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Circulating markers of inflammation and infection as adjuncts to a clinical scoring system in predicting outcome in cancer patients with febrile neutropenia.

By

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For my Wuzzie and Koby, with Love

Summary

Infections remain the major cause of serious complications in cancer patients. Cancer patients receiving chemotherapy often develop febrile neutropenia and are at high risk for development of bacterial or fungal infections, which are the major cause of morbidity and mortality in these patients, with an overall in-hospital mortality rate of 9.5% in the USA. Febrile neutropenia is a life- threatening complication which can lead to septic shock and death, but the majority of patients can survive provided they receive appropriate management. Immediate hospitalisation with rapid administration of broad-spectrum antimicrobial agents is the standard management for these patients. However, in neutropenic patients, fever can be caused not only by infection, but also by non-infective causes. In this setting, there is a critical requirement for the development of strategies, such as the Talcott system and Multinational Association of Supportive Care in Cancer (MASCC) risk-index score, which enable early identification of patients at high risk for serious infective complications.

The laboratory research presented in this dissertation was designed to:
i) to compare the performance of a conventional ELISA procedure with the highly sensitive Bio-Plex® Suspension Bead Array System using a limited group of cytokines, namely IL-1β, IL-6 and IL-8; and ii) identify host-derived, systemic markers of inflammation or infection (CRP, SAA, PCT, sTREM-1, HMGB-1, IL-1α, IL-1β, IL-6, IL-7, IL-8, IL-12, INF-γ, TNF, G-CSF, GM-CSF, and VEGF) which, either individually or in combination, can be used to distinguish between infective and non-infective causes of pyrexia in cancer patients, as well as to predict outcome in patients with chemotherapy-induced neutropenia.

To fulfil these objectives, unthawed serum specimens from 48 patients recruited to an earlier study (Uys *et al*, 2007) were analysed. All patients had a histologically confirmed malignancy and had presented with febrile neutropenia, either with an oral temperature of >39°C in a single measurement, or > 38°C on two occasions within a 24 hour period lasting at least one hour, together with an absolute neutrophil count of ≤ 0.5 x 10^9 /L as a result of chemotherapy. The original study was approved by Ethics Committee of the University of Pretoria in November 2000, while approval for

performance of the additional assay on the stored serum specimens as an extension of the original study was granted by the same committee in February 2010. Informed consent was obtained from all patients prior to enrolment in the original study.

The results demonstrated that the Bio-Plex® Suspension Bead Array System and the conventional ELISA are well correlated, although the former has greater sensitivity.

With respect to the second component of the study, all of the biomarkers tested were elevated in patients with FN. When these patients were classified into those at low- and high- risk for development of complications, (using clinical scores), IL-6, PCT and sTREM-1 demonstrated the best discriminatory potential. The sensitivity, specificity, positive predictive value (PPV), likelihood ratios and area under the ROC curve of each of these biomarkers was evaluated with respect to prediction of response to empiric antimicrobial therapy, resolution without complications, development of serious complications, and mortality. Soluble TREM-1, and to a lesser extent PCT, but not IL-6, predicted each of these events with reasonable accuracy, particularly with respect to the development of serious complications or death.

Soluble TREM-1 and PCT, individually or in combination, hold promise as objective biomarkers which could be used to complement clinical prediction-making process in patients presenting with febrile neutropenia.

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List of Abbreviations

α	Alpha
AA	Amino acid
Akt	Alternative name for PKB
AML	Acute myeloid leukemia
ANC	Absolute neutrophil Count
APR	Acute phase reactant
β	Beta
BPI	Bactericidal/permeability increasing protein
C	Complement
CAMs	Cell adhesion molecules
CD	Cluster of differentiation
CDI	Clinically documented infection
CLL	Chronic lymphocytic leukemia
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
CUP	Carcinoma of unknown primary site
DAG	Diacylglycerol
DISC	Dearth-inducing signaling complex
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal regulated kinase
FADD	Fas-associated death domain
FMLP	N-formyl-methionyl-leucyl-phenylalanine
FUO	Fever of unknown origin
HOCL	Hypoclorous acid
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GRB	Growth factor receptor binding protein
HD	Hodgkin's disease
HDL	High-density lipoprotein
HMGB1	High-mobility group box1 protein

H_2O_2	Hydrogen peroxide
IgG	Immunoglobulin G
IFN	Interferon
IL	Interleukin
i.v	Intravenous
JAMs	Junctional adhesion molecules
LPS	Lipopolysaccharides
LR	Likelihood ratio
LYMPH	Lymphocytes
MAPK	Mitogen-activated protein kinase
MASCC	Multinational Association for Supportive Care in Cancer
MBAA	Multiplex bead array assays
MDI	Microbiologically documented infections
MEKK1	Mitogen-activated protein kinase/extracellular signal-regulated kinase
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
NHL	Non Hodgkin's Lymphoma
NK	Natural killer
NSCLC	Non small cell lung cancer
O_2^-	Superoxide
O.D.	Optical density
$OH^{\cdot}$	Hydroxyl radical
PAF	Platelet-activating factor
PAMP	Pathogen-associated molecular pattern
PCT	Procalcitonin
P13-K	Phophoinositide 3-kinase
PE	Phycoerythrin
PECAM-1	Platelet endothelial cell adhesion molecule-1
phox	Phagocyte oxidase
PKC	Protein kinase C
PLA_2	Secretory phospholipase A2
PLC	Phospholipase C
POD	Peroxidase
PPV	Positive predictive value

PR3	Proteinase 3
PS	Performance status
RAGE	Receptor for advanced glycation end- products
ROC	Area under the curve
ROS	Reactive oxygen species
SAA	Serum amyloid A
SCLC	Small cell lung cancer
S.E.M	Standard error of mean
SOS	Son of sevenless
sTNF R1	Soluble tumor necrosis factor receptor 1
RT	Room temperature
TGF	Transforming growth factor
TLR	Toll-like receptors
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor
TREM-1	Triggering receptor expressed on myeloid cells-1
VEGF	Vascular endothelial growth factor
WCC	White cell count

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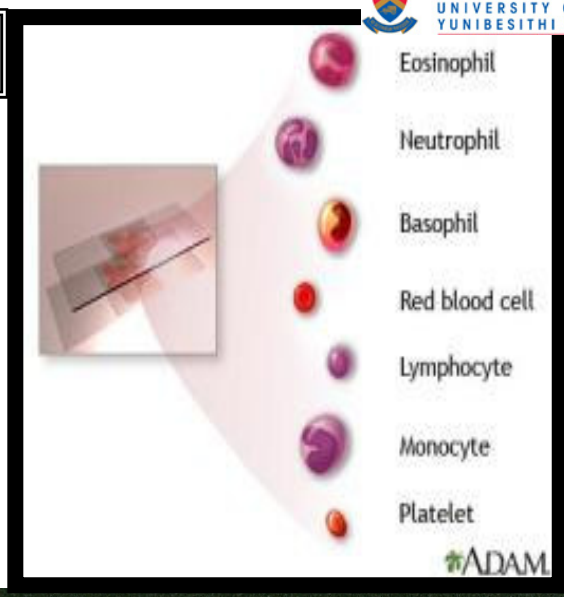

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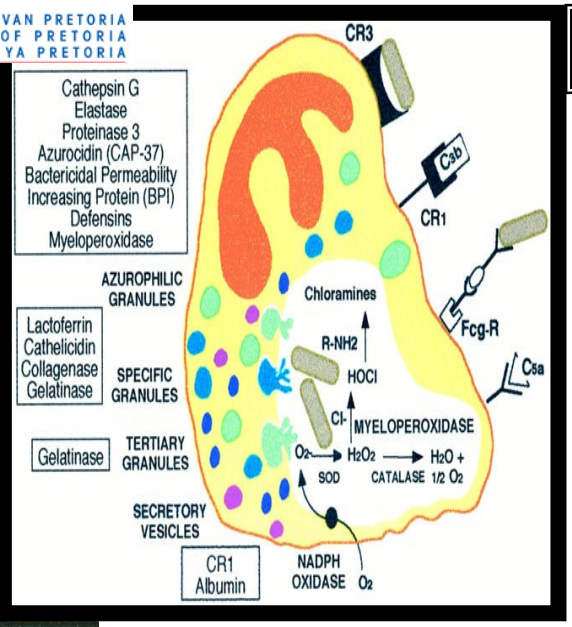

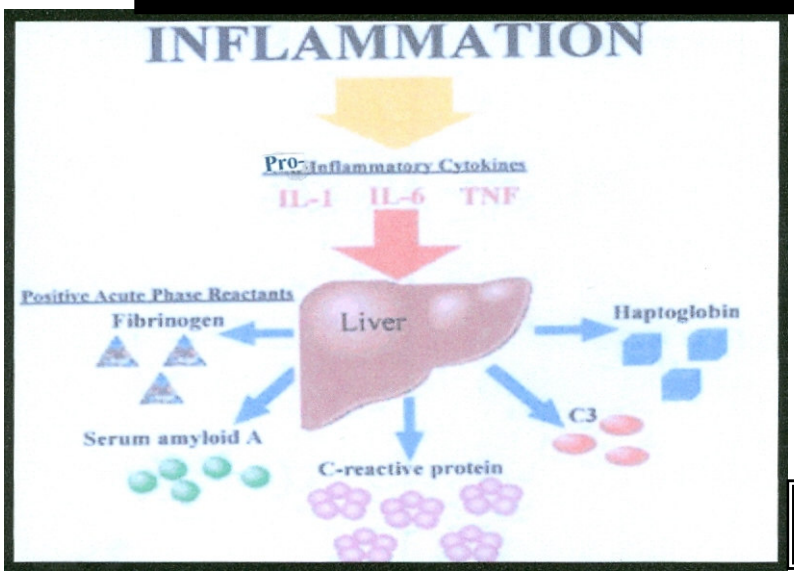

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1. Full blood count is run on cancer patients as a means of measuring hydration status, anemia and the body's ability to form blood clots and fight infection retrieved 24 August 2010, http://www.animalcancerspecialist.com/Vocabulary.html
2. Neutophil effector mechanisms involved in defence against pathogens and in the inflammatory process (Witko-Sarsat *et al*, 2000).
3. Stimulation and synthesis of positive acute-phase reactants during inflammation Source: 13 May /content/2003/00/45/09/450937/450937_fig.html)

Chapter 1

Literature Review

1.1 Febrile Neutropenia

In patients with cancer, defects of the immune response against infection are due to several causes acting either concomitantly or sequentially, with major roles being played by the underlying disease and by the medical therapies developed to treat it (Viscoli et al., 2005).

Anti-cancer chemotherapeutic agents not only affect malignant cells, but also destroy normal proliferating cells necessary for host defence. Cancer patients receiving chemotherapy therefore often develop neutropenia and are at high risk of bacterial or fungal infections. Infection in neutropenic patients may be difficult to diagnose and inadequately treated infection can rapidly lead to a fatal outcome. Early diagnosis is therefore crucial (Durack & Street, 1991). The majority of these patients, if neutropenic for more than one week, will develop fever due to infection (Schimpff, 1986; Buchheidt et al., 2003). Febrile neutropenia is a life- threatening complication which can lead to septic shock and death, but the majority of patients can survive provided that they receive prompt, appropriate antimicrobial therapy. Therefore immediate hospitalisation with rapid administration of broad-spectrum antimicrobial agents, close monitoring for development of complications, and evaluation of response to therapy is the standard management for these patients (Bodey et al., 1985; Hughes et al., 1990; Klastersky et al., 2007). However, in neutropenic patients, fever can be caused not only by infection, but also by the underlying disorder, or the administration of drugs or blood products (von Lilienfeld-Toal et al., 2004).

Many investigations have indicated that febrile neutropenic patients are a heterogeneous population and not all are at the same risk of developing serious medical complications or death (Elting, 1998). Clinical studies performed on patients' characteristics have led to the identification of Risk Models, which are based on combinations of risk factors which enable clinicians to classify patients as high- or low- risk (Jones et al., 1996; Lucas et al., 1996).

Several clinical studies involving neutropenic patients with predicted low risk have demonstrated the feasibility of newer approaches, such as outpatient therapy after early discharge from hospital or outpatient status for the entire febrile episode using oral antibiotics, which have been proven to be as effective as intravenous antibiotics (Talcott et al., 1988; Klastersky et al., 2000; Johnson et al., 2008; Rolston et al., 2009). A better understanding of the aetiology of febrile neutropenia and early identification of the presence of infection will enable a more discerning approach to antimicrobial chemotherapy in the setting of avoidance of enormous costs and anxiety to patients.

1.1.1 Fever and Neutropenia

Febrile neutropenia is a common syndrome in patients with haematological malignancies (Giamarellos-Bourboulis et al., 2001). Fever in neutropenic patients may be the only manifestation of infection. Other clinical signs or symptoms (pain, swelling, erythema) indicating an infectious process may be absent or unremarkable due to a lack of neutrophils and consequent attenuation of inflammatory processes (Talcott et al., 1988; Von Lilienfeld-Toal et al., 2004).

A fever can be defined as a single recording of an oral temperature of 38.3℃ or a rectal temperature of 39℃ (Hughes et al., 2002), or an oral temperature of at least 38℃ or a rectal temperature of 38.6℃ on two occasions within a 24 hour period lasting for at least one hour (Nutt, 2009).

Neutropenia can be defined as an absolute neutrophil count (ANC) of <500cells/mm^3 or an ANC< 1,000 cells/mm^3 with a predicted decline to <500 cells/mm^3. The infection rate and severity are inversely related to the ANC (Bodey *et al.*, 1966; Lucas *et al.*, 1996; Chauhan, 2009).

Lower ANCs are associated with more frequent and severe infections, and conversely, higher ANCs correlate with less frequent and severe infections. Patients with neutrophil counts <500 cells/mm^3, are at significantly increased risk for

infection compared with those with counts of <1,000cells/mm^3. Similarly, patients with counts of <100cells/mm^3 are at even greater risk for infection than those with counts of <500cells/mm^3. The duration of neutropenia is also an important determinant of risk of infection. Patients with a low ANC and prolonged neutropenia (eg.10 days) are at even higher risk of infection (Dale et al., 1979).

1.1.2 Epidemiology of Febrile Neutropenia

Myelosuppresion continues to represent the major dose-limiting toxicity associated with systemic cancer chemotherapy. Fever is a well-known complication in neutropenic patients with cancer, which prompts immediate hospitalization for evaluation and the administration of empiric broad-spectrum antibiotics (Hughes et al., 2002),

The most frequent by encountered Gram-negative pathogens are:

- *Escherichia coli*
- *Klebsiella species*
- *Pseudomonas aeruginosa*
- *Enterobacter species*
- *Acinetobacter species*

Uncommon, but significant Gram-negative pathogens include:

- *Serratia marcescens*
- *Flavobacterium meningosepticum*
- *Aeromonas hydrophila*

The most frequently encountered Gram-positive pathogens are:

- *Staphylococcus aureus*
- *Staphylococcus epidermidis*
- *Staphylococcus haemolyticus*
- *Staphylococcus hominis*
- *Enterococcus species*
- *Streptococcus pyogenes*

- *Viridans streptococci*

Uncommon, but significant Gram-positive pathogens include:

- *Bacillus species*
- *Streptococcus pneumoniae*

(Kannangara, 2006).

Febrile neutropenic episodes are clearly still associated with substantial morbidity and mortality with high medical cost, with serious medical complications reported in about 21% of patients (Talcott et al., 1988). More recently, Kuderer *et al* (2006) reported on an inpatient mortality of 9.5% in the USA.

1.1.3 Management of Febrile Neutropenia

Major advances have been achieved in the medical sciences in the last decades, such that patients with illnesses previously considered untreatable may now receive appropriate therapy and survive. However, this achievement often involves aggressive and invasive procedures and therapies which in turn lead to multiple disruptions in immunological protection and therefore to increased susceptibility to opportunistic infections.

Success in the management of febrile neutropenia requires prompt recognition of, and reaction to potential infection. Vital to this is educating outpatients to monitor symptoms including body temperature, as well as clear written instruction on when and how to contact the appropriate service in the event of concerns (Marti et al., 2009).

For more than 30 years the standard management for all patients developing febrile episodes while neutropenic has been in-patient treatment with broad spectrum intravenous (i.v.) antibiotics (Hughes et al., 1990; Innes et al., 2005). However, recent evidence has prompted a re-assessment of febrile neutropenia, with increasing realisation that such intensive treatment may not be necessary or appropriate for all patients.

1.1.4 Initial Assessment

An initial assessment of circulation and respiratory function with vigorous resuscitation where necessary should be followed by careful examination for potential foci of infection. Signs and symptoms of infection in neutropenic patients can be minimal, particularly in those receiving corticosteroids. Blood from a peripheral vein and any indwelling venous catheters, as well as sputum, urine and skin swabs, where clinically indicated, should be sampled for culture before the prompt institution of empirical broad-spectrum antimicrobial therapy (Table 1).

Table 1.1 Initial assessment and investigation.

Note presence of indwelling i.v catheter
Symptoms or signs suggesting an infection focus
Respiratory system
Gastrointestinal tract
Skin
Perineal region/ genitourinary
Oropharynx
Central nervous system
Investigations
Routine blood testing to assess bone marrow, renal and liver function
Coagulation screen
C-reactive protein
Blood cultures (minimum two sets) including cultures from i.v. catheter
Urinalysis and culture
Sputum microscopy and culture
Stool microscopy and culture (if diarrhoea present)
Skin lesions (aspirate/ biopsy/swab)
Chest radiograph (if respiratory symptoms present or outpatient therapy considered).

(Faizal *et al.,* 2006).

Risk assessment is important in deciding whether febrile neutropenic patients can be treated as inpatients or outpatients, or whether oral or intravenous antibiotics can be used. Two classification systems are:

i) the Talcott classification (Talcott et al., 1992) of risk group,

ii) the scoring system proposed by the Multinational Association for Supportive Care in Cancer (MASCC) group (Klastersky et al., 2000). Both systems use serious medical complications as the endpoint for risk prediction, but the sensitivity of the Talcott classification is limited and the misclassification rate is high. The MASCC group scoring system is based on patient history, age, outpatient status, clinical status at presentation. (Klastersky et al., 2000) see table 2.

Table 1. 2. MASCC scoring index

Characteristic	score
Burden of illness:no or mild symptoms	5
No hypotension	5
No chronic obstructive pulmonary disease	4
Solid tumor or no previous fungal infection	4
No dehydration	3
Burden of illness: moderate symptoms	3
Outpatient status (at on set of fever)	3
Age <20 years	2

Scores of >21 indicate low risk of complications.

(Klastersky et al., 2006).

1.1.5 Initial antibiotic therapy

Empirical antibiotic therapy in febrile neutropenic cancer patients became well established in the 1970s, because high mortality was seen when antibiotics were not provided (Schimpff et al., 1971). Early antibiotic therapy is warranted in febrile neutropenic patients because infection can progress rapidly. Commonly used antibiotics are shown in Table 3. Figure 1 summarizes the standard management of febrile neutropenia.

Table 1 3 Empiric antibiotic therapy

Monotherapy
Cephalosporins
• Antipseudomonal third-generation cephalosporin (ceftazidime)
• Fourth generation cephalosporin (cefepime)
Carbapenems
• Imipenem
• Meropenem
Combination therapy
Penicillins
• Piperacillin + tazobactam
• Ticarcillin+ clavulanic acid + aminoglycosides (amikacin, gentamycin, tobramicin).
• Carbapenems + aminoglycosides
• Antipseudomonal cephalosporin + aminoglycoside.

(Faizal *et al.*, 2006).

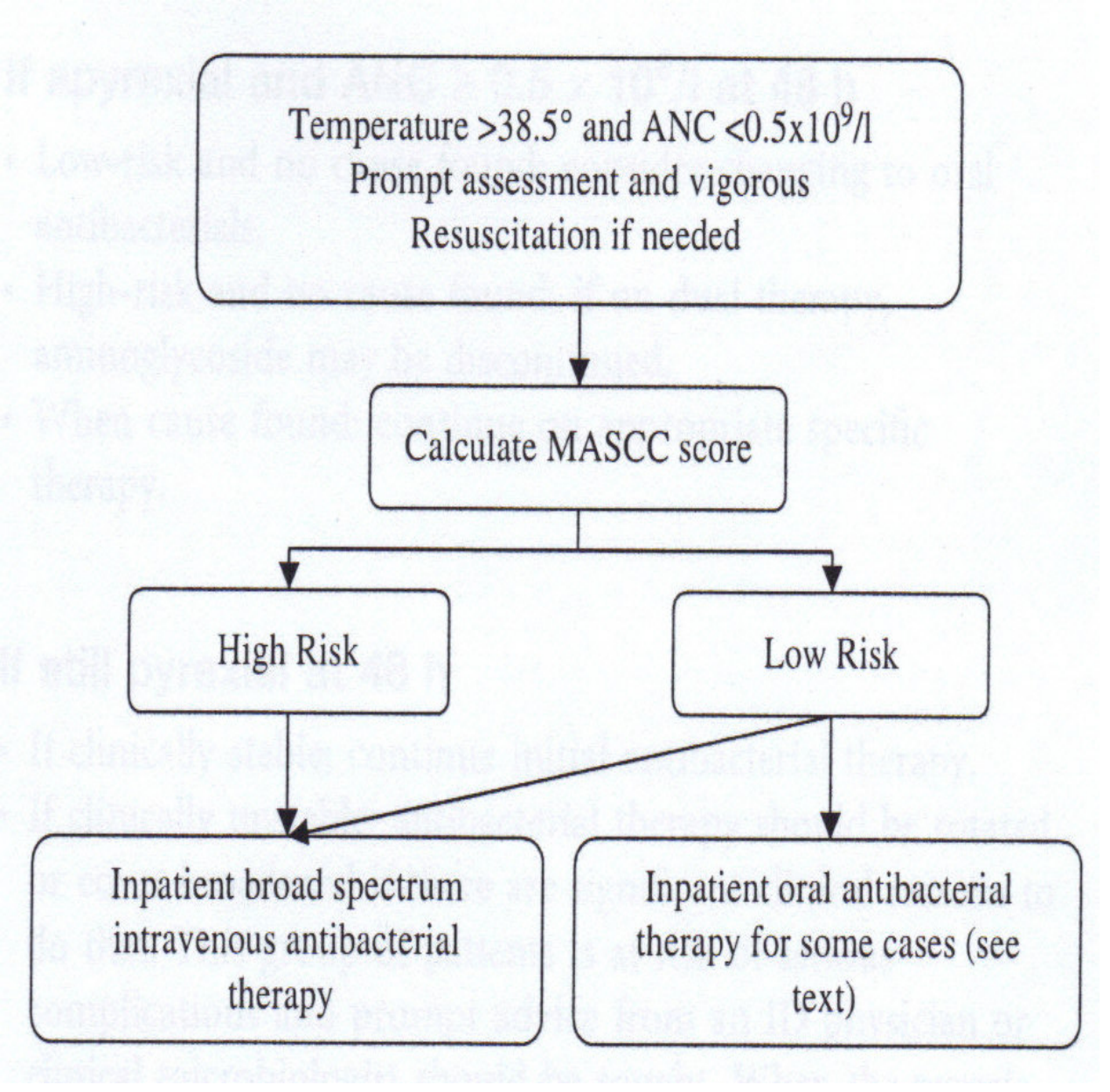


Figure 1.1 Initial management of febrile neutropenia (Marti et al., 2009).

Figure 1.2 The cartoon illustrates how an immune response is generated. After a microorganism invades the body, (e.g. Bacteria). The T- and B-cells recognize the foreign organism, following capture, and proceed to kill the microbial pathogens

(retrieved 07 June 2010, http://www.vetscite.org/publish/articles/000088/print.html).

1.2 Neutrophils

Neutrophils are the most abundant immune cells found in human blood. These cells quickly arrive at the site of infection and form the first line of defence against invading microorganisms as shown in Figure 2. Pluripotent cells divide into two more specialized cells, a lymphoid progenitor and myeloid progenitor cell. The lymphoid progenitor gives rise to T- and B-lymphocytes and the myeloid cell to basophils, eosinophils and neutrophils which are known collectively as polymorphonuclear leukocytes (Hayashi et al., 2003).

