figure 7.1 IL-6 and sICAM-1 as diagnostic markers of sepsis.
The groups given in the legend are hypothetical and may not relate to the true clinical picture for these patients. The patients were grouped by the parameters given in table 7.3

Table 7.3 Test markers in the diagnosis of sepsis in Gram-positive BC's

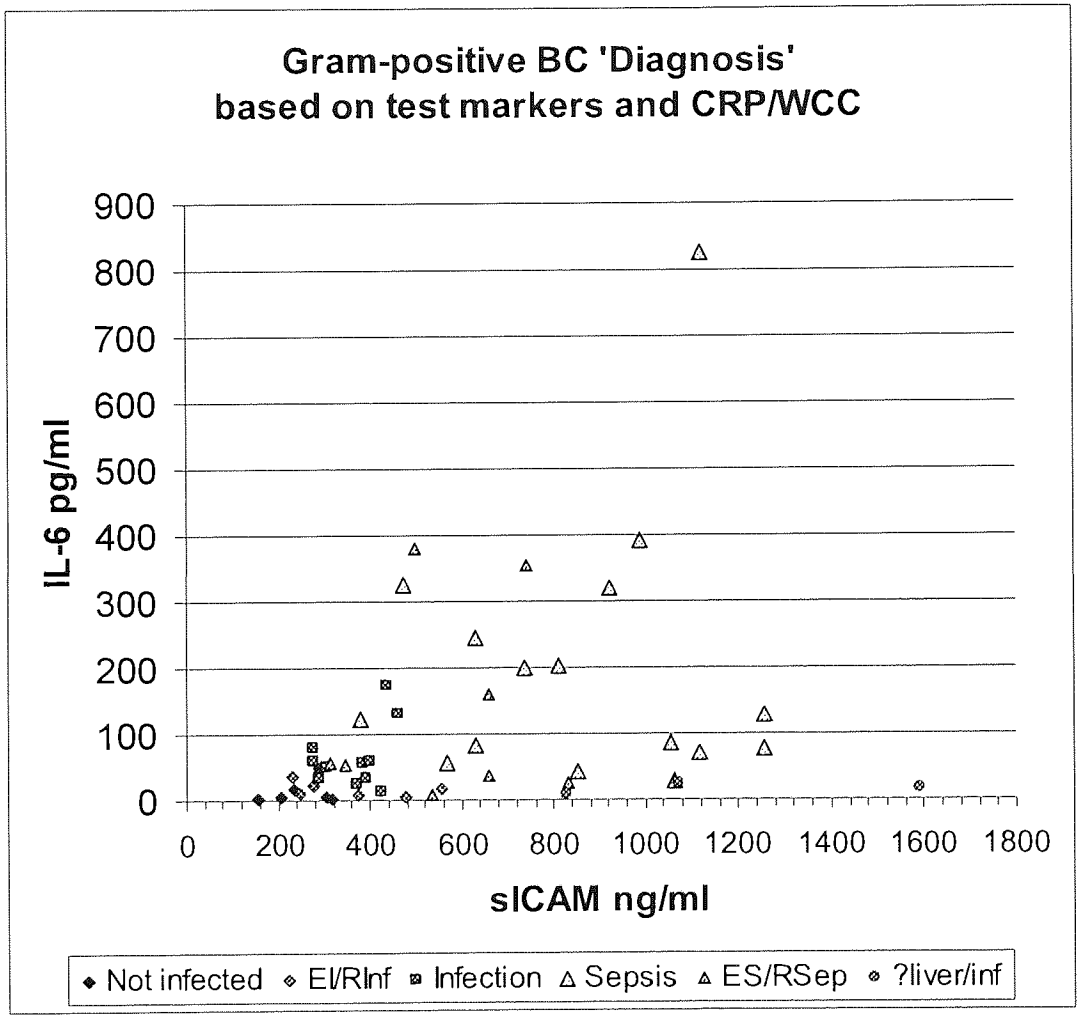
Test markers G+ve	IL-6 pg/ml	sICAM-1 ng/ml	Anti-lipid S
	range	range	range
Non-infected	1.8 - 38.3	157 - 534	
Infections	18.4 - 173	276 - 556	9350 – 15,100
Early/resol sepsis	26.9 - 159	570 - 1255	
Sepsis	41.6 - 827	427 - 1255	2950 – 91,815
(?liver disease)	9.4 - 30	829 - 1590	

Early/resol – early sepsis or resolving sepsis. The liver disease notion is based on the evidence of septic patients with liver disease from the previous trials in phase 1, 2 and 3.

Anti-lipid S anti-body was not used as a diagnostic marker, but as a differential marker. The value of this marker arises when a patient presents with a possible sepsis;

a high anti-lipid S titre strongly implies a Gram-positive sepsis as opposed to a Gram-negative sepsis, or that a positive BC with a GPC is less likely to be a contaminant.

The next stage in the process of defining sepsis using the markers as diagnostic tools was to include the additional information of serum CRP levels and the WCC. CRP levels were obtained for all the patients. Their biochemical records were checked so that it was known if the test CRP level was still increasing or if it had started to decrease (see figures 7.5/6/7). Consequently 16 'infected' cases became 11 'infected' in figure 7.2.



EI/RI, -early infection or resolving infection. ES/RSep, -early or resolving sepsis.

Figure 7.2 Diagnostic patterns from the test-markers when CRP and WCC were added.

An extra category was possible with the addition of CRP and is included in figure 7.2, a group with possible 'early infection/ resolving infection'.

The addition of CRP caused a change in the diagnosis for an arbitory liver patient who became an early sepsis or resolving sepsis case. Later the liver cases were clinically diagnosed as pneumonia, aspiration pneumonia, a chest infection and a wound infection.

There was another possible category not included in the graph that may differentiate some patients in the 'not infected' group into 'an inflammatory response' group. This group would contain patients with normal markers but elevated IL-6.

