MBL and L-ficolin

levels and genotypes in community-acquired pneumonia

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MBL en L-ficolin

spiegels en genotypen bij thuis opgelopen longontsteking

(met een samenvatting in het Nederlands)

Proefschrift

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CONTENTS

GLOSSARY

Adaptive immunity	Those cells, molecules and mechanisms of the immune system that act on danger signals in an antigen-specific manner. The adaptive immune system recognizes pathogens through specific antigenic epitopes associated with a pathogen. Responses in adaptive immunity are shaped over time by repeated challenge, becoming more targeted and they posses mechanisms of memory of earlier immune responses by memory cells. It is the counterpart of innate immunity.
Allele	A particular DNA sequence of a gene. When a gene is polymorphic, the genetic variants are called alleles.
Complement	A system of more than 30 fluid phase and cell-associated components, which act in a cascade-like network that is involved in opsonization and lysis of microbes, cellular recruitment and activation, and immune clearance of immune complexes. The complement system is part of the innate immune system and can be triggered by components of both innate and adaptive immunity.
Ficolins	A group of proteins that share the structure of a collagenous triple helix and a fibrinogen-like C-terminal domain. They are found in a wide range of species. Human ficolins L-ficolin, H-ficolin and M-ficolin resemble MBL in structure and function.
FCN2	The gene encoding human L-ficolin.
Genotype	The genetic constitution. Since humans are diploid and carry two copies of each gene on two homologous chromosomes, the genotype refers to the combination of two alleles or haplotypes.
Haplotype	A combination of co-occurring SNPs that are located at different sites on a gene. Haplotypes represent the fact that genetic variations at different sites of a gene are linked because they are situated on the same DNA fragment.
Heterozygous	Carrying different alleles of a gene on the two homologous chromosomes.
Homozygous	Carrying identical alleles of a gene on both homologous chromosomes.
Innate immunity	Those cells, molecules and mechanisms of the immune system that act on danger signals in a non-specific, generic manner. The innate immune system recognizes pathogens through evolutionary conserved general patterns associated with these pathogens. Responses in innate immunity are fixed, as they are not further shaped by repeated challenge and they do not posses mechanisms of memory of earlier immune responses. It is the counterpart of adaptive immunity.

MASP	MBL-associated serine protease. MASPs bind to MBL and ficolins. With their serine protease domains, MASP-2 and MASP-1 enzymatically cleave complement components C4 and C2, setting of the complement cascade. MASP-3 and the splicing variants Map44 and Map19 do not show this enzymatic activity and are thought to have a regulatory role.
MBL	Mannose-binding lectin. MBL is a multimeric pattern recognition receptor circulating in plasma that binds to invading pathogens and activates the complement system. Proteins similar to MBL are found in a wide range of species.
MBL2	The gene encoding MBL.
Multimeric	Consisting of multiple subunits, called monomers. Higher order multimers consist of many monomeric subunits, while lower order multimers or oligomers consist of a few.
Polymorphism	The occurrence of more than one allele of a gene within a population, representing genetic diversity in a population.
SNP	Single nucleotide polymorphism (SNP, pronounced as “snip”). A DNA sequence variation consisting of only one variant nucleotide.

1
General introduction

General introduction

Lung immune defenses

To resist the constant challenge of potentially infectious agents, the lungs posses innate and adaptive immune defense mechanisms that prevent colonization and clear invading pathogens (Figure 1).[1] Structural defenses form the first barrier for *invading* pathogens. The anatomical design of the nose causes such an airflow that large particles (>5µm) are caught on the nasal mucosa before entering the lower airways. Smaller particles can be trapped in the mucus overlying the respiratory epithelium. By the coordinated movement of the underlying cilia, mucus and particles are removed mechanically by forcing them towards the mouth. The cough and sneeze reflexes also help to remove depositions from the airways.[2]

If pathogens succeed in passing through physical and mechanical barriers, the innate immune system is called upon. Soluble antimicrobial molecules including the various components of the complement system, alveolar macrophages, neutrophils, natural killer cells and dendritic cells are active in the first minutes of colonization and invasion. They restrain infection by their intrinsic antimicrobial activity and the regulation of inflammation and activation of the adaptive immune response.

Soluble enzymes like lysozyme and peroxidase posses antibacterial activity. Defensins are secreted by epithelial cells and neutrophils and posses activity against Gram-positive and Gram-negative bacteria, mycobacteria, fungi and viruses.[3] They bind to the microbial cell membrane, forming pores that lead to lysis of the cells. Pentraxins such as C-reactive protein (CRP), serum amyloid P and PTX3 bind to microbial structures, thus opsonizing microbes for phagocytosis and complement mediated lysis.[4]

The complement system consists of more than 30 fluid phase and cell-associated components, which act in a cascade-like network that plays a pivotal role in innate immunity, as it is involved in opsonization and lysis of microbes, cellular recruitment and activation, and immune clearance of immune complexes. The complement system can be triggered by components of innate and adaptive immunity.[5,6]

The most well-known activator of complement in innate immunity is mannose-binding lectin (MBL), a plasma protein belonging to the collectin family. Collectins are oligomeric proteins with a collagenous region and C-type lectin domains, capable of binding saccharide moieties on microbial surfaces.[7] The collectins surfactant proteins A and D are found in high concentrations in the alveoli of the lungs, functioning as opsonins and agglutinins of pathogens in the lower airways. MBL not only opsonizes microbes for phagocytosis, but subsequently can activate the complement system as well. The ficolins share some structural and functional properties with MBL. Instead of recognizing microbial structures through a C-type lectin domain, they possess a fibrinogen-like domain binding to acetylated moieties also found on microbes.[8,9] Binding of ficolins leads to opsonization and complement activation as well.[10] The complement system, as well as its activators MBL and the ficolins, will be discussed in more detail below.

Besides soluble components, the innate immune system also encompasses cellular elements. In the lungs, alveolar macrophages and neutrophils can kill invading microbes via phagocytosis and subsequent production of reactive oxygen species and lytic

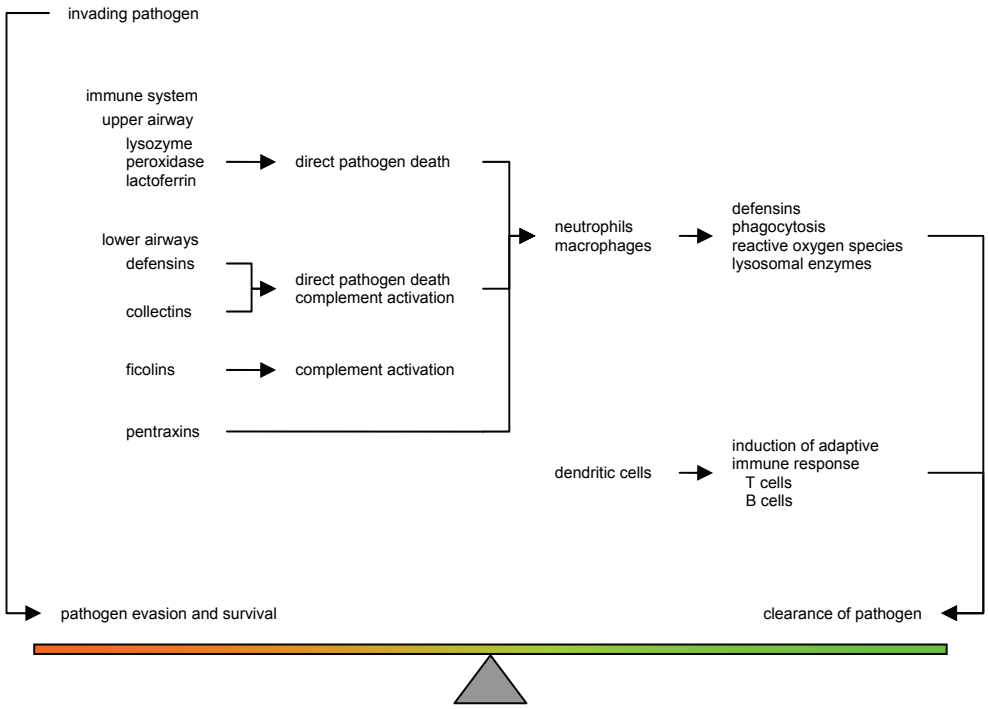

Figure 1 | ***Pulmonary defences against invading pathogens***
The lungs harbour innate and adaptive immune mechanisms to clear invading pathogens. Ultimately, the outcome is determined by the inoculum and virulence of the pathogen and the effectiveness of the immune responses, either leading to survival and spreading or to clearance of the pathogen.

enzymes.[11] Natural killer (NK) cells screen for infected lung cells and can induce killing of these cells by release of perforins and granzymes.[12] The current view is that the main function of perforins is to allow endocytosis of granzymes, which induce apoptosis of the target cell.[13]

The recognition of pathogens by the innate immune system involves the interaction between pathogen-associated molecular patterns (PAMPs) and pattern recognition receptors expressed on mammalian cells. The saccharide moieties recognized by the collectins are an example of such PAMPs. Toll-like receptors are a family of pattern recognition receptors, recognizing a broad scala of extracellular PAMP's such as lipopolysaccharide, lipoteichoic acid and peptidoglycan, and intracellular PAMP's such as viral double-stranded or single stranded RNA and bacterial DNA.[14] Membrane-bound TLRs on innate immune cells initiate an intracellular activation cascade after binding a specific PAMP, ultimately leading to translocation of NF-κB to the nucleus, that initiates the transcription of proinflammatory cytokine genes.[14]

TLRs are expressed on a variety of cells in the lung compartment but especially on dendritic cells. Lung dendritic cells play a role as antigen-presenting cells in immunity, autoimmunity and allergy.[15,16] They can be divided into two types: conventional dendritic

cells (cDCs) and plasmacytoid dendritic cells (pDCs).[17,18] cDCs are the main antigen-presenting cells, providing the link between the innate and adaptive immune response. When they arrive in the lungs, they mainly express receptors associated with antigen uptake.[17] cDCs sample the environment, not only in the lung parenchyma, but also in the alveolar space, as these cells are capable of migrating back and forth over the alveolar epithelium.[19] After antigen uptake and activation by TLR stimulation or proinflammatory cytokines, cDCs mature to function as antigen-presenting cells, expressing less peripheral tissue homing receptors (CCR1, CCR5, CCR6) and more receptors regulating the migration towards lymph nodes (CCR7).[17,20] In the lymph nodes the processed antigens are presented to T cells. cDCs also can modulate ongoing immune responses and recruit inflammatory cells towards the lungs.[18,21] pDCs are less important as antigen-presenting cells, but play a role in the antiviral response by producing type 1 cytokines.[17]

Adaptive immune responses of the lung build upon these innate immune responses. They are guided by innate immunity and simultaneously shape innate responses as they develop. The key concepts of adaptive immunity are specificity and memory, providing an antigen-specific immune response that increases in efficiency with every renewed contact.

Several subsets of T lymphocytes play a role in the pulmonary adaptive immune response. The most common type expresses a T cell receptor (TCR) with αβ variable chains. This type of T lymphocyte also makes up for 65-70% of peripheral circulating lymphocytes and expresses the highest diversity in TCR repertoire.[22] Immature, TCR-negative precursors of αβ T lymphocytes migrate to the thymus, guided by P-selectin and CXCL12.[23,24] Under the influence of chemokines expressed in the microenvironments of the thymus, they rearrange and subsequently express the TCR. The T lymphocytes undergo positive selection to ensure interaction with the major histocompatibility complex (MHC) to be able to interact with 'self' MHC molecules, and subsequently negative selection to prevent too strong interactions leading to autoreactivity.[25] Early during T lymphocyte development the cells express both CD4 and CD8 molecules (double positive). In a later stage of development, CD4 is lost on part of the cells which then become single positive for CD8. CD8 can interact with the constant region of MHC class I molecules and thus stabilize the interaction between TCR and peptides expressed in MHC class I. The cells that develop into single CD4 positive T lymphocytes interact with peptides presented in MHC class II.

The T lymphocytes leave the thymus as naive $CD8^+$ cytotoxic T lymphocytes or naive $CD4^+$ helper T lymphocytes, homing for lymph nodes by the expression of L-selectin and CCR-7 and "waiting" for dendritic cells to carry the right epitope expressed on their MHC and stimulate them towards their first active function in immunity.[25,26] This primary immune response of naive T lymphocytes therefore takes place in organized lymphoid tissue. Bronchus-associated lymphoid tissue (BALT) allows for an efficient priming process to take place in the lungs. It facilitates specific pulmonary immune surveillance and immune responses, as the combination of adhesion molecules needed for lymphocyte entry into BALT (activating L-selectin/PNAd, α4β1 integrin/VCAM-1 and LFA-1 pathways) are unique, supporting tissue-specific homing to the lungs.[27] Once activated, naive T lymphocytes develop into effector T lymphocytes. Effector αβ T lymphocytes express adhesion receptors that permit them to enter specifically into inflammatory sites.[28] There, they need far less stimuli to become activated than their naive counterparts and express their effector functions of cytolysis and cytokine release to regulate immune responses.[29] T lymphocytes that express γδ variable chains express a much more limited TCR repertoire, presumably recognizing auto-antigens expressed during stress and pathogen-derived

isoprenylpyrophosphates.[30] Although they only account for 1-5% of circulating lymphocytes, they are more common in the gut mucosa and the airway epithelium, presumably playing a role in host defense in early infection and in immunoregulation.[31] When inflammation resolves, T cell memory is maintained in the absence of antigenic stimuli by homeostatic proliferation of matured cells, driven by IL-15 for memory $CD8^+$ cytotoxic T lymphocytes and IL-7 for $CD4^+$ helper T lymphocytes.[32]
The development of B lymphocyte mediated antibody responses to protein antigens is orchestrated by $CD4^+$ helper T lymphocytes (Th). The interaction between activated Th and naive B lymphocytes leads to maturation into short-lived plasma cells that produce antibodies and the formation of germinal centers in secondary lymphoid tissue.[33] In the latter compartment, B lymphocytes further diversify and variants with high-affinity antibodies are selected to become memory B lymphocytes or long-lived plasma cells, both representing B cell memory. Besides their role in acute inflammation, B lymphocytes also play an important role in the prevention of infection by continuous production of mucosal IgA, which is driven by continuous stimulation of B lymphocytes by dendritic cells and alveolar macrophages continuously sensing antigen and PAMP's in the alveolar space.[34] The secretory IgA is transported via the polymeric Ig receptor (pIgR) of the epithelial cells to the apical surface.[35] There, it is anchored to the mucus lining the airway epithelium by the secretory component of pIgR, capable of binding pathogens and neutralizing them within intracellular vesicular compartments of epithelial cells.[36]
The memory function of adaptive immunity ensures a fast secondary response of pulmonary T and B lymphocytes upon antigen rechallenge. Functional pulmonary dendritic cells, B and T lymphocytes can persist for months after infection, ensuring a growing local immunological memory and accelerated future immune responses.[37,38]

Complement

Over 30 proteins and glycoproteins, mainly produced by hepatocytes, constitute the complement system in which one protein interacts with another one, leading to a process of sequential activation called the complement cascade. The sequence of activation begins with pattern recognition of non-self (e.g., PAMPs) and altered self (e.g., epitopes expressed as a result of apoptosis or necrosis) and ends with direct lysis, opsonization and cellular recruitment and activation, leading to inflammation and clearance of the recognized elements.[6,39]

Routes of complement activation

In humans, there are three pathways of complement activation, triggered either by antigen-specific antibodies from adaptive immunity or pattern recognition receptors from the innate counterpart (Figure 2). The so-called classical pathway starts with the binding of antibodies to their antigens, forming immune complexes. The C1qrs complex of complement docks onto the immune complex via the C1q component. This triggers the C1r and C1s components to perform the first enzymatic reaction in the cascade: the cleavage of C4 and C2. The cleavage products form the C4b2a enzyme complex, which in turn acts as a C3 convertase to form C3b. The C3b molecule is the initiator of the downstream pathway of the complement activation route leading to the production of anaphylatoxins C3a and C5a, and the formation of the membrane attack complex C5-C9

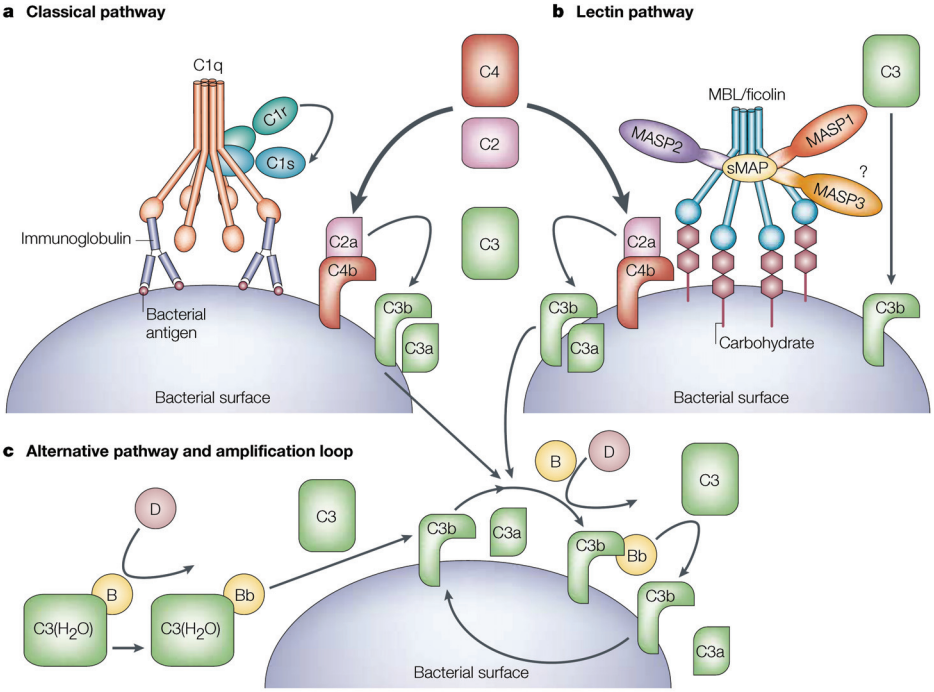

Figure 2 | ***The three routes of complement activation***

The activation routes of the classical (a), lectin (b), and alternative pathway (c) are depicted. They are triggered by antibodies bound to surface antigen, pathogen-associated patterns, or membrane-bound C3b, respectively. All three pathways lead to C3 activation, which triggers the final common pathway: the formation of the C5-C9 membrane attack complex (Fujita et al.*).*[54]

lysing the target cell. [6,39,40] This final downstream pathway is common to all three activation routes.

The alternative pathway is initiated by the deposition of trace amounts of C3b, arising from continuous low-grade hydrolysis of C3 in plasma, on all adjacent cell surfaces by covalent binding to hydroxyl groups. The membrane-bound C3b binds factor B, and the subsequent C3bB complex is activated by factor D to form C3bBb, which is the C3 convertase of the alternative pathway.[6,39] Properdin plays a central role in alternative pathway activation. This multimeric protein binds to the target surface either directly or via C3b with one subunit, serves as a surface scaffold for deposition of other C3b, C3bB and C3bBb molecules with the other subunits, promotes C3b interactions with factor B and stabilizes the ultimately produced C3 convertase C3bBb.[41] Besides a direct activation route of the complement cascade, the alternative pathway can also serve as an amplification loop once C3b is formed initially via the other two routes.[6,39]

The lectin pathway of complement activation was the most recent to be unraveled. An antibody-independent defect of yeast opsonization was first described in two families with recurrent infections.[42] This defect was further shown to occur in about 5% of the healthy adult population but was associated with unexplained frequent infections, diarrhea and atopy in small children.[43,44] The opsonic defect could be repleted by addition of normal

plasma both *in vitro* and *in vivo*, showing it was not an inhibitor.[42,43] Later, mannose-binding lectin (MBL) was identified as the defective opsonic protein.[45] MBL turned out to be a pattern recognition receptor resembling C1q, but capable of binding directly to pathogens without the need for antibodies.[46] Similar to C1r and C1s activity in the classical pathway, the MBL-associated serine proteases (MASPs) are triggered to cleave C4 and C2 after binding of MBL to mannan residues, leading to the generation of the C3 convertase C4b2a and thereby setting off the complement cascade.[46] The lectin pathway was named after MBL, the first known antibody-independent, complement activating pattern recognition receptor.

Later, the ficolins were to be discovered, originally identified as transforming growth factor-β1 binding proteins on pig uterus membranes with collagen- and fibrinogen-like domains.[47] In humans, ficolins have been identified in different settings and given different names initially: elastin-binding protein EPB-37, corticosteroid-binding protein hucolin and the molecularly cloned plasma lectin P35 all turned out to be the same protein, later designated L-ficolin because it is mainly produced in the liver.[48-50] M-ficolin was identified by cloning of oligonucleotides encoding a similar protein as L-ficolin and was therefore originally named P35-related protein.[51] As it was mainly found on monocytes, it was renamed to M-ficolin. H-ficolin was originally called Hakata-atigen, after the city where it was discovered to be an antigen which was recognized by autoantibodies in patients with systemic lupus erythematosus.[52] Later, it turned out to share its structure with the ficolin family. Ficolins were shown to activate the lectin pathway via the same serine proteases as MBL.[53] Since their discovery, ficolins have been identified in many species, including humans, rodents, pigs, hedgehogs and ascidians.[54]

Evolution of the complement system

The complement system is highly conserved among species during evolution (Figure 3). Although complement components are not interchangeable *in vitro* between species, complement functionality and its components show a high degree of resemblance. C3 and factor B analogues have been identified in a wide range of vertebrates and invertebrates, up to the sea urchin, suggesting that the central part of the complement system originated 1 billion years ago.[55] Furthermore, complement also induces opsonophagocytosis by ascidian haemocytes via a C3-receptor analogue.[56] MBL-like lectins, ficolins, associated serine proteases and lectin pathway activity have been demonstrated in ascidians, suggesting that the lectin pathway successfully defended invertebrates and vertebrates already early during evolution, before the development of adaptive immunity in jawed vertebrates.[57] Sharks and higher vertebrates have complement systems containing all three activation routes also known in man.[57] The molecular patterns recognized by the different complement systems are in their turn highly conserved between pathogens, suggesting this is the constraining force behind complement evolution.

