Characterization of the acute phase response in critically ill children

Dr Rachel Sarah Agbeko

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Portex Unit of Paediatric Anaesthesia, Pain Research, Critical Care, Respiratory Medicine, Physiology & Physiotherapy

Institute of Child Health

University College London

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Declaration of Content

I, Rachel Sarah Agbeko, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated below.

Signed ……..……

ACE I/D and rs4341 genotyping:

Ms KaWah Li, Laboratory technician (performed the genotyping)

Professor Steve E Humphries, BHF Professor of Cardiovascular Genetics

Centre for Cardiovascular Genetics, Rayne Institute, Department of Medicine, University College London, London, UK.

TLR4 D299G genotyping paediatric cohort:

Mr. Matthew Rose-Zerilli, Research Technician (performed the genotyping)

Dr John Holloway, Reader Infection, Inflammation & Repair

Human Genetics Division, University of Southampton, UK

TLR4 adult data:

Dr Shu Ye, Reader in Human Genetics

Clinical Pharmacology, William Harvey Research Institute,

Barts and the London School of Medicine (released the data)

David Pontefract, Human Genetics Division, University of Southampton, UK (performed the data collection for adult platelet count)

Clinical data Southampton PICU:

Ms Kerry Illing, Medical student (performed the data collection)

Dr John Pappachan, Consultant and Honorary Senior Lecturer,

Paediatric Intensive Care Unit, Southampton University Hospitals NHS Trust

Comparison dataset genotyping and clinical data

PICU samples 1-147

Dr Katy Fidler, SpR (performed the genotyping)

Department of Infectious Diseases & Microbiology,

Institute of Child Health

University College London

Dr Peter Wilson, SpR (performed the clinical data collection)

Ms Helen Tighe, Research Nurse (performed the clinical data collection)

Paediatric Intensive Care Unit, Great Ormond Street Hospital NHS Trust London

Comparison CICU dataset genotyping and clinical data

Dr Meredith Allen, SpR (performed the genotyping)

Ms Annette McQuillan, Research Nurse (performed the clinical data collection)

Cardiac Intensive Care Unit, Great Ormond Street Hospital NHS Trust London

MBL healthy controls

Dr Marina Johnson, Research Associate (performed the genotyping serum levels)

ICH-Immunobiology Unit

Department of Infection and Immunity

Heart rate variability analysis

Ms Helen Tighe, Research Nurse (performed some of the clinical data collection)

Paediatric Intensive Care Unit, Great Ormond Street Hospital NHS Trust London

Mr Kevin Vinecore (design of PDAS physiological signal analysis system)

Mr Timothy Ellis (extraction and analysis preparation of heart rate data)

Professor James McNames, Professor and Chair (Fast Fourier analysis of heart rate data)

Electrical and Computer Engineering

Portland State University

All other genotyping was outsourced to a commercial genotyping facility KBiosciences (Hoddesdon, Herts, UK).

I was responsible for the study design, governance, quality control and analysis of the genotyping and physiological systems studies. In addition, I recruited patients, extracted DNA, created the PICU DNA library and working arrays, identified the candidate genes and their SNPS, performed the dbSNP search and DNA sequence identification, derived the MBL haplotypes from the individual MBL SNPs, performed the MBL ELISA in the new PICU cohort and screened the heart rate data for artefacts.

Abstract

Humans come into contact and interact with potential infective agents. The innate immune system is the first line of response to ward off infection. Innate immunity is, in part, under genetic control. This genetic control may help us understand the differences between individuals in preventing infection or limiting infectious and inflammatory illness.

Systemic inflammation is a complex disorder that is difficult to define. Current definitions are derived from consensus meetings. A need has been expressed for a more useful definition of systemic inflammation.

The work presented here identifies some of the underlying hereditability in limiting or being more vulnerable to severe infectious and injurious insults. Individual differences in complement activation potential and endotoxin recognition underlie part of the observed differences in a systemic inflammatory response to severe infection and injury.

An exploratory study using heart rate variability as a non-invasive method to distinguish infectious systemic inflammation from sterile systemic inflammation was inconclusive.

Chapter 1 gives the background to this study and an introduction to the approaches taken in this thesis.

Chapter 2 describes in detail the methods used in the genetic association study and physiological systems analysis.

Chapter 3 goes into some detail about the potential pitfalls in genotyping association studies and how these were addressed in the current study. The areas of genotyping quality, linkage disequilibrium, ethnicity, sample size and validation of previously done work are discussed using *MBL-2* and *ACE* as examples.

Chapter 4 is a description of the work done on genetic variability in the endotoxin receptor complex and how in may result in the host response to severe infection and physical insults. TLR4 polymorphisms were associated with lower platelet counts in severe inflammation. The reasons for this are unclear but may point to a direct effect of the TLR4 pathways on platelets or indicate that platelet counts are a more sensitive marker of systemic inflammation than SIRS criteria. These data support the view that variation in TLR4 function influences the early inflammatory response. This phenomenon may be one aspect of reduced fitness in the capacity to respond appropriately to an insult.

Chapter 5 reports the central role of complement in the acute phase response. Polymorphisms in two out of the three complement activation pathways were shown to have potential modifying effects in paediatric systemic inflammation. This chapter reports that polymorphisms in the *CFH* gene may modulate the acute inflammatory response and corroborates the previously reported finding that *MBL-2* variant genotypes are a risk factor for the early occurrence of SIRS/sepsis in a large cohort of paediatric critical care patients, independent of other potentially important functional polymorphisms in the complement and innate immunity system. A better understanding of how these polymorphisms operate at the pathophysiological level is needed before these findings can be translated to clinically useful therapeutic modalities. This study demonstrates that genetic polymorphisms associated with reduced complement activation may be associated with early SIRS/sepsis. This is consistent with a view that appropriate complement activation occurring early following an infectious or inflammatory insult protects children from early SIRS/sepsis.

Chapter 6 assesses the usefulness of full *MBL-2* genotyping and compares the *MBL-2* genotype and MBL serum levels between a cohort of healthy children and a cohort of

critically ill paediatric patients. *MBL2* genotyping did not render more information with regards to MBL serum level when all promoter and structural polymorphisms were identified over and above structural polymorphisms and the XY promoter polymorphism. The children admitted with infection did not have a surplus of MBL deficient genotypes as compared with healthy children. This suggests that MBL deficient genotypes do not predispose to severe infection.

MBL serum levels in SIRS or sepsis were lower compared with critically ill children without systemic inflammation. MBL levels were most reduced in the acute phase response in those genotypes with intermediate serum levels, which may reflect a consumption of MBL in critical illness and an inability to maintain pre-insult MBL serum levels.

Chapter 7 explores a novel way to discriminate SIRS from sepsis by means of heart rate variability analysis. In this small paired sample study no differences were seen in LF metrics to differentiate sterile SIRS from sepsis. Neither was there a difference in LF metrics between those children who went on to develop a nosocomial infection and those who did not. Normalised HF was significantly higher in sterile SIRS vs. sepsis. These preliminary finding require further validation and a longitudinal approach in a larger cohort.

Finally, Chapter 8 discusses the findings of this thesis in the context of interpretation and of the findings and potential future approaches.

This thesis supports the view that better metrics are required to discriminate systemic inflammation as well as the concept that in children control of an inflammatory threat is aided by a vigorous capacity to respond.

Acknowledgments

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Chapter 1 Introduction

1.1 Paediatric intensive care

Paediatric intensive care is the medical subspecialty that cares for the sickest acutely ill children in the population. The severity of illness is such that vital organ functions require external support. Supportive measures include intubation and ventilation, vaso-active medication, renal replacement therapy and mechanical cardiovascular support. Invasive and non-invasive devices are routinely used to monitor heart rate, blood pressure, cardiac output, oxygen saturation and intracranial pressure. Monitoring and interventions assess and support vital organ functions, ultimately aiming to promote healing and recovery. In the UK, paediatric intensive care units admit approximately 17000 children per year.(PICANet 2010)

Early in the course of illness the critically ill child will have mounted an acute phase response. This physiological state is an activation of many regulatory systems, including the autonomic nervous system, innate immune system and the neuro-endocrine axis. The goal of this host response is to contain and eliminate the threat induced by infection or injury. The acute phase response may lead to systemic inflammation and organ dysfunction.

1.2 The Acute Phase Response

The initial response to an invading pathogen or to severe injury is an immediate activation of the host's innate immunity, autonomic nervous system and neuro-endocrine axes (Cavaillon and Annane 2006).

The ultimate goals of this complex response are to prevent the host from being overwhelmed by infection and allow for resolution and repair. For this to succeed,

extensive cross-talk is necessary between the activated systems, attempting a concerted effort to contain and neutralise the threat. Examples of failure to control the initial threat are bacterial septic shock and more locally, uncontrolled intracranial hypertension after traumatic brain injury.

In the minutes to hours after injury or microbial invasion the immune system is triggered; acute phase proteins are synthesized, neutrophils and platelets are activated, monocytes produce cytokines. The autonomic nervous system responds with activation of both the sympathetic and parasympathetic components. Last, a neuro-endocrine response is triggered to maintain vital organ function (Vanhorebeek, Langouche et al. 2006)

These responses are ubiquitous in critically ill patients. Patients differ, however, in their capacity to mount an effective response. The key question is how to explain these differences. Answers to this question will help guide interventions, ultimately improving outcome. To define and understand better the clinical course of critically ill patients one needs to address the insult (severity, type etc), the patient (predisposition, response) and the outcome (organ dysfunction). (Angus, Burgner et al. 2003, Gerlach, Dhainaut et al. 2003, Vincent, Opal et al. 2003, Vincent, Wendon et al. 2003)

1.3 Systemic inflammation and multiple organ dysfunction syndrome

The Systemic Inflammatory Response Syndrome (SIRS) is a generalised response to an insult. SIRS is a clinical description of a complex entity that underlies much of PICU related morbidity. Multi organ dysfunction syndrome (MODS) secondary to systemic inflammation remains the leading cause of death in infants and children admitted to paediatric intensive care units (PICUs).(Leteurtre, Martinot et al. 2003, Tantalean, Leon et al. 2003) (Proulx, Joyal et al. 2009) In an attempt to delineate SIRS

as a measurable entity a group of researchers in intensive care came to a consensus definition. (1992) In essence, SIRS in all its complexity was scaled back to four domains (heart rate, respiratory rate, temperature and white cell count). Systemic inflammation secondary to infection was called sepsis. Although sterile SIRS and infectious SIRS (or sepsis) only differ in the etiology from a definition point of view, there are preliminary data that support a different mechanism underlying the seemingly similar host response. Using micro-array technology Allantaz and colleagues differentiated systemic juvenile arthritis, i.e. sterile SIRS, from systemic inflammation secondary to several infectious agents, i.e. sepsis. (Allantaz, Chaussabel et al. 2007) The natural history of sterile SIRS following trauma is different from that following infection: many children admitted to PICU after trauma develop SIRS, but very few progress to MODS.(Calkins, Bensard et al. 2002, Wood, Partrick et al. 2010) Paediatric MODS invariably occurs early (within 48 hrs) in the PICU course of admission, usually in an overwhelming fashion (Tantalean, Leon et al. 2003). This pattern is different from that seen in adults, in which a later, more sequential onset is the norm (Moore and Moore 1995). Extrapolating data and studies from adults to the paediatric population may not serve our patients well. Specific paediatric data are required to understand better the etiology and course of our sickest children.
It is appreciated that the innate immune system plays a central role in inciting SIRS and MODS after infectious (Medzhitov 2007) and injurious (Hietbrink, Koenderman et al. 2006, Stahel, Smith et al. 2007) insults.

1.3.1 Epidemiology of paediatric sepsis

Sepsis is defined as systemic inflammation with proven or suspected infection (Levy, Fink et al. 2003).

Globally, the burden of paediatric sepsis is high: annually millions of children die with severe infections. This includes severe malaria, severe pneumonia, severe diarrhoea and severe measles. (Mangia, Kissoon et al. 2009)

In the United States paediatric intensive care units admit an estimated 42000 children with severe sepsis/year.(Watson, Carcillo et al. 2003) Each year the United Kingdom sees approximately 1000 children with severe sepsis admitted to PICUs.(PICANet 2010) Although mortality has reduced in the past decade in paediatric sepsis, it remains high, with an associated mortality of about 20%. This does not include the unknown number of children with sepsis who die before they reach a PICU. A recent UK study showed that children with sepsis accepted to PICU are young (median age 13.6 months) and predominantly male (58%). In 70% of cases sepsis was microbe positive, with 54% bacterial and 16% viral diagnosis. Meningococccal disease was diagnosed in over a third (36%), followed by pneumococcal disease (14%) and RSV (9%). Most children showed organ dysfunction on meeting the critical care team: 92% were ventilated, 69% received inotropic support, 12% got renal replacement therapy and 8% received ECMO.(Inwald, Tasker et al. 2009). Some of this organ dysfunction and mortality may be a result of suboptimal care.(Ninis, Phillips et al. 2005, Launay, Gras-Le Guen et al. 2010)Nonetheless, after many interventional studies in large patient cohorts failed, including paediatric studies (Levin, Quint et al. 2000, Nadel, Goldstein et al. 2007), there is an understanding that individuals differ widely in both their predisposition and response to insults (Levy, Fink et al. 2003). Given its primordial role in the initial host response the innate immune system may be one important source of this variability.

1.4 The innate immune system

1.4.1 Outline and function

The innate immune system is the first line of defence to pathogens after physical barriers have been breached. Innate immunity can be viewed as a system for recognising exogenous ‘danger signals’ on potential pathogens (Oppenheim and Yang 2005). This process of ‘pathogen-associated molecular pattern’ (PAMP) recognition triggers a range of host responses. This is a well-appreciated role of the innate immune system. More recently, the innate immune system is recognized also to trigger an inflammatory response after injury. This occurs by recognizing endogenous danger signals. These ‘danger associated molecular patterns’ (DAMPS) are intracellular components that are released into the extracellular environment after sterile insults such as trauma.(Matzinger 2002) Innate immunity is thus central to infectious as well as injurious insults.(Miyake 2007)

Figure 1-1 gives a condensed view of key actors in the first response to pathogen and danger recognition.

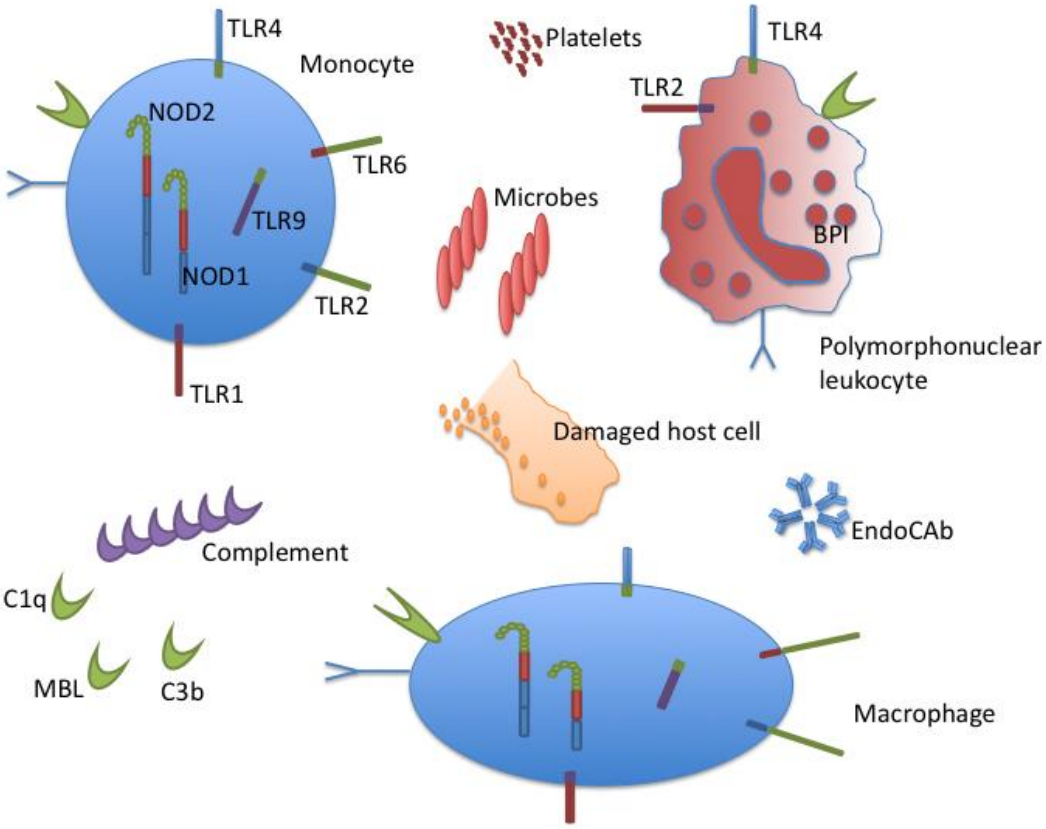


Figure 1-1 Innate pattern recognition overview

Both microbes and endogenous cells can trigger recognition. This process occurs at cell surface, intra-cellularly and in the circulation. Cell surface recognition receptors include Toll Like Receptors (TLR). Intracellular pattern recognition receptors include TLRs and Nucleotide-binding oligomerization domain-containing proteins (NODs). Examples of circulating proteins are Complement and natural antibodies such as Endotoxin core antibodies (EndoCAb).

Abbreviations: C1q complement component 1 q subcomponent. MBL Mannose Binding Lectin; C3b Complement component 3 b.

Innate immunity is evolutionary ancient and found in nearly all multicellular organisms. In humans the innate immune system is largely present at birth. This contrasts with the adaptive immune system that is found in vertebrates only and which takes years to build its repertoire. Given that the adaptive immune system evolved in the presence of innate immunity, these systems do not operate in isolation from each other; rather the innate immune system presents to and instructs the adaptive part of immunity.(Medzhitov 2007)

An ideal first-line response to invading microbes would readily recognise and promptly neutralise infectious agents without widespread collateral tissue injury. The innate immune system achieves these objectives to some degree by a combination of circulating molecules that either cause direct pathogen lysis or prime pathogens for phagocytosis and by phagocytic cells themselves.

Innate immunity encompasses immune cells, such as tissue macrophages, neutrophils and monocytes and also cells that are primarily known for other functions: platelets and endothelial cells. Complex networks of circulating mediators including cytokines, chemokines, the complement cascade, collectins, defensins and coagulation factors are integral parts of innate immunity. Recent work has highlighted apparent contributions from neuro-humoral and autonomic nervous systems via the α7 nicotinic acetylcholinergic receptor (NACHR7).(Borovikova, Ivanova et al. 2000, Wang, Yu et al. 2003)

The afferent, or ‘sensing’, limb of the innate immune system consists of pattern recognition molecules (PRMs) that recognise typical molecular structures on pathogenic micro-organisms or endogenous substances. For example, TLR4 recognizes the Gram-negative bacterial cell wall component lipopolysaccharide (Poltorak, He et al. 1998) and endogenous fibrinogen (Smiley, King et al. 2001); TLR2 recognizes the Gram-positive cell wall component lipoteichoic acid

(Yoshimura, Lien et al. 1999) and endogenous human heat shock protein 60 (HSP 60) (Asea, Rehli et al. 2002). Mannose binding lectin (MBL) binds to non-human patterns of sugars such as mannose on yeast(Turner 2003)but also endogenous ligands (Takahashi, Ip et al. 2006).

The importance of these danger signals is illustrated by the number of names competing in the literature to describe them: pathogen-associated molecular patterns (PAMPs), danger associated molecular patterns (DAMPs), and most recently '*alarmins'* (Oppenheim and Yang 2005, Bianchi 2007). Whatever name is used, the importance is that when such patterns encounter pattern recognition molecules of the innate immune system, they initiate and/or potentiate a cascade of events, which is aimed at rapid killing of invading pathogens or re-establishing immune homeostasis. Pre-existing cell surface components (e.g. CD14 and TLR4) and humoral (e.g. Mannose Binding Lectin) elements recognize danger signals. After ligation these sentinel modalities then induce an inflammatory response that includes cell signalling, cytokine production, opsonization and phagocytosis. This means that no critically ill child is admitted to intensive care without activation of its innate immune system. Infection, trauma, ischemia-reperfusion and cardiopulmonary bypass sequelae are all mediated largely by the innate immune system. (Wood, Partrick et al. 2010)

Our understanding of the immune system has thus evolved from a modality to distinguish tissues that are "infectious non-self" from "non-infectious-self" (Janeway 1992) to an even more complex tool that recognizes 'dangerous*'* and 'non-dangerous*'* matter (Matzinger 2002).

The different types of pattern recognition molecules have the capacity to recognize specific motifs that signify danger or infection. They do not operate individually, but cooperate in between themselves, not only intra recognition group, such as the endotoxin recognition complex, but also across different types of recognition

molecules and cell types. For example: TLR2 co-locates with TLR6 or TLR1 in recognizing different bacterial lipopeptides (Gay and Gangloff 2007), TLR4 on platelets and polymorphonuclear leukocytes form neutrophil extracellular traps (NETS) (Clark, Ma et al. 2007) and CD14 interacts with complement receptor 3 (CR3) in the recognition and response to yeast (Brandhorst, Wuthrich et al. 2004).

The following paragraphs outline pertinent components of the innate immune system which play an integral part in paediatric SIRS.

1.4.2 The Complement pathway

Complement is a group of proteins that is central in infection and inflammation. Its major tasks are killing micro-organisms directly via the membrane attack complex and indirectly via opsonization and readying for phagocytosis; plus activation of the coagulation cascade and inflammation via anaphylatoxins. This component of innate immune system is a cascade of events, in which early components are activated and subsequently cleave other components.

Three pathways activate previously inactive proteins. (Figure 1-2) The classical, alternative and lectin pathways converge in the activation of C3, from where a common pathway leads to further Complement activation. The lectin-complement pathway facilitates pathogen removal via carbohydrate recognition mediated phagocytosis. The alternative complement pathway is a continuously activated bactericidal humoral mechanism. (Holers 2008) The classical pathway activates when antibody-antigen complexes bind to C1, which in turn leads to generation of C1q. (Walport 2001)

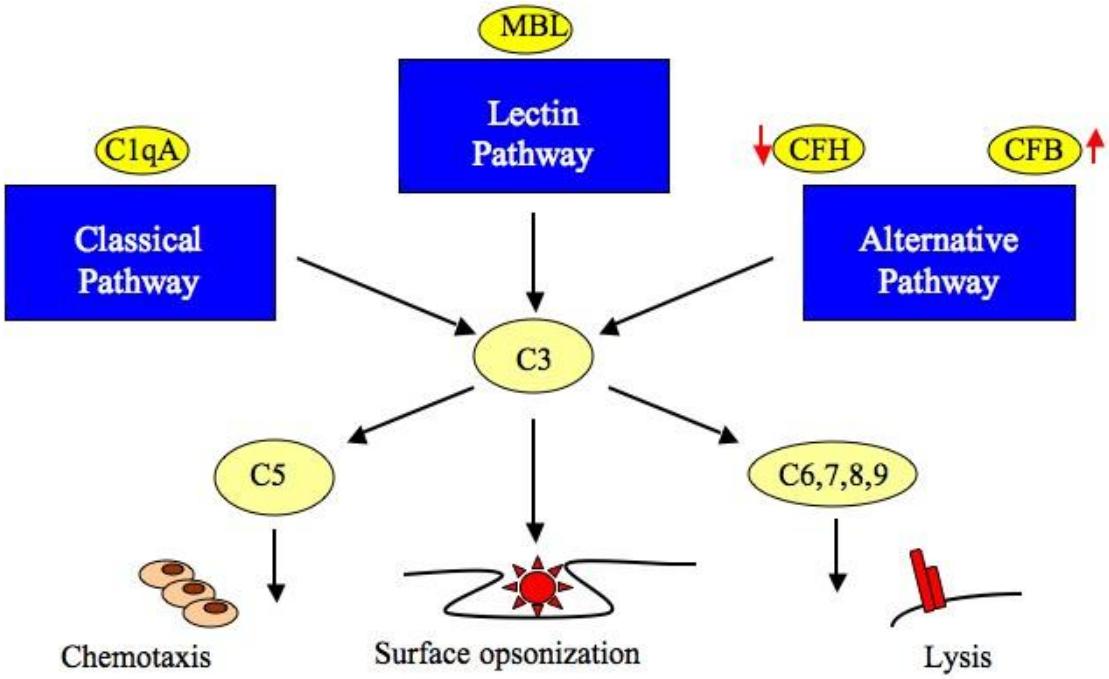


Figure 1-2 Complement activation

The three pathways that activate the complement cascade are initiated and/or modulated by C1qA, MBL, CFH and CFB. These pathways converge at C3, from where the downstream complement proteins lead to chemotaxis, opsonization and cell wall lysis.

The role of complement in combating invasive bacterial disease is well established. (Figueroa and Densen 1991) Children with complement deficiencies are at higher risk of contracting meningococcal disease. This is illustrated by the relationship between the incidence of meningococcal disease and the prevalence of complement deficiency in an international comparison. When the incidence of meningococcal disease is low, such as in Japan, the prevalence of complement deficiency in meningococcal disease is high. Conversely, when there is a high incidence of meningococcal disease, such as in Denmark, the prevalence of complement deficiency in meningococcal disease is low (Figueroa and Densen 1991). This suggests that in areas with low exposure to the micro-organism, those that do exhibit the disease have predisposing factors such as complement deficiency. Figure 1-3.

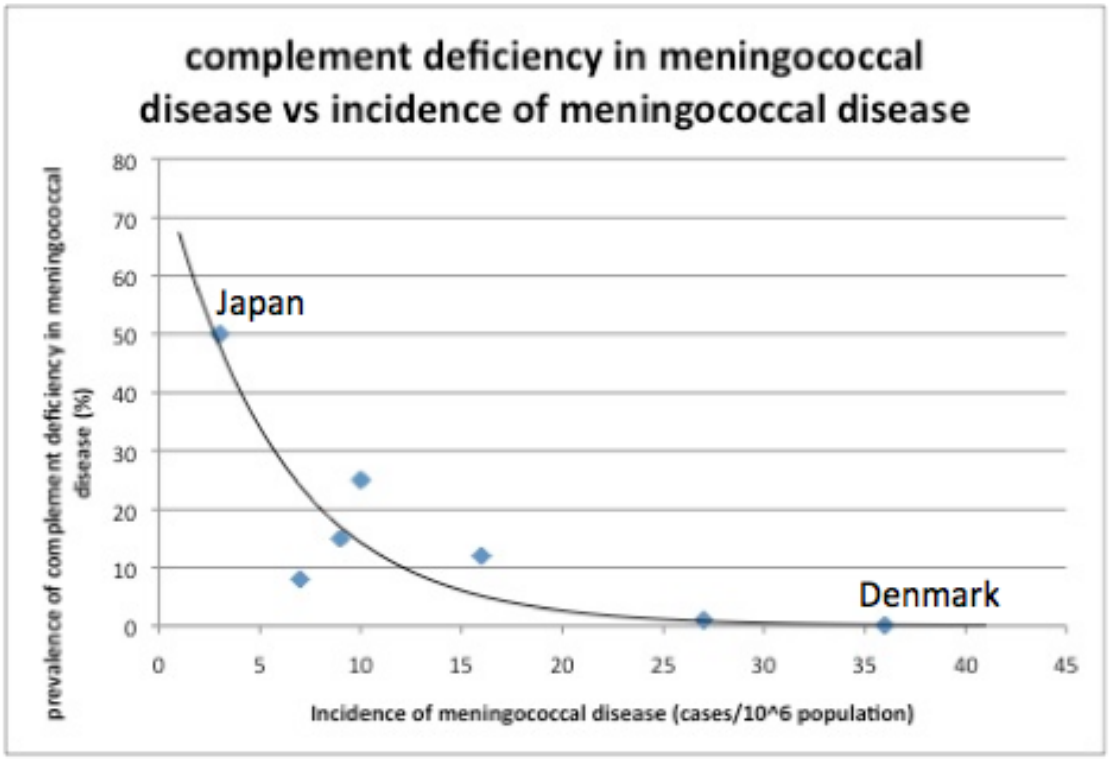


Figure 1-3 Relationship of prevalence of meningococcal disease and complement deficiency

Amended from (Figueroa and Densen 1991).

Complement activation occurs not only in infectious insults, but also in sterile inflammation such as trauma. (Ganter, Brohi et al. 2007)

Part of the complement activation cascade are sugar-recognizing *collectins*, molecules which contain collagenous structures and C-type carbohydrate recognizing domains (CRD) such as mannose binding lectin (MBL). These collectins also include surfactant proteins A and D (SP-A and SP-D), but these do not take part in complement activation. MBL is a liver derived acute phase reactant whereas SP-A and SP-D are synthesized in the lung. The main determinant of MBL levels is genotype, whereas SP-A and SP-D increase significantly with inflammatory stress. Collectins bind to many microbes: viruses, bacteria, fungi and protozoa and prepare organisms for phagocytosis (opsonization) and activate complement pathways. The ficolins, L-ficolin, M-ficolin, H-ficolin are similar but they have different structures with a fibrinogen-like domain. Both MBL and the ficolins initiate the lectin pathway of complement activation via mannose binding lectin associated serine proteases (MASPs).(Holmskov, Thiel et al. 2003)

1.4.3 Cellular Pattern Recognition Receptors: Toll Like Receptors (TLR) and Nod Like Receptors (NLR)

1.4.3.1 Toll Like Receptors

Toll like receptors (TLRs) are evolutionary preserved from the worm *Caenorhabditis elegans* and strikingly homologous to Toll, a gene product essential to *Drosophila* immunity. The TLRs are trans-membrane glycoproteins that are characterized by an extracellular binding domain with varying numbers of leucine-rich-repeat (LRR) motifs and an intracellular cytoplasmic signalling domain homologous to that of the

interleukin 1 receptor (IL-1R). This Toll/IL-1R homology (TIR) domain sits beneath the plasma membrane and interfaces primarily with the key signalling adaptor myeloid differentiation primary response gene 88 (MyD88).(O'Neill and Bowie 2007) There are at least 10 related receptors in humans (Table 1-1).

Toll-like receptor-2 recognizes the cell wall components lipoteichoic acid (LTA) and peptidoglycan of Gram positive bacteria, whereas TLR5 recognizes flagellin on *Salmonella enterica*. Viral matter is recognized by TLR3, TLR7 and TLR8. Toll-like receptors also combine in heterodimers and thus generate wider ligand specificity, for instance TLR6/TLR2 recognizes the fungal cell wall component zymosan. Intracellular Toll-like receptor-9 recognizes bacterial DNA, which is distinct from mammal DNA by way of the presence of unmethylated CpG dinucleotides. Mitochondrial DNA is one alarmin that signals through TLR9. This pathway may explain some of the systemic inflammation after tissue injury. (Zhang, Raoof et al. 2010)

One TLR may also recognise different pathogen components; TLR4 not only binds LPS, but also the structurally unrelated fusion protein of respiratory syncytial virus (RSV) (Kurt-Jones, Popova et al. 2000) and *Plasmodium falciparum* glycosylphosphatidylinositol (GPI) (Krishnegowda, Hajjar et al. 2005). Fibrinogen, heat shock proteins, reactive oxygen species, hyaluronic acid and haeme are all ligands for TLR4 in the absence of endotoxin (Takeda, Kaisho et al. 2003, Taylor, Trowbridge et al. 2004, Zhang, Shan et al. 2005, Figueiredo, Fernandez et al. 2007). These interesting observations suggest that these pathways contribute to the on-going immune response from other insults such as trauma rather than being specific to individual pathogens. This fits with a danger model of immunity, rather than a strict pathogen non-self and non-pathogen self model.

Toll-like receptors are expressed on many immune and non-immune cells, including macrophages, platelets and cardiac myocytes. This expression is modulated rapidly in response to pathogens and cytokines. Many of these TLRs, e.g. TLR1, 2, 4 and 5, are expressed on the cell wall, others are found intracellularly, e.g. TLR3, 7 and 9. (Gay and Gangloff 2007)

PRR	PAMP	Organism	Endogenous ligand	Location
TLR1-TLR2	Triacyl lipopolypeptide	Gram pos bacteria		Cell surface
TLR2-TLR6	Diacyl lipopeptide LTA Zymosan	Mycoplasma Gram pos bacteria Fungus		Cell surface
TLR2	Porins Peptidoglycan Haemagglutinin protein	Neisseria Gram pos bacteria Measles	Hyaluronic acid HSP HMGB1	Cell surface
TLR3	dsDNA	Virus		Intracellular vesicles
TLR4	LPS Envelope proteins	Gram neg bacteria RSV	Fibrinogen HSP ROS Hyaluronic acid Haeme HMGB1	Cell surface
TLR5	Flagellin	Bacteria Salmonella		Cell surface
TLR6				Cell surface
TLR7	ssRNA	RNA virus		Intracellular vesicles
TLR8	ssRNA	RNA virus		Intracellular vesicles
TLR9	CpG DNA DNA Malaria hemozoin	Bacteria DNA virus Parasites		Lysosomes
TLR10	Unknown	Unknown		B lymphocytes

Table 1-1 TLR Classification

PRR Pattern recognition Receptor; PAMP Pathogen Associated Molecular pattern; LTA Lipoteichoic Acid; HSP Heat Shock protein; HMGB1 High-mobility group protein B1; ds DNA double stranded deoxyribonucleic acid; RSV Human respiratory syncytial virus; ROS reactive oxygen species; ssRNA single stranded Ribonucleic acid; CpG DNA Cytosine phosphate Guanine deoxyribonucleic acid. Reviewed in (Pandey and Agrawal 2006)

1.4.3.1.1 Toll Like Receptor 4

The archetypal pathogen-associated molecular pattern (PAMP) is the Gram-negative bacterial cell wall component lipopolysaccharide (LPS), to which humans are exquisitely sensitive. (Sauter and Wolfensberger 1980) Endotoxin or LPS is recognised by the humoral factor lipopolysaccharide binding protein (LBP) (Schumann, Leong et al. 1990) and the cell membrane receptor toll-like receptor 4 (TLR4) (Poltorak, Smirnova et al. 1998). Lipopolysaccharide binding protein (LBP), a liver-derived glycoprotein, binds to LPS and shuttles it to an immune cell surface, such as a monocyte. The monocyte receives the LBP/LPS compound on a complex consisting of TLR4 (Medzhitov, Preston-Hurlburt et al. 1997, Poltorak, Smirnova et al. 1998), CD14 (Wright, Ramos et al. 1990) and MD2 (Shimazu, Akashi et al. 1999) (Figure 1-4).

TLR4 signalling depends on the ligand being recognized. Microbial ligands include the lipid A component of LPS, which is present on the gram negative cell wall (Raetz and Whitfield 2002), RSV fusion protein (Kurt-Jones, Popova et al. 2000), and endogenous ligands including heat shock proteins (Ohashi, Burkart et al. 2000)and fibrinogen (Smiley, King et al. 2001).

On binding, the trans-membrane receptor TLR4 signals to intracellular components, which, in turn, leads to NFκB mediated downstream gene expression and cytokine activation. (Figure 1-5). Thus, a Gram-negative infection such as meningococcemia gives rise to a rapid non-specific response which aims to kill the inoculum and contain the infection.

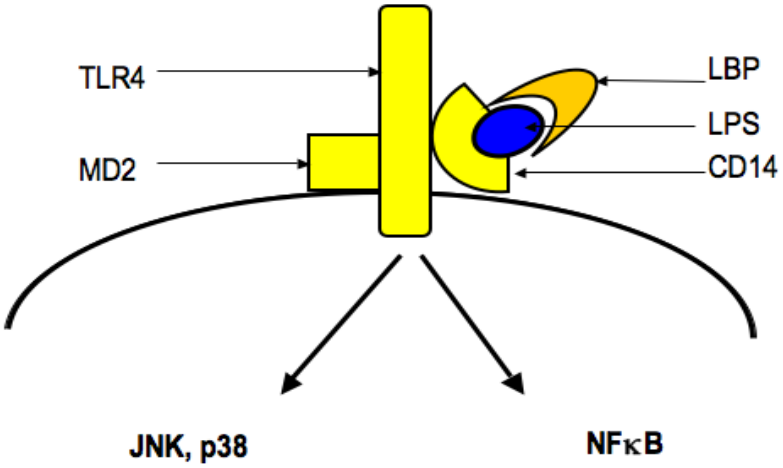


Figure 1-4 TLR4 complex

Lipopolysaccharide (LPS) is shuttled to the endotoxin receptor complex by lipopolysaccharide binding protein (LPB). CD14 independent signalling via TLR4 is possible for endogenous danger signals such as heat shock protein 70. LPS induced signalling on monocytes is optimal when all the components, i.e. MD-2, CD14, and TLR4 are available. TLR4 transduces signals via intracellular components to activate the transcription factor nuclear factor κB (NFκB), MAP kinases p38, and C-Jun N-terminal kinases (JNK). These then activate the inflammatory cascade via gene transcription. (Guha and Mackman 2001)

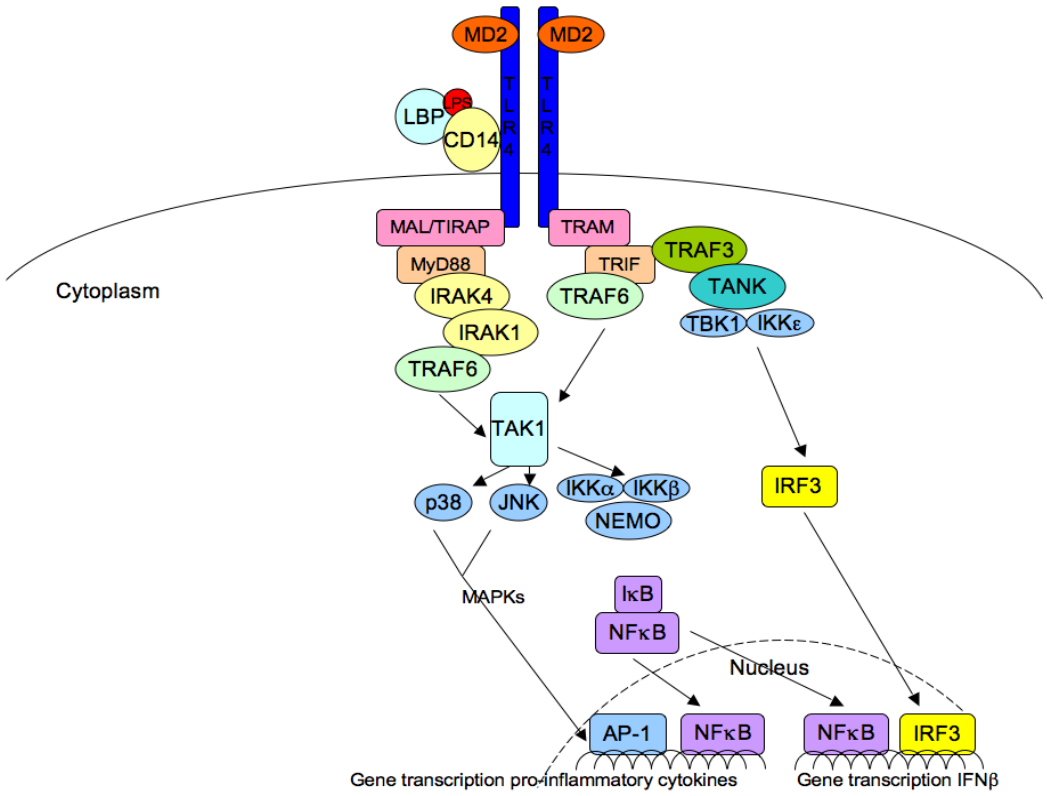


Figure 1-5 TLR signalling process

(taken from(Agbeko and Peters 2011))

LBP shepherds LPS to the TLR4 complex. On ligation, TLR4 dimerizes and activates the two distinct MyD88- and MyD88-independent TRIF-dependent pathways. The early inflammatory phase is characterized by the MyD88-dependent pathway. This pathway activates MAPKinase and NFκB mediated pro-inflammatory gene induction. The TRIF-dependent pathway will activate late phase NFκB and IRF3 mediated gene expression resulting in a more endotoxin tolerant response. (Kawai and Akira 2006, Biswas, Bist et al. 2007, Mogensen 2009)

LBP, Lipopolysaccharide binding protein; LPS, Lipopolysaccharide (endotoxin); CD14, Cluster of Differentiation 14; MD2, Myeloid differentiation protein 2; TLR4, Toll-like Receptor 4; MyD88, Myeloid differentiation primary-response protein 88; MAL/TIRAP, MyD88 adaptor like/Toll-interleukin 1 receptor (TIR) domain containing adaptor protein; IRAK4, Interleukin 1 receptor associated kinase 4; IRAK1,

Interleukin 1 receptor associated kinase 1; TAK1, transforming growth factor β-associated kinase 1; TRIF, TIR domain-containing adaptor protein inducing Interferon β; TRAM, TRIF related adaptor molecule; TRAF6, Tumour necrosis factor receptor associated factor 6; TRAF3, Tumour necrosis factor receptor associated factor 3; TANK, TRAF family member associated NFκB activator; TBK1, TANK binding kinase 1; IKKε, inhibitor of nuclear factor κB kinase ε; ; IKKα, inhibitor of nuclear factor κB kinase α; IKKβ, inhibitor of nuclear factor κB kinase β; p38, p38 kinase; JNK, c-Jun N-terminal kinase; MAPKs, mitogen activated protein kinases; NEMO, nuclear factor κB essential modulator; IRF3, Interferon regulatory factor 3; IκB, inhibitor κB; NFκB, Nuclear factor κB ; AP-1, activator protein 1.

1.4.3.2 Nod Like Receptors

A different type of pattern recognition receptors (PRR) recognizes pathogens after invasion in the cytosol. Of particular relevance to the intensivist are the nucleotide binding oligomerization domain and leucine rich repeat containing molecules (NLRs). The best studied of these proteins, Nucleotide Oligomerization Domain 1 NOD1 and NOD2, both contain N-terminal caspase recruitment domain (CARD) domains and are specialised in detection of bacterial peptidoglycan components. Two types of activation occur: NOD1 and NOD2 ligation causes their oligomerization, which in turn induces downstream gene expression via NFκB activation. Alternatively, NLRs activate caspase-1 activating complexes, also known as inflammasomes, which in turn mature cytokines IL1-β and IL18.(Inohara and Nunez 2003) Inflammasomes may be seen as sensors for danger. For instance, loss of cell integrity activates the inflammasome and hence the potent pro-inflammatory cytokine IL-1β. The significance of this pathway is increasingly recognized. Recent data shows the inflammasome to be integral to the pathogenesis of severe *S. aureus* infections.(Munoz-Planillo, Franchi et al. 2009)

1.4.4 Downstream signalling

Ligation of a cell surface TLR will activate a sequence of events, ultimately leading to gene transcription and translation to form proteins. These proteins, in turn, activate and dampen processes to allow for an appropriate host defence. Figure 1-5 provides an example of downstream signalling in response to LPS binding to the TLR4 complex. Toll-like receptors dimerize and change in conformation; this allows for recruitment of adaptor molecules to the TIR domain of the receptor. Four adaptor molecules are

known: MyD88, Mal (TIRAP), TICAM1 and TRAM. Different TLR ligands induce selected recruitment of these adaptor molecules explaining some of the distinct responses after TLR ligation.

MyD88 is essential for all downstream signalling via TLRs (except for TLR3). In addition, Mal is essential for TLR2 and TLR4 signalling, arguably the most important TLRs in critical care.

After activation of these adaptor molecules IL-1receptor associated kinase 4 (IRAK4) and IRAK1 are recruited. These, in turn catalyze a cytoplasmic cascade that ultimately leads to mitogen-activated protein kinases (MAP kinases) p38, ERK1, ERK2 and consequently NFκB mediated gene transcription in the cell nucleus. A MyD88 independent pathway exists, which has a role in DC maturation and interferon α/β production.(O'Neill 2008)

1.4.5 Cytokines

Cytokines are polypeptide intercellular messengers. Cytokines have actions stimulating or inhibiting the immune response. 'Pro-inflammatory' cytokines include TNFα, INFγ, IL-1β, IL-6 and IL-8, whereas IL-10 and IL-4 are thought to have predominantly anti-inflammatory properties. This dichotomy of pro- and anti-inflammatory cytokines does not do justice to the pleiotropic functions of these proteins.(Cavaillon, Adib-Conquy et al. 2003) For instance, dependent on timing and location, the prototypical pro-inflammatory cytokine TNFα may have a function in pro-inflammation or in resolution of inflammation. One key issue is the appropriateness of the TNFα action – an injection of TNFα to a healthy volunteer causes temperature and signs of systemic inflammation (and perhaps shock if the dose is high). This is the *cost* of TNFα without the potential *benefit* of immune activation

to kill an invading micro-organism. The cost-benefit of a cytokine such as TNFα is dependent on the scale of the infective insult being faced. Meta-analysis of animal models and human trials with agents that block TNFα or its receptor suggest that TNFα is useful when the insult is mild, but harmful when the insult is very severe.(Eichacker, Parent et al. 2002) This dose-dependent complexity is typical of cytokine responses.

One extreme example of a pro-inflammatory overactivation is secondary hemophagocytic lymphohistiocytosis. This multisystem disease results from a persistence of the inflammatory response to a systemic viral infection beyond its useful phase. The bone marrow and liver fail as they are infiltrated by activated cells derived from monocytes/macrophages. This typically occurs in patients with a congenital predisposition in which lymphocyte apoptosis is abnormal. The resultant abnormal persistence of the inflammatory response induces excessive monocyte/macrophage activation. (Castillo and Carcillo 2009)

The prototypical anti-inflammatory cytokine is Interleukin 10 (IL10). IL10 inhibits the activity of monocytes, macrophages. It reduces HLA-DR expression and limits production of the pro-inflammatory chemokines IL8, MIP-2, RANTES, MCP-1, MCP-2 and cytokines IL1, IL6, TNFα, IL12 and IL18. (Moore, de Waal Malefyt et al. 2001)

Thus it is a key regulatory cytokine with a function to limit a potentially harmful pro-inflammatory response that may in itself overwhelm the host in its attempt to neutralise infectious threat. (Couper, Blount et al. 2008)

Bearing in mind that the dichotomy in pro- and anti-inflammatory cytokines is crude, this classification may be useful in describing an overall inflammatory state or capacity to respond to threats. Table 1-2 summarizes the primary cytokines in sepsis and injury.(Cavaillon, Adib-Conquy et al. 2003, Perl, Chung et al. 2006)

Cytokine	Function
TNFα	Pro-Inflammatory
MCP1	Pro-Inflammatory
MIP 1β	Pro-Inflammatory
RANTES	Pro-Inflammatory
INFγ	Pro-Inflammatory
IL1	Pro-Inflammatory
IL6	Pro-Inflammatory
IL8	Pro-Inflammatory
TGFβ	Anti-Inflammatory
IL1ra	Anti-Inflammatory
IL4	Anti-Inflammatory
IL10	Anti-Inflammatory

Table 1-2 Cytokines in systemic inflammation

TNFα Tumor Necrosis Factor alpha, MCP1 monocyte chemotactic protein-1, (also known as Chemokine (C-C motif) ligand 2 (CCL2) MIP 1β Macrophage inflammatory protein-1β (also known as Chemokine (C-C motif) ligand 4 or CCL4), RANTES Regulated on Activation, Normal T Cell Expressed and Secreted (also known as Chemokine (C-C motif) ligand 5 (CCL5), INFγ Interferon gamma, IL Interleukin, TGFβ transforming growth factor beta

Cytokines appear in the circulation within hours after a severe insult. There are different time frames for the plasma profile of the different compounds. Initially the systemic inflammatory response syndrome and compensatory anti inflammatory response syndrome were thought to occur sequentially. (Figure 1-6) Longitudinal studies, however, have shown that SIRS and CARS occur simultaneously, albeit maybe in different compartments of the body (Figure 1-7).

After a severe insult TNFα occurs very early and decreases quickly, whereas IL10 may stay elevated over a longer time frame. The circulatory compartment may be hyporeactive to inflammatory stimuli, while simultaneously the tissues might still be in an active inflammatory phase.(Cavaillon and Annane 2006)

CARS may be seen as a reduced capacity to respond to acute inflammatory threats. This state has been also called anergy or immunoparalysis. Monocytes sequester the surface molecule HLA-DR and these monocytes have reduced capacity to respond to noxious ligands, such as LPS. (Docke, Randow et al. 1997) This is relevant to the paediatric population as well. Children with reduced monocyte surface HLA-DR after cardiopulmonary bypass were at risk of developing nosocomial infections.(Allen, Peters et al. 2002)This immunoparalysis may be a target for therapeutic intervention. In a small open label study children with multiple organ dysfunction were given GM-CSF. Monocyte capacity to produce TNFα was restored and the children in the intervention arm suffered significantly less nosocomial infections.(Hall, Knatz et al. 2011)

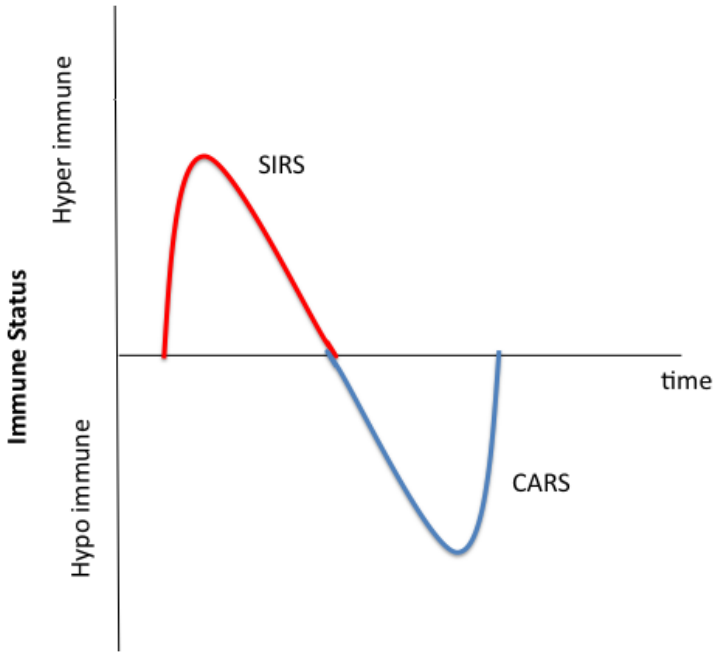


Figure 1-6 SIRS and CARS traditional view

The traditional model of the Systemic Inflammatory Response Syndrome (SIRS) and the Compensatory Anti Inflammatory Response Syndrome (CARS) is of a sequential change from excessive pro-inflammatory to excessive anti-inflammatory state.(Bone 1996)

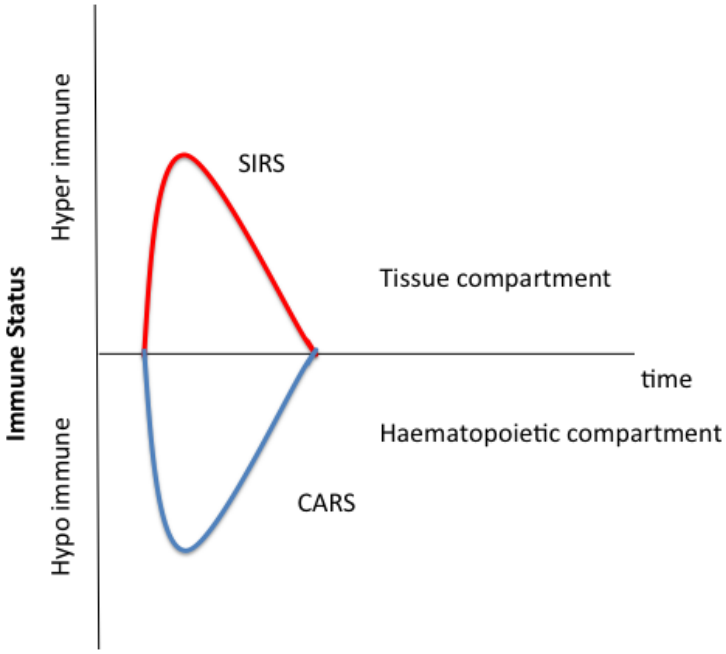


Figure 1-7 SIRS and CARS simultaneous model

The current understanding of the Systemic Inflammatory Response Syndrome (SIRS) and Compensatory Anti inflammatory Response Syndrome (CARS) is that both states occur simultaneously. Different compartments may, however, be in different inflammatory states.(Cavaillon and Annane 2006)

1.5 Crosstalk between systems

1.5.1 Coagulation and platelets

The innate immune system does not operate in isolation. It informs and interacts with the phylogenetically younger adaptive immune system. In addition, coagulation, neuro-endocrine, cardiovascular and autonomic nervous systems all influence, and are influenced by, immune responses. At the simplest level this is shown by many molecules having important properties in multiple systems, e.g. acetylcholine is a neurotransmitter as well as a paracrine regulator of lymphocytes, and epinephrine stimulates the bone marrow to release neutrophils into the circulation.

Several examples of well-characterised "crosstalk" between these systems are outlined below.

Plasminogen activator inhibitor 1 (PAI-1) is a potent inhibitor of fibrinolysis. It achieves this response by inhibiting both tissue and urinary type plasminogen activator.(Lijnen 2005) Levels are increased after trauma and sepsis, especially so in severe meningococcal sepsis.(Hazelzet, Risseeuw-Appel et al. 1996) Inflammatory mediators TNFα, IL-1 and IL-6, complement 5a, and LPS all act to increase PAI-1 production. In turn, PAI-1 contributes to a pro-coagulant state and inhibits neutrophil apoptosis. Although this may help to contain inflammation at the site of infection, genotypes associated with high levels of PAI-1 production are associated with worse outcome in septic shock.(Hermans and Hazelzet 2005) The PAI1 4G/5G insertion deletion promoter polymorphism influences PAI1 plasma concentration such that 4G is associated with higher concentration.(Eriksson, Kallin et al. 1995) This implies a direct link between how readily the immune system triggers an increased clotting tendency in critical illness and a poor outcome.

Similarly, the inflammatory mediator IL-6 stimulates release of the potent coagulation activator tissue factor (TF) from activated endothelial cells, monocytes and macrophages. This promotes thrombin formation, which, in turn, converts fibrinogen to fibrin. Thrombin and fibrin generation are increased in inflammation, in part, because fibrinolysis is impaired due to increased activity of PAI-1, but also secondary to diminished activated protein C (APC) and tissue factor pathway inhibitor (TFPI).(Levi and van der Poll 2008) These processes have been the targets for numerous clinical trials of drugs with anticoagulant / pro-fibrinolytic actions – all aiming to achieve anti-inflammatory effects by targeting coagulation systems. Unfortunately, results have been disappointing. This is most likely a reflection of the complexity of sepsis; it requires more than one pathway to be manipulated to change clinical outcome.

An example of a novel interaction between the coagulation and immune systems is that TLR4-activated platelets interact with neutrophils to trap and kill bacteria in so-called 'neutrophil extracellular traps' (NETs*). In vitro* studies showed that LPS as well as plasma from septic adults could induce this phenomenon.(Clark, Ma et al. 2007) Other investigators have shown platelets to be integral to the immune system. For example, platelet CD40 ligation activated platelet-leukocyte adhesion, which promotes the inflammatory process.(Inwald, McDowall et al. 2003)

1.5.2 The autonomic nervous system

The nervous system is an integral part of inflammation. At a local level, inflammatory responses induce pain- one of the defining elements of inflammation. Regionally, sympathetic and parasympathetic activation will, in general, inhibit inflammation.

Centrally, the hypothalamic-pituitary-adrenal axis has an overarching immunomodulatory role by activating many neuro-endocrine responses.

Importantly, immune cells carry the receptors for neurotransmitters, neuropeptides and neurohormones, including adrenergic and cholinergic receptors. These receptors may respond in an auto- or paracrine manner. Alternatively, the autonomic nervous system and innate immunity may be linked directly by the "cholinergic anti-inflammatory reflex". *In vivo* sepsis models suggest that vagal stimulation may directly dampen TNFα production via NACHR7 receptors on macrophages.(Borovikova, Ivanova et al. 2000)

The sympathetic-immune interface is another area of growing interest. At a local level sympathetic activation may have pro- as well as anti-inflammatory effects. Based on current understanding, the most prominent general effect is inhibition of inflammation. (Elenkov, Wilder et al. 2000) Adrenergic activation via a β2 mediated pathway down regulate pro-inflammatory cytokines TNFα, IL6 and IL1, while upregulating anti-inflammatory cytokines such as IL10.(de Montmollin, Aboab et al. 2009)

As this is a new field and many questions remain unanswered, it is of little surprise that there exists debate about the clinical significance of neural modulation of the immune response. (Sternberg 2006)

One way to measure a modulatory effect of the autonomic nervous system on the immune response is correlating heart rate variability with markers of immune activation. Heart rate variability (HRV) has been shown to correlate with CRP. CRP levels, arguably a measure of systemic inflammation were lower in individuals who showed higher vagal tone as measured by HRV.(Thayer and Fischer 2009)HRV mirrored response to anti-viral therapy in a longitudinal study on hepatitis C.(Osztovits, Horvath et al. 2009) Earlier work had shown that women who had survived a myocardial infarction exhibited higher IL6 levels and concomitant reduced

HRV. (Janszky, Ericson et al. 2004)Both CRP and IL6 serum levels were inversely related to HRV indices in a large cohort of healthy adults. This prompted the authors to state that vagal autonomic activation counteracts inflammation.(Sloan, McCreath et al. 2007)

HRV was not universally found to correlate with an immune response. No association was found with HRV indices and NK cells in response to mental stress.(Owen and Steptoe 2003)Conversely, in an adult cohort of critically ill patients, mortality associated with multiple organ dysfunction (MODS) was correlated with reduced HRV.(Schmidt, Muller-Werdan et al. 2005) It may be that there is a threshold effect in severity of insult or stressor.

1.6 Host factors in the acute inflammatory response

In paediatric critical care all children will exhibit an acute phase response, by nature and/or severity of the insult that required them to be admitted to the PICU. The severity of their morbidity varies not only with severity of insult, but also individually. Premature death from infectious disease is attributable to genetic influences to a higher extent than cardiovascular disease or cancer. In a landmark adoptee study Sorensen showed that the relative risk for premature death (before the age of 50 years) due to infectious disease was 5.81 (95% confidence interval 2.47 to 13.7) (Sorensen, Nielsen et al. 1988). In other words, in 1988 the case was made that susceptibility to severe infectious disease has a strong genetic background. Twenty years later some of the specific genetics has been elucidated, but many question remain. (Opal 2005) Other compelling evidence that the immune response is, at least in part, under genetic control comes from our knowledge of primary immune deficiencies. (Ballow 2008). Conversely, the hereditary periodic fever syndromes such as Familial Mediterranean

Fever are characterized by a too sensitive innate immunity response.(Bodar, Drenth et al. 2009) These rare disorders are increasingly better genetically characterized, but form but a small part of the genetic variability in the general population. One method to further understand hereditary differences is the candidate gene approach. With the advent of methods to identify subtle changes in genes coding for components in the innate immune system it became possible to search for specific genetic predisposition to an inappropriate innate immune response. Gene variations occur as single nucleotide polymorphisms (SNPs), insertion or deletion or copy number variation.

1.6.1 Candidate gene study- Single Nucleotide Polymorphisms

Genetic variability is, for a large part, made up of Single Nucleotide Polymorphisms (SNPs). (Wang, Fan et al. 1998) A SNP is a variation in a gene at 1 base pair. Figure 1-8 shows an example.

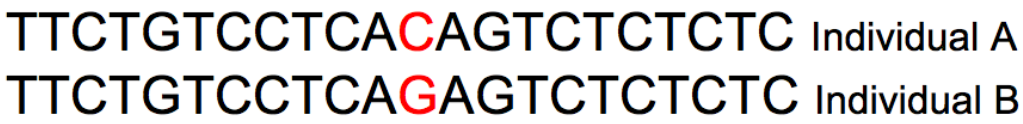


Figure 1-8 Single Nucleotide Polymorphism

Nucleotide sequence differs at the nucleotides printed in red. T denotes Thymidine, C Cytidine, G Guanosine, and A Adenosine.

In this example individual A carries a C allele and individual B a G allele. Dependent on where the SNP is located in the gene, this may or may not have functional consequences to the gene product. The protein itself may be altered, or it may result in a change in the amount of protein production, or there may be no discernible change at all. The latter category is still of interest, as this type of SNP might be in linkage disequilibrium with a SNP that does have an impact on protein synthesis.

1.6.2 Gene structure examples

A good example of a gene with several polymorphisms is *MBL2*. **Figure 1-9** shows a schematic of the gene with polymorphisms in the promoter region and in exon 1. These changes give rise to differences in expression of the protein and structural changes that interfere with the subunits forming hexamers.

