The influence of Mannose-Binding Lectin polymorphisms in children undergoing cardiopulmonary bypass surgery

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Abstract

Genetic factors may influence the outcome from surgery. Mannose-Binding lectin (MBL) is an important factor in innate immune defense. MBL gene polymorphisms result in deficiency of the encoded protein and increase susceptibility to infection. The objective of this study was to investigate the relationship between *MBL-2* exon 1 polymorphisms and outcome of children after cardiopulmonary bypass (CPB) surgery. Two hundred and forty four patients were recruited to this study. Patient's *MBL-2* genotype was determined and compared with respect to sepsis development, length of stay in intensive care and duration of mechanical ventilation. The exon 1 polymorphisms were more common in the patients with sepsis compared to the non-sepsis group (36% vs. 47%). It was observed a higher proportion of *MBL-2* variant alleles in the patients who required prolonged stay compared to the short stay group (38% vs. 51%). Similarly, *MBL-2* variant alleles were more common in those who required prolonged ventilation compared to those who required less ventilation (33% vs. 50%). Three was a significant association between *MBL-2* genotype and the duration of ventilation (p = 0.033). The data from this study showed that *MBL-2* exon 1 polymorphisms may play an important role in the outcome of children undergoing surgery.

List of Tables

List of Figures

Abbreviations

APS	Ammonium Persulfate solution
CICU	cardiac intensive care unit
CHD	Congenital heart disease
CPB	Cardiopulmonary Bypass
CRD	C-terminal carbohydrate-recognition domain
DNA	Deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
h	hours
IL-1	interleukin-1
IL-6	interleukin-6
IL-10	interleukin-10,
MAC	Membrane attack complex
MASPs	MBL-associated serine proteases
MBL	Mannose-binding lectin
$MgCl_2$	Magnesium chloride
min	minutes
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PRRs	Pattern-recognition receptors
SIRS	Systemic inflammatory response syndrome
TBE	Tris-Borate-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
TNF- α	tumor necrosis factor-α
UHG	Universal heteroduplex generator

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1. Introduction

1.1. Congenital heart disease

Congenital heart disease (CHD), in a definition proposed by Mitchell et al. (Mitchell, Korones, & Berendes 1971), is "a gross structural abnormality of the heart or intrathoracic great vessels that is actually or potentially of functional significance". CHD is the most common of all major birth defects and the incidence of moderate and severe forms of CHD is about 6/1000 births (Table 1). However, this figure has been rising steadily until recently when incidences of 12 to 14/1000 live births, have been reported in the literature (Hoffman & Kaplan 2002)

Table 1.1. Incidence of common type of CHD per Million live births (Adapted from Hoffman & Kaplan 2002)

Cardiac Lesion	Number of studies
Ventricular septal defect	43
Patent ductus arteriosus	40
Atrial septal defect	43
Atrioventricular septal defect	40
Pulmonic stenosis	39
Aortic stenosis	37
Coarctation of the aorta	39
Tetralogy	41
Complete transposition of great arteries	41
Hypoplastic right ventricle	32
Tricuspid atresia	11
Ebstein's anomaly	5
Pulmonary Atresia	11
Hypoplastic left ventricle	36
Truncus	30
Double outlet right ventricle	16
Single ventricle	23
Total anomalous pulmonary venous connection	25
All cyanotic	37
All CHD	43
Bicuspid aortic valve	10

Over the past two decades, medical and surgical advances have dramatically increased the survival rates of patients with CHD. In particular paediatric cardiac surgery has advanced with the development of the cardiopulmonary bypass (CPB) technique.

1.2. Development of Cardiopulmonary Bypass

In 1953, John Gibbon performed the first successful open heart surgery in the world using a heart-lung machine to repair an intracardiac defect. This achievement stimulated rapid development of the knowledge base and equipment necessary for accurate diagnoses and successful intracardiac operations (Edmunds, Jr. 2002). However, subsequent attempts to correct congenital heart defects were met with high morbidity and mortality rates. In 1971, Barrat-Boyes reported: "Unfortunately, until recently, the cardiac surgeon has been unable to provide safe corrective surgery in the first year of life". This year, the situation was altered with the introduction of a new method by a team of Japanese surgeons. The new technique employed hypothermic circulation arrest and when utilised during intracardiac repair appeared to be highly satisfactory in infancy, regardless of age or weight or severity of symptoms (Barratt-Boyes 1971).

1.2.1. The CPB surgery

In CPB the Heart-Lung Machine (HLM) is used to "replace" the heart and lungs during surgery. This device is commonly used in heart surgery because of the difficulty of operating on the beating heart. Operations requiring the opening of the chambers of the heart require the use of the HLM to support the circulation during that period. The HLM is composed of two main functional units, the pump (roller pump or centrifugal pump) and the oxygenator. In the roller pump system, a console usually comprised of several

rotating motor-driven pumps, peristaltically "massage" tubing made of silicone rubber, or PVC. This action gently propels the blood through the tubing. Many HLMs now employ a centrifugal pump for the maintenance and control of blood flow during CPB. By altering the speed of revolution (RPM) of the pump head, blood flow is produced by centrifugal force. This type of pumping action is considered to be superior to the action of the roller pump by many because it is thought to produce less blood damage (hemolysis, etc.). The oxygenator is a device designed to transfer gas to and from the blood. Today's oxygenators are made of materials that allow gas diffusion across a membrane (polypropylene, silicone, etc.) This allows for oxygenation of de-oxygenated blood and removal of CO_2 from the venous blood (Figure 1.1) (wikipedia website: http://en.wikipedia.org/wiki/Heart-lung_machine).

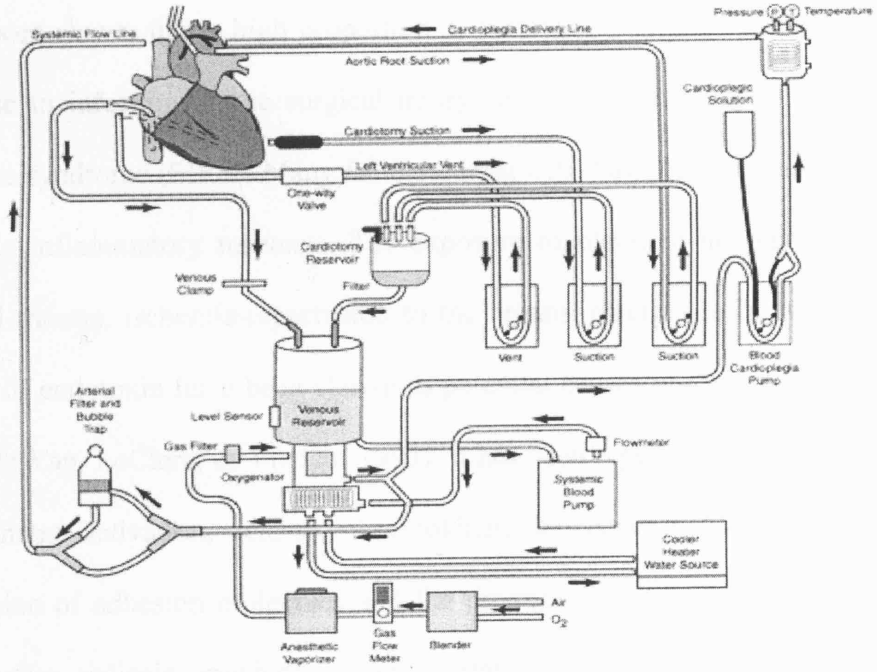

Figure 1.1. Components of cardiopulmonary bypass (wikipedia website: http://en.wikipedia.org/wiki/Heart-lung_machine).

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1.2.2. CPB and outcome

There has been a progressive reduction in the mortality associated with repair of congenital heart defects. Data from a study performed in the United Kingdom in 2000-1 revealed 91.8% survival after CPB surgery in children (Gibbs et al. 2004). While the majority of the patients recover promptly from open heart surgery, a small group of children have persisting ventilatory requirements and require prolonged intensive care, which itself is associated with significant mortality and morbidity (Allen et al. 2002;Brown et al. 2003).

1.2.3. The inflammatory response to CPB

It has been shown that a high proportion of patients develop a systemic inflammatory response to infection or the surgical injury, which is termed systemic inflammatory response syndrome (SIRS). Many factors during CPB have been assumed to lead to this systemic inflammatory response. The exposure of blood to nonphysiologic surfaces, surgical trauma, ischemia-reperfusion to the organs, changes in body temperature and release of endotoxin have been shown as potential causes of this syndrome (Fransen et al. 1998;Wan, LeClerc, & Vincent 1997). It has been reported that these factors induce complement activation, release of cytokines, leukocyte activation along with the expression of adhesion molecules and the production of various substances including oxygen-free radicals, arachidonic acid metabolites, platelet-activating factor (PAF), nitric oxide (NO) and endothelines (Figure 1.2) (Wan, LeClerc, & Vincent 1997).

Many studies have been focused on the pro-inflammatory response to CPB however more recent work has been shown that an anti-inflammatory response may also be initiated during and after CPB. Current understanding suggests that a balance between the pro-inflammatory and anti-inflammatory response is important for outcome following CPB surgery (McBride et al. 1996;Zeni, Freeman, & Natanson 1997).

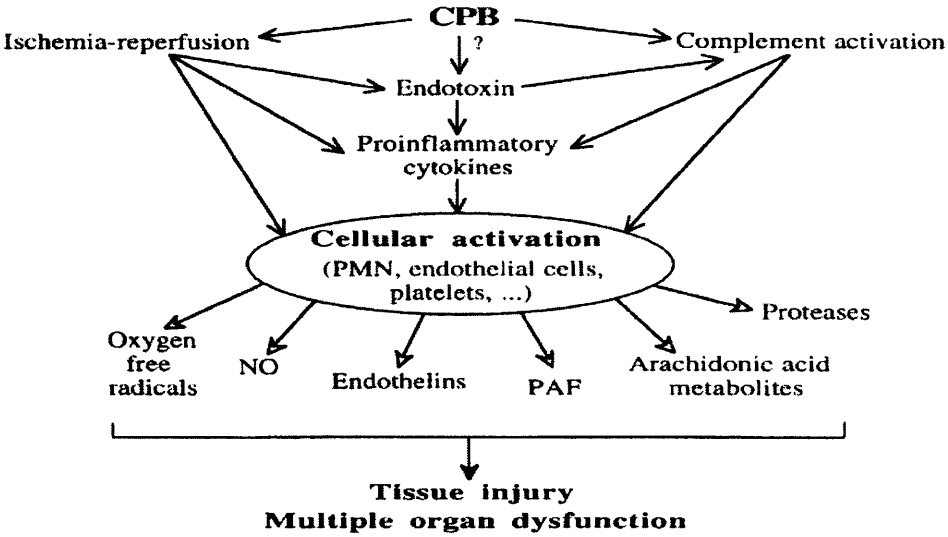


Figure 1.2. A schematic representation of the inflammatory response to CPB (Adapted from Wan, LeClerc, & Vincent 1997).