Neutrophils have a 14-day development in the bone marrow and stay temporarily in a storage pool before being released into the blood, where they spend 12-14 hours in transit from a circulating pool (axial stream) into a marginating pool (contact with blood vessel walls). In the absence of any bacterial infection, neutrophils enter reticulo-endothelial organs, such as the liver, or even return to the bone marrow to undergo apoptosis (programmed cell death).

1.2.1 Neutrophil activation

1.2.1.1 *Neutrophil recruitment to the inflammatory site*

Circulating neutrophils contact and transiently interact with endothelial cells, resulting in a rolling and release motion. The rolling step is mediated by neutrophil L-selectin, and by E- and by P- selectins expressed on inflamed endothelial cells. Selectins also contribute to signalling. Interaction of neutrophils with P-selectin facilitates neutrophil degranulation, superoxide (O_2^-) production and polarization in response to platelet-activating factor (PAF) and bacterial peptides such as N-formyl-methionyl-leucyl-phenylalanine (FMLP). Crosslinking of L-selectins on neutrophil primes the cells for increased O_2^- production and calcium influx in response to chemoattractants and stimulates adhesion (Burg & Pillinger, 2001).

The endothelium of inflamed microvessels produces chemoattractants such as platelet-activating factor, leukotriene B4 and various chemokines, immobilized via a

"presentation molecule" (proteoglycan) on the luminal surface of endothelial cells. Among these chemokines, interleukin 8 (IL-8) specifically attracts neutrophils while having no effect on monocytes (Premack & Schall, 1996; Rollins, 1997) and being unable to promote lymphocyte transmigration through endothelium (Roth et al., 1995).

Exposure of circulating neutrophils to chemoattractant gradients results in the conversion from neutrophil rolling to tight adhesion to endothelium. This firm neutrophil adhesion to endothelial cells appears to involve the interaction of leucocyte integrin of the β_2 subfamily (CD11a, CD11b, CD11c) and a common β-subunit (CD18). Two important β_2 integrins on the neutrophil are CD11a/CD18 (LAF-1) and CD11b/CD18 (MAC-1, CR3) (Fischer et al., 1983; Anderson et al., 1984; Sligh et al., 1993; Burg & Pillinger, 2001).

CD11b/CD18 integrin interacts with GPI-anchored membrane proteins, such as FcγRIIIb (CD16b), the LPS receptor, CD14, or the urokinase receptor uPAR (CD87). FcγRIIIb interaction with CD11b/CD18 promotes antibody-dependent phagocytosis (Todd & Petty, 1997), while CD14 interaction with CD11b/CD18 only occurs in the presence of LPS and LPS-binding protein and may play a role in the generation of inflammatory mediators (Todd & Petty, 1997; Zarewych et al., 1996).

After firm adhesion, neutrophil transmigration occurs at the borders of endothelial cells, where discontinuities of tight junctions are observed, in a process called diapedesis. Two cell adhesion molecules of the Ig-superfamily (CAMs) have been shown to be involved in leukocyte transmigration:

i) The platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31) and

ii) The cell junction adhesion molecules (JAMs 1,2,3) (Muller Kobold et al., 1998, Vaporciyan et al., 1993, Martin-Padura et al., 1998). In a study reported by Ortolan in 2006, it was shown that CD157 also plays an important role in neutrophil diapedesis. CD157 is expressed by endothelial cells with the highest density at intercellular junctions. It is also expressed by neutrophils, and CD157-deficient neutrophils from patients with paroxysmal nocturnal haemoglobinuria are characterized by severely impaired diapedesis (Ortolan et al., 2006).

Neutrophils migrate in tissues by haptotaxis (along a gradient of immobilized, rather than soluble chemoattractants). These chemoattractants, which are produced by bacteria, dying cells, or by various stromal and epithelial cells of inflamed tissues, are bound to extracellular matrix components. *In vitro* models show that leukocytes move through complex chemoattractant fields by migrating in a multistep process in response to one source after the other. This process is summarised in Figure 3 (Witko-Sarsat et al., 2000).

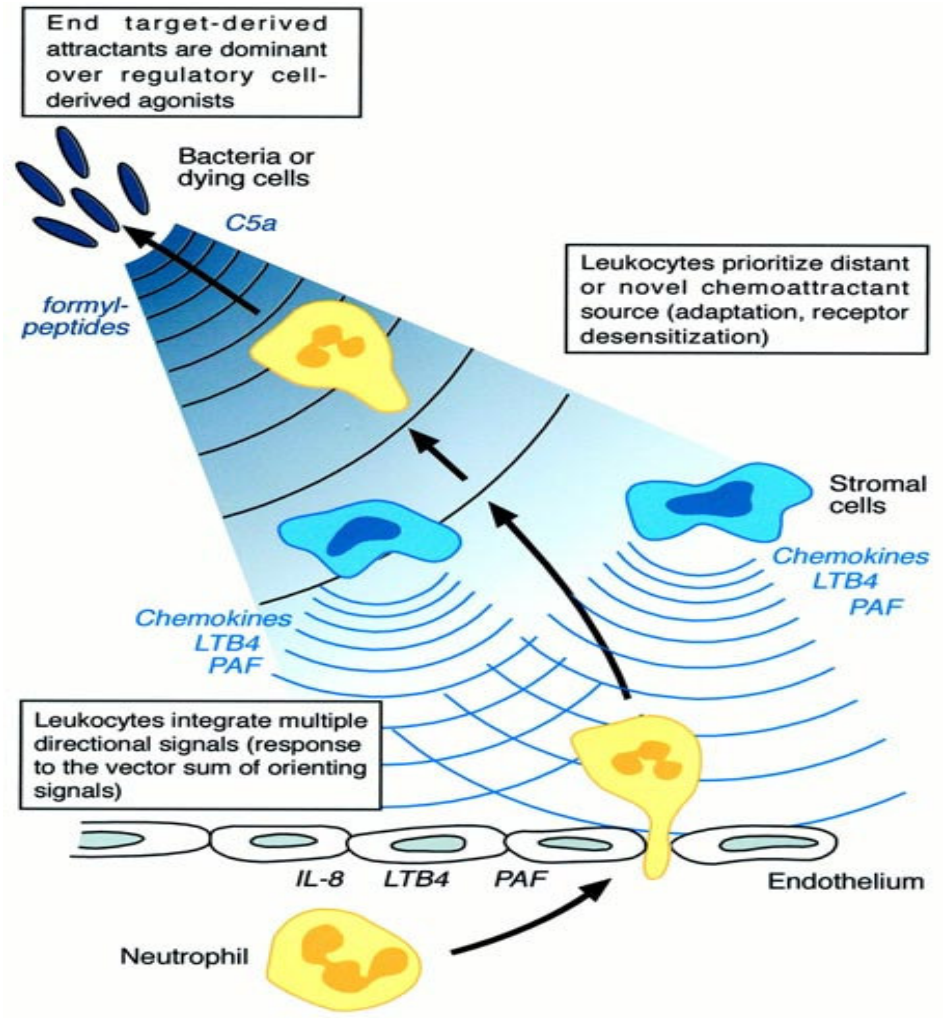


Figure 1.3. The schematic view of the chemotactic migration of leukocytes towards the site of inflammation. Neutrophils move through the endothelium and within tissues by responding to successive combinations of chemoattractant gradients. Chemoattractants are released by endothelial cells, by activated stromal cells (macrophages, epithelial cells), and by the inflammatory targets ie, bacteria or dying cells. The direction of neutrophil movement is first guided by the steepest local chemoattractant gradient and is then regulated by successive receptor

desensitization and attraction by distant agonists. Finally, end-target attractants are dominant over regulatory cell-derived agonists (Witko-Sarsat et al., 2000).

1.2.1.2 *Phagocytosis, degranulation and bacterial killing*

Antibacterial activity requires the delivery of antimicrobial substances to target cells which either prevent colonization of host tissue by the target cell, prevent the growth of the target cell, or kill the target cell. The phagocyte uses four mechanisms of delivery of antimicrobial substances:

- Phagocytosis
- Secretion/ degranulation
- Apoptosis/ cytolysis
- The respiratory burst

During neutrophil phagocytosis, recognition of micro-organisms is facilitated by coating them with serum proteins called opsonins. The process of coating the particle, such that the phagocyte may ingest it, is known as opsonization. There are two main serum-derived pathways for preparing bacterial and fungal particles for phagocytic ingestion. The first one is complement-dependent neutrophil phagocytosis, which is also receptor- mediated, with the integrin CD11b/CD18 acting as an iC3b receptor. Then iC3b receptors (CR3, CR4) which are stored in the gelatinase-containing granules are brought to the surface of the phagocyte. CR3 and CR4 bind to iC3b and enable the phagocyte to ingest microbial targets. The second one is Fc receptor which mediates antibody specific endocytosis. The most important opsonic Fc receptors are those which bind IgG. (Mitchell et al., 1994; Takano et al., 2000).

Binding of the opsonized particle triggers engulfment. Pseudopods are extended around the particles forming the phagocytic vacuole. The membrane of the vacuole then fuses with the membrane of a lysosomal granule, resulting in discharge of granule contents into the phagolysosome and degranulation of the neutrophil (Burg & Pillinger, 2001).

Phagocytosis results in the release of lysosomal enzymes not only within the phagolysosome (intraphagolysosomal secretion), but also potentially into the extra-cellular space (extracellular secretion) with resulting cell injury and matrix degradation. Seconds after a neutrophil ingests a particle, it begins to secrete granule components namely azurophilic or primary, specific or secondary, tertiary or gelatinase, and secretory vesicles into the phagosome. Neutrophil stimulation causes extracellular granule secretion in the following order: secretory vesicle, tertiary, specifc and azurophilic granules (Henderson & Chappel, 1996; Burg & Pillinger, 2001).

There are many components within the phagocyte granules (lysosomes) which exert antimicrobial effects. Some of these components are as follows:

- Defensins

  Defensins are major components of the azurophil granules of neutrophils and render the target cell membrane more permeable. They consist of a group of low molecular weight arginine/cysteine-enriched cationic peptides (3000-4000 Daltons). They are present in phagocytic vacuoles at a concentration of 1mg/ml (Ganz et al., 1990; Ganz & Weiss, 1997).

- Bactericidal/permeability increasing protein (BPI)

BPI is a 59 kD cationic protein which is only found in neutrophils. It is cytotoxic to many Gram-negative bacteria at nanomolar concentrations. Its N-terminal domain allows binding to LPS and its C-terminal mediates bacterial attachment to neutrophils. Binding of BPI causes an increase in the permeability of the outer membrane of Gram-negative bacteria and hydrolysis of bacterial phospholipids (Iovine et al., 1997; Elsbach, 1998).

- Proteinase 3 (PR3)

PR3 is a major component of the azurophil granules. It has been shown to enhance cleavage and activation of TNF and IL-1β from LPS-stimulated Human monocytes leukemia THP-1 cells (Coeshott et al., 1999; Witko-Sarsat et al., 1999).

- Elastase

Is another potent serine protease, which degrades the outer membrane protein which is highly conserved among Gram-negative bacteria (Weinrauch et al., 1998).

- Secretory phospholipase A_2 (PLA_2)

PLA_2 is a neutrophil granule protein with potent bactericidal activity. It synergizes with BPI for intracellular bacterial killing (Weiss et al., 1994).

- Matrix Metalloproteinases

They are stored in latent form within granules, are released as inactive pro-enzymes and require calcium for activation. Latent collagenase can be directly activated by hypoclorous acid (HOCL), wereas progelatinase seems to require both oxidant- and serine protease-dependent activation (Knauper et al., 1996; Burg & Pillinger, 2001).

- Phagocyte-derived, antimicrobial reactive oxygen species

The oxidative antibacterial effects are mediated by two main biochemical entities, the NADPH oxidase system and myeloperoxidase (MPO). MPO is localized in the azurophilic granules. The NADPH oxidase system spans the cytoplasm and plasma membrane (Weiss et al., 1994).

- The H_2O_2- Myeloperoxidase(MPO) system

MPO is a heme protein stored in the azurophilic granules of neutrophils and monocytes. It amplifies the toxic potential of H_2O_2 by producing reactive intermediates and also catalyses the formation of hypochlorous acid, a potent oxidant with bactericidal activity. MPO-derived oxidants are also critically involved in the modulation of signalling pathways (Lau et al., 2005).

1.2.1.3 *Respiratory burst and the NADPH oxidase system*

During phagocytosis a sudden increase in oxygen consumption occurs which is called the respiratory burst. This increase in oxygen consumption is due to the activity of the NADPH oxidase which catalyses the reaction which generates superoxide O_2^-, which is rapidly converted to other reactive oxygen species (ROS).

The NADPH oxydase system consists of different components: $p47^{phox}$ (phox= phagocyte oxidase), $p67^{phox}$, and $p40^{phox}$, are found in the resting neutrophil as a cytosolic complex; rac-2, a cytosolic, ras-related protein; $p22^{phox}$ and $gp91^{phox}$ are membrane bound proteins, which are the subunits of cytochrome b_{558}.

Upon activation of neutrophils, cytosolic $p47^{phox}$ becomes phosphorylated and migrates to the plasma membrane were it is associated with cytochrome b_{558} to assemble the active oxidase. This enzymatic comlex is thus able to generate superoxide anion (O_2^-):

$$NADPH + 2\ O_2 \rightarrow NADP^+ + 2\ O_2^- + 2H^+$$

Superoxide can dismutate into hydrogen peroxide (H_2O_2)

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

(Babior, 1984; Nathan, 1987; Segal & Abo, 1993; Robinson & Badwey, 1995; Henderson & Chappel, 1996b; Dahlgren & Karlsson, 1999).

There are three intermediates in the reduction of O_2 to H_2O_2, namely O_2^-, H_2O_2 and hydroxyl radical ($OH^{\bullet}$)

Hydroxyl radical is one of the most powerful oxidants and may therefore contribute to the toxic activity of phagocytes. It is a highly reactive, oxygen-centred radical with a half life in cells of 10^{-9} seconds. Reduction of H_2O_2 resulting in the formation of hydroxyl radical results from several mechanisms, the most important being: (Klebanoff & Headley, 1999).

Fenton reaction: $H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH^{\cdot}$

Haber-Weiss reaction: $H_2O_2 + O_2^- \rightarrow O_2 + OH^- + OH^{\cdot}$

MPO-catalyzed reaction: $H_2O_2 + Cl^- \rightarrow HOCL + OH^-$

$HOCL + O_2^- \rightarrow OH^{\circ} + O_2 + Cl^-$

Neutrophil activation is summarized in Figure 1. 4.

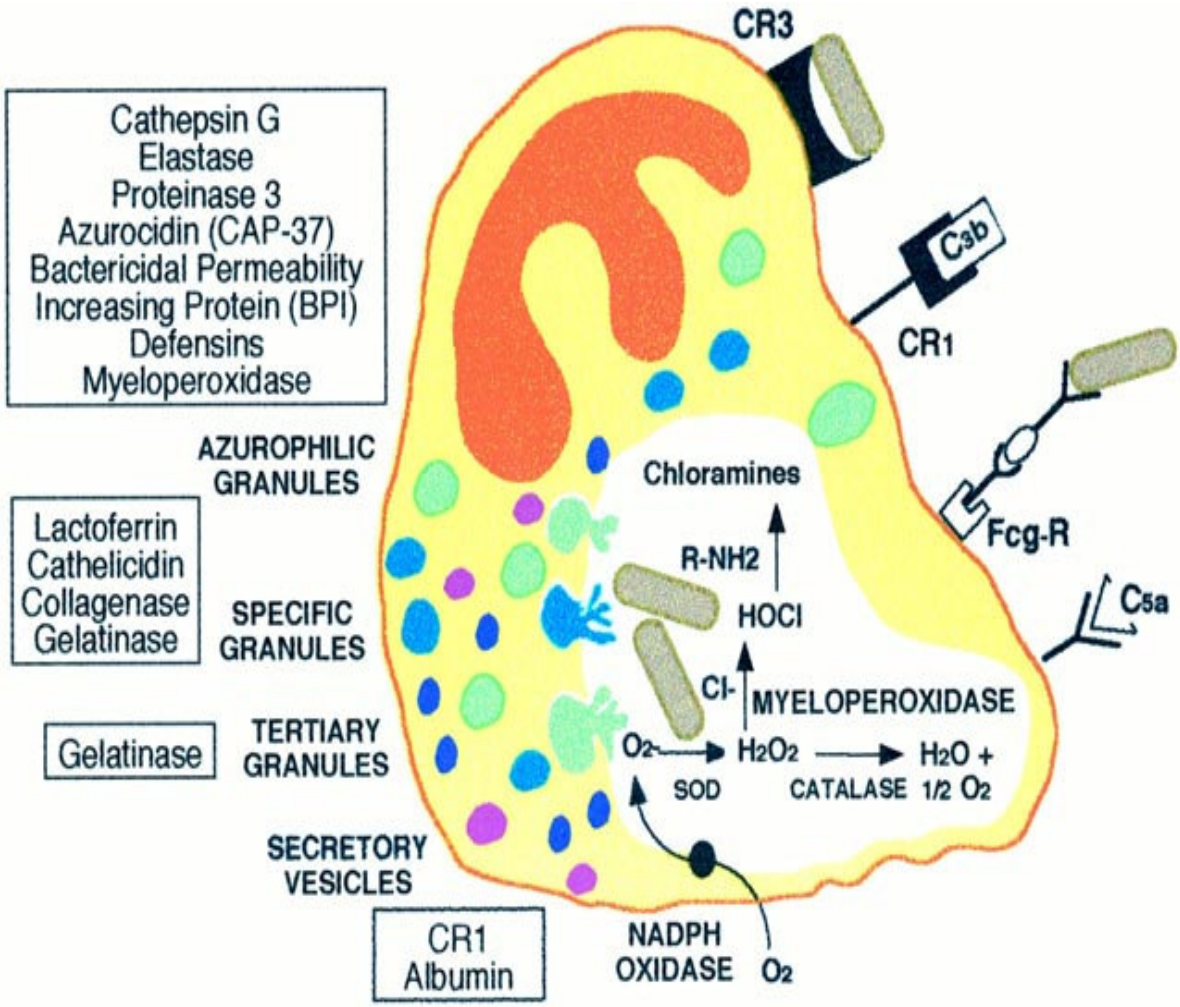


Figure 1. 4. Neutrophil effector mechanisms involved in defense against pathogens and in the inflammatory process. Neutrophil effector systems are mobilized following phagocytosis of pathogen.Complement opsonins C3b and C4b are recognized by CR1 and CR3. IgG opsonins are recognized via the immunoglobulin receptors (FcγR). The first microbicidal pathway is the oxidative reponse, which consists of the production of radical oxygen species following NADPH-oxidase complex activation, including superoxide anion (O_2^-), H_2O_2 and via myeloperoxidase, HOCl and chloramines. The 2nd pathway is non-oxygen-dependent and consists of the release in the phagolysosome or in the intracellular medium of preformed proteins stored in granules. Serine proteases, antibiotic proteins and myeloperoxidase are contained in azurophilic granules. Metalloproteinases (collagenase & gelatinase) and antimicrobial proteins (lactoferrin and cathelicidin) are contained in specific granules. Gelatinase is also contained in tertiary granules (Witko-Sarsat et al., 2000).

1.2.2 Neutrophil apoptosis

Neutrophil apoptosis and subsequent ingestion by macrophages is the major mechanism for clearing neutrophils that have been recruited to the inflamed site and thus for promoting resolution of the inflammation (Cox et al., 1995; Savill, 1997).

Apoptotic neutrophils are non-functional; they are unable to move by chemotaxis, generate a respiratory burst, or degranulate and there is a clear down-regulation of cell surface receptors, preventing them from transducing signals. The rate of apoptosis may be accelerated, as well as delayed. The delayed apoptosis correlates with severity of clinical sepsis and multiple organ dysfunctions (Keel et al., 1997; Matute-Bello et al., 1997).

Accelerated apoptosis is triggered via Fas receptor. Activation of this receptor results in the formation of the death-inducing signalling complex (DISC), which contains CD95, FADD and procaspase-8 (Scaffidi *et al*, 1998). Another regulator of neutrophil apoptosis is phosphoinositide 3-kinase (PI3-K). Neutrophil apoptosis under basal, as well as LPS-stimulated conditions was increased in PI3-K-/- mice. These mice had a decreased amount of activated Akt, phosphorylated CREB, and $NF_{-K}B$ nuclear translocation (Yang et al., 2003).

Finally, phagocytosis of apoptotic neutrophils actively inhibits the production of IL-1beta, IL-8, IL-10, GM-CSF, TNF, leukotriene C4 and thromboxane B_2 by human macrophages. This active suppression of inflammatory mediator production is presumably an important step in the resolution of inflammation (Fadok et al., 1998).

1. 3. Inflammantory Response

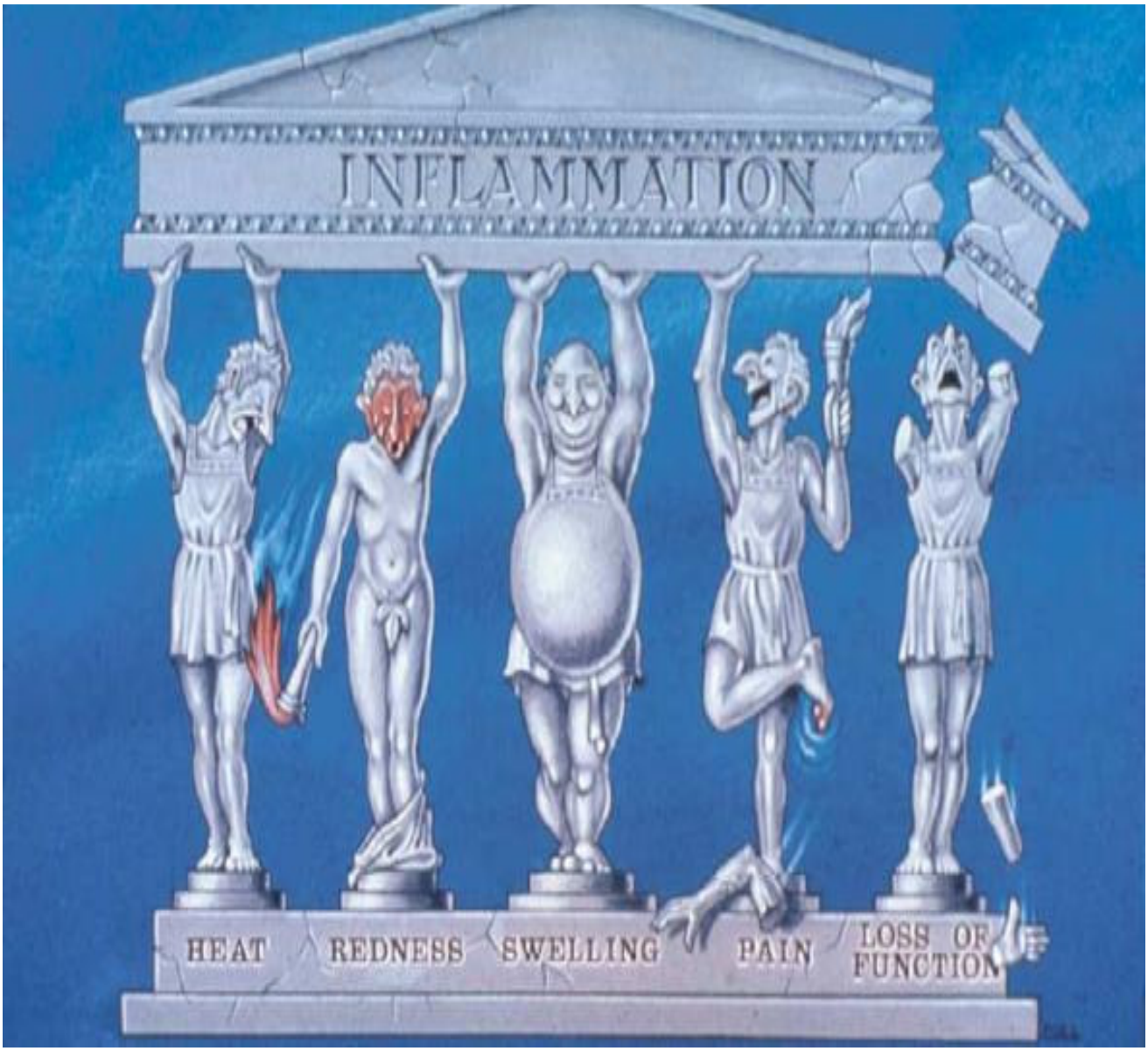


Figure 1.5. displays signs of inflammation. Inflammation is a signal-mediated response to cellular insult by infectious agents, toxins and physical stresses. The sequence of inflammatory events is: Acute phase; agents: cytokines; events: pro-inflammatory cytokines; sequence: signs /symptoms-inflammation is variably accompanied by fever, redness, swelling, pain and tissue/organ dysfunction, retrieved

(07 June 2010, http://www.nature.com/nri/journal/v2/n10/fig_tab/nri915_F1.html).