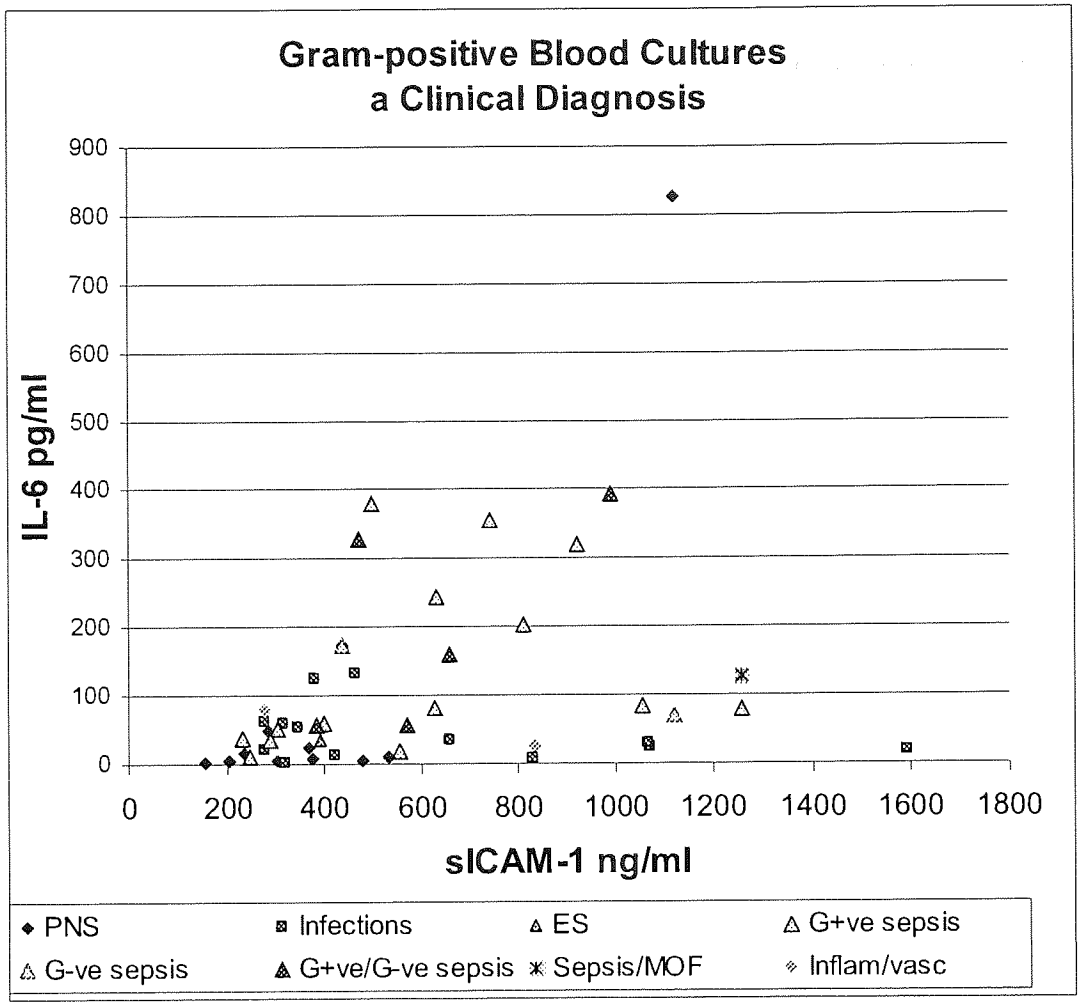
Table 7.4 Number of patients in each group according to the test-markers; test-markers with CRP/WCC and clinical with CRP/WCC for Gram-positive BC.

Diagnosis of G+ve BC	IL-6 and sICAM-1	IL-6/sICAM-1 and CRP/WCC	Clinical and CRP/WCC
Non-infected or PNS	11	6	11
Non-infection inflammatory response	-		2
Early infection/resolving inf	-	6	
Infections	16	11	13
Early S/Resol sepsis	**5**	**8**	**1**
Sepsis	**14**	**16**	**20***
(?liver disease)	4	3	**1** (with sepsis)
Severe sepsis/MOF	**-**		**1**
Number of patients	50	51	49
Mortality			13/50-**26%**

None of the patients diagnosed with sepsis in the test marker group were lost just deployed; some diagnosed 'septic' using the test markers alone became 'early sepsis' when the CRP values were added and several of the test-marker infections cases became sepsis. The clinical diagnosis required patients notes for a valid diagnosis and these were not always available therefore only 49/51 results are given in the clinical diagnosis column. * this result contained 14 with Gram-positive sepsis, 5 with a mixed BC and 1 Gram-negative case.

The major difference between the test marker evaluations and the second evaluations with the test-markers and CRP/WCC values was-confidence. They confirmed the test markers and fine tuned the diagnosis.

The clinical diagnosis given by a Consultant Microbiologist on all the patients in this phase was used to validate the test marker's ability to predict and diagnose septic cases and to see if these markers could help in the diagnoses of uncertain sepsis when a BC is positive with a low virulence microorganism. The results of the clinical diagnosis are given in figure 7.3.



PNS-probably not significant. ES-early sepsis. Inflam/vasc-inflammation or vasculitis.

Figure 7.3 The Clinical diagnosis of patients in phase 4 with a Gram-positive BC.

The categories and diagnosis for the patients in figure 7.3 are based on a patient's clinical details and microbiological interpretations. Clinical terminology such as PNS means 'probably not significant' and refers to the relevance of an isolate in the culture in question and not to a patient's overall condition.

There were three septic groups in the clinical diagnosis graph (figure 7.3), those with a Gram-positive sepsis, a Gram-negative sepsis (inferred from other microbiological evidence apart from BC's) and those patients with sepsis caused by both a Gram-positive and Gram-negative microorganisms. All these septic patients had a unifying triangle symbol to show the sepsis group as a whole, but each group was assigned a different colour. One patient with sepsis and MOF was given a separate symbol.

7.4.3 Test-marker diagnosis, patients with GNR

The patients with a Gram-negative microorganism in their BC were 'diagnosed' by applying the same septic tune/shape test to their IL-6 and sICAM-1 results as given in table 7.5

Table 7.5 Test markers in the diagnosis of sepsis in Gram-negative BC's

Test markers G-ve	IL-6 pg/ml	sICAM-1 ng/ml	Anti-lipid S
	range	range	range
Non-infected	7.0- 31	171-591	
Infections	14.9-994	402-657	
Early/resol sepsis	15.9 -311	329-666	
Sepsis	117-2360	467-1085	
liver disease (?)	71	2191	
Severe sepsis	799-1641	709-2052	

Early/resol sepsis, early or resolving sepsis.
Several of the Gram-negative patients had anti-lipid S titres (6/50), one was later diagnosed with a Gram-negative and a Gram-positive sepsis.