Complement and inflammation

The complement system functions as a bridge between innate and adaptive immunity in infection. The binding of C1q to antibodies opsonizing pathogens links innate effector functions to adaptive sentinel receptors. B cells are triggered more easily by the specific antigen binding their B cell receptors in the presence of activated complement components, especially via the complement receptor type 2 (CD21), linking the activation of adaptive immunity to soluble innate receptor molecules.[58] Toll-like receptors (TLRs) are

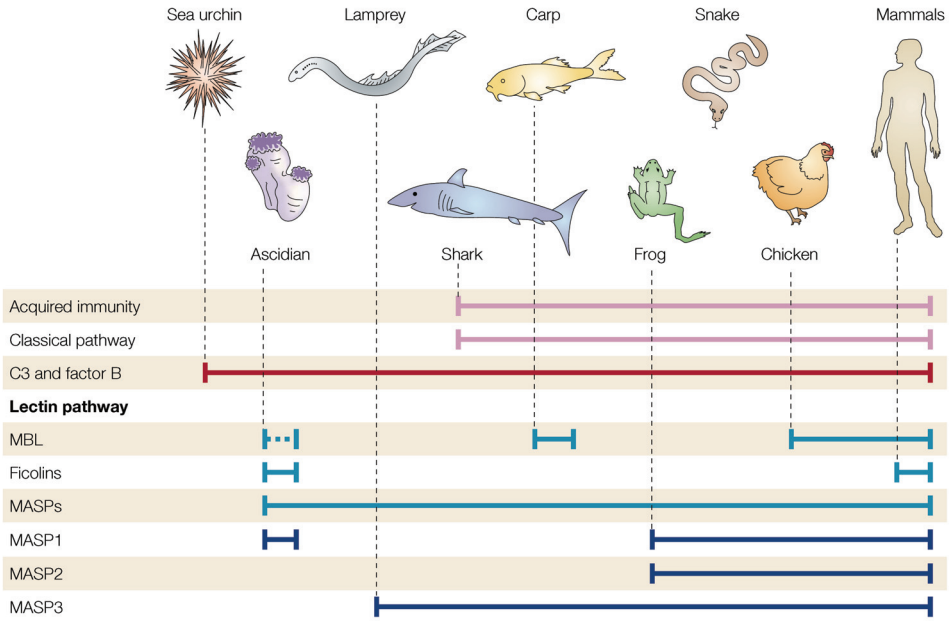

Figure 3 | ***Complement system components in different species***
The presence of complement components in a wide range of species suggests that the complement system has originated approximately 1 billion years ago, well before adaptive immune systems, and has been conserved during evolution (Fujita et al.*).*[54]

the major membrane-bound pattern recognition receptors bridging innate and adaptive immunity. Recently, a role for MBL as a co-receptor for TLRs in the phagosome was reported. In an *in vitro* model, MBL bound to lipoteichoic acid mediated engulfment of *S. aureus* into phagosomes, where it complexed with TLR2 for enhanced ligand delivery and modified the signaling of the TLR2/6 heterodimer for a proinflammatory response, thereby suggesting a role for opsonins in the spatial coordination and amplification of other innate defenses.[59]
Besides an inflammatory function in infection, complement also has anti-inflammatory effects in the sense of clearing structures capable of evoking ongoing inflammation. Immune complexes are tagged by C1q for uptake and removal by macrophages in spleen and liver.[60] Patterns of 'altered self' are recognized in the case of apoptosis and necrosis.[61,62] Fragments of apoptotic cells are recognized by classical pathway components either directly or via pentameric C-reactive protein (CRP), and their clearance by macrophages is complement dependent.[63-66] Tissue injury and necrosis after oxidative stress exposes molecular structures such as phospholipids and mitochondrial proteins capable of activating complement directly via C1q and MBL,[67,68] or indirectly via natural antibodies or pentameric CRP.[69,70]

Regulation of complement activity
Together with the self-amplifying, almost explosive nature of the complement cascade, its dual role of pro- and anti-inflammatory mechanism in inflammation requires tight

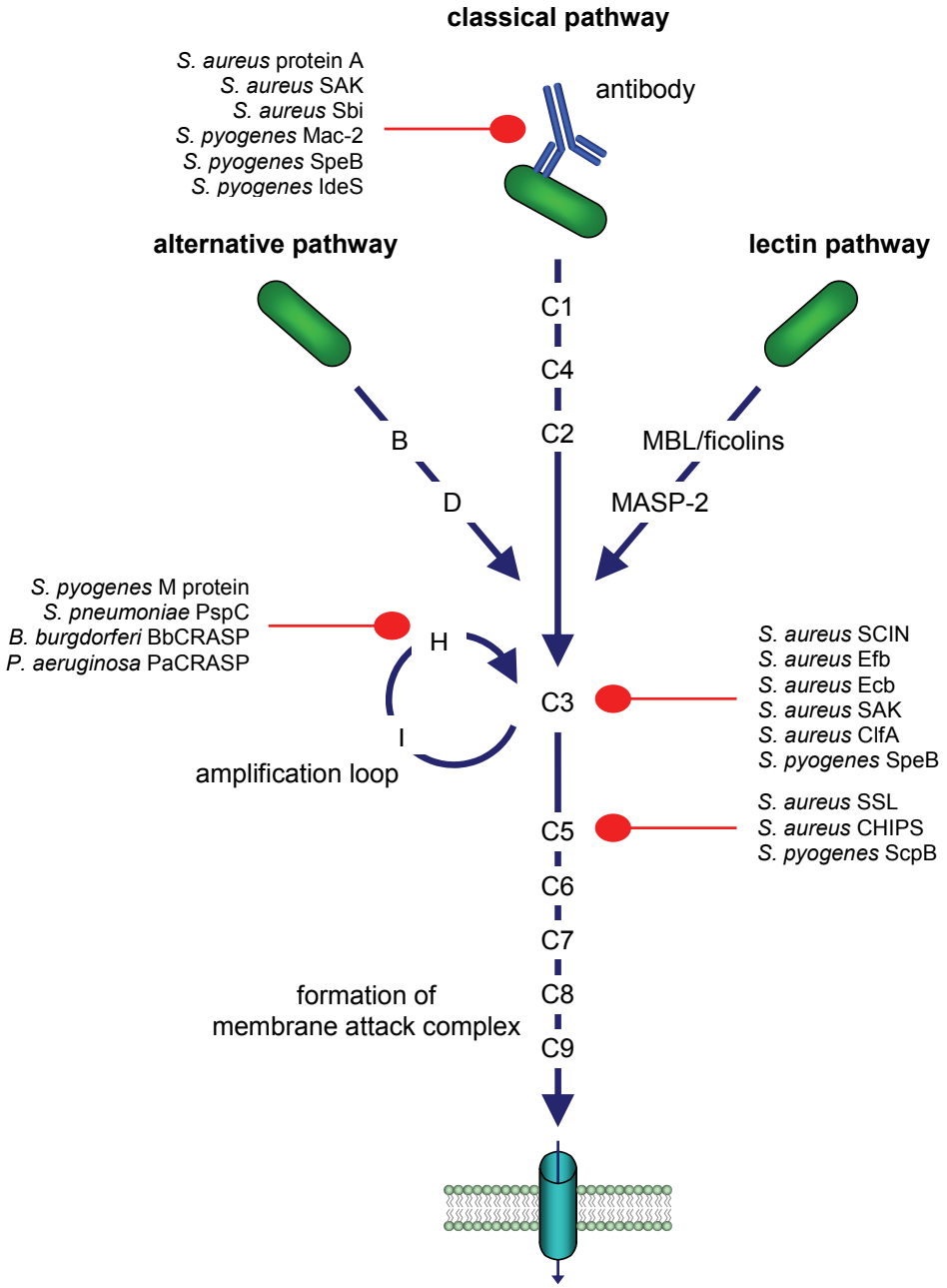

Figure 4 | ***Bacterial inhibitors of complement***

Bacterial peptides can interact with complement on various levels of the cascade to inhibit or regulate complement activity to enhance bacterial virulence. To date, the best described interactions occur at the level of IgG (inactivation and interference with Fc-receptor binding), C3 (direct cleavage, C3 convertase modulation and modulation of host regulatory proteins) and C5 (direct cleavage and interference with receptor binding) (modified from Rijkers et al. *and Laarman* et al.*).*[90,91]

regulation of the activity of the complement system itself to constrain its action to where it is needed and prevent unwanted damage to healthy tissues.[71]
On the surface of normal host cells, C3 mediated complement activation is inhibited by the simultaneous presence of complement control proteins. On surfaces lacking these inhibitory molecules, e.g. pathogen surfaces, apoptotic host cells expressing 'altered self' surface structures, and the glomerular basement membrane, the amplification loop of C3b deposition proceeds unabated.[72]
Most importantly, the amplification loop starting with C3b is inhibited by the binding of factor H and subsequent inactivation to iC3b by factor I.[39] Factor H competes with factor D for binding of C3b in a balance of inhibition and further activation of the complement system. Polyanions like sialic acid present on host cells favor factor H binding, thus inhibiting all down-stream complement pathways on normal host tissues.[72,73] Membrane-bound proteins like complement receptor type I (CD35) and membrane cofactor protein (CD46) also contribute in inactivating C3band thereby can protect cells from inadvertent damage by complement.[39] Classical pathway activation is restrained by C1 inhibitor which acts directly by inactivating C1s and C1r.[71] Apoptotic and necrotic cells lack this expression of complement inhibitors, enabling complement to tag 'altered self' for clearance by macrophages.[5] However, excessive complement activation is prevented by the simultaneous binding of the inhibitors C4 binding protein (C4BP) and factor H on most endogenous targets.[74,75] Surface-bound, monomeric CRP can facilitate this regulatory mechanism via its interactions with factor H and C4BP, resulting in inhibiting of cytolysis via all three complement pathways.[76-78] However, like pentameric CRP, monomeric CRP also binds and activates C1. The net result is the activation of the classical pathway up to C3 cleavage, without significant activation of the terminal lytic pathway.[79]
Dysregulation of complement activation causes inflammatory collateral damage and plays an important role in the development of autoimmunity. When immune complexes cannot be cleared, the binding of C1q sets off chronic activation of complement and excessive inflammation, leading to destruction of the basement membrane in the case of immune complex glomerulonephritis.[5] In autoimmunity, complement shows its two-sided face. Swift clearance of apoptotic and necrotic fragments via complement is thought to prevent the development of autoimmunity, as autoantigens are cleared before an adaptive immune response can develop against the accumulated endogenous antigens, for example in systemic lupus erythematosus (SLE).[5,80] However, complement can also aggravate autoimmune pathology as it is an important contributor to tissue injury, also in SLE.[81]

Pathogen evasion of the complement system

Many pathogens use or evade the complement system to enhance their virulence. Viruses and intracellular bacteria can enter a target cell directly via complement receptors and cell-bound regulatory proteins.[82] HIV and mycobacteria activate complement, leaving them coated with C3b and then enter their host cell via C3b receptors.[83] EBV enters B lymphocytes by binding to complement receptor 2.[84]
Protection against complement activity can be achieved by physical barriers like capsules impermeable to the membrane attack complex (e.g., *Streptococcus pneumoniae* and other polysaccharide encapsulated bacteria), binding of endogenous inhibitors like factor H by *S. pyogenes* and CD55 and CD59 by HIV,[85,86] expressing homologues to endogenous inhibitors like the vaccine virus complement-control protein[87] and producing non-homologous inhibitors like the chemotaxis inhibitory protein and staphylococcal

complement inhibitor of *S. aureus*.[88,89] In fact, more and more bacterial inhibitors of complement are found, indicating that complement evasion is a widespread virulence strategy (Figure 4).[90,91]

Complement deficiencies

Complement deficiencies can disrupt the fine balance between activation and inhibition, having impact on inflammatory processes in infection and autoimmunity.[92] Pyogenic infections with *H. influenzae* and *S. pneumoniae* occur in patients with deficiencies in the classical pathway and C3.[39] Deficiency of inhibitory molecules of the alternative amplification loop factor H and factor I leads to membranoproliferative glomerulonephritis.[93] Deficiencies in classical pathway components C1/C2/C4 are associated with SLE.[80] This seems paradoxical, as clinical exacerbations of SLE in C1q sufficient patients are characterized by the massive use of C1q.[80] It is therefore remarkable that C1q deficiency leads to a clinical syndrome with all features of SLE.

In patients with deficiencies in the membrane attack complex and in the MBL pathway, recurrent infections with *Neisseria meningitides* have been described, which is further discussed below.[94-96]

Mannose-binding lectin

Mannose-binding lectin (MBL) is a soluble pattern recognition receptor capable of activating complement. It is mainly synthesized in the liver and secreted into the circulation at a median concentration of 1.5 µg/ml and ranging from 5 ng/ml to 5 µg/ml.[97] It has also been detected at other sites, including middle ear fluid, synovial fluid during inflammation, amnionic fluid and nasopharyngeal secretion.[98,99] At these sites, it is capable of recognizing non-self in the first minutes of infection, tagging pathogens for opsonophagocytosis and setting off the complement cascade to initiate lysis and inflammation.[54] All these characteristics of MBL functionality are influenced by its level as well as its structural properties, which in turn are shaped by transcriptional control and affected by genetic polymorphisms, as will be discussed below.

Structure and function of MBL

MBL is a multimeric protein composed of helical subunits containing three identical polypeptide chains of 25 kDa (Figure 5).[100,101] Each polypeptide chain contains four distinct regions: a cysteine-rich N-terminal domain, a collagenous domain, a short α-helical coiled-coil domain and the C-type carbohydrate recognition domain (CRD). The structure of a collagenous domain with a C-type CRD is shared with other members of the collectin family (see below).[102] Three polypeptide chains assemble through their collagenous domains into structural subunits, which in turn form higher order oligomers, ranging from dimers to hexamers (Figure 5b).[103]

The multimeric structure is stabilized by the hydrophobic properties of the collagenous region and the disulfide bonds between the cysteine-rich N-terminal domains of different polypeptide chains, giving rise to a bouquet-like structure, also referred to as a bundle of tulips (Figure 6).[101]

Several structural properties of the multimeric MBL protein are crucial for full functionality. First, the selectivity of carbohydrates recognized by the CRD is anchored

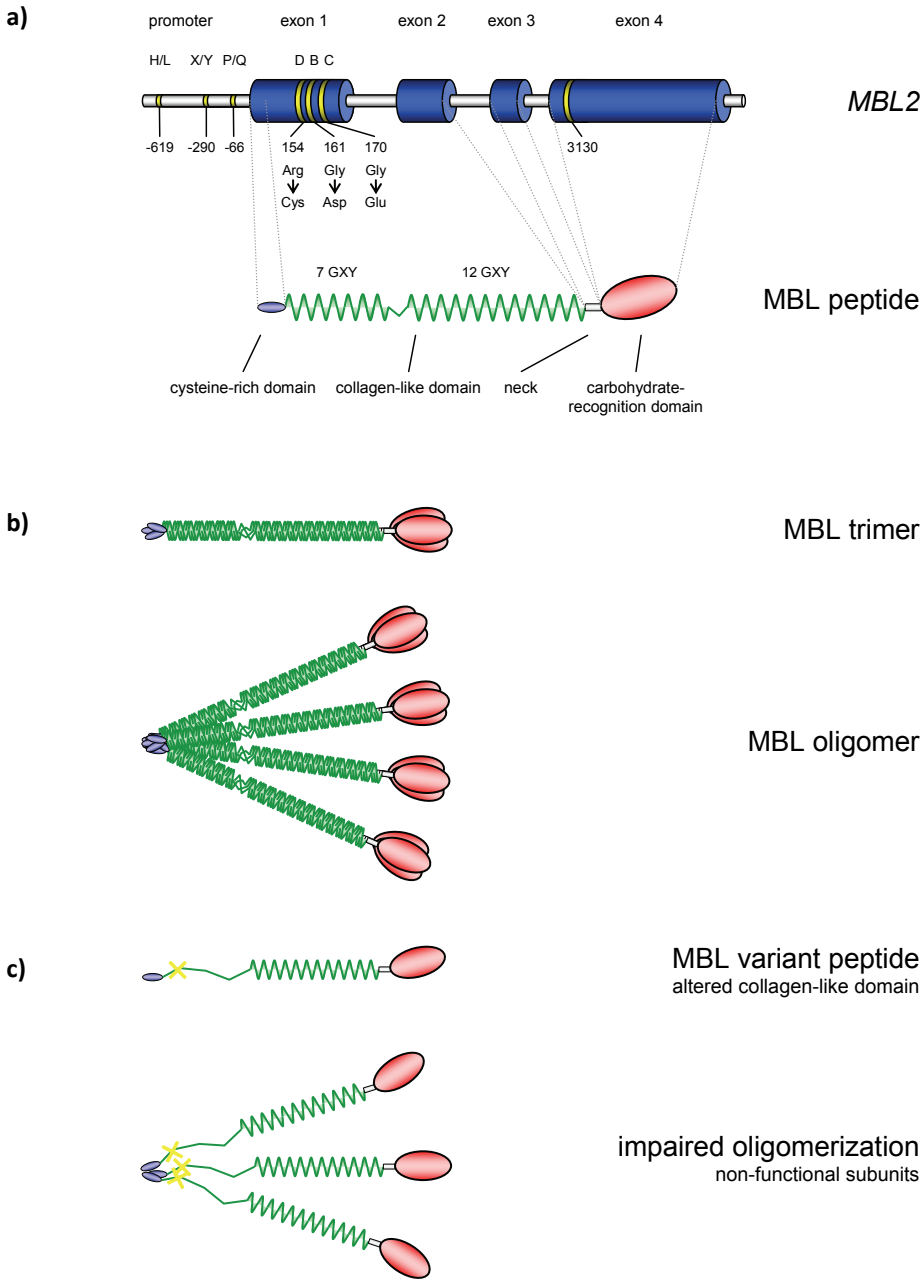

Figure 5 | ***The structure of MBL***

a) MBL2 *consists of 4 exons, encoding a cysteine-rich domain, a collagen-like domain, a neck and the carbohydrate recognition domain (CRD). SNP's in the promoter region, exon 1 and exon 4 influence the plasma MBL activity. b) In wild-type MBL ('A' allele), three polypeptide chains engage in a collagen helix to form trimers, which in turn form higher order oligomers. c) In variant peptides, resulting from one of three coding SNP's in exon 1, higher order oligomerization is impaired, leading to non-functional subunits having dramatically lower ligand binding avidity and complement activation properties than wild-type MBL.*

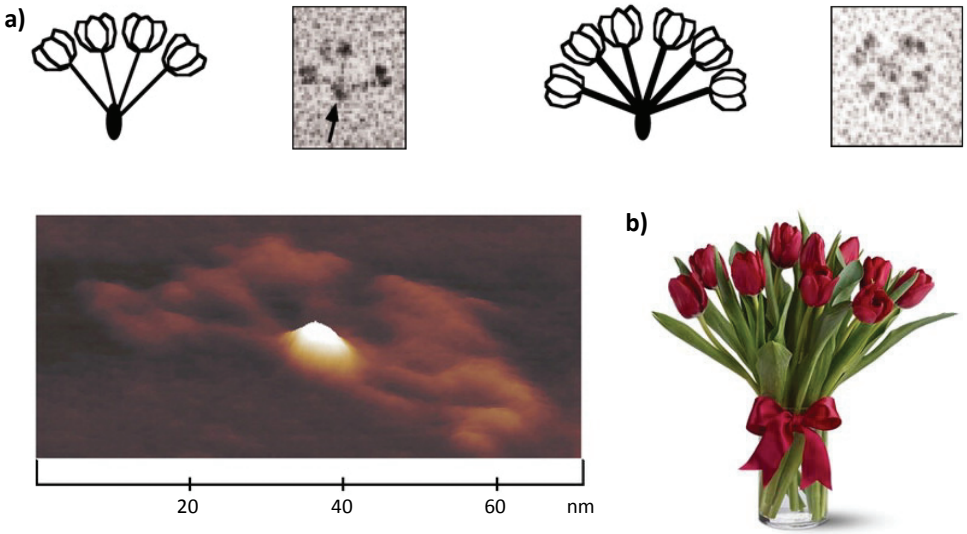

Figure 6 | ***MBL oligomers***
a) Electron microscopy and atomic force microscopy of two higher order MBL oligomers of four and six trimeric subunits (Holmskov et al.*).*[10] *b) A bundle of tulips.*

within the Glu-Pro-Asn amino acid sequence. This motif favors the calcium-dependent binding of the CRD specifically to sugar moieties with equatorial 3- and 4- hydroxyl groups in the pyranose ring such as D-mannose, glucose, L-fucose, N-acetyl-glucosamine (GlucNac) and N-acetyl-mannosamine.[104,105] All these ligands are abundantly present on a wide range of microorganisms, whereas most human carbohydrate structures are terminated by D-galactose or sialic acid. MBL does not bind to these latter structures, thus preventing lectin pathway activation on normal host cells.
Second, the multimeric structure of MBL provides the basis for high-avidity binding of pathogen-associated molecular patterns. The CRDs of the three polypeptide chains in a subunit are positioned at a fixed distance of 45 Å, matching the distance of repeated ligands on microbial surfaces. The affinity of a single CRD for its ligand is very weak, with a dissociation constant in the order of 10 mM.[106] However, the binding of multiple CRDs of one MBL subunit to a matching ligand pattern and the clustering of such subunits into a multimeric structure provides high avidity binding of MBL in the order of 2 nM.[106]
Third, the multimeric structure is also needed for efficient complement activation. Homodimers of the MBL-associated serine proteases (MASP-1, MASP-2, MASP-3, sMAp/MAp19 and MAp44) bind to the collagenous triple helix of multimeric MBL by their CUB1-EGF domains[107-110] and together they are present in plasma as MBL-MASP complexes.[111] After binding of MBL to its ligand, MASP-2 is activated by autocleavage, activating the serine protease domain, which in turn cleaves C4 and to a lesser extend C2 to form the C3 convertase C4bC2a and start the complement cascade (Figure 2).[112] MASP-1 is also capable of cleaving C2, thus complementing MASP-2 activity and enhancing C3 formation.[113] MASP-1 also has some C3 cleaving activity,[114] although the physiological relevance of this activity is questioned.[97] MASP-3 and MAp44 are two splice variants of the

MASP1 gene and MAp19 is a splice variant of the *MASP2* gene.[108-110] They do not show the serine protease activity in complement activation like their counterparts, either by having an alternative serine protease domain (MASP-3) or by lacking this domain completely (MAp19 and MAp44).[108,109,115] They were therefore suggested to play a regulatory role.[97] Indeed, MASP-3 significantly inhibited H-ficolin mediated C4 deposition and physiologically relevant competitive inhibition of complement was demonstrated for MAp44.[108,109,116] The structure and genetics of the different MASP molecules is depicted in figure 7.