1.3.1. Acute phase reactants (APR)

The acute phase response is the immediate set of host inflammatory reactions that counteract challenges such as tissue injury, infection and trauma, which occur during acute and chronic inflammatory reactions of both infective and non-infective origin. Its role is to isolate and neutralize pathogens and prevent further pathogen entry, while minimizing tissue damage and promoting repair processes, thereby permitting host homeostatic mechanisms to rapidly restore normal physiological function (Pepys & Baltz, 1983). It is characterised by elevated levels of acute phase reactants, such as C-reactive protein (CRP), and serum amyloid A (SAA).

Under normal circumstances, the liver synthesizes a characteristic range of plasma proteins at steady state concentrations. Although most APRs are synthesized by hepatocytes, some are produced by other cell types including monocytes, endothelial cells, fibroblasts and adipocytes. The major APRs can increase to 1000-fold over normal levels. The negative APRs are decreased in plasma concentration during the acute phase response to allow in the liver to increase the synthesis of induced APRs. A list of APRs is shown in Table 1. 4.

TABLE 1. 4. Human Plasma Acute Phase Proteins

Groups	Individual proteins
Positive APRs	
Major ARPs	Serum amyloid A, C-reactive protein, Serum amyloid P components
Complement poteins	C2, C3, C4, C5, C9, factor B, C1 Inhibitor, C4 binding protein.
Coagulation proteins	Fibrinogen, antithrombin 3, Alpha-2-antiplasmin, vitronectin, Pal-1, Protein S, urokinase, plasminogen
Proteinase inhibitors	α_1-Antitrypsin,α_1-antichymotrypsin, α_2-Antitrypsin, heparin cofactor II, Plasminogen activator inhibitor I
Metal-binding proteins	Haptoglobin haemopexin, Ceruloplasmin, Manganese superoxide dismutase
Negative APRs	Albumin, pre-albumin, Transferrin, apoAI, apoAII, HS glycoprotein, α-trypsin inhibitor, Histidine-rich glycoprotein.

(Adapted from (Gruys et al., 2005).

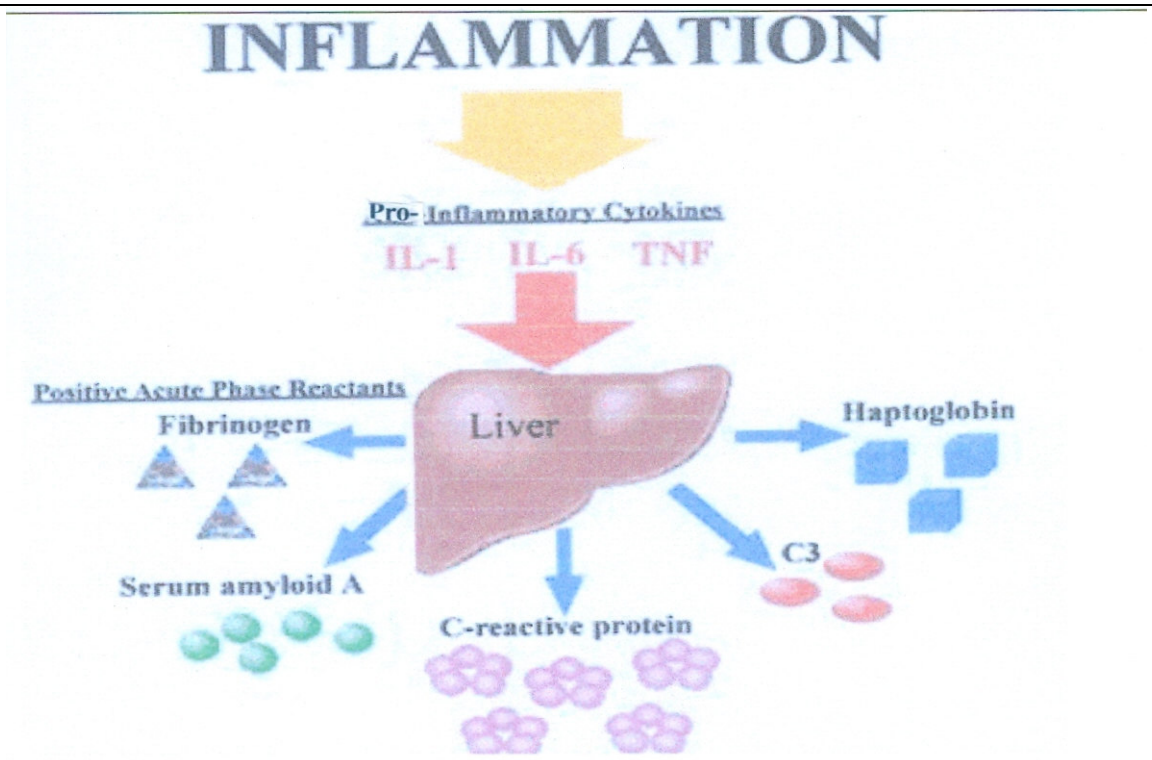


Figure 1.6. Stimulation and synthesis of positive acute-phase reactants during inflammation. Inflammation caused by infection or tissue damage stimulates the circulating inflammation-associated cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor (TNF)-α. These cytokines stimulate hepatocytes to increase the synthesis and release of positive acute-phase proteins, including CRP. IL-6 is the major cytokine simulus for CRP production.
Source: 13 May 2010
(www.medsape.com/content/2003/00/45/09/450937/450937_fig.html).

1.3.1.1 C-reactive protein (CRP)

CRP is an acute-phase protein released by the liver (see Fig. 1.6) after the onset of inflammation or tissue damage. It consists of five identical 23kDa sub-units, and belongs to a family of pentamers called pentraxins. It was the first acute phase protein to be described and is an exquisitely sensitive systemic marker of inflammation and tissue damage (Pepys & Baltz, 1983). It has both pro- and anti-inflammatory effects (Marik, 2002).

CRP is synthesised by hepatocytes and once released into the circulation can bind to activated lymphocytes, as well as neutrophils, activating the classical complement pathway, acting as an opsonin and promoting phagocytosis (Gabay & Kushner, 1999; Povoa, 2002). Other acute-phase proteins include proteinase inhibitors and coagulation, complement and transport proteins, but the only molecule that displays sensitivity, response speed, and dynamic range comparable to those of CRP is serum amyloid A protein (Pepys & Hirschfield, 2003). Synthesis of CRP, as well as that of SAA (described below), is initiated by interleukin-6 (IL-6).

1.3.1.2 Serum Amyloid A (SAA)

The SAA family was originally considered to comprise only a single circulating precursor of the amyloid A protein from which its name is derived. It is encoded by genes in chromosome 11 and consists of two isotypes with molecular weights of 11,685 Da (Uhlar & Whitehead, 1999). The amyloid protein is the principal component of the secondary amyloid plaques that may be deposited in major organs as an occasional consequence of chronic inflammatory disease (see Figure 1.7) (Husby *et* al., 1994). SAA proteins are a family of apolipoproteins found predominantly associated with high-density lipoprotein (HDL) in plasma, with different isoforms being unequally expressed constitutively and in response to inflammatory stimuli.

As compared to the currently widely used C-reactive protein, SAA is frequently a more sensitive marker of inflammation, particularly in some conditions, while it has the advantage of also being involved in the acute-phase response in species other than humans such as mice (Malle & De Beer, 1996).

SAA binds to cell membranes, but is immunosuppressive, inhibiting both B-cell and macrophage function (Urieli-Shoval et al., 1994; Benigni et al., 1996).

In febrile neutropenia, SAA appeared to be more sensitive than CRP in the early detection of bacteraemia, although according to (Riikonen et al., 1993), high levels of SAA were related to the febrile response, but did not correlate reliably with documented bacterial aetiology.

In a review by Lannergard in 2003, SAA and CRP levels were correlated in patients with infectious diseases. SAA levels correlated significantly with CRP levels for the entire study population. SAA was more sensitive than CRP for the detection of minor inflammatory stimuli in the viral and low CRP groups. Results also indicated that SAA and CRP discriminate between bacterial and viral infections, depending on the infecting organism.

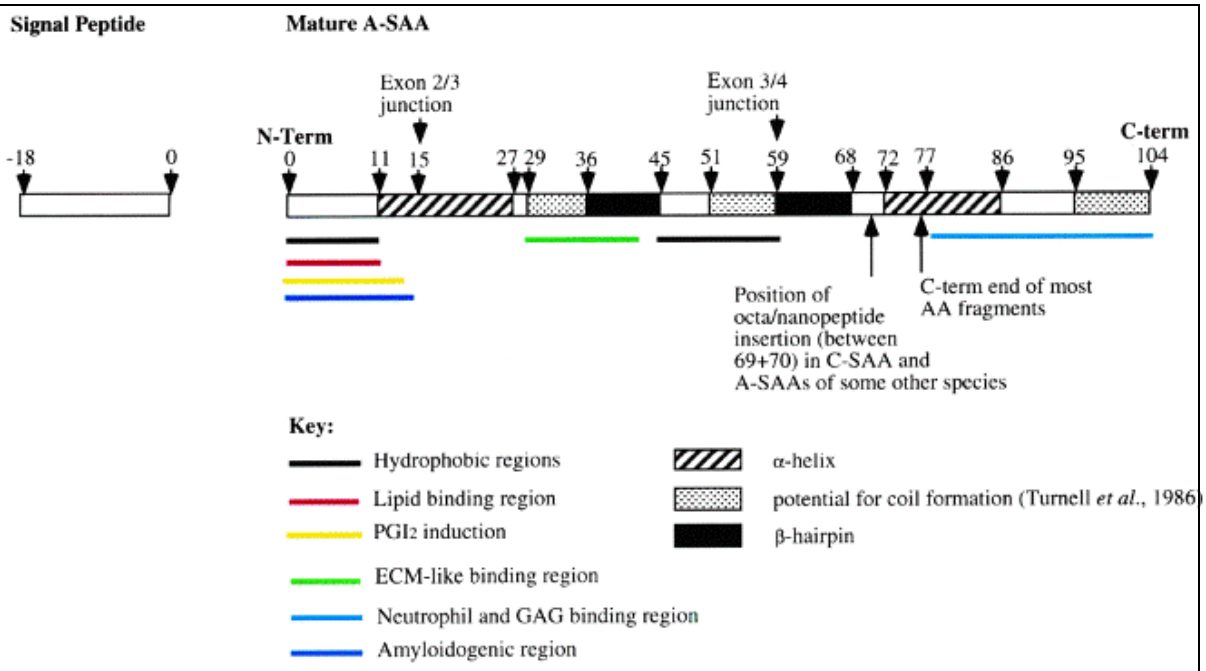


Figure 1.7. **The structure of human A-SAA protein.** The 18-amino acid signal peptide is shown (-18 to 0) together with the 104 mature protein (1-104). The most frequently observed C-terminus of the amyloid A protein is indicated at the residue 76. Regions of potential functional importance are indicated by underlining in colour, while structurally important regions are indicated by shading/hatching. (Taken from Turnell et al., 1986).

1.3.2 Procalcitonin (PCT)

Procalcitonin is a 116 amino acid protein with a sequence identical to that of the prohormone of calcitonin (32 amino acids) (see Figure 8) (Le Moullec et al., 1984). Under normal metabolic conditions, the prohormone undergoes intracellular proteolytic processing and the hormonally active calcitonin is secreted by the C-cells of the thyroid gland (Adema & Baas, 1992). However, in severe bacterial infections and sepsis, intact procalcitonin is found in blood. PCT is synthesized and released by cells including macrophages and monocytic cells of various organs, e.g. the liver in response to infections (Bracq et al., 1993). The main stimulus for PCT is the systemic action of bacterial endotoxins. Viral diseases, autoimmune diseases, neoplastic disorders and local and organ-related bacterial infections do not induce PCT (Oberhoffer et al., 1999).

The association between PCT and infection was first investigated by Assicot *et al* in 1993. It has been proven to be a very sensitive marker for systemic bacterial infections and sepsis in non-leukopenic patients, while serum concentrations seem to correlate well with the severity of infection.

Enguix in 2001 evaluated the value of SAA, CRP and PCT as diagnostic markers of bacteriological sepsis in critically ill children and neonates. In children the PCT concentration was shown to be a better diagnostic marker of sepsis, but had the same value as SAA and CRP in neonates. Cut-off concentrations for optimum prediction of sepsis were PCT> 6.1ng/ml, CRP >23 mg/l and SAA> 41 mg/l (Enguix et al., 2001).

In a recent study, procalcitonin (PCT) and CRP were correlated in a pediatric population with febrile neutropenia and cancer, dividing the patients into low- and high-risk groups (Martinez-Albarran et al., 2009). Upon reviewing the literature it seems that there is a discrepancy regarding the predictive value of CRP in patients with febrile neutropenia. Nevertheless, there are studies that indicate that CRP and PCT are of comparable utility in determining the severity of infection in these patients (Erten et al., 2004). Martinez-Albarran concluded that CRP may be a better

screening test in the emergency setting, because of its overall better sensitivity and feasibility, but it may not be the better predictor at the onset of febrile neutropenia.

Engel et al., 1994 & Fleischhack et al., 2000, concluded that in pediatric patients, the diagnostic value of PCT was superior to that of CRP, IL-2, IL-6, IL-8 and TFN. On admission, PCT offered the best discrimination between mild (fever of unknown origin) and localized infection and serious infections like bacteraemia. In a similar study conducted by Uys *et al* 2007, it was also concluded that PCT was the most useful laboratory parameter in predicting those patients with severe microbial infection, and was best correlated with MASCC score, followed in descending order by IL-6, CRP, SAA, IL-8, IL-10 AND IL-1β. A more recent study also concluded that PCT seems to be a more accurate predictor at the onset of febrile neutropenia and an important criterion in defining high-risk groups of patients with febrile neutropenia (Martinez-Albarran et al., 2009).

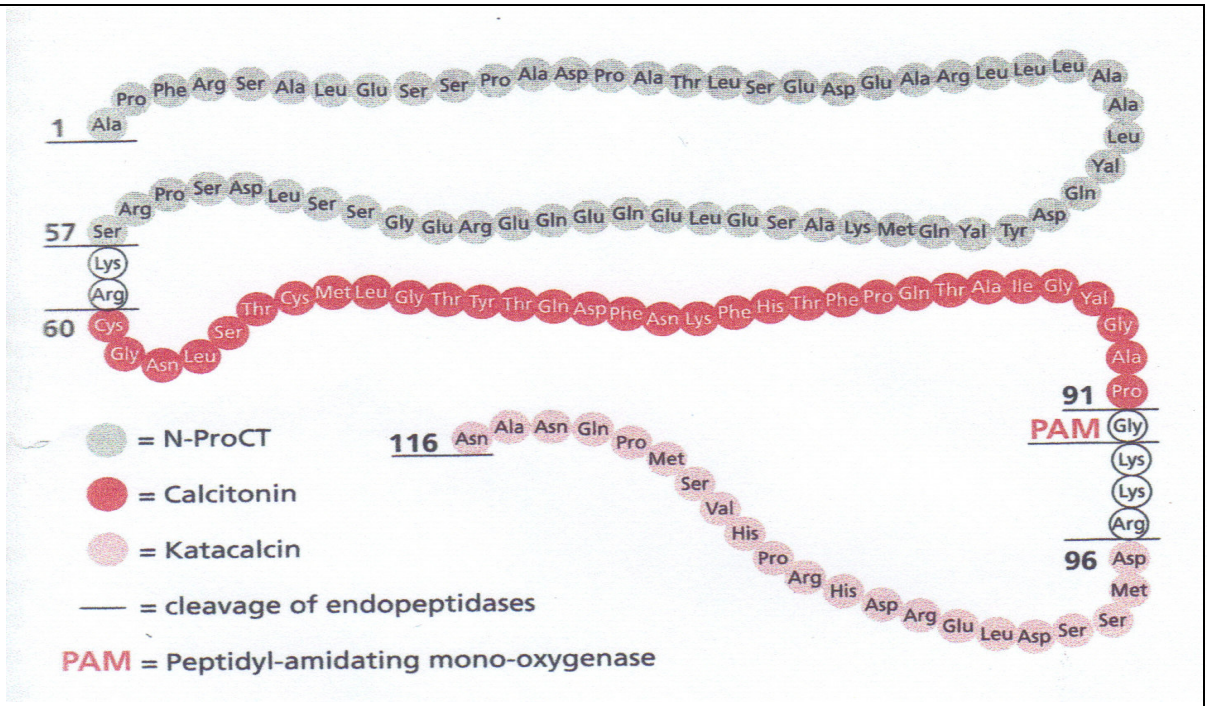


Figure 1.8 Schematic description of the amino acid sequence of PCT (Le Moullec et al., 1984).

1.3.3 Triggering receptor expressed on myeloid cells (TREM-1)

TREM-1 is part of the TREM family of receptors, which belong to the immunoglobulin superfamily and are involved in the inflammatory response.It is expressed on the surface of neutrophils, monocytes and macrophages during acute inflammatory responses. Innate immunity is crucial for host survival during the early stages of infection. Pathogen detection is achieved through pathogen recognition receptors, such as the Toll-like receptors (TLR), which activate innate immune cells to clear the pathogen and shape the adaptive immune response (Bouchon et al., 2001).

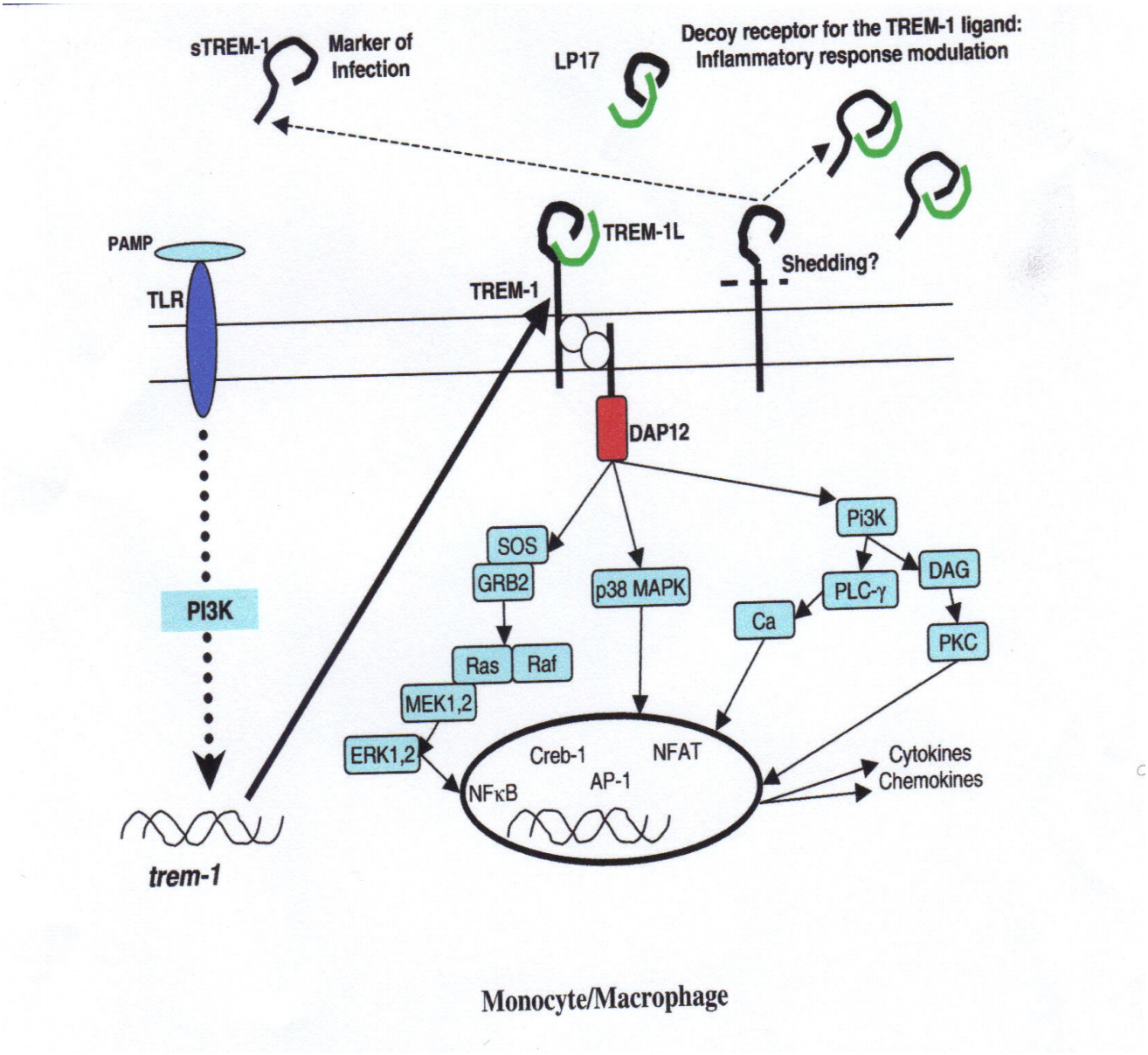
Human TREM-1 consists of an extracellular region of 194 amino acid residues (aa), a membrane-spanning domain of 29 aa and a short cytoplasmic tail of 5 aa. The spanning region contains a Lysine (Lys) residue, which forms a salt-bridge with an Asp residue of the transmembrane domain of immunoreceptore DAP12, allowing TREM-1 to associate with its adaptor (Bouchon et al., 2000; Lanier & Bakker, 2000)(Figure 1.9). Cleavage of the extracellular portion of TREM-1 on the membrane of activated neutrophils and monocytes results in TREM-1 shedding. Soluble TREM-1 (sTREM-1) is detectable in various body fluids and it functions as a decoy receptor for TREM-1 ligand. A recombinant form of sTREM-1 inhibits the lethal effects of lipopolysaccharide (LPS) *in vivo* (Bouchon et al., 2001).

Members of the TREM-family modulate the innate immune response either by amplifying or dampening TLR-induced signals (Nathan & Ding, 2001; Kelker et al., 2004, Ford & McVicar, 2009a). TREM-1 activation triggers pro-inflammatory cytokine/chemokine release, increases surface expression of all cell activation markers, stimulates the release of myeloperoxidase, and increases neutrophil and monocyte survival at sites of inflammation, thus amplifying the acute inflammatory responses (Bouchon et al., 2000; Bouchon et al., 2001).

TREM-1 as a biological marker for infection has been investigated intensively in critically ill patients with infections. (Gibot et al., 2004) determined plasma sTREM-1 levels at admission in critically ill patients with suspected infection. Plasma levels of CRP, PCT and sTREM-1 were significantly elevated in patients with sepsis relative

to patients with non-infective causes of systemic inflammatory response syndrome (SIRS). The findings indicate that sTREM-1 is a useful and sensitive marker of infection and inflammation. Also Passini et al., 2004 demonstrated that the outer membrane of peripheral neutrophils in septic patients expresses high levels of TREM-1. (Phua et al., 2006), also found that serum sTREM-1 levels of patients with pneumonia, chronic obstructive pulmonary disease (COPD), and those with asthma excerbations were elevated compared with controls. The most significant increase was seen in patients with pneumonia and Anthonisen type 1 COPD exacerbations, rather than Anthonisen type 2 and 3 COPD, or asthma exacerbations. They concluded that the serum soluble form of TREM-1 has a moderate, but insufficient degree of accuracy, as a surrogate marker for the need for antibiotics in lower respiratory tract infections.