Table 7.6 Number of patients in each group according to the test-markers; test-markers with CRP/WCC and clinical with CRP/WCC for Gram-negative BC

Diagnosis of G-ve BC	IL-6 and sICAM-1	IL-6/sICAM-1 and CRP/WCC	Clinical with CRP/WCC
Non-infected or PNS	6	4	1
Infections	7	11	9
Early /Resol sepsis	**10**	**7**	**2**
Sepsis	**13**	**15**	**35**
(?liver disease)	1	1	-
Severe sepsis	**6**	**7**	-
inflammation	6	4	2
Mortality			16/49*-**32.6%**

Inflamm.-inflammation. Inf-infection. Early S/Resol- early or resolving sepsis. The clinical assessment diagnosed more cases with sepsis than the test markers or the test markers with CRP/WCC. 'Infections' in the test marker and clinical diagnosis included bacteraemias. The 'liver' case proved to be sepsis secondary to a UTI as the primary focus of infection. *n=50 but 1 patient self-discharged and was excluded.

The test markers diagnosed 29 patients with some form of Gram-negative sepsis (early or severe). The test markers plus the CRP/WCC also diagnosed a total of 29 cases whereas the clinical diagnosis (with CRP/WCC) found 37 cases with sepsis. There was more agreement between the test markers (19 cases) and the clinical diagnosis of patients (23) with Gram-positive sepsis. This discrepancy is discussed briefly in 7.5.

7.4.4 The test-marker rules for a diagnosis of sepsis

What is this balance between the markers that suggest one patient has an infection and the other is septic, or not septic or infected, is there a quantifiable ratio? sICAM-1 is the pivotal test marker around which the other markers balance, and there appears to be a ratio, sometimes one in ten, in others it is 1:3. CRP plays an important role by balancing the effect of these different ratios, table 7 lists some of these combinations to demonstrate this hypothesis. If the ratio is 3:1 (IL-6/sICAM-1) as can be seen for *n*5 in table 7.7 then the sICAM-1 level has to well above the normal range (115-306ng/ml) and the imbalance is balanced for sepsis if the CRP level is increasing, as opposed to low and decreasing.

Table 7.7 IL-6, sICAM-1 and CRP proposed rules for a diagnosis of sepsis.

patient	IL-6	sICAM-1	CRP	Ratios IL-6/sCAM-1
***p*13-sepsis**	85	1053	547	**1:10**
***p*36-sepsis**	319	921	159	**1:3**
***p*40-sepsis**	204	812	70u	**1:4**
***n*8-sepsis**	1984	1408	555	**1:1**
***n*13-sepsis**	119	627	114	**1:6**
***n*5-sepsis**	1604	588	95u	*** same (early)**
***n*16-sepsis**	117	740	334	*** patient (late)**
***u*42**	166	1175	8u	

u –up. This indicates that the next time serum CRP was tested the level had increased. *samples 11 days apart and show early and late sepsis in a 71 year old.

Commentary on the patients in table 7.7; generally the IL-6 level in patient *p*13, when compared with the sICAM-1 concentration is too low for sepsis but the high level of CRP puts this case in the septic group. If the CRP level for *p*13 had been in the 20-30 range it would have changed the picture and indicated something different, for instance patient *u*37 in table 7.8 had liver failure caused by a drug over-dose, she was *not* septic. *p*36; this test-marker profile was more usual for sepsis, with a one in three ratio and a CRP approximately half that of IL-6. *p*40; again demonstrates a ratio, not the same as the other two but still balanced and the CRP is going up. Figures 7.4/5/6 show the serum levels of CRP over time in various patients.

Patients with a Gram-negative sepsis can have very high levels of the test-markers as shown by patient *n*8 in table 7.7 and the 1:1 ratio may reflect severe sepsis.

Patient *n*5 and *n*16 proved to be the same patient and 11 days separated the first sample from the second. It is interesting to see how the marker concentrations have

changed over time yet still say 'sepsis'. Unusually her IL-6 is twice as high as the sICAM-1 but her CRP is increasing and that balances the profile by inferring an infection as opposed to an inflammatory response/disease or this ratio may indicate the initiating stage of sepsis (early). This patient was discharged home 5 weeks later.

The WCC is not ignored in these assessments but as many patients had WCC within the normal range or below the normal range it becomes less important in the diagnostic process. Patient *u*42 (in table 7.7) was clinically diagnosed as a probable Gram-negative sepsis a confirming elevated WCC of 21 (x 10^9/l) whereas the CRP level was within the normal range but climbing.

The key marker is sICAM-1, if it is low or in the normal range then the case is unlikely to be sepsis. IL-6 is an excellent serum marker but it can be elevated for many reasons such as an MI. An example of this can be seen in a control patient (*c*18). However when IL-6 is elevated with an elevated sICAM-1 in a certain ratio-it appears to indicate sepsis.

To demonstrate how the profiles of the test markers may diagnose sepsis (table 7.7) other combinations of the test markers in table 7.8 are listed, these profiles don't suggest sepsis and the follow up notes and clinical diagnosis showed this to be true.

7.4.5 Serum test markers in patients without sepsis

The following results represent patients from the PUO group who did not have sepsis. These include; patient *u*32 with radiation induced inflammation, *u*31 had an inflammatory heart disease, *u*7 acute inflammation caused by gallstones, *u*37 drug induced (ecstacy) liver failure, another example of excessively elevated sICAM-1 associated with liver damage. *u*53 is a post-chemotherapy pyrexia.

Table 7.8 Variations in the test markers in miscellaneous conditions

	IL-6	sICAM-1	Anti-lipid S	CRP	WCC
*u*32	**66.9**	308	0	**132**	5.8
*u*31	**89**	253	0	**66**	9.3
*u*7	7.6	**941**	0	**33u**	6.7
*u*37	**39**	**2055**	0	**59**	11
*u*53	**28**	255	0	**72**	3

The markers that are elevated are highlighted in bold. The only one with a possible sepsis profile is *u*37 but the markers are out of balance for sepsis, IL-6 is too low and sICAM-1 in comparison is too high.

7.4.6 Patients with a pyrexia of unknown origin (PUO)

There were 56 cases of PUO enrolled into this study but only 41 remained for analysis as 15 patients' notes were unavailable for a clinical diagnosis. The array, range and unusual combination of the test marker levels in these patients demonstrated interesting clues for deciphering the meaning of the test markers.

