Structural and functional studies of mannose binding lectin (MBL) and the lectin pathway of complement in children with cancer

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Abstract

Infection remains a major cause of morbidity and hospitalisation in children receiving chemotherapy treatment for cancer. Among patients with the same diagnosis and treatment regimen, not all suffer equally from infectious complications. This suggests that as yet unidentified host factors may contribute to increased susceptibility to infection. Deficiency of mannose binding lectin (MBL), a pattern recognition receptor of the innate immune system, has been proposed as one such factor but clinical studies have been inconclusive. MBL works in concert with MBL associated serine proteases (MASPs) to activate the lectin pathway of complement. Functional activity of the pathway has not been investigated in children with cancer to date.

Children undergoing chemotherapy were recruited to cross sectional observational and longitudinal studies with details of febrile neutropenia (FN) episodes recorded prospectively. MBL gene polymorphisms were characterized by heteroduplexing and reverse hybridization. MBL protein and MASP levels were measured and functional activity of the lectin pathway was quantified. High oligomer structure of MBL was analysed by Western blotting. Transcriptional regulation of MBL in response to infection and inflammation was studied. The MBL promoter was cloned and its activity investigated using luciferase assays.

A major finding is that individuals in possession of MBL variant alleles suffered from more frequent and longer episodes of FN over the study period compared to wildtype individuals. Functional analysis enabled identification of specific deficiencies within the pathway in patients deemed MBL sufficient by their genotype and protein level. Changes in MASP levels were noted in response to chemotherapy and complement function was observed to increase during FN episodes. Structural analysis revealed variability in MBL higher order oligomer structure during the acute phase of FN and promoter studies further highlighted the complexity of MBL regulation.

The results presented in this thesis provide further evidence that MBL deficiency increases the frequency and the duration of FN in the largest paediatric cancer cohort studied to date. The complex interplay between other pathway components appears crucial to MBL function but their influence on clinical outcome is not yet fully understood.

Declaration

I, Rachel Dommett, 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 in the thesis.

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Chapter1

Introduction

1.0 Introduction

In the western world approximately 1 in every 600 children develops a malignancy in the first 15 years of life (Stiller, 2007). During the past 30-40 years there have been marked improvements in outcome and now over 70% of children diagnosed with cancer enjoy disease free survival in excess of 5 years (Sankila et al., 2006). Improvements in the prevention and treatment of infections have contributed to the reduction in mortality. However infection related to immunosuppression is still a cause of treatment-related death and remains a major cause of morbidity and hospitalisation in children receiving chemotherapy (Basu et al., 2005).

Children with the same underlying disease and on the same treatment protocol vary in their susceptibility to infection; the reasons for this are currently unknown (Graubner et al., 2008). This thesis aims to explore the influence of host innate immunity, particularly the role of Mannose Binding Lectin (MBL) and the lectin pathway of complement activation, to increased susceptibility to infection in children with cancer.

1.1 Susceptibility to infection during chemotherapy treatment

Establishing the fine balance between effective treatment and tolerable toxicity is an ongoing challenge in oncology because the intensive treatment protocols employed to achieve improvements in outcome are complicated by such profound immunosuppression. Therapy induced neutropenia is considered the most important risk factor for infection in cancer patients. The relationship between the degree of neutropenia and the risk of bacterial and fungal infections was first recognised nearly forty years ago. Bodey *et al* studied the infectious complications experienced by 52 patients treated with cytotoxic chemotherapy for acute leukaemia (Bodey et al., 1966).

They observed that as granulocyte counts fell (particularly below $0.5x10^9$/L), the frequency, duration and severity of infections dramatically increased (Figure 1.1).

Figure 1.1 Incidence of severe infection by absolute neutrophil count (ANC $x10^9$/L) and duration of neutropenia (adapted from Bodey et al., 1966).

The degree and duration of neutropenia is generally determined by the intensity of treatment and the patient's underlying disease. Clinical observations show that the majority of patients experience neutropenic episodes but interestingly not all suffer equally from associated complications implying that neutropenia is just one of many contributing factors. The various modalities of treatment used to conquer the underlying disease have different effects on host defence. Alterations in skin/mucosal barriers, such as occur with the use of central venous catheters (CVCs), defects in adaptive immunity and in other arms of innate defence are all likely to play a role in susceptibility to infection (Figure 1.2).

Figure 1.2 Alterations in host defence in patients receiving treatment for cancer (from Lehrnbecher et al., 1997).

When managing children, the situation is complicated further by the maturity (or immaturity) of their underlying immune system (Schultz et al., 2004; Hartel et al., 2005). The younger the patient the more immunologically naïve he/she is and the more susceptible to both common and opportunistic infections. In a well child, development of the immune system results from exposure to common 'wild-type' infections and following vaccination. At present the effect of cancer and subsequent chemotherapy treatment on a developing immune system is not fully understood.

1.2 Management of treatment related infection

Many infections in cancer patients result from microorganisms that form part of the normal host flora colonizing the skin and the gastrointestinal (GI) mucosa. In the 1960s and 1970s gram-negative bacteria were responsible for the majority of infections seen

and mortality rates were as high as 70% (Bodey et al., 1978). In 1971, Schimpff introduced the use of empirical intravenous broad spectrum antibiotics which resulted in a marked reduction in infection related mortality (Schimpff et al., 1971). More recent studies suggest that gram-positive organisms have become more common, especially coagulase-negative *Staphylococcus* (Duncan et al., 2007), which is thought to be associated with increasing use of indwelling catheters. Current management dictates that when a patient with known neutropenia (ie. absolute neutrophil count < 0.5-1.0 x 10^9/L) develops a fever (ie. > 38.0°C for more than 4h or > 38.5°C on one occasion) he/she is admitted to hospital for intravenous antibiotic treatment. Duration of admission is influenced by the speed of fever resolution, isolation of microorganisms and clinical wellbeing of the patient. With such an approach, mortality from infection in paediatric febrile neutropenic episodes is now around 1% (Hann et al., 1997).

In 1997, Hann *et al* reported that no micro-organism could be isolated in 49% of the febrile episodes in children on chemotherapy (Hann et al., 1997), raising the question of whether a significant number of patients were being overtreated. Ongoing debate about which antibiotics to use, the role of monotherapy and the potential role of outpatient based therapy coupled with increasing acknowledgement of the heterogenous nature of the patient group has resulted in efforts to modify management.

Attention has turned to the area of risk stratification (RS), by which patients at high risk of serious infection may be selected for intensive treatment and patients at low risk of serious infection may have their antimicrobial treatment appropriately reduced. The identification of prognostically different groups in oncology is not new e.g. the use of minimal residual disease testing (Goulden et al., 2001), but the use of RS in the management of infection in paediatric patients is still in its infancy. Such an approach potentially reduces the risk of nosocomial infection and the development of bacterial resistance and it has obvious cost benefits. It would also enable children to spend more

time at home with their families with the aim of improving quality of life. Such strategies are discussed in detail in Chapter 3.

The safe application of alternative management strategies in clinical practice is dependent on the ability to accurately identify 'low risk' patients. Current strategies rely on clinical variables, neutrophil count and microbiological evidence of infection. Improved understanding of the complex interplay between other immunological risk factors and their specific contribution to infection susceptibility would be invaluable. The identification of specific "host defence factors" which may influence infection risk in these patients, would enable us to tailor management and potentially reduce further the burden of infection treatment in appropriate patients. Potential therapeutic options for the future may lie in the replacement of these immunomodulatory factors.

1.3 The immune system

The immune system is composed of innate and adaptive components, which together form major interactive defence forces in the fight against infection. The adaptive immune system is highly specific in response to both host and foreign antigens utilising B and T lymphocytes and generating immunological memory (Janeway et al., 2004; Medzhitov 2007). However, this response to foreign antigens is slow, since it takes three to seven days before clonal selection and expansion of lymphocytes occurs ensuring a specific immune response. In contrast, the innate immune system forms the first line of host defence and offers protection within the first minutes, hours or days of exposure to a potential pathogen (Beutler 2004; Medzhitov 2007). It is an evolutionarily ancient form of immunity originally thought to be non-specific in its action. However it is becoming increasingly apparent that this response has considerable specificity and is able to discriminate between pathogens and self. Defects in the adaptive immune system

are likely to influence the way we deal with an infection but defects in the innate response are more likely to influence susceptibility and is the focus of the present study.

1.4 The innate immune system

Unlike the adaptive immune response the innate response does not recognise every antigen it encounters. Instead it utilises an array of pattern recognition receptors (PRRs) to recognise highly conserved structures present on pathogens termed pathogen associated molecular patterns (PAMPs) or more appropriately microbe associated molecular patterns (MAMPs). PAMPs/MAMPs are unique to microbes and therefore prevent recognition of self. PAMPs/MAMPs share a number of common features and are usually essential for the survival or pathogenicity of microorganisms (Janeway, Jr., 1989; Akira et al., 2006). Examples of PAMPs/MAMPs include bacterial lipopolysaccharide (LPS), peptidoglycan and mannans.

1.4.1 Pattern recognition receptors (PRRs)

PRRs are expressed on effectors cells of both the immune and non-immune system (Medzhitov and Janeway, Jr., 1997; Medzhitov 2007). Once a PAMP/MAMP is recognised the PRR is able to signal to its effectors enabling an appropriate response. PRRs can be distinguished structurally, according to their protein family of origin or relating to their function i.e. signalling, endocytic or secreted. The main functions of PRRs include: opsonization, activation of complement and coagulation cascades, phagocytosis, production of antimicrobial peptides (AMPs), activation of pro-inflammatory signalling pathways and induction of apoptosis (Medzhitov and Janeway, Jr., 2000; Hoebe et al., 2004). Examples of the different PRRs and their functions are illustrated in Table 1.1.

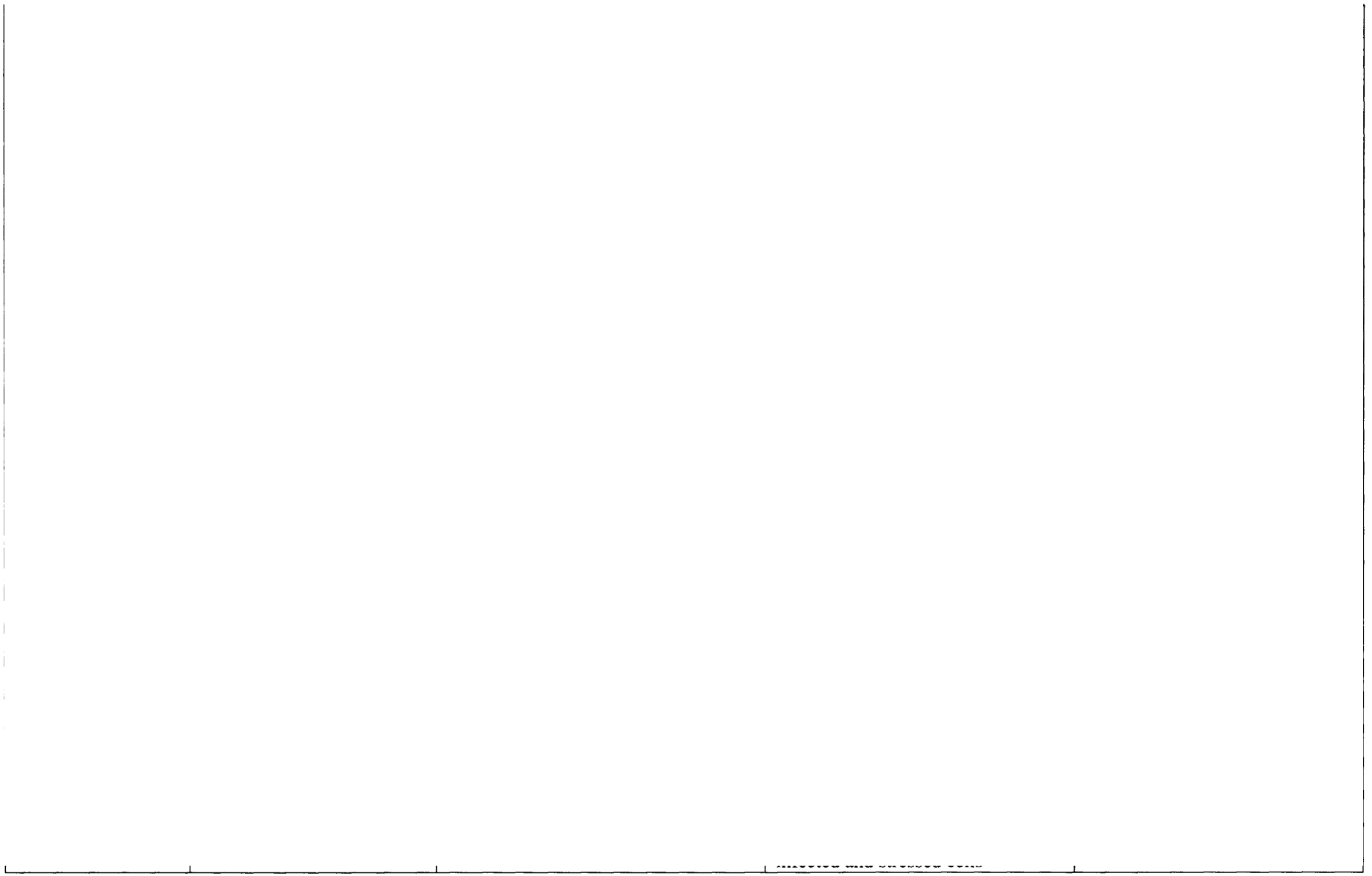


Table 1.1 Pattern recognition receptors and their major functions in host defence (adapted from Medzhitov, 2001)

1.4.2 Effector mechanisms of innate immunity

The innate response is rapid leading to activation of effector mechanisms immediately after exposure to a potential pathogen. These mechanisms consist of two major components: recruitment and/or activation of leukocytes (eg. neutrophils, monocytes/macrophages, mast cells and eosinophils) which are capable of combating invading pathogens and the release and/or activation of a variety of extracellular humoral mediators (eg. complement, cytokines and antimicrobial molecules). Evidence suggests that cellular effectors of innate responses ie. neutrophils, macrophages, dendritic cells (DCs) and natural killer (NK) cells are all affected by cancer treatment and diminished. At times of immunosuppression, it has been proposed that non-cellular and/or humoral innate host defences which are less affected by disease and treatment may assume greater importance (Lehrnbecher et al., 1997).

The predictive value of innate inflammatory markers such as C-reactive protein (CRP), procalcitonin and cytokines including Interleukins (IL) such as IL-6 and IL-8, tumour necrosis factor alpha (TNF-α) and interferon gamma (INF-γ) during febrile neutropenic episodes has been explored in a number of studies (Abrahamsson et al., 1997; de Bont et al., 1999; Lehrnbecher et al., 1999; Fleischhack et al., 2000; Oude Nijhuis et al., 2002; Lehrnbecher et al., 2004; Stryjewski et al., 2005). As yet no single marker with sufficient predictive value or discriminative power to be used in clinical practice has been identified (Hartel et al., 2007).

Many studies to date have investigated the potential role of immune candidate genes in susceptibility to infectious diseases. For example; genetic variability in cytokine loci has been shown to influence both the risk of acquiring infection and the risk of developing severe complications in many non-malignant and malignant diseases (van Deventer, 2000; Dickinson et al., 2004; Lu et al., 2005). IL-10 gene polymorphisms have been associated with severity of illness in patients with community acquired

pneumonia (Gallagher et al., 2003), pneumococcal septic shock (Schaaf et al., 2003) and rate of progression of Human Immunodeficiency Virus (HIV) (Shin et al., 2000; Smolnikova and Konenkov, 2002). An association between a high producing IL-6 promoter polymorphism and frequency and type of infection in patients with Acute Myeloid Leukaemia (AML) has been reported but a number of other innate genes explored in the same patient group showed no effect (Lehrnbecher et al., 2005).

There is a growing body of evidence to suggest that genetic deficiency of the humoral innate defence molecule and PRR, Mannose Binding Lectin (MBL), influences infection susceptibility and severity in patients undergoing treatment for cancer and the specific studies are discussed in detail in Section 1.8. Despite somewhat conflicting results and a number of unanswered questions about its specific role; MBL replacement therapy has been developed and is currently being investigated in this patient population (Petersen et al., 2006). Further work is required to establish exactly how this protein is operating in these patients in order to ensure the safe and appropriate application of MBL replacement in the future.

MBL is now known to have a number of different functions and does not work in isolation. In combination with MBL associated serine proteases (MASPs) it activates the lectin pathway of complement, also activated by Ficolins, which are a structurally and functionally related protein family. This thesis describes investigations of MBL and the lectin pathway of complement in children undergoing treatment for cancer. The components of the pathway and their effect on health and disease are now discussed in more detail.

1.5 Mannose Binding Lectin (MBL)

MBL is a member of the Collectin family; collagenous calcium-dependent lectins with a C-terminal lectin or carbohydrate-recognition domain (van de Wetering et al., 2004; Holmskov et al., 2003). The existence of mammalian serum lectins was first predicted by Robinson and colleagues (Robinson et al., 1975) and MBL protein was first isolated in cytosolic fractions of rabbit liver (Kawasaki et al., 1978). Subsequently, MBL was isolated from both human and rat liver (Wild et al., 1983). More recently extra-hepatic transcription of MBL in the small intestine and testis tissue has also been reported (Seyfarth et al., 2006).

Other human collectins include; Surfactant proteins A and D (SP-A, SP-D) which possess similar structural characteristics to MBL and are found predominantly in the lung and other mucosal sites (Holmskov et al., 2003), collectin liver 1 (CL-L1) (Ohtani et al., 1999) and collectin placenta 1 (CL-P1) (Ohtani et al., 2001) (Figure 1.3). Two other collectins: conglutinin, collectin of 43kDa (CL-43) and collectin of 46 kDa (CL-46) have so far only been detected in bovidae (Hansen et al., 2002).

Figure 1.3 The subunit structures of Collectins

(from Holmskov et al., 2003)

1.5.1 Structural aspects of MBL

MBL consists of multimers of a single polypeptide chain of 25KDa, which on post-translational modification (including hydroxylation and glycosylation) attains a molecular weight of ~32KDa (Jensen et al., 2007a). Each chain comprises four distinct regions encoded by different exons of the *MBL-2* gene as illustrated in Figure 1.4 and discussed in 1.5.3.

Each chain has a C-terminal calcium dependent carbohydrate recognition domain (CRD), a short α-helical hydrophobic neck region (coiled-coil configuration), a collagenous region containing 19 Gly-Xaa -Xaa triplets and a cysteine rich N-terminal region (Figure 1.4). Three polypeptide chains form a triple helix within the collagenous region, further stabilised by non covalent hydrophobic interactions (Sastry et al., 1989) and inter-chain disulphide bonds (Drickamer et al., 1986; Colley and Baenziger, 1987) within the N terminal cysteine rich region. The triple helix is the basic building block of circulating MBL. MBL oligomers ranging from dimers to hexamers (and higher) are found in serum (Dahl et al., 2001). X-ray crystallography and electron microscopy suggest a sertiform or bouquet like structure for MBL (similar to C1q) most likely due to an interruption in the collagenous region giving rise to a kink/hinge (Lu et al., 1990; Holmskov et al., 2003; Jensen et al., 2005;). The ability of MBL to bind microorganisms and activate complement depends on the presence of higher order oligomer structure (tetramers and above) (Larsen et al., 2004). Importantly, trimer and tetramers are the most abundant oligomer forms in serum (Teillet et al., 2005).

Each CRD binds a calcium ion enabling it to form coordination bonds with the 3- and 4-hydroxyl groups of specific sugars; mannose, glucose, L-fucose, N-acetyl-mannosamine (ManNAc) and N-acetyl-D-glucosamine (GlcNAc) (Weis et al., 1991; Weis and Drickamer, 1994). The three CRDs in each structural subunit are separated by a constant 45Å distance (Sheriff et al., 1994). Clustering of the structural subunits

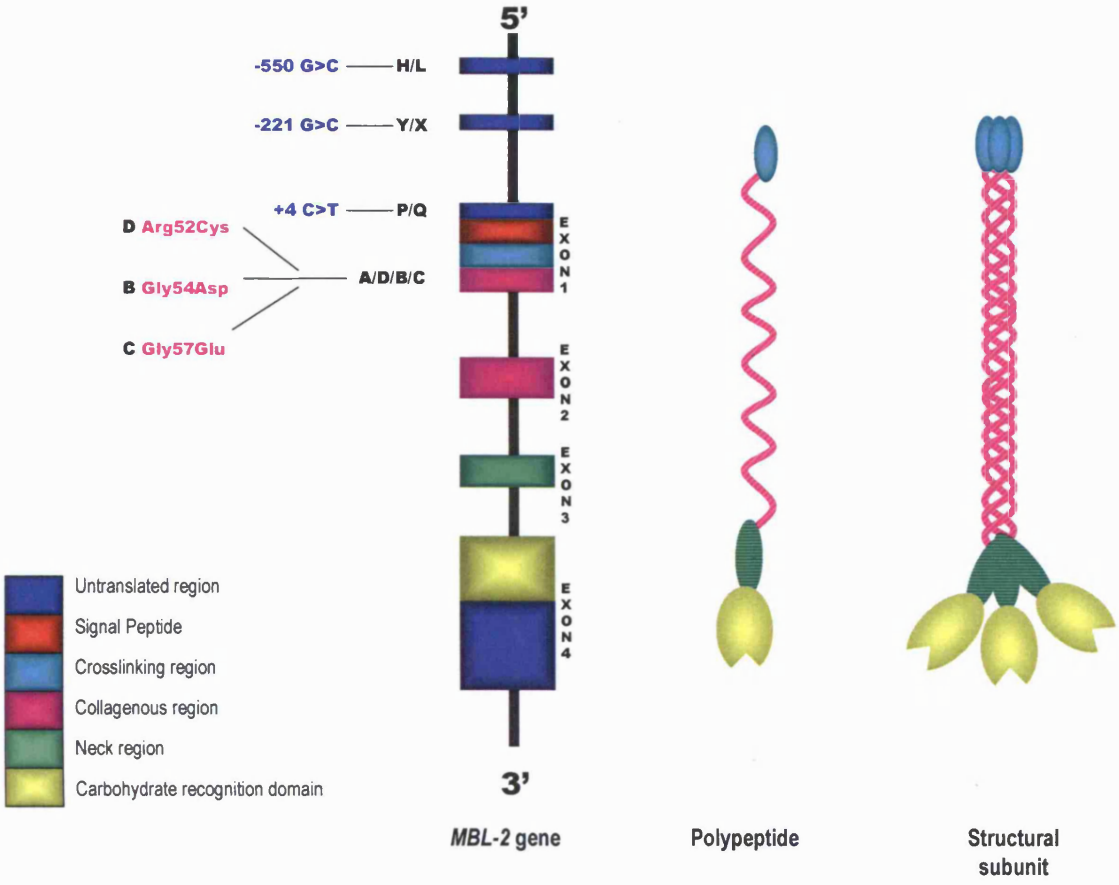


Figure 1.4 Structure of the human *MBL-2* gene and the encoded protein product. Positions of the exon1 and promoter polymorphisms are shown. Three identical 32KDa MBL polypeptides form a collagenous triple helix.

provides a flat platform, which permits binding of MBL to the arrays of repeating sugar groups on microbial surfaces. Although the binding affinity of each individual CRD-sugar interaction is relatively low at 10^{-3}M (Iobst et al., 1994) the formation of higher order oligomers provides multiple CRDs which are able to bind simultaneously with high avidity.

MBL is a major pattern recognition molecule of the innate immune system. It primarily recognises specific sugar groups (as above) on the surface of microorganisms (enabling it to distinguish self from non-self). It has been shown to bind to a wide range of bacteria, viruses, fungi and protozoa (Table 1.2) but can also bind phospholipids, nucleic acids (Palaniyar et al., 2004) and non-glycosylated proteins.

Microbes	References
Fungi	
Aspergillus fumigatus	(Neth et al., 2000)
Candida albicans	(Tabona et al., 1995; Neth et al., 2000)
Cryptococcus neoformans	(Schelenz et al., 1995)
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 Emmerik et al., 1994)
Fusobacterium (several species)	(Townsend et al., 2001)
Haemophilus influenzae	(van Emmerik et al., 1994; Neth et al., 2000)
Klebsiella aerogenes	(Neth et al., 2000)
Leptotrichia buccalis	(Townsend et al., 2001)
Listeria monocytogenes	(van Emmerik et al., 1994)
Mycobacterium avium	(Polotsky et al., 1997)
Neisseria meningitidis	(van Emmerik et al., 1994; Neth et al., 2000)
Proprionibacterium acnes	(Townsend et al., 2001)
Pseudomonas aeruginosa	(Davies et al., 2000)
Salmonella montevideo	(Kuhlman et al., 1989)
Staphylococcus aureus	(Neth et al., 2000)
Streptococcus pneumoniae	(Neth et al., 2000)
Veillonella dispar	(Townsend et al., 2001)
Protozoa	
Cryptosporidium parvum	(Kelly et al., 2000)
Plasmodium falciparum	(Klabunde et al., 2002)
Trypanosoma cruzi	(Kahn et al., 1996)
Viruses	
Influenza A	(Kase et al., 1999; Saifuddin et al., 2000; Hart et al., 2002; Ji et al., 2005)
HIV	(Saifuddin et al., 2000; Hart et al., 2002; Ji et al., 2005)
Herpes simplex 2	(Fischer et al., 1994; Gadjeva et al., 2004)
SARS-CoV	(Ip et al., 2005)

Table 1.2 MBL binds to a range of microbial surfaces

Using flow cytometry Neth *et al* demonstrated MBL binding to clinically relevant bacterial isolates from immunocompromised children and noted differences in binding within some species (Neth et al., 2000). The role of specific structures on microorganisms (e.g. the capsule) which permit or prevent binding to MBL has been explored (Krarup et al., 2005). Early studies suggested lipopolysaccharide (LPS) may play a crucial role in MBL binding and function (Kawakami et al., 1982). Other mechanisms which enable microorganisms to avoid recognition include lipooligosaccharide (LOS) sialyation (Jack et al., 1998; Devyatyarova-Johnson et al., 2000).

1.5.2 Functional aspects of MBL

MBL is implicated in diverse processes including complement activation, complement-independent opsonophagocytosis, inflammation, recognition of altered self-structures and apoptotic cell clearance.

1.5.2.1 MBL and complement activation

Complement was first described in the 1890s by Jules Bordet as a heat labile protein in serum that 'complemented' heat stable antibodies in the killing of bacteria. It is now known to be a highly developed host-defence system involved both in innate and antibody-mediated immunity (Walport, 2001a; Walport, 2001b; Gros et al 2008). It consists of over 30 protein components involved in a cascade of reactions which ultimately result in opsonization of pathogens, chemotaxis and activation of leucocytes and direct killing of pathogens via the membrane-attack complex (MAC). There are three activation pathways, namely the classical (Cooper, 1985), alternative (Pillemer et al., 1954) and lectin (Fujita et al., 2004) which converge into one common final lytic pathway (Figure 1.5).

Figure 1.5 Activation of the classical, alternative and lectin pathways.

Alternative pathway inhibitors are not illustrated (Fujita, 2002).

A role for MBL in host defence was first proposed in 1987 when Ikeda *et al* observed that the protein was able to activate the classical pathway of complement (Ikeda et al., 1987). However, it is now clear that MBL activates the novel third pathway of complement, often termed the MBL Lectin pathway, in an antibody and C1-independent fashion. This pathway is also activated by Ficolins which are discussed in detail in Section 1.6.

MBL circulates in association with a group of MASPs (Sorensen et al., 2005). In 1992 a novel complement enzyme thought to generate C3 convertase (C4bC2a), associated with classical pathway activation was identified (Matsushita and Fujita, 1992).

However, this activity was later found to be mediated by MASP2 (Thiel et al., 1997) and the original enzyme is now known as MASP1. Subsequently a small fragment of MASP2 termed sMAP or Map19 was reported (Stover et al., 1999; Takahashi et al., 1999). A third MASP (MASP3) has also been identified (Dahl et al., 2001). The MASPs are described in more detail in Section 1.7.

MBL (or Ficolins) binding to microorganisms leads to auto-activation of MASP2. MBL/MASP2 complexes initially cleave C4 to produce C4b and C4a. C4b remains attached near to the MBL/MASP complex and recruits C2 which is also cleaved by MASP2 (Wallis et al., 2007). This results in formation of C2b and C2a, the latter of which remains attached to C4b to form C3 convertase (C4b2a). The C3 convertase is indistinguishable in specificity to that found in the other two activation pathways of complement (Feinberg et al., 2003). Recent studies suggest that both MASP1 and MASP2 are required for the generation of C3 convertase with MASP3 acting as a competitive inhibitor (Dahl et al., 2001; Moller-Kristensen et al., 2007).

Interaction between MBL and the alternative pathway has been proposed (Brouwer et al., 2006) and one report suggests an MBL dependent C2 bypass mechanism for alternative pathway- mediated C3 activation (Selander et al., 2006).

1.5.2.2 Opsonophagocytosis

In 1968, Miller *et al* reported a plasma associated defect of phagocytosis in a child with severe recurrent infections, failure to thrive and diarrhoea (Miller et al., 1968). In-vitro work revealed a failure of the child's plasma to opsonise heat killed bakers yeast (*Saccharomyces cerevisiae*). This defect was later detected in the sera of children with recurrent, unexplained infections (Soothill and Harvey, 1976) and chronic diarrhoea of infancy (Candy et al., 1980). Interestingly, studies revealed a relatively high frequency of this defect in the general population (~5%). This defect was subsequently shown to be due to decreased deposition of C3b on yeast surfaces, linking it to the complement

system (Turner et al., 1981). Finally, the opsonic defect was found to be associated with low levels of MBL (Super et al., 1989).

1.5.2.3 Cell receptors for MBL

MBL interacts directly with cell surface receptors and promotes opsonophagocytosis (Kuhlman et al., 1989). MBL has been shown to neutralize influenza A virus (Kase et al., 1999). A number of putative MBL-binding proteins/receptors have been proposed including cC1qR/calreticulin (Malhotra et al., 1990), $C1qR_p$ (Tenner et al., 1995), CR1 (Klickstein et al., 1997; Ghiran et al., 2000) and α2β1 Integrin (Edelson et al., 2006). MBL may share a common receptor with C1q (Oroszlan et al., 2007). Down stream signalling pathways also remain elusive. The TLR/NF-κB pathway has been implicated in downregulation of the pro-inflammatory cytokine response to LPS by SP-A and C1q but not MBL (Alcorn and Wright, 2004; Yamada et al., 2004). At present, it is unclear whether MBL acts as a direct opsonin or acts as a modulator of other complement pathways and/or antibody mediated phagocytosis.

1.5.2.4 MBL in inflammation

The role of MBL as a modulator of inflammation appears to be complex and, accordingly, its mechanism(s) of action remains unclear. Early work suggested that MBL could inhibit TNFα output by monocytes in response to streptococcal rhamnose glucose polymers (a molecule related to LPS) and cryptococcal membrane glycoprotein (Soell et al., 1995; Chaka et al., 1997). However increased TNFα release was demonstrated in response to other organisms including *Candida*, *Leishmania chagasi* and HIV-1 gp120 protein (Ghezzi et al., 1998; Santos et al., 2001; Heggelund et al., 2005). Further work suggests that the effect of MBL on cytokine production may be concentration dependent. This concept was addressed in studies by Jack *et al* using *Neisseria meningitidis* incubated with increasing concentrations of MBL before being added to MBL deficient whole blood. Release of TNF-α, IL-1β and IL-6 from

monocytes was enhanced at MBL concentrations below 4μg/ml but suppressed at higher concentrations (Jack et al., 2001). Further studies have supported the finding that MBL can modulate the pro-inflammatory cytokine response (Nadesalingam et al., 2005; Fraser et al., 2006). MBL also appears to promote production of the anti-inflammatory cytokine IL-10 (Sprong et al., 2004; Fraser et al., 2006).

Clinical studies of critically ill patients requiring intensive care management have shown that individuals who are MBL deficient are more likely to develop Systemic Inflammatory Response Syndrome (SIRS) and progress to septic shock and death (Garred et al., 2003a; Fidler et al., 2004), findings which may well relate to the proinflammatory cytokine response. It should also be noted that chronic inflammation is now increasingly accepted to be a risk factor for myocardial infarction (MI) and a recent study by Saevarsdottir *et al* has found that patients with high MBL levels have a decreased likelihood of suffering a myocardial infarction - again suggesting a potential role for MBL in modulating the inflammatory response (Saevarsdottir et al., 2005).

1.5.2.5 The role of MBL in the recognition of altered self and apoptosis

A role for MBL in the clearance of apoptotic cells has been proposed (Ogden et al., 2001; Palaniyar et al., 2003; Palaniyar et al., 2004). Defects in the clearance of apoptotic cells have been implicated in the pathogenesis of certain autoimmune conditions although the precise role of MBL, if any, remains elusive. For example, MBL deficient mice display defective apoptotic cell clearance, and yet do not develop autoimmune diseases (Stuart et al., 2005).

In animal studies MBL has been implicated in the pathophysiology of ischemia reperfusion injury due to its ability to recognize altered self-structures. Studies by Stahl *et al* have proposed the lectin pathway as a mediator of this process in certain organs and the absence of MBL/MASP pathway activation appears to afford protection in these disease models (Hart et al., 2005; Walsh et al., 2005). Different roles for the MBL and

classical pathways in skeletal muscle ischaemia reperfusion injury have been demonstrated with the MBL pathway mediating the cytotoxic effects of reperfusion injury and the classical pathway mediating vascular injury (Chan et al., 2006). However, the relevance of these findings to human health needs to be established.

Changes in cell surface structures during oncogenic transformation appear to promote binding of MBL to cancer cells (Hakomori, 2001) where the protein can mediate cytotoxic effects including MBL-dependent cell mediated cytotoxicity (MDCC) (Ma et al., 1999; Nakagawa et al., 2003). The relative importance of such mechanisms in tumour immunology is, at present, unknown.

1.5.3 Genetics of human MBL

Although there are two human MBL genes, *MBL1* is a pseudogene and only *MBL2* encodes a protein product. The functional *MBL2* gene is located on chromosome 10q11.2-q21 (Sastry et al., 1989; Taylor et al., 1989) in a cluster encoding SP-A and SP-D (Kolble et al., 1993). MBL comprises four exons as illustrated in Figure 1.4. Exon 1 encodes the signal peptide, the N-terminal cysteine-rich region and part of the glycine rich collagenous region. Exon 2 encodes the remainder of the collagenous region and exon 3 encodes the α-helical 'neck' region. The fourth exon encodes the carbohydrate recognition domain. The promoter region contains a number of regulatory elements which are known to affect transcription (Naito et al., 1999).

The complete nucleotide sequence of the *MBL2* gene was established in two British children with recurrent infections and low MBL protein levels (Sumiya et al., 1991). In both individuals a point mutation was observed in codon 54 (GGC → GAC, glycine to aspartic acid). Familial studies confirmed the defect was inherited in an autosomal dominant fashion. Since then further mutations in exon 1 have been identified. The

codon 57 mutation (GGA → GAA, glycine to glutamic acid) was identified in a sub-Saharan African population (Lipscombe et al., 1992) followed by the mutation in codon 52 (GCG → GTG, arginine to cysteine) originally identified in both Caucasian and Kenyan populations (Madsen et al., 1994). These point mutations are commonly referred to as variants B, C and D with A indicating the wildtype (WT). The B variant occurs at a gene frequency of ≈25% in Eurasian populations. In contrast, the C variant is rare in Eurasians but common in sub-Saharan African populations with frequencies of 50-60%. Population studies suggest that the B variant mutation may have arisen 20,000-50,000 years ago (Turner et al., 2000).

The effect of exon 1 mutations on protein function is an active area of research. Impairment of oligomer structure and MASP binding/activation results in functional deficiency. B and C mutations result in replacement of critical axial glycines in the triple helix by dicarboxylic acids which result in distortion and destabilisation of helix formation (Sumiya et al., 1991; Persikov et al., 2004). In contrast the D mutation results in replacement of an arginine with a cysteine. An extra cysteine is likely to cause adventitious disulphide bond formation affecting higher oligomer formation (Wallis and Drickamer, 1999).

Three polymorphisms, H/L, X/Y and P/Q at positions -550, -221 and +4 in the promoter region of the *MBL2* gene have also been reported (Madsen et al., 1995; Madsen et al., 1998). Subsequently, four common haplotypes LXP, LYP, LYQ and HYP are known to occur. Of these, HYP, associated with medium to high levels of MBL and LXP, associated with low levels of the protein, appear to have the most profound effect. These promoter haplotypes are in strong linkage disequilibrium with the exon 1 mutations resulting in seven common extended haplotypes, namely, HYPA, LYPA, LYQA, LXPA, HYPD, LYPB and LYQC. Figure 1.6 illustrates the frequency of these various haplotypes in selected populations and highlights the degree of ethnic variation. Other

Figure 1.6 MBL haplotype frequencies in different populations.

Haplotype frequency data are taken from published population studies.
1=Chiriguanos, Argentina (Madsen et al., 1998), 2=Mapuche, Argentina (Madsen et al., 1998), 3=Eskimos, Greenland (Madsen et al., 1998), 4= Caucasians, Spain (Lozano et al., 2005), 5=Caucasians, Denmark (Madsen et al., 1998), 6=Mozambique (Madsen et al., 1998), 7=Kenya (Madsen et al., 1998), 8=Korea (Lee et al., 2005a), 9=Japan (Matsushita et al., 1998), 10=Warlpiri, Australia (Turner et al., 2000).

rare haplotypes have also been described (Boldt and Petzl-Erler, 2002) and additional polymorphisms in other areas of the gene are also under investigation (Bernig et al., 2004; Bernig et al., 2005).

Combined structural gene and promoter polymorphisms result in dramatic variation in MBL concentration even in apparently healthy individuals (Caucasian range <20-10000 ng/ml) (Figure 1.7). Such variation in MBL concentration is observed when levels are measured using antibody capture techniques utilising antibodies that preferentially detect higher (functional) MBL oligomers. Recent work using alternative capture

Figure 1.7 Correlation between MBL levels and genotype

Genotypes are shown in a simplified form using just the exon1 and XY promoter genotypes. The O allele represents any of the exon1 variant alleles, B,C or D (adapted from Christiansen et al., 1999).

antibodies which detect both large and small oligomers now suggests that levels may not vary so dramatically in individuals in possession of variant MBL but just that their MBL has reduced complement activating ability (Garred et al., 2003b).

MBL may also act an acute phase reactant (Ezekowitz et al., 1988). Studies have shown MBL levels can increase up to 3 fold during the acute phase, the response being variable between individuals (Thiel et al., 1992; Dean et al., 2005; Van Till et al., 2006). It should be noted that during an acute phase response individuals heterozygous or homozygous for MBL mutations appear unable to achieve the protein levels of those possessing a WT genotype. More recently it has been suggested that MBL synthesis may be subject to hormonal regulation (Sorensen et al., 2006).

Approximately one third of the Caucasian population possess genotypes conferring low levels of 'functional' MBL with approximately 5% having very low levels i.e homozygotes or compound heterozygotes. No absolute level of MBL deficiency has been defined. Genotype and phenotype show a relatively strong correlation and studies often use just one measure to infer deficiency.

1.5.4 MBL gene evolution

Although two MBL genes are present in the human genome only *MBL2* encodes the protein. This is in contrast to rodents and rhesus monkeys where MBL occurs in two distinct forms (Mogues et al., 1996; Hansen et al., 2000). The two human MBL genes are most likely due to a gene duplication event (Sastry et al., 1995) and the potential mechanisms responsible for silencing the *MBL1* gene are under debate. An intron 1 splicing defect leading to two stop codons in exons 3 and 4 of the *MBL1* gene may be one such mechanism (Guo et al., 1998). More recently, Seyfarth *et al* identified glycine substitutions in codon 53 of the *MBL1* gene which bear a close resemblance to those found in codon 54 of *MBL2* (Seyfarth et al., 2005). Such substitutions were also found in other higher primates including chimpanzees and gorillas but not in more distant primates such as the rhesus monkey. It has been proposed that both the *MBL1* and *MBL2* genes have been selectively silenced by the same molecular mechanisms, but skewed in time resulting in overall downregulation of MBL levels in the present human population.

1.5.5 The MBL Paradox

The high frequency of variant alleles observed in certain populations suggests that functional MBL deficiency may well be advantageous. Certain intracellular parasites

use C3 opsonization and C3 receptors on monocytes/macrophages to enter their host. Therefore any reduction in complement-activating function of the host may reduce the probability of parasitization. Studies of visceral leishmaniasis revealed that patients are more likely to have high MBL levels than uninfected controls (Santos et al., 2001; Alonso et al., 2007). Presence of MBL deficiency has also been shown to confer protection against lepromatous or borderline lepromatous leprosy (Garred et al., 1994; Dornelles et al 2006; de Messias et al., 2007). An alternative explanation of the unexpectedly high frequency of low MBL phenotype individuals found in many tropical regions is that excessive complement activation can result in immunopathologically mediated host damage and therefore any mechanism that reduces complement activation may be beneficial (Lipscombe et al., 1992).

1.5.6 MBL polymorphisms and disease

The clinical significance of MBL deficiency was initially studied in young children with recurrent infections, where it was suggested that MBL may provide protection during the 'window of vulnerability'(approximately during 6-24 months of age), i.e. the period when maternal IgG antibody levels have waned and the infant's own adaptive immune response is still immature (Super et al., 1989; Summerfield et al., 1997; Koch et al., 2001). However numerous studies have shown that MBL plays a role throughout life, as an ante-antibody, a critical component of first line host defence (Ezekowitz, 1991; Summerfield et al., 1995). MBL deficiency has been implicated in both disease susceptibility and as a modulator of severity although it is often difficult to make such a distinction. Table 1.3 highlights a selection of studies to illustrate the diverse evidence that has been accrued.

CONDITION	OUTCOME	REFERENCE
Meningococcal infection	Increased susceptibility in patients who were homozygous for MBL variant alleles- Hospital study 7.7% cf. 1.5% in controls, Community study- 8.3% cf. 2.7% in controls	Hibberd et al., 1999
	Increased frequency of MBL variant genotypes in patients compared to controls (32% Vs 8%)	Faber et al., 2007
Mycoplasma infection	Increased frequency of MBL deficiency in patients with primary antibody deficiencies and Mycoplasma compared to controls	Hamvas et al., 2005
Invasive *Pneumococcal* infection	12% patients were homozygous for MBL variants cf. 5% controls	Roy et al., 2002; Moens et al., 2006
	Small but significantly increased risk of infection in individuals with variant genotypes	
SARS	Increased frequency of MBL deficient genotypes in patients compared to controls	Ip et al., 2005; Zhang et al., 2005
HIV- susceptibility	Susceptibility was 8% in HIV infected homozygous patients, vs. 0.8% in healthy controls and 0% among high-risk controls	Garred et al., 1997
HIV- disease progression	Duration of survival for patients with variant alleles and those with WT alleles, 11 vs. 18 mths	
Chronic hepatitis B	D variant allele in 27% of white patients with hepatitis B and in 4% of healthy uninfected controls	Thomas et al., 1996
	B variant allele in 44% of Chinese patients with cirrhosis and in 23% of healthy uninfected controls	Yuen et al., 1999; Chong et al., 2005
	Increased occurrence of cirrhosis and hepatocellular carcinoma in individuals in possession of MBL variant alleles	
Chronic hepatitis C -IFN responsiveness	LX promoter or B variant allele was present in 60.7% of unresponsive patients vs. 38.5% of responsive patients.	Matsushita et al., 1998
Plasmodium falciparum	B and C variant alleles were more common in patients from Gabon with severe malaria	Luty et al., 1998
Susceptibility to *Crytosporidium parvum*	MBL variant alleles were more common among HIV infected patients	Kelly et al., 2000
Chronic necrotising pulmonary aspergillosis	MBL variant alleles were present in 70% of patients Vs. 26% of control subjects	Crosdale et al., 2001
Cystic fibrosis	Lung function significantly impaired in patients with variant MBL alleles. Association between MBL variant alleles and *Burkholderia cepacia* and *Pseudomonas aeruginosa* infection. MBL deficiency associated with earlier onset of infection and faster decline of pulmonary function	Garred et al., 1999b; Dorfman et al., 2008
	Severity of liver disease in CF patients is more severe in patients with variant MBL alleles	Gabolde et al., 2001
	Homozygosity for MBL variant alleles is associated with reduced lung function and more frequent hospital admissions in adults	Davies et al., 2004
Rheumatoid arthritis	MBL variant alleles are associated with increased severity and early onset of disease	Graudal et al., 1998; Ip et al., 2000
SLE	Increased frequency of variant alleles in patients. Over-representation of Homozygotes for MBL variant alleles in Danish SLE patients, who had more significant renal involvement and infections	Garred et al., 1999a, 2001; Lee et al., 2005b
	Homozygosity for MBL variant alleles is associated with an increased risk of arterial thrombosis	Ohlenschlaeger et al., 2004
Systemic Inflammatory response syndrome (SIRS) - Adults	MBL variant alleles were associated with an increased risk of developing sepsis, severe sepsis, septic shock and death.	Garred et al., 2003a
SIRS- Children	MBL variant alleles were associated with an increased risk of developing SIRS and of progression to sepsis and septic shock.	Fidler et al., 2004
Myocardial infarction	Individuals with high MBL levels had a decreased likelihood of suffering a myocardial infarction.	Saevarsdottir et al., 2005

Table 1.3 Clinical associations between MBL and disease

The impact of such a common immunodeficiency on the wellbeing of the general population has been questioned. Dahl *et al* studied 9245 Danish Caucasian adults as part of the Copenhagen Heart Study. Morbidity (any hospitalisation and infection) and mortality were recorded for 24 and 8 years, respectively. After adjustment for potential confounders, neither the relative risk for hospitalisation per se, nor hospitalisations for infection or death was significantly different between MBL deficient and MBL sufficient individuals (Dahl et al., 2004). Similar findings in unselected adults admitted to hospital with infections have been reported (Tacx et al., 2003). Studies by Roos *et al* offered one potential explanation for these observations. They found that antibody-mediated activation of the classical pathway of complement compensated for impaired target opsonization via the MBL pathway in MBL deficient individuals (Roos et al., 2004). The high prevalence of MBL variant alleles without a major effect on population fitness suggests that MBL may be relatively redundant in the healthy host (Verdu et al., 2006). However MBL deficiency appears to assume greatest clinical importance when it occurs in combination with defects in other arms of the immune system (Aittoniemi et al., 1998). One such population are patients on treatment for malignancy discussed in detail in Section 1.8.

1.6 Ficolins

The lectin pathway of complement is also activated by Ficolins. Ficolins are a group of proteins that consist of lectin domains linked to collagen and fibrinogen-like domains (Lu and Le, 1998). They were originally identified as transforming growth factor-β1 (TGF-β1) binding proteins on porcine uterus membranes (Ichijo et al., 1991; Ichijo et al., 1993). Ficolins have now been identified in several species including rodents (Fujimori et al., 1998), hedgehogs (Omori-Satoh et al., 2000) and invertebrates (Kenjo et al., 2001).

In human serum, two ficolins have been isolated: L-ficolin/P35 (Matsushita et al., 1996; Lu et al., 1996) and H-ficolin or Hakata antigen, (Fujimori et al., 1998; Akaiwa et al., 1999) encoded by the *FCN2* and *FCN3* genes respectively. They bind to MASPs and activate the lectin pathway (Matsushita et al., 2000a). L- and H-ficolins are synthesised by hepatocytes, although H-ficolin has also been detected in bronchial/alveolar fluid and in bile (Matsushita et al., 1996; Akaiwa et al., 1999). A third human ficolin, M-ficolin is found on peripheral blood mononuclear cells (PBMCs), polymorphonuclear cells and type II lung epithelial cells and is encoded by the *FCN1* gene. It can also bind MASPs and activate the lectin pathway (Lu et al., 1996; Teh et al., 2000; Frederiksen et al., 2005; Liu et al., 2005).

1.6.1 Ficolin structure and function

Ficolins are structurally similar to MBL, as illustrated in Figure 1.8 and consist of three identical polypeptide subunits which form functional oligomers. Their fibrinogen-like domains enable specific binding to N-acetyl-glucosamine (GlcNAc) residues on microbial surfaces (Matsushita et al., 1996; Sugimoto et al., 1998; Fujimori et al., 1998; Ohashi and Erickson, 1997; Garlatti et al., 2007) and a number of non sugars (Krarup et al., 2004).

L-ficolin can bind lipoteichoic acid (LTA) from the cell wall of gram-positive bacteria (Lynch et al., 2004) and binding to a number of gram-positive and gram-negative organisms has now been demonstrated (Krarup et al., 2005; Aoyagi et al., 2005) . H-ficolin does not bind to *Staphylococcus aureus* or *Streptococcus pneumoniae* but can bind and inhibit growth of *Aerococcus viridans* (Tsujimura et al., 2002) and M-ficolin has been shown to bind specifically to *Staphylococcus aureus* (Liu et al., 2005). In addition to complement activating function there is also evidence to suggest that L- and

Figure 1.8 Domain and oligomeric structure of mannose-binding lectin and ficolins. (from Fujita, 2002).

H-ficolin participate in the clearance of dying cells by binding to DNA on apoptotic and necrotic cells (Jensen et al., 2007b; Honore et al., 2007).

1.6.2 Ficolins and disease

At present the impact of Ficolin deficiency on clinical disease is largely unknown. L-ficolin and H-ficolin are present in the serum at mean concentrations of ~10μg/ml and 15μg/ml, respectively, which vary up to 10 fold in healthy blood donors (Kilpatrick et al., 2003a) and there is no accepted level indicating deficiency to date. H-ficolin has been reported to be elevated in systemic lupus erythematosus (SLE) (Yoshizawa et al., 1997) and acute hepatitis and decreased in hepatic cirrhosis (Fukutomi et al., 1996). A study investigating L-ficolin levels in 313 Polish children with respiratory infections found levels below the lower limit of control values in 6% of patients (p=0.03) and were most strongly associated with children having atopic disorders (11%; p=0.002). The authors concluded that L-ficolin may have a role in protection from microorganisms complicating allergic diseases (Atkinson et al., 2004). Polymorphisms have been identified in the L-ficolin/*FCN2* gene which correlate with low serum levels and altered function (Hummelshoj et al., 2005; Herpers et al., 2006; Munthe-Fog et al., 2007). A limited number of studies have investigated ficolin polymorphisms and susceptibility to disease. FCN1 single nucleotide polymorphisms (SNPs) have been implicated in the development of Rheumatoid arthritis and FCN2 SNPs have been linked with Behcets disease (Vander Cruysson et al., 2007; Chen et al 2007).

1.7 MBL associated serine proteases (MASPs)

As discussed previously, MASPs are a family of serine proteases found in serum in complex with both MBL and Ficolins. They are primarily synthesised in the liver but mRNA has recently been detected at other sites; MASP1 and MASP2 mRNA in the small intestine and testis tissue and MASP3 mRNA in brain, spleen, lung and thymus (Schwaeble et al., 2002; Seyfarth et al., 2006).

1.7.1 MASP gene and protein structure

The three MASPs and Map19 are generated from two genes by alternative splicing (Figure 1.9). The *MASP1* gene is located on chromosome 3q27-28 (Sato et al., 1994) and consists of 17 exons of which 16 encode MASP1 mRNA. Exons 1-10 encode 5 N-terminal domains and exons 12-17 encode the serine protease domain. Two splice variants of the gene generate MASP1 and MASP3. They share exons 1-10, with exon 11 encoding the serine protease domain of MASP3 (Dahl et al., 2001). MASP2 and MAp19 (or sMAP) are generated by alternative splicing of the *MASP2/MAp19* gene located on chromosome 1p36.2-3 (Stover et al., 1999; Stover et al., 2001). MAp19 and MASP2 share exons 1-4 with the C terminal of MAp19 encoded by a unique exon encoding four amino acids, EQSL, followed by a stop codon.

Figure 1.9 Genomic organisation and protein structure of MASPs and MAp19

The exon-intron structure of the MASP and MAp19 genes. The asterisks identify potential glycosylation sites and the arrows show the Arg-Ile bond for activation. (adapted from Schwaeble et al., 2002).

MASPs share structural homology with C1r and C1s (Sato et al., 1994; Endo et al., 1996; Arlaud et al., 1998). The N terminal structure is composed of two CUB (Uegf, a member of the epidermal growth factor family and bone morphogenic protein I) domains (C1r/C1s like domains), separated by an epidermal growth factor (EGF) like domain. In combination these domains are responsible for calcium dependent interaction with MBL or ficolins (Chen and Wallis, 2001; Cseh et al., 2002; Feinberg et al., 2003).

Two complement control protein (CCP) domains complete the non catalytic component, or A chain. A small linker region covalently bonds the A chain to the B chain or serine protease domain.

MASPs are synthesised as inactive presursors, which circulate as zymogens activated when MBL and ficolins bind to carbohydrates. On binding, MASPs are cleaved at the peptide bond (arginine-isoleucine) in the N terminal part of the serine protease domain. The active protease domain remains covalently attached to the MASP molecule by a disulphide bond.

The crystal structures of rat and human MASP2 have been elucidated (Feinberg et al., 2003). The rat CUB1-EGF-CUB2 fragment exhibits an elongated structure responsible for binding to MBL and Ficolin and the CCP and serine protease domains determine the substrate specificity and recognition (Ambrus et al., 2003; Rossi et al., 2005; Girija et al., 2007). The recombinant human MASP2 CCP2/SP joint was found to be flexible most likely allowing for auto-activation (Harmat et al., 2004). Human MASP1-3 and MAp19 form homodimers which independently associate with MBL in a calcium dependent complex (Thielens et al., 2001). Figure 1.10 illustrates a model of MASP2 activation by MBL. On binding, changes in MBL structure enable conformational change at the flexible regions of the MASP resulting in activation.

Figure 1.10 Model of MASP2 activation by MBL (from Wallis, 2007).

Studies to date suggest MASP complexes may not interact with the same MBL or Ficolin molecule even though there is similarity in their binding sites, with lys55 playing a critical role in function (Mayilyan et al., 2005; Teillet et al., 2007). MBL oligomers differentially activate complement (Wallis and Drickamer, 1999) and appear to associate with different MASPs suggesting diversity in function is likely (Dahl et al., 2001). MASP1 and MAp19 have been shown to associate with trimers possessing C3 cleavage activity. In contrast, MASP2 associates with tetramers responsible for C4 cleavage and MASP3 forms complexes with higher (tetramer and above) MBL oligomers. Importantly, *MBL2* exon 1 polymorphisms affect MASP binding by destabilizing oligomer formation and also prevent conformational changes occurring in the bound MBL/MASP complex inhibiting MASP activation (Wallis and Dodd, 2000, Wallis 2007).

1.7.2 MASP activity

Autoactivation of MASP2 on MBL or Ficolins binding to a target ligand leads to activation of the lectin pathway of complement (Gal et al., 2005) (Section 1.5.2.1.). The

role of MAp19 or sMAP is not clearly understood but recent work suggests that it competes with MASP2 to bind MBL and attenuates lectin pathway activation in mouse models (Iwaki et al., 2006).

The role of MASP1 in the activation of the lectin pathway of complement is somewhat controversial. MASP1 has been shown to cleave both C2 and C3 (Matsushita and Fujita, 1992; Matsushita and Fujita, 1995; Matsushita et al., 2000b; Chen and Wallis, 2004) but has no activity towards C4. Studies using purified and recombinant MASP1 have resulted in contrasting results (Wong et al., 1999; Rossi et al., 2001; Ambrus et al., 2003;) questioning the biological significance of MASP1 mediated C3 cleavage. However, recent data suggest that despite different modes of action both MASP1 and MASP2 are required for the generation of efficient C3 convertase (Moller-Kristensen et al., 2007). Studies investigating the physiological substrate for MASP1 have shown that it can cleave Factor XIII and fibrinogen, catalysing the formation of crosslinked fibrin (Hajela et al., 2002). It has been suggested that MASP1 may provide a link between the complement system and the coagulation system, thus playing a role in limiting spread of infection (Hajela et al., 2002; Gal et al., 2007).

Understanding of MASP3 function remains limited. MASP3 forms complexes with MBL oligomers and can act as a competitive inhibitor of both MASP1 and MASP2 activity but does not auto-activate (Dahl et al., 2001; Moller-Kristensen et al., 2007). Recombinant MASP3 does not cleave C2, C3 or C4 and on activation does not complex with C1 inhibitor (Zundel et al., 2004). MASP3 has been shown to cleave insulin-like growth factor binding protein (IGFBP) 5 and a synthetic substrate for plasmin and kallikreins but the biological significance of these effects is unknown (Cortesio and Jiang, 2006).

1.7.3 MASP genetics and deficiency

To date MASP2 is the only family member investigated in human disease. In 2003, the first case of MASP2 deficiency resulting in non-functional lectin-mediated complement activation was reported (Stengaard-Pedersen et al., 2003). Despite normal levels of MBL, the patient suffered from recurrent infections and chronic inflammatory disease. Sequence analysis of the *MASP2/MAp19* gene revealed that the patient was homozygous for a mutation in exon 3 which resulted in the substitution of an aspartic acid for a glycine at position 105 in mature MASP2 (D105G) preventing formation of functional MBL-MASP2 complexes. Approximately 0.3% of the population are expected to be homozygous for the D105G mutation and gene frequencies have been shown to vary between diseased and healthy populations (Carlsson et al., 2005). Other MASP SNPs have been reported which also show ethnic diversity (Lozano et al., 2005; Thiel et al., 2007). MASP2 may be a disease modifier in Cystic fibrosis (Olesen et al., 2006).

1.8 The lectin pathway of complement in patients with cancer.

As discussed previously, MBL may be relatively redundant in the healthy host, assuming greatest clinical importance when it occurs in combination with defects in other arms of the immune system such as those experienced by patients on treatment for cancer. To date, the majority of studies involving patients with cancer have investigated associations between MBL polymorphisms and infectious complications (Table 1.4). Ficolin levels have been analysed in only one study to date (Kilpatrick et al., 2003b) and four studies have explored MASPs (Ytting et al., 2004; Ytting et al., 2005a; Ytting et al., 2005b; Granell et al., 2006).

There have also been reports that MBL polymorphisms may be a risk factor for the development of malignancy. In children, there has been one study that suggests that MBL polymorphisms are a risk factor for development of childhood acute lymphoblastic leukaemia (ALL), particularly with early age of onset (Schmiegelow et al., 2002). There have also been studies conducted in adults with solid tumours implicating an increased risk in MBL variants (Baccarelli et al., 2006; Bernig et al., 2007).

1.8.1 The lectin pathway and infectious complications in cancer patients

In 2001, two studies were published which implicated low MBL as a risk factor for increased infectious complications in patients on chemotherapy. Neth *et al* reported that MBL deficiency has an important adverse influence on the duration of FN episodes in 100 children receiving chemotherapy for malignancies. Children with variant MBL alleles suffered twice as many days of FN over a six month period when compared to those with a WT genotype (20.5 days vs. 10 days, p=0.014) (Neth et al., 2001). The median duration of each FN episode was also significantly higher in patients who were MBL deficient. This study suggested that determination of the MBL status of all children receiving chemotherapy for malignancies would be beneficial to identify those at higher risk of infections. Another simultaneous study investigated 54 adult patients who were on chemotherapy for various haematological malignancies. They found a significant association between low concentrations of MBL and serious infections, defined as pneumonia, bacteraemia or both, contracted within three weeks of starting chemotherapy. Patients with an MBL concentration of 0.5μg/ml or less were at highest risk of severe infection. They suggested that MBL replacement in patients having chemotherapy could reduce susceptibility to infection (Peterslund et al., 2001).

Study	Patient population (n)	Design	MBL Assay	Follow up	Outcome measures	Conclusions
PAEDIATRIC						
Neth et al 2001	100 (55- ALL, 12-AML)	Prospective	Genotype Level	6 months	Frequency and duration of infection	Episodes in patients with MBL mutations were twice as long as wildtype
Lausen et al 2006	136 ALL	Retrospective	Genotype	50d	Frequency of infections	MBL deficiency had no effect on frequency of infections during ALL induction therapy
Frakking et al 2006	110 total, 66 with FN. 38% Haem, 45% Solid 17% Lymphoma	Prospective	Genotype	2-46 months median 13	Infection parameters, duration, type. ICU admission	Infectious parameters did not differ between MBL-deficient and MBL-sufficient neutropenic children
Schlapbach et al 2007	94 total, 63 with 177 FN episodes. 34% ALL	Retrospective	Level	Cumulative 81.7 years	Frequency of FN, type of infection	Very low MBL levels associated with more frequent FN episodes, mainly due to severe bacterial infections
ADULT						
Peterslund et al 2001	54 Haem (13%-AML)	Retrospective	Level	3 weeks	Clinically significant infection	Significant association between low MBL and serious infections related to chemotherapy
Mullighan et al 2002	97 Donor-Recipient pairs allogeneic SCT	Retrospective	Genotype	16 months	Risk of major infection pre and post neutrophil recovery	MBL genotype influences the risk of infection following allogeneic SCT
Rocha et al 2002	107 Donor-Recipient pairs HLA-identical sibling BMT	Prospective	Genotype	6 months	Severe infection, Neutrophil recovery, Mortality	No effect of MBL genotype observed
Bergmann et al 2003	80 AML	Prospective	Level Function	1 month	Fever, severe infection and mortality	MBL levels had no influence on the occurrence or course of infections in AML patients
Kilpatrick et al 2003	128 Haem (23% AML) (67% BMT conditioning)	Prospective	Level	1-4 months	Severity of infection, Duration of FN	MBL did not have a major influence on susceptibility to infection
Horiuchi et al 2005	113 Haem, treated with high dose chemo and Auto SCT	Retrospective	Genotype	100 days post SCT	Major bacterial infection	Low MBL producing genotypes, B/B and B/LXA, were associated with major bacterial infection
Molle et al 2006	113 Multiple myeloma, high dose chemo and Auto SCT	Retrospective	Genotype	180 days post SCT	Severe infections	MBL wild-type patients had a significantly reduced risk of septicaemia during the AutoSCT
Molle et al 2006	133 Multiple myeloma, 390 chemotherapy cycles	Retrospective	Genotype	28 days	Septicaemia	Indications of a reduced septicaemia in wild-type compared with variant MBL2 patients
Vekemans et al 2007	255 Haem, 569 chemotherapy cycles. FN in 200 patients	Prospective	Genotype Level	1 chemo cycle, max 45days	Ratio of duration of FN to duration of neutropenia Severe infection	MBL deficient patients had more severe infections and first severe infection was earlier than in MBL wild-type patients

Table 1.4 Summary of studies investigating the influence of MBL on infectious complications in patients on chemotherapy

ALL, Acute Lymphoblastic Leukaemia. AML, Acute Myeloid Leukaemia. BMT, Bone marrow transplant. Auto SCT, Autologous stem cell transplant. ICU, Intensive care unit.

induction phase of treatment. During this 50 day period they found no association between frequency of infection and MBL genotype (Lausen et al., 2006). The other two studies have investigated more heterogenous groups of paediatric oncology patients. Frakking and colleagues were unable to demonstrate a difference between the infectious parameters in their MBL deficient and sufficient patients but they did not dismiss the potential use of MBL replacement in such patients in the future as their patient numbers may have been too small to demonstrate an effect (Frakking et al., 2006). In contrast Schlapbach *et al* did observe that very low MBL levels were associated with more frequent FN episodes which were more likely to be due to severe bacterial infections (Schlapbach et al., 2007).

Three studies to date have investigated the role of MASPs in adults with colorectal cancer. Using a MBL/MASP2 assay measuring C4 cleavage, patients were found to have increased complement activation function compared to healthy controls (Ytting et al., 2004) but this MBL/MASP2 activity was not predictive of post-operative infections or long term prognosis (Ytting et al., 2005a). However MASP2 concentration prior to surgery was an independent prognostic marker with high levels predicting recurrence and poor survival. No association between MASP2 concentration and postoperative infection was found (Ytting et al., 2005b). A further study of adults undergoing allogeneic stem cell transplantation has demonstrated that patients heterozygous for the MASP2 D105G allele had an increased risk of invasive fungal infection (Granell et al., 2006).

The role of MBL and the lectin pathway of complement in this heterogenous patient group remains unclear as all the studies discussed have asked different questions and compared a number of different outcome measures. Supporting evidence for a role for MBL in the context of chemotherapy induced neutropenia has been investigated using a

mouse model. MBL double knockout mice (*MBLA*$^{-}$*/MBLC*$^{-}$) infected with *Staphylococcus aureus* IV showed an increased death rate when compared to WT littermates; 48h post-inoculation all MBL null-mice had died compared to 55% survival of WT mice. Interestingly, when mice were inoculated via the intraperitoneal route this difference was not observed until the mice were rendered neutropenic following administration of cyclophosphamide. The neutropenic MBL-null mice displayed enhanced bacterial accumulation in organs compared to the neutropenic MBL WT mice. By day 8 post inoculation the neutropenic MBL WT mice had sterilized their blood but there was persistent bacteraemia in the neutropenic MBL-null mice despite a recovery of circulating neutrophils. Treatment with recombinant MBL restored activation of the lectin pathway of complement and correlated with decreased bacterial accumulation in the tissues (Shi et al., 2004).

1.9 MBL replacement therapy

MBL replacement therapy is now available and is being tested in patients. MBL replacement was first attempted when plasma infusions were given to a patient and found to correct the 'common opsonic defect' (Miller 1968). Since then affinity-purified MBL has been safely given to patients, resulting in normalization of ELISA detectable MBL and complement mediated opsonic activity (Valdimarsson et al., 1998). A phase one study showed half life to vary between 18 and 115h (Valdimarsson et al., 2004). Recombinant MBL (rMBL) has been developed and has undergone phase 1 tolerability and pharmacokinetic studies with promising results (Petersen et al., 2006). It has advantages over the purified form in terms of production/availability and the risks associated with the use of blood products. The product is licensed and the results of phase 2/3 trials are awaited. Its use in animal studies has been encouraging, demonstrating positive effects of reconstitution (Shi et al., 2004; Gadjeva et al., 2004;

Moller-Kristensen et al., 2006). It has been proposed as a potential therapeutic agent against control of infection in immunocompromised patients but further work is required to define exactly who could benefit and whether MBL could be used as a prophylactic or 'rescue' treatment.

1.10 Aims of the thesis

The work described in this thesis explores several aspects of the MBL lectin pathway of complement in a population of paediatric oncology patients.

We hypothesise that innate 'host defence factors', specifically MBL and components of the lectin pathway of complement, influence infection risk and severity in children treated with chemotherapy for cancer.

The specific aims of this thesis were:

1) To explore the influence of MBL gene polymorphisms on the susceptibility and severity of infection in children treated for cancer.

2) To study the effects of chemotherapy and/or infection on MBL structure and lectin pathway function.

3) To investigate the transcriptional regulation of MBL gene expression.

The following schematic briefly outlines the results chapters of this thesis.

Chapter 3
FN management by RS
Clinical outcome data collected for the PINE project on all FN episodes in SE England

Children on active treatment for cancer in SE England recruited to MBL/lectin pathway studies and consented for access to PINE data (n=283)

Chapter 4
MBL genotyping of total study cohort (n=283)

Chapter 5
Disease association study investigating the impact of *MBL2* genotype on FN outcome utilising clinical data from the PINE project
(n=261)

Chapter 6
Cross sectional observational study investigating components of the lectin pathway of complement in a subgroup of the total patient cohort
(n=183)

Chapter 7
Longitudinal observational study investigating MBL phenotype, function and structure throughout chemotherapy and subsequent FN
(n=21)

Chapter 8
Transcriptional regulation of MBL and its promoter activity in response to infection/inflammatory stimuli at molecular level

Chapter 2

Materials and Methods

2.0 Materials and Methods

2.1 Study populations

Specialist management of children with cancer in the South East of England is provided by four Paediatric Oncology Centres (POCs). These consist of the Royal London Hospital (RLH) and the Royal Marsden Hospital (RMH) which care for children of all ages in the south and east of the region. In the north and west of the region Great Ormond Street Hospital for Sick Children (GOSH) cares for children under 13 years of age and University College Hospital (UCLH) cares for children over 13 years and adolescents. In partnership with the POCs, over forty Paediatric Oncology Shared Care Units (POSCUs) provide supportive care (including FN management) for patients close to their home. Figure 2.1 shows a simplified schematic of this system illustrating that different POSCUs link with different Thames POCs.

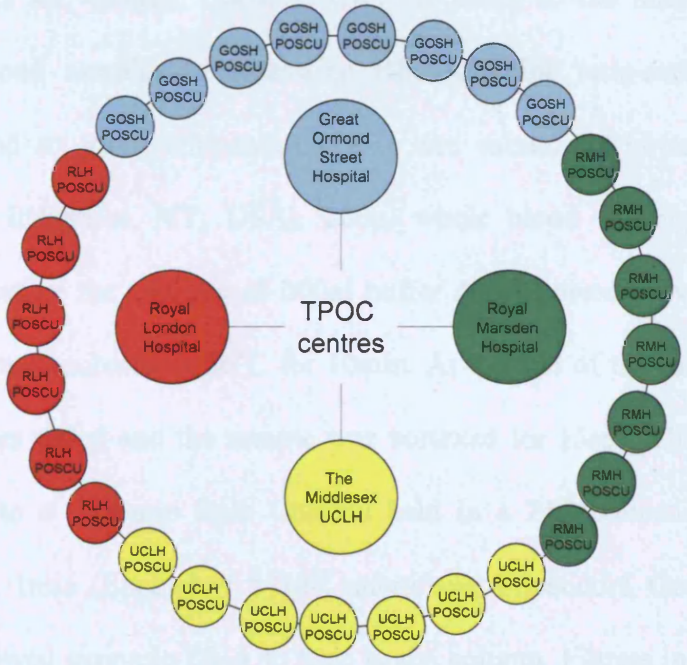


Figure 2.1 Illustration of POC and POSCU structure.

It should be noted that not all POSCUs are represented in Figure 2.1 and that some POSCUs link with more than one POC due to patient age or for geographical reasons. Patients recruited into the studies presented in this thesis were managed in these four POCs. Chapter 3 describes the PINE (Paediatric Infections during Neutropenic Episodes) project, a multicentre audit of FN episodes occurring in both the POCs and POSCUs. Clinical data obtained from this project was used in the analysis of the MBL/lectin pathway studies presented. Chapter 4 introduces the patient population investigated. Different subsets of this total population are described in Chapters 5-7. All patients had blood samples taken for MBL genetic analysis and those involved in the lectin pathway studies also had blood taken for protein analyses. The specific populations will be introduced in the relevant chapters.

2.2 DNA extraction from whole blood

Genomic DNA was extracted from whole blood using a commercial kit (QIAamp DNA blood mini kit, Qiagen, Crawley, UK) according to the manufacturer's instructions. Whole blood samples in potassium ethenediamine tetra-acetic acid (EDTA) were equilibrated to room temperature (RT) and mixed by vortexing (Vortex Genie-2, Scientific Industries, NY, USA). 200µl whole blood was mixed with 20µl Qiagen Protease before the addition of 200µl buffer AL followed by vortexing for 15sec. The mixture was incubated at 56°C for 10min. At the end of the incubation 200µl absolute ethanol was added and the sample was vortexed for 15sec. The resulting solution was loaded onto a QIAamp Spin Column held in a 2ml collection tube. Centrifugation (8000rpm, 1min (Eppendorf 5415R microfuge, Eppendorf, Germany)) was performed which allowed genomic DNA to bind to the column. Filtrate in the collection tube was discarded and a fresh collection tube attached to the column. 500µl buffer AW1 was added to the column prior to centrifugation (8000rpm, 1min). The wash step was

repeated with 500µl buffer AW2 and the sample was centrifuged (13000rpm for 3min). The filtrate was discarded and the spin column was placed in a new tube and re-spun (13000rpm, 1min) to ensure complete removal of wash buffer AW2. Genomic DNA was eluted by the addition of 200µl buffer AE with 5min equilibration at RT before centrifugation (8000rpm, 1min). Eluted DNA was appropriately aliquoted and stored at -20°C until further use. The concentration of resuspended DNA was determined by measuring absorbance (U-1800 Spectrophotometer, Digilab Hitachi, Tokyo, Japan) at 260nm and 280nm. The amount of DNA (µg/µl) was calculated as follows:

[(absorbance at 260nm) x (dilution factor) x 50] / 1000.

Purity of DNA was calculated using the ratio, A_{260}/A_{280}. Pure DNA gives a ratio of 1.8. Routinely, 5-10µg DNA was obtained from 200µl of whole blood.

2.3 MBL genotyping

In the present study two methods of genotyping have been utilised to detect mutations in Exon1 and the promoter region of the *MBL-2* gene.

2.3.1 Heteroduplexing

Briefly, this method involved the use of a universal heteroduplex generator (UHG), a synthetic DNA molecule based on the target genomic sequence, containing insertions and deletions (Wood and Bidwell, 1996). Genomic DNA polymerase chain reaction (PCR) product was combined with UHG PCR product, and the two were allowed to anneal to produce characteristic heteroduplexes for different alleles. These heteroduplexes display different electrophoretic mobility on a polyacrylamide gel, thus enabling identification of a subject's genotype (Jack et al., 1997). A general outline of the steps involved and the characteristic band patterns obtained by this technique are shown schematically in Figure 2.2 and each step is described in detail below.

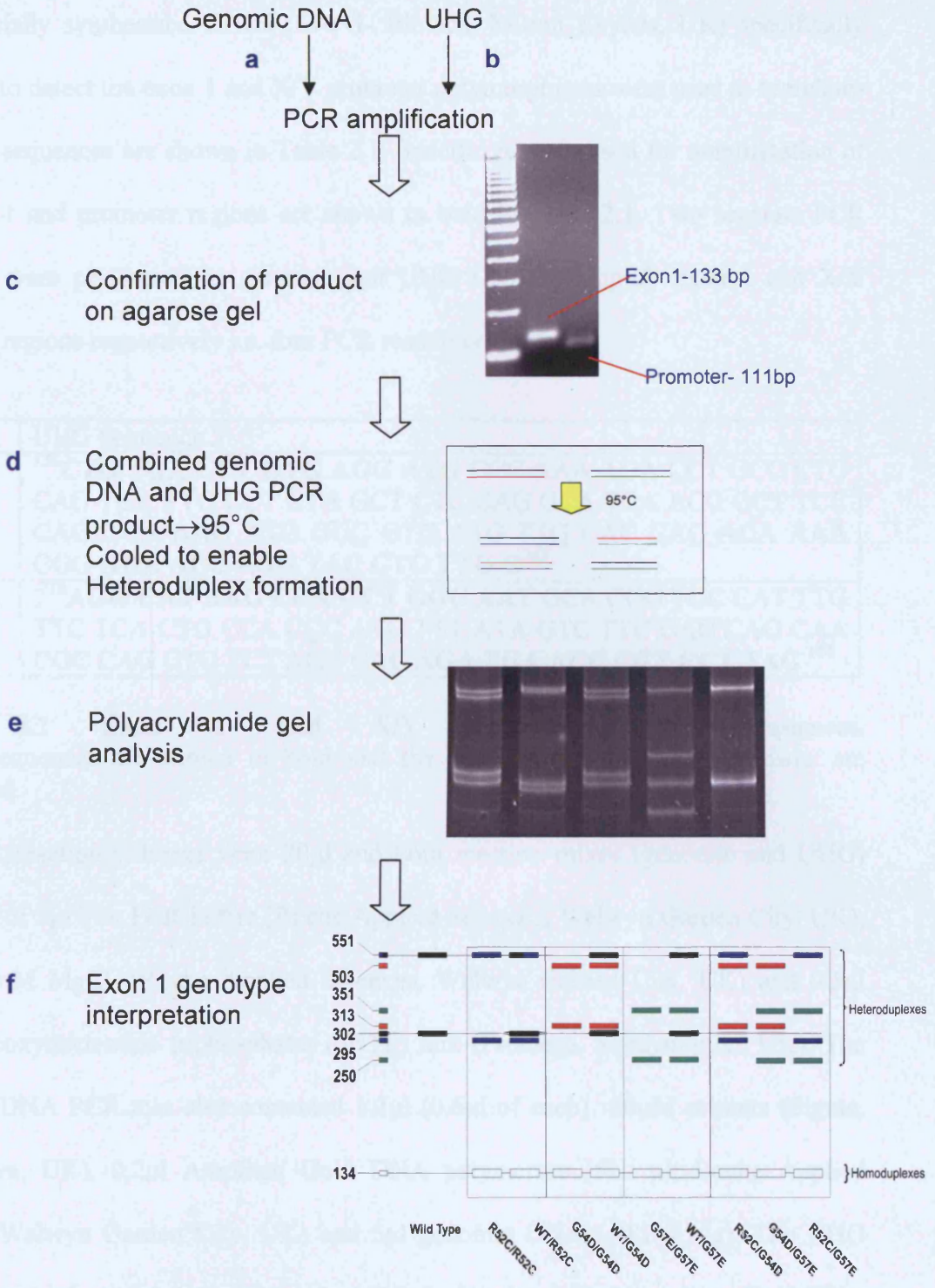


Figure 2.2 Schematic representation of the Heteroduplexing technique.

a) PCR amplification of genomic and UHG DNA

Commercially synthesised UHGs (MWG- Biotech, Milton Keynes, UK) specifically designed to detect the exon 1 and X/Y promoter polymorphisms were used as templates and their sequences are shown in Table 2.1. Specific primers used for amplification of the exon 1 and promoter regions are shown in bold in Table 2.1. Two separate PCR reactions were performed on genomic and UHG DNA to amplify exon 1 and X/Y promoter regions respectively i.e. four PCR reactions in total.

	UHG Sequence 5'-3'
Exon 1 133bp	^{136}**CTG TGA CCT GTG AGG ATG** CCC AAA AGA CCT GCC CTG CAG TGA TTG CCT GTA GCT CTC CAG GCA TCA ACG GCT TCC CAG GCA AAG ATG GGC GTG ATG TTG CAC CAG AGA AAA GGG **GGA ACC AGG TAC GTG TTG G**^{267}
Promoter 111bp	$^{-270}$**AGG CAT AAG CCA GCT GGC AAT** GCA CGG TCC CAT TTG TTC TCA CTG CCA CCC ATG TTT ATA GTC TTC CAG CAG CAA CGC CAG GTG TCT AGG CAC **AGA TGA ACC CCT CCT TAG**$^{-155}$

Table 2.1 Exon 1 and X/Y promoter UHG sequences. Primer sequences are shown in bold and the positions of the polymorphisms are underlined.

The PCR reaction volumes were 20µl and both reaction mixes (genomic and UHG) consisted of 2µl 10x PCR buffer (Roche Applied Sciences, Welwyn Garden City, UK), 1.2µl 25mM $MgCl_2$ (Roche Applied Sciences, Welwyn Garden City, UK) and 0.5µl 10mM deoxynucleotide triphosphates (dNTP) mix (Promega, Southampton, UK). The genomic DNA PCR mix also contained 1.2µl (0.6µl of each) 50µM primers (Sigma, Gillingham, UK), 0.2µl Amplitaq Gold DNA polymerase (5U/ µl; Roche Applied Science, Welwyn Garden City, UK) and 5µl genomic DNA (~0.1-0.5µg). The UHG PCR mix contained 0.6µl 50µM primers (0.3µl of each), 0.12µl Amplitaq Gold DNA polymerase (5U/ µl) and 20pg UHG per reaction. The remaining volume was made up with sterile water (Sigma, Gillingham, UK). Mastermixes were prepared for multiple reactions to minimize inter-sample variation.