1.2.4. CPB and sepsis

SIRS and sepsis are the major causes of morbidity and mortality in intensive care units (ICUs) (Angus & Wax 2001). Mortality from sepsis is often attributable to a dysfunctional innate immune response with recurrent episodes of sepsis and/or

excessive systemic inflammation (Oberholzer, Oberholzer, & Moldawer 2001). However, there have been many controversies regarding the definition of SIRS and sepsis. In August 1991, a consensus conference of the American College of Chest Physicians and the Society of Critical Care Medicine developed common definitions to clarify the terminology used to describe the spectrum of disease that results from severe infection. The definitions discussed in this conference are shown in Figure 1.3 (Robertson & Coopersmith 2006).

Systemic Inflammatory Response Syndrome (SIRS): An inflammatory response to a wide variety of clinical insults manifested by two or more of
- temperature >38°C or <36°C
- heart rate >90
- respiratory rate >20 or $PaCO_2$ <32 mm Hg
- WBC count >12K or <4K, or >10% immature (band) forms.

Sepsis: SIRS caused by infection

Severe Sepsis: sepsis with at least one organ dysfunction or hypoperfusion

Septic Shock: severe sepsis associated with hypotension that is resistant to adequate fluid resuscitation

Bacteremia: the presence of viable bacteria in the blood stream

Multiple Organ Dysfunction Syndrome (MODS): impairment of two or more organ systems in an acutely ill patient where homeostasis cannot be maintained without therapeutic intervention

Figure 1.3. Consensus definitions of a spectrum of clinical entities that result in organ failure (Adapted from Robertson & Coopersmith 2006).

Despite an knowledge of the inflammatory response to sepsis there is still lack of effective therapies targeting the inflammatory response to sepsis (Holmes, Russell, & Walley 2003). More recently, genetic factors have been recognised as important elements in the inflammatory response which may be associated with susceptibility to and outcome from sepsis (De, Torres, & Reeves 2005). Much of the work has been focused on the effect of various inflammatory cytokines polymorphisms, such as tumor necrosis factor-α (TNF- α), interleukin-1 (IL-1), interleukin-6 (IL-6) and interleukin-10

(IL-10), in the outcome from sepsis and surgery (Holmes, Russell, & Walley 2003). In addition, it has also been reported that polymorphisms in the mannose-binding lectin gene (MBL), are associated with the development of sepsis and fatal outcome in adult and paediatric patients (Fidler et al. 2004;Garred et al. 2003;Gordon et al. 2006). The role of MBL and its importance for innate immune defense, the genetic polymorphisms and the plasma levels of the encoded protein will be discussed in the following sections.

1.3. The Immune System

Living organisms have strong defence mechanisms against invading microorganisms as survival strategies. The immune system collaborates in recognition and elimination of pathogens as a part of both the innate and adaptive immune systems. The innate immune system is an evolutionarily ancient form and crucial for the first line of defence, which was thought to be a non-specific immune response characterised by phagocytosis. It is becoming increasingly apparent that the innate immunity has considerable specificity and is capable of discriminating between pathogens and self and permits an immediate response (Endo, Takahashi, & Fujita 2006). The adaptive immune system includes the generation of specific antibodies via the process of clonal selection that not only targets antigens unique to the pathogen, but also retains the immunological memory of the antigen, thereby preventing reinfection. This system is highly specific, however the response to foreigner antigens is slow, since it takes three to seven days before clonal selection and expansion of lymphocytes occurs (Medzhitov & Janeway, Jr. 1998)

1.3.1. Innate immune system

Unlike the adaptive immune response the innate response does not recognise every antigen it encounters. Instead, it relies on a well-defined, limited unchanging pool of germline-encoded pattern-recognition receptors (PRRs) that can bind highly conserved structures present on pathogens known as pathogen-associated molecular patterns (PAMPs). Recognition of PAMPs involves recognition of carbohydrates on the surfaces of bacteria, fungi, viruses and protozoans which are distinct from the carbohydrates found in eukaryotes, thereby facilitating the discrimination of self from non-self. Once the PRRs recognise the corresponding PAMP on a pathogen, they immediately trigger effector cells to destroy the invading microorganism (Takahashi et al. 2006).

1.3.1.1. Pattern Recognition Receptors

PRRs are expressed on many effector cells of the innate immune system including macrophages, neutrophils and dendritic cells or are secreted in the serum (Beutler 2004). PRRs can be divided into three classes: signalling, endocytic and secreted. Secreted PRRs function as opsonins by binding to microbial cell walls and tagging them for recognition by the complement system and phagocytes (Medzhitov & Janeway, Jr. 2000). One of the best characterised secreted receptor is MBL. MBL not only binds to microbial carbohydrates (mannose and N-acetylglucosamine sugar motifs) to initiate the lectin pathway of complement activation, but also binds phospholipids, nucleic acids and non-glycosylated proteins (Estabrook et al. 2004;Kilpatrick 1998;Medzhitov & Janeway, Jr. 2000).

1.3.2. The complement system

The complement system plays a major role in host defence involving both the innate and adaptive immune system. It consists of a large number of plasma proteins that react with infectious pathogens to mark them for destruction by phagocytes and induce a series of inflammatory responses that help to fight infection. Approximately one-third of these proteins are directly involved in one of three enzyme cascade pathways, while the remaining proteins have regulatory functions. The complement cascade can be activated through three distinct pathways named the classical, alternative and lectin pathway. These pathways depend on different molecules for their initiation, but they converge to generate the same set of effector molecules, resulting in the opsonization of pathogens, the recruitment of inflammatory cells and direct killing of pathogens via the membrane-attack complex (MAC), which creates a pore in the cell membranes of some pathogens that can lead to their death (Walport 2001).

1.3.2.1. The activation pathways

The classical pathway can be initiated by the binding of C1q (a protein belonging to the collectin family) the first protein in the complement cascade, directly to the pathogen surface or during an adaptive immune response by the binding of C1q to antibody:antigen complexes, which is a link between the effector mechanisms of innate and adaptive immunity (Endo, Takahashi, & Fujita 2006). The activation of the alternative pathway can be initiated when a spontaneously activated complement component binds to the surface of a pathogen occurring in the absence of specific antibody (Walport 2001). Recently discovered, the lectin pathway is activated following the recognition of PAMPs by either MBL or ficolins, and the subsequent activation of

associated enzymes, MBL-associated serine proteases (MASPs) that have been termed MASP-1, MASP-2 and MASP-3 and a protein with no protease activity sMAP or MAP19 (Fujita 2002;Garred et al. 2006).

1.3.3. MBL and the complement system

The lectin pathway is activated by the binding of MBL or ficolins (group of proteins containing both a collagen-like and fibrogen-like domain) associated with MASPs to the microbial surface. Engagement of ligand by MBL activates MASP-2, which then cleaves C4 and C2 proteins of the complement to generate a C3 convertase from C2b bound to C4b (Figure 1.4). C3 convertase activity is essential in complement activation, representing the point of convergence of all three complement pathways and from which the amplified final common pathway of complement activation occurs. This enzyme initiates the cascade by cleaving the C3 protein into large amounts of C3b and C3a (Walport 2001). It has also been reported that MASP-1 can directly cleave C3 (Matsushita et al. 2000). C3a, together with downstream products, such as C5a, is a mediator of inflammation and phagocyte recruitment. C3b is the principal effector molecule of the complement system. It opsonizes pathogens by binding to their surfaces and targeting them for internalisation and destruction by phagocytes bearing C3b receptors. C3b also promotes the generation of C5b, a peptide fragment that initiates the late event of complement activation, the assembly of the MAC (Walport 2001).

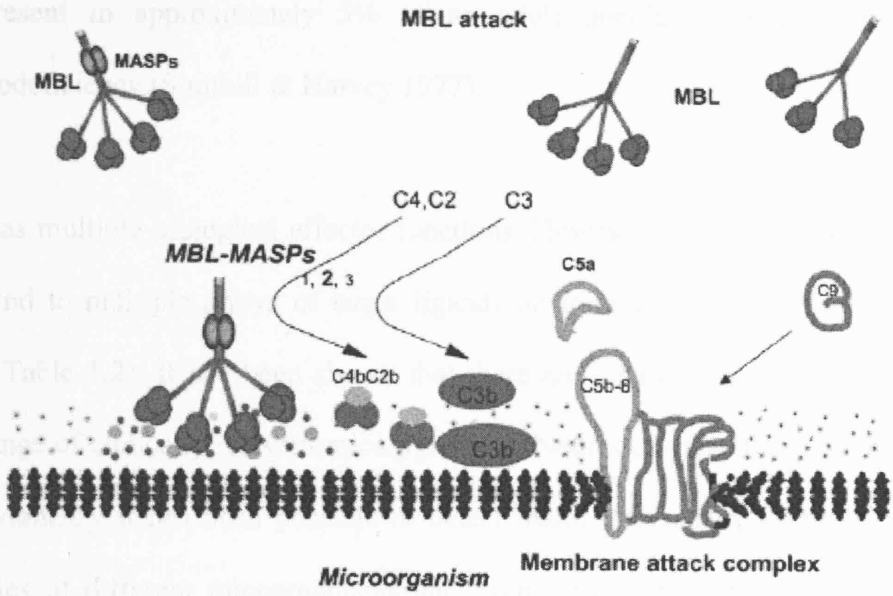


Figure 1.4. The lectin pathway of complement. MBL associated with MASPs binds to arrays of mannose groups on the surface of the microorganims and mediating the formation of the membrane attack complex (Adapted from (Garred et al. 2006).

1.3.4. MBL and the immune system

The contribution of MBL to the immune system is not limited to complement activation. The mechanisms responsible for MBL interactions with inflammatory pathways are still largely unknown. However, it has been shown that there are MBL receptors on phagocytic cells (Tenner, Robinson, & Ezekowitz 1995a), may function as an opsonin (Kuhlman, Joiner, & Ezekowitz 1989) and modulate cytokine production (Soell et al. 1995).

The opsonic defect was first demonstrated in 1976 when low serum levels of MBL were associated with opsonic defects in children experiencing repeated, unexplained infections and failure to thrive. Serum from these children failed to opsonize the yeast *Saccharomyces cerevisiae* with C3 (Soothill & Harvey 1976). A similar opsonic defect

was present in approximately 5% of an adult population without any obvious immunodeficiency (Soothill & Harvey 1977).

MBL has multiple biological effector functions. However primarily to that its function is to bind to multiple arrays of sugar ligands on microbial surfaces (refer to section 1.3.1) (Table 1.2). It has been shown that there are interactions between MBL and a wide range of clinically relevant micoorganisms. Neth *et al.* have reported that by using flow cytometry it has been possible to detect major differences in the MBL binding capacities of different micoorganisms and even differences among organisms of the same genus (Neth et al. 2000).