Diagnostic agreements for the PUO's relates to *all* the clinical diagnosis that is, 'septic', or 'not septic', 'infection' or 'not infected'. The test markers agreed with the clinical diagnosis even in terms of an inflammatory response – not an infection.

PUO patients often had unusual IL-6 and sICAM-1 combinations unlike the balance found in septic patients and they were more difficult to interpret. However using the ratio rules as for sepsis (proposed in section 7.4.4) it was found that 38/41 patients diagnosed with the test markers alone matched exactly the predictions made when CRP/WCC was added. Furthermore 29/41 matched the clinical diagnosis and the remaining cases differed largely by degree.

An example of non-agreement between the clinical diagnosis and the test-marker diagnosis occurred when a clinical diagnosis was 'not septic' and the test marker diagnosis was 'infection' but there is agreement on the level of septic/not septic. Both agreed that this patient was not septic.

The test markers were able to predict occult infections. There were 2 cases of infection with the slow growing *Mycobacterium* spp. Which were eventually isolated three weeks after the test serum date, yet the test markers diagnosis was 'infection' and matched the clinical diagnosis.

Two samples from the same patient (*u*55 and *u*56) were clinically diagnosed as Gram-negative sepsis, whereas the test markers diagnosed one as sepsis and the other as an early sepsis. It was not known that these results were from the same patient during the diagnosing process, and it was not known therefore that the 'early sepsis' was diagnosed for the earlier serum sample (three days earlier). These examples are given in table 7.8.1.

Table 7.8.1 Patient *u*55 and *u*56 test markers

IL-6	sICAM-1	anti-lipid S	CRP	WCC
35	763	0	128	nk
143	627	0	90	16.3

E/sepsis, early sepsis. nk-not known.This patient had a Gram-negative sepsis. The early sample (*u*56, was collected *after* the later sample *u*55).

7.4.7 Serum CRP levels over time in three patients with sepsis.

The following graphs demonstrate that serum CRP follows a pattern that is reproducible in different patients.

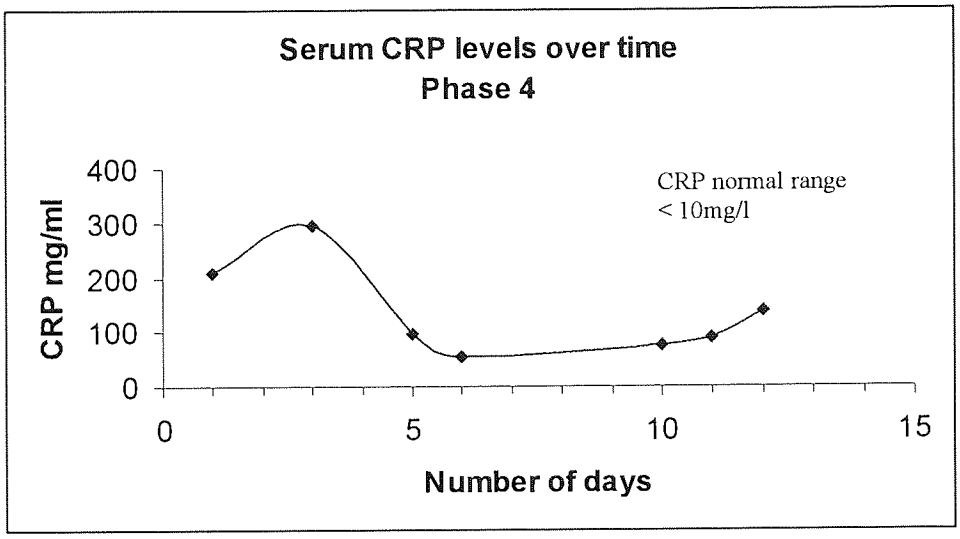


Figure 7.4 Patient *n*41 and *p*51 serum CRP levels over time
*n*41 and *p*51 are the same person. This patient was very septic with an initial Gram-positive and Gram-negative BC when her CRP was 207mg/l.

The patient represented by her CRP levels in figure 7.4 had a GNR (day 5) and a GPC in her BC on day 6. A *Candida* spp., was isolated in a BC on day 12 and she died on day 13. Her corresponding test markers are shown in table 7.9, they record the steady decrease in all the markers of infection in response to antimicrobial therapy (the serum antimicrobial activity test on day 6 produced a 12mm zone), then CRP starts to increase again due the developing Candidaemia.

Table 7.9 Test markers over time in patient *n*41

Day/month	IL-6	sICAM-1	CRP	WCC
29/05	**687**	**795**	207	**22.3**
31/05	nd	nd	297	23.0
3/06	77	**614**	98d	12.3
4/06	**18.4**	**556**	54	10.7
10/06	nd	nd	76u	
11/06	nd	nd	91u	
12/06	nd	nd	130u	
13/06				MOF-died

MOF, multiple organ failure. nd, not done.d, down. u, up.
Table 7.9 shows the serum level of the test markers over time and the levels of other markers. The patient had BC positive with a *Candida* spp., on the day her CRP was 130. This patient was negative for anti-lipid S IgG.

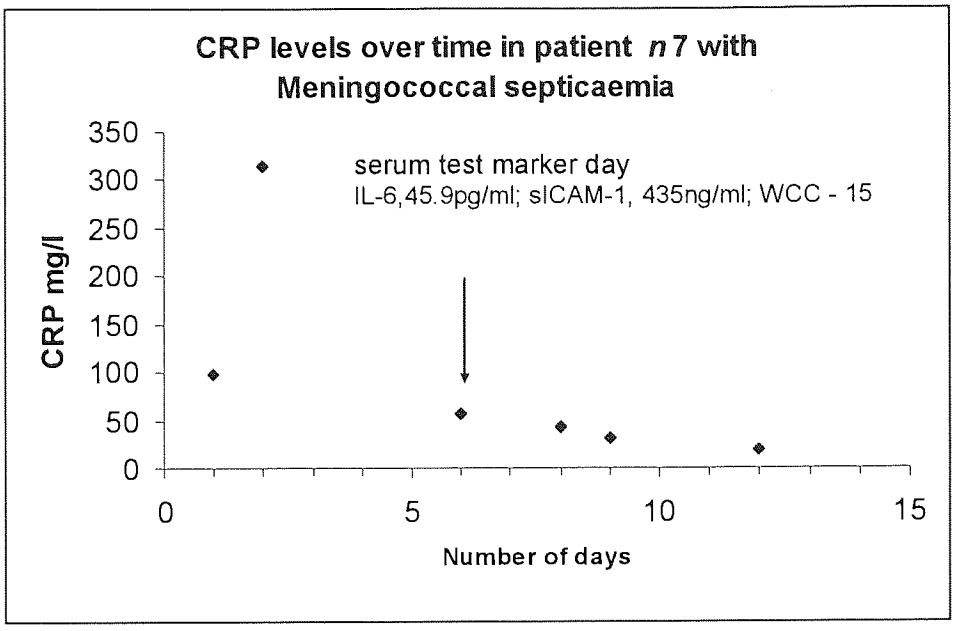


Figure 7.5 CRP levels in a case of meningococcal septicaemia

This patient had a GNC (*Neisseria. meningitides*) infection with a CNS in her BC on day 1 in the graph and this infection was resolving on day 6 when the test serum was collected.