PCR amplification was carried out in a thermal cycler (Peltier Thermal Cycler, MJ Research, BioRad, Hemel Hempstead, UK) as follows:

a)	95°C for 15min	-1 cycle
b)	95°C denaturation for 45sec	
	56°C annealing for 45sec	- 35 cycles
	72°C extension for 45sec	
c)	72°C for 10min	- 1 cycle

The PCR products were analysed using 2% agarose (Invitrogen Life Technologies, Paisley, UK) gels prepared in 1 x Tris-borate-EDTA (TBE) buffer (Sigma, Gillingham, UK). Ethidium bromide (1μg/100ml; Sigma, Gillingham, UK) was added to the cooled gel mix, before pouring onto a casting tray. PCR samples were run at 100V for 1h and visualized using the AlphaImager system (Alpha Imager, Multimage Light Cabinet, Alpha Innotech Corporation, Essex, UK). Gel electrophoresis allowed visual quantification of PCR products.

b) Heteroduplex generation and analysis

Approximately equal amounts of the relevant genomic and UHG PCR products were combined and heated to 95°C for 5min on a thermal cycler. The mix was then allowed to cool to RT over 2h to enable greater specific annealing prior to analysis by polyacrylamide gel electrophoresis (PAGE). Routinely 20% non-denaturing polyacrylamide gels were prepared (Table 2.2).

30% Acrylamide /Bis acrylamide mix (National Diagnostics, UK)	10ml
10x TBE Buffer	3ml
Sterile water	2.5ml
10% Ammonium Persulfate solution (National Diagnostics, UK)	150µl
TEMED (N,N,N',N'- tetramethylethylenediamine) (Sigma, Gillingham, UK)	15µl

Table 2.2 20% polyacrylamide gel composition of a 16x16 cm gel

10µl of heteroduplexing product was loaded into each well. The promoter heteroduplexing products were analysed on 16x16cm gels (150V, 15h, 16°C). The exon1 products were analysed using the Protean II system (BioRad Laboratories, Hemel Hempstead, UK) for optimal separation (200V, 5h, 4°C). Better resolution was obtained when gels were run in 2x TBE buffer. Gels were stained in 1xTBE containing ethidium bromide for 10-15min on an orbital shaker (Rotatest, Denley, UK) and visualized and photographed using an AlphaImager.

2.3.2 Innogenetics line probe assay- INNO-LiPA *MBL-2*

This method relies on genomic DNA: probe DNA hybridization technology that enables detection of exon 1 and all 3 promoter polymorphisms using a commercial kit (INNO-LiPA *MBL2* Amplification and INNOLiPA *MBL2*, INNOGENETICS N.V., Belgium). Amplified biotinylated genomic DNA was chemically denatured and the separated DNA strands hybridized with specific oligonucleotide probes immobilized on membrane-based strips. A stringent wash step allowed removal of any mismatched hybridization. Addition of streptavidin (conjugated with alkaline phosphatase) allowed avid binding to the biotin moiety of the hybrid DNA. Finally, incubation with an alkaline phosphatase substrate solution containing chromogen resulted in a purple/brown precipitate. The resulting probe pattern could then be translated into its appropriate genotype. Figure 2.3 represents a brief outline of the technique and each step is described in detail below.

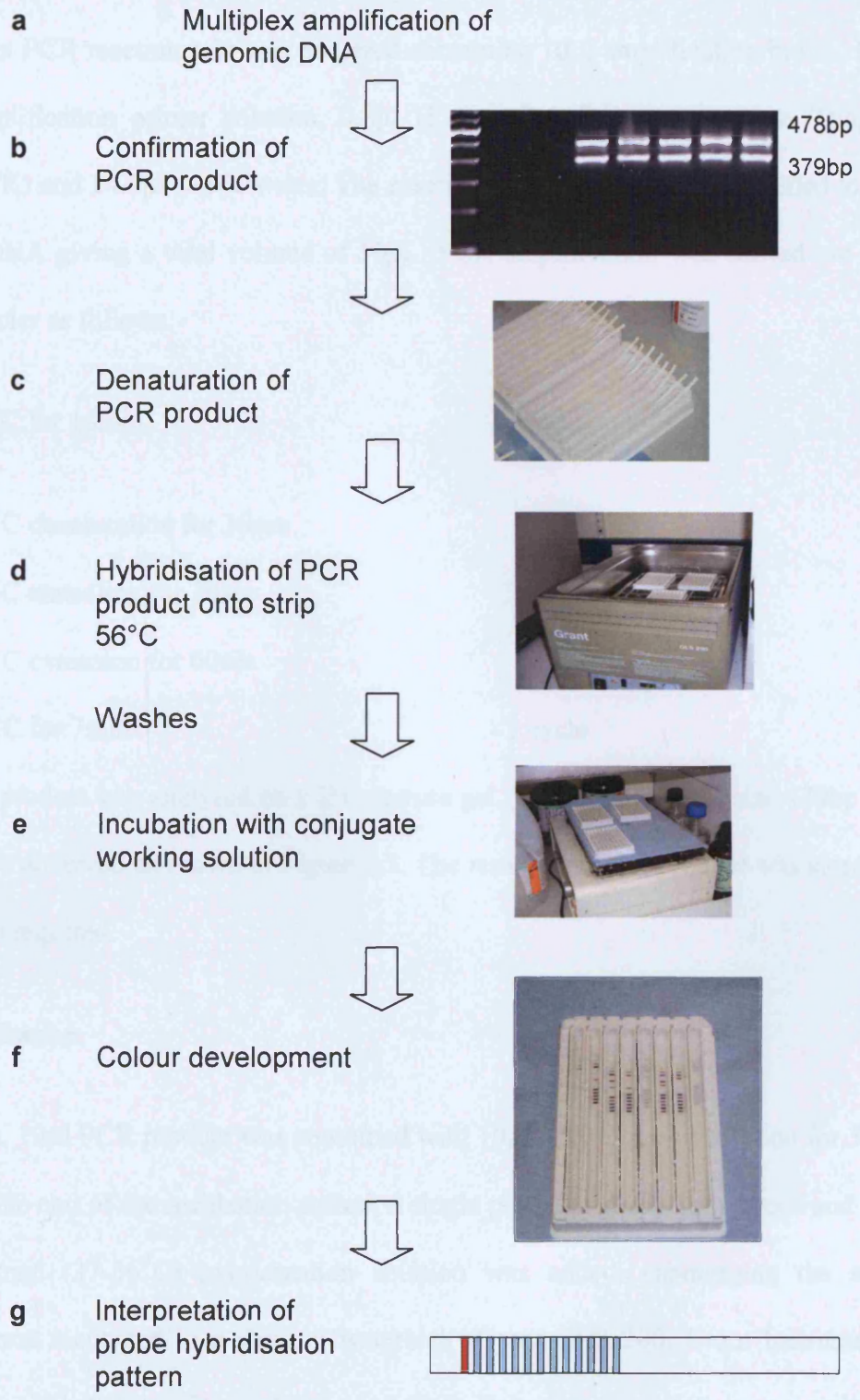


Figure 2.3 Schematic representation of the INNO-LiPA method

a) Amplification of the promoter and exon 1 regions of the *MBL2* gene

A multiplex PCR reaction mix was prepared containing 10µl amplification buffer, 10µl *MBL2* amplification primer solution, 0.3µl HotStar Taq DNA polymerase (Qiagen, Crawley UK) and 24.7µl sterile water. The reaction mix was vortexed and added to 5µl genomic DNA giving a total volume of 50µl. PCR amplification was carried out in a thermal cycler as follows:

a)	95°C for 15min	-1 cycle
b)	95°C denaturation for 30sec 56°C annealing for 30sec 72°C extension for 60sec	- 35 cycles
c)	72°C for 7min	- 1 cycle

10µl PCR product was analysed on a 2% agarose gel. Two amplicons of size 478bp and 379bp were observed as shown in Figure 2.3. The remaining PCR product was stored at -20°C until required.

b) Hybridisation

In a trough, 10µl PCR product was combined with 10µl of denaturing solution for 5min at RT. At the end of the incubation period, a single probe strip was introduced and 2ml of prewarmed (37-56°C) hybridization solution was added, submerging the strip. Troughs were incubated in a shaking waterbath (Grant OLS 200, Grant Instruments, Cambridge, UK) (80 rpm, 30min 56°C +/- 0.5°C). Hybridization solution was aspirated using a vacuum (Vaccubrand GmbH, Wertheim, Germany) and 2ml pre-warmed stringent wash solution (37-56°C) was added and placed on a shaking platform (Rotatest, Denley, UK) (60 +/- 30sec, RT). This wash step was repeated once more and

was followed by a final 10min wash step involving re-incubation in the shaking water bath at 56°C.

c) Colour development

The remaining steps were carried out at RT on a shaking platform. After the 10min stringent wash, liquid was aspirated and each strip was washed twice for 1min in 2ml rinse working solution. Finally, 2ml conjugate working solution was added and the strips were incubated for a further 30min. The substrate working solution was prepared 10min prior to requirement by addition of substrate buffer to substrate BCIP/NBT (100x solution). Each strip was washed twice for 1min in 2ml rinse working solution and once in 2ml substrate buffer. 2ml substrate working solution was added to each strip and incubated for 30min, allowing colour development to take place. The reaction was stopped by washing the strips twice in 2ml sterile water for at least 3min/wash. Strips were removed and blotted dry prior to interpretation. To validate each strip the conjugate control must be positive. The strips contain 6 mutant type probes (M- types) and 6 wild type probes (W). The presence of a visible line at a particular probe position indicates that the corresponding sequence is present in one or both alleles (Figure 2.4).

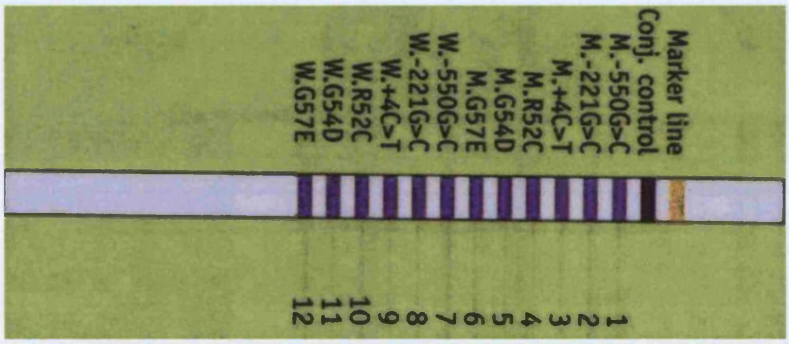


Figure 2.4 Schematic of INNO-LiPA MBL2 assay strip showing presence of all probe sequences

As discussed previously, linkage disequilibrium between polymorphisms in the promoter and exon 1 region give rise to seven common haplotypes, which give rise to 28 possible haplotype combinations. Table 2.3 highlights the haplotype combinations corresponding to each positive probe pattern.

Haplotype combinations	**Positive probe numbers**											
LXPA/LYQC	1	2	3			6		8	9	10	11	12
LXPA/LYQA	1	2	3					8	9	10	11	12
LXPA/HYPD	1	2		4			7	8	9	10	11	12
LXPA/LYPB	1	2			5			8	9	10	11	12
LXPA/HYPA	1	2					7	8	9	10	11	12
LXPA/LYPA	1	2						8	9	10	11	12
LXPA/LXPA	1	2							9	10	11	12
LYQC/HYPD	1		3	4		6	7	8	9	10	11	12
LYQA/HYPD	1		3	4			7	8	9	10	11	12
LYPB/LYQC	1		3		5	6		8	9	10	11	12
LYQA/LYPB	1		3		5			8	9	10	11	12
LYQC/HYPA	1		3			6	7	8	9	10	11	12
LYPA/LYQC	1		3			6		8	9	10	11	12
LYQC/LYQC	1		3			6		8		10	11	
LYQA/LYQC	1		3			6		8		10	11	12
LYQA/HYPA	1		3				7	8	9	10	11	12
LYQA/LYPA	1		3					8	9	10	11	12
LYQA/LYQA	1		3					8		10	11	12
LYPB/HYPD	1			4	5		7	8	9	10	11	12
LYPA/HYPD	1			4			7	8	9	10	11	12
LYPB/HYPA	1				5		7	8	9	10	11	12
LYPA/LYPB	1				5			8	9	10	11	12
LYPB/LYPB	1				5			8	9	10		12
LYPA/HYPA	1						7	8	9	10	11	12
LYPA/LYPA	1							8	9	10	11	12
HYPD/HYPD				4			7	8	9		11	12
HYPD/HYPA				4			7	8	9	10	11	12
HYPA/HYPA							7	8	9	10	11	12

Table 2.3 Haplotype combinations and probe patterns

2.4 Analysis of MBL and the Lectin pathway

2.4.1 Buffers and solutions used in protein/functional assays and Western blotting.

General Buffers	
Phosphate buffered saline (PBS)	One PBS tablet (Oxoid, UK) to 100ml MilliQ water (Millipore, Watford, UK)
Tris buffered saline (TBS)	10mM TrisHCL, 140mM NaCl, 15mM NaN_3, pH 7.4
PBS/Tween (PBS/T)	PBS with 0.05% Tween 20 (polyoxyethylene (20) sorbitan monolaurate) (BDH, Poole,UK)
TBS/Tween (TBS/T)	TBS with 0.05% Tween 20
MASP/C4/C3 assay buffers	
Blocking solution	TBS, 1mg/ml Human Serum Albumin (HSA) (Serum Statens Institute, Denmark)
Coating buffer	15mM Na_2CO_3, 35mM $NaHCO_3$, 0.02% NaN_3. pH 9.6
MASP2 sample buffer	TBS/T, 10mM EDTA, 0.86mM NaCl, 100µg/ml heat aggregated normal human IgG (Serum Statens Institute, Denmark)
MASP2 secondary buffer	TBS/T, 5 mM $CaCl_2$, 1% bovine serum, 1% heat aggregated normal human IgG
Rosa buffer	20mM TrisHCl, 1M NaCl, 0.05% Triton X-100, 10mM $CaCl_2$, 1mg/ml HSA. pH 7.4
MASP3 secondary buffer	TBS/T, 5 mM $CaCl_2$, 1% bovine serum
B1 buffer	4mM Barbital, 145mM NaCl, 2mM $CaCl_2$, 1mM $MgCl_2$ pH 7.4
B2 buffer	As for B1 buffer with addition of 0.05% Tween 20
Enhancement buffer	0.57%(v/v) acetic acid, 0.1%(v/v) Triton X-100, 1% (w/v) 6000 polyethylene glycol Immediately prior to use 15µM 2-naphtoylfluoroacetone (2-NTA)(β-NTA) and 50µM Tri-n-octylphosphine oxide (TOPO) was added
TBS/T/Ca^{2+} Wash buffer	TBS/T, 5 mM $CaCl_2$
TBS/T/EDTA	TBS/T, 25mM EDTA
Western blotting buffers	
Blocking buffer	PBS, 4 % non fat milk (Marvel, UK)
Electrode buffer (10x)	30g Tris, 144g Glycine, 10g SDS, 1L MilliQ water
Sample buffer (4x)	3.8ml MilliQ water, 1ml 05.M Tris (pH6.8), 0.8ml Glycerol, 1.6ml SDS, 0.2% Bromophenol blue
Transfer buffer	3.03g 25mM Tris, 14.4g 0.2M Glycine, 20% Methanol. 4°C (1L)

Table 2.4 Buffers and solutions used in protein/functional assays and Western blotting. Reagents are from Sigma, Gillingham, UK unless stated otherwise.

2.4.2 Antibodies used in protein/functional assays and Western blotting

Name	Company	Product code
anti-human C3c, rabbit polyclonal	Dako, Ely, UK	A0062
anti-human C4, mouse monoclonal IgG clone 162-2	The Antibody Shop, Copenhagen, Denmark	HYB 162-02
anti-human C4, mouse monoclonal IgG clone 162-4	The Antibody Shop, Copenhagen, Denmark	HYB 162-04
anti-human MASP1/3, mouse monoclonal IgG clone IE2	Hycult biotechnology, Netherlands	HM 2092
anti MASP2, rat monoclonal IgG clone 8B5	Hycult biotechnology, Netherlands	HM 2190
anti MASP2/MAp19, rat monoclonal IgG 6G12	Hycult biotechnology, Netherlands	HM 2191
anti MASP3 38:12-3	Gift of J.C.Jensenius, University of Aarhus, Denmark	
anti human MBL, mouse monoclonal IgG clone 131-1	The Antibody Shop, Copenhagen, Denmark	HYB 131-01
anti mouse Ig/HRP, goat polyclonal	Dako, Ely, UK	PO447

Table 2.5 Antibodies used in protein/functional assays and Western blotting

2.4.3 Protein assays

2.4.3.1 Determination of MBL protein concentration by ELISA

MBL protein levels were determined by symmetrical sandwich enzyme-linked immunosorbent assay (ELISA) using a commercial kit (MBL Oligomer ELISA kit, Antibody Shop, Copenhagen, Denmark) according to manufacturer's instructions.

The kit provides a 96 well plate pre-coated with a monoclonal antibody against MBL. Bound MBL is detected with the same antibody labelled with Biotin, followed by development with horse-radish peroxidase (HRP) - conjugated streptavidin and a chromogenic substrate.

Serum samples were diluted, dependent on known genotype (wild-type-1 in 200, variant alleles 1 in 20), using sample diluent. Cell culture supernatant samples were left undiluted. MBL standards (0-40ng/ml) and test samples (100µl) were applied to wells in

duplicate and incubated on a shaking platform (200 rpm, 1h, RT). Unbound material was removed by washing with 300µl wash solution (x3) and blotted dry. This procedure was followed in all wash steps. Biotinylated monoclonal antibody (100µl) was added to each well, incubated on a shaking platform (200 rpm, 1h, RT) followed by washing of unbound antibody. Addition of HRP-Streptavidin and incubation on a shaking platform (200rpm, 1h, RT) allowed complex formation with the biotinylated antibody. Any unbound material was removed by washing. Chromogenic peroxidase substrate, tetramethylbenzidine (TMB; 100µl) was added and incubated for 15min at RT in the dark followed by addition of 50µl stop solution. Colour intensity was measured at 450nm using an ELISA reader (MRX, Dynatech Laboratories Ltd, Sussex UK) within 30min of termination of the reaction. Optical density readings for the standard samples were used to construct a calibration curve from which concentrations of MBL in the test samples were determined. The interassay coefficient of variation (CV%) for the high and low internal controls was 7% and 10.5% respectively.

2.4.3.2 Determination of MASP2 protein concentration by TRIFMA

Microtiter wells (FluoroNunc plate, NUNC, Roskilde, Denmark) were coated with 0.7µg anti-MASP2 antibody (clone 8B5, which captured the CCP 1/2-SP fragment [or C-terminal part] of MASP2) in 100µl coating buffer and incubated overnight at 4°C. Coating buffer was removed and the wells incubated (1h, RT) with 200µl blocking solution to block non-specific binding sites. Each subsequent step was followed by a wash step which involved three washes using TBS/T. Test serum samples and 4 internal controls were diluted 1 in 25 with MASP2 sample buffer. A pool of plasma for use as standard (608 ng/ml) was serially diluted. Negative control wells received buffer only. Wells were washed and incubated with 100µl of diluted test and standard samples (in duplicate) overnight at 4°C. MASP2 was detected using a biotinylated anti-MASP

2/MAp19 antibody (clone 6G12, specific to the N-terminal end of MASP2 and also MAp19, 1:2000 in MASP2 secondary buffer, 100µl/well) incubated for 2h at RT. The assay was developed using time resolved immunofluorometry described in section 2.4.4.3. The interassay coefficient of variation (CV %) for the high, medium and low internal controls was 1.3, 8.14 and 4.23% respectively.

2.4.3.3 Determination of MASP3 protein concentration by TRIFMA

Microtiter wells were coated with anti-MASP1/3 antibody (clone IE2, which recognises the heavy/A chain common to both MASP1 and MASP3, 2µg/ml in PBS, 100µl/well) and incubated overnight at RT. The coating solution was removed prior to addition of 200µl blocking solution for 1h at RT. Test serum and 3 internal controls were diluted 100 fold in Rosa buffer. Negative control wells received buffer only. Pooled plasma as standard (533 ng/ml) was serially diluted. Wells were washed three times with TBS/T prior to addition of 100µl of sample (in duplicate) incubated overnight at 4°C. Each subsequent step from this point on was followed by a wash step which involved three washes using TBS/T/Ca^{2+}. Bound MASP3 was detected using 100µl biotinylated anti-MASP3 antibody (38:12-3, specific to MASP3, 0.5µg/ml in MASP3 secondary buffer) incubated for 2h at RT. Following a wash step the assay was developed as described in section 2.4.4.3. The interassay coefficient of variation (CV%) for the high, medium and low internal controls was 1.1, 10.5 and 13.25% respectively.

2.4.4 Functional assays

2.4.4.1 MBL/MASP2 complex activity/C4b deposition assay

This assay assesses the ability of an individual's MBL/MASP2 complex to cleave C4 by detection of C4b deposition (solid phase C4 activation product) (Petersen et al., 2001).

The addition of exogenous C4 overrrides any contribution of the C4 present in the test serum sample.

Microtiter wells were coated with 1μg of mannan in 100μl coating buffer overnight at 4°C, prior to blocking as described above. Test serum samples were diluted in Rosa buffer (high ionic strength to prevent classical pathway activity), according to known MBL concentration as measured by ELISA (section 2.4.3.1), i.e. 1 in 200 if MBL serum concentration <4μg/ml and 1 in 400 if >4μg/ml. Four plasma samples were used as internal controls and diluted accordingly. Pooled standard plasma (assigned the value 1000munits/ml) was serially diluted. Wells were washed with TBS/T and incubated with 100μl of test and control samples (in duplicate) overnight at 4°C. Each subsequent step was followed by a wash step (three washes with TBS/T/Ca^{2+}). After sample removal and washes, human C4 (0.98 mg/ml, NatImmune, Denmark, in 100μl B1 buffer) was added for 1.5h at 37°C. After washing, bound C4b was detected by incubating with a biotinylated anti-C4b antibody (clone 162-2, 25ng/100μl TBS/T/Ca^{2+}) for 1.5h at RT. Following a wash step the assay was developed as described in section 2.4.4.3. The interassay coefficient of variation (CV%) for the high and medium internal controls was 15.8 and 9% respectively.

2.4.4.2 C3 cleavage assay/C3b deposition

This assay assesses both MASP1 and MASP2 mediated activation of C3 via the MBL lectin pathway by detection of the C3 cleavage product C3c.

Mannan coated wells were prepared and blocked as described above. Test serum samples and internal controls (x4) were diluted (1 in 100 in B2 buffer). Pooled plasma (1000munits/ml) was used as standard and was serially diluted. Wells were washed with TBS/T and then incubated with 100μl of dilute sample (in duplicate) for 1h at 37°C in a humidified box to allow C3 cleavage. Each subsequent step was followed by wash steps

(x3) using TBS/T/Ca^{2+}. C3 cleavage was detected using a biotinylated anti C3c antibody (200ng/ml, diluted 1:1000 in TBS/T/Ca^{2+}, 100µl/well) incubated overnight at 4°C. Following a wash step the assay was developed as described in section 2.4.4.3. The interassay coefficient of variation (CV %) for the high and medium internal controls was 2.6 and 15.3% respectively.

2.4.4.3 Time Resolved ImmunoFluoroMetric Assay (TRIFMA)

Time resolved fluorometry is more sensitive than enzyme based immunoassays for measuring compounds present at low concentrations. It utilises the fluorescent label Europium which has a long decay time, whose emissions can be detected at a given wavelength following pulsed light excitation (Hemmila et al., 1984).

Following a final wash step, wells were incubated with 10ng Europium (Eu^{3+}) - labelled streptavidin in 100µl TBS/T/EDTA for 1.5h at RT. 200µl enhancement buffer was added to each well and incubated for 5min on a shaking platform. The low pH of the enhancement buffer allows dissociation of Eu^{3+} from streptavidin. Triton X-100, 2-NTA and TOPO micelles capture Eu^{3+} ions free in solution. Excitation of this complex was detected using a fluorometer (1420 Multilabel counter Victor 3, Perkin Elmer, USA). Standard sample readings were used to construct a calibration curve from which concentrations in the test samples were determined.

2.4.4.4 Wieslab total complement screen

This semi-quantitative assay is designed to screen all three pathways of complement activation (classical, MBL and alternative) simultaneously using an ELISA based detection method which is commercially available (Wieslab, Lund, Sweden).

Instructions and reagents were provided. Briefly, all reagents (unless stated otherwise) were equilibrated to RT prior to use. Serum was thawed on ice and mixed by vortexing.

The positive control (lyophilised pooled human sera) was reconstituted by adding 200µl distilled water, incubated on ice for 5min, vortexed until all material had dissolved and kept on ice prior to use. Positive and negative controls were diluted in the same way as the serum samples of interest. Serum was diluted in pathway specific diluent and incubated accordingly as shown in Table 2.6.

Pathway	Dilution	Incubation at RT	Plate coating
Classical	1 in 101	≤ 1h	IgM
MBL	1 in 101	≥ 15min	Mannan
Alternative	1 in 18	≤ 1h	LPS

Table 2.6 Pathway specific dilution factors, incubation times and plate coatings

Samples were added to the appropriate pre-coated wells (100µl/well) in duplicate and incubated for 60-70min at 37°C. Diluent alone was used to calculate background activity. This step was followed by three washes using diluted wash solution. Alkaline phosphatase-labelled antibody to human C5b-9 was then added to each well (100µl) for 30min, RT. After further washes (x3), 100µl substrate was added for 30min, RT and absorbance read at 405nm (within 30min) using an ELISA plate reader.

Results were calculated by subtracting diluent alone value from all the other pathway results and using the following equation to calculate specific activity;

Pathway activity =

$$100\% \times \frac{(\text{mean } A_{405} \text{ (Sample)} - \text{mean } A_{405} \text{ (negative control)})}{(\text{mean } A_{405} \text{ (positive control)} - \text{mean } A_{405} \text{ (negative control)})}$$

The interassay coefficient of variation (CV%) for the internal control samples is shown in Table 2.7.

	Sample	mean	SD	CV %
Classical	S1	96	5.2	5
	S2	98	4.2	4
MBL	S1	49	6.8	13
	S2	0	-	-
Alternative	S1	83	5.8	7
	S2	92	4.5	5

Table 2.7 Wieslab Total Complement Screen interassay variation

2.4.5 Western blotting of MBL

This technique was used to explore inter and intra-individual differences in higher order MBL oligomers present in serum. Samples were thawed on ice and diluted in sample buffer according to the desired dilution. Dilution factors differed between individuals according to their known MBL serum concentrations (as calculated by ELISA, section 2.4.3.1) and the same dilution factor was applied to all sequential samples from an individual. In general serum was diluted to ensure 1ng MBL protein (~ 5µl) was loaded onto the gel. Purified MBL (230ng/ml, a gift from M.Johnson, Institute of Child Health, London) was used as an internal control. Samples were subjected to non-reducing SDS PAGE (4% stacking and 6% resolving, Table 2.8) in a Mini-Protean 3 cell unit (BioRad Laboratories, Hemel Hempstead, UK).

6% Resolving Gel (10 ml)	**Volume (ml)**
Water	5.3
30% Acrylamide mix (Protogel, National Diagnostics, UK)	2.0
1.5 M Tris (pH 8.8)	2.5
10% SDS	0.1
10% Ammonium persulfate	0.1
TEMED	0.008

4% Stacking Gel (10 ml)	**Volume (ml)**
Water	7.2
30% Acrylamide mix	1.33
1.0 M Tris (pH 6.8)	1.25
10% SDS	0.1
10% Ammonium persulfate	0.1
TEMED	0.01

Table 2.8 Composition of Tris SDS gels

5µl of rainbow molecular weight (MW) marker (Amersham Biosciences, Little Chalfont, UK) was always included for MW determination and as a visual check for transfer efficiency. Gels were electrophoresed at 110V (BioRad PowerPac 3000, BioRad Laboratories, Hemel Hempstead, UK) for 5h at 4°C. Prior to transfer, blotting paper (VWR, Lutterworth, UK), nitrocellulose membrane (Hybond C extra, Amersham Biosciences, Little Chalfont, UK) and the gel were allowed to equilibrate in chilled transfer buffer for at least 10min. Proteins were transferred using a wet transfer cell system (BioRad Laboratories, Hemel Hempstead, UK) at 400mA for 90min at 4°C. Post transfer, non-specific binding was blocked by incubating the membrane in blocking buffer overnight at RT with gentle shaking. This was followed by 2h incubation with primary anti human MBL antibody (clone 131-01, 1:5000), in blocking buffer on a rotary shaker at RT.

After 3-4 washes in PBS/T for 10min each, the blot was exposed to secondary antibody (goat anti-mouse Ig/HRP, 1:2000), for 2h on a rotary shaker at RT. Blots were washed as described before detection using an enhanced chemiluminescence (ECL) reaction for 5min (ECL plus, Amersham Biosciences, Little Chalfont, UK) followed by autoradiography (MXB film, Kodak, UK). The membranes were developed from 5sec to 5min as required (XD2 developer and XF2 fixer solutions, Photosol, Essex, UK).

2.5 Transcriptional regulation of MBL and Promoter analysis

2.5.1 Cell culture

2.5.1.1 Human hepatocellular carcinoma cell-lines

The human hepatocellular carcinoma cell-lines Huh7 and HepG2 (gift from M. Jacobs, Royal Free Hospital, London) were used to investigate transcriptional regulation of MBL. These cell-lines were maintained in Dulbeccos modified Eagles medium (with 4500mg/L Glucose and GlutaMAX™) (Gibco, Paisley, UK) supplemented with 10%

fetal calf serum (FCS) (Insight technologies, London, UK), 100 units/ml penicillin and 100μg/ml streptomycin (PAA laboratories, Yeovil, UK) (complete media). Cells were maintained at 37°C in 5% CO_2 in a humidified incubator (Galaxy R, Wolf laboratories, York, UK).

2.5.1.2 Cell Passage of adherent cell-lines

Confluent Huh7 and HepG2 monolayers were routinely passaged every 3-4 days. Cells were washed with sterile PBS before adding 1x Trypsin-EDTA (Gibco, Paisley, UK) to cover the cell surface for 1-2.5min at 37°C which allowed the adherent cells to detach. Trypsin activity was immediately inhibited by addition of complete media. The resultant cell suspension was spun (1000rpm, 10min, 4°C) and cell pellet resuspended in complete media. The cell suspension was passed through a 23 gauge needle (Terumo, Leuven, Belgium) to ensure no cell clumps remained. Cells were seeded and maintained in 75cm^2 culture flasks, 6 or 96 well plates at a concentration as required.

2.5.1.3 Counting viable cells

10μl of cell suspension was removed and added to 10μl of 0.4% trypan blue (Sigma, Gillingham, UK) for 1min. 10μl of this mixture was placed onto a haemocytometer counting chamber (Neubauer, Weber, Sussex, UK), and observed under a microscope (Zeiss, Germany) at a magnification of x40. The unstained viable cell count was:

The number of cells (within a 25 box field) x dilution factor = Total number of cells x 10^4 per ml.

2.5.1.4 Freezing cells

For long term storage, confluent adherent cell-lines were washed with sterile PBS and trypsinised as described above. Cells were centrifuged (1000rpm, 5min, 4°C) and

resuspended in freezing media (80% FCS, 10% complete media and 10% sterile dimethyl sulphoxide [DMSO, Sigma, Gillingham, UK]). 1ml cell suspension aliquots were transferred into cryovials (Nunc, Roskilde, Denmark) and slowly chilled and stored at -80°C.

2.5.1.5 Thawing cells from -80°C storage

Cryovials were removed from storage and placed into a water bath at 37°C to permit rapid thawing. Thawed cells were added to complete media previously warmed to 37°C followed by centrifugation (1000rpm, 5min, 4°C) to remove DMSO/ glycerol. The cell pellet was resuspended in complete media, before proceeding with culturing in 25cm^2 tissue culture flasks.

2.5.2 Cytokine stimulation studies

This series of experiments were undertaken in media supplemented with 0.5% FCS, in the presence of antibiotics. Cells were maintained in this media overnight prior to and during the experimental timeframe. All recombinant cytokines (Peprotech, London, UK) and *E.coli* LPS (Sigma, Gillingham, UK) were reconstituted according to the manufacturer's instructions.

2.5.3 Total RNA isolation

Supernatant was aspirated from adherent cells for protein analysis and stored at -80°C until required. Total RNA isolation was carried out using a monophasic solution of phenol and guanidine thiocyanate (TRIZOL® (Invitrogen, Paisley, UK)), of which 1ml was added directly to 1 well of a 6 well plate. The cell suspension was gently shaken for 10min at RT to allow homogenization of cellular contents. Further homogenisation was achieved by repeated dispersion of the solution through a syringe and needle (25 gauge).

The resultant Trizol solution was allowed to stand at RT for 5min to permit greater dissociation between nucleic acid-protein complexes. 200µl chloroform (Sigma, Gillingham, UK) was added for every 1ml Trizol used and the mixture was vortexed thoroughly for 15sec before centrifugation (13000rpm, 25min, 4°C). This procedure allowed organic extraction of the total RNA from the remaining cellular debris. The resultant top aqueous layer (comprising RNA) was aspirated (approximately 400µl) and transferred into a fresh microcentrifuge tube. RNA was precipitated by the addition of an equal volume of isopropanol (Sigma, Gillingham, UK). Extracted RNA was stored at -20°C. Total RNA was pelleted by centrifugation (13000rpm, 20min, 4°C) and washed with ice-cold 70% ethanol (BDH Laboratories, Poole, UK). The RNA pellet was air-dried to remove traces of ethanol prior to the addition of RNase- free water.

2.5.3.1 Spectrophotometric quantification of total RNA

Appropriate amounts of resuspended RNA solution were diluted in water and the concentration determined by absorbance (U-1800 Spectrophotometer, Digilab Hitachi, Tokyo, Japan) at 260nm and 280nm. The amount of RNA (µg/µl) was calculated as follows:

[(absorbance at 260nm, A_{260}) x (dilution factor) x 40] / 1000

The ratio of the readings at 260nm and 280nm (A_{260}/A_{280}) was used to estimate the purity of the RNA, with a ratio of 2.0 signifying a pure sample.

2.5.4 Reverse transcription

Reagents used in sections 2.5.4 and 2.5.5 were from Invitrogen, Paisley, UK, unless stated otherwise.

1-5µg of total cellular RNA was transcribed to complementary DNA (cDNA) using Moloney murine leukaemia virus reverse transcriptase (MMV-RT). In a typical MMV-

RT reaction, 1µl (0.5µg) oligo-dT was added to 5µg RNA and heated to 70°C for 10min followed by immediate chilling to permit specific annealing between the oligo-dT and poly-A tail of mRNA molecules. The remaining components of the reaction comprised: 4µl 5x first strand buffer, 2µl 0.1M dithiothreitol (DTT), 1µl 10mM dNTP mix, 0.5µl sterile water and 0.5µl 100U enzyme (Superscript II reverse transcriptase). The reaction was allowed to proceed at 42°C for 1h and was terminated by heat inactivation at 70°C for 10min.

2.5.5 Polymerase Chain Reaction (PCR)

cDNA obtained was used in subsequent PCR reactions. Reaction volumes were 30µl, comprising: 3µl 10 x PCR Buffer [200mMTris-HCl (pH8.4), 500mM KCL], 0.9µl 50mM $MgCl_2$, 0.6µl 10mM dNTP mix, 1.2µl of 20pmol of each oligonucleotide primer and 0.2µl 0.1 units Taq polymerase. Primer sequences are listed in Table 2.9. The remainder consisted of RNase free water to a total volume of 30µl.

	Sense	**Antisense**	**Product size (bp)**
MBL	^{129}ACT GTG ACC TGT GAG GAT GCC CA^{151}	^{400}CTT TCT GAG GCA GCC AGG CTA CTA TCA CC^{372}	271
GAPDH	^{630}CTA CTG GCG CTG GCA AGG CTG T^{652}	^{990}GCC ATG AGG TCC ACC ACC CTG CTG^{966}	360

Table 2.9 Sequences of synthetic oligonucleotide primers utilised in the study

Mastermixes were prepared for multiple reactions to minimize inter-sample variation. The contents of the PCR reactions were mixed by brief centrifugation. PCR amplification was carried out in a thermal cycler as follows:

a) 95°C for 5min -1 cycle

b) 95°C denaturation for 1min 30sec

58°C annealing for 1min 30sec - 34-36 cycles

72°C extension for 1min 30sec

c) 72°C for 5min - 1 cycle

Subsequently, PCR products were visualized on 2% agarose gels. Semi-quantitative analyses were conducted by densitometric measurements of bands via normalization to the house-keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), using the Alpha Imager system.

2.5.6 Cloning of the MBL promoter

All reagents used were from Promega, Southampton, UK or Invitrogen, Paisley, UK unless stated otherwise. Data shown in Section 8.2.2.

The initial step for cloning the MBL promoter sequence was specific amplification of a 2933bp fragment 5' of the ATG codon. Primers were designed with restriction enzyme sites tagged at the 5' end of both sense and antisense primers as listed below and highlighted in bold type.

Sense (5'-3')

GCGC**AAGCT**TCTGATGGCTTTAGGCATGTGGCTCTG ***Hind* III**

Antisense (5'-3')

GTC**CCATGG**TCCTCACCTTGGTGTGAGAAAACTC ***Nco* 1**

The full sequence was analysed for potential restriction enzyme sites using the NEBcutter program, http://tools.neb.com/NEBcutter2/index.php.

Genomic DNA from an individual of MBL genotype LYPA/HYPA, was used as a template (10µl/reaction). PCR reaction volumes were 50µl and comprised: 5µl 10x

PCR buffer, 4µl 25mM $MgCl_2$, 1µl 10mM dNTPs, 1µl 50µM primers (0.5µl of each), 1µl Amplitaq Gold DNA polymerase (5U/ µl) and 10µl genomic DNA. The remainder consisted of sterile water. 3x 50µl reactions were prepared and amplification was carried out in a thermal cycler as follows:

a)	95°C for 5min	-1 cycle
b)	95°C denaturation for 1min 30sec	
	60°C annealing for 1min 30sec	- 36 cycles
	72°C extension for 3min	
c)	72°C for 5min	- 1 cycle

Amplified DNA fragments (1% agarose gel in Tris-acetate EDTA buffer (TAE)) were cut under a UV illuminator (UVP, Hannover, Germany) and subjected to purification using the Qiagen QIAquick Gel Extraction Kit (Qiagen, Crawley, UK). Briefly, three volumes of Buffer QG were added to one volume of gel (100mg≈ 100µl) before incubating them together for 10min at 50°C to dissolve the gel. The resulting mixture was added to a QIAquick column and spun (13000rpm, 1min). To ensure removal of all traces of agarose a further 500µl Buffer QG was added to the column, spun for 1min as before and then followed by a wash step using 750µl Buffer PE and one further spin. The column was transferred to a clean 1.5ml polypropylene tube and DNA elution was achieved by addition of 50µl Buffer EB (10mM Tris-Cl, pH8.5) followed by incubation for 1min at RT prior to a final 1min spin.

2.5.6.1 Enzyme digest and ligation of the MBL promoter sequence into the pGL3 basic vector

Both DNA and vector were subjected to sequential enzyme digestion to release sticky ends. Hind III digest was followed by Nco1 due to differing salt buffer concentrations.

Both digests were performed for 2h at 37°C in a thermal cycler. A 50µl reaction volume was used for Hind III digestion of the genomic DNA PCR product and comprised 5µl 10x REACT 2 buffer, 1µl Hind III enzyme, 25µl DNA (~250ng) and 19µl water. Hind III digestion of the vector was performed in a 25µl reaction comprising; 2.5µl 10x REACT 2 buffer, 1µl Hind III enzyme, 0.5µl DNA (~500ng) and 21µl water. Both digested DNA and vector were purified using a Qiagen PCR Purification Kit (Qiagen, Crawley, UK) prior to the second digestion and before ligation.

Digestion of the purified genomic DNA PCR product with Nco1 was performed in a 50µl reaction volume comprising: 5µl 10x REACT 3 buffer, 1µl Nco1 enzyme, 28µl DNA and 16µl water. Nco1 digestion of the vector was performed in a 40µl reaction comprising; 4µl 10x REACT 3 buffer, 1µl Nco1 enzyme, 28µl DNA and 7µl water.

Ligation is an enzymatic reaction that allows joining of a target DNA fragment with a second DNA (i.e. vector). T4 DNA ligase repairs breaks in the dsDNA backbone and can covalently rejoin annealed cohesive ends in the reverse of a restriction enzyme reaction, to create new DNA molecules. The reaction was performed utilising different amounts of MBL promoter and vector to obtain optimum product. Reactions were vector alone (control) or PCR product + vector (1:3) carried out at 14°C overnight in a thermal cycler. In a total volume of 20µl the PCR + vector reaction comprised: 1.5µl ~12.5ng HindIII/Nco1 digested vector, 12µl~60ng HindIII/Nco1 digested PCR product, 4µl 5x DNA ligase reaction buffer, 1 µl~0.1units T4 DNA Ligase and 1.5µl Water. The vector only reaction comprised: 1.5µl ~12.5ng cut vector, 4µl 5x DNA ligase reaction buffer, 1 µl~0.1units T4 DNA Ligase and 13.5µl Water. Ligated DNA was observed by the presence of higher molecular weight product on agarose gel.

The pGL3 basic vector, a promoterless luciferase reporter vector was utilised. It contains a multiple cloning site and an ampicillin resistance gene. Luciferase is an enzyme which catalyzes a light producing reaction. The luciferase gene is downstream

of the promoter sequence of interest and the regulatory potential of this sequence is measured by subsequent luciferase expression as described in section 2.5.7.

2.5.6.2 Transformation

Transformation is a process by which bacteria can uptake foreign DNA. In the laboratory *E.coli* (Recombinase negative) cells are routinely used and can be made 'competent' by specific pre treatment e.g. with calcium which destabilises the bacterial membrane allowing for uptake of exogenous DNA.

Ampicillin (100μg/ml) containing (Luria Bertani) agar/broth was prepared according to standard microbiological techniques. Plates were stored (for a maximum of 2 weeks) at 4°C until required. Competent JM109 *E.coli* cells were removed from -80°C and thawed on ice. Ligated DNA (1-10ng) was added to 75μl of competent cells respectively and incubated on ice for 10min. A gentle flicking action was performed to encourage DNA binding to JM109 cell membranes. Cells were heat-shocked at 42°C for 50sec and immediately chilled on ice for 2min. This step allows entry of exogenous DNA into cells. 900μl LB broth with 10mM Mg^{2+} and D-glucose was added to each transformation reaction and the mixture was incubated for 1h at 37°C (to allow cells to recover) on a shaking platform at low speed. Ampicillin (100μg/ml) containing LB agar plates were brought to RT and 100μl of the undiluted transformation mix was plated out and incubated overnight at 37°C.

Plates were checked for colonies the following morning and numbers per plate were compared to assess transformation efficiency. Selected colonies were screened for presence of MBL promoter insert in vector. A single colony was picked using a bacterial loop and added to LB broth in small culture volumes. After 4h, 100μl of broth was pelleted and resuspended in 100μl water (to lyse the bacteria). 10μl of the resulting

solution was used in a 25µl PCR reaction to confirm presence of MBL promoter sequence inserts.

Positive plasmid broths were utilised for long term storage. 70% Broth and 30% Glycerol (autoclaved) stocks were transferred to Nunc cryovials (Nunc, Roskilde, Denmark) and slowly chilled before freezing at -80°C.

2.5.6.3 Sequence analysis of MBL promoter construct

Plasmid DNA was prepared for sequencing using a Qiagen Mini-prep kit (Qiagen, Crawley, UK) which is based on a modified alkaline lysis procedure. An aliquot of the resulting plasmid DNA was run on a 1% agarose gel to determine yield and purity and quantity by spectrophotometry. Multiple DNA samples were digested with *Hind* III and *Nco*1 enzymes to confirm the presence of the insert in the cloned vector.

Sequencing of plasmid DNA using commercial primers spanning the vector was performed at the UCL core facility.

Sense 5'-3' GGACTCGTTCCAAACTGGG

Antisense 5'-3' CCAGGGCGTATCTCTTCATAGC

Analysis of over 600bp confirmed complete homology with the published sequence and, specifically, all three MBL promoter polymorphism sites were present. Large scale preparation and purification of plasmid DNA was performed using the Qiagen Maxi prep kit (Qiagen, Crawley, UK).

2.5.7 Transient transfection studies

In order to study transcriptional regulation of MBL, it's promoter construct was transiently transfected into liver cell-lines. Transfection is a method used to introduce DNA into eukaryotic cells.

HepG2 and Huh7 cell-lines were set up in 96 well plates at a density of 3 and 1.5 x 10^4 cells/well respectively in 100µl complete media without antibiotics. Transfections were conducted the following day when cells were 70-80% confluent using Lipofectamine 2000™ (Invitrogen, Paisley, UK). Plasmid DNA was stored in aliquots at a concentration of 0.5µg/µl. Each transfection comprised a total DNA content of 230ng. This included:

a) Firefly luciferase construct under investigation i.e MBL or IL-8 promoter (positive control for cell line, kindly provided by Dr A Bowie, Trinity College, Dublin, 60 ng/well)
b) HSV TK Renilla luciferase construct (kindly provided by Dr A Bowie, Trinity College, Dublin), to correct for transfection efficiency (20 ng/well)
c) Empty vector (pcDNA3.1; Stratagene, Cambridge, UK) to ensure a total of 230ng DNA was added (150ng/well)

Each experimental condition was performed in triplicate. Mastermixes were prepared of different promoter constructs under investigation. The protocol used was adapted from the Invitrogen Lipofectamine 2000™ manual. For each well, DNA to be transfected was prepared as follows: 230ng of DNA was added to 25µl OptiMEM® media (Gibco, Paisley, UK), in a separate tube 1µl Lipofectamine 2000 was added to 25µl OptiMEM. The Lipofectamine/OptiMEM mix was incubated for 5min at RT before combining it with the DNA/OptiMEM mix. The mixture of DNA, Lipofectamine and Optimem (50µl total) was incubated for 20min at RT to allow DNA-Lipofectamine complexes to form prior to addition to resident media. The transfection reaction was left to proceed for 22-24h, media was subsequently removed and replaced with 200µl media (DMEM, 0.05% serum) containing relevant stimulants (e.g. cytokines and LPS). Control wells received media alone. Experiments were terminated by removal of media followed by addition of

50µl reporter lysis buffer. The plate was incubated for 10-15min on a shaking platform before freezing at -80°C until analysis.

Prior to analysis, plates were thawed on ice and 20µl cell lysate was transferred to two white luciferase assay plates (Labtech International, East Sussex, UK). One plate was used for analysing Renilla luciferase activity, where 40µl of its substrate Coelentrazine (Insight technologies, London, UK) was added. The second plate was used to assess luciferase activity to which 100µl firefly luciferase substrate was added. A 96-well plate luminometer (LUCY1 luminometer, Anthos Labtech Instruments, Austria) was utilised for measuring luciferase activity. Ratio of Luciferase to Renilla activity was calculated for each condition in triplicate. Mean values were compared to control and expressed as relative fold induction.

2.6 Statistical analysis

The clinical data, MBL genotypes and protein assay data were entered onto an SPSS database and analysed using SPPS v.12 statistical software. Comparisons between groups were analysed using Mann Whitney U, Kruskal Wallis and Chi square tests where appropriate. Paired t-tests were used to assess change in MBL and MASP levels and function pre- and post-chemotherapy. The mean and standard error of the mean of experimental data (n=3) was calculated and plotted on graphs accordingly.

Chapter 3

Design and implementation of a febrile neutropenia management strategy incorporating risk stratification

3.0 Introduction

This thesis explores the role of MBL and the lectin pathway of complement in children with cancer who are immunosuppressed by their treatment and are at risk of infection. In this chapter the clinical management of infection/FN is discussed. Outcome data collected as part of the large scale PINE audit conducted in the South East of England was utilised in the disease association studies presented in this thesis. This audit project was run in collaboration with the NHS Audit, Information and Analysis Unit (London Specialised Commissioning Group) who coordinated data collection and entry. The candidate was specifically involved in the development of the new infection/FN management protocol and the analysis of audit data, jointly with Mrs J Geary (PINE project co-ordinator), under the supervision of Dr J Chisholm (Clinical lead).

A patient who is febrile (i.e. temperature > 38.0°C for more than 4 hours or > 38.5°C on one occasion) and neutropenic (ie. absolute neutrophil count < 1.0 x 10^9/L or < 0.5 x10^9/L) is presumed to have an infection until proven otherwise and requires medical assessment and broad spectrum antibiotics. The routine use of empirical intravenous antibiotics is the gold standard of treatment and has lead to a dramatic reduction in mortality from infection (Schimpff et al., 1971). However, FN still remains a major cause of morbidity and is the second most common reason for inpatient (IP) admission after chemotherapy (Chisholm and Dommett, 2006). In comparison to adults, children with FN have been reported to have a lower mortality rate (4% and 1%, respectively) and fewer documented infections (28% and 19%, respectively). Overall incidence of bacteraemia is similar (24% and 22%) but fever of unknown origin is more common (35% and 49%, respectively) (Hann et al., 1997). This means that in approximately half of all paediatric FN episodes there is no documented clinical or microbiological site of infection. These patients may be at low risk of life threatening complications and the episodes may be suitable for outpatient management.

Improvements in the identification of FN episodes in which there is a low risk of adverse outcome has enabled the development of 'low risk' management protocols which safely reduce the need for prolonged hospital admission (Ammann et al., 2005). The implications of reduced hospital stay are advantageous for the patient, their family and also the hospital, in terms of quality of life and cost savings (Ahmed et al., 2007). Early work on risk assessment focused on identifying adult cancer patients at lower risk of infection. Talcott and colleagues reviewed 261 medical records of patients with FN. They identified four subgroups of patients, three of which were considered to be at higher risk of infection (patients already hospitalised when fever developed, outpatients with comorbidity and outpatients with cancer 'not in remission') and one group of 'low risk' patients (Talcott et al., 1988). Their risk prediction model utilized clinical information available on the first day of FN and they went on to validate it in a prospective trial. Of 444 patients with FN, 34% of patients in one of the high risk groups were found to suffer from serious medical complications compared to only 5% in the low risk group (Talcott et al., 1992). However the criteria, although effective at predicting adverse outcome, were not sufficient to discriminate between bacterial infections and other causes of fever. More recently, the Multinational Association for Supportive Care in Cancer (MASCC) developed a new clinical risk index, which identifies adult patients at low risk of medical complications during resolution of fever (Klastersky et al., 2000).

The criteria used to identify low risk adult episodes cannot be directly translated into paediatric practice but several studies have been conducted in paediatric populations addressing the same issues (Aquino et al., 1997; Klaassen et al., 2000; Santolaya et al., 2004; Rondinelli et al., 2006) To date there is no universally agreed, validated, set of parameters for identifying low risk paediatric episodes. The most important adverse outcomes in children are intensive care admission and death but the majority of

paediatric studies to date have focussed on prediction of bacteraemia and identified absolute monocyte count, temperature, high CRP, hypotension, relapsed leukaemia, low platelet count and chemotherapy within the previous 7 days, as independent risk factors (Rackoff et al., 1996; Klaassen et al., 2000; Santolaya et al., 2001). Clinical variables including; predicted degree and duration of neutropenia, type and status of underlying disease, associated systemic symptoms indicative of severe infection, proven bacteraemia, and fever and neutrophil count at the time of presentation have all been used to select episodes which are suitable for management on low risk strategies.

The successful identification of low risk episodes has subsequently lead to the development of a number of different management strategies for use in such episodes which have either been piloted or validated against standard IP treatment;

a) **Ambulatory/Outpatient intravenous therapy following a period of hospitalisation**. This has been reported to be safe and enabled discharge from hospital within 24-36h with continued IV treatment in the outpatient setting (Wiernikowski et al., 1991; Santolaya et al., 2004). Santolaya *et al* were able to demonstrate that there was no significant difference in outcome between patients managed on either ambulatory or continued IP therapy (Santolaya et al., 2004).

b) **Ambulatory intravenous therapy from onset of FN episode**. A number of small single centre studies have demonstrated that once daily IV Ceftriaxone was effective in stable children without significant comorbidity (Preis et al., 1993; Kaplinsky et al., 1994; Mustafa et al., 1996). IP admission was required in up to 19% of episodes which all resolved without significant morbidity or mortality. These strategies have not yet been proved equivalent to standard management.

c) **Step-down strategies**. These involve a period of IP care (either a single dose of IV antibiotics or admission for a period of IV antibiotics) followed by a change to oral antibiotics. High rates of success without modification have been reported in two

studies where patients were randomised to either continued IV treatment or oral Cefixime after 48-72h (Paganini et al., 2000; Shenep et al., 2001). Studies utilising shorter observation periods have reported relatively low re-hospitalisation rates but higher rates of treatment modification (Mullen et al., 1999a; Aquino et al., 2000; Paganini et al., 2003).

d) **Oral therapy from the outset**. Two small studies have reported the use of oral antibiotics from the outset in groups of very carefully selected patients with low hospitalisation rates (6-9%) and no significant difference in outcome when compared with IV treatment (Petrilli et al., 2000; Malik, 1997). The majority of these studies have been conducted in single centres with a maximum of six centres involved in the minority. There have been no reports of the safe implementation of such strategies in a large multicentre setting, to date.

As mentioned above (Chapter 2), the specialist care of children with cancer in the South East of England is provided by four tertiary Paediatric Oncology Centres (POCs) in London working in collaboration with over forty Paediatric Oncology Shared Care Units (POSCUs) based at district general hospitals (Figure 2.1). Importantly, the POCs and POSCUs use unified supportive care guidelines ensuring that all children are managed in the same way. This is in contrast to other parts of the United Kingdom where management practice is diverse (Phillips et al., 2007). There has been an ongoing interest in the management of infection in the shared care setting in the South East of England. An audit of FN episodes in POSCUs conducted in 2001/2002 showed that the recommended empirical antibiotic regimen was safe, levels of antibiotic resistance were low and median inpatient stay was 5 days (Duncan et al., 2007).

Following on from this a new FN management protocol incorporating RS was designed and a step-down strategy was deemed most suitable for this multicentre healthcare

setting. This chapter describes the design of the new protocol and the results from the PINE project relating to RS and low risk management.

3.1 Methods

3.1.1 Risk Stratification Criteria

A literature review was conducted of all relevant adult and paediatric studies and RS criteria, excluding patients from low risk management, were identified (Figure 3.1). The categories included; age, medical conditions requiring IP treatment independent of FN, high risk diagnosis or treatment regime, previous admission to PICU during FN and social concerns which could be patient or carer related. The specific criteria were discussed with the PINE working party, a group of POC and POSCU clinicians and microbiologists, before a unified decision was reached.

3.1.2 Patient assessment

All patients were admitted to hospital for a minimum of 48h. On admission a full history was taken, clinical examination performed and risk assessment (low or standard) made using the RS checklist (Figure 3.1). Blood cultures were taken prior to commencing empirical antibiotics and other relevant investigations were carried out according to the clinician's assessment. At 48h, a full blood count was taken and repeat risk assessment performed. This assessment also incorporated the RS checklist, absolute neutrophil count (ANC) and blood culture results. A 48h ANC of $<0.1 \times 10^9$/L and/or positive blood cultures excluded the patient from low risk management irrespective of the RS checklist. Presence of any of the RS criteria on admission or at 48h also excluded them from low risk management.

Febrile Neutropenia- Risk Stratification Checklist

Hospital _____ Date of Admission / /

Criteria excluding patients from Low Risk protocol

	T all relevant exclusion criteria On Admission	At 48h
Age		
< 1 year		
Associated medical conditions requiring hospitalization		
Shock or compensated shock		
Haemorrhage		
Dehydration		
Metabolic instability		
Altered mental status		
Pneumonitis		
Mucositis(unable to tolerate oral fluids or requiring IV analgesia)		
Respiratory distress/compromise		
Perirectal or other soft tissue abscess		
Rigors		
Irritability/Meningism		
Organ failure		
Cancer associated comorbidities		
ALL at diagnosis/relapse <28 days		
ALL not in remission >28 days		
AML		
Infant ALL		
Intensive B-NHL protocols		
Allogenic BMT or Autologous PBSC transplant		
Sequential high dose chemotherapy with PBSC rescue		
History		
PICU admission during last FN episode		
Non adherence- Social concerns		
Patient		
Inability to tolerate oral antibiotics		

If one or more exclusion criteria are present follow Standard Febrile Neutropenia Protocol

Figure 3.1 Risk Stratification criteria

The final decision regarding low risk management lay with the attending clinician as patients had to be considered 'clinically well' but this did not mean they had to be apyrexial (Figure 3.2). Patients with a temperature ≥38°C could still be discharged on the low risk protocol. Episodes excluded from low risk management continued on the standard risk protocol (Figure 3.3).

3.1.3 Low risk management

In low risk episodes, patients stopped IV antibiotics, were discharged and continued oral Co-Amoxiclav (Augmentin) at home until they had been afebrile for 48h (Figure 3.4). If, at any time after discharge, the patient's condition deteriorated or there was parental concern, the patient was reviewed in hospital. A phone call was made at 72h to monitor progress and a clinical review was performed at 96h, either at home or in hospital depending on local arrangements. At the 96h review an ANC was repeated, vital signs were checked and any late cultures were chased. A patient remaining febrile (>38°C) at 96h was readmitted to hospital to recommence IV antibiotics, including an antifungal agent, as per the standard risk protocol. If the temperature was settling and the patient remained clinically well they returned home and discontinued antibiotics as planned.

The new protocol was implemented in all POCs and POSCUs in April 2004 and audited from the outset. Following liason with the local ethics committee, consent for data collection was not required. The clinical outcome data was recorded using audit proformas (Figures 3.5 and 3.6).

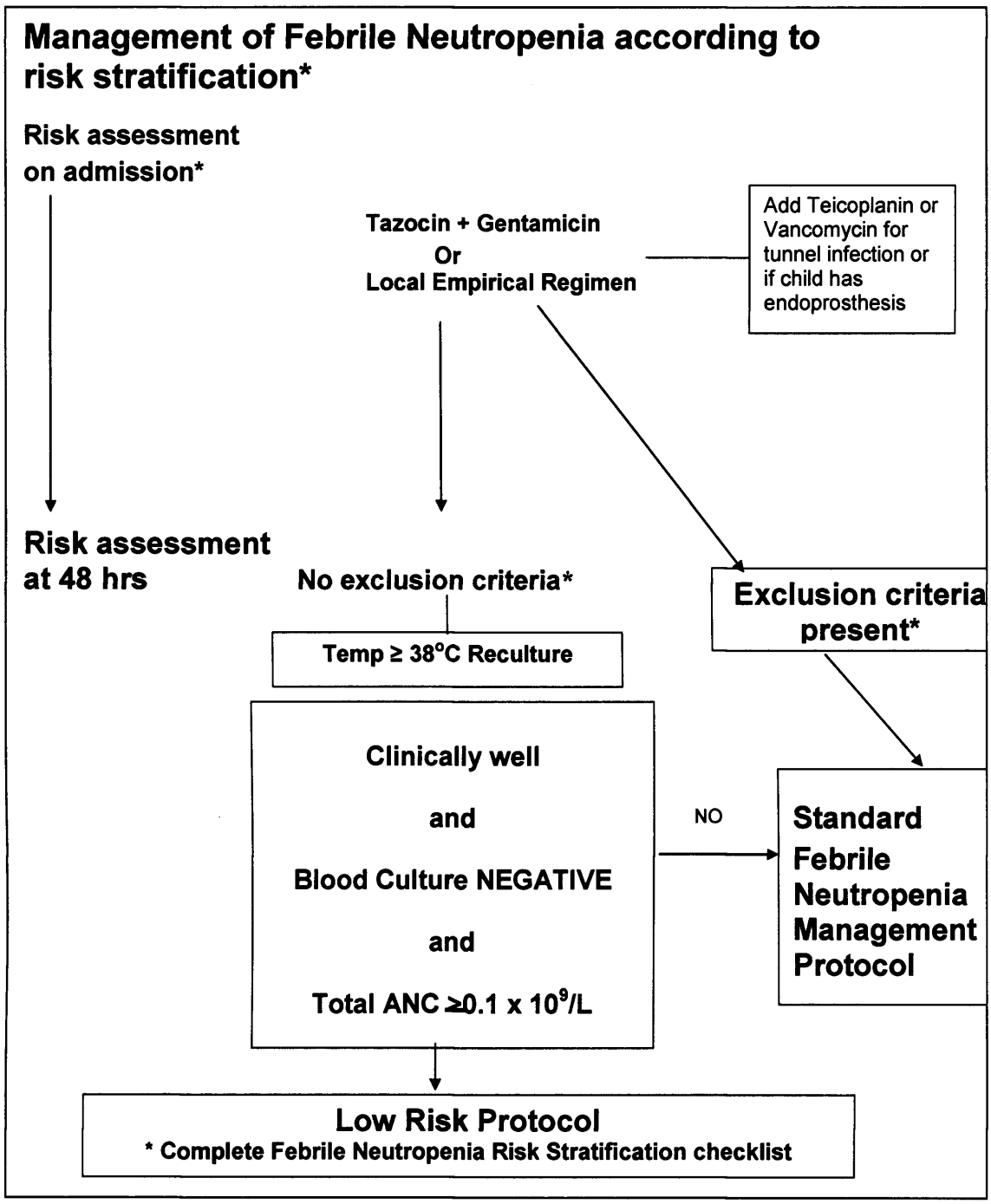


Figure 3.2 Protocol for selection of patients eligible for low risk management

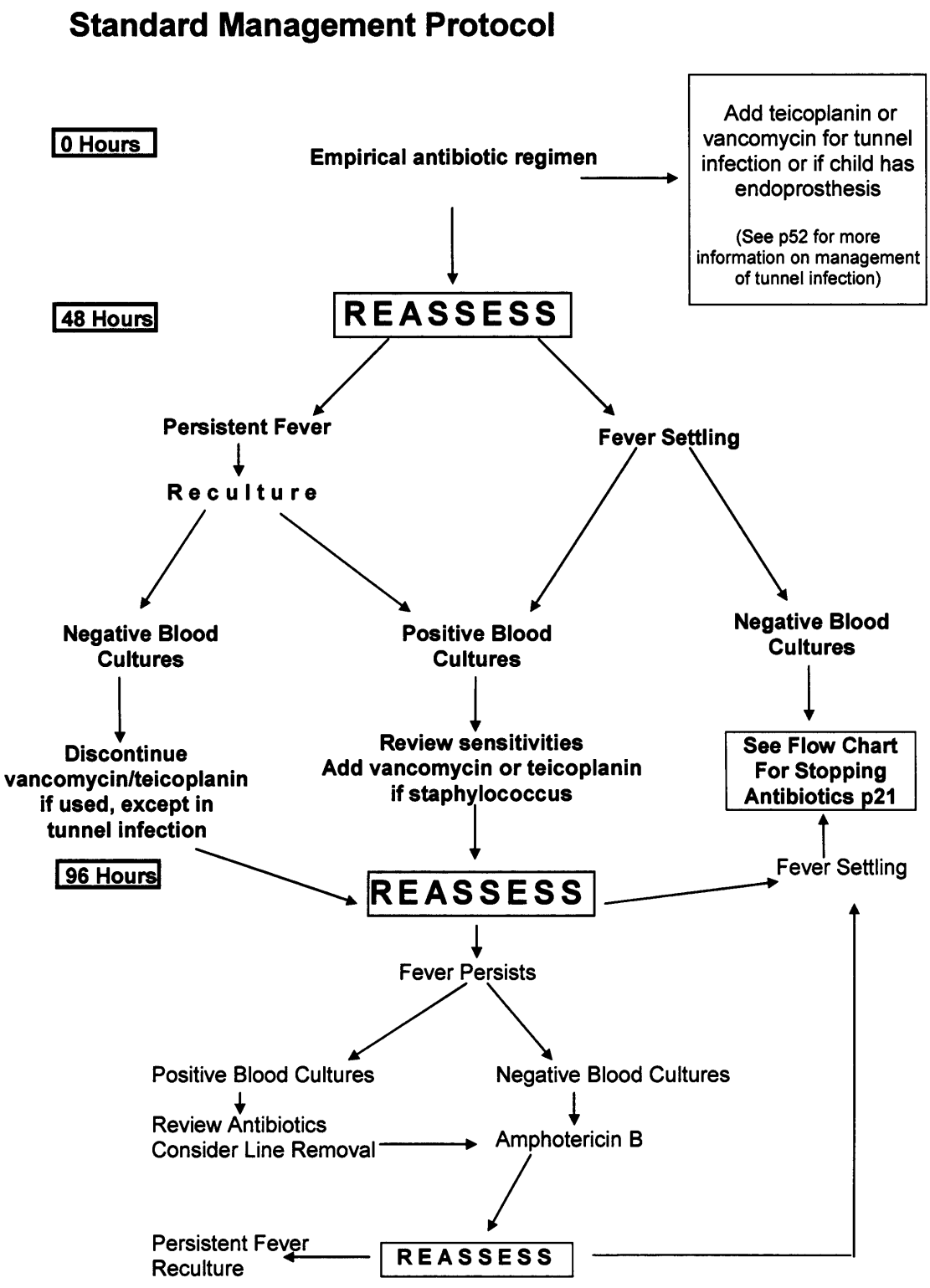


Figure 3.3 Management protocol for Standard risk patients

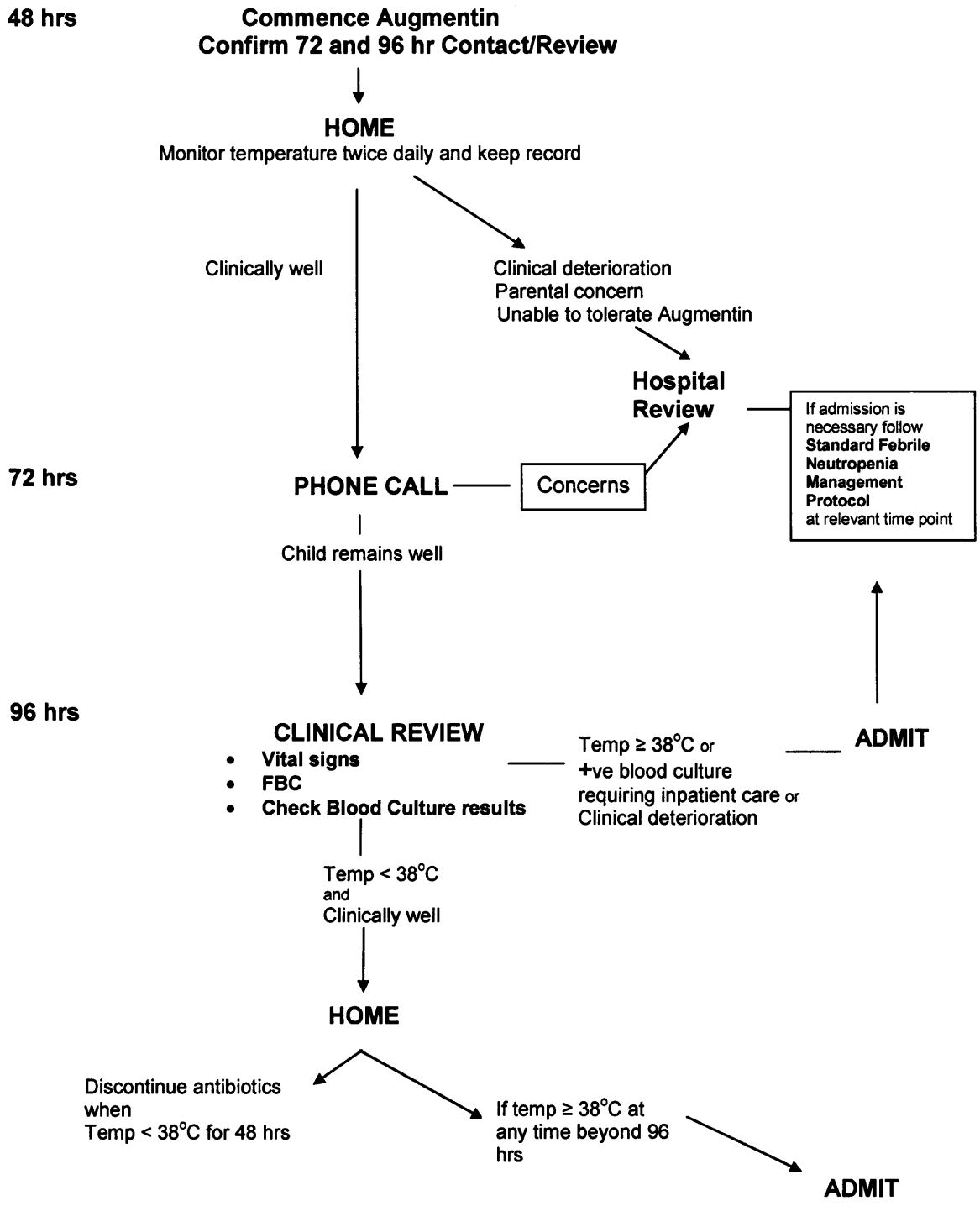


Figure 3.4 Outpatient management protocol for low risk patients

Audit, Information and Analysis Unit (London, Kent, Surrey & Sussex)

No in Series

PAEDIATRIC INFECTIONS IN FEBRILE NEUTROPENIC EPISODES (PINE) PRESENTING TO SHARED CARE UNITS WORKING WITH THE TPOC's

TO BE COMPLETED AT 48 HOURS

Please complete a separate form for each admission during the period 1st April 2004 - 31st March 2005

Name of Hospital		Tertiary Centre for this patient *(please circle)*	GOS / RMH / RLH / Middx
Patient's Date of Birth & sex *(please circle)*	/ / M F	Date of Admission	/ /
Oncology Diagnosis		Risk Stratification Checklist completed	Yes No

Symptoms reported on admission *(please ✓)*

Fever only		Line related	
Respiratory		Skin	
Gastrointestinal		Urine	
Central nervous system (CNS)		Other	

Clinical/Radiological evidence of infection *(please ✓)*

Fever only		Line related	
Respiratory		Skin	
Gastrointestinal		Urine	
Central nervous system (CNS)		Septic shock	
Cardiac signs		Other	

Admission Antibiotics used *(please ✓)*

Gentamicin	☐	Amikacin	☐	Ceftriaxone	☐
Piptazobactam	☐	Ciprofloxacin	☐	Ceftazadime	☐
Teicoplanin	☐	Vancomycin	☐		
Other					

Fluid Bolus Required *(please circle)* Yes No

Admission results ANC **48 hour results** ANC

Positive culture results

Date	Site *(please ✓)*		Organism	Sensitivities	Resistances
	Blood				
	Urine				
	Skin				
	Other				

PLEASE FAX RISK STRATIFICATION CHECKLIST

Risk assessment *(please circle)* **On admission** LOW STANDARD **At 48 hours** LOW STANDARD

LOW RISK PATIENTS ONLY

Managed on Low Risk protocol *(please circle)* Yes No * Total inpatient nights nights

Discharge Antibiotics *(please ✓)*

Augmentin	☐
Ciprofloxacin	☐
Clarithromicin	☐
Other	

* If managed on Standard risk protocol * Reason

Figure 3.5 Clinical outcome data collection sheet 1

Audit, Information & Analysis Unit (London, Kent, Surrey & Sussex)

No in Series

PAEDIATRIC INFECTIONS IN FEBRILE NEUTROPENIC EPISODES (PINE) PRESENTING TO SHARED CARE UNITS WORKING WITH THE TPOC's

TO BE COMPLETED AT END OF EPISODE

Please complete a separate form for each admission during the period 1st April 2004 - 31st March 2005

Name of Hospital

Tertiary Centre for this patient *(please circle)* — GOS / RMH / RLH / Middx

Patient's Date of Birth & sex *(please circle)* — / / — M F

Date of Discharge — / /

Patients assessed and managed as LOW RISK at 48 hours

Outpatient Management

Hospital review required Other than planned at 96 hours *(please circle)* — Yes * No

Outcome *(please circle)* — Returned Home / Admitted **

If Yes *, No. of days Post Discharge — days

Date / /

Indication *(please ✓)*

Clinical Deterioration	
Persistent Fever	
Unable to tolerate oral antibiotics	
Parental Concern	

96 hour review — ANC

Total No of days on oral antibiotics — days

Positive culture results **If changed from 48 hours**

Date	Site *(please ✓)*		Organism	Sens	Resist
	Blood				
	Urine				
	Skin				
	Other				

**** If Readmission required at any time**

No of days post Discharge — days

Date / /

Indication *(please ✓)*

Clinical Deterioration	
Persistent Fever	
Unable to tolerate oral antibiotics	
Parental Concern	

Change/addition of antibiotic required ***(please circle)*** — Yes * No

* Details

Total Antibiotic Days — days

Final Discharge Date — / /

Total inpatient nights Original + readmission — nights

Patients assessed and/or managed as STANDARD RISK AT 48 hours

Additional Positive Blood Culture results *(please circle)* — Yes No

Positive culture results

Date	Site *(please ✓)*		Organism	Sens	Resist
	Blood				
	Urine				
	Skin				
	Other				

Change/addition of antibiotic required *(please circle)* — Yes * No

* Details

Total inpatient nights — nights

All Patients

Transferred to POSCU *(please circle)* — Yes No

PICU admission required *(please circle)* — Yes No

Death *(please circle)* — Yes No

Figure 3.6 Clinical outcome data collection sheet 2

3.2 Results

3.2.1 Patient and episode characteristics

PINE data was returned from 44 out of 47 eligible hospitals between April 2004 and March 2005. Of these, 40 were POSCUs and 4 were POCs. 762 FN episodes were eligible for analysis from 368 patients, of which 213 (28%) episodes were initiated in POCs and 549 (72%) were initiated in POSCUs. The median patient age at first admission for FN was 5 years 7 months (range 1 month to 17 years 6 months). Patient numbers stratified by diagnosis, mean number of FN episodes and their distribution between POC and POSCU are detailed in Table 3.1. Acute Lymphoblastic Leukaemia (ALL) is the commonest cause of childhood cancer and ALL patients represented 51% of the total patient population and accounted for 50% of the total FN episodes captured. The majority of ALL episodes (82.3%) were initiated in POSCUs. In contrast, the second largest group of patients captured in the audit were those with Acute Myeloid Leukaemia (AML) (9%) and the majority of their episodes (89%) initiated in POCs.

Diagnosis	Patients n (%*)	Mean no. of episodes	Episodes initiated in a POC n (%*)	Episodes initiated in a POSCU n (%*)	Total episodes N (%*)
ALL	188 (51)	2	68 (32)	316 (58)	384 (50)
AML	33 (9)	3	89 (42)	11	100 (13)
Non-Hodgkins Lymphoma	28 (8)	2	21 (10)	35 (6)	56 (7)
Hodgkins Lymphoma	8	1	3	6	9
PTLD†	1	2	0	2	2
Brain tumour	18	2	3	28	31
Bone tumour	20	2	3	36 (7)	39 (5)
Neuroblastoma	20	2	15 (7)	19	34
Wilms tumour	14	3	4	31	35
Soft Tissue Sarcoma	12	2	2	25	27
Germ Cell tumour	2	1	1	1	2
Other	24 (7)	2	4	39 (7)	43
Total	**368**	**2**	**213**	**549**	**762**

Table 3.1 Patient diagnosis, mean number of FN episodes and their distribution between POC and POSCU. *Percentages are quoted for the 4 largest groups in each category †PTLD, Post Transplant Lymphoproliferative Disease

3.2.2 Risk stratification and management

The differences in the patient populations treated for FN in POCs and POSCUs were clearly demonstrated when analysing the RS checklists detailing the criteria excluding patients from low risk management (Table 3.2). Overall the POC FN patient population consists of those treated on high risk protocols e.g. AML, B cell Non Hodgkins Lymphoma (NHL) and Stage IV Neuroblastoma (requiring PBSC) and ALL patients at the time of diagnosis or relapse, all excluded from low risk management.

Exclusion criteria	**On admission**			**At 48h**		
	POC (%)	**POSCU (%)**	**Total (%)**	**POC (%)**	**POSCU (%)**	**Total (%)**
Age < 1 yr only	**1 (0.5)**	**7 (1.3)**	**8 (1.0)**	**2 (0.9)**	**8 (1.5)**	**10 (1.3)**
Age < 1 yr in combination	17 (8.0)	3 (0.55)	20 (2.6)	16 (7.5)	2 (0.36)	18 (2.4)
Diagnosis only	**147 (69)**	**38 (6.9)**	**185 (24.3)**	**147 (69)**	**41 (7.5)**	**188 (24.7)**
Diagnosis in combination	37 (17.4)	12 (2.2)	33 (4.3)	35 (16.4)	10 (1.8)	45 (5.9)
Medical conditions only	**9 (4.2)**	**46 (8.4)**	**55 (7.2)**	**7 (3.3)**	**47 (8.6)**	**54 (7.1)**
Medical conditions in combination	25 (11.7)	16 (2.9)	41 (5.4)	18 (8.5)	13 (2.4)	31 (4.0)
Patient history only	**3 (1.4)**	**14 (2.6)**	**17 (2.2)**	**4 (1.9)**	**15 (2.7)**	**19 (2.5)**
Patient history in combination	4 (1.9)	12 (2.2)	16 (2.1)	3 (1.4)	10 (1.8)	13 (1.7)
Other	5 (2.3)	6 (1.1)	11 (1.4)	5 (2.3)	9 (1.6)	14 (1.8)
No exclusion criteria	**9 (4.2)**	**401 (73)**	**410 (53.8)**	**12 (5.6)**	**376 (68.5)**	**388 (50.9)**
No documentation	0	16 (2.9)	16 (2.1)	1 (0.5)	37 (6.7)	38 (5.0)

Table 3.2 Exclusion criteria recorded on the RS checklist at 0 and 48h in both POC and POSCUs.
For criteria shown in **bold**, actual percentages are detailed but for criteria in non bold percentages are cumulative and not exclusive i.e. total ≠ 100%

Diagnosis was the most common reason, both independently or in combination with other criteria, for exclusion from low risk management in the POCs. In contrast, the most common reason for exclusion from low risk in POSCU episodes was medical

conditions that required IP treatment independently of FN. These included respiratory compromise and shock i.e. reflecting a patient who was clinically unwell. However, the majority of POSCU episodes had no exclusion criteria both on admission (73%) and at 48h (68.5%) compared to the minority of POC episodes (4.2 and 5.6%, respectively). Overall 388 episodes (50.9%) had no exclusion criteria on the RS checklist at 48h.

Clinicians recorded any clinical or radiological evidence of infection and this was documented in 628 episodes (data was missing from 134 forms). Overall, evidence of infection was reported in 258 episodes (41%) and fever only was reported in 370 (59%). Evidence of respiratory and GI infection was most common, reported in 13 and 11% of episodes, respectively.