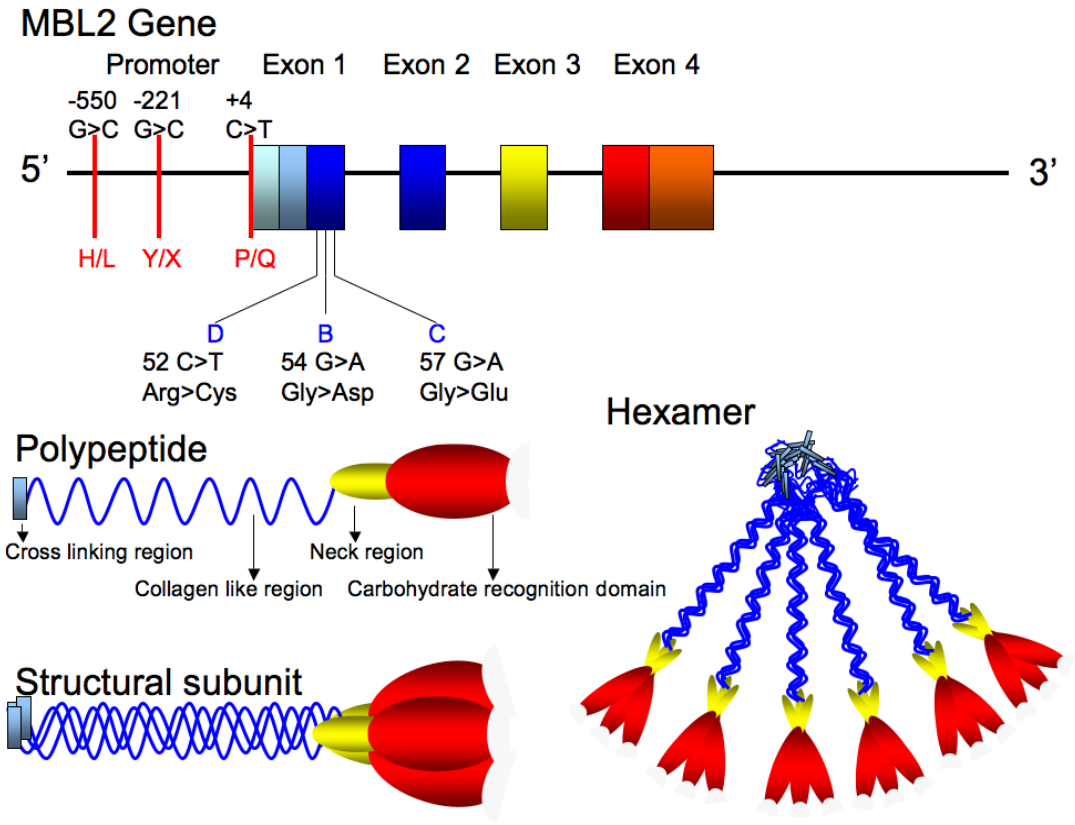


Figure 1-9 MBL2 gene and gene products

The *MBL2* gene consists of a promoter region and four exons. The promoter region holds three polymorphisms as does exon 1. The gene product is a polypeptide that conforms to a structural subunit, which in itself organises itself in multimers.

1.6.3 SNPs in Critical Illness

Early work on genetic polymorphisms showed that a high TNFα and low IL-10 pattern, suggesting a vigorous pro-inflammatory potential, was associated with better outcome in meningococcal disease.(Westendorp, Langermans et al. 1997) Conversely, more recent work suggested that a polymorphism associated with high TLR1 mediated inflammatory cytokine production was strongly associated with increased mortality and organ dysfunction in sepsis.(Wurfel, Gordon et al. 2008)

Genetic association studies in areas with more uniformity in disease definition and better understanding of the pathophysiology of disease have isolated some effects of polymorphisms. For instance, NOD2 variations are clearly associated with an increased susceptibility to Crohn's disease.(Hugot, Chamaillard et al. 2001) Interestingly, in adults, this same mutation was shown to increase risk for bacteraemia, especially in combination with TLR4 variants.(Henckaerts, Nielsen et al. 2009) Although of dubious methodological rigour this finding was mirrored in children.(Tekin, Dalgic et al. 2012)

The innate immune system is complex with many redundancies. It may be unrealistic to identify those parts that have a discernible effect on outcome in the complex outcome called critical illness by analysing variance in individual mediators. This is effectively the same issue that has confound randomized clinical trials in severe sepsis.(Dellinger, Vincent et al. 2008) Given these issues, design and execution of genetic association studies need to be held against robust and reproducible end-points and rigorous quality markers.(Clark and Baudouin 2006) (Little, Higgins et al. 2009) Despite all these difficulties, there are some observations that contribute to our understanding of innate immune variability in critical illness. The following is a sample of studies that illustrate some of these issues.

A recent systematic review and meta-analysis showed susceptibility to pneumococcal disease with mannose binding lectin (MBL) deficiency (odds ratio 2.57(95% CI 1.38-4.8) but not for the single TLR2 polymorphism Arg753Gln. It may be that complement activation is a more important part of innate immunity than TLR2 or that this specific TLR2 polymorphism does not exert a strong enough difference in response. Similarly, no evidence was found for a susceptibility to meningococcal disease for TLR4 Arg299Gly or the PAI1 4G/5G insertion deletion promoter polymorphism. (Brouwer, de Gans et al. 2009) However, the PAI1 4G/5G polymorphism was shown to influence the *severity* of disease in meningococcal septic shock: children who were homozygous for the high producing 4G were more likely to have vascular complications or to die. (Haralambous, Hibberd et al. 2003) It may be more interesting to study the influence of genetic differences on the specific pattern of disease severity rather than the overall outcome which reflects the sum of so many pathophysiology processes.

Genetic variation does not necessarily confer an increased risk for susceptibility or severity of disease. It may well exert a beneficial effect. The adaptor molecule Mal (TIRAP) may be one such an example. This molecule is an essential part of TLR2 and TLR4 downstream signalling. In a large multi-ethnic cohort (n= 6106) the effect of Mal polymorphism S180L was studied with respect to invasive pneumococcal disease, malaria and tuberculosis. (Khor, Chapman et al. 2007) Heterozygosity was associated with reduced disease severity. The authors postulated that this was due to reduced NFκB medicated cytokine response. Homozygous variant allele carriers would thus mount an inadequate antimicrobial response and wildtype carriers would mount an excessive response.

A better-known heterozygote protection against infectious disease is sickle cell trait that protects against malaria. Homozygous sickle cell anaemia however predisposes to

increased susceptibility to infectious disease. Children with sickle cell anaemia were 25 times more likely to present with bacteremia than control subjects in a recent study from sub-Saharan Africa.(Williams, Uyoga et al. 2009)

There are many studies of genetic variability in critical illness but very few provide compelling evidence of genetic variation influencing outcome. One reason may be the complexity of the clinical phenotype: i.e. heterogeneity in patients, grouped as "SIRS", "sepsis", or "ARDS". These definitions may be insufficiently precise in clinical critical illness where patient differ widely in, for example, the contribution of chronic disease to the acute state. (Marshall 2008)

1.7 Outcome - Challenges in measuring the acute phase response

Given that the acute phase response is a complex dynamic physiological state it is a challenge to define it accurately. One possibility is the international consensus definition of "SIRS".(Levy, Fink et al. 2003) The Systemic Inflammatory Response Syndrome (SIRS) was defined for adults according to parameters outside the norm for heart rate, respiratory rate, temperature and white cell count. This configuration of clinical findings is derived from a consensus meeting between a small group of critical care investigators. It discriminates poorly and its use has been debated heavily. To date, however, there is no superior measure.(Marshall 2008) Validity of the SIRS construct increased with the findings of a genetic basis for a predisposition to SIRS.(Carcillo 2004)

Attempts to better delineate the clinical SIRS phenotype include molecular techniques such as gene array analysis (Johnson, Lissauer et al. 2007). These are time consuming and costly modalities. Importantly, the complex interplay of immunology and autonomic nervous system is not reflected in these techniques. Rather than trying to

answer the question by looking at inflammatory mediators a different approach is to focus on features that reflect the sum of these components. One potential method is physiological signal analysis. Physiological signals such as heart rate are dynamic and reflect physiological states. Heart rate variability (HRV) is the dynamic of beat-to-beat changes in heart rate. HRV metrics are thought to be a reflection of health in general (Goldberger 2001) as well as acute inflammatory disease states (Gang and Malik 2002).

1.7.1 Physiological signal analysis- Heart Rate Variability

Heart rate variability is the normal beat-to-beat variation in heart rate. Changes in this normal variation occur with aging and disease. There exist many metrics to describe HRV, most of which have been applied in critical care medicine. (Bravi, Longtin et al. 2011) For the purpose of this thesis, the application of HRV is limited to one metric, i.e. power spectral analyis. Power spectral analysis quantifies heart rate variability across the frequency spectrum. This measure has been shown to identify and predict sepsis in several patient cohorts (Ellenby, McNames et al. 2001, Moorman, Lake et al. 2006, Ahmad, Ramsay et al. 2009) and predict mortality in trauma (Norris, Morris et al. 2005).

1.8 Focussed Therapeutic Interventions

Numerous attempts have been made to intervene in the acute response to infection either with agents that block elements of the acute response or those which add endogenous or innate immune or anticoagulant molecules (e.g. recombinant BPI (Levin, Quint et al. 2000) or activated protein C (Goldstein, Nadel et al. 2006). None have shown convincing evidence of benefit. There are powerful arguments that even

very effective agents would have failed in the methodology of inclusive randomised clinical trials because of wide variability in inclusion severity criteria and control group mortality between centres.(Eichacker, Parent et al. 2002) (Wong, Cvijanovich et al. 2008) It may be that it is just not feasible to intervene with a single agent in the complex system with the redundancy as discussed above. Or it may be that anti-inflammatory drugs cause harm to many patients who were already at very low risk of bad outcomes. One way forward would be to characterise the inflammatory and coagulation responses in individual patients and target intervention to the specific needs of that patient.

1.9 Aims of this thesis

This thesis addresses two areas of investigation into the acute phase response in children. The first is a prospective observational cohort study taking a candidate gene approach on the influence of genetic polymorphisms on the acute phase response. The second is a pilot study characterizing the acute phase response in terms of heart rate variability as an integrated biomarker of systemic inflammation.

Chapter 3 aims to describe potential pitfalls in interpreting results from a large genetic association study.

Chapter 4 aims to identify variation in the acute phase response associated with endotoxin recognition.

Chapter 5 describes variation in complement activation as a reason for variation in systemic inflammation.

Chapter 6 specifically probes the interplay between MBL genotype and phenotype in systemic inflammation.

Chapter 7 aims to distinguish infective from sterile systemic inflammation based on physiological signal analysis.

Chapter 2 Materials and General Methods

2.1 Clinical methods

2.1.1 Recruitment

The Great Ormond Street Hospital for Children NHS Trust / Institute of Child Health and Southampton & South West Hampshire Local Research Ethics Committees approved this study. Written parental informed consent was obtained in accordance with the Declaration of Helsinki guidelines(Association).

2.1.2 Inclusion and exclusion criteria

Children aged 0 to 18 years with at least one organ system failure for 12 hours or longer (or death within 12 hours of admission).

In order to identify a cohort that would allow for potential underlying genetic differences to become apparent mild severity of illness and known causes for a more severe illness course were excluded. Thus children were excluded in the following instances: anticipated short stay on ICU (<24 hours) or multiple congenital abnormalities, known immunodeficiency, haematological or lymphoid malignancies, systemic immunosuppressive drug therapy other than corticosteroids, known severe central neurological or neuromuscular disease, persistent pulmonary hypertension of the newborn, weight less than 2.2 kg, corrected gestational age less than 37 weeks and non-accidental injury. Multiple congenital abnormalities, pulmonary hypertension and known immunodeficiency were permitted in the subset of children admitted for cardiac surgery if they were part of the congenital heart disease phenotype.

2.1.3 Recruitment centres

The majority of this work pertains to patients recruited from Great Ormond Street Hospital for Children NHS Trust general PICU in London, UK. Other recruitment areas are the cardiac PICU (CICU) at Great Ormond Street Hospital for Children NHS Trust, the PICU at Southampton General Hospital NHS Foundation Trust and the Wessex Cardiothoracic Unit at Southampton General Hospital NHS Foundation Trust.

2.1.4 Recruitment time periods

Subjects were recruited consecutively in three recruiting time-periods from three tertiary paediatric intensive care units between 2000 and 2006 and one tertiary adult cardiothoracic unit prior to 2002, see Table 2-1. The time periods are discontinuous due to availability of research staff. Recruiters were research nurses and critical care physicians involved in the project

Population	Time period 1	Time period 2	Time period 3
GOSH General	Feb 2002-Dec 2002	March 2003-June 2004	May 2005-Dec 2006
GOSH Cardiac[a]	Jan 2000-July 2002	March 2003-Dec 2006	
Southampton PICU[a]			Nov 2005-Nov 2006
Southampton Adult[a]	Prior to 2002		

Table 2-1 Recruitment time periods

[a]Part of the TLR4 gene association study only; GOSH Great Ormond Street Hospital for Children NHS Trust.

2.1.5 Blinding

Clinical variables were collected while blinded to the genotype or physiological data and vice versa.

2.2 Population definition

2.2.1 Admission diagnosis

The on-call admitting consultant PICU clinician made the admission diagnosis. On the basis of this primary diagnosis, children in the study were then allocated to the groups '*Bypass'* (elective cardiac surgery requiring cardiopulmonary bypass), '*Infection'* and '*Non-infection'* (including elective general or non-bypass cardiac surgery, trauma, and other). Diagnoses were verified at a later time point.

2.2.2 Ethnicity

Participants came from three tertiary paediatric intensive care units in South East England, UK. The demographics of the units differ as regards to the population they serve. One hospital (Great Ormond Street Hospital for Children) serves as an international, national and regional referral centre for specialist paediatric care. The other recruiting hospital (Southampton General Hospital, Southampton, UK) has a regional function only.

It was thus expected that the ethnic background would be inhomogeneous. To account for this possible confounder ethnicity data were collected. This was done according to parental self-reported ethnic heritage as routinely collected in the National Health Service in the UK and then recoded according to the continental classification (Risch, Burchard et al. 2002) as adopted by the Hapmap consortium (2003).

2.2.2.1 Population stratification

The population in this study reflects a predominantly urban population with high level of immigration. This population is thus expected to be heterogeneous. The frequency of many genetic variants is not equal in all parts of the world.(Bamshad, Wooding et al. 2004) Population stratification is the observation of a systematic difference in polymorphic allele frequencies between sub-populations in a given cohort due to differences in ancestry. Identification of sub-populations based on ancestry may be done based on self-reported ethnicity or based on a variety of genetic markers, such as microsatellites(Bowcock, Ruiz-Linares et al. 1994, Rosenberg, Pritchard et al. 2002) *Alu* insertion polymorphisms (Bamshad, Wooding et al. 2003) or SNPs (Li, Absher et al. 2008).

If the risk of disease varies between ethnic groups, any other ethnically linked characteristic, either genetic or environmental, will appear to be associated with the disease. Controlling for ethnicity is one way to reduce bias. Interestingly, it was found that with increasing numbers of ethnic groups bias tended to decrease rather than increase, despite expected larger differences in allele frequency.(Wacholder, Rothman et al. 2000)

2.2.2.2 Definition of ethnicity

In the absence of genetic ancestral markers a surrogate measure for ancestry is self-reported ethnicity. Researchers have used the terms race and ethnicity interchangeably to denote an individual's heritage in studies focussed on health risk. Neither race nor ethnicity, however, are terms with generally agreed upon definitions. There are

complex connotations that reflect culture, history, socio-economic, and political status and ancestral geographic origins.(Long and Kittles 2003) The current understanding is that all human populations come from a common ancestral group, geographically located in East Africa, with subsequent migration to Eurasia and the Americas. (Goldstein, Ruiz Linares et al. 1995, Long and Kittles 2003) Differences occurred as a consequence of history and geography. Generally speaking, the human genetic variation encountered outside Africa is a subset of variation seen within Africa. With increasing geographical distance from Africa there is a decrease in heterozygosity and population specific alleles. African populations show higher genetic variation and less linkage disequilibrium (non-random association of alleles) than others.(Reich, Cargill et al. 2001, Tishkoff and Williams 2002)

Nevertheless, most variation, approximately 85%, is seen within populations, rather than between them.(Lewontin 1972, Rosenberg, Pritchard et al. 2002, Serre and Paabo 2004)

Notwithstanding the above, it is possible to cluster populations by geographic region and assign individuals to predefined group based on continental geography. Using 377 microsatellite DNA markers in 52 global populations it was possible to cluster individuals in five groups that correspond to geographical regions: Africa, Middle East and Europe, Asia, Oceania and Americas. It was also noted that high levels of inbreeding and genetic drift could lead to clustering, as exemplified by a sixth cluster specific to one Pakistani population. Individuals from populations from geographically intermediate locations showed partial membership in more than one cluster. Thus populations are more of a continuum rather than discrete entities. (Cavalli-Sforza 1994, Serre and Paabo 2004)

If all four grandparents came from the same part of the world, based on genetic variation, one can predict an individual's geographical origins to the geographical regions Africa, Europe, East Asia, Oceania-Pacific and the Americas as used by the Hapmap project. In many cases this also correlates with self-identified race or ethnicity.(Rosenberg, Pritchard et al. 2002)

The categorisation of people into continental ancestry is thus possible, but is more crude than the continuum that is closer to reality. For the purpose of analysing the data taking into account the different allele frequencies at sub-population level the study cohort was divided into continental ancestry groups.

In order to differentiate within this population self-reported ethnicity was used as a surrogate marker for genetic ancestry. Although some studies have shown a good correlation between self-reported ethnicity and geographical ancestry (as opposed to current geographical location) (Tang, Quertermous et al. 2005) there remains the real possibility that is not the case in the current study. The self-identified ethnic categories as reported in the NHS are imperfect proxies for geographical ancestry as they also contain self-identified culture, history, socio-economic, and political status. As such it is a socially determined categorisation and may not reflect genetic diversity. Each population has a unique genetic and social history. Individuals carry within them the genetic result (allele frequency) of ancestral patterns of migration, mating practices, reproductive bottlenecks and expansions and stochastic variation. Admixture occurs in varying degree not only due to geographical distance, but also socio-cultural barriers. The effect of admixture is compounded when the disease of interest is more prevalent in one subgroup within a given population, because alleles that are more prevalent in this subgroup can appear to be associated with the disease, even when unlinked to the disease promoting variant. Thus confounding or population

stratification needs to be guarded against in case-control studies. This is especially so as the many categories as collected routinely in the UK NHS (based on the UK 2001 census) have been paired down to four categories in the current study.

2.2.2.3 Functional polymorphisms across ethnicities

Most polymorphisms examined in the above-mentioned studies were neutral, non-functional. Functional polymorphisms also differ in frequency between groups. These differences may be larger, because functionality infers some natural selection.(Bamshad, Wooding et al. 2004) Examples of such monogenic traits that differ across populations in frequency are Δ508-CFTR (cystic fibrosis) (Schrijver, Oitmaa et al. 2005), HbS (sickle cell anaemia)(Streetly, Latinovic et al. 2010) and HEXA 1278insTATC (Tay Sachs disease)(Bach, Tomczak et al. 2001). Other functional polymorphisms differ in frequency across populations with less well-known relationships between functionality and differences in morphology or morbidity. NOD2/CARD15 polymorphisms have been associated with Crohns Disease in Caucasians, but not in African-American and Hispanic children. (Kugathasan, Loizides et al. 2005) Similarly, the ACE I/D polymorphism is in linkage disequilibrium with the functional gene responsible for different levels of ACE in European populations (Rigat, Hubert et al. 1990), but not in individuals of African descent (Payne, Dhamrait et al. 2007). The frequency of common and private polymorphisms differed widely in a mixed population in the US, consisting of self-identified African, Asian, Latino/Hispanic, and European Americans. (Guthery, Salisbury et al. 2007) Therefore, knowledge of geographical ancestry is of importance when assessing presumed functional SNPs in relation to health outcomes. There remains, however, a requirement to substantiate differences based on ancestry in a

robust scientific way. Given our social history where science has been used to rationalize discrimination between human groups one should be aware of the social ramifications in the nuances of reporting findings, including the possibility of perpetuating racism. (Knerr, Ramos et al. 2010)

A different approach is to ignore (self-assigned) ethnicity and use microsatellite markers to cluster individuals into groups of homogeneous genetic structure by means of a computer programme named STRUCTURE.(Wilson, Weale et al. 2001).

One method is to genotype a population for a panel of unlinked ancestry informative SNP markers, to adjust for spurious associations caused by stratification. Allele frequencies, which differ substantially between ancestral populations can then be used to detect, quantify and correct hidden population structure when relying on self-identified ethnicity. (Shriver, Mei et al. 2005) This analysis was not done because of the added time and expense that these methods would incur.

2.2.2.3.1 UK 2001 Census definitions of ethnic groups

The United Kingdom 2001 Census broke down the population into the following 16 discrete groupings:(2008)

White

- British
- Irish
- Other white

Mixed

- White and Black Caribbean
- White and Black African
- White and Asian

Other mixed

Asian or Asian British

Indian

Pakistani

Bangladeshi

Other Asian

Black or Black British

Caribbean

African

Other Black

Chinese or other ethnic group

Chinese

Other ethnic group

As of April 2001 the National Codes were introduced into the NHS Data Model and Dictionary under Data Set Change Notices DSCN 2/2001 in accordance with the ONS 2001 Population Census Ethnic categories. The basis of this categorisation in the NHS was to follow the government standard. Thus it is a categorisation for administrative reasons. Local NHS trusts may add more detailed ethnic categorisation, however, these still need to fit in the larger categories above. (Department of Health 2005)The Great Ormond Street Hospital for Children NHS Trust (GOSH) has broadened ethnic categorization to include those as depicted in Table 2-2. This categorization was then translated to the continental division.(Risch, Burchard et al. 2002)

GOSH Categorisation	**Continental Ancestry**
White	Caucasian
White British/UK	Caucasian

White Irish	Caucasian
White European	Caucasian
White Other	Caucasian
Turkish/Turkish Cypriot	Caucasian
Jewish	Caucasian
Maltese	Caucasian
Greek/Greek Cypriot	Caucasian
Mixed	Other/Mixed
White and Black Caribbean	Other/Mixed
White and Black African	Other/Mixed
White and Asian	Other/Mixed
White and Black Other	Other/Mixed
Asian	Caucasian
Indian	Caucasian
Bangladeshi	Caucasian
Pakistani	Caucasian
Other Asian	Asian
Black	African
Black Caribbean	African
Black African	African
Black British/UK	African
Black - Other	African
Chinese/Other	Asian
Middle Eastern/Arab States	Caucasian
Arab	Caucasian
Japanese	Asian
North African	Caucasian
Other Ethnic Group	Other/Mixed

Table 2-2 Translation of ethnicity classification

GOSH Great Ormond Street Hospital

2.3 Clinical severity scores

Measuring severity of critical illness is essential for phenotype description. It allows for comparison and benchmarking. The scores that can be used across the spectrum of critically ill children are the mortality prediction scores Paediatric Index of Mortality (PIM) (Shann, Pearson et al. 1997), PIM2 (Slater, Shann et al. 2003) and Pediatric Risk of Mortality (PRISM) III (Pollack, Patel et al. 1996). For organ dysfunction scores there exist the Pediatric Logistic Organ Dysfunction score (PELOD) (Leteurtre, Martinot et al. 1999) and the Paediatric-Multiple Organ Dysfunction Score (P-MODS) (Graciano, Balko et al. 2005).

The PIM and PIM2 scores were validated in Australia, New Zealand and the United Kingdom; the PRISM III score is validated in the USA.

The only validated organ dysfunction score is PELOD, with participating centres from France, Switzerland and Canada. (Leteurtre, Martinot et al. 2003)

PIM2 is based on a combination of type of illness and admission, generating low risk and high risk categories in addition to physiological and intervention data in the first hour of encounter with a critical care team, see Table 2-3. (Slater, Shann et al. 2003)

Item	Response
Elective Admission	Yes/no
Recovery post procedure	Yes/no
High risk diagnosis	Yes/no
Low risk diagnosis	Yes/no
Pupils fixed and >3mm	Yes/no/unknown
Mechanical ventilation	Yes/no
Systolic blood pressure	Value
Base Excess	Value
FiO_2*100/PaO_2	Value

Table 2-3 PIM2 score

The PIM2 score gives a value of 1 to "yes" answers and a 0 for "no or unknown". High risk diagnoses are cardiac arrest preceding ICU admission, severe combined immune deficiency, leukaemia or lymphoma after first induction, spontaneous cerebral haemorrhage, cardiomyopathy or myocarditis, hypoplastic left heart syndrome, HIV infection, liver failure is the main reason for ICU admission, neuro-degenerative disorder. Low risk diagnoses for the main reason for ICU admission are asthma, bronchiolitis, croup, obstructive sleep apnoea and diabetic keto-acidosis.

The predicted death rate is calculated with the following equation:

Logit = (-4.8841) + (values * Beta) + (0.01395 * (absolute (SBP-120))) + (0.1040 * (absolute base excess)) + (0.2888 * (100*FiO2/PaO2))

Predicted death rate = $e^{Logit}/ (1+e^{Logit})$ (Slater, Shann et al. 2003)

PELOD gives a score of 0, 1, 10 or 20 for criteria in domains of organ dysfunction, see Table 2-4.

The score has been criticized for being discontinuous. Because it is not possible to have a score between 14 and 20 or between 24 and 30 there is no prediction of mortality between 3.1% and 16.2% or between 40% and 80% respectively.(Garcia, Eulmesekian et al. 2006). The PELOD score has nonetheless performed well in several cohorts in determining severity of illness (Leteurtre, Martinot et al. 2003, Leclerc, Leteurtre et al. 2005).

Domain	Maximum Potential Score
Cardiovascular	20
Heart rate	10
Blood pressure	20
Neurologic	20
GCS	20
Pupillary reaction	10
Pulmonary	10
PaO_2/FiO_2	10
$PaCO_2$	10
Mechanical ventilation	1
Haematologic	10
WBC count	10
Platelet count	1
Renal	10
Serum Creatinine	10
Hepatic	1
SGOT (AST)	1
Prothrombin Time/ INR	1

Table 2-4 PELOD score

GCS Glasgow Coma Scale, WBC White Blood Cell, SGOT serum glutamic oxaloacetic transaminase, AST Aspartate transaminase, INR International Normalised Ratio. Heart rate, blood pressure and serum creatinine are scored according to age. The scores corresponding with the variables are then entered into the following equation: Logit = -7.64 + 0.30*(PELOD) . Predicted death rate = $1 / (1 + e^{-Logit})$. (Leteurtre, Martinot et al. 2003)

This study uses the PIM, PIM2 (as appropriate for recruitment time period) and PELOD to score risk of mortality and severity of organ dysfunction respectively.

2.4 Definition of outcome measures

The Systemic Inflammatory Response Syndrome (SIRS) was defined according to the most recent consensus statement for adults (Levy, Fink et al. 2003) and amended for paediatric normal values (Fidler, Wilson et al. 2004). The four variables that are taken into account are white cell count, temperature, heart rate and respiratory rate. SIRS is defined as ≥ 2 variables outside age specific values, one of which has to be either temperature or white cell count as follows:

White cell count > $12x10^9$ or < $4x10^9$;

Temperature >38°C or < 36 °C;

Heart rate: newborn to 3 months 95-145/min, 3-12 months 110-175/min, 1-3 years 105-170/min, ≥3-7 years 80-140/min, ≥7-10 years 70-120/min and ≥10 years 60-100/min.

Respiratory rate was not included, given the high number of children who are ventilated on the GOSH PICUs.

Paediatric specific criteria for SIRS and sepsis were published in 2005 (Goldstein, Giroir et al. 2005). The above criteria, however, were maintained for two reasons. First, to be able to build on work already done in this area (Fidler, Wilson et al. 2004, Stephens, Fidler et al. 2006) and second because there was no experience with these amended criteria.

Children with SIRS and proven (with microbiological evidence) or suspected infection (clinical diagnosis and antibiotic treatment in line with sepsis) were classified as sepsis.

The differences that occur between these definitions are depicted in Figure 2-1, Figure 2-2, and Table 2-5.

Generally speaking, heart rate, white cell and temperature cut off points are more liberal in the 2005 criteria, thus it is more difficult to attain a definition of SIRS based on heart rate, white cell count and temperature.

For some children only white cell count and heart rate were available to score as abnormal because their temperature was artificially controlled and the respiratory score was not taken into account for any of the children. This therefore means that scoring SIRS was set at a higher threshold.

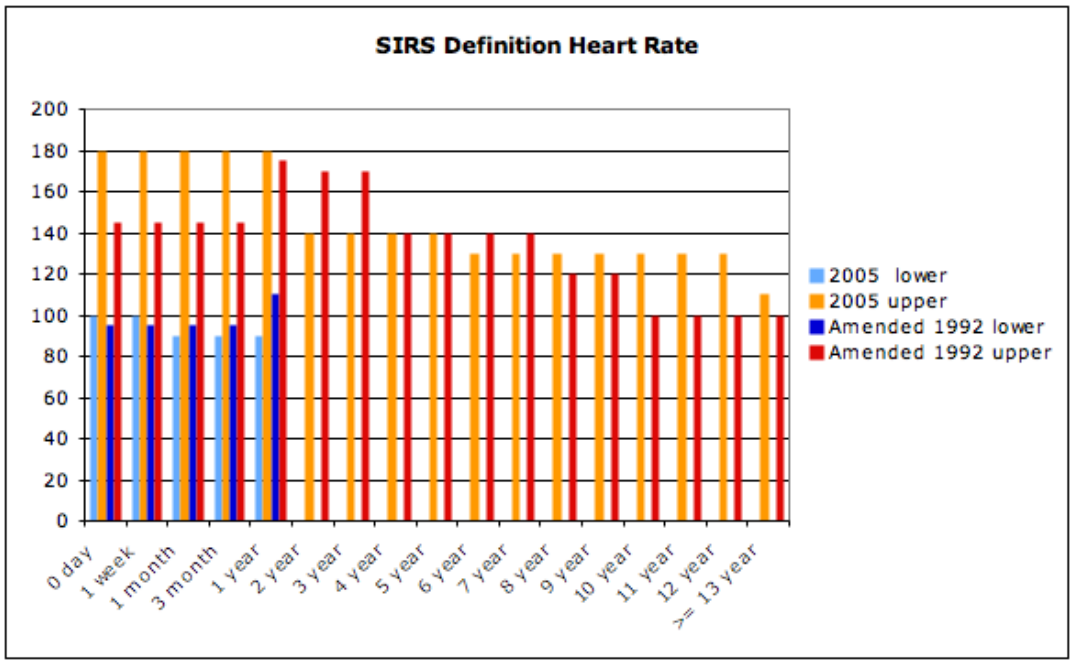


Figure 2-1 SIRS definitions heart rate values

SIRS denotes Systemic Inflammatory Response Syndrome. Heart rate values by age for the two definitions of SIRS. 2005 refers to (Goldstein, Giroir et al. 2005); Amended 1992 refers to (Fidler, Wilson et al. 2004) and (Bone, Sibbald et al. 1992)

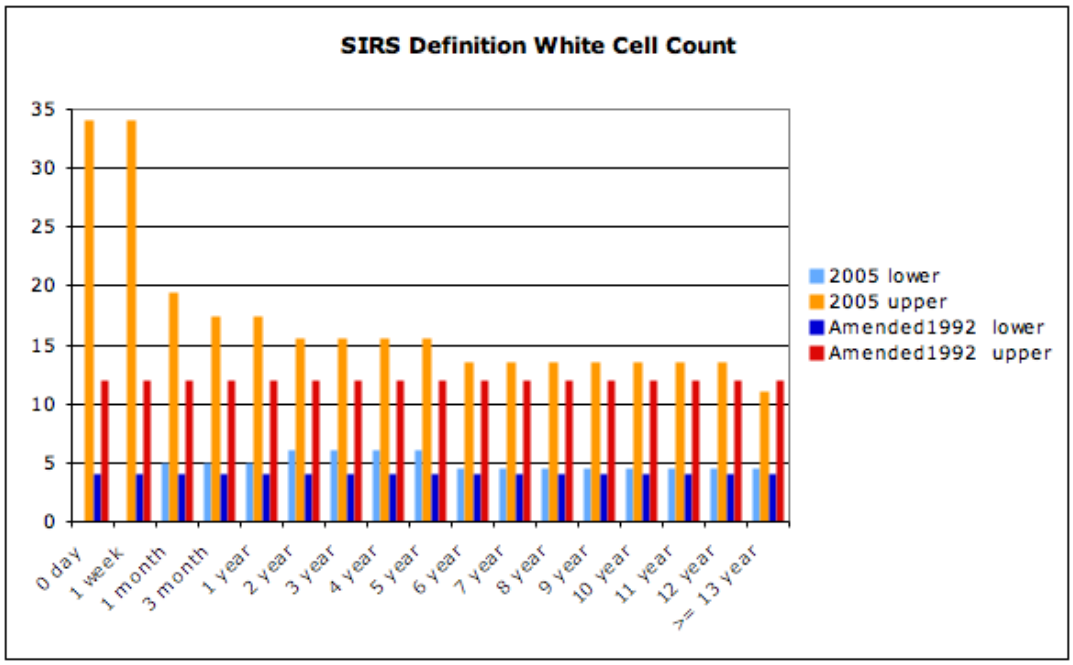


Figure 2-2 SIRS definitions white cell count

SIRS denotes Systemic Inflammatory Response Syndrome. White cell count values by age for the two definitions of SIRS. 2005 refers to (Goldstein, Giroir et al. 2005); Amended 1992 refers to (Fidler, Wilson et al. 2004) and (Bone, Sibbald et al. 1992)

Criterium	Amended 1992	2005
Temperature	> 38°C or < 36 °C	> 38.5°C or < 36 °C

Table 2-5 SIRS definitions temperature

SIRS denotes Systemic Inflammatory Response Syndrome. Temperature values are given for the two definitions of SIRS. Amended 1992 refers to (Fidler, Wilson et al. 2004) and (Bone, Sibbald et al. 1992); 2005 refers to (Goldstein, Giroir et al. 2005)

Platelet count data on admission was defined as the first platelet count result after admission to the intensive care unit (paediatric data) or prior to angiography (adult data). These data were extracted from the electronic medical records.

2.5 Clinical database

Detailed and accurate phenotypic data on each patient were essential for interpreting the results. This included patient demographic data, clinical and laboratory diagnostic results and daily review of the Intensive Care bedside electronic charting system (Care Vue, Hewlett Packard, USA).

Patient data were entered and stored in a purpose designed password protected Access database (Microsoft Office'98/XP) according to the data protection policies of University College London and Great Ormond Street Hospital. Two research nurses and one clinician entered the data prospectively.

For quality control purposes, a random sample of 5% was audited by one of the research nurses. All entries for heart rate, temperature and white cell count were twice checked against SIRS criteria. First platelet count on admission to PICU was verified before final analysis.

All were blinded to genotype and heart rate variability data.

2.6 Laboratory methods

2.6.1 Blood sample collection and processing

Blood (2 to 3 mls) was collected from subjects from an indwelling intravenous or intra-arterial catheter. The sample was divided into ethylenediaminetetraacetic acid (EDTA) and plasma collection tubes. Whole blood was stored at -80°C until DNA extraction. Plasma samples were spun immediately, aliquoted into two nunc tubes and frozen at -80°C until analyzed.

2.6.1.1 Preparation of samples for genotyping

Samples were stored at -80°C as whole blood in EDTA until processed. DNA was extracted from thawed whole blood according to the manufacturer's instructions (QIAamp DNA blood midi kit, Qiagen, Crawley, UK). DNA collected at Southampton PICU (analysed for TLR4 D299G only) was extracted from blood by a simple salt extraction method (Miller, Dykes et al. 1988).

After extraction DNA samples were resuspended in 450 μl sterile water and frozen at -80°C. These samples constituted the DNA library.

Samples previously prepared as above, resuspended in sterile water and frozen at -80°C were defrosted (PICU samples 1-147; CICU samples array A and B).

DNA content was assessed by spectroscopy (Digilab Hitachi U-1800 spectrophotometer). From the DNA library sample 20 μl was diluted with 480 μl water and read at 260 nm (DNA) and 280 nm (total protein) absorbance. The expected optical density (OD) 260:280 ratio for DNA lies between 1.8 and 2.0. (Glasel 1995)

For each well the DNA content was calculated with the formula:

[DNA] in ng/μl= OD (at 260 nm) x dilution (1:20) x 50

assuming that one OD unit at 260 nm equals 50 ng of double-stranded DNA/μl.

DNA content was standardized to 250 µl 15 ng/µl in 96 well stock arrays. From these stock arrays, 96 well working arrays were made at 50 µl 15 ng/µl.
Genotyping was done from these working arrays. This process minimized contamination and the number of freeze-thaw cycles of the DNA library.

DNA storage was done as follows:

1. DNA library in nunc tubes at 450 µl (accessed twice only, once to determine DNA content and once to create stock arrays)
2. Stock array DNA at 15 ng DNA/µl in volume of 250 µl per well in 96 well array
3. Working 96 well array at 15 ng DNA/µl in volume of 50 µl per well

2.6.2 Reagents and materials

Name	Company	Product code
MBL ELISA kit	Bioporto Diagnostics	KIT 029
MilliQ Water from Millipore Q plus purification system	Millipore	
Nuclease free water	Severn Biotech	20-9000-01
QIAMP DNA midikit extraction kit (100)	Qiagen	51185

Table 2-6 Media and kits

Name	Company	Product code
ELISA plate reader Multiscan EX	Thermo Electron Corporation	
Laptop NX260X	Gateway	0037264721
Laptop M675	Gateway	0034386339
Patient Monitor	Philips	Merlin CMS
RS232 board	Philips	M1170A
Socket serial card dual PC PCMCIA RS232 I/O	Socket	SOC0703081 Mfr port # SL0703-081
Socket serial cable	Socket	Mfr # 8100-00016
Spectrophotometer	Digilab Hitachi	U-1800
Spectrophotometer	Labtech International	Nanodrop ND-1000 v 3.1.0
T25-pin RS232 serial adapter cable	Oregon Electronics	

Table 2-7 Equipment

2.7 Candidate gene study

One method to study genetic variability in relation to the host response is the candidate gene approach. The premise is that an association between an allele and a phenotype is due to either a functionality of the genetic polymorphism itself or it is in tight linkage disequilibrium with a polymorphism with a functional effect. Linkage disequilibrium is the extent to which alleles at two or more loci are associated with each other. The third possibility is that of confounding or selection bias.(Silverman and Palmer 2000) A major concern is the possibility of random chance leading to a false association between genetic polymorphsims and phenotype. Candidate gene studies therefore require rigorous statistical analysis.

2.7.1 Background

Patients vary in their susceptibility and response to injury and infection. This variation is explained, in part, by age, gender, underlying chronic illness or environmental factors such as crowding or pathogen virulence. Genetic heterogeneity is one aspect that may explain some of the observed variation in presentation and course of illness. Premature death due to infection was found to have a strong genetic component, even more so than cardiovascular disease. The relative risk for an offspring to die of an infectious cause if their parent had died prematurely (before the age of 50) of an infectious disease was 5.81 (95% confidence interval 2.47 to 13.7). This compared with 4.52 (1.32 to 15.4) for cardiovascular and cerebrovascular causes. (Sorensen, Nielsen et al. 1988)This earlier work was updated recently.(Petersen, Andersen et al. 2010)

The next step is to identify the specific genes and genetic variability that may underlie this hereditability. The mapping of the human genome and its variation allows potential identification of specific genetic variability in the context of disease.(2003)

2.7.2 Number and variation in genes

The number of candidate genes is immense. Humans are estimated to be 99.9% identical at the DNA sequence level. Initially it was estimated that the number of genes encoding for proteins was about 30.000 (approximately twice that of a worm)(Lander, Linton et al. 2001) Only recently has the ENCODE project elucidated that the human genome contains about 21,000 protein-coding genes and that there are 4 million regulating areas. 80% of the human genome has biochemical functions.(Consortium, Dunham et al. 2012) Only a fraction of the total genome, 10 million of 3 billion nucleotides, accounts for variability among populations.(Tishkoff and Kidd 2004)

Any one of these genes encoding a protein that contributes to inflammatory processes is a candidate gene to explain interindividual differences in the host response to infection or injury. Also, there exist in these genes structural variants of different kinds: e.g. single nucleotide polymorphisms (SNPs), copy number variations (CNVs), and insertions and deletions.(Feuk, Marshall et al. 2006)

This thesis focuses mainly on SNPs as most genetic variability was thought to arise from coding SNPs in exons. The National Center for Biotechnology Information (NCBI), United States National Library of Medicine and National Institutes of Health (NIH), curates the Single Nucleotide Polymorphism Database (dbSNP). The latest build of this database (June 26 2012), holds over 53 million SNPs, 38 million of which are validated and 36 million of which have frequencies (dbSNP build 137, Genome Build 37.3) (NCBI 2012). The original definition of a SNP included a

frequency of at least 1% in a population. The current database hosts submissions without frequency data. Thus many sequence variants ultimately may not qualify as SNPs.(Day)

2.7.3 Contribution of single genes to phenotype in complex disease

The acute phase response to injury and infection is complex. Many processes occur simultaneously and consecutively in a network that includes the innate immune, coagulation and neuro-endocrine systems. There are two models for understanding genetic contributions to complex diseases: There is the mono-genic model, in which one allele change in a single gene explains much of the phenotypic difference. One example is the T>A substitution in codon 6 of the β globin gene resulting in the amino acid valine rather than glutamic acid, which, in turn, leads to malaria infection protective Haemoglobin S(Ingram 1957). More recent examples include mutations in the *NOD2* gene in Crohn's disease (Cuthbert, Fisher et al. 2002) and the Y402H *CFH* amino acid substitution in age related macular degeneration (Klein, Zeiss et al. 2005). The second model is that of many genes exerting additive small influences on the phenotype. (Pritchard 2001, Hill, Goddard et al. 2008) Recently it was proposed that genetic variant secondary to SNPs may account for much of the susceptibility to common diseases(Bodmer and Bonilla 2008, 2010, Yang, Benyamin et al. 2010). To focus on candidate genes with the most potential the following questions were asked in accordance with recommendations from the literature regarding gene association study design (Ioannidis, Ntzani et al. 2001, Tabor, Risch et al. 2002):

1. Is there biological plausibility of association?
2. Is it likely that the candidate gene contributes to the phenotype?
3. Does the SNP likely have a functional biological effect on the protein?
4. Is the SNP prevalent enough to be able to make an association?
5. Is there evidence of association from previous studies?

Thus candidate genes were identified for this gene association study of the acute phase response.

2.7.4 Functional consequences of polymorphisms

The protein coding gene structure lies between the boundaries of transcription start and end. The coding region contains codons, the sequences of three nucleotides from which the amino acids are translated. It starts with the initiation codon, usually the nucleotide sequence ATG (Adenine, Thymine, Guanine) and ends with a termination codon (TAA, TAG or TGA). On both sides of the coding region there are DNA sequences that are transcribed, but not translated: the untranslated regions 5'UTR and 3'UTR. The coding regions consist of exons interrupted by introns. Exons translate to m-RNA, while intervening sequences, introns, are not expressed. The regulatory promoter region contains specific sequences including the TATA box that are binding sites for transcription factors.(Roeder 2005)

A SNP may occur in any part of a gene, i.e. in the promoter, intron, exon or 3'UTR part (Figure 2-3). In the event the SNP is part of the promoter it may result in increased or decreased translation, in turn, leading to an increased or decreased amount of produced protein. A change in an exonic nucleotide may be silent, i.e. there is no resultant amino-acid change. A change may result in gain or loss of function. Intronic and 3' UTR SNPS may affect RNA stability. In addition, there is the possibility that it is not the SNP under investigation, but another SNP in complete linkage disequilibrium that is the cause of the observed change in biological function.(Tabor, Risch et al. 2002)

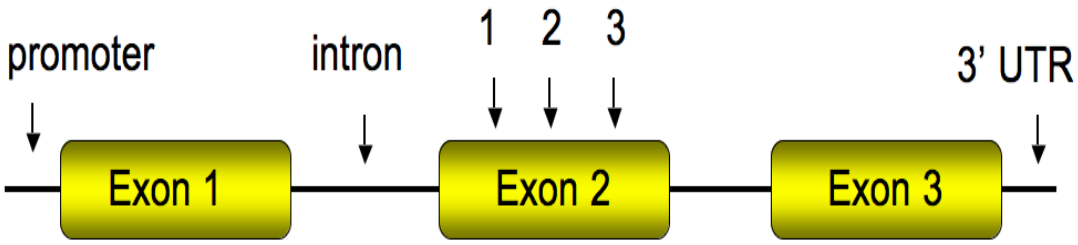


Figure 2-3 Potential positions for SNPs

Schematic of a gene with potential positions for polymorphisms. Three types of SNPS may occur in an exon. 1 depicts a silent change, 2 a resultant amino-acid change with gain of function and 3 an amino-acid change with loss of function.

2.7.5 The International Union of Pure and Applied Chemistry (IUPAC) nomenclature

The IUPAC Amino acid nomenclature was used to denote changes (if any) to the amino acid sequence secondary to a given SNP (see Table 2-8 and Table 2-9). (1984) (Cornish-Bowden 1985)

Abbreviation	Code	Amino acid name
Ala	A	Alanine
Arg	R	Arginine
Asn	N	Asparagine
Asp	D	Aspartic acid (Aspartate)
Cys	C	Cysteine
Gln	Q	Glutamine
Glu	E	Glutamic acid (Glutamate)
Gly	G	Glycine
His	H	Histidine
Ile	I	Isoleucine
Leu	L	Leucine
Lys	K	Lysine
Met	M	Methionine
Phe	F	Phenylalanine
Pro	P	Proline
Ser	S	Serine
Thr	T	Threonine
Trp	W	Tryptophan
Tyr	Y	Tyrosine
Val	V	Valine

Table 2-8 IUPAC Nomenclature for amino acids

IUPAC Code	Meaning
M	A/C
R	A/G
W	A/T
S	C/G
Y	C/T
K	G/T

Table 2-9 IUPAC Code for nucleotide change

2.7.6 Identification of candidate genes

A literature search was performed to identify functional common single nucleotide polymorphisms (SNPs) in the innate immune system. The innate immune system was approached in terms of genes that contribute to the acute phase response. In addition, expert advice was obtained on potential candidate genes.

To optimize predictive power of this study an arbitrary cut-off point of > 5% was chosen as the minor allele frequency in the Caucasian population.

Polymorphisms of interest were defined as:

1. Part of the innate immune system
2. Minor allele frequency >5% in the Caucasian population
3. Established functionality or association with relevant inflammatory disease

The *a priori* assessment whether a specific polymorphism would be likely to have a significant impact on phenotype was assessed as best as could be at that time, on the basis of known functionality, previous association studies and presumed importance in the acute phase response.

The set of chosen genes was divided into the following categories:

1. Complement activation
2. Pattern Recognition Receptors
3. Cytokines
4. Regulatory Receptors
5. Acute phase proteins
6. Other

Complement Activation Cascade

MBL-2, lectin pathway activation;

CFH, alternative pathway inhibition;

CFB, alternative pathway activation;

C1qA, classical pathway activation;

Pattern Recognition Receptors

TLR2; Lipoteichoic Acid (LTA) on gram-positive bacteria, endogenous ligands

Endotoxin recognition complex

TLR4, signal transduction of LPS, endogenous ligands;

CD14, binds LPS to TLR4-MD2 complex;

LBP shuttles LPS to CD14;

Cytokines

TNFα, pro-inflammatory;

IL6, pro-inflammatory;

IL10, anti-inflammatory;

Regulation

CHRNA7, regulates cytokine production, down-regulates TLR4;

ADRB2, regulates cytokine production;

MHC2TA, regulates HLA-DR expression;

PTPN22, regulates T Cell response;

SELS, protects against metabolic stressors

Acute Phase Reactants

CRP, C-Reactive Protein potentiates pro-inflammatory reponse;

ADM, Adrenomedullin is pluripotent, binds to CFH;

PAI-1; Plasminogen Activator Inhibitor-1 pro-inflammatory and inhibits fibrinolysis

Other

ACE, Angiotensin Converting Enzyme vaso-active;

APOE; Apolipoprotein E cholesterol carrier lipoprotein

This list is limited for several reasons. There are many more genes that would qualify as part of innate immunity with a putative important role in the acute phase response. Two examples of genes not included in the analysis follow here:

MD2, (LYP 96) binds LPS to TLR4 in the endotoxin recognition complex and is pivotal for a robust response (Fitzgerald, Rowe et al. 2004), however, no common SNPs were identified.

The IL1 group is a potent pro-inflammatory cytokine family. It includes agonists IL1α, IL1β and the IL1 receptor antagonist IL1ra. There are several polymorphisms in all three genes, with a complex interplay (Waterer and Wunderink 2003). Given that genetic variation in this cytokine was difficult to interpret, this cytokine was not chosen in the gene set.

Most importantly, adding genes "on spec" without a robust hypothesis would decrease the power of the study because of undue correction for multiple testing.(Hattersley and McCarthy 2005)

2.7.6.1 Identification of nucleotide sequence

The positions of the polymorphisms were established using NCBI dbSNP ID rs numbers on the NCBI SNP website, dbSNP build 126, Genome Build 36.1 (May 4, 2006). Current URL:(2010) The website identifies the position of the requested polymorphism using the unique identifiying rs number. (Figure 2-4)

The thus obtained nucleotide sequence output was then emailed from the NCBI website in a FASTA text file format. Figure 2-5 shows an example for rs1800871 (IL10).

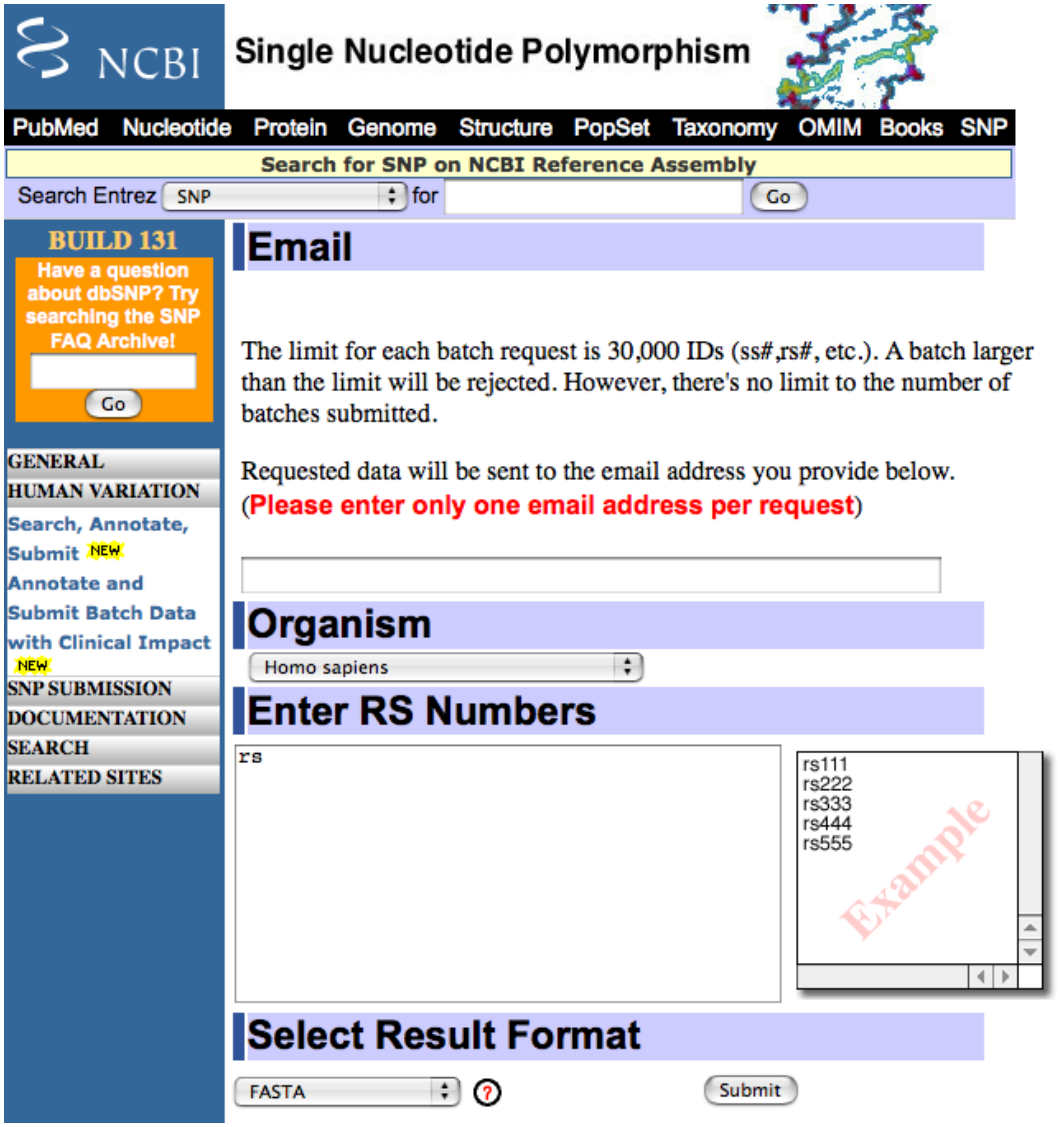


Figure 2-4 NCBI SNP identification website

```
>gnl|dbSNP|rs1800871 rs=1800871|pos=401|len=801|taxid=9606|mol="genomic"|class=1|alleles="C/T"|build=126
CCTTCCCCAG GTAGAGCAAC ACTCCTCGCC GCAACCCAAC TGGCTCCCCT TACCTTCTac acacacacac acacacacac
acacacacac acacacacac acaAATCCAA GACAACACTA CTAAGGCTTC TTTGGGAAGG GGAAGTAGGG ATAGGTAAGA
GGAAAGTAAG GGACCTCCTA TCCAGCCTCC ATGGAATCCT GACTTCTTTT CCTTGTTATT TCAACTTCTT CCACCCCATC
TTTTAAACTT TAGACTCCAG CCACAGAAGC TTACAACTAA AAGAAACTCT AAGGCCAATT TAATCCAAGG TTTCATTCTA
TGTGCTGGAG ATGGTGTACA GTAGGGTGAG GAAACCAAAT TCTCAGTTGG CACTGgtgta cccttgtaca ggtgatgtaa
Y
atctctgtgc ctcagtttgc tcactataaa atagagaCGG TAGGGGTCAT GGTGAGCACT ACCTGACTAG CATATAAGAA
GCTTTCAGCA AGTGCAGACT ACTCTTACCC ACTTCCCCCA AGCACAGTTG GGGTGGGGGA CAGCTGAAGA GGTGGAAACA
TGTGCCTGAG AATCCTAATG AAATCGGGGT AAAGGAGCCT GGAACACATC CTGTGACCCC GCCTGTACTG TAGGAAGCCA
GTCTCTGGAA AGTAAAATGG AAGGGCTGCT TGGGAACTTT GAGGATATTT AGCCCACCCC CTCATTTTTA CTTGGGGAAA
CTAAGGCCCA GAGACCTAAG GTGACTGCCT AAGTTAGCAA GGAGAAGTCT TGGGTATTCA TCCCAGGTTG GGGGGACCCA
```

Figure 2-5 Nucleotide sequence for IL10 SNP rs1800871

Nucleotides are denoted by AGTC and the polymorphism by Y.

(A Adenine; G Guanine; T Thymine; C Cytosine; Y (C/T)).

Nucleic acid sequences were then counted 50 base pairs upstream and downstream of the thus identified polymorphic site. If rs numbers were not available for the position then Genbank reference numbers, original manuscripts and/or personal communication with the authors describing the polymorphism were used.
These sequences were imported to an excel spreadsheet and emailed to the commercial facility KBiosciences (Hoddesdon, Herts, UK).
This facility then designed and validated the oligonucleotides on the basis of the nucleic acid sequences.
Sealed plate arrays were couriered to the genotyping facility on dry ice. Telephone contact with the facility established arrival in good order. Samples were not contaminated during transport. The 50μl samples were diluted 1:4 to allow for all SNPS to be run.
Table 2-10 summarizes the chosen genes, locus, positions SNP names and functionality as described in the literature as well as common haplotypes.

Gene	Locus	Position	SNP	rs number	Function	Haplotype	
MBL2	10q11.2-q21	Exon 1	"D" 52C→T Arg>Cys	5030737	T less	HYPA: high LYQA: wildtype	(Dommett, Klein et al. 2006)
MBL2		Exon 1	"B" 54G→A Gly>Asp	1800450	A less	LYPA: intermediate LXPA: intermediate	
MBL2		Exon 1	"C" 57G→A Gly>Glu	1800451	A less	HYPD: low LYQC: low	
MBL2		Promoter	-221 G/C X/Y	7096206	G less	LYPB: low	
MBL2		Promoter	-550 C→G L/H	11003125	C less		
MBL2		Promoter	+4 C→T P/Q (KB G→A)	7095891	NA		
C1QA	1p36.12	Exon 2	276G>A; Gly70GGA	172378	AA deficient		(Racila, Sontheimer et al. 2003)
CFB	6p21.3	Exon 1	L9H Leu9His T→A	4151667	A low In signal peptide		(Gold, Merriam et al. 2006)
CFB		Exon 2	R32Q 95 G→A or C→T Arg32Gln	641153	A reduced hemolytic activity;		

		Exon 2	R32W 94 C→T Arg32Trp	12614	Trp no effect Functionality -		
CFH	1q32	Exon 9	Tyr402His T>C; Y402H; 1277T→C	1061170	C defective C allele high risk AMD		(Haines, Hauser et al. 2005)
ADM	11p15.4	Promoter	-1984A>G	3814700	G high		(Li, Staessen et al. 2006)
TLR2	4q32	TIR domain Exon 3	Arg753Gln R753Q G2258A G→A	5743708	A has reduced response (missense) ?Signalling function↓		(Lorenz, Mira et al. 2000)
TLR2		Intron 1	TagSNP 16933A→T	4696480	AA more infections		(Sutherland, Walley et al. 2005)
TLR2		Exon 3; Pseudo snp (Malhotra, Relhan et al. 2005)	Arg677Trp C2029T	N/A	Reduced intracellular signalling;		(Kang and Chae 2001, Bochud, Hawn et al. 2003)
TLR4	9q33.1	Exon 3	Thr399Ile C>T	4986791	T low	299G/399I low	(Arbour, Lorenz et al. 2000)
TLR4		Exon 3	Arg299Gly A>G	4986790	G low		
CD14	5q31.1	Promoter	C-159T;	2569190	TT high		(Hubacek, Rothe et

			C-260T				al. 1999)
LBP	20q11.23-q12	Exon 3	Pro97Pro C/T	2232582	T worse outcome		(Hubacek, Stuber et al. 2001, Barber and O'Keefe 2003)
CHRNA7	15q13-q14	Promoter_6	-46G→T	No rs numbers	T low		(Leonard, Gault et al. 2002)
		Promoter_5	-86 C→T		T low		
		Promoter_4B	-92 G→A	AF029837 sequence used	A low		
		Promoter_4A	-143G→A		A low		
		Promoter_3	-178 -G		- low		
		Promoter_2	-194G→C		C or T low		
		Promoter_1	-241A→G		G low		
ADRB2	5q31-q32	Exon1	Arg16Gly A/G	1042713	Gly/Gly and Gly/Arg downregulates Arg is WT		(Brodde and Leineweber 2005)
ADRB2		Exon1	Gln27Glu C/G	1042714	Glu/Glu Very resistant to downreg Gln is WT		
ADRB2		Exon1	Thr164Ile C>T	1800888	TT less affinity binding		
ADRB2		‘5 LC (leader cistron); at translational level influence	-47T>C Arg-19Cys	1042711 (identified later)	CC greater expression		(McGraw, Forbes et al. 1998)

					Gly16 dominant over Glu27; Arg16/Glu27 double mutant completely resistant to downregulation CysGlyGln haplotype more susceptible to b agonist desensitization		(Oostendorp, Postma et al. 2005)
IL10	1q31-q32	Promoter	-1082G→A	1800896	G/A	GCC/GCC high	(Turner, Williams et al. 1997)
IL10		Promoter	-819C→T	1800871	C/N		
IL10		Promoter	-592C→A	1800872	C/N		
					High/low		
MHC2TA	16p13	Promoter III (B cells)	-168A→G	3087456	G high		(Swanberg, Lidman et al. 2005)
IL6	7p21	Promoter	-174G→C	1800795	G/C (CC low)		(Terry, Loukaci et al. 2000)
IL6		Promoter	-572G→C	1800796	G/G	GGG/GGG high CGA/CGA low	
IL6		Promoter	-597G→A	1800797	G/A		
					High/Low		
DCP1= ACE	17q23.3	Intron 16	287 bp Alu rpt	4646994	DD high (50%)		(Rigat, Hubert et al. 1990)
DCP1= ACE		Exon 17	Thr776Thr G2350A	4343	GG lowest GA int AA high		(Glenn, Du et al. 2009)

					(19%); complete LD with rs4646994		
DCP1= ACE		Promoter	A-240T	4291	AA lowest AT interm TT high (6%)		(Zhu, Bouzekri et al. 2001)
DCP1= ACE		Intron 16		4341	In complete LD with rs4646994		(Tanaka, Kamide et al. 2003)
(Serpine-1) PAI-1	7q21.3-q22	Promoter	-765 4G/5G	1799889	4G/4G high		(Dawson, Wiman et al. 1993)
TNF (TNF alpha)	6p21.3	Promoter	-308 G→A	1800629	AA high		(Kroeger, Carville et al. 1997)
SELS (SEPS1)	15q26.3	Promoter	-105G→A	28665122	A		(Curran, Jowett et al. 2005)
PTPN22 (Lyp)	1p13.3-p13.1	Exon 14	R620W C1858T	2476601	TT high Inhibitor T-cell activation		(Bottini, Musumeci et al. 2004, Zuo, Lubischer et al. 2004)
CRP	1q21-23		-717A/G	2794521	Tag snp		Personal communication Dr Aroon Hingorani
CRP			-305G/A	3093062	Tag snp		
CRP			-286C/T/A	3091244	Tag snp		
CRP			+1444C/T	1130864	Tag snp		
CRP		1. Exon 2 C/T	+2302G/A	1205	Tag snp		

		untrans					
CRP			+4899T/G	3093077	Tag snp		
APOE	19q13.2	Exon 4	Arg112Cys Arg130Cys C/T	429358		ε2, ε3, ε4 E2 Cys^{112}-Cys^{158} E3 Cys^{112}-Arg^{158} E4 Arg^{112}-Arg^{158}	(Teasdale, Nicoll et al. 1997)
APOE		Exon 4	Arg158Cys Arg176Cys C/T	7412			

Table 2-10 Candidate gene characteristics

The table contains polymorphisms that were previously mistyped. This information became apparent after the genotyping requests had already been made.