Graph 7.5 shows how serum CRP can increase in one day from 98 to 315 mg/l. The pattern of CRP's disappearance from the serum is the usual finding in resolving infections; another example is given in figure 7.6.

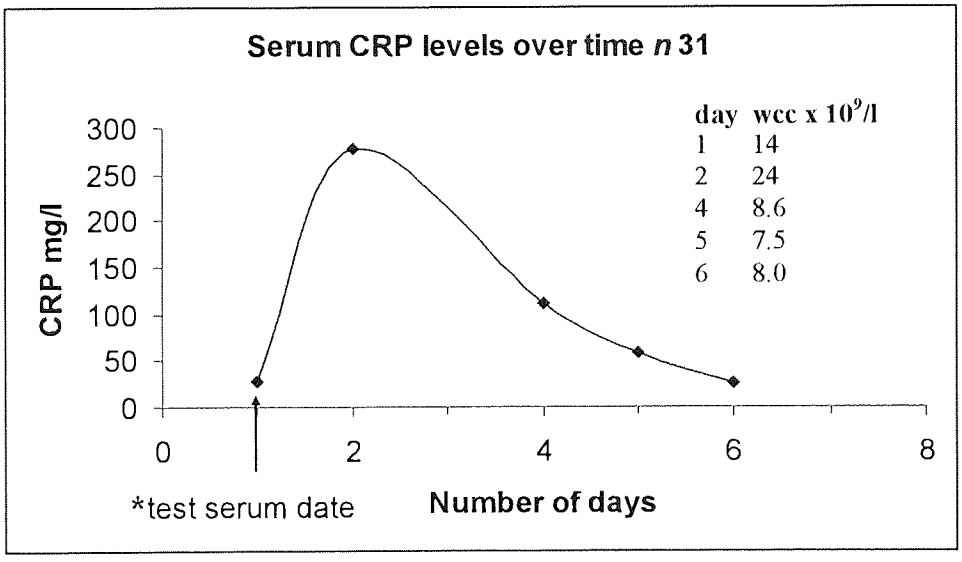


Figure 7.6 Serum CRP levels in a patient with a UTI septicaemia

Patient *n*31 was admitted to the ITU with a UTI and chest infection that developed into septicaemia. Her WCC is included on the graph to show that the CRP values and WCC reached their peak levels at the same time, day 2. (*IL-6/sICAM-1 -994pg/ml-380ng/ml).

7.5 DISCUSSION

This phase of the project was fundamentally different from all the other trials in the study. Instead of testing potential serum markers for sepsis and evaluating them in terms of statistical sensitivity and specificity, this phase put the recorded sensitivities and specificities to the test in a semi-blind trial. The test markers had to demonstrate their intrinsic ability to diagnose patients with sepsis based on the concentration of the markers in a patient's serum.

The purpose of this trial and the project as a whole was to find a serum marker that would assist clinicians in the diagnosis of sepsis, particularly when the clinical picture is complicated by underlying disease and positive blood cultures that may not represent a true bacteraemia but a contaminant. This latter point is highlighted with force by the blood culture results from the control patients. 36 positive results from 50 patients none of whom it can be safely said had bacteria in their blood stream. When 72% of blood cultures are producing growth when there is no truth in any of them-the problem is defined. How can a clinician be certain that a BC result is truly adding to the clinical picture for a given patient?

The control blood cultures were taken in operating theatres. An area in the hospital committed to the highest and most rigorous standards of disinfection and sterilisation, where staff are gloved and covered from head to foot in protective clothing to protect the patient from undue bacterial contamination during surgery.

The microorganisms isolated from the control BC's were Gram-positive skin and environmental bacteria. It could be argued that theatre staff being accustomed to working in areas of low microbial contamination may not appreciate the importance of using strict aseptic techniques when inoculating BC's with patient's blood. Whereas ward staff who are working in opposite conditions appreciate the care that must taken to avoid contaminating clinical samples and are therefore practiced in using aseptic techniques.

On the other hand if the control results do reflect the level of contamination of BC's being sent to microbiology for processing from all over the hospital it becomes even more imperative to find a serum marker or markers that can add empirical evidence to back up a clinical diagnosis of sepsis.

Over 200 patients were involved in phase 4, 50 controls, 50 patients with a GNR and 50 with a GPC in their BC's. In addition 56 patients with a PUO were enrolled into this phase to test the potential of the test markers on a more stringent level –when BC's

are negative and there is no other microbial evidence of infection that could be causing pyrexia.

To evaluate if the test markers assist in the diagnosis of sepsis they had to pass a stand alone test, do they diagnose sepsis without the help of CRP and the WCC? The results for the BC's with GPC are illustrated graphically in figures 7.1, for the test markers alone, 7.2 for the test markers with CRP and WCC and finally the clinical diagnosis in figure 7.3. The test markers showed an elegant gradation of infection to sepsis that was not as apparent when CRP and the WCC were added to the diagnostic picture (figure 7.2). There was an 82.6% agreement (for sepsis) between the test markers and the clinical diagnosis of patients with a GPC in their BC's. The test markers identified 19 cases of sepsis and the clinical diagnosis 23.

In the GNR cases there was less agreement. The test markers defined 29 patients as having sepsis whereas the clinical diagnosis identified 37 cases, giving an agreement of 78 %. The addition of CRP and WCC to the test markers in the GNR set caused a general shuffling, an early sepsis to sepsis and inflammatory responses to become infections. Many of the clinical cases of sepsis were in the test-markers infected group as bacteraemias. A Gram-negative bacteraemia has a 50-60% chance of resulting in septic shock as opposed to 5-10% of patients with a Gram-positive bacteraemia (Rangel-Frausto, 1999). Consequently a GNR in the blood is never considered lightly in the clinical setting.