Positive blood cultures were present in 223 (29%) episodes at 48h and positive cultures from any source were present in 254 (33%) episodes. Approximately two thirds of blood culture isolates were gram-positive bacteria of which 70.5% were *Staphylococcus epidermidis* and other coagulase-negative *Staphylococci*. Gram-negative bacteria accounted for 28.3% of isolates and fungal organisms accounted for just 2.2%. The mean ANC in POSCU episodes rose between admission and 48h from 0.26 to 0.42 $x10^9$/L in comparison to POC episodes where it fell slightly from 0.13 to 0.12 $x10^9$/L, reflecting the relative degree of immunosuppression in the different populations and their capacity for count recovery.

On admission 403 (53%) episodes were identified as eligible for low risk management and at the 48h assessment 212 (28%) remained eligible. Of the 191 episodes which changed from low to standard risk, positive blood cultures and ANC < 0.1 $x10^9$/L were solely responsible in 69 and 50 episodes, respectively. Positive blood cultures and/or low ANC were responsible for the change of risk assignment in 74% (142/191) of these episodes. Other reasons for a change in risk assessment are detailed in Table 3.3.

Reason for change	Episodes, n (%)
Positive blood culture only	69 (36)
ANC < 0.1	50 (26)
Other clinical reasons	7 (4)
Positive blood cultures and ANC < 0.1	23 (12)
Exclusion criteria at 48h (not present at admission)	25 (13)
Inappropriate risk assignment	15 (8)
No reason given	2 (1)
Total	**191**

Table 3.3 Reasons for change from low to standard risk at 48h

Of the remaining 212 episodes eligible for low risk management, 143 episodes were actually managed as low risk. This left 69 patients who were managed as standard risk and the reasons given for this decision are shown in Table 3.4. The only reason given in 6 episodes was that the patient had a persistent fever but this should not have excluded the child from low risk management. A number of the reasons given were unclear or not given at all.

Reason for standard management	Episode n (%)
Clinical decision	35 (51)
Persistent fever only	6 (9)
Confusion over ANC threshold	4 (6)
No reason/unclear	24 (35)
Total	69

Table 3.4 Reasons given for decision to manage patients eligible for low risk on the standard risk protocol

Overall, 19% (143/762) of the total episodes were managed appropriately on the low risk protocol. A further 18 patients who should have been managed as standard risk were managed as low risk, giving a total of 161 episodes of which 160 were initiated in POSCUs. The recommended oral antibiotic, Co-Amoxiclav, was prescribed in 71% of these episodes. Of the remaining episodes; other antibiotics were used in 9%, no antibiotics were prescribed on discharge in 15% and there was no documentation in 5%. The median number of oral antibiotic days was 5 and total antibiotic days was 7.

Hospital review was required in 17 patients before the planned 96h assessment. At the 96h assessment, the ANC was recorded in 110 (68.3%) episodes and of these only 8 had an ANC < 0.1 $x10^9$/L.

Readmission was required in a total of 11 of the episodes managed as low risk. The readmission rate in patients who had been treated appropriately on the low risk protocol was 5.6% (8/143) compared to 16.6% (3/18) in those who had been managed inappropriately. For six episodes the number of days post discharge was not recorded. For the remaining episodes, the mean number of days between discharge and readmission was 1.4 (median 1, range 1-2). The most common reason for readmission was clinical deterioration which was recorded in 6 episodes. Other reasons included; persistent fever, parental concern, inability to tolerate oral antibiotics and development of herpes zoster. Eight of the readmission episodes required a change in antibiotics although none had any new positive blood culture results. All patients requiring readmission made a satisfactory recovery and spent on average 4 extra nights in hospital. The total number of IP nights and antibiotic days for all patients managed on the low risk protocol are summarised in Table 3.5.

	Episode Type	Median	Mean	Range	No data
Total inpatient nights	All Low Risk Patients	2	2.5	1 - 4	0
	Non-readmitted patients	2	2.5	1 - 4	0
	Re-admitted patients	6	6.8	3 - 14	0
Total antibiotic days	All Low Risk Patients	7	6.0	2 - 20	5
	Non-readmitted patients	7	5.8	2 - 11	4
	Re-admitted patients	8.5	9.8	6 - 20	1

Table 3.5 IP nights and antibiotic days for patients managed on the low risk protocol

The median number of IP nights for all low risk patients was 2 in comparison to standard risk patients who spent 7 nights in hospital (data not shown). The median stay in POSCU standard risk patients was 5 nights, identical to that reported in the previous audit (Duncan et al., 2006) and significantly shorter than the median IP stay in POC standard risk episodes which was 11 nights. It should also be noted that no patients

managed on the low risk protocol required transfer to their POC because of FN and there were no PICU admissions or deaths in any low risk patients.

3.3 Discussion

This chapter describes the safe implementation of a new low risk FN management strategy in over 40 hospitals across the South East of England. It is the first report of the use of RS and its applicability in such a large paediatric multicentre setting, made possible by the established partnership between our POCs and POSCUs and the use of shared management protocols.

A number of previous studies have been conducted in relatively selective single centre populations. An advantage of our study is that we have captured a patient population that represents the full spectrum of paediatric haemato-oncology diagnoses, from ALL to the less common e.g. Germ cell tumours and Anaplastic Large Cell Lymphoma (ALCL), by utilising data collected in both POCs and POSCUs. The location of supportive care of such patients is largely governed by the intensity of their treatment protocol; for example AML patients receive 6 months of intensive chemotherapy and spend most of this time as an IP in their POC whereas ALL patients receive less immunosuppressive treatment over 2-3 years, generally as an outpatient with much of the supportive care delivered at the POSCU. This was demonstrated by the distribution of FN episodes initiated in POCs or POSCUs (Table 3.1). The majority of solid tumour patient episodes were also managed in POSCUs which also reflects the nature of their treatment and the fact that they have normally been discharged from their POC after chemotherapy before their ANC falls and they become febrile and neutropenic.

It has been proposed that between one-third and one-half of children with FN at are low risk of serious infection (Orudjev and Lange, 2002) but the proportion suitable for treatment on low risk strategies is dependent on the specific selection criteria. Our

protocol enabled early discharge in 19% of total episodes after 2 IP nights and if the POSCU episodes are considered separately, 29% (160/549) were managed on the low risk protocol. A recent study by Quezada *et al*, reported that 26% of their patient population were eligible for management on a step-down strategy similar to ours but that only 56% of these actually received any outpatient care (Quezada et al., 2007). It is important to note that their patients were all managed in a large American oncology centre and did not include many low risk diagnoses e.g.'good risk' ALL or low stage Wilm's tumour. Patients were required to have a declining fever to qualify for outpatient management, which is in contrast to our protocol and meant that their patients spent on average 3.5 days as an IP and 3.5 days as an outpatient. Interpretation of results from other similar studies must include the eligibility/exclusion criteria used and patients studied. Some very encouraging results have been reported in studies which have been highly selective of their patient population (Malik, 1997; Petrilli et al., 2000) and may not be comparable to our practice.

A step-down strategy was chosen because it enabled a period of IP admission and an informed discharge decision utilising both blood culture results and ANC in order to optimise both patient safety and experience. The 48h observation period ensured the identification of patients with significant gram negative sepsis who most commonly deteriorate within the first 24h of admission; there were no late gram negative infections in any episodes identified as low risk in our study. Positive blood cultures alone were responsible for the change to standard management in 36% of episodes but it could be debated whether all patients with gram positive infections should have been excluded from management on a low risk management strategy as they may have been adequately treated with once daily IV or even oral antibiotics at home. Knowledge of blood culture results at the time of discharge should prevent the need for readmission of patients who require prolonged IV antibiotics to treat a microbiologically defined

infection although in some studies positive blood cultures are not considered an absolute indication for IP management (Mullen et al., 1999a). Reassuringly none of the re-admitted low risk patients had any new positive cultures. The 48h ANC was used to assess evidence of impending bone marrow recovery which is important for predicting uneventful recovery of FN (Jones et al., 1994; Lucas et al., 1996). ANC alone was responsible for the change to standard management in 25% of episodes. Interestingly, of those patients discharged on the low risk protocol with an ANC ≥ 0.1 at 48h, 7% had an ANC < 0.1 at 96h but continued to make an uneventful recovery which may indicate that clinical wellness may be more relevant in predicting outcome in this low risk group. Most importantly none of the episodes managed on the low risk protocol required PICU admission and there were no deaths.

Timing of risk assessment or step-down is very important and has repercussions for both the patient and healthcare system. The accuracy of risk assignment needs to be high if low risk patients are to be discharged after a short IP stay or a single dose of antibiotics, in order to prevent the need for re-admission. For some parents, the uncertainty of outpatient management may be more problematic or inconvenient than prolonged IP admission especially if co-ordinating the care of other children. Readmission rates were low (6%) in our patients discharged appropriately on the low risk protocol. Some patients were readmitted because of parental concern which may in part relate to the change in protocol because using the old protocol patients were only discharged when afebrile. Parental confidence is paramount to the success of these strategies and it remains unclear whether more radical protocols encompassing oral antibiotics from the onset of FN would be widely accepted (Sung et al., 2004).

Any patient with FN in the outpatient setting requires rigorous follow up, whether that is ambulatory or at home. Our patients were contacted at home, 24h after discharge and reviewed at 48h to ensure that the episode was resolving. Most of the patients requiring

readmission presented within 48h of discharge therefore the 96h review served more to reassure parents than to detect actual deterioration. The ability to follow up these episodes was dependent on relevant personnel e.g. paediatric community nurses; these services were not universally available and may have limited or prevented the use of the low risk protocol in some centres. Reduction in treatment costs for care delivered in the outpatient (Mustafa et al., 1996b; Ahmed et al., 2007) or home setting (Raisch et al., 2003) has been demonstrated and early step-down to oral antibiotics after a single dose of IV has been shown to be less costly than outpatient IV therapy in a randomised study (Mullen et al., 1999b). A full cost-benefit analysis has not been performed for the current study but such information will inform future practice.

Overall, adherence to the new management strategy was good with the majority of hospitals utilising the low risk protocol appropriately. 'Health care system competence' has been identified as a significant risk factor in the treatment of low risk FN (Mullen, 2001). This competence relates to the ability to identify patients correctly in the first instance and the ability to adequately follow up patients as discussed. We conducted a review of all the audit proformas in order to assess the accuracy of both risk assignment and subsequent management. We identified 18 patients who were managed inappropriately on the low risk protocol predominantly due to incorrect risk assignment, 3 of whom required readmission. This could have been due to poor understanding of the protocol or over confidence in low risk management. It is also possible that there may have been some parental pressure for early discharge in some cases. Fortunately none of these episodes had an adverse outcome and all resolved promptly after re-admission. When introducing any new protocol one cannot underestimate the importance of educating the personnel who will be interpreting and using it.

Further refinement of the RS process should improve our ability to identify episodes suitable for management on low risk protocols which may ultimately prevent admission

altogether. Acute phase proteins such as procalcitonin and proinflammatory cytokines such as IL-8 and IL-6 have been investigated for their ability to identify individuals with FN who have significant infection, or as markers of clinical deterioration, with differing results (de Bont et al., 1999; Lehrnbecher et al., 2004; Lilienfeld-Toal et al., 2004; Persson et al., 2005; Hodge et al., 2006; Secmeer et al., 2007). In 2005, Oude Nijhuis *et al* reported their ability to identify a subpopulation of FN patients using both clinical parameters and plasma IL-8 levels in whom they were able to withhold antibiotic treatment safely (Oude Nijhuis et al., 2005). As discussed in Chapter 1 we may also be able to utilise genetic information about a patient's infection susceptibility profile in the RS process (Oude Nijhuis et al., 2002) and MBL may be a potential candidate gene.

In summary we have designed and implemented a new management strategy for FN in over 40 hospitals utilising RS based on clinical criteria. This has enabled early discharge from hospital and ongoing management with oral antibiotics in 19% of all FN episodes and 29% of those initiated in POSCUs. The protocol was safe and resulted in a low re-admission rate in those treated correctly. Approximately 66% of the patients captured in the PINE project were recruited into the MBL/lectin pathway studies with consent to access PINE data. These patients are described in the subsequent chapters and the PINE data was specifically used in the analysis of the studies reported in Chapters 5 and 7.

Chapter 4

MBL genotyping in children with cancer

4.0 Introduction

Our knowledge of an association between MBL deficiency and disease has improved significantly since 1989 when Super *et al* observed that low levels of mannan-binding protein (now known as MBL) were associated with 'a common defect of opsonisation' (Super et al., 1989). The identification of three mutations in exon 1 of the *MBL2* gene over the following 5 years, and the later discovery of polymorphisms within the promoter region are now known to account for the majority of inter-individual variation in MBL protein levels (Sumiya et al., 1991; Lipscombe et al., 1992; Madsen et al., 1994; Madsen et al., 1995). When analysing for disease association, genotype offers more information than serum protein level alone and both measures should ideally be utilised (Garred et al., 2003b). Genotype is also a constant measure, unlike serum levels which can change depending on the current health of the individual.

An ideal method for genotyping large cohorts of patients requires it to be accurate, fast and cost effective. A number of methods for genotyping the *MBL2* gene have been described, some of which are listed in Table 4.1. All the methods listed rely on PCR but vary in the number of reactions and subsequent steps required which influence their applicability. In the present study two different methods, heteroduplexing, a technique developed and established in our laboratory and the INNO-LiPA *MBL2* assay, a commercial kit, were utilised to genotype the patient cohort. The INNO-LiPA method had previously been validated against other genotyping methods including PCR with sequence specific primers (PCR-SSP) (Steffensen et al., 2000) and real time PCR (Steffensen et al., 2003). However this study is the first to validate the INNO-LiPA assay against heteroduplexing.

The focus of this study is the influence of MBL on infection related morbidity in children with cancer. Studies to date have reported conflicting findings (Section 1.8)

Genotyping method	Reference
Amplification refractory mutations system (ARMS)	(Davies et al., 1995)
PCR and restriction fragment length polymorphism (RFLP)	(Lipscombe et al., 1992; Madsen et al., 1994)
PCR and restriction fragment length polymorphism (RFLP) and site directed mutagenesis	(Madsen et al., 1998)
Heteroduplexing	(Jack et al., 1997)
Denaturing gradient gel electrophoresis (DDGE)	(Gabolde et al., 1999)
PCR using sequence specific primers (SSP)	(Steffensen et al., 2000)
PCR-SSP and sequence specific oligonucleotide probes (SSOP)	(Boldt and Petzl-Erler, 2002)
Oligonucleotide ligation assay	(Roos et al., 2003)
Real-time PCR with fluorescent hybridization probes	(Steffensen et al., 2003)
TaqMan® assay using minor groove binder probes	(Van Hoeyveld et al., 2004)
Multiplex PCR	(Skalnikova et al., 2004)
PCR + Pyrosequencing	(Roos et al., 2006)
INNO-LiPA reverse hybridization	(Nuytinck and Shapiro, 2004)
DNA Microarray-based on-chip PCR	(Mitterer et al., 2005)

Table 4.1 *MBL2* genotyping methods

but sample sizes have been relatively small (~ 100 patients/study) which may explain the differing results. The present study was designed to investigate MBL and the lectin pathway in a larger patient population. This chapter describes two different MBL2 genotyping methods and compares the practicalities of their use. The genotyping for the study population was investigated and the potential interaction between MBL genotype and diagnosis highlighted.

4.1 Methods

4.1.1 Patient population

A cross sectional observational study was performed during a one year period (April 2004- March 2005). It was designed to capture all children on active treatment for cancer treated at the 4 London POCs.

Inclusion criteria

1. Age 0-16 years inclusive.

2. Currently receiving chemotherapy/on active treatment for any solid tumour or a haematological malignancy in remission.

Exclusion criteria

1. Patients who had received an allogenic bone marrow transplant (BMT) or autologous peripheral blood stem cell (PBSC) transplant.

2. Leukaemia at first diagnosis (< 28 days) or relapse, not in remission.

4.1.1.1 Sample and data collection

Blood samples were collected from children when they were clinically well and attending their POC for a routine outpatient clinic or at the time of admission for routine treatment. Informed, written consent from the parent/guardian (or the child where applicable) with verbal assent from the child was obtained for blood sampling and access to data from the PINE audit project.

1ml of whole blood was collected and stored at -20°C until required for DNA extraction. Blood was collected through a central venous line, where available, at a time when blood was being collected for other reasons. Clinical information (age, sex, diagnosis and ethnic status) was obtained on all patients.

The MBL and Lectin pathway studies were approved by the Research Ethics committees of the 4 London POCs, Section 2.1.

4.1.2 MBL2 Genotyping

4.1.2.1 Heteroduplexing

The heteroduplexing method is described in detail in Section 2.3.1. Briefly, a universal heteroduplex generator (UHG), a synthetic DNA molecule based on the MBL genomic sequence, containing appropriate insertions and deletions is used as a template (Wood and Bidwell, 1996). Patient genomic DNA PCR product was combined with UHG PCR product, and the two were allowed to anneal to produce characteristic heteroduplexes for different alleles. These heteroduplexes display different electrophoretic mobility on PAGE, thus enabling identification of a subject's genotype (Jack et al., 1997).

4.1.2.2 INNO- LiPA assay

The methodology is described in detail in Section 2.3.2. Briefly, the method relies on genomic DNA: probe DNA hybridization technology that enables detection of exon 1 and all 3 promoter polymorphisms using a commercial kit (INNO-LiPA *MBL2* Amplification and INNOLiPA *MBL2*, INNOGENETICS N.V., Belgium). Amplified biotinylated genomic DNA was chemically denatured and the separated DNA strands were hybridized with specific oligonucleotide probes immobilized on membrane-based strips. Addition of streptavidin (conjugated with alkaline phosphatase) allowed avid binding to the biotin moiety of the hybrid DNA. Finally, incubation with an alkaline phosphatase substrate solution containing chromogen resulted in a purple/brown precipitate. The resulting probe pattern could then be translated into its appropriate genotype.

4.1.2.3 Terminology

MBL2 genotyping data can be expressed in a number ways encompassing the different exon 1 and promoter polymorphisms. To ease interpretation exon 1 variant alleles (B,C & D) are often referred to collectively as the 'O' allele and heterozygotes for variant

alleles (A/O) are often grouped with homozygotes or compound heterozygotes (ie. two different variant alleles) (O/O). The INNO-LiPA assay provides a full haplotype combination encompassing the exon 1 alleles, A,B,C and D and the three promoter polymorphisms X/Y, H/L and P/Q for more detailed characterisation (Nuytinck and Shapiro, 2004).

4.2 Results

4.2.1 Heteroduplexing

Initial experiments revealed problems with the existing heterpduplexing methodology which hindered interpretation of the results. A number of experiments were undertaken to optimise the technique prior to patient sample analyses. Genomic DNA from laboratory staff volunteers was used for these studies. The quality and amount of Universal Heteroduplex Generator (UHG) required was optimised as variability in heteroduplex clarity with different UHGs (1 & 2) was observed as illustrated in Figure 4.1. Degradation of UHG was eliminated and the optimal concentration required established. Variation in band pattern (i.e. additional non-specific bands) was observed with different UHG preparations and this effect was also concentration dependant. Optimum band resolution was achieved using 20pg UHG/PCR reaction (Figure 4.2, lanes 1a, 2a and 3a).

MBL Genotyping was undertaken using the optimum concentration of UHG 1 (20pg/reaction). Examples of representative heteroduplexing are shown in Figure 4.3 and 4.4.

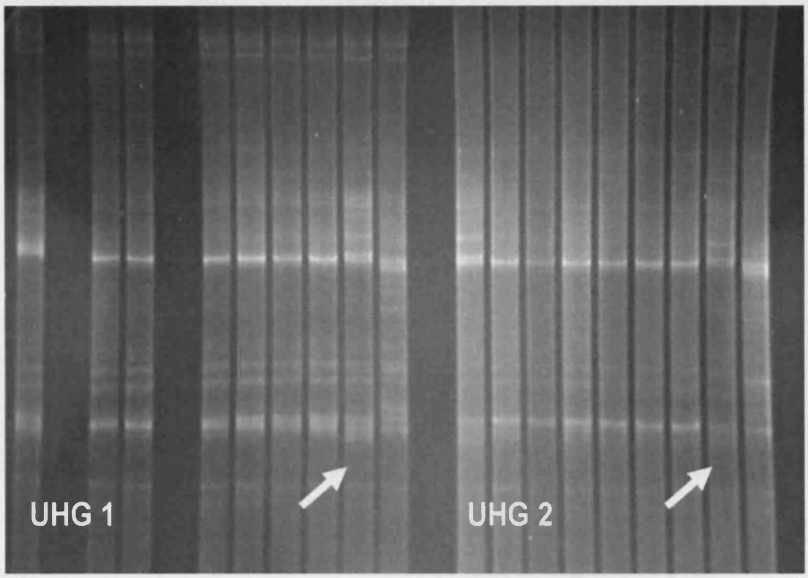


Figure 4.1 Exon 1 heteroduplex band patterns using different UHG preparations (1&2) on 9 DNA samples.
Arrows highlight an example of the variation in band pattern seen with the same DNA sample combined with different UHG preparations. Multiple non-specific bands prevent accurate interpretation of the genotype.

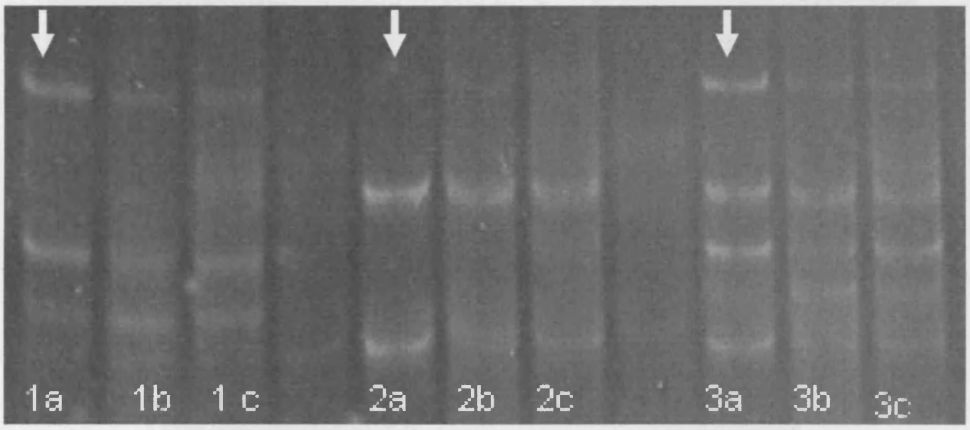


Figure 4.2 Promoter samples heteroduplexed with different UHG preparations of varying concentration, a) UHG 1- 20pg/reaction (optimal concentration highlighted with arrows) b) UHG 1- 200pg/reaction c) UHG 2- 100 pg/reaction

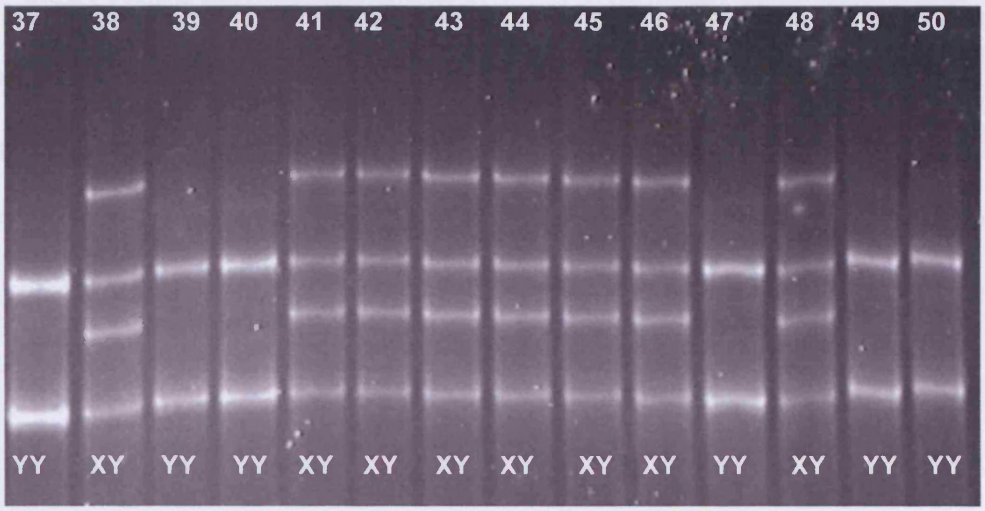
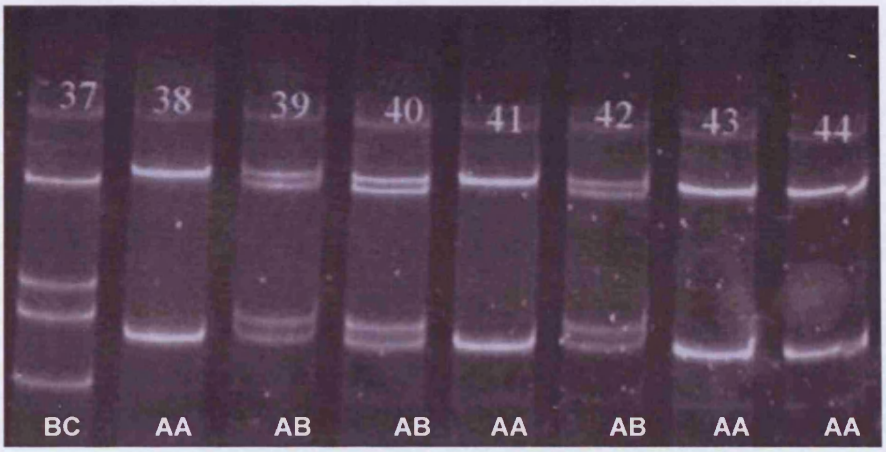


Figure 4.3 XY promoter polymorphism heteroduplexing results for patients 37-50. Specific genotype results are detailed with corresponding band patterns.



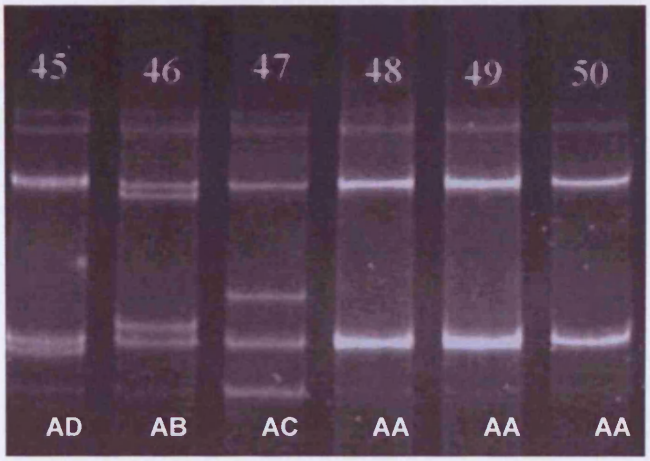


Figure 4.4 Exon 1 polymorphism heteroduplexing results for patients 37-50. Specific genotype results are detailed with corresponding band patterns.

4.2.2 INNO-LiPA *MBL2* assay

All patient samples were amplified at the first attempt but two samples failed to adequately hybridize generating an uninterpretable result on the nitrocellulose strip. Increasing DNA concentration in the initial PCR reaction did not improve the results and at present the reason for this failure is unknown. The results for samples 37-50 are illustrated in Figure 4.5 (heteroduplexing shown previously, Figures 4.3 and 4.4).

Figure 4.5 INNO-LiPA strip probe patterns and corresponding haplotype combination for patients 37-50

4.2.3 Comparison of Genotyping methods

Both methods revealed the same genotype result for each sample analysed making the new INNO-LiPA method as reliable as the heteroduplexing method. It should be noted that heteroduplexing of the H/L and P/Q promoter polymorphisms was not performed and therefore only data obtained for the exon1 and XY polymorphisms can be compared. This is the first time the INNO-LiPA method has been validated against heteroduplexing. Table 4.2 details a comparison of the two methods highlighting the differences in timing and cost.

	Heteroduplexing	**INNO-LiPA**
Polymorphism analysis	Exon 1 XY promoter	Exon 1 XY, HL, PQ promoters
Procedure	4 PCRs	1 multiplex PCR
Duration	2h Heteroduplex formation 5-15h Gel analysis	3h Amplification step
Results	283/283	281/283
Cost/sample	< £5	~ £30

Table 4.2 Comparison of heteroduplexing and INNO-LiPA genotyping methods

4.2.4 *MBL2* gene and haplotype frequencies

MBL2 genotyping data was available on 283 children with cancer recruited in the London POCs between April 2004 and March 2005.

4.2.4.1 *MBL2* exon1 allele and genotype frequencies

Exon 1 allele frequencies in the study population were as follows; wild-type (A) 0.76, codon 54 (B) 0.11, codon 52 (D) 0.08 and codon 57 (C) 0.04. Figure 4.6 illustrates the exon 1 allele frequencies for the total group and specific ethnic groups within the study population (data for the mixed race group and "other" group is not shown).

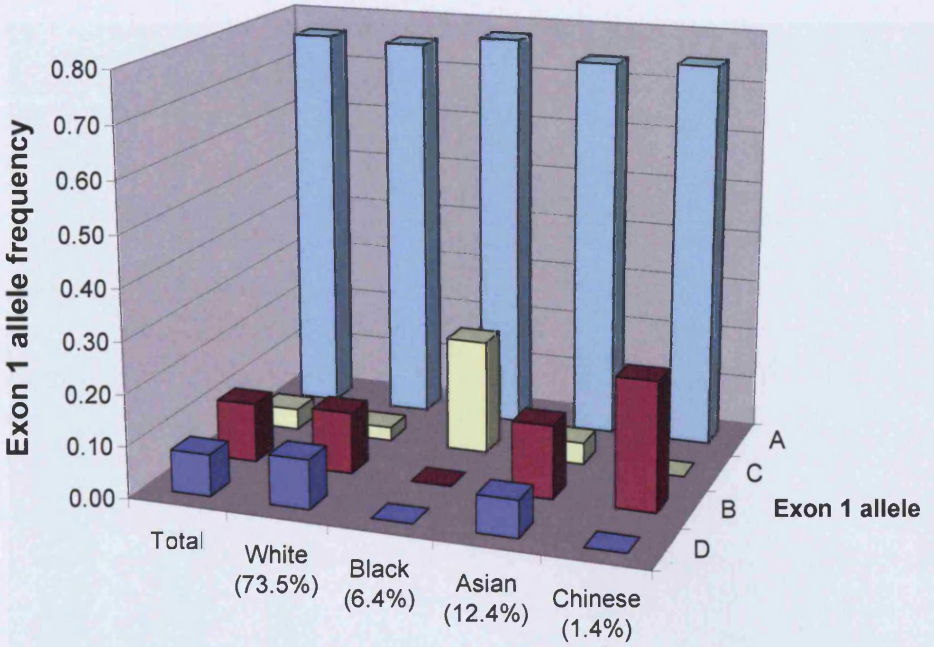


Figure 4.6 Exon 1 allele frequencies for the total group and specific ethnic groups.

The A or WT allele was predominant and present in similar frequencies throughout the different ethnic groups. The D allele was observed in white, asian and mixed race patients. The C allele was the only variant allele observed in black patients and the B allele was the only variant allele observed in chinese patients. The patient cohort was ethnically diverse (26.5% non-white) and representative of South East England.

Exon 1 genotype frequencies were; AA, 0.576; AB, 0.184; AD, 0.12; AC, 0.071; DB, 0.025; BC, 0.007; CD, 0.004; BB, 0.007; DD, 0.007, (Figure 4.7). The exon1 genotype frequencies obeyed the Hardy-Weinberg expectations i.e. there was no significant difference between observed and expected populations, chi square= 1.679 (non significant) (Table 4.3).

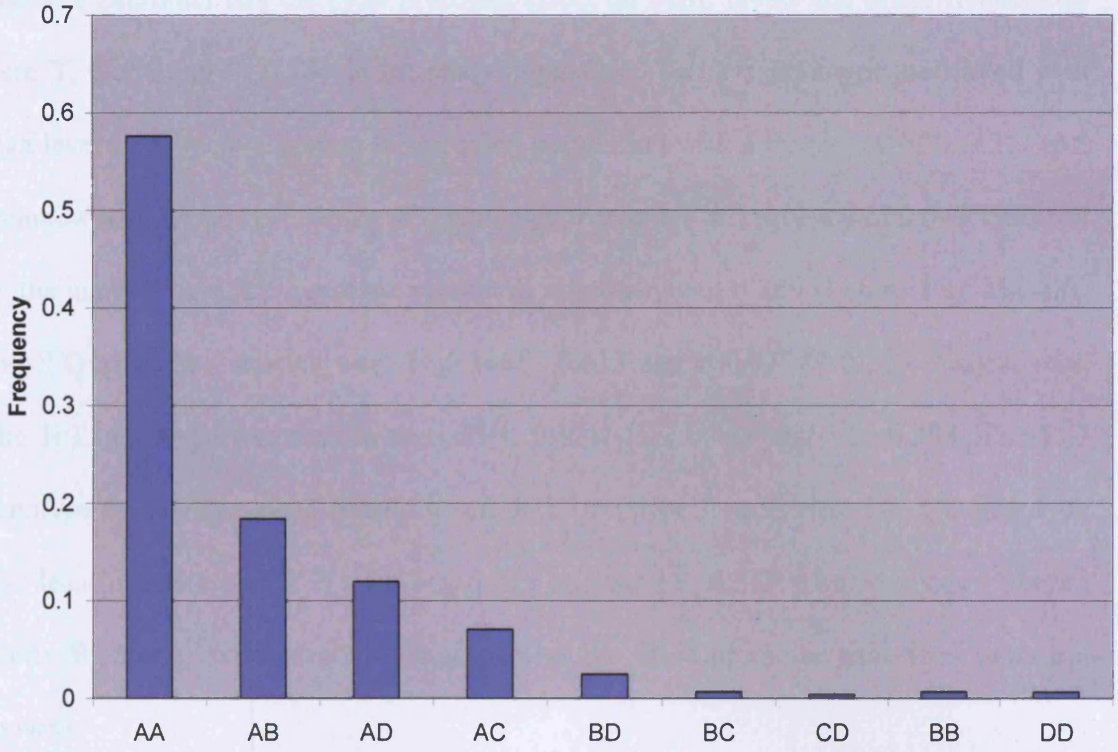


Figure 4.7 Exon 1 genotype frequencies

Genotype	Observed		Expected	
	Number	Frequency	Number	Frequency
AA	163	0.576	165	0.582
AB	52	0.184	50	0.175
AD	34	0.120	35	0.124
AC	20	0.071	18	0.063
BD	7	0.025	6	0.020
BC	2	0.007	3	0.009
BB	2	0.007	3	0.012
CD	1	0.004	2	0.006
CC	0	0.000	0	0.001
DD	2	0.007	2	0.007
Total	283		283	

Table 4.3 Observed and expected MBL exon1 genotypes in the study population

4.2.4.2 MBL2 promoter allele and genotype frequencies.

The X/Y promoter has the most profound effect on MBL levels and allele frequencies were Y, 0.756 and X, 0.244 in the study population. The YY genotype associated with high levels was most common in our study population with a frequency of 0.562. Least common was the low producing XX genotype, present at a frequency of 0.049 followed by the intermediate XY genotype present at a frequency of 0.389 (Figure 4.8). The H/L and P/Q allele frequencies were H 0.366/L 0.633 and P 0.777/Q 0.223, respectively. The H/L genotype frequencies were HH, 0.121; HL, 0.491 and LL, 0.388. The P/Q genotype frequencies were similar to the X/Y genotype frequencies. The QQ genotype was least frequent at 0.057, with PQ, 0.331 and the PP, 0.612. The population obeyed Hardy-Weinberg expectations with respect to the three promoter genotypes (data not shown).

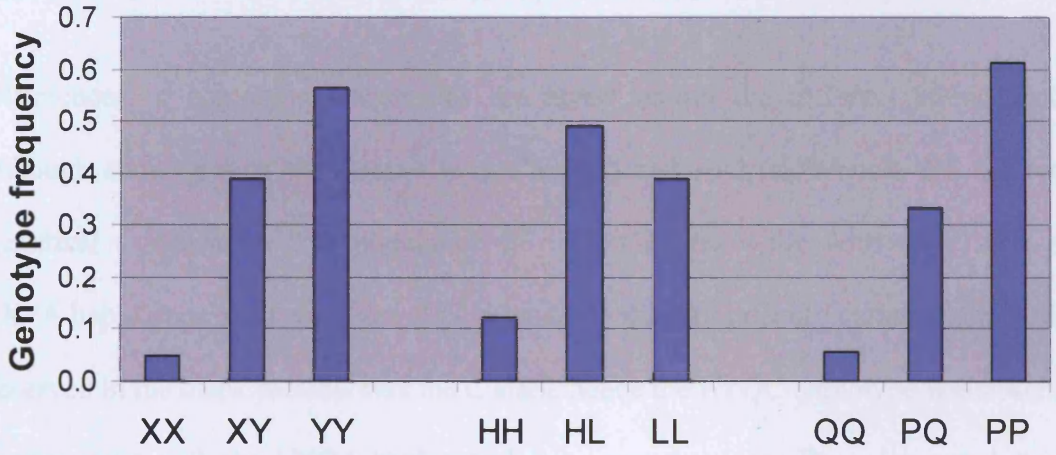


Figure 4.8 Promoter genotype frequencies

4.2.4.3 *MBL2* haplotype frequencies

Due to linkage disequilibrium between the polymorphisms in the promoter and exon 1 regions, only seven common haplotypes are described leading to 28 possible haplotype

combinations. Using the INNO-LiPA method we were able investigate the haplotype frequencies in the population (table 4.4).

	HYPA	LYPA	LYQA	LXPA	HYPD	LYPB	LYQC	Frequency of homozygous-variant alleles
Total population	**0.28**	0.06	0.18	**0.24**	0.08	0.11	0.04	0.05
White (73.5%)	**0.29**	0.05	0.17	**0.25**	0.10	0.12	0.03	0.06
Black (6.4%)	0.17	0.17	**0.31**	0.14	0.00	0.00	**0.22**	0.00
Asian (12.4%)	**0.30**	0.04	0.19	**0.21**	0.07	0.14	0.04	0.03
Chinese (1.4%)	**0.38**	**0.25**	0.13	0.00	0.00	**0.25**	0.00	0.20
Mixed (4.6%)	0.31	0.08	0.15	0.35	0.04	0.04	0.04	0.00
Other (4.6%)	0.30	0.00	0.20	0.30	0.00	0.20	0.00	0.00

Table 4.4 MBL haplotype and homozygote frequencies for the total population and stratified by ethnicity. Dominating haplotypes are indicated in bold.

Differences in haplotype frequencies are noted within the different ethnic groups although some groups were small (e.g. Chinese) and such differences did not reach statistical significance. The population is predominantly white with the HYPA and LXPA haplotypes most common. As discussed previously the only variant exon 1 allele observed in the black patients was the C allele hence the LYQC haplotype was observed in this group with the LYQA haplotype being most common. The asian population is predominantly Indian or Bangladeshi and bears similarities to the white population in terms of haplotype frequencies. The Chinese population is very small but of note the only variant haplotype present is LYPB. 26 of the possible haplotype combinations were present in the study population and the relative frequencies are shown in Figure 4.9. Of the exon 1 WT genotypes the LXPA/HYPA haplotype combination was most frequently observed in the cohort. The most common exon1 heterozygote combination

was the LYPB/HYPA and the most common homozygote or compound heterozygote combination was LYPB/HYPD.

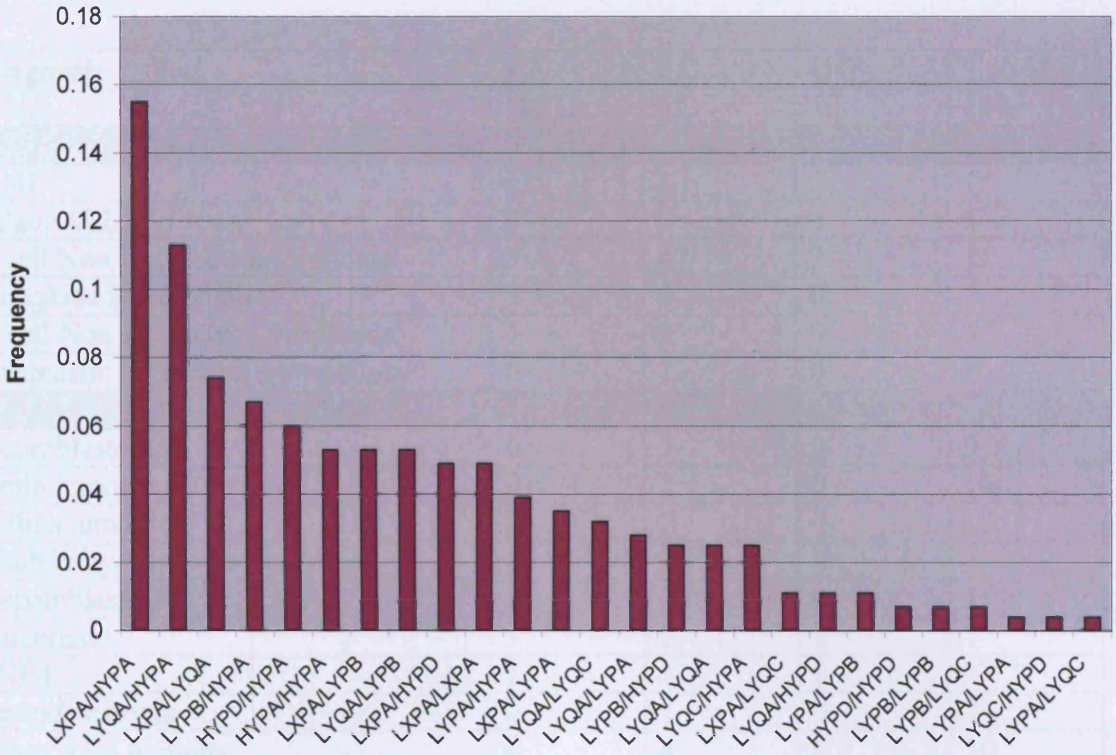


Figure 4.9 Haplotype combination frequencies.

4.2.5 Genotype and Disease

To explore any potential association between MBL genotype and oncological diagnosis, genotype frequencies within the different diagnostic groups were analysed (Table 4.5). Due to small numbers within some diagnostic categories, haematological diagnoses other than ALL were combined as were all solid tumour patients. Exon 1 variant alleles collectively are represented by the O allele (Figure 4.10). ALL patients and the 'other haematological' diagnoses group did not differ significantly from the total patient population. Of note within the AML group there was a higher frequency of wildtype individuals (11/15, 0.73) compared to other groups. Marked variation within the solid tumour patients was observed and overall there was a higher frequency of heterozygotes

compared to the other groups. There was no evidence of a higher proportion of homozygotes in any of the diagnostic groups.

Diagnosis	AA	AO	OO	Total
ALL	105	65	10	180
AML	11	4	0	15
Infant ALL	4	2	0	6
T cell Non Hodgkins Lymphoma	5	4	2	11
Hodgkins Lymphoma	2	0	0	2
B cell Non Hodgkins Lymphoma	1	3	0	4
Anaplastic Large Cell Lymphoma	0	1	0	1
Neuroblastoma	6	3	0	9
Brain tumours	9	7	0	16
Wilms tumour	6	7	0	13
Rhabdomyosarcoma/Rhabdoid	3	2	0	5
Hepatoblastoma	1	0	0	1
Sarcomas	3	2	2	7
PNET	1	1	0	2
Retinoblastoma	0	1	0	1
Other solid tumours	6	4	0	10
				283

Table 4.5 Exon 1 genotype frequencies and diagnosis

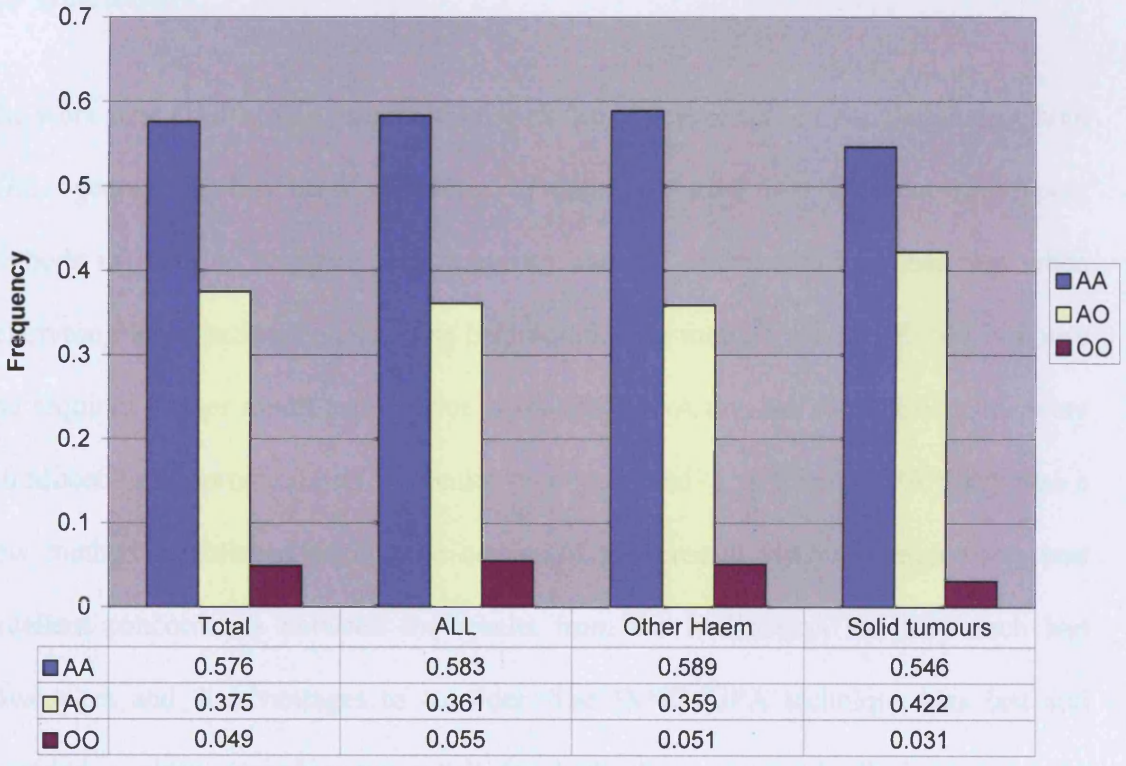


Figure 4.10 Exon 1 genotype frequencies in different diagnostic groups

More detailed investigation of the specific haplotype frequencies revealed differences between the different diagnostic groups but these did not reach significance (Table 4.6). The solid tumour group had the highest frequency of LYPB and LYQC haplotypes and the lowest frequency of homozygotes but was also the most ethnically diverse with a non-white population of 34%.

	% non-white	HYPA	LYPA	LYQA	LXPA	HYPD	LYPB	LYQC	Frequency of homozygous-variant alleles
Total population	26.5	0.28	0.06	0.18	0.24	0.08	0.11	0.04	0.05
ALL	25	0.27	0.06	0.18	0.24	0.08	0.11	0.04	0.05
Other Haem	21	0.38	0.03	0.18	0.18	0.10	0.09	0.04	0.05
Solid tumours	34	0.25	0.06	0.16	0.28	0.06	0.13	0.05	0.03

Table 4.6 MBL haplotype and homozygote frequencies for the total population and stratified by diagnostic group.

4.3 Discussion

The work described in this chapter represents the largest oncology population on whom *MBL2* genotyping has been performed to date. We used two different genotyping methods in order to compare their accuracy and the practicalities of their use when genotyping large patient cohorts. The heteroduplexing method was developed in-house and required further modification prior to routine use. A number of modifications were introduced and reproducibility of results was optimized. The INNO-LiPA assay was a new method established during the course of the present study. Overall there was excellent concordance between the results from the two assays but they each had advantages and disadvantages to consider. The INNO-LiPA technique was fast and provided a clear, unambiguous result for both the exon1 and all three promoter

polymorphisms. Of the 283 samples genotyped, only two samples failed to produce an interpretable pattern, the reasons for which remain unclear. The heteroduplexing method gave less information and required more bench time. The interpretation of results was potentially more prone to error due to the close proximity of the band patterns but the clear advantage of the method was the lower reagent cost which may therefore make it a more suitable technique for use in resource poor settings. It should be noted that the cost of personnel may also dictate preferred method of choice.

The 283 paediatric cancer patients represent an ethnically diverse population from the South East of England undergoing treatment for a wide range of diseases. In order to answer questions as to whether *MBL2* variant alleles are over or under represented in our study population we would have needed to recruit a matched control group. Defining an appropriate control group is itself a difficult task and then matching them for both disease and ethnicity is even harder. This is an ongoing issue in paediatric studies and particularly when studying relatively uncommon diseases. Many studies utilize adult blood donor populations which are not an equivalent population. Due to the lack of detailed genotyping information in previously published studies, comparisons between similar patient populations have not been made.

The best control group available to us for comparison is from the Avon Longitudinal Study of Pregnancy and Childhood (ALSPAC). This cohort is part of a European-wide World Health Organisation prospective study designed to determine factors influencing health and development of children in Europe. Approximately 14,000 expectant British mothers and their subsequent children were recruited. *MBL2* exon 1 genotype frequencies have been published on a subgroup of these patients (n=302) (Mead et al., 1997). Differences observed between our population and this 'healthy UK paediatric population' are predominantly explained by the ethnic diversity within the two samples.

The proportion of non-white subjects was 26.5% in our study group representative of populations in London and the South East of England compared to < 5% in ALSPAC where patients were recruited from Bristol and the South West of England. The most marked difference is seen in the C allele frequency commonly associated with black Africans which is 0.008 in ALSPAC and 0.04 in the study group. Overall the AA, AO and OO frequencies from Mead's data were 0.596, 0.357 and 0.046 respectively which are very similar to our study group, 0.576, 0.375 and 0.049.

The full haplotype frequencies we observed were in keeping with a predominantly Caucasian population (Crosdale et al., 2000; Dahl et al., 2004) with high frequencies of both HYPA and LXPA haplotypes. Despite studying small groups clear differences were seen in variant allele distribution between specific populations. In our black patients the C allele (LYQC haplotype) was dominant and in our Chinese population the B allele was the only variant allele observed. These findings reflect similar homogenous ethnic groups e.g. African and Korean populations where the LYQC and LYPB haplotypes have been reported to be disproportionately high, respectively (Madsen et al., 1998, Lee et al., 2005a). Our asian population was predominantly Indian or Bangladeshi and gene frequencies were similar to those observed in white patients. To date there is very little data available from Indian populations with whom we can make comparison. The INNO-LiPA assay enabled us to generate a full haplotype combination and 26 of the possible 28 combinations were present in our patients albeit some in small numbers. The use of such detailed genetic information in disease association studies is limited by patient numbers. The majority of studies use exon 1 genotype with or without the X/Y promoter to enable meaningful statistical analysis.

The potential influence of MBL genotype on disease predisposition was addressed using this data set (Tables 4.5 and 4.6, Figures 4.10). In the patient cohort presented the

frequency of MBL homozygosity in the ALL patients was 5.5% compared to 4.9% in the total patient cohort and 4.6% in the ALSPAC cohort. The genotype frequencies observed in the other diagnostic groups must be interpreted with caution due to the small sample sizes. The solid tumour patients have been combined but this means that they form a group of very diverse diseases which cannot be interpreted as a homogenous group. In 2002, Schmiegelow *et al* reported an increased frequency of MBL deficiency among children with ALL (Schmiegelow et al., 2002). The frequency of MBL variant allele homozygosity was 8.8% in the patients (n=137) compared to 2.2% in an adult blood donor population (n=250). Their explanation for this finding was based on the 'Two hit' model, where individuals have an underlying genetic mutation (present *in utero*) which when combined with a second hit, which may be an infection, they develop childhood leukaemia (Greaves, 1999). Schmiegelow proposed that children with MBL mutations suffering from more frequent and/or severe infections experienced more proliferative stress on their developing immune system, potentially leading to critical leukaemogenic DNA damage. This is in contrast to the original hypothesis by Greaves that proposed that exposure to common infections in early childhood may protect against ALL by promoting normal maturation of the immune system. Lack of early exposure to infection rendered the immune system unmodulated. Subsequent infection with common microbes that occurs in a biologically abnormal timeframe for which the immune system is inappropriately programmed, leaves such children at risk of leukaemogenesis (Greaves, 1997). To support this a large UK case-control study looking at day care and social activity during the first year of life as a measure of potential exposure to infection supports the hypothesis that early exposure to infection reduces the risk of subsequent ALL (Gilham et al., 2005). Our data is not in agreement with Schmieglow's findings. The frequency of exon 1 variant allele

homozygosity in all the diagnostic groups; ALL, other haematological malignancies and solid tumours, is similar or lower compared to the ALSPAC cohort.

With regard to solid tumours, a study of Polish adults has reported that the HYD haplotype is specifically associated with gastric cancer risk and the authors suggest that this may be related to the influence of MBL on *Helicobacter pylori* infection (Baccarelli et al., 2006). There has also been data published which suggests that MBL may influence breast cancer risk (Bernig et al., 2007). We did observe an overrepresentation of MBL variant alleles in the solid tumour group but this was small, the group was heterogenous and we would be wary to draw any firm conclusions from uncontrolled data. Larger homogenous groups are required to investigate the role of specific variant haplotypes in predisposition to paediatric solid tumours.

In summary we have performed detailed genotyping of the *MBL2* gene on 283 paediatric oncology patients utilising two techniques giving concordant results. Differences in genotype frequencies were observed between the patients and a healthy paediatric cohort which reflect the ethnic variation within the two groups. The distribution of MBL genotypes in ALL patients was in keeping with the general population but assessment of other diagnostic groups was limited by small patient numbers. This study group is investigated in more detail in following chapters.

Chapter 5

Influence of MBL on infectious complications in children with cancer

5.0 Introduction

The role of MBL in defence against infection following chemotherapy remains unclear (Klein and Kilpatrick, 2004). Studies to date have reported conflicting findings but their differing designs and size have made comparisons difficult, fuelling the ongoing debate. Oncology patient populations are heterogenous. Their underlying disease and subsequent treatment renders them immunosuppressd but to differing degrees (Graubner et al., 2007). Such differences are compounded further by variability in treatment intensity and supportive care measures employed such as use of colony stimulating factors and prophylactic antibiotics (predominantly used in adult practice).

It remains important to establish whether MBL has a role in some or all of these complex patients. Such information may be particularly useful in FN risk assessment and for the selection of patients who may benefit from MBL replacement therapy in the future.

A drawback of studies investigating the role of MBL in oncology patients to date has included their lack of applicability because the majority have been conducted in single, specialist oncology centres. Our study was multicentred and designed to capture all FN episodes in both POCs and POSCUs, hoping to provide a true reflection of the burden of infection in these patients. The study was run in parallel with the PINE project described in Chapter 3 which provided the clinical data used in the analysis. The aims of this study were to explore the influence of MBL genotype on FN frequency and severity in a large paediatric cohort by recruiting all children on active treatment for cancer in the South East of England over a one year period.

5.1 Methods

5.1.1 Patient population

The patients recruited into this study are described in Section 4.1.1. Clinical information (age, sex, diagnosis and ethnic status) was obtained on all patients and in addition to this, information on RS criteria (Figure 3.1) and outcome measures (Figures 3.5 and 3.6) was collected on the subset of patients admitted with FN during the study period through the PINE project (Chapter 3).

The MBL and Lectin pathway studies were approved by the Research Ethics committees of the 4 POCs, section 2.1. Permission was sought from the Caldicott Guardians at each Paediatric Oncology Shared Care Unit (POSCU) in order to access data collected for audit purposes for use in research.

5.1.2 Definitions

FN was defined as neutrophils <1.0 x 10^9/L **and** fever >38°C for more than 4h or on 2 occasions at least 4h apart **or** fever >38.5°C on one occasion **or** clinical suspicion of sepsis in the absence of fever e.g. unexplained abdominal pain or generally unwell. Episodes where there was clinical evidence of infection (including radiological evidence) as assessed by the attending clinician, and/or microbiological evidence of infection are termed clinical/microbiological infections. Episodes where there was no clinical or microbiological evidence of infection are termed fever of unknown origin (FUO). Episode duration refers to the number of inpatient nights.

5.1.3 Outcome measures

The primary endpoint was frequency of documented clinical/microbiological infections in relation to MBL genotype. The secondary endpoints were; a) duration of FN episode, b) Paediatric Intensive Care Unit (PICU) admission and c) death, in association with MBL genotype.

5.1.4 Data collection and analysis

Data was collected on FN episodes as part of the PINE project. In addition, a review of patient records was conducted in order to assess the accuracy of the PINE data and also collect information on any FN episodes not captured through the PINE project. Information collected on these additional episodes of FN was not always complete and did not include details about risk stratified management. Data were analysed on the number of episodes for which specific information was available and therefore this number varied for different parameters.

5.1.4.1 MBL genotype

All analyses have compared the influence of MBL genotype on different outcome variables. Data have been analysed using exon 1 genotype alone and heterozygotes and homozygotes have been grouped together owing to the small number of homozygotes, giving an abbreviated genotype, AO/OO.

Patients and episodes have also been grouped in different analyses as follows:

5.1.4.2 By Episode- Subjects experienced different numbers of FN episodes during the study period which may not have all been independent of each other. In order to examine the data on a per patient basis both the first and longest FN episodes were analysed. The same episode was included in each analysis (first and longest) for those patients who experienced only one episode of FN.

5.1.4.3 By Diagnosis- In order to explore the effect of MBL on FN in different diagnostic groups, patients were separated into the ALL group and Non ALL group. The ALL group includes patients with ALL and T-NHL because of similarities in their treatment protocols. The Non-ALL group includes patients with other haematological malignancies and solid tumours (Table 4.5).

5.1.4.4 By Risk- Clinicians risk stratified patients according to clinical criteria (Figure 3.1), both on admission and at 48h which dictated their subsequent management as

either low or standard risk as described in Chapter 3. The 'Exclusions' group refers to patient episodes automatically excluded from low risk management by virtue of an underlying diagnosis/treatment regime or patient age <1yr, that rendered the patient at higher risk of adverse outcome. In the analysis of the influence of MBL genotype on episode duration the 'Exclusions' episodes are compared to all other episodes termed 'No exclusions'.

5.1.5 Statistical analysis

In order to detect a 20% difference in infection rate between patients with MBL WT (AA), and those with one or more variant alleles (AO and OO) with 90% power and 5% significance we required a sample size of 300. This was based on previous research, and assumes a prevalence of 58% of patients with an AA genotype, and an estimated sepsis rate of approximately 40% (Neth et al, 2001).

Frequencies between groups were compared by the Mann Whitney U and Chi square tests, where appropriate. Linear by linear chi square tests were used to test for a trend in frequency of outcome across ordered exposure variables. If the number was smaller than five in any cell a Fisher's exact test was performed.

5.2 Results

5.2.1 Patient characteristics

Of the 283 patients recruited into the study, 269 were eligible for analysis. Fourteen patients were excluded from the analysis because clinical data were not available. A total of 513 FN episodes were analysed from 211 subjects (zero episodes were identified in 58 subjects), of which 421 (72%) were PINE episodes and 92 (28%) were traced from clinical notes. Table 5.1 shows details of the patient cohort. 57% (153) of subjects were wildtype (AA), 39% (104) were heterozygous for variant alleles (AO) and 4% (12) were homozygous for variant alleles. The hetero and homozygotes were

analysed together. The cohort consisted of 148 boys and 121 girls whose median age at recruitment was 5.34 years. Both age and sex distributions were independent of genotype as shown in Table 5.1.

	Total group n=269	**AA n=153 (57%)**	**AO/OO n= 116 (43%)**	**p value between AA & AO/OO**
Age				
Median (years)	5.3	5.2	5.7	0.254
Range	0.6-16.6	0.6-16.6	1-16.6	
Sex				
Male	148 (55%)	84 (54.9 %)	64 (55.2%)	0.965
Female	121 (45%	69 (45.1 %)	52 (44.8%)	
Diagnosis				
ALL (+ T NHL)	178 (66%)	101 (66%)	77 (66%)	
Other Haem	24 (9%)	17 (11%)	7 (6%)	0.552
Solid tumours	67 (25%)	35 (23%)	32 (28%)	
Months on treatment (median)	8	8.2	8.3	0.919
Ethnicity				
White	194 (72.1%)	109 (71.2%)	85 (73.3%)	
Black	18 (6.7%)	10 (6.5%)	8 (6.9%)	
Asian	35 (13%)	18 (11.8%)	17 (14.7%)	0.570
Chinese	5 (1.9%)	3 (2%)	2 (1.7%)	
Mixed	12 (4.5%)	10 (6.5%)	2 (1.7%)	
Other	5 (1.9%)	3 (2%)	2 (1.7%)	

Table 5.1 Patient characteristics of the total group and according to MBL genotype

170 (63%) subjects had ALL and have been grouped together with 8 T cell-NHL patients for the purposes of the analysis. This 'ALL' group accounted for 66% of patients. The remaining patients formed the 'Non ALL' group; 22 (9%) had other haematological malignancies and 69 (25%) had solid tumours. Median time on treatment at recruitment into the study was 8 months (IQR 15 months). No association between diagnosis or ethnicity and abbreviated MBL genotype was evident (Table 5.1).

5.2.2 MBL genotype and frequency of FN

In order to investigate the effect of MBL genotype on susceptibility to infection, the number of episodes per patient over the study period was analysed. FN signifies infection until proven otherwise and approximately 50% of episodes would be expected to have no evidence of either clinical or microbiological infection, i.e. fever of unknown origin (FUO). For this reason the number of FN episodes has been analysed as a total (Table 5.2) and also separated by type i.e. clinical/microbiological infection or FUO (Table 5.3 and 5.4) respectively. Of the 269 subjects, 58 (21.6%) had no FN episodes during the study period. Of these 37 (24.2%) were AA and 21 (18.1%) AO/OO.

	Total group n (%)	**AA n (%)**	**AO/OO n (%)**	**p value between AA & AO/OO**
Number of FN episodes (n=269)				
0-1	131 (48.7)	82 (53.6)	49 (42.2)	0.048
2-4	126 (46.8)	66 (43.1)	60 (51.7)	
≥ 5	12 (4.5)	5 (3.3)	7 (6.1)	
Median (range)	2 (0-8)	1 (0-6)	2 (0-8)	0.074

Table 5.2 Total FN episode frequency stratified by MBL genotype

Overall, patients in possession of MBL variant alleles were found to experience more FN episodes than wildtypes, with a median number of 2 compared to 1 (p=0.074). As the number of FN episodes per patient increased the distribution of MBL genotypes shifted. An increased proportion of wildtype individuals had zero or one episode compared to individuals in possession of variant alleles who appeared more likely to experience multiple episodes, p=0.048, shown graphically in Figure 5.1.

Analysis of the number of episodes with documented clinical and/or microbiological evidence of infection, a subset of the total FN episodes, revealed a similar trend. A higher proportion of MBL hetero- and homozygotes experienced multiple infections,

e.g. 14.6% (AO/OO) compared to 8% (AA) with three or more infections. This trend is illustrated in Figure 5.2.

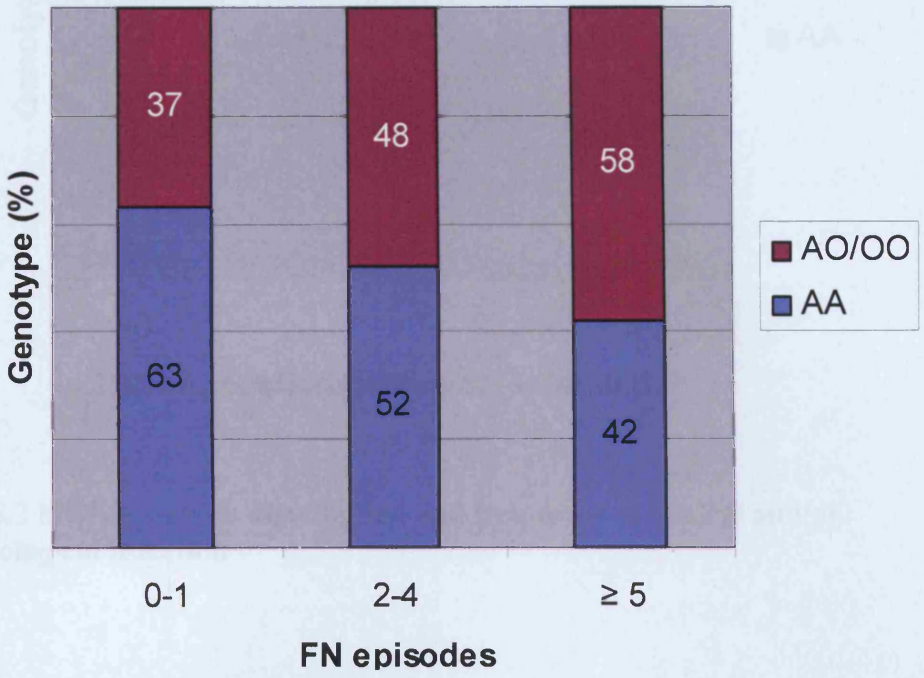


Figure 5.1 MBL genotype distribution and frequency of FN

Number of Clinical / Microbiological infections (n=240)	**Total group n (%)**	**AA n (%)**	**AO/OO n (%)**	**p value** between AA & AO/OO
0	103 (42.9)	64 (46.7)	39 (37.9)	0.045
1	75 (31.3)	44 (32.1)	31 (30.1)	
2	36 (15)	18 (13.1)	18 (17.5)	
≥ 3	26 (10.8)	11 (8)	15 (14.6)	
Median (range)	1 (0-5)	1 (0-4)	1 (0-5)	0.064

Table 5.3 Clinical/Microbiological infection frequency stratified by MBL genotype

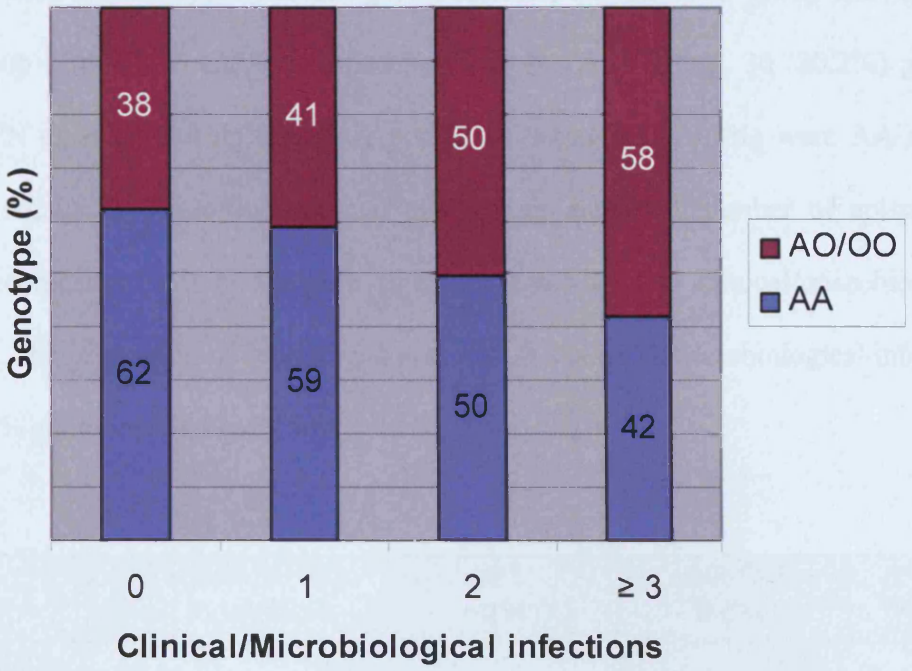


Figure 5.2 MBL genotype distribution and frequency of clinical and/or microbiological infection

In contrast, no significant relationship was found between the number of FUO episodes and MBL genotype (Table 5.4).

	Total group n (%)	AA n (%)	AO/OO n (%)	p value between AA & AO/OO
Number of FUO episodes (n=240)				
0	125 (52.1)	76 (55.5)	49 (47.6)	0.349
1	67 (27.9)	36 (26.3)	31 (30.1)	
2	33 (13.8)	16 (11.7)	17 (16.5)	
≥ 3	15 (6.3)	9 (6.6)	6 (5.8)	
Median (range)	0 (0-5)	0 (0-5)	1 (0-4)	0.262

Table 5.4 FUO episode frequency stratified by MBL genotype

Patients were also analysed according to diagnosis, i.e. the ALL group and the Non ALL group (Tables 5.5 and 5.6 respectively). In the ALL group, 36 (20.2%) patients had no FN episodes during the study period, of which 24 (23.8%) were AA and 12 (15.6%) AO/OO. There was a trend towards an increased number of episodes in AO/OO compared to AA for both total FN episodes and clinical/microbiological episodes. The proportion of AO/OO patients with ≥ 2 clinical/microbiological infections was 29.8% compared to 17.2% AA.

	Total group n (%)	**AA n (%)**	**AO/OO n (%)**	**p value between AA & AO/OO**
ALL patients				
Number of FN episodes (n=178)				
0-1	88 (49.4)	55 (54.5)	33 (42.9)	0.113
2-4	84 (47.2)	43 (42.6)	41 (53.2)	
≥ 5	6 (3.4)	3 (3)	3 (3.9)	
Number of Clinical / Microbiological infections (n=154)				
0	66 (44.2)	42 (48.3)	26 (38.8)	0.109
1	51 (33.1)	30 (34.5)	21 (31.3)	
2	22 (14.3)	9 (10.3)	13 (19.4)	
≥ 3	13 (8.4)	6 (6.9)	7 (10.4)	

Table 5.5 Episode frequency stratified by MBL genotype in the ALL group

There was also a similar weaker trend in Non ALL patients, e.g. the proportion of AO/OO patients with ≥ 2 clinical/microbiological infections was 36% compared to 28% for AA patients. These differences did not reach statistical significance for either diagnostic group. In the ALL and Non ALL groups no association was found between the number of FUO episodes and genotype (p=0.363 and p=0.520, data not shown).

	Total group n (%)	AA n (%)	AO/OO n (%)	p value between AA & AO/OO
Non ALL patients				
Number of FN episodes (n=269)				
0-1	43 (47.3)	27 (51.9)	16 (41)	0.216
2-4	42 (46.2)	23 (44.2)	19 (48.7)	
≥ 5	6 (6.6)	2 (3.8)	4 (10.3)	
Number of Clinical / Microbiological infections (n=240)				
0	35 (40.7)	22 (44)	13 (36.1)	0.287
1	24 (27.9)	14 (28)	10 (27.8)	
2	14 (16.3)	9 (18)	5 (13.8)	
≥ 3	13 (15.1)	5 (10)	8 (22.2)	

Table 5.6 Episode frequency stratified by MBL genotype in the Non-ALL group

Findings reported in the next chapter (Chapter 6) suggest that MBL from YAYD individuals exhibited functional activity which was similar to wildtype MBL. For this reason the total group were re-analysed with the YAYD individuals combined with AA individuals and compared to all other AO/OO subjects. Although similar trends were observed (Table 5.7) they did not reach significance when compared to the original analyses shown in Tables 5.2 and 5.3.

	Total group n (%)	AA + YAYD n (%)	AO/OO n (%)	p value between AA & AO/OO
Number of FN episodes (n=269)				
0-1	131 (48.7)	89 (51.4)	42 (43.8)	0.102
2-4	126 (46.8)	79 (45.7)	47 (49)	
≥ 5	12 (4.5)	5 (2.9)	7 (7.3)	
Median (range)	2 (0-8)	1 (0-6)	2 (0-8)	0.140
Number of Clinical / Microbiological infections (n=240)				
0	103 (42.9)	72 (46.5)	31 (36.5)	0.099
1	75 (31.3)	49 (31.6)	26 (30.6)	
2	36 (15)	18 (11.6)	18 (21.2)	
≥ 3	26 (10.8)	16 (10.3)	10 (11.8)	
Median (range)	1 (0-5)	1 (0-4)	1 (0-5)	0.080

Table 5.7 Episode frequency stratified by MBL genotype, AA + YAYD compared to all other heterozygotes and homozygotes, AO/OO.

5.2.3 MBL level and frequency of FN

The influence of MBL level on episode frequency was also explored in the subset of patients for whom serum samples were available. Three groups were analysed, those with levels >1000ng/ml, 500-1000ng/ml and <500ng/ml. The results were comparable to analysis by genotype group (Table 5.2, Figure 5.1), i.e. patients with lower levels of MBL (most likely to be AO/OO) were more likely to experience multiple episodes of FN during the study period, (Table 5.8 and Figure 5.3). A similar relationship was also observed for Clinical/Microbiological infections, except in the group experiencing ≥ 3 episodes, who appeared to have higher MBL levels (Table 5.8 and Figure 5.4). No relationship between the number of FUO episodes and MBL level was demonstrated (data not shown).

	Total group N (%)	**>1000ng/ml n (%)**	**500-1000 ng/ml n (%)**	**<500 ng/ml n (%)**	**p value**
Number of FN episodes (n=180)					
0-1	79 (43.4)	52 (49.5)	10 (37.0)	17 (35.4)	0.036
2-4	89 (49.4)	48 (45.7)	16 (59.3)	25 (52.1)	
≥ 5	12 (6.7)	5 (4.8)	1 (3.7)	6 (12.5)	
Clinical / Micro infections (n=162)					
0	67 (41.4)	48 (50.0)	7 (31.8)	12 (27.3)	0.045
1	49 (30.2)	26 (27.1)	7 (31.8)	16 (36.4)	
2	23 (14.2)	9 (9.4)	4 (18.2)	10 (22.7)	
≥ 3	23 (14.2)	13 (13.5)	4 (18.2)	6 (13.6)	

Table 5.8 Episode frequency stratified by MBL level

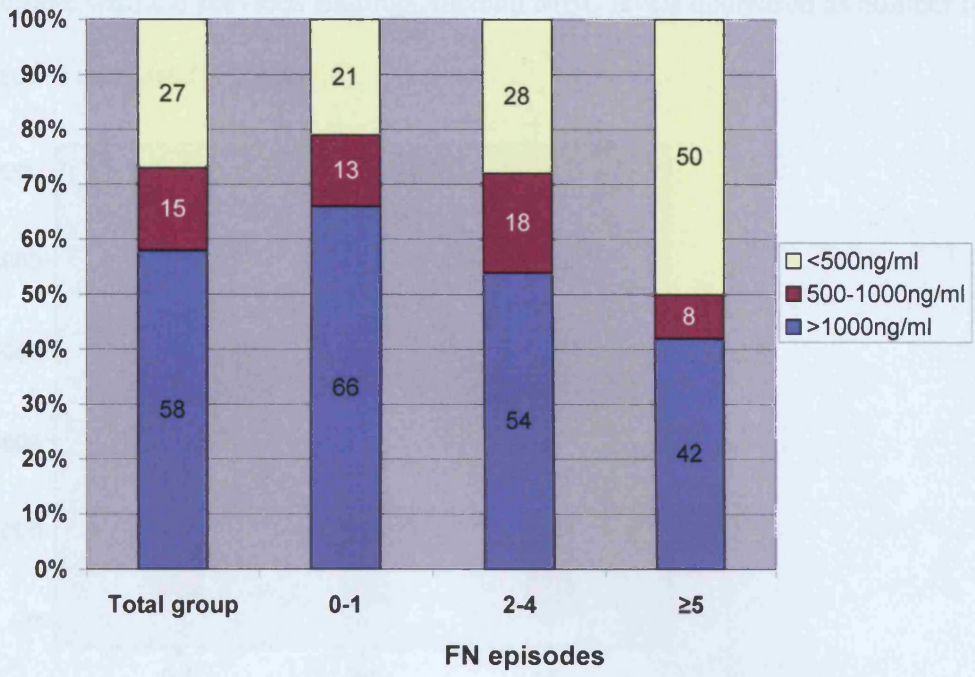


Figure 5.3 MBL level distribution and frequency of FN episodes

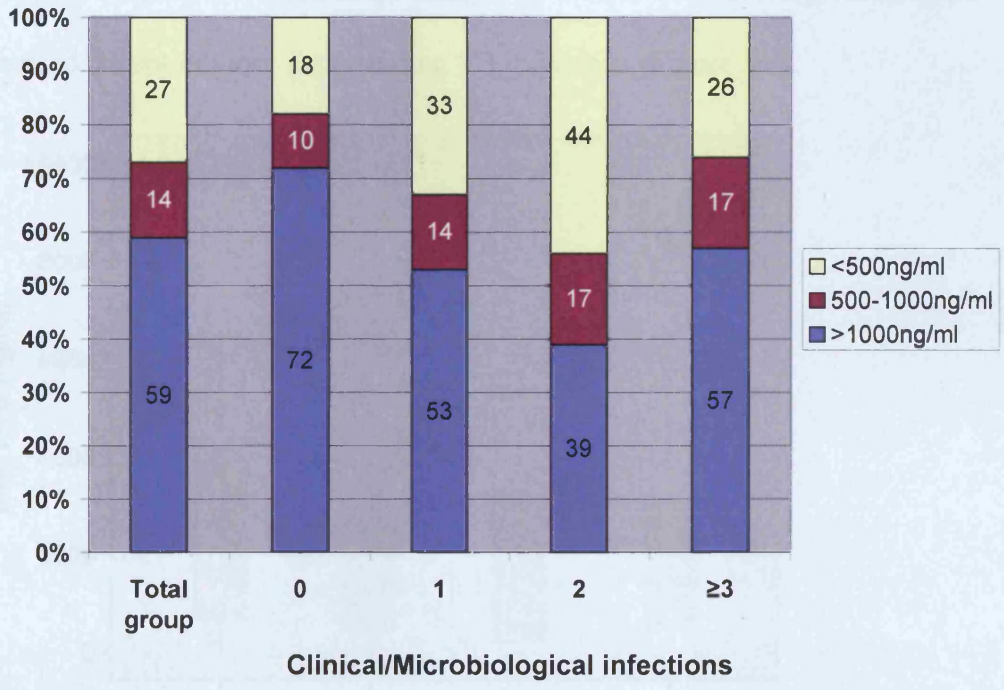


Figure 5.4 MBL level distribution and frequency of clinical/microbiological infections

In agreement with the previous findings, median MBL levels decreased as number of FN episodes increased (Figure 5.5).

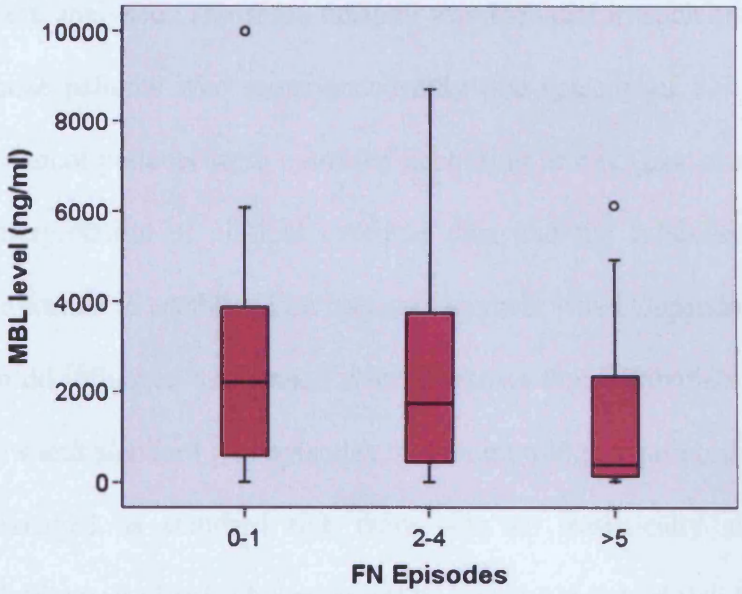


Figure 5.5 MBL levels stratified by FN episodes group (median indicated by a line, boxes represent interquartile range and bars represent outliers)

Median MBL also decreased as the number of Clinical/Microbiological episodes increased except in those experiencing ≥ 3 infections (Figure 5.6).