LBP P97P: Originally thought to affect outcome in adults with sepsis (Hubacek, Stuber et al. 2001), but found to be identified wrongly (Barber and O'Keefe 2003).

TLR2 Arg677Trp was originally thought to occur in Korean patients with tuberculosis at higher frequency (Kang and Chae 2001), but found to be identified incorrectly (Malhotra, Relhan et al. 2005).

The CHRNA7 promoter polymorphism choices were based on a study by(Leonard, Gault et al. 2002). Subsequent work showed the variant frequency to be very low.(Stephens, Logel et al. 2009) (Table 2-11)

Ultimately 52 SNPs in 24 genes were identified. These SNPs were hypothesized to modify the primary outcome measures as outlined in Table 2-12.

SNP	Position	Frequency Caucasian	Frequency African American
Promoter_6	-46G→T	0.003	0.097
Promoter_5	-86 C→T	0.064	0.004
Promoter_4B	-92 G→A	0.006	0.004
Promoter_4A	-143G→A	Rare in original already	Rare in original already
Promoter_3	-178 -G	0.004	0.076
Promoter_2	-194G→C	0.042	0.02
Promoter_1	-241A→G	0.001	0.0

Table 2-11 CHRNA7 Promoter variant frequencies

SNP Single Nucleotide Polymorphism

Gene	Category
MBL2	Early SIRS
C1QA	Early SIRS
CFB	Early SIRS
CFH	Early SIRS
ADM	Modified HRV
TLR2	Early SIRS
TLR4	Early SIRS/ platelet count on admission
CD14	Confounder
LBP	Confounder
CHRNA7	Confounder
ADRB2	Modified HRV
IL10	Confounder
MHC2TA	Nosocomial infection
IL6	Confounder
DCP1= ACE	AHRF
(Serpine-1) PAI-1	Confounder
TNF (TNF alpha)	Confounder
SELS (SEPS1)	Early SIRS
PTPN22 (Lyp)	Early SIRS
CRP	Early SIRS
APOE	Worse neurological outcome after severe TBI

Table 2-12 Candidate genes linked to outcome measure

HRV, heart rate variability; AHRF, acute hypoxic respiratory failure; TBI traumatic brain injury

2.8 Genotyping methods

2.8.1 KASPAR technology (KBioscience)

Most SNPs were determined by the KASPar assay system (KBioscience, Herts, UK). KBioscience is a commercial genotyping facility. The company has developed a fluorescent-based competitive allele-specific PCR method.

The principle works as follows:

Based on the genotyping sequence that was sent to the facility they designed oligonucleotide probes to synthesize allele specific primers.

The DNA was distributed in 96 well plates. DNA was diluted and pipetted onto working plates. KASP®mix was then added to the DNA. This mix contains two competitive allele-specific tailed forward primers, one common reverse primer and a combination of components to allow the PCR reaction to work. This included the universal fluorescent reporting system and their proprietary KTaq polymerase, which is a recombinant thermostable DNA polymerase. The polymerase originated from the thermophilic bacterium Thermus aquaticus and was obtained from high level gene expression in E coli.

The plates were then sealed and put through the following thermocycle:

(1) 95° for 15 minutes
(2) 95° for 30 seconds
(3) 61° for 30 seconds
(4) 72° for 1 minute
Steps (2) to (4) are repeated 34 times
(5) 72° for 5 minutes

(1) 94 °C for 15 minutes
Hot-start enzyme activation
(2) 94 °C for 20 seconds
Touchdown over 65-57°C for 60 seconds
10 cycles (dropping 0.8°C per cycle)
(3) 94°C for 20 seconds

57°C for 60 seconds
26 cycles

After cooling to room temperature the plates were read on a fluorescent reader.

The genotyping results were generated in a comma separated text file (.csv) listing each of the samples with each SNP and its genotype. These then could be imported in excel for further analysis. In addition, these results could be viewed graphically using the SNP viewer tool (KBioscience).

Figure 2-6 shows the excel file for SNP rs1800871 (IL10) and Figure 2-7 shows the SNP viewer graph for SNP rs1800871 (IL10).

Genotyping-340.001-01.xls

	A	B	C	D	E	F	G	H
646	Data							
647	DaughterPlate	MasterPlate	MasterWell	Call	X	Y	SNPID	SubjectID
4418	KB124468	PICU3	A01	C:C	14.45856	1.93062	gnl|dbSNP|rs1800871	200
4419	KB124468	PICU3	B01	C:C	14.49676	1.66266	gnl|dbSNP|rs1800871	201
4420	KB124468	PICU3	C01	T:C	12.02378	5.64068	gnl|dbSNP|rs1800871	202
4421	KB124468	PICU3	D01	C:C	14.42769	1.81885	gnl|dbSNP|rs1800871	203
4422	KB124468	PICU3	E01	T:C	12.04311	5.77906	gnl|dbSNP|rs1800871	204
4423	KB124468	PICU3	F01	C:C	14.92352	1.82109	gnl|dbSNP|rs1800871	205
4424	KB124468	PICU3	G01	C:C	14.50441	1.69563	gnl|dbSNP|rs1800871	206
4425	KB124468	PICU3	H01	T:C	11.68395	5.7847	gnl|dbSNP|rs1800871	207
4426	KB124468	PICU3	A02	C:C	14.56679	1.82809	gnl|dbSNP|rs1800871	208
4427	KB124468	PICU3	B02	C:C	14.44525	1.79892	gnl|dbSNP|rs1800871	209
4428	KB124468	PICU3	C02	C:C	14.65672	1.82731	gnl|dbSNP|rs1800871	210
4429	KB124468	PICU3	D02	C:C	14.44976	1.75339	gnl|dbSNP|rs1800871	211
4430	KB124468	PICU3	E02	C:C	14.87389	1.8649	gnl|dbSNP|rs1800871	212
4431	KB124468	PICU3	F02	C:C	14.38203	1.73778	gnl|dbSNP|rs1800871	213
4432	KB124468	PICU3	G02	C:C	14.78227	1.78493	gnl|dbSNP|rs1800871	214
4433	KB124468	PICU3	H02	C:C	14.46167	1.64748	gnl|dbSNP|rs1800871	215
4434	KB124468	PICU3	A03	C:C	14.55325	1.84342	gnl|dbSNP|rs1800871	216
4435	KB124468	PICU3	B03	C:C	14.5128	1.67207	gnl|dbSNP|rs1800871	217
4436	KB124468	PICU3	C03	C:C	14.81726	1.70894	gnl|dbSNP|rs1800871	218
4437	KB124468	PICU3	D03	C:C	15.29333	1.75377	gnl|dbSNP|rs1800871	219
4438	KB124468	PICU3	E03	T:T	3.57569	7.29111	gnl|dbSNP|rs1800871	220
4439	KB124468	PICU3	F03	C:C	14.83795	1.70296	gnl|dbSNP|rs1800871	221
4440	KB124468	PICU3	G03	T:C	12.13011	6.04684	gnl|dbSNP|rs1800871	222
4441	KB124468	PICU3	H03	C:C	14.60604	1.5947	gnl|dbSNP|rs1800871	223

Figure 2-6 Excel file raw data for rs1800871

The output from KBioscience shows the name of the daughter plate as created by KBioscience, the name of the masterplate created by me, the position of the masterwell, the interpretation of genotype, the X and Y value to be viewed in the programme SNP viewer, the SNPID rs number and the subject ID.

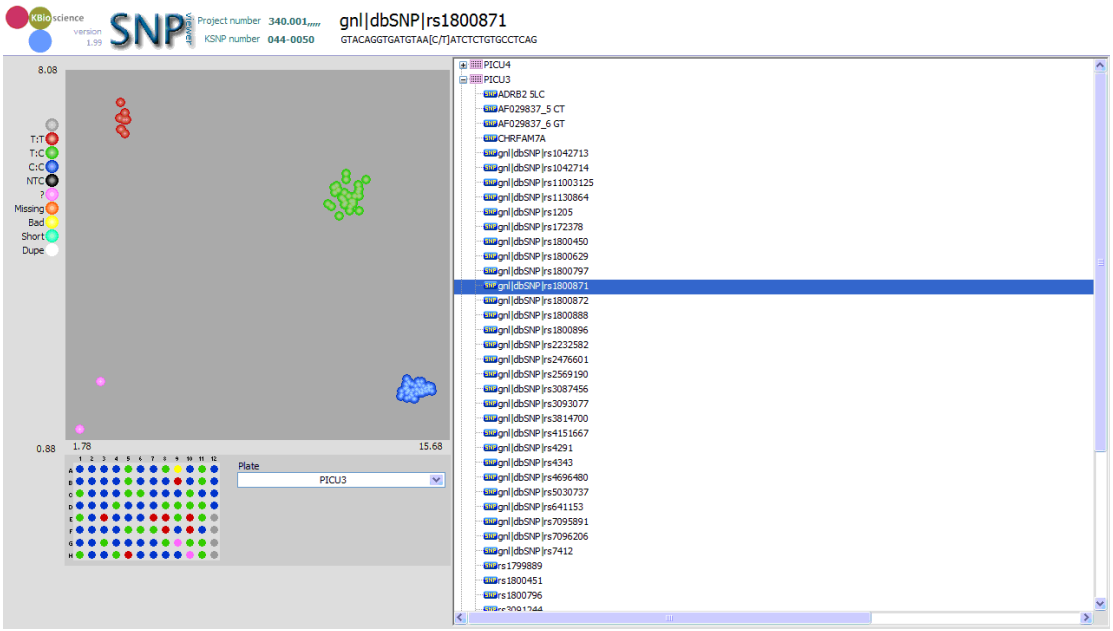


Figure 2-7 Graphic representation of results for SNP rs1800871

Red dots denote genotype T:T, green dots T:C, blue dots C:C, pink dots could not be called and yellow dots denote insufficient quality DNA. The bottom left corner shows the master array plate (PICU3 in this instance).

2.8.2 Taqman Method

Taqman methodology is a fluorescent real-time PCR assay. The polymorphisms TLR4 D299G (rs 4986790) and ACE rs4341 were analysed with this technique.

TLR4

The TLR4 D299G polymorphism was determined by a TaqMan assay (Applied Biosystems, Warrington UK, Assay ID: C_11722238_20) according to the manufacturers protocol.

TaqMan (Applied Biosystems, Warrington UK) primers and probes were as follows:

Context sequence:

GCATACTTAGACTACTACCTCGATG**[A/G]**TATTATTGACTTATTTAATTGTT TG

Forward primer: 5'-TGACCATTGAAGAATTCCGATTAGCA

Reverse primer: 5'-ACACTCACCAGGGAAAATGAAGAA

Probe (wild-type allele): (FAM label) 5'-TACCTCGATG*A*TATTATT-MGB

Probe (variant allele): (VIC label) 5'-CCTCGATG*G*TATTATT-MGB

ACE

The *ACE* polymorphism rs4341 C>G, is reported to be in 100% linkage disequilibrium (LD) with the ACE I/D polymorphism rs4646994 (Tanaka, Kamide et al. 2003) (CC with II and GG with DD). One DNA array plate (PICU plate 1, n=91) was genotyped for both *ACE* rs4341 and *ACE* I/D rs4646994 to confirm this LD in the current patient cohort.

TaqMan (Applied Biosystems, Warrington UK) primers:

Forward -CCTTACAAGCAGAGGTGAGCTAA

Reverse -CCATCACATTCGTCAGATCTGGTA

and probes:

VIC -TCAAGCCATTCAAAC

FAM -CAAGGCATTCAAAC

A specific set of primers amplifies a DNA region to allow specifically designed probes to anneal within that DNA region. A fluorophore is attached to the 5'-end of the oligonucleotide probe and a quencher is attached at the 3'-end. The fluorophore 6-carboxyfluorescein (FAM) labeled probe detects one allele and the (VIC) labeled probe detects the other potential allele. The emission spectrum peaks are 530 nm for FAM 550 nm for and VIC respectively. The cycler's light source excites the fluorophore via Fluorescence Resonance Energy Transfer (FRET). The quencher molecule then quenches this emitted fluorescence.

Taq polymerase extends the primer and synthesizes the nascent strand, while the 5' to 3' exonuclease activity of the polymerase degrades the probe that has annealed to the template. This degradation of the probe causes the fluorophore to be released from it and also breaks the close proximity to the quencher. In turn, the quenching effect ceases to be which allows the fluorophore to fluoresce. This way, the fluorescence that is detected in the real-time PCR thermal cycler is directly proportional to the fluorophore released and the amount of DNA template present.

Fluorescent reporter probes only detect DNA that contains the probe sequence. This significantly increases specificity because it enables quantification even in the presence of non-specific DNA amplification.

2.8.3 Three Primer PCR method

The specifications are outlined as supplied by the Department of Cardiovascular Genetics, Rayne Institute, University College London, London, UK.

The ACE I/D polymorphism protocol is a 3 primer PCR method that generates two PCR products. Counter intuitively, the larger product corresponds with the deletion allele and the smaller product with the insertion allele. The protocol is depicted in **Figure 2-8** and the nucleotide sequence with primers and PCR products is given in Figure 2-9.

Cardiovascular Genetics - Genotyping form

ROP Number:

Gene Name (Genotype) ACE insertion -deletion genotype (i16)

Operator: Ros

- **5' Oligo : FH 76 Sequence: CAT CCT TTC TCC CAT TTC TC; 5' insertion Oligo : FH77 Sequence: TGG GAT TAC AGG CGT GAT ACAG; 3' oligo : FH78 Sequence: ATT TCA GAG CTG GAA TAA AAT T**

PCR Cocktail 20µl PCR. For 100 reactions/2ml

10 x dNTP. Mix	200µl
50mM]$MgCl_2$	100µl (final 2. 5mM)
BSA	60 µl
Oligo FH 76	4 µl (400 pmol/ µl)
Oligo FH 77	2µl (100 pmol/ µl)
Oligo FH 78	10 µl (100 pmol/ µl)
Taq Polymerase	8 µl
dH_2O	µl to total vol of 2000 µl

- Cover each PCR with 20 µl mineral oil
- ALTERNATIVELY do 10µl PCR and use heated lid- then add dye straight to plate and save time!

PCR Conditions

1.	95°C	5 min
2.	95°C	45sec
3.	54°C	45sec
4.	72°C	30sec
5.	72°C	5 min

Specify number of cycles where appropriate (step 1 x1) (cycles 2,3,4 x 35) (step 5 x1)

- **Machine**
- omnigene
- **Program usually called 41**
- **Ref: Q. J. M. 87:211**

Enzyme digest conditions

N/A

Electrophoresis conditions

- 7.5 % MADGE gel
- Run time: 0.5 hours
- Voltage: max V

Electrophoresis

- "MADGE" dye
- Loaded 5 µl of PCR product

Any other comments

- Always use an ID +ve control for PCR.
- Yes, the deletion allele product is larger than the insertion allele product!

PCR product sizes

- 85 bp (deletion allele)
- 65 bp (insertion allele)

Digest product sizes

- N/A

Figure 2-8 ACE I/D polymorphism protocol

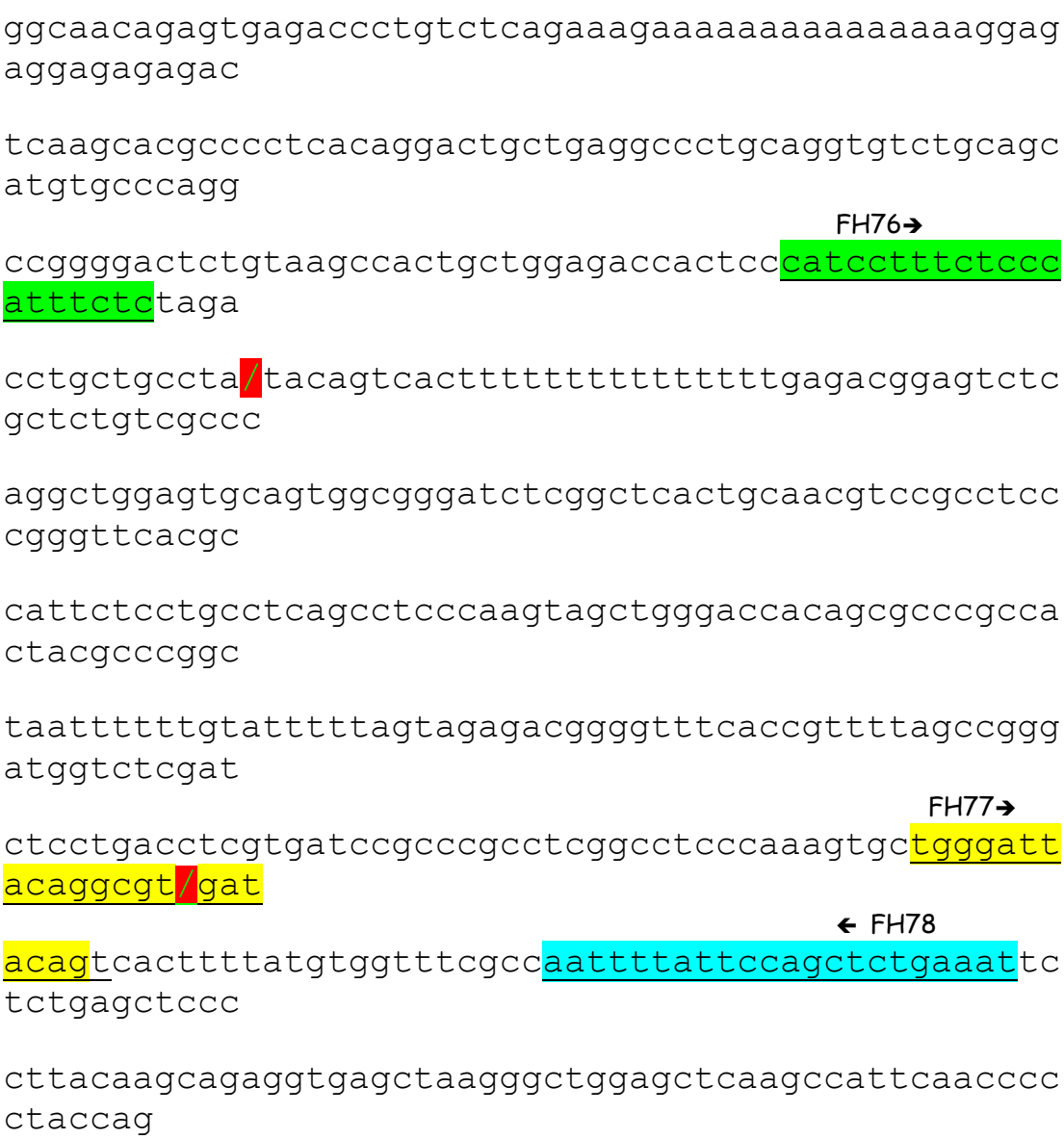


Figure 2-9 ACE I/D polymorphism 3 primer PCR method

- fh76 5’ primer
- fh77 5’ primer
- fh78 3’ primer

PCR products

- 85bp deletion allele
- 65bp insertion allele

ACE intron 16 I/D polymorphism (rs4646994). 3 primer PCR method. Insertion

marked by /.

The genotyping for this project was therefore done using several techniques at different facilities. I performed DNA extraction and analysis.

A subset of the current cohort had previously been genotyped by other researchers. These historical genotyping data were not available to those currently conducting the actual genotyping. This subset consisted of PICU patients number 1-147. The researchers or facility involved in prior genotyping are denoted by their initials in Table 2-13. These results were available to me and used as a quality control measure.

Gene	RSnumber	Facility	Subset prior genotyped
PTPN22	2476601	Kbioscience 1	
C1QA	172378	Kbioscience 1	
CRP	3093077	Kbioscience 1	
CRP	1205	Kbioscience 1	
CRP	1130864	Kbioscience 1	
CRP	3091244	Kbioscience 1	
CRP	3093062	Kbioscience 1 and 2	
CRP	2794521	Kbioscience 1 and 2	
IL10	1800872	Kbioscience 1	
IL10	1800871	Kbioscience 1	
IL10	1800896	Kbioscience 1	KF
CFH	1061170	Kbioscience 2	
TLR2	N/A	*	
TLR2	4696480	Kbioscience 1	
TLR2	5743708	Kbioscience 1	Innogenetics, Belgium
CD14	2569190	Kbioscience 1	Innogenetics, Belgium
ADRB2	1042711 (5'LC)	Kbioscience 1	
ADRB2	1042713	Kbioscience 1	
ADRB2	1042714	Kbioscience 1	
ADRB2	1800888	Kbioscience 1	
TNFA	1800629	Kbioscience 1	KF
CFB	4151667	Kbioscience 1	
CFB	641153	Kbioscience 1	
IL6	1800797	Kbioscience 1	
IL6	1800796	Kbioscience 1	
IL6	1800795	Kbioscience 2	KF
PAI-1	1799889	Kbioscience 1	KF
TLR4	4986790	Southampton	Innogenetics, Belgium
TLR4	4986791	Kbioscience 1	Innogenetics, Belgium
MBL2	1800451	Kbioscience 1	KF
	1800450	Kbioscience 1	KF
	5030737	Kbioscience 1	KF
	7095891	Kbioscience 1	
	7096206	Kbioscience 1	KF
	11003125	Kbioscience 1	
ADM	3814700	Kbioscience 1	
CHRNA7	-241A→G	Kbioscience 1 and 2	
	-194G→C	Kbioscience 1 and 2	

	#NAME?	Kbioscience 1 and 2	
	-143G→A	Kbioscience 1 and 2	
	-92 G→A	Kbioscience 1 and 2	
	-86 C→T	Kbioscience 1	
	-46G→T	Kbioscience 1	
CHRFAM7A	TG/-	Kbioscience 1	
CHRFAM7A	TG/-	Kbioscience 1	
SELS (SEPS1)	28665122	Kbioscience 1 and 2	
MHC2TA	3087456	Kbioscience 1	
DCP1= ACE	4291	Kbioscience 1	
DCP1= ACE	4646994	Rayne	KF
DCP1= ACE	4341	Rayne	
DCP1= ACE	4343	Kbioscience 1	
APOE	429358	Kbioscience 1 and 2	
APOE	7412	Kbioscience 1	
LBP	2232582	Kbioscience 1	

Table 2-13 Genotyping facilities

2.8.4 Collaboration

I performed DNA extraction, sample handling and despatching, quality control and data analysis. Genotyping was done by the commercial facility KBioscience and the following collaborators (bolded):

ACE genotyping:

Ms KaWah Li, **Laboratory Technician**

Professor Steve E Humphries, BHF Professor of Cardiovascular Genetics

Centre for Cardiovascular Genetics, Rayne Institute, Department of Medicine,

University College London, London, UK.

TLR4 D299G genotyping:

Mr Matthew Rose-Zerilli, **Research Technician**

Dr John Holloway, Reader Infection, Inflammation & Repair

Human Genetics Division, University of Southampton, UK

TLR4 adult data:

Dr Shu Ye, Reader in Human Genetics

Clinical Pharmacology, William Harvey Research Institute,

Barts and the London School of Medicine

David Pontefract, Human Genetics Division, University of Southampton, UK

Clinical data Southampton PICU:

Ms Kerry Illing, Medical student

Dr John Pappachan, Consultant and Honorary Senior Lecturer,

Paediatric Intensive Care Unit, Southampton University Hospitals NHS Trust

Comparison dataset genotyping and clinical data

PICU samples 1-147

Dr Katy Fidler, **SpR**

Department of Infectious Diseases & Microbiology,

Institute of Child Health

University College London

Dr Peter Wilson, SpR

Ms Helen Tighe, Research Nurse

Paediatric Intensive Care Unit, Great Ormond Street Hospital NHS Trust London

Comparison CICU dataset genotyping and clinical data

Dr Meredith Allen, SpR

Ms Annette McQuillan, Research Nurse

Cardiac Intensive Care Unit, Great Ormond Street Hospital NHS Trust London

2.8.5 Hardy Weinberg Equilibrium

In a randomly mating infinite size population there exists a relationship between allele frequency and genotype frequencies at an autosomal locus. This equilibrium is expected in a diploid population that mates randomly and is free from evolutionary forces, e.g. genetic drift, mutation and migration. The expected genotype frequencies major homozygote: heterozygote: minor homozygote for a bi-allelic gene under this law are p^2:2pq:q^2.(Gillespie 2004) Deviations from this equation are thought to occur because of genotypic errors or population characteristics such as recent migrations or

mixture of sub-populations that do not completely interbreed.(Schaid, Batzler et al. 2006)

2.8.6 Linkage disequilibrium

Because several genes harboured more than one SNP the potential of linkage disequilibrium occurs. Linkage disequilibrium (LD) is the extent to which alleles at two or more loci are associated with each other; it may occur within one gene but also between genes (Hajeer and Hutchinson 2001). LD among alleles arises from various influences: mutation, random genetic drift, migration, and selection in response to environmental factors. Thus different patterns of LD may occur across bio-geographical groups. This may be of most relevance to genes with a strong selection advantage. Resistance to malaria is one example: variants in the genes *G6PD*, *HBE1*, *Duffy* (*FY*) are associated with malaria resistance.(Williams 2006)

Measures of LD are D', r^2, which are the correlation coefficient between two alleles at different loci and the logarithm of the odds (LOD) score. The Hapmap Consortium views a LOD score over +2 (i.e 100 to 1 odds) to indicate evidence for linkage.

If markers occur at significantly different rates in the affected group vs. the non-affected group, then one can assume that the allele itself or an allele in LD with it is associated with the disease.

The software package Haploview (Barrett, Fry et al. 2005) was used to investigate linkage disequilibrium. Briefly, two separate data files were created in text format: one with the genotyping results as bi-allelic data and one with the chromosome position of the SNPs. The human genome build 37.1 and the rsnumbers (2010) formed the basis for the SNPs' chromosome positions. Haploview could then analyse these two files and calculate Hardy Weinberg equilibrium, LD, haplotype blocks as well as quality indicators such as percentage of successful genotyping.

LD was calculated for the whole cohort as well as for Caucasians only.

Linkage data was formatted in the Haploview Linkage Format textfile:

FamilyID Individual ID FatherID MotherID Sex Outcome Gene1Allele1 Gene1Allele2

Missing data were entered as 0.

The SNP position data file is depicted in Table 2-14.

Gene	Rsnumber	Chromosome	Chromosome position
PTPN22 (Lyp)	2476601	1p13.3-p13.1	114377568
C1QA	172378	1p36.12	22965438
CRP	3093077	1q21-23	159679636
CRP	1205		159682233
CRP	1130864		159683091
CRP	3091244		159684665
CRP	3093062		159684684
CRP	2794521		159685096
IL10	1800872	1q31-q32	206946407
IL10	1800871		206946634
IL10	1800896		206946897
CFH	1061170	1q32	196659237
TLR2	4696480	4q32	154607126
TLR2	5743708		154626317
CD14	2569190	5q31.1	140012916
ADRB2	1042711	5q31-q32	148206348
ADRB2	1042713		148206440
ADRB2	1042714		148206473
ADRB2	1800888		148206885
TNFA	1800629	6p21.3	31525175
CFB	4151667	6p21.3	31896215
CFB	641153		31896371
IL6	1800797	7p21	22766221
IL6	1800796		22766246
IL6	1800795		22766645
PAI-1	1799889	7q21.3-q22	100769710:100769711
TLR4	4986790	9q33.1	120475302
TLR4	4986791		120475602
MBL2	1800451	10q11.2-q21	54531226
MBL2	1800450		54531235

MBL2	5030737		54531242
MBL2	7095891		54531461
MBL2	7096206		54531685
MBL2	11003125		54532014
ADM	3814700	11p15.4	10324658
*CHRNA7**	-241A→G	15q13-q14	30109849
*CHRNA7**	-194G→C		30109896
*CHRNA7**	-178 -G		30109912
*CHRNA7**	-143G→A		30109947
*CHRNA7**	-92 G→A		30109998
*CHRNA7**	-86 C→T		30110004
*CHRNA7**	-46G→T		30110044
*CHRFAM7A**	67158670		30237167:30237168
CHRFAM7A	67158670		30665281:30665282
SELS (SEPS1)	28665122	15q26.3	101817727
MHC2TA	3087456	16p13	10970902
DCP1= ACE	4291	17q23.3	61554194
DCP1= ACE	4646994		61565904:61565905
DCP1= ACE	4341		61565990
DCP1= ACE	4343		61566031
APOE	429358	19q13.2	45411941
APOE	7412		45412079
LBP	2232582	20q11.23-q12	36979265

Table 2-14 Haploview SNP position data

*Based on ref_assembly 36.3 (Stephens, Logel et al. 2009)

SNPs in red were not entered into the linkage analysis because of inability to genotype (APOE, SELS, CHRNA7) or because they were not bi-allelic (PAI-1, CHRFAM7A, ACE I/D).

Haploview criteria for LD blocks are based on (Gabriel, Schaffner et al. 2002) and Hardy Weinberg Equilibrium (HWE) calculations are based on (Wigginton, Cutler et al. 2005). The output of the Haploview programme is both graphical as well as textual. The legend for the graphical display is depicted in

.

	D' < 1	D' = 1
LOD < 2	White	Blue
LOD ≥ 2	Shades of pink/red	Bright red

Table 2-15 Legend colour coding haploview

LD Values: measures of linkage disequilibrium

r^2 is the raw r^2 value, the square of the correlation coefficient for a given marker pair. SNPs that have not been separated by recombination have $r^2 = 1$. Lower r^2 values correlate with a lower degree of LD, implying that some recombination would have taken place in this population.(Hill and Robertson 1966)

D' shows the raw D' value, which is the normalized covariance for a given marker pair. A D' value of 1, i.e. complete LD indicates that two SNPs have not been separated by recombination. Lower values indicate evidence of recombination in the history of that population. Only D' values near 1 are a reliable measure of LD. Lower values are difficult to interpret because sample size strongly influences the value of D'.(Lewontin 1988) No clear cut off point below 1 has been defined to indicate alleles not to be in LD (Ardlie, Kruglyak et al. 2002), although a D' value > 0.5 has been used as a cut off point for assessing potential LD.(Reich, Cargill et al. 2001)

The default score for Haploview shows D'/LOD, which displays D' and the log odds score for linkage disequilibrium between a given marker pair as a measure of confidence of the value of D'. (Insitute 2010)

2.9 Quality Control

Phenotype databases:

Designated members of the team (n=6) entered data onto the database. The complete records of subjects were audited in 5% of the cases. Data and conclusions for all outcome measures were double checked against source data. All study numbers were double checked against hospital numbers.

Genotyping:

All EDTA blood samples were labelled immediately with study number and/or subject name. After DNA-extraction, samples were collected in pre-labelled vials and stored in a racking system until use. Where possible, DNA handling, i.e. DNA extraction, library and array plate assembly, was done in the presence of two researchers.

Five percent of samples were analysed *in duplo*. Genotype data was available for a limited number of SNPs (*ACE* I/D, *PAI-1 4G/5G*, *MBL-2 X/Y promoter and gene*, *IL10 -1082 G/A*, *IL6 -174 G/C*, *TNFα -308 G/A* and *TLR4* Arg299Gly) that had been previously genotyped in a subset of the population (n=147). In the current analysis for *ACE* one array plate (n=91) was genotyped twice using the three primer PCR method for the I/D polymorphism as well as the Taqman method for rs4341.

2.10 Statistical analysis

The statistical analysis is described in the methods section of each chapter separately.

2.11 Physiological signal analysis

The autonomic nervous system plays an integral part in the systemic inflammatory

response.(Borovikova, Ivanova et al. 2000, Elenkov, Wilder et al. 2000) One way to quantify autonomic nervous system activity is the measurement of heart rate variability. Heart rate variability is the dynamic of beat-to-beat differences in heart rate. This measurement is well established as a marker of severity of disease in cardiac failure (Ho, Moody et al. 1997) and overall fetal distress (1997). More recently it has been used as predictor of sepsis in premature infants (Moorman, Lake et al. 2006) and adult haematopoetic stem cell transplantation (Ahmad, Ramsay et al. 2009) and as a measure of multi-organ dysfunction in adults (Schmidt, Muller-Werdan et al. 2005) and children (Tibby, Frndova et al. 2003).

Inflammation is a key part of these disease entities, which has prompted research groups to investigate HRV as a correlate of systemic inflammation. This yielded insight into cytokine production, e.g. IL-6 levels were shown to be inversely related to HRV (Aronson, Mittleman et al. 2001, Janszky, Ericson et al. 2004).

2.11.1 HRV metrics

There are several methods to quantify HRV. Most research groups have used time or frequency domain metrics.

A time series can be plotted graphically as a set of measurements in two-dimensions. Time is usually on the horizontal axis, and the magnitude of the measurable quantity is on the vertical axis. One can represent any periodic function of time as a sum of sines and cosines. This is called the Fourier series expansion of the function or frequency domain.

Heart rate variability or the pattern of change in heart rate over time can be expressed in the frequency domain. The variation in heart rate over time may be depicted by a

time series of ECG tracing sequential RR' intervals. These RR' intervals in a given time period are a sum of oscillations with distinct frequencies. These functions can be expressed in a frequency domain. The French mathematician Jean-Baptiste Fourier described the transformation of a time domain to a frequency domain in 1807. The resultant spectrum analysis contains the sum of each sine and cosine within the signal. The relative contribution of each wave is determined by its amplitude (or power) and the frequency with which it is represented in the signal.(Seely and Macklem 2004)

Computer programs can perform this fast Fourier transformation. The resultant power spectrum density plot depicts the relative contribution of the different frequencies in the original time series. The square of the contribution of each frequency is the power of that frequency to the total spectrum. The total power of the spectrum analysis, which is the area under the curve of the power spectrum is equal to the variance in the time domain.(Electrophysiology 1996)

2.11.1.1 Fast Fourier Transform Analysis

Power spectrum analysis of heart rate was first performed by (Sayers 1973). Although the power spectrum of a heart rate time series is a cumulative of all frequencies in that epoch with an infinite number of frequencies, there exists a physiological classification of predefined frequencies. In 2-5 minute ECG recordings these peaks are in the following range: very low frequency (VLF, cycle ≤ 0.04 Hz or a cycle length of ≥ 25 seconds); low frequency (LF, cycle 0.04-0.15 Hz or a cycle length of 6-25 seconds) and high frequency (HF, cycle 0.15-0.4 Hz or a cycle length of 2.5-6 seconds). In adults these discrete frequency peaks were associated with temperature

and humoral regulation and sympathetic/parasympathetic and vagal respiratory rhythms modulating heart rate respectively (Stauss 2003).

These discrete bandwidths have been adopted as standards in adults (Electrophysiology 1996). Children and infants show peaks at different bandwidths, which represents their different age-dependent physiology. It is, therefore, not useful to use the same bandwidths as those used in adults. Unfortunately no validated classification for children exists. The areas in which differences are most prominent are the bandwidth of the respiratory rhythm and the baseline heart rate, both of which are inversely related to age. Investigators thus have used different cut-off points that reflect these differences. Chapter 7 discusses these choices in more depth.

In order to transform the time series to the frequency domain without the introduction of artifact the following need to be adhered to:

The sampling frequency must be at least twice the highest frequency present in the sample (Nyquist theorem) in order to prevent aliasing. Aliasing is the phenomenon where the number of sampling points is insufficient to reproduce the original waveform and the ensuing frequency is different from the original waveform.

The signal must be stationary. In other words, over the sampled time period there must not be a significant change in mean and standard deviation of the signal.

The sample must be free of artifacts. The raw data must be inspected for premature atrial and/or ventricular beats, movement artifacts or lead dysfunction.

The heart rate variability data analysis was performed in collaboration with Mr Timothy Ellis, MSc candidate and Dr James McNames, Professor - Electrical and

Computer Engineering, Portland State University, OR, USA.

Chapter 3 Potential pitfalls in genotyping

3.1 Introduction

This chapter aims to discuss several areas of potential error in large genotyping studies and how these potential errors were addressed in the current study.

3.2 Genotyping quality

There were n= 318 samples available for the PICU cohort. This included n=3 unintentional duplicates (i.e. children that had been recruited before, but not recognized as such) and 12 intentional duplicates.

To address potential contamination because of transport one array was tested for admixture of DNA. This array showed no contamination (Figure 3-1).

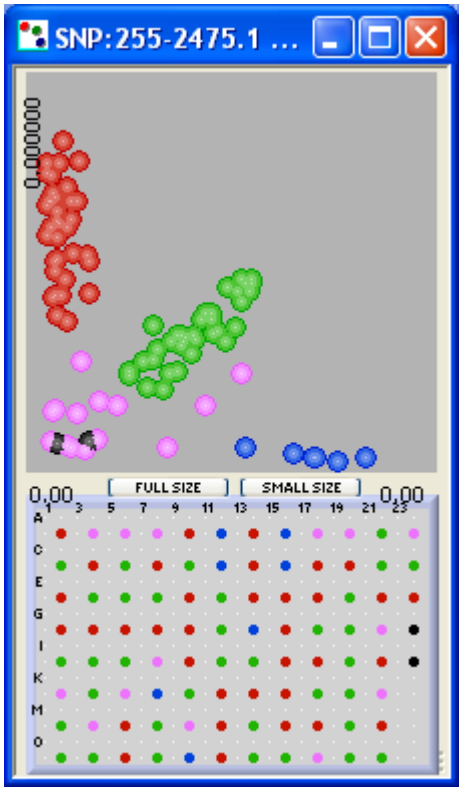


Figure 3-1 KBioscience test plate at 1:4 dilution

KBioscience test plate at 1:4 dilution. Array PICU 1 shows no contamination. All wells have a single output, correlating with the colours as depicted.

The KBioscience facility was consistently unable to yield results n= 9 samples. Most of these samples (7/9) contained less than 15 ng/μl DNA.

The initial candidate gene set consisted of 52 SNPs in 24 genes. The genotyping facility KBioscience was requested to genotype 50 SNPs. The initial results yielded genotypes on 38 SNPs (one SNP was inadvertently genotyped twice, thus yielding 39 results). A second attempt was made using oligos on the other DNA strand, which yielded 2 more genotypes. Then a third attempt was made using the Taqman method for one SNP, which failed also.

Southampton laboratory and the Rayne Institute genotyped one SNP each. Thus 42 SNPs were successfully typed and 10 SNPs in 5 candidate genes remained unanalysed. The unanalysed SNPs are shown in Table 3-1.

Identification	Gene	Kaspar both DNA strands	Taqman	Reason
rs429358	APOE	Not worked	Not worked	Unknown
rs2794521 -717A/G	CRP	Not worked	Not available	Unknown
rs3093062 -305G/A	CRP	Not worked	Not available	Unknown
rs28665122	SELS	Not worked	Not available	Unknown
AF029837_1 -241 A/G	CHRNA7	Not worked	Not available	Extremely high CG content
AF029837_2 -194 G/C	CHRNA7	Not worked	Not available	Extremely high CG content
AF029837_3 -178 -/G	CHRNA7	No assay designable	Not available	Unknown
AF029837_4 -143 G/A	CHRNA7	Not worked	Not available	Extremely high CG content
AF029837_4' -92 G/A OR C/T	CHRNA7	Not worked	Not available	Extremely high CG content
arg677trp R677W	TLR2	Not worked	Not available	Pseudo SNP

Table 3-1 SNPs that failed genotyping

The rs3091244 -286 C/T/A CRP tri-allelic SNP was genotyped as bi-allelic (C/T). Because the A allele is very rare in Caucasians, the bi-allelic analysis results would be a fair approximation in this ethnic group only.

Given their interest and expertise in CRP genotyping, the UCL Rayne laboratory was requested to perform genotyping on the outstanding CRP SNPs. Unfortunately, the time line was such that it precluded timely analysis.

Subsequent to further reading it transpired that TLR2 R677W is not a true SNP and therefore unclassifiable. The initial report pertained to variation in TLR2 exon 3 duplication. (Malhotra, Relhan et al. 2005)
The two remaining SNPs in the genes that remained of interest (APOE and SELS) unfortunately could not be genotyped. APOE was tried with three different approaches and SELS with two. Time restrictions precluded further pursuit.

Analysis of SELS would have allowed for testing the hypothesis whether this regulatory gene would have modified the outcome SIRS. SELS codes for Selenoprotein S, which is a transmembrane protein found in plasma membranes and endoplasmic reticulum. Selenoprotein S is thought to be involved in the prevention of ER stress reactive oxygen species production and as such modifies signaling secondary to severe insults. (Huang, Rose et al. 2012)Crucially, SELS protects the functional integrity of the ER against potential metabolic stressors. In this sense, low expression of SELS may increase inflammatory cytokines through increased ER stress. The SELS promoter polymorphism (-105 G-A) impaired Selenoprotein S expression and was strongly associated with higher circulating levels of IL-1b, TNF-a

and IL-6.(Curran, Jowett et al. 2005)If added to the overall logistic regression model in the TLR4 analysis pertaining to SIRS, there may have been an increase in explanation of genetic variability on the outcome of SIRS.

Analysis of APOE would have allowed for an analysis of nosocomial infection in those children admitted with a non-infectious insult. The APOE gene codes for three isoforms of the protein, ε2, ε3, and ε4. The wild type isoform is apoE3 and the two variant isoforms differ by a single amino acid substitution. These polymorphisms have been shown to be functional (Mahley and Rall 2000) in that the low-density lipoprotein (LDL) receptor binding affinities may be ranked as ε4 > ε3 >>> ε2. APOE wildtype was associated with reduced incidence of severe sepsis post elective surgery (Moretti, Morris et al. 2005). It is currently unclear what the underlying mechanisms might be, although lymphocyte activity has been implicated.

3.3 Aggregate results

The potential for spurious conclusions in genotyping analysis is increased if there are subjects that have not been included in the analysis because of inability to genotype. Therefore an overall assessment was done to ascertain the percentage calls per SNP. Table 3-2 shows the aggregate data on KBioscience's results. Next, the yield of the individual SNPS was analyzed. Table 3-3 and Table 3-4 show the calls made per SNP.

KBioscience 1			
Plate	SNPs	Subjects	Percentage called
CICU1	39	92	98.9
CICU2	39	80	98.5
CICU3	39	84	98.3
CICU4	39	91	98.1
CICU5	39	80	98.5
PhD Array 1A	39	87	99.0
PhD Array 2B	39	88	98.7
PICU1	39	86	98.5
PICU2	39	77	98.7
PICU3	39	91	99.0
PICU4	39	42	98.8
KBioscience 2			
Plate	SNPs	Subjects	Percentage called
CICU1	2	91	98.3
CICU2	2	75	99.3
CICU3	2	81	96.9
CICU4	2	90	99.4
CICU5	2	79	97.4
PhD Array 1A	2	86	97.6
PhD Array 2B	2	88	97.7
PICU1	2	84	98.8
PICU2	2	72	100
PICU3	2	88	99.4
PICU4	2	39	100

Table 3-2 Aggregate genotype data KBioscience

The origin of samples was denoted CICU for Cardiac Intensive Care Unit; PICU for Paediatric Intensive Care Unit; PhD Array for Cardiac Intensive Care Unit PhD work M Allen.

SNP	Gene	Calls	Percentage called	Absolute missed
rs4291	ACE	846	89.8	96
rs7412	APOE	887	94.1	55
rs1061170	CFH	895	95.0	47
CHRFAM7A	CHRFAM7A	907	96.2	35
rs3091244	CRP	916	97.2	26
rs641153	CFB	920	97.6	22
AF029837_6 GT	CHRNA7	921	97.7	21
rs172378	C1QA	921	97.7	21
rs1800795	IL6	921	97.7	21
rs2569190	CD14	922	97.8	20
rs4343	ACE	923	97.9	19
AF029837_5 CT	CHRNA7	930	98.7	12
ADRB2 5LC	ADRB2	932	98.9	10
rs1042713	ADRB2	932	98.9	10
rs1130864	CRP	932	98.9	10
rs1800450	MBL2	932	98.9	10
rs1800797	IL6	932	98.9	10
rs1800872	IL10	932	98.9	10
rs1800888	ADRB2	933	99.0	9
rs2232582	LBP	934	99.1	8
rs5743708	TLR2	934	99.1	8
rs1800896	IL10	935	99.2	7
rs1799889	PAI-1	935	99.2	7
rs11003125	MBL2	936	99.3	6
rs1800871	IL10	936	99.3	6
rs3087456	MHC2TA	936	99.3	6
rs3814700	ADM	936	99.3	6
rs1800796	IL6	936	99.3	6
rs4696480	TLR2	936	99.3	6
rs4986791	TLR4	936	99.3	6
rs1205	CRP	937	99.4	5
rs4696480	TLR2	937	99.4	5
rs7095891	MBL2	937	99.4	5
rs1800451	MBL2	937	99.4	5
rs2476601	PTPN22	938	99.5	4
rs3093077	CRP	938	99.5	4
rs5030737	MBL2	938	99.5	4

rs1042714	ADRB2	939	99.6	3
rs1800629	TNFA	939	99.6	3
rs4151667	CFB	939	99.6	3
rs7096206	MBL2	941	99.8	1
Total		942	100	

Table 3-3 KBioscience aggregate genotyping results per SNP

SNP	**Gene**	**Calls**	**Percentage called**	**Absolute missed**
rs4986790	TLR4	965	98.2	17
rs4646994	ACE	86	94.5	5
rs4341	ACE	901	97.0	27

Table 3-4 Aggregate data genotyping Southampton and Rayne Institute

3.3.1 PICU failed samples

There were 8 samples that showed a very high failure rate (≥41 SNPs), 3 of those samples were duplicates. A further 13 samples failed ≥ 3 genotyping, 2 of which were duplicates. (Figure 3-2)

Failure was defined as an inability to assign the identity of a nucleotide at the location of the polymorphic site.

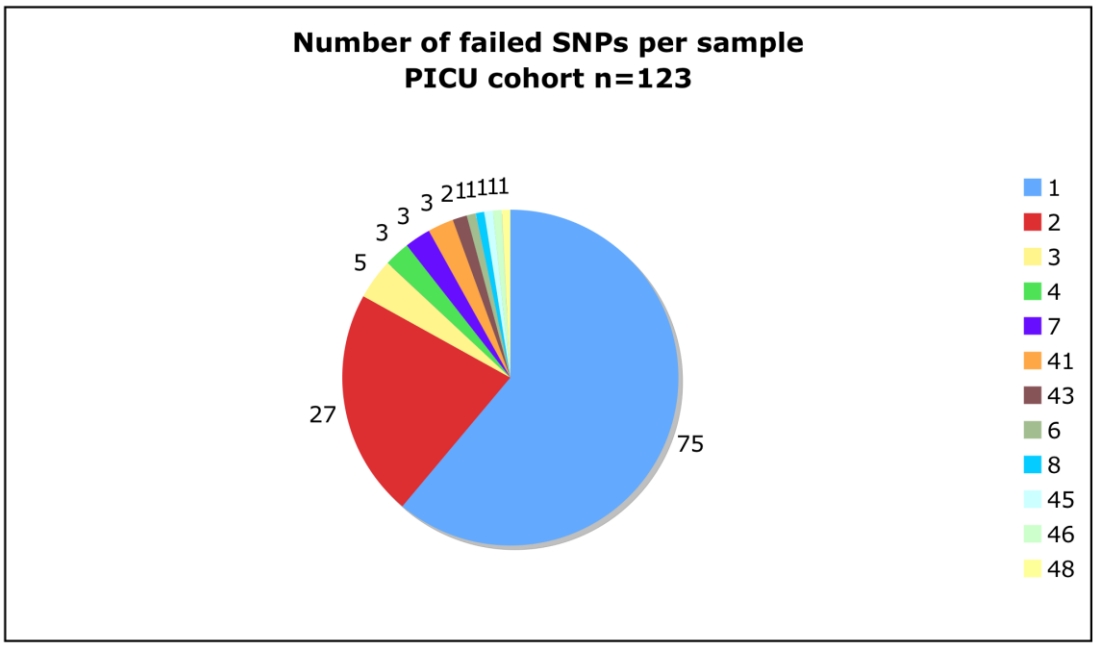


Figure 3-2 Failed PICU samples

The total number of children was 299, of these, 123 samples showed failed genotyping in ≥1 SNP.

The potential effect of these failures is allele dropout, thus influencing HWE; change in population with regards to clinical characteristics and reduction of quality control efficacy. Most failures were due to low DNA content and most were samples obtained at the early stages of the study, 15/21 (71%).

Given that the failures are thought to be random (secondary to reduced amount of DNA availability) there should not be a systematic change in the early population. One potential effect might have occurred, which is that the influence of the latter part of the PICU cohort exerts a higher degree of influence on the overall outcome measures. However this seems unlikely, given that there were 5 major dropouts in the early cohort (n=165) and 1 major dropout in the late cohort (n=134), p = 0.23, Fishers exact test.

The number of children recruited in the PICU cohort was n=305. Of these, 3 were repeat admissions and 3 did not have a blood sample available. The cohort thus consisted of n=299 children.

3.4 Hardy Weinberg Equilibrium (HWE)

Heterozygosity and Hardy Weinberg Equilibrium were calculated using the Tufts University Pharmacogenomics Laboratory HWE calculator and confirmed by Haploview (Barrett, Fry et al. 2005).

Given the heterogeneity of the cohorts, integral to a cosmopolitan city London with high migration and interbreeding there was a real possibility that SNPs may not have been in HWE, even when accounting for ethnicity.

In fact, for the PICU cohort the following genes were not in HWE: CRP rs3091244; CFB rs641153; MHC2TA rs3087456 and IL6 rs1800796.

This was further explored by categorizing the cohort into the sub populations according to ethnicity. Table 3-5, Table 3-6 and Table 3-7 show this for the PICU cohort.

CRP rs3091244	**PICU**	**Caucasian**	**African**	**Asian**	**Other/Unknown**
CC	132	96	12	9	15
CT	275	89	10	7	3
TT	122	26	8	1	10
χ^2 p value	7.32 <0.01				
CFB rs641153 (R32Q)	PICU	Caucasian	African	Asian	Other/Unknown
CC	234	173	22	13	26
CT	42	29	9	3	1
TT	14	9	4	1	0
χ^2 p value	29.2 <0.001	19.7 <0.001			
IL6 rs1800796 (-572G>C)	PICU	Caucasian	African	Asian	Other/Unknown
GG	235	177	28	8	22
GC	48	30	6	7	5
CC	10	5	2	2	1
χ^2 p value	11.9 <0.001	6.3 <0.02			
MHC2A rs3087456	PICU	Caucasian	African	Asian	Other/Unknown
AA	121	105	0	5	11
AG	108	77	14	6	11
GG	65	31	22	6	6
χ^2 p value	16.6 <0.001	6.7 <0.02			

Table 3-5 PICU cohort SNPs not in HWE.

Red denotes the population that is not in HWE.

CRP rs3091244	**PICU**	**Caucasian**	**African**	**Asian**	**Other/Unknown**
CC	122	94	10	9	9
CT	130	94	15	7	13
TT	35	24	6	1	4
CFB rs641153 (R32Q)	PICU	Caucasian	African	Asian	Other/Unknown
CC	224	167	20	12	26
CT	62	42	13	4	1
TT	4	3	2	1	0
IL6 rs1800796 (-572G>C)	PICU	Caucasian	African	Asian	Other/Unknown
GG	229	174	26	8	21
GC	60	36	9	7	6
CC	4	2	1	2	1
MHC2A rs3087456	PICU	Caucasian	African	Asian	Other/Unknown
AA	104	97	2	4	10
AG	142	94	11	8	13
GG	48	23	23	5	5

Table 3-6 PICU cohort expected genotype distribution if in HWE

The number of heterozygotes is lower than expected.

Table 3-7 Heterozygosity compared in PICU cohort

SNP	Observed	Expected	P value
CRP rs3091244	0.32	0.43	$1.1*10^{-11}$
CFB rs641153 (R32Q)	0.14	0.21	$6.0*10^{-14}$
MHC2A rs3087456	0.38	0.46	$1.8*10^{-6}$
IL6 rs1800796 (-572G>C)	0.16	0.20	$1.5*10^{-9}$

P value derived from Tufts University Pharmacogenomics Laboratory HWE Calculator

Given that the different ethnic populations were in HWE for CRP rs3091244, the reason that the total PICU cohort was not in HWE for this SNP may have been the lumping together of ethnic groupings. This was not the case for CFB R32Q and MHC2A rs3087456, both SNPs were not in HWE in the Caucasian group.

A different explanation may have been a skewing of the population because of allelic dropouts. The number of missed genotypes, however, was low (1 per plate), therefore allelic dropout is very unlikely (Table 3-8.)

Plate	Samples	rs641153
PICU1	86	85
PICU2	77	76
PICU3	91	90
PICU4	42	41

Table 3-8 CFB R32Q rs641153 dropouts PICU cohort

Next, genotyping error was assessed by viewing the results spatially in SNPviewer. There were no “close calls” and genotypes were well separated in three groups (homozygous for wildtype, heterozygotes and homozygous for minor allele). Figure 3-3 shows this for CFB R32Q rs641153.

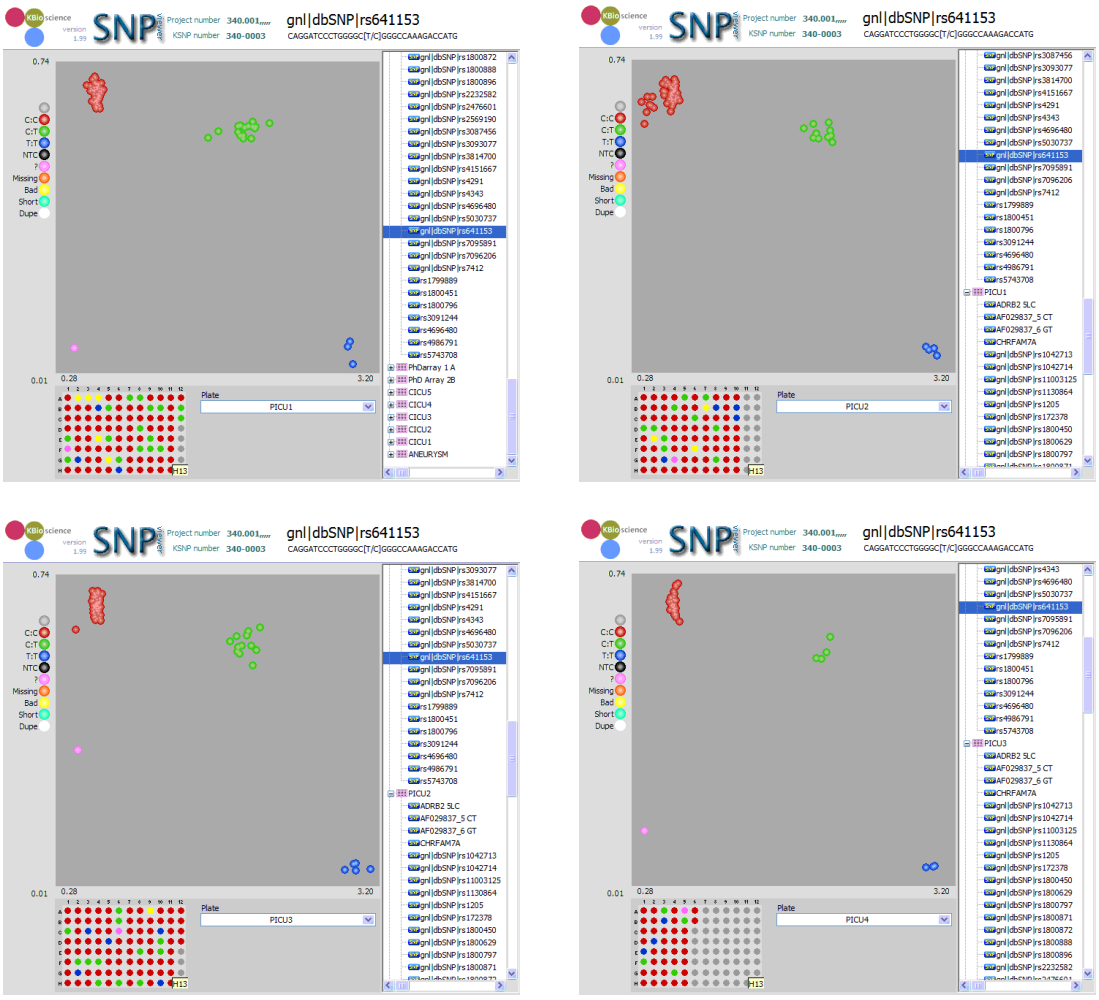


Figure 3-3 CFB R32Q visual inspection PICU arrays 1-4

Red dots denote CC, green dots CT, pink dots unclassifiable and blue TT

Finally, the minor allele (T) frequency for CFB R32Q rs641153 was assessed against what is known in the literature. For cohorts of European descent frequencies have been quoted between 0.06 and 0.12 (Maller, George et al. 2006, Ferrara, Merriam et al. 2008, 2010) and for cohorts of African descent 0.25 (2010).

In the PICU cohort overall T allele frequency was 0.12 for the Caucasian and African subgroups it was 0.11 and 0.24 respectively. The Cardiac cohort showed similar allele frequencies (0.11 and 0.22 for Caucasians and Africans respectively). These results concur with previously published results.

Thus a genotyping error is unlikely. This is specifically of concern for this SNP, because of the existence of a non-functional SNP at the adjoining position 94 C>T (rs12614) leading to CFB R32W.

Thus the allele frequency is as expected, but the number of homozygotes is too high. What remain as explanations are the following possibilities: selection, inbreeding and the Wahlund effect (subpopulations that do not interbreed). Given the recruiting strategy ("allcomers" from anywhere in the UK) the most likely reason is that the Caucasian population is actually made up of sub-populations that are each in good Hardy-Weinberg equilibrium and the deviation is due to pooling of these separate populations. It may be that "Caucasian" was too heterogeneous for these two SNPs.

To test this hypothesis the PICU Causasian population was re-analysed for Hardy Weinberg Equilibrium for the original ethnic category White British UK only, thus excluding the other Caucasian subjects from the following descent: Middle Eastern, Arab States, Pakistani, Bangladeshi, Indian, White Irish, White, White European, White other, Jewish and Malteser for rs641153 (CFB R32Q) allele frequencies.

This showed that allele frequencies in the White British UK group were still not in HWE: CC/CT/TT 124/22/7 (expected 119/32/2), χ^2 14.4, *P* value 0.00014.

Only if the London area were not taken into account was the British UK White population in HWE (n=79).

It has been postulated previously that urban areas are not the most optimal for genetic association studies, given the high likelihood of cryptic sub populations (Cooper, Tayo et al. 2008).

Population stratification does occur in European populations. Based on microarray genotype data of approximately 10.000 SNPs two axes of stratification were observed: the main one was north-south, but there was also an east-west trend.(Bauchet, McEvoy et al. 2007)

Thus cryptic subpopulation, or the Wahlund effect may be the underlying reason for the results of the rs641153 (CFB R32Q) allele frequencies. Why this was then not the case for other SNPs remains unexplained.

Another explanation for exclusion of the London population to arrive at a White British population that is in HWE is that the resultant cohort is very small (n=79), which allows for an easier fit to HWE.

Finally, it may be that the departure of HWE is not a technical or sampling error and neither a statistical fluke. It may be that the heterozygote population is more robust than the homozygotes and thus are protected from being admitted to PICU.

To explore this hypothesis the PICU cohort was stratified according to admission diagnosis. The two groups were defined according to primary inflammatory/infectious process or primary trauma/elective surgery. This would allow testing for reduced innate robustness in predisposition to severe inflammatory processes, assuming that trauma and elective surgery are random admission events from an inflammatory point of view.

This generated a potential explanation for departure of HWE for IL6 rs1800796 (-572G>C) and MHC2A rs3087456. Both SNPs still showed a large departure from HWE in the infection/inflammation group, but not at all in the trauma/elective surgery group (Table 3-9).