Disease association studies have shown that MBL deficiency is associated with increased risk of infections during early childhood, in immunocompromised patients and is a risk factor for critically ill patients to develop sepsis (Garred et al. 2003;Koch et al. 2001). In addition, low levels of MBL in autoimmunity have been related to diseases like systemic lupus erythematosus and rheumatoid arthritis (Garred et al. 2000;Garred et al. 2001)

Table 1.2. Selected microorganisms that have been shown to bind to MBL (Adapted from (Dommett, Klein, & Turner 2006)

	Reference
Bacteria	
Actinomyces israelii	Townsend et al. 2001
Bifidobacterium bifidum	Townsend et al. 2001
Burkholderia cepacia	Davies et al. 2000
Chlamydia pneumoniae	Swanson et al. 1998
Escherichia coli	van Emmeik et al. 1994
Haemophilus influenzae	Neth et al. 2000, van Emmeik et al. 1994
Kleibsiella aerogenes	Neth et al. 2000
Leptotrichia buccalis	Townsend et al. 2001
Listeria monocytogenes	van Emmeik et al. 1994
Mycobacterium avium	Polotsky et al. 1997
Mycoplasma pneumoniae	Hamvas et al. 2005
Neisseria meningitides	Neth et al. 2000, van Emmeik et al. 1994
Proprionibacterium acnes	Townsend et al. 2001
Pseudomonas aeruginosa	Davies et al. 2000
Salmonella montevideo	Kuhlman e tal. 1989
Staphylococcus aureus	Neth et al. 2000
Streptococcus pneumoniae	Neth et al. 2000
Viruses	
Influenza A	Saifuddin et al. 2000, Hart et al. 2002, Ji et al. 2005
Human immunodeficiency virus	Saifuddin et al. 2000, Hart et al. 2002, Ji et al. 2005
Herpes simplex 2	Fisher et al. 1994, Gadjeva et al. 2004
Severe acute respiratory syndrome	Ip et al. 2005
Fungi	
Aspergillus fumigatus	Neth et al. 2000
Candida albicans	Neth et al. 2000, Tabona et al. 1995
Cryptococcus neoformans	Schelenz et al. 1995
Protozoa	
Cryptosporidium parvum	Kelly et al. 2000
Plamodium falciparum	Klabunde et al. 2002
Trypanosoma cruzi	Kahn et al. 1996

1.4. Mannose Binding Lectin

MBL is a member of the collectin (collagen and lectin) family of proteins that consists of a collagen-like domain and a carbohydrate recognition domain (Holmskov, Thiel, & Jensenius 2003). It is produced in the liver and it responds as an acute-phase reactant (Thiel et al. 1992). Human MBL (*MBL-2*) is derived from a single gene located on chromosome 10 (Sastry et al. 1989b). Genetic polymorphisms are associated with low levels of the encoded protein (Madsen et al. 1995).

1.4.1. Structure

The MBL protein is a complex of triple helix structures and each polypeptide chain contains four domains: a 21 amino acid N-terminal cyteine-rich region involved in oligomerization by formation of inter and intra-subunit disulphide bonds; a 59 amino acid collagen-like domain consisting of 20 tandem repeats of Glycine-X-Y (where X and Y represent any amino acid) that account for the long stalk of the molecule; a 30 amino acid α-helical, hydrophobic neck region, which is crucial for initiating the oligomerization; a 188 amino acid C-terminal carbohydrate-recognition domain (CRD).

Human MBL seems to consist of oligomers, each with three identical polypeptide chains of 25 kD. The three polypeptides fold together to form the structural subunit and 3-6 of these subunits join to form a mature protein. MBL circulates in plasma as a mixture of dimers, trimers, tetramers, pentamers and hexamers (Dahl et al. 2001;Garred, Larsen, Seyfarth, Fujita, & Madsen 2006). The overall structure of MBL is shown in Figure 1.5.

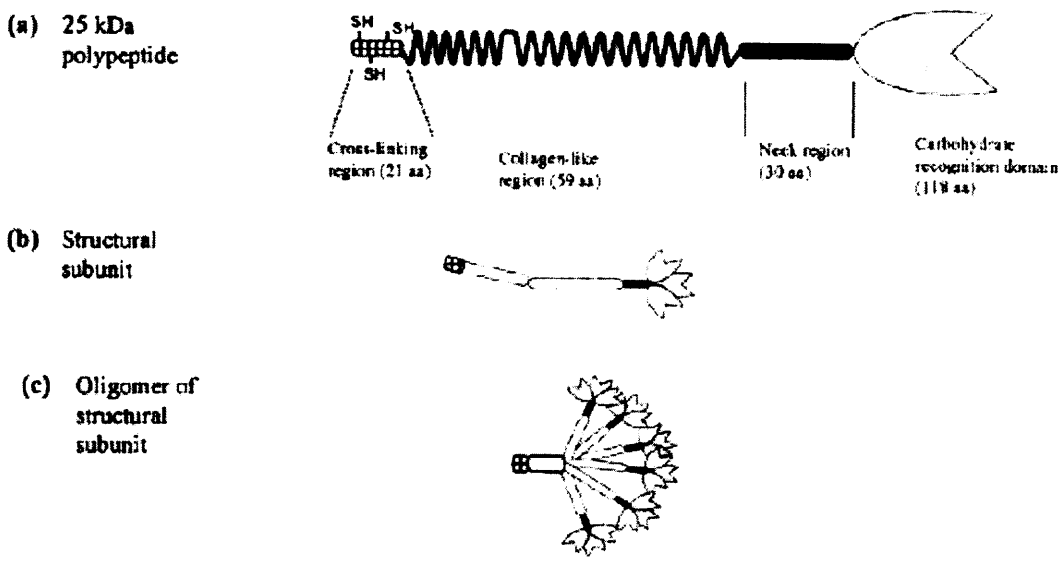


Figure 1.5. The 25 kD MBL polypeptide consisting of four domains and the oligomeric structure (Adapted from Presanis, Kojima, & Sim 2003).

1.4.2. Binding

Through its CRD, MBL binds carbohydrates with 3- and 4- hydroxyl groups in the presence of calcium in the MBL CRD (Weis, Drickamer, & Hendrickson 1992). MBL binds to mannose, glucose, fructose and N-acetylglucosamine whereas carbohydrates that do not have this geometry such as galactose and sialic acid, which are sugars of most mammalian glycoproteins, have undetectable affinity for MBL. This allows discrimination between self and non-self (Holmskov, Thiel, & Jensenius 2003).

1.4.3. Acute phase reactant

MBL is a serum protein mainly produced by hepatocytes. Recently it has been revealed that extra-hepatic transcription of human MBL-2 is taking place, especially in small intestine and testis tissue (Seyfarth, Garred, & Madsen 2006). MBL is known as an acute phase protein, its synthesis is induced in response to stress and inflammatory

stimuli (Ezekowitz, Day, & Herman 1988). Studies have shown that serum levels increase up to three fold during an acute phase response (Holmskov, Thiel, & Jensenius 2003). A study using the HuH-7 hepatocyte cell line revealed that MBL plasma levels, which are in part determined genetically, may also be influenced by growth hormone thyroid hormones (T3 and T4) and the IL-6 cytokine (Riis et al. 2005;Sorensen et al. 2006).

1.4.4. Complement and Modulation of inflammatory responses

MBL has a broad role in innate immunity, although its role is not limited to complement activation it is also a modulator of inflammation. There is evidence of direct interactions of MBL with phagocytic cells to promote phagocytosis and cytokine production (Soell et al. 1995;Tenner, Robinson, & Ezekowitz 1995b).

It has been shown that MBL modulates the profile of cytokines in response to a microbial signalling molecule, Lipopolysaccharide, including the diminution of the pro-inflammatory cytokines IL-1α and IL-1β and increased levels of the anti-inflammatory cytokine IL-10 (Fraser et al. 2006). Also, the addition of high concentrations of MBL to the blood of MBL-deficient donors decreased the production of IL-6, IL-1β and TNF- α, whereas lower concentrations increased the production of IL-6 and IL-1β (Jack et al. 2001).

1.5. Genetics

The structure of the functional human *MBL-2* gene was published in 1989. However, an expressed *MBL1* pseudogene has also been found (Guo et al. 1998). In mice, two different forms of MBL are encoded by two distinct functional genes known as *mbl-a* and *mbl-c*, which are positioned on chromosome 14 and 19, respectively (White et al. 1994).

Analysis of the *MBL-2* gene revealed that it is comprised of four exons and that it is located on the long arm of chromosome 10 at 10q11.2-q21(Sastry et al. 1989a). Exon 1 encodes the cysteine rich N-terminal domain and part of the collagen-like region and exon 2 encodes the remainder of the collagen-like region. Exon 3 encodes the α-helical coil neck region and exon 4 the carbohydrate binding domain. Exon 0 is not translated into protein. Upstream of the gene are the regulatory promoter elements (Figure 1.6) (Naito et al. 1999;Sastry et al. 1989a).

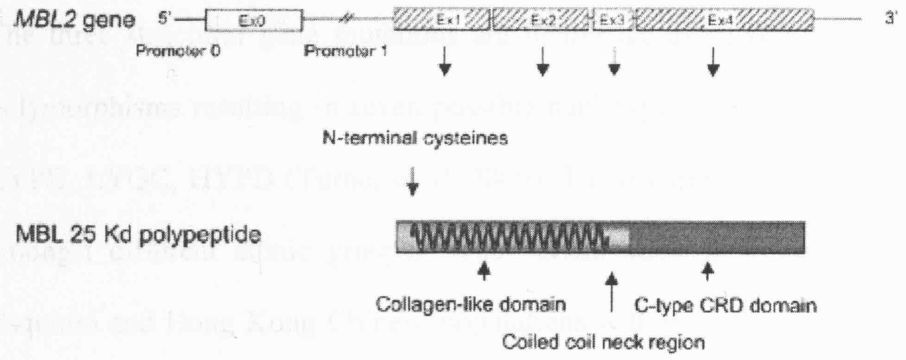


Figure 1.6. MBL-2 gene structure and the corresponding encoded MBL polypeptide regions (Adapted from (Garred, Larsen, Seyfarth, Fujita, & Madsen 2006).

1.5.1. MBL-2 polymorphisms

Low levels of MBL in circulation are strongly associated with the presence of three point mutations in exon 1. Such mutations are located in codons 54 (allele B), 57 (allele C), and 52 (allele D), which are termed B, C and D variants, respectively. The three variant alleles collectively are termed O with A representing the wild-type allele (Lipscombe et al. 1995;Madsen et al. 1994). Both alleles B and C result in the substitution of a glycine to aspartic acid and glutamic acid, respectively. A nucleotide substitution in allele D causes exchange of an arginine residue with a cysteine. The three single point mutations compromise the assembly of functional MBL oligomers. In addition, three polymorphisms in the promoter region of the *MBL-2* gene also affect the expression of MBL. The H/L, X/Y and P/Q are located at the positions -550, -221 and +4, respectively. Of these the X/Y variant profoundly influences the expression of the protein (Madsen et al. 1995).

The three structural gene mutations are in linkage disequilibrium with the promoter polymorphisms resulting in seven possible haplotypes: HYPA, LYQA, LYPA, LXPA, LYPB, LYQC, HYPD (Turner et al. 2000). The occurrence of these haplotypes varies amongst different ethnic groups. The variant allele B is common in Caucasoids, Esquimo and Hong Kong Chinese populations with gene frequencies ranging between 0.11 and 0.17 (Lau et al. 1996;Lipscombe et al. 1996). The mutant allele C is found almost exclusively within populations from Africa with a frequency reaching 0.29 (Sullivan et al. 1996). The allele D is the least common of the structural mutations and is present in both Black and Caucasoid populations, with gene frequencies of 0.05 in both populations (Madsen et al. 1994).