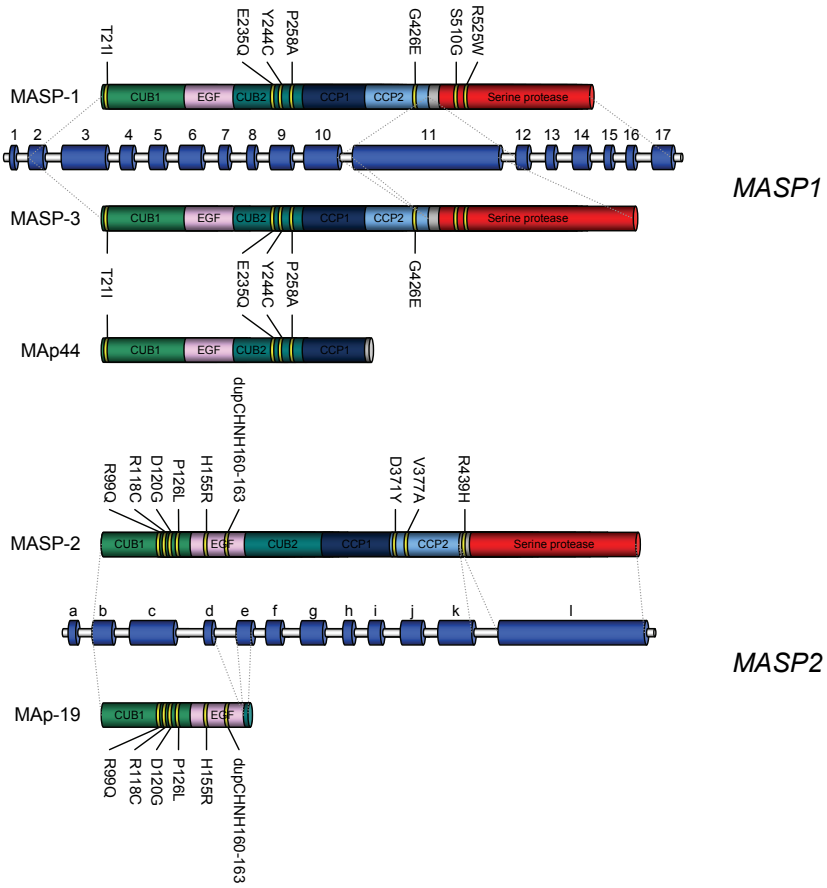

Figure 7 | ***Protein and gene structure of MASPs, MAp44 and MAp19***

The three MASPs, MAp19 and MAp44 are splicing variants of two MASP genes. The genes contain a CUB1 domain (an acronym of C1r/C1s, Uegf and Bone morphogenic protein-1, which are the proteins in which this domain was described originally), an EGF domain (Epidermal Growth Factor), a second CUB domain, two CCP domains (Complement Control Protein), an activation peptide and an SP domain (serine protease). Coding SNP's have been found in both MASP genes (indicated). MASP-1 and MASP-3 share an identical A-chain encoded by exons 1-10 of the MASP1 *gene on chromosome 3q27-28. Exon 11 encodes the SP domain of MASP-3, while exons 12-17 encode the SP domain of MASP-1. MAp44 is truncated after the first CCP domain, with 17 amino acid residues. The* MASP2 *gene on chromosome 1p36.2-3 has 12 exons. Exons 1-4 (originally named a-d) encode the N-terminal domain of both MASP-2 and MAp19. Exon 5 (e) encodes the C-terminal domain of Map19 and exons 6-12 (f-l) the SP domain of MASP-2.*

Gene structure and polymorphisms of *MBL2*

MBL is encoded by the four exons of the *MBL2* gene located on chromosome 10.[117] Exon 1 encodes the 5'-untranslated region (5'-UTR), the signal peptide, the cysteine-rich domain and the first seven copies of the collagenous glycine (GXY) motif (Figure 5a). The remaining part of the collagenous region is encoded by exon 2. Exon 3 continues with the neck region and exon 4, the largest exon, holds the sequence for the CRD. Next to the *MBL2* gene lies the *MBL1* gene. *MBL1* is a pseudogene, presumed to be arisen together with *MBL2* from gene duplication of a common ancestral gene.[118]

Sequencing of the complete *MBL2* gene in different American ethnic groups revealed a total of 87 polymorphic sites.[119] Single nucleotide polymorphisms (SNPs) in the promoter region and exon 1 of *MBL2* have a large impact on the level of circulating multimers of MBL.[120] Coding SNPs in exon 1 ('0' alleles D, B and C versus wild-type A allele) lead to non-functional MBL monomers and lower order oligomers in homozygotes, impairing early complement activation.[100,121] All three coding variants lead to an amino-acid substitution (codon 52 Arg/Cys, codon 54 Gly/Asp and codon 57 Gly/Glu for the D, B and C alleles, respectively) disrupting the collagenous triple helix and disabling the formation of higher order multimers.[100,121]

Three promoter variants affect the expression of the *MBL2* gene. The X/Y promoter SNP at position -290 influences MBL plasma levels in heterozygotes by controlling transcription of the functional A allele, and thus the balance between wild-type polypeptide chains capable of higher order oligomerization and incapable variant chains.[120] Although no consensus cut-off level for MBL-deficiency is established, genotypes 0/0 and XA/0 display MBL levels <0.2 mg/ml and are generally considered deficient.[120,122] Other promoter SNPs are found at positions -619 (H/L) and -66 (P/Q), but have a less pronounced effect on levels of functional MBL.[120,123] In children with otitis media, the 3130G>C SNP located in exon 4, was shown to significantly increase functional MBL levels in patients with the XA haplotype.[123]

Large differences exist between ethnic groups worldwide in *MBL2* haplotype frequencies (Table 1).[120,121] The promoter Y allele is most prevalent in Caucasians, Africans and Eskimo's. However, in Eskimo's the variant X allele is rare compared to the other ethnic groups. The exon 1 D allele is largely confined to Caucasian and northern East-African populations. The B allele is common in Caucasians, Asians and South-American Indians. It is present at a low frequency in northern East-Africans, while it is virtually absent in Sub-Saharan West-Africans. The C allele is almost restricted to Sub-Saharan West-Africans, with a low prevalence in Caucasians and absence in Asians and South-American Indians.

Since the promoter X allele is linked to the exon 1 A allele, the rare occurrence of the X allele is reflected in the low frequency of the XA haplotypes in Eskimo's, whereas this haplotype is fairly common in Caucasians and Africans (Table 1).[120] Although the occurrence of genetic MBL deficiency through homozygosity for exon 1 variants (genotype 0/0) is similar in Caucasians and Eskimo's, the frequency of functional MBL deficiency in Caucasians is expected to be higher, since the MBL-deficient genotype XA/0 is more prevalent in this population. In Africa, large differences between ethnic groups are observed in haplotype frequencies, representing the high genetic diversity of this continent. As a result, large differences in the prevalence of MBL-deficient genotype 0/0 are observed, ranging from 1 to 14%.[121]

The high frequency of variant *MBL2* haplotypes and the conservation of three different SNPs in exon 1 exerting the same influence on MBL functionality have led to the

Table 1 | MBL2 *allele frequencies in different ethnic populations*

The proportion of the alleles, haplotypes and genotypes of MBL2 *are given for different ethnic groups.*[120,121]

	***MBL2* allele frequencies**						***MBL2* haplotype**			***MBL2* genotype (%)**		
	promoter		**exon 1**									
Ethnic groups	**Y**	**X**	**A**	**B**	**C**	**D**	**YA**	**XA**	**Y0**	**A/A**	**A/0**	**0/0**
Europeans												
Danish Caucasians	0.76	0.24	0.79	0.12	0.03	0.06	0.56	0.24	0.20	60	36	4
British Caucasians			0.77	0.14	0.02	0.07				60	36	4
Sub-Saharan Africans												
East-Africans (Kenya)	0.73	0.27	0.68	0.03	0.24	0.05	0.47	0.23	0.30	51	35	14
West-Africans (Ghana)			0.68	0.004	0.32	0				47	43	10
San Bushmen (Namibia)			0.90	0.03	0.07	0				79	20	1
Xhosa (South Africa)			0.73	0	0.27	0				51	44	5
Asians												
Chinese (Hong Kong)			0.89	0.11	0	0				78	20	2
Japanese (Kyoto)			0.77	0.23	0	0				59	36	5
Australia and Oceania												
Papua (New Guinea)			0.99	0.01	0	0				97	3	NF
Aboriginals (Australia)			1.00	0	0	0				100	NF	NF
Americans												
Eskimos (East-Greenland)	0.97	0.03	0.88	0.12	0	0	0.88	0.03	0.09	78	18	4
Chiriguanos Amerindians (Argentina)			0.58	0.42	0	0				30	56	14
Quechua Amerindians (Peru)			0.20	0.80	0	0				7	28	65

hypothesis that the evolutionary distribution of MBL-deficiency has been shaped by balancing selection, where simultaneous positive and negative selective pressure favor heterozygous individuals.[124] Two studies that compared SNPs in *MBL2* and the flanking regions in various ethnic populations to examine this hypothesis have revealed conflicting results, either showing evidence of balancing selection,[119] or evidence of neutral selection, where genetic drift and human migration are the sole forces behind haplotype spreading.[125] However, in the latter study balancing selection was observed in other primates, suggesting that selective forces on *MBL2* haplotypes have diminished in importance only in human primates. The evolutionary pattern of genetic drift and shift that followed, could point to immunological redundancy, compensating for MBL-deficiency.[125]

Disease and pathogen associations with MBL deficiency

Ever since MBL-deficiency was associated with an opsonic defect in children with unexplained infection risk, diarrhea and atopy,[43,45] a high and still increasing number of clinical association studies has been performed. MBL deficiency was linked to both increased and decreased susceptibility to infectious and autoimmune diseases, and was identified as a disease modifying factor in such diseases.[126,127]

The wide range of pathogens recognized by MBL would suggest that deficiency caused an increased susceptibility to infections in general. However, MBL deficiency is one of the most common immunodeficiency states, with 5-10% of the population having MBL levels lower than 0.1 µg/ml. The majority of MBL-deficient individuals are apparently healthy and do not display an increased morbidity or mortality.[128,129] Besides the apparent random expansion of *MBL2* haplotypes described above, this too suggests that the redundant nature of the immune system compensates for MBL deficiency, for example via antibody-mediated classical pathway activation or phagocytosis.[130] Indeed, when other components of the immune system are lacking or impaired, the effect of MBL deficiency on susceptibility to infections is observed more clearly, e.g. in neutropenic patients and neonates with immature immunity.[131-135]

MBL-deficiency has also been associated with an increased susceptibility to infections with encapsulated bacteria. This association was found especially with meningococci.[94,95,136] MBL can bind strongly to meningococci, subsequently activate complement and increase their killing, which would explain the increased incidence of meningococcal infection when MBL is absent.[137] However, the outcome in neisserial meningitis is favorable in MBL-deficient patients, as the collateral damage of inflammation on surrounding tissues is less pronounced.[136,138] Circulating markers of complement activation (i.e., C3bc, terminal complement complex C5-C9, C3bBbP, C4bc, C3bBbP) and diffuse intravascular coagulation were lower in MBL-deficient patients, indicating that MBL is critical for the systemic activation of complement during meningococcal septic shock.[95]

In contrast, a recent study showed no effect of MBL *in vitro* on the serum bactericidal activity and opsonophagocytic activity by granulocytes on a *N. meningitidis* 44/76 strain, while a large effect of post-vaccination antibodies was observed.[139] It was hypothesized that a high degree of sialylation of the test strain reduced the binding of MBL, since sialic acids shield the target motifs for MBL.[140] During infection, MBL could still play a role, since *N. meningitidis* shows reduced sialylation by phase switching when becoming invasive.[141]

As another explanation, older studies already have shown that in order for MBL to mediate serum bactericidal activity *in vitro* against *N. gonorrhoeae*, the neisserial test

strain should be preincubated with MBL before adding the test serum.[142]

In pneumococcal disease, the association with the capsulated pathogen is more controversial and conflicting results have been published.[143-145] Kronborg and colleagues reported no differences in genotypical MBL deficiency between 140 patients with invasive pneumococcal disease and 250 healthy controls.[146] In this study, 66% of the patients had an identified underlying illness and this was associated with death in pneumococcal disease.[145,146] No effect of MBL genotypes on outcome was reported, but unfortunately no exact data on genotype groups and adjustment for other risk factors were described. Roy and colleagues found an associated of genotypical MBL deficiency in two different study cohorts with a total of 337 patients and 1032 controls.[144] Again, no effect on morbidity was reported, but no adjustment for comorbidity was performed. When data of both studies were combined, a small but significant effect of genetic MBL deficiency was observed on the risk of invasive pneumococcal disease (OR 2.3 [1.5-3.5], p<0.0001).[145] Perez and colleagues concluded that MBL genotypes were not associated with severity of pneumococcal pneumonia, but genotype A/A was more at risk of developing bacteremia.[147] However, this study did not investigate promoter polymorphisms and the analysis was done grouping genotype A/0 together with 0/0 as MBL deficient, thereby incorrectly classifying genotype YA/0 as MBL deficient. To address the question of MBL genotype influence on outcome in pneumococcal disease, Eisen and colleagues conducted a meta-analysis of four previous studies with a total of 186 patients with pneumococcal disease of whom data on comorbidity was available.[143] Genotypical MBL deficiency was shown to increase the risk of death among these patients in multivariate analysis (OR 5.62 [1.27–24.92], p=0.02).

Apparently, the disadvantages of MBL-deficiency described above must have been nullified by other evolutionary advantages, as three different point mutations in exon 1, all leading to MBL deficiency, have been conserved during evolution in different populations.[119] Suggested advantages are the decreased susceptibility to intracellular pathogens including several parasites like malaria, the diminished inflammatory response causing less collateral tissue damage and less progression towards certain autoimmune states.[119,148] From an evolutionary point of view, the impact of MBL deficiency on susceptibility for parasitic infections would be the most relevant.

Naturally occurring MBL deficiency in animals analogous to the human situation is not known. In primates, *MBL2* is a well conserved gene, with 99% homology in amino acid sequence of the protein between humans, chimpanzees and gorillas.[149] The few differences do not result in changes of structure or function of MBL. In higher order primates, no variants have been found analogous to the human variant '0' alleles, although only a few individuals per primate species were investigated.

However, mechanisms of gene silencing of the closely related *MBL1* pseudogene (*MBL1P1*) have been described in humans and higher order primates similar to human *MBL2* exon 1 polymorphisms (the '0' alleles).[150] Several glycine substitutions were found in the collagenous region of the putative MBL1 protein in man, chimpanzee, gorilla and orangutan. These substitutions are thought to disrupts the collagenous backbone of MBL1 analogous to the glycine substitution in codon 54 in human *MBL2*. These substitutions are thought to have arisen from specific, but independent events, as they are not the result of gene-conversion between *MBL1* and *MBL2* and they were not found in lower order primates. Although other, possibly more influential mechanisms of gene silencing (i.e. splicing variants and premature stop codons) have also been described for *MBL1P1*, these

data taken together suggest that evolutionary pressure has down-regulated MBL1 and MBL2 functionality by similar mechanisms in higher order primates.[121,149,150] Effects on pathophysiology of primate *MBL1* variants have not been described.
In other mammals, the two genes of MBL still exist and polymorphisms have been described that influence the level and functionality of the two MBL proteins. In mice, multiple SNPs were identified that alter the collagenous region and the carbohydrate recognition domain, presumably altering the multimeric structure and the ligand binding characteristics.[151] Polymorphisms in porcine MBL genes have been associated with decreased complement activity and a higher risk of infection.[152,153] Polymorphisms of *MBL1* in cattle breeds correlated with the risk of mastitis and milk production, suggesting it might be a candidate gene to screen for in breeding programs.[154]
In knock-out murine models, MBL deficiency was associated with an increased risk of infectious diseases, such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* infections, and with decreased clearance of apoptotic cells.[155] However, like in man, MBL deficiency also had protective effects, as it enhanced survival in a model of septic peritonitis and reduced the risk of reperfusion injury after cerebral ischaemia.[155] Further studies in animal models could assist in unravelling the role of MBL in pathophysiology and in understanding the existence of polymorphisms.

Ficolins

Ficolins are a group of proteins that share the structure of a collagenous triple helix and a fibrinogen-like C-terminal domain. They are found in a range of species, from invertebrate ascidians to humans (Table 2).[156] Three human ficolins are found in plasma (L-ficolin, H-ficolin and M-ficolin) and on leukocytes (M-ficolin). They resemble MBL in structure and function (Figure 8).[97,156,157] Like MBL, ficolins have a multimeric structure, are capable of binding patterns of acetyl groups found on microbes and exert MASP-dependent complement activation.[50,115,158-160]

L-ficolin

L-ficolin is mainly synthesized in the liver and is present in plasma in a concentration range of 1-13 µg/ml.[50,161] Next to abundant expression in liver, low expression of the L-ficolin *FCN2* mRNA was also found in the bone marrow, the intestine and the fetal lung.[162] The structural and functional resemblance between MBL and L-ficolin hints to a similar role for L-ficolin in infection as MBL.

Structure and function of L-ficolin

L-ficolin is a multimeric protein with strong resemblance to MBL in structure and function. Like MBL, it is composed of collagen-like helical subunits containing three identical polypeptide chains of 35 kDa (Figure 8).[50] Instead of the CRD found in MBL, a fibrinogen-like (FBG) domain is found at the C-terminal end of each ficolin polypeptide, separated from the collagenous stem by a short linker region. The subunits assemble into a higher order oligomer held together at the cysteine-rich N-terminal domains by cross-linking the subunits via disulfide bridges (Figure 9).[54] A tetrameric structure was proposed for ficolins by electron micrographs of pig ficolin (Figure 9b and c).[163] Later, the same tetrameric structure was found for L-ficolin.[164]

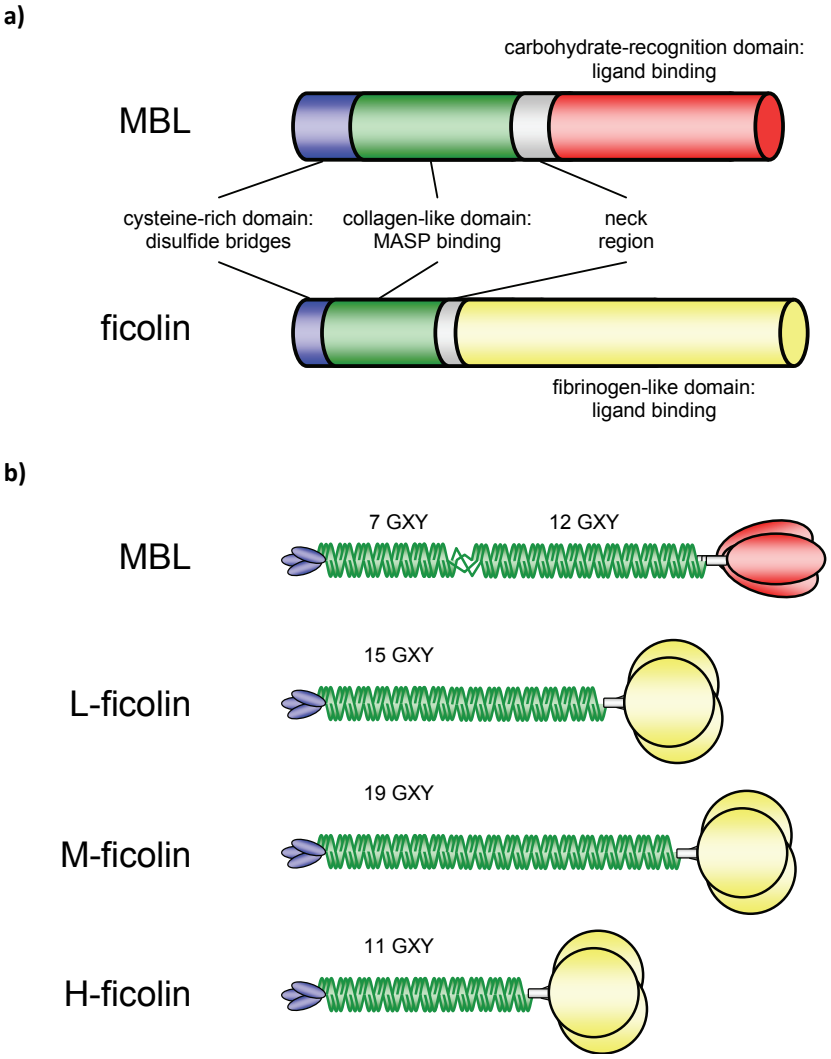

Figure 8 | ***Structural resemblance of MBL and ficolins***
a) MBL and ficolins share a similar protein structure, with the four domains having similar functionality. The major difference concerns the nature of the ligand binding domains: a carbohydrate-recognition domain (CRD) in MBL and a fibrinogen-like domain (FBG) in ficolins. b) Three polypeptide chains engage to form trimers through a collagen-like helix, which differs in length between the four proteins. The trimeric structural subunits end in three ligand-binding domains.