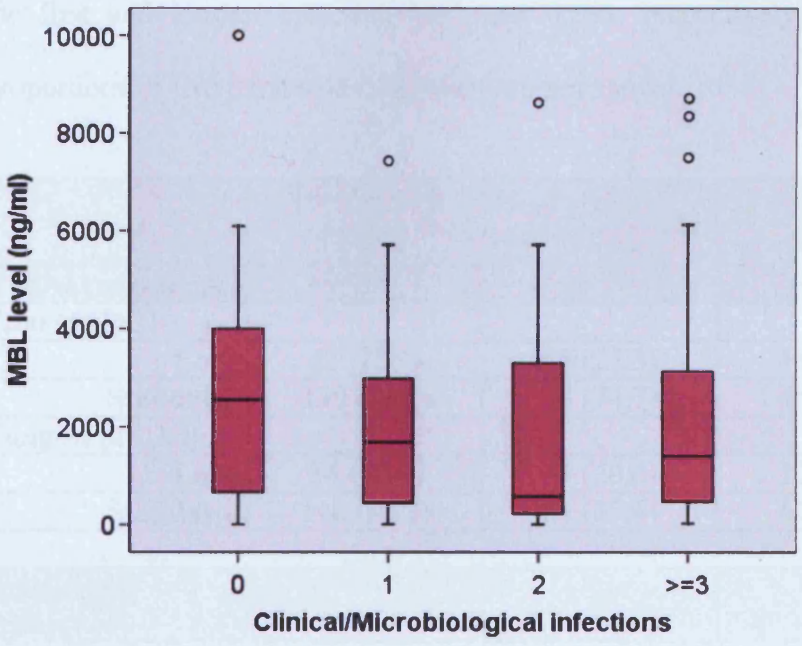


Figure 5.6 MBL levels stratified by Clinical/Microbiological infections group (median indicated by a line, boxes represent interquartile range and bars represent outliers)

5.2.4 MBL genotype and FN management

In order to examine the data on a per patient basis both the first and longest FN episodes were analysed. The same episode was included in each analysis (first and longest) for those patients who experienced only one episode of FN (n=73). Using the new FN protocol patients were managed according to risk (low or standard). In order to enable interpretation of clinical outcome data and the influence of MBL genotype it was important to establish that risk management was independent of genotype, as this alone could influence outcome. Table 5.9 shows the distribution of MBL genotypes between low and standard risk episodes. Although a higher proportion of AO/OO episodes were managed as standard risk there was no statistically significant difference in the distribution of episodes managed as low and standard risk between the AA and AO/OO subjects in both the first and longest episodes. This was also the case for the number of 'Exclusions' episodes i.e. those excluded from low risk management by virtue of their age (under 1yr) or diagnosis/treatment regimen (e.g. AML, B-NHL) (Table 5.9). The proportion of patient episodes excluded was very similar in the genotype groups in both the first and longest episodes (p=1 and 0.965, respectively). Actual numbers and proportions of 'No exclusions' episodes are not shown.

	Total group n (%)	**AA n (%)**	**AO/OO n (%)**	**p value between AA & AO/OO**
Risk management				
First (n=182)				0.697
Low	43 (23.6)	25 (25.3)	18 (21.7)	
Standard	139 (76.4)	74 (74.7)	65 (78.3)	
Longest (n=182)				0.580
Low	34 (18.7)	21 (20.6)	13 (16.3)	
Standard	148 (81.3)	81 (79.4)	67 (83.7)	
'Exclusions' group				
First (n=208)	55 (26.4)	30 (26.5)	25 (26.3)	1
Longest (n=209)	52 (24.9)	29 (25.4)	23 (24.2)	0.965

Table 5.9 FN episode risk adapted management and MBL genotype

5.2.5 MBL genotype and clinical outcome measures

Differences were observed in clinical outcome measures between the first and longest episodes for the total cohort which reflect the need for longer inpatient stays when patients are more unwell or have a microbiologically defined infection, shown in Table 5.10. During longest episodes: fluid bolus requirement (a surrogate measure of circulatory system compromise), frequency of positive blood cultures, change/addition of antibiotics, CVL removal and PICU admission were higher than in the first episodes analysed. MBL genotype did not appear to influence the majority of these outcome measures.

Positive blood cultures were present in 29.4% and 33.8% of the first and longest episodes respectively. Interestingly when analysing the episodes by genotype, wildtype individuals had more episodes with positive blood cultures than AO/OO individuals and this reached significance in the longest episodes, p=0.044. Gram positive organisms accounted for approximately 2/3rds of blood culture isolates (63-71%), which is in keeping with previous reports (Hann et al., 1997). However, a higher proportion of the common gram positive isolates (*Staphyloccus epidermidis* and Coagulase negative *staphylococcus)* were isolated in AA compared to AO/OO individuals. The remaining isolates consisted of predominantly gram negative isolates (27-31%) and a small proportion of fungal organisms (2-5.6%). There were a higher number of both gram positive and negative isolates in AA episodes, in line with the higher proportion of positive blood cultures but this was not statistically significant.

When patients remain febrile for 96h they are commenced on antifungal agents as part of routine management. No difference was observed between the need for antifungals and MBL genotype in either first or longest episodes (p=0.822 and 0.719, respectively). Changes or additions to first line antibiotics are made in episodes with positive blood cultures, dependent on antibiotic sensitivities and resistances, or in those not responding

	Total group	AA n (%)	AO/OO n (%)	p value between AA & AO/OO
Fluid bolus required at admission				
First (n=182)	6 (3.3%)	4 (4.0%)	2 (2.4%)	0.844
Longest (n=181)	15 (8.3%)	11 (11.0%)	4 (5.0%)	0.230
Positive blood culture				
First (n=194)	57 (29.4%)	36 (34.3%)	21 (23.6%)	0.141
Longest (n=195)	66 (33.8%)	43 (40.6%)	23 (25.8%)	0.044
Gram +ve isolate				
First	42 (71.0%)	26	16	0.437
Longest	45 (63.4%)	29	16	0.238
Gram –ve isolate				
First	16 (27.0%)	11	5	0.377
Longest	22 (31.0%)	16	6	0.114
Fungal isolate				
First	1 (2.0%)	1	0	-
Longest	4 (5.6%)	2	2	1.0
Antifungal commenced				
First (n=188)	26 (13.8%)	15 (14.9%)	11 (12.6%)	0.822
Longest (n=187)	45 (24.1%)	23 (22.5%)	22 (25.9%)	0.719
Change of 1st line antibiotics required				
First (n=188)	85 (45.7%)	49 (48.0%)	37 (43.0%)	0.589
Longest (n=188)	112 (59.6%)	62 (60.2%)	50 (58.8%)	0.967
CVC removed				
First (n=202)	4 (2.0%)	3 (2.7%)	1 (1.1%)	0.744
Longest (n=202)	10 (5.0%)	5 (4.5%)	5 (5.5%)	1
Transfer from POSCU to POC for FN				
First (n=210)	7 (3.3%)	5 (4.3%)	2 (2.1%)	-
Longest (n=210)	13 (6.2%)	7 (6.0%)	6 (6.3%)	-
PICU admission				
First (n=210)	3 (1.4%)	2 (1.7%)	1 (1.1%)	1
Longest (n=211)	7 (3.3%)	5 (4.3%)	2 (2.1%)	0.615

Table 5.10 Clinical outcome measures and MBL genotype

to first line treatment (including addition of antifungal agents). No differences were observed in the requirement for change of first line antibiotics between the genotype groups. The number of patients requiring CVC removal and/or transfer from POSCU to POC because of FN was relatively low in the episodes recorded and there was no over representation of individuals with variant MBL genotypes.

In this study no relationship between MBL variant genotype and PICU admission was demonstrated. Numbers were small but of the 7 admissions during the longest episodes, 5 were in wildtype individuals. Overall there were a total of 9 PICU admissions in 269 subjects, 7 AA and 2 AO/OO. No deaths during the study period were related to FN episodes.

5.2.6 MBL genotype and FN duration

Duration of inpatient (IP) stay was used as a surrogate measure of the severity of FN and analysed for both the first and longest episodes. The whole group were analysed and also divided into the two diagnostic categories: ALL and Non ALL. Within the whole group and the two diagnostic categories all episodes were subsequently analysed together and also separated into the 'Exclusions' group (those automatically excluded from low risk by virtue of age or underlying diagnosis) and the 'No exclusions' group (all other episodes which may have been managed as low or standard risk for clinical reasons at the time of admission). Longer inpatient stays were observed in the 'Exclusions' group in all analyses reflecting the prolonged neutropenia experienced when treated on intensive treatment protocols (Table 5.11).

Longer IP stays were observed in AO/OO compared to AA individuals in the majority of analysis groups, predominantly in the longest episodes. The differences between the genotype groups were greatest in the 'Exclusions' group, specifically in the Non-ALL patients, but none of these differences reached statistical significance.

The influence of MBL genotype on episode duration in ALL patients was minimal.

When the group was analysed as a whole, AO/OO patients spent one night longer in hospital than AA in both the first and longest episodes. When the group was separated into 'No Exclusions' and 'Exclusions' this difference was lost (first episodes) or reduced (longest episodes) which may reflect the many confounding variables that can

IP nights	First episode			Longest episode		
	AA	AO/OO	p value	AA	AO/OO	p value
Total group (n=)	108	89		109	91	
Mean	6.5	7.5		7.6	9.2	
Median	**5**	**6**	0.301	**6**	**6**	0.190
'No exclusions' (n=)	78	63		80	69	
Mean	5.3	5.5		6.2	7.4	
Median	**4**	**4**	0.609	**5**	**6**	0.211
'Exclusions' (n=)	30	24		29	22	
Mean	9.6	11.7		11.6	15.1	
Median	**7.5**	**10.5**	0.155	**12**	**14.5**	0.223
ALL (n=)	72	61		72	62	
Mean	5.7	7.1		6.6	8.3	
Median	**4**	**5**	0.120	**5**	**6**	0.100
'No exclusions' (n=)	59	47		62	50	
Mean	5.2	5.8		6.2	7.2	
Median	**4**	**4**	0.281	**5**	**5.5**	0.184
'Exclusions' (n=)	13	14		10	12	
Mean	7.9	11.2		8.9	12.9	
Median	**7**	**7**	0.306	**8.5**	**9**	0.390
Non ALL (n=)	36	28		37	29	
Mean	7.9	7.9		9.6	11.2	
Median	**6**	**6**	0.849	**8**	**10**	0.650
'No exclusions' (n=)	19	18		18	19	
Mean	5.6	4.7		6.1	7.8	
Median	**5**	**4**	0.407	**5.5**	**6**	0.760
'Exclusions' (n=)	17	10		19	10	
Mean	10.7	13.8		12.9	17.6	
Median	**8**	**12.5**	0.217	**13**	**15.5**	0.198

Table 5.11 Mean and median (bold) IP nights for different patient groups stratified by MBL genotype

contribute to length of IP stay, independent of risk, which may mask any effect of MBL genotype. In contrast, the Non ALL group consists of patients with a wide range of diagnoses. Solid tumour patients vary considerably in their risk and severity of FN. Within this group are also AML and B NHL patients who are rendered significantly immunosuppressed by their treatment regimes and subsequently experience prolonged FN.

A comparison of median inpatient stay for all episodes for the different diagnostic groups is illustrated in Figure 5.7, and highlights the variability between different diagnoses and the two genotype groups. It should be noted that not all diagnoses were represented in each of the two genotype groups.

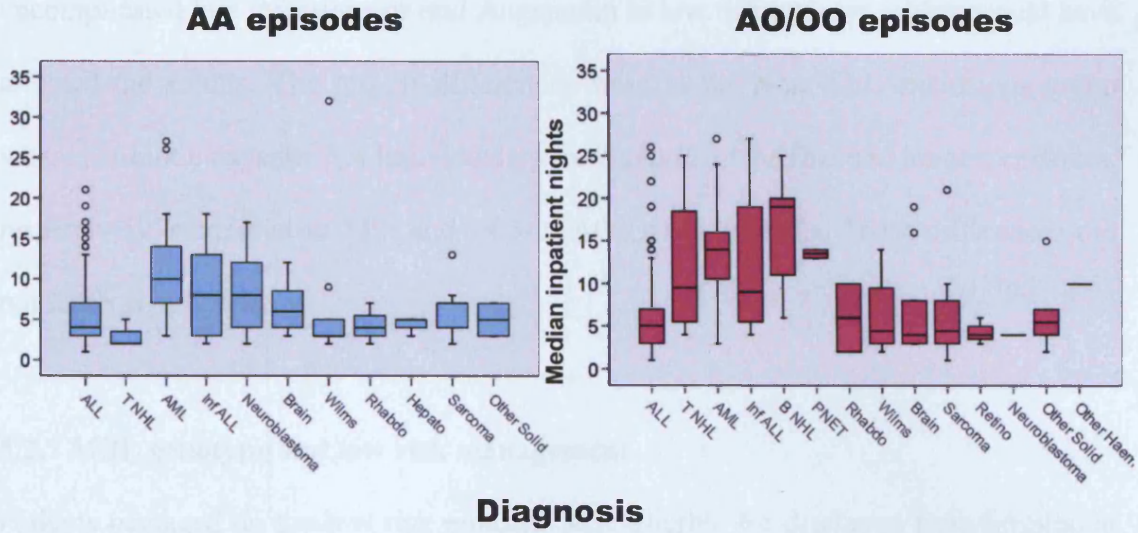


Figure 5.7 Median inpatient nights for all captured episodes stratified by diagnosis and genotype

Overall the Non ALL group contained diagnoses/patients some of which were at very high risk and some at relatively lower risk of adverse outcome which could explain the lack of difference between IP nights in the total group, first episode where they are likely to even each other out. The Non ALL/'No exclusions' group represents lower risk episodes and the shorter IP stays in AO/OO in the first episodes may be due to confounding factors. The Non ALL 'Exclusions' group predominantly consists of patients with AML, infant ALL, B NHL and those post peripheral blood stem cell Transplant (e.g. Stage IV Neuroblastoma). Due to sample sizes there was no statistically significant difference between genotype groups but the results do suggest that MBL may be exerting a greater effect in these higher risk episodes where AO/OO subjects spent on average 4.5 days longer in hospital than AA individuals.

Number of antibiotic days was also used as a surrogate measure of FN severity. Trends were similar to those for IP nights but the effect of MBL deficiency was less pronounced (data not shown). The data are generally more difficult to interpret as they do not necessarily reflect whether the patient was unwell. A number of clinically well patients would have continued antibiotics at home e.g. intravenous Teicoplanin for uncomplicated line infections or oral Augmentin in low risk patients, which would have affected the results. The largest differences were in the Non ALL exclusions group where antibiotic days for AA individuals were 7 and 12 in the first and longest episodes, respectively, compared to 11.5 and 14.5 in AO/OO individuals. These differences did not reach significance.

5.2.7 MBL genotype and low risk management

Patients managed on the low risk protocol were eligible for discharge from hospital at 48h to continue oral antibiotics at home until their fever resolved. Patients who deteriorated clinically or had persistent fever at 96h were readmitted at any point post early discharge. It was hypothesised that MBL deficiency may be a risk factor for readmission. Of the 421 PINE episodes, 115 (27%) were managed as low risk and only 9 (7.8%) required readmission. These readmissions were in 5 AA and 4 AO/OO individuals. The reasons given for readmission included ongoing fever, clinical deterioration and parental concern, of which the latter is assumed to be MBL independent.

5.3 Discussion

This study was designed to explore the role of MBL in FN episodes in a paediatric oncology population. The principle aim was to investigate the effect of MBL genotype on susceptibility and severity of FN. This is the largest study of its kind to date in terms

of both patient numbers and participating centres which included both oncology units and shared care/district general hospitals. We have shown that individuals in possession of MBL variant alleles suffer more episodes of FN compared to wildtype (Table 5.2). These episodes are generally of longer duration with the most notable differences observed in patients with diagnoses and/or treatment regimens which render them at increased risk of severe infection i.e. the 'Exclusions' group.

Our data indicates that MBL deficiency may increase susceptibility to FN, as the proportion of individuals experiencing multiple episodes of FN was higher for AO/OO than AA patients (Figure 5.1). More specifically it appears to influence frequency of actual clinical and/or microbiological infections as opposed to FUO episodes i.e. MBL appears to be important in protection against bacterial infections. Our initial analysis stratified patients by genotype i.e. AA Vs AO/OO but we were also able to demonstrate similar findings when analysing by MBL level in the subpopulation of patients for whom serum was available (Table 5.8, Figure 5.3). This is in agreement with Schlapbach *et al* who observed that patients with very low levels of MBL were at increased risk of FN caused by severe bacterial infections (Schlapbach et al., 2007). They categorized their patients by MBL level into three groups; <100ng/ml, 100-1000ng/ml and > 1000ng/ml, different from this study. They reported that when compared to individuals with MBL levels of 100-1000ng/ml, patients with very low (<100ng/ml) and normal levels (>1000ng/ml) had more episodes of FN which were of longer duration. They also reported that children with MBL levels >1000ng/ml were more frequently hospitalized for FN due to episodes of FUO. This study involved 94 patients of whom 10 had levels <100ng/ml and they were heterogenous in terms of patient age, diagnosis and chemotherapy intensity.

Findings reported in the next chapter (Chapter 6) suggested that individuals in possession of the YAYD genotype had MBL levels and function which were more

similar to wildtype than other heterozygotes. Interestingly when the YAYD individuals were analysed with the wildtype group and compared with the other heterozygotes and homozygotes the trend was weaker implying that the YAYD group behave clinically more like other heterozygotes.

MBL appears to not only influence susceptibility to FN but also how an individual handles an infection i.e. their duration of illness. Neth et al reported an effect of MBL on duration of fever during neutropenic episodes, with AO/OO individuals spending twice as many days with fever and neutropenia over the first six months of cancer treatment compared to AA. Our study also supports a role for MBL in modulating duration of FN episodes. We used IP nights as a surrogate measure of FN duration because capturing information on actual fever duration is difficult, particularly in a multicentre setting. We were unable to collect such detailed information due to the involvement of over 40 hospitals. The data are subject to a number of confounding variables which are complicated further in a multicentre study. There may be a number of reasons why patients undergoing treatment for malignancy experience a prolonged inpatient stay for FN e.g. Hospital staff may have different levels of experience in caring for such patients and hence different levels of confidence in decision making regarding discharge which may contribute to prolonged admissions and parental confidence may vary at different points during treatment and influence the duration of inpatient treatment. Essentially using the new protocol, patients were discharged when clinically well +/- afebrile dependent on RS. Despite the potential effects of the confounding factors discussed which may dilute any biological effect we did find a difference in the number of IP nights when comparing AA and AO/OO individuals although this did not reach statistical significance. This was a secondary analysis and our study was not powered to detect differences in duration of episode. MBL deficient patients generally spent longer in hospital with their FN but the differences were less

pronounced than those demonstrated by Neth for fever days because we used IP nights however this may be a more relevant measure from the patient's perspective.

Identifying within which patient groups MBL exerts its (most significant) effect is important when considering which patients may gain most benefit from replacement therapy. Previous studies have been criticised for failing to consider the intensity of chemotherapy regimens when analysing patient episodes. It has been proposed that MBL could prove to be most beneficial in those patients at lower risk of infection whose capacity for granulocyte recovery is better (Klein and Kilpatrick, 2004). This theory was supported by Schlapbach et al who showed that very low MBL levels were most relevant to FN outcome under transiently myelosuppressive chemotherapy e.g. treatment for solid tumours and ALL (Schlapbach et al., 2007). The issue of chemotherapy intensity was analysed by separating episodes by diagnosis (ALL Vs Non ALL) and the presence or absence of Exclusion criteria which selects out patients on the most intensive regimes. The data suggest that MBL may be operating in ALL patients but its effect was not dramatic. This may reflect the fact that ALL patients, especially during maintenance treatment, do not generally suffer from severe infections and commonly present with mild upper respiratory tract infections which are viral in nature. Differences in the recognition and binding of MBL to different pathogens may in part explain these findings. Evidence therefore indicates that MBL deficiency (as assessed by genotype) may be most important in those patients at highest risk of severe FN e.g. AML and B NHL patients who experienced longer FN episodes than their MBL sufficient peers. This is in contrast to findings reported by Bergmann *et al* who studied 80 adult AML patients for 28 days post induction chemotherapy. They found no effect of MBL deficiency on frequency of severe infections or duration of fever but it should be noted that only 16/80 patients were considered deficient (Bergmann et al., 2003). They hypothesised that the effect of MBL was overshadowed by the profound

disturbance in phagocytic function experienced in these patients. This explanation implies that the main role of MBL is initiation of opsonophagocytosis but this may not be its only function. Its role in the modulation of inflammation may also be important, particularly in the resolution of episodes.

Analysis of ALL patients alone demonstrated a trend towards an increased frequency of FN episodes which were generally longer in the AO/OO group compared to AA (Table 5.5, Table 5.11). Lausen *et al* retrospectively analysed FN data on 137 children during their first 3 months of induction therapy for ALL (Lausen et al., 2006). In this homogenous group they reported that MBL deficiency did not influence frequency of infections. In the present study 178 patients were investigated including 170 children with ALL who were grouped together with T NHL patients due to similarities in treatment protocol and subsequent risk of FN. FN episodes captured during both the first and last few months of their treatment (which spans up to 3 years) were investigated. The first few months are when patients are deemed at highest risk of infection but this does vary from patient to patient throughout their treatment (Graubner et al., 2007). Interestingly our data from first FN episode captured during the study period showed no difference in duration for AA and AO/OO subjects. A proportion of these episodes would have been from patients at the time of diagnosis and may reflect the relatively standard length of IP admission for a newly diagnosed ALL patient. Current protocols require a repeat bone marrow aspirate on day 8 after diagnosis and most patients remain an inpatient until this time. The current one year study period has provided a more global overview of the influence of MBL deficiency in children with ALL which may have been overlooked by Lausen *et al* when focussing on just the first three months of treatment.

The duration of the study period may well be important when assessing the true influence of MBL on infection morbidity in such patients. Previous studies that have

reported an effect of MBL deficiency on infection outcome (Peterslund et al., 2001; Neth et al., 2001; Mullighan et al., 2002; Horiuchi et al., 2005; Schlapbach et al., 2007) have generally included longer follow up periods than those who have found no effect (Kilpatrick et al., 2003; Bergmann et al., 2003; Frakking et al., 2006) with the study by Peterslund *et al* being the exception. As previously discussed the studies have asked different questions of their heterogenous groups of patients making comparisons difficult but it can be said that the studies of longer duration have been more informative. The only study to demonstrate an effect of MBL on infections after chemotherapy in a single patient episode was conducted by Peterslund and colleagues which simply compared the severity of infections. The impact of this study was great as it highlighted oncology patients as potential beneficiaries of MBL replacement therapy and has fuelled the development of recombinant MBL.

One of the weaknesses of this study was its cross-sectional design. We aimed to capture patients on active treatment during a one year period rather than following individual patients prospectively for a year. Some patients were diagnosed before the study started and some patients were diagnosed during the one year period which meant that some patients started and/or finished their treatment during the study period i.e. did not have FN episodes for the full year period and we have made the assumption that they balance each other out. If patients were not equally distributed we may have introduced bias into our sample and skewed our findings in either direction, potentially diluting the effect of MBL deficiency. Different diagnostic groups receive different management e.g. patients with ALL receive 2-3 years of treatment (predominantly as an out-patient) during which the degree of treatment related immunosuppression varies compared to patients with AML who receive 6 months of intensive treatment which requires inpatient admission and patients with solid tumours who receive treatment of differing duration and intensity. Patients from most of the diagnostic groups were present in our sample with

no marked difference between the wild type and variant populations. Of note 11 of the 15 patients with AML possessed a wildtype genotype. The advantage of this study design was that it enabled us to recruit a large number of patients who had many FN episodes over the one year period which were available for analysis. Frakking *et al* were unable to demonstrate an effect of MBL deficiency in their study of a mixed group of paediatric oncology patients which may be due to the fact that they analysed a single FN episode from just 66 patients (Frakking et al., 2006). This meant that there were only 39 patients in their high MBL genotype group and 26 in the medium/low group making analysis and interpretation limited.

MBL genotype did not appear to influence the type of bacterial infection that patients experienced which is in keeping with a number of other studies. The finding of more episodes of bacteraemia in wildtype subjects is in keeping with the results of a recent study of adults with community acquired pneumonia (Perez-Castellano et al., 2006). A greater risk of developing bacteraemia was reported in wildtype subjects with the authors hypothesising that MBL may favour entry of bacteria via the bloodstream. The number of reported fungal infections was low in our population preventing any meaningful analysis. Choi et al were unable to demonstrate an association between MBL polymorphisms and chronic disseminated candidiasis in adult leukaemia patients (Choi et al., 2005) but MBL deficiency was found to be a predictive factor for invasive fungal infection in adults after stem cell transplantation (Granell et al., 2006). Viral infections are currently poorly investigated and reported and therefore no comment can be made on whether MBL is important. It remains unclear how many episodes classified as FUO may be viral in origin. A relationship between MBL deficiency and intensive care admission in the paediatric oncology population has never been convincingly demonstrated. Of the 9 episodes requiring transfer to PICU in this study, 7 were in AA subjects which is in keeping with two other studies (Frakking et al., 2006;

Schlapbach et al., 2007). This is in contrast to findings of Neth *et al* who reported that 3 out of 4 patients who went to PICU were AO/OO individuals (Neth et al., 2001). These divergent findings do not correlate with work demonstrating an effect of MBL deficiency on incidence of SIRS in intensive care patients and are likely to reflect the multifactorial reasons for PICU admission in oncology patients in which MBL may play only a part (Garred et al., 2003; Dalton et al., 2003; Fidler et al., 2004).

The final question of this study was whether MBL genotype would be a useful addition to RS to aid the accurate identification of patients suitable for low risk management. It was hypothesised that MBL deficiency may contribute to the need for readmission following early discharge but we have been unable to conclusively answer this question. Of those patients who did require readmission, 5 were AA and 4 were AO/OO but the reasons for readmission make interpretation difficult. As previously discussed the new protocol was introduced and audited from the outset. Confidence in the low risk management protocol differed between hospitals and parents as did the practicalities of its use which may have resulted in inappropriate or under use and the subsequent need for readmission. MBL genotype may form part of the RS process in the future but larger studies are required to delineate its specific role.

In conclusion, this large multicentre study has demonstrated that MBL genotype (and protein level) appears to influence both frequency of FN and duration of IP stay for FN in children with cancer. It has highlighted that MBL genotype may be most important in patients at risk of severe FN but due to relatively low patient numbers in some analysis groups this finding must be interpreted with caution. Previous studies that have dismissed a relationship between MBL and infections in cancer patients have often involved inadequate numbers of patients from which to draw such conclusions. It remains unclear exactly how MBL is exerting its effect in oncology patients with different diagnoses and therefore further analysis of large homogenous groups is

required to answer such questions. MBL deficiency may contribute to susceptibility but may have most impact on modulation of inflammation and subsequent resolution of these episodes. It may be most suited as an adjunct to infection management with the aim of accelerating recovery and reducing potential interruptions in treatment. It must be remembered that MBL is just one component of the lectin pathway and further studies are required in order to clarify the role of the other components e.g. MASPs and Ficolins, in infection susceptibility in children with cancer.

Chapter 6

MBL structure and lectin pathway function in children with cancer

6.0 Introduction

Assessment of MBL 'deficiency' has been the focus of many studies over the last 17 years but the methodology used has differed. Many studies have relied on genotype data alone but this may be considered an over simplified measure of this complex pathway. More detailed characterisation of the pathway is of importance, particularly with the prospect of selecting patients for MBL replacement therapy. MBL, at least in terms of complement activation, does not function in isolation and identification of other defects within the pathway would prevent inappropriate use of recombinant MBL in potential subjects.

Identification of the most clinically relevant measure is obviously paramount when conducting and analysing disease association studies and in the case of MBL has led to the development of a number of assays (Petersen et al., 2001; Kuipers et al., 2002; Seelen et al., 2005). Measurement of MBL 'functional activity' is complement specific and should improve our understanding of its disease specific actions. However it should be noted that MBL has other functions which are not quantified by these assays (Dommett et al., 2006).

The contribution of individual MASPs to lectin pathway function can also be assessed using functional assays (Sorensen et al., 2005). Polymorphisms in the genes encoding these serine proteases have been identified and their clinical importance is currently under investigation (Stengaard-Pedersen et al., 2003; Lozano et al., 2005; Thiel et al., 2007). The involvement of Ficolins in this system is an evolving story, and to date, our understanding of ficolin deficiency and disease remains in its infancy.

The aim of this work was to characterise components of the MBL lectin pathway and their combined functional activity in children with cancer. Firstly, potential associations between MBL genotype, protein levels and functional activity were assessed.

Investigation of MBL structure, specifically the presence of higher order oligomers, added to this global assessment. Unexplained functional deficiencies in some individuals further led to investigations of their MASP levels and function.

6.1 Methods

6.1.1 Patient population

The patients used in this study are a subset of those described in Section 4.1.1, which were recruited at Great Ormond Street Hospital. An additional sample of whole blood was collected and centrifuged (2500rpm, 15min). Serum was aliquoted and stored at -80°C until required. Blood samples were taken when the subjects were clinically well, i.e. not during an acute infective episode. Actual sampling took place either on the day of admission for inpatient chemotherapy when patients were well enough to commence treatment or when they were attending the outpatient department for routine follow up. All investigations were performed using a fresh aliquot of serum.

6.1.2 Measurement of MBL concentration

The ELISA method described in Section 2.4.3.1 was used to determine the MBL levels in serum.

6.1.3 Wieslab total complement screen

The Wieslab total complement screen method described in Section 2.4.4.4 was used to investigate lectin (Figure 6.1), classical and alternative pathway function.

6.1.4 Western blotting of MBL in serum

This method was developed and optimised as part of this study. The final method used in these studies is described in Section 2.4.5.

6.1.5 Measurement of MASP2 levels

The TRIFMA method described in Section 2.4.3.2 was used to determine the MASP2 levels in serum.

6.1.6 Genotyping of MASP2

Analysis of the D105G SNP was performed by Innogenetics, Belgium using a multi probe assay system similar to that used in the INNO-LiPA MBL2 assay.

6.1.7 Measurement of MASP3 levels

The TRIFMA method described in Section 2.4.3.3 was used to determine the MASP3 levels in serum.

6.1.8 MBL/MASP2 complex activity /C4b deposition

The C4b deposition assay described in Section 2.4.4.1 was used to investigate MBL/MASP2 complex activity (Figure 6.1). Recombinant MBL (rMBL) was added to a number of samples with low activity. rMBL was a gift from NatImmune A/S, Copenhagen, Denmark and was prepared as previously described (Vorup-Jensen et al., 2000). For reconstitution studies rMBL was added to serum at a concentration of 1000ng/ml.

6.1.9 C3 cleavage activity/C3b deposition

The C3 assay described in Section 2.4.4.2 was used to investigate activation of C3 by MBL/MASP2 and MBL/MASP1 complexes (Figure 6.1). rMBL was added to a number of samples prior to assessment of C3 cleavage activity.

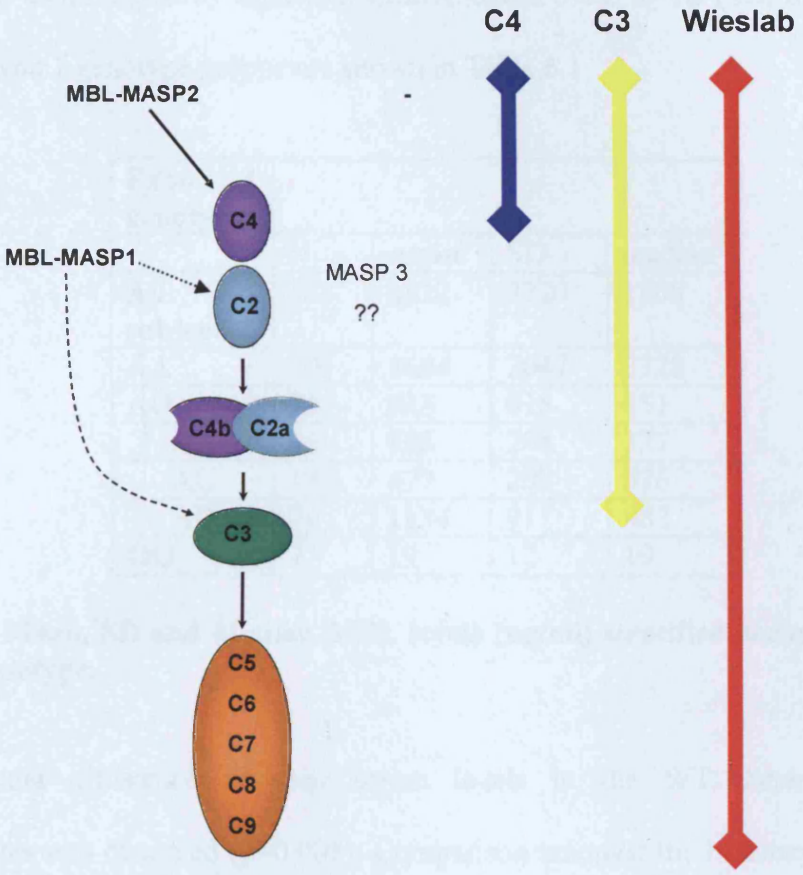


Figure 6.1 Schematic of MBL lectin pathway and its components assessed by the different functional assays utilised in this study. The blue bar (C4) corresponds to the components assessed by the MBL/MASP2 complex activity or C4b deposition assay, the yellow bar (C3) corresponds to components assessed by the C3 assay and the red bar (Wieslab) corresponds to components assessed by the Wieslab MBL Lectin pathway screening assay.

6.2 Results

Serum samples were analysed from 183 subjects recruited from Great Ormond Street Hospital.

6.2.1 Relationship between MBL levels and Exon 1 genotype

The mean MBL level for the entire cohort was 2322ng/ml (SD 2220, median 1808ng/ml) as measured by sandwich ELISA. Mean MBL levels (SD, median) for the different exon 1 genotype groups are shown in Table 6.1.

Exon 1 genotype				
	N	**mean**	SD	median
All subjects	183	**2322**	2220	1808
AA	103	**3684**	2047	3328
AO	73	**623**	615	451
AB	36	**336**	208	327
AC	13	**477**	209	576
AD	24	**1134**	917	982
OO	7	**19**	12	19

Table 6.1 Mean, SD and Median MBL levels (ng/ml) stratified according to MBL Exon 1 genotype.

A significant difference between serum levels in the WT, heterozygotes and homozygotes was observed ($p<0.001$). Comparison amongst the heterozygotes showed AD individuals to have the highest levels, followed by AC and AB (Table 6.1).

6.2.2 Relationship between MBL levels and promoter genotype

The XY promoter has the most profound effect on serum levels and its influence in both WT and heterozygotes is illustrated in Figure 6.2.

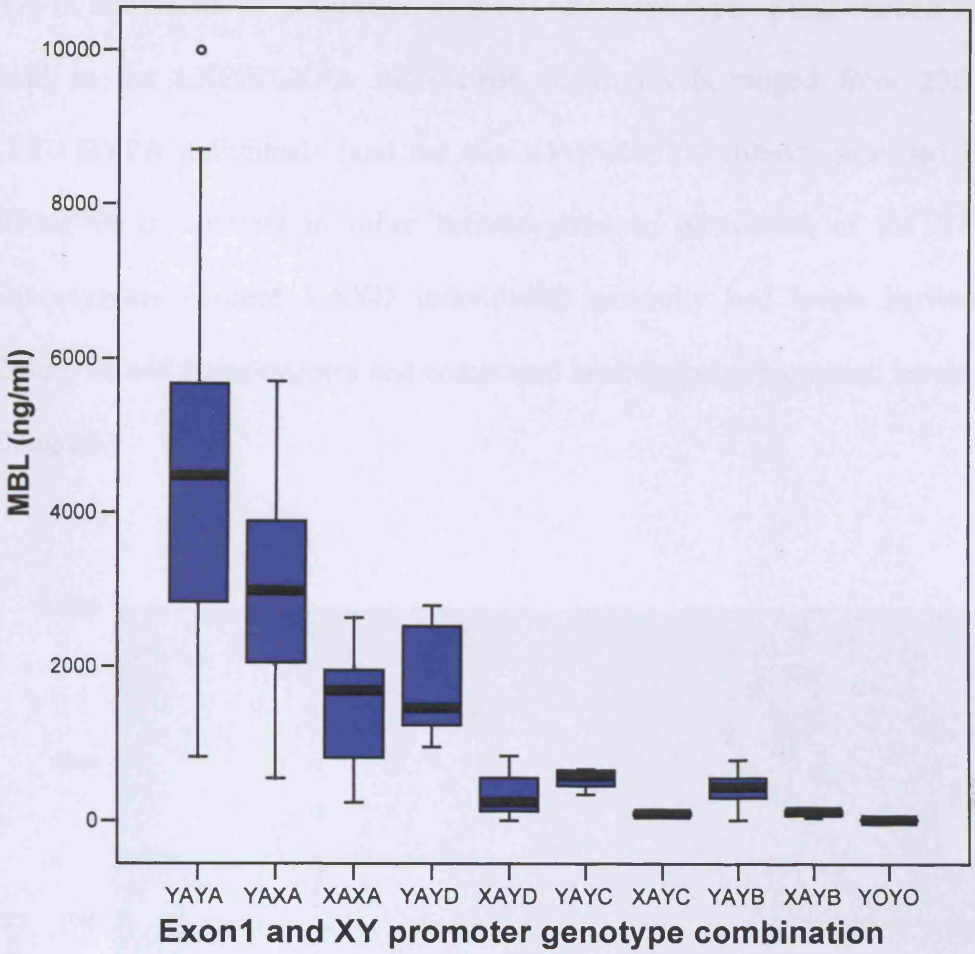


Figure 6.2 MBL serum concentration stratified according to Exon 1 and XY promoter genotype (median indicated by a line, boxes represent interquartile range and bars represent outliers).

The median MBL levels in YAYA, XAYA and XAXA individuals were 4484, 2989 and 1692 ng/ml respectively. In heterozygous individuals the presence of the YY promoter is associated with increased average levels in all groups compared to those in possession of the XY promoter and this effect is most pronounced in the AD individuals (AD, YY=1466 ng/ml and XY= 245ng/ml, AC 579 and 96, AB 443 and 127 ng/ml respectively). When combining the heterozygotes, the median levels in the YAYO group were 576ng/ml compared to 138ng/ml in the XAYO group. When stratified by full haplotype combination a good correlation was observed with MBL levels (Figure 6.3). The majority of WT individuals had levels above 1000ng/ml and the highest levels

were in individuals in possession of the HYPA haplotype. Most marked variation was noted in the LXPA/LXPA individuals where levels ranged from 235-2453ng/ml. HYPD/HYPA individuals (and the one LYQA/HYPD subject) also had levels above 1000ng/ml in contrast to other heterozygotes in possession of the YY promoter. Heterozygotes (except YAYD individuals) generally had levels between 100 and 1000ng/ml and homozygotes and compound heterozygotes possessed levels of less than 100ng/ml.

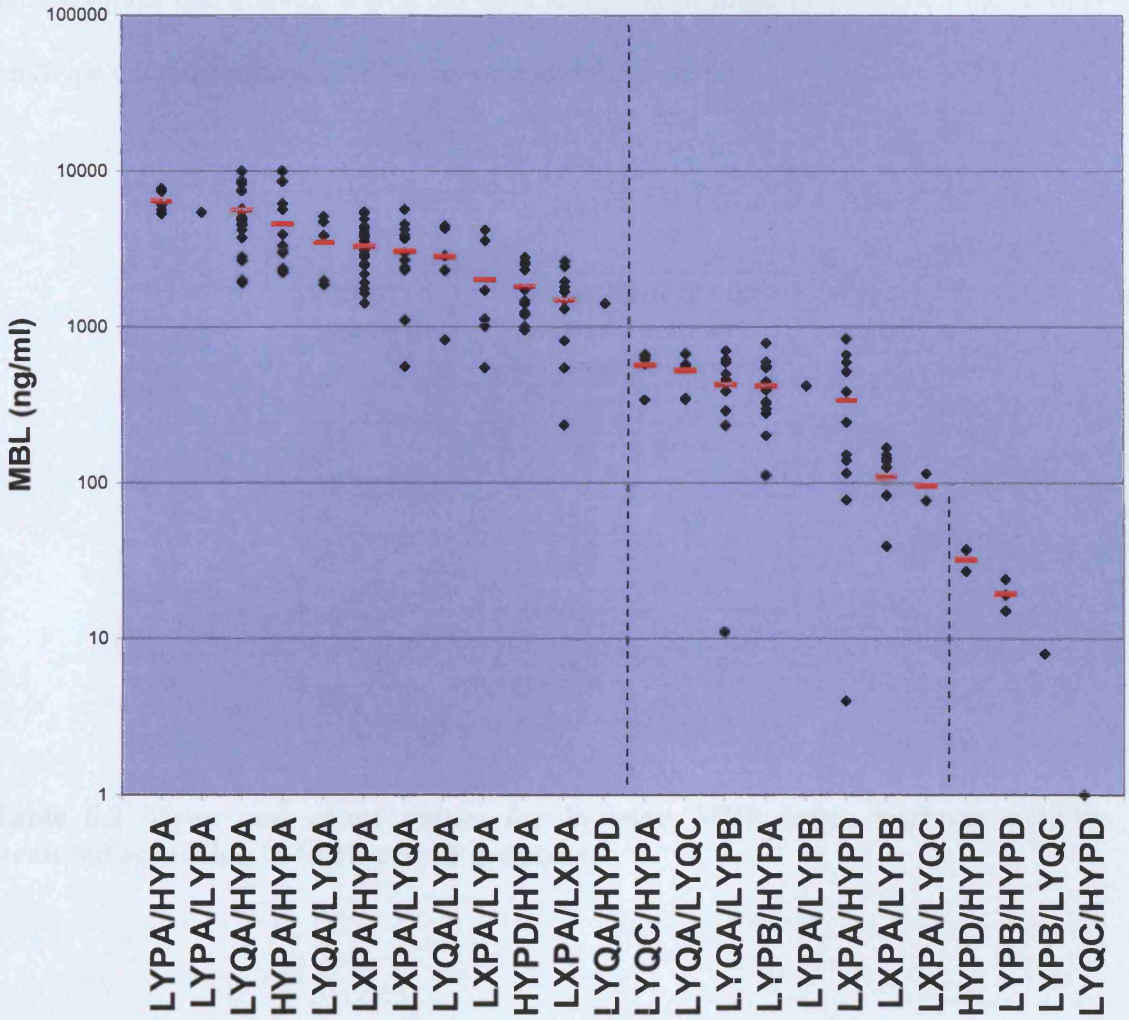


Figure 6.3 MBL serum levels and full haplotype combination.
Black dashed lines delineate WT and YAYD individuals from other heterozygotes and compound heterozygotes or homozygotes. Red lines represent mean levels.

6.2.3 Relationship between MBL level and function

Functional assessment of the MBL lectin pathway was initially investigated using the Wieslab MBL lectin pathway screen which assesses the ability of MBL complexes to activate complement as determined by detection of terminal components of the pathway, C5b-9. Individuals exhibiting <10% activity compared to the positive control were deemed deficient (Seelen et al., 2005). The MBL lectin pathway activity stratified by exon 1 (Table 6.2) and XY promoter genotypes is shown in Figure 6.4. Deficient activity was observed in 82% (60/73) of heterozygotes and all homozygotes. Thirteen heterozygotes had activity above the 10% level and of these 11 possessed the YAYD genotype combination, with MBL levels above 1000 ng/ml.

Exon 1 genotype	**Wieslab MBL lectin pathway activity % (of positive control)**		
	n	mean	range
All subjects	183	36	0-193
AA	103	56	0-193
AO	73	12	0-133
AB	36	2	0-10
AC	13	4	0-19
AD	24	30	0-133
OO	7	1	0-3

Table 6.2 Mean and range values for Wieslab MBL lectin pathway activity stratified according to MBL exon 1 genotype.

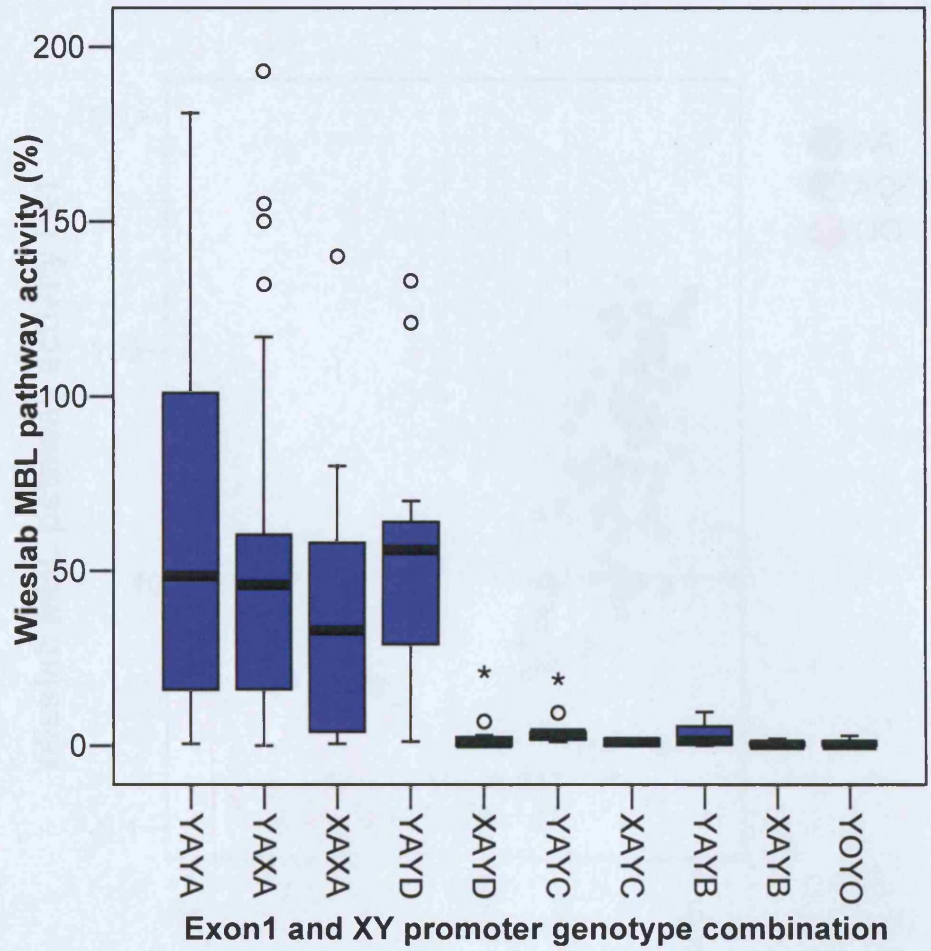


Figure 6.4 MBL lectin pathway activity (median indicated by a line, boxes represent interquartile range and bars outliers) stratified according to exon 1 and XY promoter genotype.

There is no agreed definition of MBL deficiency but WT subjects generally exhibit MBL levels >1000ng/ml as shown in Figure 6.3. When MBL lectin pathway activity was plotted against MBL level (Figure 6.5), AA subjects were predominantly found in the upper right area i.e. >10% activity and MBL level >1000ng/ml. The AO subjects were mainly seen in the middle lower area, i.e. <10% activity and MBL levels between 100-1000ng/ml. The OO individuals were observed in the lower left area, i.e. <10% activity and MBL levels <100ng/ml.

The data identified a subset (n=20) of WT individuals with low lectin pathway activity i.e. <10%. Of these, 5 subjects had MBL levels below 1000ng/ml but 15 had levels >1000ng/ml and warranted further investigation.

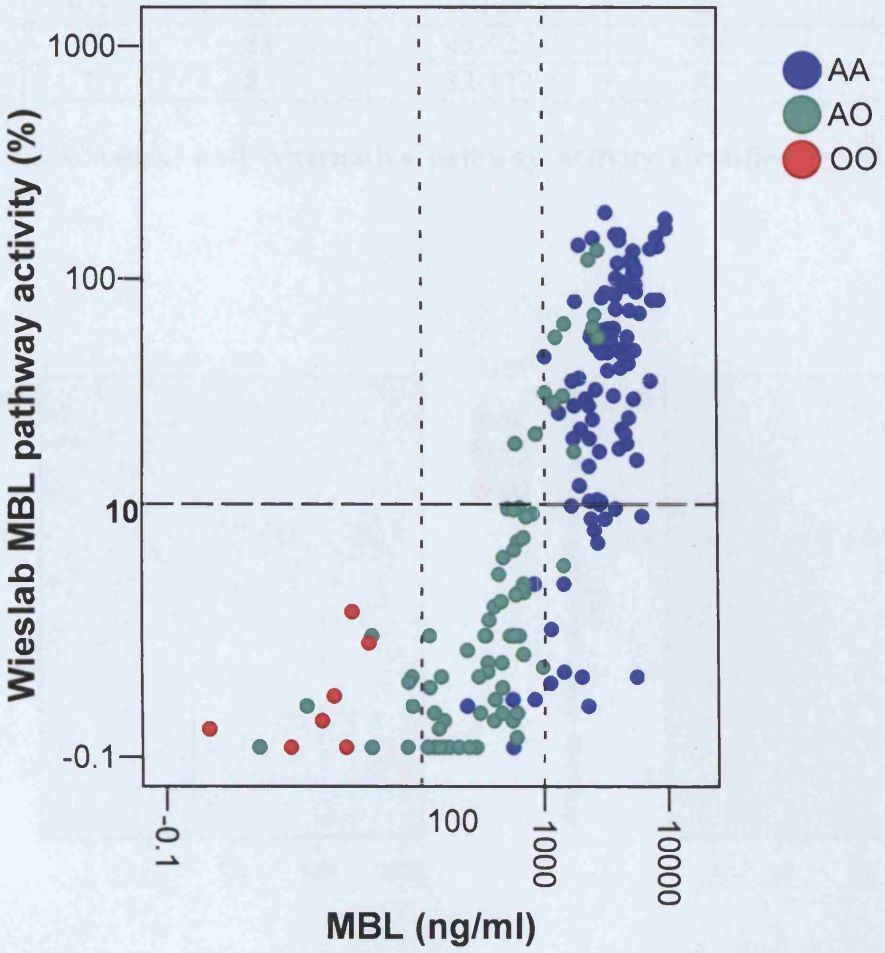


Figure 6.5 MBL levels plotted against MBL lectin pathway activity. The horizontal dashed line marks 10% activity. The vertical dashed lines mark MBL levels of 100ng/ml and 1000ng/ml.

Whether the lack of activity was due to a defect in the MBL lectin component of the pathway or due to a defect in the terminal pathway was unclear. In order to clarify this both classical and alternative pathway were screened in these individuals as these also rely on an intact terminal pathway.

Results for the entire cohort for the classical and alternative pathway assays stratified by MBL exon 1 genotype are shown in Table 6.3. Mean pathway activity was relatively uniform between the MBL genotype groups. There was no relationship between classical and alternative pathway function and MBL level as illustrated in Figure 6.6.

		Classical pathway (%)		Alternative pathway (%)	
	n	mean	range	mean	range
AA	103	86	4-137	86	33-113
AO	73	88	43-127	89	13-117
OO	7	83	32-133	82	54-100

Table 6.3 Classical and Alternative pathway activity stratified by MBL exon 1 genotype

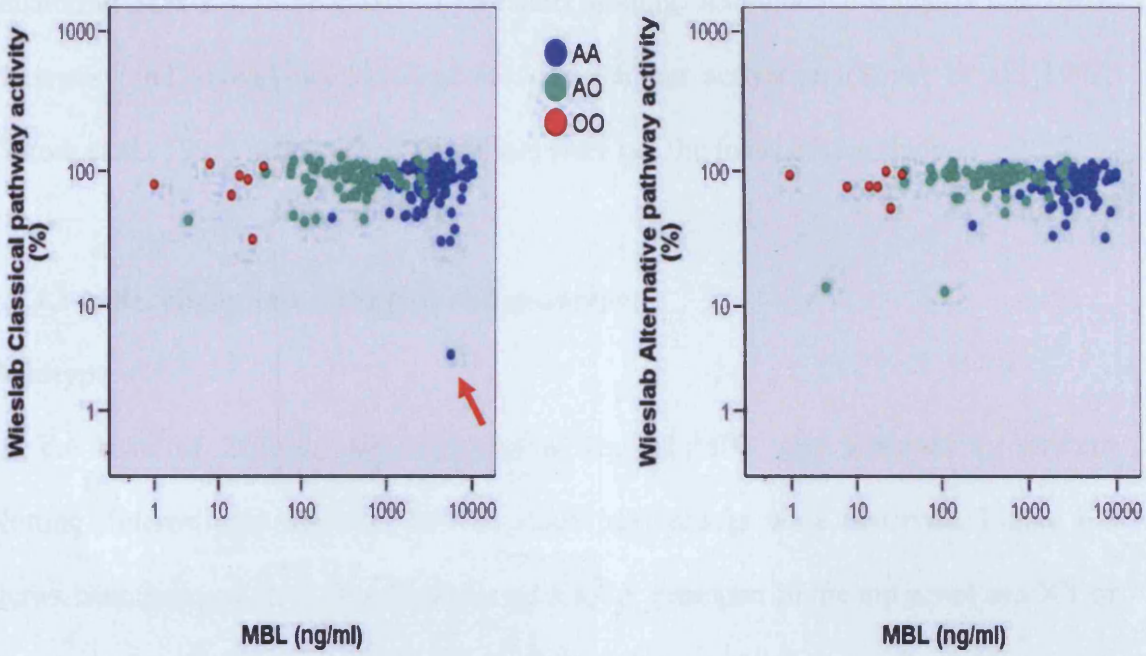


Figure 6.6 MBL levels plotted against Classical and Alternative pathway activity. Red arrow indicates individual with deficient classical pathway activity.

One *MBL2* WT individual exhibiting low lectin patheway function also showed deficiency in classical pathway activity (4%, highlighted by red arrow in Figure 6.6) but not alternative pathway activity. This suggests a defect common to both the MBL lectin and classical pathways e.g. this could occur at the C2/C4 level. As all other individuals had no detectable deficiency in either the classical or the alternative pathways using this screening tool, deficiency in lectin specific components was assumed.

Our next line of investigation was to examine MBL structure. Differences in MBL oligomeric structure which correlate with functional activity have been demonstrated in

hetero and homozygotes (Dean et al., 2005) however detailed structural analysis in WT individuals has not been performed.

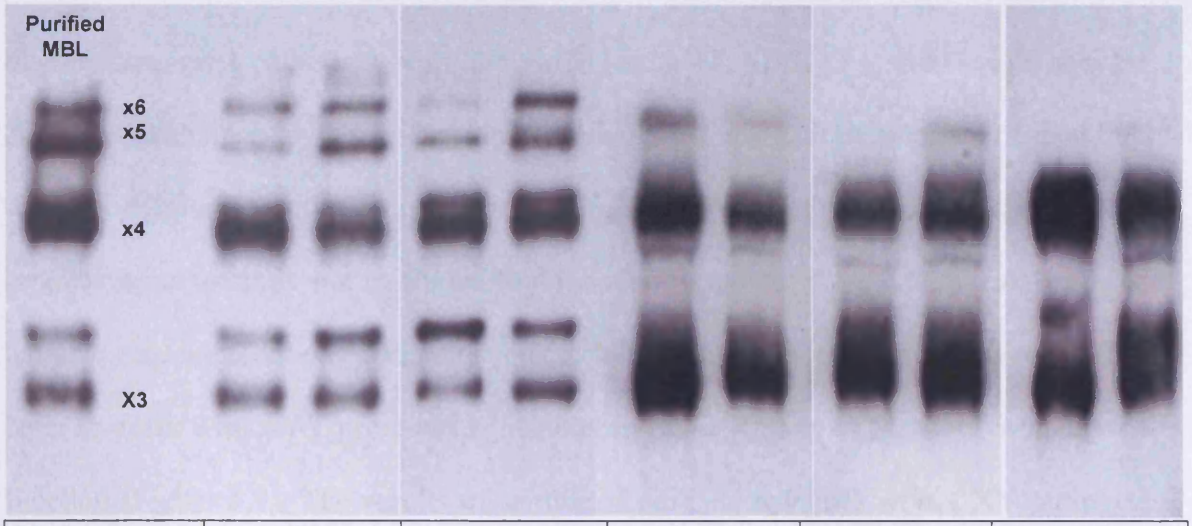
6.2.4 Relationship between MBL structure and function

To explore the influence of MBL structure on complement activating function, the presence of MBL higher order oligomers (trimer and above) was analysed by non-denaturing SDS-PAGE followed by Western blotting. As evidence suggests that MBL tetramers (and above) are required for complement activation (Super et al., 1992; Yokota et al., 1995), lower order oligomers were not the focus of this study.

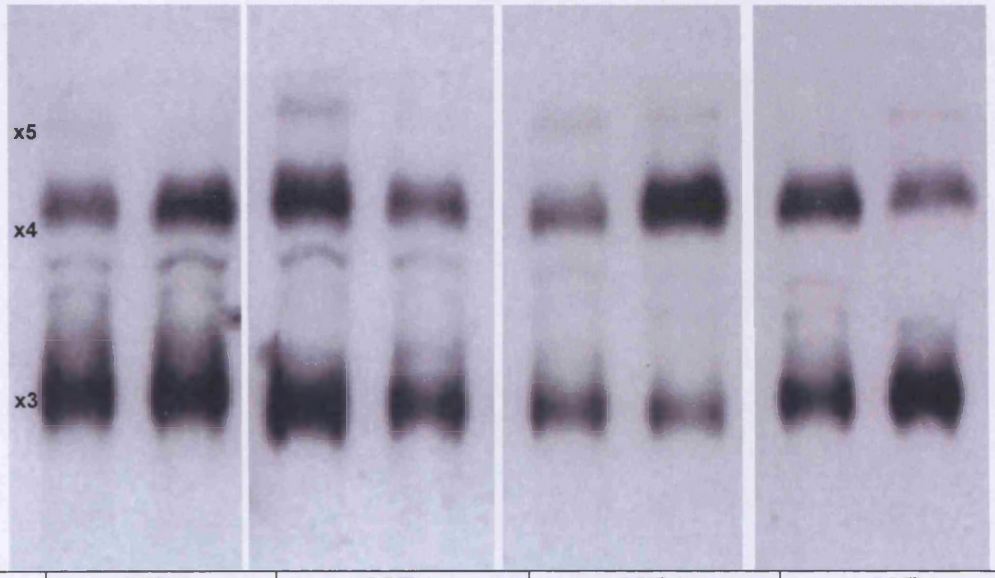
6.2.4.1 MBL oligomeric structure and genotype

Wildtype

On the basis of ELISA, serum containing 1ng of MBL was analysed by western blotting. Interestingly, marked interindividual differences were observed. Figure 6.7 shows band patterns for WT individuals with a YY promoter in the top panel and XY or XX promoter in the bottom panel. Purified MBL (positive control) yielded bands presumed to represent trimer, tetramer, pentamer and hexamer at approximately 288, 384, 480 and 576 KDa, respectively. The tetramer band was the most abundant. In some samples a band was also noted between the trimer and tetramer at ~320 KDa, whose structure is unclear. Due to the low band intensity seen for pentamers and hexamers, when compared to trimers and tetramers, the blots were exposed for different durations in order to visualize these bands optimally. The gel images shown represent one exposure time and individuals with the same genotype were run on the same gel to enable direct comparison.



Haplotype combination	HYPA HYPA		LYQA HYPA		LYPA HYPA		LYQA LYPA		LYQA LYQA	
MBL level (ng/ml)	8622	6189	8343	7096	5671	6087	2306	4450	5122	4727
Wieslab function	138	9	151	110	95	71	28	97	120	19

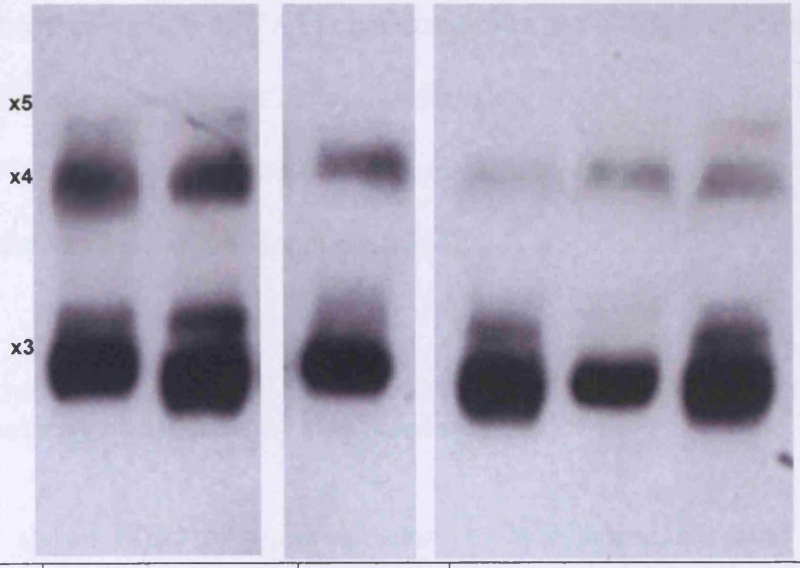


Haplotype combination	LXPA HYPA		LXPA LYQA		LXPA LYPA		LXPA LXPA	
MBL level (ng/ml)	2806	3328	3692	2678	4203	3581	1692	815
Wieslab function	10	48	31	51	155	58	36	4

Figure 6.7 SDS-PAGE separation of MBL oligomers followed by blotting with anti-MBL and development by ECL. Examples from 18 individuals are shown with their corresponding MBL genotype, level and functional activity as measured by the Wieslab assay. Purified MBL is shown for comparison and bands corresponding to trimers (x3), tetramers (x4), pentamers (x5) and hexamers (x6) are indicated.

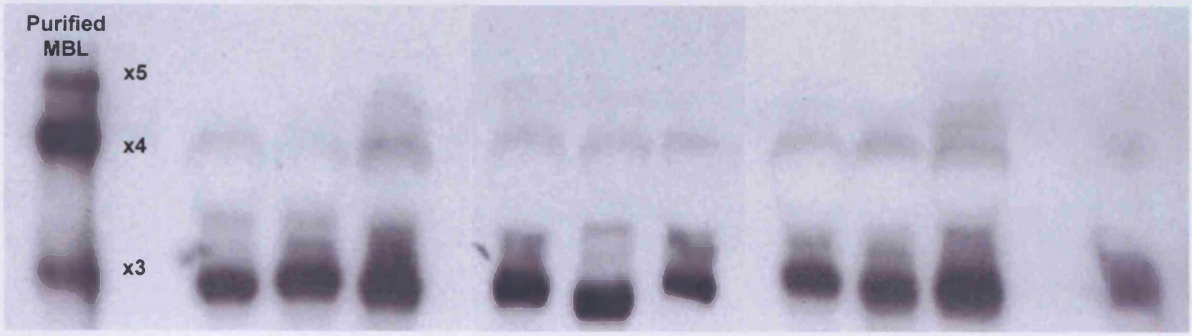
Heterozygotes

Analysis of 1ng of MBL protein from exon 1 heterozygotes revealed differences in oligomer patterns compared with WT. MBL from HYPD/HYPA individuals showed closest resemblance to WT and contained mainly trimers and tetramers corresponding with good complement function (Figure 6.8). One LYQA/HYPD subject appeared to produce some tetramer but exhibited low function (Lane 3; Figure 6.8). Weak bands of tetramer size were observed in AD subjects with a XY promoter (Figure 6.8) and other heterozygotes with a YY promoter which corresponded with markedly low complement function (Figure 6.9). The results of samples from heterozygotes with a XY promoter (except XAYD, Figure 6.8), with very low MBL concentration were the most difficult to interpret due to poor clarity of the trimer band (Figure 6.9). Uninterpretable results were obtained from homozygotes (data not shown).



Haplotype combination	HYPD HYPA		LYQA HYPD	LXPA HYPD		
MBL level (ng/ml)	2601	2332	1414	519	843	664
Wieslab function (%)	70	121	2	2	21	7

Figure 6.8 MBL oligomers from subjects with an AD genotype.
Corresponding MBL level and functional activity is shown for 6 individuals.



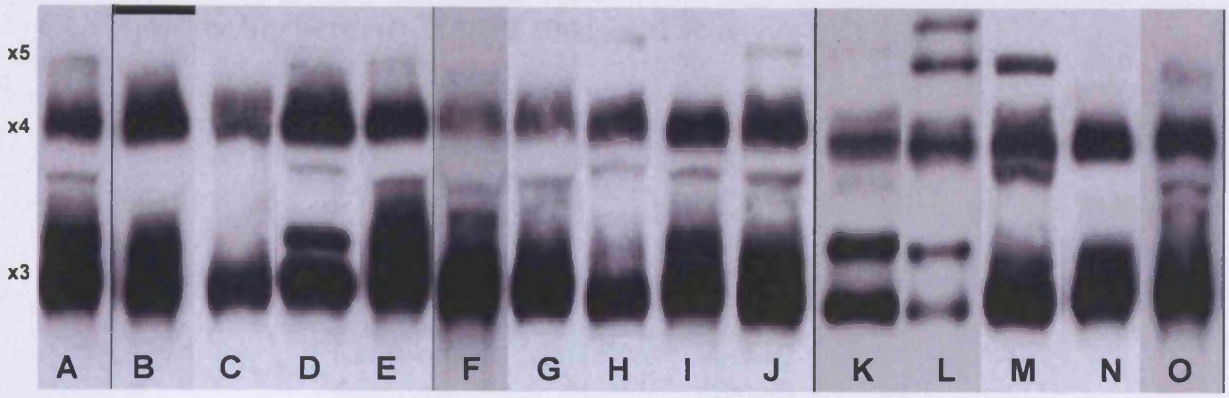
Haplotype combination	LYQC HYPA				LYQA LYQC				LYPB HYPA				LXPA LYPB
MBL level (ng/ml)	665	644	341		344	674	576		597	549	557		152
Wieslab function (%)	2	4	1		1	4	19		7	<1	6		<1

Figure 6.9 MBL oligomeric structure in MBL2 variant allele heterozygotes. Corresponding MBL level and functional activity is shown for 10 individuals.

Overall, with the exception of YAYD heterozygotes, the absence of tetramer correlated very well with low complement activating function as assessed by the Wieslab screen (Lanes 4-6 Figure 6.8 and Figure 6.9). The presence of a strong trimer band however was not indicative of good functional capacity.

6.2.4.2 Analysis of *MBL2* WT individuals with MBL level > 1000ng/ml but low functional activity as assessed by the Wieslab assay

Figure 6.10 shows MBL oligomer structure in WT individuals who exhibited low functional activity. MBL tetramers were observed in all samples tested. Trimer and tetramer bands were clearly saturated making quantification impossible. The MBL oligomer patterns observed in these individuals did not appear to explain their low functional activity unlike the patterns observed in certain heterozygotes.



Subject	MBL genotype	MBL level (ng/ml)	MBL lectin pathway (%)
A	HYPA/HYPA	2240	0.5
B	LXPA/LYQA	1104	0.9
C	LYQA/HYPA	5569	1.0
D	LYQA/HYPA	1990	1.0
E	LXPA/HYPA	1421	1.1
F	LXPA/LYPA	1120	2.2
G	LXPA/HYPA	1419	4.0
H	LXPA/LYQA	2689	6.5
I	LXPA/HYPA	2525	7.5
J	HYPA/HYPA	3098	8.5
K	HYPA/HYPA	2377	8.5
L	HYPA/HYPA	6189	8.8
M	LXPA/HYPA	3761	9.5
N	LXPA/HYPA	1646	9.8
O	LXPA/HYPA	2806	10

Figure 6.10 MBL oligomeric structure in exon 1 WT individuals (MBL protein level >1000ng/ml) with low Wieslab functional activity.

This prompted the investigation of other components of the lectin pathway which may be contributing to the low functional activity. As discussed, MBL works in concert with MASPs. At present, there is no assay available for MASP1 quantification; assays are available for MASP2 and MASP3. The role or significance of the latter is unclear, although there is a suggestion that it may act as a competitive inhibitor of MASP1 and MASP2 activity (Dahl et al., 2001; Moller-Kristensen et al., 2007). Additional functional assays were also used to assess function at different levels in the MBL lectin pathway in order to investigate the influence of specific MASP deficiencies and assess their correlation with the results obtained using the Wieslab screen.

6.2.5 Relationship between MASP2 levels and genotype

Levels of MASP2 in relation to MBL exon 1 genotype are shown in Table 6.4. The mean MASP2 level for the cohort was 442 ng/ml. 20 (11%) individuals had MASP2 levels <200ng/ml.

Exon 1 genotype		**MASP2 (ng/ml)**	
	N	Mean	Range
All subjects	183	442	54-1474
AA	103	378	54-1294
AO	73	527	115-1474
AB	36	584	138-1252
AC	13	483	222-839
AD	24	465	116-1474
OO	7	496	239-834

Table 6.4 Mean and range MASP2 levels stratified by MBL exon 1 genotype

MASP2 levels were noted to be lower in the *MBL2* WT individuals when compared to the hetero and homozygotes but this was not statistically significant. No clear relationship was observed between MBL and MASP2 levels (r^2=0.04) (Figure 6.11).

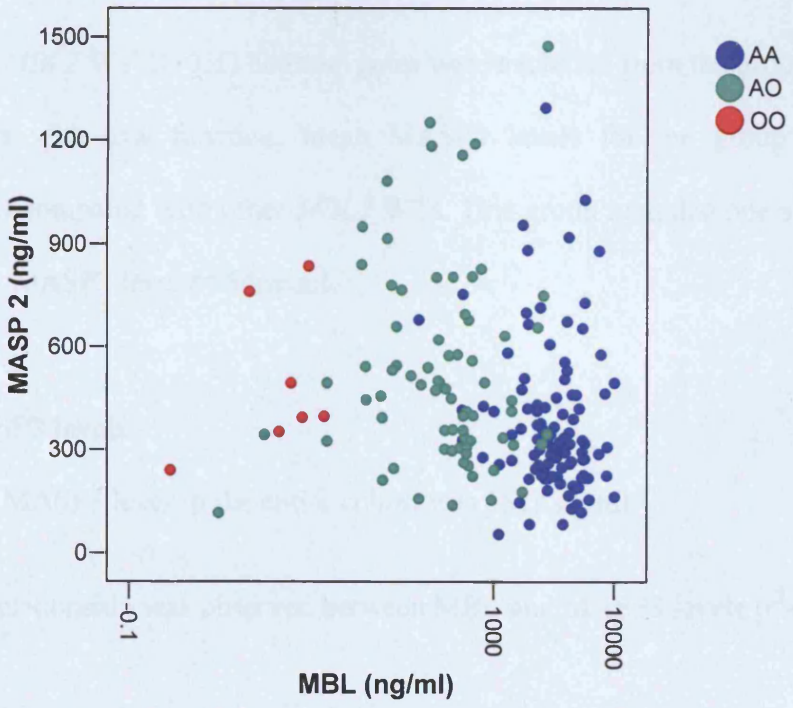


Figure 6.11 MBL serum concentrations plotted against MASP2 levels.

Of note, mean MASP2 levels in the 15 *MBL2* WT subjects with low Wieslab function were 197ng/ml, compared to 378ng/ml in the total WT group.

6.2.5.1 MASP2 SNP analysis

The total patient population (n=283) as described in Chapter 4 was genotyped for a well known SNP in exon 3 of the MASP2 gene, D105G. This work was performed in collaboration with Innogenetics NV, Belgium. This SNP prevents MASP2 forming complexes with MBL and the ficolins (Sorensen et al., 2005). There were no homozygotes found but 15 heterozygotes were identified, giving an allele frequency of 5.3%.

Serum samples were available on 6 of the 15 heterozygotes and their mean MASP2 level was 129ng/ml (range 83-162) compared to 452 ng/ml (range 54-1474) in the MASP2 D105G WT individuals. Of the 6 MASP2 D105G heterozygotes in this study population, 3 were *MBL2* WT and 3 were heterozygotes. Two of the *MBL2* WT individuals had low MBL lectin pathway function and one had very high function.

When the *MBL2* WT/D105G heterozygotes were excluded from the group of *MBL2* WT individuals with low function, mean MASP2 levels for the group remained low (215ng/ml) compared with other *MBL2* WTs. This group included one subject who had a very low MASP2 level of 54ng/ml.

6.2.6 MASP3 levels

The mean MASP3 level in the entire cohort was 6518 ng/ml.

No clear relationship was observed between MBL and MASP3 levels (r^2=0.067) (Figure 6.12).

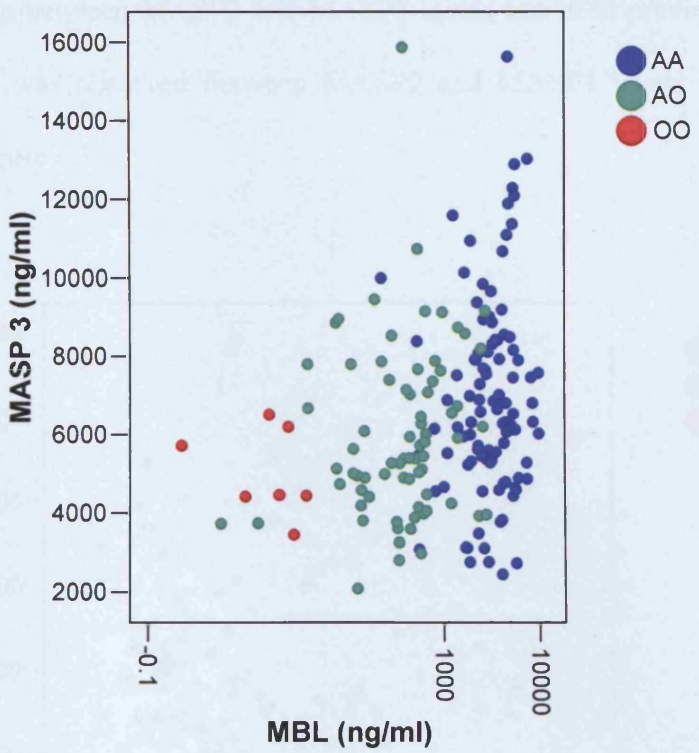


Figure 6.12 MBL serum concentrations plotted against MASP3 levels.

When stratified by MBL exon 1 genotype the highest MASP3 levels were noted in the *MBL2* WT individuals and the lowest in the homozygotes, in contrast to MASP2 (Table 6.5). Mean MASP3 levels in the 15 *MBL2* WT subjects with low function were 7302ng/ml, compared to 6975ng/ml in the total *MBL2* WT group.

Exon 1 genotype		**MASP3 (ng/ml)**	
	n	mean	Range
All subjects	183	6518	2089-15880
AA	103	6975	2445-15638
AO	73	6014	2088-15880
AB	36	6197	2088-15880
AC	13	4973	2806-9150
AD	24	6305	3725-9162
OO	7	5035	3452-6520

Table 6.5 Mean and range MASP3 levels stratified by MBL exon 1 genotype

No relationship between MASP2 and MASP3 levels has been previously reported and no correlation was observed between MASP2 and MASP3 levels in this population (r^2=0.052) (Figure 6.13).

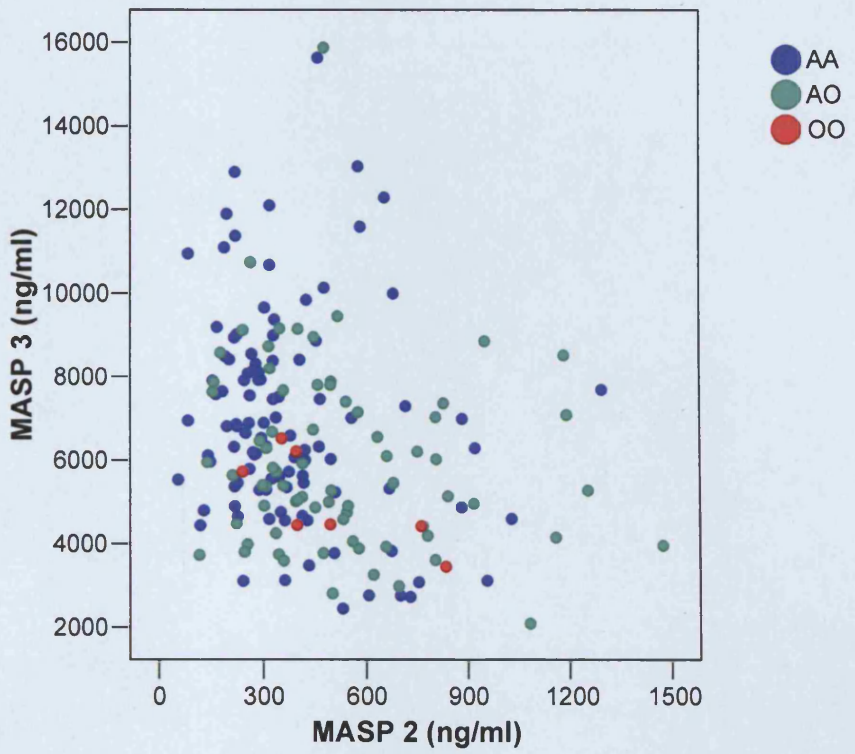


Figure 6.13 MASP2 levels plotted against MASP3 levels

6.2.7 MBL/MASP2 complex activity- C4b deposition assay

This assay assesses the first step in MBL complement activation i.e. whether MBL/MASP2 complexes in serum are able to cleave complement component C4, reported to be unaffected by lack of MASP1 and MASP3 (Moller-Kristensen et al., 2007). The results shown in Table 6.6 are from *MBL2* WT subjects and a subset of heterozygotes with sufficient MBL levels. The levels of activity followed a similar trend to MBL levels, i.e. AA>AD>AC>AB. The additional influence of the XY promoter is illustrated in Figure 6.14.