SNP	Presumed reduced robustness	Presumed random occurrence
IL6 rs1800796 (-572G>C)	Infection/Inflammatory	Trauma/Elective postoperative
GG	131	104
GC	21	27
CC	8	2
Total	160	133
χ^2; p value	20.5; <0.001	NS
MHC2A rs3087456	Infection/Inflammatory	Trauma/Elective postoperative
AA	68	53
AG	51	57
GG	42	23
Total	161	133
χ^2; p value	19.6; <0.001	NS

Table 3-9 Sub classification IL6 departure HWE in PICU cohort

Red denotes departure from HWE.

To test this hypothesis further the independent cardiac cohort was queried for MHC2A rs3087456 HWE. If the abovementioned hypothesis of a random sterile inflammatory insult in relation to HWE holds true then this cohort should be in HWE, corrected for ethnicity as needed. Indeed this was the case (Table 3-10). The notion that MHC2A rs3087456 homozygosity reduces innate robustness in the face of acute inflammation/infection was further strengthened.

MHC2TA	Cardiac	Caucasian	African	Asian	Other/Unknown
1	247	237	2	3	5
2	225	199	9	3	14
3	85	46	30	1	8
dropped	22	16	1	1	4
χ^2; p value	11.2; <0.001				

Table 3-10 MHC2TA distribution in cardiac cohort

Red denotes not in HWE. Cardiac cohort in HWE when adjusted for ethnicity.

Similarly, IL6 rs1800796 (-572G>C) was not in HWE for the whole PICU cohort. Genotyping for this SNP did not show ambiguous calling (Figure 3-4) and the success rate for genotyping was high (n=1 dropout). Subgroup analysis showed the genotypes to be in HWE for all but the Caucasian subgroups (Table 3-9).

Comparing the PICU cohort with a cohort of white Europeans (n=100) (Muller-Steinhardt, Ebel et al. 2007) showed that the Caucasian subgroup was similar in genotype frequencies and that the African subgroup differed significantly from both groups ($P<0.0001$, χ^2). Therefore the initial HWE result for this polymorphism is probably, in part, attributable to population stratification. The Caucasian subgroup, however, was still not in HWE. Again, the departure from HWE may be due to reduced innate robustness as described above.

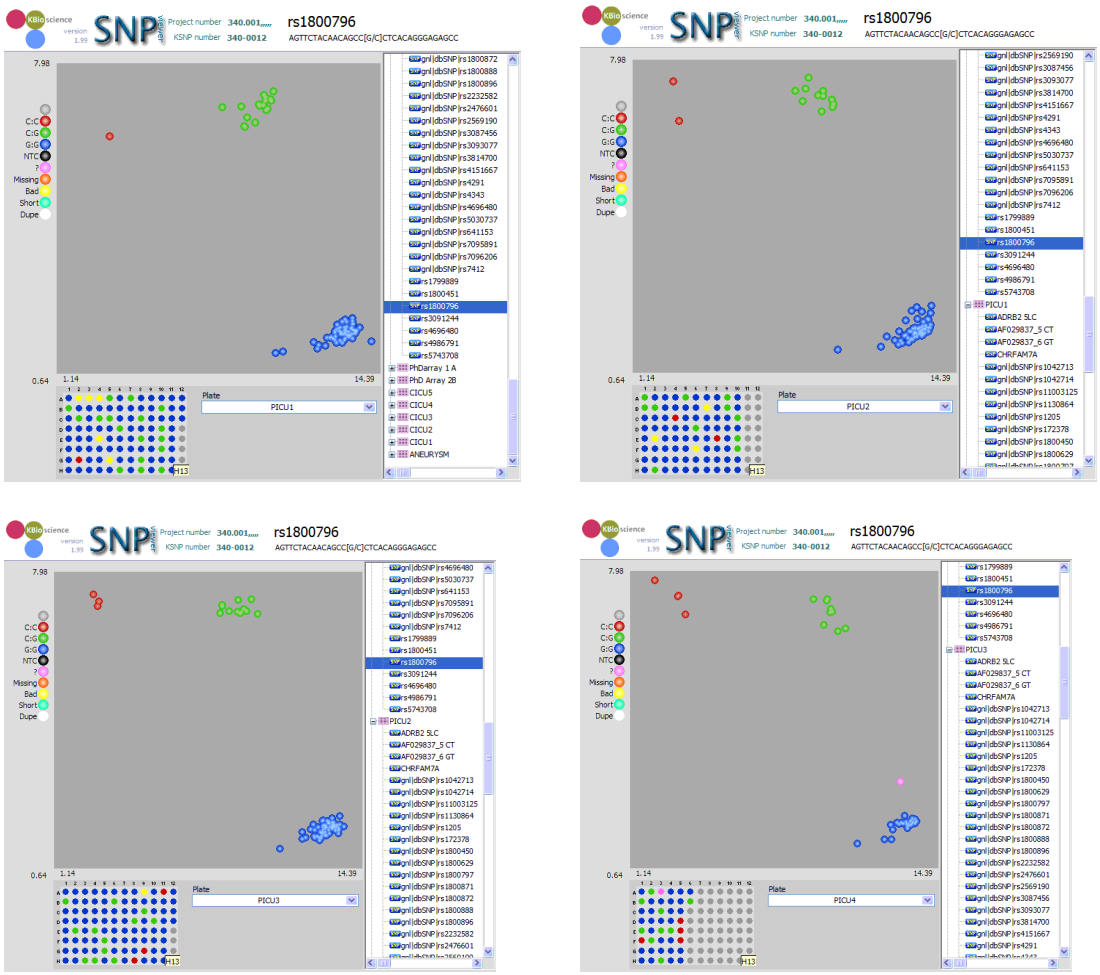


Figure 3-4 IL6 rs1800796 results for PICU 1-4

The results for IL6 1800796 show no ambiguous calling.

MHC2TA was not analysed further for this thesis. The outcome measure "nosocomial infection" was not pursued. The hypothesis that homozygosity for MHC2TA rs3087456 carries a penalty for reduced robustness in the face of an acute infectious or inflammatory event is potentially of interest. Given the limited influence of single SNPs on complex outcomes it is, however, unlikely.

In summary, the observed departures in HWE may be due to genotyping error (CFB R32Q), population stratification (CRP rs3091244) or reduced innate robustness, (IL6 rs1800796 (-572G>C) and MHC2TA rs3087456).

3.5 Linkage Disequilibrium

Linkage disequilibrium (LD) is the non-random association of alleles at several loci. Haploview is a freely available software program that is developed and maintained by the Broad Institute of Massachusetts Institute of Technology (MIT) and Harvard University (Barrett, Fry et al. 2005). The program allows for calculation and graphical display of linkage disequilibrium between SNPs. Haploview criteria for LD blocks are based on (Gabriel, Schaffner et al. 2002) and HWE calculations are based on (Wigginton, Cutler et al. 2005).

"These empirical assessments led to the following validated block definitions. In all cases, the outer-most marker pair was required to be in strong LD with an upper confidence limit (CU) that exceeds 0.98, and a lower confidence limit (CL) that exceeds 0.7.

Two marker rules: pairs of markers with confidence bounds of 0.8<CL/0.98<CU, and spanning no more than 20 kb in the European and Asian samples, and no more than 10 kb in the Yoruban and African-American samples.

Three markers rules: sets of three consecutive markers for which all pairs have

confidence bounds of 0.5<CL/0.98<CU and span <30 kb in the European and Asian samples, and for which all pairs have 0.75<CL/0.98<CU and span <20 kb in the Yoruban and African-American samples.

Four markers: the fraction of informative pairs in strong LD (0.7<CL/0.98<CU) was required to be > 95%, and to span no more than 30 kb.

For runs of five or more markers, the fraction of informative pairs in strong LD must be >95%, and were allowed to span any distance." (Gabriel, Schaffner et al. 2002).

Linkage disequilibrium for the PICU cohort was determined as follows:

Analysis settings: 299 subjects, 40 SNPs in 18 genes.

HWE p-value cut off 0.001 and minimum genotype percentage 50%.

This yielded the following results:

Five individuals were excluded because of insufficient percentage known genotype and four SNPs were excluded, two (MHC2TA rs3087456 and CFB R32Q rs641153 because of violation of HWE), one because it was not polymorphic (CHRNA7 -46G>T AF029837_6 GT CHRNA7) and one because of insufficient genotyping data (45.9% for ACE I/D rs4646994).

This resulted in 630 comparisons for identification of LD between SNPs. Of these 630 comparisons 16 qualified for strong LD. These 16 pairs showed a high D', defined as 0.7< Confidence Interval (CI) low and 0.98< CI high as well as a LOD score > 2. Table 3-11 depicts these 16 SNPs in 6 genes.

The r^2 values range between 0.095 and 1. Those SNP pairs that have an r^2 value of 1 are thought not to have separated by recombination. In essence, from a point of genotyping, one of the SNPs from such a pair would be superfluous. For SNP pairs in which the r^2 value is <1, recombination has occurred.

The Logarithm of the odds (LOD) score is a measure of LD. The Hapmap Consortium applies a cut off LOD score over +2 (i.e 100 to 1 odds) as evidence for linkage. (2005)

D’ is reported to measure only recombinational history and r^2 to summarize both recombinational and mutational history (Devlin and Risch 1995, Jorde 2000).

Locus 1	Locus 2	D'	LOD	CI low	CI high	r^2	Distance
rs1800797(IL6-597G>A)	rs1800795(IL6-174G>C)	0.99	97.85	0.96	1	0.971	424
rs1800451(MBL57C)	rs7095891(MBL+4PQ)	1	8.63	0.78	1	0.095	235
rs5030737(MBL52D)	rs11003125(MBL-550LH)	1	10.25	0.83	1	0.131	772
rs7095891(MBL+4PQ)	rs7096206(MBL-221XY)	1	11	0.85	1	0.103	224
rs7095891(MBL+4PQ)	rs11003125(MBL-550LH)	1	19	0.91	1	0.204	553
rs7096206(MBL-221XY)	rs11003125(MBL-550LH)	1	11.78	0.86	1	0.135	329
rs4341(ACE)	rs4343(ACE)	1	82.78	0.97	1	0.745	41
rs1042711(ADRB25LC)	rs1042713(ADRB2Arg16Gly	1	44.93	0.95	1	0.398	92
rs1042711(ADRB25LC)	rs1042714(ADRB2Gln27Glu)	1	128.28	0.98	1	1	125
rs1042713(ADRB2Arg16Gly	rs1042714(ADRB2Gln27Glu)	1	45.87	0.95	1	0.397	33
rs1205(CRP)	rs1130864(CRP)	1	17.28	0.9	1	0.16	858
rs1205(CRP)	rs3091244(CRP)	0.953	21.53	0.85	0.99	0.206	2432
rs1130864(CRP)	rs3091244(CRP)	0.97	68.39	0.92	1	0.703	1574
rs1800872(IL10)	rs1800871(IL10)	1	118.73	0.98	1	1	227
rs1800872(IL10)	rs1800896(IL10)	1	35.65	0.94	1	0.32	490
rs1800871(IL10)	rs1800896(IL10)	1	35.35	0.94	1	0.321	263

Table 3-11 Linkage Disequilibrium PICU cohort quantitative measures

Blocks were identified in the following genes: IL6, MBL2, ACE, ADRB2, CRP and IL10. The TLR4 SNPs showed some LD (D' 0.78 (CI 0.61-0.9), LOD15.55 and r^2 0.49). This is to a lesser extent than one would expect, given that the literature quotes these two SNPs to be commonly co-segregated.

Figure 3-5 shows the overall LD depicted in colour coded diamonds. Bright red diamonds without a number denote a D' of 1.

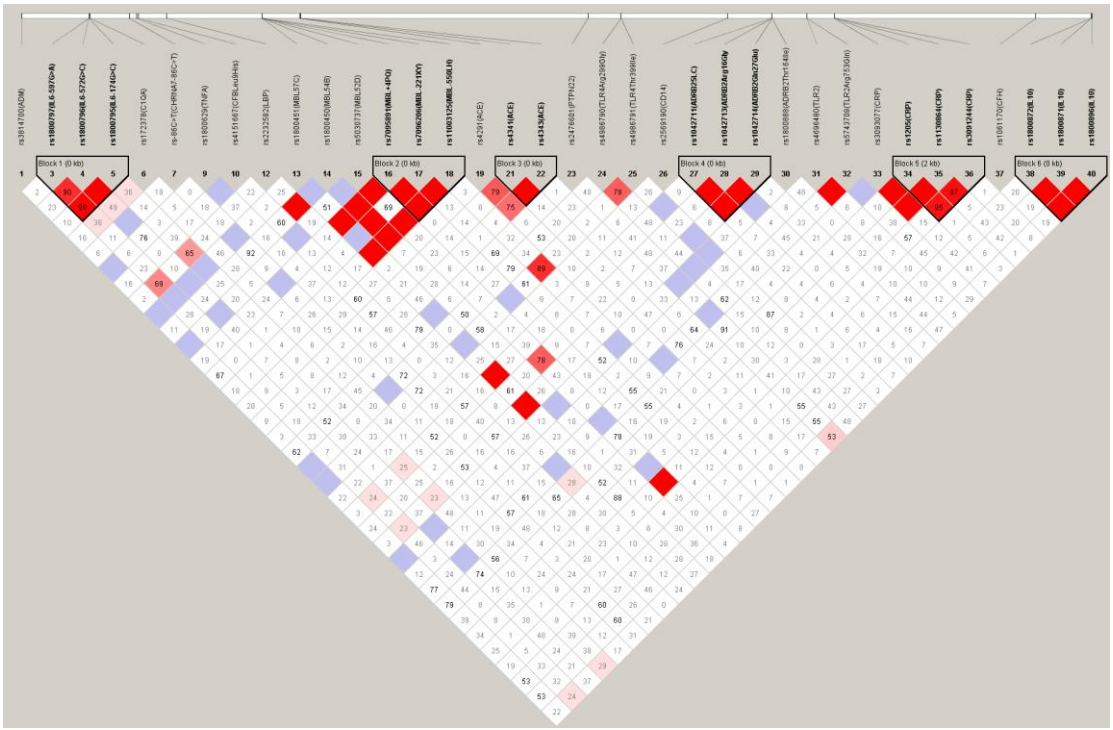


Figure 3-5 Linkage Disequilibrium in colour coding

PICU (n=299) Color Scheme LD plot

	D' < 1		D' = 1	
LOD < 2	White		Blue	
LOD ≥ 2	Shades of pink/red	34	Bright red	98

Linkage disequilibrium blocks that were thus identified within genes are:
IL6, MBL2 (except MBL57C and 52D), ACE, TLR4, ADRB2, TLR2, CRP (except for rs3093077 and rs3091244) and IL10.

In this cohort two pairs of SNPs were identified to be in complete LD with each other: rs1042711(ADRB25LC) with rs1042714(ADRB2Gln27Glu); D' =1(CI 0.98 - 1), LOD =128.28, r^2 =1 and rs1800872(IL10) with rs1800871(IL10); D'=1 (CI 0.98-1), LOD = 118.73, r^2 =1.

Rather than relying on general terms given in the literature it is advisable to assess the extent of LD for the actual cohort, especially if of mixed heritage.
The strong LD between rs1800795 (IL6 -174G>C) and rs1800797 (IL6 -597G>A) as previously reported in Caucasians (r^2 0.95)(Hamid, Rose et al. 2005) was validated in the current PICU cohort across ethnicities (see Table 3-12). This, however, cannot be said for the TLR4 LD.
In addition, aside from genotyping errors, LD parameters are not only influenced by population admixture but also by population size. To illustrate this Figure 3-6 shows the LD plot for the same SNPs as in Figure 3-5, but this time for the combined cardiac and general population. The number of LD blocks is higher in this larger cohort of n= 878 vs. n=299. Of note, the difference in LD may also be due to a higher proportion of Caucasians in the cardiac cohort (424/494 vs. 216/299, P <.0001, χ^2), see Table 3-12.

Ethnicity	PICU	CICU
Caucasian	216 (72)	424 (86)
African	36 (12)	39 (8)
Asian	18 (6)	6 (1)
Mixed/Unknown	29 (10)	25 (5)
Total	299	494

Table 3-12 PICU and CICU cohorts according to ethnicity

Absolute numbers, percentage in brackets.

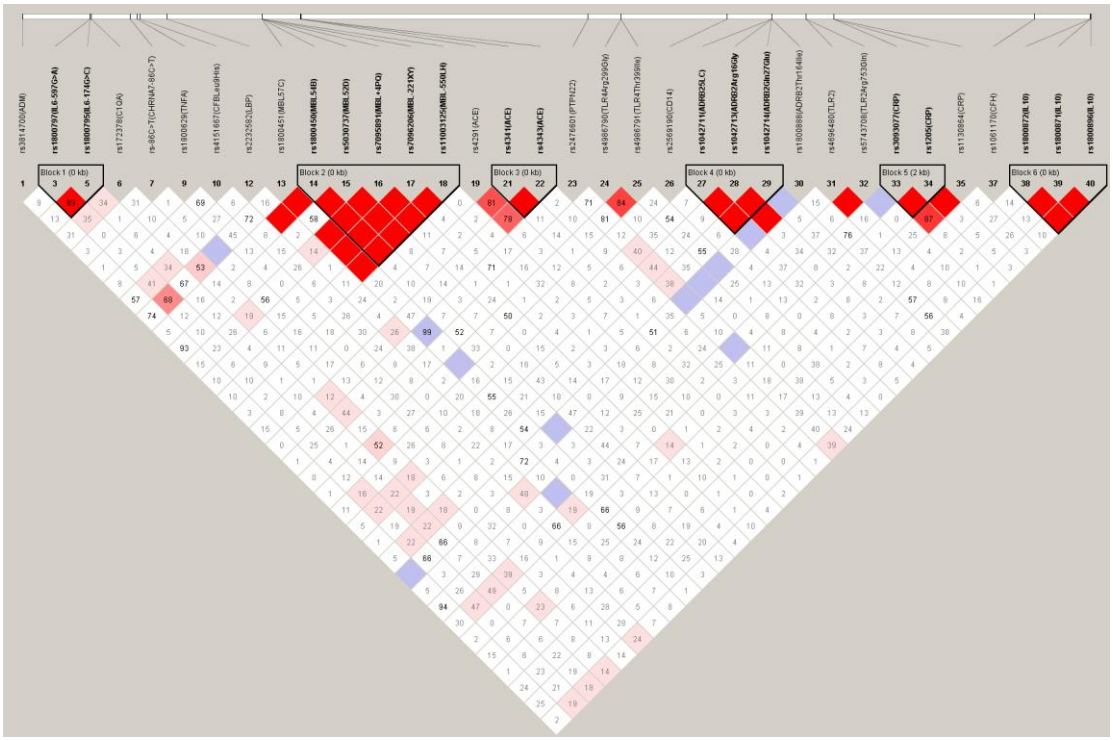


Figure 3-6 Linkage Disequilibrium in PICU and CICU cohorts combined

(n= 793)

3.5.1 MBL2

To illustrate how cohort size and ethnic background influences linkage disequilibrium magnitude and probability, the MBL2 SNP LD blocks are used here (Figure 3-8). Results are based on the raw genotypes of subjects of mixed ethnicity recruited from paediatric intensive care (n= 299) and cardiac intensive care (n=494).

As a reminder of the MBL2 polymorphisms **Figure 1-9** reproduced here as Figure 3-7.

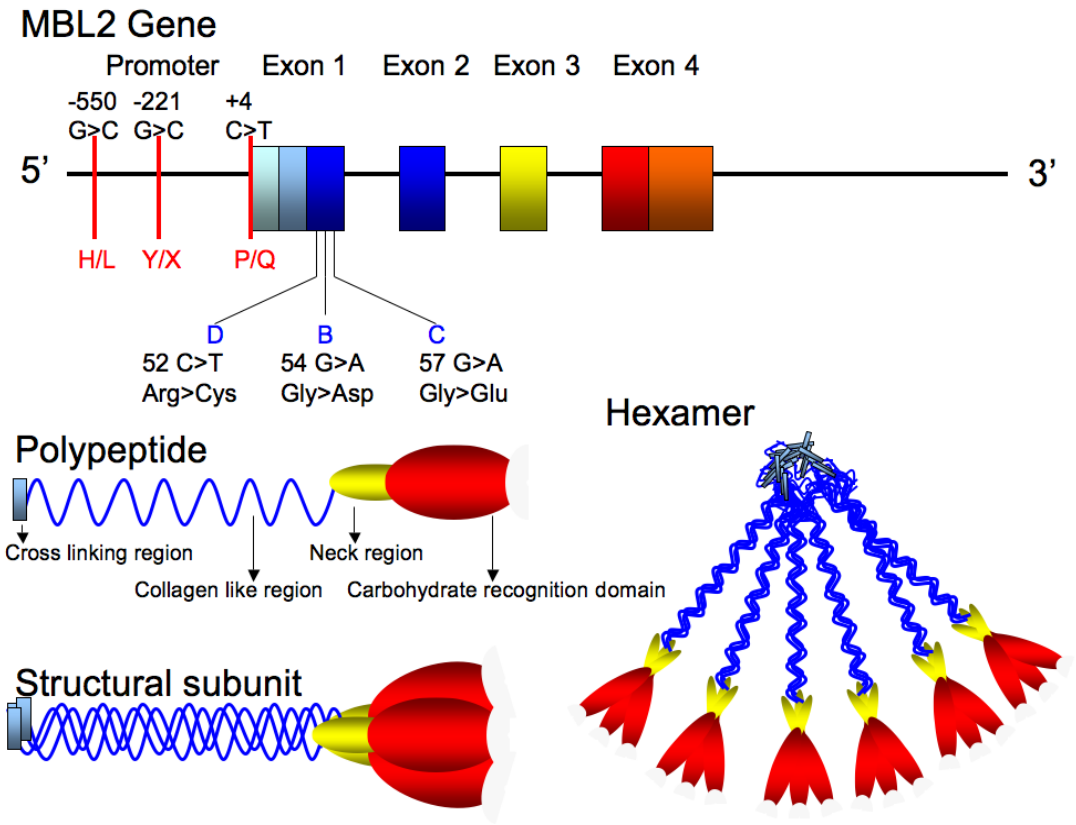
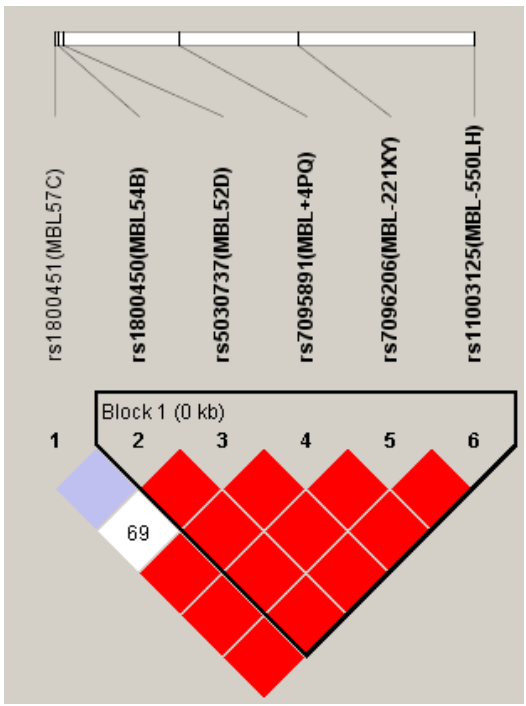
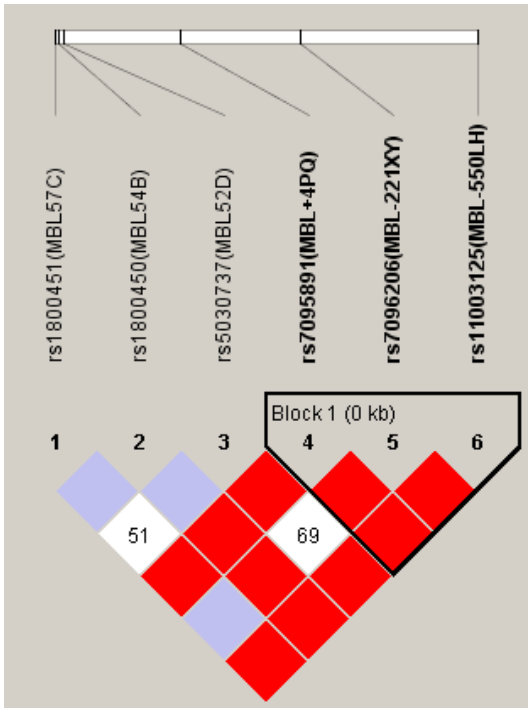


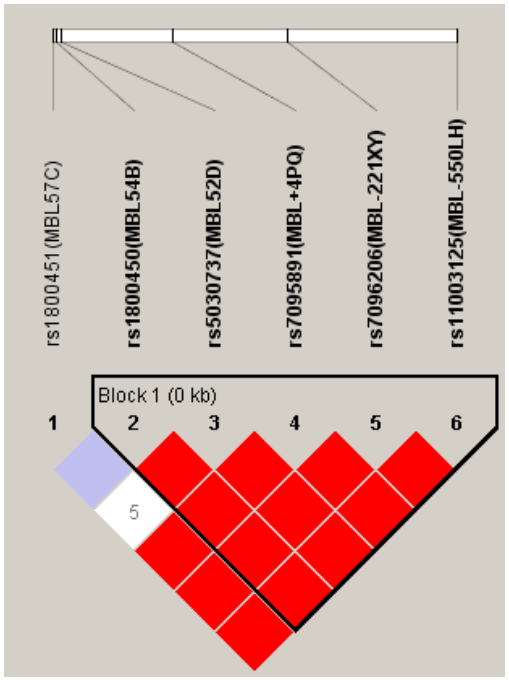
Figure 3-7 MBL 2 Gene and polymorphisms



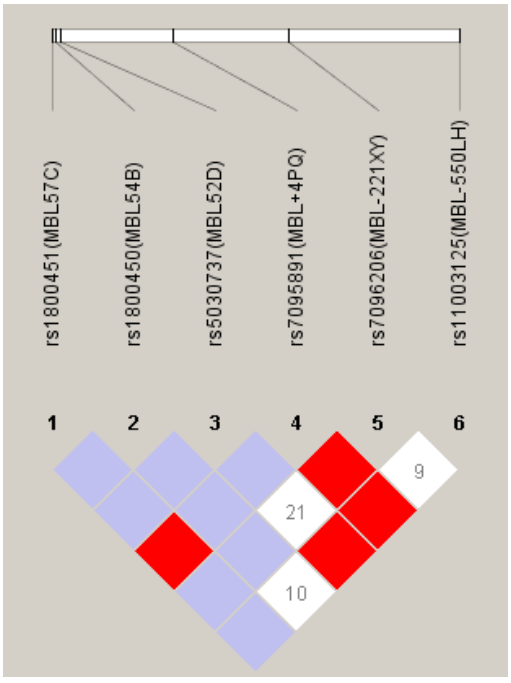
All (n=793)



PICU (n=299)



Caucasian (n= 640)



African (n=75)

Figure 3-8 MBL2 Linkage Disequilibrium in PICU and CICU cohorts compared according to ethnicity

Interestingly the two SNPs that were least in LD (57C and 52D) are the two that are very close in distance (16 base pairs) but phylogenetically most removed from each other. (Figure 3-9 from (Garred, Larsen et al. 2006))

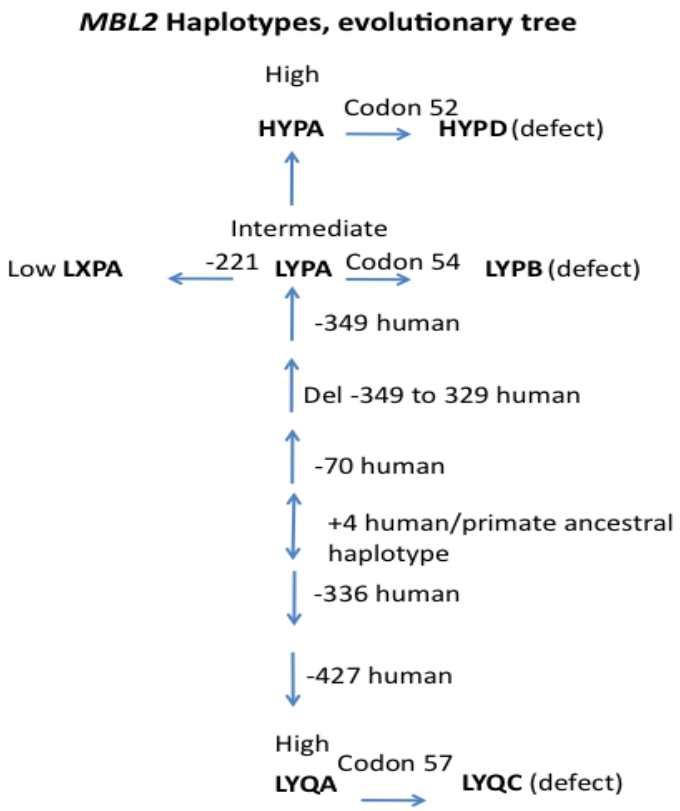
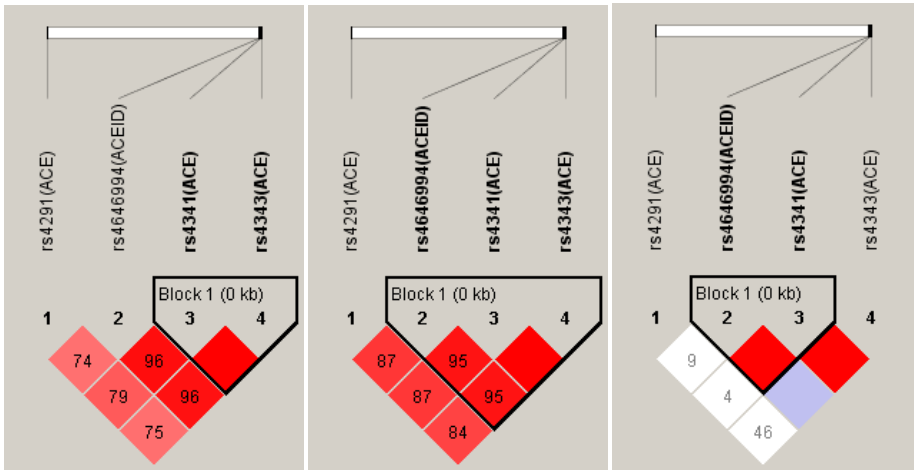


Figure 3-9 MBL2 Evolutionary Tree

An unexpected LD occurred between rs2232582 LBP and rs1800450 MBL 54B (D' 1.0, LOD 5.3). Given that this LD did not occur with any of the other MBL SNPs it may well be a false positive. More so, because MBL2 lies on Chromosome 10 and LBP on Chromosome 20 and thus doesn't qualify for LD as it is commonly understood. LD, however, may occur between loci on different chromosomes, possibly due to other mechanisms than permutations.(Huttley, Smith et al. 1999) Interestingly this LBP SNP was previously found to impact outcome in sepsis in a Caucasian adult cohort. (Hubacek, Stuber et al. 2001)Although this finding was not replicated in a different adult cohort (Barber and O'Keefe 2003), one might speculate that the first finding may be attributed to MBL B rather than LBP. The MBL B genotype has indeed been found to modify sepsis in both adults (Garred, Strom et al. 2003, Gordon, Waheed et al. 2006) and children (Fidler, Wilson et al. 2004).

3.5.2 Linkage disequilibrium in ACE

The linkage disequilibrium for ACE in the PICU cohort is shown in Figure 3-10.



All (n=299) Caucasians (n=216) Africans (n=36)

Figure 3-10 Linkage disequilibrium for ACE in PICU cohort

In Caucasians the ACE I/D Alu polymorphism (rs4646994; rs1799752; rs4340; rs13447447**)** is thought to be in tight LD with the ACE allele that is actually responsible for the hereditary difference in ACE activity. In Africans, however, this is less the case.(Payne, Dhamrait et al. 2007)

ACE is a very illustrative candidate gene to highlight the many potential pitfalls in elucidating the underlying susceptibility or quantitative trait locus (QTL) of complex inherited diseases. Many studies have identified ACE as a modulator of cardiovascular disease, e.g. (Pfeffer, Braunwald et al. 1992, Lewis, Hunsicker et al. 1993). ACE inhibition is an integral part of the pharmacopoeia available to medicine today. Serum ACE levels are fairly static when measured repeatedly in the same individual over time (Dux, Aron et al. 1984), but can differ up to five fold between individuals (Alhenc-Gelas, Richard et al. 1991). The first study to identify that ACE serum levels are under considerable genetic control was done in 87 healthy French families.(Cambien, Alhenc-Gelas et al. 1988)The authors speculated that there may be polymorphisms responsible for these familial differences. Soon after the ACE gene was located to chromosome 17q23 (Mattei, Hubert et al. 1989) and a genetic marker was found that accounted for half the variance in ACE serum levels in healthy French Caucasians. This marker was identified as a Mendelian inherited intronic ~250 base pair long Insertion (I)/Deletion (D) polymorphism. The allele frequencies were 0.406 for the I and 0.594 for the D allele in 80 subjects. (Rigat, Hubert et al. 1990)These alleles accounted for 47% of the variance in ACE serum levels in an additive way. Highest levels were seen with the homozygous D allele and lowest with the homozygous I allele. The I/D polymorphism was located to intron 16 of the ACE gene and measured to be 287 bp long. The same group investigated this relationship in a separate healthy cohort of 404 Caucasian subjects from the same geographical

area near Nancy, France. They concluded that the I/D polymorphism was a marker for a causative allele that explained 44% of the variance in serum ACE levels.(Tiret, Rigat et al. 1992)

Further independent validation in a British Caucasian cohort showed this.

In contrast to Caucasians the ACE I/D polymorphism was not highly associated with serum ACE level in a family study of Black African Nigerians (n=1343). This study found that two polymorphisms accounted for 19% (A2350G; A11860G; rs4343) and (A-240T; A-262T; rs4291) 6% of the variance in ACE activity respectively. In fact the A allele for A-240T was associated with increased serum ACE levels (Zhu, Bouzekri et al. 2001), which is opposite to that found in British (Keavney, McKenzie et al. 1998) and French (Villard, Tiret et al. 1996) populations. A2350G (rs4343) is a silent substitution in exon 17. It has been proposed as being in complete LD with the I/D polymorphism in Caucasians, A corresponding to I (Abdollahi, Huang et al. 2008).

A few studies have addressed ACE polymorphisms other than the I/D in populations of African descent. Payne et al showed in South African Xhosa that the I/D polymorphism accounted for 0.7% and the A22982G polymorphism (rs4363) for 3.7%. (Payne, Dhamrait et al. 2007)In a study of n=376 Kenyans it was found that the I/D polymorphism accounted for 13% and the A22982G for 24% in variance of ACE serum levels, with A carriers having lower ACE serum levels. In Europeans there was complete LD between the I/D and A22982G genotype, I corresponding to A and D with G respectively.(Scott, Moran et al. 2005) Consequently, the A allele has been associated with lower circulating ACE levels than the G allele (Cox, Bouzekri et al. 2002). Another study, however, found that the A allele was associated with higher ACE levels.(Zhu, McKenzie et al. 2000)

Given that the polymorphism is located in an intron, it was deemed unlikely that this polymorphism, in itself, was directly responsible for the observed serum ACE level differences, but rather in linkage disequilibrium with a polymorphism that had regulatory effects on the ACE gene.

The ACE I/D polymorphism is technically challenging to genotype. Therefore an alternative was sought: The SNP rs4341 is in 100% LD with the ACE I/D SNP rs4646994.(Tanaka, Kamide et al. 2003)

3.5.3 ACE serum and tissue levels are under genetic control

ACE levels in healthy adults show large inter-variability that was postulated to be, in part, under genetic control(Cambien, Alhenc-Gelas et al. 1988). Subsequently it was found that a ~250 bp insertion/deletion polymorphism caused two different sized alleles. The insertion and larger allele was designated I and the deletion (shorter) allele D.

Family studies confirmed mendelian inheritance of the polymorphism. The observed allele frequencies in 80 healthy Caucasian French subjects were 0.406 for the I allele and 0.594 for the D allele. Genotype distribution was .18 for II, .46 for ID and .36 for DD, in Hardy Weinberg Equilibrium.

ACE genotypes showed a significant additive relationship between the polymorphism and the serum levels, explaining 47% of the variance in serum levels.(Rigat, Hubert et al. 1990)The mean serum ACE level was found to be nearly twice as high in homozygous DD genotypes when compared with II genotypes.(Rigat, Hubert et al. 1990)Tissue levels showed a similar association(Costerousse, Allegrini et al. 1993).

This correlation is strongly dependent on ethnicity. White children with DD genotype showed about twice as high ACE serum levels compared with II genotype, whereas in black children no association was found (Bloem, Manatunga et al. 1996) Similarly, in South African blacks no association was found with the ACE I/D polymorphism and ACE serum level, however ACE serum levels were significantly associated with the ACE A22982G (rs4363) genotype.(Payne, Dhamrait et al. 2007)

3.5.4 ACE I/D genotype distribution in different ethnic populations

The distribution of the I and D alleles differs markedly between populations of Caucasian, Asian and African descent (Table 3-13). This requires that the ethnicity of the populations studied is taken into account before conclusions may be drawn about predispositions.

Ethnicity	Country	Sample size (n)	Insertion	Deletion	Reference
Caucasian	French	199	.427	.573	(Rigat, Hubert et al. 1992)
Caucasian	French	35	.47	.53	(Costerousse, Allegrini et al. 1993)
Caucasian	British	1906	.49	.51	(Marshall, Webb et al. 2002)
Caucasian	Flemish	1461	.496	.504	(Staessen, Wang et al. 2001)
Asian	Japanese	100	.58	.42	(Nakai, Itoh et al. 1994)
Asian	Japanese	5014	.65	.35	(Higaki, Baba et al. 2000)
Caucasian	USA	139	.46	.54	(Bloem, Manatunga et al. 1996)
Black	USA	62	.36	.64	(Bloem, Manatunga et al. 1996)
Black	South Africa	200	.32	.68	(Payne, Dhamrait et al. 2007)

Table 3-13 ACE I/D distribution in different populations

3.5.5 Genotyping methods for ACE I/D polymorphism

In Caucasians the markers for differences in ACE level traditionally has been the ACE 287 kb *Alu* Insertion Deletion polymorphism (rs4646994) in intron 16.

The first genotyping method used the cDNA of 15 subjects and identified a two-allele restriction fragment length polymorphism (RFLP), identified by the restriction enzyme *Hind* III. The larger allele was designated I (Insertion) and the shorter D (Deletion).(Rigat, Hubert et al. 1990)

After this initial discovery a PCR genotyping process was developed. In the presence of the insertion the PCR product is 490 bp long and in the absence of the insertion the PCR product is 190 bp long.(Rigat, Hubert et al. 1992)

This 2 primer genotyping process for the ACE I/D polymorphism has been noted to be difficult, time consuming and prone to errors, especially mistyping IDs for DDs. (Shanmugam, Sell et al. 1993). A modification was therefore required to identify the mistaken ID genotypes from DD genotypes.(Lindpaintner, Pfeffer et al. 1995)

An alternative is to use a 3 primer process, which precludes mistyping. This method yields a PCR product that is 84 bp long for the ACE D allele and 65 bp for the ACE I allele. Counter-intuitively, the PCR product for the I (insertion) is actually shorter than the D (deletion).(Evans, Poirier et al. 1994)

The method is still cumbersome and time consuming. Therefore alternative markers were sought. The ACE polymorphism rs4341 (G/C) is in complete linkage disequilibrium with the ACE I/D polymorphism and can be genotyped with specific primer techniques.(Tanaka, Kamide et al. 2003)

After this discovery, another SNP (rs4343) was found to be in complete LD with both rs4341 as well as the ACE I/D SNP in Caucasians.(Glenn, Du et al. 2009)

3.5.6 Interpretation errors in ACE I/D polymorphism results

The Department of Cardiovascular Genetics, Rayne Institute, University College London, London, UK where the rs4341 polymorphism was genotyped for this project had previously identified the C allele to correspond with the I polymorphism. (personal communication Prof S.E.Humphries)

The original article, however, identified the rs4341 G allele to be in LD with the I allele (Figure 3-11), (Tanaka, Kamide et al. 2003)). Of note, the ACE I/D polymorphism was identified as rs4340 in this paper (which corresponds to rs4646994, rs1799752, rs13447447).

dbSNP ID	Region	Allele 1/ allele 2	Allele frequency
rs4340	intron 16	I/D	0.628/0.372
rs4341	intron 16	G/C	0.621/0.379

Figure 3-11 ACE rs4341 and ACE I/D association

From (Tanaka, Kamide et al. 2003)

The described allele frequencies for ACE I/D are in keeping with previous studies describing Asian populations (Table 3-13).

The method used in this paper has been cited 44 times, but the method has been actually used by few other groups (Muthumala, Montgomery et al. 2007, Weger, Hofer et al. 2007, Glenn, Du et al. 2009),(Lely, Heerspink et al. 2010),(Gu, Kelly et al. 2010, Camos, Cruz et al. 2012). None specified the linkage between the alleles explicitly.

To further confound matters, a subset of the current PICU cohort (n=141) had been previously genotyped for the ACE I/D allele using the 3-primer method (Fidler 2007). These results were opposite to that of the current interpretation of the rs4341 data. Unfortunately, the raw data were not available to make a direct comparison.

The discrepancy between the Rayne laboratory interpretation of the rs4341 genotyping results vs. the original rs4341 literature and historical PICU genotyping required resolution.

Two methods were used to clarify this issue. First, a subset of the PICU cohort (n=91) was genotyped again for the actual ACE I/D polymorphism using the three primer method. This confirmed that the allele matching between rs4341 and ACE I/D was exactly opposite to the one described by Tanaka et al. The rs4341 C allele corresponded with the Insertion allele and the G allele with the Deletion allele.

Second, linkage analysis and allele frequencies were calculated for three ACE polymorphisms: the I/D polymorphism, rs4341 and rs4343.

The reason to choose rs4343 was the finding that this polymorphism was in complete linkage disequilibrium LD with both the I/D as well as rs4341. Moreover, a direct comparison of alleles had been performed, linking the rs4343 A allele to the I

polymorphism (Kehoe, Katzov et al. 2004, Abdollahi, Huang et al. 2008). Last, the rs4343 G allele was associated with higher plasma ACE levels (Zhu, Bouzekri et al. 2001).

Figure 3-12 shows the Haploview LD plot for 4 ACE polymorphisms (n=147 with the original ACE I/D results) for the Caucasians in the PICU cohort. This confirms a 100% LD between rs4341 and rs4343 (D' 1, LOD score 81.09 and r^2 0.9). The LD for the original ACE I/D results is lower for both rs4341 and rs4343 (D' 0.95, LOD score 32 and r^2 0.8). This may be a result of genotyping errors. The promoter polymorphism rs4291 will not be discussed here.

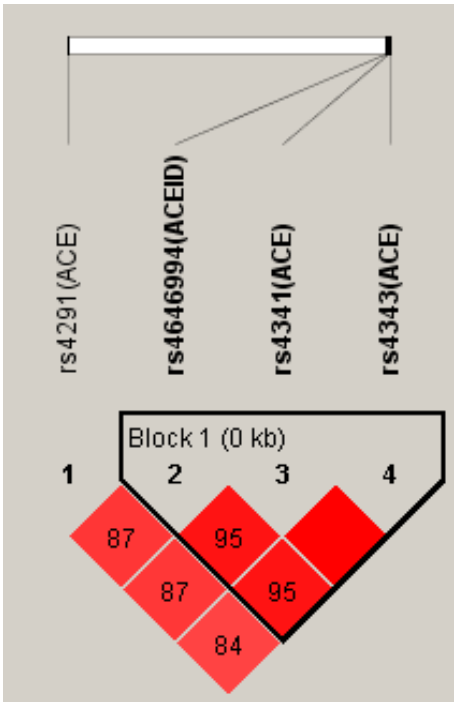


Figure 3-12 ACE Linkage Disequilibrium

Given that rs4341 and rs4343 were indeed in complete LD a comparison was then made with allele frequencies.

This showed that the allele frequencies for rs4343 G and rs4341 G were the same.

Given that three independent groups have shown the rs4343 G to be associated with the D genotype, based on the allele frequency and LD results, rs4341 G is associated with ACE D.

Only very recently did a Japanese group (Takeuchi, Yamamoto et al. 2012) specify which rs4341 allele is in LD with the I or D genotype. This group identified the rs4341 G allele as the risk allele, corresponding with the ACE risk D allele. The risk G allele frequency was .35 in a cohort of n=21851 Japanese subjects. This risk allele frequency is in keeping with previously described D allele frequencies (Table 3-13). These recent results are in conflict with the original manuscript.

In summary, the original paper on rs4341 must have mistakenly equated the rs4341 G allele with the ACE I/D I allele. The original 3 primer PICU genotyping results for the ACE I/D polymorphism were probably misinterpreted because of the counter intuitiveness of labelling a shorter allele I (insertion) rather than D (deletion).

This conundrum was resolved by re-genotyping a PICU cohort subset as well as by using linkage disequilibrium in combination with data from the literature. These results were recently confirmed in a large cohort of Japanese subjects.

3.6 Conclusions

Quality control for large genotyping studies is essential. Methods include Hardy Weinberg Equilibrium and linkage disequilibrium to identify potential genotyping errors.

Chapter 4 Polymorphisms in the endotoxin recognition receptor complex

4.1 Endotoxin is a key Pathogen Associated Molecular Pattern (PAMP)

Lipid A or Endotoxin is the component of the Gram-negative bacteria cell wall that the innate immune system recognizes. It is highly immune stimulatory, even at low concentrations.(Sauter and Wolfensberger 1980, Warren, Fitting et al. 2010)

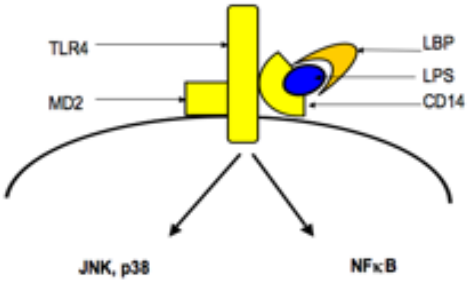
4.2 TLR4 activation in platelets and white cells

Recognition of endotoxin occurs via the endotoxin recognition receptor complex consisting of Toll-Like receptor 4 (TLR4), and the proteins CD14 and Myeloid Differentiation 2 (MD2); Lipopolysaccharide binding protein (LBP) delivers endotoxin to this complex.(Schumann, Leong et al. 1990, Wright, Ramos et al. 1990, Poltorak, He et al. 1998, Shimazu, Akashi et al. 1999)

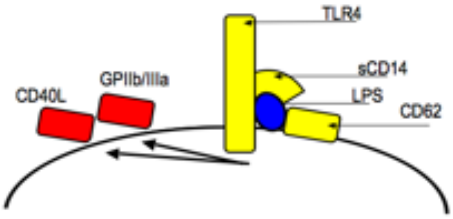
Many human cell types including monocytes/macrophages, endothelium and platelets express functional TLR4 (Zarember and Godowski 2002, Andonegui, Kerfoot et al. 2005, Dauphinee and Karsan 2006). Monocyte TLR4 ligation initiates a cascade of intracellular signalling events culminating in gene transcription and the ensuing production of inflammatory mediators (Figure 4-1). Activated TLR4 recruits adapter molecules such as MyD88 and TRIF, which in turn continues signalling via the cytoplasmic IKK complex to phosphorylate IKB and thus activates NFκB. NFκB then translocates to the nucleus to upregulate the transcription of inflammatory genes, including TNFα and Interleukins. These compounds then contribute to the clinical picture of systemic inflammation and in severe cases, shock and multiple organ

dysfunction.

Events relating to platelet endotoxin binding are less well described, but include soluble CD14 and CD62P (P-Selectin) in addition to TLR4 (Figure 4-1)(Cognasse, Lafarge et al. 2006, Stahl, Svensson et al. 2006). Recently, platelets were shown to contain the intracellular signalling apparatus commensurate with TLR4 signalling. In addition to TLR4, platelets expressed MyD88, IκB-a and NFκB p65, TRIF, TBK-1, IRAK-1, JNKs, MAPk, TRAF3, TRAF6, IRF-3 and IKK-I.(Berthet, Damien et al. 2010)



A.



B.

Figure 4-1 The endotoxin recognition complex on A. white cells and B. platelets
TLR4 Toll Like Receptor 4, MD2 Myeloid differentiation protein 2, LBP Lipopolysaccharide binding protein, LPS Lipopolysaccharide, CD14 Cluster of Differentiation 14, JNK c-Jun N-terminal kinase, p38 p38 kinase, NFκB Nuclear factor κB, CD40L Cluster of Differentiation 40 Ligand, GPIIb/IIIa Glycoprotein IIb/IIIa, sCD14 soluble CD14, CD62 Cluster of Differentiation 62

Thrombocytopenia, production of TNFα(Aslam, Speck et al. 2006) and enhancement of neutrophil bactericidal properties via neutrophil extracellular traps or NETs(Clark, Ma et al. 2007) are both thought to occur as a consequence of platelet TLR4 ligation. More evidence that platelets play an active role in LPS mediated immune response was gained in an ex vivo human model. LPS induced platelet secretion of dense and α granules (ATP release and P-selectin expression), and enhanced platelet aggregation through a TLR4/MyD88- and cGMP/PKG-dependent pathway.(Zhang, Han et al. 2009)

A different group investigated the nature of pro-inflammatory compounds in an in vitro human model. Here, LPS activated a kinase cascade in platelets leading to stimulated IL-1b production. Moreover, LPS signaling promoted the production of pro-inflammatory platelet microparticles that expressed caspase-1–dependent IL-1b.(Brown and McIntyre 2011)

4.3 TLR4 recognizes endogenous ligands

Although initially seen as the endotoxin receptor, TLR4 is now also implicated in an endotoxin independent response to injury. Despite discussion that endogenous signalling via TLR4 ligation may be due to LPS contamination (Tsan and Gao 2004), fibrinogen, heat shock proteins, reactive oxygen species, hyaluronic acid and haeme are all ligands for TLR4 in the absence of endotoxin (Takeda, Kaisho et al. 2003, Taylor, Trowbridge et al. 2004, Zhang, Shan et al. 2005, Figueiredo, Fernandez et al. 2007).

An appropriate host response to the common (Lockhart, Brennan et al. 2008) transient bacteraemia secondary to tooth brushing is not to mount a systemic response (Bahrani-Mougeot, Thornhill et al. 2008). Conversely, systemic inflammation occurs

in response to severe infection and injury. Given the multitude of potential ligands in both infection as well as injury the TLR4 complex is an essential step in the development of inflammation. Individuals differ in their inflammatory response to insults. The pivotal role of the TLR4 complex means that it is an important candidate site as a source of some of this variability. This concept is consistent with previous observations indicating that circulating anti-endotoxin core antibodies act as a modifier of SIRS in children.(Stephens, Fidler et al. 2006)

4.4 TLR4 Polymorphisms may contribute to variability in host response

Some of the variability in host response to infections was explained by human TLR4 polymorphisms that are thought to have a deleterious effect on host response to meningococcal disease.(Smirnova, Mann et al. 2003) The two common single nucleotide polymorphisms (SNPs) are D299G and T399I. Both lie in the extra-cellular domain and are thought to cause less effective endotoxin binding and hence a decreased sensitivity to endotoxin.(Rallabhandi, Bell et al. 2006) Functionality of these SNPs has been shown (Arbour, Lorenz et al. 2000, Fageras, Hmani-Aifa et al. 2004) but also contested (Erridge, Stewart et al. 2003, Heesen, Bloemeke et al. 2003, van der Graaf, Kullberg et al. 2005).

Those studies that showed the SNPs to have direct functional consequences were studies that explored this functionality in airway related models.

Arbour et al showed that adults inhaling LPS showed different responsiveness as measured by FEV_1. Eleven subjects, two of whom were homozygous for the TLR4 SNP D299G showed reduced bronchial reactivity when compared with 73 subjects with the wild type genotype (P .029). The authors used a one-tailed test. Had they

used a two-tailed test, however, it would still have been significant (P .035, Fisher's exact).

Airway epithelial cells, harvested from subjects with known TLR4 genotypes were stimulated with LPS and differed in their production of IL1α, where TLR4 wildtype exhibited higher responsiveness than variant genotypes. Last, the authors transfected THP-1 cells and measured NFκB activity following activation with LPS. This showed WT to have the highest activation, TLR4 SNP D299G the lowest and TLR4 T399I an intermediate activation. Over expression of TLR4 wildtype alleles in variant cells restored LPS responsiveness. Thus, functionality was observed in airway cells and lung physiology.

A study on TLR4 polymorphisms and its potential role in paediatric asthma showed that children with TLR4 variant genotypes were more likely to be asthmatic and PBMCs from children with variant genotypes were less responsive to stimulation with LPS. IL12(p70) production was lower in PBMC isolates from TLR4 variant carriers than from wildtype.(Fageras, Hmani-Aifa et al. 2004)

In contrast, there exist studies that have demonstrated no difference in LPS responsiveness between TLR4 wildtype and variant status.

Three TLR4 wildtype and 3 TLR4 variant subjects were identified from a cohort of 80 Scottish adults. Monocytes from these individuals were isolated and challenged with LPS at different concentrations and from different bacteriae (*Escherichia coli*, *Neisseria meningitidis*, *Bacteroides fragilis*, *Yersinia pestis*, *Chlamydia trachomatis*, *Porphyromonas gingivalis*, and *Pseudomonas aeruginosa*. No difference was observed in the capacity to produce IL1β.(Erridge, Stewart et al. 2003)

A review published in 2008 identified 25 studies that had compared functionality of the two common TLR4 variants to TLR4 wildtype genotype.(Ferwerda, McCall et al.

2008) From these studies one may conclude that, aside from questionable statistical choices, before a robust answer can be given to whether or not TLR4 variants have functional consequences the following other parameters need to be addressed: cell type, type and purity of LPS, LPS dose, timing of data collection, choice of phenotype, compound heterozygosity and other genetic variability.

Thus it is difficult enough to identify mechanistic and biological substrates for TLR4 polymorphisms. This is even more the case in the clinical arena.

There exist few clinical studies with convincing data that TLR4 SNPs D299G and T399I influence the course of critical illness in general (Imahara and O'Keefe 2004). The early clinical studies that did show a difference in clinical outcome were hampered by small sample size (n=77)(Agnese, Calvano et al. 2002), (n=159) (Barber, Aragaki et al. 2004), (n=91) and questionable choices in statistics (Lorenz, Mira et al. 2002). More specific associations were made between TLR4 polymorphisms and an increased vulnerability to bacteraemia in critically ill patients (Henckaerts, Nielsen et al. 2009) and aspergillosis in stem cell transplantation patients (Bochud, Chien et al. 2008).

4.5 Definition of outcome measures

The observed inconsistencies between studies may reflect no functional relevance of the TLR4 SNPs. Deficiencies in study design, or alternatively, any signal from altered endotoxin recognition could be drowned in the noise of other elements of host variability and environmental determinants in the acute inflammatory response.

Further, the limitations of SIRS as an end-point to describe the acute inflammatory response have long been recognised in adults.(Vincent 1997, Marshall, Vincent et al. 2003) This concern is compounded in the paediatric age group given the variability in

the age-related normal values of the components that define SIRS (heart rate, respiratory rate and white cell count). Paediatric SIRS criteria had only been established recently (Goldstein, Giroir et al. 2005), and had not been validated at the time of this study. A second marker of severe inflammation, namely platelet count on admission was therefore chosen.

This outcome measure was chosen for several reasons:

Platelets actively contribute to systemic inflammation (Levi and Lowenberg 2008). Clinically, platelet count has been recognized as a sensitive marker of severity of critical illness in both adults (Akca, Haji-Michael et al. 2002) and children (Peters, Ross-Russell et al. 2001, Nguyen and Carcillo 2006). Thrombocytopenia was found to be associated with mortality in a recent prospective observational cohort study.(Krishnan, Morrison et al. 2008) Endotoxin has been shown to directly reduce platelet count (Ohtaki, Shimauchi et al. 2002) to which TLR4 activity may be relevant, as shown in both mice (Aslam, Speck et al. 2006) and humans (Clark, Ma et al. 2007).

4.6 Potential confounding polymorphisms in other genes

Systemic inflammation is under genetic control of several mediators.(Calvano, Xiao et al. 2005) Other genetically variable mediators in the innate immune system were analysed in order to establish whether the influence of genetic variability in the endotoxin recognition complex was robust in the context of multiple variability in the innate immune system.

The rationale underlying the choice from many candidate genes is given in paragraph 2.7.6.

Briefly, these genes were chosen because of reported functionality, commonality in the population, modulation of the TLR4 complex and/or previous association studies in relevant cohorts.

4.7 Validation

Finally, to validate the findings in this cohort, the relationship was assessed between the TLR4 polymorphisms and platelet count on admission in a cohort of 1170 adults with atherosclerosis. This is a well-described previously genotyped cohort with a severe inflammatory insult.(Yang, Holloway et al. 2003)

4.8 Methods

4.8.1 Subjects

Between 2000 and 2006 children were recruited consecutively in three recruiting time-periods from three tertiary paediatric intensive care units. Inclusion and exclusion criteria are outlined in paragraph 2.1.2.

Subjects were categorized by primary diagnosis into '*Bypass'*, '*Infection'* and '*Non-infection'.* Patients admitted for heart surgery on cardiopulmonary bypass were assigned to the category '*Bypass',* those children with an infectious process as the admitting diagnosis were assigned to the category '*Infection'* and the category '*Non-infection'* was used in case of all other admission diagnoses, mainly trauma and elective surgery.

4.8.2 Identification of polymorphisms

A literature search revealed functional common single nucleotide polymorphisms (SNPs) in the endotoxin recognition complex. TLR4 D299G and TLR4 T399I and the CD14 promoter -159C>T were identified. LBP P97P was included as a common SNP of unknown functionality, which may influence outcome from sepsis. (Table 4-1) Polymorphisms in MD2 were rare SNPs and thus not included.

The polymorphisms in TLR4 D299G and T399I are both located in the extracellular domain of the molecule. They are each thought to reduce the "docking" properties of the ligand, with the largest effect being when both polymorphisms are present. (Rallabhandi, Bell et al. 2006)

Human in vitro studies show functionality in varying degrees. Airway epithelial cells and macrophages stimulated with LPS secreted less IL1α and TNFα when polymorphic for TLR4 than when wildtype. The clinical substrate consisted of reduced sensitivity to inhaled LPS.(Arbour, Lorenz et al. 2000)

In contrast, no difference was found between TLR4 wildtype or polymorphic monocyte production of TNFα or IL10 after stimulation with LPS, Aspergillus fumigatus, Cryptococcus neoformans, or heat shock protein 60. (van der Graaf, Kullberg et al. 2005)

Several clinical studies have shown differences in outcome in severe inflammatory and infectious diseases associated with TLR4 polymorphisms. Most show increased severity for those with the polymorphic genotype. These include, but are not restricted to RSV bronchiolitis (Tal, Mandelberg et al. 2004), invasive aspergillosis infection (Bochud, Chien et al. 2008), atherosclerosis(Kiechl, Lorenz et al. 2002) and sepsis(Agnese, Calvano et al. 2002, Lorenz, Mira et al. 2002, Barber, Aragaki et al. 2004, Henckaerts, Nielsen et al. 2009, Shalhub, Junker et al. 2009). Other studies did

not show associations between TLR4 polymorphisms and sepsis (Feterowski, Emmanuilidis et al. 2003, Jessen, Lindboe et al. 2007).

To account for genetic variability in other areas SNPs that have either been shown to have an effect on the acute inflammatory response or are thought to have modifier effects on TLR4 activity were analysed: Mannose Binding Lectin (MBL-2) (Fidler 2007), CHRNA7 (Leonard, Gault et al. 2002, Hamano, Takahashi et al. 2006) , IL-6 (Ahrens, Kattner et al. 2004), IL-10 (Nakada, Hirasawa et al. 2005), TNFα (Westendorp, Langermans et al. 1997)and PAI-1 (Hermans and Hazelzet 2005).

Gene name[a]	**Gene locus**	**Region**	**Nucleotide change and position**	**Amino acid change**	**NCBI SNPid**[b]	**Effect of SNP; Presumed mode of inheritance**
TLR4	9q32-q33	Exon 3	A>G +896	D299G	4986790	Less transcription of NFκB; decreased response to inhaled LPS(Arbour, Lorenz et al. 2000); Dominant
TLR4		Exon 3	C>T+ 1196	T399I	4986791	Less binding of ligand(Rallabhandi, Bell et al. 2006); Dominant
CD14	5q31.1	Promoter	C>T-159 (also known as -260)	Not applicable	2569190	Higher expression of mCD14[c] on monocytes, higher plasma level of sCD14 [d] increased response to LPS(Baldini, Lohman et al. 1999,

						Hubacek, Skodova et al. 2004); Recessive
LBP	20q11.23-q12	Exon 3	T>C+291	P97P	2232582	Unknown; Unknown, may be associated with greater mortality in sepsis (Hubacek, Stuber et al. 2001, Barber and O'Keefe 2003)

Table 4-1 Main features of the SNPs in the endotoxin recognition complex

[a]Components of the endotoxin recognition complex: TLR4 toll-like receptor 4; CD14, Cluster of Differentiation 14 molecule, codes for both [c] membrane bound protein mCD14 as well as [d] soluble protein sCD14; LBP, Lipopolysaccharide binding protein. [b]NCBI reference sequence number found at http://www.ncbi/nlm.nih.gov/SNP

4.8.3 Outcome measures

The primary outcome measures were: '*development of SIRS in the first three days of intensive care stay*' and '*platelet count on admission'*. SIRS was defined according to the 1992 ACCP-SCCM consensus (1992), with adjustments as described previously (Fidler, Wilson et al. 2004, Stephens, Fidler et al. 2006) and given in detail in paragraph 2.4.