The FBG-domain is the ligand-binding domain specifying the binding selectivity of L-ficolin.[165] Acetyl groups are recognized on carbohydrates (e.g., GlucNAc, GalNAc) as well as non-carbohydrates (e.g., N-acetylglycine).[159,165] Microbes can be bound via surface acetyl groups or via lipoteichoic acid on *Staphylococcus aureus* and fungal 1,3 beta-D-glucan, but not mannose.[159,166,167] Crystal structure analysis of the FBG domain identified four distinct

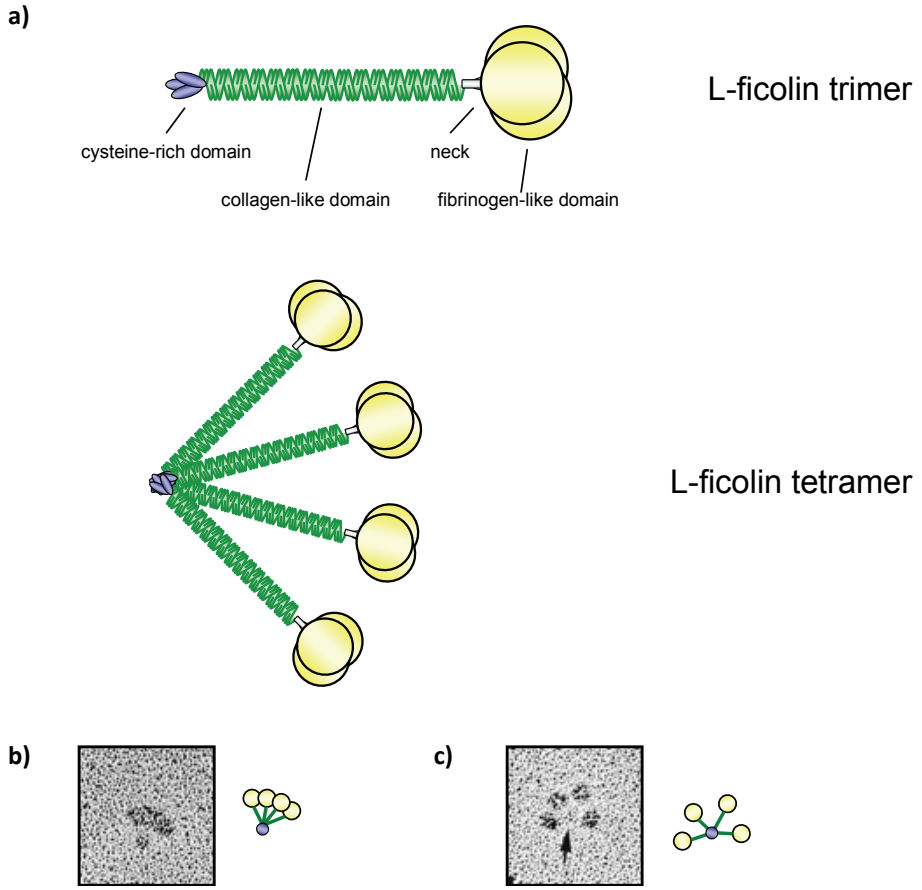

Figure 9 | ***Multimeric structure of L-ficolin***
a) Three L-ficolin polypeptides form a trimeric subunit harbouring three fibrinogen-like domains at the C-terminus. Four of these subunits organize in an L-ficolin tetramer, held together by disulfide bonds in the N-terminal cysteine-rich domains. The tetrameric structure was proposed by electron micrographs of rotary shadowed pig ficolin in a) closed and b) maximally disrupted states. The arrow indicates the central hub formed by the N-terminal domains (figures b) and c) modified from Ohashi et al.*).*[163]

binding sites of L-ficolin, while M-ficolin and H-ficolin only had one.[168-170] Therefore, it appears that the binding specificity of L-ficolin is broader than of the other two ficolins.[171] The multiple FBG domains in the oligomeric structure provide the basis for high avidity pattern recognition of the ligands described above. Complement activation by L-ficolin requires the same interaction between the collagenous region and MASPs as with MBL.[115,158,160]

Gene structure and polymorphisms of *FCN2*
L-ficolin is encoded by the *FCN2* gene on chromosome 9.[172] The *FCN2* gene consists of 8 exons (Figure 10), encoding a short 5'-UTR, the signal peptide and the first part of the

Species	Ficolins	mRNA expression	Protein identified	Binding substance	Function
Human	L-ficolin	liver	serum/plasma	GlcNAc (acetyl group); β-(1⟩3)-D -glucan; N-acetylneuraminic acid; lipoteichoic acid; C-reactive protein; fibrinogen; fibrin; DNA; elastin; corticosteroid	complement activation; opsonin
	H-ficolin	liver, lung, glioma cell	serum/plasma, bile duct, bronchus, alveolus	GlcNAc, GalNAc, fucose; poly-saccharides; lipopolysaccharide	complement activation; opsonin
	M-ficolin	lung, monocyte, spleen	plasma, monocyte, neutrophil, alveolar epithelial cell	GlcNAc, GalNAc; sialic acid	complement activation; phagocytic receptor
Mouse	Ficolin A	liver, spleen	serum/plasma	GlcNAc, GalNAc; elastin	complement activation
	Ficolin B	bone marrow, spleen	macrophage	GlcNAc, GalNAc; sialic acid	opsonin
Pig	Ficolin α	liver, lung, bone marrow	serum/plasma, uterus membranes	GlcNAc; lipopolysaccharide, lipoteichoic acid, elastin; TGF-β1	antiviral activity
	Ficolin β	neutrophil, bone marrow	neutrophil, uterus membranes	TGF-β1	ND
Hedgehog	Erinacin	ND	muscle	metalloprotease	antihemorrhagic activity
Xenopus	XeFCN1	liver, spleen, heart	serum	GlcNAc, GalNAc	ND
	XeFCN2	lung, spleen, leukocyte	NI	ND	ND
	XeFCN3	ND	NI	ND	ND
	XeFCN4	lung, spleen	NI	ND	ND
Ascidian	p40	hepatopancreas	hemolymph plasma	GlcNAc, GalNAc	ND
	p50	hepatopancreas	hemolymph plasma	GlcNAc	ND

Table 2 | ***Characteristics of ficolins of different species***

Ficolins are found in a wide range of species, from ascidians to higher vertebrates. They share their structure and carbohydrate-binding properties (modified from Matsushita et al.*).*[156]
(ND = not determined; NI = not identified)

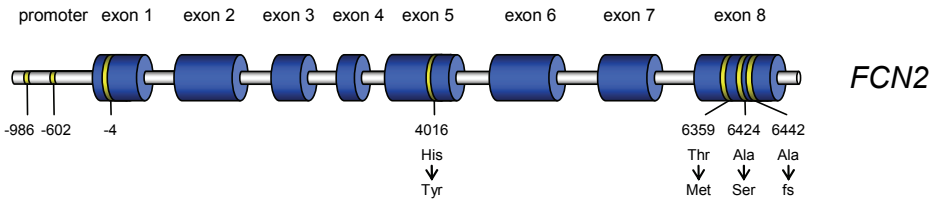

Figure 10 | ***Gene structure and SNP's of FCN2***
The FCN2 *gene consists of 8 exons, encoding a 313 amino-acid polypeptide chain, including a leader sequence of 25 amino-acids. Non-synonymous SNP's are indicated, as well as three SNP's in the promoter and 5'-UTR influencing L-ficolin plasma levels. The numbers indicate the position relative to the ATG start site. (fs = frame shift)*

cysteine-rich N-terminal domain (exon 1), the remainder of the N-terminal domain and the collagenous domain (exon 2-3), the linker region (exon 4) and the FBG domain followed by the 3'-UTR (exon 5-8). Three splicing variants are known, encoding a protein variant lacking part of the collagen-like domain or the FBG-like domain.[172] Whether this leads to non-functional protein *in vivo* is uncertain, as these translational products have not been detected yet. The similarities between *FCN2* and the M-ficolin gene *FCN1* in localization on chromosome 9, exon structure and sequence suggest that these genes have arisen from gene duplication.
We found the *FCN2* gene harboring 10 SNPs in a study group of 188 Dutch Caucasians, ranging in allele frequency between 1 and 45% (Chapter 2), comparable to frequencies found in a Danish cohort.[173,174] Two conserved coding SNPs were found in exon 8, leading to amino acid substitutions within the fibrinogen-like domain (Thr236Met and Ala258Ser) in the near proximity of amino acid motifs highly conserved among several proteins in many species. Although this conserved part harboring the coding SNPs is not the proposed binding site of L-ficolin, these SNPs alter the affinity of L-ficolin for its substrates, either decreasing (Thr236Met) or increasing (Ala258Ser) the binding to GlucNac.[169,174] Polymorphisms in the promoter region of *FCN2* at -986, -602 and -4 have been associated with different L-ficolin plasma levels.[174-176]

Disease and pathogen associations with L-ficolin
In vitro studies showed that L-ficolin can bind to bacteria and molecular patterns associated with altered self, with subsequent complement activation. Pathogens like *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Streptococcus pneumoniae* are recognized by L-ficolin.[159,166,177] Direct complex formation of L-ficolin and CRP enhanced the deposition of CRP on bacteria and boosted complement activation.[178] L-ficolin, like MBL and H-ficolin, also binds to altered self structures on apoptotic cells, linking these molecules to the disposal of self antigens.[179,180] These observations all suggest a role for L-ficolin in infectious diseases and autoimmunity.
Only a limited number of clinical association studies have been performed with L-ficolin, either at the expression level or on *FCN2* genotypes. No strong association was found between L-ficolin and chemotherapy-related infections, respiratory infections in children, adult pneumococcal disease or malaria.[181-185] A protective effect of high-producing *FCN2* genotypes was found in leprosy.[186] Low levels of L-ficolin were associated with premature, low birth weight neonates, and perinatal infections.[187]

Community-acquired pneumonia

When the pulmonary defense mechanisms fail, pneumonia can develop. Community-acquired pneumonia (CAP) is defined as an acute symptomatic infection of the lower airways that is acquired outside a hospital or nursing home.[188] The incidence of CAP ranges from 8-15 per 1000 persons per year, with the highest incidence in young children and the elderly. In the Netherlands, an incidence of 5-10 per 1000 persons per year was estimated.[189] Most cases occur during winter months. A large number of risk factors for acquiring CAP have been identified, including age >65 years, male sex, African race, and host conditions that may lead to impairment of pulmonary defense mechanisms. The latter include decreased level of consciousness leading to (micro)aspiration, smoking, alcohol consumption, pulmonary edema (for example by toxic inhalations), malnutrition, obstruction of the airways, decreased mechanical clearance of the airways and immunosuppression or immune deficiency.[190]

Community-acquired pneumonia is caused by a wide variety of pathogens, with *S. pneumoniae* accounting for up to 40% of cases (see below).[188] Since different etiological pathogens require different anti-microbial treatment, a panacea for CAP is not available. However, treatment outcome is associated with timely and appropriate antibiotic therapy.[191,192] Therefore, initial treatment should be aimed at the most likely causative agents of pneumonia in an individual patient. This challenge in treatment choice is reflected in the diagnostic challenge of rapid pathogen detection and of stratifying patients to predict the causative microorganism at admission. Several prediction models have been published, guiding practitioners in their choice of treatment for CAP.[188,189,193,194]

Since the development of pneumonia reflects an interplay between pathogen-related virulence factors and host factors determining susceptibility, the risk-assessment includes not only symptoms, but host factors as well. In the last years, genetic host factors influencing susceptibility to CAP have become an increasing field of research. Likewise, host factors influencing the outcome of CAP have been studied to identify patients requiring extra supportive care or more specific therapy at an early stage of disease.

Etiological pathogens of community-acquired pneumonia

Bacteria, viruses and parasites all can cause community-acquired pneumonia.[188] Data on pathogens in non-hospitalized patients show heterogeneity, as the methods to detect the causative agent greatly differ between studies.[188,193] Overall, *Streptococcus pneumoniae* (8.4-36.0%), *Haemophilus influenzae* (1.1-10.2%), *Mycoplasma pneumoniae* (1.3-26.2%) and influenza viruses (6.0-8.1%) were reported as the main causative agents of CAP in general practice.[193] In a large proportion of patients (45.3-53.7%) in these studies, no causative pathogen was found.

In hospitalized patients, the etiology of CAP shows the same pattern in studies from different countries.[193] Generally, *S. pneumoniae* (18.4-41.8%), *H. influenzae* (3.4-8.0%) and *M. pneumoniae* (5.4%-12.6%) were most frequently detected, comparable to results in ambulatory patients. However, in severe CAP requiring ventilation at the intensive care unit, a higher frequency of *Legionella pneumophila* and *Pseudomonas spp.* was reported compared to non-ventilated patients (15.1% vs. 7.1% and 6.6% vs. 1.0%, respectively).[195] Also, *Staphylococcus aureus* was detected more frequently in ICU patients (7.0-8.7%). Influenza viruses were detected in 2.3-5.4% of patients with severe CAP. In about one third to half of the patients, no etiologic agents could be detected. One study showed that

Etiology	Boersma n = 90	Bohte n = 334	v.d. Eerden n = 260	Braun n = 302	Endeman n = 199
S. pneumoniae	38	27	37	34	30
H. influenzae	2	8	10	12	7
M. catarrhalis	1	1	2	1	
S. aureus	1	1	5	3	3
Legionella spp.		2	5	8	5
Enterobacteriaceae	2		2	2	3
M. pneumoniae	4	6	8	24	5
Chlamydophila spp.	6	3	<1	4	1
Coxiella burnetii				1	<1
influenza A/B,	7	4	2	22	7
other viruses	4	3	2	10	6
M. tuberculosis	1			1	
B. pertussis				18	
other			3	10	1
none	38	49	24	13	36

Table 3 | ***Etiologic agents of CAP isolated from hospitalized patients in the Netherlands***
Percentages for the different etiologic micro-organism are given for six studies on hospitalized patients in the Netherlands.[197-201]

in 32.7% of these patients, *S. pneumoniae* was found in the lungs by transthoracic needle aspiration.[196] Recently, careful monitoring of changes in specific anti-pneumococcal antibody levels also suggested a higher involvement of *S. pneumoniae*, again emphasizing the importance of pneumococci as the main etiological pathogen (Van Mens, personal communication, submitted 2010).

In the St. Antonius Hospital in Nieuwegein we have conducted a large study in 199 patients presenting with CAP. Data on the etiology of CAP from this study confirm the reported pathogen frequencies from earlier Dutch studies (Table 3).[197-201] By use of a stringent protocol for detection by culture, PCR and serology, an etiological agent could be detected in 64% of all patients.[201] In the case of an identified microorganism, therapy was more likely to be switched from extended spectrum antibiotics to small spectrum antibiotics, emphasizing the relevance of thorough diagnostic efforts.[201] Although the frequency of *Coxiella burnetii* infection was low (0-1%) in the Dutch studies mentioned above, the incidence of Q fever and associated pneumonia has increased in the last two years due to a large, ongoing outbreak of multiple genotypes of *C. burnetii* in the Netherlands.[202,203]

Aim and outline of the thesis

Under normal physiological conditions, the pulmonary defense mechanisms described above are capable of clearing the lungs from microbes and particles, keeping the lower respiratory tract sterile. The development of pneumonia therefore represents a breach in the pulmonary defense mechanism either by an extraordinary large inoculum or high virulence of a pathogen, or a defect in the host defense itself.[204-206]

In this thesis, the interplay between invading pathogens, host defense genes and their encoded proteins are studied in the context of community-acquired pneumonia and two activators of the lectin pathway of complement: MBL and L-ficolin. By studying genotypes, protein levels and functionality in a cohort of hospitalized patients with CAP and in an outbreak of legionellosis, we aim to obtain more insight into the reciprocal effect of these complement proteins and pneumonia on each other, in the light of the genetic background of the patients.

Both MBL and L-ficolin are part of the humoral innate immune system. The innate immune system is designed for immediate activation upon microbial challenge, the first phase of infections like pneumonia. Other components of the innate immune system show a sharp increase or decrease in concentration and/or activity upon microbial encounter, a phenomenon termed the acute phase response. Therefore, this thesis not only investigates steady state conditions such as genetic polymorphisms and etiologic pathogens, but also the dynamics of MBL and L-ficolin levels in the acute phase of pneumonia in light of genetic polymorphisms and the etiology and course of disease.

Chapter 1

An introduction is given in the main topics of the thesis. After summarizing the different components of innate and adaptive immunity of the lung, the complement system of innate immunity is further looked into. Routes of activation, its role in inflammation, regulatory mechanisms and deficiencies are discussed. Two initiators of the lectin pathway of complement activation, MBL and L-ficolin, are the subject of investigation of this thesis and their structure, functional properties and genetics appear to be very similar. In this thesis, these two proteins are investigated in the context of pneumonia. A brief summary of CAP and its etiological agents are given.

Chapter 2

The first question to be answered was whether the *FCN2* gene is polymorphic. For *MBL2*, polymorphisms were already known. The homology between structure and functionality of MBL and L-ficolin led us to ask whether *FCN2* also harbored SNPs possibly influencing protein level and function. With the use of denaturing gradient gel electrophoresis, we identified 10 SNPs, two of which were coding polymorphisms leading to amino acid substitution in the ligand-binding domain of L-ficolin.

Chapter 3

This chapter describes the modification of a hemolytic assay for the quantification of MBL activity. The redundancy of activation pathways of the complement system can cause technical problems *in vitro* when trying to measure a single component of the system. In the hemolytic MBL assay, complement activation by the antibody-mediated classical pathway was shown to interfere with measurements of MBL activity. Modification of the

assay by addition of anti-C1q antibodies inhibited this unwanted interference.

Chapter 4
In this chapter, the role of *MBL2* genotypes in CAP in relation to the causative agent was the subject of study. MBL binds different pathogens with different avidity, and pathogens have different ways to cause infection, for example by entering host cells. Therefore, it was investigated whether *MBL2* genotypes were correlated with the type of etiologic pathogen and with outcome. MBL deficiency was not a risk factor for developing pneumonia in this cohort. MBL-deficient genotypes were observed more frequently in viral (co)infections. No correlation was found between pneumococcal pneumonia and genotypes.

Chapter 5
In a large clonal outbreak of legionellosis, the influence of *MBL2* genotypes as a risk factor for acquiring the disease was studied. Furthermore, the effect of the disease on MBL function in relation to the *MBL2* genotype was investigated. Genetic MBL deficiency was not a risk factor for developing legionellosis in this cohort. A large proportion of patients with MBL-sufficient genotypes showed absence of MBL functionality during legionellosis. This was not observed in the control subjects, suggesting that the deficiency in MBL activity in legionellosis is an effect of the disease rather than a risk factor for acquiring it.

Chapter 6
We have further studied the genetic polymorphism of *MBL2* and the behavior of MBL levels during CAP in relation to these genetic properties. By incorporating genotypes in the analysis of the behavior of MBL during the acute phase of pneumonia, it was shown that MBL is an acute phase reactant. However, MBL levels can only rise during the acute phase response if the genotype allows rapid upregulation of the production. With low-producing promoter genotypes, consumption of MBL during pneumonia outweighs MBL production.

Chapter 7
Do *FCN2* polymorphisms exert similar effects on L-ficolin levels during pneumonia as shown for MBL in chapter 6? In an analogous study design, it was shown that the *FCN2* promoter SNP -986G>A, and to a lesser extend -4A>G influence protein levels in both the acute and convalescent phase, but do not influence acute-phase responsiveness.

Chapter 8
The data from the previous chapters are further discussed in the context of recent literature. Hypotheses on cause and effect of the observed behavior of MBL and L-ficolin are proposed together with suggestions for further research on the investigated topics.

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2

Coding and non-coding polymorphisms in the lectin pathway activator L-ficolin gene in 188 Dutch blood bank donors

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Abstract

Human L-ficolin (FCN) is a serum lectin characterized by a collagen-like and a fibrinogen-like domain that can activate the lectin pathway of complement. Structural and functional similarities to mannose-binding lectin (MBL) suggest a role for L-ficolin in innate immunity. Structural polymorphisms in the *MBL2* gene lead to functional deficiency of MBL. Polymorphisms in the *FCN2* gene have not been studied previously.

We developed ten denaturing gradient gel electrophoresis (DGGE) assays to screen a total of 188 Dutch Caucasians for polymorphisms in *FCN2*. Total gene screening in this large cohort revealed ten single nucleotide polymorphisms (SNPs).

Interestingly, two conserved coding SNPs were found in exon 8, leading to amino acid substitutions within the fibrinogen-like domain. Fibrinogen-like domains are highly conserved among several proteins in many species. As this domain is responsible for binding of L-ficolin, these newly found coding polymorphisms could alter the affinity of the protein for its substrates and possibly alter the ability of L-ficolin to recognize invading microorganisms.

Introduction

Ficolins are lectins with a typical domain structure of a cystein-rich N-terminus, a collagenous region, a short neck region and a fibrinogen-like COOH-terminus.[1] Their fibrinogen-like (FBG) domains show similarities to the COOH-terminal halves of fibrinogen α and γ chains and have a common specificity for binding N-acetylglucosamine (GlcNAc).[2,3] Ficolins have been identified in vertebrates and invertebrates. In humans, three ficolins are known. M-ficolin is expressed on the surface of monocytes, while L-ficolin and H-ficolin (Hakata antigen) are present in serum.[4]

Human L-ficolin is synthesized in the liver and serum levels vary between 1 to 12 μg/ml.[5,6] It has a bouquet-like multimeric structure of four or more subunits composed of three identical polypeptides which form a collagenous triple helix.[2,7] The collagenous region can bind MBL-associated serine proteases (MASPs) that subsequently set off the complement cascade by cleaving C4 and C2.[3,8] This activation occurs after L-ficolin has bound carbohydrate structures through its FBG-domain.[5] The multimeric structure of the protein ensures high-avidity binding of multiple FBG-domains to carbohydrate structures on microorganisms. The multimeric structure is maintained by disulfide bonds in the N-terminal domain.[9] Besides complement activating properties, L-ficolin also plays a role in innate immunity by enhancing phagocytosis of bound microorganisms.[3,10]

In structure and function L-ficolin resembles MBL, a serum collectin first known to activate the lectin complement pathway. MBL also displays a sertiform structure held together by disulfide bonds and it can activate complement through binding of MASPs to its collagen-like domain.[11] Although in MBL the COOH-terminus is a carbohydrate recognition domain (CRD) instead of a FBG-domain, both bind carbohydrate patterns on microorganisms.[11] Several polymorphisms in the gene encoding MBL are known, leading to functional deficiency of the protein.[12] MBL deficiency has been correlated to increased risk of infection with capsulated bacteria and infections in children and neutropenic patients.[12]
The *FCN2* gene encoding L-ficolin is located on chromosome 9p34 and consists of eight exons.[13] The first exon encodes the 5' untranslated region (5'-UTR), a signal peptide and the N-terminal domain. The second and third exon encode the collagenous domain. The fourth exon encodes a neck region preceding the globular FBG-domain, which is encoded by exons five to eight. This last exon also encodes the 3'-UTR.
We searched for polymorphisms of *FCN2* in 188 healthy unrelated Dutch Caucasians using denaturing gradient gel electrophoresis (DGGE). This technique uses differences in melting behavior of DNA fragments to allow rapid and low-cost screening for unknown genetic variants.[14] Ten DGGE assays were developed for the identification of polymorphisms and their frequencies in the eight exons and intron-exon boundaries of *FCN2* in this population. A total of ten SNPs were identified. Two conserved coding SNPs were found in exon 8 encoding the fibrinogen-like domain, which is responsible for substrate binding of L-ficolin.