Exon 1 genotype	MBL/MASP2 complex activity- C4b deposition (munits/ml)		
	n	mean	range
All subjects	156	694	54-3476
AA	102	872	138-3476
AO	54	357	54-2870
AB	23	218	54-440
AC	12	266	62-394
AD	19	581	68-2870
OO	-		

Table 6.6 Mean and range values for MBL/MASP2 complex activity stratified by MBL exon 1 genotype

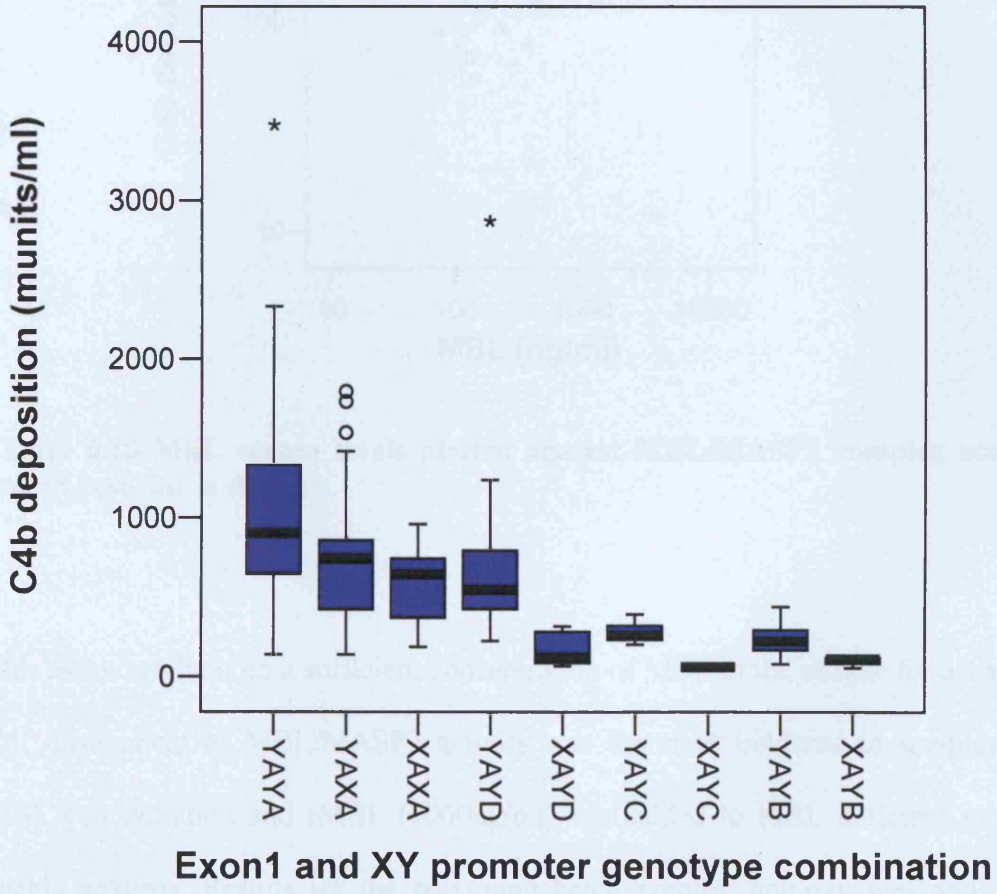


Figure 6.14 MBL/MASP 2 complex activity (median indicated by a line, boxes represent interquartile range and bars outliers) stratified according to Exon 1 and XY promoter genotype

Marked variation in activity was observed among WT individuals and activity in YAYD individuals was noted to be higher than other heterozygotes with the YY promoter. A scatterplot of MBL level against C4b deposition shows good correlation (r^2=0.817) between level and activity (Figure 6.15).

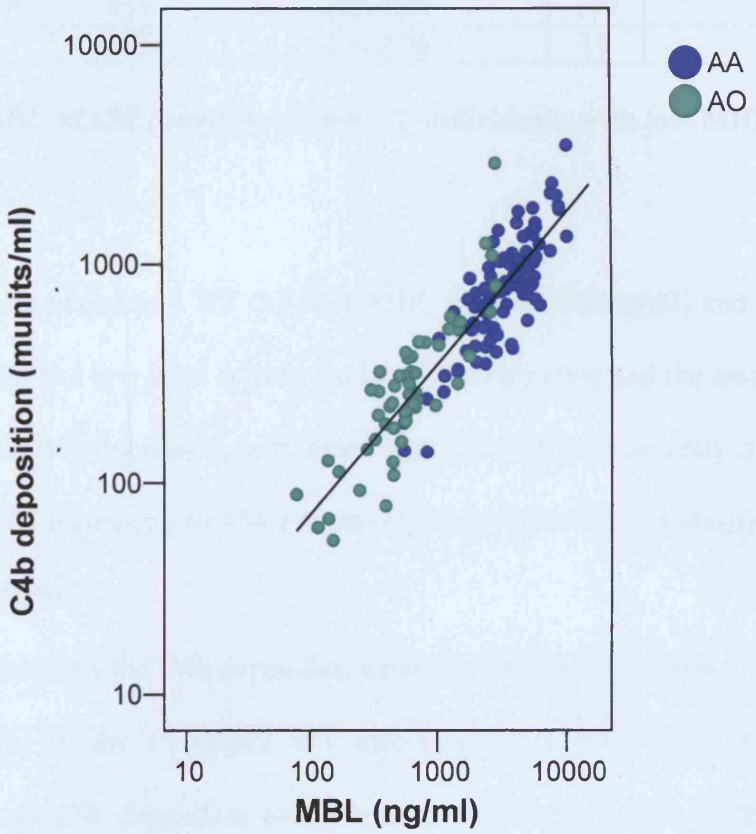


Figure 6.15 MBL serum levels plotted against MBL/MASP2 complex activity. A line of best fine is shown

This assay is reliant on a sufficient concentration of MBL in the sample for activation of C4. Assessment of MBL/MASP2 activity was therefore hindered in samples of low MBL concentration and rMBL (1000ng/ml) was added to MBL deficient samples to enable analysis. Results for the remaining heterozygotes, homozygotes and one WT individual whose MBL levels were low requiring addition of rMBL are shown in Table

6.7. The levels of activity observed in the homozygotes were similar to heterozygote levels shown in Table 6.6.

Exon 1 genotype	MBL/MASP2 complex activity- C4b deposition (munits/ml) +rMBL			MBL level (ng/ml)	
	n=	Mean	Range	mean	range
AA	1	542	-	235	-
AO	19	454	182-800	179	4-843
OO	7	382	314-536	19	1-37

Table 6.7 MBL/MASP2complex activity in individuals with low MBL levels

rMBL was also added to 3 WT (all with MBL levels <1000ng/ml) and 16 heterozygote samples which had low level activity on initial investigation and the assay was repeated. In all cases activity increased, with mean activity on the initial assay of 128 munits/ml (range 54-192) increasing to 454 munits/ml (range 306-642), implicating low MBL as the limiting factor.

Correlation between the C4b deposition assay and the Wieslab assay was good, r^2=0.76 (Figure 6.16). Of the 15 *MBL2* WT individuals with low Wieslab lectin pathway function, mean C4b deposition was 466munits/ml, compared to 872munits/ml in the total WT population. The plot (Figure 6.16) clearly identifies a WT individual with low Wieslab activity but high C4b deposition (872munits/ml); this is the individual with low classical pathway activity (marked with a red arrow). This confirms that MBL/MASP2 complex activity is good and that the defect is likely to be at the C4/C2 level. When this individual is removed from the analysis the mean C4b deposition remains low (437munits/ml) for the *MBL2* WT/low Wieslab cohort.

Interestingly poor correlation was noted between MASP2 levels and C4b deposition, r^2=0.028, data not shown.

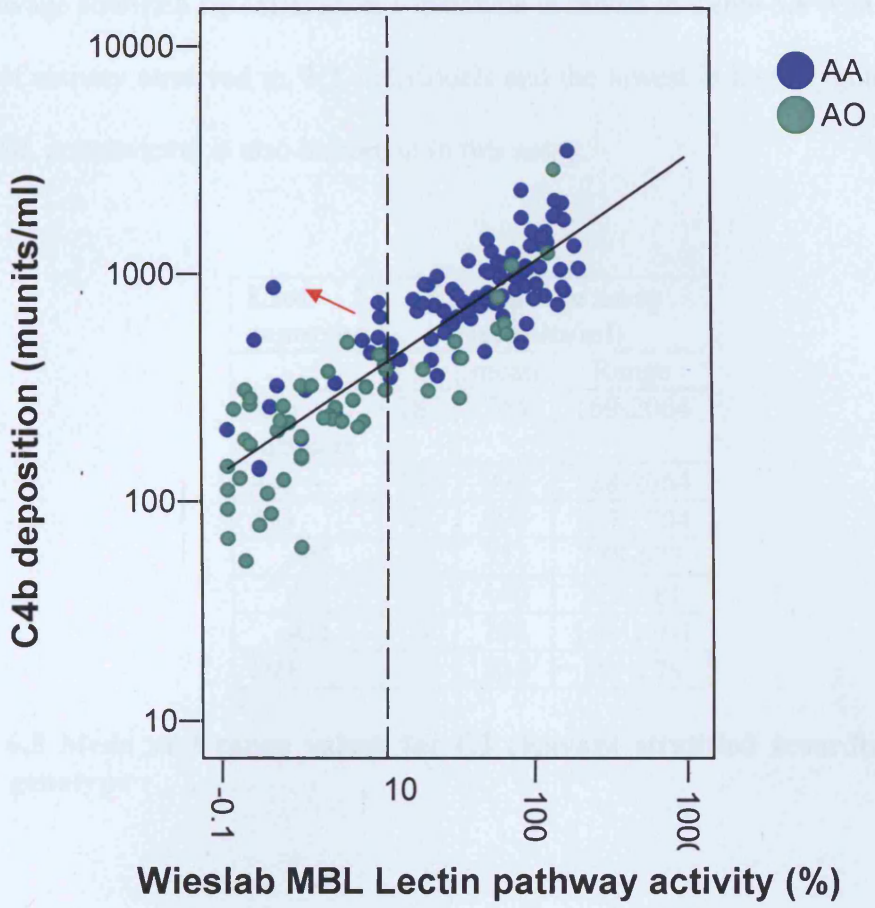


Figure 6.16 X/Y plot comparing MBL/MASP2 complex activity versus Wieslab MBL lectin pathway activity.
The vertical dashed line marks 10% activity as measured by the Wieslab assay. A line of best fit is shown. The red arrow represents individual with presumed deficiency at C4/C2 level.

6.2.8 C3 cleavage assay- C3b deposition

This assay assesses the ability of MBL complexes to cleave C3, detected by the presence of C3b, reflecting the influence of MASP2 and also MASP1 within the lectin pathway. It is newly developed and as yet there is no published data utilizing this assay. C3 cleavage stratified by MBL exon 1 genotype is shown in Table 6.8 with the highest levels of activity observed in WT individuals and the lowest in homozygotes implying that MBL protein level is also important in this assay.

Exon 1 genotype	**C3 cleavage assay (munits/ml)**		
	n	mean	Range
All subjects	183	743	169-2064
AA	103	994	188-2064
AO	73	496	169-1704
AB	36	352	188-627
AC	13	440	222-681
AD	24	705	169-1704
OO	7	226	181-279

Table 6.8 Mean and range values for C3 cleavage stratified according to MBL exon 1 genotype

The additional effect of the XY promoter is illustrated in Figure 6.17. Marked variability in levels of activity was observed in WT individuals. In the heterozygotes, the XY promoter had the most profound effect in AD individuals, in keeping with results from the other assays.

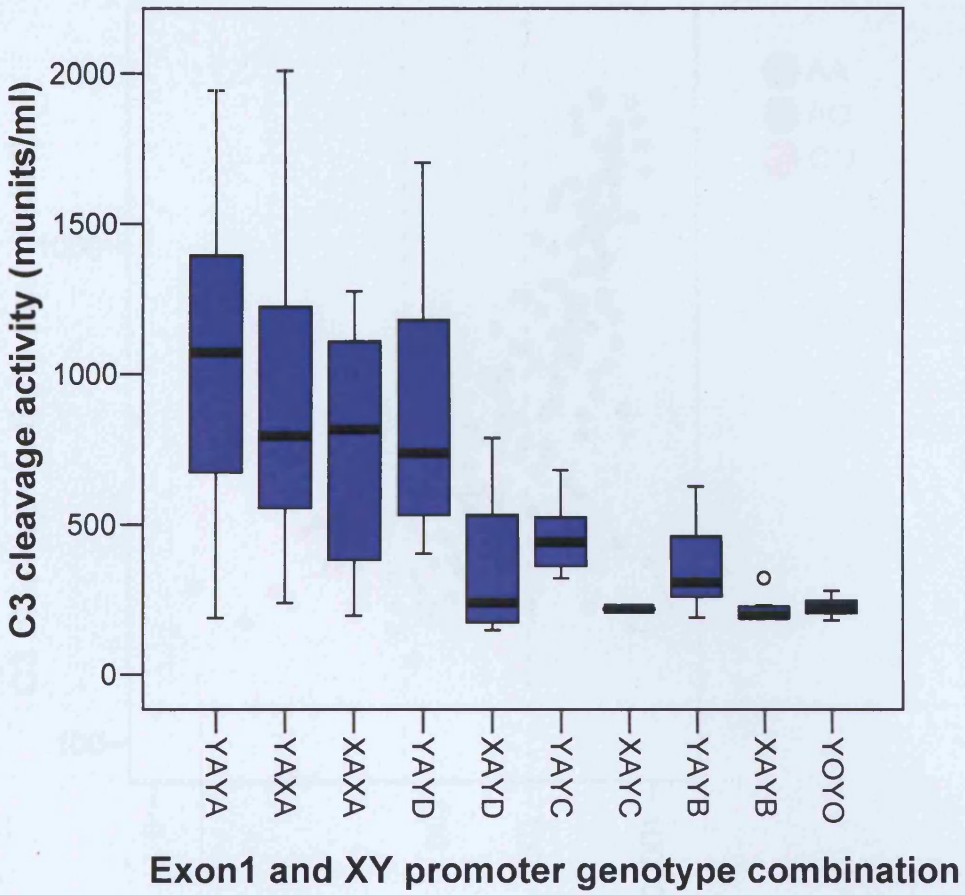


Figure 6.17 C3 cleavage activity (median indicated by a line, boxes represent interquartile range and bars outliers) stratified according to Exon 1 and XY promoter genotype

Potential relationship between MBL level and C3 cleavage is shown in Figure 6.18 (r^2=0.65). The scatter plot displays two WT outliers with relatively high MBL levels but low C3 activity who were not identified by the C4b deposition assay. This implies that their MASP2 function is adequate but that they may be MASP1 deficient.

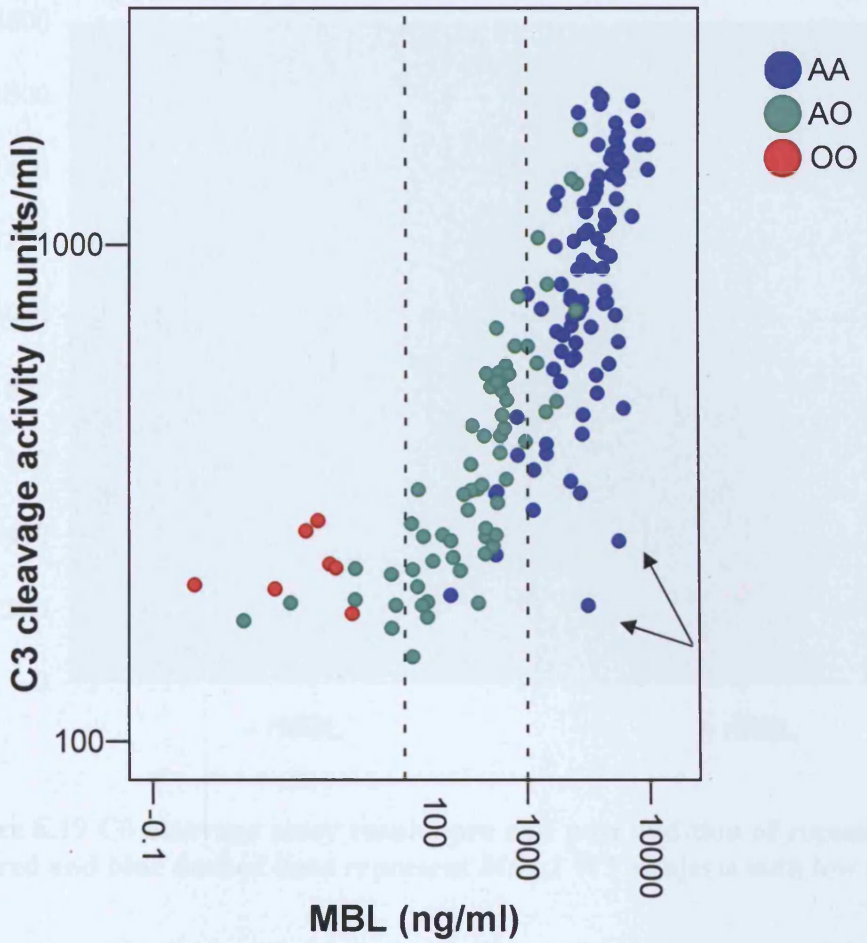


Figure 6.18 MBL levels plotted against C3 activity on a log scale. The vertical dashed lines mark MBL levels of 100ng/ml and 1000ng/ml. Outliers are marked with an arrow.

rMBL was also added to 66 samples with low C3 cleavage activity to override the influence of low MBL within the assay system. The majority of these samples were from homo and heterozygotes but also included 10 of the 15 *MBL2* WT subjects with low Wieslab lectin pathway activity. Figure 6.19 shows the change in C3 cleavage following the addition of recombinant MBL. The mean percentage increase in C3 cleavage was 55% (IQR 45-67).

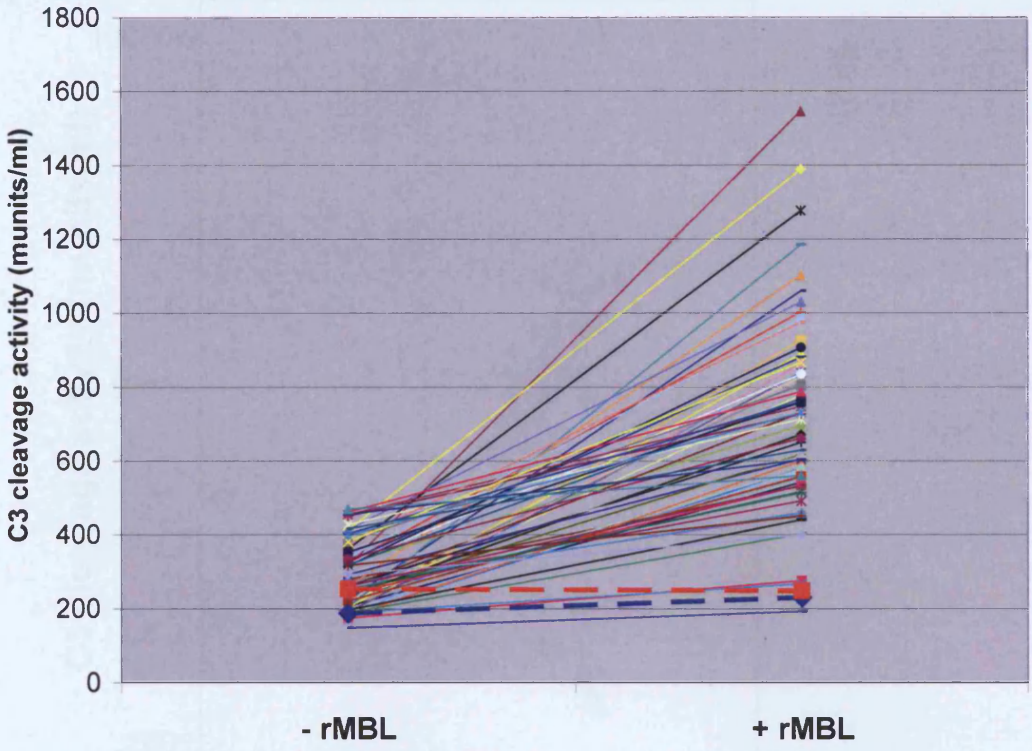


Figure 6.19 C3 cleavage assay results pre and post addition of recombinant MBL. The red and blue dashed lines represent *MBL2* WT subjects with low function.

Interestingly 2 of the 10 WT individuals with low function showed minimal increments following the addition of rMBL (red and blue dashed lines). In particular the subject identified as having low lectin and classical pathway activity showed no increase at all (shown as a red dotted line). The blue dashed line represents a subject who may be MASP1 deficient. These individuals are also highlighted as outliers (marked with red and blue arrows, respectively) when the C4b deposition assay was plotted against the C3 cleavage assay results, r^2=0.77 (Figure 6.20). Interestingly, these individuals would not have been detected using the C4b deposition assay alone. The yellow arrow identifies the individual with a low MASP2 level who was not heterozygous for the D105G polymorphism who also has relatively low C3 cleavage activity and C4b deposition.

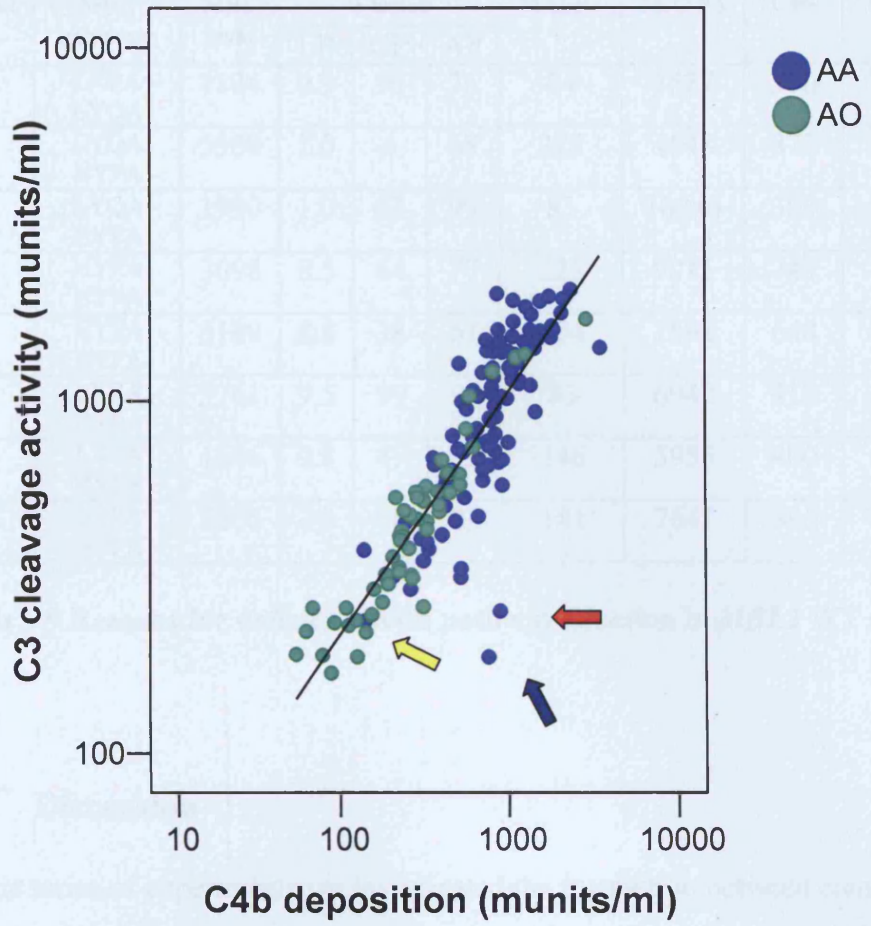


Figure 6.20 X/Y plot comparing results obtained from the MBL/MASP2 complex activity assay and the C3 cleavage activity assay. A line of best fit is shown. The yellow, red and blue arrows highlight *MBL2* WT subjects of interest.

On review of the cumulative results of the different assays utilized we were able to propose a reason for deficient function in 8 of the 15 *MBL2* WT subjects with low Wieslab function (Table 6.9).

Subject	MBL genotype	MBL level	Wieslab			MASP2	MASP3	C4b	C3b	Defect
			LP	CP	AP					
B	LXPA LYQA	1104	0.9	50	76	54	5527	260	293	Low MASP2
C	LYQA HYPA	5569	1.0	4	68	225	4648	872	254	C2/C4, low classical
D	LYQA HYPA	1990	1.0	63	99	83	10946	300	407	D105G heterozygote
J	HYPA HYPA	3098	8.5	64	77	223	9015	748	188	?MASP1 deficiency
L	HYPA HYPA	6189	8.8	38	61	154	7898	648	470	Low MASP2
M	LXPA HYPA	3761	9.5	99	95	83	6942	412	504	D105G heterozygote
N	LXPA HYPA	1646	9.8	47	72	146	5955	400	563	Low MASP2
O	LXPA HYPA	2806	10	66	93	181	7641	396	417	Low MASP2

Table 6.9 Reasons for deficient lectin pathway function in *MBL2* WT subjects

6.3 Discussion

In this series of experiments we investigated the interaction between components of the lectin pathway in a population of children receiving treatment for malignancy. We used different assays to quantify and investigate the activity of various components which has enabled detailed evaluation of potential 'deficiencies' in the lectin pathway. We found MBL to be a major contributor and our results also highlight other deficiencies that can compromise MBL's activity.

MBL levels for the study group were in keeping with other paediatric populations (M.Johnson, Institute of Child Health, London [personal communication], Neth et al., 2001). Marked differences in MBL levels were observed between individuals in possession of WT and variant MBL alleles which influenced subsequent function. We also confirmed the influence of the XY promoter (Madsen et al., 1995; Minchinton et al., 2002). Its effect was evident on both protein levels and functional activity and of particular note in AD individuals where the largest differences were seen between the

YY and XY groups (Figure 6.2 & 6.4). The influence of the H/L and P/Q polymorphisms was minimal in comparison.

The Wieslab total complement screen assay enabled us to assess MBL lectin, classical and alternative pathway activity simultaneously, thus offering greater insight into potential deficiencies of complement components which participate in more than one pathway. The semi-quantitative nature of the Wieslab assay is a major disadvantage and at present should be viewed primarily as a 'screening' methodology. Despite lack of sensitivity a relationship between MBL lectin pathway functional activity and MBL levels was observed, further variability in functional activity between *MBL2* exon 1 WT individuals was of particular interest. We identified a group of individuals who in the majority of studies to date would have been presumed to have good MBL activity as assessed by genotype and/or protein level. This study showed that despite a WT genotype and MBL level of >1000ng/ml, deficiency in MBL lectin pathway complement function can exist (Figure 6.5), thus highlighting deficiencies in other components of the pathway e.g. MASPs (Figure 6.21). Our findings are in agreement with Carlsson *et al* who also noted marked variation in pathway activity in MBL sufficient control and diseased individuals (Carlsson et al., 2005). However the data contrast with results from another study of adult blood donors where all individuals with MBL levels >1000ng/ml had >10% pathway activity (Seelen et al., 2005). Analysis of the classical and alternative pathways allowed us to identify one *MBL2* WT subject with low function who also had apparent deficiency in the classical pathway implicating a defect at the C2/C4 level. However, more individuals with a similar phenotype need to be identified to confirm our findings of one individual. The majority of subjects had sufficient classical and alternative pathway activity which may be compensating for MBL deficiency. We were interested to explore the sensitivity of the three assays and

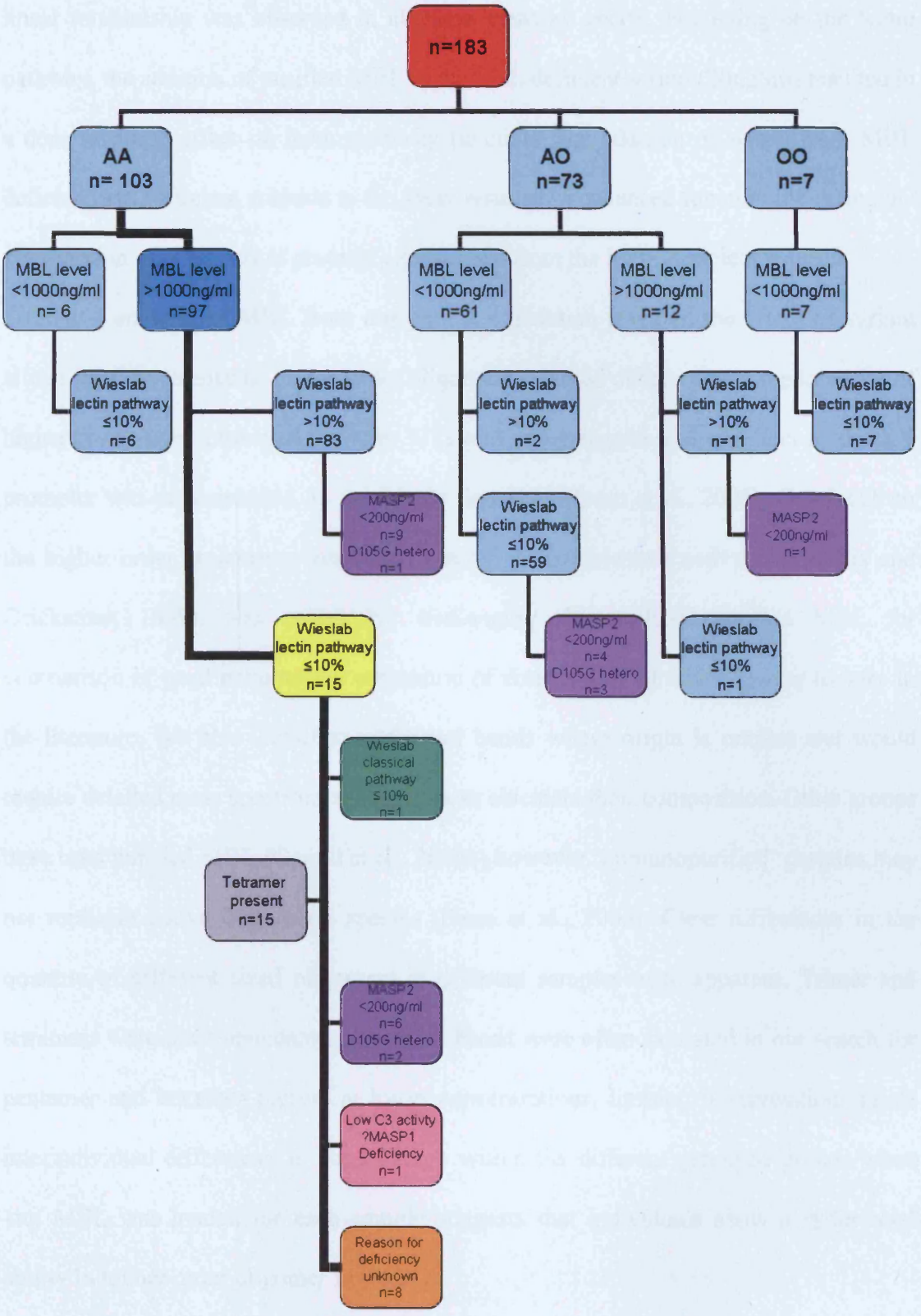


Figure 6.21 Flow chart illustrating investigation of *MBL2* WT subjects with low lectin pathway activity as assessed by the Wieslab screen.
Subjects with low MASP2 levels are also shown.

performed a number of preliminary experiments in order to investigate this. A non-linear relationship was observed in all three pathway assays. Focussing on the lectin pathway, the addition of purified MBL to an MBL deficient serum (20ng/ml) resulted in a dose response effect on lectin pathway function. The addition of serum from MBL deficient and sufficient subjects to the assay resulted in enhanced function indicating the contribution of other serum proteins e.g. MASPs from the MBL deficient subject.

Structural analysis of MBL from our patient population revealed the effect of variant alleles on the presence of higher order oligomers. Marked differences in the intensity of higher bands were observed between WTs and heterozygotes and an effect of the XY promoter was demonstrated, as previously described (Dean et al., 2005). Our focus on the higher order structures, considered crucial for complement activation (Wallis and Drickamer, 1999), was technically challenging. We utilized purified MBL for comparison of band patterns and estimation of size, reports of which appear to vary in the literature. We also identified additional bands whose origin is unclear and would require detailed mass spectrometry analysis to elucidate their composition. Other groups have used purified MBL (Garred et al., 2003b) however 'immunopurified' proteins may not replicate native oligomeric species (Dean et al., 2005). Clear differences in the quantity of different sized oligomers in different samples were apparent. Trimer and tetramers were most abundant hence these bands were often saturated in our search for pentamer and hexamer present at lower concentrations, limiting interpretation. These interindividual differences in band pattern within the different genotype groups when 1ng MBL was loaded for each sample suggests that individuals show a differential ability in higher order oligomer formation.

Possession of the B and C variant alleles had the most profound effect on structure, levels and function which is in keeping with previous reports (Madsen et al., 1995; Minchinton et al., 2002). Codon 54 (B) and 57 (C) variants result in glycine substitution

which disrupts the Gly-Xaa-Yaa structure in the triple collagenous helix, an effect likely to have profound effects on protein stability (Sumiya et al., 1991; Larsen et al., 2004). Not surprisingly this mutation also appears to influence binding of MASPs (Wallis et al., 2004; Wallis et al., 2005). Dean *et al* observed differences in oligomer pattern in heterozygotes in possession of the B allele compared to those in possession of the C allele and proposed a unique model of their structure and effective MASP binding sites (Dean et al., 2005). We did not observe such marked variation between our AB subjects compared to ACs which is in agreement with other reports (Heise et al., 2000). In contrast, individuals in possession of D variant alleles appeared to have consistently higher MBL levels and functional capacity. Structurally at codon 52 (D), an arginine is substituted with a cysteine which has been reported to cause the formation of adventitious disulphide bonds (Wallis and Cheng, 1999). The size difference caused by the substitution is also proposed to affect the stability of the molecule leading to accelerated degradation (Madsen et al., 1994). In addition, it has been suggested that variant D chains may combine with normal A chains enabling formation of stable higher order oligomers (Garred et al., 2003b). This was evident in our study as serum from some AD individuals showed tetramer and pentamer formation, but whether these complexes are composed solely of normal A chains or a mix of A and D chains is unknown. In AB and AC heterozygotes, the presence of trimer and minimal tetramer was noted but whether it consists of mixed chains also remains unclear (Wallis and Cheng, 1999).

An apparent lack of higher order oligomers did not appear to account for the low functional activity in the group of *MBL2* WT subjects identified by the Wieslab MBL lectin assay. This led us to investigate the role of MASPs within the system. As described previously we were unable to quantify MASP1. Mean MASP2 level in the study population (442 ng/ml) was similar to that reported in 350 Danish blood donors

(mean = 440ng/ml, range 125-1152, Dr S Thiel, personal communication). Studies measuring MASP2 levels have been performed in adult populations but there are no published cohorts of healthy children to date. Further studies are required to compare levels to age matched controls in order to investigate any paediatric or disease specific variation. Ytting and colleagues reported higher MASP2 levels in adults with colorectal cancer when compared to controls (415ng/ml and 368ng/ml respectively) and somewhat unexpectedly found that MASP2 level had independent prognostic value for survival (Ytting et al., 2005). Like MBL, MASP2 mRNA is found primarily in the liver with expression in the small intestine and testis also noted (Seyfarth et al., 2006). It is unknown how levels are regulated during illness.

MASP2 binds to both MBL and Ficolins and it was therefore no surprise that we were unable to demonstrate a clear relationship between MBL and MASP2 levels. The use of a high ionic strength buffer and the presence of EDTA in the assay ensures that MASP2 dissociates from MBL and Ficolins, i.e. to enable measurement of MASP2 in isolation (Moller-Kristensen et al., 2003). It is currently unknown exactly what proportion of MASP2 circulates free in serum (if any) (Thiel et al., 2000; Moller-Kristensen et al., 2003) and whether it exhibits equal binding to both MBL and Ficolins. The affinity of MASP2 for different sized oligomers and whether it binds equally to variant chains also remains uncertain (Chen and Wallis, 2001; Dean et al., 2005). This would also explain the lack of correlation between MASP2 levels and C4b deposition in our cohort.

Genotyping of the D105G mutation in exon 3 of the MASP2 gene revealed a variant allele frequency of 5.3%, comparable to an allele frequency of 3.6% reported for healthy Danish volunteers (Sorensen et al., 2005). Carlsson *et al* investigated a cohort of 112 Swedish cystic fibrosis patients, of which 44 were under the age of 18, and reported an allele frequency of 6.3% in patients and 1.3% in adult blood donors (Carlsson et al., 2005). In the same cohort minimal differences in levels were observed between the

patients and controls for both D105G heterozygotes (157ng/ml and 166ng/ml respectively) and WTs (380ng/ml and 352ng/ml respectively). The D105G heterozygotes identified in our study also had markedly lower levels than WT individuals (129ng/ml and 452ng/ml respectively).

The influence of the MASP2 D105G mutation on function using the different assays was variable in the 6 individuals who were heterozygous. The three *MBL2* WT/D105G heterozygotes had C4 activity of 300, 412 and 1332 munits/ml with MBL levels of 3761, 1990 and >10,000 ng/ml respectively. The polymorphism did not appear to influence activity in the subject with a very high MBL level but may have had an effect on MBL/MASP2 complex activity in the other two subjects who display levels similar to those observed in MBL heterozygotes (Table 6.6). The 3 *MBL2* heterozygote/D105G heterozygotes had C4 activity of 178, 226 and 232munits/ml (after addition of recombinant MBL) with MBL levels of 451, 957 and 234ng/ml respectively. Interpretation of these levels is limited as numbers were small and MBL level obviously influenced results, making interpretation of activity from joint MBL and MASP2 (D105G) heterozygotes difficult, warranting further studies. Overall 15 *MBL2* WT subjects had MASP2 levels <200ng/ml, of which 6 had low functional activity. There were individuals with low MASP2 levels and low functional activity who were not heterozygous for the D105G mutation, suggesting other mutations within the MASP2 gene may also modulate expression (Lozano et al., 2005; Thiel et al., 2007). There were also individuals with low MASP2 levels but good pathway function which may reflect the contribution from MASP1 (Moller-Kristensen et al., 2007).

Our knowledge of MASP3 function remains limited. Production of a specific monoclonal antibody to MASP3 has enabled measurement of serum levels but there are no published data on MASP3 levels. The only data available are on 350 healthy Danish

controls (Dr S Thiel, personal communication) and on comparison, MASP3 levels were found to be higher in our paediatric oncology cohort (mean 4193ng/ml and 6518ng/ml respectively). These levels are approximately 10 fold higher than MASP2 levels, reasons for which are unknown. Measurement of MASP3 in other populations is required in order to attempt any interpretation of our finding. No clear relationship was observed between MASP2 and 3 levels; there may be a relationship between their function if MASP3 is involved in inhibition of MASP2 activity, but current assays cannot confirm this. A relationship between MASP2 and MASP1 enzyme activity has been reported, implicating an inverse relationship between the two (Mayilyan et al., 2005). Evidence for the presence of separate populations of MBL/MASP complexes which may also have unique MASP binding sites is growing (Teillet et al., 2007). The interaction between the three MASPs and also MAp19 and their affinity for MBL requires further investigation.

Assessment of complement activation by MBL using the C4b deposition assay has long been the method of choice for quantifying MBL functional activity, but the assay only assesses the first step in the activation pathway, providing no information on downstream events. C4b deposition in our WT subjects with low MBL lectin pathway function was also low in comparison with their WT peers, but without definitions of deficiency this is difficult to interpret. The C3 cleavage assay was designed to assess the activity of MBL, MASP2 and MASP1 (Moller-Kristensen et al., 2007). This assay should also reflect the potential inhibitory activity of MASP3 and therefore provides a more comprehensive measure than C4b deposition. Overall, good correlation between the three functional assays was evident. By plotting individual results together we were able to identify a small number of individuals with clear defects at specific levels of the cascade. The effect of low (or high) MASP levels in otherwise MBL sufficient individuals was difficult to analyse. We have identified an individual with possible

MASP1 deficiency who had low C3b deposition (despite addition of rMBL) but good C4b deposition. However, we are also left with a number of subjects with more subtle 'deficiencies' or 'excesses' e.g. MASP3, which are likely to be influencing the Wieslab screening assay results. We did not measure C2, C4 or C3 levels and it is possible that these components may also be contributing to low function in some individuals but this would have been detected by the total Wieslab screen. It should be noted that Ficolins also activate complement via the lectin pathway although these assays are presumed to be MBL specific because they rely on binding to a mannan coated surface. There has been a recent report which suggests that Ficolins may be able to bind mannan in specific conditions (Sung et al., 2006) and further studies are required to improve our understanding of their contribution.

The Wieslab MBL lectin pathway screen identified all patients (except 2 heterozygotes) with MBL levels <1000ng/ml as deficient and from our in depth investigation it also identified WT subjects with potentially low MASP levels as having deficient function.

In summary, in this study we attempted to gain further insight into the complexities of MBL lectin pathway function. Our findings raise important issues of how to classify patients in disease association studies as we have identified a group of *MBL2* WT individuals with MBL levels in the normal range but yet exhibited deficient complement function. These results also provide evidence that MBL from AD individuals (specifically YAYD) behaves more like WT than other heterozygotes in the functional assays used. Complement activation is not the only function of MBL but in certain disease processes this may well be its principal role and therefore analysis using functional assay data may prove more relevant than genotype or MBL protein level. A number of questions remain unanswered in relation to the role of Ficolins in the pathway and the true impact of MASP deficiencies. Investigations in other healthy and

diseased populations will improve our knowledge of these different MASP 'abnormalites'.

Chapter 7

Study of MBL structure and lectin pathway function in response to chemotherapy and during febrile neutropenia

7.0 Introduction

The regulation of MBL production and function during disease remains poorly understood. Unlike the classic acute phase protein, CRP whose levels increase up to 1000 fold in response to infection (Gabay and Kushner, 1999), changes in MBL level are minimal, increasing between 1.5 and 3 fold (Thiel et al., 1992; Van Till et al., 2006). A recent study investigating MBL acute phase activity in patients admitted with sepsis and septic shock observed that 41% of individuals maintained consistent MBL levels throughout their admission and 31% demonstrated an actual positive acute phase response (≥25% increase), the majority of whom were wildtypes. The remaining patients demonstrated a negative acute phase response (≥25% decrease) (Dean et al., 2005).

A limited number of studies have investigated changes in MBL protein level in response to chemotherapy and during subsequent FN with most concentrating on its role as an acute phase reactant. (Neth et al., 2001; Frakking et al., 2006). Increases in MBL protein level were observed in children with FN, specifically in *MBL2* wildtype (AA) individuals who showed significant increases by day 7 of their febrile neutropenic episode, responses that declined by day 14 ($p=0.004$) (Neth et al., 2001). Interestingly, levels in patients with variant MBL alleles (A/O, O/O) did not alter significantly and it has been suggested that ability to increase MBL may be essential to control infection during neutropenic episodes.

A number of chemotherapy drugs are known to be hepatotoxic which may influence the regulation of MBL during chemotherapy but interestingly MBL levels in oncology patients have been reported to be higher than in controls (Bergmann et al., 2003). The effect of liver impairment on MBL production is unclear as higher levels have been reported in hepatitis (Kilpatrick et al., 2003a; Brown et al., 2007). Impact of treatment on the oligomeric nature of circulating MBL is unknown. This may be important as

higher order oligomers of rat MBL-A induce better complement activation (Wallis and Drickamer, 1999). One may therefore hypothesize that during the acute phase response increased production of higher order oligomers is likely to be beneficial, contributing to microbial clearance, whereas a reduction in response to chemotherapy might be detrimental.

In this chapter we investigated potential changes in MBL protein levels and in its oligomeric structure in response to chemotherapy and subsequent FN episodes. In addition the dual effect of MASP activity and MBL modulation on complement function was studied.

7.1 Methods

7.1.1 Patient population

This study was restricted to patients who fulfilled the inclusion criteria in section 4.1.1 and in addition whose treatment was based solely at GOSH, where they received all their shared/supportive care. Serum was collected prior to commencement of chemotherapy, on the final day of chemotherapy and days 7, 14 and 21 (as appropriate) after treatment. If patients developed FN/infection they had samples taken on day 1, 7 and 14 of the episode. Details of chemotherapy administered and neutrophil counts during the inpatient episode were also noted. Clinical information about FN/infections during the episode was recorded in conjunction with the PINE project. This study was approved by the Research Ethics committee at Great Ormond Street Hospital and the Institute of Child Health.

7.1.2 Measurement of MBL concentration

The ELISA method described in Section 2.4.3.1 was used to determine the MBL levels in serum.

7.1.3 Measurement of MASP2 levels

The TRIFMA method described in Section 2.4.3.2 was used to determine the MASP2 levels in serum.

7.1.4 Measurement of MASP3 levels

The TRIFMA method described in Section 2.4.3.3 was used to determine the MASP3 levels in serum.

7.1.5 MBL/MASP2 complex activity /C4b deposition

The C4b deposition assay described in Section 2.4.4.1 was used to investigate MBL/MASP2 complex activity.

7.1.6 C3 cleavage activity

The C3 cleavage assay described in Section 2.4.4.2 was used to investigate activation of C3 by MBL/MASP2 and MBL/MASP1 complexes.

7.1.7 Wieslab total complement screen

The Wieslab total complement screen method described in Section 2.4.4.4 was used to investigate MBL lectin, classical and alternative pathway function.

7.1.8 Western blotting of MBL in serum

This method was developed and optimised as part of this study. The final method used in these studies is described in Section 2.4.5.

7.19 Statistical analysis

Due to small sample sizes paired data was compared using Wilcoxon signed rank tests.

7.2 Results

7.2.1 Patient characteristics

Twenty one patients were recruited into the study and monitored throughout 27 courses of chemotherapy (episodes) in total. In 6 patients, 2 episodes of chemotherapy were

investigated. Samples were taken pre and post chemotherapy in every patient and additional samples were taken in those who remained as inpatients at Great Ormond Street Hospital during the episode. The majority of patients had AML and were *MBL2* wildtype (Table 7.1). Patients remained in hospital for different periods of time after chemotherapy depending on their clinical progress and therefore the number of samples collected differed between individuals and chemotherapy episodes. The specific samples used for analysis were those taken, pre and post chemotherapy and on days 1, 7 and 14 of FN.

Patient initials	**Diagnosis**	**Chemotherapy***	***MBL2* genotype**
JM	AML	ADE 2 and MIDAC	LYQA/HYPA
NH	AML	ADE 2 and MIDAC	HYPD/HYPA
JC	AML	ADE 2 and HIDAC	LYQA/HYPA
JB	AML	ADE 1 and ADE 2	LXPA/HYPA
AF	AML	ADE 2	HYPA/HYPA
KP	AML	ADE 2 and MACE	HYPD/HYPA
JD	AML	MACE	LXPA/HYPA
BC	AML	ADE 2 and MACE	LYPA/HYPA
MN	AML	MIDAC	LYQA/LYPB
GM	Rhabdoid tumour	Vincristine, Carboplatin, Cyclophosphamide, Etoposide	LXPA/LYPB
MP	Wilms tumour Stage IV	Etoposide, Carboplatin	LXPA/HYPD
DG	Hepatoblastoma	Doxorubicin, Carboplatin	LXPA/LYQA
SC	Ectomesenchymoma	VIDE	LYPB/HYPA
CYY	Stage IV Neuroblastoma	Etoposide, Cyclophosphamide, Vincristine (Day 20)	LYPA/HYPA
GK	Orbital Rhabdomyosarcoma	IVA	LXPA/HYPA
EG	Pelvic PNET (relapsed)	Etoposide, Ifosfamide	LXPA/LYPB
CJ	B NHL (Group B)	COPADM 2	LYQA/LYQC
SLP	Ependymoma	Cisplatin	LXPA/LXPA
KS	Pleuropulmonary blastoma	Doxorubicin, Vincristine	LYQA/HYPA (D105G hetero)
EMC	Wilms tumour (relapsed)	Carboplatin, Etoposide	LXPA/HYPA
VB	PNET	VIDE	LYQA/HYPA

Table 7.1 Diagnosis, chemotherapy regime and *MBL2* genotype

* Chemotherapy drug combinations:-
ADE- Daunorubicin, Cytarabine, Etoposide
MIDAC- Mitoxantrone, Cytarabine
HidAC- High dose Cytarabine
MACE- Cytarabine, Amsacrine, Etoposide

VIDE- Vincristine, Doxorubicin, Ifosfamide, Etoposide
IVA- Ifosfamide, Vincristine, Actinomycin D
COPADM- Cyclophosphamide, Vincristine, Prednisolone, Adriamycin, Hydrocortisone and Methotrexate

7.2.2 Effect of chemotherapy on the MBL lectin pathway

7.2.2.1 MBL levels

The analysis of all samples taken pre and post chemotherapy revealed no significant change in median MBL levels (Table 7.2). This was also the case when the AA and AO episodes were analysed individually.

MBL	Total group			AA episodes			AO episodes		
	n	median value (ng/ml)	p	n	median value (ng/ml)	P	n	median value (ng/ml)	p
Pre	27	2410	0.866	17	3325	0.943	10	527	0.959
Post		2487			3630			509	

Table 7.2 Changes in median MBL level in response to chemotherapy.

Episodes in patients on treatment for AML receiving similar chemotherapy were also analysed as a separate group and no significant difference in MBL levels pre and post chemotherapy were observed (data not shown). Individual fluctuations in levels are shown in Figure 7.1, illustrating the interindividual variation. Overall, levels increased during 13 episodes with a mean increase of 21% (median 12, range 2-56 %) and levels decreased in 14 episodes with a mean decrease of 17% (median 15, range 1-41%).

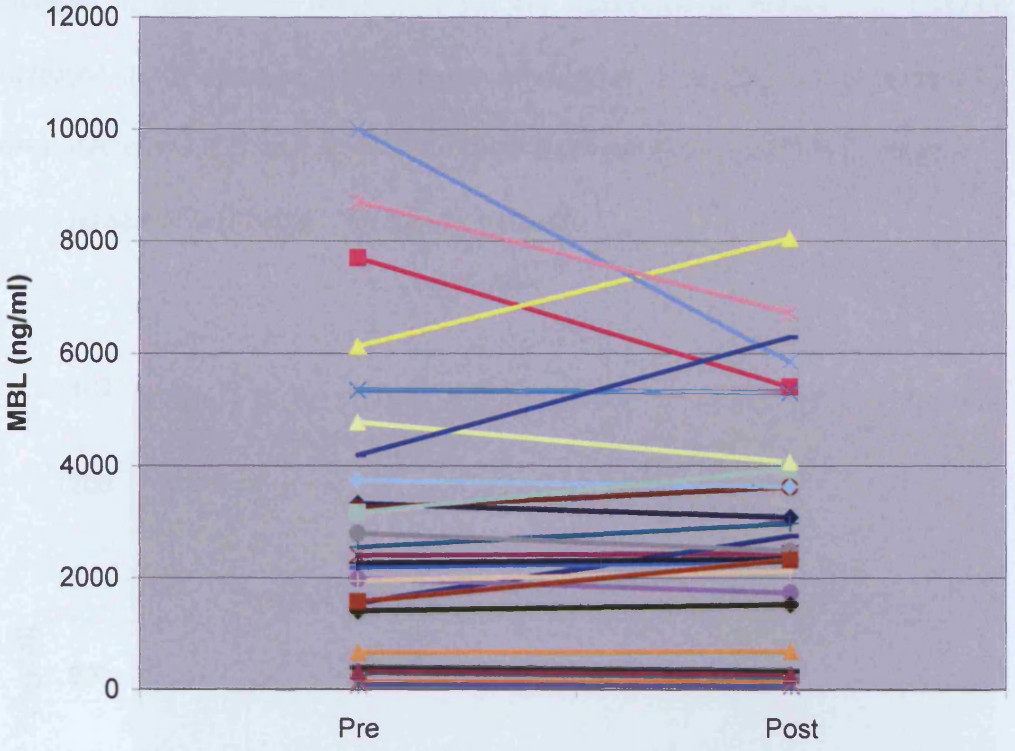


Figure 7.1 Changes in MBL level pre and post chemotherapy.
Each line represents a different patient episode.

7.2.2.2 MASP2 levels

Median MASP2 levels increased in all groups in response to chemotherapy (Table 7.3).

MASP2	Total group			AA episodes			AO episodes		
	n	median value (ng/ml)	p	n	median value (ng/ml)	p	n	median value (ng/ml)	p
Pre	27	346	**0.001**	17	307	**0.003**	10	390	**0.037**
Post		406			407			393	

Table 7.3 Changes in median MASP2 levels in response to chemotherapy.

Changes in individuals MASP2 levels are illustrated in Figure 7.2. Overall, levels increased in 21 episodes with a mean increase of 34% (median 24, range 2-153 %), levels decreased in 5 episodes with a mean decrease of 9% (median 8, range 4-14%) and levels remained unchanged during one episode.

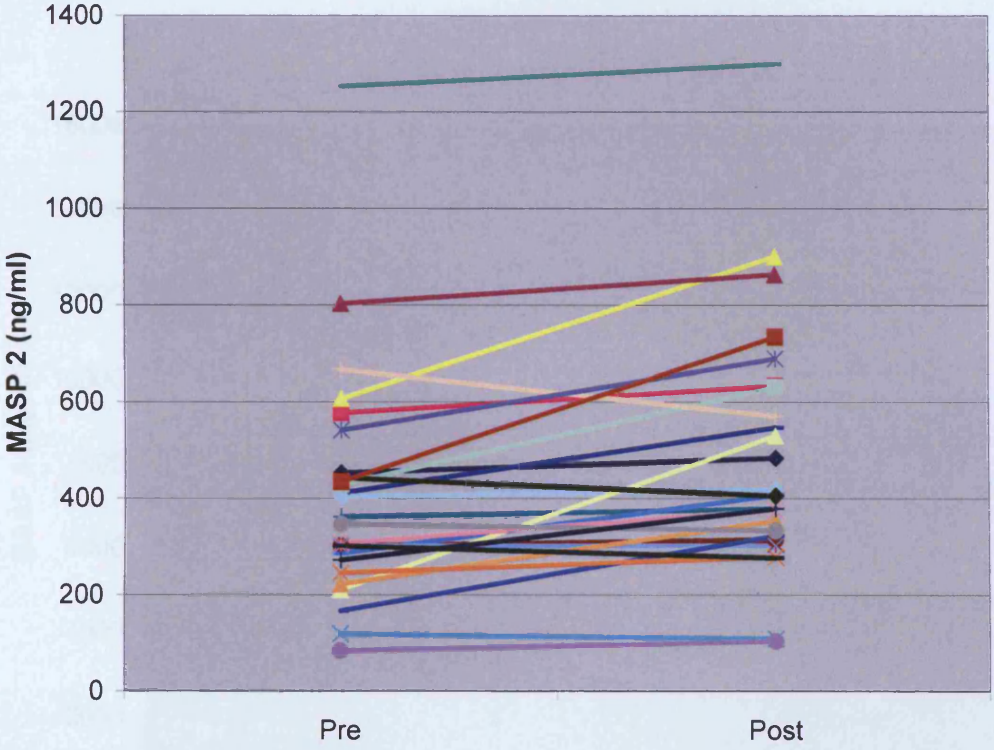


Figure 7.2 Changes in MASP2 level pre and post chemotherapy.
Each line represents a different patient episode.

7.2.2.3 MASP3 levels

As discussed in Chapter 5, there is no published data on MASP3 levels in either healthy or diseased populations. Interestingly, MASP3 levels fell in 26 of the 27 chemotherapy episodes studied, irrespective of MBL genotype and in the remaining episode levels were unchanged (Table 7.4 and Figure 7.3). Levels decreased by a mean of 32% (median 33, range 2-67%).

MASP3	Total group			AA episodes			AO episodes		
	n	median value (ng/ml)	p	n	median value (ng/ml)	p	n	median value (ng/ml)	p
Pre	27	6726	**<0.001**	17	7918	**<0.001**	10	5087	**0.005**
Post		4215			4647			3536	

Table 7.4 Changes in median MASP3 levels in response to chemotherapy.

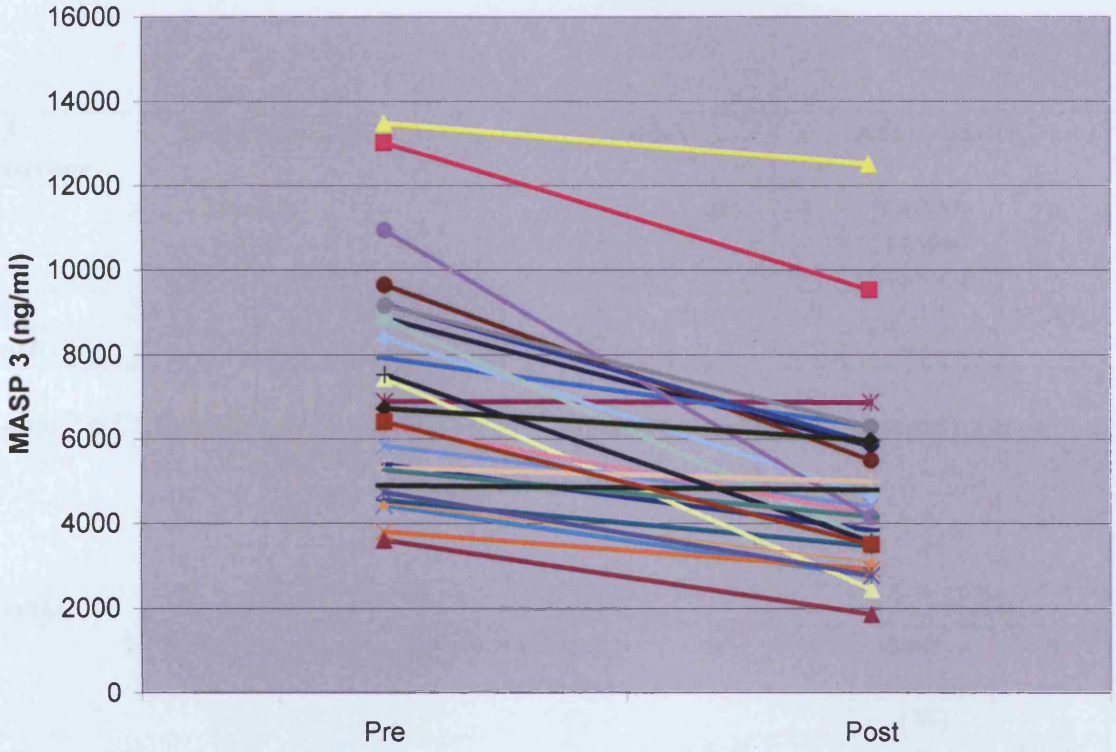


Figure 7.3 Changes in MASP3 level pre and post chemotherapy.
Each line represents a different patient episode

We utilized three functional assays (described in Chapter 5) to investigate the effect of chemotherapy on pathway function. The analysis of all samples taken pre and post chemotherapy revealed no significant change in functional activity irrespective of assay used (Tables 7.5-7.7). It should be noted that data from all three assays was not available for all 27 chemotherapy episodes.

C4b deposition	Total episodes			AA episodes			AO episodes		
	N	median value (munits/ml)	p	n	median value (munits/ml)	p	n	median value (munits/ml)	p
Pre	22	779	0.733	17	874	0.723	5	500	0.893
Post		885			900			598	

Table 7.5 Changes in median MBL/MASP2 complex activity - C4b deposition.

C3 cleavage	Total episodes			AA episodes			AO episodes		
	n	Median value (munits/ml)	p	n	median value (munits/ml)	p	n	median value (munits/ml)	p
Pre	27	992	0.848	17	1380	0.768	10	439	0.508
Post		1035			1237			426	

Table 7.6 Changes in median C3 cleavage activity in response to chemotherapy.

Lectin	Total episodes			AA episodes			AO episodes		
	n	median activity (%)	p	n	median activity (%)	p	n	median activity (%)	p
Pre	23	47	0.765	17	73	0.865	7	5	0.892
Post		45			79			22	

Table 7.7 Changes in median MBL lectin pathway activity in response to chemotherapy.

7.2.2.4 MBL/MASP2 complex activity- C4b deposition

Individual changes in MBL/MASP2 complex activity are illustrated in Figure 7.4. Overall, activity increased in 12 episodes with a mean increase of 49% (median 15, range 1-161%) and activity decreased in 10 episodes with a mean decrease of 12% (median 12, range 3-31%).

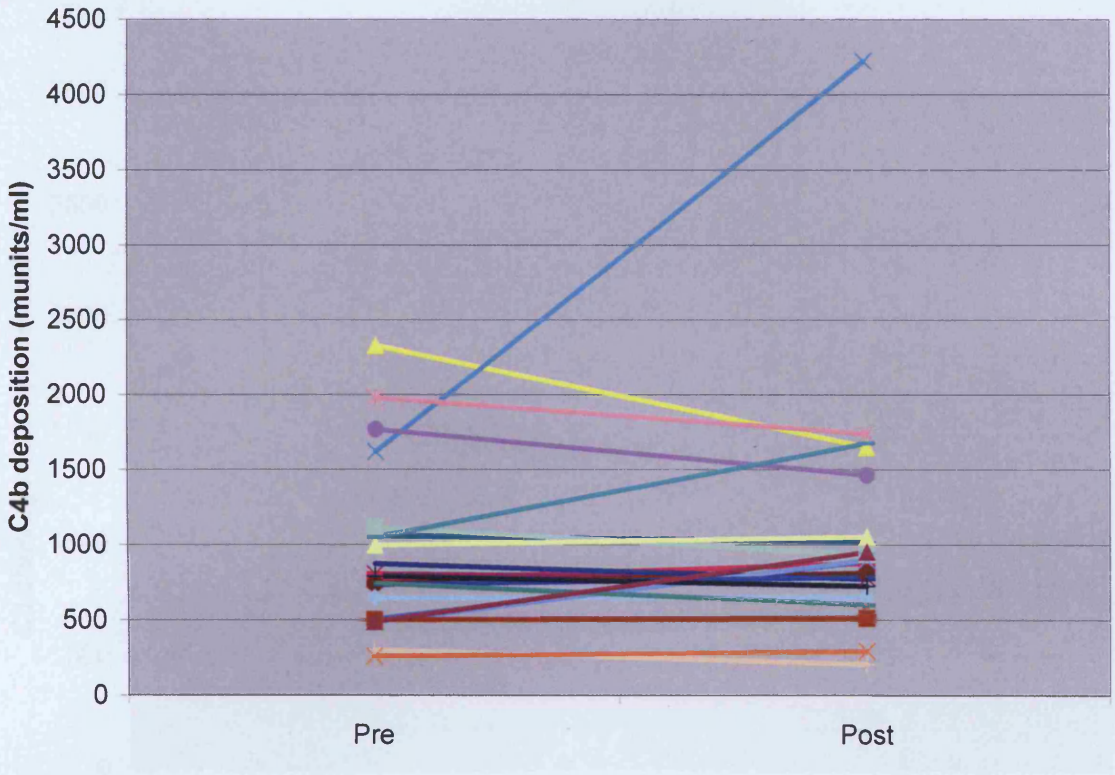


Figure 7.4 Interindividual variations in MBL/MASP2 complex activity

7.2.2.5 C3 cleavage activity

Individual changes in C3 cleavage activity are illustrated in Figure 7.5. Overall, activity increased in 13 episodes with a mean increase of 34% (median 16, range 3-93%) and levels decreased in 14 episodes with a mean decrease of 22% (median 17, range 5-44%).

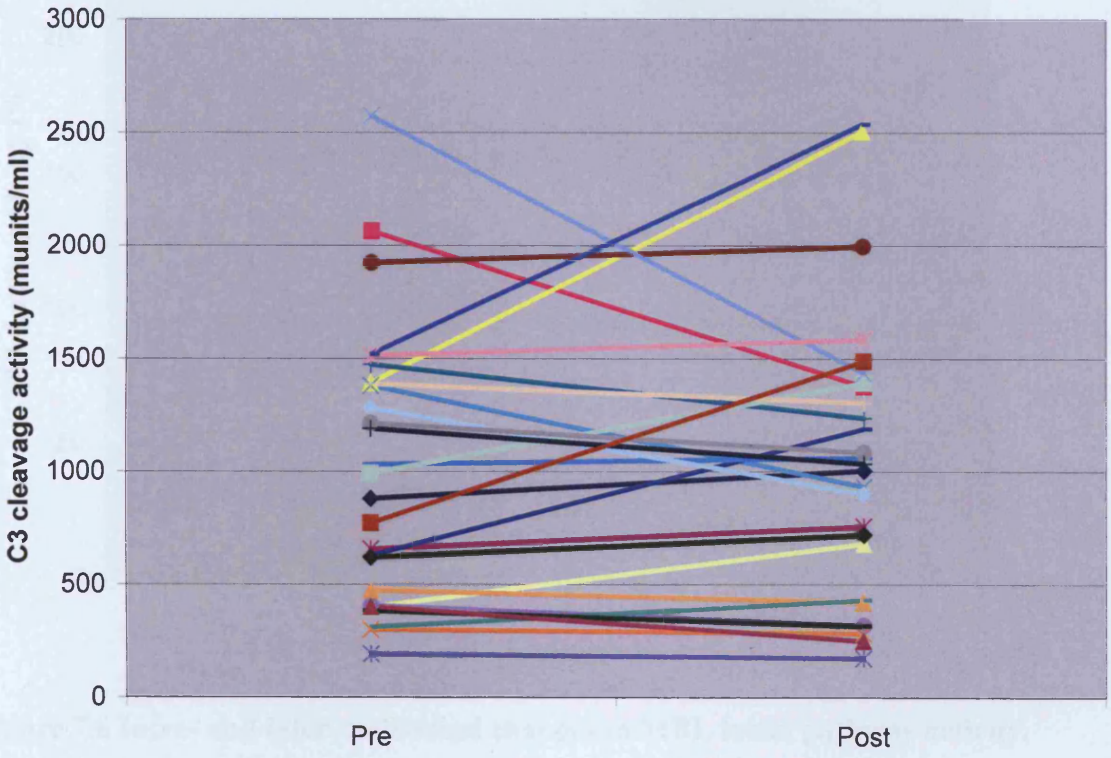


Figure 7.5 Interindividual variations in C3 cleavage activity in response to chemotherapy.

7.2.2.6 MBL Lectin pathway activity as assessed by the Wieslab Total Complement Screen

Individual changes in MBL lectin pathway activity are illustrated in Figure 7.6. Marked fluctuations in activity were observed with activity increasing in 9 episodes, decreasing in 11 episodes and remaining unchanged in 3 episodes. In contrast classical and alternative pathway activity showed less variation (Figures 7.7 and 7.8).

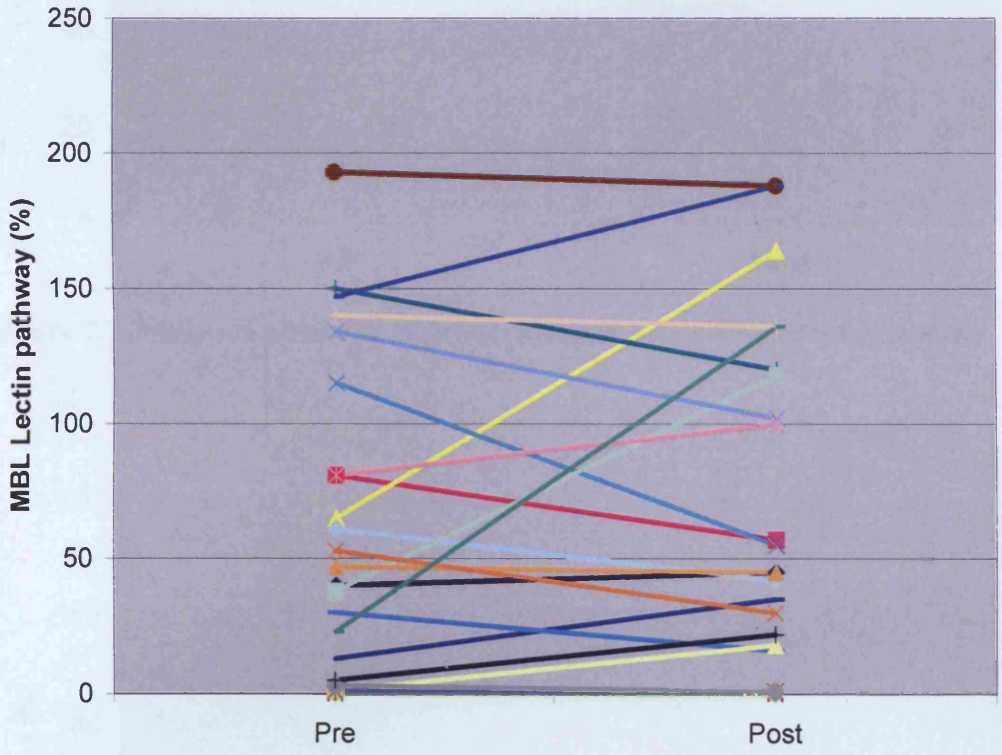


Figure 7.6 Intra- and inter- individual changes in MBL lectin pathway activity.

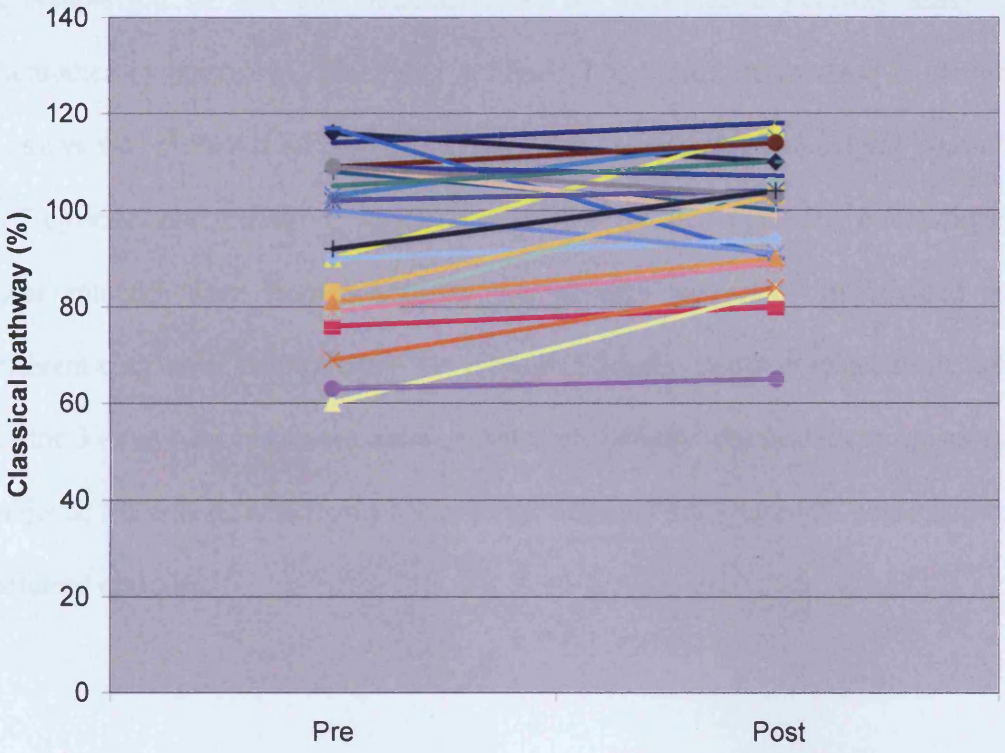


Figure 7.7 Intra- and inter- individual changes in classical pathway activity.

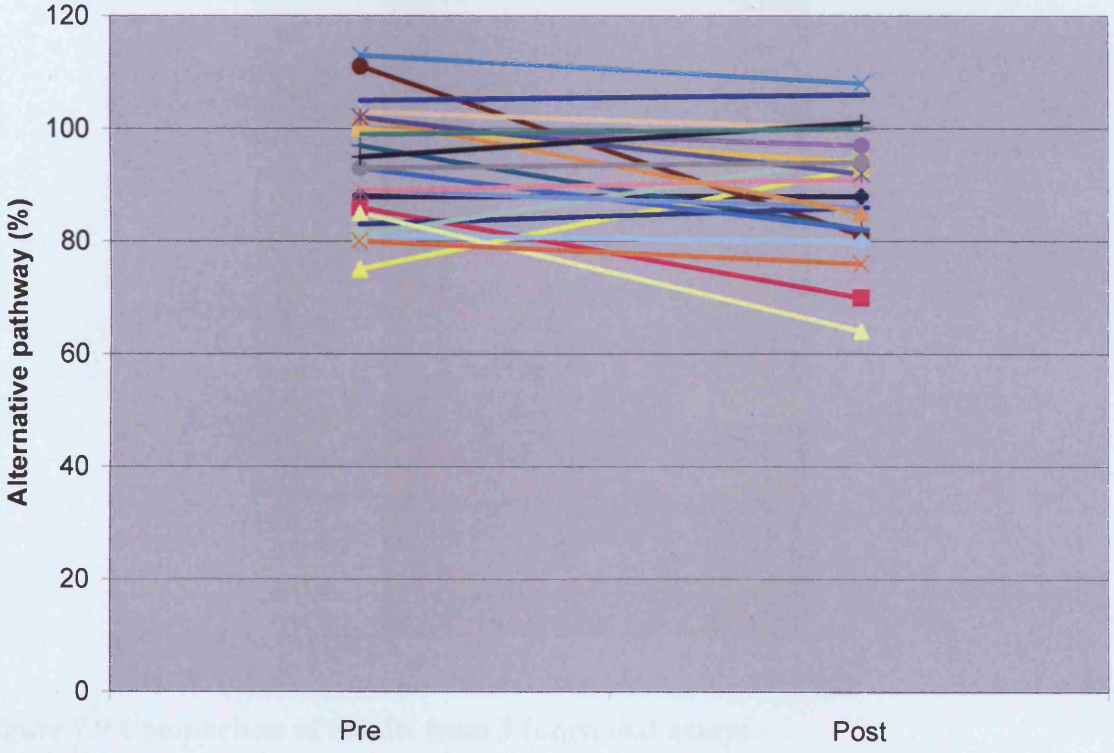


Figure 7.8 Intra- and inter- individual changes in alternative pathway activity.

A comparison of the data obtained from all three lectin pathway assays for 20 chemotherapy episodes is illustrated in Figure 7.9. A uniform increase in activity in all 3 assays was observed during 8 episodes. A uniform decrease in activity was observed in 7 episodes and variability in results was observed in 5 episodes. Interestingly 12 of these episodes were from 6 subjects, i.e. patients who were investigated during 2 different courses of chemotherapy. In these individuals results were generally consistent for the 3 assays but responses did vary between different chemotherapy episodes in two subjects, BC and JC which may relate to the different drug regimens administered in the different episodes.

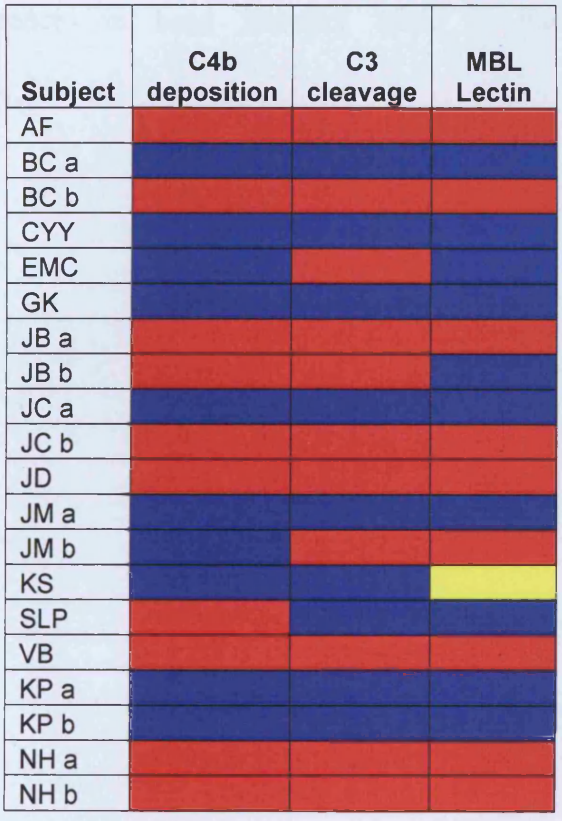
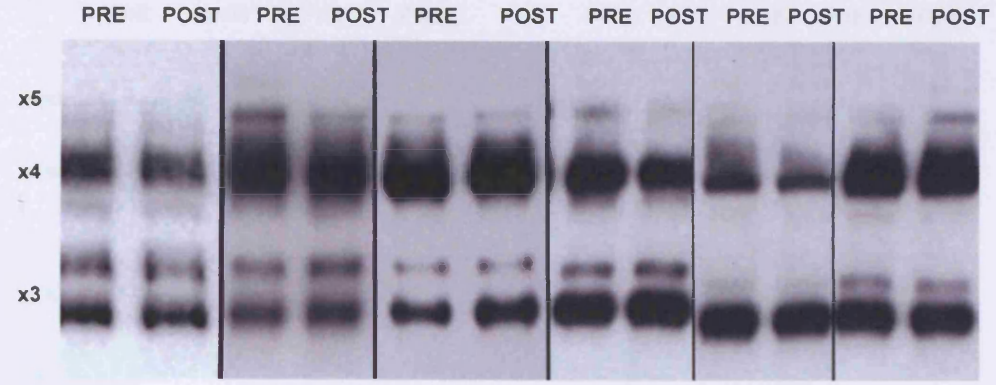


Figure 7.9 Comparison of results from 3 functional assays.
Red shading indicates increased activity, blue shading indicates decreased activity and yellow shading indicates no change in activity in response to chemotherapy.

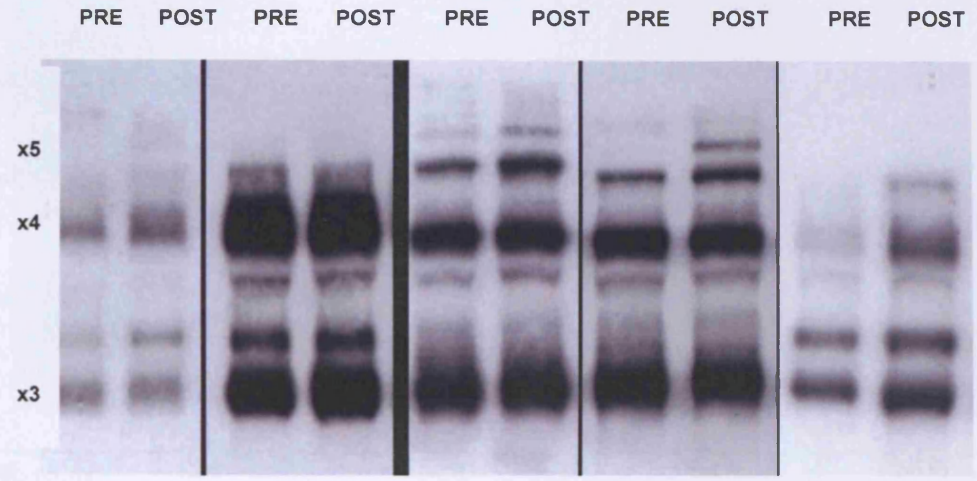
7.2.3 Analysis of MBL structure in samples pre and post chemotherapy

MBL structure was analysed as previously described (Section 6.2.4). The same dilution factor was applied to all sequential serum samples from an individual and 1ng of MBL protein was loaded for each sample and run on the same gel to permit comparison.

Differences in higher oligomer band intensity were noted in samples which were taken on the start and finish days of chemotherapy (Figures 7.10 and 7.11) but such results are semiquantitative and must be interpreted with caution. Saturation of the trimer and tetramer bands made interpretation difficult but there was no clear pattern identified between pre and post chemotherapy samples in episodes from individuals who had received the same chemotherapy drug combinations. It remains unknown whether interindividual differences in band intensity relate to the effect of different chemotherapy regimes.