Duration of ventilation, length of PICU stay and development of nosocomial infection in the first week of intensive care stay were secondary outcome measures.

4.8.4 Validation cohort

A cohort of 1170 Caucasian patients, diagnosed to have coronary artery stenosis by angiography (> 50% stenosis in ≥1 major epicardial coronary artery) recruited in the Southampton Atherosclerosis Study, had previously been genotyped for the TLR4 polymorphism D299G. A subset of patients (n=777) was also genotyped by Taqman analysis for both the D299G and T399I polymorphisms. Given substantial time and financial constraints associated with recruiting a large equivalent paediatric cohort for validation purposes this cohort was a readily available relevant dataset.

A subset of the Southampton Atherosclerosis Study had a history of recent myocardial infarction. This is a severe inflammatory insult to which TLR4 and platelets are relevant (Kiechl, Lorenz et al. 2002, Frossard, Fuchs et al. 2004, Lundberg and Hansson 2010).

To validate the observations in paediatric inflammation the hypothesis was tested that TLR4 variant carriers, with a history of recent myocardial infarction as a severity threshold for inflammation, would show lower platelet counts on admission.

This cohort was recruited consecutively from the Wessex Cardiothoracic Unit, Southampton General Hospital, as part of the Southampton Atherosclerosis Study. The original cohort consisted of 1501 Caucasian subjects admitted for interventional or diagnostic coronary angiography. Of these, 1170 had documented coronary atherosclerosis of >50% diameter in at least one major coronary artery. The demographic data are shown in Table 4-2.

Variable	
Age (years)	63.29 (9.96)
Gender (male/female)	896/274
Current and ex-smokers	872/298
Body mass index (kg/m^2)	27.5 (4.2)
Plasma cholesterol (mmol/l)	5.1 (1.0)
Plasma triglyceride (mmol/l)	1.8 (1.2)
Hyperlipidemia	957/213
Hypertension	524/646
Type I Diabetes	37/1133
Type II Diabetes	119/1051
Family history of CAD	566/604

Table 4-2 Demographics adult validation cohort

(n=1170) Continuous variables are expressed as mean and (standard deviation)

Adapted from (Ye, Dunleavey et al. 2003).

All members of the cohort had been genotyped already for the TLR4 D299G polymorphism, by means of a tetra-primer amplification refractory mutation system (ARMS) polymerase chain reaction (PCR) assay to amplify the polymorphic region of TLR4. The methods described below are described in (Yang, Holloway et al. 2003).

"Primers that were used to genotype the polymorphism were:

Outer upper 5'-CCTGAACCCTATGAACTTTATCC-3'

Outer lower 5'-GTTAACTAATTCTAAATGTTGCCATC-3'

Inner upper (Asp allele) 5'-GCATACTTAGACTACTACCTCGAaGA-3'

20 ng of genomic DNA was amplified in a total reaction volume of 10 ml containing dNTP 0.2 mM, MgCl2 4 mM, primers (outer upper, 0.1 mM; outer lower, 0.1 mM; inner upper, 2 mM; inner lower, 2 mM), AmpliTaq Gold DNA polymerase (0.025 U/ml) and buffer (Applied Biosystems, Warrington, UK). The PCR cycling conditions were 95 8C for 5 min; then 9 cycles of 94 8C for 30 s, X 8C for 30 s (where X is initially 72 8C, decreasing 1 8C per cycle to 64 8C), 72 8C for 30 s; then 31 cycles of 94 8C for 30 s, 64 8C for 30 s and 72 8C for 30 s; and finally 72 8C for 10 min. A constant PCR product (385 bp) and allele-specific products (Gly 292 bp, Asp 147 bp) were resolved by micro-array diagonal gel electrophoresis. Two researchers independently scored the genotypes and representative genotypes were confirmed by DNA sequencing."

The TLR4 genotyping results in absolute numbers are shown in Table 4-3.

	Original publication	Current data
TLR4 AA (Wildtype)	1256	743
TLR4 AG or GG	144	84
TLR4 CC (Wildtype)	n/a	755
TLR4 CT or TT	n/a	77

Table 4-3 TLR4 SNPs genotyping results adult cohort

The original publication by (Ye, Dunleavey et al. 2003) analysed only TLR4 D299G.

For the current study both TLR4 D299G as well as T399I genotyping was available.

These distributions are in HWE and are as expected for Caucasian ethnicity.

4.8.5 Statistical analysis

Hardy Weinberg equilibrium was tested by means of the χ^2 -test. Univariate analyses were performed by χ^2, Mann-Whitney *U*, Student's T test or one-way ANOVA as appropriate. A binomial regression analysis incorporated genotype parameters and clinically relevant parameters. Statistical significance was set at a two-tailed *p* value < 0.05. All statistical analyses were performed using SPSS version 13.0 for Mac (SPSS, Chicago, IL, USA).

4.9 Results

4.9.1 Recruitment

The total number of children that was recruited amounted to n= 913, (n= 579 from the cardiac intensive care at GOSH, n= 299 from the PICU at GOSH and n= 35 from the PICU at Southampton).

4.9.2 Genotyping

Genotyping was successful for 906 (99.2%) critically ill children.

All SNPs were in Hardy-Weinberg equilibrium stratified to ethnic background and allele frequencies were as previously described (Smirnova, Hamblin et al. 2001).

TLR4 genotypes varied across ethnicity. The children with an Asian background (n= 26) showed only wildtype TLR4 genotype, whereas TLR4 D299G was common (n=10; 12%) and T399I was observed only once (1.2%) amongst those of African descent (n= 78). In the Caucasian group (n= 747) both variant alleles were present (D299G n=72 (9.6%); T399I n=74 (9.9%)) and mostly occurred together, i.e. showed cosegregation. (Figure 4-2).

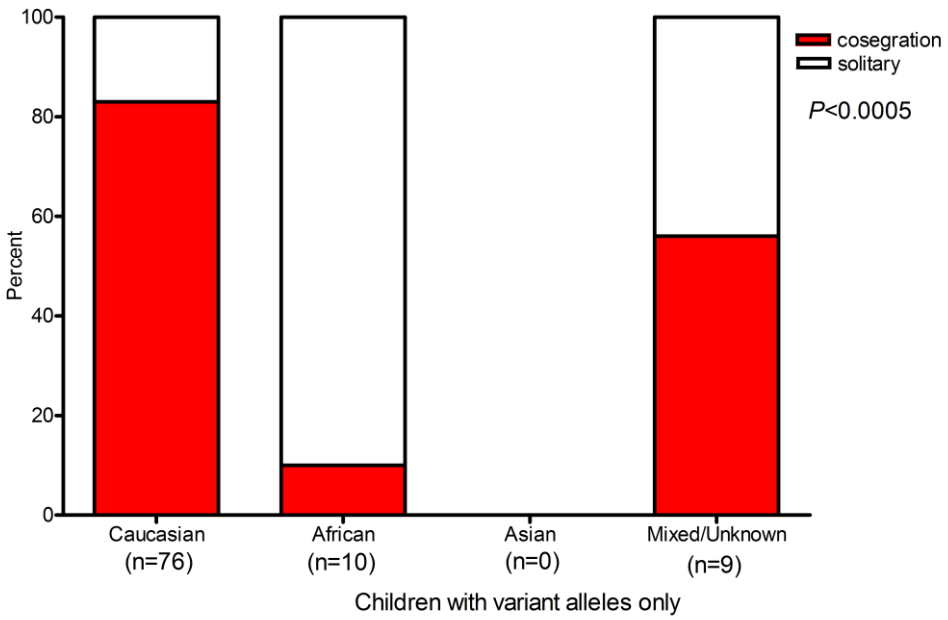


Figure 4-2 Cosegregation of TLR4 alleles

P value derived from χ^2 test

4.9.3 Development of SIRS in the first 3 days of intensive care stay

Overall, SIRS developed in 594/913 (65%) subjects. Significantly more children in the *Infection* group developed SIRS (143/179; 79.8%) than in the *Non-infection* (126/199; 63.3%) or *Bypass* group (325/535; 60.7%) (Table 4-4).

Characteristic	**All (n=913)**	**Non-SIRS (n=319)**	**SIRS (n=594)**	**p value[a]**
Precipitating event				< 0.0005
Bypass	536	211	325	
Non-Infection	198	72	126	
Trauma	104	28	76	
Cardiac surgery (Non-Bypass)	43	18	25	
Elective postoperative	36	23	13	
Other	15	3	12	
Infection	179	36	143	
PIM score expected mortality in % [b]	2.45 (0.17-98)	2.10 (0.17-88.6)	2.80 (0.24-98)	<0.0005
Observed 28 day mortality % (n)	3.17 (29)	1.56 (5)	4.0 (24)	0.04
Age in months	17 (0-218)	11 (0-218)	22 (0-207)	<0.0005
Sex ratio (M/F)	1.34 (523/390)	1.35 (183/136)	1.33 (340/254)	0.97

Ventilator days	2.0 (0-362)	1.9 (0-106)	2.9 (0-362)	0.002
Length of stay (days)	3.7 (0.1-362)	2.9 (0.1-106)	3.8 (0.1-362)	0.001
Nosocomial infection in week 1 [c]	51/688	14/267	37/421	0.08
Platelet count	171±4	162±6	177±5	0.04
Ethnicity				0.09
Caucasian	747	273	474	
African	78	18	60	
Asian	26	7	19	
Mixed/Unknown	62	21	41	

Table 4-4 Clinical characteristics for the total paediatric cohort stratified according to early development of SIRS

Continuous values are expressed as medians and range or means ± SEM; categorical values as counts and percentages. [a]Comparison between SIRS and Non-SIRS. Mann-Whitney *U* or Student's t-test (continuous variables); Pearson χ^2 test (categorical variables) [b]Based on PIM Paediatric index of Mortality(Shann, Pearson et al. 1997) [c]Not documented in infection group.

Subjects with wildtype or variant genotypes for either of the TLR4 variant alleles, alone or in combination, did not differ in the development of SIRS; 66% (68/103) of the subjects with a TLR4 mutation developed SIRS, versus 65% (522/803) with the wildtype genotype (Figure 4-3).

The *Bypass* and *Infection* groups (Figure 4-4 and Figure 4-5) showed no difference in the rate of SIRS when stratified according to TLR4 genotypes.

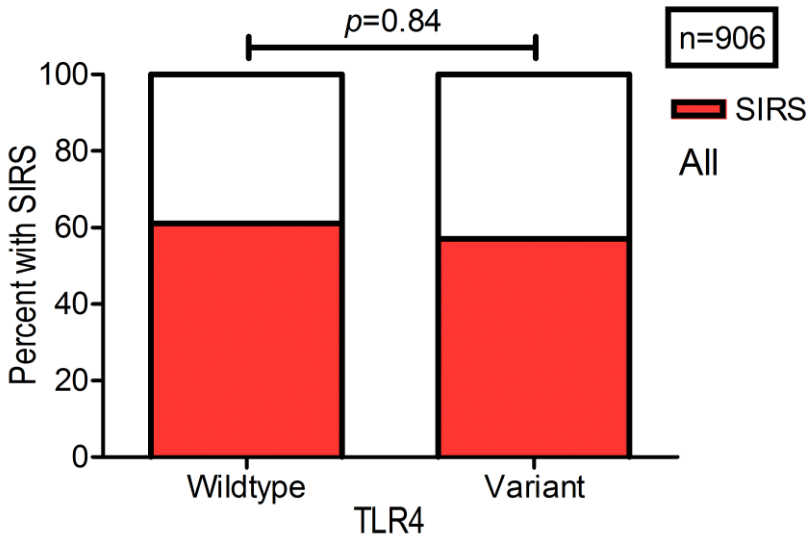


Figure 4-3 TLR4 wildtype vs. TLR4 variant genotypes PICU

Univariate analysis for TLR4 polymorphism variant alleles as a risk factor for SIRS in the total paediatric cohort. P value derived from χ^2 test.

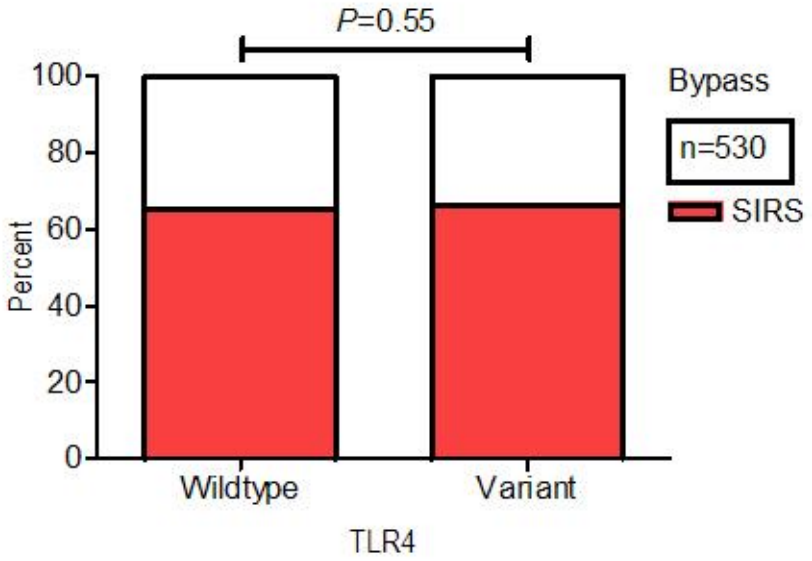


Figure 4-4 TLR4 genotypes in Bypass group

Univariate analysis for TLR4 polymorphism variant alleles as a risk factor for SIRS in the bypass group. P value derived from χ^2 test.

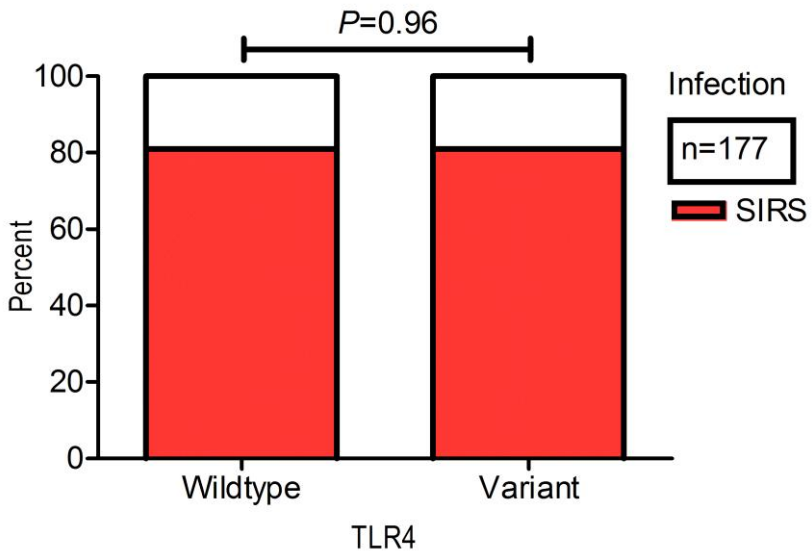


Figure 4-5 TLR4 genotypes in Infection group

Univariate analysis for TLR4 polymorphism variant alleles as a risk factor for SIRS in the infection group. P value derived from χ^2 test.

In the *Non-infection* group there was a trend for carriers of the TLR4 variant alleles to be at an increased risk for developing SIRS; OR 2.4 (95% CI 0.84-6.6, $p = 0.09$) (Figure 4-6**)**. After multivariate analysis, adjusting for basic demographics, and other polymorphisms in the endotoxin recognition complex, CHRNA7, PAI-1, MBL-2 and the genes encoding cytokines IL-6, TNFα and IL-10 this just reached statistical significance ($p = 0.03$) with an OR 5.4 (95% CI 1.1 - 26.5).

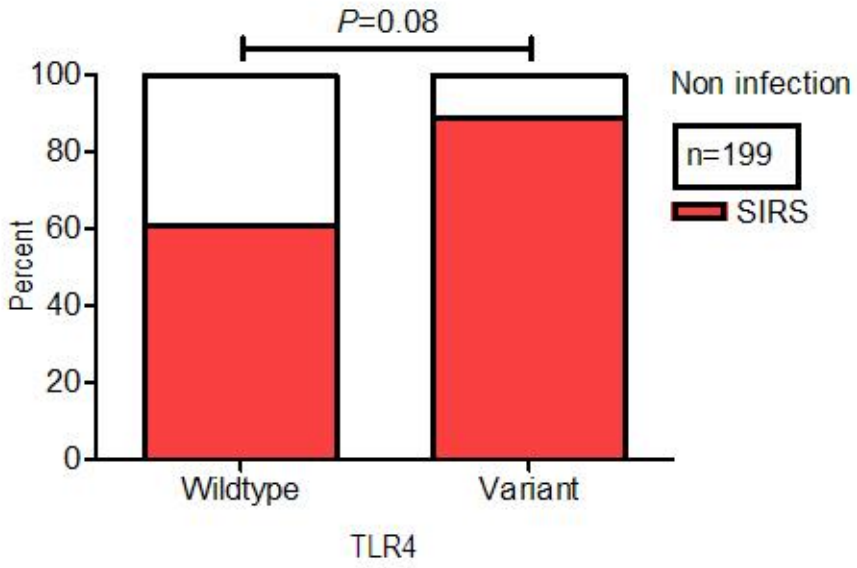


Figure 4-6 TLR4 genotypes in non infection group

Univariate analysis for TLR4 polymorphism variant alleles as a risk factor for SIRS in the Non infection group. P value derived from χ^2 test.

4.9.4 Platelet Count

There was a marked difference in admission platelet count between subjects with wildtype and those carrying the 299G and/or 399I TLR4 variant in the total paediatric cohort: (mean ± SEM: 174 ± 4 vs. 142 ± 7, $p = 0.0001$) (Figure 4-7).

The impact of TLR4 genotype on platelet count showed a "dose response" or additive pattern. Those children with wildtype genotype had the highest platelet count, whereas those with two or more variant alleles had the lowest platelet counts and children with 1 variant allele had an intermediate phenotype. (Figure 4-8).

Consequently, the proportion of children with actual thrombocytopenia (platelet count <150 x 109) was significantly higher amongst those carrying one or more variant TLR4 alleles compared to wildtype (OR 1.7 (95% CI 1.1-2.7, p = 0.01) (Figure 4-9 and Table 4-5).

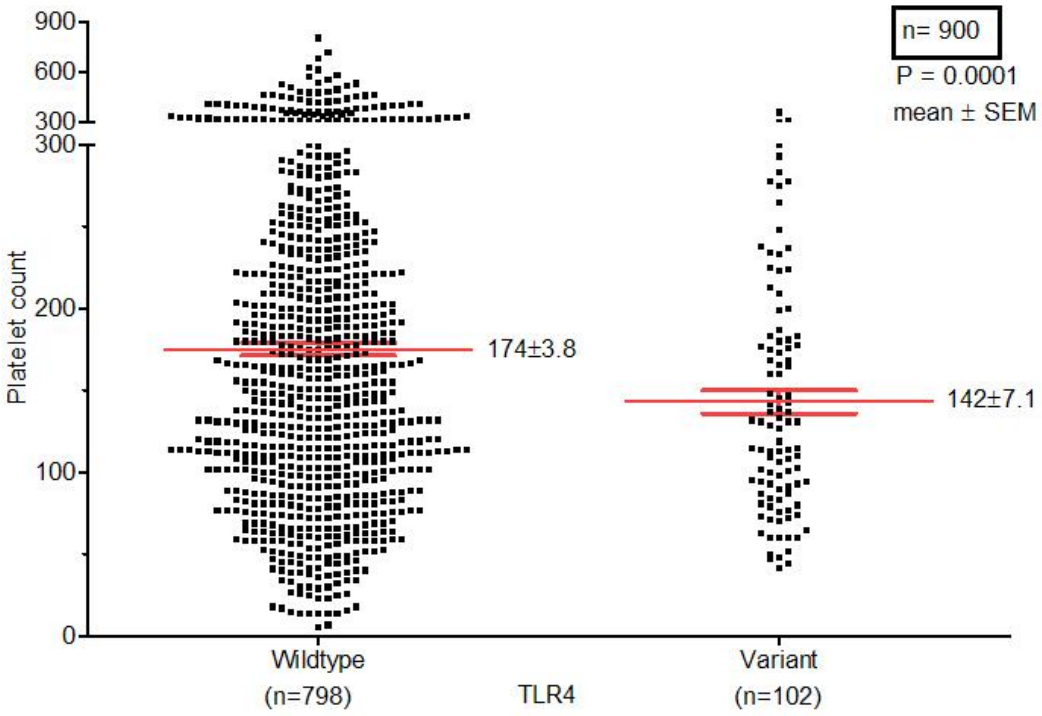


Figure 4-7 Platelet count for the total paediatric group stratified by TLR4 genotype

P value derived from Student's t-test.

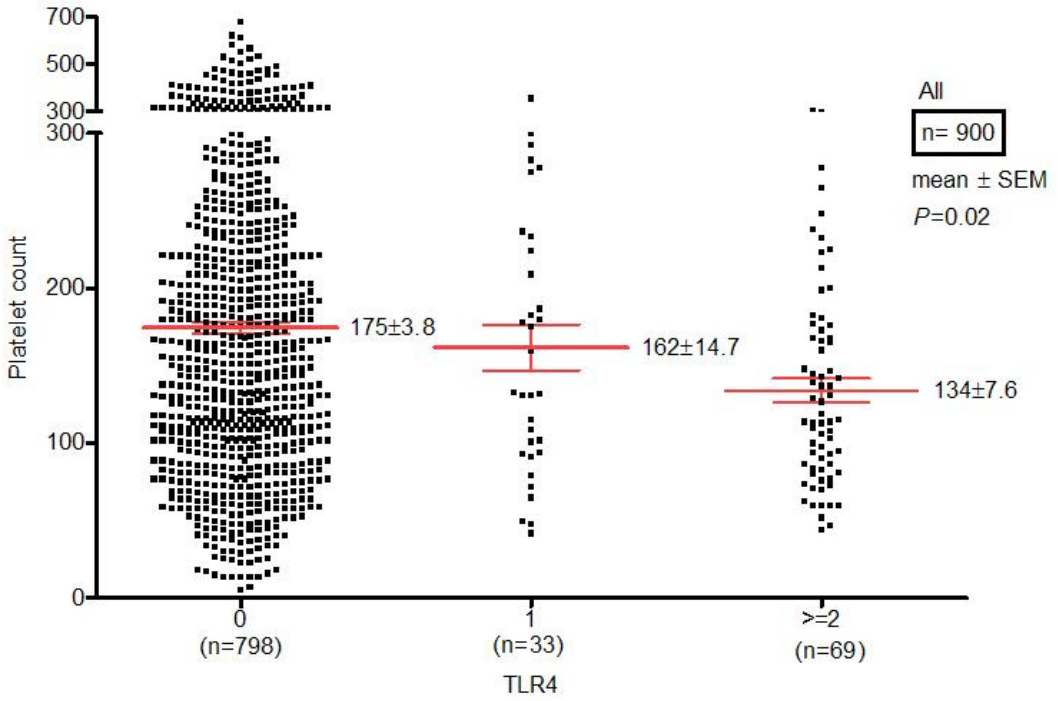


Figure 4-8 One Way ANOVA analysis for platelet count per TLR4 variant allele PICU cohort

One-Way ANOVA analysis for platelet count per TLR4 variant allele (0,1 or ≥2) for the total paediatric cohort.

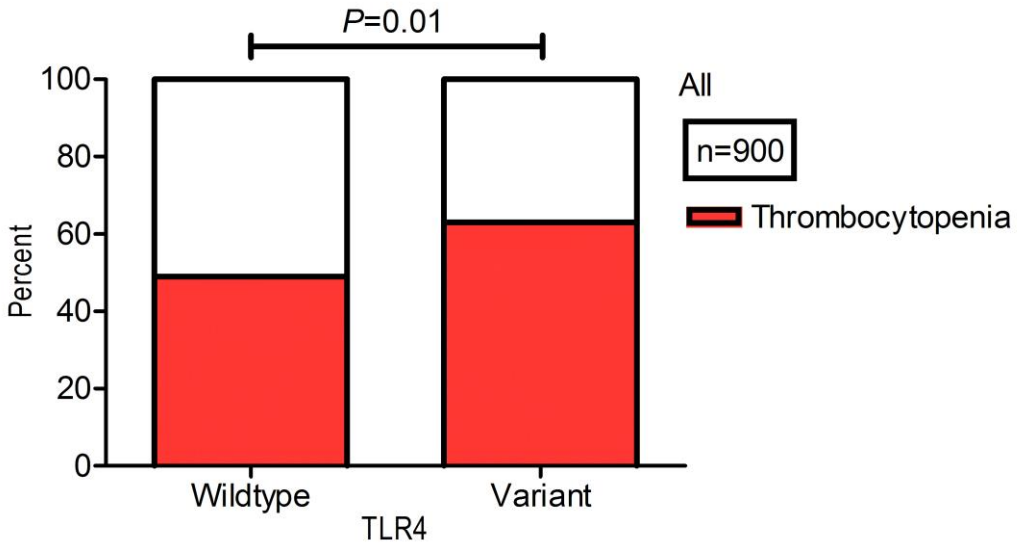


Figure 4-9 Thrombocytopenia by TLR4 genotype PICU cohort

Thrombocytopenia (platelet count <150x10^9) stratified by TLR4 genotype for the total paediatric cohort (Odds ratio 1.7 (95% C.I. 1.1-2.6, χ^2 test)).

Independent variable	**Univariate** [a]	**Adjusted (logistic regression analysis)** (Adjusted for: Age (logged), sex, neutrophil count, SNPs in CD14, LBP, TNFα, IL6 and IL10; CHRNA7, PAI-1 and MBL-2)	**Multiple logistic regression analysis** (Adjusted for: Age (logged), sex, neutrophil count, SNPs in CD14, LBP, TNFα, IL6 and IL10; and CHRNA7, PAI-1, MBL-2) plus risk factors identified on univariate analysis i.e: TLR4 SNPs, ethnicity, primary diagnosis, severity of illness (PIM), and SIRS)
TLR4 any variant	1.7 (1.1-2.6) p =0.01	1.9 (1.2-3.0) p = 0.007	2.2 (1.2-3.9) p = 0.01
TLR4 variant number [b]			
1	1.1 (0.5-2.2) p = 0.79	1.4 (0.6-3.1) p = 0.39	2.6 (0.9-7.0) p = 0.06
≥2	2.2 (1.3-3.7) p = 0.003	2.1 (1.2-3.6) p = 0.01	2.0 (0.99-4.0) p = 0.05
SIRS	0.79 (0.6-1.03) p = 0.09	0.72 (0.5-0.97) p = 0.03	1.0 (0.7-1.5) p = 0.9
Primary diagnosis [c]			
Cardiac Bypass	7.6 (3.3-17.2) p <0.0005	8.7 (3.5-21) p < 0.0005	9.9 (3.9-25) p < 0.0005

Ethnicity [d]			
African	0.4 (0.2-0.7) p = 0.001	0.4 (0.2-0.7) p = 0.005	0.4 (0.2-0.7) p = 0.003
Asian	0.1 (0.1-0.9) p = 0.03	0.3 (0.1-0.7) p = 0.01	0.6 (0.2-2) p = 0.37

Table 4-5 Independent variables for thrombocytopenia in the PICU cohort

Thrombocytopenia (<150*10^9/l) Results show odds ratios, 95% Confidence Interval and *p* value. [a] χ Square test. [b] vs. wildtype *TLR4*. [c]vs. elective postoperative. [d]vs. Caucasian

Interestingly, the effect that TLR4 polymorphisms exerted differed between the precipitating event groups. No difference in platelet count could be seen in the *Bypass* group (Figure 4-10).

In the *Non-infection* group, children with variant TLR4 alleles had lower admission platelet counts than wild type (Figure 4-11).

A trend in this direction was also evident in the smaller *Infection* group (Figure 4-12).

The risk for thrombocytopenia on admission in the whole population associated with TLR4 variants remained after multiple regression analysis. It was independent of any combination of: primary diagnosis, demographic characteristics, neutrophil count, SIRS, PIM score, and other endotoxin complex and PAI-1, MBL-2, CHRNA7, IL-6, IL-10 or TNFα genotypes (OR 2.2 (95% CI 1.2- 3.9), $p = 0.01$).

Polymorphisms in LBP or CD14 did not alter the risk for development of SIRS or platelet count (Table 4-6).

The secondary clinical outcome measures, i.e. length of stay, length of ventilation or nosocomial infection did not show any stratification according to genotype (Table 4-7).

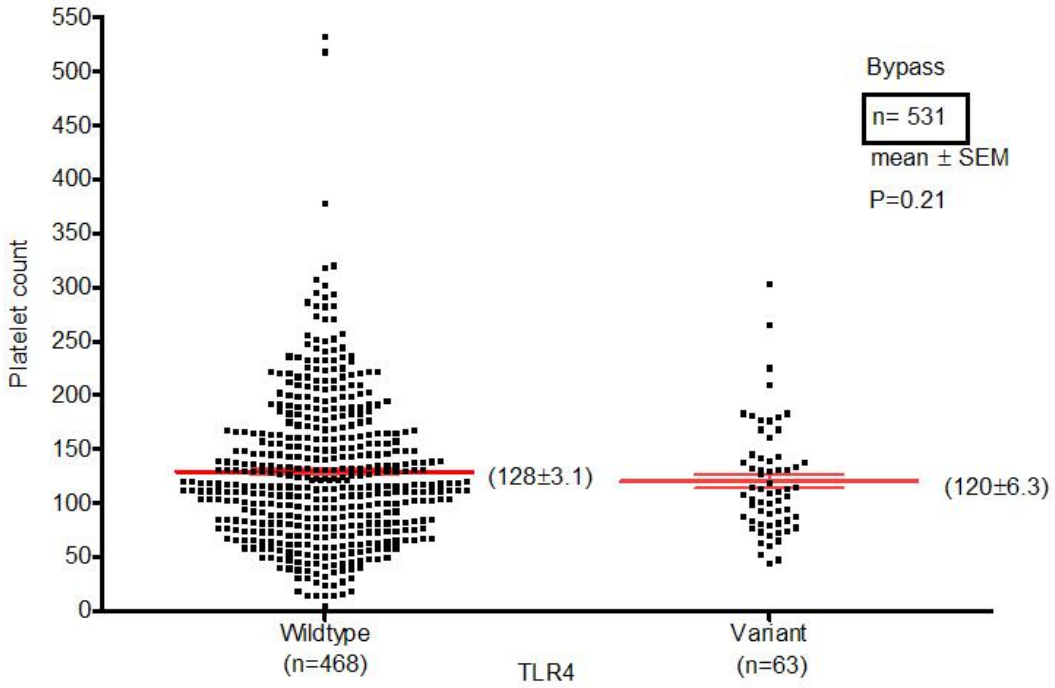


Figure 4-10 Platelet count per TLR4 genotype in PICU Bypass group

P value is derived from Student t-test

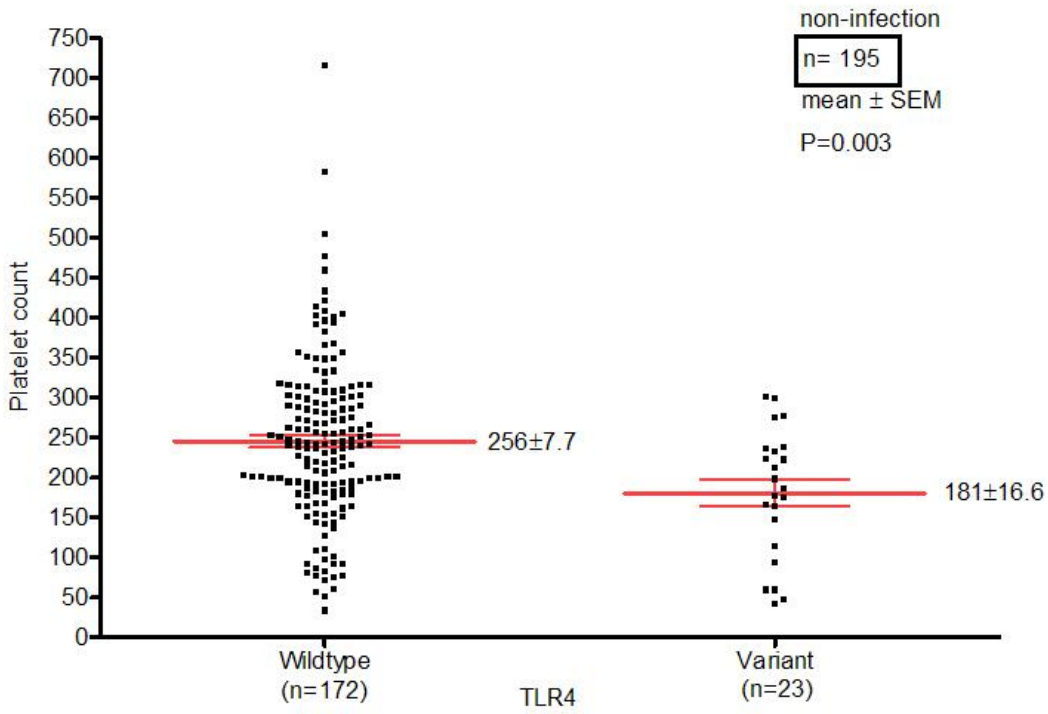


Figure 4-11 Platelet count per TLR4 genotype in Non-infection group

P value is derived from Student t-test

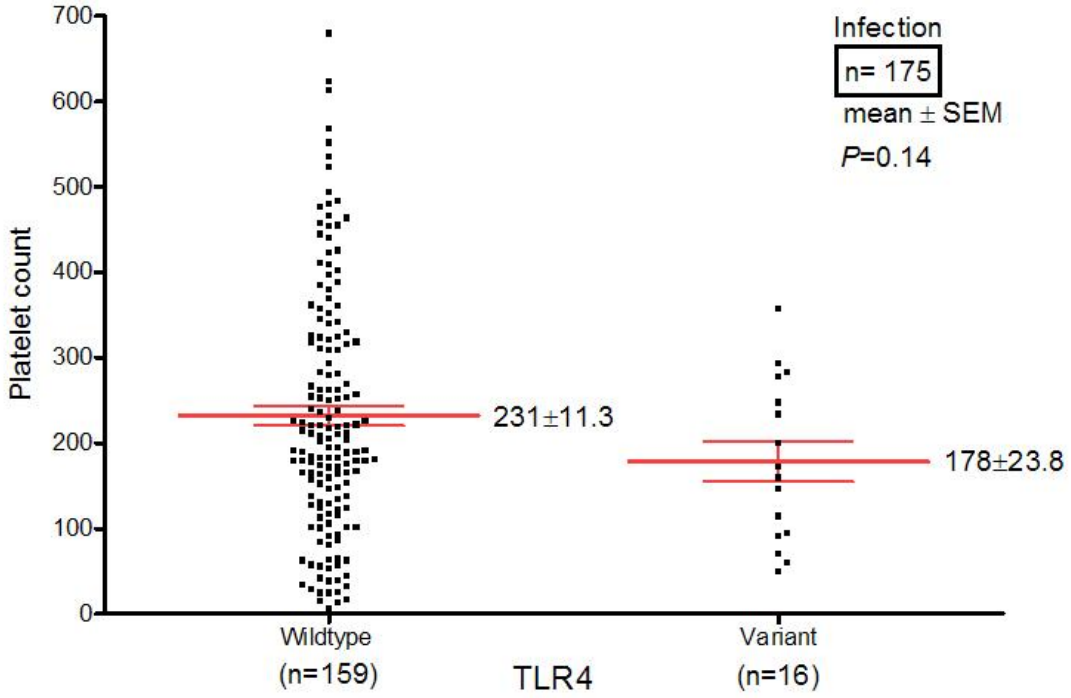


Figure 4-12 Platelet count per TLR4 genotype in Infection group

P value is derived from Student t-test

SNP	Non SIRS	SIRS	P value[a]	Platelet count[b]	P value[c]
CD14 C-159T					
CC or CT	232	412	Recessive 0.27	170±4.1	Recessive 0.6
TT	62	133		166±8.0	
LBP P97P					
TT	199	370	Additive 0.85	167±4.4	Additive 0.46
CT	81	155	Dominant 1.0	172±7.0	Dominant 0.34
CC	17	27	Recessive 0.62	186±15.7	Recessive 0.28

Table 4-6 Primary outcome measures according to polymorphisms in the endotoxin recognition complex

[a]P value derived from χ^2 test, [b]Mean ± SEM [c]P value derived from Student's t-test or One Way ANOVA.

SNP[a]	28 day mortality (n) no/yes	Expected mortality in %[b]	Length of stay (days)[c]	Ventilator days[d]	Nosocomial infection week 1[e] (n) no/yes
TLR4 D299G/T399I					
WT	772/25	2.4 (0.1-98)	3.6 (0.1-362)	2.0 (0-362)	561/44
Variant	97/4	2.1 (0.6-88)	3.8 (0.2-136)	2.0 (0-136)	72/7
	P=0.55	P=0.41	P=0.26	P=0.08	P=0.64
CD14 C-159T					
CC or CT	622/20	2.4 (0.1-98)	3.5 (0.1-362)	2.0 (0-362)	475/33
TT	187/8	2.3 (0.1-75)	3.7 (0.1-83)	2.0 (0-61)	131/18
	P=0.5	P=0.75	P=0.08	P=0.2	P=0.03[f]
LBP P97P					
TT	549/18	2.3 (0.1-98)	3.1 (0.1-135)	2.0 (0-135)	413/31
CT	230/6	2.3 (0.3-75)	3.8 (0.1-107)	2.8 (0-91)	176/16
CC	41/3	3.1 (0.1-90)	4.6 (0.8-362)	3.0 (0-362)	27/4
	P=0.33	P=0.41	P=0.26	P=0.08	P=0.44

Table 4-7 Clinical outcome measures according to genotype in the endotoxin recognition complex

Univariate analysis by Mann-Whitney *U* (continuous variables) or Pearson χ^2 test (categorical variables). [a]Single Nucleotide Polymorphism, [b]Based onPaediatric index of Mortality (PIM) score, median and range; [c, d]Median and range; [e]Not documented in infection group, [f]Not significant in adjusted logistic regression analysis; P=0.3 (adjusted for clinical co-variates and polymorphisms).

4.9.5 Validation cohort

Out of the original 1170 patients, both phenotypic data (age 63.3 ± 0.3 (mean ± SEM), sex ratio (M/F) (888/276) and TLR4 D299G genotype was available for 1065 patients. Of these patients 573 (54%) had experienced a recent (within 3 months) myocardial infarction. In this group those subjects with TLR4 variant genotypes had lower platelet counts than those with the wildtype genotype: 217 ± 7.0 vs. 237±2.8 (mean±SEM) $p = 0.021$, Figure 4-13.

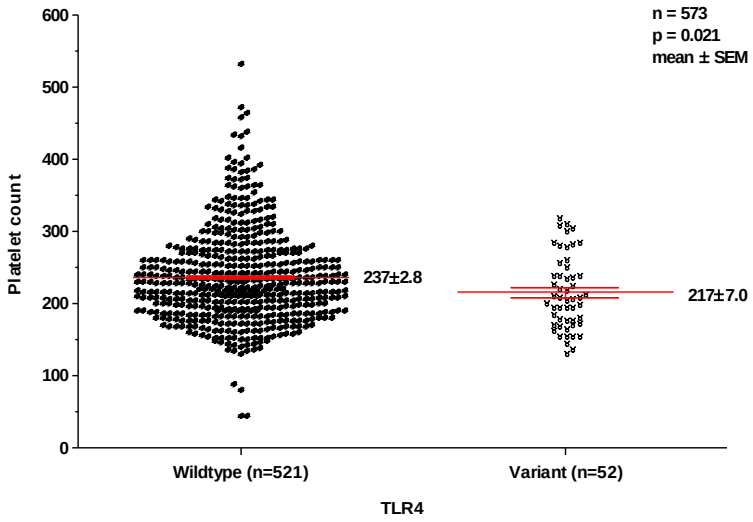


Figure 4-13 Platelet count per TLR4 genotype in adult validation cohort

Platelet count for adults with a history of myocardial infarction, stratified by TLR4 genotype wildtype vs. variant. P value derived from Student's t-test.

Because of the high prevalence of allele cosegregation in this Caucasian cohort, this study was not powered to assess a "gene dose effect". There was no significant difference in platelet count in the group that had not suffered a recent myocardial infarction, indeed the platelet count in the TLR4 variant group was higher than in the wildtype group: variant 244 ± 10 vs. wildtype 229 ± 3.5 (mean±SEM), $p = 0.14$. Age and gender are known to influence platelet count.(Miller, Jayachandran et al. 2007) The current cohort confirmed this; females had higher platelet counts than males (254 2.0 ±vs. 226 ± 5.0 (mean ± SEM), p <0.0001) and platelet count was inversely correlated with age (p<0.0001). Thrombocytopenia (platelet < 150 x 10^9/l) occurred in 53 cases and there was no difference between TLR4 genotype status.

4.10 Discussion

This cohort of more than 900 critically ill children shows little impact of common functional polymorphisms in the endotoxin receptor on the development of SIRS. Conversely, there was a clear and unequivocal effect of TLR4 genotype on platelet count. Polymorphisms associated with reduced TLR4 function exerted a dose-dependent effect; the lowest platelet counts were seen in the haplotypes associated with lowest TLR4 function. The stepwise association between TLR4 polymorphisms and lower platelet counts may imply that these two SNPs reduce TLR4 function by addition or interaction.

So-called "common" co-segregation for the D299G and T399I polymorphisms is found only in Caucasians. Therefore studies that analyse either SNP in isolation will be suboptimal in multiethnic cohorts. (Ferwerda, McCall et al. 2008). This issue may underlie some of the variability in the existing literature.

The main question to ask in interpreting this data is:

How can a clinically small (175 vs. 143), but statistically highly significant difference in platelet count related to TLR4 haplotype, be reconciled with the lack of effect on SIRS?

The two observations appear to be contradictory, but perhaps they offer some insight into acute inflammatory processes and the limitations of the definitions of systemic inflammation currently employed for clinical research.

The apparent conflict arises from the view that platelets are an integral part of the acute inflammatory response (Klinger and Jelkmann 2002) with an important relationship between thrombocytopenia and severity of illness across all age ranges (Vanderschueren, De Weerdt et al. 2000, Peters, Ross-Russell et al. 2001, Akca, Haji-Michael et al. 2002, Ragazzi, Pierro et al. 2003). Numerous observations suggest that platelets actively contribute to the intensity of the acute inflammatory response rather than being bystanders that are consumed as a 'para-phenomenon'.(Davi and Patrono 2007, Zarbock, Polanowska-Grabowska et al. 2007) Mechanisms include acceleration of recruitment of neutrophils and monocytes by formation of heterotypic complexes as well as production of inflammatory mediators including CD40 ligand, tissue factor, RANTES and matrix metalloproteinases.(Gawaz, Langer et al. 2005)

If platelet activation is inseparable from the acute inflammatory response, why might reduced endotoxin responsiveness affect platelet count but have no detectable effect on clinical markers of systemic inflammation?

The simplest explanation would be a type II error. SIRS may be an insufficiently discriminatory measure and therefore despite the numbers of cases reported here, this

study may be inadequately powered. The limitations of a clinical diagnosis of SIRS are well documented.(Vincent 1997) In part this is due to therapy-induced changes on the variables temperature, heart rate and respiratory rate that constitute clinical SIRS. The label SIRS thus may poorly reflect the underlying state of immune activation. In children this is compounded by the normal developmental changes in heart rate, respiratory rate and white cell counts. The paediatric criteria for SIRS take into account these age-related changes, but these consensus decisions equally have not been validated in large cohorts. So, there is no "gold standard" for SIRS.

Next, the study included only children with a precipitating insult of sufficient severity to require admission to intensive care for organ support. Any variability in the development of SIRS that may be attributable to TLR4 polymorphisms may be swamped by the severity of this level of insult. The patient cohort thus may well be too sick to identify what we now know are subtle changes due to common genetic polymorphisms. Common genetic polymorphisms in complex disease are expected to have but a small influence with odds ratios < 2. (Stranger, Stahl et al. 2011)

A recent clinical study in stem cell transplant patients demonstrated TLR4 variant alleles to be a risk factor for Aspergillosis infection.(Bochud, Chien et al. 2008) Unlike the study's mixed ICU population at risk for SIRS, this was a specific complication in a very high-risk population; hence a more favourable 'signal-to-noise' ratio might be expected. In other words, the phenotype description is of utmost importance. With a more specific phenotype, the potential increases to ascertain a genotype association.

The fact that SIRS is a binary outcome measure compounds these limitations. In contrast, admission platelet count is a near continuous measure that is unlikely to be

confounded dramatically by therapy. Dichotomizing data leads to reduction of power because of loss of information and reduction in variability.(Altman and Royston 2006)

Alternatively, it may be that platelet count is just a more sensitive measure of the degree of inflammation than the crude indices used to define SIRS.

More recent evidence to support the hypothesis that platelets are a sensitive (or even sentinel) marker of inflammation was found in the observation in mice that a single intravenous low dose LPS induced a TLR4-dependent increase in basal P-selectin positive platelets 24 hours later and a corresponding increase in reticulated platelets two days later, indicating a sustained reactive platelet response. Of note, this activation and reduction in life span occurred without measurable changes in serum TNFα.(Jayachandran, Miller et al. 2010)

Then there is the possibility that the effect on platelet count was specific to platelets, rather than it being part of systemic inflammation.

This possibility has credibility, because importantly, no influence of MBL-2 genotype on platelet count was demonstrated, while others and we have previously described an increased risk of SIRS in children carrying MBL-2 genotypes associated with reduced plasma levels of the pattern recognition molecule Mannose Binding Lectin. Therefore, another explanation for the discordant results between the impact of TLR4 polymorphisms on development of SIRS and platelet count requires consideration.

Rather than these results reflect the degree of complexity and specificity of the two end-points themselves, it may be that these results reflect the difference in complexity of the inflammatory pathways relevant to the two end-points. Development of SIRS is a complex process with multiple pathways and thus a high level of redundancy.

Inherited dysfunction or therapeutic inhibition of single elements are therefore unlikely to have a large impact on overall outcome.(Bone 1996, Zeni, Freeman et al. 1997) Indeed, TLRs intrinsically have different redundancies. Intracellular TLRs are less tolerant to reduced function than ligand binding cell surface ones.(Barreiro, Ben-Ali et al. 2009)

Specifically, the eukaryotic nucleated intra-cellular signalling response to TLR ligation has been described in great detail as an example of a biologically robust process.(Oda and Kitano 2006) In contrast to the main effector cells of systemic inflammation (monocyte/macrophages and endothelial cells), platelets express a very limited range of surface receptors for inflammatory mediators and have a limited response repertoire due to the absence of genomic DNA.(Davi and Patrono 2007) Thus, it is biologically plausible that endotoxin-TLR4 binding causes a direct, non-redundant response of platelet activation and consumption that is independent of the development of SIRS.

The demonstration of functional TLR4 expression on platelets (Andonegui, Kerfoot et al. 2005, Shashkin, Brown et al. 2008) and the observation that adults with TLR4 variant genotypes have significant differences in platelet function (Patrignani, Di Febbo et al. 2006) provide some support for this concept.

To validate this concept an independent cohort with a relevant inflammatory insult was required. In the absence of a relevant independent paediatric cohort a convenience cohort consisting of adults with atherosclerosis and a recent inflammatory insult (e.g. myocardial infarction) was identified. Reduced severity of atherosclerosis has previously been associated with TLR4 variant genotypes (Kiechl, Lorenz et al. 2002, Patrignani, Di Febbo et al. 2006). Indeed, adults with coronary artery stenosis and a recent history of myocardial infarction who carried TLR4 variant

alleles had platelets counts, which, on average, were 20 x 10^9/l lower than their wildtype counterparts.

Finally, clinical trial evidence further supports the possibility of the impact of this pathway on platelet count. In a double blind multi-centre randomised study of 393 children with severe meningococcal disease, the anti-endotoxin molecule recombinant bactericidal/permeability increasing protein fragment ($rBPI_{21}$) had no significant effects on mortality (although morbidity was better with less amputations and better functional recovery). However, thrombocytopenia was less severe in children receiving $rBPI_{21}$ (control group 36% transfused platelets vs. 25% r-BPI_{21} group, p=0.03).(Levin, Quint et al. 2000)

Unfortunately, none of the other large anti-endotoxin clinical trials comment specifically on the effect of therapy on platelet count or platelet transfusion. (McCloskey, Straube et al. 1994, Derkx, Wittes et al. 1999, Vincent, Laterre et al. 2005)

The TLR4 antagonist Eritoran study in sepsis was stopped for futility. No data as of yet are available on morbidity and haematological parameters such as platelet count and transfusions.

4.11 Limitations

This study has several limitations. The paediatric cohort is heterogeneous. Different precipitating events in nature and time course before admission may have led to the inability to determine a differentiation in SIRS. Given the inherent non-protocolized nature of this observational study, there was no attempt to describe SIRS by way of humoral biomarkers of inflammation, such as cytokines. Future strictly protocolized

studies may elucidate the temporal pattern of circulating cytokines in relation to genetic profile.

The validation cohort differed in age, inflammatory insult and ethnic mix, and thus is not perfect.
Myocardial infarction, however, is an inflammatory process of substantial severity with a relevance to platelet count and TLR4. Despite this and also that the observed difference on platelet count in this cohort is small (although statistically significant) and the counts are in the normal range, this result still corroborates the findings in the paediatric cohort.

Polymorphisms in endogenous BPI (Michalek, Svetlikova et al. 2007) and at least two components of TLR4 induced intracellular signalling, i.e. Mal (Khor, Chapman et al. 2007) and IRAK4 (Ku, von Bernuth et al. 2007) may change susceptibility or outcome in severe infections. These polymorphisms were not included in this study and thus this study might be labelled as incomplete. Given the ever-evolving knowledge in this area, however, all similar studies are hampered by this phenomenon.

Last, these findings require a mechanistic approach in human *ex vivo* experiments or *in vivo* knock out mice models to determine the underlying processes. Future studies will need to focus on the specific underlying pathophysiological mechanisms.

4.12 Conclusion

In conclusion, TLR4 polymorphisms are associated with lower platelet counts in severe inflammation. The reasons for this are unclear but may point to a direct effect of the TLR4 pathways on platelets or indicate that platelet counts are a more sensitive marker of systemic inflammation than SIRS criteria. These data support the view that variation in TLR4 function influences the early inflammatory response. This phenomenon may be one aspect of reduced fitness in the capacity to respond appropriately to an insult.

4.13 Future work

Future strictly protocolized studies may elucidate the temporal pattern of circulating cytokines in relation to genetic profile.

Future studies will need to focus on the specific underlying pathophysiological mechanisms.

Baseline platelet count and percentage reticulated platelets are 20% lower in TLR4 deficient mice.(Jayachandran, Brunn et al. 2007) An attempt was made to identify whether the lower platelet count in the TLR4 variant groups, as observed in both cohorts was secondary to the acute phase response or rather a pre-existing state in these individuals. Two healthy adult cohorts were identified from the literature where platelet counts may have been collected as part of the protocol, the Italian and the UK Caerphilly Prospective Study (CaPS). Contact was made by email. One research group declined to respond. The other group showed some enthusiasm, but was unable to generate the desired data. Thus it remains to be seen whether TLR4 variant populations exhibit a baseline loss of redundancy in their capacity to respond to day-to-day minor insults, such as bacteraemias secondary to brushing teeth.

What is the evolutionary meaning of TLR4 polymorphism differences across ethnicities. The debate is ongoing whether this is genetic drift or infectious pressure. One group states that given the similar frequencies of TLR4 polymorphisms in populations with different infectious pressures exerted by malaria, it is likely genetic drift that accounts for the prevalence of TLR4 polymorphisms (Greene, Moormann et al. 2009) Others however makes the claim that in African populations the prevalence of the TLR4 D299G variant is 10-20x higher than in populations from other countries.(Ferwerda, McCall et al. 2008)Moreover, TLR4 polymorphsims were shown to be protective against malaria with a consistent protective effect in the D299G variant in three case control studies with nearly 4500 African children with severe malaria. The identification of the malaria parasite GPI anchor molecules as ligands for human TLR4 receptors supports the role of TLR4 in malaria. (Hill 2006)

Chapter 5 Genetic variability in complement activation

5.1 Complement Activation Cascade

Complement activation is a key early event in the host response to injury (Fosse, Pillgram-Larsen et al. 1998) and infection (Jack, Klein et al. 2001). When appropriately activated, this powerful cascade contains, kills and clears pathogens and host debris without leading to a detrimental systemic "runaway" response. Three major activation pathways exist: the classical, alternative and lectin pathway. (Figure 5-1)

Classical pathway

C1q, a complex molecule made up of three different polypeptide chains (A, B and C) initiates the classical pathway. The protein is a defence collagen, as are others such as Mannose Binding Lectin (MBL), surfactant protein A, surfactant protein D and ficolin. They share a collagen-like domain that is in continuity with a noncollagen like sequence. This *globular carboxyl* head recognizes specific pathogen associated molecular patterns. It enables the host to mount an immediate response by activating the classical (e.g. C1q) or lectin pathway (MBL and ficolin) through auto-activation of their associated serine proteases.(Sim and Tsiftsoglou 2004)

Alternative pathway

The alternative complement pathway is a continuously activated antimicrobial defence mechanism that also amplifies the other two pathways (Holers 2008). Strong negative feedback mechanisms exist because of the potentially destructive nature of

unchecked complement activation. Complement Factor H (*CFH*) is an important part of this regulation. Circulating CFH levels are high and increase during acute inflammation, thus preventing potentially dangerous excessive complement activation.(Rodriguez de Cordoba, Esparza-Gordillo et al. 2004) Complement Factor B (CFB) has the opposite effect; it competes with CFH thereby enhancing the alternative pathway. (Lachmann and Hughes-Jones 1984)

Lectin Pathway

Mannose Binding Lectin (MBL) initiates the lectin pathway. MBL and C1q have similar structures and functions; they recognize and bind to pathogen-associated membrane patterns and have bactericidal properties. MBL associates with MBL-associated serine proteases (MASPs) on binding with micro-organisms, which, in turn, leads to cleavage of C4 and C2 to form C3 convertase. (Dommett, Klein et al. 2006)

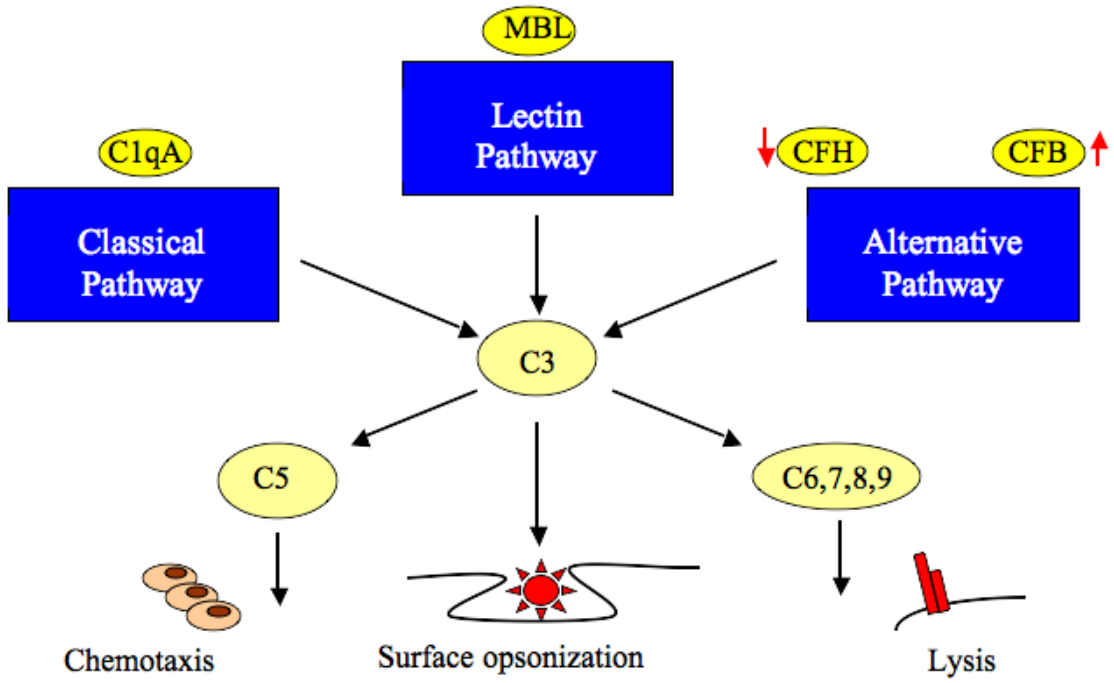


Figure 5-1 Complement activation cascade

The three pathways that activate the complement cascade are initiated and/or modulated by C1qA, MBL, CFH and CFB. These pathways converge at C3, from where the downstream complement proteins lead to chemotaxis, opsonization and cell wall lysis.

5.2 Genetic variation in complement function

Complement deficiencies are well known to predispose to infection or inflammatory disease.(Walport 2001) An ever increasing number of studies report altered risk to disease or functional change associated polymorphisms in the genes encoding the proteins in all three complement activation pathways (Holers 2008, Heurich, Martinez-Barricarte et al. 2011).

Given the many proteins involved in the complement cascade there are many candidate genes. The following polymorphisms were chosen based on the concept of final complement activation and known potential disease modifiers:

MBL-2, lectin pathway activation;

CFH, alternative pathway inhibition;

CFB, alternative pathway activation;

C1qA, classical pathway activation; C1q is the recognition subcomponent of the C1 complex

Host genotype determines 63% of the variation observed in CFH levels.(Esparza-Gordillo, Soria et al. 2004) The *CFH* Y402H polymorphism results in reduced CFH function (Schmidt, Herbert et al. 2008) and is implicated in the aetiology of the inflammatory eye disease age-related macular degeneration (Klein, Zeiss et al. 2005) and possibly myocardial infarction (Kardys, Klaver et al. 2006), although this is questioned (Zee, Diehl et al. 2006). *CFB* is located on the major histocompatibility complex (MHC) class III region on chromosome 6 (Regueiro and Arnaiz-Villena 1988). Polymorphisms within this gene are also associated with age related macular degeneration (Gold, Merriam et al. 2006, Maller, George et al. 2006). A functional polymorphism in *C1qA* have been associated with decreased complement activity in relation to dysregulated inflammation in subacute cutaneous lupus erythematosus

(SCLE) (Racila, Sontheimer et al. 2003) an increased risk for reduced rate of metastases in breast cancer (Racila, Racila et al. 2006) and most recently, an increased risk for mortality in breast cancer (Azzato, Lee et al. 2010).

The most studied component of complement activation in relation to sepsis is mannose binding lectin (MBL). Serum MBL levels are predominantly determined by genotype in *MBL2*. Common structural and promoter polymorphisms associated with low serum levels are risk factors for infections in vulnerable individuals such as young children (Summerfield, Sumiya et al. 1997), neutropenic children (Neth, Hann et al. 2001). There exists, however, ongoing debate with regards to *MBL2* deficiency as a risk factor for severe infection. A recent case control study and meta-analyis did not find any association with MBL deficient genotypes and invasive meningococcal disease (Bradley, Bourke et al. 2012) and neither did a genome wide association study that included rs1800450, which is the most common MBL deficient genotype (B) in people of European ancestry (Davila, Wright et al. 2010).

MBL-2 polymorphisms and reduced serum levels were associated with the development of early SIRS/sepsis in a relatively small cohort of critically ill children(Fidler, Wilson et al. 2004) and susceptibility to and worse outcome in adults with sepsis (Gordon, Waheed et al. 2006). This association of SIRS/sepsis with MBL deficiency may reflect the known interactions with pro-inflammatory cytokine production. Low dose of MBL enhanced the production of IL1β and IL6 in monocytes, whereas high dose MBL profoundly reduced pro-inflammatory cytokine production.(Jack, Read et al. 2001) Alternatively, MBL deficiency may exert a direct effect on the rate and extent of complement activation.

The importance of the three pathways in relation to SIRS is unknown, however it seemed plausible that irregularities in the pattern recognition or regulation components would influence the magnitude of cascade activation and thus initial host response to injury and infection.

To further determine the importance of the other complement activation pathways in the development of SIRS/sepsis in paediatric critically ill patients a larger cohort was genotyped for functional polymorphisms in *MBL-2*, *CFH*, *CFB* and *C1qA*.