Materials and Methods

Isolation of DNA
Genomic DNA was isolated from whole blood obtained from 188 unrelated Dutch Caucasian blood bank donors (mean age 45 years, female to male ratio 1:1) using Qiagen Maxi DNA isolation Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany).

Design of DGGE assays
To optimize specificity of the primers the sequences of *FCN2* (accession nr. D49353 and D63155-D63160) and the closely related *FCN1* (BC020635) gene were aligned to chromosome 9 (AL603650) in the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). DGGE assays were developed following guidelines previously described.[15] Gene fragments suitable for DGGE were chosen from *FCN2* exons and intron-exon boundaries by simulating the melting behavior of the fragment using MELT94 (http://web.mit.edu/osp/www/melt.html). In most cases a 41-bp GC-clamp was used. However, in some cases a high melting temperature of the fragment required the use of a 59-bp GC-clamp (sequence kindly provided by dr. Hofstra, University of Groningen). Primer annealing temperatures and internal loops were analyzed with OLIGO software (MBI inc., Cascade, USA). Primer sequences were checked for specificity using BLAST.

PCR assays
Primers and PCR conditions are listed in table 1. PCR was performed in a total volume of 25 µl containing SuperTaq PCR buffer, 2.5 units SuperTaq polymerase, dNTPs (all Sphaero

Assay exon	Primerset	T_{ann} (cycles)	Gradient
1	F: [clamp1]-GGC ACC TTT TGA AGC AAA R: ccg gCC CTG GAG TGC CCT TAC	57°C (40)	50-70%
2	F: cgc gcg cgC AGG TGA CAC TGA GTG GC R: [clamp1]-AAC TCA GCA TCT GGT TTC AA	57°C (40)	50-70%
3	F: [clamp1]-TTT GAT CCT GGG CTG G R: AAG ACC CGC CTC ACG	57°C (40)	30-90%
4	F: [clamp1]-AGA AAA TGG TGT CCG CG R: gcg gcg AGT GTG GGG GTG GTC AGT	57°C (40)	50-70%
5	F: cgg cgc ggc CCT GCT TCT TCC TCC CA R: [clamp2]-ata AGA GAA TTC CAG AGT GTG TTC TCC C	57°C (40)	55-75%
6	F: [clamp1]-GGC TCC TGT CCC CTG GCT R: GCT CTT GTG TTC CAG GCA	67°C (40)	20-80%
7.1	F: ccg cGC CCT TCC GCT GAG A R: [clamp1]-TCT CCG CCT CGT CG	57°C (40)	35-55%
7.2 (1^{st})	F: ccg cGC CCT TCC GCT GAG A R: GGC GGA AGC CCA GGC CAC A	57°C (20)	-
7.2 (2^{nd})	F: [clamp2]-CAA CTA CCA GTT TGC TAA GTA CAG R: CCC CAA GCA GAC ACT CA	57°C (40)	20-80%
8.1	F: [clamp1]-GCC AGG CCT CAG GTA TAA A R: TGA CCC TGA GGC CG*	57°C (40)	20-50%
8.2	F: GAC CTG CCT AGC CCA G R: [clamp3]-AGC AAG ACA AAC CCA ATT T	57°C (40)	20-80%

Table 1 | ***Primers, PCR conditions and denaturing gradients to genotype the 8 exons of*** **FCN2**
*In the primer sequences, the gene sequence is given in capitals. Added nucleotides are given in undercase. [clamp1]: ccg ccc gcc gcg ccc cgc gcc cgg ccc gcc gcc ccc gcc cc. [clamp2]: cgc gcc cgc cgc gcc ccg cgc ccg gcc cgc cgc ccc cgc ccg cgc cgc cgc ccc gcg cc. [clamp3]: reverse complement of [clamp1]. * Screening was performed using overlapping primers. To ensure optimal visualization of heteroduplexes, patterns were confirmed with an alternative reverse primer (CCA CCA AGC TCC CTG AAA C).*

Q, Leiden, The Netherlands), 500 nM of forward and reverse primer (Isogen, Maarssen, The Netherlands), 400 µg/ml BSA (New England BioLabs, Beverly, MA, USA) and 2.5 µl template DNA. After initial denaturation (10 min, 95˚C), 40 PCR cycles (30 s at 95˚C, 30 s at 57˚C and 30 s at 72˚C) were passed, followed by a final elongation step (10 min, 72˚C). For exon 6 the annealing temperature was 67˚C to ensure specificity. For exon 7.2 a nested protocol was developed to ensure specificity and DGGE suitability.

Denaturing Gradient Gel Electrophoresis (DGGE)
PCR products were mixed with loading buffer (1:5) and 15 µl was run on a DGGE DCODE system (BioRad, Hercules, CA, USA) at 60 V for 16 h using a 9% polyacrylamide gel with an optimized denaturing gradient (Table 1) of ureum and formamide. After electrophoresis the gels were stained in ethidium bromide for 30 minutes, washed in distilled water and viewed under UV light.

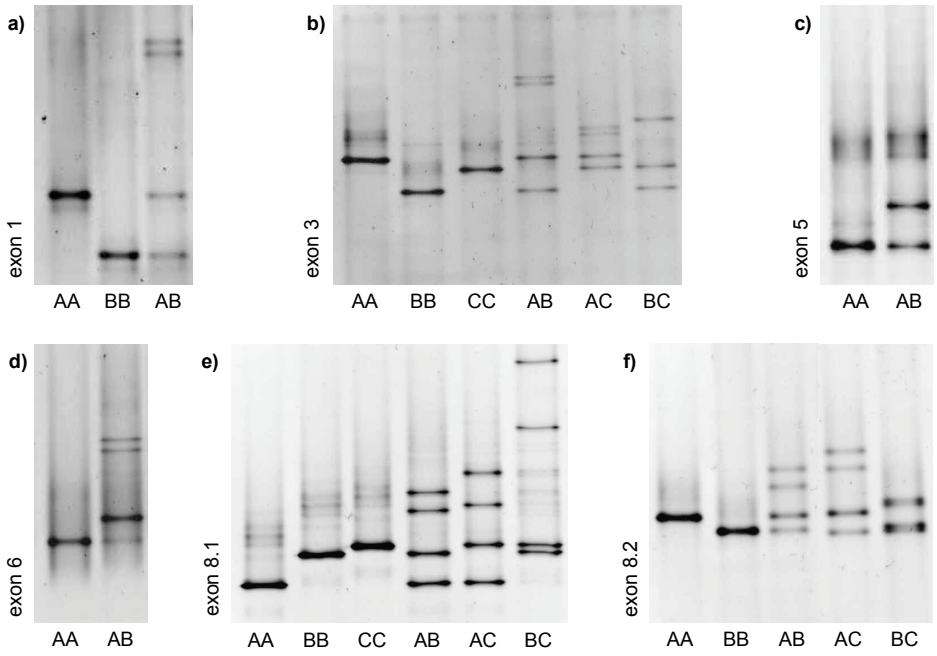

Figure 1 | ***Migration patterns and genotypes of DGGE assays to genotype* FCN2**
In assays for exons 1 (a), 5 (c) and 6 (d) two haplotypes were found. In assays for exons 3 (b), 8.1 (e) and 8.2 (f) three haplotypes were found. Per assay, haplotypes were named A (wildtype), B and C in order of frequency. Per genotype, the number of individuals is given. The total number of Dutch Caucasians screened was 188.

Sequencing of samples

At least two samples of each genotype pattern were sequenced in a Big Dye terminator reaction (Applied Biosystems, Foster City, CA, USA) on an ABI 377 sequencer following the manufacturer's instructions. Because of the high homology between *FCN1* and *FCN2* we preferred to use the same primer sequences used in the DGGE reaction for both the PCR and sequence reaction rather than designing a new pair of outer primers (Table 1). Sequence data were analyzed with Staden Package (http://staden.sourceforge.net/).

Data analysis and haplotype analysis

For all SNPs the Hardy-Weinberg equilibrium was verified by analysis in the Pearson's chi-square test. Haplotypes were calculated by combining data on all exons in PL-EM software.[16] All sequence data were submitted to NCBI's dbSNP database (SS28476277-28476289).

Results

Ten DGGE assays were developed to genotype all exons of *FCN2*. Table 1 shows the optimized conditions for PCR and DGGE to show distinct patterns for each genotype. For

Location*	SNP†	Substitution	Frequency‡
5'UTR (1B)	c.-4A>G	-	0.27 (101)
intron 2 (3B)	c.213-7A>G	-	0.33 (123)
exon 3 (3B)	c.222T>C	synonymous	0.33 (123)
intron 3 (3C)	c.269+10G>A	-	0.26 (97)
exon 5 (5B)	c.337C>T	p.His113Tyr	0.01 (2)
intron 6 (6B)	c.560+44G>A	-	0.14 (52)
exon 8 (8.1B)	c.707C>T	p.Thr236Met	0.27 (103)
exon 8 (8.1C)	c.772G>T	p.Ala258Ser	0.14 (51)
3'UTR (8.2B)	c.987T>G	-	0.45 (169)
3'UTR (8.2C)	c.987_988insG	-	0.01 (2)

Table 2 | ***Location, amino acid substitution and frequency of SNPs in* FCN2**

** The corresponding DGGE assay number and haplotype is given between brackets. Per assay, haplotypes were named A (wildtype), B and C in order of frequency. Linkage of SNPs in assays for exon 3 and 8.1 are clarified in the main text.*

† The location of SNPs is given relative to FCN2 cDNA (D49353).

‡ Since the first two SNPs in the assay for exon 3 are linked in this study population, and the multiple SNPs in all assays are mutually exclusive, the SNP frequency also is the frequency of the haplotype mentioned in the first column. The number of observed alleles is given between brackets. The total number of alleles screened was 376.

exons 1 to 6 a single PCR fragment was sufficient to cover the entire exon in the DGGE assay. The natural melting curves of exons 7 and 8 did not allow a single low-melting domain to be designed for the whole exon. Therefore, these exons were split in two overlapping fragments (named exon 7.1 and 7.2, respectively 8.1 and 8.2). In case of exon 7.2 a nested PCR was necessary to ensure the combination of specificity for *FCN2* (achieved with the first PCR) and suitability for DGGE (second PCR).

Screening of all exons of *FCN2* showed variation in DGGE patterns of the PCR products of exons 1, 3, 5, 6, 8.1 and 8.2 (Figure 1). To validate the DGGE results, at least 2 samples of the different DGGE patterns of all exons were sequenced. This revealed a total of seven non-coding SNPs in the 5'-UTR and 3'-UTR, in introns 2, 3 and 6 and in exons 3 and 8 (Table 2). One coding SNP was found in exon 5 leading to the substitution of histidine with tyrosine in the first part of the fibrinogen-like domain. In exon 8 two coding SNPs were found which result in the substitution of threonine with methionine respectively alanine with serine in the COOH-terminal part of the FBG-domain. Furthermore, two heterozygotes carrying two different incidental mutations were found (exon 1 (c.-5A>T) and exon 6 (c.543C>T); DGGE patterns not shown). In the cDNA sequence of L-ficolin (D49353) three consecutive thymidines are reported starting from position c.999T. In accordance with the genomic sequence (AL603650), we found only two consecutive thymidine residues.

Frequencies of the SNPs were calculated from genotype frequencies (Table 2). All homozygous and heterozygous allele combinations of exons 1, 3, 6 and 8 were found. For the SNP in exon 5 and the insertion in exon 8 only 2 heterozygotes and no variant

homozygotes were identified. All genotype frequencies of individual SNPs were in Hardy-Weinberg equilibrium.
Haplotype analysis of all SNPs showed thirteen haplotypes. In this study population, linkage was observed between three SNPs near exon 3, where three haplotypes were found: c.213-7A c.222T c.269+10G (A, wildtype, 41%), c.213-7A>G c.222T>C c.269+10G (B, 33%) and c.213-7A c.222T c.269+10G>A (C, 26%). This linkage also is demonstrated by the limited number of DGGE patterns, i.e. haplotypes found in this population compared to the number of SNPs in this assay. In the first part of exon 8, two coding SNPs accounted for three haplotypes in a mutually exclusive manner: c.707C c.772G (A, wildtype, 59%), c.707C>T c.772G (B, 27%) and c.707C c.772G>T (C, 14%). Similarly, three haplotypes were produced by two SNPs in the 3'-UTR: c.987T (A, wildtype, 54%), c.987G (B, 45%) and c.987_988insG (C, 1%). In all other assays, either no variation or one SNP was found, giving rise to one (only wildtype) or two haplotypes respectively (Table 2). In the latter cases, the haplotype frequency is the same as the SNP frequency.

Discussion

Here we describe ten DGGE assays for the identification of polymorphisms in the *FCN2* gene encoding the human lectin-pathway activator L-ficolin. In this setting, DGGE proved to be a suitable and simple method to screen for unknown genetic variations, which could be unambiguously detected by the formation of heteroduplexes (Figure 1). Once the different DGGE patterns were validated by sequencing, samples could be genotyped by DGGE alone. This offers a simple and less expensive strategy to screen for polymorphisms than sequencing. Compared to SNP-PCR this technique allows the detection of multiple SNPs and unknown genetic variations on a single PCR fragment. A previously described DGGE assay to genotype exon 1 of *MBL2* indeed was able to detect all three known SNPs and detected an incidental mutation in codon 44.[17]
We found ten polymorphisms and two incidental mutations in *FCN2* in 188 Dutch Caucasian blood bank donors and described their frequencies. Although seven of these SNPs were already reported in the dbSNP database, they were not all validated and frequencies were not known, as they were not studied in a population. Overall, SNPs were found in all regions of the gene and with allele frequencies ranging from 0.01 to 0.45 these genetic variations are highly conserved.
Polymorphisms were found in the 5'-UTR and 3'-UTR of *FCN2*. Polymorphisms in the upstream and downstream untranslated regions of other genes have been described to influence protein levels by translational control.[18] Serum levels of L-ficolin are reported to vary between 1 to 12 µg/ml, with most samples (>90%) ranging between 2.5 and 5.5 µg/ml.[5,6] In agreement a short length of the 5'-UTR, as in *FCN-2*, usually indicates a constant and high rate of translation. Therefore, the relevance of these two SNPs in this particular context has to be further investigated.
Silent and intronic SNPs were found in exons 3 and 8 and in introns 2, 3 and 6. In recent years, the effect of intronic mutations and non-coding exonic mutations on splicing events has become more appreciated.[19,20] Three splicing variants (SV1-3) are known for L-ficolin.[13] The SV1 variant lacks the entire exon 2 sequence and has an extended 3'-UTR. Since the reading frame is not altered this results in a deletion of 38 amino acids in the collagen-like domain. In SV2 the fifth intron is inserted. The resulting additional sequence harbors a

stop codon leading to 39 additional amino acids replacing a large part of the fibrinogen-like domain. In SV3 the fourth and fifth introns are inserted. The in-frame stop codon at the beginning of the inserted intron 4 also leads to a truncated form of the protein, lacking the fibrinogen-like domain. It seems very likely that these splicing variants affect the function of the protein, as either the MASP-activating domain or the pattern-recognition domain is affected in SV1 and SV2/SV3 respectively. Whether the SNPs in *FCN2* can affect the splicing machinery and alter the probability of production of these SV1-3 variants, needs to be further investigated.

Three coding SNPs were found in exons 5 and 8 encoding the fibrinogen-like domain of L-ficolin. Fibrinogen-like domains are highly conserved throughout several proteins in many species. These domains consists of approximately 220-250 residues with 26 invariant, mostly hydrophobic residues and at least 46 highly conserved residues. Although the frequency of the coding SNP in exon 5 is low, the two variations in exon 8 are conserved. Of these latter two SNPs, the first SNP results in the substitution of threonine with the hydrophobic methionine. The second SNP substitutes alanine with serine. Both amino acid substitutions occur within the near proximity of the invariant and conserved residues. As the fibrinogen-like domain of L-ficolin is responsible for pattern recognition, it is of interest to investigate whether the genetic variation in this domain alters the affinity or specificity of carbohydrate binding on microorganisms.[5] Such possible influence could affect the recognition of invading microorganisms and thereby influence one of the first lines of defense in innate immunity.

Very recently, comparable data were described on *FCN2* SNPs found with sequencing of the gene in a Danish study population.[21] Using DGGE in our Dutch study population we could confirm six of the described SNPs, including the two coding SNPs in exon 8. The frequency of these coding SNPs were lower in the Danish study. Most discordances in observed SNPs could be explained by a low allele frequency and non-overlapping targets. Linkage of the SNPs in intron 2 and exon 3 was not observed in the Danish study population. With recent screening of a new study population indeed we found two new DGGE patterns representing other haplotypes (data not shown). We therefore conclude that the linked haplotype is the most prevalent in our study population, however linkage is not exclusive.

The study of Hummelshøj *et al.* also described the correlation of *FCN2* SNPs with functional properties of L-ficolin. The 5'-UTR SNP indeed correlated with increased serum levels of the protein. An increased affinity for N-acetylglucosamine was found for the variant fibrinogen-like domain p.Ala258Ser. The p.Thr236Met variant decreased the affinity. It would be of interest to see whether these differences are also observed with other acetylated molecules and with complement activation by complete microorganisms.

In conclusion, ten polymorphisms were found in the *FCN2* gene encoding human L-ficolin, a protein of innate immunity and conserved widely among species. Two conserved coding SNPs are especially interesting as they occur in the substrate binding domain. A possible relationship between the genotype and biological activity of L-ficolin towards microorganisms is currently under investigation, as this protein appears to play a role as an activator of complement in innate immunity.

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3

Hemolytic assay for the measurement of functional human mannose-binding lectin

A modification to avoid interference from classical pathway activation

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Hemolytic assay for the measurement of functional human mannose-binding lectin

A modification to avoid interference from classical pathway activation

Abstract

Diagnostic assays for measurement of functional mannose-binding lectin (MBL) in serum are widely performed as part of immune status assessment. Classical pathway mediated complement activity can interfere in these functional MBL assays. Here we describe classical pathway interference incidentally occurring in a previously described hemolytic MBL assay and the modification of this assay to prevent this artifact by addition of anti-C1q antibodies. Classical pathway interference in functional MBL assays can and should be inhibited to prevent that MBL deficiency is overlooked and patients are misdiagnosed.

Introduction

Low levels of functional mannose-binding lectin (MBL), impairing complement activation via the lectin pathway, result from single nucleotide polymorphisms in the *MBL2* gene and have been associated with a variety of infectious and autoimmune diseases.[1,2] Therefore, diagnostic procedures to determine MBL levels and lectin pathway activity are widely performed as part of immune status assessment.

In a previously described hemolytic assay, the lectin pathway of complement is activated by binding of MBL to mannan on *Saccharomyces cerevisiae.*[3] Bystander-hemolysis of indicator erythrocytes is measured and compared to a standard sample to calculate the level of MBL. In this assay, all down-stream complement components are provided by addition of MBL-deficient serum. Therefore the rate limiting step is the amount of functional MBL in the test sample, independent of deficiencies or depletion of down-stream complement components. As both the ligand-binding and complement-activating properties of MBL are assessed simultaneously, this assay can quantitatively measure the level of functional MBL.

Interference by classical pathway activity in functional MBL assays has been postulated.[4] In the hemolytic MBL assay described above, immune complexes could lead to MBL-independent complement activation, for example by antibodies against *S. cerevisiae*. Such interference could lead to false positive test results in patient samples. Consequently MBL deficiency will not be recognized and these patients will be misdiagnosed.

During the laboratory diagnostic procedures for patients with suspected complement deficiencies we have identified a series of patients with an MBL-deficient genotype (XA/0 or 0/0) but with hemolytic activity in the hemolytic MBL assay. We have used those serum samples to modify the hemolytic assay. Here we describe the effect of classical pathway interference in the hemolytic MBL assay and the modification of this assay to prevent this artifact by addition of anti-C1q antibodies blocking classical pathway activity.

Materials and Methods

Human blood samples

Serum and EDTA blood samples from 11 patients with an MBL-deficient genotype (XA/0 or 0/0) but activity in the unmodified hemolytic MBL assay (see below) were used. Furthermore, samples from 18 patients with MBL-sufficient genotypes (A/A and YA/0) were used. All samples were originally sent to the laboratory for genotyping and functional characterization of MBL as part of immune status assessment. MBL-deficient serum was obtained from a volunteer with MBL-deficient genotype 0/0. Human pooled serum (HPS) was prepared from serum of 20 healthy unrelated volunteers. All sera were stored at -70°C.