There is, however, almost no literature on sTREM-1 and febrile pneutropenia.



Figer 1.9. **Overview of the role of Trem-1 in sepsis.** DAG, diacyglycerol; ERK, extracellular signal regulated kinase; GRB, growth factor receptor binding protein; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase; PAMP, pathogen-associated molecular pattern; P13K , phosphatidylinositol 3-kinase; PKC, protein kinase C; PLC, phospholipase C; SOS, son of sevenless; TLR Toll-like receptor; TREM triggering receptor expressed on myeloid cells; TREM-1L, TREM-1 ligand (Gibot, 2005).

1.3.4 High-mobility group box 1 protein (HMGB1)

HMGB1 is present in almost all eukaryotic cells. It was isolated over 30 years ago from calf thymus as an abundant, chromosomal protein with important structural functions in chromatin organization (Goodwin et al., 1973). It is a 215 amino acid protein. HMGB1 is highly conserved among species, with over 98% identity between rodents, bovine and human proteins (Bustin et al., 1990; Yotov & St-Arnaud, 1992).

Structurally, it is composed of three domains, two of which are composed of 80 amino-acid domains referred to as “HMG boxes A and B”. These two domains are strongly positively charged and the third carboxyl terminal (acid tail) domain is extremely negatively charged (Figure .10). The A and B boxes of HMGB1 can interact with DNA, leading to distortion and bending of the double helix structure (Bustin et al., 1990; Read et al., 1993; Weir et al., 1993; Hardman et al., 1995).

Functionally, A and B boxes are the DNA binding regions of the protein which facilitate gene transcription by stabilizing nucleosome formation. The B box contains the cytokine-like activity and stimulates pro-inflammatory responses in monocytes / macrophages, initiating the production of TNF, IL-1β and IL-6 (Andersson & Tracey, 2003; Palumbo et al., 2004; Liu et al., 2009). This activity is antagonized by the recombinant A box. The first 21 amino acid residues of the recombinant B box represent the minimal peptide sequence that retains the cytokine-like activity. The protein structure involved in the binding of HMGB1 with receptor advanced glycation end products (RAGE) is located between amino acid residues 150 and 183 (Ulloa et al., 2002; Sasahira et al., 2005; Balasubramani et al., 2006) (Figure.1.10).

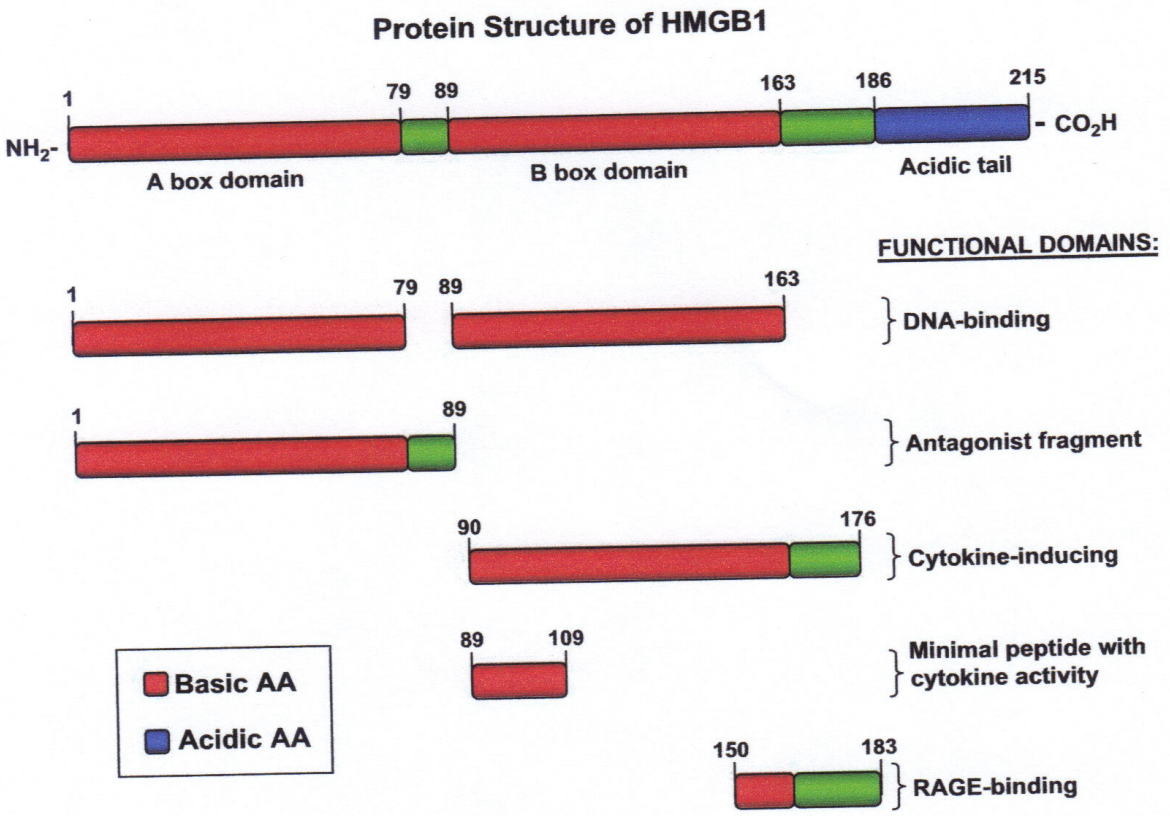


Figure 1.10. Structure of HMGB1 protein. HMGB1 is a 215- amino acid (AA) protein of ~ 30 kDa (Ellerman et al., 2007).

HMGB1 can be secreted by cells of the innate immune system, such as macrophages in response to microbial products and functions as a late mediator in LPS-induced toxicity, appearing in plasma after between 8 and 32 hours. When released into the extracellular milieu by necrotic or inflammatory cells, HMGB1 acts as a cytokine and there are two separate ways for HMGB1 to be secreted from a cell (Figure. 1.11). HMGB1 can be passively released from the nuclei of the necrotic, disintegrating or damaged cells (Gardella et al., 2002). HMGB1 is also actively released into the extacellular milieu by various types of cells including activated macrophages/monocytes, pituicytes, endothelial cells, neutrophils, epithelial cells, dendritic cells, smooth muscle cells and erythroleukemic cells, although the precise mechanism remains unknown (Scaffidi et al., 2002).

HMGB1 does not have a leader sequence and therefore is not processed via the endoplasmic reticulum/Golgi pathway (Gardella et al., 2002). Because it is released by living and dead cells in various organs and tissues, detection of HMGB1 may or may not reflect disease activity and as such is difficult to use as a unique diagnostic marker. Successful detection of HMGB1 in biological samples (serum, plasma, spinal fluid, synovial fluid, etc.) has been described in various disease conditions such as *Plasmodium falciparum malaria*. Alleva *et al*. reported the presence of HMGB1 in African children manifesting cerebral symptoms as a consequence of severe *P. falciparum* malaria. The sera from all malaria cases contained significantly more HMGB1 than that of controls (Alleva et al., 2005).

(Fang et al., 2002) reported that up-regulation of HMGB1 expression may be involved in the pathogenesis of endogenous endotoxin-mediated multiorgan damage secondary to major burns. In chronic inflammatory diseases such as arthritis, elevated HMGB1 has been reported in synovial fluid from animal models as well as human patients with arthritis (Taniguchi et al., 2003). Ellerman et al., 2007 reported on the potential role of HMGB1 In cancer. He found it puzziling how one protein could be responsible for so many roles, both within and outside cells. He concluded that HMGB1 clearly plays a role in cancer development and metastasis, with RAG-HMGB1 signalling promoting spread of most tumour types. However, the full extent of that role remains cryptic along with the possibility of its interaction with other signalling systems.

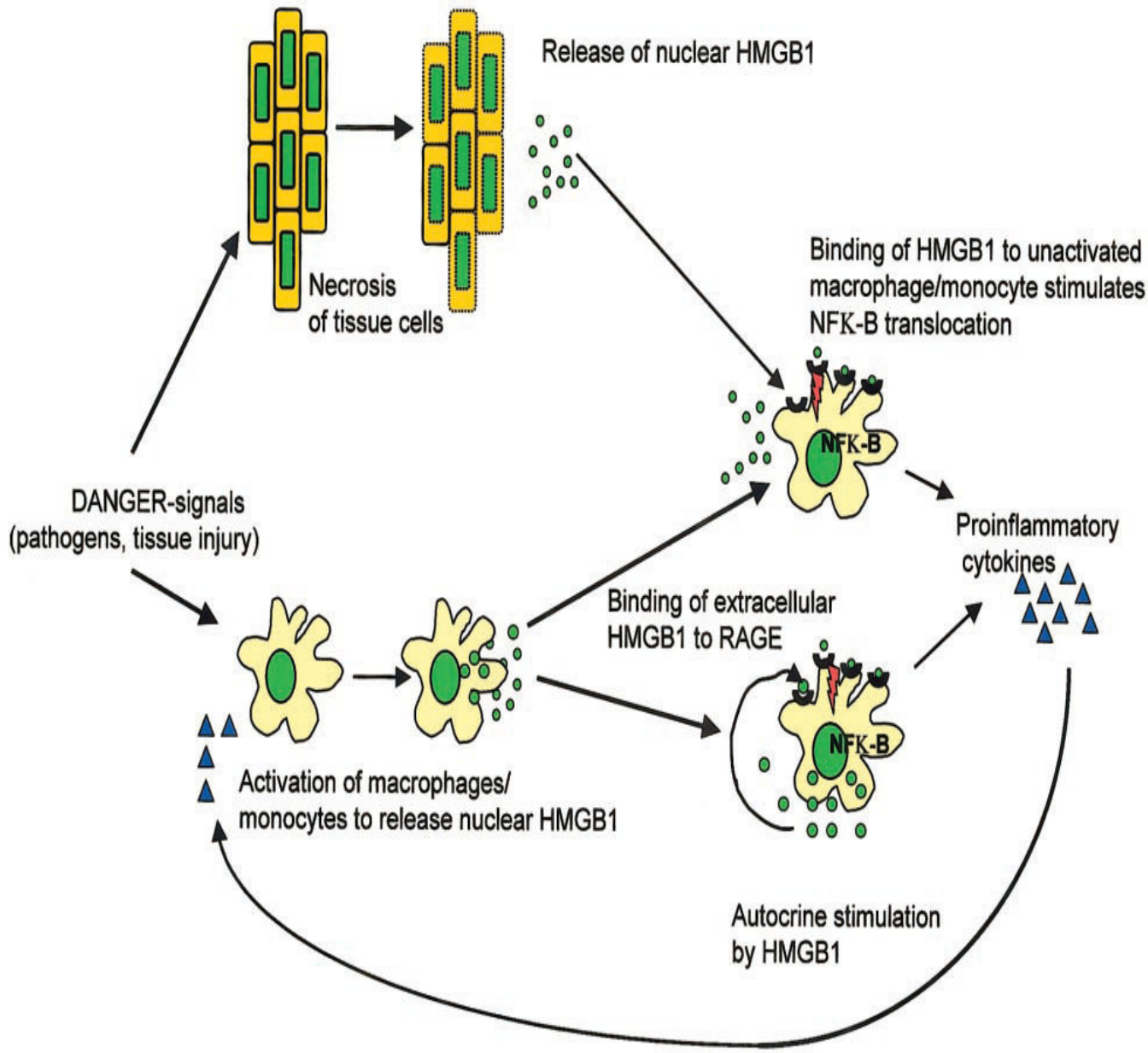


Figure 1.11. Schematic illustration of potential pathways for HMGB1 release leading to inflammatory responses. HMGB1 can be released extracellularly by passive secretion from any necrotic cell, or by active secretion from activated macrophages/ monocytes (Andersson & Tracey, 2003).

1.3.5 Cytokines

Cytokine is the generic term for small proteins which are produced by the cells of the immune system and which act as immunomodulating agents. They may act locally, either on other cell types (paracrine), or on the same cell type (autocrine), or systemically (endocrine). Cytokines can be grouped as lymphokines, interleukins, interferons, chemokines and growth factors. Cytokines have several important characteristics:

- The same cytokines may be made by a number of different cells.
- The same cytokine may have different effects in different circumstances (this is called `pleiotropy').
- Different cytokines may have the same activity depending on the situation (`redundancy').
- Cytokines often act together and increase the effects of one another (`synergy,). They may also act as antagonists.
- Most cytokines have either paracrine or autocrine effects.

They tend to be very potent (Opal & DePalo, 2000).

They can also be classified into pro-inflammatory (IL-1, IL-6, IL-7, IL-8, TNF) (Dinarello, 2000) and anti-inflammatory (IL-4, IL-10, IL-11, IL-13, IL-16, IFN-α, TGF-β, IL1ra) cytokines (Opal & DePalo, 2000), cytokines. A summary of selected immune cytokines and their activities is shown in Table 1.5.

Table 1.5. Selected functions of some Cytokines.

Cytokine	Producing cell	Target cell	Function
GM-CSF	Th1 cells	Progenitor cells	Growth and differentiation of monocytes and DC
IL-1α IL-1β	Monocytes Macrophages B cells DC	T cells B cells NK cells various	Co-stimulation Maturation and proliferation Activation Inflammation, acute phase response, fever
IL-2	Th1 cells	Activated T and B cells, NK cells	Growth, proliferation, activation
IL-3	T cells NK cells	Stem cells Mast cells	Growth and differentiation Growth and histamine release
IL-4	T cells	Activated B cells Macrophages T cells	Proliferation and differentiation IgG1 and IgE synthesis MHC Class II Proliferation
IL-5	T cells	Activated B cells	Proliferation and differentiation IgA synthesis
IL-6	Monocytes Macrophages T cells Stromal cells	Activated B cells Plasma cells Stem cells various	Differentiation into plasma cells Antibody secretion Differentiation Acute phase response
IL-7	Marrow stroma Thymus stroma	Stem cells	Differentiation into progenitor B and T cells
IL-8	Macrophages Endothelial cells	Neutrophils	Chemotaxis
IL -10	T cells	Macrophages B cells	Cytokine production Activation
IL 12	Macrophages B cells	Activated T cells NK cells	Differentiation into CTL (with IL-2) Activation

IFN-α	Leukocytes	Various	Viral replication MHC 1 expression
INF-β	Fibroblasts	Various	Viral replication MHC 1 expression
IFN-γ	Th1 cells NK cells	Various Macrophages Activated B cells Th2 cells Macrophages	Viral replication MHC expression IgG class switch to IgG2a Proliferation Pathogen elimination
TGF-β	T cells, Monocytes	Monocytes, Macrophages Activated Macrophages Activated B cells Various	Chemotaxis IL-1 synthesis IgA synthesis Proliferation
TGF-α	Macrophages, Mast cell, NK cells	Macrophages Tumor cells	CAM and cytokine expression Cell death
TGF-β	Th1 and Tc cells	Phagocytes Tumor cells	Phagocytosis, Cell death
TNF-α	Macrophages, Mast cells, NK cells	Macrophages Tumor cells	CAM and cytokines expression Cell death
TNF-β	T cells	Phagocytes Tumor cells	Phagocytosis, Cell death

(Adapted from (Ashman & Papadimitriou, 1995; Feldmann, 2008).

1.3.5.1 Interleukin 1(IL-1)

IL-1 consists of two distinct, but related molecules, IL-1α and IL-β, which are both 17.5 kDa proteins. Each is encoded by a separate gene. IL-1β is secreted by macrophages and epithelial cells, and induces expression of adhesion molecules on endothelium, and synthesis of acute phase proteins, and IL-6 (Peakman & Vergani, 1997). This cytokine also increases the expression of adhesion molecules on endothelial cells to enable transmigration of leukocytes. IL-1 also leads to the increase of body temperature, and may promote synthesis of acute phase reactants by hepatocytes, but is less effective than IL-6.

IL-1 receptor antagonist (IL-1ra)

IL-1ra is a protein which binds to the same receptor on the cell surface as IL-1, preventing IL-1 from sending a signal to this cell.

1.3.5.2 Interleukin-6 (IL-6)

IL-6 is a protein which acts both as a pro-inflammatory and an anti-inflammatory cytokine. It is one of the most important mediators of fever and the acute phase response. It is secreted by T cells and macrophages to stimulate the immune response to trauma, especially burns or other tissue damage leading to inflammation. In terms of host response to foreign pathogens, IL-6 has been shown, to be required for resistance against the bacterium *Streptococcus pneumoniae* (van der Poll et al., 1997).

The literature regarding IL-6 in febrile neutropenia as a sensitive inflammatory marker is contradictory. In the case of circulating IL-6, values were elevated at onset of fever, but no correlation could be detected between levels of this cytokine and documented bacterial aetiology (Riikonen et al., 1993). IL-6 measured at onset of febrile neutropenia was reported to enable identification of the low-risk group of neutropenic patients (de Bont et al., 2000). Although it may be a useful marker of infection in neutropenic patients, especially in patients with Gram-negative

bacteraemia, it is still regarded as inferior to PCT (Ruokonen et al., 1999; Fleischhack et al., 2000; Uys et al., 2007).

1.3.5.3 Interleukin-7 (IL-7)

IL-7 is a hematopoietic growth factor secreted by the stromal cells of the red marrow and thymus. It stimulates the differentiation of hematopoietic stem cells into lymphoid progenitor cells and is important for the proliferation, survival, development and homeostasis of B- and T-lymphocytes and NK (natural killer) cells.

1.3.5.4 Interleukin-8 (IL-8)

This is an 8 kDa non-glycosylated protein of 72 amino acids. It is produced by mononuclear cells, and other leukocyte types (myeloid precursors, NK cells, neutrophils, eosinophils, and mast cells), various tissue cells (fibroblasts, endothelial cells, epithelial cells) and tumor cells. The primary function is the induction of chemotaxis in its target cells and to recruit neutrophils to the site of infection.

During febrile neutropenia, neutrophils and monocytes are unlikely to be the main source of IL-8 (Ostermann et al, 1994). TNF or endotoxin probably causes its release from other cells such as macrophages, endothelial cells and epithelial cells (Huber et al., 1991; von Asmuth et al., 1993).

1.3.5.5 Interleukin-12 (IL-12)

IL-12 is a T-cell stimulating factor, which can stimulate growth and function of these cells. IL-12 also mediates enhancement of the cytotoxic activity of NK cells and cytotoxic T-lymphocytes. IL-12 also has anti-angiogenic activity, blocking the formation of new blood vessels.

1.3.5.6 Granulocyte colony-stimulating factor (G-CSF)

G-CSF is a cytokine produced by a number of different tissues to stimulate the bone marrow to produce granulocytes and stem cells. It also stimulates the survival, proliferation, differentiation, and function of neutrophil precursors and mature neutrophils.

1.3.5.7 Granulocyte-macrophage colony-stimulating factor (GM-CSF)

GM-CSF is a protein that functions as a white blood cell growth factor, stimulating the production of neutrophils, eosinophils, basophils and monocytes.

1.3.5.8 Interferons

These are proteins of both the innate and adaptive immune systems and are induced at an early stage in viral (Type I) and microbial (Type II) infection. There are two types of interferons:

Type I interferon:
Interferon-alpha- is produced by virus-infected leukocytes, while interferon-beta is produced by virus-infected fibroblasts, and virus-infected epithelial cells.

Type II interferon:
Interferon – gamma (IFN-γ)- is produced by activated T-cells and NK cells.
INF-γ has antiviral, antimicrobial, immunoregulatory, and anti-tumor properties.

1.3.5.9 Vascular endothelial growth factor (VEGF)

This growth factor is an important signalling protein involved in both vasculogenesis and angiogenesis, and the growth of blood vessels from pre-existing vasculature. Its function is to create new blood vessels during embryonic

development, new blood vessels after injury, and new vessels to bypass blocked vessels (http://www.copewithcytokines; 30.01.2009).

Inflammation is a protective response to infection by the immune system that requires communication between different classes of immune cells to coordinate their action. Acute inflammation is an important part of the immune response, but chronic inappropriate inflammation can lead to destruction of tissues in autoimmune disorders and perhaps neurodegenerative or cardiovascular disease. Secreted cytokine proteins provide signals between immune cells to coordinate the inflammatory response. Figure 1.12 shows the summary of cytokines and the inflammatory response.

1.3.5.10 Tumor necrosis factor (TNF)

TNF is secreted by macrophages, monocytes, neutrophils, T-cells, and NK-cells following their stimulation by bacterial lipopolysaccharides. It is involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction. Cells expressing CD4 secrete TNF. The synthesis of TNF is induced by many different stimuli, including interferons, IL-2, and GM-CSF.

1.3.6 Receptor for Tumor necrosis factors (TNF)

Tumor necrosis factors (TNFs) are pleitropic cytokines that are considered primary modifiers of the inflammatory and immune reactions of animals produced in response to injury or infection. TNF was originally discovered in sera of animals and was found to cause hemorrhagic necrosis of some transplantable mouse and human tumors and to exhibit primarily cytotoxic activities against tumor but not normal cells in vitro (Carswell et al., 1975; Old, 1985).

The TNF family consists of two proteins designated TNF-α, also called cachectin (Beutler & Cerami, 1987), and TNF-β, also called lymphotoxin; they play a necessary and beneficial role as mediators of host resistance to infections and tumor formation. However over-production or inappropriate expression of these

factors can lead to variety of pathogical conditions, including wasting, systemic toxicity and septic shock (Paul & Ruddle, 1988).

The actions of TNFs are produced subsequent to binding of the factors to the cell surface receptors. Two distinct TNF receptors have been identified:

- TNF RII - Type A / Type α with molecular weight of 75 kDa
- TNF RI – Type B / Type β with molecular weight of 55 kDa.

Both receptor types show affinity binding of either TNF-α or TNF-β (Brockhaus et al., 1990; Rossol et al., 2007).

Several groups have identified soluble TNF binding proteins in human serum and urine (Olson *et al*, 1989; Engelmann *et al*, 1990), that can neutralize the biological activities of TNF-α and TNF-β. Two types have been identified: sTNF RI and sTNF RII which arises as a result of shedding of the extracellular domain of the receptors and concentrations of about 1-2 ng/mL are found in the serum of healthy individuals (Aderka et al., 1992).

Elevated levels of sTNF-R1 have been reported in association with disease states such as cancer (Keller, 1993), human immunodeficiency virus (HIV) infection (Salehian et al., 1993, Salazar-Gonzalez et al., 1997), sepsis (Gatanaga et al., 1990; Girardin et al., 1992; Kalinkovich et al., 1992), other catabolic situations (Van der Poll et al., 1991), and has recently been involved in the pathophysiology of obesity (Bullo et al., 2002).