In this study, as in the majority of the published studies, only exon 1 mutations and X/Y promoter polymorphism were analysed.

1.5.2. MBL levels in serum

Humans show considerable variations in the circulating MBL levels from 5 ng to more than 10 μg/ml, but the level of MBL in each individual is quite stable throughout life. Individuals heterozygous for these polymorphisms (A/O) have reduced levels of MBL, whereas in those who are homozygous or compound heterozygous (O/O) circulating MBL is almost absent (Thiel, Frederiksen, & Jensenius 2006;Turner 1996). Genotype has the largest influence on circulating MBL levels. However Crosdale et al reported that not all low-level MBL producers could be linked to the presence of specific haplotypes. This indicates that other factors may also contribute to the levels of circulating MBL (refer to section 1.4.2) (Crosdale et al. 2000).

1.6. Statement of the Aims

The mortality rate for children undergoing open heart surgery is now less than 5% and the majority recover rapidly. However, a small group of children have persisting ventilatory requirements and require prolonged intensive care reasons for which remain unknown (Allen et al. 2002). The presence of *MBL-2* variant alleles and low serum MBL levels have been associated with the development of sepsis and fatal outcome in adult patients admitted to intensive care and in children with SIRS (Fidler et al. 2004;Garred et al. 2003;Gordon et al. 2006).

We hypothesised that MBL is a risk factor for the development of sepsis and influences outcome after CPB surgery. The purpose of this study was to investigate:

- The influence of *MBL-2* gene polymorphisms on the outcome in children admitted to intensive care after a cardiopulmonary bypass surgery.
- Whether *MBL-2* variant alleles confer increased risk of sepsis.
- Whether MBL deficient individuals are more likely to require prolonged intensive care and mechanical ventilation.

2. Materials and Methods

2.1. Study population

Patients included in this study were from part of a larger study investigating genetic risk factors for adverse outcome from CPB. Recruitment of the patients was conducted over an 18-month period and included children up to 16 years old undergoing elective CPB. In this study, complete phenotypic and genotypic data was available on 244 patients. Clinical information obtained from the Great Ormond Street Hospital database was recorded for each patient. Patient demographic details, such as age, sex and ethnicity were included in this study. In addition, the following outcome measures were included: sepsis, length of stay in cardiac intensive care unit (CICU) and duration of ventilation (Figure 2.1 and 2.2). Sepsis was defined as presence of positive blood cultures and positive diagnosis for SIRS. Short stay was arbitrarily defined for this study as < 48 hours in intensive care unit. Local research ethics committee approval was obtained.

CV ☐ Lab ☐ Allan's Database ☐ PATS ☐ Data Entered ☐

Genetic Variability & Inflammatory Response Study

Hospital #:	Date of birth:	Gender: Male ☐ Female ☐	*Study Number*
Gestation: (if under 2 years)	Ethnicity:		

Date of admission to hospital: _____ Age on admission to hospital: _____

Cardiac diagnosis *(CCAD)*:

Age cardiac defect detected:

Previous medical interventions *(e.g. cardiac catheter, balloon septostomy)*:

Date:

Previous surgical interventions *(e.g. PDA ligation, modified BT shunt)*:

Date:

Bloods: Pre-op: ☐ ___/___/___ Day 1: ☐ ___/___/___

Post-op: ☐ ___/___/___ Day 2: ☐ ___/___/___

Genetics: ☐ ___/___/___ Day 3: ☐ ___/___/___

Figure 2.1 Demographic details and clinical outcome data collection sheet 1

Intensive Care Summary

Diagnosis of SIRS: No: ☐ Yes: ☐

Diagnosis of sepis: No: ☐ Yes: ☐

(SIRS + positive blood cultures)

(In view of the frequency with which these children receive ventilation and inotropic support, RR and HR are not included, **temp <36°C** or **>38°C** [not artificially controlled], **WCC<4,000** or **>12,000 cells/mm^3**)

Date: Site: Organism:

Date: Site: Organism:

Date: Site: Organism:

Intubation:

Date of extubation:

Time of extubation:

Total time of intubation: (hrs)

Reintubated: No: ☐ Yes: ☐

Reason:

Date & time intubated:

Date & time re-extubated:

ITU drugs: Inotropes: No: ☐ Yes: ☐

Adrenaline: ☐ bolus ☐ infusion, *(max dose)*

Discharge from intensivists:

Date of discharge from CICU:

Time of discharge from CICU: (hrs)

Total time in CICU: (hrs)

Transfered to another ICU: ☐

Readmited: No: ☐ Yes: ☐

Outcome at 28 days: Alive: 1 Deceased: 2

Cause of death:

Figure 2.2 Demographic details and clinical outcome data collection sheet 5

2.2. DNA extraction from whole blood

Genomic DNA was extracted from whole blood using a commercial kit (QIamp DNA blood mini kit, Qiagen, Crawley, UK) according to the manufacturer's instructions, which was performed by Dr Merethid Allen. Final DNA concentration was adjusted to15 ng/µl and stored in aliquots at -80° C until further use.

2.3. MBL-2 genotyping

In this study a heteroduplex method for genotyping was used for the detection of the *MBL-2* structural gene mutations in exon 1 and the X/Y promoter region polymorphism. This method was based on the generation of DNA heteroduplexes. In this procedure universal heteroduplex generators (UHGs), synthetic DNA molecules covering the section of exon 1 site of the three point mutations and the X/Y promoter regions. This synthetic DNA and the patient's genomic DNA were amplified by polymerase chain reaction (PCR) using sequence specific primers. Equal amounts of both UHG and genomic DNA PCR products were then combined and allowed to anneal to produce the characteristic heteroduplexes, which are unique for the allele present. The heteroduplexed products were electrophoresed on a non-denaturing polyacrylamide gel enabling identification of the patient's genotype (Jack et al. 1997). Each step for the heteroduplex genotyping is described in detail bellow.

2.3.1. PCR amplification of genomic DNA

Two separate PCR reactions were prepared to amplify the genomic DNA samples with primers spanning the three exon 1 mutations and the X/Y promoter region, respectively. The primer pairs used for PCR amplification of genomic DNA are shown in Table 2.1.

Table 2.1. Primer sequences for amplification of exon 1 and X/Y promoter regions and PCR product sizes in base pairs (bp).

Target region	Sense primer/Antisense primer	Product size (bp)
Exon 1	5'-CCAACACGTACCTGGTTCC-3'	113
	5'-CTGTGACCTGTCAGGATGC-3'	
X/Y Promoter	5'-AGGCATAAGCCAGCTGGCAAT-3'	111
	5'-CTAAGGAGGGGTTCATCTG-3'	

Two different PCR master mixes were used for amplification of the exon 1 and X/Y promoter. For the exon 1 the PCR mix comprised the following reagents and respective volumes:

Table 2.2. PCR mix for amplification of exon 1 for genomic DNA

Reagent	Volume (µl)
10x PCR buffer (Roche Applied Science, East Sussex, UK)	2.0
25mM Magnesium chloride ($MgCl_2$)(Roche Applied Science, East Sussex, UK)	1.2
10mM deoxynucleotide triphosphates (dNTPs) mix (Promega, UK)	0.5
50µM primer Forward (Sigma, UK)	0.6
50µM primer Reverse(Sigma, UK)	0.6
Amplitaq Gold DNA polymerase (5U/µl)(Roche Applied Science, East Sussex, UK)	0.2
Sterile water (Sigma, UK)	9.9
Genomic DNA	5.0

For the X/Y promoter region amplification a PCR ready to use mix was used, to which the following reagents were added and respective volumes:

Table 2.3. PCR mix for amplification of promoter for genomic DNA

Reagent	Volume (µl)
Master Mix (Biomix Red, Bioline,UK)	12.5
50µM primer Forward	0.5
50µM primer Reverse	0.5
Sterile water	7.5
Genomic DNA	5.0

The genomic DNA was amplified by PCR carried in a thermal cycler (Peltier Thermal Cycler, MJ Research, BioRad, UK). PCR cycle conditions for both exon 1 and promoter are desribed in Table 2.4. PCR products were stored at 4 °C until further use.

Table 2.4. PCR conditions for amplification of exon 1 and X/Y promoter regions

Temperature °C	Duration	Number of cycles
95	15 minutes	1
95	45 seconds	
56	45 seconds	35
72	45 seconds	
72	10 minutes	1

2.3.2. PCR amplification of UHG

Separate PCR amplifications were performed for amplification of the commercially synthesised UHGs (MWG-Biotech, Milton Keynes, UK) to identify the exon 1 and X/Y promoter polymorphisms (Table 2.5). The primer pairs used for amplification of the UHG were the same as the ones described in section 2.3.1 (Table 2.1).

Table 2.5. Nucleotide sequences for the UHG spanning the exon 1 mutations and promoter polymorphisms

UHG	Sequence
Exon 1	5'-CTGTGACCTGTGAGGATGCCCAAAAGACCTGCCCTG
133 bp	CAGTGATTGCCTGTAGCTCTCCAGGCATCAACGGCT
	TCCCAGGCAAAGATGGGCGTCATGTTGCACCAGAGA
	AAAGGGGGAACCAGGTACGTGTTGG-3'
Promoter	5'-AGGCATAAGCCAGCTGGCAATGCACGGTCCCATTTG
111 bp	TTCTCACTGCCACCCATGTTTATAGTCTTCCAGCAG
	CAACGCCAGGTCTCTAGGCACAGATGAACCCCTCCT
	TAG-3'

Two different PCR master mixes were used for the amplification of exon 1 and the promoter. The PCR mixes were prepared as a stock for multiple reactions. For the exon 1 the PCR mix comprised the following reagents and respective volumes:

Table 2.6. PCR mix for amplification of exon 1 for UHG

Reagent	Volume (µl)
10x PCR buffer	100
25mM $MgCl_2$	60
10mM dNTPs mix	25
50µM primer Forward	18
50µM primer Reverse	18
Amplitaq Gold DNA polymerase (5U/µl)	6
Sterile water	873.5
UHG template	1.5

For the promoter amplification a PCR ready to use mix was used, to which the following reagents were added and respective volumes:

Table 2.7. PCR mix for amplification of promoter for UHG

Reagent	Volume (µl)
Master Mix	12.5
50µM primer Forward	0.35
50µM primer Reverse	0.35
Sterile water	11.5
Genomic DNA	0.3

Exon 1 UHG and promoter UHG were amplified by PCR which was carried in a thermal cycler. The cycling conditions used were as described in section 2.3.1 (Table 2.4). PCR products were stored at 4 °C until further use.