Genotyping of MBL2

Combined haplotypes of single nucleotide polymorphisms (SNP) in the promoter and exon 1 of *MBL2* were determined in a previously described denaturing gradient gel electrophoresis (DGGE) assay.[5] *MBL2* genotypes were considered MBL-deficient (XA/0 and 0/0) or MBL-sufficient (A/A and YA/0).[6,7]

Blocking of classical pathway activity by anti-C1q in the CH50 assay

To determine the concentration of anti-C1q needed to inhibit classical pathway activity, classical pathway complement activity was assessed in the presence of anti-C1q monoclonal antibodies in 4 randomly chosen sera. The sera were diluted 1:10 in

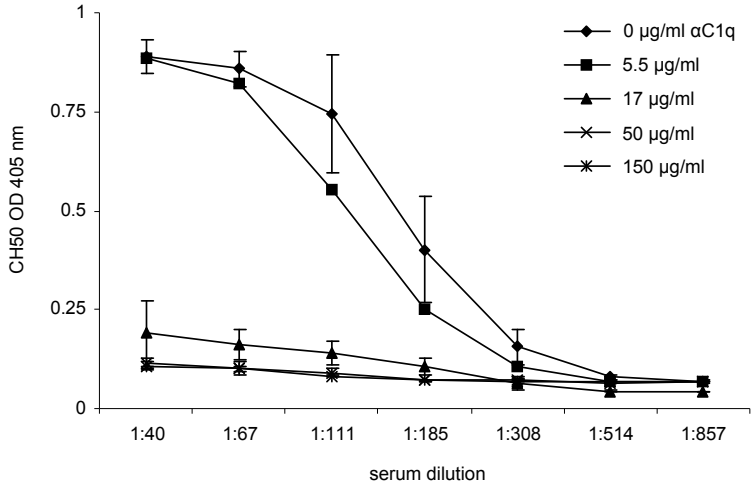

Figure 1 | ***Dose-dependent inhibition of the CH50 assay by anti-C1q***

The anti-C1q antibody mAb 2204 inhibited the classical pathway mediated hemolyis dose-dependently in the CH50 assay. Classical pathway mediated hemolysis was completely inhibited at 50 µg/ml. The mean hemolysis (OD 405 nm) of four randomly selected sera is depicted.

veronal-buffered saline with 0.15 mM Ca^{2+} and 0.5 mM Mg^{2+} (VBS^{++} buffer) at a final volume of 100 µl containing 0, 5.5, 17, 50 or 150 µg/ml anti-C1q monoclonal antibodies mAb 2204 (Sanquin, Amsterdam, The Netherlands).[8] and preincubated on ice for 15 min. Classical pathway activity was assessed by measuring hemolysis of sheep erythrocytes in a CH50 assay in a dilution series of the test sera (dilution range 1:40 to 1:857).[9]

Blocking of classical pathway activity by anti-C1q in the hemolytic MBL assay

To examine the interference from classical pathway activity in the hemolytic MBL assay described by Kuipers et al. (2002), hemolytic activity was assessed in absence and presence of anti-C1q. All sera were diluted 1:10 in VBS^{++} buffer containing 0 or 50 µg/ml anti-C1q antibodies (mAb 2204) and incubated on ice for 15 minutes. Per 96 wells round-bottom microtiter plate, a reaction mix was prepared of 10 ml VBS^{++} containing *S. cerevisiae* ($3x10^5$ cells/well), MBL-deficient serum providing all complement components except MBL (15 µl/ml), anti-C1q to inactivate C1q in the MBL-deficient serum (7.5 µg/ml) and chicken erythrocytes as target cells (10^7 erythrocytes/well). The reaction mix was prepared and preincubated on ice for 15 min. MBL was activated by *S. cerevisiae* after adding 100 µl of the reaction mix to a dilution series of the test serum (volume 50 µl; dilution range 1:10 to 1:3162) in the round-bottom wells.

Complement mediated hemolysis of chicken erythrocytes was measured by spectrophotometry (absorbance at 405 nm). Hemolysis of the test samples was expressed as a percentage of hemolysis compared to HPS. Hemolysis <25% was considered deficient.[3]

Determination of MBL protein levels

Serum levels of the multimeric MBL protein were determined in a commercially available ELISA (Sanquin, Amsterdam, the Netherlands).[10] In short, MBL bound to coated mannan was quantified with the use of an anti-MBL antibody recognizing the multimeric form only.

Results

Anti-C1q antibody mAb 2204 inhibited classical pathway complement activity dose-dependently in four random sera, with full inhibition at 50 µg/ml (Figure 1). Therefore, the 50 µg/ml anti-C1q antibody concentration was chosen to inhibit all classical pathway activity in subsequent experiments with the hemolytic MBL assay.

All 18 samples from individuals with MBL-sufficient genotypes A/A or YA/0 showed hemolysis >25% in the unmodified hemolytic MBL assay, i.e. without anti-C1q addition (Figure 2). Only 1 of these samples (genotype YA/0) showed deficient hemolysis (<25%) upon addition of anti-C1q. The MBL protein level in this sample was <0.2 µg/ml.

A striking result was found in sera from 11 patients with MBL-deficient genotypes XA/0 and 0/0. In the unmodified assay in absence of anti-C1q, hemolysis exceeded 25% in all of these samples, suggesting functional MBL activity (Figure 2). Addition of anti-C1q completely inhibited hemolysis in all samples, indicating the absence of functional MBL activity in these sera. MBL protein levels were below 0.2 µg/ml in all samples with MBL-deficient genotypes.

Measurements in the modified hemolytic assay were linearly correlated with the MBL protein levels (Spearman's rho 0.969, $p<0.001$).

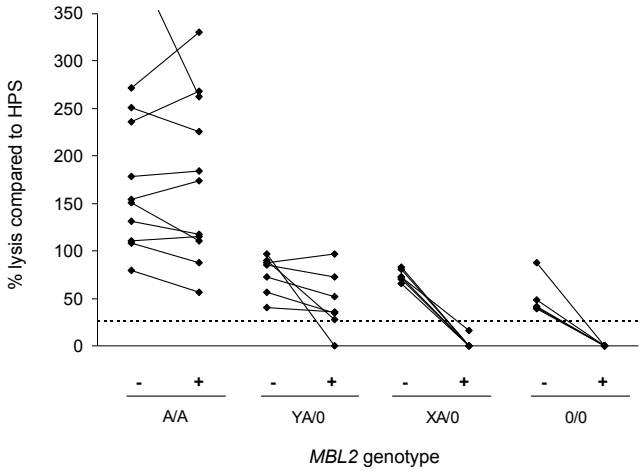

Figure 2 | ***Effect of anti-C1q antibody mAb 2204 on hemolysis in the hemolytic MBL assay***
The effect of the anti-C1q antibody mAb 2204 on hemolysis in the hemolytic MBL assay was analyzed in routine clinical samples per genotype. The cut off value for deficient hemolysis (<25%) is plotted. In subjects with MBL-deficient genotypes XA/0 and 0/0, the hemolysis >25% observed in the unmodified MBL assay was inhibited to <25% by anti-C1q in the modified assay.

Discussion

The hemolytic MBL assay is designed to measure functional MBL levels by the ability of MBL in the test serum to bind its ligand and to activate complement, independent of depletion or deficiencies of down-stream complement components.[3] It is based on the principle of bystander hemolysis. Because binding of C1q to immune complexes and subsequent classical pathway activation would also result in hemolysis, classical pathway activity could interfere with the hemolytic MBL assay.

The anti-C1q monoclonal antibody mAb 2204 inhibited classical pathway activity dose-dependently in the CH50 assay and thus should be able to prevent classical pathway interference in the MBL assay. The concentrations of anti-C1q found to completely inhibit classical pathway activity in our study are in the same range as the suggested 20 µg/ml by Roos et al. (2003). Since the anti-C1q antibody is directed against the globular head of C1q and inhibits the binding of C1q to IgG, its blocking effect is specific for the classical pathway activity.[8,11]

In clinical samples, the classical pathway interference could lead to an overestimation of MBL-mediated hemolysis. Especially in patients with MBL-deficient genotypes, this interference would lead to a false test result of sufficient functional MBL levels. Addition of 50 µg/ml anti-C1q completely inhibited this artifact, as the functional results of the modified MBL assay were in complete accordance with the MBL protein levels of these individuals and no hemolytic activity was found in patients with MBL-deficient genotypes.

In conclusion, the hemolytic MBL assay can measure functional MBL levels without interference from down-stream complement deficiencies. Classical pathway interference in this and other functional MBL assays can and should be blocked by anti-C1q to prevent that MBL deficiency is overlooked and patients are misdiagnosed.

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4

Mannose-binding lectin genotypes in susceptibility to community-acquired pneumonia

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Mannose-binding lectin genotypes in susceptibility to community-acquired pneumonia

Abstract

Community-acquired pneumonia (CAP) is most frequently caused by *Streptococcus pneumoniae*, *Haemophilus influenzae*, atypical pathogens, and respiratory viruses. Susceptibility to CAP can be increased by single-nucleotide polymorphisms (SNPs) within the mannose-binding lectin (MBL) gene. We questioned whether MBL polymorphisms are associated with the susceptibility to and outcome of CAP in relationship to the most common respiratory pathogens.

All adult patients presenting with CAP in a 23-month period were included in this study. Frequencies of SNPs were determined for the promoter X/Y and the three coding SNPs in exon 1 (A/0). Six genotypes were constructed representing patients with sufficient and deficient serum levels of MBL. The results of the patients with CAP were compared with control subjects.

In 199 patients and 223 control subjects, MBL genotypes were determined. There were no differences in MBL genotype frequencies between patients with CAP in general, pneumonia caused by *S. pneumoniae* or *H. influenzae*, and control subjects. The frequency of MBL-sufficient genotypes was non-significantly higher in patients with pneumonia with *Legionella spp.* and *Mycoplasma pneumoniae*. In *Legionella spp.*, the sufficient YA/YA genotype was significantly more frequent than in control subjects (odds ratio [OR], 5.43; confidence interval [CI], 1.32 to 22.41; p 0.02). The frequency of the MBL-deficient genotype was significantly higher in patients with viral (co)infections (OR, 2.36; CI, 1.06 to 5.26; p = 0.03) and non-significantly higher in patients with pneumococcal pneumonia and viral (co)infections. MBL genotypes had no effect on outcome.

In conclusion, MBL genotypes play a limited role in pneumococcal pneumonia. MBL-sufficient genotypes were more frequently found in a small group of patients with atypical pneumonia, and MBL-deficient genotypes were more frequently found in patients with viral (co)infections.

Introduction

Community acquired pneumonia (CAP) is the most common infectious disease requiring hospitalization in the Western world. In spite of improving antibiotic regiments, CAP is still a disease with a significant mortality and morbidity.[1] CAP is caused by a variety of micro-organisms. Most frequently isolated micro-organisms are *Streptococcus pneumoniae*, *Haemophilus influenzae*, atypical pathogens (*Legionella species* and *Mycoplasma*

pneumoniae) and influenza viruses.[2-6] Although some micro-organisms are found in patients with specific risk factors, such as Gram-negative bacteria in patients with a history of COPD, many patients suffer from CAP in absence of these risks. Genetic susceptibility to a specific pathogen causing CAP may play a role in these patients.[7]

Mannose-binding lectin (MBL) is a calcium dependent collagenous serum lectin produced by the liver. MBL binds to repeating carbohydrates (mannan or N-acetylglucosamine) on the surface of different micro-organisms and is therefore called a pattern-recognition receptor. After binding, MBL mediates complement activation and opsonophagocytosis by activation of different mechanisms.[8-14] MBL deficiency is associated with an increased general risk of severe infection in immune compromised patients.[15-19] In other patient groups, MBL deficiency is associated with recurrent respiratory infections and infections with capsulated bacteria.[20-22] Furthermore, MBL deficiency increases the risk of bacteraemia, sepsis and fatal outcome.[24-27]

MBL deficiency is common and caused by single nucleotide polymorphisms (SNPs) in the promoter and coding regions of the *MBL2 gene* on chromosome 10.[28-32] We investigated whether MBL polymorphisms are associated with the susceptibility to CAP in relationship to the most frequent causative pathogens (*S. pneumoniae*, *H. influenzae*, *Legionella spp.* and *M. pneumoniae* and viral (co-)infections with influenza A and B, and herpes simplex virus 1). Furthermore we report the effect of the MBL polymorphisms on clinical course.

Materials and Methods

Patients and controls

All patients (>18 years) with CAP presenting in the period October 2004 - August 2006 in our general 600-bed teaching hospital were included in this study. Patients with a history of recent hospitalization (<30 days) or a congenital or acquired immunodeficiency (including patients treated with prednisone 20 mg per day for more than three days) were excluded. Pneumonia was defined as a new infiltrate on the chest X-ray and two out of six clinical signs of pneumonia (cough, production of sputum, signs of consolidation on respiratory auscultation, temperature >38 or <35 degrees Celsius, leukocytosis (white blood count (WBC) >10 G/l) or leukopenia (WBC <4 G/l) or more than 10% rods in the differential count and C-reactive protein (CRP) 3 times above the upper limit of normal (5 mg/dl). The chest X-ray was interpreted by the resident at the first aid department. Within 24 hour after presentation, the chest X-ray was evaluated by an experienced radiologist, who was not aware of the clinical course of the patient. The following data were collected at presentation: age, sex, history of COPD and Fine-score. A DNA-sample was taken at admission and stored for further analysis. The control group consisted of 223 Caucasian white sex-matched blood bank donors from the same geographical area as the patients. This study was approved by the local Medical Ethics Committee and informed consent was obtained from each patient.

Pathogen identification

Of all patients sputum (if available) and blood (two samples) were cultured. Polymerase chain reactions (Taqman real-time PCR) were performed in sputum in order to detect DNA from *M. pneumoniae*, *Legionella pneumophila* and/or *Chlamydophila pneumoniae*.[33] Urine samples were taken for antigen testing on *S. pneumoniae* (Binax *S. pneumoniae* kit) and *L.*

Patient characteristic	n (%)
Male	123 (62)
Age (mean, SD)	63 (17)
COPD	61 (31)
Hospital mortality	10 (5)
ICU admission	21 (11)
Length of hospitalstay (median, range)	11 (3-153)
Bacteraemia	18 (9)
Fine-class	
I	30 (15)
II	34 (17)
III	53 (27)
IV	55 (28)
V	27 (14)
Identified micro-organism	
Unidentified	72 (36)
S. pneumoniae	60 (30)
H. influenzae	14 (7)
Legionella spp.	9 (5)
M. pneumoniae	9 (5)
S. aureus	6 (3)
Other Gram-negative bacteria[1]	8 (4)
Other atypical micro-organism[2]	3 (2)
Other *Streptococcus spp.*	2 (1)
Influenza virus (A and B)	13 (7)
Other respiratory viruses[3]	11 (6)
Herpes simplex virus type 1	9 (5)

Table 1 | ***Characteristics of 199 patients with community acquired pneumonia***
[1] *A. calcoaceticus , E. Coli, Klebsiella spp., P. aeruginosa and S. maltophilia*
[2] *Chlamydophila spp. and C. burnetii*
[3] *adenovirus, para-influenzavirus 1 and 3, and respiratory syncytial virus*

pneumophila (Binax *Legionella* urine antigen test).[34,35] Sampling for serologic testing on the presence of antibodies against *M. pneumoniae*, *Coxiella burnetii* or respiratory viruses (influenza A and B, parainfluenza viruses 1, 2 and 3, adenovirus and respiratory syncytial virus) was done at day 1 and 10 (Complement fixation test), for *Legionella spp.* on day 1 and 30 (IgM/IgG virion/serion ELISA Clindia).[36] Pharyngeal samples were taken for viral culture (influenza and parainfluenza viruses and herpes simplex virus type 1 (HSV1)). Viral pneumonia was defined as a positive test for a respiratory virus (culture or seroconversion positive) in combination with negative cultures, PCRs, serologic and antigen tests for bacterial micro-organisms.

Genotyping of MBL

Genotyping was done after closing of inclusions. Genotyping of *MBL2* (GeneID 4153) was

done for the promoter X/Y SNP (rs7096206) and the exon 1 SNPs on codon 52 (rs5030737; D52C; D variant), 54 (rs5030737; G54D; B variant) and 57 (rs1800451; G57E; variant C). The promoter SNP is denoted as X/Y, with Y as wild type. The three coding SNPs in exon 1 D, B en C are all denoted by 0 versus wild type A. These SNPs are combined in 6 genotypes: YA/YA, YA/XA, XA/XA, YA/0, XA/0 and 0/0. MBL serum levels are sufficient in YA/YA, YA/XA, XA/XA and YA/O and deficient in XA/0 and 0/0. Genomic DNA was extracted from whole blood using a QIAamp DNA Blood Kit (Qiagen, Westburg, the Netherlands). Combined MBL X/Y promoter and exon 1 genotypes were determined using a previously described denaturing gradient gel electrophoresis (DGGE) assay with slight modifications in a nested PCR protocol. Two PCR assays specific for the promoter X genotype (forward primer ATT TGT TCT CAC TGC CAC C; reverse primer GAG CTG AAT CTC TGT TTT GAG TT; 25 cycles, annealing temperature 63°C) or Y genotype (forward primer TTT GTT CTC ACT GCC ACG) were run. The PCR products were diluted 1:100 in distilled water. MBL exon 1 was amplified from these dilutions with an extra GC-clamp attached (forward primer with clamp CCG CCC GCC GCG CCC CGC GCC CGG CCC GCC GCC CCC GCC CCG TGT TCA TTA ACT GAG ATT AAC CTT C; reverse primer CAG AAC AGC CCA ACA CG). The amplified DNA was run overnight on a 6% polyacrylamide gel containing a denaturing gradient linearly increasing from 35% to 55% formamide and urea. All exon 1 genotypes had different patterns of migration. The corresponding X/Y promoter genotype could be inferred from the presence or absence of a product in the nested PCR.

Statistics

Statistical analysis for the frequencies of the different genotypes was done by Pearson's Chi-square tests or Fisher's exact tests. Logistic regression analysis was performed with univariate statistically significant results and age as a known risk for CAP. A difference of $p<0.05$ was considered statistically significant. All calculations were done by using SPSS version 11.0.

Results

Initially 255 patients were considered eligible for this study; in 201 cases a new infiltrate on chest X-ray was confirmed by an experienced radiologist. In one patient DNA isolation failed (Gram-negative pathogen) and in one patient exon 1 analysis failed (unidentified micro-organism and XY promoter genotype), leaving 199 patients for further analysis. Patient characteristics are shown in Table 1. In 127 cases (64%) the causative micro-organism of CAP was identified. Most frequent identified micro-organisms were: *S. pneumonia*, *H. influenzae*, *Legionella spp.*, *Mycoplasma pneumoniae* and *S. aureus.* Viruses were isolated in 33 patients, either as causative micro-organism of CAP (n = 16), or as co-infection (n = 17). The 223 controls were blood bank donors derived from the same geographical region as the patients.

Table 2 shows the frequency of the six MBL haplotypes and the two functional groups of genotypes, coding for sufficient (YA/YA, YA/XA, XA/XA and YA/0) and deficient (XA/0 and 0/0) MBL serum levels. As shown, frequencies did not differ between patients with CAP in general and controls. Furthermore, we found no differences in frequencies of MBL genotypes between sexes, ages (decades) and Fine-classes within patients, and between patients and controls.

MBL genotype	Controls	CAP	Causative micro-organism					
			S. pneumoniae	*H. influenzae*	*Legionella spp.*	*M. pneumoniae*	Viral	Influenza A/B
Total	223	199	60	14	9	9	9	13
Genotype								
YA/YA	60 (27)	55 (28)	16 (27)	3 (21)	6 (67)[1]	5 (56)	1 (11)	2 (15)
YA/XA	46 (21)	45 (23)	16 (27)	3 (31)	1 (11)	3 (33)	1 (11)	3 (23)
XA/XA	12 (5)	11 (6)	6 (10)	2 (14)	0	0	0	2 (15)
YA/0	66 (30)	51 (26)	12 (20)	4 (29)	2 (22)	1 (11)	3 (33)	2 (15)
XA/0	26 (12)	27 (14)	6 (10)	2 (14)	0	0	3 (33)	3 (23)
0/0	13 (6)	10 (5)	4 (7)	0	0	0	1 (11)	1 (8)
Genotype group								
Sufficient[2]	184 (83)	162 (81)	50 (83)	12 (86)	9 (100)	9 (100)	5 (56)	9 (69)
Deficient[3]	39 (18)	37 (19)	10 (17)	2 (14)	0	0	4 (44)	4 (31)

Table 2 | ***Mannose-binding lectin genotypes in community-acquired pneumonia***

The frequency of the MBL genotypes (n, %) is given for controls and patients with CAP in general, and for patients grouped by the causative micro-organism.

[1] *YA/YA versus non-YA/YA in patients with CAP caused by Legionella spp. compared to controls OR 5.43, CI [1.32-22.41]; p = 0.02*

[2] *A/A + YA/0*

[3] *XA/0 + 0/0*

Table 2 also shows the frequencies of MBL genotypes in patients with CAP caused by *S. pneumoniae*, *H. influenzae*, *Legionella spp.* and *M. pneumoniae.* No differences were found in frequency of MBL genotypes between patients with CAP caused by *S. pneumoniae* and *H. influenzae* and controls. The frequency of genotypes coding for sufficient MBL levels was higher in patients with CAP caused by *Legionella spp.* or *M. pneumonia* than in controls, but this difference did not reach the level of statistical significance. The frequency of the YA/YA genotype in patients with *Legionella* pneumonia was significantly higher than in controls (6/9 (67%) vs. 60/223 (27%), respectively; OR 5.43 CI [1.32-22.41]; p = 0.02). In the three patients in whom other atypical pathogens (*C. psitacci* and *C. burnetii*) were isolated, one had a MBL-deficient genotype.

The six patients with pneumonia with *S. aureus* consisted of 4 patients with a MBL-sufficient genotype and 2 with a deficient genotype. The 72 patients with negative pathogen isolation consisted of 12 (17%) patients with a deficient and 60 (83%) with a sufficient genotype.

Viral pneumonia was diagnosed in 9 patients. The frequency of MBL deficiency was higher in patients with viral pneumonia (Table 2; 4/9; 44%) compared to controls (39/184; 18%), but this difference did not reach the level of statistical significance (p = 0.06). Pure influenza pneumonia was diagnosed in 6 patients, of which 2 were MBL-deficient (33%). In 7 patients only HSV-1 was found. Since this is an uncommon causative agent of CAP and

MBL genotype	Patients	Mortality	ICU admission	Bacteraemia	Length of hospital stay
Total	199	10 (5)	21 (11)	18 (9)	11 (3-153)
Sufficient[1]	162	8 (5)	18 (11)	15 (9)	11 (3-69)
Deficient[2]	37	2 (5)	3 (8)	3 (8)	11 (5-153)

Table 3 | ***Mannose-binding lectin genotypes and outcome of community-acquired pneumonia***
The outcome parameters of community-acquired pneumonia did not differ between patients with MBL-sufficient and deficient genotypes. The number of patients is given per parameter, with percentages between brackets. The length of hospital stay is given in days (median, range).
[1] *A/A + YA/0*
[2] *XA/0 + 0/0*

the risk of contamination originating from oral mucosa is high, they were not considered as having a viral pneumonia.
Positive viral tests were present in a total of 33 patients. Influenza (A and B) was the most frequently isolated and/or cultured virus and frequencies of the MBL genotypes in these patients are shown in the last column of Table 2. The frequency of MBL-deficient genotypes was higher in patients with influenza, but this difference did not reach the level of statistical significance. In the 33 patients in whom any virus was identified (influenza A and B, parainfluenza 1 and 3, HSV-1, respiratory syncytial virus en adenovirus), the frequency of MBL-deficient genotypes (n = 11/33, 33%) was significantly higher than in controls (OR 2.36, CI [1.06-5.26]; p = 0.03), but this difference did not remain statistically significant in the logistic regression model. In younger patients (< 55 year) with viral co-infections the frequency of MBL-deficient genotypes was significantly higher compared to controls (4/9 (44%) versus 20/138 (15%); OR 4.72, CI [1.17-19.23]; p = 0.04). MBL-deficiency was more often present in patients with HSV-1 (4/9 (44%) versus 39/184 (18%); p = 0.06). In patients with CAP caused by *S. pneumoniae* and viral co-infection, the frequency of MBL-deficiency was 3/13 (23%) compared to 39/184 (18%) in controls and 7/40 (18%) in patients with pneumococcal pneumonia without viral co-infection, but these differences did not reach the level of statistical significance. Similar statistically non-significant results were found for patients with pneumococcal pneumonia and influenza A or B co-infection: MBL-deficiency was found in 2/5 (40%) patients, 39/184 (18%) controls and 8/40 (17%) patients with pneumococcal CAP and negative influenza testing (non-influenza viruses or virus negative).
Mortality, number of patients admitted to the ICU, number of patients with bacteraemia or length of hospital stay did not differ between patients with a sufficient or deficient MBL genotype (Table 3).