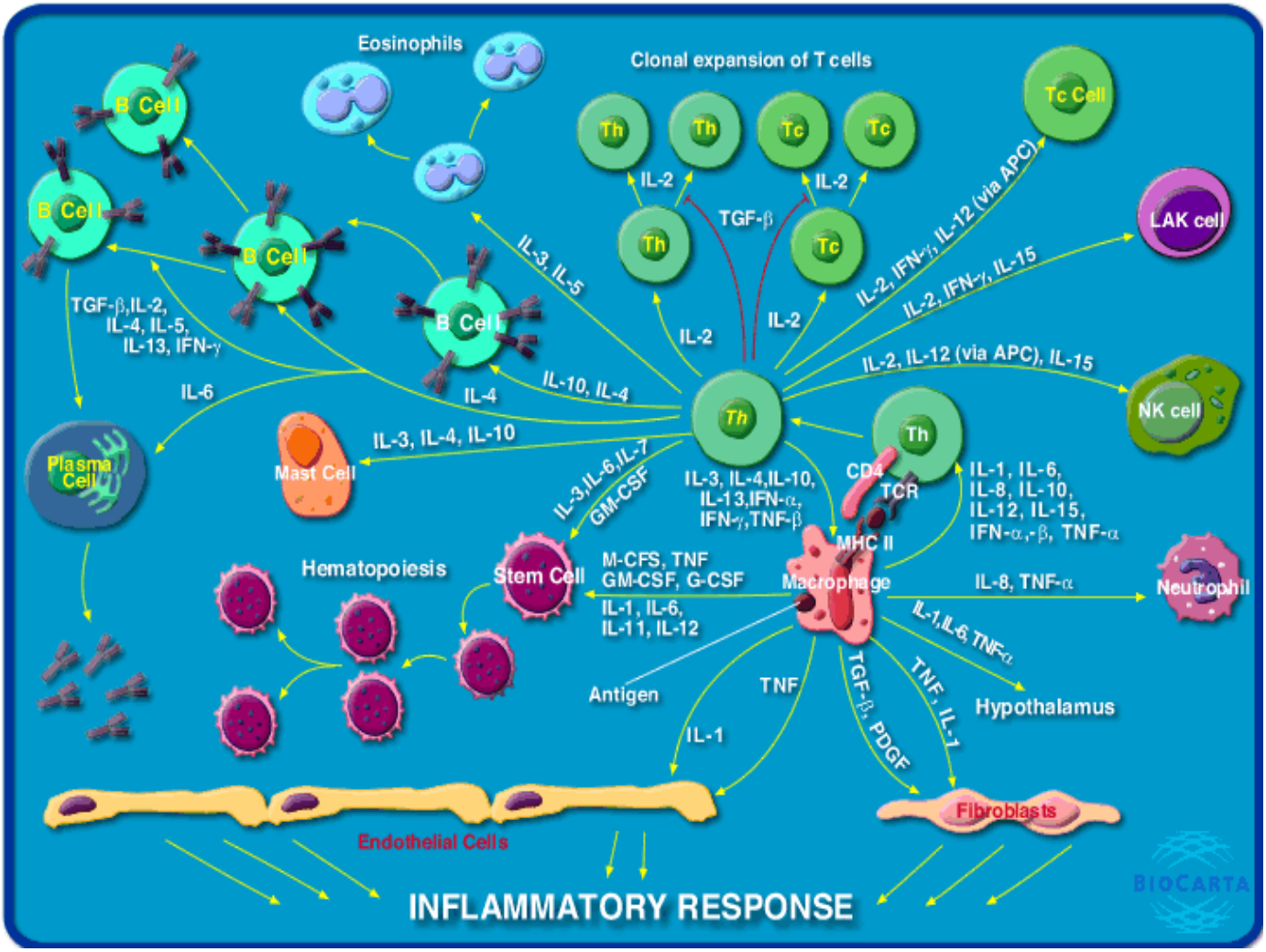


Figure 1.12. Secreted cytokines and Inflammatory response. Some cytokines such as IL-1, IL-6 (see IL-6 pathway) and TNF (see TNFR1 and TNFR2 pathways) act to broadly provoke the inflammatory response while others act on specific types of immune cells. Macrophages and other phagocytic cells provide a front-line defense against bacterial infection. Macrophages stimulate the inflammatory responses of neutrophils, fibroblasts, and endothelial cells in response to infection by secreting IL-1 and TNF. IL-1 and TNF cause fever through alteration of the body temperature set-point in the hypothalamus. Fibroblasts and endothelial cells respond to IL-1 and TNF by recruiting more immune cells to the site of inflammation. Secreted IL-8 is a chemokine that attracts neutrophils to sites of infection. Macrophages also present antigen to T helper cells that play a central role in coordinating immune responses. T helper cells induce clonal expansion of T cells that respond to antigen, with IL-2 as a key mediator of T cell proliferation and activation (see IL-2 pathway). TGF-beta is a negative regulator of proliferation in many cells, have anti-inflammatory actions in some settings (see TGF-beta

pathway). The cytotoxic activity of natural killer cells (NK cells) and lymphokine-activated killer cells (LAK cells) toward virus-infected or tumor cells is stimulated by IL-2 and other cytokines. T helper cells secrete IL-3 and IL-5 to stimulate eosinophil proliferation and activation (see IL-5 pathway). Eosinophils are involved in the immune response to parasitic infection. T helper cells are required to stimulate B cell responses, with the cytokines IL-10, IL-4 and other cytokines regulating the clonal selection and differentiation of antigen-specific B cells to form antibody-secreting plasma B cells and memory cells. In addition to inducing activation and proliferation of specific differentiated immune cells, cytokines act on hematopoeitic stem cells, causing their proliferation and differentiation into the full range of immune cells. Retrieved
(23/08/2010 http://kugi.kribb.re.kr./KUGI/Pathways/BioCarta/h_inflamPathway).

1.4 Hypothesis and Objectives

1.4.1 Hypothesis

The hypothesis to be tested is that the differentiation between infective and non-infective causes of pyrexia in patients with chemotherapy-induced neutropenia can be facilitated by the inclusion of measurements of systemic, host-derived markers of inflammation / immune activation as adjuncts to a clinical scoring index *viz* the Multinational Association of Supportive Care in Cancer (MASCC) clinical scoring system or the Talcott system.

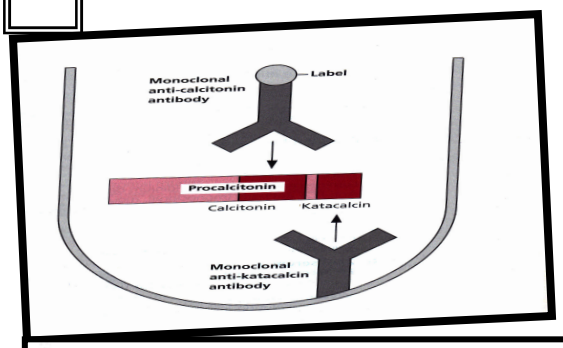
1.4.2 Objectives

- To identify host-derived, systemic markers of inflammation or infection which, either individually or in combination, can be used to distinguish between infective and non-infective causes of pyrexia in cancer patients, as well as to predict outcome in patients with chemotherapy-induced neutropenia.

- To compare the performance of conventional ELISA procedures with the highly sensitive Bio Plex ®Suspension Bead Array System using a limited group of cytokines, namely IL-1β, IL-6 and IL-8.

The major difference between this and the previous study reported by Uys *et al* (2007) was the inclusion of a series of additional biomarkers (sTREM-1, HMGB1, Intereukins- 7 and 12, G-CSF, GM- CSF, INF-g, TNF-a, VEGF and sTNF R1).

1



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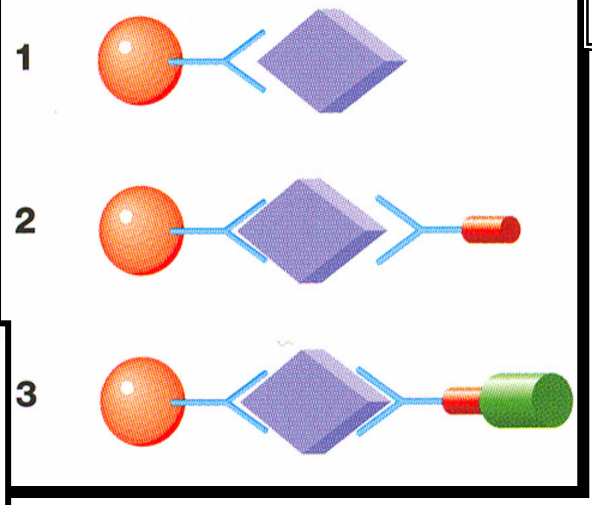
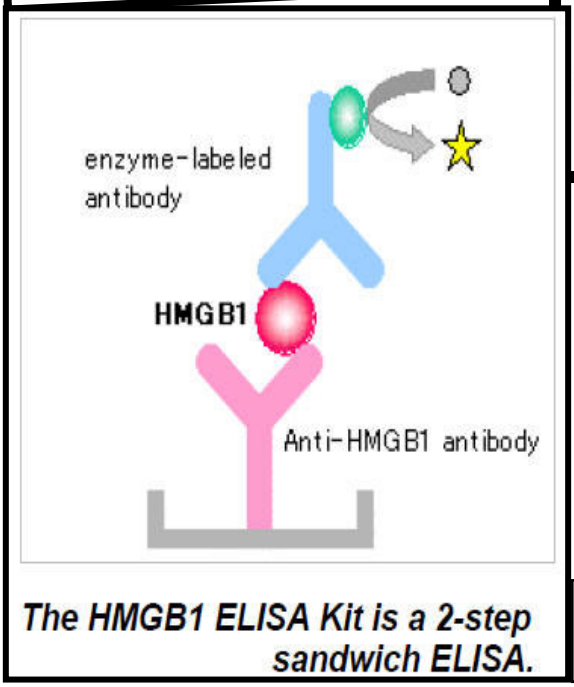




The HMGB1 ELISA Kit is a 2-step sandwich ELISA.

3

1. Summarised scheme of procedure used to detect PCT (Adapted from PCT manual)
2. In a typical Bio-Plex assay, a captured molecule conjugated to a color-coded bead binds to a target analyte (Bio-Plex® System and technology manual.).
3. Summarised scheme of procedure used to detect HMGB1 retrieved 25 August 2010 http://stanford.edu/~drm1987/reoviridae.html

Chapter 2 Study group and Methods

STUDY GROUP AND METHODS

2.1 STUDY DESIGN

As alluded to earlier, the design of the study is retrospective with the primary objective of evaluating the usefulness of measurements of the above-mentioned circulating cytokines / chemokines / growth factors / markers of infection / acute phase reactants as potential adjuncts to a clinical scoring system (MASCC) in the early identification of infection and prediction of outcome in cancer patients with chemotherapy-induced febrile neutropenia.

2.2 STUDY GROUP

2.2.1 Ethical Consent

The study is an extension of the study originally conducted at the Medical Oncology Centre of Rosebank in Johannesburg. The study was approved by the Research Ethics Committee of the Faculty of Health Science, University of Pretoria. The purpose and objective of the study were explained to the patients and written informed consent was obtained from each patient, prior to enrolment into the study. (Protocol: IPA-44,154/2000, Amendment 27/05/2009, approved 8/02/2010).

2.2.2 Study group

The study group consisted of 48 patients with histologically confirmed cancer who presented with chemotherapy-induced febrile neutropenia, either with an oral temperature of > 39℃ in a single measurement, or 38℃ in two consecutive measurements at least four hours apart, together with an absolute neutrophil count of ≤ 0.5 $x10^9$/L as a result of chemotherapy. The control group consisted of 10 healthy individuals. All patients were admitted to hospital on presentation with febrile neutropenia and treated as inpatients receiving empiric antibiotic treatment until resolution of fever or outcome.

2.2.3 Control Group

The control group consisted of healthy adults volunteers. The purpose of the study was explained to the volunteers, and this group consisted of three females and seven males of average age of forty years.

2.2.4 Criteria for Eligibility

Patients were eligible for the study if they had a histologically confirmed diagnosis of cancer with febrile neutropenia as a result of chemotherapy. Chemotherapeutic agents and combinations are detailed in table 2.1.

2.2.5 Classification of febrile episodes

The febrile episodes were classified as microbiologically documented infections (MDI) with or without bacteraemia, clinically- documented infection (CDI), and unexplained fever (FUO) (Hamidah et al., 2008).

2.2.6 Definition of Bacteraemia

Bacteraemia was defined as the presence of live bacterial pathogens in the bloodstream.

2.2.7 Definition of Success or failure of empiric antibiotic therapy

The treatment was regarded as a success if fever and clinical signs of infections resolved, and if blood or infection sites were cleared from isolated pathogens without change in the empiric antibiotic regimen and maintenance of the response for at least seven days after discontinuation of antimicrobial therapy.The treatment was regarded as failure if:

i) The primary infection recurred within one week after discontinuation of the antibiotics therapy
ii) Death from the primary infection

iii) An addition to or modification of the antibiotic therapy, such as anti-fungal treatment (Hamidah et al., 2008).

2.2.8 Collection of data

Each patient underwent evaluation consisting of a physical examination, blood counts, blood cultures, chest X-ray and urine analysis after obtaining detailed history and informed consent.

Data from the original study were collected in a confidential manner using patients' initials and institution file numbers only. Information collected included

1. Demographic data
2. Underlying oncological data
    - Pertinent information regarding chemotherapy
3. Febrile neutropenia data
    - History of previous infections and febrile neutropenic episodes
4. Calculation of MASCC risk-index score (Klastersky et al., 2000)
    - Focus on the prognostic variables and the risk index score
5. Treatment of febrile neutropenia
6. Infection documentation
    - Documented microbial infection
    - Microbiologically-documented infection with bacteraemia
    - Clinically documented infection
    - Fever of unknown origin

7. Outcome
    - Response to empiric therapy (success/ failure)
    - Final outcome : Resolution without complication
      : Resolution with complication
      : Death before resolution

      (Uys et al., 2004).

2.2.9 Collection of samples

All investigations were done on stored serum, which had been frozen at -70°C during the original study. For the present investigation, only stored serum which had never been thawed was used.

Table 2.1. Chemotherapeutic agents received prior to the febrile neutropenic episode

Diagnosis	Chemotherapeutic agents
Solid tumours	
Breast cancer	Doxorubicin/ Cyclophosphamide
	Docetaxel/ Doxorubicin
	Cyclophosphamide/ Doxorubicin/ 5-Fluorouracil
	Cisplatinin/ Trastuzumab ± Paclitaxel
	Docetaxel
	Cisplatinin/Raltitrexed
	Paclitaxel/ Liposomal Doxorubicin
Ovarian Cancer	Docetaxel/ Carboplatin
	Ifosfamide/ Etoposide
	Carboplatin/Liposomal Doxorubicin
	Topotecan
NSCLC	Cisplatinin/ Vinorelbine
	Cisplatinin/ Etoposide
Sarcoma	Ifosfamide/ Doxorubicin
	Cisplatinin/ Doxorubicin
Colon cancer	Irinotecan/ 5-Fluorouracil/ Leecovonrin
CUP	Carboplatin/ Etoposide
	Carboplatin/ Liposomal Doxorubicin
	Ifosfamide/ Etoposide
Anal canal	5-Fluorouracil/ Cisplatinin
	5-Fluorouracil/ Mitomycin C
SCLC	Carboplatin/ Etoposide

Testicular cancer	Bleomycin/ Etoposide/ Cisplatinin
Stomach cancer	Cisplatinin/ 5-Fluorouracil
Cervix cancer	Cisplatinin/ 5-Fluorouracil
Oesophagus	Cisplatinin/ 5-Fluorouracil
Carcinoid tumour	Cisplatinin/ Etoposide
Melanoma	Cisplatinin/ Velbe/ Dacarbazine/ Interleukin-2/ Interferon-α2b
Mesothelioma	Cisplatinin/ Ifosfamide/ Cyclophosphamide
Haematological Malignancies	
NHL	Cyclophosphamide/ Vincristine/ Doxorubicin/ Prednisone ± Rituximab/ Interferon-α2b
	Etoposide/Cisplatinin/ Cytarabine/ Methylprednisilone
	Ifosfamide/ Etoposide
HD	Doxorubicin/ Bleomycin/ Velbe/ Dacarbazine
	Ifosfamide/ Etoposide
Myeloma	Melphalan/ Prednisone
	Cyclophosphamide
AML	Mitoxantrone/ Cytaranine/ Etoposide
	Daunorubicin/ Cytarabine
CLL	Chlorambucil/ Prednisone
	Fludarabine

(Uys et al., 2004)

2.3 Methods

2.3.1 Soluble Triggering Receptor Expressed on Myeloid Cells-1 (sTREM-1)

This assay employs the quantitative sandwich enzyme immunoassay technique, using reagentssupplied by R & D system Europe, Ltd. A monoclonal antibody specific for TREM-1 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any TREM-1 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TREM-1 is added to the wells, following a wash to remove any unbound antibody-enzyme reagent. A substrate solution is then added to the wells and colour develops in proportion to the amount of sTREM-1 bound in the initial step. The colour development is stopped and the intensity of the colour is measured as optical density in a microplate spectrophotometer at a wavelength of 450 nm and the sTREM-1 concentrations are determined from the standard curve using the software laboratory data system, Graphpad Prism. Results are presented as pg/ml serum.

2.3.1.1 Reagent preparation

- All reagents must reach room temperature before use
- Wash Buffer: 20mL of Wash buffer concentrate was diluted into deionised or distilled water to prepare 500mL of wash buffer.

- Substrate Solution: Colour reagents A and B were mixed together in equal volumes within 15 minutes of use.

- sTREM-1 Standard: TREM-1 standard was reconstituted with 1.0 mL of deionised or distilled water. This reconstitution produced a stock solution of 40,000pg/mL. The standard was mixed to ensure complete reconstitution and allowed to stand for a minimum of 15 minutes prior to making dilutions.

- 900µL of Calibrator Diluent RD5-18 was pipetted into the 4000pg/mL tube and 500µL of Calibrator Diluent RD5-18 was pipetted into the rest of the tubes (as shown in Figure 2.1). The stock solution was used to produce known concentrations of sTREM-1 from which the standard curve was constructed (Figure 2.1). Each tube was mixed thoroughly before the next transfer. The 4000pg/mL standard served as the high standard and the calibrator diluent served as the zero standard (0pg/mL).

- The normal value for sTREM-1 for this study was < 90pg/ml.

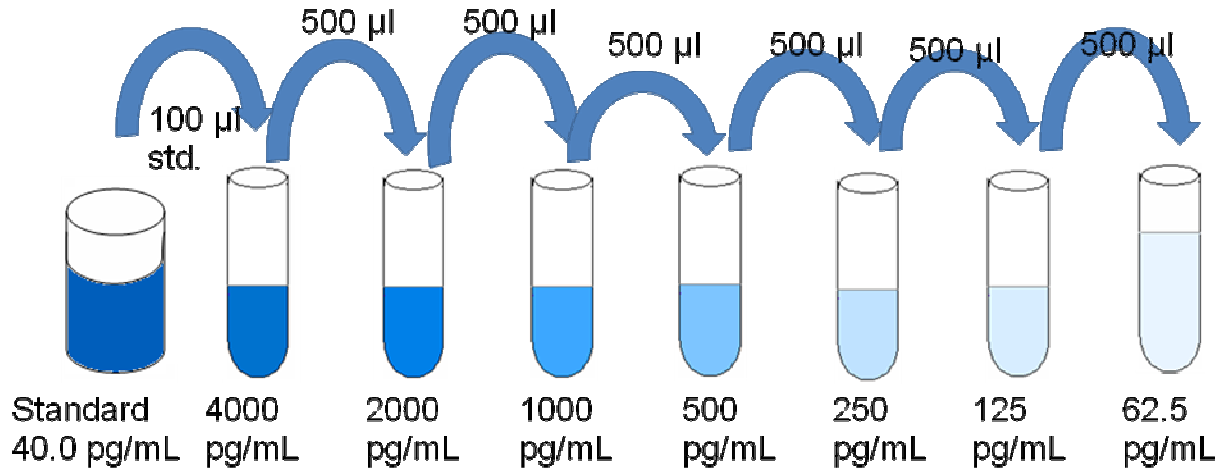


Figure 2.1. Stock dilution to produce dilution series from which the standard curve was constructed (sTREM package insert).

Table 2.2. Summarised test procedure for measurement of sTREM-1 in serum

procedure	Volume/ Well	Time
Pipette 50µL of Assay Diluent RD90 to each well	50µL	
Add 50µL of standard, control or sample per well. Cover with adhesive strip and incubate at room temperature	50µL	2 hours
Aspirate each well and wash, repeating the process with wash buffer	4 x 400µL	
Add 200µl of TREM-1 Conjugate to each well. Cover with a new strip and incubate at room temperature	200µL	2 hours
Repeat the aspiration/ wash as in step 3.		
Add 200µL of Substrate solution to each well incubate at room temperature. **Protect from light**	200µL	30 minutes
7. Add 50µL of Stop solution, the colour should change	50µL	

The optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm.

2.3.2 Procalcitonin (PCT)

PCT was assayed by an immunoluminescence procedure, using the B.R.A.H.M.S chemiluminometer and reagents provided by B.R.A.H.M.S Aktiengesellschaft, (Henningsdorf, Germany).

Capture and detection of PCT is based on the application of two PCT-specific monoclonal antibodies, which bind to PCT at two different sites. The first of these antibodies, which binds to epitopes on the katacalcin region, is coated to PCT assay tubes and is used as a capture antibody. The second antibody binds to the calcitonin region of the PCT molecule. This antibody is labelled with a chemiluminescent substrate (acridine derivate) and is used in the detection and quantitation of bound PCT. Chemiluminescence is activated by addition of H_2O_2 (0.5%). The resultant chemiluminescence which originates from H_2O_2-mediated oxidation of the luminescent substrate is detected using a chemiluminometer, the magnitude of chemiluminescence being directly proportional to the amount of bound (captured) PCT in the assay tubes. The laboratory determination of PCT is summarised diagrammatically in Figure 2.2.

The normal value for circulating PCT is <0.5ng/ml.

Test procedure is explained in Table2.3.

Table 2.3. Summarised test procedure for measurement of PCT in serum

procedure	Volume/ Well	Time
Pipette 20µL of PCT standards or serum sample into test tubes	20µL	
Add 250µL acridine-labelled second monoclonal antibody into test tube and mix	250µL	10 min
Cover test tubes with adhesive foil and incubate on the rotator at 250 rpm (protected from light) at room temperature		1 hour
Invert tubes and drain on clean blotting paper	1mL	10 min
Place tube in luminometer and start measurement with automatic injection of 300µL LUMItest Basiskit	300µL	1 sec

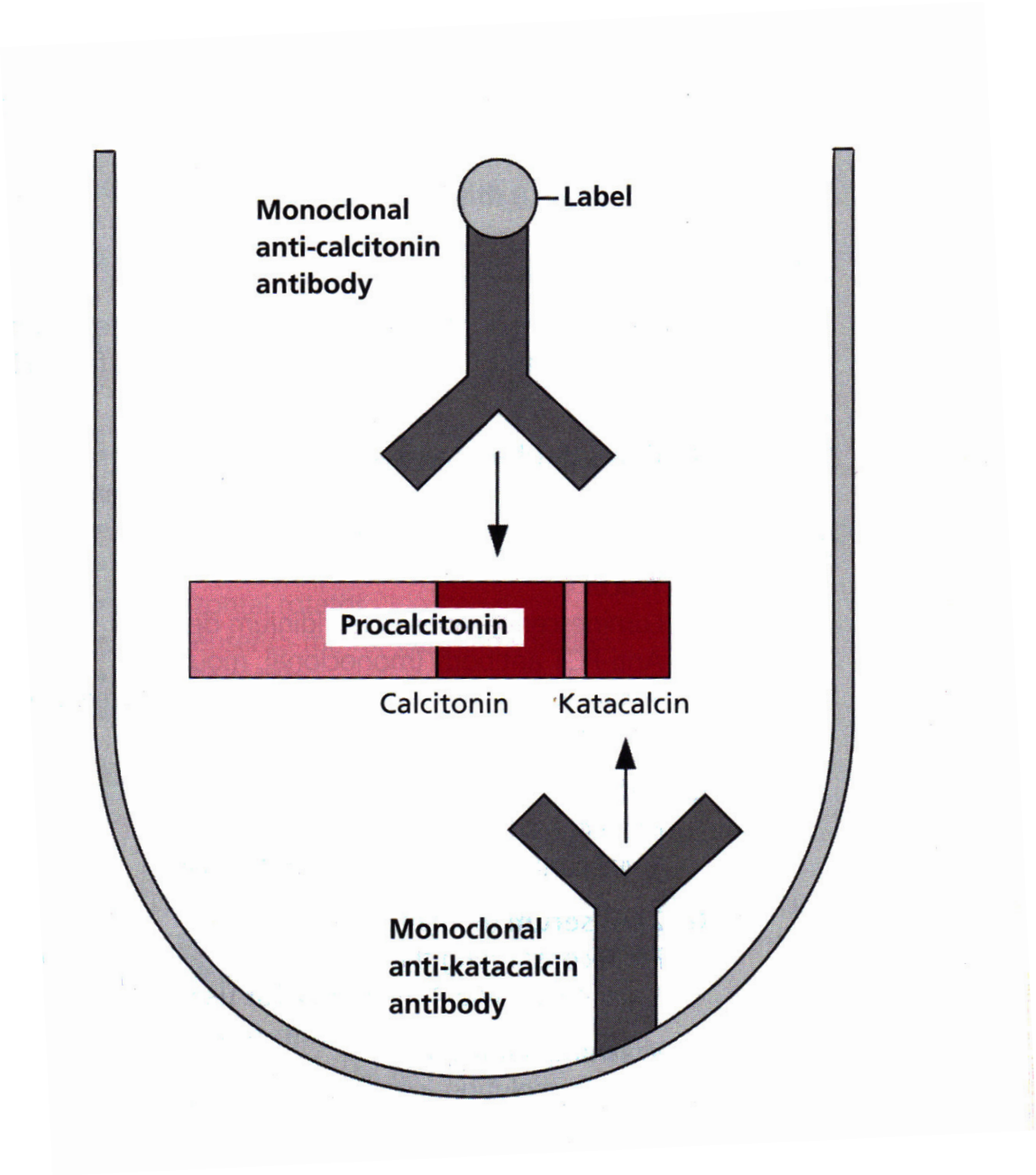


Figure 2.2. Summarised scheme of procedure used to detect PCT (Adapted from PCT manual).