2.3.3. PCR products analysis by Agarose gel electrophoresis

In order to ensure that DNA amplification had been successful, PCR products were analysed on a 2% agarose gel and then quantified. The gel was prepared by dissolving 2g of agarose (Invitrogen Life Technologies, Paisley, UK) in 100 ml of 1x Tris-Borate-EDTA (TBE) buffer (Sigma, UK). Ethidium bromide (1µl/100ml; Sigma, UK) was added to the cooled gel and poured onto a casting tray. 1 µl of 6x loading dye (Sigma, UK) was added to 4 µl of the PCR product and loaded onto the gel. The samples were then run in 1x TBE buffer at 120V for 30 minutes. PCR products were visualised using a UV transilluminator (Alpha Imager, Essex, UK) and photographed.

2.3.4. Heteroduplexing

Approximately equal amounts of genomic and UHG PCR products were combined and heated to 95°C (Peltier Thermal Cycler, MJ Research, BioRad, UK) for 5 minutes and then allowed to cool to room temperature (RT) over 2 hours to enable the heteroduplexes to form.

2.3.5. Heteroduplex analysis by 20% Polyacrylamide gel electrophoresis

Heteroduplex mixes were run on 20% polyacrylamide gels.

Exon 1 heteroduplexes were run on 20% polyacrylamide minigels (Mini-Protean, Bio Rad, UK) and were prepared as follows:

Table 2.8. 20% polyacrylamide gel composition for exon 1

Reagent	Volume*
30% Acrylamide/Bis acrylamide mix (Severn Bithec Ltd, UK)	6.7ml
10x TBE buffer	2.0ml
Sterile water	1.7ml
10% Ammonium Persulfate solution(APS) (National Diagnostics, UK)	100µl
N,N,N',N'-tetramethylethylenediamine (TEMED) (Sigma, UK)	10µl

* Volumes to prepare two gels.

Gel components were combined and left to set for approximately 15 minutes at RT. On removal of combs, the wells were rinsed with 2x TBE and then the samples were loaded. 2 µl of 6x loading dye was added to the heteroduplex mix and the entire mixture loaded onto the gel. The gels were run for 4 hours at 200V in 2x TBE buffer and the gel containers were placed in an ice bath. Gels were stained in 1x TBE containing ethidium

bromide 0.5µg/ml for 10-15 minutes on an orbital shaker (Rotatest, Denley, UK). Heteroduplexes were visualised on a UV transilluminator and photographed.

Promoter heteroduplexes were run on 20% polyacrylamide gels and were prepared as follows:

Table 2.9. 20% polyacrylamide gel composition for promoter

Reagent	Volume*
30% Acrylamide/Bis acrylamide mix	20ml
10x TBE buffer	6ml
Sterile water	5ml
10% Ammonium Persulfate solution(APS)	300µl
N,N,N',N'-tetramethylethylenediamine (TEMED)	30µl

* Volumes to prepare two gels.

The gels were prepared as above and were also equally visualised and photographed, except that the gels for promoter heteroduplex mixes were run for 15 hours at 150V, 16°C in 2x TBE buffer.

2.4. MBL-2 genotyping and haplotyping

Allele frequencies for both exon 1 and X/Y promoter polymorphisms were estimated by allele counting. There was no control population in this study. Exon 1 allele and genotype frequencies were compared with a previously published study of 302 UK white children, known as the Avon Longitudinal Study of Pregnancy and Childhood (ALPSPAC) (Mead et al. 1997). The X/Y promoter allele frequencies were taken from a

study of a Caucasoid adult population for comparison (Madsen et al. 1995). The three *MBL-2* structural gene mutations B, C and D are in linkage disequilibrium with the promoter region polymorphism X/Y, and only Y associates with each of the mutations (Madsen et al. 1995). Haplotypes were determined by combining the exon 1 and X/Y promoter genotypes and frequencies were compared with a Danish population (Garred et al. 2001;Garred et al. 2003). Exon 1 variant alleles (B, C and D) were referred as O. Patients were classified according to their MBL exon 1 mutations as wild-type (AA), heterozygous (AO), or homozygous (OO).

2.5. Statistical analysis

The frequency and outcome in patients without gene mutations (AA) were compared with data for patients with a mutation (AO/OO). Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS; version 12.0.1). A p value of < 0.05 was considered indicative of statistical significance.

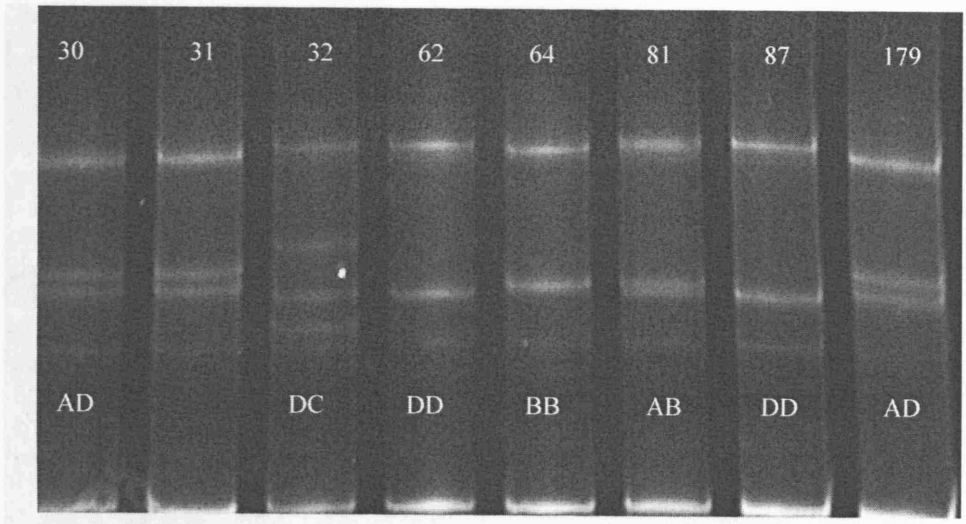
3. Results

This study is part of a previous larger study investigating genetic risk factors for adverse outcome of children undergoing CPB. The previous study investigated the role of functional cytokine polymorphisms, such as IL-6, IL-10 and TNF-α. Over the 18 months of study recruitment, 450 children underwent elective cardiac repair involving cardiopulmonary bypass. Two hundred and forty four of those were included in this study. *MBL-2* genotype, demographic details and CICU outcome for this patient cohort were investigated in this study.

3.1. Detection of MBL-2 polymorphisms

Heteroduplexing was used to detect the *MBL-2* genotypes of the 244 patients. The method permitted the identification of the exon 1 and X/Y promoter polymorphisms. Both exon 1 and X/Y promoter genotypes were interpreted by comparison with known reference band patterns. An example of the heteroduplexing results for exon 1 and X/Y promoter and the respective heteroduplex patterns are shown in Figures 3.1 and 3.2 (patient's genotype data in appendix).

A



B

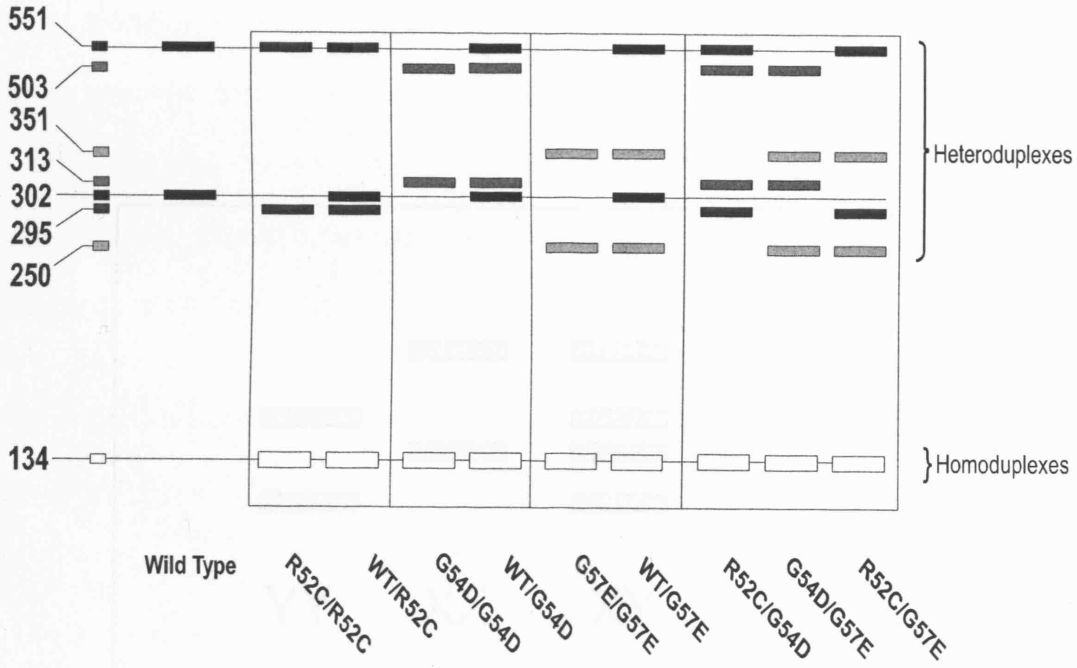
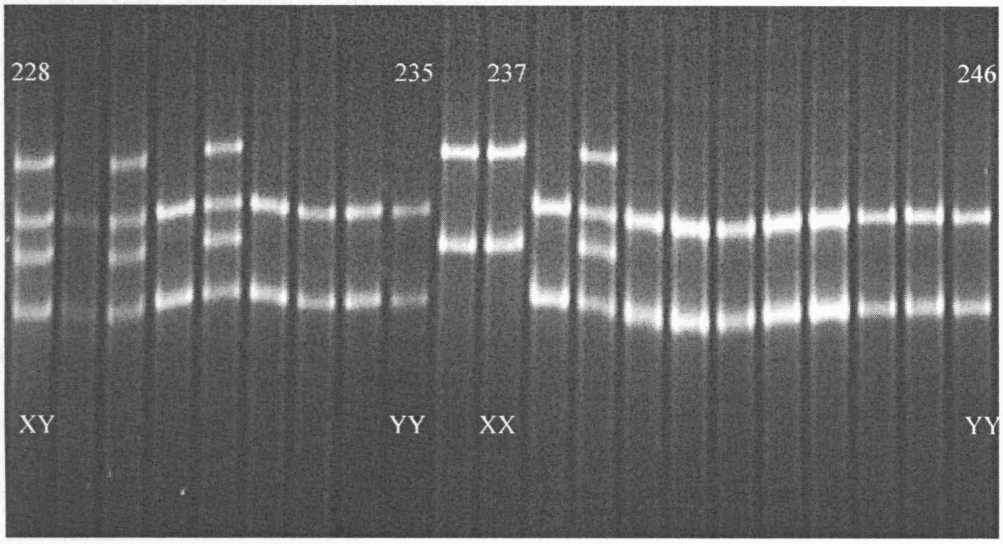


Figure 3.1. A. MBL-2 exon 1 genotypes for different patients **B.** Schematic of heteroduplex patterns for exon 1 polymorphisms. WT=wild type (A); R52C=codon 52 mutation (D); G54D=codon 54 mutation (B); G57E=codon 57 mutation (C).