It has been shown that the markers are able to draw a distinction between patients with an early-sepsis as opposed to sepsis see table 7.9. If that is true, then many of the patients diagnosed by the test markers with an early infection or 'infection' could be reflecting that the test markers are sensitive to degrees of infection, from infected to borderline early sepsis, early sepsis and sepsis. The test markers would be invaluable to clinicians as they would be able to monitor serological changes and show an individual's disease progression (or resolution) and also the patient's response to treatment. However for this to become a reality the assays would have to be routinely practiced in the hospital setting as opposed to being confined to research laboratories.

Given that markers such as CRP can increase in the serum from 20 mg/l to approximately 300mg/l within 24 hours (see figure 7.5) then the progression of an infection developing into sepsis may occur equally as fast, indeed this abrupt rise in serum CRP levels could be diagnostic for such an event. Given that the search for a diagnostic marker for sepsis has been exercising research centres for over a decade, it

was surprising to see how effective CRP and the WCC are in the diagnosis of infection and sepsis in otherwise uncomplicated cases(chapter 6).

As a single marker CRP demonstrated high sensitivities and specificities (98% and 96% respectively see table 6.2.1) at a cut off level of 40mg/l in Gram-negative sepsis but was less useful in Gram-positive cases (93% sensitivity and 92% specificity) at a lower cut-off level 20mg/l.

Although CRP and WCC are the gold standard serum markers of infection they are not infallible. CRP can be elevated in patients without an apparent infection and this undermines its diagnostic value. However the routine practice of waiting for evidence of an infection in terms of an increase in temperature before requesting a CRP test should be ignored. Particularly as it is also routine practice in hospitals to administer paracetamol which may well suppress any such evidence. The CRP test is very inexpensive and should be requested routinely and often thereafter.

In cases of leukaemia where an aberrant WCC is expected, CRP levels are always requested if an infection is suspected. In other situations asking for the serum level of CRP is often dependant upon seeing an elevated WCC (or pyrexia, as previously mentioned).

Figure 7.6 shows the WCC in relation to the CRP in a patient with a UTI septicaemia. This is a classic example of a septic episode resolving over time, with the CRP and WCC levels peaking at the same time and then dropping incrementally and in step. However due to the fact that many patients have under performing immune responses due to disease or drugs the WCC is often a poor indicator of infection.

Conversely cases occur where there is both an elevated CRP and WCC and an infective cause for this response remains elusive. Other markers are still needed to cover every possible contingency.

Positive anti-lipid S titres were particularly helpful in the PUO group of patients; as elevated IgG levels helped in the diagnosis of abscesses, ongoing but culture negative UTI septicaemia and occult infections.

PUO's with normal test markers and normal CRP/WCC (yet by definition all had a pyrexia) included patients (*u*8, *u*47 and *u*50) with viral pneumonia; hypokalaemia secondary to administration of a diuretic (Bendrofluazide), and an inflamed insect bite. Examples of PUO's with raised test markers but not septic are given in table 7.8.

Apart from being a potential differential marker for Gram-positive sepsis elevated serum levels of anti-lipid S IgG may assist the clinician in deciding if a positive

BC under diagnostic consideration is the result of contamination, catheter line colonisation or evidence for a Gram-positive infection from a focus other than the blood. IL-6 with anti-lipid S antibodies gave the highest sensitivity and specificity (100% and 93%, table 6.2.3) for a diagnosis of sepsis in patients with a Gram-positive sepsis.

Every marker (9) assayed in this project has been shown to be singly elevated in different control patients at the proposed cut-off levels for a diagnosis of sepsis. Consequently it was established that two markers would be required for a diagnosis of this condition. However one control patient without sepsis and elevated levels of two markers confounded the concept of cut-off levels.

This chapter was concerned with not only testing the intrinsic value of the markers in diagnosing sepsis but with finding and establishing relationship patterns between the test markers that would categorically define this condition, even in patients with underlying disease.

It has been shown that a ratio balance is required and that CRP levels assists in this process. It was also evident from section 7.4.4 (the test-marker rules for a diagnosis of sepsis) that sICAM-1 was the pivotal marker, the fulcrum for IL-6 and CRP and that there was a degree of interpretation required.

In answer to the question 'could these markers assist the clinician in the diagnosis of sepsis?' the answer given is an irrefutable 'yes' by a clinical example from this study. Patient *p*15 was sent home, any increase in temperature was probably masked by paracetamol and mental confusion caused by sepsis was probably covered by a degree of inherent dementia; his septic screen-test markers were IL-6 827 pg/ml, sICAM-1 1120 ng/ml. He was re-admitted as an emergency the following day. If the serum level of these markers been known his sepsis would have been revealed.

CHAPTER 8 GENERAL DISCUSSION

Sepsis, defined as a systemic inflammatory response in the presence of infection can readily develop into septic shock, multiple organ failure and death. Indeed it is the leading cause of death in intensive care units (10 to 50%). Consequently there has been much research on the pathophysiology of sepsis. From these various research projects an array of serum proteins and cytokines have been cited in the literature as having the potential to be diagnostic markers for sepsis.

The aim of this project was to evaluate nine of these potential serum markers in the clinical setting. Patients with a Gram-negative or Gram-positive sepsis were enrolled into this study and their serum was test for 9 of these potential markers. Although many of the markers have been tested in clinical studies as opposed to animal models of sepsis or *in vitro* studies, none had tested this particular group of nine markers on the same serum sample from patients with sepsis.

The fundamental purpose behind the aim of these evaluations was to find a marker or markers for sepsis would assist doctors to diagnose sepsis in cases were sepsis was uncertain due to confounding conditions such a the patients underlying disease. There are many occasions when sepsis fails to present clinically with the criteria necessary for sepsis as described by the diagnostic guidelines (table 1.1).

If such a marker could be identified that was reliable in the clinical setting then the next purpose would be to translate the test kit designed for research into a routinely available diagnostic test for sepsis in the hospital laboratories.

Four hundred and forty-five patients were eventually enrolled into this project. The trials were conducted in four phases; each phase proceeded from the findings in the previous phase.