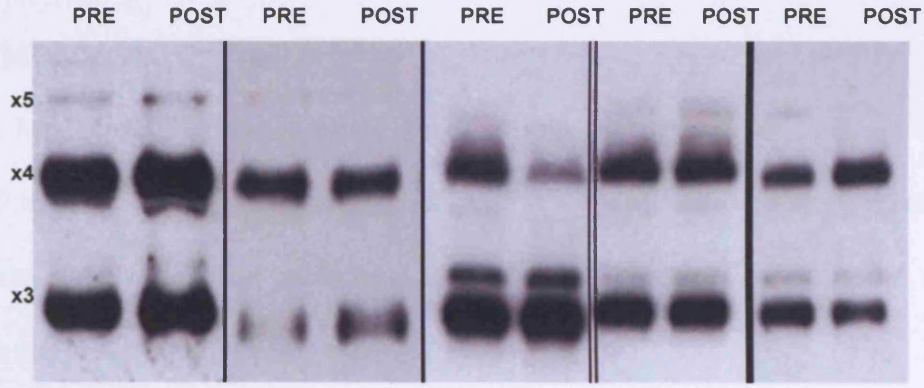


Subject	AF		BCa		BCb		CYY		EMC		GK	
Haplotype combination	HYPA HYPA		LYPA HYPA		LYPA HYPA		LYPA HYPA		LXPA HYPA		LXPA HYPA	
Pathway function	40	45	81	57	65	164	115	55	193	188	151	120

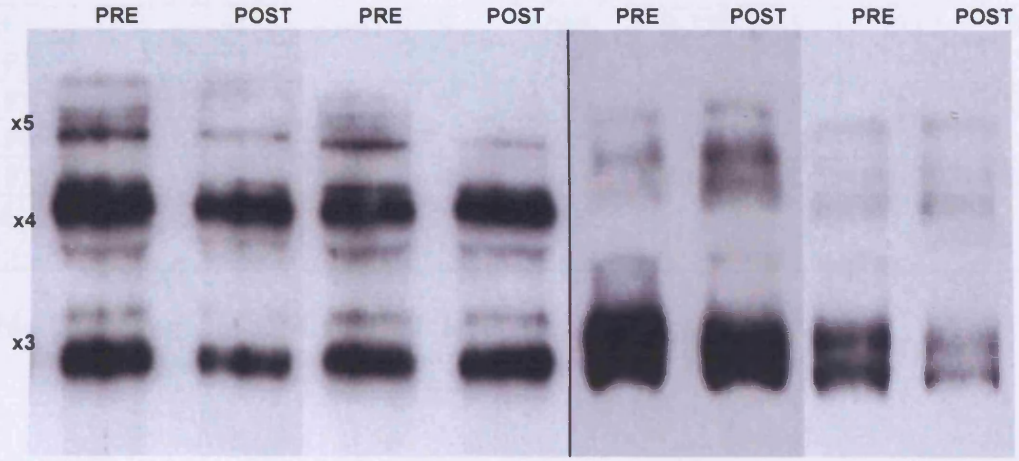


Subject	JBa		JBb		JCa		JCb		JD	
Haplotype Combination	LXPA HYPA		LXPA HYPA		LYQA HYPA		LYQA HYPA		LXPA HYPA	
Pathway function	13	35	30	16	61	42	38	118	1	18

Figure 7.10 MBL structure pre and post chemotherapy during 11 episodes. Bands corresponding to presumed trimer (x3), tetramer (x4) and pentamer (x5) are indicated and corresponding MBL lectin pathway activity is shown.



Subject	JMa		JMb		KS		SLP		VB	
Haplotype Combination	LYQA HYPA		LYQA HYPA		LYQA HYPA		LXPA LXPA		LYQA HYPA	
Pathway Function	134	102	81	100	1	1	140	136	147	188



Subject	KPa		KPb		NHa		NHb	
Haplotype Combination	HYPD HYPA		HYPD HYPA		HYPD HYPA		HYPD HYPA	
Pathway function	47	45	53	30	5	22	23	136

Figure 7.11 MBL structure pre and post chemotherapy during 9 episodes. Bands corresponding to presumed trimer (x3), tetramer (x4) and pentamer (x5) are indicated and corresponding MBL lectin pathway activity is shown.

7.2.4 MBL/lectin pathway activity during FN

7.2.4.1 MBL levels

Median MBL levels measured at the specific timepoints throughout FN are shown in Table 7.8. The number of samples at each timepoint differed and therefore the Wilcoxon signed rank test was used to compare differences between the available samples e.g post chemotherapy and day 1 FN (n=14) etc.

MBL	Total group			AA episodes			AO episodes		
	n	median value (ng/ml)	p	n	median value (ng/ml)	p	n	median value (ng/ml)	p
Post	14	3357	0.463	9	4066	0.779	5	2337	0.686
D1 FN		2631			5100			1598	
D1 FN	11	2587	0.155	7	5100	0.237	4	1607	0.465
D7 FN		3592			6326			2045	
D7 FN	6	3074	**0.046**	4	5183	0.068	2	1128	0.655
D14 FN		2592			4206			822	

Table 7.8 Changes in median MBL level during FN.

The overall trend was that median MBL levels increased between day 1 and 7 of FN but this difference was not significant in any group. By day 14 median levels had fallen and this reached significance in the total episodes and the AML episodes (data not shown). Individual fluctuations in levels are shown in Figure 7.12, illustrating the marked interindividual variation. In those episodes where samples were available at both day 1 and day 7 FN, MBL levels increased in 8/11 (5, AA and 3 AO) and decreased in 3 (2, AA and 1, AO). The maximum increase was observed in a YAYD individual whose MBL level increased 2.4 fold or 140%.

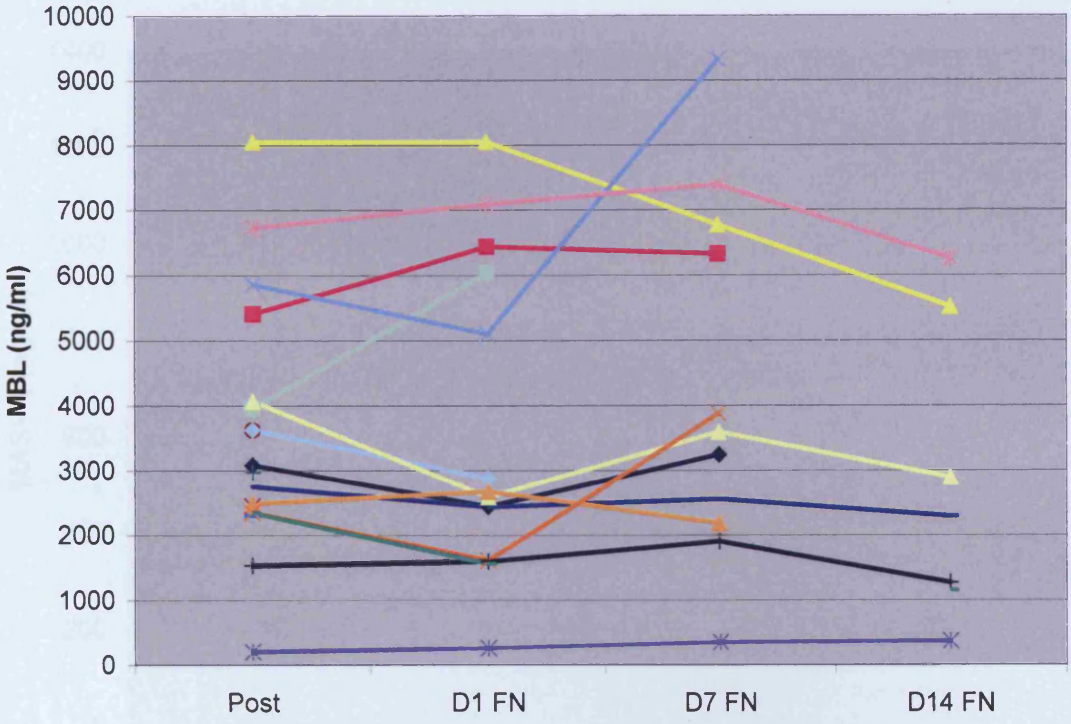


Figure 7.12 Changes in MBL level post chemotherapy and during FN. Each line represents a different patient episode.

7.2.4.2 MASP2 levels

Median MASP2 levels peaked on day 1 of FN and fell between days 1 to 7 and again from days 7 to 14 in the total group and in AA episodes (Table 7.9 and Figure 7.13). Levels in heterozygotes peaked on day 7. Changes in levels in heterozygotes were difficult to interpret as samples were only available from two episodes at all 4 timepoints.

MASP2	Total group			AA episodes			AO episodes		
	n	median value (ng/ml)	p	N	median value (ng/ml)	p	n	median value (ng/ml)	p
Post	14	507	0.701	9	530	0.484	5	406	0.686
D1 FN		521			537			505	
D1 FN	11	572	0.594	7	572	0.310	4	496	0.465
D7 FN		455			452			570	
D7 FN	6	471	0.173	4	405	0.465	2	928	0.180
D14 FN		436			376			817	

Table 7.9 Changes in median MASP2 levels during FN

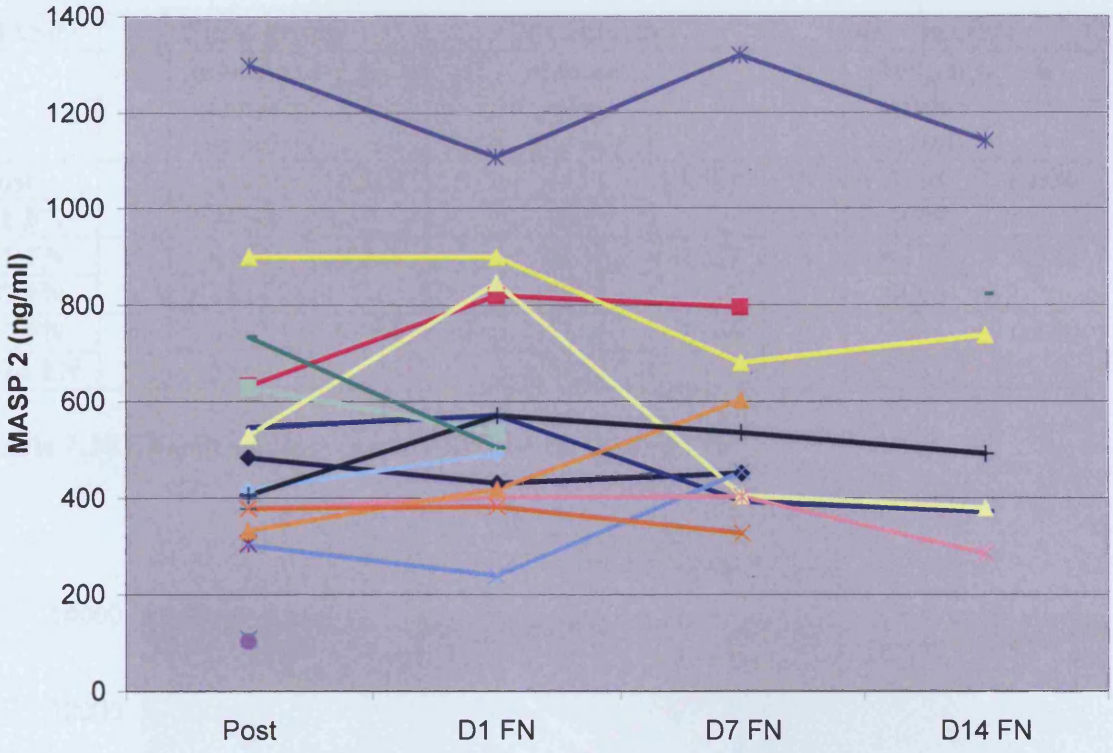


Figure 7.13 Changes in MASP2 level post chemotherapy and during FN. Each line represents a different patient episode.

7.2.4.3 MASP3 levels

As discussed, mean MASP3 levels fell in 26/27 episodes in response to chemotherapy, and then continued to fall throughout the episode in some individuals at different rates but were generally increasing to pre chemotherapy levels by day 14 FN (Table 7.10 and Figure 7.14). MASP3 levels were generally higher in *MBL2* AA individuals compared to the AO subset as previously observed in Chapter 6.

MASP3	Total group			AA episodes			AO episodes		
	n	median value (ng/ml)	p	n	median value (ng/ml)	p	n	median value (ng/ml)	p
Post	14	4315	0.382	9	4414	0.484	5	4164	0.080
D1 FN		4430			4823			3689	
D1 FN	11	4174	0.328	7	4177	0.237	4	4187	0.465
D7 FN		4814			5674			4065	
D7 FN	6	4315	0.046	4	5244	0.144	2	2851	0.180
D14 FN		5542			5750			5071	

Table 7.10 Changes in median MASP3 levels during FN.

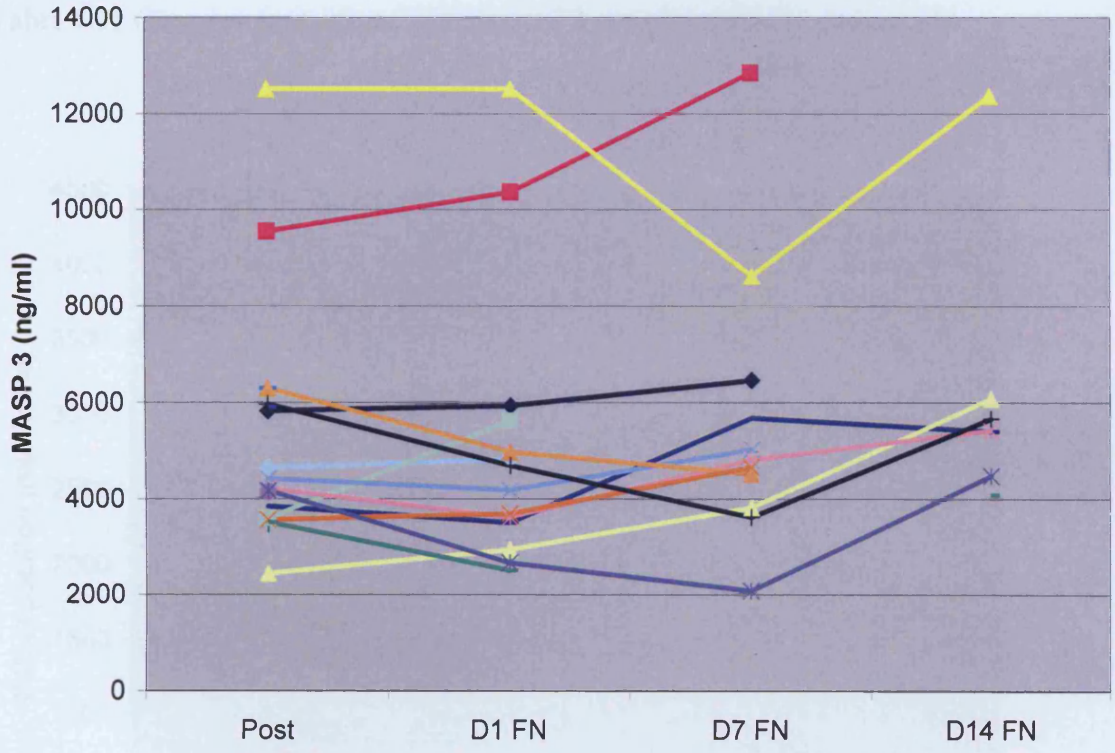


Figure 7.14 Changes in MASP3 level post chemotherapy and during FN. Each line represents a different patient episode.

7.2.4.4 MBL/MASP2 complex activity- C4b deposition

In all groups, median activity peaked on day 7 of FN and decreased significantly by day 14, showing a similar trend to MBL levels (Table 7.11 and Figure 7.15). MASP2 levels

increased in response to chemotherapy but this was not reflected by a significant increase in C4b deposition. As discussed in Chapter 6, MASP2 does not interact solely with MBL and C4b deposition activity correlates well with MBL levels alone.

C4b	Total group			AA episodes			AO episodes		
	n	median value (munits/ml)	p	n	median value (munits/ml)	p	n	median value (munits/ml)	p
Post	13	936	0.754	9	1052	0.575	4	662	0.715
D1 FN		924			1068			640	
D1 FN	10	867	0.139	7	966	0.398	2	670	0.109
D7 FN		1233			2120			1162	
D7 FN	5	1162	**0.042**	4	1610	**0.068**	1	-	-
D14FN		876			1172			-	

Table 7.11 Changes in median MBL/MASP2 complex activity during FN

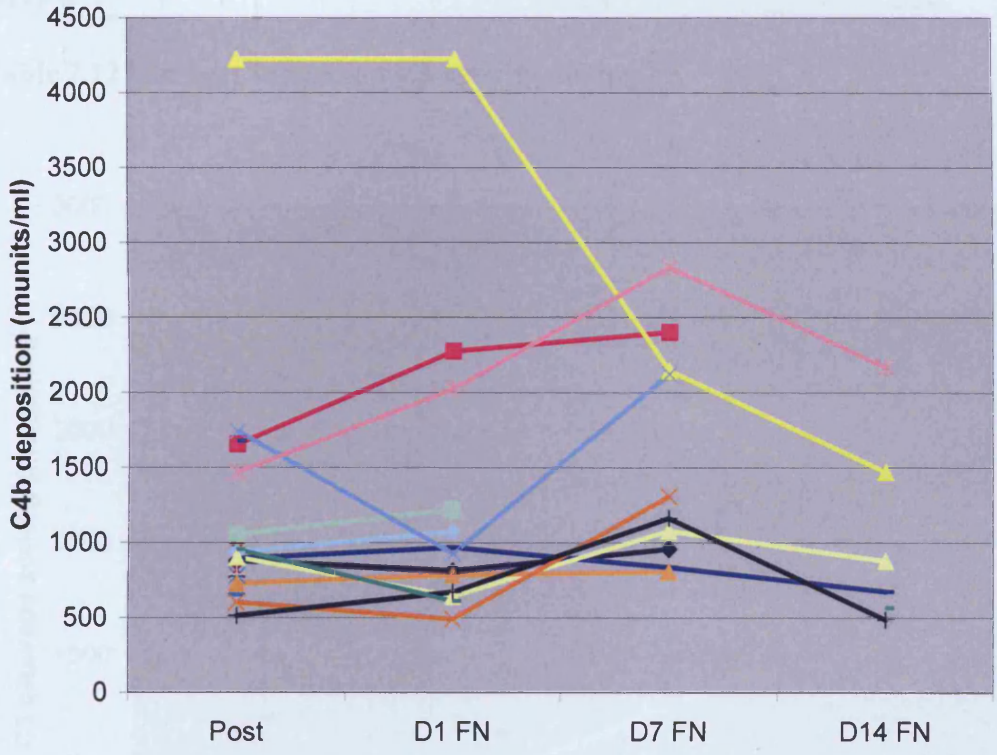


Figure 7.15 Interindividual variations in MBL/MASP2 complex activity post chemotherapy and during FN

7.2.4.5 C3 cleavage activity

A similar trend was observed in C3 cleavage activity as that for C4b deposition. This was expected as the two assays showed good correlation (Chapter 6). Activity peaked at day 7 of FN and decreased by day 14 (Table 7.12). Individual fluctuations in activity are shown in Figure 7.16.

C3	Total group			AA episodes			AO episodes		
	n	median value (munits/ml)	p	n	median value (munits/ml)	p	N	median value (munits/ml)	p
Pre	27	992	0.848	17	1380	0.768	10	439	0.508
Post		1035			1237			426	
Post	14	1136	0.889	9	1376	0.726	5	1035	0.500
D1 FN		916			1396			780	
D1 FN	11	836	0.131	7	996	0.237	4	808	0.465
D7 FN		1531			1936			1475	
D7 FN	6	1221	**0.046**	4	1479	0.068	2	808	0.655
D14FN		672			1058			564	

Table 7.12 Changes in median C3 activity during FN

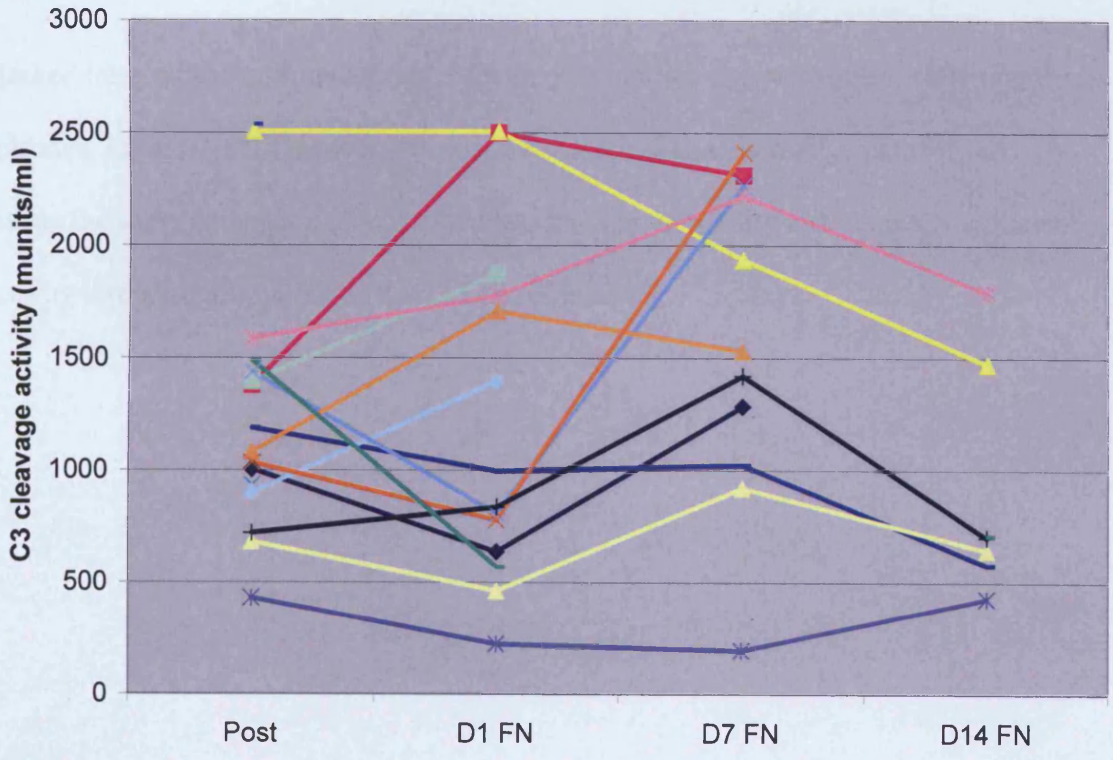


Figure 7.16 Interindividual variations in C3 cleavage activity in response to chemotherapy.

7.2.4.6 MBL lectin pathway activity as assessed by the Wieslab Total Complement Screen

The trend in MBL lectin pathway activity again mirrored that seen for the C4 and C3 assays i.e. median levels peaked on day 7 of FN and fell by day 14 (Table 7.13). Similar trends were seen for both AA and AO episodes.

	Total group			AA episodes			AO episodes		
	n	**median activity (%)**	**p**	**n**	**median activity (%)**	**p**	**n**	**median activity (%)**	**p**
Pre	23	47	0.765	17	73	0.865	7	5	0.892
Post		45			79			22	
Post	14	45	0.638	9	57	0.674	5	30	1.000
D1 FN		43			62			12	
D1 FN	11	42	0.059	7	42	0.176	4	27.5	0.285
D7 FN		97			97			81	
D7 FN	6	69	**0.046**	4	69	0.068	2	65	0.655
D14 FN		11			29			8.5	

Table 7.13 Changes in median MBL lectin pathway activity during FN.

Marked intraindividual fluctuations in lectin pathway activity were observed within episodes, Figure 7.17. This was in contrast to classical and alternative pathway activity during the same episodes which showed less marked variability and generally sufficient activity throughout, Figures 7.18 and 7.19 respectively.

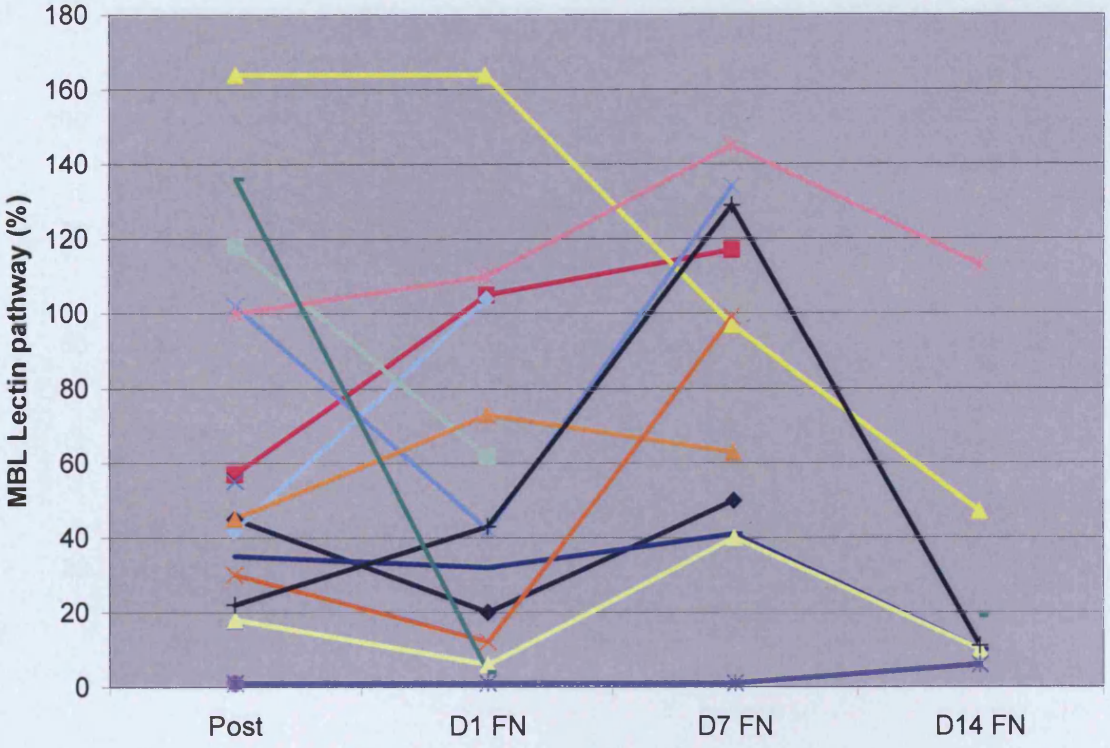


Figure 7.17 Intra- and inter- individual changes in MBL lectin pathway activity post chemotherapy and during FN.

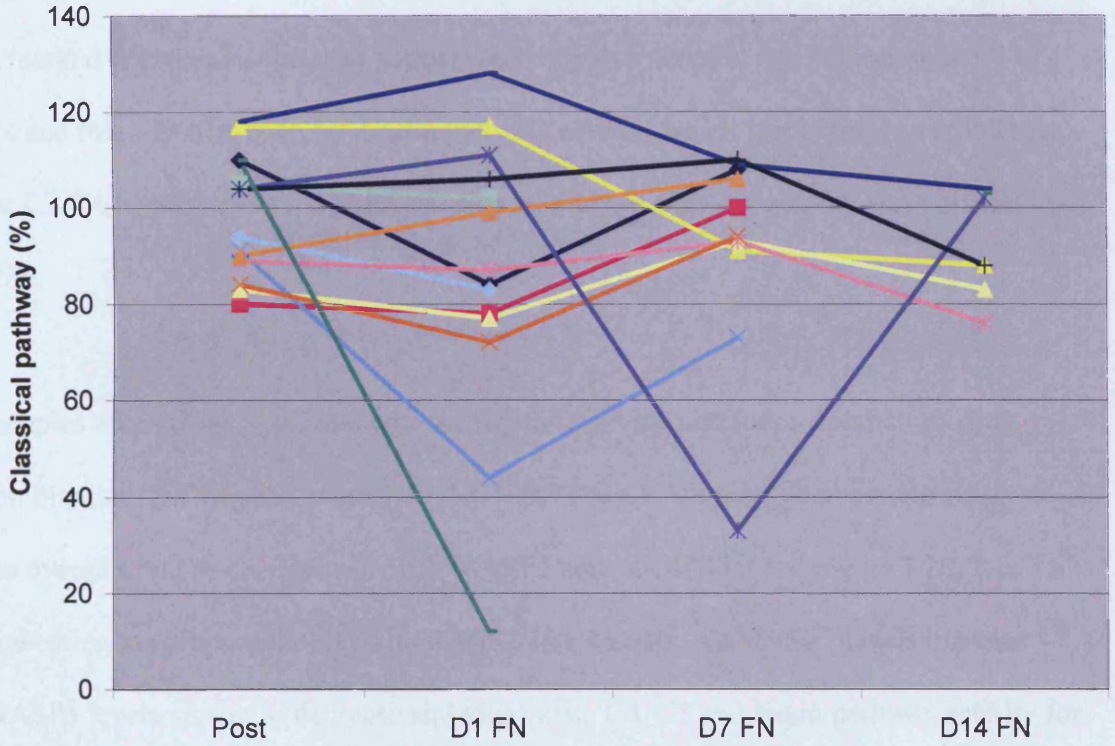


Figure 7.18 Intra- and inter- individual changes in classical pathway activity post chemotherapy and during FN.

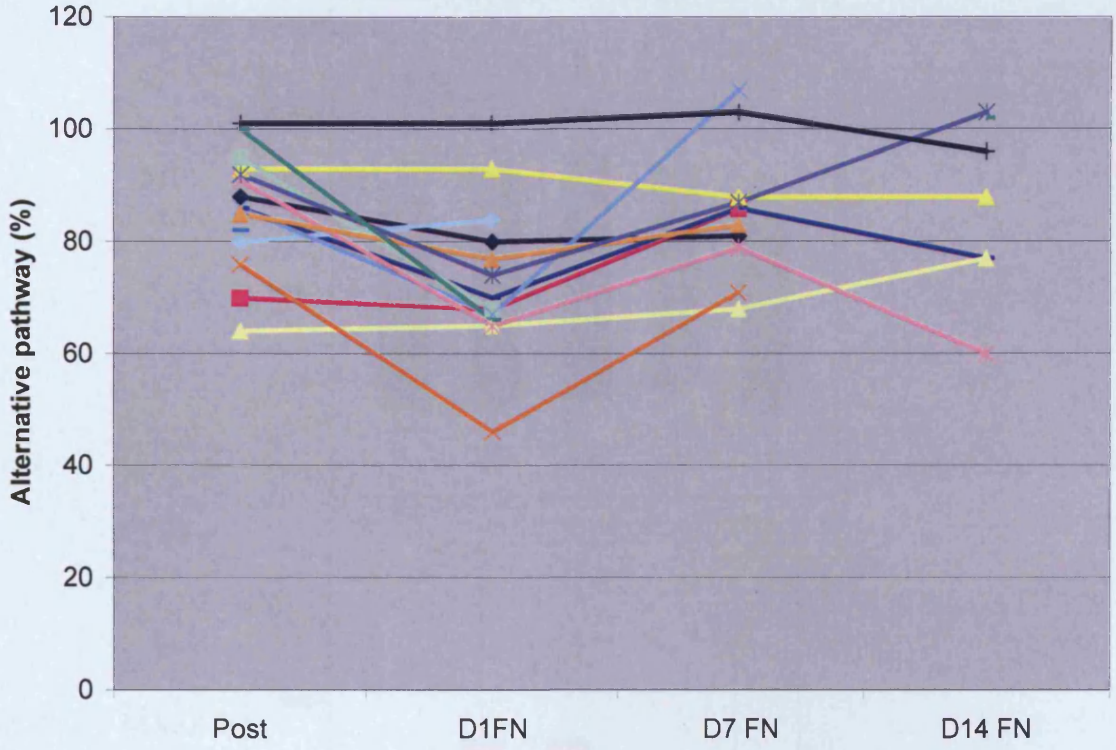


Figure 7.19 Intra- and inter- individual changes in alternative pathway activity post chemotherapy and during FN.

A marked decrease in classical pathway activity was noted in one subject on day 1 of FN and this was also seen for Lectin pathway activity which would imply a problem at the C2/C4 level at this time point because alternative pathway activity was sufficient at 66%.

Samples taken from 6 patients with AML, all receiving similar chemotherapy drug combinations, of whom 4 were *MBL2* AA and 2 were AO have been used to illustrate the overall trend in changes of MBL, MASP2 and MASP3 levels, Figure 7.20. It is interesting to compare the trend in MASP2 and 3 levels. As MASP2 levels increase MASP3 levels appear to decrease and vice versa. C4, C3 and lectin pathway activity for the same 6 AML patients is shown in Figure 7.21.

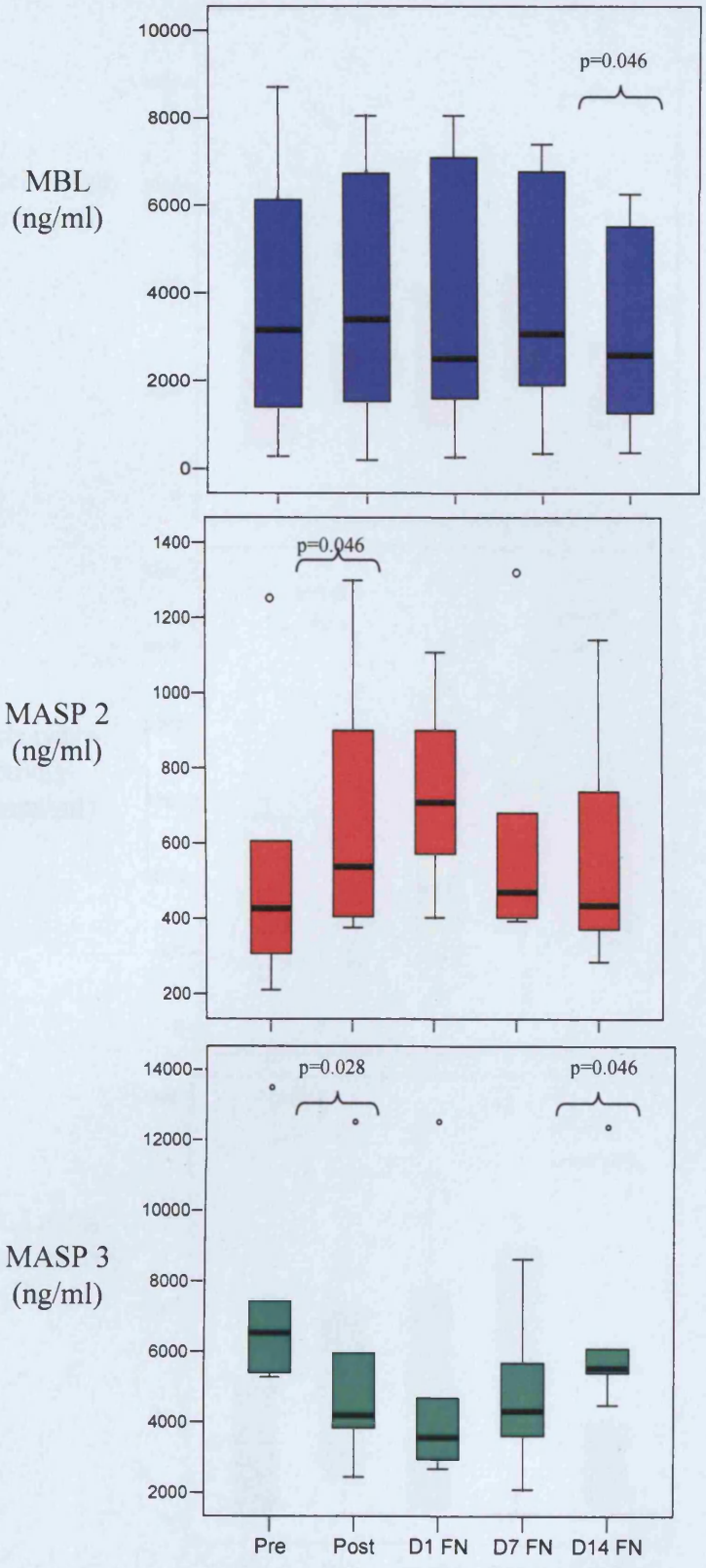


Figure 7.20 Changes in MBL, MASP2 and MASP3 levels in 6 patients on treatment for AML (4 AA, 2 AO).

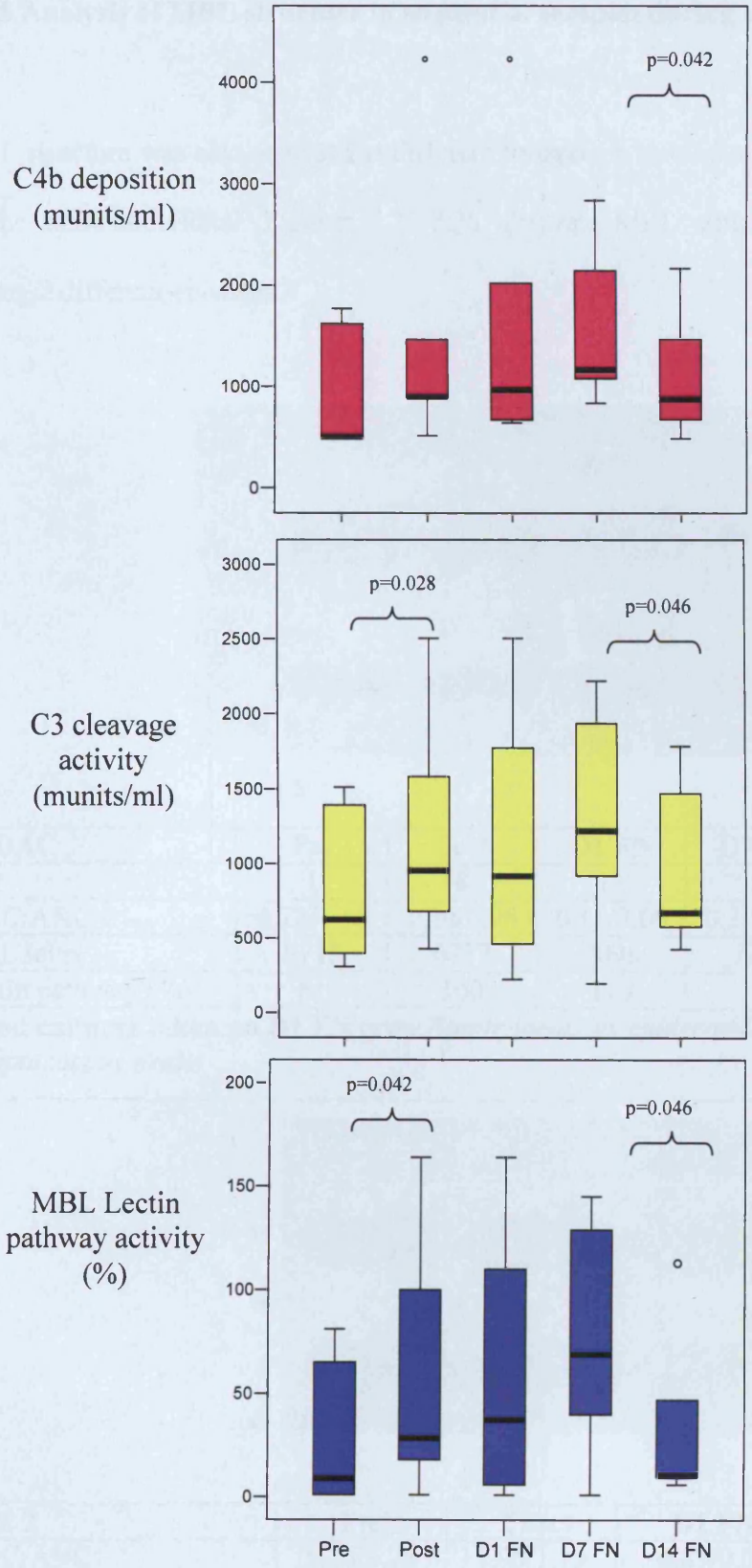
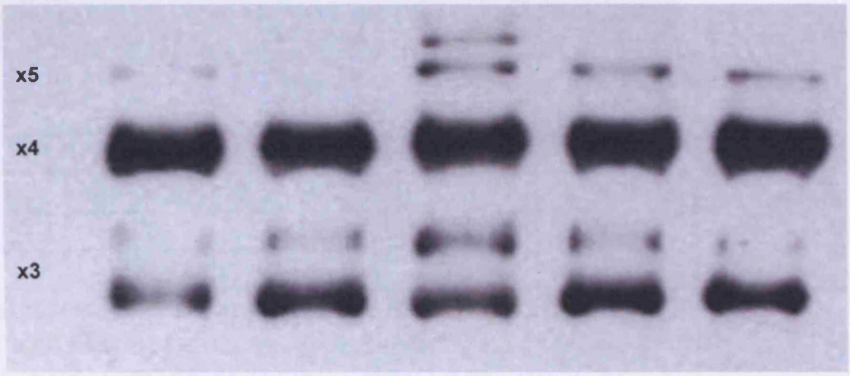


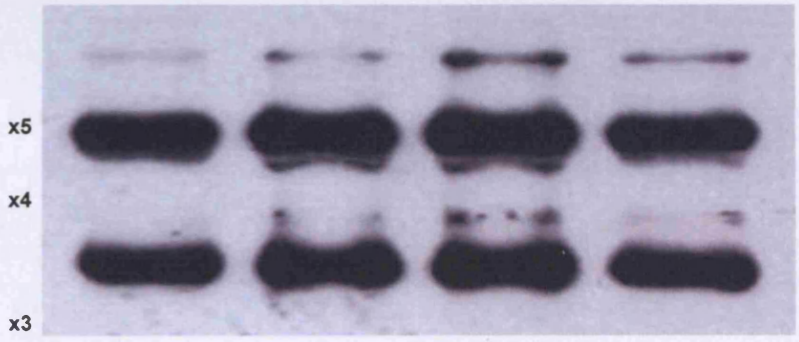
Figure 7.21 Changes in C4, C3 and Wieslab lectin pathway activity in 6 patients on treatment for AML (4 AA, 2 AO (except C4 results, where only 1 AO is represented).

7.2.5 Analysis of MBL structure in sequential samples during chemotherapy and FN

MBL structure was also analysed at different timepoints throughout 2 different episodes in the same individual. Figures 7.22-7.25 illustrate MBL structure in 4 individuals during 2 different episodes.

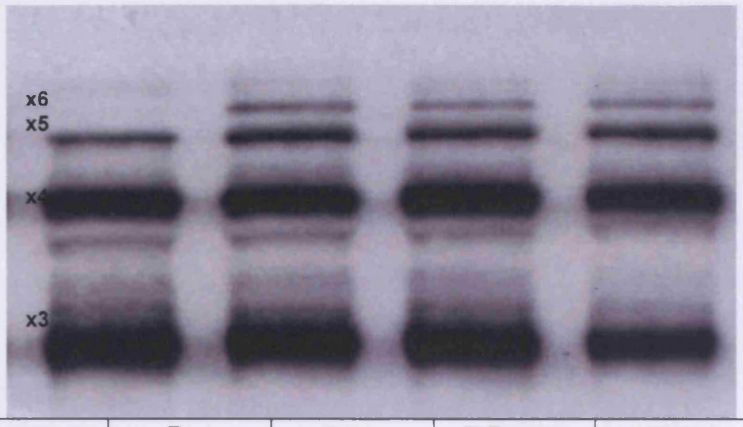


MIDAC	Pre	Post	**D1 FN**	D7 FN	D14 FN
Day	1	8	11	18	25
WCC/ANC	4.72/1.65	1.34/0.98	0.11/0.00	0.37/0.01	0.72/0.04
MBL level	8712	6737	7096	7398	6258
Lectin pathway (%)	81	100	110	145	113
Blood cultures taken on D1 FN grew *Staphylococcus epidermidis* and *Streptococcus oralis*					

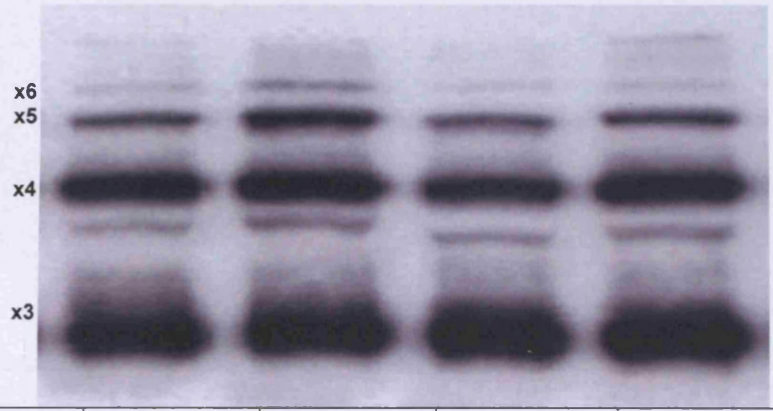


ADE 2	Pre	Post	**D1 FN**	D7 FN
WCC/ANC	5.24/3.78	1.95/1.19	0.89/0.01	3.51/0.17
Day	1	9	16	23
MBL level	10000	5860	5100	9324
Lectin pathway (%)	134	102	44	134
Blood cultures taken on D1 FN grew *Staphylococcus epidermidis*				

Figure 7.22 MBL structure and function in patient JM (LYQA/HYPA) undergoing treatment for AML during MIDAC and ADE 2 chemotherapy.

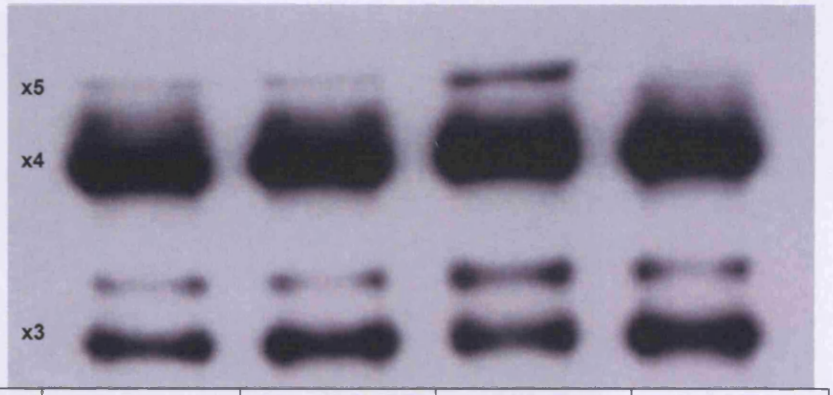


HiDAC	Pre	**Post**	D7 post chemo	**D1 FN**
Day	1	7	14	21
WCC/ANC	5.68/3.06	2.83/2.81	0.77/0.71	0.28/0.02
MBL level	3175	3952	4241	6041
Lectin pathway (%)	38	118	76	62
Febrile but not neutropenic on D1 post chemo and blood cultures grew *Enterococcus* and *Staphylococcus epidermidis* D1 FN blood cultures grew *Staphylococcus epidermidis*				

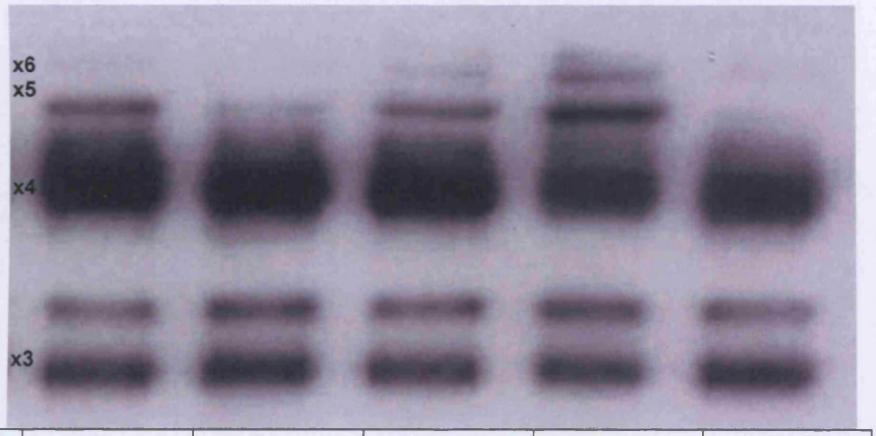


ADE 2	Pre	**Post**	D7 post chemo	**D1 FN**
Day	1	7	14	21
WCC/ANC	4.84/1.41	2.06/1.78	1.06/0.01	1.33/0.34
MBL level	3751	3630	2177	2882
Lectin pathway (%)	61	42	9	104
Febrile on finish day of chemotherapy, not neutropenic and negative blood cultures. When FN, no growth on blood cultures				

Figure 7.23 MBL structure and function in patient JC (LYQA/HYPA) undergoing treatment for AML during HiDAC and MIDAC chemotherapy.

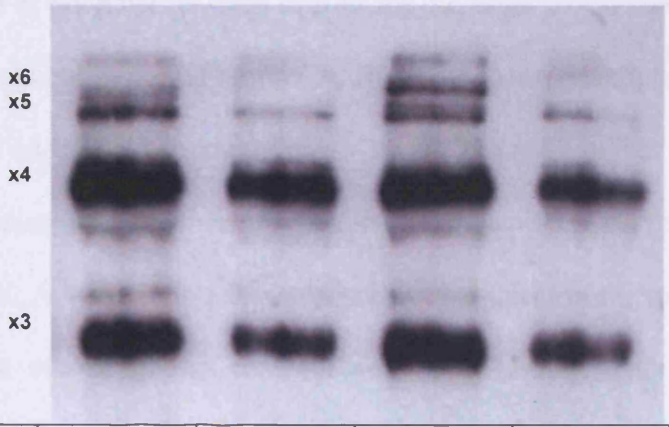


HiDAC	Pre	Post/D1 FN	**D7 FN respiked**	D14 FN
Day	1	9	17	24
WCC/ANC	5.96/1.99	1.24/0.02	0.89/0.01	3.93/0.34
MBL level	6132	8054	6774	5519
Lectin pathway (%)	65	164	97	47
No growth on blood cultures throughout episode				

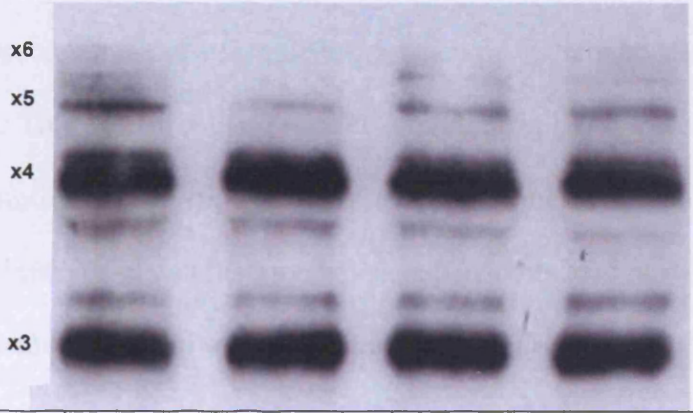


MIDAC	Pre	Post	D7 post chemo	**D1 FN**	D7 FN
Day	1	7	14	17	23
WCC/ANC	7.68/3.37	3.58/1.87	3.29/0.03	1.31/0.00	5.28/0.82
MBL level	7715	5409	7626	6443	6326
Lectin pathway (%)	81	57	132	105	117
Blood cultures taken on D1 FN grew *Klebsiella aeruginosa.* Patient required CVL removal					

Figure 7.24 MBL structure and function in patient BC(LYPA/HYPA) undergoing treatment for AML during HiDAC and MIDAC chemotherapy.



MACE	Pre	Post	**D1 FN**	D7 FN
Day	1	7	10	17
WCC/ANC	3.02/0.78	1.73/0.26	0.45.0.01	1.04/0.00
MBL level	2264	2349	1616	3881
Lectin pathway (%)	53	30	12	99
Blood cultures taken on D1 FN grew *Staphylococcus epidermidis*				



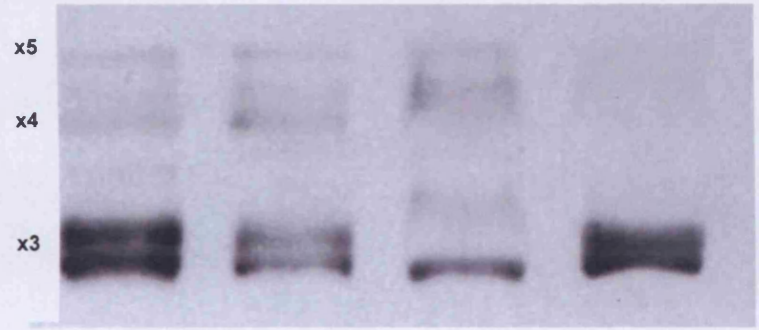
ADE 2	Pre	Post	**D1 FN**	D7 FN
Day	1	8	16	22
WCC/ANC	4.0/1.16	2.02/1.27	0.82/0.05	1.31/0.03
MBL level	2796	2487	2675	2186
Lectin pathway (%)	47	45	73	63
Blood cultures taken on D1 FN showed no growth				

Figure 7.25 MBL structure and function in patient KP (HYPD/HYPA) undergoing treatment for AML during MACE and ADE 2 chemotherapy.

Each individual shows different band patterns as expected from the earlier studies described in Chapter 6, but patterns were similar for the same individual when receiving different courses of chemotherapy. Figure 7.22 shows sequential samples for patient JM undergoing MIDAC and ADE2 chemotherapy. During MIDAC, higher order oligomers corresponding to presumed pentamer and hexamer are clearly observed in the sample taken on day 1 of FN. A very weak pentamer band was evident in the pre chemotherapy sample but was not visible in the post chemotherapy sample. This pentamer band persisted during FN but at decreased intensity. During ADE2 chemotherapy the pentamer band is also visible and increases in intensity, peaking again on day 1 of FN. It is difficult to comment on changes in intensity of the tetramer band as it is saturated. Variability in the intensity of the pentamer band and appearance of hexamer bands are also observed in patient JC, Figure 7.23. During HiDAC chemotherapy hexamer is visible in the post chemotherapy sample taken when the patient became febrile although not neutropenic. This hexamer band was also evident in the two subsequent samples. During ADE2, similar variability was observed with the hexamer band intensity highest on the day of finishing chemotherapy which again coincided with the patient being febrile but not neutropenic and on day 1 of actual FN. Figure 7.24 also shows changes in higher oligomers in patient BC which correspond to FN episodes with or without any positive cultures. Samples taken from a HYPD/HYPA individual are shown in Figure 7.25 and variability is observed in the higher order oligomers similar to that seen in the wildtype individuals previously described. Pentamer was evident in both pre chemotherapy samples but decreased in intensity in the post chemotherapy samples. Hexamer and pentamer appeared most abundant in the samples taken on day 1 of FN.

Distinct higher order oligomers were not observed in all HYPD/HYPA individuals. Figure 7.26 shows the MBL higher order oligomers detected in samples from patient

NH during MIDAC chemotherapy. The most prominent bands detected corresponded with the trimer and an associated higher molecular weight band. The sample taken on day 2 of FN shows a single low band compared to double/split bands seen at the other timepoints. There is some signal detected at tetramer level. At this timepoint the patient was very unwell, *Streptococcus viridans* was grown from blood cultures and admission to PICU was required. This also corresponded with low lectin and classical pathway activity. No sample was available at day 7 FN as the patient required removal of their CVL but by day 14 FN MBL structure appeared similar to that observed in the pre chemotherapy sample.



HiDAC	Pre	Post	**D2 FN**	D14 FN
Day	1	6	13	22
WCC/ANC	7.01/3.96	2.55/2.3	0.05/0.0	4.17/0.81
MBL level	1584	2337	1559	1153
Lectin pathway (%)	23	136	4	19
Classical (%)	105	110	12	103
Alternative (%)	99	100	66	102
Blood cultures taken on day 1 FN grew *Streptococcus viridans*				

Figure 7.26 MBL structure and complement function in patient NH undergoing HiDAC chemotherapy.

In total 14 episodes of FN were analysed and an increase in higher order oligomer band intensity was apparent in 10 of these episodes coinciding with the onset of FN.

Sequential samples from healthy volunteers were also analysed to act as controls for comparison. Figure 7.27 shows samples from 2 *MBL2* wildtype individuals taken at weekly intervals during which time individuals were well and at work. Two different exposures of each blot are shown to demonstrate the lack of variability between different samples.

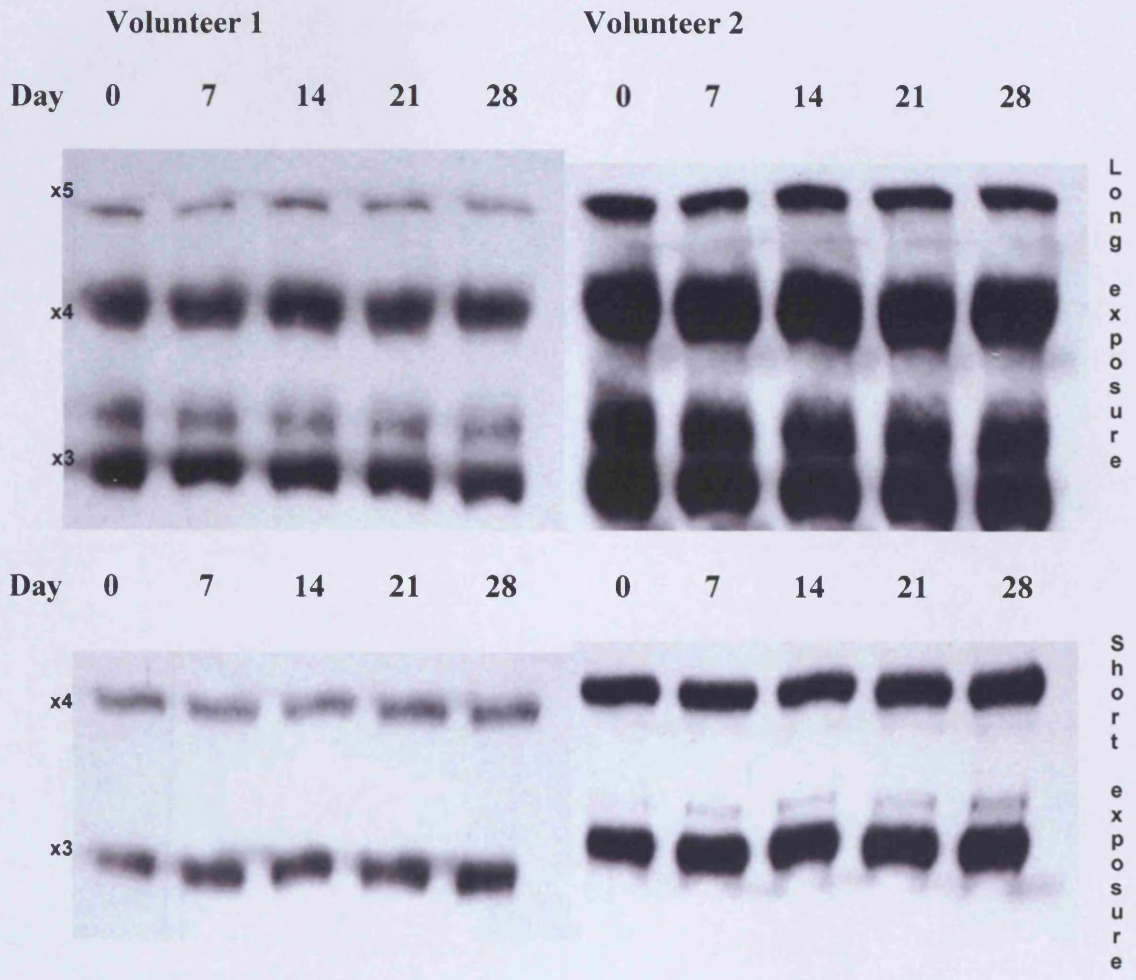


Figure 7.27 MBL oligomers in serial samples taken from two healthy volunteers. Long and short exposures (top and bottom panel respectively) are shown for comparison.

7.3 Discussion

The work presented in this chapter represents the most detailed study to date investigating changes in MBL structure and MBL lectin pathway function in response to chemotherapy. Patient numbers were small and there was no control group which does limit interpretation but a number of interesting findings warrant further investigation and could be important when selecting patients for MBL replacement therapy.

Our findings suggest that median MBL levels are not significantly affected by a number of different chemotherapy combinations (Table 7.2). In those patients who went on to develop FN, average MBL levels increased between day 1 and 7 but this did not reach significance. However median levels did decrease significantly from day 7 to 14. These results are in agreement with previous paediatric studies which have shown minimal changes in levels in response to chemotherapy and increases during FN (Neth et al., 2001; Frakking et al., 2006). We did not observe dramatic increases in levels during the 'acute phase' (maximum 2.4 fold) supporting the view that MBL is not a classic acute phase protein. The timing of sampling may be crucial to the detection of any acute rise in levels but other studies using more frequent sampling do not suggest that this is the case (Ytting et al., 2006).

There are no published studies investigating changes in MASP levels in response to chemotherapy or during FN episodes. Our data suggests that MASP2 levels increase after chemotherapy and peak on day 1 of FN, decreasing thereafter. Changes in levels by up to 400% were observed in some subjects. MASP2 has not been proposed as an acute phase reactant (Moller-Kristensen et al., 2003) but our data suggest that a single level taken at diagnosis is not predictive of changes observed during times of stress. MASP2 levels have been measured in adults undergoing surgery for colorectal cancer and a mean increase of 10% was noted on day 8 post surgery when compared to pre-

operative levels (Ytting et al., 2006). In contrast, MASP3 levels decreased in all subjects in response to chemotherapy ($p<0.001$) and had increased again by the end of the episode. MASP2 and MBL are predominantly hepatic in origin unlike MASP3 which has been detected in a number of different tissues (Seyfarth et al., 2006). The results presented would suggest that MASP3 levels may be affected by chemotherapy and that reduced levels could be implicated in susceptibility to infection but the significance of this finding remains unknown.

Functional activity of MBL and the lectin pathway in sequential samples assessed by the C4b deposition, C3 and Wieslab assays showed similar trends. This was expected as the results of the different assays showed good correlation with each other (Chapter 6). Median functional activity generally peaked on day 7 of FN and decreased thereafter (Figure 7.8). This may suggest that the lectin pathway was operating in response to FN and activity increased when required most. Marked fluctuations in MBL/lectin pathway activity were observed in some individuals (Figures 7.5-7.7). Interestingly, classical and alternative pathway activity was more consistent throughout the episodes which may reflect increased stability of their components or increased sensitivity of the lectin pathway assays.

The analysis of MBL structure in sequential samples using western blotting has revealed some interesting findings. Interindividual variation was noted in response to chemotherapy but (presumed) trimer and tetramer remained markedly intact. The detection of higher bands at the start of a FN episode was somewhat unexpected and we can only speculate on its significance. One hypothesis is that at this time of presumed infection i.e. FN, the production of higher order MBL2 oligomers, which are reported to be more efficient at complement activation, would be beneficial to the host in combating infection. Detectable MBL levels do not appear to increase significantly at these times but the ability to regulate oligomer formation may be more important to the

host. Such differences in band pattern were not observed in sequential samples taken from healthy volunteers. The control of oligomer formation and whether such tight control is even possible are currently unknown. It is understood that MBL matures post translationally into higher order oligomeric forms within the endoplasmic reticulum prior to secretion (Heise et al., 2000). Evidence from work investigating rat MBL A suggests that formation of higher oligomers occurs over a number of hours but the degree of regulation of this process has not been investigated. A number of chaperone proteins have been shown to interact with collagenous proteins during their assembly but it is unclear if they can influence the higher oligomeric forms. One other explanation may be that body temperature influences the process. The changes in higher oligomers correspond to febrile episodes which may affect the maturation of the protein. Such differences were not observed in healthy subjects over similar time periods. Alternatively the higher bands detected on the immunoblot may purely relate to a change in size of oligomer e.g. tetramer, which is then bound to a target organism in the bloodstream. A recent study has demonstrated that conformational changes occur in the quaternary structure of MBL upon binding to surface immobilized ligands (Dong et al., 2007).

We should also consider the issue of degradation which may affect the band patterns detected. Previous studies have suggested that both large and small oligomers are more prone to degradation and that middle sized oligomers are more stable and therefore crucial to complement activation (Yokota et al., 1995). In our study the higher bands were clearly visible at specific timepoints and no obvious degradation was observed.

There are a number of technical issues which must be considered when interpreting the results. The detectable bands are presumed to correlate with MBL trimer, tetramer, pentamer and hexamer but actual quantification is difficult. A band detected between the trimer and tetramer in some individuals at ~325 KDa remains of unknown origin

and detailed mass spectrometry of this specific band would be required to identify it. The small number of samples investigated were predominantly from *MBL-2* AA subjects and just two YAYD subjects and an increased sample size would be required to confirm the findings. The comparison of sequential samples was possible because an equal amount of MBL was loaded for each timepoint calculated from the MBL concentration measured by ELISA. The accuracy of the ELISA measurements could therefore affect the interpretation of the Western blot.

In the current study we have demonstrated variability in the activity of the MBL lectin pathway of complement in response to chemotherapy and FN in a small group of children on treatment for cancer. Investigations into MBL structure at these times suggest that higher order oligomers are more abundant at the onset of FN in some individuals and the reasons for this are currently unknown. The changes in complement function are presumed to be influenced by the combination of changes in both MBL and MASP levels and potentially MBL structure. Our small and heterogenous sample limits further interpretation of these findings but future work will be important in deciphering the role of replacement therapy after chemotherapy treatment.

Chapter 8

Transcriptional regulation of MBL

8.0 Introduction

Although the potential role of MBL during various infectious and inflammatory diseases has been studied in great detail, our current understanding of the regulation of MBL remains severely limited. MBL is a soluble protein produced mainly by hepatocytes, though extrahepatic transcription has been reported in the small intestine, testis tissue and in mononuclear cells (Downing et al., 2003; Seyfarth et al., 2006). It is well established that polymorphisms in the *MBL2* gene result in variation in the amount and/or function of the protein. For example: three point mutations in exon 1 impair formation of functional oligomers and the ability to activate downstream events. In conjunction with exon 1 polymorphisms, MBL expression is regulated by polymorphisms in the promoter region, with the XY polymorphism exerting the most profound effect.

Data presented in Chapter 7 show interindividual variation in MBL protein levels in response to chemotherapy and during FN which indicates that non-genetic mechanism(s) may influence its regulation. MBL is also suggested to be an acute phase reactant, adding further complexity to its regulation. An acute phase reactant is defined as a protein whose levels change by at least 25% in response to a stimulus e.g. inflammation (Morley and Kushner, 1982). An acute phase response is a systemic reaction to disturbances in homeostasis due to infection, tissue injury/trauma, surgery, neoplastic growth or an immunological disorder. The acute phase response (APR) is orchestrated by the release of cytokines (especially IL6) and activation of vascular and inflammatory cells. These in turn influence the production of acute phase reactants, such as C reactive protein (CRP), by hepatocytes (Kushner, 1993; Gabay and Kushner, 1999).

Characterisation of the *MBL2* gene and its promoter region has revealed similarities common to other acute phase reactants. Sequence analysis also highlights homology

with regions of the Drosophila heat shock consensus sequence, three regions similar to glucocorticoid-responsive elements and a sequence present in a cytokine responsive element found in serum amyloid A protein (SAA) (Sastry et al., 1989; Taylor et al., 1989). The MBL promoter was first characterised by Naito and co-workers and they found hepatocyte-specific nuclear factor (HNF)-3 sites within the sequence (Naito et al., 1999). Binding of transcription factor to these sites resulted in up regulation of MBL transcription; in contrast, activation of the glucocorticoid responsive elements resulted in down regulation. IL-6 responsive elements were also identified but their role in MBL transcriptional regulation needs further characterisation. Using the Huh7 hepatoma cell-line Arai *et al* found IL-6, dexamethasone and heatshock increased MBL gene expression (Arai et al., 1993). Investigations into the effect of other pro-inflammatory cytokines revealed that IL-1 inhibited transcription with minimal effect of TNFα and IFNγ. More recently, Sorensen *et al* reported thyroid and growth hormones can also influence MBL synthesis (Sorensen et al., 2006).

In vivo studies have reported a 1.5 to 3 fold increase in MBL serum levels following major surgery and compared this response to changes in CRP levels. A dramatic 1000 fold increase in CRP levels was noted, whereas MBL levels increased modestly and slowly as previously seen for C3 and ceruloplasmin (Thiel et al., 1992). Due to the very modest increase in MBL levels, defining MBL as an acute phase reactant may be an oversimplification.

Our knowledge regarding MBL's regulation during infection and inflammation remains limited and the purpose of the present series of experiments was to gain further insight into MBL transcriptional regulation.

8.1 Methods

8.1.1 Cell culture

Cell lines were maintained as described in Section 2.5.1. Cells were grown to confluency prior to stimulation studies (Section 2.5.2).

8.1.2 Reverse Transcription Polymerase Chain Reaction (RTPCR)

RNA was isolated from cell preparations prior to reverse transcription and then used in PCR. PCR products were visualised on agarose gels and semi-quantitative analysis was performed using densitometry. Specific details of the techniques used are detailed in Sections 2.5.3-2.5.5.

8.1.3 Cloning of the MBL promoter

The stepwise approach used to clone the MBL promoter is described in full in Section 2.5.6.

8.1.4 Transient transfection studies

The MBL promoter/luciferase construct was transiently transfected into liver cell-lines prior to stimulation studies. On termination of the experiments, luciferase activity was measured and normalised to Renilla. Full details are described in Section 2.5.7. The IL-8 promoter construct was used as a positive control for the cell-line.

8.2 Results

8.2.1 MBL gene expression in Hepatoma cell-lines

Initial experiments were conducted to confirm the genotype of the two hepatoma cell-lines. Genotyping (using the heteroduplexing method) revealed the HepG2 cell-line was heterozygous for a mutation in codon 54 (A/B) and the Huh7 cell-line was homozygous for wildtype alleles (A/A) (Figure 8.1). This is in agreement with a previous study (Dumestre-Perard et al., 2004). Both cell-lines were homozygous for the Y promoter (not shown) and their full haplotype combinations analysed using the INNO-LiPA assay were HYPA/HYPA, normally associated with high MBL levels and LYPB/HYPA, associated with medium to low levels.

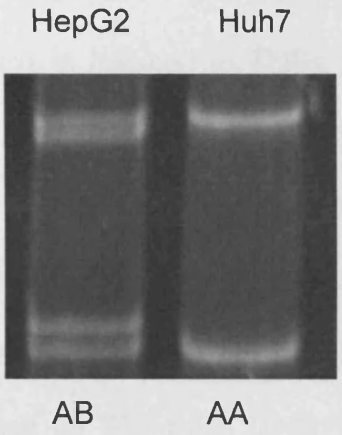


Figure 8.1 Exon 1 heteroduplexing results for HepG2 and Huh7 hepatoma cell-lines

MBL mRNA was detected in both cell-lines and also in primary hepatocytes (kindly provided by Dr P Smith, Institute of Child Health, London). The two cell-lines investigated showed constitutive MBL expression with the wild type Huh7 cells expressing at least 3 fold more mRNA when compared to the Hep G2 cell-line, measured by densitometry. This confirmed the influence of genotype on MBL mRNA expression as expected (Figures 8.2 and 8.4).

Cytokines that are produced at inflammatory sites, predominantly by macrophages and monocytes, are the chief stimulators of the production of acute phase proteins. Therefore we wished to understand how this cytokine milieu in an ongoing inflammatory episode may influence MBL expression. The potential effect of pro-inflammatory cytokines including IL-1β, TNFα, INFγ and IL-6 on MBL mRNA expression was investigated. IL-10 and IL-22 were chosen as representative of 'anti-inflammatory' responses. *E.coli* LPS was used to mimic the effect of Gram-negative sepsis.

Expression of MBL in response to the various stimuli in both Huh7 and HepG2 cell-lines is shown in Figures 8.2-8.5. In the Huh7 cell-line a significant reduction in IL-1β mediated mRNA expression was noted 24h post stimulation. A trend for decrease in MBL expression was also noted in response to TNFα. IL-6 and IL-10 had minimal effect on expression however an increase was noted in response to IL-22 which was greater at 8h compared to 24h. In contrast in Hep G2 cell-line effects of IL-1β on MBL mRNA expression were modest, reaching no statistical significance. IL-6 had the greatest effect on MBL expression in the HepG2 cell-line and a trend for increase in MBL expression was also noted for TNFα and IL-22. Minimal effect of IL-10 was observed but LPS did modulate expression at 24h.

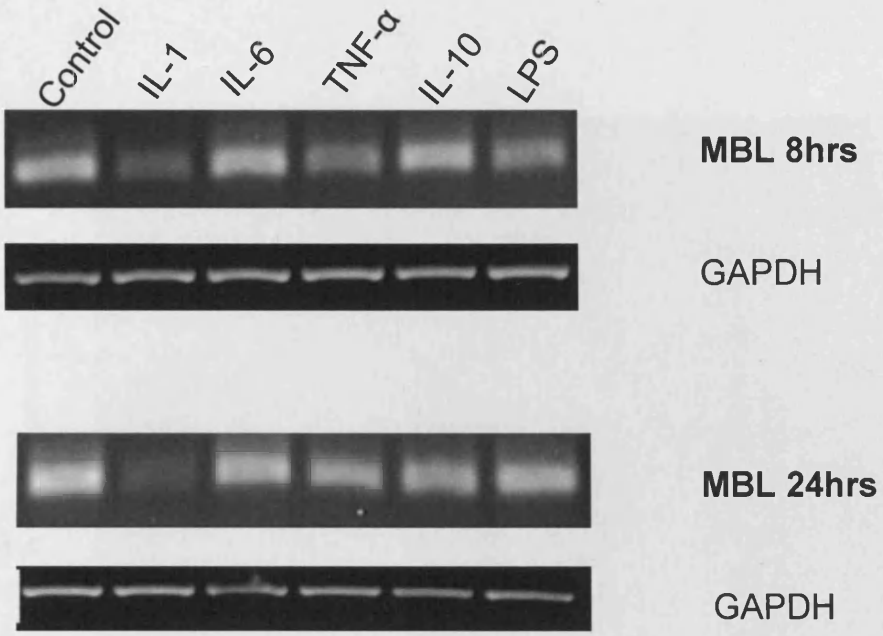


Figure 8.2 Expression of MBL in Huh7 cell line

RNA from Huh7 cells was amplified by RT-PCR with primers specific for MBL and GAPDH. The constitutive expression of MBL in this cell line was found to be further modulated by cytokines. Results shown are representative data from 3 experiments performed at 8hrs and 24hrs.

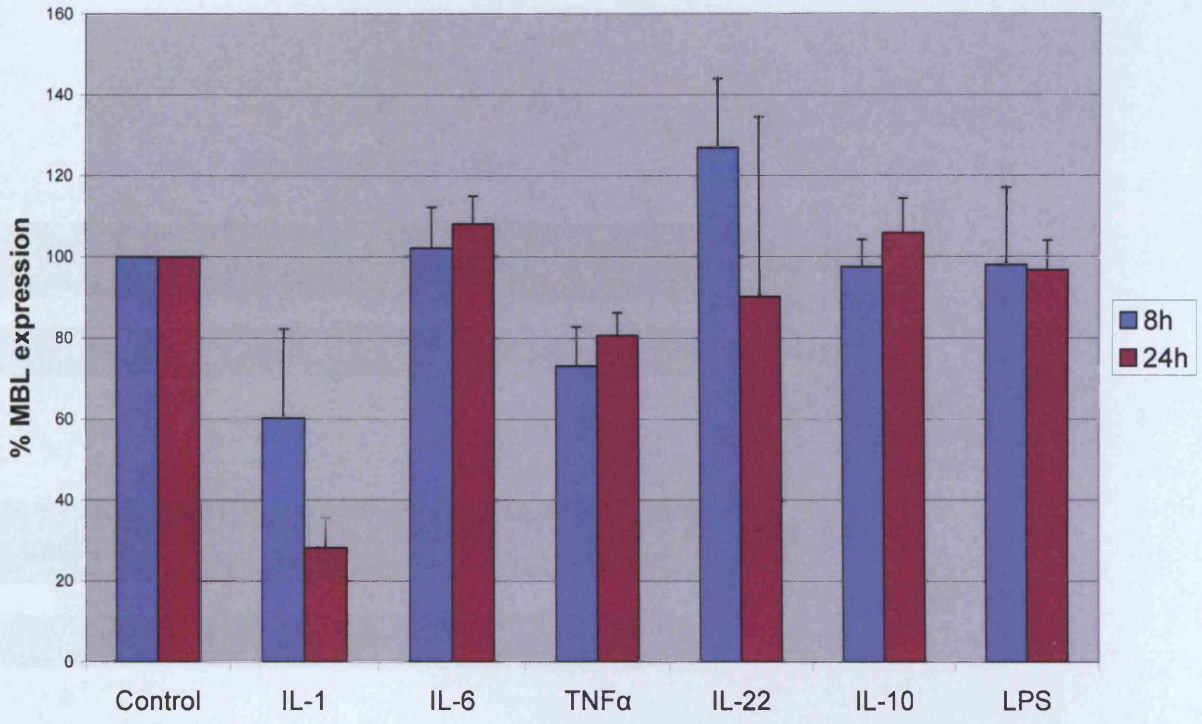


Figure 8.3 Regulation of MBL mRNA expression in Huh7 cells

Graphical representation of the mean MBL mRNA expression at 8 and 24h. Data shown are from three independent experiments and results are presented as mean ± S.E.

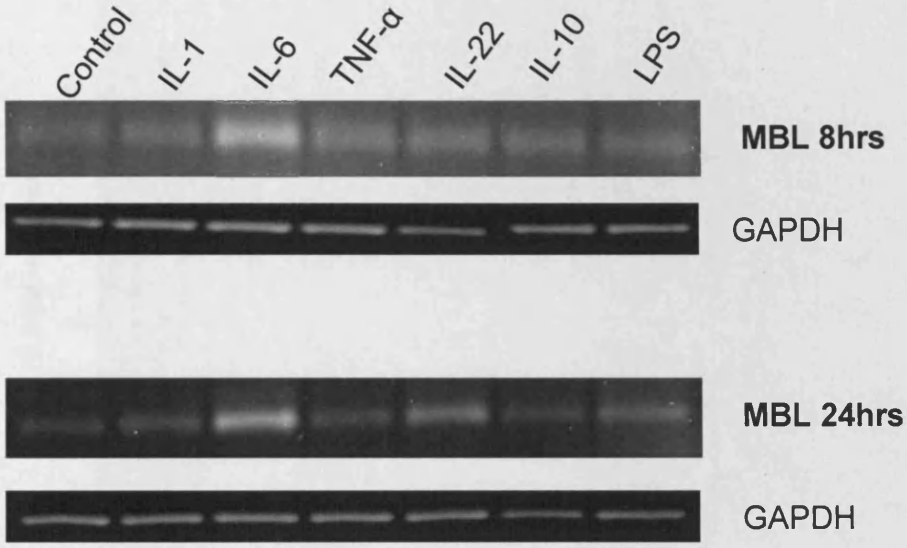


Figure 8.4 Expression of MBL in HepG2 cell line

RNA from HepG2 cells was amplified by RT-PCR with primers specific for MBL and GAPDH. MBL gene expression was constitutive and further modulated by cytokines. Results shown are representative data from 3 experiments performed at 8 and 24hrs.