2.3.3 Acute-phase reactants

These proteins were not reanalysed in the present study and the results from the previous study were used in which serum levels of C-reactive protein (CRP) and serum amyloid A (SAA) were analysed using the Dade Behring BN II nephelometer, validated by N/T Rheumatology Controls SL/2&2 and the N Latex SAA kit and SAA controls (Marburg Gmbh, Germany).

2.3.3.1 Assay procedure for C-Reactive protein (CRP)

Plasma levels of CRP were analysed using the Dade Behring BN II nephelometer, validated by N/T Rheumatology Controls SL1 and 2 (Marburg GmbH, Germany). The polystyrene particles coated with monoclonal antibodies to CRP are agglutinated when mixed with sample containing CRP. The intensity of the light in the nephelometer depends on the CRP content of the sample and therefore the CRP concentration can be determined versus dilutions of a standard of a known concentration. The normal value for circulating CRP is ≤ 5µg/ml (CRP package insert).

2.3.3.2 Assay procedure for Serum Amyloid A (SAA)

Plasma levels of SAA were analysed using the Dade Behring BN II nephelometer (Marburg GmbH, Germany). The N Latex SAA kit and SAA controls were used. The polystyrene particles coated with antibodies to human SAA are aggregated when mixed with samples containing SAA. These aggregates scatter a beam of light passed through the mixture. The intensity of the scattered light is proportional to the concentration of the relevant protein in the sample. The result is evaluated by comparison with a standard of known concentration. The normal value for circulating SAA is ≤ 6.8 µg/ml (Dade Behring N Latex SAA package insert).

2.3.4 Cytokines

Cytokines namely (Interleukins- 1β,-6,-7, -8 and -12, G-CSF, GMCSF, INF-g, TNF-a, and VEGF were measured using the Bio-Plex suspension array system. Interleukins -1β, -6 and -8 were measured in the previous study. This system utilizes the Luminex^{R}xMAP^{R} Multiplex technology (This technology is explained in detail in Chapter 3.3.1.2).

2.3.4.1 Sample preparation.

- Prepare the thawed serum samples for analysis by diluting 1 volume of the human serum with 3 volumes of Bio-Plex human serum sample diluent.
- Reconstitute and dilute the cytokine standards in the appropriate Bio-Plex species-specific standard diluent and incubate on ice for 30 minutes.

2.3.4.2 Assay Procedure

- Prepare the sample and cytokine standard dilutions according to the package insert.
- Turn on the Bio-Plex system at least 30 minutes prior to reading a plate.
- Prepare multiplex beads working solution 25x beads. Protect baeds from light. Keep all tubes on ice until ready for use.
- Vortex the anti-cytokine conjugates beads (25x) and prepare the conjugate beads.
- Pre-wet a 96-well filter plate with 100µl of Bio-Plex assay buffer. Place the pre-wetted filter plate on a calibrated filter plate vacuum manifold. Remove the buffer by vacuum filtration. Dry the bottom of the filter plate thoroughly with a clean paper towel.
- Vortex the multiplex bead working solution for 15-20 seconds and pipette 50 µl into each well. Remove the buffer by vacuum filtration.
- Dispense 100 µl of Bio-Plex wash buffer into each well. Remove the buffer by vacuum filtration. Repeat this step. Blot the bottom of the filter plate once

with paper towel to prevent cross-contamination. Place the filter plate on the plastic plate holder included with the kit.

- Gently flick the bottom of each diluted standard and sample tube 3-5 times. Pipette 50 µl of diluted standard or sample per well. Cover the plate with the plate sealing tape provided. Place the filter plate onto a microplate shaker and cover with aluminium foil and shake for 30 sec, then incubate at room temperature for 60 minutes at 250 rpm.
- At the end of the first incubation, place the plate on a flat surface and remove the sealing- tape.
- Wash 3 times with 100µl of Bio-Plex wash buffer. Remove the buffer by vacuum filtration after every wash and blot the bottom of the plate with a clean paper towel and place the plate on the plastic holder.
- Prepare detection antibody solution.
- Vortex the Bio-Plex detection antibody working solution gently and add 25 µl to each well. Cover the plate with a new sheet of sealing tape. Place the filter plate and plastic plate holder onto a microplate shaker and cover with aluminium foil and shake for 30 sec, then incubated at room temperature for 30 minutes.
- Wash 3 times with 100 µl of Bio-Plex wash buffer. Remove the buffer by vacuum filtration after every wash and blot the bottom of the plate with a clean paper towel and place the plate on the plastic holder.
- Prepare streptavidin- phycoerythrin (PE).
- Vortex the 1x streptavidin-PE vigorously and add 50 µl to each well, cover the plate with a new sealing tape and foil and incubate on a shaker for 10 minutes at room temperature.
- Wash 3 times with 100 µl of Bio-Plex wash buffer. Remove the buffer by vacuum filtration after every wash and blot the bottom of the plate with a clean paper towel and place the plate on the plastic holder.
- Resuspend the beads in each well with 125 µl of Bio-Plex assay buffer, mix tray for 30 seconds.
- Then read plate on the Bio-Plex system (summary of the method in figure 2.3).
- The normal value for circulating cytokines is <10pg/ml.

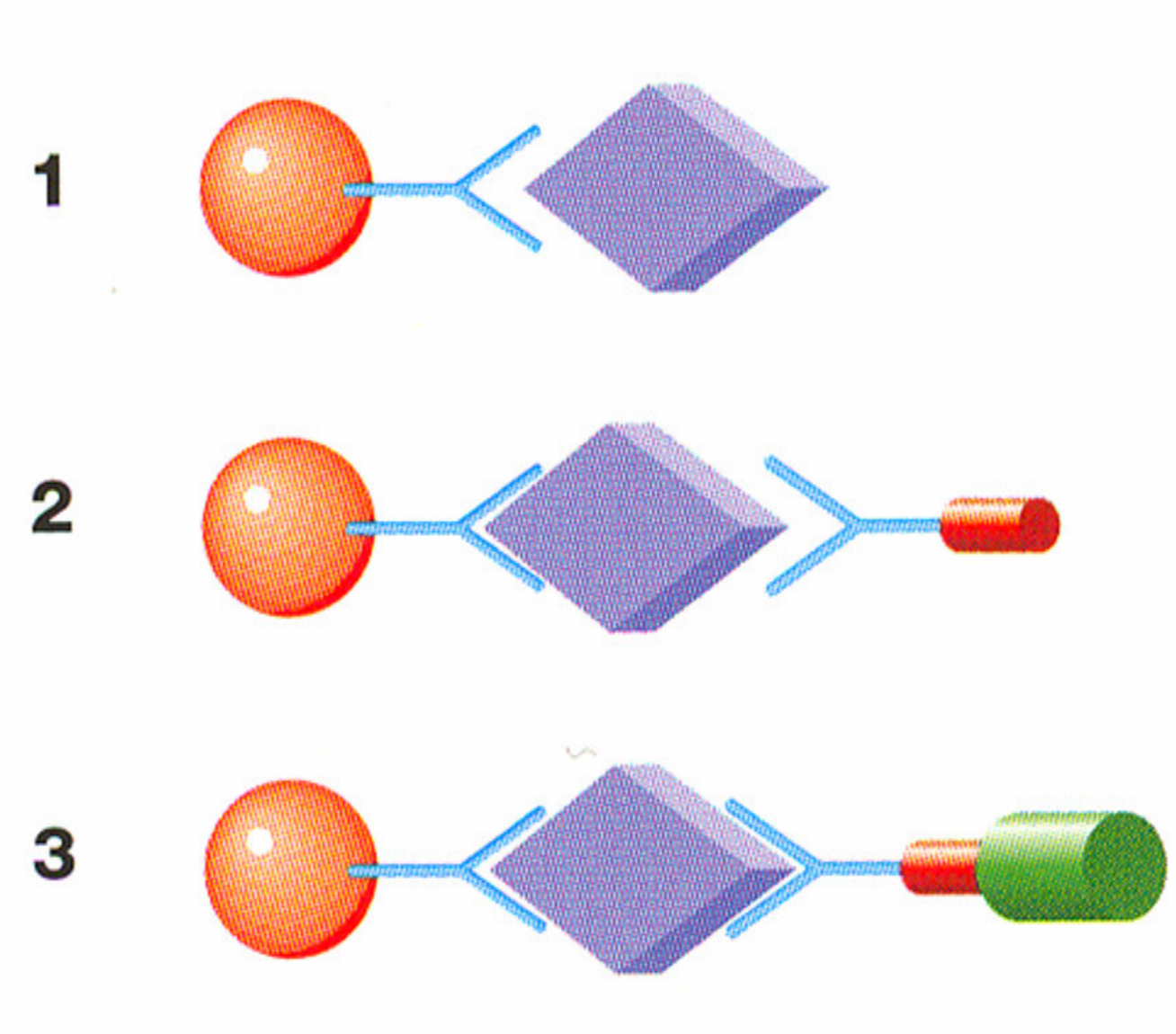


Fig. 2.3 In a typical Bio-Plex assay, a captured molecule conjugated to a color-coded bead binds to a target analyte (1) followed by binding with biotinylated detection antibody (2) and a reporter molecule, streptavidin- PE (3) (Bio-Plex® System and technology manual).

2.3.5 Soluble tumor necrosis factor receptor 1 (sTNF R1)

This sTNF R1 enzyme linked immunosorbent assay (ELISA) applies a technique called quantitative sandwich immunoassay, utilises kit supplied by Roche diagnostics GmbH Mannheim. Germany. A monoclonal antibody specific for sTNF R1 has been pre-coated onto a microplate. Standards and samples are then added to the appropriate microtiter plate wells and any sTNF R1 present is bound by the immobilized antibody. The microplate wells are thoroughly washed to remove any unbound substances, an enzyme-linked polyclonal antibody specific for sTNF R1 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of sTNF R1 bound in the initial step. The enzyme-substrate reaction is terminated by the addition of a stop solution and the color change is measured spectrophotometrically at a wavelength of 450 nm with the results expressed as pg/mL.

2.3.5.1 Assay procedure summary

- Prepare all reagents, working standards, and samples before starting the assay.
- Add 50 µL of assay diluent HD1-7 to each well.
- Add 200 µL of standard, control, or sample to the appropriate wells. Cover with the adhesive strip, mix well and incubate for two hours at room temperature (RT).
- Aspirate each well and wash, repeating the process twice for a total of three washes. Wash by filling each well with wash buffer (400 µL). After the last wash, remove any remaining wash buffer by inverting the plate and blot it agaist clean paper towels.
- Add 200 µL of sTNF R1 conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at RT.
- Repeat the aspiration/wash step.
- Add 200 µL of sTNF R1 substrate solution to each well. Incubate for 20 minutes at RT. Protect from light.

- Add 50 µL of stop solution to each well.
- Read the optical density (O.D) of each well at 450 nm using spectrophotometer within 30 minutes.

2.3.6 High-Mobility Group Box 1 Protein

This assay employs a two-step sandwich enzyme-linked immunosorbent assay (ELISA), using kit from Oonodai, Sagamihara-shi, Kanagawa, Japan. The wells of the microtiter strips are coated with a polyclonal antibody specific for HMGB1. Diluted samples, standards and controls are added to the wells where HMGB1 in the samples will bind to the immobilized antibody. Unbound serum is washed off and a peroxidase-conjugated anti-HMGB1 monoclonal antibody is added which will bind to a different epitope on HMGB1. After another wash step, substrate is added and broken down by the peroxidase, resulting in a color the intensity of which is proportional to the HMGB1 concentration. Stop solution (sulfuric acid) stops the enzymatic reaction and the intensity of the colour is measured as optical density in a spectrophotometer at 450 nm, with the results being expressed as ng/ml. The HMGB1 concentrations are determined from the standard curve using the software laboratory data system, Graphpad Prism.

2.3.6.1 Assay Procedure

- Add 100µL of sample diluent to each well.
- Add 10 µL of sample diluent to zero well.
- Add 10 µL of standard, control and samples to the appropriate wells.
- After shaking the plate with a plate mixer, cover all wells tightly using plate seal and incubate for 20-24 hours at 37℃.
- Wash the wells 5 times with wash solution (400 µL/well) . After the final wash, turn over the plate and gently tap 4/5 times on a lint-free paper towel to remove any remaining wash buffer.
- Add 100 µL of POD-conjugate solution to each well.
- Cover all wells tightly with plate seal and incubate for 2 hours at 25℃.

- Wash the wells 5 times. After the final wash, turn over the plate and gently tap to remove any remaining buffer.
- Clean the back of the well. Be careful not to scratch the wells as this may interfere with the measurements.
- Add 100 µL of substrate solution to each well. Incubate for 30 minutes at room temperature.
- Add 100 µL of stop solution to each well in the same sequence and at the same time intervals to the addition of substrate solution.
- Read the absorbance of each well, using the spectrophotometer at 450 nm.

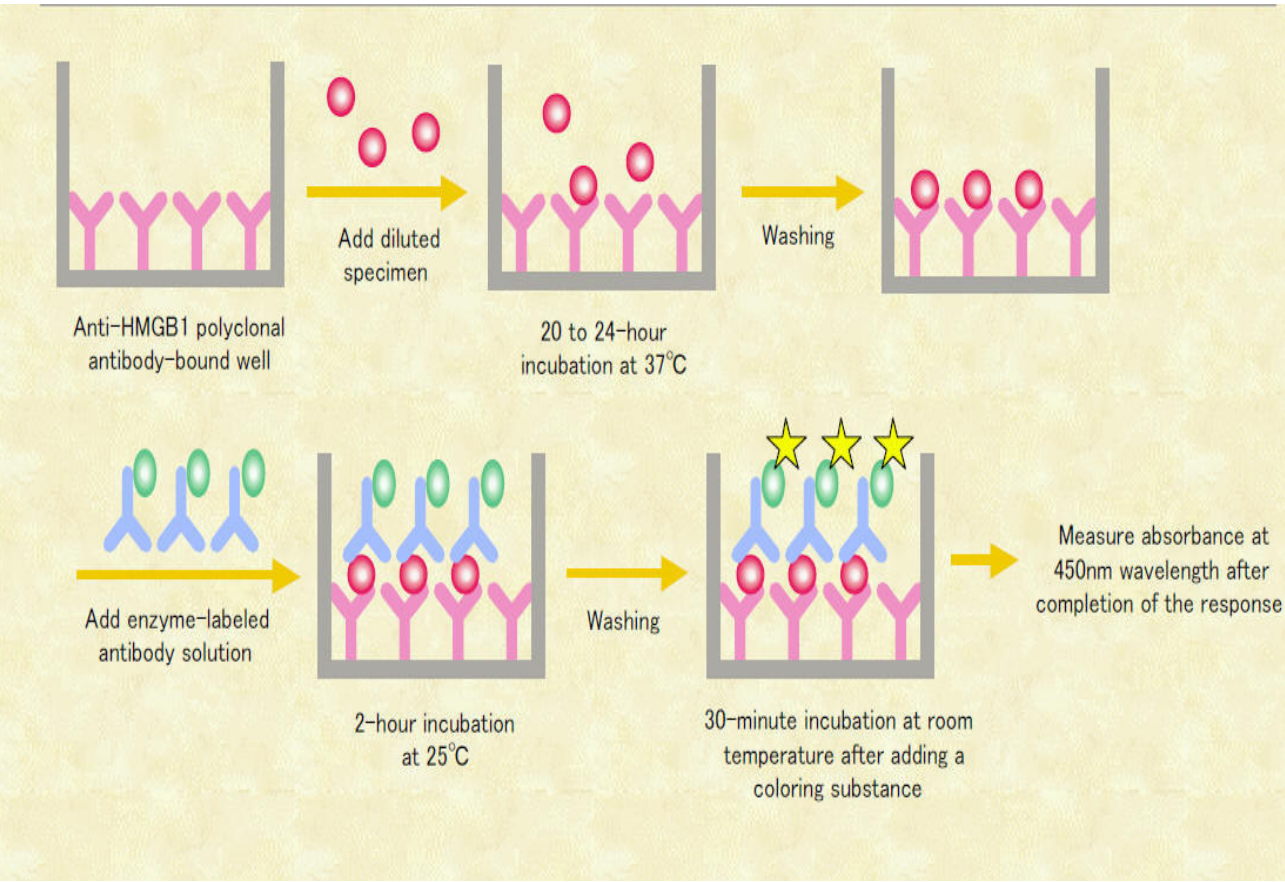
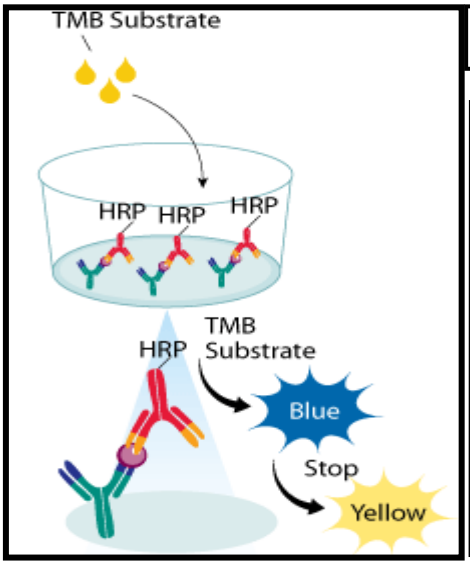


Figure 2.4. Summarised scheme of procedure used to detect HMGB1 (HMGB1 Elisa kit II package insert).



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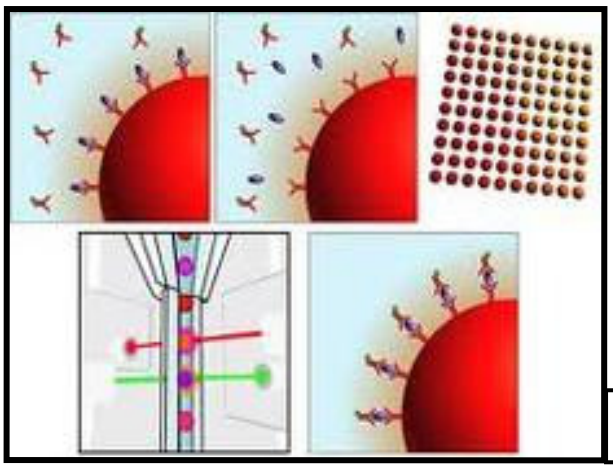


2

1. General summarised scheme of ELISA principle retrieved 24 August 2010
http://www.rndsystems.com/product_detail_objectname_QuantikineAssayPrinciple.aspx
2. Summarised procedure of Bio-Plex principle retrieved 25 August 2010
http://www.upci.upmc.edu/luminex.about.cfm

Chapter 3

Comparison of the Bio-Plex® and Roche Diagnostics ELISA systems for the dectection and quantitation of the concentration of IL-1β, IL-6 and IL-8 in serum specimens from patients with febrile neutropenia.

3.1 Introduction

Cytokines, as mentioned in the previous chapter, are small soluble proteins that are immune mediators and together constitute the communication network of the immune system, and play important roles in diverse physiological functions, including inflammation, cell differentiation, promoting chemotaxis, and generating an acute phase response (Ray et al., 2005). Each cytokine has a variety of functions that may be additive, similar to, or inhibitory to the activities of other cytokines. Elevated levels of cytokines may indicate inflammation or disease progression. Although cytokine functions are complex, cytokine profiles are highly relevant to the parameters of an immune response. Cytokine measurement and ratios of different cytokines in a biologic system have become critical indicators of normal and disease states. Therefore the accurate measurement of biomarkers is essential for the diagnosis, monitoring, progression and evaluation of disease (Kellar & Douglass, 2003; Ray et al., 2005).

A number of methodologies can be used to measure cytokines. The most common one is the enzyme-linked immunosorbent assay (ELISA), which allows measurement of different cytokines with high sensitivity and specificity. However, this well-developed methodology requires significant sample volumes for each analyte, is labour intensive, and is restricted to analysing one cytokine target at a time, which limits its usefulness when simultaneous measurement of multiple cytokine targets is required. Recently, several multiplex protein analysis technologies have been developed including slide/microarray-based assays (Wiese et al., 2001; Pickering et al., 2002) and flow cytometry-based assays (Oliver et al., 1998; Cook et al., 2001; Pickering et al., 2002). The suspension bead array technology employed in the Bio-Plex system (Luminex xMAP technology) allows multiple cytokines to be measured simultaneously in a single small volume of sample in a microplate well (Elshal & McCoy, 2006).

3.2 Objective

The aim of this study was to compare the performance of a conventional ELISA procedure with the highly sensitive Bio Plex ®Suspension Bead Array System using a limited group of cytokines, namely IL-1β, IL-6 and IL-8.

3.3 Patients

The group of patients is described in chapter 2.2.

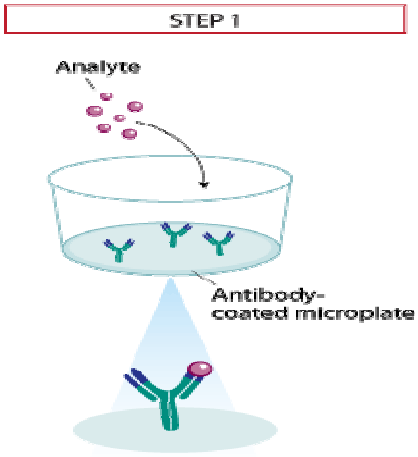
3.4 Methods

3.4.1 Analyses of inflammatory cytokines: IL-1β, IL-6 and IL-8

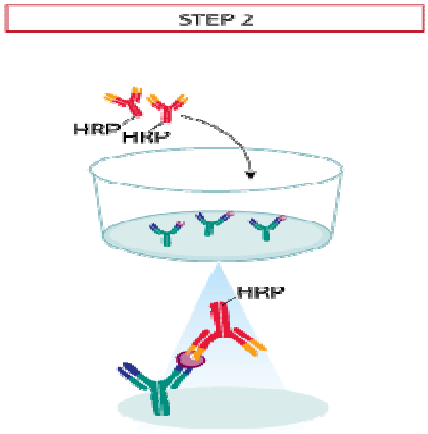
3.4.1.1 Enzyme linked immunosorbent assay (ELISA)

In the previous study, serum levels of all cytokines were measured by ELISA-assay. The method of choice was the cormmercial test kits “human- Interleukin ELISA” for the cytokines IL-1β, IL-6 and IL-8 from Roche Diagnostics GmbH, Mannheim, Germany, IL-10 was done on the previous study (Uys et al.,2007), but IL-10 was not included the current study. The assay is based on the quantitative “sandwich enzyme-immuno-assay” principle, using two murine monoclonal antibodies, directed against two different epitopes of the specific cytokine, illustrated in Figure 3.1.

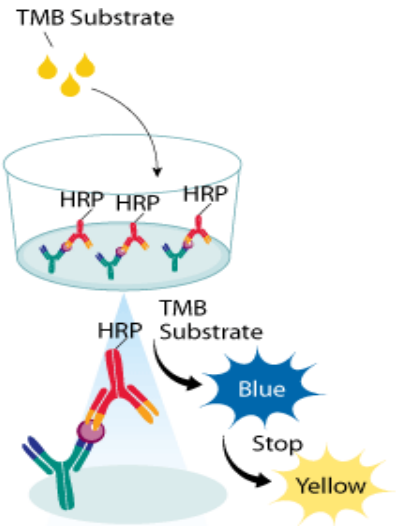
For all cytokines, the principle of the ELISA assay was identical; Table 3.1 shows the summary of the working procedure.



microplate pre-coated with capture antibody is provided. Samples or standards are added and any analyte present is bound by the immobilized antibody. Unbound materials are washed away



A second HRP-labeled antibody (detection antibody) is added and binds to the captured analyte. Unbound detection antibody is washed away.