A



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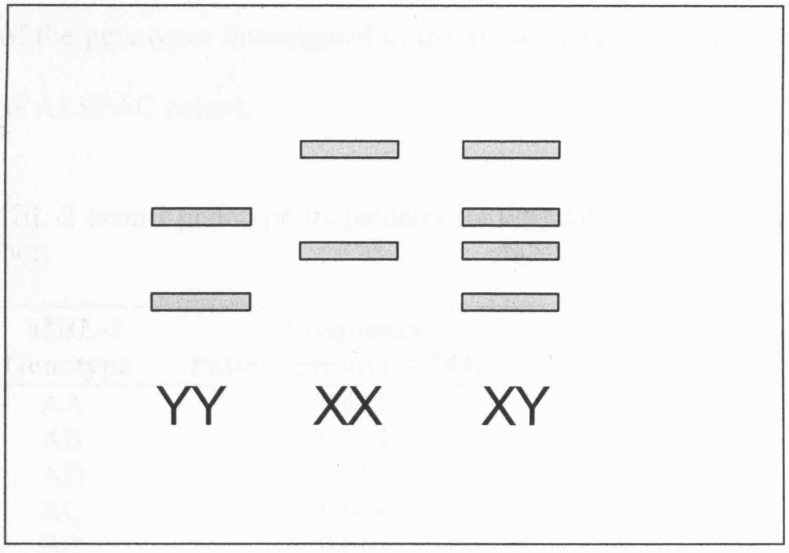

Figure 3.2. A. X/Y promoter genotypes for patients 228-246 **B.** Schematic of heteroduplex patterns for X/Y promoter.

3.2. Prevalence of MBL-2 genotypes

Allele, genotype and haplotype frequencies for both exon 1 and X/Y promoter polymorphisms were estimated and compared with previously published study cohorts.

Exon 1 allele frequencies observed in this study population were as follows: A 0.782, B 0.117, D 0.070 and C 0.031. In the ALSPAC study, an UK paediatric white population, the allele frequencies were: A 0.775, B 0.144, D 0.066 and C 0.015. Differences in allele frequencies were observed when comparing the two studies. In the ALSPAC study 2.4% of the population were non-white (Mead et al.1997), whereas in this study 29.1% of the patients were non-white, reflecting the ethnically diverse population. Of those the majority were Asians and Arabs, however there is limited data on *MBL-2* allele or genotype frequencies in such populations. Exon 1 genotype frequencies obtained in this study were also compared with the ALSPAC cohort (Table 3.1). The frequencies of the genotypes investigated in the study population were similar to those reported in the ALSPAC cohort.

Table 3.1. MBL-2 exon 1 genotype frequencies for the study population and the ALSPAC cohort

MBL-2 Genotype	Frequency Patient group (n=244)	Frequency ALSPAC (n=302)
AA	0.611	0.596
AB	0.184	0.228
AD	0.111	0.103
AC	0.049	0.026
BB	0.016	0.023
DD	0.008	0.010
BC	0.008	0.003
DB	0.008	0.010
DC	0.004	0.000

The X/Y promoter allele frequencies in the study population were: Y 0.797 and X 0.0203. The promoter polymorphism frequencies were compared with a Caucasian adult population, in this study the frequencies were: Y 0.763 and X 0.238 (Madsen et al. 1995). The frequencies of the X/Y promoter genotypes investigated in the study population were similar to the reported frequencies. The X/Y promoter genotype frequencies in this cohort were YY 0.635, XY 0.324 and XX 0.041. There is no published data for X/Y promoter genotype.

The haplotype frequencies obtained in this study were compared with a Danish study (Table 3.2). The frequencies of the haplotypes investigated in the study population were slightly different to those reported in the Danish control population (Garred et al. 2003).

Table 3.2. MBL-2 haplotype frequencies for the study population and the Danish cohort

MBL-2 gentotype	Frequency Patient group (n=244)	Frequency Danish cohort (n=250)
YA/YA	0.320	0.288
YA/XA	0.250	0.292
XA/XA	0.041	0.048
YA/YO	0.270	0.212
XA/YO	0.074	0.132
YO/YO	0.045	0.028

3.3. Patient characteristics

Patient demographic details were obtained from the Great Ormond Street Hospital database. Demographic details of the whole study group are shown in Table 3.3. In addition, an association between *MBL-2* exon 1 genotypes and patient characteristics is presented.

Of the 244 patients, 131 (53.7%) were males. The median age was 11 months (range 0 months – 202 months) and a high percentage of the patients 173 (79.9%) were white. No statistical association was found between age, gender, ethnicity and *MBL-2* exon 1 genotypes.

Table 3.3. Characteristics for the whole study group and stratification by MBL-2 exon 1 genotype

	All cases (n=244)	AA (n=149)	AO/OO (n=95)	*p* value
Male	131 (53.7%)	79 (53.0%)	52 (54.7%)	0.793
Age, median (months)	11 (0-202)	11 (0-199)	10.5 (0-202)	0.737
White	173 (70.9%)	103 (69.1%)	70 (73.7%)	0.443

3.4. Outcome

Patient outcome details were obtained from the Great Ormond Street Hospital database. Outcome measures included in this study were: sepsis, length of stay in CICU and duration of ventilation. Outcome measures of the whole study group are shown in Table 3.4. In addition, an association between *MBL-2* exon 1 genotypes and patient's outcome is presented.

The median duration of mechanical ventilation was 26 hours (range 0 h – 2563 h) and the median length of stay in CICU was 68.5 hours (range 16 h – 2590 h). In the majority of patients 154 (63.9%) the length of stay was > 48 hours, with the remainder, 87 (35.7%) requiring less than 48 hours.

During the intensive care stay, 70 patients (28.7%) developed sepsis, of those 61 (25%) were long stay patients. (Table 3.4). Six patients (2%) died postoperatively, all of them were within the long stay group and four developed sepsis (patient outcome details in appendix).

Table 3.4. Outcome for the whole study group and stratification by MBL-2 exon 1 genotype

	All cases (n=244)	AA (n=149)	AO/OO (n=95)	*p* value
Sepsis	70 (28.7%)	37 (24.8%)	33 (34.7%)	0.096
Length of stay, median (h)	68.5 (16-2590)	149 (100%)	95 (100%)	0.197
Ventilation, median (h)	26 (0-2563)	149 (100%)	95 (100%)	0.266

3.5. MBL-2 genotype and outcome

In this analysis, only *MBL-2* exon 1 genotype has been used due to small numbers in different groups when using haplotype, which limits interpretation of results. In order to avoid small groups, the three structural variant alleles (B, C and D) which have a significant effect on MBL concentrations were grouped in one category, termed "O". Thus, two groups of MBL genotype were defined: the AA group (wild type alleles) and the AO/OO group (hetero/homozygotes).

The influence of *MBL-2* exon 1 genotype on outcome after CPB surgery was assessed. Outcome measures examined in this study were sepsis, length of stay in CICU and duration of ventilation.

3.5.1. MBL-2 genotype and sepsis

Of the 244 patients recruited, 70 (28.7%) developed sepsis after surgery. Within this group a higher proportion of heterozygotes 33 (34.7%) was observed compared to homozygotes 37 (24.8%) (Table 3.4). Figure 3.3 presents an analysis of the proportion of patients with *MBL-2* exon 1 mutations and the development of sepsis. The proportion of heterozygotes was increased in the sepsis group (47.1%) compared to the non-sepsis group (35.6%). Wild type alleles were more common in patients who did not develop sepsis. However, there was no significant difference in *MBL-2* exon 1 genotype percentage between septic and non-septic patients ($p = 0.096$).

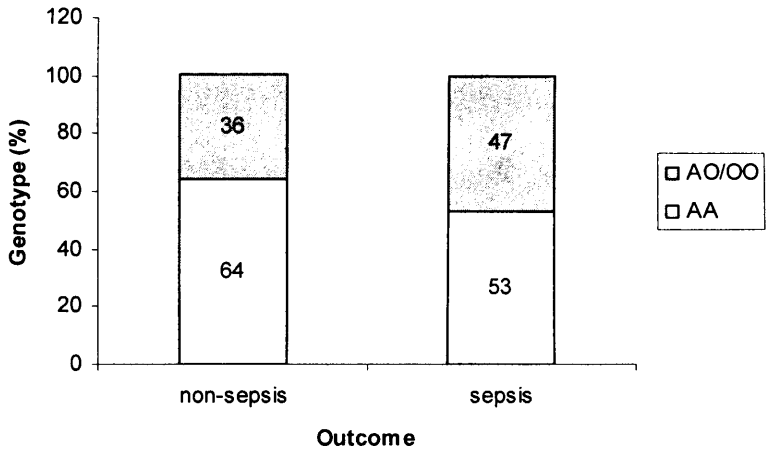

Figure 3.3. Relationship between MBL-2 genotype and the development of sepsis.

3.5.2. MBL-2 genotype and length of stay in CICU

In order to investigate the effect of *MBL-2* exon1 genotype on the inpatient stay, patients were divided into three groups; short stay, defined as < 48 hours in CICU, 48-120 hours and long stay > 120 hours.

In the long stay group there was a higher percentage of hetero/homozygotes (50.8%) compared to wild type homozygotes (49.2%). The proportion of heterozygotes increased with increased period of stay comparing between short and long stay. However, this trend was not observed in the 48-120 hours group. Overall, there was no association between the length of stay and genotype ($p = 0.152$).

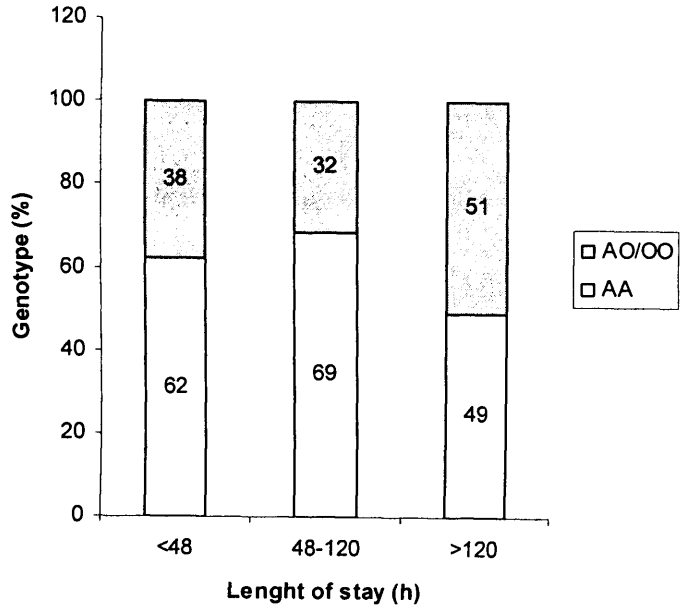

Figure 3.4. Relationship between MBL-2 genotype and the length of stay

3.5.3. MBL-2 genotype and ventilation

In order to investigate the effect of MBL-2 exon 1 genotype on ventilation, the patients were divided in three groups. The same time periods used for length of stay were applied.