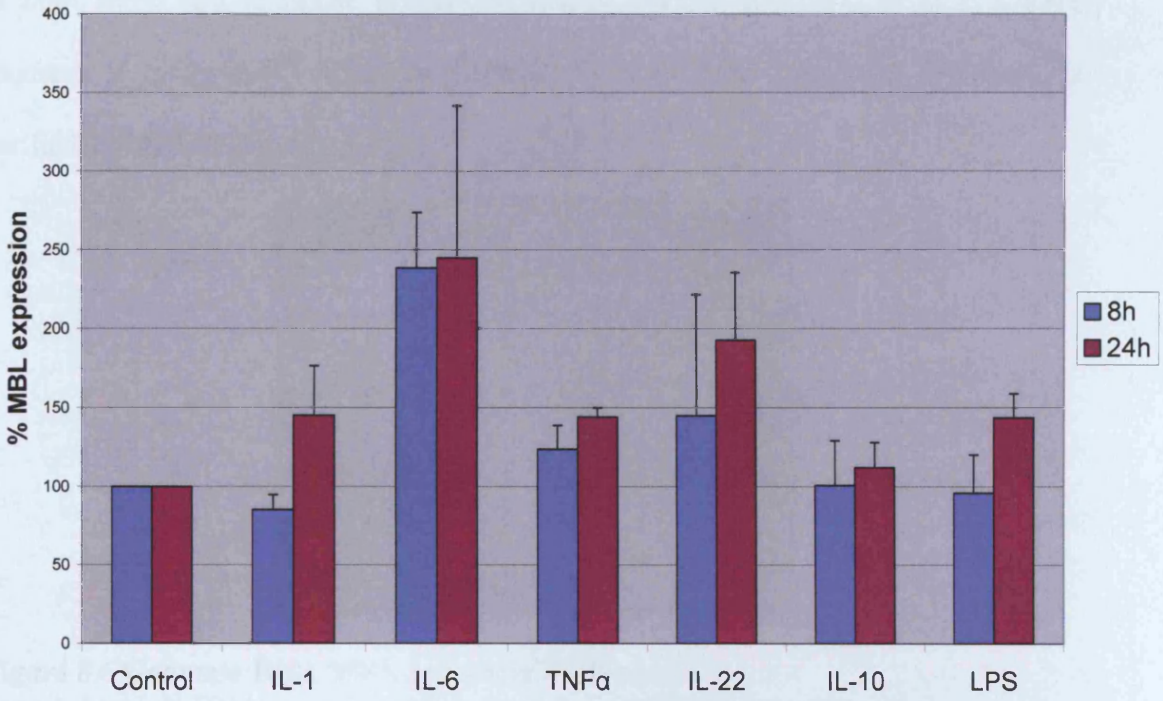


Figure 8.5 Regulation of MBL mRNA expression in HepG2 cells

Graphical representation of the mean MBL mRNA expression at 8 and 24h. Data shown are from three independent experiments and results are presented as mean ± S.E.

Due to contrasting findings noted in the two cell-lines, we wished to further confirm our data using the more sensitive methodology of promoter-luciferase assays. This required cloning of the MBL promoter.

8.2.2. Cloning of the MBL promoter

The MBL promoter of choice was from an individual with the HYPA/LYPA haplotype combination. Primers were designed with restriction enzyme sites tagged at the 5' end of both sense and antisense primers to enable specific amplification of a 2933bp fragment 5' of the ATG codon, as described by Naito et al. Figure 8.6 illustrates the purified 2.93KB PCR product.

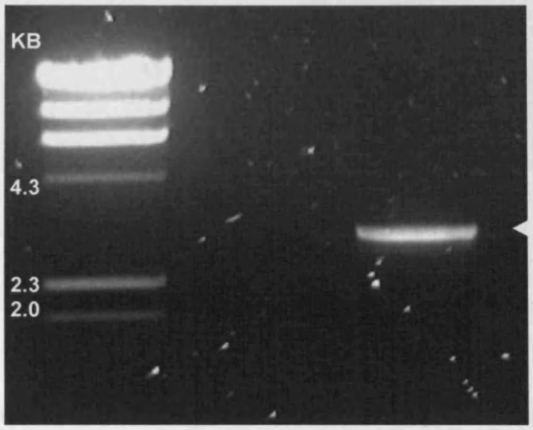


Figure 8.6 Genomic DNA MBL promoter PCR product.
Lane 1, Lambda DNA/Hind III marker. Lane 2, 2.93KB MBL promoter PCR product

Enzyme digestion of the MBL promoter and a promoterless luciferase reporter vector, the pGL3 basic vector, was performed using Hind III and Nco1 restriction enzyme (Figure 8.7).

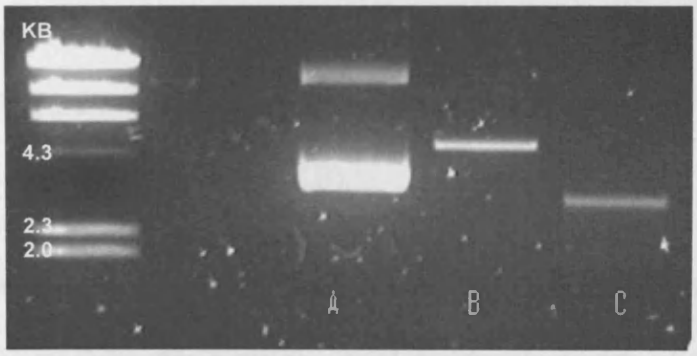


Figure 8.7 Restriction digest products.
Lambda DNA/Hind III marker shown. Lane A- uncut vector, Lane B- cut vector ~5KB, Lane C- cut PCR product 2.93KB.

This step was followed by successful ligation of the PCR product into the vector, observed by the presence of higher molecular weight bands (lanes G and H) (Figure 8.8).

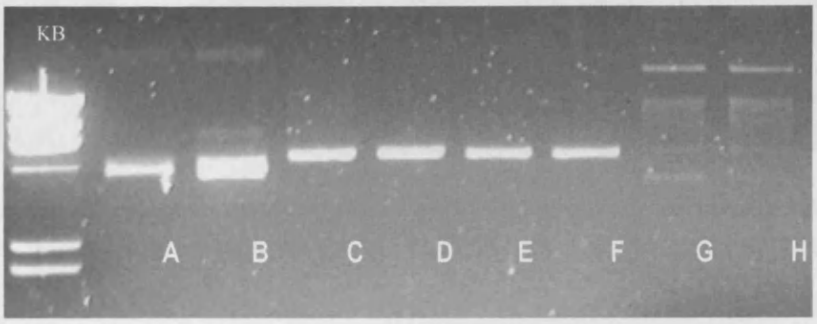


Figure 8.8 Ligation results.
Lambda DNA/Hind III marker shown (KB). Lane A&B- Vector, Lane C&D single cut HindIII and Nco1 respectively, Lanes E&F double cuts, Lanes G&H ligation product.

Transformation was performed using competent JM109 *E.coli* cells and subsequent colonies were screened for the presence of the MBL promoter insert (Figure 8.9).

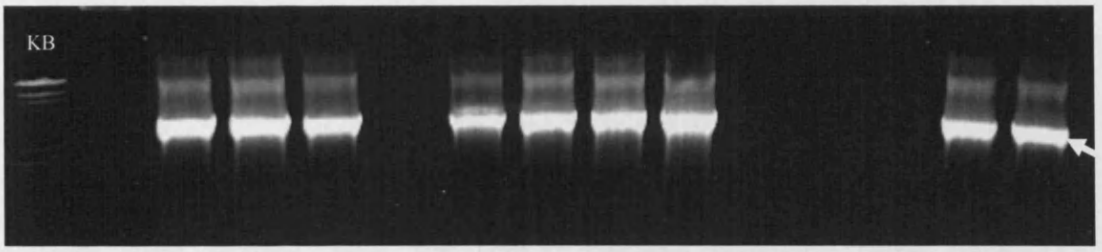


Figure 8.9 PCR results confirming presence of MBL promoter construct in 9 colonies.
Lambda DNA/Hind III marker shown (KB).

Double digests were subsequently performed to confirm the presence of the insert in the cloned vector. Figure 8.10 shows the results of the double digests of four independent colonies. All four showed the presence of the correct sized fragments for vector and insert. A third higher band represents uncut plasmid DNA.

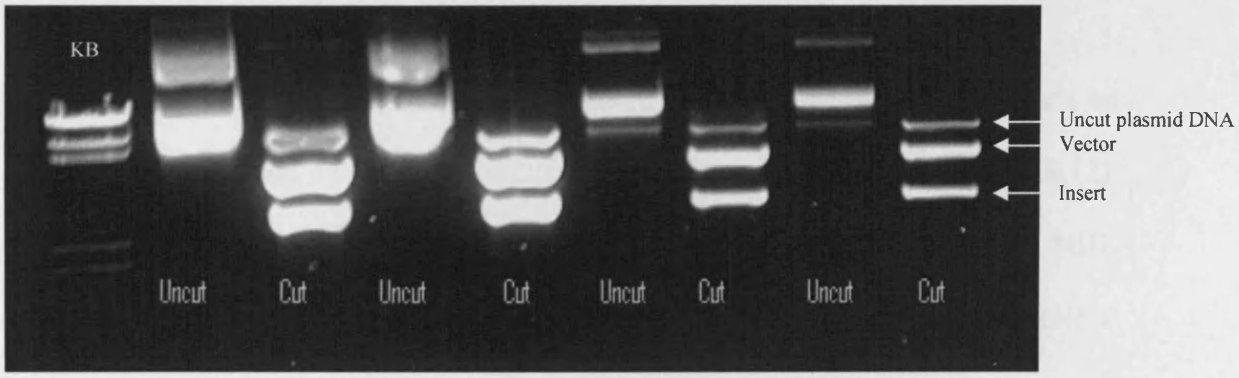


Figure 8.10 Double digests of 4 colonies showing presence of vector and insert in cut samples. A third higher band in the cut samples represents uncut plasmid DNA.

Sequencing of plasmid DNA using commercial primers spanning the vector was performed at the UCL core facility. Analysis of over 600bp confirmed complete homology with the published sequence and, specifically, all three MBL promoter polymorphisms were present.

8.2.3 Preliminary transfection studies

Once cloned, preliminary studies were performed to establish the optimum promoter concentration that would be used in transfection experiments. Both Huh7 and HepG2 cell-lines were studied, with the HepG2 cell-line providing better transfection efficiency. All subsequent studies were conducted in HepG2 cells. Following transfection cells were stimulated for 8 and 24h with cytokines and LPS alone and/or in combination.

8.2.4 MBL promoter/luciferase studies

IL-1β, IL-6 and LPS showed a slight propensity for MBL promoter activation, between 1.2 and 1.5 fold induction during the first 24h (Figure 8.11). None of these effects were statistically significant. TNFα was the most potent cytokine tested with at least 2-fold

induction at 8h increasing to 4 fold at 24h. In contrast IFNγ showed a slight reduction when compared to control.

As *in-vivo*, cytokines are likely to act in concert, we hypothesised greater effects on MBL gene expression in the presence of combinations of stimulants (Figure 8.12). Interestingly IL-6 in combination with IL-1 and LPS showed an additive effect on MBL transcription. No additive effect of IL-6 was noted in the presence of TNFα. Importantly, addition of a third stimulant in any form did not enhance MBL gene expression, highlighting the tight transcriptional regulation of this critical innate immune molecule.

The IL-8 promoter was used as a positive control for the cell-line and over 50 fold induction was observed following IL-1 stimulation (data not shown).

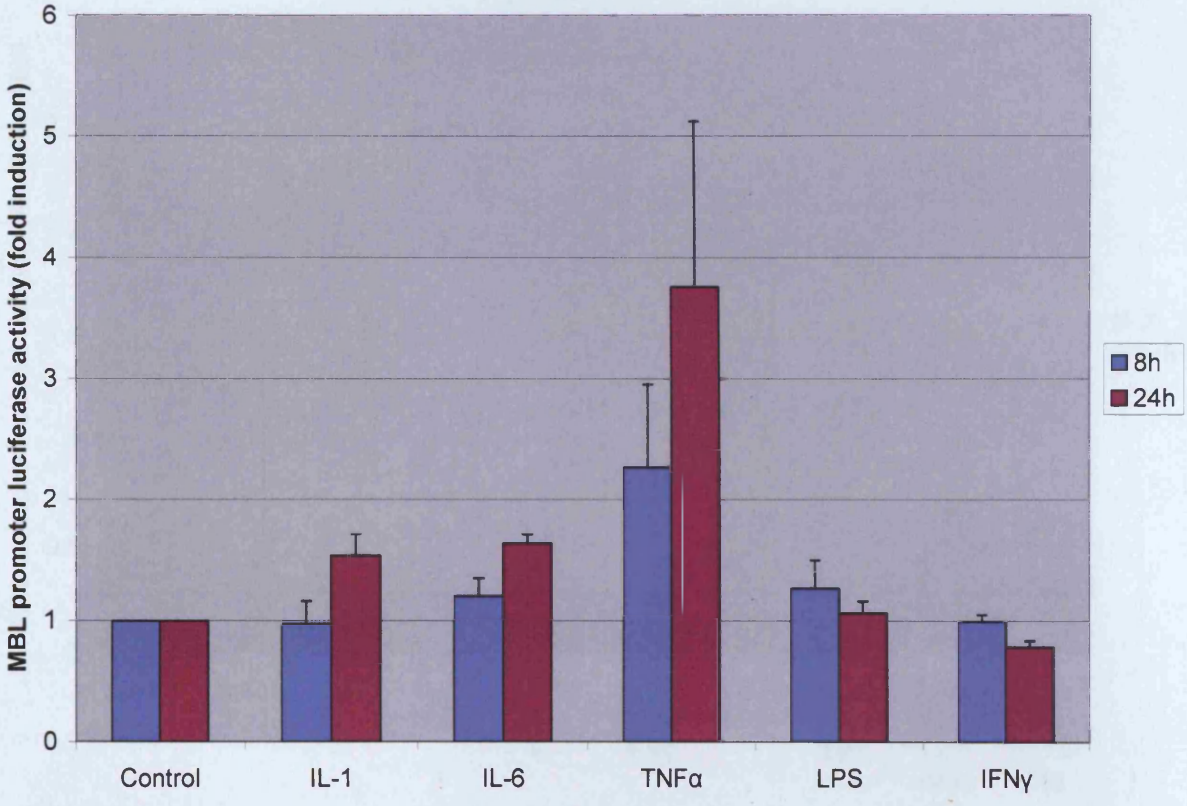


Figure 8.11 MBL promoter activity in response to cytokine/LPS stimulation in HepG2 cells.

Promoter luciferase activity was assessed by normalisation to Renilla luciferase. Data are expressed as n-fold changes in luciferase activity when compared with unstimulated, control cells. Error bars indicate ± S.E. of values representative of 3 experiments conducted in triplicate.

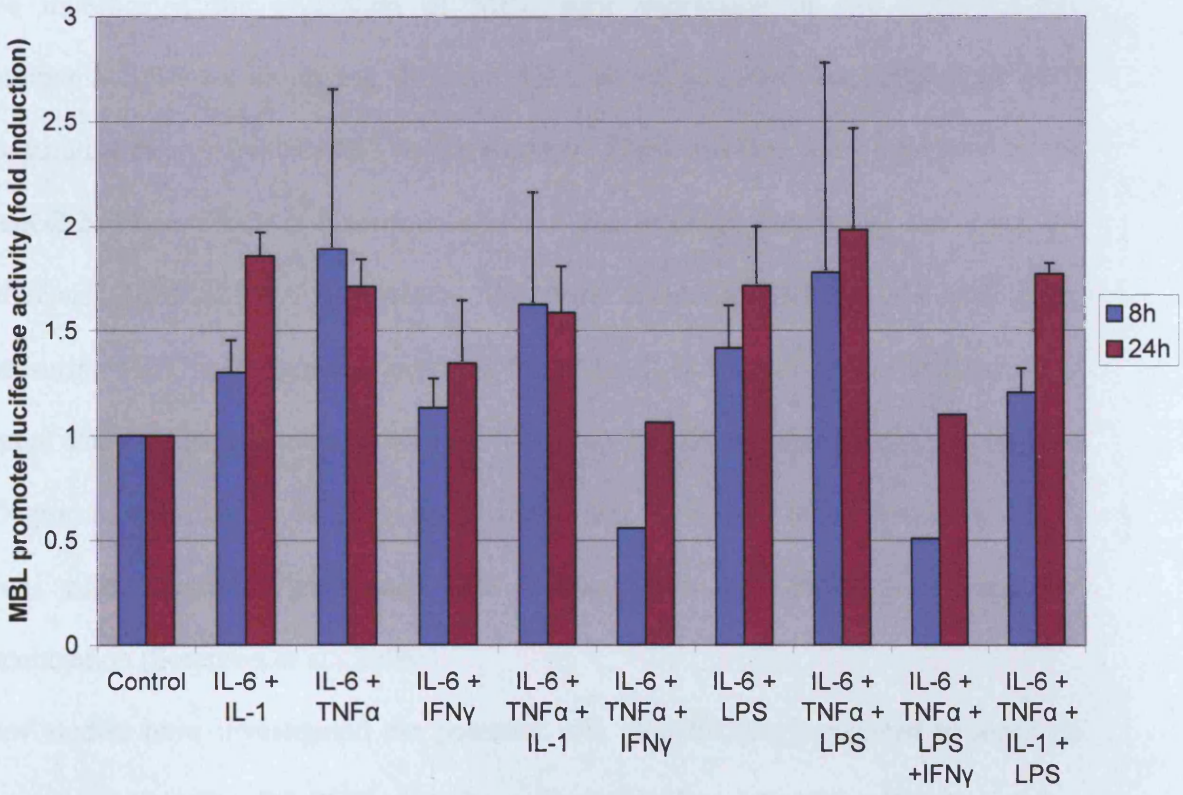


Figure 8.12 MBL promoter activity in response to stimulation with cytokine/LPS combinations in HepG2 cells.

Promoter luciferase activity was assessed by normalisation to Renilla luciferase. Data are expressed as n-fold changes in luciferase activity when compared with unstimulated, control cells. Error bars indicate ± S.E. of values representative of 3 experiments conducted in triplicate (where error bars are not shown experiments had been performed only once).

8.3 Discussion

To date there have been a limited number of studies investigating MBL transcriptional regulation with only one study investigating promoter function. The work presented in this chapter was undertaken to further delineate the regulation of MBL gene expression and its potential role as an acute phase reactant.

We investigated the regulation of MBL gene expression in two hepatocellular carcinoma cell-lines exhibiting different MBL genotypes. We found higher (3 fold) constitutive expression of MBL in the wild-type Huh7 cell-line when compared to the HepG2 cell-line which is heterozygous for a variant allele, confirming that genotype influences MBL mRNA expression. We were unable to confirm this finding by measuring MBL in the supernatant using the MBL ELISA described in Chapter 2 but a recent study utilising a more sensitive MBL assay (TRIFMA) with a detection limit of 30pg/ml reported higher MBL levels in supernatant from Huh7 cells when compared to Huh1 cells, another heterozygous liver cell line with the LYPB/LYQA haplotype combination (Sorensen et al., 2006).

Few studies have investigated the potential role of cytokines implicated in sepsis in regulating MBL. The RT-PCR experiments in the Huh7 and HepG2 cell-lines revealed contrasting findings. These may relate to genuine differences between the cell-lines, the limited sensitivity of the technique and/or their markedly different levels of constitutive MBL expression which influences interpretation of results. IL-6, IL-1 and TNF-α were chosen as pro-inflammatory cytokines and the observed upregulation in response to IL-6 and down-regulation with IL-1 is in agreement with a previous report (Arai et al., 1993). The effect of IL-6 on MBL gene expression was greatest in the HepG2 cell line. TNF-α also modulated MBL expression in the HepG2 cell line. IL-10 has anti-inflammatory effects and IL-22 is a member of the same cytokine family. IL-22 has previously been reported to up-regulate mRNA expression of acute phase reactants

(serum Amyloid A, α1-antichymotrypsin and haptoglobin) (Dumoutier et al., 2000). For this reason, we also wished to investigate if IL-22 and IL-10 influenced MBL expression. We found IL-22 induced MBL expression in a time dependant manner with greater effects noted in HepG2 cells but IL-10 exerted no observable effect on MBL transcriptional regulation. An effect of *E.coli* LPS was only observed in the HepG2 cell line at 24h. A recent study by Sorensen *et al* was able to detect MBL in culture supernatants of Huh7 cells stimulated with IL-6 for 3 days (Sorensen et al., 2006). A dose dependent increase in MBL was observed with a maximum two fold increase in cells stimulated with 1μg/ml IL-6 which is above levels normally used in experiments investigating acute phase activity and it could also be argued that 3 days is beyond the time of an innate response.

Promoter studies in HepG2 cells revealed upregulation with TNF-α and IL-6. Interestingly the only other study investigating the promoter reported no significant effect of IL-6, TNF-α or IFN-γ on MBL transcriptional activity (Naito et al., 1999). Naito *et al* used deletion constructs to conclude these findings in contrast to the stimulation experiments reported. Of note, when using combinations of cytokines and LPS, TNF-α alone remained the most potent stimulant.

Detailed analysis of the promoter sequence identifies one potential NFκB binding site through which you would expect IL-1, IL-6 and TNF-α cytokines to be operating but there is no published data from deletion construct studies to confirm this (Dr M Haston, Institute of Child Health, London, personal communication). Synergistic effects were observed when some cytokines were combined and generally effects were greater at 24 than 8h. IL-1 appeared to exert a mild inhibitory effect in some combinations as did IFN-γ. This IL-1 effect may be time dependent as it has previously been noted to down regulate gene expression at 12 and 24h but upregulate it at 48h (Arai et al., 1993).

Overall, promoter activity (fold induction) observed with the different stimulants was relatively low. In contrast, the IL-8 promoter was used as an internal control for the cell line and over 50 fold induction was observed in response to IL-1 stimulation. Naito *et al* made deletion constructs which enabled identification of a number of both negatively and positively acting elements within the promoter sequence implicating a complex system which responds to a variety of stimuli (Naito et al., 1999). The construct utilized in the current study contained all of these elements and results are likely to represent their combined overall effect. Variation in MBL levels observed in individuals in response to infection has been reported to be up to 3 fold (Thiel et al., 1992). Murine MBL acute phase responses have also been studied and in response to LPS stimulation, MBL A levels were reported to increase two fold (after 32h) but no increase in MBL C was observed (Liu et al., 2001). The protein is also subject to a number of post translational modifications and therefore the relevance of such findings to the *in-vivo* situation remains unclear. Measurement of actual protein levels may be the most useful measure of the acute phase response in future studies. Our use of 8 and 24h timepoints may have been too early to assess the maximum response but as already discussed we would argue that these would reflect an innate response and longer timepoints would not.

This work adds support to the idea of a complex system of regulation involved in MBL expression which varies in individuals depending on their exposure to specific stimuli. Further work cloning different promoters, e.g. LXP/LXP, would be very useful to enable a comparison of the response to the stimuli used in this series of experiments, to further delineate individual variations in response. Gene deletion/directed mutagenesis experiments may also enable identification of novel transcription factor binding sites.

Chapter 9

General discussion and future prospects

9.0 General discussion and future prospects

The successful treatment of cancer in children comes at a price. Both disease and treatment render children immunosuppressed and susceptible to infection which causes significant morbidity and mortality. Treatment related infection is the second commonest reason for admission to hospital during treatment for cancer and has significant implications for both the health service and the quality of life of the patient and their family. Work to improve the treatment of infection is ongoing, specifically the ability to identify people at lower risk of infection who may be suitable for less intensive treatment with the goal of spending less time in hospital. What remains clear is that children with the same disease, treated on the same protocol vary in their susceptibility to infection. Identifying the reasons for this variability could prove critical to optimising management. Focus on the immune system may hold the key to this variation. It has been proposed that the innate immune system, our first line of defence against infection, may assume greatest importance at such times. MBL has been proposed in a number of studies to play a role in infection susceptibility. Genetically defined deficiency has been investigated but the findings of such studies have been variable leading to controversy in the field. MBL has warranted greater interest because recombinant protein has been produced and the oncology population have been proposed as potential candidates for therapy. However, a number of questions remain unanswered which are paramount to delineating the role of MBL replacement therapy in the future.

To address the issues remaining in the field we have undertaken a number of studies in a population of children with cancer. In collaboration with the 4 specialist oncology centres in London we have developed a new management strategy for FN which has been introduced in over 40 hospitals. Our cohort has provided us with the largest group of children with cancer in whom MBL gene polymorphisms have been studied in

relation to infection susceptibility to date. MBL genotyping has been performed using 2 different techniques and the benefits of these have been compared for their potential use in large scale studies. Protein work has been performed on a subgroup of these patients to explore MBL and MASP protein levels and functional activity of the lectin pathway of complement. Techniques have been developed to explore changes in MBL structure in response to both chemotherapy and during FN episodes. We have also explored the effects of chemotherapy and infection in a longitudinal manner enabling us to question the mechanisms behind MBL's potential protective effect. The regulation of MBL expression is largely unknown and we have also investigated transcriptional control mechanisms.

9.1 The role of risk stratification (RS) in FN management in children

RS is not a new concept but it is relatively under used in paediatric populations because there are no universally agreed definitions. We were able to introduce of a new protocol (Chapter 3) which now enables approximately 20% of patients to be discharged after 48h in hospital compared to previous median stays of 5 days for uncomplicated FN. Safe introduction of such a protocol into multiple centres with differing experience and confidence in the management of these children was the main priority. The data in this study represented the first year of its introduction and with time we would expect confidence in the application of the low risk policy to improve. Acceptance of the new management strategy by patients and their families is equally important. Studies have suggested that parental anxiety may limit uptake of outpatient FN management strategies, emphasising the need for good support and education (Sung et al., 2004, Laddie and Chisholm, 2007).

Our RS methodology concentrated on clinical variables and the patients underlying disease and treatment. Work is ongoing to improve the accuracy of the process and the

inclusion of potential immune modifiers or genetic information about our immune system may optimise this further. The use of cytokine measurements such as IL8 has been reported and Oude Nijhuis *et al* described a cohort of patients treated for FN as outpatients selected using a combination of clinical data and IL8 measurements (Oude Nijhuis et al., 2005). At present such work is limited to research centres as these measurements are not routinely undertaken. The advantage of genetic information is that it can be obtained at the time of diagnosis of cancer and utilised in future RS processes. Figure 9.1 represents a proposed hierarchy of RS criteria utilising potential innate immune modifiers. These include patient specific factors that remain relatively constant throughout treatment (shown in blue) and episode specific factors that further influence the decision making process in any given FN episode (shown in red).

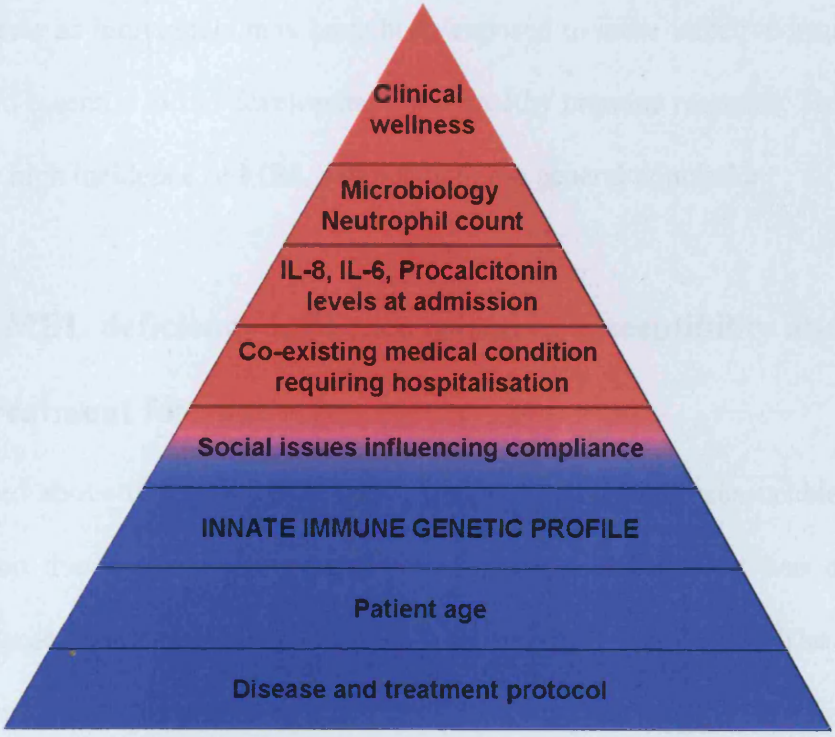


Figure 9.1 Hierarchy of factors influencing RS.
Base of pyramid (blue) represents patient specific factors, top of pyramid represents episode specific factors (red).

9.2 Does MBL deficiency predispose children to cancer?

It has been proposed that MBL deficiency may predispose people to cancer. For this purpose *MBL2* genotyping was performed (Chapter 4). Our data represents the largest population of children with cancer studied to date. We did not observe marked differences between our population and a similar paediatric cohort (Mead et al., 1997); any differences observed related to ethnicity and could not be explained by underlying disease. A link between MBL deficiency and childhood ALL has been reported but this work has not been supported by further studies (Schmiegelow et al., 2002). As expected our largest single disease group was ALL and the population was similar to the total group. The aetiology of childhood leukaemia remains unknown but an abnormal immune response to a common infection in a previously unexposed individual is proposed (Greaves 2006). With this regard MBL deficiency may actually have a protective role as individuals may have been exposed to more infective insults in early life, deemed essential to the development of a healthy immune response. This may also support the high incidence of MBL deficiency in the general population.

9.3 Does MBL deficiency influence infection susceptibility and severity during treatment for cancer?

As discussed above (Chapter 1) paediatric studies to date have been unable to answer this question due to study design and patient numbers. Our study was designed to capture a larger cohort and study patients over a longer time course. The group was heterogenous and the data was not truly prospective but the overall information suggests unequivocally that patients heterozygous or homozygous for MBL mutations experienced more FN episodes with a median of 2 *versus* 1 compared to wild type and these FN episodes were more likely to represent clinically or microbiologically defined infections. This supports the concept that MBL is likely to be important in bacterial

infections, however the individual infections found in the present study did not implicate a propensity for specific infections within our MBL deficient group (Chapter 5). The duration of hospitalisation, used as a surrogate marker of severity of FN, was prolonged in patients deemed MBL deficient. Our analyses identified that this effect was most profound in patients at higher risk of severe infection by virtue of their underlying disease or treatment. Such endpoints are subject to a number of confounding variables but our findings were relatively consistent between groups, i.e. that the MBL deficient patients spent longer in hospital with FN compared to their MBL sufficient peers.

As the name suggests FN encompasses neutropenia in a febrile patient and implies a state of infection until proven otherwise. The contribution of MBL to this process is not in prevention of neutropenia or from a patient becoming febrile. Our data suggest that MBL sufficiency/deficiency influences the incidence of documented infection. If this is the case it may prevent infection developing or simply modify the clinical consequences e.g. lower the risk of prolonged/severe infection. Such an effect is supported by animal studies which have demonstrated that neutropenic MBL WT mice were able to clear their bacterial load faster than their MBL deficient litter-mates (Shi et al., 2004). The interpretation of data relating to paediatric intensive care unit admission in our population and in previously studied populations remains unclear. It may be inappropriate to extrapolate our findings to this cohort as there are often many reasons why patients get admitted to PICU (Meyer et al., 2005) which do not purely reflect their ability to respond to infection.

The use of MBL gene status in RS was inconclusive (Chapter 5) due to the group size. We hypothesised that MBL deficient patients would be more likely to require readmission to hospital following early discharge however the safety of our protocol resulted in very low readmission rates overall. Further work would be required in order

to answer this question studying a larger cohort over a longer follow up period. It may be more useful in future studies to select patients from specific diagnostic groups at specific stages in their treatment for management with oral antibiotics from the outset. One such group would be children with ALL on maintenance therapy who are less likely to have a clinically or microbiologically defined infection and less likely to require prolonged hospital admission.

The heterogeneity of patients, in terms of diagnosis and treatment over the one year study period is likely to affect the validity of our findings. The potential use of MBL status in FN management is dependent on the definition of 'risk' and it is important to accept that this will vary over time. The influence of MBL during such episodes is also likely to differ depending on the degree of immunosuppression. This is explained by the concept of environmentally determined genetic expression (EDGE), i.e. genetically encoded differences in expressed proteins react differently under increasingly extreme environmental conditions. The resulting phenotype or pathology is determined through a combination of the functional magnitude of the genetic change and the severity of the environmental stimulus. Rare genetic disorders represent one extreme resulting in a phenotype while extreme environmental conditions represent the other and the majority of diseases lie between these two extremes (Figure 9.2).

This concept supports studies suggesting that the MBL gene is relatively redundant in a healthy host and implies that the environmental stimulus of different chemotherapy regimes coupled with MBL deficiency will inevitably result in different phenotypes over time. When interpreting our results we have compared patients with differing levels of risk in terms of developing FN or severe infection and despite this we have observed a relationship with MBL status. Using ALL as an example we have compared patients undergoing intensification with those on maintenance, two very different states of immuno-competence. Further prospective studies of disease specific populations are

Figure 9.2 The EDGE concept.
Differing phenotypes or pathologies are reached through a combination of the severity of the genetic change and the severity of the environmental stimulus (Kallianpur, 2005).

warranted in order to prove the influence of MBL deficiency on infection burden during treatment for cancer.

9.4 Assessment of MBL or Lectin Pathway deficiency?

Our disease association studies utilised MBL genotype and levels (Chapter 5) but the question remains as to whether MBL genotype/level is the most clinically relevant measure to use. MBL does not work in isolation and the concept of one gene one effect would be somewhat surprising, hence our studies progressed to explore other components of the Lectin Pathway (Figure 9.3). Assessment of the functional activity of the three complement activation pathways identified a group of *MBL2* WT subjects with protein levels in the normal expected range (>1000ng/ml) who were deficient in function. This group was able to form higher order oligomers (Figure 6.10), therefore structural differences did not explain the observed defect in function. We hypothesised that downstream components, the MASP enzymes may be responsible for the lack of activity in this group.

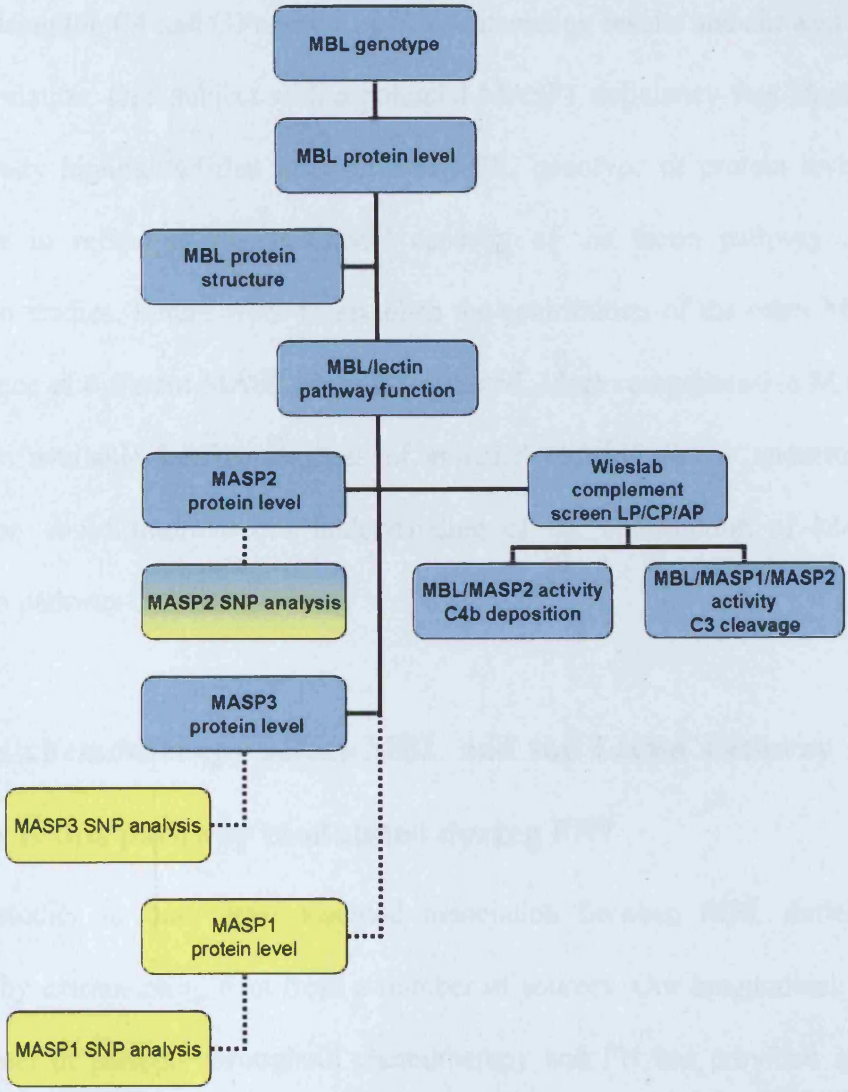


Figure 9.3 Investigation of MBL specific lectin pathway activation
Blue boxes represent parameters analysed in the current study. Yellow boxes represent additional investigations which would contribute valuable information to future studies of abnormal pathway function.

Very little data is available on MASP levels in paediatric or diseased populations. Polymorphisms in the MASP2 gene have been reported which correlate with low protein levels. We investigated the MASP2 D105G polymorphism and a number of heterozygotes were identified with corresponding low protein levels. Other polymorphisms are likely to be responsible for low MASP2 levels observed in a number of other individuals. MASP3 was found to be present at high levels when compared to MASP2, the reason for this is unknown. Assessment of the different steps in the

pathway using the C4 and C3 assays provided interesting results and showed good inter assay correlation. One subject with a potential MASP1 deficiency was identified. The present study highlighted that utilisation of MBL genotype or protein level alone is inadequate in reflecting the functional capacity of the lectin pathway in disease association studies. Future work to establish the contribution of the other MASPs and the influence of different MASP SNPs is warranted. More comprehensive MASP2 SNP analysis is available but the genetics of *masp1/3* remains poorly understood. Such information could improve our understanding of the contribution of MASP1 and MASP3 to pathway dynamics.

9.5 Does chemotherapy affect MBL and the Lectin Pathway function and how is this pathway modulated during FN?

Clinical studies to date have assumed association between MBL deficiency and infection by extrapolating data from a number of sources. Our longitudinal study of a small subset of patients throughout chemotherapy and FN has provided insight into potential mechanism(s) that may be involved. We found chemotherapy did not markedly affect MBL production and this is in agreement with previous findings (Neth et al., 2001). Marked individual variation was observed during different episodes of chemotherapy which may relate to different chemotherapy drugs used. There was also no obvious effect of chemotherapy on higher order oligomer structure. Variation was seen from sample to sample but due to saturation this was difficult to interpret.

Our study is the first to investigate MASP levels in this context and maybe an important component contributing to changes in functional activity. Increase in MASP2 levels and marked decreases in MASP3 were observed in response to chemotherapy; in fact 26 out of 27 patients showed a decrease in MASP3 levels (Figure 7.3). Current understanding of MASP3 function is limited. It is present in a variety of tissues and is

not just hepatic in origin. Despite dynamic changes in MASP2 and MASP3 protein levels marked changes in functional activity with chemotherapy were not observed. Interpretation of these findings is limited by different durations of chemotherapy courses and different combinations of drugs used.

In contrast, differences in MBL structure and functional activity throughout FN episodes were observed. MBL higher order oligomers rather than the amount of total protein may be critical during FN. During a number of episodes (and consistent within the same individual) an additional higher band was identified on the first day of FN. One may hypothesise that patients suffering from infections respond by increasing production of higher order oligomers which have enhanced complement function. Of the two HYPD/HYPA subjects studied, one also showed the presence of higher oligomers on day 1 of FN. Further studies are required to explore how MBL heterozygotes respond to infection.

MBL pathway function appeared to peak on day 7 of the FN episode implying that it was playing a role within the resolution of these episodes. As the majority of our subjects in the study were MBL WT we were unable to test the hypothesis that heterozygotes were unable to increase their functional activity and hence suffered prolonged episodes. Variability in classical and alternative pathway function was less apparent throughout the period post-chemotherapy. This may relate to the sensitivity of assays utilised or may imply that components of these pathways are less affected by treatment and disease. The significant reduction in MASP3 levels during chemotherapy returned to pre-chemotherapy levels during the episode and MASP2 levels peaked on the first day of FN, falling thereafter. Results from Chapter 7 highlight the variability in MBL during stress but also imply that changes in other components of the pathway e.g. MASP2 are likely to influence function. Further work is required to delineate the complex control of these proteins and their interactions.

Very little attention has been given to the transcriptional control of the MBL gene unlike other APRs e.g. CRP. Findings from Chapter 8 investigating the transcriptional regulation of the MBL protein using promoter studies revealed complex regulation of the system. Promoter activity was relatively low in keeping with changes observed in MBL protein levels in response to infection or inflammation. The influence of IL-6 and IL-22 with known effects on liver homeostasis was apparent and the signalling pathways involved are currently under investigation. Our results are in agreement with previous studies i.e. that MBL does not behave like a classical acute phase reactant such as CRP.

9.6 MBL replacement therapy

The work presented supports a role for MBL and the lectin pathway of complement in the regulation of infections in children with cancer and highlights the pitfalls of defining MBL deficiency by genotype or protein level alone. The question remains of how and when MBL replacement therapy may be a viable option. The present study emphasizes the need for potential candidates to undergo full assessment of lectin pathway function prior to consideration for therapy as we found patients who were WT for MBL genotype with normal expected MBL protein levels but an inactive lectin pathway. The identification of individuals with MASP2 deficiency is of particular relevance as administration of additional MBL would be fruitless in such subjects. Further work to identify subjects with other MASP deficiencies is also important in order to optimise the effects of rMBL in the most appropriate subjects. In terms of disease groups it seems most relevant to target those at increased risk of clinically significant infection on highly immunosuppressive drug regimens. Previous studies have suggested that any effect of MBL is overshadowed in such situations and that patients who are less immunocompromised by treatment may benefit more (Bergmann et al., 2003; Klein and

Kilpatrick, 2004). Our data, using inpatient stay as a proxy measure of severity suggest that MBL deficiency assumes greatest relevance in patients at higher risk of clinically significant infection by virtue of their disease or treatment protocol but our numbers were too small to show significance. Improvements in supportive care and the universal use of broad spectrum antibiotics has resulted in the vast majority of patients recovering from FN without significant complications. We must await proof of concept studies in order to answer the question of who would benefit most.

Issues regarding patient selection rely somewhat on the decisions regarding administration. Phase I trials suggest that rMBL has a half life of ~31 hours and repeat infusions are required to maintain adequate plasma concentrations (>1000ng/ml) (Petersen et al., 2006). Whether MBL should be used as a prophylactic measure or a rescue treatment is open to debate. Our data suggest that MBL deficient patients suffer more episodes of FN with specifically clinically or microbiologically defined infections. This is in agreement with adult studies which have linked MBL deficiency with severe infections in similar patients (Peterslund et al., 2001; Horiuchi et al., 2005; Molle et al., 2006 ; Vekemans et al., 2007). The administration of MBL replacement throughout chemotherapy treatment is unfeasible and would be very costly. The use of MBL replacement as rescue treatment is the alternative but the same issues remain i.e. frequency, duration and cost. Similarities can be drawn with the use of G-CSF in such patients. The practicalities of administration i.e. duration and frequency of infusions and the overall cost of the procedure may limit use in the long run.

The safety of any such procedure is of paramount importance in an already vulnerable population. The use of recombinant protein has overcome a number of safety concerns inherent to a plasma purified product which may originate from a number of donors with the risk of transinfection. Another risk with any recombinant protein is the development of antibodies but data suggests that this is not an issue with either

recombinant or plasma purified products (Valdimarsson et al., 2004; Petersen et al., 2006). MBL has a number of functions and there appear to be advantages and disadvantages to its replacement in an MBL deficient subject (Dommett et al., 2006) (Figure 9.4).

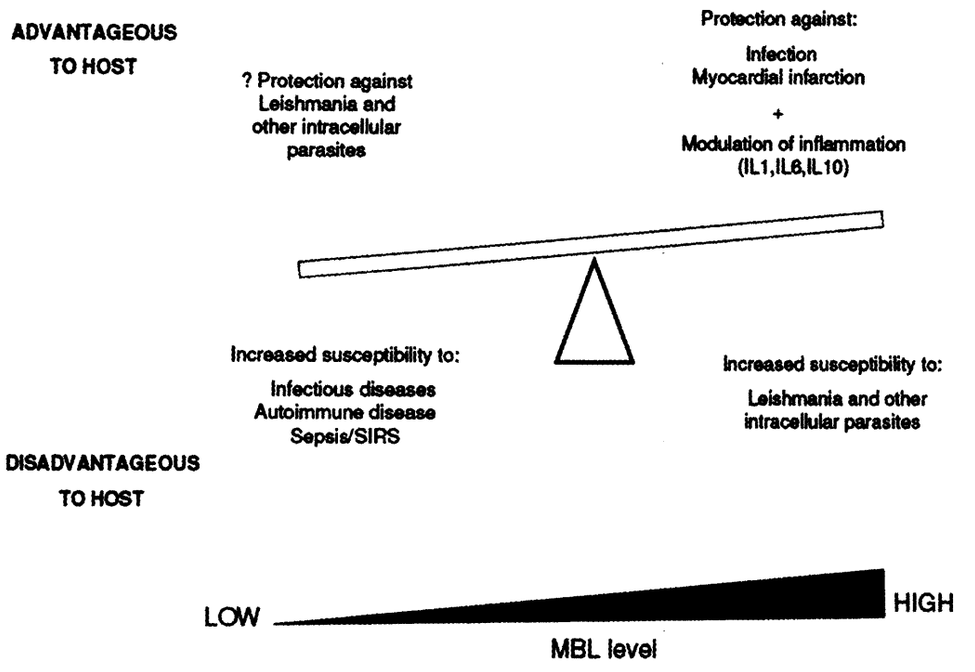


Figure 9.4 Schematic representation illustrating how both high and low serum MBL levels may impact the health of a given host.

Lack of complement activating function is obviously undesirable in terms of infection management but equally enhanced complement activation can be deleterious to the host, e.g. ischaemia reperfusion injury (Hart et al., 2005; Walsh et al., 2005). Phase I studies using rMBL report no nonspecific complement activation following reconstitution to normal plasma MBL levels as measured by C3d concentrations. Overall no safety or tolerability concerns were raised when using rMBL in healthy subjects (Petersen et al., 2006). The results of Phase II and III trials which may modify our future practice are eagerly awaited.

9.7 Unanswered questions and future direction

The present project set out to address whether MBL gene status is the right indicator to influence our management of FN using RS. Our results suggest that MBL status does influence infection risk but that MBL status alone is an oversimplified measure of this system. Our studies imply that the lectin pathway is active within our patient population using the functional assays available. Further work to investigate the clinical relevance/applicability of these measures is warranted. These assays incorporate the role of MASPs but it must be remembered that these enzymes also work in concert with Ficolins which in turn also activate the lectin pathway. Future work should include the investigation of Ficolins within the pathway in order to delineate their role and contribution to host defence as they may assume greater importance (Hummelshoj et al, 2007) (Figure 9.5).

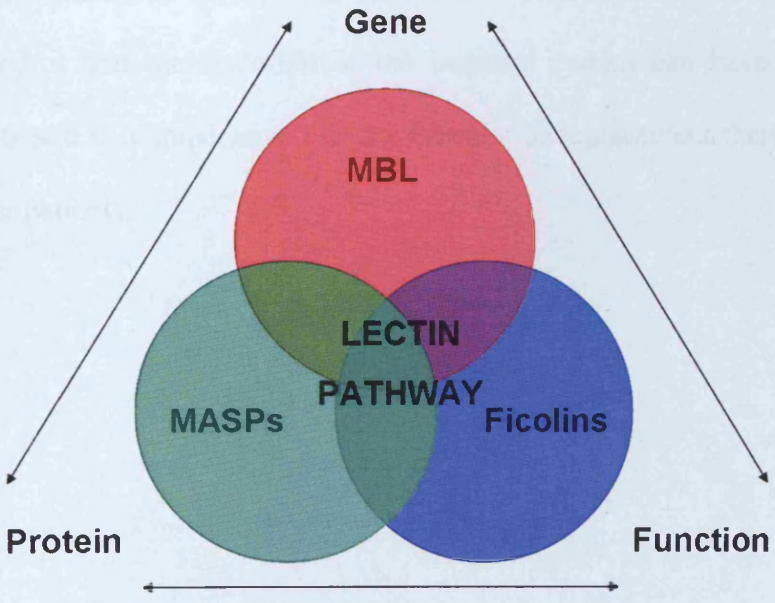


Figure 9.5 Illustration of components of the lectin pathway.
Future studies should incorporate genetic, protein and functional analysis of the MASPs, Ficolins and MBL.

Our current understanding of the role of MBL higher order oligomers to MBL function remains limited. If MBL deficient patients are unable to increase production of higher order oligomer in order to fight infection MBL replacement at such a time may be very valuable. Variation between the YAYD individuals also warrants further studies as these individuals are obviously a unique population who can raise their levels to that of WT but clinically behave like other heterozygotes.

The role of a lectin pathway genetic profile incorporating MBL, MASP and Ficolin polymorphisms may be most informative to future practice. Identifying pathway deficiencies within patients at the beginning of their treatment for cancer and modifying their infection management accordingly may improve both quality of life for the patient and also improve the efficacy of their chemotherapy treatment preventing interruptions and delays in therapy (Figure 9.1). The use of MBL replacement as a supportive measure in infection management is an exciting prospect. The practicalities of its use and the cost effectiveness of such an intervention remain under investigation. We should be mindful that manipulation of the immune system can have positive and negative effects and it is imperative that the benefits of replacement therapy outweigh any risks to our patients.

Abbreviations

Å	Angstrom
AA	*MBL2* Exon 1 wildtype
ALL	Acute Lymphoblastic Leukaemia
AML	Acute Myeloid Leukaemia
AMPs	Antimicrobial Peptides
ANC	Absolute Neutrophil count
AO	*MBL2* Exon 1 heterozygote
ATP	Adenosine triphosphate
BIR	Baculoviral inhibitor-of-apoptosis-protein repeat
BMT	Bone Marrow Transplant
bp	Base pair
C	Cysteine
CARD	Caspase-recruitment domain
cDNA	Complementary DNA
CRD	Carbohydrate recognition domain
CRP	C reactive protein
CVC	Central venous catheter
D	Aspartic acid
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DTT	Dithiothreitol
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
FCS	Fetal Calf Serum
FN	Febrile Neutropenia
G	Glycine
GAPDH	Glyceraldehyde-3 phosphate dehydrogenase
GI	Gastrointestinal
hBD	Human beta defensins

HIV	Human immunodeficiency virus
HRP	Horse radish peroxidase
IFN-γ	Interferon gamma
IL	Interleukin
IRAK	IL-1 receptor-associated protein kinase
KDa	Kilodalton
LBP	Lipopolysaccharide binding protein
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
LTA	Lipoteichoic acid
μ	micro
MAC	Membrane attack complex
MAMPs	Microbe associated molecular patterns
MAP	Mitogen-activated protein
MARCO	Macrophage receptor with collagenous structure
MASP	MBL associated serine protease
MBL	Mannose Binding Lectin
MCP	Membrane cofactor of proteolysis
mM	millimolar
mRNA	Messenger ribonucleic acid
MW	Molecular weight
NAIP	Neuronal apoptosis inhibitor protein
NALP	NACHT, LRR, PYD containing protein
NF-κB	Nuclear factor-κB
NHL	Non Hodgkins Lymphoma
NK	Natural killer
nM	Nanometre
NOD	Nucleotide binding oligomerization domain protein
OD	Optical density
OO	*MBL2* Exon 1 homozygote or compound heterozygote
p	Pico

PAGE	Polyacrylamide gel electrophoresis
PAMPs	Pathogen associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBSC	Peripheral blood stem cell
PCR	Polymerase chain reaction
PINE	Paediatric infections during neutropenic episodes
PKR	Double stranded RNA (dsRNA)-activated protein kinase
POC	Paediatric Oncology Centre
POSCU	Paediatric Oncology Shared Care Unit
PRRs	Pattern recognition receptors
rMBL	Recombinant Mannose Binding Lectin
RPM	Revolutions per minute
RS	Risk stratification
RT	Room temperature
SAP	Serum Amyloid protein
SCT	Stem cell transplant
SDS	Sodium dodecyl sulfate
SIRS	Systemic Inflammatory Response syndrome
SLE	Systemic Lupus Erythematosus
SNP	Single Nucleotide Polymorphism
SP-A	Surfactant protein A
SP-D	Surfactant protein D
TBS	Tris buffered saline
TLR	Toll like receptor
TNF-α	Tumour necrosis factor alpha
TPOC	Thames Paediatric Oncology Centre
TRAF6	Tumour necrosis factor receptor-activated factor 6
UHG	Universal Heteroduplex generator
UV	Ultra violet
μ	micro
WT	Wildtype

References

Abrahamsson,J., Pahlman,M., and Mellander,L. (1997). Interleukin 6, but not tumour necrosis factor-alpha, is a good predictor of severe infection in febrile neutropenic and non-neutropenic children with malignancy. Acta Paediatr. *86*, 1059-1064.

Ahmed,N., El-Mahallawy,H.A., Ahmed,I.A., Nassif,S., El-Beshlawy,A., and El-Haddad,A. (2007). Early hospital discharge versus continued hospitalization in febrile pediatric cancer patients with prolonged neutropenia: A randomized, prospective study. Pediatr. Blood Cancer *49*, 786-792.

Aittoniemi,J., Baer,M., Soppi,E., Vesikari,T., and Miettinen,A. (1998). Mannan binding lectin deficiency and concomitant immunodefects. Arch. Dis. Child *78*, 245-248.

Akaiwa,M., Yae,Y., Sugimoto,R., Suzuki,S.O., Iwaki,T., Izuhara,K., and Hamasaki,N. (1999). Hakata antigen, a new member of the ficolin/opsonin p35 family, is a novel human lectin secreted into bronchus/alveolus and bile. J. Histochem. Cytochem. *47*, 777-786.

Akira,S., Uematsu,S., and Takeuchi,O. (2006). Pathogen recognition and innate immunity. Cell. *124*, 783-801.

Albiger,B., Dahlberg,S., Henriques-Normark,B., and Normark,S. (2007). Role of the innate immune system in host defence against bacterial infections: focus on the Toll-like receptors. J. Intern. Med. *261*, 511-528.

Alcorn,J.F. and Wright,J.R. (2004). Surfactant protein A inhibits alveolar macrophage cytokine production by CD14-independent pathway. Am. J. Physiol Lung Cell Mol. Physiol *286*, L129-L136.

Alonso,D.P., Ferreira,A.F., Ribolla,P.E., de,M.S., I, do Socorro Pires e Cruz, ecio de,C.F., Abatepaulo,A.R., Lamounier,C.D., Werneck,G.L., Farias,T.J., Soares,M.J., and Costa,C.H. (2007). Genotypes of the mannan-binding lectin gene and susceptibility to visceral leishmaniasis and clinical complications. J. Infect. Dis. *195*, 1212-1217.

Ammann,R.A., Simon,A., and de Bont,E.S. (2005). Low risk episodes of fever and neutropenia in pediatric oncology: Is outpatient oral antibiotic therapy the new gold standard of care? Pediatr. Blood Cancer *45*, 244-247.

Ambrus,G., Gal,P., Kojima,M., Szilagyi,K., Balczer,J., Antal,J., Graf,L., Laich,A., Moffatt,B.E., Schwaeble,W., Sim,R.B., and Zavodszky,P. (2003). Natural substrates and inhibitors of mannan-binding lectin-associated serine protease-1 and -2: a study on recombinant catalytic fragments. J. Immunol. *170*, 1374-1382.

Aoyagi,Y., Adderson,E.E., Min,J.G., Matsushita,M., Fujita,T., Takahashi,S., Okuwaki,Y., and Bohnsack,J.F. (2005). Role of L-ficolin/mannose-binding lectin-associated serine protease complexes in the opsonophagocytosis of type III group B streptococci. J. Immunol *174*, 418-425.

Aquino,V.M., Buchanan,G.R., Tkaczewski,I., and Mustafa,M.M. (1997). Safety of early hospital discharge of selected febrile children and adolescents with cancer with prolonged neutropenia. Medical and Pediatric Oncology *28*, 191-195.

Aquino,V.M., Herrera,L., Sandler,E.S., and Buchanan,G.R. (2000). Feasibility of oral ciprofloxacin for the outpatient management of febrile neutropenia in selected children with cancer. Cancer *88*, 1710-1714.

Arai,T., Tabona,P., and Summerfield,J.A. (1993). Human mannose-binding protein gene is regulated by interleukins, dexamethasone and heat shock. Q. J. Med. *86*, 575-582.

Arlaud,G.J., Volanakis,J.E., Thielens,N.M., Narayana,S.V., Rossi,V., and Xu,Y. (1998). The atypical serine proteases of the complement system. Adv. Immunol. *69*, 249-307.

Atkinson,A.P., Cedzynski,M., Szemraj,J., St Swierzko,A., Bak-Romaniszyn,L., Banasik,M., Zeman,K., Matsushita,M., Turner,M.L., and Kilpatrick,D.C. (2004). L-ficolin in children with recurrent respiratory infections. Clin. Exp. Immunol. *138*, 517-520.

Baccarelli,A., Hou,L., Chen,J., Lissowska,J., El-Omar,E.M., Grillo,P., Giacomini,S.M., Yaeger,M., Bernig,T., Zatonski,W., Fraumeni,J.F., Jr., Chanock,S.J., and Chow,W.H. (2006). Mannose-binding lectin-2 genetic variation and stomach cancer risk. Int. J. Cancer *119*, 1970-1975.

Basu,S.K., Fernandez,I.D., Fisher,S.G., Asselin,B.L., and Lyman,G.H. (2005). Length of stay and mortality associated with febrile neutropenia among children with cancer. J. Clin. Oncol. *23*, 7958-7966.

Bergmann,O.J., Christiansen,M., Laursen,I., Bang,P., Hansen,N.E., Ellegaard,J., Koch,C., and Andersen,V. (2003). Low levels of mannose-binding lectin do not affect occurrence of severe infections or duration of fever in acute myeloid leukaemia during remission induction therapy. Eur. J. Haematol. *70*, 91-97.

Bernig,T., Boersma,B.J., Howe,T.M., Welch,R., Yadavalli,S., Staats,B., Mechanic,L.E., Chanock,S.J., and Ambs,S. (2007). The mannose-binding lectin (MBL2) haplotype and breast cancer: an association study in African-American and Caucasian women. Carcinogenesis *28*, 828-836.

Bernig,T., Breunis,W., Brouwer,N., Hutchinson,A., Welch,R., Roos,D., Kuijpers,T., and Chanock,S. (2005). An analysis of genetic variation across the MBL2 locus in Dutch Caucasians indicates that 3' haplotypes could modify circulating levels of mannose-binding lectin. Hum. Genet. *118*, 404-415.

Bernig,T., Taylor,J.G., Foster,C.B., Staats,B., Yeager,M., and Chanock,S.J. (2004). Sequence analysis of the mannose-binding lectin (MBL2) gene reveals a high degree of heterozygosity with evidence of selection. Genes Immun. *5*, 461-476.

Beutler,B. (2004). Innate immunity: an overview. Mol Immunol. *40*, 845-59.

Bodey,G.P., Buckley,M., Sathe,Y.S., and Freireich,E.J. (1966). Quantitative relationships between circulating leukocytes and infection in patients with acute leukemia. Ann. Intern. Med. *64*, 328-340.

Bodey,G.P., Rodriguez,V., Chang,H.Y., and Narboni (1978). Fever and infection in leukemic patients: a study of 494 consecutive patients. Cancer *41*, 1610-1622.

Boldt,A.B. and Petzl-Erler,M.L. (2002). A new strategy for mannose-binding lectin gene haplotyping. Hum. Mutat. *19*, 296-306.

Bottazzi,B., Garlanda,C., Salvatori,G., Jeannin,P., Manfredi,A., and Mantovani,A. (2006). Pentraxins as a key component of innate immunity. Curr. Opin. Immunol. *18*, 10-15.

Brouwer,N., Dolman,K.M., van,Z.R., Nieuwenhuys,E., Hart,M., Aarden,L.A., Roos,D., and Kuijpers,T.W. (2006). Mannan-binding lectin (MBL)-mediated opsonization is enhanced by the alternative pathway amplification loop. Mol. Immunol. *43*, 2051-2060.

Brown,G.D., Gordon,S. (2001). Immune recognition. A new receptor for beta-glucans. Nature. *413*, 36-7.

Brown,K.S., Keogh,M.J., Tagiuri,N., Grainge,M.J., Presanis,J.S., Ryder,S.D., Irving,W.L., Ball,J.K., Sim,R.B., and Hickling,T.P. (2007). Severe fibrosis in hepatitis C virus-infected patients is associated with increased activity of the mannan-binding lectin (MBL)/MBL-associated serine protease 1 (MASP-1) complex. Clin. Exp. Immunol. *147*, 90-98.

Candy,D.C., Larcher,V.F., Tripp,J.H., Harries,J.T., Harvey,B.A., and Soothill,J.F. (1980). Yeast opsonisation in children with chronic diarrhoeal states. Arch. Dis. Child *55*, 189-193.

Carlsson,M., Sjoholm,A.G., Eriksson,L., Thiel,S., Jensenius,J.C., Segelmark,M., and Truedsson,L. (2005). Deficiency of the mannan-binding lectin pathway of complement and poor outcome in cystic fibrosis: bacterial colonization may be decisive for a relationship. Clin. Exp. Immunol *139*, 306-313.

Chaka,W., Verheul,A.F., Vaishnav,V.V., Cherniak,R., Scharringa,J., Verhoef,J., Snippe,H., and Hoepelman,A.I. (1997). Induction of TNF-alpha in human peripheral blood mononuclear cells by the mannoprotein of Cryptococcus neoformans involves human mannose binding protein. J. Immunol. *159*, 2979-2985.

Chamaillard,M., Girardin,S.E., Viala,J., and Philpott,D.J. (2003). Nods, Nalps and Naip: intracellular regulators of bacterial-induced inflammation. Cell Microbiol. *5*, 581-592.

Chan,R.K., Ibrahim,S.I., Takahashi,K., Kwon,E., McCormack,M., Ezekowitz,A., Carroll,M.C., Moore,F.D., Austen,W.G. (2006). The Differing Roles of the Classical and Mannose-Binding Lectin Complement Pathways in the Events following Skeletal Muscle Ischemia-Reperfusion. J. Immunol. *177*, 8080 - 8085.

Chen,C.B. and Wallis,R. (2004). Two mechanisms for mannose-binding protein modulation of the activity of its associated serine proteases. J. Biol. Chem. *279*, 26058-26065.

Chen,C.B. and Wallis,R. (2001). Stoichiometry of complexes between mannose-binding protein and its associated serine proteases. Defining functional units for complement activation. J. Biol. Chem. *276*, 25894-25902.

Chen,.X, Katoh,Y., Nakamura,K., Oyama,N., Kaneko,F., Endo,Y., Fujita,T., Nishida,T., and Mizuki,N. (2006). Single nucleotide polymorphisms of Ficolin 2 gene in Behçet's disease. J Dermatol Sci. *43*, 201-5.

Chisholm,J.C. and Dommett,R. (2006). The evolution towards ambulatory and day-case management of febrile neutropenia. Br. J. Haematol. *135*, 3-16.

Choi,E.H., Taylor,J.G., Foster,C.B., Walsh,T.J., Anttila,V.J., Ruutu,T., Palotie,A., and Chanock,S.J. (2005). Common polymorphisms in critical genes of innate immunity do not contribute to the risk for chronic disseminated candidiasis in adult leukemia patients. Med. Mycol. *43*, 349-353.

Chong,W.P., To,Y.F., Ip,W.K., Yuen,M.F., Poon,T.P., Wong,W.H., Lai,C.L., and Lau,Y.L. (2005). Mannose-binding lectin in chronic hepatitis B virus infection. Hepatology *42*, 1037-1045.

Christiansen,O.B., Kilpatrick,D.C., Souter,V., Varming,K., Thiel,S., and Jensenius,J.C. (1999). Mannan-binding lectin deficiency is associated with unexplained recurrent miscarriage. Scand. J. Immunol. *49*, 193-196.

Colley,K.J. and Baenziger,J.U. (1987). Post-translational modifications of the core-specific lectin. Relationship to assembly, ligand binding, and secretion. J. Biol. Chem. *262*, 10296-10303.

Cooper,N.R. (1985). The classical complement pathway: activation and regulation of the first complement component. Adv. Immunol. *37*, 151-216.

Cortesio,C.L. and Jiang,W. (2006). Mannan-binding lectin-associated serine protease 3 cleaves synthetic peptides and insulin-like growth factor-binding protein 5. Arch. Biochem. Biophys. *449*, 164-170.

Crosdale,D.J., Ollier,W.E., Thomson,W., Dyer,P.A., Jensenious,J., Johnson,R.W., and Poulton,K.V. (2000). Mannose binding lectin (MBL) genotype distributions with relation to serum levels in UK Caucasoids. Eur. J. Immunogenet. *27*, 111-117

Crosdale,D.J., Poulton,K.V., Ollier,W.E., Thomson,W., and Denning,D.W. (2001). Mannose-binding lectin gene polymorphisms as a susceptibility factor for chronic necrotizing pulmonary aspergillosis. J. Infect. Dis. *184*, 653-656.

Cseh,S., Vera,L., Matsushita,M., Fujita,T., Arlaud,G.J., and Thielens,N.M. (2002). Characterization of the interaction between L-ficolin/p35 and mannan-binding lectin-associated serine proteases-1 and -2. J. Immunol. *169*, 5735-5743.

Dahl,M., Tybjaerg-Hansen,A., Schnohr,P., and Nordestgaard,B.G. (2004). A population-based study of morbidity and mortality in mannose-binding lectin deficiency. J. Exp. Med. *199*, 1391-1399.

Dahl,M.R., Thiel,S., Matsushita,M., Fujita,T., Willis,A.C., Christensen,T., Vorup-Jensen,T., and Jensenius,J.C. (2001). MASP-3 and its association with distinct complexes of the mannan-binding lectin complement activation pathway. Immunity. *15*, 127-135.

Dalton,H.J., Slonim,A.D., and Pollack,M.M. (2003). MultiCenter outcome of pediatric oncology patients requiring intensive care. Pediatr. Hematol. Oncol. *20*, 643-649.

Davies,E.J., Snowden,N., Hillarby,M.C., Carthy,D., Grennan,D.M., Thomson,W., and Ollier,W.E. (1995). Mannose-binding protein gene polymorphism in systemic lupus erythematosus. Arthritis Rheum. *38*, 110-114.

Davies,J., Neth,O., Alton,E., Klein,N., and Turner,M. (2000). Differential binding of mannose-binding lectin to respiratory pathogens in cystic fibrosis. Lancet *355*, 1885-1886.

Davies,J.C., Turner,M.W., and Klein,N. (2004). Impaired pulmonary status in cystic fibrosis adults with two mutated MBL-2 alleles. Eur. Respir. J. *24*, 798-804.

de Bont,E.S., Vellenga,E., Swaanenburg,J.C., Fidler,V., Visser-van Brummen,P.J., and Kamps,W.A. (1999). Plasma IL-8 and IL-6 levels can be used to define a group with low risk of septicaemia among cancer patients with fever and neutropenia. Br. J. Haematol. *107*, 375-380.

de Messias-Reason,I.J., Boldt,A.B., Moraes Braga,A.C., Von Rosen Seeling Stahlke,E., Dornelles,L., Pereira-Ferrari,L., Kremsner,P.G., and Kun,J.F. (2007). The association between mannan-binding lectin gene polymorphism and clinical leprosy: new insight into an old paradigm. J Infect Dis. *196*, 1379-85.

Dean,M.M., Minchinton,R.M., Heatley,S., and Eisen,D.P. (2005). Mannose binding lectin acute phase activity in patients with severe infection. J. Clin. Immunol *25*, 346-352.

Dean,M.M., Heatley,S., and Minchinton,R.M. (2005). Heteroligomeric forms of codon 54 mannose binding lectin (MBL) in circulation demonstrate reduced in vitro function. Mol. Immunol.

Devyatyarova-Johnson,M., Rees,I.H., Robertson,B.D., Turner,M.W., Klein,N.J., and Jack,D.L. (2000). The lipopolysaccharide structures of Salmonella enterica serovar Typhimurium and Neisseria gonorrhoeae determine the attachment of human mannose-binding lectin to intact organisms. Infect. Immun. *68*, 3894-3899.

Dickinson,A.M., Middleton,P.G., Rocha,V., Gluckman,E., and Holler,E. (2004). Genetic polymorphisms predicting the outcome of bone marrow transplants. Br. J. Haematol. *127*, 479-490.

Dommett,R.M., Klein,N., and Turner,M.W. (2006). Mannose-binding lectin in innate immunity: past, present and future. Tissue Antigens *68*, 193-209.

Dong,M., Xu,S., Oliveira,C.L., Pedersen,J.S., Thiel,S., Besenbacher,F., and Vorup-Jensen,T. (2007). Conformational changes in mannan-binding lectin bound to ligand surfaces. J. Immunol. *178*, 3016-3022.

Dorfman,R., Sandford,A., Taylor,C., Huang,B., Frangolias,D., Wang,Y., Sang,R., Pereira,L., Sun,L., Berthiaume,Y., Tsui,L.C., Paré,P.D., Durie,P., Corey,M., and Zielenski,J.(2008) Complex two-gene modulation of lung disease severity in children with cystic fibrosis. J Clin Invest. *118*, 1040-1049.

Dornelles,L.N., Pereira-Ferrari,L., and Messias-Reason,I. (2006).Mannan-binding lectin plasma levels in leprosy: deficiency confers protection against the lepromatous but not the tuberculoid forms. Clin Exp Immunol. *145*, 463-8.

Downing,I., Koch,C., and Kilpatrick,D.C. (2003). Immature dendritic cells possess a sugar-sensitive receptor for human mannan-binding lectin. Immunology *109*, 360-364.

Drickamer,K., Dordal,M.S., and Reynolds,L. (1986). Mannose-binding proteins isolated from rat liver contain carbohydrate-recognition domains linked to collagenous tails. Complete primary structures and homology with pulmonary surfactant apoprotein. J. Biol. Chem. *261*, 6878-6887.

Dumestre-Perard,C., Ponard,D., and Colomb,M.G. (2004). Analysis of low molecular weight intracellular associations of a human mannan binding lectin (MBL). Mol. Immunol. *40*, 795-801.

Dumoutier,L., Van Roost,E., Colau,D., and Renauld,J.C. (2000). Human interleukin-10-related T cell-derived inducible factor: molecular cloning and functional characterization as an hepatocyte-stimulating factor. Proc Natl. Acad. Sci U. S. A *97*, 10144-10149.

Duncan,C., Chisholm,J.C., Freeman,S., Riley,U., Sharland,M., and Pritchard-Jones,K. (2007). A prospective study of admissions for febrile neutropenia in secondary paediatric units in South East England. Pediatr. Blood Cancer *49,* 678-81.

Edelson,B.T., Stricker,T.P., Li,Z., Dickeson,S.K., Shepherd,V.L., Santoro,S.A., and Zutter,M.M. (2006). Novel collectin/C1q receptor mediates mast cell activation and innate immunity. Blood *107*, 143-150.

Elomaa,O., Kangas,M., Sahlberg,C., Tuukkanen,J., Sormunen,R., Liakka,A., Thesleff,I., Kraal,G., and Tryggvason,K. (1995). Cloning of a novel bacteria-binding receptor structurally related to scavenger receptors and expressed in a subset of macrophages. Cell *80*, 603-609.

Endo,Y., Sato,T., Matsushita,M., and Fujita,T. (1996). Exon structure of the gene encoding the human mannose-binding protein-associated serine protease light chain: comparison with complement C1r and C1s genes. Int. Immunol. *8*, 1355-1358.

Ezekowitz,R.A. (1991). Ante-antibody immunity. Current Opinion in Immunology *1*, 60-62.

Ezekowitz,R.A., Day,L.E., and Herman,G.A. (1988). A human mannose-binding protein is an acute-phase reactant that shares sequence homology with other vertebrate lectins. J. Exp. Med. *167*, 1034-1046.

Ezekowitz,R.A., Sastry,K., Bailly,P., and Warner,A. (1990). Molecular characterization of the human macrophage mannose receptor: demonstration of multiple carbohydrate recognition-like domains and phagocytosis of yeasts in Cos-1 cells. J. Exp. Med. *172*, 1785-1794.

Faber,J., Schuessler,T., Finn,A., Murdoch,C., Zenz,W., Habermehl,P., Meyer,C.U., Zabel,B.U., Schmitt,H., Zepp,F., and Knuf,M. (2007). Age-dependent association of human mannose-binding lectin mutations with susceptibility to invasive meningococcal disease in childhood. Pediatr. Infect. Dis. J. *26*, 243-246.

Feinberg,H., Uitdehaag,J.C., Davies,J.M., Wallis,R., Drickamer,K., and Weis,W.I. (2003). Crystal structure of the CUB1-EGF-CUB2 region of mannose-binding protein associated serine protease-2. EMBO J. *22*, 2348-2359.

Fidler,K.J., Wilson,P., Davies,J.C., Turner,M.W., Peters,M.J., and Klein,N.J. (2004). Increased incidence and severity of the systemic inflammatory response syndrome in patients deficient in mannose-binding lectin. Intensive Care Med. *30*, 1438-1445.

Fischer,P.B., Ellermann-Eriksen,S., Thiel,S., Jensenius,J.C., and Mogensen,S.C. (1994). Mannan-binding protein and bovine conglutinin mediate enhancement of herpes simplex virus type 2 infection in mice. Scand. J. Immunol. *39*, 439-445.

Fleischhack,G., Kambeck,I., Cipic,D., Hasan,C., and Bode,U. (2000). Procalcitonin in paediatric cancer patients: its diagnostic relevance is superior to that of C-reactive protein, interleukin 6, interleukin 8, soluble interleukin 2 receptor and soluble tumour necrosis factor receptor II. Br. J. Haematol. *111*, 1093-1102.

Frakking,F.N., van,d.W., Brouwer,N., Dolman,K.M., Geissler,J., Lemkes,B., Caron,H.N., and Kuijpers,T.W. (2006). The role of mannose-binding lectin (MBL) in paediatric oncology patients with febrile neutropenia. Eur. J. Cancer *42*, 909-916.

Fraser,D.A., Bohlson,S.S., Jasinskiene,N., Rawal,N., Palmarini,G., Ruiz,S., Rochford,R., and Tenner,A.J. (2006). C1q and MBL, components of the innate immune system, influence monocyte cytokine expression. J. Leukoc. Biol. *80*, 107-116.

Frederiksen,P.D., Thiel,S., Larsen,C.B., and Jensenius,J.C. (2005). M-ficolin, an innate immune defence molecule, binds patterns of acetyl groups and activates complement. Scand. J. Immunol *62*, 462-473.

Fritz,J.H., Ferrero,R.L., Philpott,D.J., and Girardin,S.E. (2006) Nod-like proteins in immunity, inflammation and disease. Nat Immunol. *7*, 1250-7

Fujimori,Y., Harumiya,S., Fukumoto,Y., Miura,Y., Yagasaki,K., Tachikawa,H., and Fujimoto,D. (1998). Molecular cloning and characterization of mouse ficolin-A. Biochem. Biophys. Res. Commun. *244*, 796-800.

Fujita,T. (2002). Evolution of the lectin-complement pathway and its role in innate immunity. Nat. Rev. Immunol. *2*, 346-353.

Fujita,T., Matsushita,M., and Endo,Y. (2004). The lectin-complement pathway--its role in innate immunity and evolution. Immunol. Rev. *198*, 185-202.

Fukutomi,T., Ando,B., Sakamoto,S., Sakai,H., and Nawata,H. (1996). Thermolabile beta-2 macroglycoprotein (Hakata antigen) in liver disease: biochemical and immunohistochemical study. Clin. Chim. Acta *255*, 93-106.

Gabay,C. and Kushner,I. (1999). Acute-phase proteins and other systemic responses to inflammation. N. Engl. J. Med. *340*, 448-454.

Gabolde,M., Muralitharan,S., and Besmond,C. (1999). Genotyping of the three major allelic variants of the human mannose-binding lectin gene by denaturing gradient gel electrophoresis. Hum. Mutat. *14*, 80-83.

Gabolde,M., Hubert,D., Guilloud-Bataille,M., Lenaerts,C., Feingold,J., and Besmond,C. (2001). The mannose binding lectin gene influences the severity of chronic liver disease in cystic fibrosis. J. Med. Genet. *38*, 310-311.

Gadjeva,M., Paludan,S.R., Thiel,S., Slavov,V., Ruseva,M., Eriksson,K., Lowhagen,G.B., Shi,L., Takahashi,K., Ezekowitz,A., and Jensenius,J.C. (2004). Mannan-binding lectin modulates the response to HSV-2 infection. Clin. Exp. Immunol. *138*, 304-311.

Gal,P., Barna,L., Kocsis,A., and Zavodszky,P. (2007). Serine proteases of the classical and lectin pathways: similarities and differences. Immunobiology *212*, 267-277.

Gal,P., Harmat,V., Kocsis,A., Bian,T., Barna,L., Ambrus,G., Vegh,B., Balczer,J., Sim,R.B., Naray-Szabo,G., and Zavodszky,P. (2005). A true autoactivating enzyme. Structural insight into mannose-binding lectin-associated serine protease-2 activations. J. Biol. Chem. *280*, 33435-33444.

Gallagher,P.M., Lowe,G., Fitzgerald,T., Bella,A., Greene,C.M., McElvaney,N.G., and O'Neill,S.J. (2003). Association of IL-10 polymorphism with severity of illness in community acquired pneumonia. Thorax *58*, 154-156.

Garlatti,V., Belloy,N., Martin,L., Lacroix,M., Matsushita,M., Endo,Y., Fujita,T., Fontecilla-Camps,J.C., Arlaud,G.J., Thielens,N.M., and Gaboriaud,C. (2007). Structural insights into the innate immune recognition specificities of L- and H-ficolins. EMBO J. *26*, 623-633.

Garred,P., Harboe,M., Oettinger,T., Koch,C., and Svejgaard,A. (1994). Dual role of mannan-binding protein in infections: another case of heterosis? Eur. J. Immunogenet. *21*, 125-131.

Garred,P., Larsen,F., Madsen,H.O., and Koch,C. (2003a). Mannose-binding lectin deficiency--revisited. Mol. Immunol. *40*, 73-84.

Garred,P., Madsen,H.O., Balslev,U., Hofmann,B., Pedersen,C., Gerstoft,J., and Svejgaard,A. (1997). Susceptibility to HIV infection and progression of AIDS in relation to variant alleles of mannose-binding lectin. Lancet *349*, 236-240.

Garred,P., Madsen,H.O., Halberg,P., Petersen,J., Kronborg,G., Svejgaard,A., Andersen,V., and Jacobsen,S. (1999a). Mannose-binding lectin polymorphisms and susceptibility to infection in systemic lupus erythematosus. Arthritis Rheum. *42*, 2145-2152.

Garred,P., Pressler,T., Madsen,H.O., Frederiksen,B., Svejgaard,A., Hoiby,N., Schwartz,M., and Koch,C. (1999b). Association of mannose-binding lectin gene heterogeneity with severity of lung disease and survival in cystic fibrosis. J. Clin. Invest *104*, 431-437.

Garred,P., Strom,J., Quist,L., Taaning,E., and Madsen,H.O. (2003b). Association of mannose-binding lectin polymorphisms with sepsis and fatal outcome, in patients with systemic inflammatory response syndrome. J. Infect. Dis. *188*, 1394-1403.