Discussion

This study shows no role of MBL genotypes in pneumoccocal pneumonia, but a possible role in viral (co-) infections. MBL-sufficient genotypes were more often observed in a small group of patients with CAP caused by *Legionella spp.* and *M. pneumoniae*. MBL genotypes had no effect on clinical outcome.
The role of MBL genotypes in invasive *S. pneumonia* disease has been reported before.[37,38]

Some laboratory findings have suggested a minor role for MBL, as MBL-binding to *S. pneumoniae* was reported to be weak.[11] The results of studies in patients with pneumococcal disease are conflicting; one study reports MBL deficiency as a risk for pneumococcal sepsis,[38] which is not confirmed by a second study.[37] We did not find an increased risk for pneumococcal CAP in patients with MBL-deficient genotypes. Genetic and geographical background of earlier two studies was similar to ours, but there are also many differences. The previously published studies have a retrospective design. Patients with positive blood cultures for *S. pneumoniae* were genotyped, making them different from our patients by origin of the *S. pneumoniae* (pneumococcal CAP versus pneumococcal bacteraemia) and the severity of disease (bacteraemia). Another explanation could be the presence of a (prior) viral (co-)infection. The frequency of MBL-deficiency in patients with positive viral testing was higher compared to controls and negative viral testing, especially for influenza, but these differences did not reach the level of statistical significance. The role of MBL genotypes in invasive *S. pneumoniae* infections remains unclear, but is limited in the susceptibility for community acquired pneumococcal pneumonia, and possibly only present in patients with a viral co-infection.

The number of patients with atypical pneumonia in our group is too small to reach levels of statistically significant differences. However, the complete absence of MBL-deficient genotypes in patients with *Legionella* and *Mycoplasma* pneumonia in our study is remarkable. In contrast to the trend observed in our cohort, MBL deficiency was overrepresented in patients with a primary antibody deficiency and Mycoplasma infections in a previous study. [39] *Legionella pneumophila* can bind MBL and in an outbreak of *Legionella* pneumonia, MBL levels in serum were reported to be low (Kuipers, thesis 2004, personal communication).[40] Although genotypes of these patients were not determined, this suggests the presence of deficient MBL genotypes. A possible explanation for the difference between this finding and ours is that low MBL serum levels could rather be caused by MBL utilization than by MBL genotypes. Or perhaps the multiple Legionella strains in our study interact differently with MBL than the strain in the clonal outbreak setting. The role of MBL deficiency in atypical pneumonia is a subject of further research.

The role of MBL genotypes in human viral infection in pneumonia is unknown. Interaction between viruses and MBL is best described for influenza A virus, which is inactivated by MBL, and there are similar reports for herpes simplex virus type 2.[41,42] We found tendencies for MBL deficiency as a risk for viral pneumonia and co-infection, including pure influenza pneumonia and co-infection with influenza A and B. Similar results were found for patients in whom HSV-1 was isolated. But HSV-1 was not regarded as a causative micro-organism of CAP and positive testing was probably due to re-activation. Positive viral testing in general showed a significant role for MBL genotypes. This significant difference disappeared in multivariate analysis, in which age was a far more effective predictor for viral co-infection than MBL genotypes. Only in young patients, a deficient MBL genotype was found to be a risk factor for viral (co-)infection.

MBL genotypes had no effect on clinical course. This study was underpowered to show the effect on the clinical endpoints death and admission to the ICU, but other severity markers (duration of stay in the hospital and positive blood cultures) did not differ either between the different genotypes. Main limitation of this study was the sample-size. Although we succeeded to identify the causative micro-organism in a majority of the patients, the number of patients with atypical pneumonia and viral co-infections was too small to

identify significant differences in frequency of genotypes. The same is true for the number of patients with *S. aureus*. In an experimental setting MBL deficient mice are highly susceptible to *S. aureus* pneumonia, but the low number of cases with *S. aureus* pneumonia prevents a thorough analysis of this correlation in our cohort.[43]

In summary, MBL genotypes possible play a pathogen dependent role in CAP. Contrary to previous reports, MBL genotypes play no role in pneumonia caused by *S. pneumoniae*. In a small group of patients with pneumonia with atypical, intracellular micro-organisms MBL-sufficient genotypes were observed more frequently. MBL-deficient genotypes were found more often in patients with viral (co-)infection, but not in multivariate analysis containing age. Only young patients with a deficient MBL genotype are at risk for viral co-infection. MBL genotypes have no effect on outcome.

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5

Deficient mannose-binding lectin-mediated complement activation despite MBL-sufficient genotypes in an outbreak of Legionella pneumophila pneumonia

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Abstract

Polymorphisms leading to deficiency of mannose-binding lectin (MBL) are associated with predisposition to infection. However, MBL deficiency can be protective against intracellular pathogens that use MBL to enter host cells. The role of MBL genotype and activity in infection with the intracellular pathogen *Legionella pneumophila* was studied in a large outbreak of legionellosis at a Dutch flower show.

A total of 141 patients, 65 exposed asymptomatic exhibition staff members and 670 unexposed blood bank donors were included for the study of *MBL2* genotypes and MBL-mediated complement activation.

Genotypic MBL deficiency was equally prevalent in patients and controls. Deficient MBL-mediated complement activation was more prevalent in patients. Even in patients with genotypes that confer MBL sufficiency, 20.6% lacked MBL-mediated complement activation. In most patients with MBL-sufficient genotypes who lacked MBL-mediated activation at the acute phase of disease, lectin pathway functionality was restored at convalescence.

In conclusion, genotypic MBL deficiency was not a risk factor for legionellosis. However, patients with legionellosis displayed deficient MBL-mediated complement activation even with MBL-sufficient genotypes. Together, these genotypical and functional data suggest that the observed deficiency of lectin pathway activation is an effect of legionellosis rather than a risk factor for acquiring it.

Introduction

Mannose-binding lectin (MBL) is a pattern recognition receptor of the innate immune system that activates complement via the lectin pathway.[1] Functional MBL is a multimeric molecule, with its subunits organized in a bouquet-like structure.[2] In its multimeric form, it binds to a variety of microorganisms, including respiratory pathogens such as influenza A virus, *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Legionella pneumophila.*[3-5] Single nucleotide polymorphisms (SNPs) in the *MBL2* gene control the level of MBL in serum. Certain combinations of SNPs can lead to MBL deficiency. Coding polymorphisms in exon 1 ("0" alleles B, C and D vs. wild-type A allele) lead to monomeric non-functional MBL

subunits. The X/Y promoter polymorphism determines the serum level of functional MBL multimers by transcriptional control of the wild-type A allele. In healthy individuals, genotypes 0/0 and LXA/0 display MBL levels <0.2 μg/ml and are considered deficient.[6]
MBL deficiency is associated with an increased risk of infection, as opsonization by complement is compromised.[7] In infections with intracellular pathogens, the role of MBL deficiency is more ambiguous, as some intracellular pathogens use opsonization by MBL to enter their host cell.[7]
Although MBL can bind to the intracellular pathogen *Legionella pneumophila*,[5] the role of MBL genotypes and activity as a risk factor for legionellosis is unclear.
We determined *MBL2* genotypes and MBL-mediated complement activation in a retrospective case-control study in the setting of a clonal outbreak of Legionnaires' disease at a flower show in the Netherlands in 1999.[8,9] Since this clonal outbreak had no pathogen variability, this patient cohort offered a unique opportunity to study the interplay between these host factors and legionellosis. The combination of genetic and functional data in this study allowed us to distinguish the influence of *MBL2* polymorphisms on MBL activity from the effect of legionellosis itself.

Materials and Methods

Patients and controls

Patient criteria were described earlier in detail.[9] In brief, *Legionella* pneumonia was diagnosed in 188 visitors or exhibition staff members of a flower show, according to the criteria described by the European Working Group on *Legionella* Infections (EWGLI).[10] Informed consent was obtained from 141 hospitalized patients and these were included in the study.
We used two control groups. In the exposed control group, asymptomatic exhibition staff members who had been exposed to *L. pneumophila* as evidenced by seroconversion were included (n=65 for seroconversion controls).[11] A second, unexposed control group consisted of a group of 670 blood bank donors composed of 223 donors for genotypical analysis and 447 different donors for functional analysis. All participants in both control groups gave informed consent.
Data collection and definitions of clinical parameters were previously described in detail.[8,9,11] DNA extracted from whole blood samples and serum samples collected earlier from these study groups were used to determine *MBL2* genotypes and MBL activity as described below. To study MBL-mediated complement activation over time, we defined acute and convalescent phase sera as follows. Serum samples drawn between day 0 and +3 after presentation at the hospital were considered acute phase samples. Samples drawn at day 20 or later were considered convalescent phase samples (IJzerman, submitted).

Genotyping of *MBL2* in whole blood samples

Combined haplotypes of the X/Y promoter and exon 1 SNPs of *MBL2* were determined using a previously described denaturing gradient gel electrophoresis (DGGE) assay with modifications in a nested PCR protocol.[12] Per sample, two PCR assays specific for the promoter X SNP (forward primer ATT TGT TCT CAC TGC CAC C; reverse primer GAG CTG AAT CTC TGT TTT GAG TT; annealing temperature 63°C; 25 cycles; 629 bp) or Y SNP

(forward primer TTT GTT CTC ACT GCC ACG; 628 bp) were run. The PCR products were diluted 1:100 in distilled water. *MBL2* exon 1 was amplified from these dilutions with an additional GC-clamp attached to one primer to meet DGGE requirements (forward primer with clamp: CCG CCC GCC GCG CCC CGC GCC CGG CCC GCC GCC CCC GCC CCT CCA TCA CTC CCT CTC CTT CTC; reverse primer: GAG ACA GAA CAG CCC AAC ACG; 241 bp).
The amplified DNA was run overnight at 75 Volts on a 6% polyacrylamide gel containing a denaturing gradient linearly increasing from 35% to 55% formamide and urea. All *MBL2* exon 1 genotypes could be distinguished by their different patterns of migration. The corresponding *MBL2* X/Y promoter haplotype could be inferred from the presence or absence of a product in the two nested PCR assays. Genotypes 0/0 and XA/0 were considered "MBL-deficient", and genotypes YA/0, XA/XA, XA/YA and YA/YA were considered "MBL-sufficient".[6,13]

Genotyping of *MBL2* in serum samples
To increase the number of patients available for genotypic analysis, genotyping from serum was used, but a stringent protocol for *MBL2* genotyping from serum was set up. Genomic DNA was isolated from 100 µl of serum with the MagNAPure LC robot (Roche Diagnostics, Mannheim, Germany) using the MagNAPure DNA Isolation Kit according to the manufacturer's protocol. To minimize the risk of genotyping errors due to minimal DNA concentrations in these samples, each sample was genotyped in two different nested PCR assays. In the first assay, a 433 bp fragment was amplified (forward primer TAT TTC TAT ATA GCC TGC ACC CA; reverse primer GAG CTG AAT CTC TGT TTT GAG TT; annealing temperature 57°C; 40 cycles) to serve as a template for the consecutive exon 1 PCR. In the second assay, the X and Y SNP-PCR products served as the first step of the nested protocol similar to the whole blood genotyping protocol described above, except for running 40 cycles. In both assays, exon 1 was amplified from 1:100 diluted PCR products and further analyzed as described above. Serum genotyping was only considered reliable when the two different PCR protocols resulted in the same genotype.

Functional MBL assay
Functional MBL levels were determined in serum samples using a hemolytic assay.[14] In this assay, functional MBL levels were determined by measuring complement mediated bystander-hemolysis evoked by binding of MBL to mannan residues on the surface of *Saccharomyces cerevisiae*. To ensure that the rate of hemolysis via the lectin pathway was not limited by shortage of complement components down-stream of MBL, MBL-deficient serum (donor genotype 0/0) was added to the test wells to provide an excess of these components. The functional MBL level in the test sample was calculated from comparison with hemolysis by a standard serum of 1.67 µg/ml MBL.[14] Since this assay measured a down-stream effect of MBL activity rather than the protein concentration itself, levels were expressed as microgram equivalents per milliliter (µg.eq/ml).
In healthy adults, levels below 0.2 µg.eq/ml are considered deficient in this hemolytic assay.[14] To confirm this cut-off value for the seroconversion controls, data on *MBL2* genotypes and MBL-mediated complement activation from this control group were analyzed using a receiver operating characteristic (ROC) curve. The cut-off value derived from the ROC-curve analysis was used to assess MBL-mediated complement activation as a predictor of MBL-deficient genotypes 0/0 and XA/0 in patients and seroconversion controls.

Statistical analysis

Patients were compared to controls to assess whether *MBL2* genotypes and MBL-mediated complement activation are risk factors for *L. pneumophila* pneumonia. The highest MBL-mediated complement activation measured in multiple serum samples per patient was used for overall analysis.

Groups were compared first by means of univariate analysis. For normally distributed continuous variables, a t-test was used after correction for inequality of variances (based on Levene tests). Categorical variables were analyzed with a Pearson's chi-square test or a Fisher's exact test. To adjust for confounders, multivariate logistic regression models using backward stepwise elimination by likelihood ratio tests were used. Where appropriate, randomly selected seroconversion controls matched by sex and age within 2 years of each legionellosis patient were used in analysis. Mean MBL functionality was compared by genotype between patients and controls using a univariate analysis of variance (ANOVA). Data were analyzed with SPSS software version 15.0 (SPSS, Chicago, USA).

Results

In this study of a clonal outbreak of legionellosis in 188 patients at a flower show, 141 hospitalized patients, 65 exposed asymptomatic exhibition staff members with seroconversion and 670 unexposed blood bank donor controls were included to assess *MBL2* genotypes and MBL-mediated complement activation.

MBL2 genotypes were determined in whole blood samples available from 78 of 141 patients, 53/65 seroconversion controls and 223/670 blood bank donors. Serum samples for genotyping were available from 111 of 141 patients. With the use of a stringent protocol, 72 patients could be genotyped reliably from these 111 serum samples. Thirty-eight patients were genotyped from both whole blood and serum samples, and no discrepancies were found between the results from these genotyping protocols. By combining whole blood and serum genotyping results, a total of 112 of 141 patients could be included in the final analysis of *MBL2* genotypes.

MBL-mediated complement activation could be determined in serum samples available from 125 of 141 patients (a total of 265 samples available), 59/65 seroconversion controls and 447/670 blood bank donors (1 sample per control). Patients and exposed controls that could not be included (because of the absence of samples) did not differ in age, sex or smoking habits from those included in the study (data not shown).

The ROC curve of data from the seroconversion controls on genotypic MBL deficiency and MBL-mediated complement activation confirmed that the previously reported cut-off value for healthy adults of 0.2 µg.eq/ml was also appropriate in this control group.[14] The ROC curve had an area under the curve of 1, and, using 0.2 µg.eq/ml as cut-off for MBL deficiency, the sensitivity and specificity were 100% in seroconversion controls. The cut-off value of 0.2 µg.eq/ml derived from the ROC-curve analysis was used to categorize MBL-mediated complement activation as sufficient or deficient.

Cases were older than those in both control groups (Table 1; t-tests, $p<0.05$). A non-significant difference in sex was observed, with more patients being male compared to seroconversion controls (2x2 χ^2 test, $p=0.13$).

MBL2 genotypes were equally distributed between patients with legionellosis, seroconversion controls and blood bank donors in univariate analysis (Table 1; 3x6 χ^2 test,

	Patients	Exposed seroconversion controls	Unexposed blood bank donors
MBL2 genotype:			
Total nr tested	112	53	223
Age ± SD (y)	63.8 ± 10.5[a]	45.0 ± 14.3	45.2 ± 12.0
M:F ratio	1.4:1	1.0:1	1.5:1
Sufficient:			
YA/YA	32 (28.6%)	19 (35.8%)	61 (27.4%)
XA/YA	27 (24.1%)	18 (34.0%)	45 (20.2%)
YA/0	30 (26.8%)	12 (22.6%)	66 (29.6%)
XA/XA	8 (7.1%)	1 (1.9%)	12 (5.4%)
Subtotal	97 (86.6%)	50 (94,3%)	184 (82.5%)
Deficient:			
XA/0	10 (8.9%)	3 (5.7%)	26 (11.7%)
0/0	5 (4.5%)	0 (0.0%)	13 (5.8%)
Subtotal	15 (13.4%)	3 (5,7%)	39 (17.5%)
MBL-mediated complement activation:			
Total nr tested	125	59	447
Age ± SD (y)	64.6 ± 10.4[a]	46.0 ± 14.3	45.0 ± 17.2
M:F ratio	1.5:1	0.8:1	1.7:1
Sufficient (≥0.2 µg.eq/ml)	89 (71,2%)	56 (94.9%)	434 (97.1%)
Deficient (<0.2 µg.eq/ml)	36 (28,8%)[b]	3 (5.1%)	13 (2.9%)

[a] *t-test, p<0.05*
[b] *3x2 χ^2 test, p<0.01*

Table 1 | ***Characteristics of patients and controls***
MBL2 *genotypes and MBL-mediated complement activation were determined in patients with legionellosis, exposed controls showing seroconversion for* Legionella pneumophila *without clinical signs of infection and unexposed blood bank donors.*

p=0.27). Also, MBL-deficient genotypes were equally distributed between the three groups (A/A and YA/0 vs. XA/0 and 0/0; 3x2 χ^2 test, p=0.082). When comparing only patients and seroconversion controls, the frequency of genotypic MBL deficiency also did not differ significantly between the two groups (OR 2.6, 95% CI [0.7-9.3]; 2x2 χ^2 test, p=0.14). When patients and seroconversion controls were matched by sex and age, genotypic MBL deficiency remained equally distributed between the two groups (3/30 patients with genotypes XA/0 or 0/0 versus 0/30 matched seroconversion controls; 2x2 Fisher's exact test, p=0.24).

In contrast, deficient MBL-mediated complement activation was more prevalent in patients than in both seroconversion controls and blood bank donors (Table 1; 3x2 χ^2 test, p<0.01). When comparing cases and seroconversion controls, the frequencies of deficient MBL-mediated complement activation differed significantly (OR 7.6, [2.2-25.7]; 2x2 χ^2 test, p<0.001). Also, smoking (OR 2.1 [1.1-4.1]; p=0.03), age (t-test, p<0.001) and diabetes mellitus (OR 1.7 [1.5-1.9]; p=0.01) were associated with legionellosis, while sex (OR 1.6 [0.9-2.9], COPD (OR 0.4 [0.1-1.2]), rheumatic diseases (Fisher's exact test; p=0.30) and malignancies (OR 1.1 [0.2-6.4]) were not. When patients and seroconversion controls were

matched by sex and age, deficient MBL-mediated complement activation remained more prevalent in patients than in matched controls (8/35 patients with MBL-mediated complement activation <0.2 µg.eq/ml versus 0/35 matched seroconversion controls; 2x2 Fisher's exact test, p<0.01). All parameters were included in the logistic regression models.
In the multivariate logistic regression model analyzing genotypic MBL deficiency between patients and seroconversion controls, only smoking (OR 13.7 [3.8-48.5]) and age (exp(B) 0.9 [0.8-0.9]) were significantly associated with Legionnaires' disease. Genotypic MBL deficiency was not associated with Legionnaires' disease.
When the ability to activate complement via MBL, rather than the MBL genotype, was analyzed in the multivariate logistic regression model, deficiency of MBL-mediated complement activation was significantly associated with Legionnaires' disease (OR 11.2 [1.6-76.9]), as were smoking (OR 13.5, [3.9-46.4]) and age (exp(B) 0.9 [0.8-0.9]).
Analyzing the combined genotypical and functional data, the mean level of MBL-mediated complement activation did not differ between patients and seroconversion controls compared by genotype (Figure 1; ANOVA, p=0.974). However, of 97 patients with MBL-sufficient genotypes, MBL-mediated complement activation was sufficient in 77 (79.4%) and deficient in 20 (20.6%). None of the seroconversion controls with MBL-sufficient genotypes showed deficient MBL-mediated complement activation (Figure 1; 2x2 χ^2 test, p=0.001).
The deficiency of MBL-mediated complement activation in patients with MBL-sufficient genotypes was observed during the acute phase of legionellosis (11/41 (26.8%) patients with MBL-sufficient genotypes) as well as the convalescent phase (14/55 (25.5%) patients). Paired serum samples were available from 35 patients with MBL-sufficient genotypes. Ten of these patients (28.5%) had deficient MBL-mediated complement activation in the acute phase. In the convalescent phase, MBL-mediated activation was restored to sufficient levels in 8/10 patients. Two patients continued to show deficient activation, at day 21 (genotype YA/YA) and day 81 (genotype YA/0) respectively.