Tetramethylbenzidine (TMB) substrate solution is added to the wells and a blue color develops in proportion to the amount of analyte present in the sample. Color development is stopped turning the color in the wells to yellow. The absorbance of the color at 450 nm is measured.

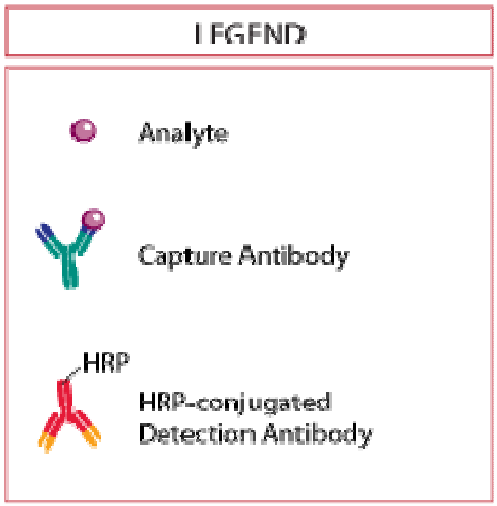


Figure 3.1. General Summarised scheme of ELISA principle: retrieved 24/08/2010 (http://www.rndsystems.com/product_detail_objectname_QuantikineAssayPrinciple.aspx).

Table 3.1: Summarised test procedure for measurement of IL-1β, IL-6 and IL-8 by ELISA.

Procedure	Volume / Well	Time
1. Pipette 20 µl of 6 standards or serum sample into duplicate wells containing captured antibody	20 µl	
2. Add 200 µl immunoreagent. Cover with adhesive foil and incubate on a shaker at 250 rpm at room temperature	200 µl	2 hours
3. Remove solution by tapping and was four times with washing buffer	4 x 300 µl	3 x 1 min
4. Add 200µl of TMB substrate solution. cover with foil and incubate in the dark on a shaker at 250 rpm at room temperature	200µl	10-30 min
5. Add 50 µl stop solution	50 µl	1 min
6. Measure absorption at 450 nm		

The absorbance values of all standards were corrected by subtracting the value of the reagent blank. The mean absorbance values for samples and standards were calculated. Sample concentrations were determined from the standard curves (Uys et al., 2007).

3.4.1.2 Bio-Plex (Luminex) system, a multiplex suspension array texhnique

The Bio-Plex array system is based on the xMAP technology developed and owned by Luminex Corp., which permits multiplexing of up to 100 different analytes within a single sample. The system uses a liquid suspension array of 100 sets of 5.5 µm polystyrene beads. The beads are filled with different ratios of two different fluorescent dyes, resulting in an array of 100 distinct spectral addresses (Figure 3.2 point number 1) (Kettman et al., 1998; Earley et al., 2002). Each set of beads can be conjugated with a different captured molecule; the conjugated beads can be mixed and incubated with the sample in a microplate well to react with specific analytes. Captured molecules can include:

- Enzyme subtrates
- DNA
- Receptors
- Antigens
- Antibodies

(Oliver et al., 1998; Etienne et al., 2000; Martins, 2002).

The principle is based on the sandwich immunoassay technique (explained indetail chapter 2.3.4). Following incubation, the contents of each microplate well are drawn into the Bio-Plex array reader, and precision fluidics align the beads in a single file through a flow cell where two lasers excite the beads individually (see Figure 3.2 point number 6). The red classification laser excites the dyes in each bead, identifying the specific bead address. The green reporter laser excites the reporter molecule associated with the bead, allowing quantitation of the captured analyte. High-speed digital signal processors and Bio-Plex Manager software record the fluorescent signals simultaneously for each bead, translating the signal into data for each bead-based assay (Carson & Vignali, 1999). The cytokine analysis kits were obtained from Bio-Rad Laboratories, Inc.

The sample preparation and assay procedure are explained in chapter 2.2.4 **(**from page 58).

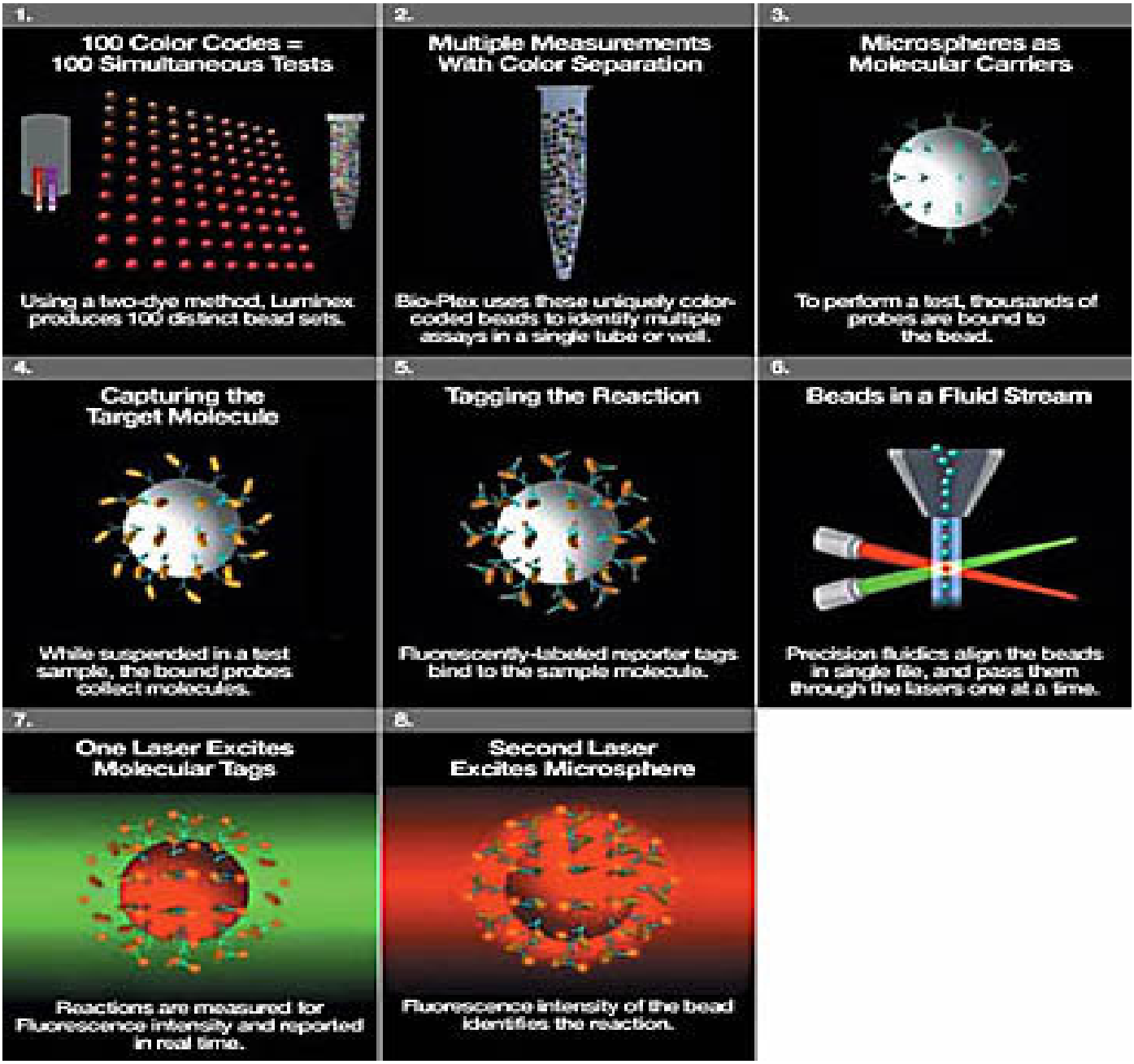


Figure 3.2 Summarised procedure of Bio-Plex principle (Retrieved 24/08/2010 http://www.cvrc.dkf.unibe.ch/content/technical_equipment/).

3.5 Results

Serum concentrations of IL-1β, IL-6, and IL-8 measured by the Bioplex® and Roche ELISA system

In the case of all three cytokines, especially IL-6 and IL-8, significantly high values were detected with the Bio-Plex® system, and correlated well using both methods. The individual values and the mean values for the entire group of 48 patients with febrile neutropenia is shown in Table 3.2 and in Figure 3.3 respectively, while the correlations between these are shown in Table 3.3.

The concentration of IL-1β was low using both systems, with a weak-to-moderate correlation between the two groups. Correlations of IL-6 and IL-8 on the other hand were much higher with the two detection system correlating moderatatly (IL-6) or stronger (IL-8) for the two systems. Importantly, the concentration of IL-6 and IL-8 were significantly higher ($p<0.0001$) when using the Bio-Plex system, demonstrating the high sensitivity of this system.

Table 3.2 The actual serum concentration values of the IL-6, IL-8 and IL-1β measured by Bio-Plex® and Roche ELISA system.

ELISA IL-6	Bio-Plex IL-6	ELISA IL-1β	Bio-Plex IL-1β	ELISA IL-8	Bio-Plex IL-8
158	841.45	7.8	11.98	269	406.25
542	2223.14	0.3	20.58	627	1476.1
18	138.58	5.9	6.88	37	48.16
43	234.43	3.6	7.81	55	89.7
51	202.26	0.1	5.47	30	27.67
232	544.57	0.3	12.42	809	1038.08
7	59.63	0	6.88	27	36.07
57	181.52	0.5	7.81	69	100.34
29	158.63	9.2	32.25	102	252.93
701	41059.3	11.5	320.72	809	80736.11
111	583.21	1.9	15.15	667	1087.58
47	39.44	0	9.2	55	55.68
230	760.98	7	34.49	536	1398.34
63	211.56	2	9.2	154	216.15
16	74.02	0.6	8.27	200	320.04
7	467.34	0	10.12	77	125.88
706	1256.21	0	37.39	173	1401.67

702	634.05	1.9	28.67	809	2442.03	
8	26.43	0	5.94	42	355.29	
38	109.92	0	8.97	512	707.91	
194	82.64	507.4	6.88	809	832.9	
59	228.71	2.7	11.04	809	3508.9	
67	191.54	0	6.41	80	151.62	
335	98.44	5.8	12.87	809	294.39	
74	239.43	0	9.66	215	215.15	
11	162.92	5.9	13.79	52	82.7	
21	128.55	0.9	7.81	164	331.35	
31	129.99	0	6.41	304	601.93	
706	114338.1	41.6	541.23	468	108933.4	
114	441.61	0.3	14.24	205	608.33	
36	112.78	0.3	4.99	56	23.94	
11	13.36	0	6.88	117	2.17	
14	67.55	0	7.34	64	73.17	
304	42342.45	23.1	360.56	809	27231.46	
47	207.27	0.6	7.81	82	188.49	
13	45.22	4.9	14.24	155	154.63	
349	317.3	0.5	14.24	809	647.23	
26	121.39	0	9.2	134	185.29	
43	112.07	3.2	4.99	466	106.65	
456	2831.55	4.2	25.53	429	1686.52	
153	8.98	0.3	9.66	158	3.8	
130	80.49	0.5	6.88	809	135.75	
543	1286.73	0	17.42	809	2106.24	
86	304.44	0.3	9.2	258	1201.26	
1.8	428.74	1	42.53	676	995.96	
3	63.95	24.6	12.42	23	139.17	
688	1187.99	0.3	17.42	809	1804.48	
26	107.05	5.9	8.97	77	418	
173.0792	4489.331	14.31042	37.72542	348.2083	5103.894	mean

Table 3.3 Correlation between Bio-Plex and ELISA results.

Cytokine	Spearman Rank Correlation (r)	# of assays
IL-1β	0.3758 P 0.0085	48
IL-6	0.6651 P < 0.001	48
IL-8	0.7582 P <0.0001	48

The correlation coefficient is the upper value and the corresponding P values underneath.

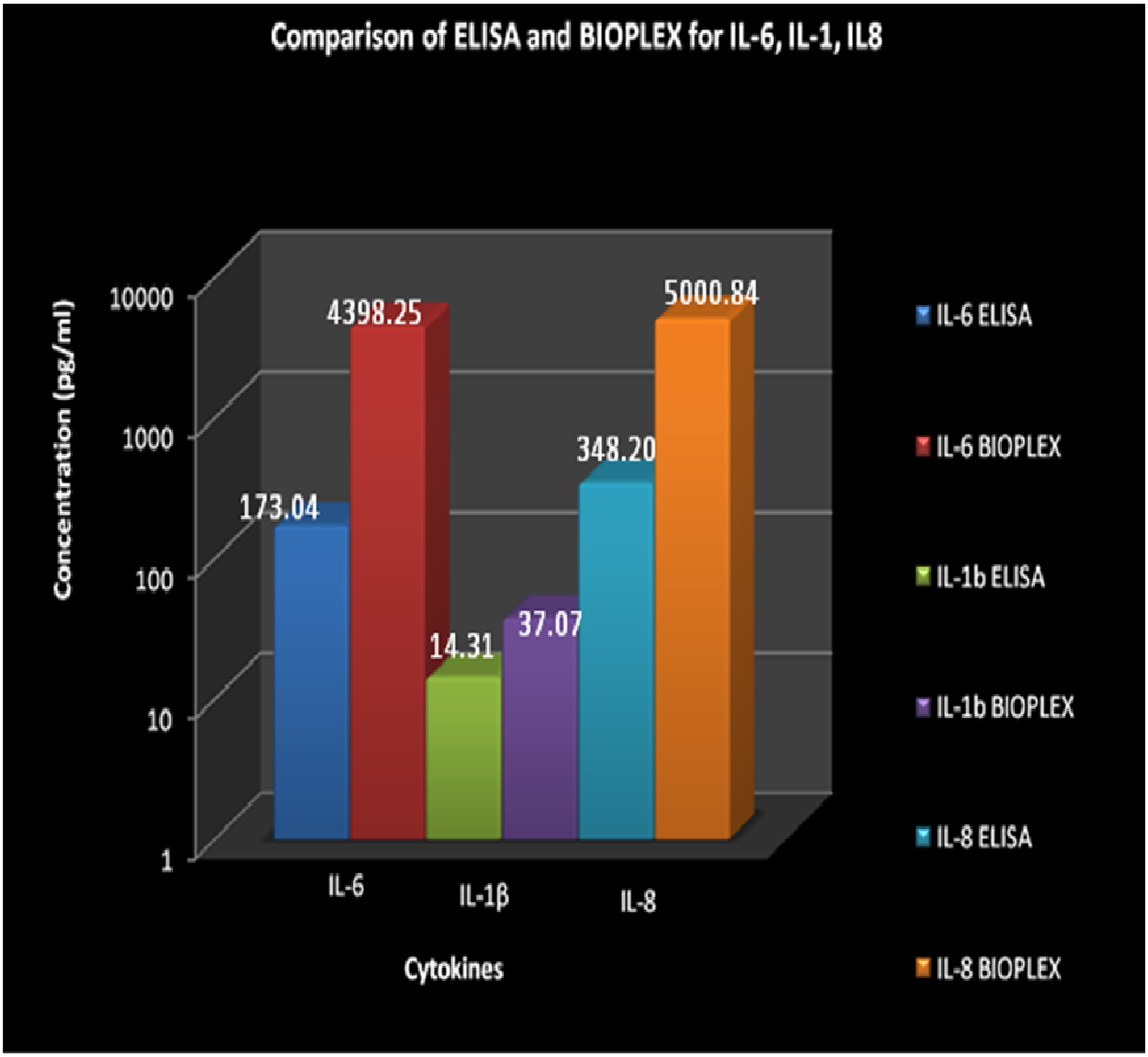


Figure 3.3 Median values for the measurements of IL-6, IL-1β, and IL-8

3.6 Discussion

The degree of agreement between the Bio-Plex and ELISA cytokine detection procedures was evaluated by measuring the correlation between three cytokine targets (IL-6, IL1β and IL-8; see Table 3.3) in 48 samples from patients with febrile neutropenia. The concentrations of the majority of the cytokines in the samples measured by ELISA compared well with those concentrations obtained fro Bio-Plex assay (see Figure 3.3). Spearman rank coefficient (r) ranged from 0.3758 to 0.7582, with significant. Excellent correlations were observed between ELISA and Luminex

determination of cytokine levels in serum for IL-6 and IL-8. In the case of IL-1β, the degree of correlation falls within the generally acceptable range which is significant, but the weak concentration may reflect the low concentrations of IL-1β.

The majority of published studies have shown good correlation between multiplex bead array assays (MBAA) and ELISA for most but not all, of the cytokines tested, but the degree of correlation has varied widely. These variations are likely the result of how these comparisons were made as well as the antibodies used in each of the assays. For example Oliver *et al*, 1997 compared MBAA to ELISA but did not state the manufacturer of the ELISA kit, or the specific antibody pair used in ELISA, a correlation coefficient of 0.912 was reported, but it was not stated if this was a Pearson's or Spearman's value.

A recent study by (Khan et al., 2004) used kits from different vendors (LINCO Research, Bio-Rad Laboratories, R&D Systems, and Bio Source International) measured the levels of IFN-gamma, IL-1β, IL-6, IL-8, and tumor necrosis factor-alpha, and compared the results with those from ELISA techniques. In this study, MBAA kits from different suppliers yielded different concentrations of cytokines, although the cytokine levels followed similar qualitative patterns. Values for IL-8 were similar in ELISA and Luminex using kits from the same manufacturer (R&D Systems).

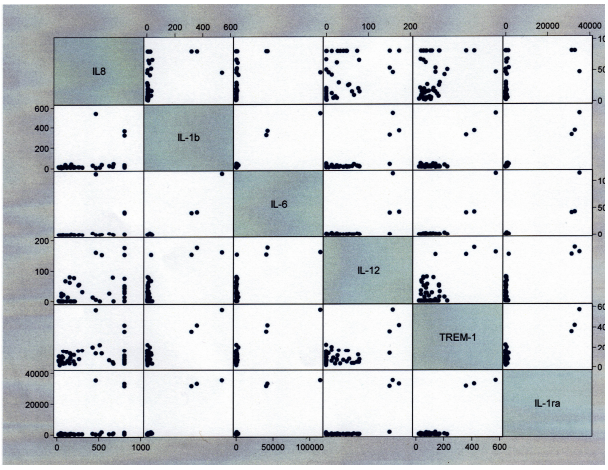
(dupont et al., 2005) examined the correlation of ELISA and MBAA techniques for quantitating a variety of cytokines in culture supernatants using kits from *viz* Linco Research Inc. and Upstate Cell Signaling Solution. They showed excellent correlation between ELISA and Luminex for seven out of nine cytokines namely (IL-1β, IL-4, IL-5, IL-6, IL-10, IFN- gamma and TNF-alpha), acceptable correlations for IL-13, and a poor correlation for IL-12. Although the correlations were generally good, a significant variation between the absolute cytokine concentrations determined by ELISA and either multiplex kit was reported.

(Ray et al., 2005) examined the validation and implementation of cytokine multiplex assays, as a replacement of ELISA techniques. He concluded that, given the

different analytic platforms, different antibody pairs, and different lots of standards, the level of correlation was “impressive”.

In conclusion, although this technology is relatively new, there are now commercial vendors for a number of multiplex analyses. The advantages of the multiplex cytokine assays over the ELISA assay include smaller sample volumes required to obtain multiple results, decreased demand for labour and supplies, and much higher sensitivity. Since the assay measures multiple analytes simultaneously, therefore, the consumables and labour time are reduced. The other advantage is reduced cost of reagents.

In this study, it was demonstrated that testing samples from cancer patients with febrile neutropenia for IL-6, IL-1β and IL-8 by ELISA and Bio-Plex assay yield eventually similar results, with the latter being superior in the case of IL-1β. As mentioned the Bio-Plex assay has broader dynamic range and allows for multiplex analysis of cytokines in a single sample. Therefore, Bio-Plex assays have an advantage over the traditional ELISA methodology when multiple samples have to be analyzed for several cytokines and when sample volume is limited. This study also shows that Bio-Plex is much more sensitive that the ELISA method.



summary of the correlation between cytokines and sTREM-1

CHAPTER 4

Circulating biomarkers of infection and inflammation as potential adjuncts to clinical scoring systems in chemotherapy-induced febrile neutropenia

4.1 Introduction

As mentioned earlier in this dissertation, infection remains the most serious complication in cancer patients with chemotherapy-associated febrile neutropenia (FN), with an overall in-hospital motality of 9.5% (Kuderer et al., 2006). On presentation with FN, strategies to identify patients at highest risk for progression to severe infection include: i) clinical scoring systems (Talcott et al., 1988; Klastersky et al., 2000; Nakagawa et al., 2009); ii) measurement of circulating, host-derived markers of inflammation and/or infection, including pro-inflammatory cytokines, acute phase reactants, and procalcitonin (PCT) reviewed by (Sakr et al., 2008); and combinations of these strategies (Jimeno et al., 2004; Uys et al., 2007).

Of the circulating biomarkers with predictive potential, those which have attracted the most attention in patients with FN are PCT, interleukin-6 (IL-6), and the acute phase reactant, C-reactive protein (Katz et al., 1992; Engervall et al., 1995; Engel et al., 1998; Lehrnbecher et al., 1999; Karan, 2002; Oude Nijhuis et al., 2003; Uys et al., 2007; Sakr et al., 2008), with some studies having reported that PCT is superior to IL-6 and/ or CRP (Katz et al., 1992; Bernard et al., 1998; Engel et al., 1999; Fleischhack et al., 2000; Sauer et al., 2000; Harbarth et al., 2001; Liaudat et al., 2001; Chirouze et al., 2002; Chan et al., 2004; Persson et al., 2005; Massaro et al., 2007; Uys et al., 2007), while others have reported equivalence (Sakr et al., 2008). Some studies have suggested that measurement of PCT in FN is best stuited to identifying patients who are low risk for developing severe infection (Persson et al., 2005; Uys et al., 2007; Prat et al., 2008; Semeraro et al., 2010), while others have found it to have high positive predictive value for development of severe infection (Jimeno et al., 2004; Massaro et al., 2007).

Recently, circulating soluble Triggering Receptor Expressed on Myeloid Cells-1 (sTREM-1) has been reported to possess diagnostic value in the detection of bacterial infection (Schultz & Determann, 2008; Ford & McVicar, 2009b; Jiyong et al., 2009). Soluble TREM-1, a member of immunoglobulin superfamily is expressed on a variety of cells of myeloid lineage of the innate system, especially neutrophils and monocytes, and appears to amplify pathogen- associated, Toll-like receptor-

mediated inflammatory responses (Ford & McVicar, 2009a). Soluble TREM-1 is released from activated neutrophils and monocytes/macrophages and its detection in body fluids is considered to be a potentially useful biomarker of bacterial infection, which may, if used in combination with PCT, improve the recognition of true infection (Schultz & Determann, 2008). Soluble TREM-1 concentrations have also been used to predict the prognosis of patients with sespsis (Han et al., 2003). However only one previous study (Lin et al., 2009), has investigated the usefulness of circulating sTREM-1 in febrile neutropenia.

The primary objectives of the laboratory research described in the current chapter were: i) to identify the potential of range of mainly pro-inflammatory cytokines/ chemokines/ growth factors, sTNFR-1, acute phase reactants, HMGB-1, PCT and sTREM-1 to distinguish between low- and high risk-patients with FN; and ii) to establish which of these has the best predictive potential in respect of outcome, supporting their inclusion as adjuncts to clinical scoring sustems such as the MASCC and Talcott systems.