Garred,P., Voss,A., Madsen,H.O., and Junker,P. (2001). Association of mannose-binding lectin gene variation with disease severity and infections in a population-based cohort of systemic lupus erythematosus patients. Genes Immun. *2*, 442-450.

Ghezzi,M.C., Raponi,G., Angeletti,S., and Mancini,C. (1998). Serum-mediated enhancement of TNF-alpha release by human monocytes stimulated with the yeast form of Candida albicans. J. Infect. Dis. *178*, 1743-1749.

Ghiran,I., Barbashov,S.F., Klickstein,L.B., Tas,S.W., Jensenius,J.C., and Nicholson-Weller,A. (2000). Complement receptor 1/CD35 is a receptor for mannan-binding lectin. J. Exp. Med. *192*, 1797-1808.

Gilham,C., Peto,J., Simpson,J., Roman,E., Eden,T.O., Greaves,M.F., and Alexander,F.E. (2005). Day care in infancy and risk of childhood acute lymphoblastic leukaemia: findings from UK case-control study. BMJ.

Girija,U.V., Dodds,A.W., Roscher,S., Reid,K.B., and Wallis,R. (2007). Localization and characterization of the mannose-binding lectin (MBL)-associated-serine protease-2 binding site in rat ficolin-A: equivalent binding sites within the collagenous domains of MBLs and ficolins. J. Immunol. *179*, 455-462.

Goulden,N., Oakhill,A., and Steward,C. (2001). Practical application of minimal residual disease assessment in childhood acute lymphoblastic leukaemia annotation. Br. J. Haematol. *112*, 275-281.

Granell,M., Urbano-Ispizua,A., Suarez,B., Rovira,M., Fernandez-Aviles,F., Martinez,C., Ortega,M., Uriburu,C., Gaya,A., Roncero,J.M., Navarro,A., Carreras,E., Mensa,J., Vives,J., Rozman,C., Montserrat,E., and Lozano,F. (2006). Mannan-binding lectin pathway deficiencies and invasive fungal infections following allogeneic stem cell transplantation. Exp. Hematol. *34*, 1435-1441.

Graubner,U.B., Porzig,S., Jorch,N., Kolb,R., Wessalowski,R., Escherich,G., and Janka,G.E. (2008). Impact of reduction of therapy on infectious complications in childhood acute lymphoblastic leukemia. Pediatr. Blood Cancer. 50, 259-63.

Graudal,N.A., Homann,C., Madsen,H.O., Svejgaard,A., Jurik,A.G., Graudal,H.K., and Garred,P. (1998). Mannan binding lectin in rheumatoid arthritis. A longitudinal study. J. Rheumatol. *25*, 629-635.

Greaves,M. (2006). Infection, immune responses and the aetiology of childhood leukaemia. Nat Rev Cancer *6*, 193-203.

Greaves,M. (1999). Molecular genetics, natural history and the demise of childhood leukaemia. Eur. J. Cancer *35*, 1941-1953.

Greaves,M.F. (1997). Aetiology of acute leukaemia. Lancet *349*, 344-349.

Gros,P., Milder,F.J., and Janssen,B.J. (2008). Complement driven by conformational changes. Nat Rev Immunol. *8*, 48-58.

Guo,N., Mogues,T., Weremowicz,S., Morton,C.C., and Sastry,K.N. (1998). The human ortholog of rhesus mannose-binding protein-A gene is an expressed pseudogene that localizes to chromosome 10. Mamm. Genome *9*, 246-249.

Hajela,K., Kojima,M., Ambrus,G., Wong,K.H., Moffatt,B.E., Ferluga,J., Hajela,S., Gal,P., and Sim,R.B. (2002). The biological functions of MBL-associated serine proteases (MASPs). Immunobiology *205*, 467-475.

Hakomori,S. (2001). Tumor-associated carbohydrate antigens defining tumor malignancy: basis for development of anti-cancer vaccines. Adv. Exp. Med. Biol. *491*, 369-402.

Hamvas,R.M., Johnson,M., Vlieger,A.M., Ling,C., Sherriff,A., Wade,A., Klein,N.J., Turner,M.W., and Webster,A.D. (2005). Role for mannose binding lectin in the prevention of Mycoplasma infection. Infect. Immun. *73*, 5238-5240.

Hann,I., Viscoli,C., Paesmans,M., Gaya,H., and Glauser,M. (1997). A comparison of outcome from febrile neutropenic episodes in children compared with adults: results from four EORTC studies. International Antimicrobial Therapy Cooperative Group (IATCG) of the European Organization for Research and Treatment of Cancer (EORTC). Br. J. Haematol. *99*, 580-588.

Hansen,S., Thiel,S., Willis,A., Holmskov,U., Jensenius,J.C. (2000) Purification and characterization of two mannan-binding lectins from mouse serum. J. Immunol. *164*, 2610-8.

Hansen,S., Holm,D., Moeller,V., Vitved,L., Bendixen,C., Reid,K.B., Skjoedt,K., and Holmskov,U. (2002). CL-46, a novel collectin highly expressed in bovine thymus and liver. J. Immunol. *169*, 5726-5734.

Harmat,V., Gal,P., Kardos,J., Szilagyi,K., Ambrus,G., Vegh,B., Naray-Szabo,G., and Zavodszky,P. (2004). The structure of MBL-associated serine protease-2 reveals that identical substrate specificities of C1s and MASP-2 are realized through different sets of enzyme-substrate interactions. J. Mol. Biol. *342*, 1533-1546.

Hart,M.L., Ceonzo,K.A., Shaffer,L.A., Takahashi,K., Rother,R.P., Reenstra,W.R., Buras,J.A., and Stahl,G.L. (2005). Gastrointestinal ischemia-reperfusion injury is lectin complement pathway dependent without involving C1q. J. Immunol *174*, 6373-6380.

Hart,M.L., Saifuddin,M., Uemura,K., Bremer,E.G., Hooker,B., Kawasaki,T., and Spear,G.T. (2002). High mannose glycans and sialic acid on gp120 regulate binding of mannose-binding lectin (MBL) to HIV type 1. AIDS Res. Hum. Retroviruses *18*, 1311-1317.

Hartel,C., Adam,N., Strunk,T., Temming,P., Muller-Steinhardt,M., and Schultz,C. (2005). Cytokine responses correlate differentially with age in infancy and early childhood. Clin. Exp. Immunol. *142*, 446-453.

Hartel,C., Deuster,M., Lehrnbecher,T., and Schultz,C. (2007). Current approaches for risk stratification of infectious complications in pediatric oncology. Pediatr. Blood Cancer *49*, 767-773.

Hashimoto,C., Hudson,K.L., and Anderson,K.V. (1988). The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. Cell *52*, 269-279.

Heggelund,L., Mollnes,T.E., Espevik,T., Muller,F., Kristiansen,K.I., Aukrust,P., and Froland,S.S. (2005). Modulatory effect of mannose-binding lectin on cytokine responses: possible roles in HIV infection. Eur. J. Clin. Invest *35*, 765-770.

Heise,C.T., Nicholls,J.R., Leamy,C.E., and Wallis,R. (2000). Impaired secretion of rat mannose-binding protein resulting from mutations in the collagen-like domain. J. Immunol. *165*, 1403-1409.

Hemmila I, Dakubu S, Mukkala VM, Siitari H, Lovgren T (1984) Europium as a label in time-resolved immunofluorometric assays. Anal Biochem 137:335-343

Herpers,B.L., Immink,M.M., de Jong,B.A., van Velzen-Blad,H., de Jongh,B.M., and van Hannen,E.J. (2006). Coding and non-coding polymorphisms in the lectin pathway activator L-ficolin gene in 188 Dutch blood bank donors. Mol. Immunol. *43*, 851-855.

Hibberd,M.L., Sumiya,M., Summerfield,J.A., Booy,R., and Levin,M. (1999). Association of variants of the gene for mannose-binding lectin with susceptibility to meningococcal disease. Meningococcal Research Group. Lancet *353*, 1049-1053.

Hodge,G., Osborn,M., Hodge,S., Nairn,J., Tapp,H., Kirby,M., Sepulveda,H., Morgan,E., Revesz,T., and Zola,H. (2006). Rapid simultaneous measurement of multiple cytokines in childhood oncology patients with febrile neutropenia: increased interleukin (IL)-8 or IL-5 correlates with culture-positive infection. Br. J. Haematol. *132*, 247-248.

Hoebe,K., Janssen,E., and Beutler,B. (2004). The interface between innate and adaptive immunity. Nat Immunol. *5*, 971-4.

Holmskov,U., Thiel,S., and Jensenius,J.C. (2003). Collections and ficolins: humoral lectins of the innate immune defense. Annu. Rev. Immunol. *21*, 547-578.

Holmskov,U.L. (2000). Collectins and collectin receptors in innate immunity. APMIS Suppl *100*, 1-59.

Honore,C., Hummelshoj,T., Hansen,B.E., Madsen,H.O., Eggleton,P., and Garred,P. (2007). The innate immune component ficolin 3 (Hakata antigen) mediates the clearance of late apoptotic cells. Arthritis Rheum. *56*, 1598-1607.

Horiuchi,T., Gondo,H., Miyagawa,H., Otsuka,J., Inaba,S., Nagafuji,K., Takase,K., Tsukamoto,H., Koyama,T., Mitoma,H., Tamimoto,Y., Miyagi,Y., Tahira,T., Hayashi,K., Hashimura,C., Okamura,S., and Harada,M. (2005). Association of MBL gene polymorphisms with major bacterial infection in patients treated with high-dose chemotherapy and autologous PBSCT. Genes Immun. *6*, 162-166.

Hummelshoj,T., Munthe-Fog,L., Madsen,H.O., Sim,R.B., and Garred,P. (2007) Comparative study of the human ficolins reveals unique features of Ficolin-3 (Hakata antigen). Mol Immunol (Epub ahead of print).

Hummelshoj,T., Munthe-Fog,L., Madsen,H.O., Fujita,T., Matsushita,M., and Garred,P. (2005). Polymorphisms in the FCN2 gene determine serum variation and function of Ficolin-2. Hum. Mol. Genet. *14*, 1651-1658.

Ichijo,H., Hellman,U., Wernstedt,C., Gonez,L.J., Claesson-Welsh,L., Heldin,C.H., and Miyazono,K. (1993). Molecular cloning and characterization of ficolin, a multimeric protein with fibrinogen- and collagen-like domains. J. Biol. Chem. *268*, 14505-14513.

Ichijo,H., Ronnstrand,L., Miyagawa,K., Ohashi,H., Heldin,C.H., and Miyazono,K. (1991). Purification of transforming growth factor-beta 1 binding proteins from porcine uterus membranes. J. Biol. Chem. *266*, 22459-22464.

Ikeda,K., Sannoh,T., Kawasaki,N., Kawasaki,T., and Yamashina,I. (1987). Serum lectin with known structure activates complement through the classical pathway. J. Biol. Chem. *262*, 7451-7454.

Iobst,S.T., Wormald,M.R., Weis,W.I., Dwek,R.A., and Drickamer,K. (1994). Binding of sugar ligands to Ca(2+)-dependent animal lectins. I. Analysis of mannose binding by site-directed mutagenesis and NMR. J. Biol. Chem. *269*, 15505-15511.

Ip,W.K., Chan,K.H., Law,H.K., Tso,G.H., Kong,E.K., Wong,W.H., To,Y.F., Yung,R.W., Chow,E.Y., Au,K.L., Chan,E.Y., Lim,W., Jensenius,J.C., Turner,M.W., Peiris,J.S., and Lau,Y.L. (2005). Mannose-binding lectin in severe acute respiratory syndrome coronavirus infection. J. Infect. Dis. *191*, 1697-1704.

Ip,W.K., Lau,Y.L., Chan,S.Y., Mok,C.C., Chan,D., Tong,K.K., and Lau,C.S. (2000). Mannose-binding lectin and rheumatoid arthritis in southern Chinese. Arthritis Rheum. *43*, 1679-1687.

Iwaki,D., Kanno,K., Takahashi,M., Endo,Y., Lynch,N.J., Schwaeble,W.J., Matsushita,M., Okabe,M., and Fujita,T. (2006). Small mannose-binding lectin-associated protein plays a regulatory role in the lectin complement pathway. J. Immunol. *177*, 8626-8632.

Jack,D., Bidwell,J., Turner,M., and Wood,N. (1997). Simultaneous genotyping for all three known structural mutations in the human mannose-binding lectin gene. Hum. Mutat. *9*, 41-46.

Jack,D.L., Dodds,A.W., Anwar,N., Ison,C.A., Law,A., Frosch,M., Turner,M.W., and Klein,N.J. (1998). Activation of complement by mannose-binding lectin on isogenic mutants of Neisseria meningitidis serogroup B. J. Immunol *160*, 1346-1353.

Jack,D.L., Read,R.C., Tenner,A.J., Frosch,M., Turner,M.W., and Klein,N.J. (2001). Mannose-binding lectin regulates the inflammatory response of human professional phagocytes to Neisseria meningitidis serogroup B. J. Infect. Dis. *184*, 1152-1162.

Janeway,C.A., Jr. (1989). Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb. Symp. Quant. Biol. *54 Pt 1*, 1-13.

Janeway,C.A., Jr. and Medzhitov,R.(2002). Innate immune recognition. Annu Rev Immunol. *20*, 197-216.

Janeway,C.A., Travers,P., Walport,M., and Schlomchik,M. (2004) Immunobiology 6th edition. Garland Science, New York and London.

Jensen,M.L., Honore,C., Hummelshoj,T., Hansen,B.E., Madsen,H.O., and Garred,P. (2007a). Ficolin-2 recognizes DNA and participates in the clearance of dying host cells. Mol. Immunol. *44*, 856-865.

Jensen,P.H., Laursen,I., Matthiesen,F., and Hojrup,P. (2007b). Posttranslational modifications in human plasma MBL and human recombinant MBL. Biochim. Biophys. Acta *1774*, 335-344.

Jensen,P.H., Weilguny,D., Matthiesen,F., McGuire,K.A., Shi,L., and Hojrup,P. (2005). Characterization of the oligomer structure of recombinant human mannan-binding lectin. J. Biol. Chem. *280*, 11043-11051.

Ji,X., Gewurz,H., and Spear,G.T. (2005). Mannose binding lectin (MBL) and HIV. Mol. Immunol. *42*, 145-152.

Jones,G.R., Konsler,G.K., Dunaway,R.P., Gold,S.H., Cooper,H.A., and Wells,R.J. (1994). Risk factors for recurrent fever after the discontinuation of empiric antibiotic therapy for fever and neutropenia in pediatric patients with a malignancy or hematologic condition. J. Pediatr. *124*, 703-708.

Kahn,S.J., Wleklinski,M., Ezekowitz,R.A., Coder,D., Aruffo,A., and Farr,A. (1996). The major surface glycoprotein of Trypanosoma cruzi amastigotes are ligands of the human serum mannose-binding protein. Infect. Immun. *64*, 2649-2656.

Kallianpur,A.R. (2005). Genomic screening and complications of hematopoietic stem cell transplantation: has the time come? Bone Marrow Transplant. *35*, 1-16.

Kaplinsky,C., Drucker,M., Goshen,J., Tamary,H., Cohen,I.J., and Zaizov,R. (1994). Ambulatory treatment with ceftriaxone in febrile neutropenic children. Isr. J. Med. Sci. *30*, 649-651.

Kase,T., Suzuki,Y., Kawai,T., Sakamoto,T., Ohtani,K., Eda,S., Maeda,A., Okuno,Y., Kurimura,T., and Wakamiya,N. (1999). Human mannan-binding lectin inhibits the infection of influenza A virus without complement. Immunology *97*, 385-392.

Kawakami,M., Ihara,I., Suzuki,A., and Harada,Y. (1982). Properties of a new complement-dependent bactericidal factor specific for Ra chemotype salmonella in sera of conventional and germ-free mice. J. Immunol *129*, 2198-2201.

Kawasaki,T., Etoh,R., and Yamashina,I. (1978). Isolation and characterization of a mannan-binding protein from rabbit liver. Biochem. Biophys. Res. Commun. *81*, 1018-1024.

Kelly,P., Jack,D.L., Naeem,A., Mandanda,B., Pollok,R.C., Klein,N.J., Turner,M.W., and Farthing,M.J. (2000). Mannose-binding lectin is a component of innate mucosal defense against Cryptosporidium parvum in AIDS. Gastroenterology *119*, 1236-1242.

Kenjo,A., Takahashi,M., Matsushita,M., Endo,Y., Nakata,M., Mizuochi,T., and Fujita,T. (2001). Cloning and characterization of novel ficolins from the solitary ascidian, Halocynthia roretzi. J. Biol. Chem. *276*, 19959-19965.

Kilpatrick,D.C., Delahooke,T.E., Koch,C., Turner,M.L., and Hayes,P.C. (2003a). Mannan-binding lectin and hepatitis C infection. Clin. Exp. Immunol. *132*, 92-95.

Kilpatrick,D.C., McLintock,L.A., Allan,E.K., Copland,M., Fujita,T., Jordanides,N.E., Koch,C., Matsushita,M., Shiraki,H., Stewart,K., Tsujimura,M., Turner,M.L., Franklin,I.M., and Holyoake,T.L. (2003b). No strong relationship between mannan binding lectin or plasma ficolins and chemotherapy-related infections. Clin. Exp. Immunol. *134*, 279-284.

Klaassen,R.J., Goodman,T.R., Pham,B., and Doyle,J.J. (2000). "Low-risk" prediction rule for pediatric oncology patients presenting with fever and neutropenia. J. Clin. Oncol. *18*, 1012-1019.

Klabunde,J., Uhlemann,A.C., Tebo,A.E., Kimmel,J., Schwarz,R.T., Kremsner,P.G., and Kun,J.F. (2002). Recognition of plasmodium falciparum proteins by mannan-binding lectin, a component of the human innate immune system. Parasitol. Res. *88*, 113-117.

Klastersky,J., Paesmans,M., Rubenstein,E.B., Boyer,M., Elting,L., Feld,R., Gallagher,J., Herrstedt,J., Rapoport,B., Rolston,K., and Talcott,J. (2000). The Multinational Association for Supportive Care in Cancer risk index: A multinational scoring system for identifying low-risk febrile neutropenic cancer patients. J. Clin. Oncol. *18*, 3038-3051.

Klickstein,L.B., Barbashov,S.F., Liu,T., Jack,R.M., and Nicholson-Weller,A. (1997). Complement receptor type 1 (CR1, CD35) is a receptor for C1q. Immunity. *7*, 345-355.

Klein,N.J. and Kilpatrick,D.C. (2004). Is there a role for mannan/mannose-binding lectin (MBL) in defence against infection following chemotherapy for cancer? Clin. Exp. Immunol. *138*, 202-204.

Koch,A., Melbye,M., Sorensen,P., Homoe,P., Madsen,H.O., Molbak,K., Hansen,C.H., Andersen,L.H., Hahn,G.W., and Garred,P. (2001). Acute respiratory tract infections and mannose-binding lectin insufficiency during early childhood. JAMA *285*, 1316-1321.

Kolble,K., Lu,J., Mole,S.E., Kaluz,S., and Reid,K.B. (1993). Assignment of the human pulmonary surfactant protein D gene (SFTP4) to 10q22-q23 close to the surfactant protein A gene cluster. Genomics *17*, 294-298.

Krarup,A., Sorensen,U.B., Matsushita,M., Jensenius,J.C., and Thiel,S. (2005). Effect of capsulation of opportunistic pathogenic bacteria on binding of the pattern recognition molecules mannan-binding lectin, L-ficolin, and H-ficolin. Infect. Immun. *73*, 1052-1060.

Krarup,A., Thiel,S., Hansen,A., Fujita,T., and Jensenius,J.C. (2004). L-ficolin is a pattern recognition molecule specific for acetyl groups. J. Biol. Chem. *279*, 47513-47519.

Kuhlman,M., Joiner,K., and Ezekowitz,R.A. (1989). The human mannose-binding protein functions as an opsonin. J. Exp. Med. *169*, 1733-1745.

Kuipers,S., Aerts,P.C., Sjoholm,A.G., Harmsen,T., and van Dijk,H. (2002). A hemolytic assay for the estimation of functional mannose-binding lectin levels in human serum. J. Immunol. Methods *268*, 149-157.

Kushner,I. (1993). Regulation of the acute phase response by cytokines. Perspect. Biol. Med. *36*, 611-622.

Laddie,J., and Chisholm,J. (2007). A survey of parent and staff views on management by risk stratification in children with febrile neutropenia. Pediatr. Blood Cancer. *49*, 508.

Larsen,F., Madsen,H.O., Sim,R.B., Koch,C., and Garred,P. (2004). Disease-associated mutations in human mannose-binding lectin compromise oligomerization and activity of the final protein. J. Biol. Chem. *279*, 21302-21311.

Lausen,B., Schmiegelow,K., Andreassen,B., Madsen,H.O., and Garred,P. (2006). Infections during induction therapy of childhood acute lymphoblastic leukemia--no association to mannose-binding lectin deficiency. Eur. J. Haematol. *76*, 481-487.

Lee,S.G., Yum,J.S., Moon,H.M., Kim,H.J., Yang,Y.J., Kim,H.L., Yoon,Y., Lee,S., and Song,K. (2005a). Analysis of mannose-binding lectin 2 (MBL2) genotype and the serum protein levels in the Korean population. Mol. Immunol *42*, 969-977.

Lee,Y.H., Witte,T., Momot,T., Schmidt,R.E., Kaufman,K.M., Harley,J.B., and Sestak,A.L. (2005b). The mannose-binding lectin gene polymorphisms and systemic lupus erythematosus: two case-control studies and a meta-analysis. Arthritis Rheum. *52*, 3966-3974.

Lehrnbecher,T., Bernig,T., Hanisch,M., Koehl,U., Behl,M., Reinhardt,D., Creutzig,U., Klingebiel,T., Chanock,S.J., and Schwabe,D. (2005). Common genetic variants in the interleukin-6 and chitotriosidase genes are associated with the risk for serious infection in children undergoing therapy for acute myeloid leukemia. Leukemia *19*, 1745-1750.

Lehrnbecher,T., Fleischhack,G., Hanisch,M., Deinlein,F., Simon,A., Bernig,T., Chanock,S.J., and Klingebiel,T. (2004). Circulating levels and promoter polymorphisms of interleukins-6 and 8 in pediatric cancer patients with fever and neutropenia. Haematologica *89*, 234-236.

Lehrnbecher,T., Foster,C., Vazquez,N., Mackall,C.L., and Chanock,S.J. (1997). Therapy-induced alterations in host defense in children receiving therapy for cancer. J. Pediatr. Hematol. Oncol. *19*, 399-417.

Lehrnbecher,T., Venzon,D., de,H.M., Chanock,S.J., and Kuhl,J. (1999). Assessment of measuring circulating levels of interleukin-6, interleukin-8, C-reactive protein, soluble Fc gamma receptor type III, and mannose-binding protein in febrile children with cancer and neutropenia. Clin. Infect. Dis. *29*, 414-419.

Lemaitre,B., Nicolas,E., Michaut,L., Reichhart,J.M., and Hoffmann,J.A. (1996). The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell *86*, 973-983.

Lilienfeld-Toal,M., Dietrich,M.P., Glasmacher,A., Lehmann,L., Breig,P., Hahn,C., Schmidt-Wolf,I.G., Marklein,G., Schroeder,S., and Stuber,F. (2004). Markers of bacteremia in febrile neutropenic patients with hematological malignancies: procalcitonin and IL-6 are more reliable than C-reactive protein. Eur. J. Clin. Microbiol. Infect. Dis. *23*, 539-544.

Lipscombe,R.J., Sumiya,M., Hill,A.V., Lau,Y.L., Levinsky,R.J., Summerfield,J.A., and Turner,M.W. (1992). High frequencies in African and non-African populations of independent mutations in the mannose binding protein gene. Hum. Mol. Genet. *1*, 709-715.

Liu,H., Jensen,L., Hansen,S., Petersen,S.V., Takahashi,K., Ezekowitz,A.B., Hansen,F.D., Jensenius,J.C., and Thiel,S. (2001). Characterization and quantification of mouse mannan-binding lectins (MBL-A and MBL-C) and study of acute phase responses. Scand. J. Immunol. *53*, 489-497.

Liu,Y., Endo,Y., Iwaki,D., Nakata,M., Matsushita,M., Wada,I., Inoue,K., Munakata,M., and Fujita,T. (2005). Human M-ficolin is a secretory protein that activates the lectin complement pathway. J. Immunol *175*, 3150-3156.

Lozano,F., Suarez,B., Munoz,A., Jensenius,J.C., Mensa,J., Vives,J., and Horcajada,J.P. (2005). Novel MASP2 variants detected among North African and Sub-Saharan individuals. Tissue Antigens *66*, 131-135.

Lu,J. and Le,Y. (1998). Ficolins and the fibrinogen-like domain. Immunobiology *199*, 190-199.

Lu,J., Tay,P.N., Kon,O.L., and Reid,K.B. (1996). Human ficolin: cDNA cloning, demonstration of peripheral blood leucocytes as the major site of synthesis and assignment of the gene to chromosome 9. Biochem. J. *313 (Pt 2)*, 473-478.

Lu,J.H., Thiel,S., Wiedemann,H., Timpl,R., and Reid,K.B. (1990). Binding of the pentamer/hexamer forms of mannan-binding protein to zymosan activates the proenzyme

C1r2C1s2 complex, of the classical pathway of complement, without involvement of C1q. J. Immunol. *144*, 2287-2294.

Lu,W., Pan,K., Zhang,L., Lin,D., Miao,X., and You,W. (2005). Genetic polymorphisms of interleukin (IL)-1B, IL-1RN, IL-8, IL-10, and tumor necrosis factor α and risk of gastric cancer in a chinese population. Carcinogenesis. *26*, 631-6.

Lucas,K.G., Brown,A.E., Armstrong,D., Chapman,D., and Heller,G. (1996). The identification of febrile, neutropenic children with neoplastic disease at low risk for bacteremia and complications of sepsis. Cancer *77*, 791-798.

Luty,A.J., Kun,J.F., and Kremsner,P.G. (1998). Mannose-binding lectin plasma levels and gene polymorphisms in Plasmodium falciparum malaria. J. Infect. Dis. *178*, 1221-1224.

Lynch,N.J., Roscher,S., Hartung,T., Morath,S., Matsushita,M., Maennel,D.N., Kuraya,M., Fujita,T., and Schwaeble,W.J. (2004). L-ficolin specifically binds to lipoteichoic acid, a cell wall constituent of gram-positive bacteria, and activates the lectin pathway of complement. J. Immunol. *172*, 1198-1202.

Ma,Y., Uemura,K., Oka,S., Kozutsumi,Y., Kawasaki,N., and Kawasaki,T. (1999). Antitumor activity of mannan-binding protein in vivo as revealed by a virus expression system: mannan-binding proteindependent cell-mediated cytotoxicity. Proc. Natl. Acad. Sci. U. S. A *96*, 371-375.

Madsen,H.O., Garred,P., Kurtzhals,J.A., Lamm,L.U., Ryder,L.P., Thiel,S., and Svejgaard,A. (1994). A new frequent allele is the missing link in the structural polymorphism of the human mannan-binding protein. Immunogenetics *40*, 37-44.

Madsen,H.O., Garred,P., Thiel,S., Kurtzhals,J.A., Lamm,L.U., Ryder,L.P., and Svejgaard,A. (1995). Interplay between promoter and structural gene variants control basal serum level of mannan-binding protein. J. Immunol. *155*, 3013-3020.

Madsen,H.O., Satz,M.L., Hogh,B., Svejgaard,A., and Garred,P. (1998). Different molecular events result in low protein levels of mannan-binding lectin in populations from southeast Africa and South America. J. Immunol. *161*, 3169-3175.

Malhotra,R., Thiel,S., Reid,K.B., and Sim,R.B. (1990). Human leukocyte C1q receptor binds other soluble proteins with collagen domains. J. Exp. Med. *172*, 955-959.

Malik,I.A. (1997). Out-patient management of febrile neutropenia in indigent paediatric patients. Ann. Acad. Med. Singapore *26*, 742-746.

Martinez-Pomares,L., Wienke,D., Stillion,R., McKenzie,E.J., Arnold,J.N., Harris,J., McGreal,E., Sim,R.B., Isacke,C.M., and Gordon,S. (2006). Carbohydrate-independent recognition of collagens by the macrophage mannose receptor. Eur. J. Immunol. *36*, 1074-1082.

Matsushita,M., Endo,Y., and Fujita,T. (2000a). Cutting edge: complement-activating complex of ficolin and mannose-binding lectin-associated serine protease. J. Immunol. *164*, 2281-2284.

Matsushita,M., Endo,Y., Taira,S., Sato,Y., Fujita,T., Ichikawa,N., Nakata,M., and Mizuochi,T. (1996). A novel human serum lectin with collagen- and fibrinogen-like domains that functions as an opsonin. J. Biol. Chem. *271*, 2448-2454.

Matsushita,M. and Fujita,T. (1995). Cleavage of the third component of complement (C3) by mannose-binding protein-associated serine protease (MASP) with subsequent complement activation. Immunobiology *194*, 443-448.

Matsushita,M. and Fujita,T. (1992). Activation of the classical complement pathway by mannose-binding protein in association with a novel C1s-like serine protease. J. Exp. Med. *176*, 1497-1502.

Matsushita,M., Hijikata,M., Matsushita,M., Ohta,Y., and Mishiro,S. (1998). Association of mannose-binding lectin gene haplotype LXPA and LYPB with interferon-resistant hepatitis C virus infection in Japanese patients. J. Hepatol. *29*, 695-700.

Matsushita,M., Thiel,S., Jensenius,J.C., Terai,I., and Fujita,T. (2000b). Proteolytic activities of two types of mannose-binding lectin-associated serine protease. J. Immunol. *165*, 2637-2642.

Mayilyan,K.R., Presanis,J.S., Arnold,J.N., Hajela,K., and Sim,R.B. (2006). Heterogeneity of MBL-MASP complexes. Mol. Immunol *43*, 1286-92.

Mead,R., Jack,D., Pembrey,M., Tyfield,L., and Turner,M. (1997). Mannose-binding lectin alleles in a prospectively recruited UK population. The ALSPAC Study Team. Avon Longitudinal Study of Pregnancy and Childhood. Lancet *349*, 1669-1670.

Medzhitov,R. (2001). Toll-like receptors and innate immunity. Nat. Rev. Immunol. *1*, 135-145.

Medzhitov,R. (2007). Recognition of microorganisms and activation of the immune response.Nature. *449*, 819-26.

Medzhitov,R. and Janeway,C.A., Jr. (1997). Innate immunity: impact on the adaptive immune response. Curr. Opin. Immunol. *9*, 4-9.

Medzhitov,R. and Janeway,C.A., Jr. (2000). Innate immunity. N. Engl. J. Med. *343*, 338-344.

Meyer,S., Gottschling,S., Biran,T., Georg,T., Ehlayil,K., Graf,N., and Gortner,L. (2005). Assessing the risk of mortality in paediatric cancer patients admitted to the paediatric intensive care unit: a novel risk score? Eur. J. Pediatr. *164*, 563-567.

Meylan,E., Tschopp,J., and Karin,M. (2006) Intracellular pattern recognition receptors in the host response. Nature. *442*, 39-44

Miller,M.E., Seals,J., Haye,R., and Levitsky,L.C. (1968). A familial plasma-associated defect of phagocytosis. Lancet 2 60-63.

Minchinton,R.M., Dean,M.M., Clark,T.R., Heatley,S., and Mullighan,C.G. (2002). Analysis of the relationship between mannose-binding lectin (MBL) genotype, MBL levels and function in an Australian blood donor population. Scand. J. Immunol. *56*, 630-641.

Mitterer,G., Bodamer,O., Harwanegg,C., Maurer,W., Mueller,M.W., and Schmidt,W.M. (2005). Microarray-based detection of mannose-binding lectin 2 (MBL2) polymorphisms in a routine clinical setting. Genet. Test. *9*, 6-13.

Moens,L., Van,H.E., Peetermans,W.E., De,B.C., Verhaegen,J., and Bossuyt,X. (2006). Mannose-binding lectin genotype and invasive pneumococcal infection. Hum. Immunol. *67*, 605-611.

Mogues,T., Ota,T., Tauber,A.I., Sastry,K.N. (1996) Characterization of two mannose-binding protein cDNAs from rhesus monkey (Macaca mulatta): structure and evolutionary implications. Glycobiology. *6*, 543-50.

Molle,I., Peterslund,N.A., Thiel,S., and Steffensen,R. (2006a). MBL2 polymorphism and risk of severe infections in multiple myeloma patients receiving high-dose melphalan and autologous stem cell transplantation. Bone Marrow Transplant. *38*, 555-560.

Molle,I., Steffensen,R., Thiel,S., and Peterslund,N.A. (2006b). Chemotherapy-related infections in patients with multiple myeloma: associations with mannan-binding lectin genotypes. Eur. J. Haematol. *77*, 19-26.

Moller-Kristensen,M., Jensenius,J.C., Jensen,L., Thielens,N., Rossi,V., Arlaud,G., and Thiel,S. (2003). Levels of mannan-binding lectin-associated serine protease-2 in healthy individuals. J. Immunol. Methods *282*, 159-167.

Moller-Kristensen,M., Ip,W.K., Shi,L., Gowda,L.D., Hamblin,M.R., Thiel,S., Jensenius,J.C., Ezekowitz,R.A., and Takahashi,K. (2006). Deficiency of mannose-binding lectin greatly increases susceptibility to postburn infection with Pseudomonas aeruginosa. J. Immunol. *176*, 1769-1775.

Moller-Kristensen,M., Thiel,S., Sjoholm,A., Matsushita,M., and Jensenius,J.C. (2007). Cooperation between MASP-1 and MASP-2 in the generation of C3 convertase through the MBL pathway. Int. Immunol. *19*, 141-149.

Morley,J.J. and Kushner,I. (1982). Serum C-reactive protein levels in disease. Ann. N. Y. Acad. Sci. *389*, 406-418.

Mullen,C.A. (2001). Which children with fever and neutropenia can be safely treated as outpatients? Br. J. Haematol. *112*, 832-837.

Mullen,C.A., Petropoulos,D., Roberts,W.M., Rytting,M., Zipf,T., Chan,K.W., Culbert,S.J., Danielson,M., Jeha,S.S., Kuttesch,J.F., and Rolston,K.V. (1999a). Outpatient treatment of fever and neutropenia for low risk pediatric cancer patients. Cancer *86*, 126-134.

Mullen,C.A., Petropoulos,D., Roberts,W.M., Rytting,M., Zipf,T., Chan,K.W., Culbert,S.J., Danielson,M., Jeha,S.S., Kuttesch,J.F., and Rolston,K.V. (1999b). Economic and resource

utilization analysis of outpatient management of fever and neutropenia in low-risk pediatric patients with cancer. J. Pediatr. Hematol. Oncol. *21*, 212-218.

Mullighan,C.G., Heatley,S., Doherty,K., Szabo,F., Grigg,A., Hughes,T.P., Schwarer,A.P., Szer,J., Tait,B.D., Bik,T.L., and Bardy,P.G. (2002). Mannose-binding lectin gene polymorphisms are associated with major infection following allogeneic hemopoietic stem cell transplantation. Blood *99*, 3524-3529.

Munthe-Fog,L., Hummelshoj,T., Hansen,B.E., Koch,C., Madsen,H.O., Skjodt,K., and Garred,P. (2007). The impact of FCN2 polymorphisms and haplotypes on the Ficolin-2 serum levels. Scand. J. Immunol. *65*, 383-392.

Mustafa,M.M., Aquino,V.M., Pappo,A., Tkaczewski,I., and Buchanan,G.R. (1996). A pilot study of outpatient management of febrile neutropenic children with cancer at low risk of bacteremia. J. Pediatr. *128*, 847-849.

Nadesalingam,J., Dodds,A.W., Reid,K.B., and Palaniyar,N. (2005). Mannose-binding lectin recognizes peptidoglycan via the N-acetyl glucosamine moiety, and inhibits ligand-induced proinflammatory effect and promotes chemokine production by macrophages. J. Immunol *175*, 1785-1794.

Naito,H., Ikeda,A., Hasegawa,K., Oka,S., Uemura,K., Kawasaki,N., and Kawasaki,T. (1999). Characterization of human serum mannan-binding protein promoter. J. Biochem. (Tokyo) *126*, 1004-1012.

Nakagawa,T., Kawasaki,N., Ma,Y., Uemura,K., and Kawasaki,T. (2003). Antitumor activity of mannan-binding protein. Methods Enzymol. *363*, 26-33.

Neth,O., Hann,I., Turner,M.W., and Klein,N.J. (2001). Deficiency of mannose-binding lectin and burden of infection in children with malignancy: a prospective study. Lancet *358*, 614-618.

Neth,O., Jack,D.L., Dodds,A.W., Holzel,H., Klein,N.J., and Turner,M.W. (2000). Mannose-binding lectin binds to a range of clinically relevant microorganisms and promotes complement deposition. Infect. Immun. *68*, 688-693.

Nuytinck, L. and Shapiro, F. (2004). Mannose-binding lectin: laying the stepping stones from clinical research to personalized medicine. Personalized Medicine *1*, 35-52.

Ogden,C.A., deCathelineau,A., Hoffmann,P.R., Bratton,D., Ghebrehiwet,B., Fadok,V.A., and Henson,P.M. (2001). C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. J. Exp. Med. *194*, 781-795.

Ohashi,T. and Erickson,H.P. (1997). Two oligomeric forms of plasma ficolin have differential lectin activity. J. Biol. Chem. *272*, 14220-14226.

Ohlenschlaeger,T., Garred,P., Madsen,H.O., and Jacobsen,S. (2004). Mannose-binding lectin variant alleles and the risk of arterial thrombosis in systemic lupus erythematosus. N. Engl. J. Med. *351*, 260-267.

Ohtani,K., Suzuki,Y., Eda,S., Kawai,T., Kase,T., Keshi,H., Sakai,Y., Fukuoh,A., Sakamoto,T., Itabe,H., Suzutani,T., Ogasawara,M., Yoshida,I., and Wakamiya,N. (2001). The membrane-type collectin CL-P1 is a scavenger receptor on vascular endothelial cells. J. Biol. Chem. *276*, 44222-44228.

Ohtani,K., Suzuki,Y., Eda,S., Kawai,T., Kase,T., Yamazaki,H., Shimada,T., Keshi,H., Sakai,Y., Fukuoh,A., Sakamoto,T., and Wakamiya,N. (1999). Molecular cloning of a novel human collectin from liver (CL-L1). J. Biol. Chem. *274*, 13681-13689.

Olesen,H.V., Jensenius,J.C., Steffensen,R., Thiel,S., and Schiotz,P.O. (2006). The mannan-binding lectin pathway and lung disease in cystic fibrosis--disfunction of mannan-binding lectin-associated serine protease 2 (MASP-2) may be a major modifier. Clin. Immunol. *121*, 324-331.

Omori-Satoh,T., Yamakawa,Y., and Mebs,D. (2000). The antihemorrhagic factor, erinacin, from the European hedgehog (Erinaceus europaeus), a metalloprotease inhibitor of large molecular size possessing ficolin/opsonin P35 lectin domains. Toxicon *38*, 1561-1580.

Oroszlan,M., Daha,M.R., Cervenak,L., Prohaszka,Z., Fust,G., and Roos,A. (2007). MBL and C1q compete for interaction with human endothelial cells. Mol. Immunol. *44*, 1150-1158.

Orudjev,E. and Lange,B.J. (2002). Evolving concepts of management of febrile neutropenia in children with cancer. Med. Pediatr. Oncol. *39*, 77-85.

Oude Nijhuis,C.S., Daenen,S.M., Vellenga,E., van der Graaf,W.T., Gietema,J.A., Groen,H.J., Kamps,W.A., and de Bont,E.S. (2002). Fever and neutropenia in cancer patients: the diagnostic role of cytokines in risk assessment strategies. Crit Rev. Oncol. Hematol. *44*, 163-174.

Oude Nijhuis,C., Kamps,W.A., Daenen,S.M., Gietema,J.A., van der Graaf,W.T., Groen,H.J., Vellenga,E., Ten Vergert,E.M., Vermeulen,K.M., de Vries-Hospers,H.G., and de Bont,E.S. (2005). Feasibility of withholding antibiotics in selected febrile neutropenic cancer patients. J. Clin. Oncol. *23*, 7437-7444.

Paganini,H., Gomez,S., Ruvinsky,S., Zubizarreta,P., Latella,A., Fraquelli,L., Iturres,A.S., Casimir,L., and Debbag,R. (2003). Outpatient, sequential, parenteral-oral antibiotic therapy for lower risk febrile neutropenia in children with malignant disease: a single-center, randomized, controlled trial in Argentina. Cancer *97*, 1775-1780.

Paganini,H.R., Sarkis,C.M., De Martino,M.G., Zubizarreta,P.A., Casimir,L., Fernandez,C., Armada,A.A., Rodriguez-Brieshcke,M.T., and Debbag,R. (2000). Oral administration of cefixime to lower risk febrile neutropenic children with cancer. Cancer *88*, 2848-2852.

Palaniyar,N., Nadesalingam,J., Clark,H., Shih,M.J., Dodds,A.W., and Reid,K.B. (2004). Nucleic acid is a novel ligand for innate, immune pattern recognition collectins surfactant proteins A and D and mannose-binding lectin. J. Biol. Chem. *279*, 32728-32736.

Palaniyar,N., Nadesalingam,J., and Reid,K.B. (2003). Innate immune collectins bind nucleic acids and enhance DNA clearance in vitro. Ann. N. Y. Acad. Sci. *1010*, 467-470.

Pearson,A.M. (1996). Scavenger receptors in innate immunity. Curr. Opin. Immunol. *8*, 20-28.

Persson,L., Soderquist,B., Engervall,P., Vikerfors,T., Hansson,L.O., and Tidefelt,U. (2005). Assessment of systemic inflammation markers to differentiate a stable from a deteriorating clinical course in patients with febrile neutropenia. Eur. J. Haematol. *74*, 297-303.

Persikov,A.V., Pillitteri,R.J., Amin,P., Schwarze,U., Byers,P.H., and Brodsky,B. (2004). Stability related bias in residues replacing glycines within the collagen triple helix (Gly-Xaa-Yaa) in inherited connective tissue disorders. Hum. Mutat. *24*, 330-337.

Petersen,S.V., Thiel,S., Jensen,L., Steffensen,R., and Jensenius,J.C. (2001). An assay for the mannan-binding lectin pathway of complement activation. J. Immunol. Methods *257*, 107-116.

Petersen,K.A., Matthiesen,F., Agger,T., Kongerslev,L., Thiel,S., Cornelissen,K., and Axelsen,M. (2006). Phase I safety, tolerability, and pharmacokinetic study of recombinant human mannan-binding lectin. J. Clin. Immunol. *26*, 465-475.

Peterslund,N.A., Koch,C., Jensenius,J.C., and Thiel,S. (2001). Association between deficiency of mannose-binding lectin and severe infections after chemotherapy. Lancet *358*, 637-638.

Petrilli,A.S., Dantas,L.S., Campos,M.C., Tanaka,C., Ginani,V.C., and Seber,A. (2000). Oral ciprofloxacin vs. intravenous ceftriaxone administered in an outpatient setting for fever and neutropenia in low-risk pediatric oncology patients: randomized prospective trial. Med. Pediatr. Oncol. *34*, 87-91.

Phillips,B., Selwood,K., Lane,S., Skinner,R., Gibson,F., and Chisholm,J.C. (2007). Variation in policies for the management of febrile neutropenia in United Kingdom Children's Cancer Study Group centres. Arch. Dis. Child. *92*, 495-8.

Philpott,D.J. and Girardin,S.E. (2004). The role of Toll-like receptors and Nod proteins in bacterial infection. Mol. Immunol. *41*, 1099-1108.

Pillemer, L, Blum, L, Lepow, I. H, Ross, O. A, Todd, E. W, and Wardlaw, A. C. (1954). The properdin system and immunity. I. Demonstration and isolation of a new serum protein, properdin, and its role in immune phenomena. Science *120*, 279-285.

Polotsky,V.Y., Belisle,J.T., Mikusova,K., Ezekowitz,R.A., and Joiner,K.A. (1997). Interaction of human mannose-binding protein with Mycobacterium avium. J. Infect. Dis. *175*, 1159-1168.

Preis,S., Jurgens,H., Friedland,C., Oudekotte-David,A.A., Thomas,L., and Gobel,U. (1993). Ceftriaxone alone or in Combination with Teicoplanin in the Management of Febrile Episodes in Neutropenic Children and Adolescents with Cancer on an Outpatient Base. Klin. Padiatr. *205*, 295-299.

Quezada,G., Sunderland,T., Chan,K.W., Rolston,K., and Mullen,C.A. (2007). Medical and non-medical barriers to outpatient treatment of fever and neutropenia in children with cancer. Pediatr. Blood Cancer. *48*, 273-7.

Rackoff,W.R., Gonin,R., Robinson,C., Kreissman,S.G., and Breitfeld,P.B. (1996). Predicting the risk of bacteremia in childen with fever and neutropenia. J. Clin. Oncol. *14*, 919-924.

Raisch,D.W., Holdsworth,M.T., Winter,S.S., Hutter,J.J., and Graham,M.L. (2003). Economic comparison of home-care-based versus hospital-based treatment of chemotherapy-induced febrile neutropenia in children. Value. Health *6*, 158-166.

Robinson,D., Phillips,N.C., and Winchester,B. (1975). Affinity chromatography of human liver alpha-D-mannosidase. FEBS Lett. *53*, 110-112.

Rocha,V., Franco,R.F., Porcher,R., Bittencourt,H., Silva,W.A., Jr., Latouche,A., Devergie,A., Esperou,H., Ribaud,P., Socie,G., Zago,M.A., and Gluckman,E. (2002). Host defense and inflammatory gene polymorphisms are associated with outcomes after HLA-identical sibling bone marrow transplantation. Blood *100*, 3908-3918.

Rondinelli,P.I., Ribeiro,K.C., and de,C.B. (2006). A proposed score for predicting severe infection complications in children with chemotherapy-induced febrile neutropenia. J. Pediatr. Hematol. Oncol. *28*, 665-670.

Roos,A., Bouwman,L.H., Munoz,J., Zuiverloon,T., Faber-Krol,M.C., Fallaux-van den Houten FC, Klar-Mohamad,N., Hack,C.E., Tilanus,M.G., and Daha,M.R. (2003). Functional characterization of the lectin pathway of complement in human serum. Mol. Immunol. *39*, 655-668.

Roos,A., Dieltjes,P., Vossen,R.H., Daha,M.R., and de Knijff,P. (2006). Detection of three single nucleotide polymorphisms in the gene encoding mannose-binding lectin in a single pyrosequencing reaction. J. Immunol. Methods *309*, 108-114.

Roos,A., Garred,P., Wildenberg,M.E., Lynch,N.J., Munoz,J.R., Zuiverloon,T.C., Bouwman,L.H., Schlagwein,N., Fallaux van den Houten FC, Faber-Krol,M.C., Madsen,H.O., Schwaeble,W.J., Matsushita,M., Fujita,T., and Daha,M.R. (2004). Antibody-mediated activation of the classical pathway of complement may compensate for mannose-binding lectin deficiency. Eur. J. Immunol. *34*, 2589-2598.

Rossi,V., Cseh,S., Bally,I., Thielens,N.M., Jensenius,J.C., and Arlaud,G.J. (2001). Substrate specificities of recombinant mannan-binding lectin-associated serine proteases-1 and -2. J. Biol. Chem. *276*, 40880-40887.

Rossi,V., Teillet,F., Thielens,N.M., Bally,I., and Arlaud,G.J. (2005). Functional characterization of complement proteases C1s/mannan-binding lectin-associated serine protease-2 (MASP-2) chimeras reveals the higher C4 recognition efficacy of the MASP-2 complement control protein modules. J. Biol. Chem. *280*, 41811-41818.

Roy,S., Knox,K., Segal,S., Griffiths,D., Moore,C.E., Welsh,K.I., Smarason,A., Day,N.P., McPheat,W.L., Crook,D.W., and Hill,A.V. (2002). MBL genotype and risk of invasive pneumococcal disease: a case-control study. Lancet *359*, 1569-1573.

Saevarsdottir,S., Oskarsson,O.O., Aspelund,T., Eiriksdottir,G., Vikingsdottir,T., Gudnason,V., and Valdimarsson,H. (2005). Mannan binding lectin as an adjunct to risk assessment for myocardial infarction in individuals with enhanced risk. J. Exp. Med. *201*, 117-125.

Saifuddin,M., Hart,M.L., Gewurz,H., Zhang,Y., and Spear,G.T. (2000). Interaction of mannose-binding lectin with primary isolates of human immunodeficiency virus type 1. J. Gen. Virol. *81*, 949-955.

Sankila,R., Martos Jimenez,M.C., Miljus,D., Pritchard-Jones,K., Steliarova-Foucher,E., and Stiller,C. (2006). Geographical comparison of cancer survival in European children (1988-1997): report from the Automated Childhood Cancer Information System project. Eur. J. Cancer *42*, 1972-1980.

Santolaya,M.E., Alvarez,A.M., Aviles,C.L., Becker,A., Cofre,J., Cumsille,M.A., O'Ryan,M.L., Paya,E., Salgado,C., Silva,P., Tordecilla,J., Varas,M., Villarroel,M., Viviani,T., and Zubieta,M. (2004). Early hospital discharge followed by outpatient management versus continued hospitalization of children with cancer, fever, and neutropenia at low risk for invasive bacterial infection. J. Clin. Oncol. *22*, 3784-3789.

Santolaya,M.E., Alvarez,A.M., Becker,A., Cofre,J., Enriquez,N., O'Ryan,M., Paya,E., Pilorget,J., Salgado,C., Tordecilla,J., Varas,M., Villarroel,M., Viviani,T., and Zubieta,M. (2001). Prospective, multicenter evaluation of risk factors associated with invasive bacterial infection in children with cancer, neutropenia, and fever. J. Clin. Oncol. *19*, 3415-3421.

Santos,I.K., Costa,C.H., Krieger,H., Feitosa,M.F., Zurakowski,D., Fardin,B., Gomes,R.B., Weiner,D.L., Harn,D.A., Ezekowitz,R.A., and Epstein,J.E. (2001). Mannan-binding lectin enhances susceptibility to visceral leishmaniasis. Infect. Immun. *69*, 5212-5215.

Sastry,K., Herman,G.A., Day,L., Deignan,E., Bruns,G., Morton,C.C., and Ezekowitz,R.A. (1989). The human mannose-binding protein gene. Exon structure reveals its evolutionary relationship to a human pulmonary surfactant gene and localization to chromosome 10. J. Exp. Med. *170*, 1175-1189.

Sastry,R., Wang,J.S., Brown,D.C., Ezekowitz,R.A., Tauber,A.I., and Sastry,K.N. (1995). Characterization of murine mannose-binding protein genes Mbl1 and Mbl2 reveals features common to other collectin genes. Mamm. Genome *6*, 103-110.

Sato,T., Endo,Y., Matsushita,M., and Fujita,T. (1994). Molecular characterization of a novel serine protease involved in activation of the complement system by mannose-binding protein. Int. Immunol. *6*, 665-669.

Schaaf,B.M., Boehmke,F., Esnaashari,H., Seitzer,U., Kothe,H., Maass,M., Zabel,P., and Dalhoff,K. (2003). Pneumococcal septic shock is associated with the interleukin-10-1082 gene promoter polymorphism. Am. J. Respir. Crit Care Med. *168*, 476-480.

Schelenz,S., Malhotra,R., Sim,R.B., Holmskov,U., and Bancroft,G.J. (1995). Binding of host collectins to the pathogenic yeast Cryptococcus neoformans: human surfactant protein D acts as an agglutinin for acapsular yeast cells. Infect. Immun. *63*, 3360-3366.

Schimpff,S., Satterlee,W., Young,V.M., and Serpick,A. (1971). Empiric therapy with carbenicillin and gentamicin for febrile patients with cancer and granulocytopenia. N. Engl. J. Med. *284*, 1061-1065.

Schlapbach,L.J., Aebi,C., Otth,M., Luethy,A.R., Leibundgut,K., Hirt,A., and Ammann,R.A. (2007). Serum levels of mannose-binding lectin and the risk of fever in neutropenia pediatric cancer patients. Pediatr. Blood Cancer. *49*, 11-6.

Schmiegelow,K., Garred,P., Lausen,B., Andreassen,B., Petersen,B.L., and Madsen,H.O. (2002). Increased frequency of mannose-binding lectin insufficiency among children with acute lymphoblastic leukemia. Blood *100*, 3757-3760.

Schultz,C., Temming,P., Bucsky,P., Gopel,W., Strunk,T., and Hartel,C. (2004). Immature anti-inflammatory response in neonates. Clin. Exp. Immunol. *135*, 130-136.

Schwaeble,W., Dahl,M.R., Thiel,S., Stover,C., and Jensenius,J.C. (2002). The mannan-binding lectin-associated serine proteases (MASPs) and MAp19: four components of the lectin pathway activation complex encoded by two genes. Immunobiology *205*, 455-466.

Secmeer,G., Devrim,I., Kara,A., Ceyhan,M., Cengiz,B., Kutluk,T., Buyukpamukcu,M., Yetgin,S., Tuncer,M., Uludag,A.K., Tezer,H., and Yildirim,I. (2007). Role of procalcitonin and CRP in differentiating a stable from a deteriorating clinical course in pediatric febrile neutropenia. J. Pediatr. Hematol. Oncol. *29*, 107-111.

Seelen,M.A., Roos,A., Wieslander,J., Mollnes,T.E., Sjoholm,A.G., Wurzner,R., Loos,M., Tedesco,F., Sim,R.B., Garred,P., Alexopoulos,E., Turner,M.W., and Daha,M.R. (2005). Functional analysis of the classical, alternative, and MBL pathways of the complement system: standardization and validation of a simple ELISA. J. Immunol Methods *296*, 187-198.

Selander,B., Martensson,U., Weintraub,A., Holmstrom,E., Matsushita,M., Thiel,S., Jensenius,J.C., Truedsson,L., and Sjoholm,A.G. (2006). Mannan-binding lectin activates C3 and the alternative complement pathway without involvement of C2. J. Clin. Invest *116*, 1425-1434.

Seyfarth,J., Garred,P., and Madsen,H.O. (2006). Extra-hepatic transcription of the human mannose-binding lectin gene (mbl2) and the MBL-associated serine protease 1-3 genes. Mol. Immunol *43*, 962-971.

Seyfarth,J., Garred,P., and Madsen,H.O. (2005). The 'involution' of mannose-binding lectin. Hum. Mol. Genet. *14*, 2859-2869.

Shenep,J.L., Flynn,P.M., Baker,D.K., Hetherington,S.V., Hudson,M.M., Hughes,W.T., Patrick,C.C., Roberson,P.K., Sandlund,J.T., Santana,V.M., Sixbey,J.W., and Slobod,K.S. (2001). Oral cefixime is similar to continued intravenous antibiotics in the empirical treatment of febrile neutropenic children with cancer. Clin. Infect. Dis. *32*, 36-43.

Sheriff,S., Chang,C.Y., and Ezekowitz,R.A. (1994). Human mannose-binding protein carbohydrate recognition domain trimerizes through a triple alpha-helical coiled-coil. Nat. Struct. Biol. *1*, 789-794.

Shi,L., Takahashi,K., Dundee,J., Shahroor-Karni,S., Thiel,S., Jensenius,J.C., Gad,F., Hamblin,M.R., Sastry,K.N., and Ezekowitz,R.A. (2004). Mannose-binding lectin-deficient mice are susceptible to infection with Staphylococcus aureus. J. Exp. Med. *199*, 1379-1390.

Shin,H.D., Winkler,C., Stephens,J.C., Bream,J., Young,H., Goedert,J.J., O'Brien,T.R., Vlahov,D., Buchbinder,S., Giorgi,J., Rinaldo,C., Donfield,S., Willoughby,A., O'Brien,S.J., and Smith,M.W. (2000). Genetic restriction of HIV-1 pathogenesis to AIDS by promoter alleles of IL10. Proc. Natl. Acad. Sci. U. S. A *97*, 14467-14472.

Skalnikova,H., Freiberger,T., Chumchalova,J., Grombirikova,H., and Sediva,A. (2004). Cost-effective genotyping of human MBL2 gene mutations using multiplex PCR. J. Immunol. Methods *295*, 139-147.

Smolnikova,M.V. and Konenkov,V.I. (2002). Association of IL2, TNFA, IL4 and IL10 Promoter Gene Polymorphisms with the Rate of Progression of the HIV Infection. Russ. J. Immunol. *7*, 349-356.

Soell,M., Lett,E., Holveck,F., Scholler,M., Wachsmann,D., and Klein,J.P. (1995). Activation of human monocytes by streptococcal rhamnose glucose polymers is mediated by CD14 antigen, and mannan binding protein inhibits TNF-alpha release. J. Immunol. *154*, 851-860.

Soothill,J.F. and Harvey,B.A. (1976). Defective opsonization. A common immunity deficiency. Arch. Dis. Child *51*, 91-99.

Sorensen,R., Thiel,S., and Jensenius,J.C. (2005). Mannan-binding-lectin-associated serine proteases, characteristics and disease associations. Springer Semin. Immunopathol. *27*, 299-319.

Sorensen,C.M., Hansen,T.K., Steffensen,R., Jensenius,J.C., and Thiel,S. (2006). Hormonal regulation of mannan-binding lectin synthesis in hepatocytes. Clin. Exp. Immunol. *145*, 173-182.

Sprong,T., Jack,D.L., Klein,N.J., Turner,M.W., van der,L.P., Steeghs,L., Jacobs,L., van der Meer,J.W., and van,D.M. (2004). Mannose binding lectin enhances IL-1beta and IL-10 induction by non-lipopolysaccharide (LPS) components of Neisseria meningitidis. Cytokine *28*, 59-66.

Steffensen,R., Hoffmann,K., and Varming,K. (2003). Rapid genotyping of MBL2 gene mutations using real-time PCR with fluorescent hybridisation probes. J. Immunol. Methods *278*, 191-199.

Steffensen,R., Thiel,S., Varming,K., Jersild,C., and Jensenius,J.C. (2000). Detection of structural gene mutations and promoter polymorphisms in the mannan-binding lectin (MBL) gene by polymerase chain reaction with sequence-specific primers. J. Immunol. Methods *241*, 33-42.

Stengaard-Pedersen,K., Thiel,S., Gadjeva,M., Moller-Kristensen,M., Sorensen,R., Jensen,L.T., Sjoholm,A.G., Fugger,L., and Jensenius,J.C. (2003). Inherited deficiency of mannan-binding lectin-associated serine protease 2. N. Engl. J. Med. *349*, 554-560.

Stiller,C. (2007). Childhood cancer in Britain incidence, survival, mortality. (Oxford: Oxford University Press).

Stover,C., Endo,Y., Takahashi,M., Lynch,N.J., Constantinescu,C., Vorup-Jensen,T., Thiel,S., Friedl,H., Hankeln,T., Hall,R., Gregory,S., Fujita,T., and Schwaeble,W. (2001). The human gene for mannan-binding lectin-associated serine protease-2 (MASP-2), the effector component of the lectin route of complement activation, is part of a tightly linked gene cluster on chromosome 1p36.2-3. Genes Immun. *2*, 119-127.

Stover,C.M., Thiel,S., Thelen,M., Lynch,N.J., Vorup-Jensen,T., Jensenius,J.C., and Schwaeble,W.J. (1999). Two constituents of the initiation complex of the mannan-binding lectin activation pathway of complement are encoded by a single structural gene. J. Immunol. *162*, 3481-3490.

Stryjewski,G.R., Nylen,E.S., Bell,M.J., Snider,R.H., Becker,K.L., Wu,A., Lawlor,C., and Dalton,H. (2005). Interleukin-6, interleukin-8, and a rapid and sensitive assay for calcitonin precursors for the determination of bacterial sepsis in febrile neutropenic children. Pediatr. Crit Care Med. *6*, 129-135.

Stuart,L.M., Takahashi,K., Shi,L., Savill,J., and Ezekowitz,R.A. (2005). Mannose-binding lectin-deficient mice display defective apoptotic cell clearance but no autoimmune phenotype. J. Immunol *174*, 3220-3226.

Sugimoto,R., Yae,Y., Akaiwa,M., Kitajima,S., Shibata,Y., Sato,H., Hirata,J., Okochi,K., Izuhara,K., and Hamasaki,N. (1998). Cloning and characterization of the Hakata antigen, a member of the ficolin/opsonin p35 lectin family. J. Biol. Chem. *273*, 20721-20727.

Sumiya,M., Super,M., Tabona,P., Levinsky,R.J., Arai,T., Turner,M.W., and Summerfield,J.A. (1991). Molecular basis of opsonic defect in immunodeficient children. Lancet *337*, 1569-1570.

Summerfield,J.A., Ryder,S., Sumiya,M., Thursz,M., Gorchein,A., Monteil,M.A., and Turner,M.W. (1995). Mannose binding protein gene mutations associated with unusual and severe infections in adults. Lancet *345*, 886-889.

Summerfield,J.A., Sumiya,M., Levin,M., and Turner,M.W. (1997). Association of mutations in mannose binding protein gene with childhood infection in consecutive hospital series. BMJ *314*, 1229-1232.

Sung,J.J., Hwa,L.H., Gerl,M.Y., Ji-Won,P., Nam-Chul,H., Jung, H.J., Matsushita, M., and Luel,L.B. (2007) Improved purification methods for human MBL/MASP and ficolin/MASP complexes and roles of Ca^{2+} ion in the ligand binding and complex association. Abstract VIth International Workshop on the First Component of Complement C1 and Collectins.

Sung,L., Feldman,B.M., Schwamborn,G., Paczesny,D., Cochrane,A., Greenberg,M.L., Maloney,A.M., Hendershot,E.I., Naqvi,A., Barrera,M., and Llewellyn-Thomas,H.A. (2004). Inpatient versus outpatient management of low-risk pediatric febrile neutropenia: measuring parents' and healthcare professionals' preferences. J. Clin. Oncol. *22*, 3922-3929.

Super,M., Gillies,S.D., Foley,S., Sastry,K., Schweinle,J.E., Silverman,V.J., and Ezekowitz,R.A. (1992). Distinct and overlapping functions of allelic forms of human mannose binding protein. Nat. Genet. *2*, 50-55.

Super,M., Thiel,S., Lu,J., Levinsky,R.J., and Turner,M.W. (1989). Association of low levels of mannan-binding protein with a common defect of opsonisation. Lancet *2*, 1236-1239.

Swanson,A.F., Ezekowitz,R.A., Lee,A., and Kuo,C.C. (1998). Human mannose-binding protein inhibits infection of HeLa cells by Chlamydia trachomatis. Infect. Immun. *66*, 1607-1612.

Tabona,P., Mellor,A., and Summerfield,J.A. (1995). Mannose binding protein is involved in first-line host defence: evidence from transgenic mice. Immunology *85*, 153-159.

Tacx,A.N., Groeneveld,A.B., Hart,M.H., Aarden,L.A., and Hack,C.E. (2003). Mannan binding lectin in febrile adults: no correlation with microbial infection and complement activation. J. Clin. Pathol. *56*, 956-959.

Takahashi,M., Endo,Y., Fujita,T., and Matsushita,M. (1999). A truncated form of mannose-binding lectin-associated serine protease (MASP)-2 expressed by alternative polyadenylation is a component of the lectin complement pathway. Int. Immunol. *11*, 859-863.

Takeda,K. and Akira,S. (2005). Toll-like receptors in innate immunity. Int. Immunol. *17*, 1-14.

Talcott,J.A., Finberg,R., Mayer,R.J., and Goldman,L. (1988). The medical course of cancer patients with fever and neutropenia. Clinical identification of a low-risk subgroup at presentation. Arch. Intern. Med. *148*, 2561-2568.

Talcott,J.A., Siegel,R.D., Finberg,R., and Goldman,L. (1992). Risk assessment in cancer patients with fever and neutropenia: a prospective, two-center validation of a prediction rule. J. Clin. Oncol. *10*, 316-322.

Taylor,M.E., Brickell,P.M., Craig,R.K., and Summerfield,J.A. (1989). Structure and evolutionary origin of the gene encoding a human serum mannose-binding protein. Biochem. J. *262*, 763-771.

Taylor,P.R., Tsoni,S.V., Willment,J.A., Dennehy,K.M., Rosas,M., Findon,H., Haynes,K., Steele,C., Botto,M., Gordon,S., and Brown,G.D. (2007) Dectin-1 is required for beta-glucan recognition and control of fungal infection. Nat Immunol. *8*, 31-8

Teh,C., Le,Y., Lee,S.H., and Lu,J. (2000). M-ficolin is expressed on monocytes and is a lectin binding to N-acetyl-D-glucosamine and mediates monocyte adhesion and phagocytosis of Escherichia coli. Immunology *101*, 225-232.

Teillet,F., Dublet,B., Andrieu,J.P., Gaboriaud,C., Arlaud,G.J., and Thielens,N.M. (2005). The two major oligomeric forms of human mannan-binding lectin: chemical characterization, carbohydrate-binding properties, and interaction with MBL-associated serine proteases. J. Immunol. *174*, 2870-2877.

Teillet,F., Lacroix,M., Thiel,S., Weilguny,D., Agger,T., Arlaud,G.J., and Thielens,N.M. (2007). Identification of the site of human mannan-binding lectin involved in the interaction with its partner serine proteases: the essential role of lys55. J. Immunol. *178*, 5710-5716.

Tenner,A.J., Robinson,S.L., and Ezekowitz,R.A. (1995). Mannose binding protein (MBP) enhances mononuclear phagocyte function via a receptor that contains the 126,000 M(r) component of the C1q receptor. Immunity. *3*, 485-493.

Thiel,S., Holmskov,U., Hviid,L., Laursen,S.B., and Jensenius,J.C. (1992). The concentration of the C-type lectin, mannan-binding protein, in human plasma increases during an acute phase response. Clin. Exp. Immunol. *90*, 31-35.

Thiel,S., Vorup-Jensen,T., Stover,C.M., Schwaeble,W., Laursen,S.B., Poulsen,K., Willis,A.C., Eggleton,P., Hansen,S., Holmskov,U., Reid,K.B., and Jensenius,J.C. (1997). A second serine protease associated with mannan-binding lectin that activates complement. Nature *386*, 506-510.

Thiel,S., Petersen,S.V., Vorup-Jensen,T., Matsushita,M., Fujita,T., Stover,C.M., Schwaeble,W.J., and Jensenius,J.C. (2000). Interaction of C1q and mannan-binding lectin (MBL) with C1r, C1s, MBL-associated serine proteases 1 and 2, and the MBL-associated protein MAp19. J. Immunol. *165*, 878-887.

Thiel,S., Steffensen,R., Christensen,I.J., Ip,W.K., Lau,Y.L., Reason,I.J., Eiberg,H., Gadjeva,M., Ruseva,M., and Jensenius,J.C. (2007). Deficiency of mannan-binding lectin associated serine protease-2 due to missense polymorphisms. Genes Immun. *8*, 154-163.

Thielens,N.M., Cseh,S., Thiel,S., Vorup-Jensen,T., Rossi,V., Jensenius,J.C., and Arlaud,G.J. (2001). Interaction properties of human mannan-binding lectin (MBL)-associated serine proteases-1 and -2, MBL-associated protein 19, and MBL. J. Immunol. *166*, 5068-5077.

Thomas,H.C., Foster,G.R., Sumiya,M., McIntosh,D., Jack,D.L., Turner,M.W., and Summerfield,J.A. (1996). Mutation of gene of mannose-binding protein associated with chronic hepatitis B viral infection. Lancet *348*, 1417-1419.

Townsend,R., Read,R.C., Turner,M.W., Klein,N.J., and Jack,D.L. (2001). Differential recognition of obligate anaerobic bacteria by human mannose-binding lectin. Clin. Exp. Immunol. *124*, 223-228.

Tsujimura,M., Miyazaki,T., Kojima,E., Sagara,Y., Shiraki,H., Okochi,K., and Maeda,Y. (2002). Serum concentration of Hakata antigen, a member of the ficolins, is linked with inhibition of Aerococcus viridans growth. Clin. Chim. Acta *325*, 139-146.

Turner,M.W., Dinan,L., Heatley,S., Jack,D.L., Boettcher,B., Lester,S., McCluskey,J., and Roberton,D. (2000). Restricted polymorphism of the mannose-binding lectin gene of indigenous Australians. Hum. Mol. Genet. *9*, 1481-1486.

Turner,M.W., Mowbray,J.F., and Roberton,D.R. (1981). A study of C3b deposition on yeast surfaces by sera of known opsonic potential. Clin. Exp. Immunol *46*, 412-419.

Valdimarsson,H., Stefansson,M., Vikingsdottir,T., Arason,G.J., Koch,C., Thiel,S., and Jensenius,J.C. (1998). Reconstitution of opsonizing activity by infusion of mannan-binding lectin (MBL) to MBL-deficient humans. Scand. J. Immunol. *48*, 116-123.

Valdimarsson,H., Vikingsdottir,T., Bang,P., Saevarsdottir,S., Gudjonsson,J.E., Oskarsson,O., Christiansen,M., Blou,L., Laursen,I., and Koch,C. (2004). Human plasma-derived mannose-binding lectin: a phase I safety and pharmacokinetic study. Scand. J. Immunol. *59*, 97-102.

van de Wetering,J.K., van Golde,L.M., and Batenburg,J.J. (2004). Collectins: players of the innate immune system. Eur. J. Biochem. *271*, 1229-1249.

van Deventer,S.J. (2000). Cytokine and cytokine receptor polymorphisms in infectious disease. Intensive Care Med. *26 Suppl 1*, S98-102.

van Emmerik,L.C., Kuijper,E.J., Fijen,C.A., Dankert,J., and Thiel,S. (1994). Binding of mannan-binding protein to various bacterial pathogens of meningitis. Clin. Exp. Immunol. *97*, 411-416.

Van Hoeyveld,E., Houtmeyers,F., Massonet,C., Moens,L., Van Ranst,M., Blanckaert,N., and Bossuyt,X. (2004). Detection of single nucleotide polymorphisms in the mannose-binding lectin gene using minor groove binder-DNA probes. J. Immunol. Methods *287*, 227-230.

Van Till,J.W., Boermeester,M.A., Modderman,P.W., Van Sandick,J.W., Hart,M.H., Gisbertz,S.S., Van Lanschot,J.J., and Aarden,L.A. (2006). Variable mannose-binding lectin expression during postoperative acute-phase response. Surg. Infect. (Larchmt.) *7*, 443-452.

Vander Cruyssen,B., Nuytinck,L., Boullart,L., Elewaut,D., Waegeman,W., Van Thielen,M., De Meester,E., Lebeer,K., Rossau,R., and De Keyser,F. (2007). Polymorphisms in the ficolin 1 gene (FCN1) are associated with susceptibility to the development of rheumatoid arthritis. Rheumatology (Oxford). *46*, 1792-5.

Vekemans,M., Robinson,J., Georgala,A., Heymans,C., Muanza,F., Paesmans,M., Klastersky,J., Barette,M., Meuleman,N., Huet,F., Calandra,T., Costantini,S., Ferrant,A., Mathissen,F., Axelsen,M., Marchetti,O., and Aoun,M. (2007). Low mannose-binding lectin concentration is associated with severe infection in patients with hematological cancer who are undergoing chemotherapy. Clin. Infect. Dis. *44*, 1593-1601.

Verdu,P., Barreiro,L.B., Patin,E., Gessain,A., Cassar,O., Kidd,J.R., Kidd,K.K., Behar,D.M., Froment,A., Heyer,E., Sica,L., Casanova,J.L., Abel,L., and Quintana-Murci,L. (2006). Evolutionary insights into the high worldwide prevalence of MBL2 deficiency alleles. Hum. Mol. Genet. *15*, 2650-2658.

Vorup-Jensen,T., Petersen,S.V., Hansen,A.G., Poulsen,K., Schwaeble,W., Sim,R.B., Reid,K.B., Davis,S.J., Thiel,S., and Jensenius,J.C. (2000). Distinct pathways of mannan-binding lectin (MBL)- and C1-complex autoactivation revealed by reconstitution of MBL with recombinant MBL-associated serine protease-2. J. Immunol. *165*, 2093-2100.

Wade,J.C., Schimpff,S.C., Newman,K.A., and Wiernik,P.H. (1982). Staphylococcus epidermidis: an increasing cause of infection in patients with granulocytopenia. Ann. Intern. Med. *97*, 503-508.

Wallis,R. (2007). Interactions between mannose-binding lectin and MASPs during complement activation by the lectin pathway. Immunobiology *212*, 289-299.

Wallis,R. and Cheng,J.Y. (1999). Molecular defects in variant forms of mannose-binding protein associated with immunodeficiency. J. Immunol. *163*, 4953-4959.

Wallis,R. and Dodd,R.B. (2000). Interaction of mannose-binding protein with associated serine proteases: effects of naturally occurring mutations. J. Biol. Chem. *275*, 30962-30969.

Wallis,R., Dodds,A.W., Mitchell,D.A., Sim,R.B., Reid,K.B., and Schwaeble,W.J. (2007). Molecular interactions between MASP-2, C4, and C2 and their activation fragments leading to complement activation via the lectin pathway. J. Biol. Chem. *282*, 7844-7851.

Wallis,R. and Drickamer,K. (1999). Molecular determinants of oligomer formation and complement fixation in mannose-binding proteins. J. Biol. Chem. *274*, 3580-3589.

Wallis,R., Lynch,N.J., Roscher,S., Reid,K.B., and Schwaeble,W.J. (2005). Decoupling of carbohydrate binding and MASP-2 autoactivation in variant mannose-binding lectins associated with immunodeficiency. J. Immunol *175*, 6846-6851.

Wallis,R., Shaw,J.M., Uitdehaag,J., Chen,C.B., Torgersen,D., and Drickamer,K. (2004). Localization of the serine protease-binding sites in the collagen-like domain of Mannose-binding protein: Indirect effects of naturally occuring mutations on protease binding and activation. J. Biol. Chem *279*, 14065-73.

Walport,M.J. (2001a). Complement. First of two parts. N. Engl. J. Med. *344*, 1058-1066.

Walport,M.J. (2001b). Complement. Second of two parts. N. Engl. J. Med. *344*, 1140-1144.

Walsh,M.C., Bourcier,T., Takahashi,K., Shi,L., Busche,M.N., Rother,R.P., Solomon,S.D., Ezekowitz,R.A., and Stahl,G.L. (2005). Mannose-binding lectin is a regulator of inflammation that accompanies myocardial ischemia and reperfusion injury. J. Immunol *175*, 541-546.

Weis,W.I. and Drickamer,K. (1994). Trimeric structure of a C-type mannose-binding protein. Structure. *2*, 1227-1240.

Weis,W.I., Kahn,R., Fourme,R., Drickamer,K., and Hendrickson,W.A. (1991). Structure of the calcium-dependent lectin domain from a rat mannose-binding protein determined by MAD phasing. Science *254*, 1608-1615.

Wild,J., Robinson,D., and Winchester,B. (1983). Isolation of mannose-binding proteins from human and rat liver. Biochem. J. *210*, 167-174.

Wiernikowski,J.T., Rothney,M., Dawson,S., and Andrew,M. (1991). Evaluation of a home intravenous antibiotic program in pediatric oncology. Am. J. Pediatr. Hematol. Oncol. *13*, 144-147.

Williams,B.R. (1999). PKR; a sentinel kinase for cellular stress. Oncogene *18*, 6112-6120.

Wong,N.K., Kojima,M., Dobo,J., Ambrus,G., and Sim,R.B. (1999). Activities of the MBL-associated serine proteases (MASPs) and their regulation by natural inhibitors. Mol. Immunol. *36*, 853-861.

Wood,N. and Bidwell,J. (1996). Genetic screening and testing by induced heteroduplex formation. Electrophoresis *17*, 247-254.

Wright,S.D., Ramos,R.A., Tobias,P.S., Ulevitch,R.J., and Mathison,J.C. (1990). CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science *249*, 1431-1433.

Wright,S.D., Tobias,P.S., Ulevitch,R.J., and Ramos,R.A. (1989). Lipopolysaccharide (LPS) binding protein opsonizes LPS-bearing particles for recognition by a novel receptor on macrophages. J. Exp. Med. *170*, 1231-1241.

Yamada,M., Oritani,K., Kaisho,T., Ishikawa,J., Yoshida,H., Takahashi,I., Kawamoto,S., Ishida,N., Ujiie,H., Masaie,H., Botto,M., Tomiyama,Y., and Matsuzawa,Y. (2004). Complement C1q regulates LPS-induced cytokine production in bone marrow-derived dendritic cells. Eur. J. Immunol. *34*, 221-230.

Yokota,Y., Arai,T., and Kawasaki,T. (1995). Oligomeric structures required for complement activation of serum mannan-binding proteins. J. Biochem. (Tokyo) *117*, 414-419.

Yoshizawa,S., Nagasawa,K., Yae,Y., Niho,Y., and Okochi,K. (1997). A thermolabile beta 2-macroglycoprotein (TMG) and the antibody against TMG in patients with systemic lupus erythematosus. Clin. Chim. Acta *264*, 219-225.

Ytting,H., Christensen,I.J., Jensenius,J.C., Thiel,S., and Nielsen,H.J. (2005a). Preoperative mannan-binding lectin pathway and prognosis in colorectal cancer. Cancer Immunol Immunother. *54*, 265-272.

Ytting,H., Christensen,I.J., Thiel,S., Jensenius,J.C., and Nielsen,H.J. (2005b). Serum mannan-binding lectin-associated serine protease 2 levels in colorectal cancer: relation to recurrence and mortality. Clin. Cancer Res. *11*, 1441-1446.

Ytting,H., Christensen,I.J., Basse,L., Lykke,J., Thiel,S., Jensenius,J.C., and Nielsen,H.J. (2006). Influence of major surgery on the mannan-binding lectin pathway of innate immunity. Clin. Exp. Immunol. *144*, 239-246.

Ytting,H., Jensenius,J.C., Christensen,I.J., Thiel,S., and Nielsen,H.J. (2004). Increased activity of the mannan-binding lectin complement activation pathway in patients with colorectal cancer. Scand. J. Gastroenterol. *39*, 674-679.

Yuen,M.F., Lau,C.S., Lau,Y.L., Wong,W.M., Cheng,C.C., and Lai,C.L. (1999). Mannose binding lectin gene mutations are associated with progression of liver disease in chronic hepatitis B infection. Hepatology *29*, 1248-1251.

Zhang,H., Zhou,G., Zhi,L., Yang,H., Zhai,Y., Dong,X., Zhang,X., Gao,X., Zhu,Y., and He,F. (2005). Association between mannose-binding lectin gene polymorphisms and susceptibility to severe acute respiratory syndrome coronavirus infection. J. Infect. Dis. *192*, 1355-1361.

Zundel,S., Cseh,S., Lacroix,M., Dahl,M.R., Matsushita,M., Andrieu,J.P., Schwaeble,W.J., Jensenius,J.C., Fujita,T., Arlaud,G.J., and Thielens,N.M. (2004). Characterization of recombinant mannan-binding lectin-associated serine protease (MASP)-3 suggests an activation mechanism different from that of MASP-1 and MASP-2. J. Immunol. *172*, 4342-4350.