Phase 1. The selected nine candidate markers were IL-6, IL-10, IL12, TNF-α, PCP, LBP, sE-selectin and sICAM-1 together with a novel differential marker for Gram-positive sepsis called anti-lipid S antibody (described in chapter 2). All the assays kits (with the exception of the anti-lipid S antibody assay) were ELISA's commercially produced by the various companies for research purposes only. The anti-lipid S assay ELISA was created by Dr. P.A. Lambert and colleagues from Aston University, (Birmingham) and developed by Dr. T. Worthington (Queen Elizabeth Hospital, Birmingham).

After receiving approval for this study from the Queen Elizabeth Hospital Ethics committee (Birmingham), serum was collected from patients (one phase at a time) separated and stored at minus 20°C until assayed.

Hospitalised patients attending the Queen Elizabeth or Selly Oak Hospitals in Birmingham and diagnosed with sepsis were enrolled into phase 1. All the patients in this phase with a Gram-positive isolate had documented CVC line sepsis caused by CNS. Patients without infection but waiting for coronary artery by-pass grafts (CABG) were enrolled as the control patients. All the markers were elevated in patients with sepsis as opposed to the controls. Patients with a Gram-negative sepsis invariably had higher serum levels of these markers than the patients with a Gram-positive sepsis. The sensitivity and specificity of the markers was calculated and comparisons made to evaluate the best marker for sepsis. It soon became clear that although many of the markers had excellent sensitivity and specificity (results in chapter 4) no single marker reliably identified all the septic patients. The literature suggested that serum PCT > 2.0 ng/ml indicates infection/sepsis. According to this proposition 27 patients in the Gram-positive group with less than 2ng/ml of PCT in their serum and 6 patients from the Gram-negative group were not septic, not even infected.

The assay results from two markers were combined to see if this would reflect the true status of the patients. Receiver operating characteristic (ROC) curves were created to establish serum cut-off levels for the markers (and the combined markers) for a diagnosis of sepsis.

IL-12 was not detected in the serum of 23/36 patients with Gram-negative sepsis and 10/33 patients in the Gram-positive group but it was detected in the serum of 17 controls therefore this marker was excluded. A similar pattern was found for the IL-10 cytokine assay and consequently excluded from further trials.

E-selectin is only expressed on activated vascular endothelium and as such any elevated level in the serum is usefully reflecting some degree of systemic inflammation and is contributing to the clinical picture. However for a marker to become realistically practical in the clinical setting, sensitivities and specificities (for a diagnosis of sepsis) would have to regularly exceed 90%. sE-selectin failed this test.

From the assay results and ROC curve analysis, five of the markers were selected for further trials they were TNF-α, IL-6, sICAM-1, LBP and anti-lipid S antibody assay.

Phase 2 (Chapter 5). From this point on in the project until the last phase (4) the study became hospital based rather than university based. This was because it became part of the project to visit the microbiology laboratory at the Queen Elizabeth (QE) Hospital and find the patients with sepsis, collect their serum which was either at the QE or in Selly Oak Hospital several miles away. Telepath searches had to be made to check potential candidates for the study. The appropriate candidates needed to demonstrate the cardinal signs of sepsis apart from their positive blood culture. These included recorded pyrexia at the time the blood culture was taken, increased WCC or CRP. Telepath searches also provided evidence of recent infections which would have excluded them from this study. In the same way the CABG patients were scrutinised for recent past infections and excluded if any were recorded as recent infections may have affected the serum levels of the test markers.

Serum was collected from 70 new patients, 26 CABG control patients and 20 patients with a Gram-positive sepsis and 26 with a Gram-negative sepsis. A strict criterion for the diagnosis of sepsis was applied and the septic patients were enrolled only after being verified as septic by a Consultant Microbiologist based at Queen Elizabeth Hospital. The isolates from the positive blood cultures in the Gram-positive group were varied unlike the previous trial when all the isolates were CNS.

The assays for IL-6 and TNF–α were changed from the high sensitivity (HS) versions, to the non-high sensitivity ELISA's produced by the same company R&D systems (Oxen, UK). The HS assays were able to detect very low concentrations of the cytokine in serum but they were less accurate at the higher concentrations detected in the serum of patients with sepsis. This was particularly true for the IL-6 assay.

The anti-lipid S antibody assay is a time delay marker as it tests for antibodies rather than the antigen lipid S. However 85% of the Gram-positive group were positive for this marker and 1 patient from the Gram-negative group, but none of the controls had positive anti-lipid S titres. Patient notes were requested to find a possible cause for the positive result in the Gram-negative case as there was no evidence of past infections with a Gram-positive microorganism on Telepath.

Interestingly this Gram-negative patient was admitted as a PUO, malaria was suspected, eventually *Salmonella paratyphoid* A was isolated from her BC and it is conjecture that gastroenteritis caused bacterial translocation from the large intestine to

the portal venous system, involving Gram-positive bacteria and hence her anti-lipid S titre.

Patients' notes became an important aspect of the project during this phase. There were several reasons for this, one being that the test markers were beginning to exert their authenticity as sepsis markers. Yet several test marker profiles suggested that the patients concerned were not septic. Recourse to the patients notes resulted in revised diagnosis, one was an infected joint and the other did have a bacteraemia but not a septicaemia.

These results underscored the need for a septic screening test as patients can present with all the clinical signs and yet not be septic. The converse is also true, patients may not present with the classical signs of sepsis and it may go undetected resulting in a poor outcome for the patient.

Test markers for sepsis had to be robust and reliable if they were to be of any practical use to the clinicians and it was important to record patients underlying disease to see if there was any possibility that they had an adverse effect on the serum level of the test markers. Although this project was not designed to control for these underlying disorders (except that some of the control patients had similar conditions such as diabetes), observations from patients notes and comparisons of the results in patients with the same or similar underlying disease was useful in allaying concerns that the test markers may show significant partiality in a disease dependent way. In particular the effects of steroids and chemotherapy treatments were examined in relation to the test marker results.

TNF–α was not carried through to the next phase as fewer patients from this trial had detectable serum levels of this cytokine. However the results from phase 1 and 2 suggested that TNF–α would be a useful prognostic marker in the ITU setting as high serum levels became associated with a poor outcome.

LBP is an impressive marker and combined with IL-6 gave some of the best sensitivities and specificities (see figure 5.6). Some of the results from phase 2 suggest that LBP could be the new CRP because it was elevated in the serum of some infected patients *before* CRP. It is also considered to be more specific for bacterial infections. The literature has implied that in septic patients LBP levels decrease and this serum protein was not carried forward to the next phase for the reasons given in 5.8.7.