In the group that did not required prolonged mechanical ventilation (< 48 h), 67% of the patients were carriers of wild type alleles (AA). Of the heterozygote individuals 33% required less than 40 h of mechanical ventilation, 43% required 48-120 h and 50% required more than 120 h. The proportion of heterozygotes increased with increasing time of ventilation. Overall, there was association between the duration of ventilation and genotype ($p = 0.033$).

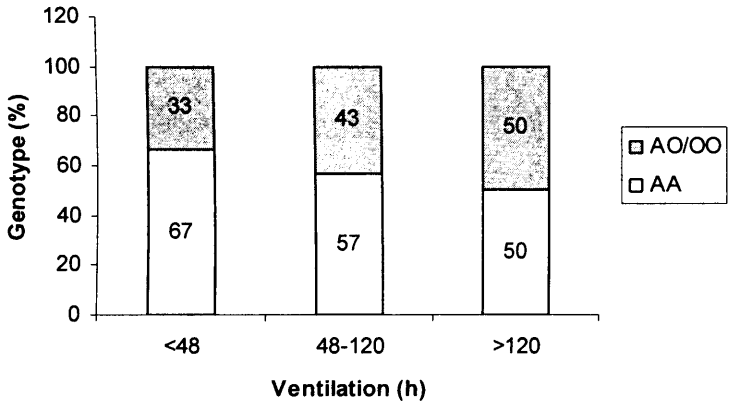

Figure 3.5. Relationship between MBL-2 variant alleles and ventilation

4. Discussion

Genetic factors may influence the outcome from surgery. Mannose-Binding lectin (MBL) is an important factor in innate immune system. It has been shown that *MBL-2* polymorphisms result in deficiency of the encoded protein and increased susceptibility to infection especially in children and the immunocompromised and is a risk factor for critically ill patients to develop sepsis (Garred et al. 2003;Koch et al. 2001).

The purpose of this study was to investigate the effect of *MBL-2* genotype on the outcome of children undergoing cardiopulmonary bypass (CPB) surgery. This study has revealed that *MBL-2* exon 1 variant alleles (AO/OO) were more common in children with sepsis compared to those who did not develop sepsis. Also, a higher proportion of patients who required prolonged stay in intensive care and ventilation (>120 h) were carrier of *MBL-2* variant alleles compared to wild-type (AA). Thus, *MBL-2* exon 1 polymorphisms seem to influence patient's outcome. There was no association between *MBL-2* genotype and the patient characteristics (age, sex and ethnicity).

This is the first association study on *MBL-2* polymorphisms in paediatric CPB patients. However, three recently papers, including critically ill children and adults, have shown that *MBL-2* variant alleles were associated with the development of sepsis or the systemic inflammatory response syndrome (SIRS) (Fidler et al. 2004;Garred et al. 2003;Gordon et al. 2006). In this study as well as in a cohort of critically ill adult patients (Garred et al.2003), about 50% of the patients who developed sepsis were carriers of *MBL-2* polymorphisms. In another study they found a strong relationship between *MBL-2* genotype and susceptibility to sepsis/septic shock. However, they did not observe influence of *MBL-2* genotype on outcome (Gordon et al. 2006). Other

studies have demonstrated the influence of cytokine polymorphisms on the outcome of sepsis (Nakada et al. 2005). The reasons for the effect of MBL on the development of sepsis remain unclear. There are numerous pathways by which complement activation can lead to enhanced inflammation. In fact *MBL-2* variant alleles, which lower complement activation, can actually be beneficial as it may reduce the inflammatory response to sepsis. Besides, MBL can also bind to apoptotic cells to facilitate their phagocytosis by macrophages therefore the altered structure of MBL in those with variant alleles might prevent effective binding and opsonization. However another mechanism by which MBL could influence the development of sepsis is through a direct effect on pro-inflammatory and anti-inflammatory cytokine production. In an *ex vivo* model low levels of MBL have been observed to enhance the production of IL-6 and IL-1β from monocytes (Jack et al. 1998). In this study only *MBL-2* exon 1 genotype was analysed. Therefore, limitations were found to explain a possible mechanism through which MBL may have operated in this study cohort. Further studies should include patient's haplotype (which includes the X/Y promoter variant) and/or MBL protein levels.

In this study, it was also observed that children who required prolonged stay and mechanical ventilation (>120) in intensive care were carriers of *MBL-2* variant alleles. The data have shown a significant association between *MBL-2* genotype and duration of ventilation. The presence of cytokine polymorphisms have also been shown to influence the patient's stay and the requirement of mechanical ventilation. In a previous study carried by Dr Meredith Allen, using the same cohort, it was shown as association between IL-6 and TNF-α polymorphisms and shorter duration of ventilation and intensive care support. Other study has also showed an association between a TNF-α

polymorphism and shorter times to extubation with lower risk of prolonged mechanical ventilation (Yende et al. 2003). The risk of death was not investigated in the present study due to the low number of deaths.

Many factors seem to influence patient's outcome from CPB surgery. The results presented in this study have shown an association between *MBL-2* exon 1 polymorphisms and outcome indicating a role for MBL in determining the host response to cardiopulmonary bypass surgery. Preoperative genetic screening for *MBL-2* may guide clinical management to reduce postoperative complications. Although, further studies are needed to clarify the effect that *MBL-2* polymorphisms may have on the outcome of surgery.

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APPENDIX

Patient's *MBL-2* XY promoter and exon 1 genotype, characteristics and outcome measures.

Sample #	PROMOTER XY	EXON 1	Age (month)	Sex	Ethnicity	Length stay (h)	Ventilation (h)	Sepsis
1	YY	AA	67	f	w	22	12	non-sepsis
2	YY	AD	10	m	non-w	96	67,5	non-sepsis
3	XY	AD	16	m	w	102	92	non-sepsis
4	XY	AA	50	m	w	24	6	non-sepsis
5	YY	AA	56	f	non-w	50	9,57	non-sepsis
6	YY	AC	62	m	w	20,8	6	non-sepsis
7	YY	AA	19	m	non-w	46	4	non-sepsis
8	XY	AA	181	f	w	23	0,15	non-sepsis
9	XY	AB	18	f	w	233	213	sepsis
10	YY	AA	70	m	w	45	17	non-sepsis
11	XY	AA	0	m	w	110	90	non-sepsis
12	YY	AD	3	f	w	120	28	sepsis
13	YY	AA	4	m	non-w	95	29	sepsis
14	XY	AA	5	m	w	94	18	non-sepsis
15	XY	AB	4	f	w	67	27	non-sepsis
16	YY	AA	25	m	non-w	21	0	non-sepsis
17	XY	AA	6	f	w	41	17	non-sepsis
18	YY	AA	12	m	w	142	83	non-sepsis
19	YY	AA	1	m	w	96	89	non-sepsis
20	YY	AD	0	m	w	2563	2563	sepsis
21	XY	AA	0	f	w	332	254	non-sepsis
22	YY	BB	135	m	w	45	12	sepsis
23	YY	AB	10	f	w	38,5	12,5	non-sepsis
24	YY	AA	95	m	non-w	428	403	sepsis
25	XY	AB	134	f	w	23	1,25	non-sepsis
26	YY	AA	26	m	w	50,5	12,5	sepsis
27	XY	AA	11	f	w	137	94	non-sepsis
28	YY	AB	12	m	non-w	93	69	non-sepsis
29	XY	AA	72	m	non-w	22	8	non-sepsis
30	YY	AB	8	f	non-w	93	64	non-sepsis
31	YY	AB	1	f	w	92	39,5	non-sepsis
32	YY	DC	41	m	non-w	47,8	4,75	non-sepsis
33	YY	AA	4	m	w	42,5	20,5	non-sepsis
34	YY	AC	20	m	non-w	44	8,42	non-sepsis
35	YY	AA	15	m	w	44	31,5	non-sepsis
36	YY	AB	3	f	w	187	96,5	non-sepsis
37	YY	AC	3	f	w	167	98,5	non-sepsis
39	XY	AA	27	f	w	63	13	non-sepsis
40	YY	AA	8	m	w	168	148	non-sepsis
41	YY	AA	4	m	w	122	93	non-sepsis
42	YY	AD	12	f	w	142	136	non-sepsis
43	YY	AB	1	m	w	88,5	55	sepsis
44	YY	AA	1	m	w	474	300	non-sepsis
45	YY	AB	1	f	w	416	416	non-sepsis
46	XY	AA	50	f	w	26	3	non-sepsis
47	YY	AA	6	f	non-w	101	76,5	sepsis
48	YY	AB	18	m	non-w	28	13	non-sepsis

Sample #	PROMOTER XY	EXON 1	Age (month)	Sex	Ethnicity	Length stay (h)	Ventilation (h)	Sepsis
49	YY	AA	0	m	non-w	646	506	sepsis
50	YY	AA	110	m	w	231	209	non-sepsis
51	YY	AA	6	m	w	39	4	non-sepsis
52	XY	AA	106	f	w	23	4	non-sepsis
53	XY	AA	11	m	w	24	5,75	non-sepsis
54	YY	AA	0	m	w	68	21,5	non-sepsis
55	YY	AA	0	m	w	551	360	non-sepsis
56	XY	AA	25	m	w	62,8	41	non-sepsis
57	YY	AA	4	f	non-w	65,5	46,3	non-sepsis
58	YY	AB	1	m	w	49,6	18,5	non-sepsis
59	YY	AD	1	m	w	91,9	87,5	non-sepsis
60	YY	AB	8	f	non-w	89,5	19,3	non-sepsis
61	XX	AA	1	m	non-w	94,3	46,5	non-sepsis
62	YY	DD	5	f	w	171	166	non-sepsis
63	XY	AA	3	m	non-w	41,3	18,8	non-sepsis
64	YY	AA	104	m	non-w	360	238	sepsis
65	YY	AB	0	m	w	52	25,7	non-sepsis
66	XY	AA	94	f	non-w	29	7	non-sepsis
67	YY	AA	0	m	non-w	46,5	23,5	non-sepsis
68	XY	AA	41	f	w	21,2	3,38	non-sepsis
69	XY	AA	1	f	w	32,3	42,3	non-sepsis
70	XY	AA	100	f	w	24,7	13,7	non-sepsis
71	YY	BC	3	m	w	53,8	50,5	non-sepsis
72	YY	AA	12	m	w	117	91,8	sepsis
73	YY	AC	0	f	non-w	1280	1280	sepsis
74	YY	AA	64	m	w	41,3	3,25	non-sepsis
75	YY	AA	2	m	w	145	123	sepsis
76	YY	AD	3	m	w	69,3	7,25	non-sepsis
77	YY	AC	26	f	non-w	48,1	16,3	non-sepsis
78	YY	AA	5	m	w	41,2	20,2	non-sepsis
79	YY	AB	2	f	w	1990	237	sepsis
80	XY	AA	6	m	w	71,5	24,5	sepsis
81	YY	AD	22	m	non-w	335	327	sepsis
82	YY	AC	7	f	non-w	198	141	sepsis
83	XX	AA	0	m	w	68,5	46	sepsis
84	YY	AC	1	m	non-w	36,8	57,3	sepsis
85	YY	AA	10	m	w	22,3	12,3	non-sepsis
86	YY	AC	6	f	w	45,3	11,8	non-sepsis
87	YY	DD	29	f	w	16,8	0	non-sepsis
88	YY	AB	30	f	non-w	40,3	33 min	non-sepsis
89	XY	AA	12	m	w	44	18,5	sepsis
90	YY	AA	83	m	w	25,8	0	non-sepsis
91	YY	AA	0	m	w	619	646	sepsis
92	XY	AA	40	f	w	91	21	sepsis
93	YY	AA	81	m	w	23,5	4,5	non-sepsis
94	YY	AA	18	f	non-w	17,5	5	non-sepsis
95	YY	AA	18	m	non-w	20,5	12,5	non-sepsis
96	XY	AB	1	m	w	544	142	sepsis
97	YY	AA	0	m	w	111	50,5	non-sepsis
98	XY	AB	0	m	w	451	166	sepsis
99	YY	AA	49	m	non-w	45	18	sepsis
100	XY	AB	7	f	w	23	6	non-sepsis
101	XY	AA	0	m	w	67,5	51,5	sepsis