5.3 Potential genetic confounders

Complement activation is only part of the host response to injury and infection. Genetic variation in other components of the innate immune system may modify outcome in critical illness.(Waterer and Wunderink 2003, Lin and Albertson 2004) To determine whether the impact of complement polymorphisms was independent of other known genetic variability in the innate immune system the cohort was also analysed for functional polymorphisms in *TNFα, IL6*, *IL10,* and *PAI-1*.

Cytokine production is a downstream event relevant to both sepsis and sterile SIRS. This process is, in part, under genetic control and this may modify outcome in critical illness. (Westendorp, Langermans et al. 1997, Galley, Lowe et al. 2003, Schaaf, Boehmke et al. 2003)

5.4 Hypothesis

Polymorphisms associated with reduced complement activation are associated with an increased incidence of SIRS in the first three days of intensive care admission.

5.5 Materials and Methods

5.5.1 Ethical approval and parental consent

The Great Ormond Street Hospital for Children NHS Trust / Institute of Child Health Local Research Ethics Committee approved this prospective observational cohort study. Parental informed consent was obtained according to the Declaration of Helsinki guidelines.

5.5.2 Subjects

Between 2002 and 2006 subjects were recruited consecutively in two recruiting time-periods from a tertiary paediatric general intensive care unit in London, UK.
The aim of the selection criteria was to include children who were sick enough to stay for longer than an overnight time period but did not have known underlying reasons for a more severe illness course. Thus, inclusion criteria were: age up to 18 years and the presence of at least one organ system failure for 12 hours or longer (Proulx, Fayon et al. 1996) (or death within 12 hours of admission). Excluded were children with multiple congenital abnormalities, known immunodeficiency, haematological or lymphoid malignancies, systemic immunosuppressive drug therapy other than corticosteroids, known central neurological or neuromuscular disease, persistent pulmonary hypertension of the newborn, weight less than 2.2 kg, corrected gestational age less than 37 weeks, repeat intensive care admission, and non-accidental injury.

Ethnicity was coded according to Hapmap classification (2003) on the basis of parental described heritage routinely collected on admission in the National Health Service (NHS).

Patients were classified by primary diagnosis as '*Infection'* and '*Non-infection'* (including elective general surgery, trauma, and other) based on the diagnosis made by the responsible admitting physician. Clinical data were collected blinded to the genotyping data.

5.5.3 Genotyping

Blood (2.5 ml) was collected in EDTA bottles and stored at -80°C until DNA extraction according to the manufacturer's instructions (QIAamp DNA blood mini or midi kit, Qiagen, Crawley, UK).

The individual *CFH, MBL-2, CFB, C1qA, TNFα, IL6, IL10* and *PAI-1* polymorphisms were determined by KASPar assay at the commercial genotyping facility K-Biosciences (Herts, UK). For quality control purposes duplicates were included (5%) as well as three blank wells per 96 well plate. The commercial genotyping facility KBiosciences (Herts, UK) has developed a competitive allele specific PCR combined with Fluorescence Resonance Energy Transfer (FRET) method (KASPar) for single nucleotide polymorphism (SNP) identification. The company itself and numerous research groups have validated this method (Weedon, McCarthy et al. 2006), including the Wellcome Trust Centre for Human Genetics (Zeggini, Scott et al. 2008).

Results from a subset of the cohort for *MBL-2* that had previously been genotyped by PCR amplification and heteroduplexing (Fidler, Wilson et al. 2004) served as further validation. This showed 100% concordance for the compared genotypes.

Genotyping was done blinded to clinical data.

5.5.4 *CFH* Genotypes analysis

The non-synonymous SNP Y402H occurs due to substitution of the wildtype T allele with the variant C allele at nucleotide position 1277. This results in an amino-acid change from tyrosine (Y) to histidine (H) at amino acid position 402. The mechanisms underlying reduced CFH functionality of the 402H variant was recently reviewed. Although not completely elucidated, the current understanding of reduced functionality centres around the CFH binding affinity. (Schmidt, Herbert et al. 2008) This loss of function translates to the clinical field: A meta-analysis showed that homozygote variant carriers (CC) were at highest risk for the inflammatory eye disease age related macular degeneration (AMD) (Thakkinstian, Han et al. 2006). A recessive model, i.e. CC vs. CT/TT genotypes, seems to be the most appropriate.

5.5.5 *MBL-2* Genotypes

The *MBL-2* gene has 6 common polymorphisms: 3 structural gene variants in exon 1 at positions 52, 54 and 57 (known as variants C, B and D respectively) and 3 promoter SNPs at positions -221, -550 and +4 (denoted as X/Y, L/H and P/Q, respectively). By convention, wildtype genes are denoted "A" and variant genes "O". Strong linkage disequilibrium causes there to be just seven common haplotypes. (Madsen, Garred et al. 1995, Madsen, Satz et al. 1998)

Full *MBL-2* haplotypes were inferred from the raw genotyping data based on these seven known haplotypes. These haplotypes then combine into 28 possible genotypes. Based on previously reported associations between plasma MBL levels and *MBL-2*

genotypes (Fidler, Wilson et al. 2004, Gordon, Waheed et al. 2006) (Madsen, Garred et al. 1995) (Madsen, Satz et al. 1998) the genotypes HYPA/HYPA, HYPA/LYQA, LYQA/LYQA, HYPA/LYPA, HYPA/LXPA, LYQA/LYPA and LYQA/ LXPA qualified as wildtype and all other genotypes as variant. Both the structural gene and the promoter variants influence plasma MBL levels. Homozygous wildtype carriers for HYPA have the highest MBL plasma levels and lowest plasma levels are found in individuals who are homozygous or compound heterozygous for gene variants LYPB and LYQC. In between these two extremes are combinations of haplotypes that result in intermediate phenotypes. The structural gene polymorphism haplotype HYPD is associated with higher plasma levels than found for the other variants, but clinically behaves as other gene-variants. The X promoter and L promoter have both been associated with lower plasma MBL levels, such that LXPA, and to a lesser extent the LYPA genotypes are associated with lower plasma MBL levels (Madsen, Satz et al. 1998). Thus homozygotes and compound heterozygotes for these haplotypes were assigned variant status. Due to sample size restrictions a detailed analysis on the basis of the 28 possible genotypes is not feasible. The genotypes were therefore categorized into two groups: wildtype and variant based on the above classification.

5.5.6 Complement activation potential

To semi-quantify complement activation potential the *MBL-2* and *CFH* haplotypes combined to “Very high”: *MBL-2* HYPA/HYPA plus *CFH* CC; “High” *MBL-2* HYPA/LYQA or LYQA/LYQA or HYPA/LXPA or HYPA/LYPA or LYQA/LYPA or LYQA/LXPA plus *CFH* CC; “Very low” *MBL-2* double structural gene variants (O/O) plus *CFH* TT; “Intermediate” all other combinations of *MBL-2* and *CFH*.

5.5.7 *CFB* Genotypes

Two SNPs in the *CFB* gene were found to be associated with reduced risk of the complement mediated inflammatory eye disease AMD: L9H in exon 1 and R32Q in exon 2.(Gold, Merriam et al. 2006) The L9H SNP is caused by a T to A allele change, leading to a leucine (L) to histidine (H) amino acid change at amino acid position 9. The R32Q polymorphism is caused by a change from G to A (antisense C to T) leading to the arginine (R) to glutamine (Q) change at amino acid position 32. Both SNPs are associated with loss of function, thus reducing the capacity to form complement C3b.(Gold, Merriam et al. 2006, Montes, Tortajada et al. 2009) They conferred a protective effect for AMD independently of each other in a co-dominant model. (Maller, George et al. 2006) The analysis done here followed this principle.

5.5.8 *C1qA* Genotypes

The SNP *C1qA*-Gly70$_{GGA}$ at nucleotide position 276 in exon 2 of the *C1qA* gene consists of a change from G to A in the codon for Gly70 (GGG to GGA). Homozygosity for the A allele was associated with significantly reduced serum C1q levels both in healthy individuals and a non-familial form of subacute cutaneous lupus erythematosus (SCLE) patients and an increased risk for SCLE (Racila, Sontheimer et al. 2003). The data was thus analyzed in recessive inheritance mode (alleles GG and GA vs. AA).

5.5.9 Possible confounding polymorphisms

Known functional SNPs in genes possibly associated with modification of critical illness were analysed. The following SNPs were chosen as potential confounding

factors: the promoter polymorphisms in the cytokines *TNFα* -308 G/A, *IL6* -174 G/C, *IL10* -1082 G/A and the insertion deletion SNP *PAI-1* 4G/5G.

Table 5-1 lists the descriptive details for the analysed SNPs. Many more genetic polymorphisms may influence outcome in critical illness. The host response to injury and infection is a complex phenomenon that encompasses thousands of genes (Calvano, Xiao et al. 2005) and thus potential polymorphisms. These SNPs were chosen as possible confounders for the following three reasons: 1) Common prevalence of the SNP (>1%); 2) Change in function associated with the SNP and 3) Direct relevance to downstream effects of complement activation (Jack, Read et al. 2001, Wojta, Kaun et al. 2002, Nauta, Castellano et al. 2004, Sprong, Jack et al. 2004, Kastl, Speidl et al. 2006).

rs number[a]	Gene	Locus	Region	Nucleotide change	Effect of SNP	Key references
5030737	MBL-2	10q11.2-q21	Exon 1	"D" 52C>T Arg>Cys	"D" reduced	(Madsen, Garred et al. 1995, Madsen, Satz et al. 1998, Fidler, Wilson et al. 2004, Gordon, Waheed et al. 2006)
1800450	MBL-2		Exon 1	"B" 54G>A Gly>Asp	"B" reduced	
1800451	MBL-2		Exon 1	"C" 57G>A Gly>Glu	"C" reduced	
7096206	MBL-2		Promoter	-221 G/C X/Y	"X" reduced	
11003125	MBL-2		Promoter	-550 C>G L/H	"L" reduced	
7095891	MBL-2		Promoter	+4 C>T P/Q	"P" depends on genotype	
1061170	CFH	1q32	Exon 9	1277T>C Tyr402His; Y402H	CC reduced	(Klein, Zeiss et al. 2005, Kardys, Klaver et al. 2006, Schmidt, Herbert et al. 2008)
172378	C1qA	1p36.12	Exon 2	276G>A; $Gly70_{GGA}$	AA reduced	(Racila, Sontheimer et al. 2003)
4151667	CFB	6p21.3	Exon 1	T>A L9H; Leu9His	AA	(Gold, Merriam et al. 2006, Maller, George et al. 2006)
641153	CFB		Exon 2	95 G>A; R32Q;	AA reduced	

				Arg32Gln		
1800795	IL6	7p21	Promoter	-174G>C	CC reduced	(Terry, Loukaci et al. 2000, Rivera-Chavez, Peters-Hybki et al. 2003)
1800896	IL10	1q31-q32	Promoter	-1082G>A	AA reduced	(Turner, Williams et al. 1997, Westendorp, Langermans et al. 1997)
1800629	TNFα	6p21.3	Promoter	-308 G>A	AA increased	(Kroeger, Carville et al. 1997, Westendorp, Langermans et al. 1997)
1799889	PAI-1	7q21.3-q22	Promoter	-765 4G/5G	4G/4G increased	(Dawson, Wiman et al. 1993, Festa, D'Agostino et al. 2003, Hermans and Hazelzet 2005)

Table 5-1 Gene and polymorphism characteristics in the complement activation cascade and potential confounders

[a]NCBI dbSNP number

5.6 Outcome measure

The outcome measure was occurrence of SIRS within the first three days of admission. SIRS was defined according to the 1992 ACCP-SCCM consensus guidelines (1992), modified for age as described previously (Fidler, Wilson et al. 2004). Briefly, a diagnosis of SIRS was made if 2 or more of the following were present: white cell count >$12x10^9$/l or < $4x10^9$/l, heart rate (beats per minute, bpm) outside age specific ranges (newborn to 3 months: 95-145 bpm, 3-12 months 110-175 bpm, 1-<3 years 105-170 bpm, >3-<7 years, 80-140 bpm, >7-<10 years 70-120 bpm, >10 years 60-100 bpm), central temperature >38.0^{o}C or < 36.0^{o}C. The respiratory score was not included in the measure as most children were expected to be ventilated. Temperature was scored only when not controlled artificially.

5.7 Statistical analysis

Hardy Weinberg equilibrium was calculated by χ^2 -test.

Univariate analysis was performed by Fisher exact, χ^2 or Mann-Whitney *U* tests as appropriate. Binary logistic regression analysis was used to determine the independent contribution to risk of the genetic and demographic covariates. In the final regression analysis a two-tailed P value < 0.01 was considered to be statistically significant. This incorporates a stringent Bonferroni correction for 5 independent candidate gene variants. All statistical analyses were performed using SPSS version 13.0 for Mac (SPSS, Chicago, IL, USA).

5.8 Results

Three hundred children were recruited into the study. Blood for genotyping was available for 299 patients. Patient clinical details are summarized in Table 5-2. Most children were ventilated (275/299; 92%) or on cardiovascular support (152/299; 51%) at admission. SIRS occurred in 223/299 (74%) subjects. Mortality was in the expected range: 21/299 (7%). All children who died were ventilated and more likely to have been on inotropes, diagnosed with SIRS and thrombocytopenia on admission. No differences were found for sex, ethnicity, entry criterium, acute lung injury or genetic polymorphism in relation to mortality.

Characteristic	All (n=299)	Non-SIRS (n=76)	SIRS (n=223)	P value[a]
Age in months	40 (0-218)	35 (0-218)	43 (0-199)	0.83
Sex (M/F)	183/116	49/27	134/89	0.49
Ethnicity (n)				0.72
Caucasian	216	58	158	
African	36	7	29	
Asian	18	5	13	
Mixed/Unknown	29	6	23	
Primary diagnosis				0.00005[b]
Non Infection	141	51	90	
Elective postop	35	23	12	
Trauma	99	28	71	
Other	7	0	7	
Infection	158	25	133	
Septic shock	62	2	60	
Lower respiratory tract infection	56	13	43	
Meningitis/encephalitis	20	8	12	
Other	20	2	18	
PELOD on admission[c]	11 (0-61)	2 (0-31)	11 (0-61)	0.001
Risk of mortality[d]	0.06 (0.007-0.98)	0.038 (0.007-0.37)	0.068 (0.008-0.98)	< 0.0005
28 day mortality n (%)	21 (7.0)	1 (1.3)	20 (8.96)	0.024
Ventilator days	4.0 (0-362)	3.0 (0-20)	4.0 (0-362)	0.007
Length of stay days	4.6 (0.1-362)	3.6 (0.4-20.3)	4.8 (0.1-362)[e]	0.020

Continuous values are expressed as medians and range. Descriptive statistics are expressed as counts and percentages for categorical values. [a]Comparison between the groups with and without SIRS by the Mann-Whitney *U* test (continuous variables); Pearson chi-square or Fishers exact (categorical variables). [b]Non-Infection vs. Infection. [c]PELOD Pediatric Logistic Organ Dysfunction (Leteurtre, Martinot et al. 2003). [d]Based on PIM Paediatric index of Mortality (Shann, Pearson et al. 1997). [e]One child remained ventilated until transfer to a chronic care facility.

Table 5-2 Description of PICU cohort and comparison of SIRS and non-SIRS patients

5.9 Genetic variation in complement activation and SIRS

5.9.1 Genotyping overall results

Genotyping for *CFH* Y402H, *CFB* L9H, *CFB* R32Q and *C1qA*-Gly70$_{GGA}$ was successful in 280, 293, 290 and 292 children respectively. A full *MBL-2* genotype analysis was completed for 294 children. All genotypes were in Hardy Weinberg equilibrium when stratified according to ethnicity and allele frequencies were in accordance with those previously described in the literature. (see Table 5-3 and Table 5-4).

Genotype	Total	Caucasian	African	Asian	Other/Unknown
Total (n)	299	216	36	18	29
CFH Y402H					
TT	112	73	16	12	11
CT	126	102	12	4	8
CC	42	29	6	1	6
CFB L9H					
TT	273	197	36	16	24
TA	19	14	0	1	4
AA	1	1	0	0	0
CFB R32Q					
CC	234	173	22	13	26
CT	42	29	9	3	1
TT	10	9	0	1	0
C1qA					
GG	73	34	21	9	9
GA	135	104	12	5	14
AA	84	74	3	2	5
MBL-2					
HYPA/HYPA	20	12	0	1	7
HYPA/LYQA	48	44	1	1	2
LYQA/LYQA	14	4	10	0	0
HYPA/LYPA	11	7	3	1	0
HYPA/LXPA	34	25	3	3	3
LYQA/LYPA	12	9	3	0	0
LYQA/ LXPA	26	18	1	4	3
LYPA/LYPA	2	1	1	0	0
LYPA/LXPA	8	7	0	0	1
LXPA/LXPA	16	10	0	2	4
HYPD/HYPA	8	6	0	1	1
HYPD/LYQA	7	7	0	0	0
HYPD/LYPA	4	3	0	0	1
HYPD/LXPA	12	10	2	0	0
LYPB/HYPA	20	15	1	3	1
LYPB/LYQA	11	11	0	0	0
LYPB/LYPA	6	5	0	0	1
LYPB/LXPA	9	7	0	0	2
LYQC/HYPA	4	3	1	0	0
LYQC/LYQA	10	2	8	0	0
LYQC/LYPA	2	1	1	0	0
LYQC/LXPA	2	0	1	0	1
HYPD/HYPD	1	1	0	0	0
LYPB/HYPD	2	1	0	0	1
LYQC/HYPD	2	2	0	0	0
LYPB/LYPB	3	2	0	1	0

Table 5-3 Complement genotype distribution according to ethnicity

Genotype	All	Caucasian	African	Asian	Other/Unknown
Total (n)	299	216	36	18	29
IL6 -174G>C					
GG	161	95	36	13	17
GC	104	91	0	3	10
CC	26	24	0	1	1
IL10 -1082G>A					
GG	62	51	3	1	7
GA	128	100	15	6	7
AA	103	61	18	10	14
TNFα -308 G>A					
GG	221	153	29	17	22
GA	67	55	6	0	6
AA	6	5	1	0	28
PAI-1-765 4G/5G					
5G/5G	80	52	22	1	5
5G/4G	139	102	12	7	18
4G/4G	72	57	1	9	5

Table 5-4 Distribution of possible confounding polymorphisms according to ethnicity

5.9.2 Complement activation components as a risk factor for SIRS

Table 5-5 shows the univariate analysis for the different complement activation candidate genes in relation to early SIRS. Given the cohort size, prevalence of SIRS and minor allele frequencies, this study was powered >80% at an α of 0.05 to detect an Odds Ratio ≥ 2 at allele level in all but the CFB candidate gene and at the dichotomy variant/wildtype for the MBL genotypes.

The *CFH* Y402H polymorphism was associated with a significant lower incidence of SIRS. Figure 5-2shows the analysis in recessive mode (CC vs. CT/TT genotypes): OR 0.4 (95% CI 0.2 -0.8, P = 0.006.

After binary logistic regression analysis this was shown to be independent of other covariates (demographic characteristics, severity of illness, diagnosis group and polymorphisms in both the complement pathway and other components of the innate immune system): adjusted OR 0.3, 95% CI 0.1-0.7, P = 0.005 (Table 5-6).

Structural variant haplotypes in *MBL-2* were seen in 34% (103/299) of children. LYPB and HYPD were the most common variant haplotypes in patients of Caucasian and Asian descent. Children of African descent carrying structural *MBL-2* gene defects showed a preponderance of LYQC carriers. Low MBL promoter variants X and/or L were less common; 8.7% (26/299) individuals carried combinations with both LXPA and/or LYPA.

Children who carried variant *MBL-2* haplotypes showed an increased risk for developing early SIRS (Figure 5-3): Unadjusted Odds Ratio 2.0, 95% Confidence Interval 1.1-3.4; P = 0.016).

Gene	Name	Genotype	Non-SIRS	SIRS	OR (95% CI)	P value[a]
CFH	Y402H	TT	23	89	0.4 (0.2-0.8)	0.006[b]
		TC	31	95		
		CC	18	24		
MBL-2		Wildtype	51	114	2.0 (1.1-3.4)	0.016[c]
		Variant	24	105		
CFB	L9H	TT	69	204	0.8 (0.3-2.1)	0.6[c]
		TA	6	13		
		AA	0	1		
CFB	R32Q	CC	60	174	2.1 (0.5-9.5)	0.3[b]
		CT	11	31	1.1 (0.6-2.3)	0.7[c]
		TT	2	12		
C1qA	$Gly70_{GGA}$	GG	18	55	1.1 (0.6-1.9)	0.9[b]
		GA	36	99		
		AA	21	63		

Table 5-5 Univariate analysis for the complement candidate genes in relation to SIRS

[a]Pearson chi square; [b]recessive; [c]dominant

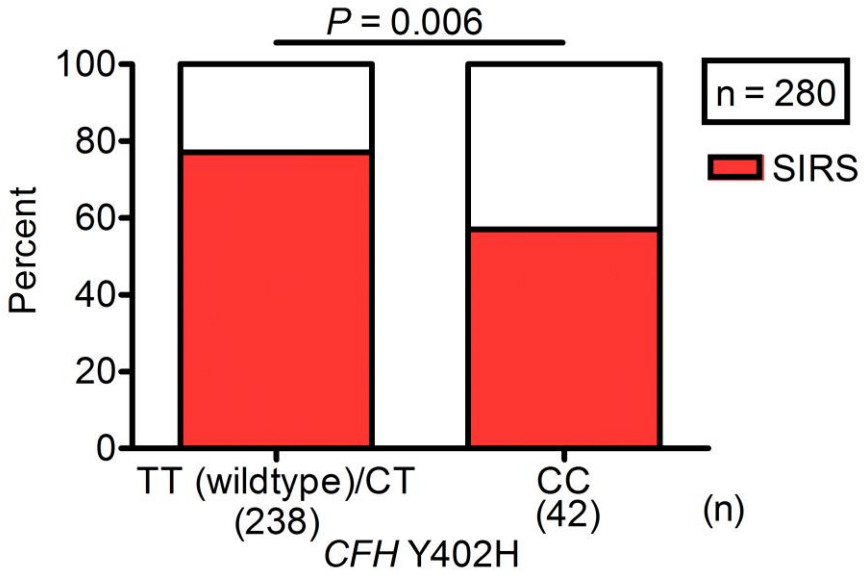


Figure 5-2 Risk for SIRS according to CFH Y402H genotype

The percentage of children developing SIRS according to Complement Factor H genotype. Univariate analysis in recessive heritability mode, P value is derived from χ^2 test.

Risk factor	Univariate analysis	Binary logistic regression analysis[a]
CFH CC variant	0.4 (0.2-0.8) P=0.006	0.3 (0.1 - 0.7) P= 0.005
MBL-2 variant	2.0 (1.1-3.4) P=0.016	2.5 (1.3 - 5.0) P= 0.008
Infection[b]		8.3 (3.0 - 24) P< 0.0005

Table 5-6 Risk factor analysis for the development of early SIRS/sepsis

[a]Adjusted for sex, age (logged), ethnicity, risk of mortality (PIM), primary diagnosis (elective postoperative, infection, trauma, other), *MBL-2*, *CFH, C1qA, CFB, IL6, IL10, TNF*α and *PAI-1* polymorphisms. [b]Versus elective postoperative. Results are shown as Odds Ratios with 95% confidence interval and P value. Only the covariates with a P value < 0.01 in the final analysis are shown. (cohort n=299)

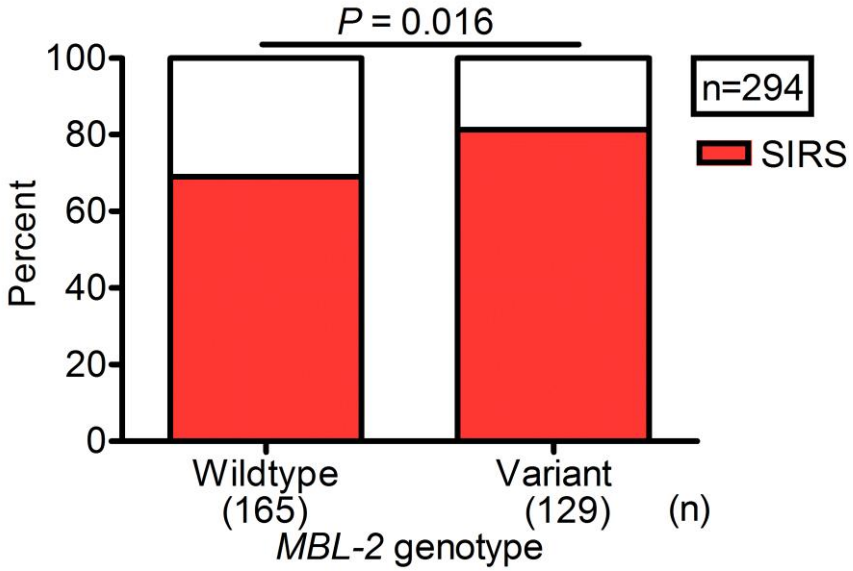


Figure 5-3 Risk factor for SIRS according to MBL2 genotype

Comparison of early SIRS development between the two *MBL-2* genotype categories.

Variant genotypes include both structural gene variants as well as promoter variants.

Univariate analysis, P value is derived from χ^2 test.

This risk increased and was significant after correction for demographic characteristics, severity of illness, diagnosis group and polymorphisms in both the complement pathway and other components of the innate immune system (adjusted OR 2.5, 95% CI 1.3-5.0, P = 0.008, Table 5-6).

In this cohort of critically ill children the odds for developing systemic inflammation were, at least in part, dependent on *CFH* and *MBL-2* genotype.
This association remained after controlling for admission reason (infectious or non-infectious). In other words, children admitted with an infectious insult were more likely to develop sepsis if they carried genotypes that are associated with reduced Complement activation potential and children admitted with a non-infectious insult were more likely to develop SIRS if they carried genotypes associated with reduced complement activation potential.

5.9.3 Sepsis and sterile SIRS may be differentially modulated

A priori the sample size prohibits quantifying effect size in the sub groups "*Non-infection*" and "*Infection*" separately with adequate statistical power. Nonetheless, to better understand the underlying pathophysiology this analysis was performed. The results suggest that the effect size for the two genotypes is larger for those children admitted with infection (Table 5-7).

	Non-infection		**Infection**	
	OR (95% CI)	P value	OR (95% CI)	P value
MBL-2 variants				
Univariate	1.8 (0.9-3.6)	0.11	2.4 (0.9-6.1)	0.07
Adjusted[a]	1.9 (0.7-5.1)	0.18	3.3 (0.9-12)	0.06
CFH CC variant				
Univariate	0.7 (0.2-1.4)	0.23	0.2 (0.1-0.7)	0.004
Adjusted[a]	0.3 (0.1-1.2)	0.11	0.12 (0.02-0.6)	0.01

Table 5-7 Risk factor analysis for the development of early SIRS/sepsis according to admitting diagnosis

Non-infection n=141 Infection n=158. OR= Odds Ratios for SIRS with 95% CI. P values derived from Pearson χ^2 analysis or logistic regression analysis. [a]Adjusted for sex, age (logged), ethnicity, risk of mortality (PIM), *MBL-2*, *CFH*, *C1qA*, *CFB*, *IL6*, *IL10*, *TNF*α and *PAI-1* polymorphisms.

5.9.4 Overall complement activation capacity and risk of SIRS

When *CFH* and *MBL-2* genotypes were combined to provide a crude measure of complement activation potential, a stepwise increase became apparent in the risk of SIRS in those genotypes with increasingly lower complement activation potential (Figure 5-4).

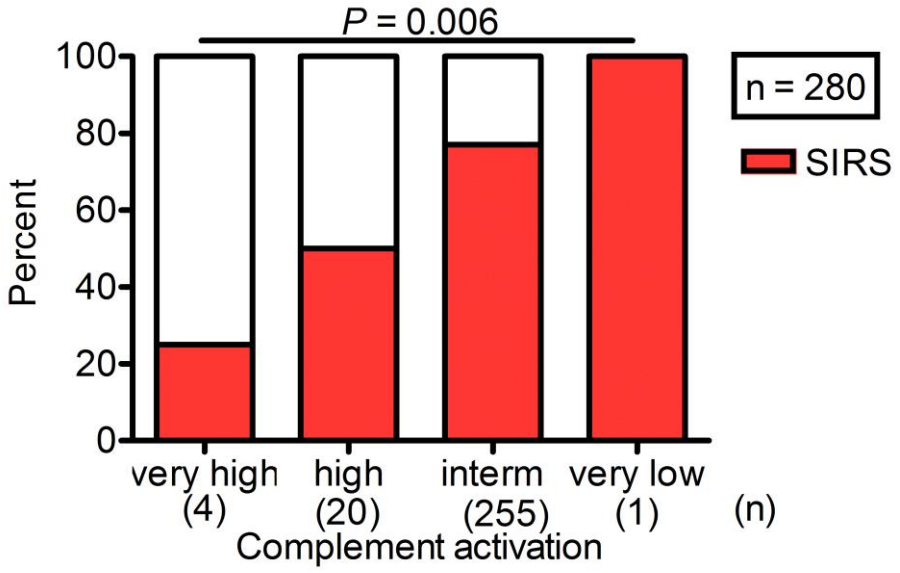


Figure 5-4 Risk for SIRS according to complement activation potential

Combination of *MBL-2* and *CFH* genotypes show an increase in the risk of SIRS according to potential of complement activation. "Very high": *MBL-2* HYPA/HYPA and *CFH* CC, "high" *MBL-2* HYPA/LYQA or LYQA/LYQA or HYPA/LXPA or HYPA/LYPA or LYQA/LYPA or LYQA/LXPA plus *CFH* CC, "very low" *MBL-2* homozygous or compound heterozygous variant (O/O) and *CFH* TT, "interm" (intermediate) all other genotypes. P value is derived from χ^2 test.

5.9.5 *CFB, C1qA* and potential confounding polymorphisms

Neither *CFB* nor *C1qA* SNPs showed any influence on the occurrence of SIRS (Table 5-5). Although *TNFα* and *PAI-1* variant carriers trended toward less SIRS in univariate analysis, in the final binary logistic analysis there was no influence of the potentially confounding polymorphisms in *TNFα, IL6, IL10* and *PAI-1* on the occurrence of SIRS (see Table 5-8).

Gene	Promoter SNP	Genotype	Non-SIRS	SIRS	OR (95%CI)	P value[a]	Adjusted OR (95% CI)[b]	Adjusted P value
TNFα	-308G>A	GG	50	171	0.6 (0.3-1.0)[c]	0.05	0.5 (0.2-1.0)[c]	0.06
		GA	23	44				
		AA	2	4				
IL6	-174G>C	GG	37	124		0.5		
		GC	30	74			0.7 (0.3-1.5)[d]	0.4
		CC	6	20			0.9 (0.3-3.3)[d]	0.9
IL10	-1082G>A	GG	18	44		0.7	1.3 (0.5-2.9)[d]	0.6
		GA	31	97			0.9 (0.4-2.1)[d]	0.7
		AA	25	78				
PAI-1	-765 4G/5G	5G/5G	17	63	0.6 (0.3-1.0)[e]	0.05	1.0 (0.5-2.1)[e]	0.9
		5G/4G	33	106	0.9 (0.4-1.7)[d]	0.7	1.1 (0.5-2.6)[d]	0.8
		4G/4G	25	47	0.5 (0.2-1.0)[d]	0.07	1.1 (0.4-2.9)[d]	0.8

Table 5-8 Analysis possible confounding polymorphisms in relation to SIRS

[a]Pearson χ^2 test; [b]Binary logistic regression analysis; Adjusted for sex, age (logged), ethnicity, risk of mortality (PIM), primary diagnosis, (elective postoperative, infection, trauma, other), *MBL*-2, *CFH*, C1qA, *CFB, IL6, IL10, TNFα* and *PAI-1* polymorphisms. [c]Dominant mode;. [d]vs. wildtype; [e]Recessive mode

Binary logistic analysis showed that the final model, incorporating sex, age (logged), ethnicity, risk of mortality (PIM), primary diagnosis (elective postoperative, infection, trauma, other), *MBL-2, CFH, C1qA, CFB, IL6, IL10, TNFα* and *PAI-1* polymorphisms explained 29% of the variance in SIRS/sepsis (r^2 Nagelkerke = 0.29), whereas the clinical characteristics alone explained only 22% (r^2 Nagelkerke = 0.22). This implies that some of the inherited variability in critical illness phenotype may be explained by genetic variability in complement activation. It also implies that further elucidating in the understanding of paediatric SIRS remains.

5.10 Discussion

These data show that polymorphisms in genes involved in the complement cascade are associated with the likelihood of developing early SIRS/sepsis in children admitted to intensive care. Homozygous *CFH* Y402H variant allele carriers were 3 times less likely to develop early SIRS compared with wildtype, whereas *MBL-2* variant genotypes conferred a two-fold increased risk of developing early SIRS. Importantly, this remained so after correcting for severity and type of illness, demographical characteristics and other polymorphisms in the innate immune system. Thus, it seems that genotypes that predispose to *less* complement activation are associated with an increased risk of SIRS/sepsis in the early part of intensive care stay.

This study did not address causality, in that no mechanistic experiments were performed. Nonetheless, the premise that the polymorphisms that were studied here indeed have altered function is supported by work that others have performed. *In vivo* data in septic patients showed that MBL deficient patients indeed had lower plasma MBL levels than did wildtype (Dean, Minchinton et al. 2005) and CFH polymorphic

patients showed higher complement activation than did wildtype (Scholl, Charbel Issa et al. 2008), which may have contributed to an altered likelihood to develop SIRS/sepsis. Interestingly, the Y402H CFH polymorphism has recently been identified as protective in group A streptococcal infections. This was postulated to be due to reduced binding affinity of group A streptococcus to CFH, thus precluding optimal intravascular bacterial growth. (Haapasalo, Jarva et al. 2008, Haapasalo, Vuopio et al. 2012)

The alternative pathway maintains a continuous low level of complement activation. This "ticking over process" is attenuated by CFH. Therefore, a polymorphism in this protein that leads to less inhibition of this pathway, could allow for the complement cascade to respond more vigorously when provoked. The finding that the *CFH* Y402H variant genotype CC exerts a strongly protective influence on the occurrence of early SIRS/sepsis is biologically interesting. The resultant amino acid change is thought to lead to a change in ligand binding properties and hence a reduced functionality in homozygous (CC) individuals especially (Schmidt, Herbert et al. 2008). Presumably, the reduced complement inhibitory properties would then lead to increased complement activation. This can be detrimental as seen in the chronic inflammatory eye disease AMD (Klein, Zeiss et al. 2005), where the tissue is chronically exposed to high complement activity. High complement activity, i.e. a vigorous containing and killing capacity could however be beneficial in the acute phase of critical illness. Early neutralisation of the offending agent might prevent downstream signaling and resultant SIRS/sepsis. Indeed, the promoter polymorphism *CFH* C-496T CC genotype, which is associated with increased CFH levels (and hence reduced complement activation potential) has been associated with a predisposition to meningococcal disease in children.(Haralambous, Dolly et al. 2006)

Similarly, low MBL levels are associated with less complement activation. This study confirms the previous observations of increased risk for SIRS/sepsis with polymorphisms associated with reduced MBL levels (Fidler, Wilson et al. 2004). The current study expanded the MBL genotyping to the full haplotype in a larger cohort.

Classification of *MBL-2* variants on the basis of the full promoter genotype can be problematic. The homozygous and heterozygous structural gene *MBL-2* polymorphisms D, B and C (denoted OO for compound heterozygotes/homozygotes and AO for heterozygotes) are invariably designated variant. However, other genotypes are also associated with low resting MBL levels, at least in healthy adults. These include the LXPA/LXPA, LYPA/LYPA and LXPA/LYPA. Median serum MBL levels associated with the LYPA/LYPA and LYPA/LYQA genotypes have been reported to be low compared with the LYQA/LYQA genotype. (Madsen, Satz et al. 1998) Subjects with the LXPA/LXPA genotype had even lower serum levels.(Madsen, Garred et al. 1995) Thus MBL deficiency is a complex defined by absolute levels, type of exon 1 variant and promoter capacity to increase levels. This study shows an increased risk for SIRS/sepsis with *MBL-2* genotypes associated with a reduced capacity to respond, either due to constitutively low MBL levels or the reduced ability to increase levels. Arguably, in the context of an infectious or inflammatory challenge, it is not merely the resting MBL levels that are important but the capacity to increase levels when needed. This may explain the high levels of MBL seen in adults (but not children) with Cystic Fibrosis and high producing *MBL-2* genotypes (Davies, Turner et al. 2004). It was postulated then that this could be

related to patients with these genotypes being able to respond to the infectious and inflammatory stimuli seen in patients with advanced disease.

The precise interplay between these polymorphic genes is unknown, let alone how this affects patients with combinations of polymorphisms in the complement cascade. A crude categorization of complement activation potential by combining of the *CFH* and *MBL-2* polymorphisms showed a direct relationship between high complement activation potential and the development of SIRS/sepsis in the early phase of critical illness. This would be consistent with the view that many cases of SIRS/sepsis result from the host failing to contain an infectious or inflammatory challenge at the earliest possible opportunity. Vigorous and early mobilisation of the complement system could help to control the infectious or inflammatory insult before initiating or propagating harmful downstream events manifested clinically as SIRS/sepsis. As such, SIRS/sepsis as a sequela of genetic polymorphism in the complement activation system may be an example of extreme sensitivity to initial conditions, a central property of complex systems such as the systemic inflammatory response (Seely and Christou 2000). Specifically, the physiological correlate for sepsis in this scenario might be that complement activity determines if an inoculum of bacteria are rapidly killed or whether some escape to enter a phase of logarithmic growth necessitating a more widespread inflammatory response.

5.11 Limitations

There are several limitations to this study.

A consequence of the open cohort study design is that data from a subset of this cohort has been analysed previously when investigating related questions (Fidler,

Wilson et al. 2004) (Stephens, Fidler et al. 2006, Plunkett, Agbeko et al. 2008) (Agbeko, Holloway et al. 2010). This limitation is compounded by the absence of an independent validation cohort. For these results to be robust the current findings need to be corroborated in an independent well-powered cohort.

The study was underpowered to assess the influence of one candidate gene in recessive analysis (*CFB)* and one possible confounder (*TNFA*). Thus it may not have been able to detect a *CFB* R32Q modifying effect due to the small number of children who were homozygous for the variant allele (Richardson, Islam et al. 2009). Also, new data on AMD shows that the *CFB* L9H polymorphism may not modify risk after all.(McKay, Silvestri et al. 2009, Richardson, Islam et al. 2009)

Thus false negative results may have occured. In contrast, it is less likely to have generated false positive results because of the observed tight confidence intervals and stringent adjustment for multiple testing.

Further, bias due to an ethnically mixed cohort and therefore different prevalence rates of polymorphisms may have influenced the study. From a purist point of view genetic association studies require an ethnically homogeneous population. This was not possible given the geographical location of the investigation (a large urban quaternary centre) Therefore ethnicity was part of the final regression analysis.

Last, SIRS as an outcome measure to assess the acute inflammatory response is an imprecise metric; clinical parameters are age dependent and sensitive to external manipulation. Other, more precise, surrogate markers would be useful, but are currently not available.

GOSH admitted 2611 children to the units in May 05-Dec 06.(PICANet, 2007) Most did not meet the inclusion criteria, some were missed or refused consent, which might elicit concern re generalizability. Currently there is no means to quantify this.

5.12 Conclusions

Despite these limitations this study shows that polymorphisms in the *CFH* gene may modulate the acute inflammatory response and corroborated the previously reported finding that *MBL-2* variant genotypes are a risk factor for the early occurrence of SIRS/sepsis in a large cohort of paediatric critical care patients independent of other potentially important functional polymorphisms in the complement and innate immunity system. A better understanding of how these polymorphisms operate at the pathophysiological level is needed before these findings can be translated to clinically useful therapeutic modalities.

In summary, this study demonstrates that genetic polymorphisms associated with reduced complement activation may be associated with early SIRS/sepsis. This is consistent with a view that appropriate complement activation occurring early following an infectious or inflammatory insult protects children from early SIRS/sepsis.

5.13 Future work

Rather than concentrate on blocking what is thought to be a harmful excessive inflammatory response the focus needs to shift to increasing early activation.

This flies in the face of current paradigms and thus requires data for the paradigm shift to happen. Corroboration for this position is found not only in the many disappointing interventional clinical trials in sepsis, but also from recent evidence that showed an inverse relationship between severity of illness and c5a levels at presentation to the emergency department with severe sepsis.(Younger, Bracho et al. 2010)

The same principle was demonstrated on a more global level in a study evaluating fever in the early stages of sepsis.(Young, Saxena et al. 2012)

Potential avenues are:

Observational cohort studies in paediatric sepsis/SIRS analysing *CFH* and *MBL2* genotype and early plasma complement activation levels.

Ex vivo models to elucidate the underlying mechanisms of CFH in paediatric sepsis.

Interventional trial to inhibit CFH or increase c5a at an early stage of injury/infection.

Interventional trial in paediatric sepsis to withhold anti-inflammatory agents vs. usual practice.

Chapter 6 Mannose binding lectin in health and critical illness

6.1 Introduction

Mannose binding lectin (MBL) is a circulating serum protein, synthesized in the liver with a key role in innate immunity.

The protein is phylogenetically old, estimated to have been around for five hundred and fifty million years, when innate immunity was developing in early invertebrates.(Flajnik 1998) It is found in mammals and birds alike. Two different MBL proteins, i.e. MBL A and MBL C, are found in many mammals, but only one type is found in humans. (Holmskov, Malhotra et al. 1994) *MBL1* is a pseudogene, assumed to have become defunct during evolution. *MBL2* encodes for the human equivalent of MBL C.

Mannose binding lectin belongs to the collectin family of proteins. This categorization is based on the underlying structure of collagenous and C-type carbohydrate recognizing domains (CRD) lectin subunits. Other collectins are Surfactant Protein A and Surfactant Protein D. All three proteins are engaged in the first line of host defence.

Collectins bind to highly conserved carbohydrate patterns on many microbes: viruses, bacteria, fungi and protozoa and prepare these organisms for phagocytosis (opsonization).(Turner and Hamvas 2000) Additionally, they activate complement pathways. The ficolins, L-ficolin, M-ficolin, H-ficolin are similar to collectins but they have different structures with a fibrinogen-like domain. Both MBL and the ficolins initiate the lectin pathway of complement activation via associated serine proteases (MASPs).(Holmskov, Thiel et al. 2003) On electron microscopy the MBL structure has a bouquet like structure, similar to complement factor c1q.(Lu 1997)

MBL is central to many innate immune function processes, including complement activation (Ohta, Okada et al. 1990), enhancing complement-independent opsophagocytosis (Tenner, Robinson et al. 1995), modulation of cytokine production (Jack, Read et al. 2001, Fraser, Bohlson et al. 2006), recognition and clearance of apoptosed or altered host cells (Stuart, Henson et al. 2006).

The liver is the main organ that synthesises MBL, although extra-hepatic derived MBL production might be relevant to local host response in the small intestine.(Seyfarth, Garred et al. 2006) MBL is configured as oligomers of two or more subunits, each of which consists of three polypeptides coiled around each other. (Holmskov, Thiel et al. 2003) Serum contains MBL multimeres up to octamers, but mostly trimers and tetramers.(Lipscombe, Sumiya et al. 1995)

MBL serum levels in humans are modulated by genetic factors (Madsen, Garred et al. 1995) and inflammatory state (Ezekowitz, Day et al. 1988). Upstream from exon 1 there are two transcription start sites, both of which have IL6 and glucocorticoid responsive elements.(Heitzeneder, Seidel et al. 2012)

There exist polymorphisms in exon 1 that change the collagen like structure of MBL such that they preclude higher order oligomerisation (polymorphisms B and C) or change the number of cysteines in the collagen like structure, such that oligomerisation is not needed to attain a stable molecule (polymorphism D). (Jensen, Weilguny et al. 2005) This reduced oligomerisation, in turn, reduces serum MBL levels. (Lipscombe, Sumiya et al. 1995) Individuals homozygous for MBL structural defects have near absent MBL serum levels.(Lipscombe, Sumiya et al. 1992)Heterozygosity is characterized by protein levels which, on average, are approximately 1/8 normal. This may be explained by the fact that heterozygous

individuals have only a 1 in 8 probability of assembling three normal peptide chains during MBL biosynthesis.(Turner 1998)

In addition to polymorphisms that lead to structural defects, promoter polymorphisms modulate the capacity to increase serum levels on provocation.(Madsen, Garred et al. 1995)

However, these polymorphisms do not explain all variation in MBL levels.

MBL deficiency is common, dependent on the population studied, MBL deficiency occurs in 20% to 80% of the population.(Garred, Larsen et al. 2006) The clinical importance of MBL deficiency has been established in young children who show an increased susceptibility to infections.(Super, Thiel et al. 1989) Other associations remain more disputed, but many report that vulnerable populations, such as infants or immuno-compromised children and adults are at greater risk of developing infections.(Eisen and Minchinton 2003)

Conversely, one might ask the question: "why does such a high prevalence of MBL deficiency exist?" Two not necessarily mutually exclusive propositions are i) a protective effect on collateral damage from complement activation to the host in inflammatory processes(Lipscombe, Sumiya et al. 1992) and ii) a protective effect against parasites that infect the host by means of C3 mediated opsonisation and intracellular uptake (e.g. Leishmania and mycobacteria) (Garred, Harboe et al. 1994).

6.2 Modulation of serum MBL levels

6.2.1 MBL2 genotype

The relationship between serum MBL levels and *MBL-2* genotype is complex and depends in part on exon 1 polymorphisms and promoter polymorphisms.

6.2.1.1 Structure of *MBL2*

The *MBL2* gene structure is given in **Figure 1-9**.

MBL2 is located on the long arm of chromosome 10 (10q11.2-q21). (Sastry, Herman et al. 1989) The protein encoding area is made up of four exons. (Taylor, Brickell et al. 1989) Exon 1 encodes the signal peptide, a cysteine rich domain, as well as part of the glycine-rich collagenous region (seven Gly-Xaa-Yaa repeats). Exon 2 encodes the rest of the glycine-rich collagenous region (twelve Gly-Xaa-Yaa repeats), which conforms to a triple helix structure. Exon 3 encodes the so-called neck region, and exon 4 encodes the carbohydrate-binding domain, that configures into a globular arrangement. (Sastry, Herman et al. 1989, Taylor, Brickell et al. 1989)

The carbohydrate-binding domain is the pattern recognition area of the protein. It recognizes microbial as well as endogenous ligands. Upstream from the protein encoding exons lies the promoter part of the gene, which affects transcription of the protein.(Madsen, Garred et al. 1995)

6.2.1.2 Polymorphisms in the *MBL2* gene and promoter region

The first identified polymorphism in exon 1 that underlies MBL deficiency is located at codon 54. The wildtype nucleotide GGC sequence is changed to GAC, which then results in a change in translation from Glycine to Aspartic acid.(Sumiya, Super et al. 1991) This is the most prevalent defect in people of European and Asian descent and is denoted “B”, (rs1800450).

The next polymorphism to be identified was the Glycine to Glutamic acid change at codon 57 because of a nucleotide change GGA to GAA. This defect is most prominent in populations of Sub-Saharan African descent and is denoted “C” (rs1800451).

Finally, a change in nucleotide at codon 52, changing the sequence from CGT to TGT results in an amino acid change Arginine to Cysteine.(Madsen, Garred et al. 1994) This variant is named “D” (rs5030737). Collectively the variant types are denoted “O” in differentiation to wildtype “A”.

The functional change in variants B and C are thought to be due to impaired oligomerisation. The B variant causes a disruption of the fifth Gly-Xaa-Yaa repeat in the collagen like domain, which may prevent formation of the triple helix (Sumiya, Super et al. 1991) and stable higher order oligomers (Garred, Larsen et al. 2003). The variant lower order oligomers show reduced binding capacity (Garred, Larsen et al. 2003) and impaired complement activation (Super, Gillies et al. 1992). The functional effect of the C variant is analogous to that of the B variant. (Lipscombe, Sumiya et al. 1992)

The D variant is somewhat different in that plasma levels are found to be higher than B and C deficient genotypes. This is mainly thought to be due to D variant protein to form stable, if dysfunctional, higher oligomers with wildtype protein.(Garred, Larsen et al. 2003)

In addition to these structural variants, there are three promoter variants that independently influence circulating MBL levels. They are located at positions -550 (H/L) with a G to C nucleotide substitution (rs11003125); -221 (X/Y) with a C to G nucleotide substitution, (rs7096206) and at +4 from the start of transcription the (P/Q) C to T nucleotide substitution, (rs7095891). The X/Y polymorphism exerts the greatest influence on plasma MBL levels.

Due to strong linkage disequilibrium only eight common haplotypes are found in the population. These haplotypes are associated with varying amount of circulating

MBL.(Madsen, Garred et al. 1995) Concentrations of MBL vary 1000 fold between the lowest deficient O/O and highest HYPA/HYPA genotypes.

Although these genetic variants explain much of MBL serum levels, they do not explain all. Notably, within wildtype genotype groups there still are log differences in MBL serum level.(Madsen, Garred et al. 1995)

6.2.2 Non genetic modulation of MBL serum levels

In addition to genetic effects, age (Thiel, Bjerke et al. 1995), inflammatory state (Ezekowitz, Day et al. 1988, Thiel, Holmskov et al. 1992) and hormonal regulation (Sorensen, Hansen et al. 2006) are known to influence MBL serum level.

6.2.2.1 MBL may be an acute phase protein

The capacity of the host to respond to MBL associated ligands is thus determined by the structure of the molecule but also by the capacity of the liver to increase levels in response to gene activation. The promoter polymorphisms (H/L, P/Q and X/Y) may thus be of additional importance in the host response to inflammatory threat, over and above constituent MBL serum levels as defined by the structural genotypes A, B, C or D.

The literature gives conflicting results regarding the extent to which MBL functions as an acute phase protein. Initial studies showed elements in the transcription region that classically would be regarded as acute phase reactants, such as IL6 and glucocorticoid responsive regions. (Ezekowitz, Day et al. 1988) Later studies in critically ill adults are less convincing. In a cohort of adults undergoing major abdominal surgery there was a robust early response in IL6 and CRP, but not in MBL. MBL levels actually reduced significantly, albeit modestly (17%) in the first 48 hours,

and then increased back to baseline between day 8 and 30 postoperatively (Ytting, Christensen et al. 2006) Patients undergoing major orthopaedic surgery did show a 1.5-3 fold increase in MBL serum levels between day 1 and 3 postoperatively.(Thiel, Holmskov et al. 1992) This contrasts with another preoperative study in adults undergoing oesophageal adenocarcinoma resection. This study showed that MBL rose 4 fold compared to baseline between day 5 and day 10 postoperatively.(Van Till, Boermeester et al. 2006)

In a more recent description of MBL levels in acute pancreatitis, a disease that induces severe SIRS, the authors showed only a slight increase in MBL serum levels by day 2 but no association with severity of illness or multi organ dysfunction. (Novovic, Andersen et al. 2011)

The data on the acute phase response to severe infection are similar. For instance, in a Spanish cohort of adults with community acquired pneumococcal pneumonia there was no difference in MBL levels in the acute phase (within 48 hours of admission) compared to recovery (at least 4 weeks after resolution). (Perez-Castellano, Penaranda et al. 2006)

These studies adjusted MBL levels for the structural but not the promoter polymorphisms. This may underestimate the true variability in MBL concentration in terms of both baseline and capacity to change MBL concentration.

The two studies that did take into account promoter polymorphisms actually did show a modified acute phase response of MBL in exon 1 wildtype genotypes. These were the X/Y polymorphism in adult community acquired pneumonia (Herpers, Endeman et al. 2009) and the HY polymorphisms in febrile neutropenia in children (Frakking, van de Wetering et al. 2006). (Herpers, Endeman et al. 2009) defined acute phase response as a change of 25% in serum value on day 1 of illness. Interestingly, only the

haplotype YA/YA showed a significant correlation with CRP levels, suggesting this to be the most responsive MBL haplotype in the early phase of acute pneumonia.

6.2.2.2 MBL serum levels differ in age groups

At birth, MBL serum levels are on average 37% of MBL serum levels at 3 months of age, which reflects that of the adult population.(Thiel, Bjerke et al. 1995) These lower levels of MBL in newborns were replicated in several larger studies (Kilpatrick, Liston et al. 1996), including one cohort of 1800 newborns (Swierzko, Szala et al. 2009). Several studies report even further reduced MBL serum levels in premature infants in comparison to term babies(Lau, Chan et al. 1995, Sallenbach, Thiel et al. 2011) although this is not universal (Swierzko, Szala et al. 2009). These lower levels may contribute to some of the vulnerability to infections in this age group. MBL deficiency as defined by means of MBL levels has been shown to be a risk factor for sepsis in newborns, but *MBL2* polymorphisms were not found to be a risk factor in a recent systematic analysis.(Israels, Frakking et al. 2010)

6.2.2.3 Hormonal regulation of MBL levels

Hepatocytes exposed to thyroid hormone and growth hormone increased MBL secretion at least three fold. Thyroid hormone was more potent than growth hormone. Interestingly, IL6 caused very little increase in MBL production.(Sorensen, Hansen et al. 2006)In critically ill adults MBL serum levels were weakly correlated with serum T3 levels but not with outcome.(Koenig, Potlukova et al. 2012)

6.2.2.4 Measurement of serum MBL

Last, the method by which MBL serum levels are measured is of importance. The amount of measured deficient MBL protein varies dependent on method. However, when applying a cut off of 500 ng/ml for MBL deficiency, these differences are of no consequence. (Frederiksen, Thiel et al. 2006)

6.3 MBL2 genotype, MBL serum levels and systemic inflammation

There are few data on the genetic and serum level interplay in paediatric sepsis/SIRS. Most studies on susceptibility to, or severity of, inflammatory disease have focussed on either MBL levels or *MBL2* genotype rather than both.

MBL levels and *MBL2* polymorphisms in paediatric sepsis and SIRS are not interchangeable. Children with *MBL2* polymorphisms were at higher risk of developing systemic inflammation in the presence or absence of infection. However, these children did not consistently show MBL deficiency as defined by MBL serum levels. (Fidler, Wilson et al. 2004) The study was limited to the structural gene and XY promoter polymorphism, thus precluding more detailed analysis on the association of promoter polymorphisms, the acute phase response and MBL levels. Thus, it is unknown whether the nuances in full haplotype are of relevance in assessing the host capacity to respond, or whether it suffices to assess the partial genotype based on exon 1 polymorphisms and the (X/Y) promoter polymorphism.

6.4 Role of MBL in paediatric critical illness

The Systemic Inflammatory Response Syndrome (SIRS) is integral to much of paediatric critical care related morbidity and mortality. (Tantalean, Leon et al. 2003, Proulx, Joyal et al. 2009) Host factors have been implicated in the variability of individual responses to insults of similar severity. *MBL2* genotype and MBL serum levels may contribute to this variability. *MBL2* deficient genotypes in adults predisposed to severe sepsis and septic shock and low serum MBL levels were associated with worse outcome.(Gordon, Waheed et al. 2006) This chapter examines the contribution of MBL variability to the host response after a severe insult and the susceptibility of children to be admitted to PICU with severe infection. MBL serum levels in critically ill children may be determined by genetically defined factors and by the capacity to counteract presumed consumption.

The three exonal and three promoter polymorphisms as well as serum MBL levels were determined in a cohort of healthy children and a cohort of children admitted to PICU.

6.5 Aims

1. To establish if full genotyping for *MBL2* is superior to partial genotyping in predicting low serum MBL levels in children admitted to the paediatric intensive care unit.

2. To determine if MBL levels in the acute phase response are higher than in health.

3. To ascertain if MBL genotypes associated with MBL deficiency predispose to PICU admission with infection.

4. To establish if MBL serum levels are higher in children admitted to PICU without SIRS than those developing SIRS.

6.6 Methods

6.6.1 Summary of sample handling in the PICU cohort

Serum levels were taken within a 48-hour time frame from admission, immediately spun and frozen -80°C until analysis. Samples 1-147 had been freeze-thawed twice before the current analysis. All other samples had not been freeze-thawed.
Two samples were taken as control for validation of high and low levels as well as process consistency. All measurements were performed in duplicate.

6.6.2 MBL ELISA process

MBL levels in subject sera were determined by a symmetrical sandwich ELISA kit (Bioporto Diagnostics, Denmark) according to the manufacturer's instructions. The MBL ELISA works according to the following principle: A monoclonal antibody against the carbohydrate recognition domain of MBL is coated on microwell plates. Serum is added to the microwell. Biotin labelled antibody detects bound MBL, incubation with added horseradish peroxidase (HRP) conjugated to streptavidin induces a colour change. Serum was defrosted and diluted 1:10, 1:100 or 1:200 dependent on the expected value from the known genotype. Samples were immediately processed and read within 5 minutes after the stop solution had been

added. This kit was chosen because of its widespread use in clinical cohort studies, thus allowing for reliable inter research group comparisons of MBL levels.

6.6.3 ELISA validation

The kit supplies MBL standards for each ELISA plate. A calibration curve was fitted by plotting the mean of the duplicate optical densities (OD) values for each MBL standard on the y axis and the corresponding MBL concentrations in ng/ml on the x axis. All samples were assayed in duplicate.

The MBL concentration in the specimen was then calculated by multiplying the obtained mean OD by the dilution factor and referencing to the calibration curve.

Results were taken to be valid if all of the following occurred:

1. a calibration curve within the specifications of the manufacturer
2. coefficient of variation between duplicate samples of <10% (standard deviation of the replicate response X 100/mean of the response)
3. adequate interplate variation, i.e. if the high, medium and low standard samples were within 10% of their expected value

6.6.4 MBL genotypes and serum levels in healthy control subjects

MBL full genotypes and serum concentrations were available from a large cohort of healthy British Caucasian children previously studied. (Johnson 2007)

This cohort of children was recruited from the Avon Longitudinal Study of Parents and Children (ALSPAC).

Serum MBL levels in the PICU cohort had been analysed for n=137 patients in 2006 (Fidler 2007). The same ELISA method as described above was used in both historical cohorts.

6.7 Results

6.7.1 General Results

A total of 531 full genotype and MBL levels were available from the ALSPAC cohort. The original ALSPAC cohort was 661 samples. The assay failed in n=3 and genotype was not fully measured in 127 [personal communication M Johnson]. Genotype distribution was in Hardy Weinberg Equilibrium and in keeping with previously described European cohorts (Madsen, Garred et al. 1995) This cohort had, in part, been genotyped for *MBL2* before.(Mead, Jack et al. 1997)

The total PICU cohort comprised of 299 patients. Serum samples were not available for 24, samples were not attempted in 32, the assay failed in 28, and full genotype data was not available for 6. Thus in the PICU cohort 233 samples were available for analysis with full genotype and MBL levels.

The two cohorts did not differ in proportion of deficient genotypes 129/294 vs. 235/531 (χ^2, $p = 0.9$).

The two control samples were comparable in measured level at the two dates (2006 and 2009) and both were representative for a low and high level (Table 6-1).

Sample	Value 2006	Value 1 2009	Value 2 2009	Mean 2009
High	3176	3255	2689	2972
Low	17	34	34	34

Table 6-1 MBL control samples

6.7.2 MBL serum levels are defined by both gene and promoter genotype in the acute phase response

MBL serum levels for the PICU cohort are shown in Figure 6-1. AA denotes wildtype, AO denotes heterozygote deficient (either B, C or D) and OO denotes homozygous or compound heterozygous deficient genotypes. There is an additive, significant difference between structural gene polymorphisms AA/AO/OO, (p <.0001, Kruskall-Wallis).

The AA genotype group was further analysed by adding in the XY promoter polymorphism. When adding in the main promoter polymorphism X/Y to the homozygous AA group there is again a differentiation in MBL serum levels for YAYA , XAYA and XAXA genotypes (p <.0001, Kruskall-Wallis) (Figure 6-2).

The last step was to fully classify *MBL2* genotype YAYA with all three promoter polymorphisms (Figure 6-3). This does not differentiate any further (p =.38, Kruskall-Wallis) except maybe for the homozygous LYPA/LYPA genotype that may be expected to have intermediate values. However, there are only two patients with this genotype, thus warranting cautious interpretation.

It would have been of interest to be able to attribute some of the variability of MBL serum levels to timing between the insult and blood sampling, given that there was a potential for this to be between 0 and 48 hours. Unfortunately this information was not available.