4.2 Patients

Stored (-20°C), unthawed serum specimens from 48/63 patients recruited to an earlier study (Uys *et al*, 2007) were analysed in the current study. All patients had a histologically confirmed malignancy and had presented with FN, either with an oral temperature of >39°C in a single measurent, or > 38°C in two consecutive measurements at least four hours apart, together with an absolute neutrophil count of ≥ 0.5 $x10^9$ /L as a result of chemotherapy. Approval to conduct the original study has been granted by the Research Ethics Committee of the Falculty of Health Sciences, University of Pretoria in Norvember 2000, while approval for the performance of the sTREM-1 assays on the stored serum specimens described in the current study (as an extension of the original study), was granted by the same committee in October 2009. Informed consent was obtained from all patients prior to enrolment in the original study.

Patients with both solid tumors and haematological malignancies were included. The anti-cancer agents used in these patients are shown in Table 2.2 (page 49). No patients had undergone radiotherapy immediately prior to the FN episode. All patients received broad-spectrum empirical antimicrobial chemotherapy (cefepime/ceftriaxone plus amikacin), except for one patient who received vancomycin, while for patients with persistent fever (7 days), amphotericin was added. On presentation, patients were catagorized using the MASCC score (Klastersky *et al*, 2006) and Talcott system (Talcott *et al*, 1988) to evaluate their risk for serious medical complications. These were hypotension, respiratory/ renal/ congestive cardiac failure, intensive care admission, confusion, bleeding requiring transfusion, electrocardiographic changes, arrhythmias requiring treatment, or an allergic reaction.

The patient characteristics with respect to age, gender, circulating leukocyte count, absolute neutrophil counts, type of malignancy, HIV status and inpatient/outpatient therapy are shown in Table 4.1.

Table 4.1 patient characteristics at initial presentation

Age (Mean ± S.E.M.)	51± 2years	
Gender	Male: 12	Female: 36
Solid Tumor	3	7
Total WCC (Mean ± S.E.M.)	0.7± 0.14	1.08 ± 0.13
Absolute Neutrophil Count (Mean ± S.E.M.)	0.13 ± 0.05	0.17 ± 0.03
HIV Status	1 positive	
Hospital Admission	10 inpatients 2 outpatients	24 inpatients 12 outpatients

S.E.M- starndard error of mean

4.3 Laboratory Methods

Circulating cytokines/ chemiokines/ growth factors (IL-1α, IL-1β, IL-1Ra, IL-6, IL-7, IL-8, IL-12, IFN-γ, TNF, G-CSF, GM-CSF, VEGF) were measured using Bio-Plex® Suspension bead array technology; sTNFR-1, HMGB-1 , and sTREM-1 by ELISA procedures; PCT by an immunoluminescence procedure; and CRP and SAA by nephelometric procedures. All of these procedures are described in detail in Chapter 2. These various biomarkers were selected according to either their apparent potential (eg. CRP, IL-6, PCT) or because they had not been previously evaluated (eg IL-7, IL-12, IFN-γ, GM-CSF, VEGF, HMGB-1, sTREM-1).

As mentioned in Chapter 2, the normal values of the cytokines, CRP, SAA, PCT, sTREM-1, HMGB-1 are <10ng/ml, ≤ 5µg/ml, ≤6.5µg/ml, <0.5ng/ml, <90pg/ml, and <10ng/ml respectively.

4.4 Study design and statistical analysis

The objective of the study was to probe the diagnostic and prognostic potential of the above biomarkers in patients with FN. Results are expressed as the mean ± S.E.M. Levels of statistical significance were calculated using the Mann-Whitney U thest for comparison of non-parametric data, and a P value <0.05 was considered significant.

Correlations between the various biomarkers and /or clinical indices were determined using the Spearman Correlation Coefficient, while sensitivity, positive predictive value (PPV), likelihood ratios and area under the curve (ROC) were used to predict response to empiric antimicrobial therapy, resolution without complications, development of serious complications, and motality.

4.5 Results

4.5.1 Serum concentrations of the various biomarkers in FN patients categorized according to low- or high-risk MASCC scores.

These results are shown in Table 4.2 (page 84). With the exceptions of SAA, IL-1β, IL-7, IL-8, IL-12, and VEGF, all of the biomarkers were significally elevated in the high-risk group, with sTREM-1, IL-6, and PCT exhibiting the most significant discriminatory potential. With respect to correlations between the cytokines, strong positive correlations ($r = \geq 0.7$, $p < 0.0001$) were noted between: i) IL-1α and/ or IL-1β with IL-6, IL-8, TNF and IFN-γ; ii) IL-6 and G-CSF; iii) TNF with IL-8, G-CSF and IFN-γ and iv) IL-12 and VEGF (see Appendix 1.1-1.9). Not surprisingly, CRP and SAA were strongly inter-correlated, and moderatetly correlated with IL-6. PCT correlated moderately with CRP and SAA ($r = 0.54/0.56$, $p < 0.0001$), sTREM-1 ($r = 0.54$, $p < 0.0001$), and IL-6 ($r = 0.49$, $p < 0.0004$), but only weakly with the other cytokines, as was the case with sTREM-1(see Appendix 2). The MASCC score correlated best with sTREM-1 ($r = 0.54$, $p < 0.0001$), PCT ($r = 0.48$, $p < 0.0006$), CRP ($r = 0.44$, $p < 0.002$) and IL-6 ($r = 0.41$, $p < 0.004$), and these correlations are shown in Appendix 3.

4.5.2 MASCC scores and values on presentation with febrile neutropenia for circulating CRP, SAA, PCT, sTREM-1, total neutrophil and monocyte counts, type of malignancy and microbial infection in 5 patients who died.

The individual results for the above-mentioned clinical and laboratory parameters for the 5 patients who died are shown in Table 4.3 (page 85). All 5 patients had septi with elevated levels of all biomarkers, with the exception of

the PCT value for patient 5 who was receiving antimicrobial prophylaxis at the time of presentation.

4.5.3 IL-6, PCT and sTREM-1 values for the various groups of FN patients categorized according to the performance status (PS), Talcott and MASCC scores, response to initial antimicrobial therapy, resolution with or without complications, presence or absence of serious complications, survival, and presence or absence of microbiologically documented infection (MDI).

Because they were found to have the most significant discriminatory value with respect to distinguishing between FN patients at low- and high- risk, IL-6, PCT and sTREM-1 were selected for more intensive analysis of their relationships with various clinical criteria ,PS, Talcott/ MASCC scores, response to antibiotics, resolution, complications, survival and presence or absence of MDI. These results are shown in table 4.4 (page 86).

Circulating sTREM-1 and serum PCT concentrations increased progressively as the PS of the patient deteriorated from PS1 to PS3, and decreased progressively from Talcott class 1 to 4 in keeping with a decline in disease severity predicted by the Talcott system. Patients who responded to initial empiric antimicrobial therapy had significantly lower sTREM-1 and PCT concentrations than those who did not and these were significantly greater for those patients who subsequently developed serious complications. Both sTREM-1 and PCT predicted the presence of bloodstream infections, but not other types of microbiologically-documented infections. The mean sTREM-1 and PCT concentrations for survivors were significantly lower than those of non-survivors.

IL-6 concentrations showed similar trends to those observed with sTREM-1, but did not reach statistical significance.

The sensitivity, specificity, positive predictive value (PPV), likelihood ratios and area under the ROC curve of circulating sTREM-1 and PCT concentrations to predict response to empiric antimicrobial therapy, resolution without complications, development of serious complications and mortality are shown in Table 4.5 (page 87). The threshold concentrations of circulating sTREM-1 and PCT selected to derive these variables were 100 pg/ml and 0.5 ng/ml respectively, and 100 or 200 pg/ml to predict mortality with sTREM-1. Soluble TREM-1 concentrations predicted each of the above events with reasonable accuracy, particularly with respect to the development of serious complications or death with positive likelihood ratios of 3 and 39 respectively. Similar results were obtained for the serum PCT concentrations.

4.6 Discussion

The results presented in this chapter demonstrate that cancer patients with chemotherapy- induced FN manifest a broad increase in a range of circulating biomakers of inflammation, including cytokines/ chemokines/ growth factors, their receptors/ receptor antagonists, acute phase reactants, and HMGB-1. This probably reflects an underlying inflammatory response due to the malignancy *per se*, and/ or an inflammatory response due to other non-infective causes, or to active microbial infection as a consequence of severe neutropenia. Interestingly, PCT and sTREM-1, considered to be markers of bacterial infection, had a more restricted occurrence. Although categorization of the group of FN patients into those with low- or high- risk MASCC scores revealed increased circulating concentrations of all of the biomarkers (with the exception of IL-7) in the high-risk group, discrimination was in most cases limited by the considerable range of results. The exceptions were IL-6, PCT, and sTREM-1, all of which were selected for more intensive analysis with respect to their relationships with clinical indices of severity and outcome. Of these, sTREM-1 and PCT were found to be the most meaningfully associated with outcome.

Previous studies have validated the Talcott grading system as a clinical predictor of outcome in patients with FN (Talcott et al., 1992). Circulating sTREM-1 concentrations correlated closely with the Talcott class, increasing progressively as the magnitude of risk escalated. This suggests that sTREM-1 concentrations increase as the patient's clinical status deteriorates, even in the presence of severe leukopenia. Soluble TREM-1 concentrations also increased as the performance status (PS) of the patients on initial evaluation deteriorated, further supporting the potential usefulness of sTREM-1 as a biomarker of underlying disease severity. Although the current study is limited by a relatively small number of patients, sTREM-1 concentrations were significantly lower in patients who survived than those who did not with a positive likelihood ratio for mortality of 39 at concentrations exceeding 200 pg/ml.

Serum PCT concentrations accurately predict outcome in patients with sepsis in the absence of neutropenia (Han et al., 2003), although conflicting results have been reported in patients with FN (Sakr et al., 2008; Sarmati et al., 2010). Despite these apparent inconsistencies, serum PCT concentrations in the current study correlated closely with those of s-TREM-1, suggesting that these biomarkers, either alone or in combination, may be useful for predicting the clinical course of patients with FN. Furthermore, in this setting, both s-TREM-1 and serum PCT predicted the presence of bloodstream infections.

In contrast to sTREM-1 and PCT, the concentrations of IL-6 and IL-8 did not accurately reflect response to treatment, complications, or risk profiles in these patients. This may be due to the short half-lives of these molecules in the circulation or individual differences in the magnitudes of systemic inflammatory response to microbial pathogens (Yamamura et al., 1991).

The findings of the current study are important considering the reliance on subjective clinical parameters incorporated into recently published prediction rules. Clinical criteria such as a “patient looks ill” (Hakim et al., 2010) may not be consistently applied by clinicians. Notwithstanding the importance of an appropriate clinical evaluation of patients with FN, sTREM-1 could be used as an adjunctive biomarker to facilitate the decision-making process in these high risk patients. Conceivably, a threshold value for sTREM-1 of 100 pg/ml could guide clinicians treating patients who do not fit clearly into low- or high-risk groups. Patients with s-TREM-1 concentrations below this threshold appeared to demonstrate a favorable outcome.

In conclusion, circulating s-TREM-1 appears to hold promise as an objective bio-marker which could be used to complement clinical prediction rules and thereby expedite the clinical decision-making process in patients presenting with FN.

Table 4.2 Values for the laboratory parameters in patients categorized according to low or high MASCC risk-index scores

Parameter	Low N=34	High N=14	P-values
CRP(µg/ml)	62,2	122,2	0.0148
SAA(µg/ml)	197,6	413,0	0.1230
PCT(ng/ml)	0,37	12.2	0.0030
sTREM-1(pg/ml)	84,8	192.79	0.0019
HMGB-1(pg/ml)	0.48	0.07	0.9504
sTNFR-1(pg/ml)	264,8	494,8	0.0097
VEGF(pg/ml)	195.73	283,27	0.5478
TNFa(pg/ml)	42,47	435,87	0.0379
INF-g(pg/ml)	37,4	486,5	0.0254
GM-CSF(pg/ml)	14,6	198,5	0.0219
G-CSF(pg/ml)	1722,1	10645,0	0.0380
IL-12(pg/ml)	22,89	55,26	0.4736
IL-8(pg/ml)	556,8	16146,8	0.0614
IL-7(pg/ml)	23,9	26,52	0.8117
IL-6(pg/ml)	307.55	14645	0.0021
IL-1α(pg/ml)	187,3	7432,9	0.0278
Il-1β(pg/ml)	11,8	100,6	0.0553

Table 4.3. Types of malignancy and microbial pathogens, as well as MASCC risk-index score and individual values of PCT, SAA, CRP, sTREM-1 and total circulating leukocyte, neutrophil and monocyte counts measured on presentation with febrile neutropenia in five patients who died.

Patient	Age/ Gender	Type of malignancy	Infection	MASCC score	SAA (µg/ml)	CRP (µg/ml)	PCT (ng/ml)	sTREM-1 (pg/ml)	Leukocyte count (x 10^9/l)	Neutrophil count (x 10^9/l)	Monocyte count (x 10^9/l)
1	57/F	Breast/ melanoma	*K pneumoniae/ Enterococcus faecalis*	16	129.0	143.0	3.59	203.5	0.94	0.34	0.46
2	43/F	Anal canal	*K pneumoniae/ Streptococcus viridans*	18	668.0	431.0	68.23	358.2	0.02	0	0
3	60/M	Gastric cancer	*E coli*	18	335.0	93.3	74.95	417	0.36	0	0
4	39/F	Cervix cancer	*E coli/ Pseudomonas aeruginosa*	10	1670.0	196.0	13.19	570.8	0.79	0	0
5*	59/F	Breast cancer	FUO#	18	34.5	26.6	0.32	169.7	0.68	0.14	0.16

* On antimicrobial prophylaxis at time of presentation.

FOU- Fever of unknown origin.

Table 4.4 Soluble TREM-1, PCT, and IL-6 values for patients categorised according to PS, Talcott class, response or lack of response to empiric therapy, resolution with or without complications, absence or presence of serious complications, survivors and non-survivors, absence or presence of MDI, and absence or presence of bloodstream infections.

	sTREM (Mean ± S.E.M.)	PCT	IL-6
All patients combined (n= 48)	116±15	3.8±2	4489±2630
Performance status 1 (n= 17)	77 ± 11	0.5 ± 0.3	332 ± 126
Performance status 2 (n= 20)	93 ± 10*	0.5 ± 0.2	317 ± 86
Performance status 3 (n= 11)	220 ± 49*	15 ± 8.5	18501 ± 10786
Talcott 1 (n = 13)	163 ± 43	7.7 ± 5.7	12428 ± 9080
Talcott 2 (n = 4)	139 ± 29	1.15 ± 0.8	1053 ± 600
Talcott 3 (n = 15)	116 ± 23	4.7 ± 4.5	2968 ± 2721
Talcott 4 (n = 16)	73 ± 9*	0.6 ± 0.3	325 ± 137
MASCC score			
Low risk (n = 34)	85 ± 8	0.4 ± 0.14	308 ± 78
High risk	193 ± 41	12 ± 6.8	14645 ± 8632
Response to empiric therapy (n = 29)	85 ± 8	0.43 ± 0.2	348 ± 90
No response to empiric therapy (n = 19)	165 ± 32*	9 ± 5	10810 ± 6476
Resolution without Complications (n = 35)	87 ± 8	0.3 ± 0.2	314 ± 76
Resolution with Complications (n = 13)	196 ± 44*	13.1 ± 7.3	15731 ± 9249
Absence of serious Complications (n = 36)	85 ± 8	0.4 ± 0.1	304 ± 72
Serious complications Developed (n = 12)	209 ± 45*	14.2 ± 7.8	17022 ± 9957
Survivors (n = 43)	89 ± 7	0.5 ± 0.2	337 ± 66
Non-survivors (n = 43)	344 ± 73*	32 ± 16.3	40140 ± 20620

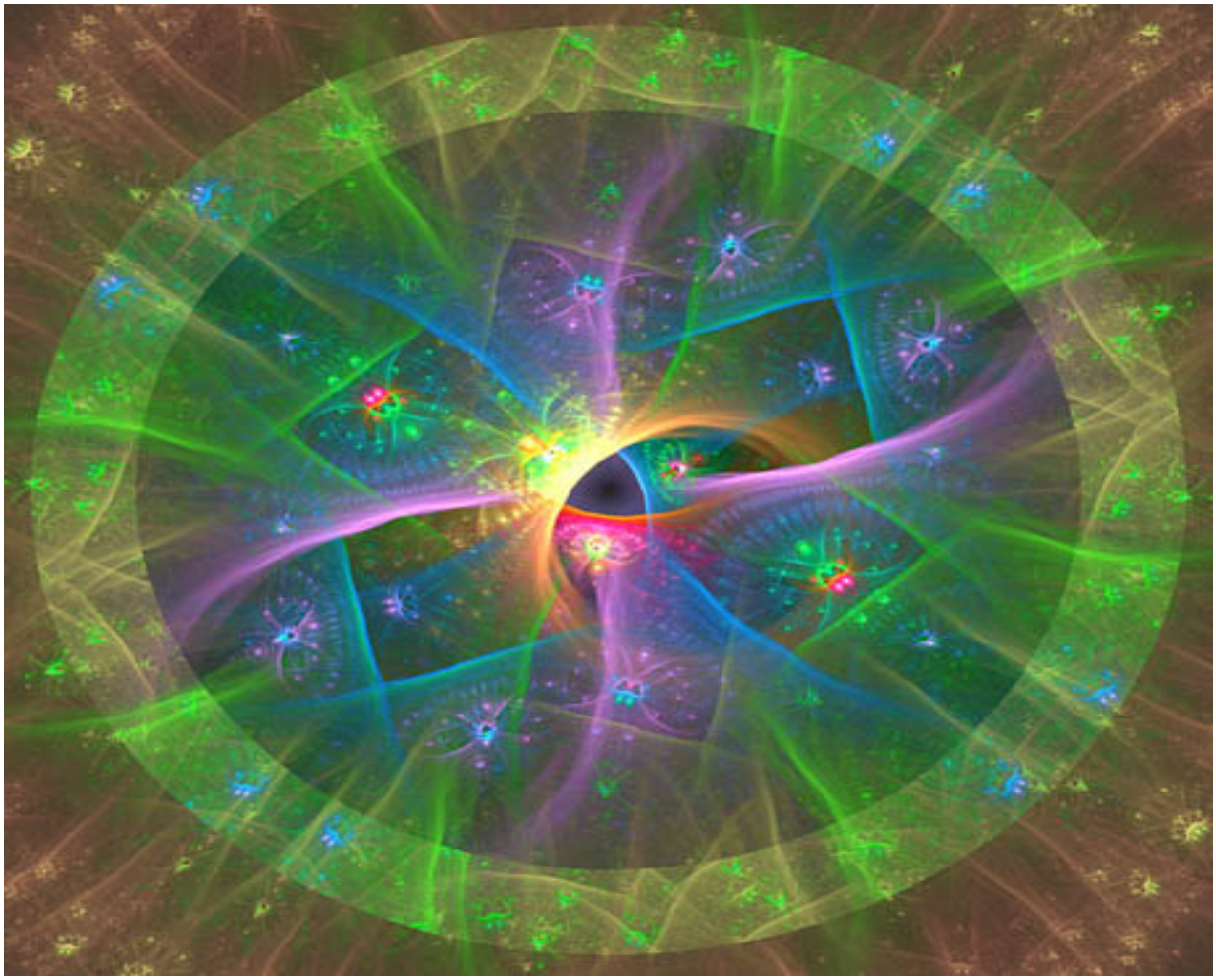
Table 4.4 continued

No documented infection Microbiologically-Documented infection	89 ± 7 94 ± 18	0.5 ± 0.18 0.62 ± 0.5	357± 81 302± 116
No bloodstream infection Bloodstream infection Present	90 ± 7 344 ± 73*	0.5 ± 0.2 32 ± 16.3	337± 66 40140 ± 20620

*P <0.05 for the comparison with each category or PS1 and 2 *versus* PS3 and class 1 *versus* class 4

Table 4.5. The sensitivity, specificity, positive predictive value (PPV), positive likelihood ratio (LR) and ROC (area under curve) of sTREM-1 and PCT to predict response to empiric therapy, resolution without complications, development of serious complications and death in patients with febrile neutropenia

	Sensitivity		Specificity		PPV		Positive LR		ROC (Area under curve)	
	sTREM-1	PCT	sTREM-1	PCT	sTREM-1	PCT	sTREM-1	PCT	sTREM-1	PCT
Response to empiric therapy	0.66	0.86	0.47	0.47	66%	71%	1.25	1.64	0.7	0.65
Resolution without complication	0.69	0.86	0.69	0.47	86%	76%	2.3	1.64	0.77	0.65
Development of serious complications	0.75	0.58	0.7	0.81	45%	50%	2.5	3	0.84	0.77
Death	0.99	0.8	0.66	0.82	25%	33%	3	4.4	0.99	0.94



J-Ring, 2007, Apophysis gallery, retrieved 15 October 2010, <http:// exper.3drecursions.com/apo/j-ring_tmb.jpg>

CHAPTER 5

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Appendix

1. Correlations between cytokines and sTREM-1

1

IL-1β	r	p
IL-1ra	0.66	<0.0001
IL-6	0.69	<0.0001
IL-7	-----	-----
IL-8	0.70	<0.0001
IL-12	0.48	0.0004
G-CSF	0.76	<0.0001
GM-CSF	0.56	<0.0001
TNF-α	0.84	<0.0001
INF-γ	0.72	<0.0001
VEGF	0.49	0.0003
STREM-1	-----	-----

2

IL-1α	r	p
IL-6	0.62	<0.0001
IL-7	-----	-----
IL-8	0.67	<0.0001
IL-12	0.38	0.0065
G-CSF	0.62	<0.0001
GM-CSF	0.65	<0.0001
TNF-α	0.72	<0.0001
INF-γ	0.79	<0.0001
VEGF	0.41	0.0033
sTREM-1	-----	-----

3

IL-6	r	p
IL-7	-----	-----
IL-8	0.73	<0.0001
IL-12	0.43	0.0020
G-CSF	0.74	<0.0001
GM-CSF	0.53	<0.0001
TNF-α	0.79	<0.0001
INF-γ	0.63	<0.0001
VEGF	0.47	0.0006
sTREM-1	-----	-----

4

IL-7	r	p
IL-8	0.30	0.0343
IL-12	0.48	0.0005
G-CSF	-----	-----
GM-CSF	-----	-----
TNF-α	-----	-----
INF-γ	-----	-----
VEGF	0.62	<0.0001
sTREM-1	-----	-----

5

IL-8	r	p
IL-12	0.51	0.0002
G-CSF	0.66	<0.0001
GM-CSF	0.46	0.0009
TNF-α	0.73	<0.0001
INF-γ	0.58	<0.0001
VEGF	0.62	<0.0001
sTREM-1	0.29	0.0424

6

IL-12	r	p
G-CSF	-----	-----
GM-CSF	0.36	0.0099
TNF-α	0.41	0.0033
INF-γ	0.33	0.0216
VEGF	0.85	<0.0001
sTREM-1	-----	-----

7

G-CSF	r	p
GM-CSF	0.48	0.0005
TNF-α	0.84	<0.0001
INF-γ	0.63	<0.0001
VEGF	0.33	0.0197
sTREM-1	------	------

8

GM-CSF	r	p
TNF-α	0.64	<0.0001
INF-γ	0.65	<0.0001
VEGF	0.35	0.0197
sTREM-1	------	------

9

	IFN- γ	VEGF	sTREM-1
TNF-α	0.77 <0.0001	0.42 0.0029	-------
INF-γ		0.34 0.0164	--------
VEGF			---------

2. Correlations between sTREM-1 SAA, CRP, PCT and cytokines

	SAA	PCT	sTREM-1	IL-6	IL-8
CRP	0.7779 <0.0001	0.5432 <0.0001	0.2418 NS	0.542 <0.0001	0.307 0.0334
PCT	0.5641 <0.0001		0.5443 <0.0001	0.4935 0.0004	0.381 0.0074
sTREM-1	NS	0.5443 <0.0001		NS	0.2912 0.0424

3. Correlations between MASCC score, sTREM-1, PCT, SAA, CRP and cytokines.

	CRP	SAA	IL-6	PCT	sTREM-1
MASCC score	r -0.4396 p 0.0018	r -0.3144 p 0.0295	r -0.4056 p 0.0043	r -0.4752 p 0.0006	r -0.5407 p <0.0001