Sample #	PROMOTER XY	EXON 1	Age (month)	Sex	Ethnicity	Length stay (h)	Ventilation (h)	Sepsis
102	YY	AA	0	m	w	178	130	sepsis
103	YY	AD	51	m	w	35,2	6,33	non-sepsis
104	XY	AA	5	f	w	690	384	sepsis
105	YY	AB	46	m	w	2590	2190	sepsis
106	XX	AA	8	f	non-w	43	17,5	non-sepsis
107	XY	AB	0	f	w	66,5	56,3	non-sepsis
108	YY	AB	5	f	w	66	27	sepsis
109	YY	AA	34	f	non-w	22	1,5	non-sepsis
110	XY	AD	27	m	w	115	45	sepsis
111	YY	AA	2	m	w	116	2,33	sepsis
112	YY	AA	22	m	w	18,7	14,7	non-sepsis
113	XY	AA	8	m	non-w	116	18,7	sepsis
114	XY	AA	0	m	w	16	26	non-sepsis
115	XY	AA	2	f	w	65	20,2	non-sepsis
116	XY	AA	26	f	w	41,8	0,08	non-sepsis
117	XY	AA	8	f	w	22,5	6	sepsis
118	YY	AB	55	f	w	14,5	o	non-sepsis
119	YY	DB	8	m	w	68,3	49,5	non-sepsis
120	YY	AB	24	m	w	16	6,25	non-sepsis
121	YY	AC	0	m	w	1898	648	non-sepsis
122	XY	AA	0	m	non-w	64	40	non-sepsis
123	YY	AB	5	m	w	94	18	non-sepsis
124	XY	AA	69	f	non-w	23,3	1,83	non-sepsis
125	XY	AB	0	m	w	188	148	non-sepsis
126	YY	AA	0	m	w	278	205	non-sepsis
127	YY	AB	0	m	w	302	233	sepsis
128	YY	BC	10	m	non-w	21	15	non-sepsis
129	XY	AA	44	f	non-w	25	0	non-sepsis
130	XY	AA	44	f	w	237	192	sepsis
131	YY	AA	12	f	w	95,3	77	non-sepsis
132	YY	AA	0	m	w	88	71	sepsis
133	YY	AA	0	m	w	120	94	sepsis
134	YY	BB	19	m	w	17	5,98	sepsis
135	YY	AB	12	m	non-w	238	138	sepsis
136	XY	AD	47	f	w	19	2,7	non-sepsis
137	YY	AA	3	m	w	115	23,5	sepsis
138	YY	AA	0	m	w	212	212	non-sepsis
139	XY	AA	0	f	w	237	142	sepsis
140	XY	AA	8	m	non-w	93,6	75,1	non-sepsis
141	XY	AA	0	f	non-w	66	39,9	non-sepsis
142	YY	AA	3	f	w	70	49	non-sepsis
143	YY	AB	79	f	w	24	1	non-sepsis
144	XY	AA	3	f	non-w	27	9,7	non-sepsis
145	XY	AD	1	m	non-w	185	47,3	sepsis
146	YY	AD	30	f	w	18,5	1,75	non-sepsis
147	YY	AD	27	m	w	163	107	non-sepsis
148	YY	AD	1	m	non-w	978	934	sepsis
149	YY	AA	64	f	w	51	16	non-sepsis
150	YY	AA	3	f	w	90	177	non-sepsis
151	XY	AA	8	f	w	549	549	sepsis
152	YY	AA	148	f	non-w	22,3	4	non-sepsis
153	YY	AA	127	f	non-w	23	27,3	sepsis
154	YY	AB	18	f	w	171	146	sepsis

Sample #	PROMOTER XY	EXON 1	Age (month)	Sex	Ethnicity	Length stay (h)	Ventilation (h)	Sepsis
155	XY	AA	3	m	w	51,7	28	non-sepsis
156	YY	AC	14	m	non-w	1360	11+	sepsis
157	YY	AA	3	f	w	71,2	46	non-sepsis
158	YY	AA	3	f	non-w	186	146	sepsis
159	XY	AA	12	f	non-w	47,2	19,5	non-sepsis
160	YY	AB	8	f	w	649	477	sepsis
161	YY	AA	0	m	w	243	141	sepsis
162	XY	AB	5	m	w	212	69,5	sepsis
163	XY	AB	8	f	w	51,8	44,8	non-sepsis
164	XX	AA	109	f	w	163	6,08	non-sepsis
165	XX	AA	12	f	w	33	9	non-sepsis
166	YY	AA	162	m	non-w	27,6	4,83	non-sepsis
167	YY	AA	4	f	w	69,5	31,9	non-sepsis
168	YY	AA	3	f	w	87,9	15,9	non-sepsis
169	YY	AB	61	m	w	26	5	non-sepsis
170	YY	AB	17	m	w	214	179	sepsis
171	YY	AA	71	f	w	52,3	18,2	non-sepsis
172	YY	DB	32	m	w	70	52,5	non-sepsis
173	YY	AA	11	m	non-w	66	14,25	non-sepsis
174	XY	AA	10	f	non-w	90	59,5	sepsis
175	XY	AA	16	f	w	70	27,5	sepsis
176	XY	AA	6	m	w	185	60,3	non-sepsis
178	YY	AD	43	m	w	136	62,8	non-sepsis
179	YY	AD	0	m	w	1560	1560	sepsis
180	YY	AD	2	f	w	74,7	11,7	sepsis
181	XY	AC	11	f	non-w	508	335	sepsis
182	YY	AA	1	m	non-w	164	127	non-sepsis
183	YY	AB	137	f	w	19,1	1,82	non-sepsis
184	YY	AD	2	f	w	28	14	non-sepsis
185	YY	AB	7	f	w	44,3	22	non-sepsis
186	XY	AA	193	f	w	49,6	1	non-sepsis
187	XY	AA	11	f	w	50,4	40,8	non-sepsis
188	YY	AD	91	m	w	50,3	17,1	non-sepsis
189	YY	AB	16	m	w	147	17,1	sepsis
190	YY	AA	3	f	w	95,5	72,6	non-sepsis
191	YY	AA	0	m	non-w	43,3	27	non-sepsis
192	YY	AA	70	m	w	86,1	11,6	sepsis
193	XX	AA	130	f	non-w	30	4,3	non-sepsis
194	YY	AD	192	f	w	30,7	8	non-sepsis
195	YY	AD	202	f	w	69,8	18,1	non-sepsis
196	XY	AA	199	m	w	47,9	32,3	non-sepsis
197	XY	AA	189	f	w	262	43,5	non-sepsis
198	XY	AA	6	m	non-w	168	49	non-sepsis
199	XY	AA	74	f	non-w	151	74,5	non-sepsis
200	YY	AA	5	f	w	74	17,5	non-sepsis
201	YY	AA	23	m	w	50,2	2,42	non-sepsis
202	XY	AA	20	m	w	88.6	21.8	sepsis
203	XY	AD	86	m	non-w	30	18,5	non-sepsis
204	YY	AA	2	f	w	140	65,1	non-sepsis
205	YY	BB	39	f	non-w	45,6	6,22	non-sepsis
206	XY	AA	14	f	w	93,4	13,3	non-sepsis
207	XY	AA	20	m	non-w	53	33,5	non-sepsis
208	XY	AA	7	f	w	67,3	37,3	non-sepsis

Sample #	PROMOTER XY	EXON 1	Age (month)	Sex	Ethnicity	Length stay (h)	Ventilation (h)	Sepsis
209	XY	AB	5	m	non-w	99,2	82,8	sepsis
210	XY	AA	50	f	w	53,2	5,85	non-sepsis
211	YY	AA	7	f	w	95,4	49,8	non-sepsis
212	YY	AB	0	m	w	144	95,4	non-sepsis
213	XX	AA	136	f	non-w	95,3	28,4	non-sepsis
214	XX	AA	113	f	non-w	46,8	21,6	sepsis
215	XY	AA	70	m	w	54,1	8,33	non-sepsis
216	YY	BB	69	f	w	44,6	13,1	non-sepsis
217	YY	AB	173	m	w	68,7	5,15	non-sepsis
218	XY	AA	142	m	w	25,6	11,9	non-sepsis
219	YY	AA	0	m	w	90,3	24,3	non-sepsis
220	YY	AA	10	f	w	26,1	6,25	sepsis
221	XY	AA	3	m	w	64,8	39,8	non-sepsis
222	YY	AA	5	m	w	103	80,8	non-sepsis
223	YY	AB	13	m	non-w	43	5,5	non-sepsis
224	YY	AA	131	f	non-w	90,7	83,2	non-sepsis
225	XY	AA	34	f	w	192	34,8	non-sepsis
226	XY	AA	102	m	w	46,8	20,3	non-sepsis
227	YY	AB	67	f	w	20,2	1,67	non-sepsis
228	XY	AD	61	m	non-w	44,8	13,8	non-sepsis
230	XY	AA	12	f	non-w	90,4	17,3	non-sepsis
231	YY	AA	127	f	w	50,5	17	non-sepsis
232	XY	AA	0	m	non-w	116	98,6	sepsis
233	YY	AA	55	f	w	23,3	5,5	non-sepsis
234	YY	AA	3	m	w	485	266	sepsis
235	YY	AA	44	f	w	77,5	13,9	non-sepsis
236	XX	AA	5	m	w	70,8	29	non-sepsis
237	XX	AA	3	f	w	87,8	11,9	non-sepsis
238	YY	AC	6	f	w	458	458	sepsis
239	XY	AA	75	m	w	42,6	10,3	non-sepsis
240	YY	AA	134	f	non-w	22,8	12,3	non-sepsis
241	YY	AD	27	m	w	65,8	23,3	sepsis
242	YY	AA	23	f	w	143	114	non-sepsis
243	YY	AB	116	m	w	79,9	4,5	non-sepsis
244	YY	AD	11	m	non-w	30,9	16,3	non-sepsis
245	YY	AD	35	f	w	28,8	5.33	non-sepsis
246	YY	AB	7	f	w	142	71,2	sepsis
247	YY	AA	194	m	non-w	27,2	5,33	non-sepsis