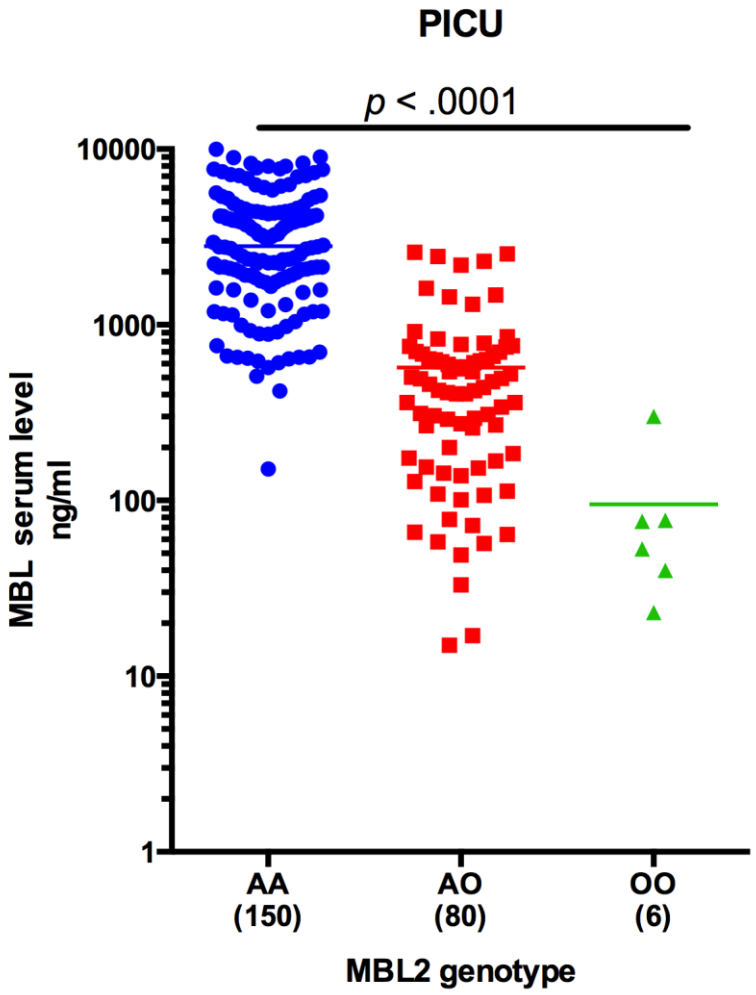


Figure 6-1 MBL serum level split in AO genotype only in the PICU cohort (n=236), Kruskal-Wallis test.

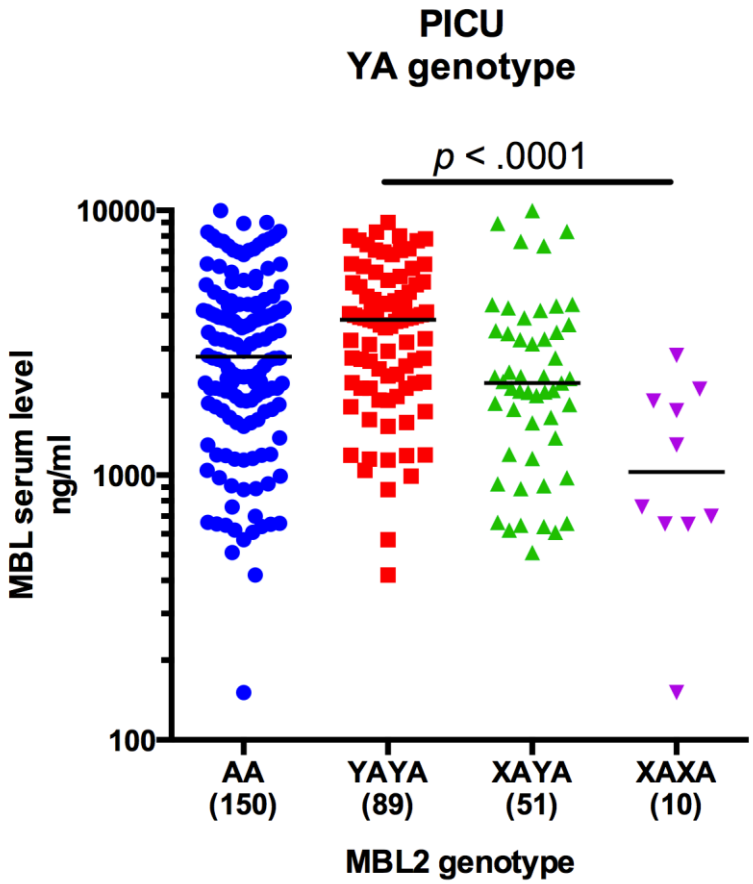


Figure 6-2 PICU cohort MBL serum levels divided according to AO and promoter XY

P value derived from Kruskal-Wallis test.

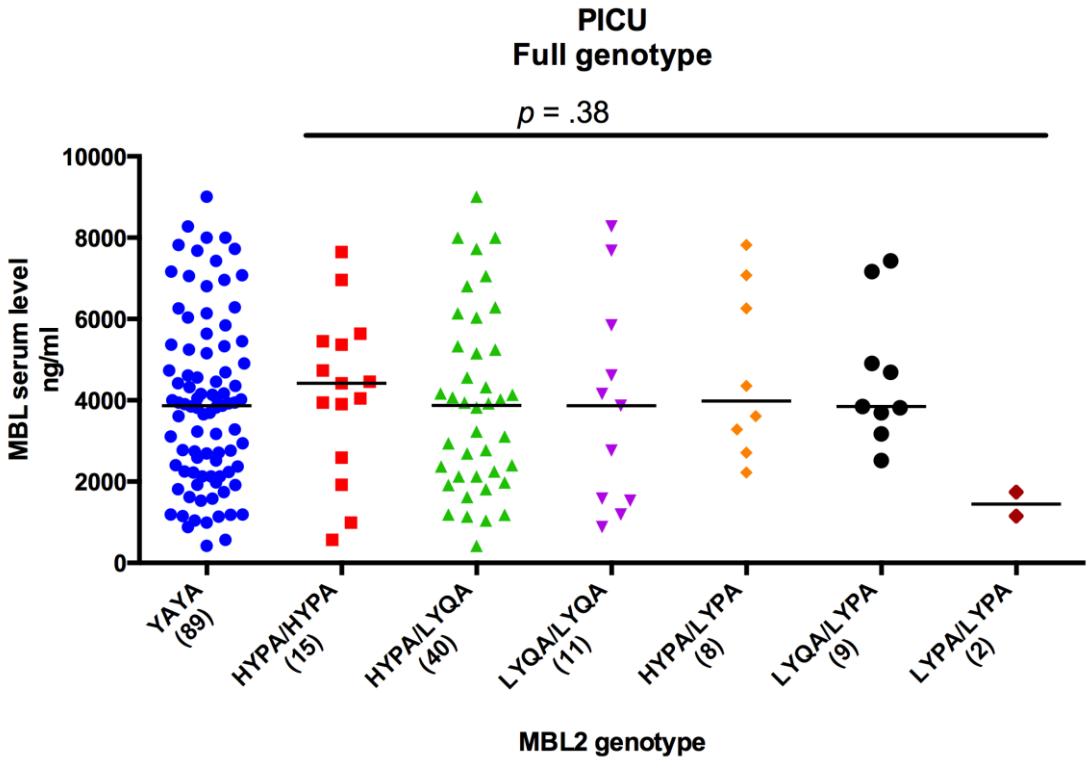


Figure 6-3 Full sub division of YAYA genotype by promoter polymorphism

The influence of the X/Y polymorphism was also observed in the heterozygote population, with the XAO sub group showing half of the YAO subgroup serum levels (Figure 6-4) (Mann Whitney, p =.007).

Further characterisation of the YAO genotypes revealed that the HYPD genotypes had significantly higher MBL serum levels than other genotypes (p=.008, Kruskal-Wallis (Figure 6-5). This observation is in keeping with previous studies.

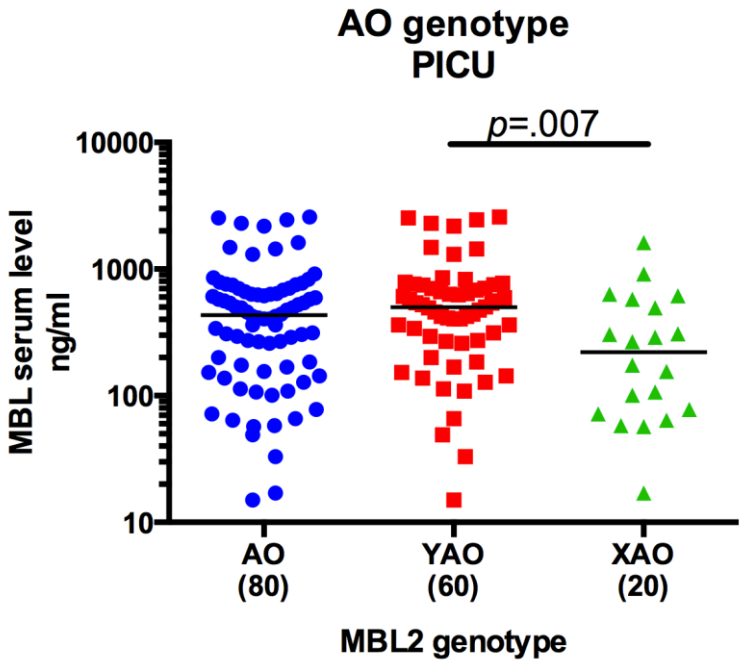


Figure 6-4 MBL serum levels for AO heterozygotes divided according to XY polymorphism

P value derived from Kruskall-Wallis

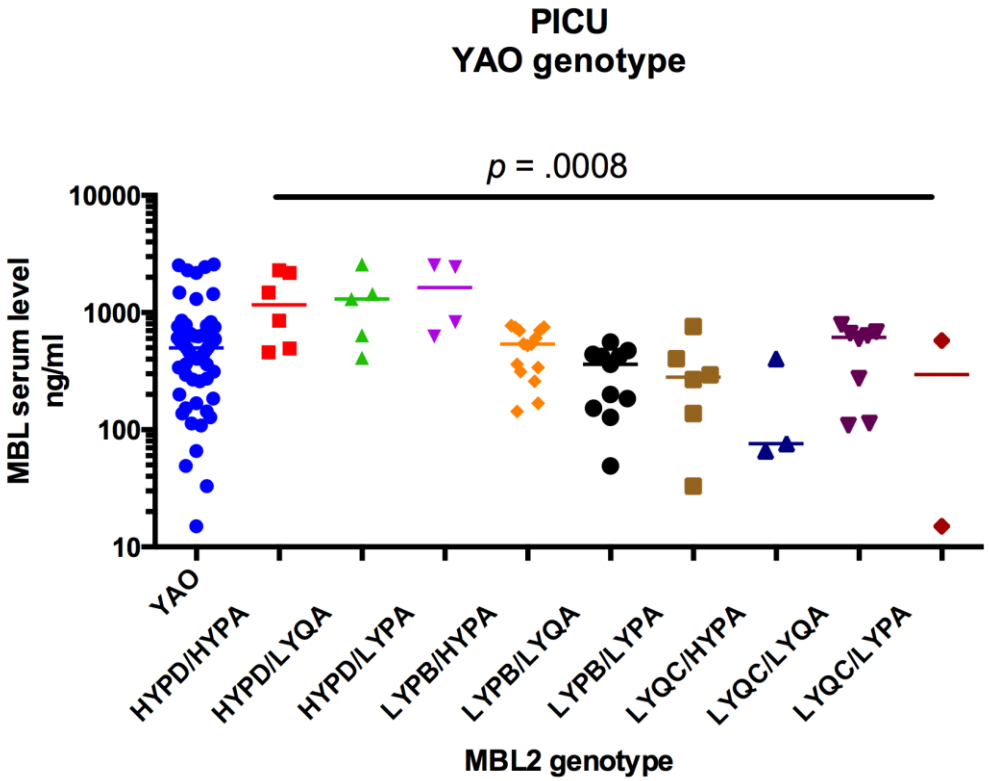


Figure 6-5 MBL serum levels in YAO genotypes subdivided according to L/H and P/Q polymorphisms

P Value derived from Kruskall-Wallis

6.7.3 MBL serum levels in the acute phase response compared with health in children

The median MBL serum level in the PICU cohort was 1753 ng/ml (IQR 3152) and 3155 ng/ml (IQR 3213) in the ALSPAC cohort. Levels in the PICU cohort were approximately half that of levels in healthy children. This difference was statistically significant ($p < .0001$, Mann-Whitney test). Levels stratified according to genotypes are shown in Table 6-2.

The intermediate genotypes XAYA, XAXA and YAO show the most difference in MBL serum levels between the PICU and ALSPAC cohorts.

Genotype	PICU	ALSPAC	P value	Absolute difference	Percentage difference
All	1753	3155	<.0001	-1402	-44
YAYA	3866	4197	0.02	-331	-7.9
XAYA	2230	3653	<.0001	-1423	-39
XAXA	1030	2589	.001	-1559	-60
YAO	498	935	<.0001	-437	-47
XAO	220	295	NS	-75	-25
OO	64	36	.03	+28	+56

Table 6-2 MBL median values by genotype for PICU and ALSPAC cohorts

P values derived from Mann-Whitney test

6.7.4 Do MBL genotypes associated with MBL deficiency predispose to a PICU admission with infection?

The occurrence of MBL deficient genotypes was not higher in the group that was admitted with infection compared with the cohort of healthy children 69/154 (44%) vs. 235/531 (44%) (χ^2, $p = 0.9$). However, overall, MBL serum levels were significantly lower in those children admitted to PICU with infection than healthy children (median 1384 vs. 3155, $p < .0001$ Mann Whitney test) Figure 6-6.

Of note, serum MBL levels were lower in the whole PICU group vs. healthy children (Table 6-2).

This suggests that MBL deficiency is not a risk factor for severe infections in children, but MBL consumption might be higher in the infection group.

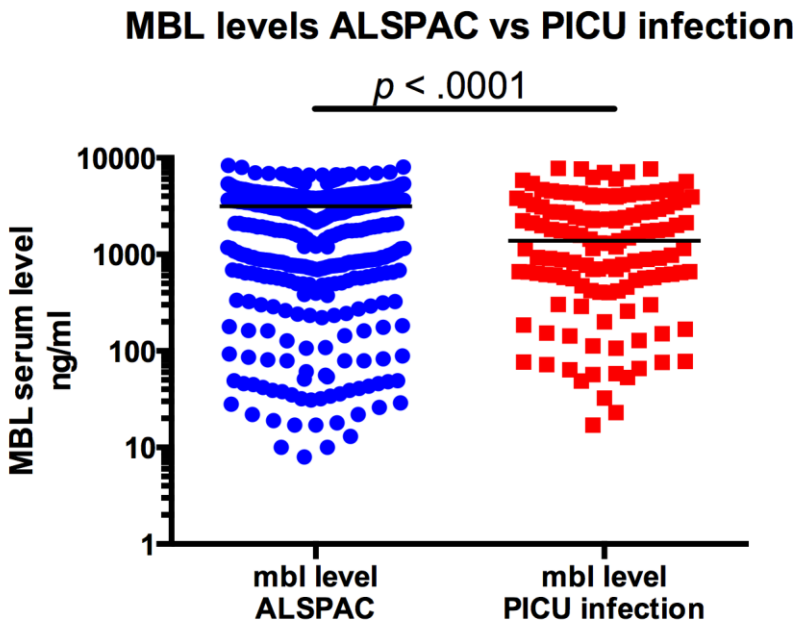


Figure 6-6 MBL levels ALSPAC vs. PICU infection

P value derived from Mann-Whitney test

Children admitted to PICU with non infectious diagnoses had lower MBL levels than healthy children, and children admitted to PICU with infection had even lower MBL levels (p <.0001, Kruskall Wallis test), Figure 6-7.

The difference in MBL levels admitted to PICU with infection vs. non-infection showed a trend to lower MBL levels in the infection group (p = .09, Mann Whitney), Figure 6-8.

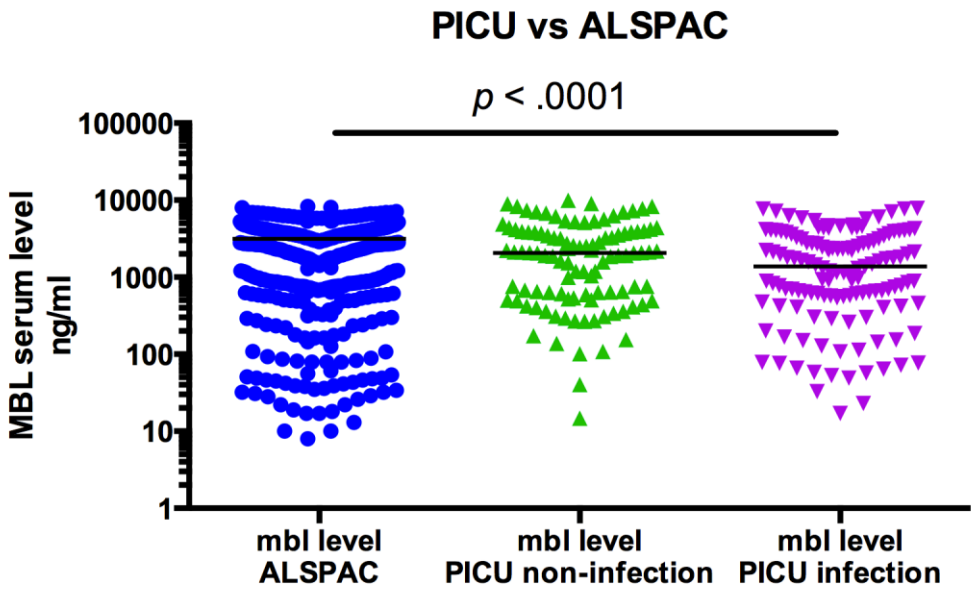


Figure 6-7 MBL levels in health, infection and sterile insults

Median MBL levels in ng/ml: ALSPAC 3155 (IQR 885, 4098), PICU non-infection 2069 (IQR 554, 3896), PICU infection 1384 (IQR 551, 3208).

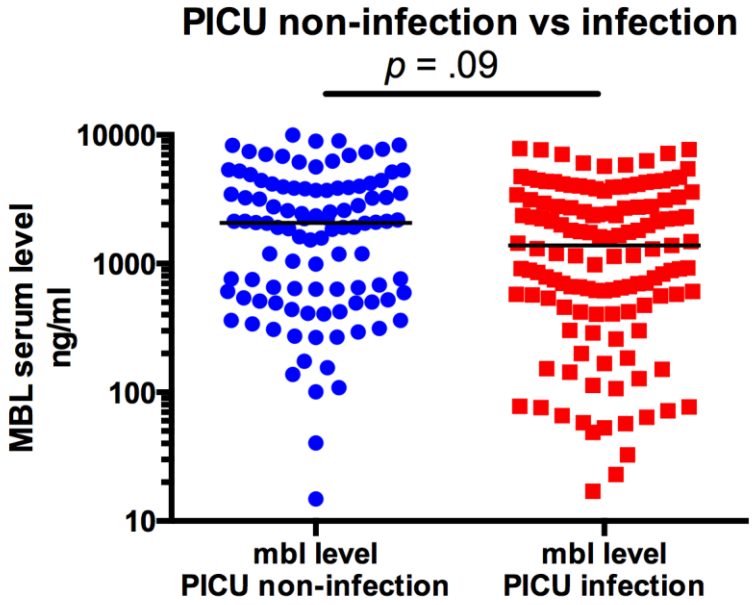


Figure 6-8 MBL levels in infection and non-infection

P value derived from Mann-Whitney test

6.8 MBL serum levels are higher in children admitted to PICU without SIRS than those developing SIRS

The PICU cohort did show lower MBL serum levels for those children who developed SIRS compared to those who did not (median 1480 vs. 2230 ng/ml, $p <$ .05, Mann Whitney, Figure 6-9).

This is mirrored by the increased risk that MBL defient genotypes pose to developing early SIRS in PICU. (Table 6-3)

MBL deficient genotypes predispose to SIRS and those children who developed SIRS had lower MBL serum levels.

Within the infection group there was a trend towards MBL levels being higher in the group that did not develop SIRS vs. those who did develop SIRS (sepsis) (Figure 6-10).

Children with sepsis had lower serum levels of MBL (median 1250 ng/ml) when compared with children with sterile SIRS (2058 ng/ml), but this did not reach significance (Figure 6-11).

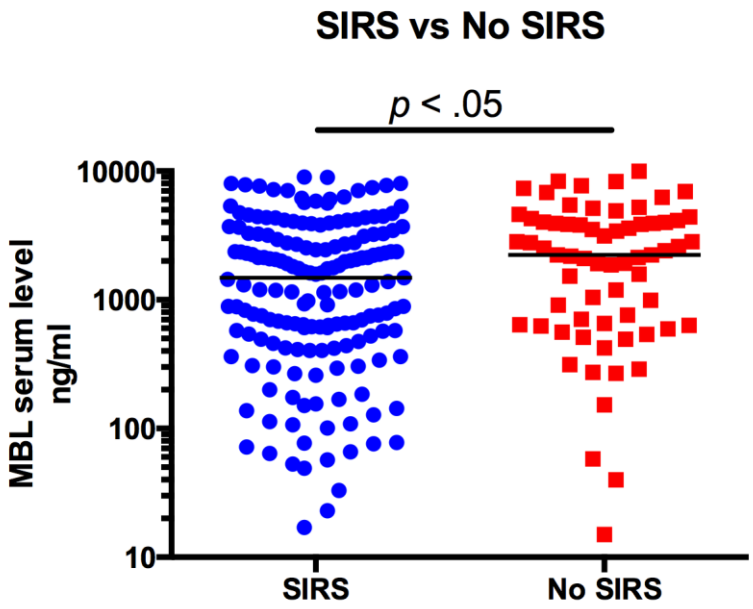


Figure 6-9 MBL levels SIRS vs. no SIRS

P value derived from Mann-Whitney test

Risk factor	Univariate analysis	Binary logistic regression analysis[a]
CFH CC variant	0.4 (0.2-0.8) P=0.006	0.3 (0.1 - 0.7) P= 0.005
MBL-2 variant	2.0 (1.1-3.4) P=0.016	2.5 (1.3 - 5.0) P= 0.008
Infection[b]		8.3 (3.0 - 24) P< 0.0005

Table 6-3 in the PICU cohort Risk factors for early SIRS/sepsis

[a]Adjusted for sex, age (logged), ethnicity, risk of mortality (PIM), primary diagnosis (elective postoperative, infection, trauma, other), MBL-2, CFH, C1qA, CFB, IL6, IL10, TNFα and PAI-1 polymorphisms. [b]Versus elective postoperative. Results are shown as Odds Ratios with 95% confidence interval and P value. Only the covariates with a P value < 0.01 in the final analysis are shown. (cohort n=299)

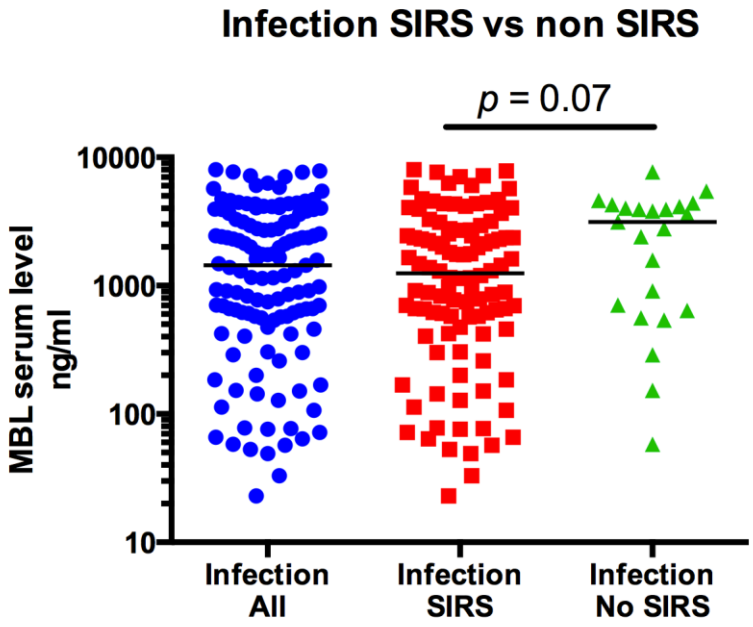


Figure 6-10 MBL levels in the infection group SIRS vs. non SIRS

P value derived from Mann Whitney test

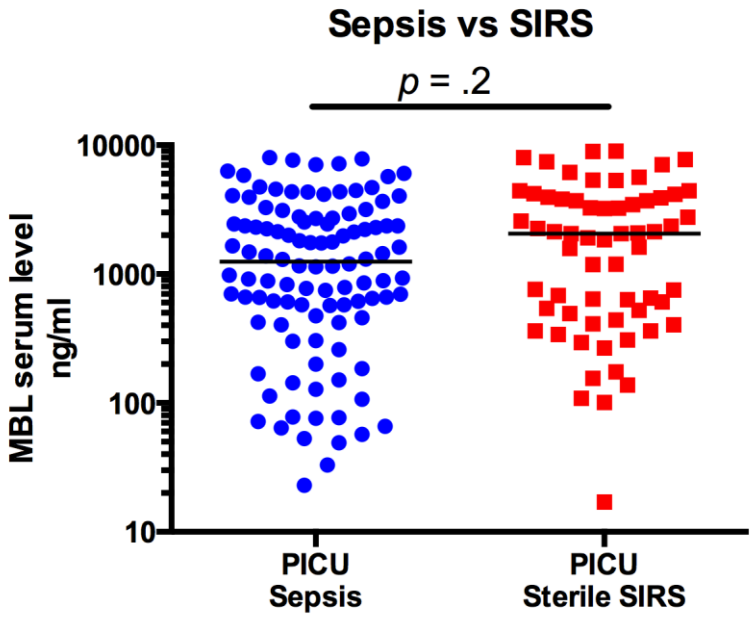


Figure 6-11 MBL levels sepsis vs. SIRS

P value derived from Mann Whitney test

In univariate analyses the association with MBL serum levels depends on *MBL2* genotype, PICU admission, and development of SIRS. In a linear regression model incorporating clinical relevant parameters, e.g. age, sex, severity of illness (PIM2 score), development of SIRS, and admission diagnosis with *MBL2* genotype showed that only *MBL2* genotype (standardized coefficient β -0.6; p <0.0001) and infection (standardized coefficient β -0.15; p =0.015) were independently associated with MBL serum level.

6.9 Summary of results

This study showed that:

Extending genotyping beyond the exonal and XY polymorphisms did not yield any further discriminatory capacity in terms of MBL serum levels in critically ill children.

MBL2 genotype distribution in a general PICU cohort was comparable to a cohort of healthy children.

MBL serum levels were lower in critical illness than in the healthy population.

Children with intermediate *MBL2* genotypes showed the greatest difference in serum MBL levels in critical illness when compared with healthy children.

MBL2 deficient genotypes predisposed to SIRS in critically ill children.

Children admitted to PICU with infection had a similar genotype distribution compared to healthy children.

MBL serum levels in children who developed early SIRS were lower than those who did not.

MBL serum levels were lowest in children admitted to PICU with infection, compared to children admitted to PICU for other reasons and highest in healthy children.

Children with sepsis had lower MBL serum levels than children with localized infection.

Children with sepsis showed similar MBL serum levels compared to children with sterile SIRS.

In multivariate analysis MBL serum levels were independently associated with infection and *MBL2* genotype.

6.10 Discussion

The results of this study suggest that MBL is integral to the acute phase response in children. Lower serum MBL levels were each associated with critical illness and SIRS. Less clear is how MBL levels and *MBL2* genotype each define predisposition and outcome.

One way to interpret the current results is that MBL serum levels are a resultant of constitutive, genetically defined factors compounded by presumed consumption. Insults large enough to warrant PICU admission would induce MBL consumption, regardless of baseline levels. Those genotypes, however, least capable of replenishing MBL back to baseline would show the highest difference when compared to health. Both constituently low as well as an inability to replenish MBL genotypes produce MBL levels that predispose to SIRS because the host is incapable to quickly contain the insult.

It may be that containing infection requires more MBL than does scavenging endogenous debris resulting from a sterile insult. This might explain why infection induces lower MBL levels than do sterile insults.

Alternatively, in this cohort, the time period between the onset of sterile insult to PICU admission and the onset of infection to PICU admission may have been

sufficiently different to explain the MBL serum level difference in terms of duration of time for MBL to be consumed, rather than type or severity of insult.

To be able to better understand MBL levels in the context of genotype and insult, further studies would require consecutive serum levels from admission to hospital until discharge and convalescence. Both infectious as well as sterile insults would need to be included, as well as timing of onset of illness.

One explanation for the differences between the two cohorts in the intermediate genotypes is different laboratory processing. However, given that three other genotype groups do not show this difference, that is not likely. Another explanation is that these intermediate genotypes are less able to maintain MBL serum levels in critical illness.

MBL is consumed in the acute phase response and this consumption may be seen more clearly in those genotypes that have intermediate levels to start with. At the very high end of the spectrum (YAYA) there is a surplus of MBL combined with a high capacity to replenish MBL, serum levels do not drop as much.

At the lower end of the spectrum (XAO and OO) levels are so low that MBL consumption in critical illness does not make much of a difference.

To more realibly answer this question serum MBL levels in the PICU cohort would need to be measured while they were well.

The current study fits within a “capacity to respond” hypothesis. The host prevents downstream effects to insults such as SIRS by being capable of inducing a robust, vigorous first response.

6.11 Limitations

The comparison of the ALSPAC cohort with the PICU cohort poses the following methodological issues:

The ALSPAC cohort is of Caucasian descent only, while the PICU cohort is ethnically mixed.

Although the same ELISA kit was used, there may be systematic differences due to different time points of sample processing.

Freeze thawing twice may have induced flaws in measurement of PICU MBL serum levels, although the two control samples do not suggest this.

Given the many potential modifiers of serum MBL levels this study falls short in evaluating potential influencers such as hormonal influences. Thyroid hormone levels do vary in critical illness, and is known as sick euthyroid syndrome.

6.12 Conclusions

MBL2 genotyping does not render more information with regards to MBL serum level when all promoter and structural polymorphisms are identified over and above structural polymorphisms and the XY promoter polymorphism.

MBL serum levels in SIRS or sepsis were lower as compared with critically ill children without systemic inflammation.

The children admitted with infection did not have a surplus of MBL deficient genotypes as compared with healthy children. This suggests that MBL deficient genotypes do not predispose to severe infection.

MBL levels are most reduced in the acute phase response in those genotypes that have intermediate serum levels, which may reflect a consumption of MBL in critical illness and an inability to maintain pre-insult MBL serum levels.

Chapter 7 Defining the Systemic Inflammatory Response Syndrome (SIRS) by means of physiological signal analysis

7.1 Introduction

Inflammation due to severe injury and infection is a complex physiological response that ultimately leads to healing and resolution. The synthesis and interplay between the inflammatory cascade, neuro-endocrine and autonomic nervous systems defines the course of systemic illness.(Sharshar, Hopkinson et al. 2005) A suboptimal or detrimental trajectory may occur at any stage in the time-period between initiation and resolution. Ultimately, the most detrimental outcome is terminal organ failure and death.

The previous chapters have focussed on how differences in initial conditions, such as polymorphisms in genotype may influence outcome in acute inflammatory processes. The occurrence of SIRS, regardless of initiating insult, is in part dependent on genetic variability in the innate immune system. (Agbeko, Fidler et al. 2010) However, these techniques are expensive, time consuming, and not point of care. At best they might identify those patients at higher risk for SIRS or sepsis, but not whether they are actually on the trajectory for developing systemic inflammation.

The current clinical definition of the systemic inflammatory response syndrome is a very unrefined reflection of the complex physiological response to injury and infection. This chapter examines whether SIRS can be characterized more specifically in terms of initiating insult.

The definition of SIRS is based on respiratory rate, heart rate, temperature and white cell count, regardless of aetiology. (1992) It is a convenient, but crude score that does not do justice to the complex underlying interactions in immune response, autonomic function and neuro-endocrine activation (Vincent 1997).

Increasingly, there is an understanding that infectious SIRS (i.e. sepsis) and sterile SIRS do not share the same clinical trajectory or molecular pathophysiology.

A large adult cohort multi-centre observational study (n= 35430) showed that although mortality did not differ between severe sepsis and sterile SIRS patients, there were significant differences in aetiology of death (mortality in adults with sepsis was more often due to severe organ dysfunction compared to the more prevalent neurological aetiology in sterile SIRS), length of ICU stay and time to peak organ dysfunction. (Dulhunty, Lipman et al. 2008) Thus, although the host response to a severe insult or infection may look the same (abnormal heart rate, respiratory status, temperature and white cell count) and end the same (death) there may be patho-physiological differences between SIRS and sepsis. These differences may then lead to different required interventions in preventing adverse outcome.

7.2 Diagnosing and differentiating SIRS and sepsis

Attempts to define SIRS more specifically include the use of inflammatory biomarkers. Acute response proteins CRP and procalcitonin were elevated and correlated with severity of organ dysfunction in both states, but levels were higher in sepsis.(Castelli, Pognani et al. 2004) A more detailed probe into the molecular basis for SIRS by means of gene array analysis showed communal but also differentiating patterns after three different types of insult (burn injury, trauma/haemorrhage and intraperitoneal LPS) leading to systemic inflammation in a mouse model. Out of 1461

probes that were differentially up- or down regulated in blood compared with sham mice, only 13 were shared between the 3 models.(Brownstein, Logvinenko et al. 2006) Similarly, gene expression profiling and liquid chromatography show that an immunological distinction can be made between sterile and infectious SIRS: up to 48 hours before developing sepsis, patients with SIRS showed differential regulation in inflammatory pathways related to innate immunity (including TLR4, TLR2, MD2), cytokine receptors, T-cell differentiation and protein synthesis)(Johnson, Lissauer et al. 2007).

Plasma levels of complement and coagulation activation factors were significantly differentiated in sepsis and sterile SIRS patients (Lissauer, Johnson et al. 2007).

A prospective multicentre observational study showed that a panel of 42 gene expression markers in the dynamics of immune function could differentiate sepsis from sterile SIRS and normal controls. This study required RNA analysis platforms and sophisticated bioinformatics and statistical analysis.(Sutherland, Thomas et al. 2011)

Uncovering the molecular fingerprint of sepsis and SIRS is of interest, but also a time consuming and costly avenue before it is translated to the bedside.

Given that the autonomic nervous system plays an integral part in SIRS (Borovikova, Ivanova et al. 2000, Elenkov, Wilder et al. 2000), measuring cardiac autonomic function is a potential alternative route. Physiological signal analysis provides a real-time and cost-effective way of assessing ICU patients for overall changes in physiology (Goldstein 2006). Physiological signal analysis has been most used in neonatology (Griffin, Lake et al. 2005), cardiology (Stein, Domitrovich et al. 2005)and intensive care (Goldstein, Kempski et al. 1996, Tibby, Frndova et al. 2003)to predict outcome, usually mortality.

Whether physiological systems analysis may be of use in the early diagnosis of sepsis has only recently been studied in neonates (Moorman, Carlo et al. 2011) and adults (Bravi, Green et al. 2012). These preliminary results suggest that sepsis may be diagnosed earlier than currently possible with conventional tools.

7.3 Heart rate variability

Heart rate variability (HRV) is a dynamic measure of beat-to-beat differences in heart rate. This normal variability reflects cardiovascular, central nervous and autonomic system interactions. Thus, HRV may be a surrogate marker for physiological state. Decreased HRV has been noted in many chronic disease states such as diabetes mellitus (Chessa, Butera et al. 2002) but also as a prognosticator for mortality after myocardial infarction (Stein, Domitrovich et al. 2005), trauma (Norris, Ozdas et al. 2006), stroke (Makikallio, Makikallio et al. 2004) and neonatal sepsis (Griffin, Lake et al. 2005).

Reduced heart rate variability has long been recognized as a sign of disease. As early as 300 AD Dr. Wang Su Ho wrote in *The Pulse Classic* that "'If the pattern of the heart beat becomes as regular as the tapping of a woodpecker or the dripping of rain from the roof, the patient will be dead in four days." (Cowan 1995).

In more modern times Goldberger reintroduced this concept of HRV as a mirror of health and aging (Goldberger 1996) (Figure 7-1)

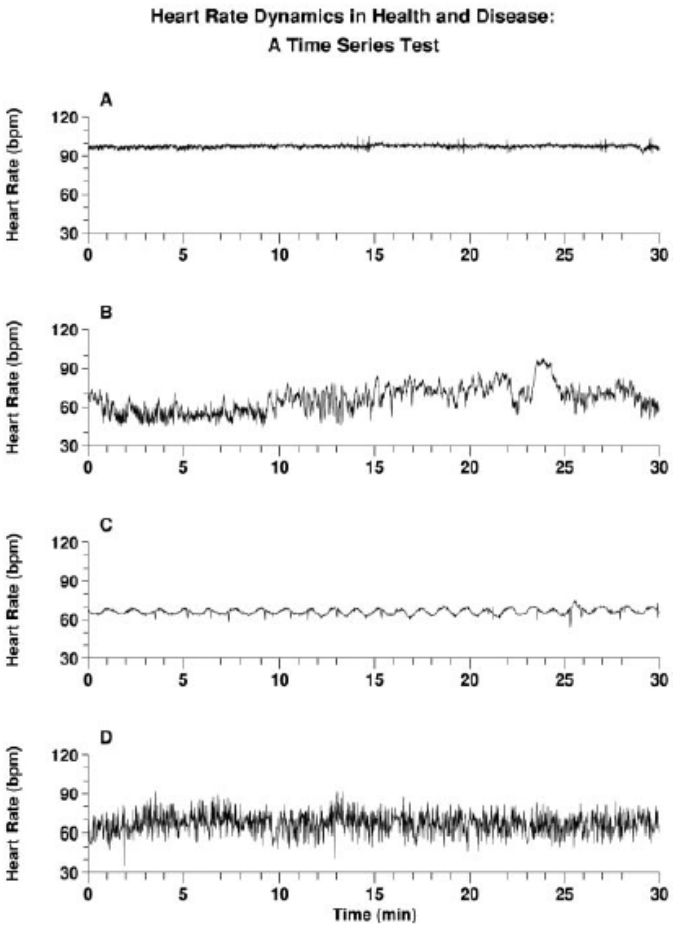


Figure 7-1 Tachogram

Legend: A and C sinus rhythm in congestive heart failure, B normal healthy adult, D atrial fibrillation. (Goldberger, Amaral et al. 2002)

Studies in critical care have repeatedly shown that loss of HRV is associated with more severe disease and mortality. (Bigger, Fleiss et al. 1993)

At the most severe end of the health spectrum markedly reduced HRV was seen in brainstem death (Goldstein, DeKing et al. 1993) (Su, Kuo et al. 2005) and multiple organ dysfunction syndrome (MODS)(Schmidt, Muller-Werdan et al. 2005). Incremental decrease in HRV in relation to severity of illness was observed cross-sectional in brain injury (Su, Kuo et al. 2005), critically ill adults (Schmidt, Muller-Werdan et al. 2005) and critically ill children (Tibby, Frndova et al. 2003). Longitudinal studies showed a correlation with severity of illness over time in critically ill children (Ellenby, McNames et al. 2001), in patients progressing to brain stem death (Rapenne, Moreau et al. 2000) and inflammatory complications after traumatic brain injury (Norris, Ozdas et al. 2006). Thus, HRV was able to predict and reflect severity of disease. A more specific application of HRV was attempting to predict onset of sepsis in neonates(Griffin, Lake et al. 2005, Moorman, Lake et al. 2006) and adults(Ahmad, Ramsay et al. 2009).

7.3.1 Quantification of heart rate variability

Many measures exist to quantify HRV. The debate is still ongoing which metrics to use and what they signify.(Bravi, Longtin et al. 2011) One view is that HRV does not differentiate well between pathophysiological states and its usefulness lies primarily in individual longitudinal changes in severity of illness (Goldstein 2006). A different view is that HRV metrics are specific to certain pathophysiological states. For instance, HRV metrics in septic adult patients were different from those with a neurological insult (Korach, Sharshar et al. 2001). Another study showed that HRV indices changed in trauma patients when sepsis developed.(Norris, Ozdas et al. 2006)

This could be explained either way: a different process developed as well as a clinical deterioration.

Techniques used for HRV analysis can be roughly divided into three areas: time domain, frequency domain and regularity/complexity domain. They have different limitations and may complement each other in interpretation. (Bravi, Longtin et al. 2011) For the purpose of this thesis I will limit myself to frequency domain analysis as this is the most used metric in critical care populations, which allows for relation to the existing body of knowledge.

7.3.2 Fast Fourier Transform Spectral Analysis

Time series, such as heart rate, may be transformed to their frequency components. Heart rate is thus a composite of sinusoidal oscillations that can be broken down to frequencies. One widely used method in heart rate variability analysis is Fast Fourier Spectral Analysis (Seely and Macklem 2004). Each sine and cosine wave has an amplitude. This amplitude determines the contribution of the waves to the signal. Thus frequency domain analysis shows the level of contribution to the signal as a function of frequency. The square of the amplitude of each frequency in the signal is called the power of that frequency. The sum of all power related to their respective frequencies or area under the curve is the total power in a signal.

Fast Fourier Transform analysis presumes the signal to be periodic, i.e. not random, and stationary. Stationary is the requirement that the signal's statistical properties such as mean and standard deviation are stable over the measured time period.(Electrophysiology 1996) Given that physiological systems are not stationary over a prolonged period of time, FFT analysis is done over short time windows of 5-15 minutes.

Defined bandwidths for adults are low frequency (LF) at 0.04-0.15 Hz and high frequency (HF) at 0.15-0.4 Hz. These spectra relate to physiology albeit that the exact mechanisms are currently still unknown. It is generally thought that LF and HF predominantly reflect cardiac autonomic function. LF is influenced by both sympathetic and parasympathetic influences related to the baroreflex. HF is characterised by parasympathetic modulation. The HF portion of the power spectrum is associated with respiratory sinus arrhythmia (RSA). These fluctuations in heart rate are a resultant of parasympathetic functions such as central vagal outflow to the heart, baseline cardiac vagal tone, the baroreflex and respiratory modulation of vagus activity. (Berntson, Bigger et al. 1997)

Ultra low frequency (ULF) at ≤ 0.003 Hz (>5 h our cycles) and very low frequency (VLF) 0.0003-0.04 Hz (> 25 second cycle length) waves are identified as well if recordings are made over 24 hour time periods. These bandwidths are not well understood, but may reflect circadian rhythms (ULF), temperature and humoral influences (Braga, da Silva Lemos et al. 2002).(Electrophysiology 1996)Sympathetic nerve discharges induce a strong circadian rhythmicity. (Barrett, Navakatikyan et al. 2001)

A typical frequency domain analysis is shown in Figure 7-2.

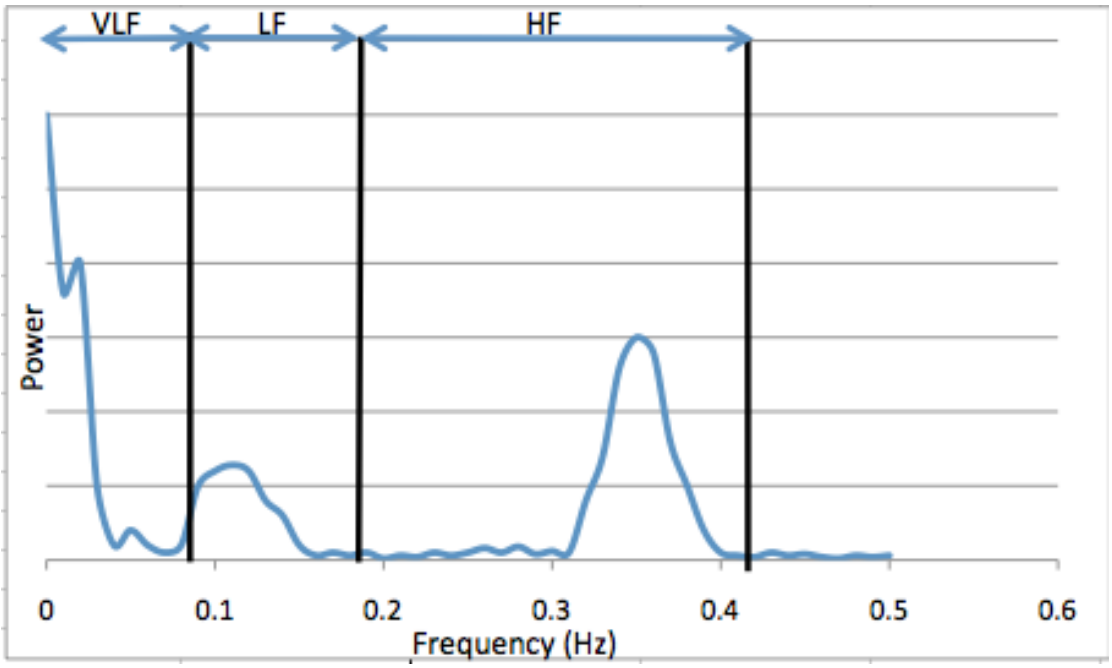


Figure 7-2 Frequency domains in heart rate variability analysis

VLF Very Low Frequency; LF Low Frequency; HF High Frequency. Power is expressed on the Y axis as spectral density in ms^2 /Hz. This ensures that the spectral amplitude is not dependent on the record length. The integration of the spectral area, i.e. the area under the curve is equal to the variance (ms^2).

One summary statistic is to compare LF and HF components of the observed frequency analysis. The normal LF/HF ratio in adults is 1.5-2 (Electrophysiology 1996). In children normal values change with age. LF, HF and total power increase between 0-6 yrs, followed by a decrease. Infants show higher indices than children and they tend to have much greater LF power. (Mehta, Super et al. 2002)

Although myocytes and hearts have an intrinsic beat, there is no variability to this rhythm. A non-rejecting denervated transplanted heart shows virtually no variability in heart rate and it's power spectrum has no peaks. The inflammatory state of graft rejection corresponded with reappearance of heart rate variability. However the appearance was not of distinct peaks of LF and HF, but rather a random "broad band" pattern. This was not a universal phenomenon, as other patients in acute rejection did not show an increase in spectral power.(Sands, Appel et al. 1989)

LF and HF power spectra change with time of day (Yamasaki, Kodama et al. 1996), gender and age (Kuo, Lin et al. 1999), behavioural state (Pagani, Furlan et al. 1989) and fitness (Cornolo, Brugniaux et al. 2005).

7.3.3 Heart rate variability and inflammation

Clinical data suggest an association between decreased HRV and systemic inflammation. A review of the literature on inflammation and HRV in cardiac patients showed that serum inflammatory markers were negatively correlated with LF HRV. (Haensel, Mills et al. 2008).

A relationship between increased plasma levels of pro-inflammatory cytokines TNFα and IL6 and reduced HRV has been shown in chronic and acute illness (Malave, Taylor et al. 2003, Gonzalez-Clemente, Vilardell et al. 2007, Sloan, McCreath et al. 2007, Tateishi, Oda et al. 2007). Administration of endotoxin to human adult

volunteers induces self-resolving clinical systemic inflammation, a concomitant cytokine response and reduced HRV (Alvarez, Katsamanis Karavidas et al. 2007). A mild form of sterile stress, such as exercise induces systemic inflammation (Bruunsgaard 2005) and reduced HRV (Yamamoto, Hughson et al. 1991).
Overall, HRV is expected to decrease with systemic inflammation.

7.3.4 Heart rate variability after trauma

Extremely low or even absent HRV was observed in those children that were brain dead (Goldstein, DeKing et al. 1993). LF was preserved in relation to HF in all but brain dead patients (Su, Kuo et al. 2005). In neurotrauma low LF/HF ratio was observed in those children with high (>30 mm Hg) ICP (Biswas, Scott et al. 2000). Marked sympathetic activation is a hallmark for traumatic brain injury and was associated with immunoparalysis (low monocyte HLA-DR expression) and nosocomial infection (Woiciechowsky, Asadullah et al. 1998). Long-term sympathetic dysautonomia in traumatic brain injury patients is associated with increased LF (Katz-Leurer, Rotem et al.)((Baguley, Heriseanu et al. 2006).

7.3.5 Heart rate variability in sepsis, SIRS and MODS

Several investigators have shown that HRV in septic shock is lower than in sepsis. One group showed that sepsis patients (including septic shock) showed lower HRV than sterile SIRS in adults (Korach, Sharshar et al. 2001). That group also observed greater LF in those patients with a neurological insult.
Schmidt showed that all HRV indices were significantly reduced in MODS, but VLF was most predictive of 28 day mortality (Schmidt, Hoyer et al. 2008).

More recently, a prospective study in ambulatory post bone marrow transplant patients a panel of HRV indices, including LF/HF were early markers of sepsis. A drop of 25% in HRV was seen 24-120 hours prior to clinical diagnosis of sepsis.(Ahmad, Ramsay et al. 2009)

A similar result was found in premature neonates. A change in heart rate characteristics preceded the onset of sepsis by 12 to 24 hours. (Griffin, O'Shea et al. 2003)This group performed a trial in 3003 premature infants, randomising the cohort to presence or absence of heart rate characteristics on the monitor display. The group that was randomised to having their heart rate characteristics displayed had significantly lower sepsis associated mortality, with an absolute risk reduction of 6.1%. This group was subjected to more blood cultures (1.8/month vs. 1.6/month), but did not receive antibiotics for a longer time period.(Moorman, Carlo et al. 2011)

7.3.6 Pathophysiology of HRV changes in systemic inflammation

The underlying mechanics of systemic inflammation induced reduction of HRV are not well understood. Reduction of vagal activity (Huston and Tracey 2011) and increase in sympathetic activity (Pagani, Montano et al. 1997) (Annane, Trabold et al. 1999) are both directly as well as indirectly implicated. The innate immune response and neuro-hormonal activity are both, in part, under autonomic control.

HRV changes may be due to a mechanistic effect of cytokines. An LPS mouse model showed a temporal effect of cytokines and HRV indices of both parasympathetic and sympathetic parameters (Fairchild, Saucerman et al. 2009). However, others found that adrenergic attenuation of the immune response to LPS as measured by plasma TNFα did not modulate autonomic changes (Jan, Coyle et al. 2009), suggesting that cytokines may not be the predominant pathophysiological substrate for altered HRV.

Instead, corticosteroids may explain part of the changes in systemic inflammation associated reduced HRV. Decreased HRV was associated with relative adrenal insufficiency (Morris Jr, Norris et al. 2007) and in a mouse model HRV increased after steroids (Fairchild, Saucerman et al. 2009). Notably, a placebo controlled human study on the effects of low dose glucocorticoids on endotoxin induced physiological changes showed that cytokines TNFα and IL6 were significantly reduced, but decreases in HRV remained.(Alvarez, Katsamanis Karavidas et al. 2007)

7.3.7 Factors attributed to modulating HRV

There are possible confounding factors when attributing differences in HRV to systemic inflammation. Their effect size in relation to systemic inflammation has however not been quantified. Some of these may be too subtle to be of interest with this magnitude of insult.

Respiratory Sinus Arrhythmia (RSA) originates from both peripheral as well as central mediated processes. RSA has been observed during apnoea and pulmonary denervation, which implies the existence of a central oscillator. Thoracic stretch receptors inhibit vagal motor outflow during inspiration, which causes an increase in heart rate. Therefore mechanical ventilation may in itself contribute to HF power.(Berntson, Bigger et al. 1997) This interaction in itself is poorly understood in critically ill patients. For instance it was shown that both LF and HF decreased in patients when ventilation strategy was changed from mandatory assisted ventilation to spontaneous breathing.(Shen, Lin et al. 2003)Mechanically ventilated premature infants showed HF power amplitudes that corresponded to the ventilator rate.(van Ravenswaaij-Arts, Hopman et al. 1995) Drugs that block and augment adrenergic and cholinergic function (Ahmed, Kadish et al. 1994) (Martinmaki, Rusko et al. 2005)

or sedative medications (Win, Fukayama et al. 2005) may influence HRV. Conversely, in patients with MODS, catecholamines did not appear to influence HRV.(Schmidt, Muller-Werdan et al. 2005)Unfortunately, there are no metrics to quantify the effect size of these potential modulators.

In healthy children, HRV indices are influenced by age. However, "normal" HRV changes with age depend, in part, on type of HRV metric, definition of HRV metric, age cut off points, sleep state, circadian rhythm (Massin, Maeyns et al. 2000), and gender. Table 7-1 shows a summary of variation HRV power spectrum values for healthy neonates, infants and children and effect of interventions/illness.

Author	Year	Age	Diagnosis	LF	HF	Observations
(Electrophysiology 1996)	1996	Adults	Standard Adults	0.04-0.15	0.15-0.4	n/a
(Fracasso, Porges et al. 1994)	1994	5-13m	Healthy	n/a	0.24-1.04	No change
(Finley and Nugent 1995)	1995	1m-24 y	Healthy	0.03-0.15	0.15-0.6	LF, HF and TP increase between 0-6 years followed by a decrease
(Massin and von Bernuth 1997)	1997	3 d-14 y	Healthy	0.04-0.15	0.15-0.4	LF/HF decreases with age
(Rosenstock, Cassuto et al. 1999)	1999	Neonates and infants	Healthy Review	0.04-0.2	0.2-1.5	HRV increases with age in first 6 months
(Villa, Calcagnini et al. 2000)	2000	9.4±2.3m and 8.93±0.65 y	Healthy	0.04-0.15	0.15-0.4	HF higher in children; LF/HF lower in children
(Acharya,	2004	10 ± 5 y	Healthy	0.04-0.15	0.15-0.4	LF/HF = 1.425±1.05

Kannathal et al. 2004)						
(Andriessen, Oetomo et al. 2005)	2005	Preterm 32.1±3.7 wk	Healthy	0.04-0.15	0.4-1.5	HF and LF increase with age
(Annane, Trabold et al. 1999)	1999	Adults	Septic shock	0.06-0.14	.2-.35	LF, HF and LF/HF lower in septic shock vs. sepsis
(Goldstein, Fiser et al. 1998)	1998	1 d- 22 y 6.8±6.2 y	Mixed PICU	0.01-0.15	0.15-2.0	Lower LF worse outcome
(Toweill, Sonnenthal et al. 2000)	2000	1m-18y 8.3±5.7y	Sepsis Septic shock	0.01-0.15	0.15-1.0	LF lower in septic shock
(Ellenby, McNames et al. 2001)	2001	6m-21 y	Septic shock	0.04-0.15	0.15-1.0	LF_n and LF/HF increase with recovery
(Lin, Wang et al. 2006)	2006	1-9 y	Enterovirus infection	0.04-0.15	0.15-0.4	LF and HF lower with worse disease LF/HF higher with worse disease
(Polson, McCallion et al. 2006)	2006	Term infants	Co-arctation	0.04-0.15	0.15-1.1	LF and HF decreased

(Pruvost, Zaaimi et al. 2006)	2006	7-18 y mean 11.7y	Epilepsy VNS	0.04-0.15	0.15-0.6	HF decreased

Table 7-1 HRV power spectrum values in infants and children

LF Low Frequency, HF High Frequency, LF_n Normalised LF, VNS Vagus Nerve Stimulator

Other known influences on HRV include circadian rhythm (Bilan, Witczak et al. 2005) and ethnicity (Urbina, Bao et al. 1998). Heritability of HRV was shown to be high (up to 70%) (Kupper, Willemsen et al. 2004, Newton-Cheh, Guo et al. 2007). In one study African American youth had higher HRV indices than did European Americans (Wang, Thayer et al. 2005).

Candidate genes to narrow this heritability down to specific genetic variability are GNAS1 (Yasuda, Matsunaga et al. 2004), eNOS (Binkley, Nunziatta et al. 2005) and ACE I/D. The ACE I/D studies give conflicting evidence: DD homozygotes have higher HF (Busjahn, Voss et al. 1998), lower HF (Thayer, Merritt et al. 2003) or the same HF (Steeds, Fletcher et al. 2002) compared with the II genotypes. In another study, ADRB2 polymorphisms and HRV complexity metrics were independently associated with mortality after trauma, but not with each other (Norris, Canter et al. 2009).

No HRV study in children has focussed on the differentiation of infective (sepsis) vs. sterile SIRS. This differentiation is relevant to early source control and antibiotic stewardship as well as therapeutic potential for anti-inflammatory or autonomic nervous system modulation.

To explore the potential of HRV to differentiate between SIRS and sepsis we studied HRV in a cohort of critically ill children.

Hypotheses:

1. Clinical SIRS can be differentiated from sterile and infectious SIRS on the basis of heart rate variability metrics. Specifically, the sepsis group is expected to have reduced LF power and LF/HF ratio compared with the sterile SIRS group.

2.Admission LF and LF/HF in the SIRS group that go on to develop nosocomial infection are higher than those who did not go on to develop nosocomial infection.

7.4 Methods

Prospective observational matched pairs study.

The total cohort consisted of n=176 consecutive paediatric patients admitted to PICU with > 1 organ failure and an expected stay > 24 hrs or death < 12 hours. From this dataset the study set was chosen based on the following criteria:

First ECG recording within 24hrs of admission;

Diagnosis of SIRS/sepsis in the first 24 hrs of admission;

Matching for age and severity of illness.

A control group was assembled with the same inclusion criteria but without SIRS.

HRV metrics analysis was done blinded to SIRS and admission diagnosis.

These data were collected as part of the genetic study outlined in paragraph 2.1.1.

7.4.1 Definitions

SIRS and sepsis were defined according to the modified criteria as described in paragraph 2.4.

Nosocomial infection was defined as initiation of antibiotics for a new onset presumed infection at the treating physician's discretion.

7.4.2 Physiological Data Acquisition System (PDAS) hardware and software

The PDAS hardware consists of a laptop computer, a standard PCMCIA serial card (Socket Communications, Inc. Newark, CA), RS232 serial interface cables (Oregon Electronics, Portland, OR) and a RS232 card fitted to the ICU monitoring devices.

A dual high-speed serial I/O PCMCIA card provided serial ports on the laptop. This card connected to a RS232 communication port on the medical devices with a custom-built T25-pin serial interface cable. The RS232 communication card provides an interface to export data from a medical device, i.e Philips Component Monitoring System (CMS) (Philips Medical Systems, Guildford Surrey, UK) patient monitor to a laptop.(Figure 7-3)

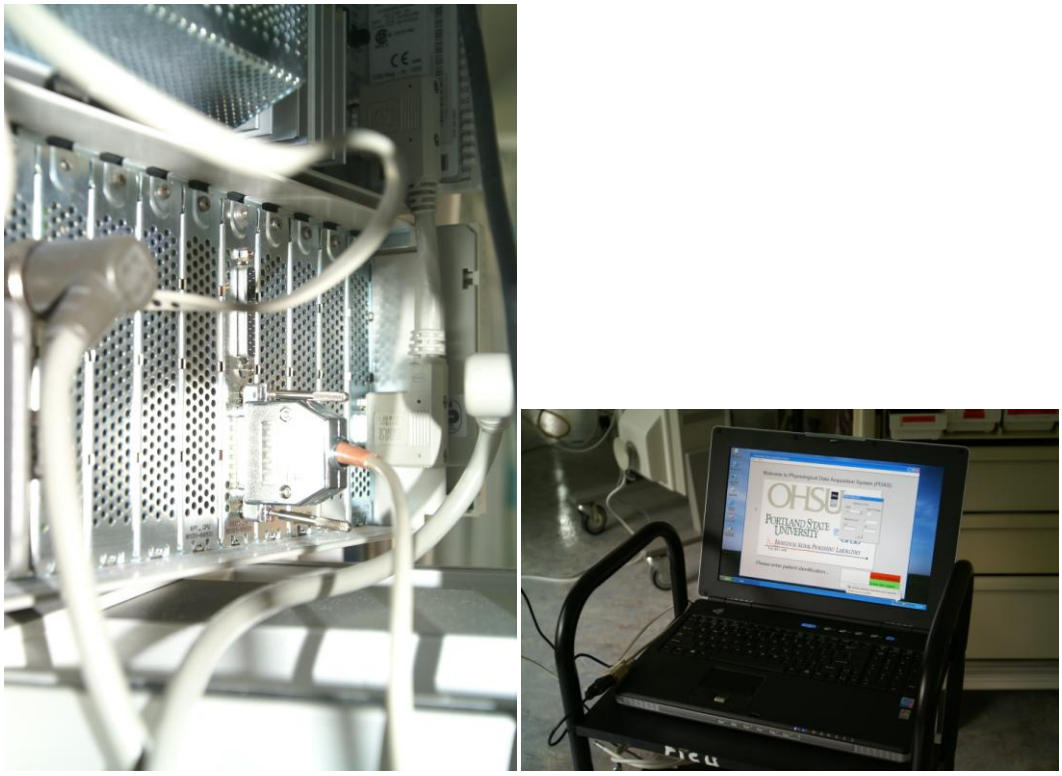


Figure 7-3 PDAS hardware setup

Back of the Merlin/CMS monitor with cable inserted in RS232 board connected to laptop.

The PDAS software is custom-made.(Vinecore, Aboy et al. 2007) It was developed at Oregon Health & Science University (OHSU) in collaboration with Portland State University. The software acquires and stores physiological signals from ICU monitoring devices and coordinates the incoming data flow from each of the physiological signals. All recorded data are transferred to text files in patient specific files and folders. These files were then imported offline for post hoc signal processing, waveform analysis and statistical analysis into Excel (Microsoft, Redmond WA) or MATLAB (The MathWorks, Natick, MA).