Phase 3 (Chapter 6). Cut-off levels for the test markers had been proposed rather than defined at the end of phase 2. However the data base was small, therefore the purpose of phase 3 was essentially to increase the data-base and establish cut-off levels for a diagnosis of sepsis. IL-6, sICAM-1 and anti-lipid S antibody assay were the remaining markers tested in this phase. New patients were enrolled so that there would be at least 50 patients in each group and a better base from which cut-off levels for the serum markers could be evaluated.

CRP was re-discovered in the previous phase as an effective marker of infection. The value of this traditional marker was explored more fully in this phase together with a patient's corresponding WCC.

Elevated CRP with distinctly elevated WCC in the presence of a positive blood culture (and pyrexia) appeared to be all that was required for a diagnosis of sepsis and the purpose of this project seemed to fade. However not all patients have elevated WCC and a rise in serum CRP occurs for reasons other than infections, in other words it lacked specificity.

The question was, could the test markers in the absence of any patient knowledge or patient diagnosis predict which patients were septic or not, merely from their test marker results? Secondly, what are the cut-off levels for a diagnosis of sepsis?

A semi-blind trial was proposed and conducted in the last phase in an attempt to answer these questions.

Phase 4 (Chapter 7).

Serum from over 200 new patients was collected for the last phase of the project. In this semi-blind trial it was proposed to control for the positive blood cultures by submitting blood from the control patients for culture. This required signed consent from the CABG controls and the collaboration of the Cardiology department and theatre staff to collect blood and inoculate BC bottles just prior to surgery.

50 patients with a GNR, 50 with a GPC with 50 patients with PUO and negative BC's were enrolled into this phase. No other patient information was initially sought.
On reflection a truly random trial would elect to secure serum samples from across the hospital population rather than going to the microbiology department. Nevertheless this was going to be a very stringent appraisal for the test markers.

The test marker results were evaluated and a 'diagnosis' was made and recorded, then the patient's CRP and WCC levels were found and this extra information was added to the test marker results. Had this added information altered the test-marker diagnosis in any way? Finally the patient's full notes were requested and a Consultant Microbiologist at the Queen Elizabeth hospital diagnosed the patients case by case in the normal way. These results were then compared with the previous two.

The test-marker diagnosis demonstrated a remarkable agreement with the clinical diagnosis. In patients with a GPC there was 82% agreement (for sepsis) between the test-marker diagnosis and the clinical diagnosis. When the CRP/WCC was added to the test markers, the results were the same as for the test-markers alone. For patients with a GNR the agreement between the test-marker results and the clinical findings was 78% (for sepsis).

For the PUO patients the test markers diagnosed 10 patients with sepsis and 2 cases of early sepsis; the test markers plus the CRP/WCC diagnosed 4 with early sepsis and 8 with sepsis and the clinical diagnosis was 15 septic patients.

When a blood-culture is positive.

An unexpected finding in the project was the 36 positive blood cultures produced from the controls which is fully discussed in chapter 7. According to Washington (1992), 10% of positive blood cultures are due to contamination, but which 10%? According to this current study that number could in fact be much higher as 72% of the control patients in phase 4 were found to produce positive blood cultures. All of these blood culture results were verified as contaminants.

How can the clinician know with certainty that the blood culture results under consideration are truly positive? Obviously the overall clinical picture should lead to appropriate decisions case by case. Nevertheless patients may be given inappropriate antibiotic treatment for a blood stream infection that they do not have.

When a blood culture is negative.

A negative blood culture can be just as misleading to the clinician. Prophylactic antibiotics will interfere with microbial growth. Blood stream infections can go undiagnosed if the volume blood cultured is less than 10ml (Mermel *et al.*, 1993).

Patients with pyrexia of unknown origin included in the phase 4 prove this point, as they all had negative blood cultures yet 15 were subsequently diagnosed as having

sepsis caused by Gram-positive or Gram-negative bacteria and 1/15 had both a Gram-negative and Gram-positive bacterial sepsis and many others had an infection that proved to be the cause of their pyrexia.

This project has clearly shown that IL-6 and sICAM-1 together can diagnose patients with sepsis. Indeed they have demonstrated that they can differentiate between inflammation and infection, early sepsis and sepsis and uncover infectious causes in PUO's with negative blood cultures. If these markers were routinely available to the clinicians they would be very helpful in the clinical setting as demonstrated by patient *p*15 (end discussion chapter 7). They would be particularly helpful to clinicians in the oncology department as serum levels of these two markers would show the difference between an inflammatory response to chemotherapy and early infection thus avoiding unnecessary antibiotic treatment.

In their present form these assays are sold on the basis that they are for research use only and they are *not* to be used in diagnostic procedures in a clinical setting (R&D systems, Oxen, UK). This may be for legal reasons but these assays could not realistically be used as a routine test as they are both time consuming and expensive. IL-6 assays take 4 hour 20 minutes to run (not including the preparation and processing time) and sICAM-1 takes just 2 and 30 minutes. There is an IL-6 strip (Knoll, Germany) used for a bedside diagnosis that gives a plus or minus result. However the plus result occurs when there is a serum level of 1000pg/ml (IL-6) and few of the septic patients from this study had serum levels that reached that high.

Further work.

Ideally the next best step to take as a follow on from this project would be to try and develop rapid tests for these two serum proteins. This is readily possible for IL-6 but for sICAM-1 that is not as likely as this molecule together with PCP, LBP (indeed all the other test markers in this project) belong to various companies who have ownership rights. It is possible that from the results of projects such as this, one of those companies would develop a diagnostic test for the rapid measurement of sICAM-1 and IL-6.

There is a large amount of patient data in this project, 50% of which came from the last phase. Due to the semi-blind trial nature of that phase there was less need initially to analyse the data in terms of underlying disease and their possible effects on the markers.

There are many ways in which all this data could be further analysed, for instance the test marker results could compared with the specific BC isolate to see if there is any correlation with the serum levels or studied in terms of age, underlying disease or outcome.

Until such time as these authentic markers of sepsis (IL-6 with sICAM or LBP with PCT) are developed for routine use in the hospital setting, it is recommended that regardless of WCC and the presence or absence of a pyrexia CRP levels are requested routinely. It is an inexpensive test that had it been performed for patient *p*15 the results would have raised the index of suspicion and prevented his discharge and subsequent re-admission the following day as it was 118 mg/l –and rising.

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