Physiological data from the Philips patient monitors were downloaded at sampling rates of 500 Hz for ECG waveforms, 125 Hz for other waveforms and parametric data at 60 Hz. Both waveform signals and parametric data were displayed real time on the laptop screen. (Figure 7-4)

Figure 7-4 Screenshot of real time data capture with PDAS

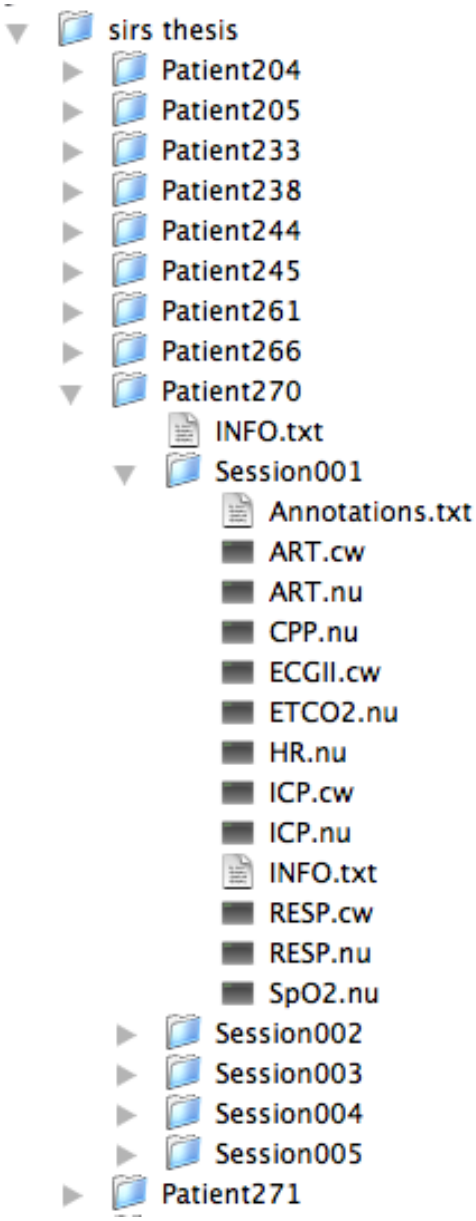
Screenshot of data capture. Four waves are depicted. From top to bottom: ECG (Electrocardiogram), ABP (Arterial Blood pressure), SpO_2 (Oxygen saturation) and AWP (airway pressure).

The CMS patient monitor's data transmission rate of 38400 bits per second (bps) limited data capture for each RS232 connection to a maximum of one 500 Hz ECG signal and three additional 125 Hz signals with their numerical values.

The software creates a hierarchical structure for data storage on the laptop's hard drive based on the specific research study, the study specific patient identification number, the recording session number and the type of signal. The data files are stored in ASCII text format files with one data point per line.

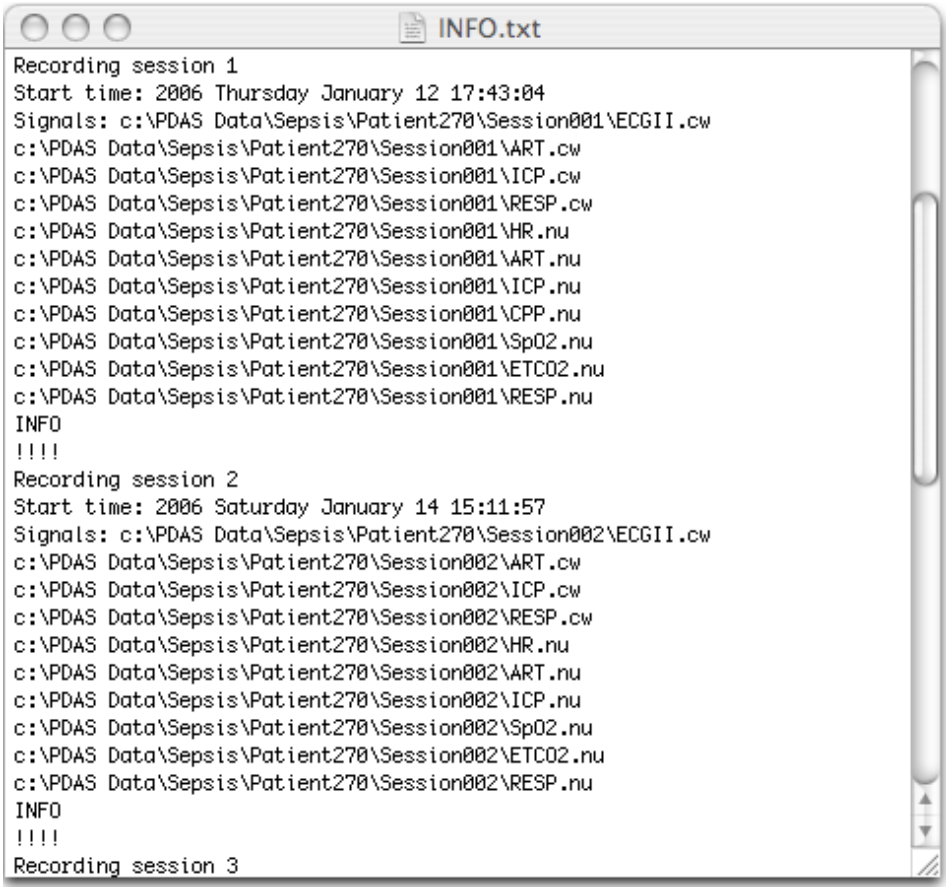
Each session generated a single information text file with meta data such as scaling values, units, and study start and stop times for each of the signals recorded, (Figure 7-5).

Clinical or study annotations and comments were entered as free-form and stored in a separate ACSII text file. The file was time-stamped and indexed to both the 125 Hz and 500 Hz data files allowing for clinical events to be associated with the physiological data.


sirs thesis
Patient204
Patient205
Patient233
Patient238
Patient244
Patient245
Patient261
Patient266
Patient270
INFO.txt
Session001
Annotations.txt
ART.cw
ART.nu
CPP.nu
ECGII.cw
ETCO2.nu
HR.nu
ICP.cw
ICP.nu
INFO.txt
RESP.cw
RESP.nu
SpO2.nu
Session002
Session003
Session004
Session005
Patient271

A

B


INFO.txt
Recording session 1
Start time: 2006 Thursday January 12 17:43:04
Signals: c:\PDAS Data\Sepsis\Patient270\Session001\ECGII.cw
c:\PDAS Data\Sepsis\Patient270\Session001\ART.cw
c:\PDAS Data\Sepsis\Patient270\Session001\ICP.cw
c:\PDAS Data\Sepsis\Patient270\Session001\RESP.cw
c:\PDAS Data\Sepsis\Patient270\Session001\HR.nu
c:\PDAS Data\Sepsis\Patient270\Session001\ART.nu
c:\PDAS Data\Sepsis\Patient270\Session001\ICP.nu
c:\PDAS Data\Sepsis\Patient270\Session001\CPP.nu
c:\PDAS Data\Sepsis\Patient270\Session001\SpO2.nu
c:\PDAS Data\Sepsis\Patient270\Session001\ETCO2.nu
c:\PDAS Data\Sepsis\Patient270\Session001\RESP.nu
INFO
!!!!
Recording session 2
Start time: 2006 Saturday January 14 15:11:57
Signals: c:\PDAS Data\Sepsis\Patient270\Session002\ECGII.cw
c:\PDAS Data\Sepsis\Patient270\Session002\ART.cw
c:\PDAS Data\Sepsis\Patient270\Session002\ICP.cw
c:\PDAS Data\Sepsis\Patient270\Session002\RESP.cw
c:\PDAS Data\Sepsis\Patient270\Session002\HR.nu
c:\PDAS Data\Sepsis\Patient270\Session002\ART.nu
c:\PDAS Data\Sepsis\Patient270\Session002\ICP.nu
c:\PDAS Data\Sepsis\Patient270\Session002\SpO2.nu
c:\PDAS Data\Sepsis\Patient270\Session002\ETCO2.nu
c:\PDAS Data\Sepsis\Patient270\Session002\RESP.nu
INFO
!!!!
Recording session 3

INFO.txt

```
Recording session 1
Start time: 2006 Thursday January 12 17:43:04
Files:

c:\PDAS Data\Sepsis\Patient270\Session001\ECGII.cw
label: II
# of samples: 16
gain: 1420.000000
offset: 1661.000000
rt_unit:  mV
Extra label: 1mV
Extra label: F HR

c:\PDAS Data\Sepsis\Patient270\Session001\ART.cw
label: ART
# of samples: 4
lower mark: 448
lower calibration mark value: 0.000000
upper mark: 2848
upper calibration mark value: 180.000000
rt_unit: mmHg

c:\PDAS Data\Sepsis\Patient270\Session001\ICP.cw
label: ICP
# of samples: 4
lower mark: 448
lower calibration mark value: 10.000000
upper mark: 2848
upper calibration mark value: 28.000000
rt_unit: mmHg

c:\PDAS Data\Sepsis\Patient270\Session001\RESP.cw
label: RESP
# of samples: 4
Extra label: 1Ohm

c:\PDAS Data\Sepsis\Patient270\Session001\HR.nu
label: HR
# of samples: 1

c:\PDAS Data\Sepsis\Patient270\Session001\ART.nu
label: ART
# of samples: 3

c:\PDAS Data\Sepsis\Patient270\Session001\ICP.nu
label: ICP
# of samples: 1

c:\PDAS Data\Sepsis\Patient270\Session001\CPP.nu
label: CPP
# of samples: 1

c:\PDAS Data\Sepsis\Patient270\Session001\SpO2.nu
label: SpO2
# of samples: 1
```

C

Figure 7-5 Hierarchical structure and meta data PDAS signals

A. Patient identified recording session number and type of signals

B. Timed recording sessions with identified signals and values

C. Individual signal labels with bandwidth of values

7.4.3 Metrics definitions

Frequency domain band spectra were defined as follows:

Low frequency (LF) 0.04-0.15 Hz;

High Frequency (HF) 0.15-1 Hz;

Total HRV power 0.04-1 Hz;

Normalised LF (LF_n) LF/(LF+HF)*100;

Normalised HF (HF_n) HF/(LF+HF)*100;

LF /HF ratio

The Low Frequency component was defined according to the taskforce definition of 0.04-.15 Hz.(Electrophysiology 1996) The High Frequency component of the spectral analysis was defined in the frequency spectrum between 0.15 and 1 Hz. The taskforce specified a frequency bandwidth between 0.15 and 0.4 Hz for adults. This takes into account the normal respiratory rate and hence RSA for adults, but not for children. Given the higher respiratory rates for children in general and infants in particular, especially when they are ill, the HF bandwidth was set between 0.15 and 1 Hz.

7.4.4 Clinical circumstances

Patients were nursed supine in bed. Routine practice has the head of the bed at 0° except in the case of traumatic brain injury, when the head of the bed is kept at 30°. Recordings took 15 minutes, during which no clinical interventions took place. Diagnosis, medications and ventilator settings were annotated bedside.
Recordings were done in the morning as much as possible.

7.4.5 Data processing

The 15 minute raw ECG files were uploaded to a custom made software programme Sympatho-Analysis 2.0. This software program was kindly shared by Ir. C.H.L. Peters, Departement of Clinical Physics, Máxima Medical Centre, Veldhoven, The Netherlands. Sympatho-Analysis was developed using LabVIEW 7.0, National Instruments, Woerden, The Netherlands.

The programme allows visual inspection of the ECG signal over the full measurement time period. Gross artefacts, disconnections and/or trends were identified. Two usable 5 minute epochs had to be available. These recordings were then transferred to the Biomedical Signal Processing Laboratory, Electrical and Computer Engineering, Portland State University, Portland, USA.

RR intervals were identified with an automated R wave peak detection tool (Mr Timothy Ellis, Biomedical Signal Processing Laboratory, Electrical and Computer Engineering, Portland State University, Portland, USA). After identification of RR intervals they were plotted to identify unexpected jumps. The power spectral density was estimated from RR intervals as described in (McNames and Aboy 2006). The programme is Matlab based, MathWorks, Natick, MA, USA.

7.4.6 Statistical analysis

Paired Student t-test or paired non-parametric Wilcoxon signed ranked test was performed for the measures LF_n and LF/HF ratio between the sepsis and SIRS groups. Independent Student t-test was performed for the measures LF_n and LF/HF ratio between SIRS and secondary infected SIRS groups.

7.5 Results

7.5.1 General Results

Based on the selection criteria the following number of patients was identified:

n=20 patients age and severity of illness matched with SIRS (n=10 sepsis and n=10 sterile SIRS) and n=4 patients with no SIRS as a control sample.

The admission session for one sepsis patient showed many artefacts, thus this patient was removed together with their age equivalent in the SIRS group. In the non SIRS group data for one patient failed analysis and one patient was mistakenly identified. This left only 2 patients in the non-SIRS group.

Table 7-2 shows the demographics according to diagnosis group.

All ECG waves were manually inspected for artefacts. (Figure 7-6)

	SIRS (n=9)	**Sepsis (n=9)**	**P value**	**Non SIRS (n=2)**
Age mean±sd[a]	106±58	107±56	0.9	94±94
Gender M:F	7:2	7:2		1:1
PIM2	0.08±0.10	0.12±0.11	0.35	0.22±0.21
Ethnicity	7 Caucasian; 1 Asian; 1 Unknown/other	6 Caucasian; 1 Black; 2 Unknown/other		2 Caucasian
28d mortality (n)	1	0		0
Ventilated	9	9		2
Inotropic support	3	5		0
Sedation	9	9		2
Nosocomial infection	5	n/a		0

Table 7-2 Demographic according to diagnosis group HRV study

[a]Age in months

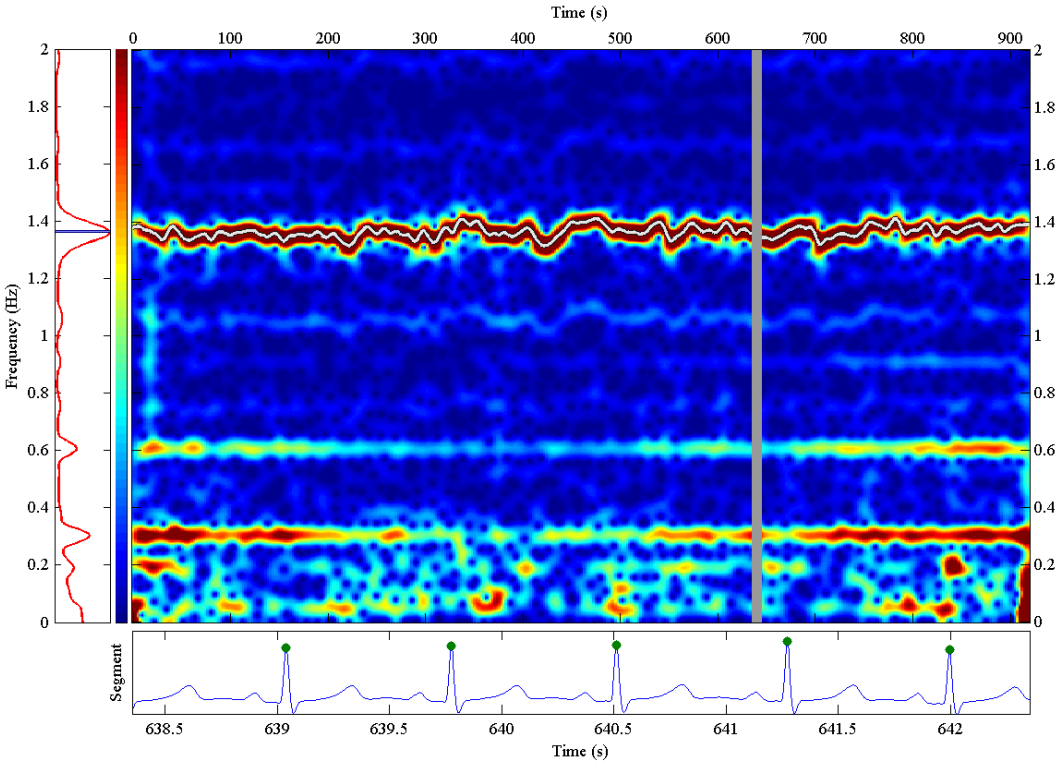


Figure 7-6 ECG tracings from waveform patient 204

The heart rate (in Hz) is superimposed on top of a spectrogram of the ECG signal. The ECG signal spectrogram is depicted in the centre. Colours indicate the relative power of the signal as it varies over time. The strongest (i.e., darkest red) band flows from left-to-right and represents the fundamental cardiac frequency.

The lighter coloured bands below the dark red band correspond to the power of the patient's breathing cycle.

A light-grey line (outlined in dark grey) is superimposed on the central image. It should more-or-less follow the dark red band on the spectrogram. This is the heart rate as identified from the RR interval data.

A randomly-selected four-second time-domain example of the ECG signal is plotted at the bottom of the image. Red dots correspond to the times of R peak detection. A vertical thick medium grey line represents the four-second corresponding portion on the central spectrogram image.

The thin vertical colour bar to the left of the central image represents how the colours on the spectrogram correspond with the relative power of the signal in frequency and time: colours near the top indicate more power, those near the bottom indicate less power.

The far-left box is a vertically-oriented plot of the power spectral density (PSD) of the signal over its entire duration. It represents the power at each frequency averaged over the length of the signal. The blue line represents the average heart rate frequency computed from the detected R-to-R intervals. This blue line should correspond with the peak of the red (PSD) line. (personal communication Mr Timothy Ellis)

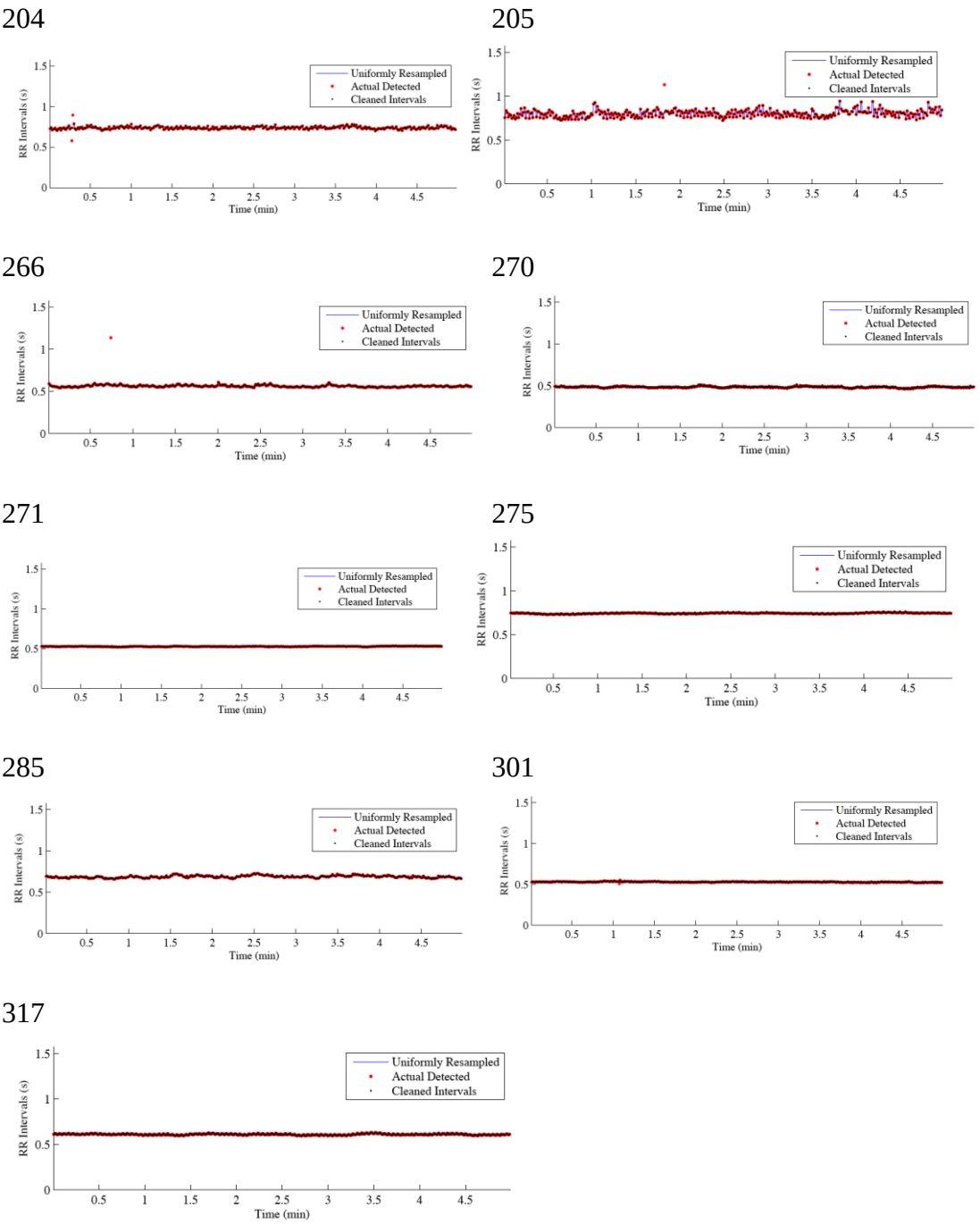
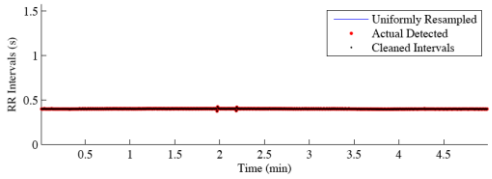


Figure 7-7 Overview five minute heart rate recording session in first 24 hours of admission sterile SIRS

Plots identified by patient number

238



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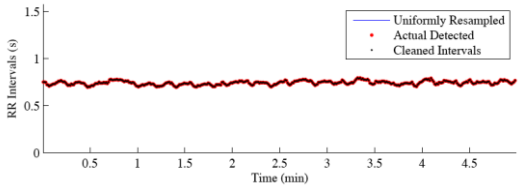

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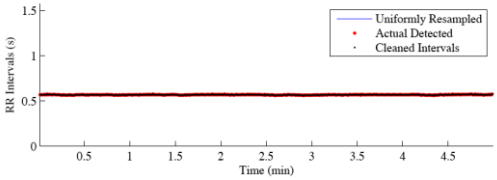

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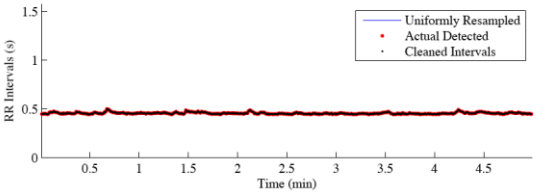

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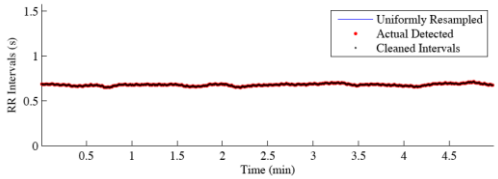

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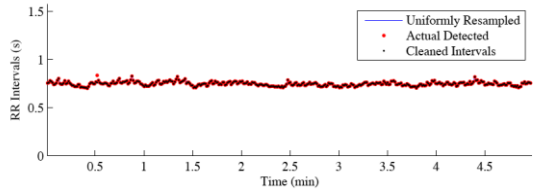

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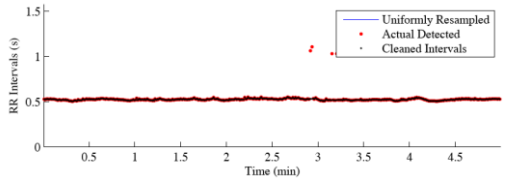

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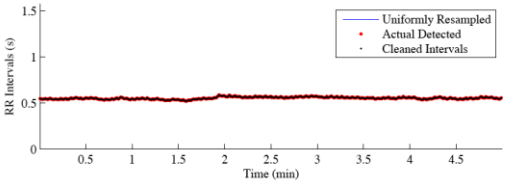

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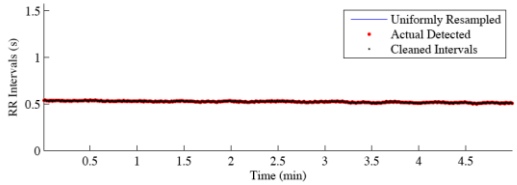
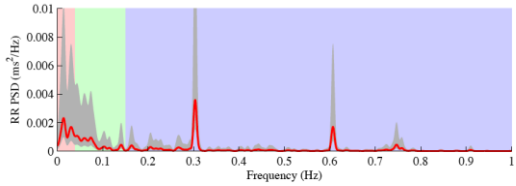


Figure 7-8 Overview five minute heart rate recording session in first 24 hours of admission sepsis

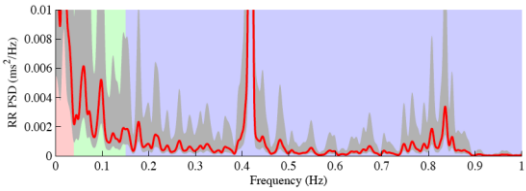
Plots identified by patient number

204



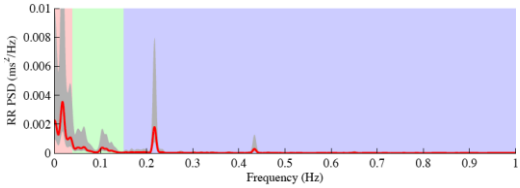
Ventilator rate and sedation n/a

205



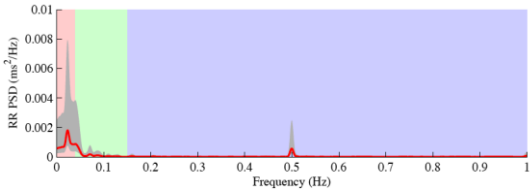
Ventilator rate and sedation n/a

266



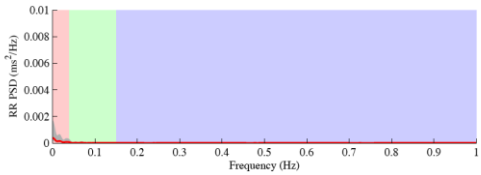
Ventilator rate 13 (0.21 Hz); midazolam/morphine

270



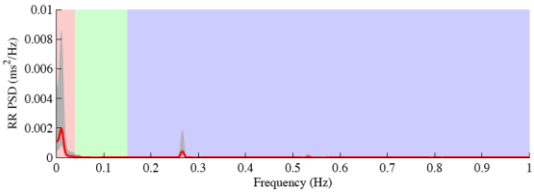
Ventilator rate 30 (0.5 Hz); muscle relaxed

271



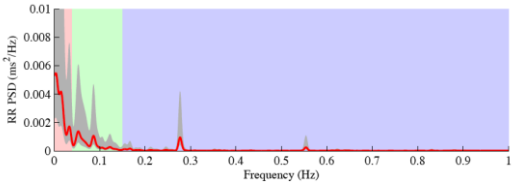
Ventilator rate 14 (0.23 Hz); muscle relaxed

275



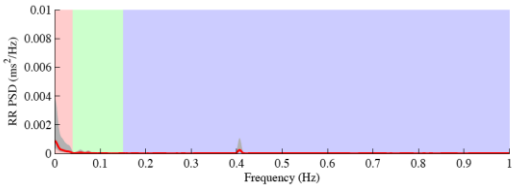
Ventilator rate 16 (0.27 Hz); muscle relaxed

285



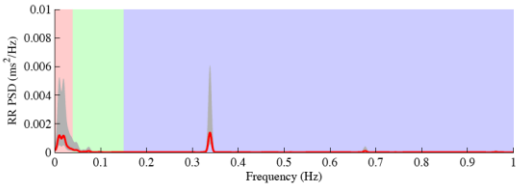
Ventilator rate 17 (0.28 Hz); midazolam/morphine

301



Ventilator rate 24 (0.4 Hz); muscle relaxed

317

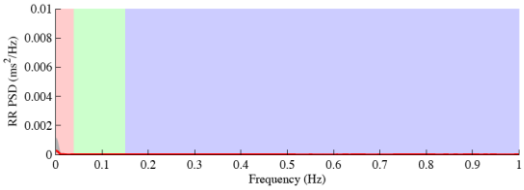


Ventilator rate 20 (0.33 Hz); muscle relaxed

Figure 7-9 Power spectrum derived from five minute RR interval measurement sterile SIRS group

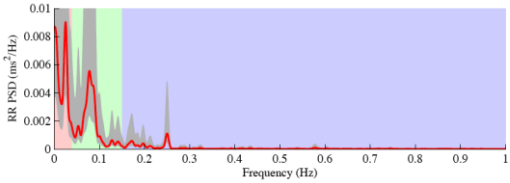
Plots identified by patient number

238



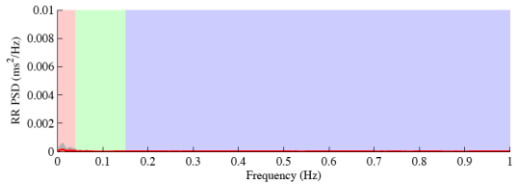
HFO; midazolam/morphine

245



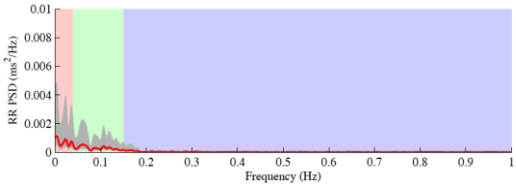
Ventilator rate 10 (0.16 Hz); midazolam/morphine

261



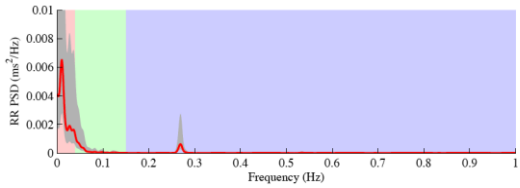
Ventilator rate 12 (0.2 Hz); midazolam/morphine

272



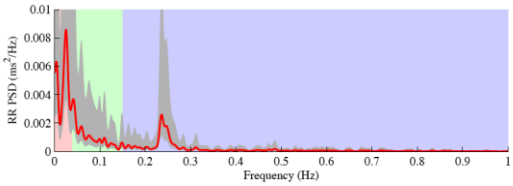
Ventilator rate 16 (0.26 Hz); midazolam morphine

282



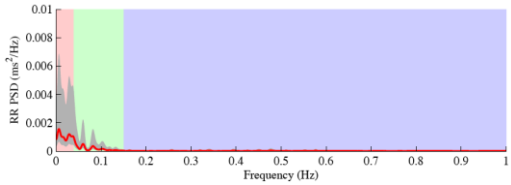
Ventilator rate 16 (0.26 Hz); midazolam morphine

284



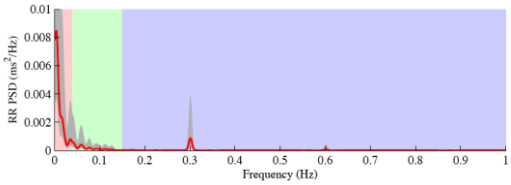
Ventilator rate and sedation n/a

297



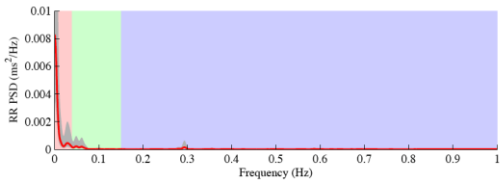
Volume support; chloralhydrate

298



Ventilator rate 20 (0.33 Hz); midazolam/morphine

327



Ventilator rate 7 (0.11 Hz); midazolam/morphine

Figure 7-10 Power spectrum derived from five minute RR interval measurement sepsis group

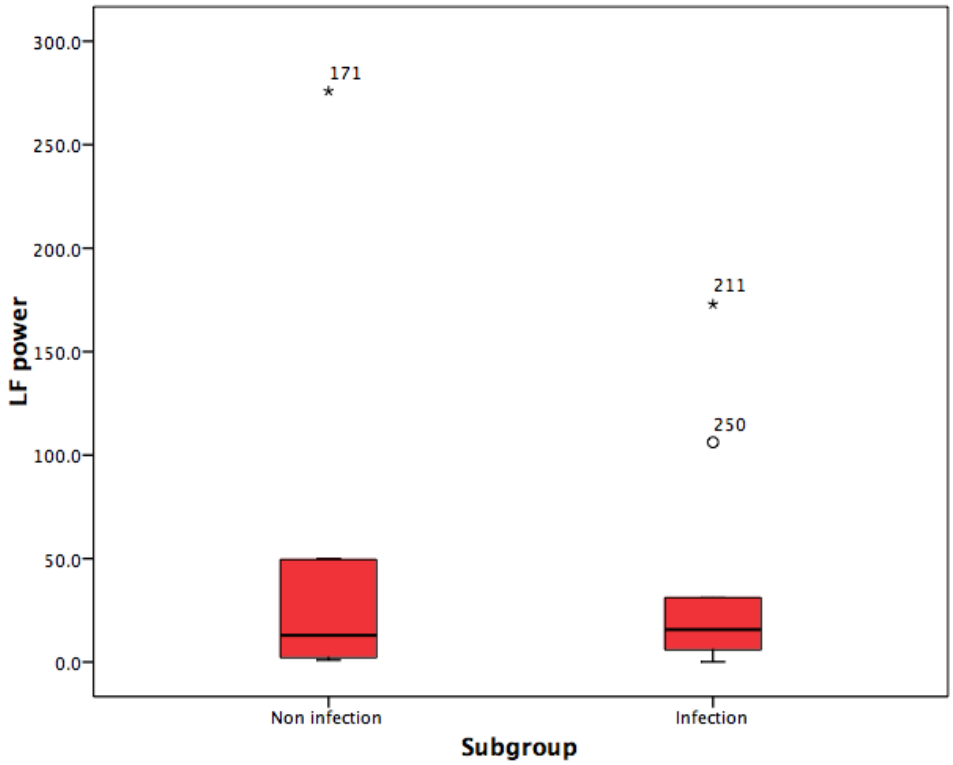
Plots identified by patient number

	SIRS (n=9)	Sepsis (n=9)	W value	P value
LF median; IQR	12.9; 48.2	15.7; 65.2	-1	NS
LF Range	0.9-275.9	0.1-172.9		
HF median IQR	16.4; 57.1	10.7; 22.7	9	NS
HF range	2-801.2	2.6-117.8		
TP median	64.7; 177.4	73.6; 245.7	3	NS
TP range	9.1-1581.7	6.7-428.8		
LFn	0.28	0.38		0.31
HFn	0.51	0.32		0.029
LHR	0.62	1.55		0.11

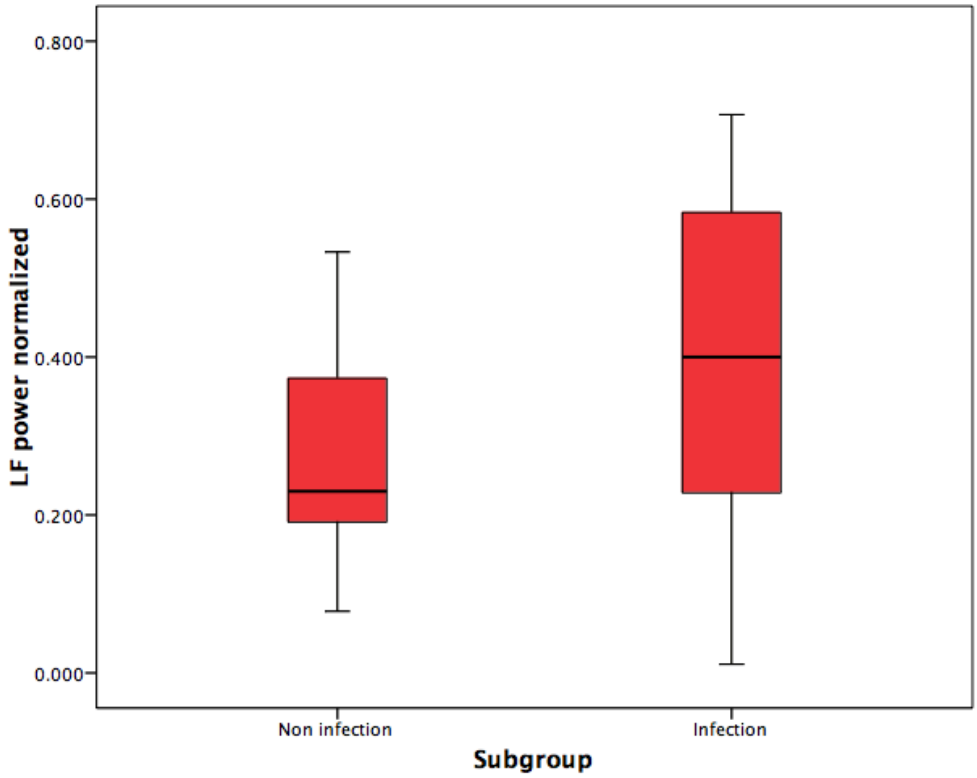
Table 7-3 HRV characteristics between sterile SIRS and sepsis groups

LF low frequency; HF high frequency; TP total power; LFn normalised LF; HFn normalised HF; LHR low frequency/high frequency ratio.

P value derived from paired Wilcoxon signed ranked test and for LFn, HFn and LHR from paired Student t-test.



A.



B.

Figure 7-11 LF analysis between sterile SIRS and sepsis groups A. LF power B. LF power normalized

LF Low Frequency. No difference between the two groups (paired Wilcoxon signed ranked test)

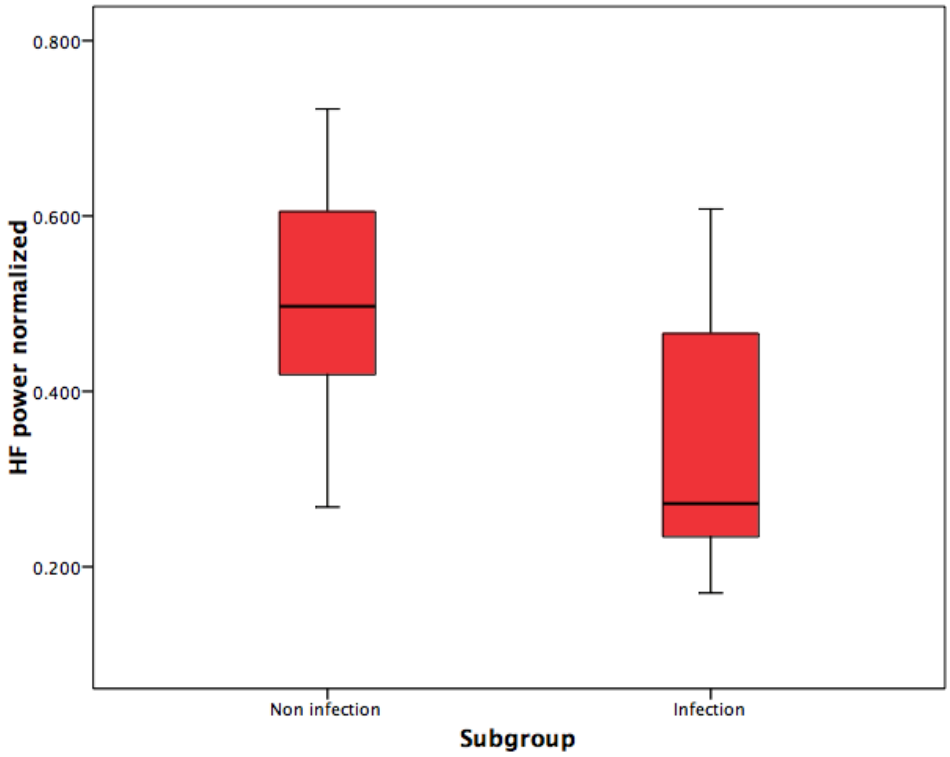


Figure 7-12 HF (normalized) power analysis between sterile SIRS and sepsis groups

HF High Frequency, SIRS Systemic Inflammatory Response Syndrome

The distribution of values for LF and HF violated the Kolmogorov-Smirnov test for normality. Thus, the paired non-parametric Wilcoxon signed ranked test was used. For this to be significant at P=0.05 on 9 pairs the W value would have to be at least 29. For LFn, HFn and HF/LF the distribution was normal, thus paired Student t-test was used. No significant difference was found between the sterile SIRS and sepsis in absolute LF power (Figure 7-11 A. and Table 7-3).

Given the large interindividual differences in absolute power, the data were also normalized. This way LFn (LF/(LF+HF)) was also assessed. This metric too did not show a significant difference between groups. If anything, the LFn was lower in the sterile SIRS group (Figure 7-11 B.).

Surprisingly, a difference was found in the HF_n spectrum. The sterile SIRS groups showed higher HF_n than the sepsis group (Table 7-3 and Figure 7-12).

7.5.2 Nosocomial infection

Nosocomial infection occurred in 5/10 patients admitted with sterile SIRS. Those that developed infection tended to be older, 137 vs.74 (p 0.08), but had no difference in severity of illness. All HRV metrics were lower in the group that developed infection, except for HF, but these were not significant (Table 7-4).

Group Statistics

	any infection in first week	N	Mean	Std. Deviation	Std. Error Mean
LF power	none	5	75.680	114.4581	51.1872
	yes	5	21.120	26.2107	11.7218
HF power	none	5	1.725E2	351.5559	157.2206
	yes	5	29.520	39.1301	17.4995
Total power	none	5	3.842E2	670.9763	300.0697
	yes	5	1.007E2	96.7211	43.2550
LF power normalized	none	5	.39960	.226299	.101204
	yes	5	.26500	.171991	.076917
HF power normalized	none	5	.44500	.176688	.079017
	yes	5	.51820	.176753	.079046
LHR	none	5	1.3560	1.60435	.71749
	yes	5	.6840	.75132	.33600

Table 7-4 HRV metrics for nosocomial infection

LF low frequency; HF High Frequency; LHR low high frequency ratio

7.6 Discussion

As previously noted by other groups there is a striking loss of HRV in paediatric critical illness. In this paired sample no differences were seen in LF metrics to differentiate sterile SIRS from sepsis. Neither was there a difference in LF metrics between those children who went on to develop a nosocomial infection and those who did not.

The strength of this study is the matching of severity of illness and age to reduce confounding. However, there still remain the small number of children, the different ventilatory requirements, sedation and inotropic medication that may have caused this study to be underpowered.

Given the new data on the importance of vagal mediated dampening of systemic inflammation in sepsis, the significantly higher normalised HF in sterile SIRS vs. sepsis was not expected. One reason might be that the HF component is a combination of patient driven and ventilator modulation. Different ventilatory requirements in the groups may have caused this difference. This is compounded by the occurrence of first order and sometimes second order harmonics in the HF spectrum in some patients.

The data were analysed with conventional frequency domain methods. This metric was chosen to allow for comparison with work that had previously been done in critical care. Also, the small sample size would not allow for multiple testing had different types of analysis been added without an a priori hypothesis to test against. Finally, rather than comparison between groups, it may be more valuable to follow individual patients longitudinally in order to capture HRV changes that correlate with the development of sepsis. The data compiled from the sterile SIRS group showed

that all HRV metrics were lower in the group who developed infection. The group was small (n=5 in each group), therefore a larger cohort might be able to answer this question.

Chapter 8 Final discussion and future work

8.1 Introduction

This work originated from the following queries:

1) why do children differ in their response to similar infectious and inflammatory insults?
2) is genetic predisposition a factor underlying individual responses in critical illness?
3) might be possible to better define the complex inflammatory state known as the Systemic Inflammatory Response Syndrome (SIRS).

It has been long recognized that individuals (James, Nicol et al. 1932) and populations (Modiano, Petrarca et al. 1996) vary in their susceptibility to severe infectious disease. Part of this variation was found to be highly hereditable (Sorensen, Nielsen et al. 1988). The challenge remained to pinpoint the specific genetic differences that underlie this heritability. When the currently reported work started several genetic association studies had been published in the domain of innate immunity and sepsis/SIRS. These studies were typically small and at risk for being underpowered. None of these studies had addressed paediatric systemic inflammation. Most studies had only assessed a single gene or single polymorphism in a very complex disease. This study was therefore conceived to reduce errors in study design and produce data that were pertinent to paediatric practice. Technology was only now available to achieve this because of simplification of the genotyping process. This permitted us to assess several polymorphisms simultaneously, within less time and at an affordable price.

This study also focussed on key pathways, rather than isolated genes. The thinking behind this approach was that given the complexity of systemic inflammation, it was unlikely that a single gene would modify outcome in critical illness in a way that we could measure. Chapter 5 illustrates the concept of the additive effect of multiple genes. Here, polymorphisms in two genes in the complement pathway incrementally increased the risk for SIRS. Conversely, as described in Chapter 4, the multi gene endotoxin recognition transmembrane complex did not modify risk for SIRS, but polymorphisms in the single gene TLR4 were associated with admission platelet count. Assessing the risk that small changes may confer in a complex system very much depends on the relationship between those seemingly small differences and the outcome of interest. The complement genes seemed to confer a linear risk to SIRS, whereas the TLR4 polymorphisms may have identified a weak point in terms of redundancy. Platelets in themselves, or as part of the larger systemic inflammatory response, may not be able to maintain responsiveness, other than at the cost of reduced numbers.

Suggested future work includes specific mechanisms for TLR4 mediated platelet activation and their role in systemic inflammation (Smith and Weyrich 2011), and containment of microbes such as NETs (Clark, Ma et al. 2007).

8.2 Probing the genetics of the host response to infection and injury

Many studies have tried to identify specific genes and variation in gene function to try to answer what the genetic variation actually is that underlies hereditary differences in response to microbes. The candidate gene approach, based on either locus identification from family studies or based on known roles of certain genes in the disease process has proven to be disappointing.

Very few, if any, polymorphic genes have been identified confidently as contributors to sepsis and SIRS. The reasons for this are many fold, not least because of methodological concerns. (Little, Higgins et al. 2009) We were able to limit the impact of some of these issues in the current study, but a number remain.

The population we studied was heterogeneous in terms of demographics (ethnicity, age, gender), but also in type of insult (injury, infection, causative organism) and pre-morbid condition (deprivation). Nonetheless, the cohort may have been large enough to be able to stratify for most of these potential confounders and still be able to identify genetic risk. The genetic risk factor however would have to have exerted a sufficiently large effect and prevalence.

Genome Wide Association Studies (GWAS) are a method to overcome some of the above-mentioned concerns. There is no burden of a priori (and potentially wrong) mechanistic hypotheses. The approach allows for many hundreds of thousands to millions of SNPs to be investigated at the same time. Conversely, statistical methods need to be robust to safeguard against false positive results. When the current study was conducted, this approach was still in its infancy. There is now more evidence that GWAS may aid understanding susceptibility to infectious disease. A GWAS in n = 7522 individuals found that two SNPs in the CFH and CFHR3 were protective against meningococcal disease. (Davila, Wright et al. 2010) The two SNPs were in complete LD with each other, but not with the functional CFH polymorphism previously associated with age related macular degeneration and in this study (Chapter 5) shown to be protective for SIRS.

Studying individual and families who have rare monogenic defects may be yet another way of better understanding of infectious and inflammatory processes. Whole

exome sequencing is currently used to identify the molecular basis for hitherto unexplained primary immune deficiencies. (Hambleton, Salem et al. 2011)

Primary immune deficiency has traditionally been defined as highly penetrant single gene mutations leading to severe and diverse childhood infections. This concept is changing to a broader understanding, in part due to identification of polymorphisms in immune-related genes that lead to susceptibility or protection against a very narrow range of pathogens in otherwise healthy individuals.(Casanova and Abel 2007)

One such example is the TLR–nuclear factor-κB (NF-κB) signalling pathway. Genetic defects downstream in this pathway include interleukin-1 receptor-associated kinase-4 (IRAK4) deficiency, myeloid differentiation primary response gene 88 (*MYD88*), NF-κB essential modulator (NEMO; also known as IKBKG, which encodes the I- κB kinase regulatory subunit IKKγ) and NFKBIA (which encodes the IκBα inhibitor of NF-κB). Children with IRAK4 and MYD88 defiency are very vulnerable to invasive pneumococcal infections, but with time there is improvement. (Picard, von Bernuth et al. 2010) The NEMO and NFKBIA deficiencies have a more severe phenotype, presumably because of an interruption of multiple innate and adaptive pathways. The hosts are more susceptible to not only encapsulated bacteria, but also atypical mycobacteria, fungi and viruses. Reviewed in (Chapman and Hill 2012).

These mutations are very rare, but gene association studies have identified common polymorphisms in this pathway that were associated with a modified susceptibility to infectious diseases. People with the Mal polymorphism were protected from invasive pneumococcal disease, malaria, bacteraemia and tuberculosis.(Khor, Chapman et al. 2007)

Subsequently to this study it was found that heterozygote carriers for the 180L allele were at lower risk of septic shock and showed an intermediate pro inflammatory cytokine response.(Ferwerda, Alonso et al. 2009)

These insights into modulation of susceptibility and response to pathogens may lead to more specific treatment of individuals with sepsis. Risk stratification based on innate capacity to clear pathogens might inform antibacterial strategy, for instance in treatment and prevention of nosocomial infection. Large sepsis trials may be stratified according to innate inflammatory response capacity, thus preventing harm by enrolling a mixed population by not including those that would be potentially harmed by an intervention. The challenge remains to not only identify these key pathways and genes, but also the appropriate timing and population.(Grau and Maennel 1997)

Both rare and common polymorphisms in key pathways and genes have been shown to modify susceptibility to invasive pneumococcal disease (TLR–NF-κB pathway) and meningococcal disease (CFH gene).

In contrast this has not been found for another pillar of innate immunity, TLR4. This gene harbours both rare and common polymorphisms. Rare polymorphisms were identified as a risk factor for susceptibility to severe meningococcal disease, whereas common polymorphisms were not.(Smirnova, Mann et al. 2003)

Performing whole exome sequencing in individuals with extreme phenotypes is one way of identifying these key genes and pathways. These extreme phenotypes may well be found in the intensive care unit. Therefore a large programme that will collect DNA samples as well as a detailed phenotype and environmental description would inform targeted interventions in what is currently ineptly named sepsis or SIRS.

8.2.1 Whole genome sequencing

The most recent advances in whole genome analysis have shown that variation between humans depends not only on common single nucleotide polymorphisms, but also on copy number variation, rare variants (0.1% present in a population), epigenetic regulation, variability in transcription sites, and distance regulation. (Genomes Project, Abecasis et al. 2010, Mills, Walter et al. 2011, Consortium, Dunham et al. 2012, Djebali, Davis et al. 2012, Genomes Project, Abecasis et al. 2012, Sanyal, Lajoie et al. 2012). On average, each person might harbour approximately 250 to 300 loss-of-function variants and 50 to 100 variants that have been associated with inherited disorders. (Genomes Project, Abecasis et al. 2010) In addition, genes may overlap and have multiple beginnings and ends. Such is the variation of regulation in transcription, that the term gene may have to be redefined.(Djebali, Davis et al. 2012)

8.3 Signal to Noise ratio

8.3.1 Replication in genetic studies

This study has shown associations between biological plausible candidate genes and conventional outcome measures. There remains, however, the issue of replication. The replication cohort for the TLR4 study described in Chapter 4 was unconventional since both age and type of insult were different. The complement study in Chapter 5 had no replication cohort at all. False positive results, therefore, cannot be excluded. Nonetheless, corroborative evidence that complement may be a key variant in host response in acute severe infection has emerged since this study was performed.(Davila, Wright et al. 2010)

8.3.2 Environmental variation

All clinical studies suffer from heterogeneity introduced by treatment and logistics. This is especially so in diseases in which there is no stringent treatment protocol. In this study this was compounded by the issue that children were admitted with a variety of diagnoses, time-lines to admission to PICU and from a multitude of healthcare areas. This alone may have introduced elements into the study that have nothing to do with the host per se or their disease, but with access to healthcare, logistics, and quality of health care provision. This may have induced such a heterogeneity in case mix that a weak signal in genetic predisposition may have been lost.

The phenotypic description was extensive, but lacked details on pre-admission data regarding duration of illness prior to admission, and timing and appropriateness of interventions such as fluid resuscitation, inotropic support, oxygenation and timely appropriate antibiotics. That these issues matter has been shown clinically (Rivers, Nguyen et al. 2001) (de Oliveira, de Oliveira et al. 2008) (Maitland, Babiker et al. 2012) (Kumar, Roberts et al. 2006) as well as influencing the immunological profile (Rivers, Kruse et al. 2007). The differences in outcome may not have been entirely because of the intervention per se, but because of different circumstances that arose from the intervention. This risk of attribution error (Ross 1977) occurs in all clinical studies, because of an inherent change in circumstance. Maybe the reduction in mortality in the first early goal directed therapy trial in the emergency department (Rivers, Nguyen et al. 2001) was not the algorithm that was used, but the expert bedside-attending who administered it. This may explain why his results were not clearly replicated in subsequent independent studies, although we are still awaiting

data from Australasian Resuscitation In Sepsis Evaluation Randomised controlled Trial (ARISE), ClinicalTrials.gov Identifier NCT00975793, the North American Protocolized Care for Early Septic Shock (ProCESS) ClinicalTrials.gov Identifier NCT00510835 and the UK Protocolised Management in Sepsis (ProMISe), UKCRN ID 9820.

We collected detailed clinical phenotype data from the time of intensive care admission. These data were collected to a high level standard. Data pertaining to the pre-admission period would not have been as accurate. Collecting detailed pre-PICU admission data would have required a complex multiple centre set-up. This was beyond the scope of the current study.

Regionalisation of retrieval services and an increasing number of dedicated paediatric emergency departments lead to data gathering for clinical research becoming more feasible.

This is due to the changing nature of organising research. There is a trend towards a more collaborative approach across departmental boundaries via such collaborations as the Paediatric Intensive Care Society Study Group (PICS-SG). In addition, the increasing acceptance of approaches such as deferred consent mean that it is possible to perform clinical research in time critical acute situations. This now applies not only in adult interventional trials (Investigators, Finfer et al. 2009), but also in English paediatric intensive care, e.g. the Catheter Infections in Children (CATCH) trial, ClinicalTrials.gov Identifier NCT01029717.

8.3.3 Description and Definition

A fundamental flaw in this study is the lack of discrete diagnoses, definitions and

scoring methods when studying sepsis and systemic inflammation. This is important because it is nigh on impossible to count reproducibly what one cannot define with discrimination. Case in point: recently it was demonstrated that the increase in sepsis-associated hospitalizations was offset by the concurrent decrease in hospital admissions with pneumonia. Coding differences over the years had introduced a false understanding.(Lindenauer, Lagu et al. 2012) Obviously both entities share communalities that might have led to spurious results in counting.

The current definitions of systemic inflammation and sepsis are derived from a consensus meeting held in 1991 by North American experts in adult critical care.(Bone, Sibbald et al. 1992) They aimed to describe sepsis to be better able to risk stratify and treat this phenomenon. The driver behind this initiative was the high incidence of failing organs and death. Ten years after the SCCM conference a second conference was held, this time it also included European researchers. The conclusion was that there was no additional evidence to change the 1992 definitions (Levy, Fink et al. 2003).

Boiling down this highly complex process to four markers of dysfunction is attractive because it allows a common language and understanding. The downside of lumping together these generic common features is the risk of ignoring important differences. Ignoring the route the patient took to end up in a classification group termed “sepsis” or “SIRS” ignores fundamental differences in pathophysiology. (Soni 2010) Many interventions in sepsis have failed. In part, this is a problem of definition.

Since 1992 we have a better understanding of the molecular basis of systemic inflammation and sepsis but we are no further in our capacity to differentiate the two at the bedside. We are still describing sepsis and SIRS rather than defining them. Rather than continue to “lump” we must also “split”.(Baue 2004)

Future work requires identifying specific causes for infection and specific host responses, not only cross sectional but also longitudinal. The success of interventions not only depends on the type of intervention, but also of timing and the state the patient is at that particular moment. Sepsis and systemic inflammation are dynamic processes. One example is fluid resuscitation. Early larger bolus fluid resuscitation allowed for less total volume and better outcome compared to smaller boluses over longer period of time.(Carcillo, Davis et al. 1991)

It is unhelpful to our patients to define rigidly systemic inflammation and sepsis in terms of dichotomous notions such as clotting/immunity, innate/adaptive; self/nonself; danger/nondanger; pro-inflammatory/anti-inflammatory. False dichotomies hinder creative thinking and potential new avenues in helping our patients. (Suffredini and Munford 2011)

The attempt to define sterile SIRS and sepsis by means of HRV was not successful. Future studies might focus on longitudinal studies to observe changes in variability that then can be correlated with infection and/or deterioration/recovery.

A critically ill patient may be seen as an entity with many dimensions and it should therefore be possible to assess their extent of similarity via multiple ways. The principle of purpose dependent ontology takes into account clarity of definition as well as purpose of definition.(Greenspan 2004)

The concepts of sepsis, SIRS, severe sepsis and septic shock require revisiting. Although convenient, it is now time to question and challenge this scheme. Twenty years have passed without this schema bringing discernible benefit to our patients.

A more useful way of classifying this group of patients might by their capacity to respond. Longitudinal studies have been instrumental in identifying a temporal change in inflammatory status. Profound immune depression is common in the course

of sepsis or injury. Immunoparalysis of monocytes (Docke, Randow et al. 1997) (Allen, Peters et al. 2002) and prolonged lymphopenia (Felmet, Hall et al. 2005) predict adverse outcome in critically ill children. Apoptosis of B and T cells (Tinsley, Grayson et al. 2003) is thought to be a key driver in the concept of the Compensatory Anti-inflammatory Response Syndrome (CARS) (Bone 1996).

Dichotomisation loses the complexity of critical illness. Rather than asking the question whether this patient is in SIRS, sepsis, severe sepsis, septic shock, or CARS maybe a more useful question is whether a patient is still robust enough to respond.

8.4 Complex systems, redundancy and resilience

The human body is continuously responding to stressors. In health, there is enough redundancy in the complex system to allow for these attacks. The capacity to maintain responsiveness is a core attribute of resilience. However, when overload has occurred with the consequence of critical illness, there is less capacity to deal with ongoing or new threats.(McEwen and Wingfield 2003) This may manifest itself as multiple organ dysfunction. Differences in initial response capacity may influence subsequent outcomes. People who have an initial vigorous capacity to respond may benefit in infection because they eliminate the pathogen swiftly, e.g. complement activation capacity (Chapter 5 and Chapter 6) Conversely, people who respond vigorously to injury may induce exaggerated longer term sequelae in immunosuppresion. (Woiciechowsky, Asadullah et al. 1998)The disappointments in trying to block “pro-inflammatory” compounds have lead to trials intervening by immunostimulation. Glutamine supplement(Ong, Eaton et al. 2012) was successful in neonates to reduce sepsis, GM-CSF (Hall, Knatz et al. 2011) reversed immunoparalysis as measured by HLA-DR but the combination of zinc, selenium, glutamine, and metoclopramide did

not reduce nosocomial infections in a paediatric critical cohort (Carcillo, Dean et al. 2012).

Although these trials focus on immunomodulation by increasing response capacity the paradigm of pro- and anti-inflammatory is not touched upon.

Small difference in initial state may have profound impact on outcome. This is a key understanding in the behaviour of complex systems.(Lorenz 1963) This non-linear behaviour might underpin the large effect some polymorphisms exert in the acute phase response, especially if they reside in an area without redundancy to overcome the disadvantage. The TLR4 study in Chapter 4 may have illustrated the concept of redundancy. There is evidence that TLR4 is directly involved in platelet activation. (Berthet, Damien et al. 2010) This, in combination with platelets' reduced capacity to maintain counts during severe acute inflammatory disease (Peters, Ross-Russell et al. 2001) may have led to identification of a fragile part in the acute host response.

Methods to capture resilience in critical care have not been developed yet. Loss of complexity may manifest itself in rhythms that are more monotonous. In critical illness monotony and decomplexification associated with worse outcome has been measured in T cell repertoire, serum glucose levels and most extensively in heart rate. Reduced heart rate variability measures were associated with worse organ failure and mortality in critically ill adults (Schmidt, Werdan et al. 2001) and children (Tibby, Frndova et al. 2003), and susceptibility to sepsis in immunocompromised adults (Bravi, Green et al. 2012). The concept of complexity and allostasis has only recently been introduced to the intensive care literature. (Cuesta and Singer 2012)

Future work might encompass assessing resilience using an HRV metric. The dataset used in this study might be used to that end by ways of complexity metrics rather than frequency analysis. More ambitious goals include performing interventional studies to

restore health by targeting regularity such as adding noise to mechanical ventilation (Graham, Gulati et al. 2011) and cardio pulmonary bypass (Graham, Warrian et al. 2002).

8.5 Conclusion

This thesis has added to the understanding of the acute phase response in critically ill children.

Genetic predisposition to systemic inflammation was identified in the complement activation cascade. This may lead to interventions increasing an individual's capacity for complement activation.

The role of TLR4 in platelet participation in the acute phase response was identified, which may allow for avenues to harness TLR4 mediated platelet activation in the acute phase response.

A better definition of systemic inflammation in the presence or absence of infection is still required. Physiological systems analysis did not generate definitive answers, but may have identified an area worth pursuing: that of longitudinal intra patient change in heart rate variability as a measure of robustness.

This thesis supports the concept that in children control of an inflammatory threat is aided by a vigorous capacity to respond.

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Appendices

Manuscripts that have been written based on the work done in this thesis.