Discussion

In this case-control study in a large clonal outbreak of legionellosis, we determined *MBL2* genotypes and MBL-mediated complement activation as potential risk factors for acquiring legionellosis. Using a stringent protocol for genotyping from serum samples, genotypes could be determined reliably in a large proportion of patients, for whom no whole blood samples were available.
Genotypic MBL deficiency could not be shown to be significantly associated with legionellosis in this cohort. In contrast, deficiency of MBL-mediated complement activation was observed more frequently in patients than in both control groups. The association of legionellosis with functional lectin pathway deficiency was independent of age, sex or smoking, as demonstrated by the univariate matched analysis and multivariate logistic regression models.
When genotypes were allocated to alternative categories, considering genotypes XA/XA and YA/0 as producing intermediate levels of MBL, or grouping them with the MBL-deficient genotypes,[15] some differences were found between seroconversion controls and blood bank donors (data not shown). However, no differences were found between patients and both control groups. Therefore, legionellosis could also not be shown to be

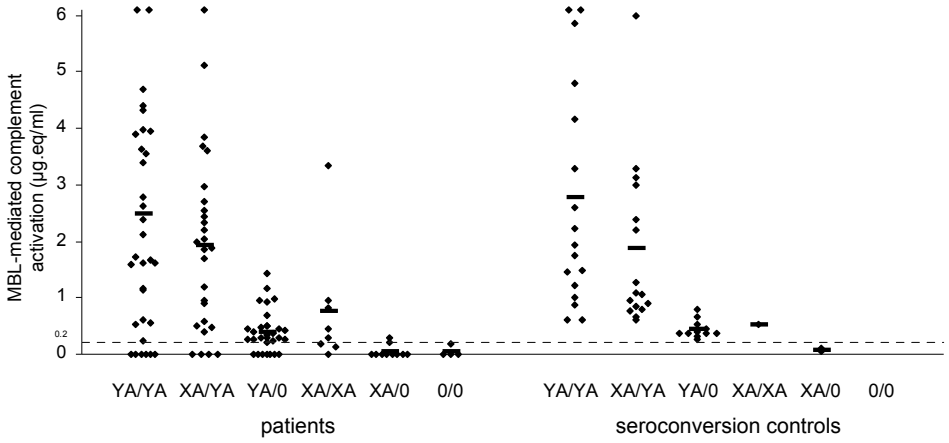

Figure 1 | ***MBL-mediated complement activation in patients and exposed controls***
MBL-mediated complement activation is depicted by genotype in patients with legionellosis and exposed seroconversion controls. The threshold for sufficient MBL-mediated complement activation is plotted (0.2 µg.eq/ml, dashed line). In patients with legionellosis, deficient lectin pathway activation was observed even in subjects with MBL-sufficient genotypes. This was not observed in seroconversion controls (2x2 χ^2 test, p=0.001).

significantly associated with alternative genotype classifications in this study.

The combination of genetic and functional data in our study allowed us to distinguish the influence of *MBL2* polymorphisms on the MBL-mediated complement activation from the effect of legionellosis itself. Recently, reduced MBL-mediated C4 deposition was reported in patients with legionellosis in an outbreak in Melbourne. The authors concluded that deficiency of MBL-mediated complement activation appeared to predispose to Legionnaires' disease.[16] In contrast to the present study, data on *MBL2* genotypes were not available in the Melbourne cohort. Since MBL-deficient genotypes were not associated with legionellosis in our study, the observed deficiency of lectin pathway activation in both our patients and the Melbourne patients more likely is an effect of legionellosis rather than a risk factor for acquiring it.

In a large number of patients, deficiency of MBL-mediated complement activation was observed even though they carried a MBL-sufficient genotype. The lack of lectin pathway activity in patients with MBL-sufficient genotypes could not be explained by exhaustion or deficiency of downstream complement factors, since complement factors other than MBL were exogenously added in the functional assay.

Lectin pathway functionality was restored at convalescence in most patients with MBL-sufficient genotypes who showed absent lectin pathway activation in the acute phase. The recovery of lectin pathway functionality supports the hypothesis that the observed functional deficiency is an effect of legionellosis rather than a risk factor.

The discrepancy between MBL-sufficient genotypes and deficient MBL-mediated complement activation was observed in patients only. The normal relationship between MBL genotypes and complement activation in seroconversion controls in our study suggests that deficient lectin pathway activation does not result from asymptomatic infection.

The MBL-mediated complement activity could have been overestimated in the hemolytic assay used in this study due to MBL-independent complement activation by immune complexes, e.g., those formed by antibodies to *S. cerevisiae.*[17] However, such an effect would also occur in individuals with a MBL-deficient genotype, and we did not observe high levels of hemolysis in these individuals in this study. Instead, classical pathway activity could have led to an underestimation of the frequency of absent MBL-mediated complement activation in individuals with MBL-sufficient genotypes. In the blood bank donors, MBL-deficient genotypes were found more frequently than deficient lectin pathway functionality. This could be due to a sampling error between the two different cohorts used for genotypical and functional analysis. Another possibility is that due to classical pathway activation, deficient lectin pathway activation was underestimated in the cohort used for functional analysis. The potential effects of classical pathway interference described above would not change the observation that in patients with legionellosis, deficient MBL-mediated complement activation was observed even in those with MBL-sufficient genotypes. Therefore, we believe that this potential interference would not affect the main conclusions of this study.

In conclusion, genotypical MBL deficiency could not be shown to be associated with legionellosis in this large clonal outbreak. However, a large number of patients with legionellosis displayed deficient MBL-mediated complement activation even though they carried MBL-sufficient genotypes. In most patients with MBL-sufficient genotypes who showed functional deficiency at the acute phase of disease, the lectin pathway functionality was restored at convalescence. Together, these genotypical and functional data suggest that the observed deficient lectin pathway activation is an effect of legionellosis rather than a risk factor for acquiring it.

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6

Acute-phase responsiveness of mannose-binding lectin in community-acquired pneumonia is highly dependent upon *MBL2* genotypes

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Acute-phase responsiveness of mannose-binding lectin in community-acquired pneumonia is highly dependent upon *MBL2* genotypes

Abstract

Mannose-binding lectin (MBL) is a pattern recognition receptor of the complement system and plays an important role in innate immunity. Whether or not MBL acts as an acute-phase response protein in infection has been an issue of extensive debate because MBL responses have shown a large degree of heterogeneity. Single nucleotide polymorphisms (SNPs) in the promoter (wild-type Y vs. X) and exon 1 (A vs. 0) of the *MBL2* gene can lead to MBL deficiency. This study investigated the influence of SNPs in the promoter and exon 1 of the *MBL2* gene on the acute-phase responsiveness of MBL in 143 patients with community-acquired pneumonia.

Acute-phase reactivity was only observed in MBL-sufficient genotypes (YA/YA, XA/YA, XA/XA and YA/0). In patients with wild-type exon 1 genotype A/A, positive acute-phase responses were associated with the presence of the YA haplotype and negative responses with its absence. Genotypes YA/0 and XA/XA produced equal levels of MBL in convalescence. In the acute phase, however, patients with genotype XA/XA displayed negative acute-phase responses more often than those with genotype YA/0. Correlation of MBL and CRP levels in the acute phase of pneumonia also depended upon the *MBL2* genotype.

In conclusion, acute-phase responsiveness of MBL was highly dependent upon the *MBL2* genotype. These data suggest that heterogeneity in protein responses in the acute phase of disease should always be viewed in the light of possible influences of genetic differences in both structural and regulatory parts of the gene.

Introduction

Mannose-binding lectin is a multimeric pattern recognition protein of innate immunity and activates the lectin pathway of complement. It binds to a variety of microorganisms, including respiratory pathogens like influenza A virus,[1] pneumococci, *Haemophilus influenzae*[2] and *Legionella pneumophila*[3] MBL deficiency has been correlated with increased risk of infection, including repeated respiratory tract infections,[4] as early opsonization is compromised.

MBL levels in serum are influenced by single nucleotide polymorphisms (SNPs) in exon 1 and in the promoter region of the *MBL2* gene. Coding SNPs in exon 1 ("0" alleles B, C and D vs. wild-type A allele) lead to non-functional MBL monomers in homozygotes, impairing early complement activation. The X/Y promoter SNP at position -220 influences MBL serum levels in heterozygotes by controlling transcription of the functional A allele.

Genotypes 0/0 and XA/0 display MBL levels <0.2 µg/ml and are considered deficient.[5,6] Other promoter SNPs are found at positions -550 (H/L) and +4 (P/Q).[5]

It has been suggested that MBL acts as an acute-phase protein responding to inflammation, consistent with its role in early infection.[7] However, this has been extensively debated since MBL responses have shown a large degree of heterogeneity. Conflicting results have been described in post-operative patients,[8-10] patients with severe infections[11] and patients with community-acquired pneumonia (CAP).[12] Although some of these studies considered *MBL2* exon 1 polymorphisms in the analysis, none of them reported the influence of the X/Y promoter SNP on MBL acute-phase responsiveness in individual patients. However, increasing MBL levels in the first 5 days of febrile neutropenia were reported to be associated with the HY haplotype in pediatric oncology patients.[13] Therefore, including the promoter SNP seems important when considering acute-phase responsiveness of MBL.

We determined MBL levels and *MBL2* genotypes in patients with community-acquired pneumonia, and analyzed whether acute-phase responsiveness was associated with the observed genotypes, including the X/Y promoter SNP.

Materials and Methods

Patients and controls

All adult patients (>18 years) presenting with CAP in our general 600-bed teaching hospital during the period from October 2004 to August 2006 were included in this prospective study as described before.[14] Patients with a history of recent hospitalization (<30 days) or a congenital or acquired immunodeficiency (including the use of prednisone 20 mg/day for more than three days) were excluded.

Pneumonia was defined as a new infiltrate on chest X-ray and the presence of two out of the following six clinical signs of pneumonia: cough, production of sputum, signs of consolidation on respiratory auscultation, temperature >38°C or <35°C, C-reactive protein (CRP) 3 times above the upper limit of normal (5 mg/dl), or a leukocyte reaction (leukocytosis (white blood count (WBC) >10 g/l), leukopenia (WBC <4 g/l) or more than 10% rods in the differential count). The chest X-ray was interpreted at presentation in the emergency department by a resident. For this study, it was evaluated the next day by an experienced radiologist who was blinded to the clinical information.

Whole blood samples were taken at day 1 of admission for DNA extraction. Serum samples were drawn at the acute phase (day 1) and during convalescence (day 30 or later) and stored for further analysis. Data on clinical parameters on the day of admission were collected and used to calculate the pneumonia severity index (i.e., Fine-score) as described before.[14,15] The Fine-score stratifies patients with CAP in categories with low (score ≤90; 0.0%-2.8% risk), medium (91-130; 8.2-12.5%) and high (≥130; >25%) risk of mortality.[15] The study was approved by the local Medical Ethics Committee and informed consent was obtained from each patient.

Genotyping of *MBL2*

Combined MBL X/Y promoter and exon 1 haplotypes of *MBL2* were determined using a previously described denaturing gradient gel electrophoresis (DGGE) assay[16] with modifications in a nested PCR protocol.[17] For each sample, two PCR assays specific for the

promoter X SNP (forward primer ATT TGT TCT CAC TGC CAC C; reverse primer GAG CTG AAT CTC TGT TTT GAG TT; annealing temperature 63°C; 25 cycles) or Y SNP (forward primer TTT GTT CTC ACT GCC ACG; same reverse primer and PCR conditions) were run. The two PCR products were separately diluted 1:100 in distilled water. *MBL2* exon 1 was amplified from these two dilutions with an extra GC-clamp attached to one primer to meet DGGE requirements (forward primer with 41-bp clamp: CCG CCC GCC GCG CCC CGC GCC CGG CCC GCC GCC CCC GCC CCT CCA TCA CTC CCT CTC CTT CTC; reverse primer: GAG ACA GAA CAG CCC AAC ACG).

The amplified DNA was run overnight on a 6% polyacrylamide gel containing a denaturing gradient linearly increasing from 35% to 55% formamide and urea. All *MBL2* exon 1 genotypes could be distinguished by their different patterns of migration. The corresponding *MBL2* X/Y promoter haplotype could be inferred from the presence or absence of a product in the in the two nested PCR assays. Genotypes 0/0 and XA/0 were considered "MBL-deficient", and genotypes YA/0, XA/XA, XA/YA and YA/YA were considered "MBL-sufficient".[5,6]

MBL ELISA

Serum levels of the multimeric MBL protein were determined using a commercially available ELISA (Sanquin, Amsterdam, The Netherlands). In short, MBL bound to coated mannan was quantified with the use of an anti-MBL antibody recognizing the multimeric form only.[13]

Statistical analysis

Data on *MBL2* genotypes and MBL protein levels in convalescence were analyzed using a receiver operating characteristic (ROC) curve. The cut-off value derived from the ROC-curve analysis was used to assess the MBL level as a predictor of MBL-deficient genotypes 0/0 and XA/0. Serum levels below this cut-off value were considered deficient.

The difference in MBL level between the acute and convalescent phase sera was analyzed by genotype to measure the influence of the *MBL2* genotype on acute-phase responsiveness of MBL. A decrease or increase of the MBL level by at least 25% in the acute phase compared to the convalescent phase was considered an acute-phase reaction.[11,18] When patients displayed deficient MBL concentrations in both the acute and convalescent phase, they were classified as not showing acute-phase responsiveness and therefore the relative change in MBL level was considered 0%.

Genotype groups were compared first by means of univariate analysis. Unpaired continuous variables were analyzed with a Student's t-test after correction for inequality of variances (based on Levene tests). Normality of the difference between two paired variables was analyzed with use of a Kolmogorov-Smirnov procedure. For paired continuous variables with normally distributed differences, a paired Student's t-test was used. Paired continuous variables without normally distributed differences were analyzed using a Wilcoxon signed ranks test. Categorical variables were analyzed with a Pearson's chi-squared test or Fisher's exact test.

To adjust for confounders, multivariate logistical regression models using backward stepwise elimination by likelihood ratio tests were used. Since the Fine-score incorporates both patient (i.e., age and gender) and clinical characteristics, this score was used as the only extra covariate.

To measure the correlation between parameters, the Spearman's rho or Pearson

MBL2 genotype	n	Age (years ± SD)	Sex (M:F)	Fine-score
Total	143	61.1 ± 16.7	1.6:1	81.2 ± 30.6
YA/YA	43	60.7 ± 15.6	1.2:1	75.5 ± 27.6
XA/YA	34	61.5 ± 17.1	2.1:1	86.2 ± 36.9
YA/0	34	61.9 ± 14.7	2.1:1	87.4 ± 26.0
XA/XA	8	58.5 ± 24.9	1.7:1	76.0 ± 37.6
XA/0	19	59.6 ± 19.3	1.4:1	78.1 ± 31.5
0/0	5	65.6 ± 16.5	0.7:1	75.6 ± 21.4

Table 1 | ***Characteristics of patients with CAP by MBL2 genotype***
No differences were found between the genotype groups in age, sex or fine-score.
n = number of patients, M = male, F = female

correlation coefficient was calculated, depending on the type and distribution of the data. Data were analyzed with SPSS software version 15.0 (SPSS, Chicago, USA).

Results

For 143 of the 201 included patients, whole blood samples and both acute and convalescent phase serum samples were available for analyzing the dynamics of MBL levels in patients with community-acquired pneumonia. In the remaining 58 patients, MBL acute-phase responsiveness could not be analyzed because no convalescent phase serum sample was available. They did not differ from the remaining study group in MBL levels on day 1 of admission (Student's t-test, p=0.25) or *MBL2* genotype (Fisher's exact test, p=0.744). They were older than the remaining study group (67.9 ± 18.5 years vs. 61.1 ± 16.7 years; mean ± SD; Student's t-test, p=0.01) and they showed a higher mortality (9/58 vs. 1/143; Fisher's exact test, p<0.001).

The ROC curve of the data on *MBL2* genotypes and MBL protein levels in convalescence suggested a cut-off value of 0.2 µg/ml for predicting genotypic MBL deficiency. The ROC curve had an area under the curve of 0.961 [95%C.I. 0.925-0.997], and using 0.2 µg/ml as cut-off for MBL deficiency, the sensitivity and specificity were maximized at 87.5% and 91.6%, respectively. Therefore, MBL serum levels below the cut-off value of 0.2 µg/ml were considered deficient.

Patient characteristics are shown in table 1. The *MBL2* genotype groups did not differ in age (ANOVA, p=0.98), sex (χ^2 test, p=0.66), or Fine-score (ANOVA, p=0.50).

In overall analysis, the mean MBL level was increased significantly in the acute phase of disease compared to convalescence (1.51 ± 1.31 vs. 1.30 ± 1.10 µg/ml, mean ± SD in acute phase vs. convalescence, respectively; Wilcoxon signed ranks test, p<0.001). When analyzed by genotype, the mean MBL level was increased significantly only in genotypes YA/YA (2.73 ± 0.86 vs. 2.23 ± 0.88 µg/ml; paired Student's t-test, p<0.001) and XA/YA (2.11 ± 0.85 vs. 1.82 ± 077 µg/ml; paired Student's t-test, p=0.02)(Figure 1). No significant differences between the two time points were found in genotype YA/0 (0.65 ± 0.91 vs. 0.62 ± 0.72 µg/ml; Wilcoxon signed ranks test, p=0.75), genotype XA/XA (0.46 ± 0.38 vs. 0.66 ± 0.38 µg/ml; paired Student's t-test, p=0.124) and genotype 0/0 (0.00 ± 0.00 vs. 0.00

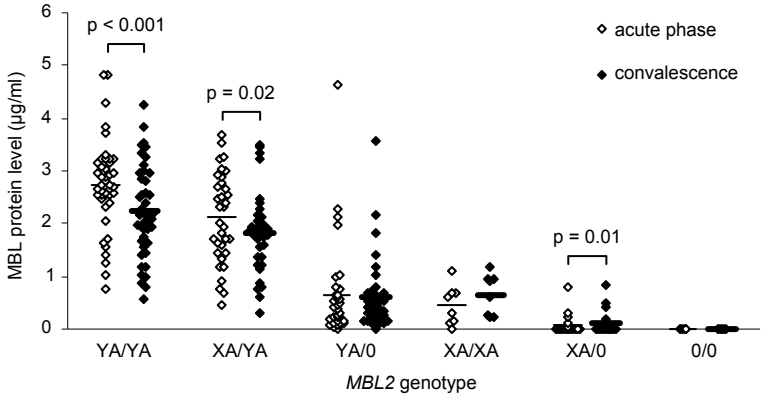

Figure 1 | ***MBL protein levels at the acute and convalescent phase of CAP by* MBL2 *genotype***
Mean MBL levels were significantly increased at the acute phase compared to convalescence in patients with genotypes YA/YA and XA/YA (paired Student's t-test). In patients with genotype XA/0, the mean MBL level was decreased slightly, yet statistically significant (Wilcoxon signed ranks test). (◇/◆= MBL level of an individual patient at the acute phase/convalescence, ▬ = mean)

± 0.00 µg/ml). Statistically, the MBL level was significantly lowered in the genotype XA/0 in the acute phase compared to convalescence, although the actual values and their difference were small (0.08 ± 0.19 vs. 0.11 ± 0.23 µg/ml; Wilcoxon signed ranks test, p=0.01).

The relative change in MBL levels in the acute phase compared to convalescence, and hence the acute-phase responsiveness, also differed between genotypes (Table 2 and Figure 2). Acute-phase responses of MBL were observed in 55 of 143 (38.5%) patients with CAP, of whom 40 (28.0%) showed a positive acute-phase response of MBL and 15 (10.5%) showed a negative response. Significantly more patients with MBL-sufficient genotypes showed acute-phase responses of MBL than patients with MBL-deficient genotypes (53/119 vs. 2/24 patients, respectively; χ^2 test, p<0.01, OR 8.8, 95% C.I. 2.0-39.3]).

In patients with wild-type exon 1 genotype A/A who displayed acute-phase responsiveness of MBL, positive acute-phase responses were observed significantly more often in patients having at least one wild-type promoter Y allele (32/36 patients with genotype YA/YA or XA/YA vs. 1/5 patients with genotype XA/XA; Fisher's exact test, p<0.01). This correlation remained significant when corrected for possible confounding by differences in severity of disease as expressed by the Fine-score in the multivariate logistic regression model (OR 32.0 [95%C.I. 2.9-361.8]).

In all A/A patients, negative acute-phase responsiveness was correlated with the absence of the promoter Y allele. MBL showed a negative acute-phase response in 4 of 8 patients (50.0%) with genotype XA/XA, but in only 4 of 77 (5.2%) patients with either genotype XA/YA (3/34 patients) or YA/YA (1/43 patients) (Table 2; Fisher's exact test; p<0.01). This correlation also remained significant in the multivariate analysis (OR 0.06 [95%C.I. 0.01-0.30]).

Mean convalescent MBL levels were similar in patients with genotypes XA/XA and YA/0 (0.66 ± 0.38 µg/ml vs. 0.62 ± 0.72 µg/ml; Student's t-test; p=0.87). Also in the acute phase,

MBL2 genotype	n	Change in MBL level (mean % ± SD)	APR (n, (%))		
			Total APR	Positive APR	Negative APR
Total	143	14.6% ± 44.2	55 (38.5%)	40 (28.0%)	15 (10.5%)
YA/YA	43	30.8% ± 37.5	20 (46.5%)	19 (44.2%)	1 (2.3%)
XA/YA	34	27.7% ± 60.0	16 (47.1%)	13 (38.2%)	3 (8.8%)
YA/0	34	3.13% ± 35.8	12 (35.3%)	7 (20.6%)	5 (14.7%)[1]
XA/XA	8	-24.1% ± 43.0	5 (62.5%)	1 (12.5%)	4 (50.0%)[1]
XA/0	19	-4.6% ± 13.6	2 (10.5%)	0 (0%)	2 (10.5%)
0/0	5	0.0% ± 0.0	0 (0%)	0 (0%)	0 (0%)

Table 2 | ***Relative change in MBL levels and acute-phase responsiveness of MBL in CAP***
The relative change in MBL levels in the acute phase of CAP compared to convalescence and acute-phase responsiveness (APR) of MBL is given by genotype. An increase (positive APR) or decrease (negative APR) of the MBL level by at least 25% in the acute phase compared to the convalescent phase was considered an acute-phase reaction. Patients displaying deficient MBL concentrations in both the acute and convalescent phase were classified as not showing acute-phase responsiveness and the relative change in MBL level was considered 0%.
[1] negative APR in genotype YA/0 vs. XA/XA; p=0.050, Fisher's exact test

the mean MBL level did not significantly differ (0.46 ± 0.38 µg/ml vs. 0.65 ± 0.91 µg/ml resp.; Student's t-test; p=0.56). However, the mean difference between MBL levels at the acute phase and convalescence differed significantly between the two genotypes (-0.20 ± 0.32 µg/ml in XA/XA vs. 0.04 ± 0.29 µg/ml in YA/0; Student's t-test; p=0.05). Furthermore, negative acute-phase responses were observed significantly more often with genotype XA/XA than with YA/0 (4/8 XA/XA vs. 5/34 YA/0; Fisher's exact test; p=0.05).
There was no significant correlation between MBL and CRP at day 1 of admission in overall analysis of all patients (Figure 3a; Spearman's rho correlation coefficient -0.049, p=0.56). However, in patients with wild-type genotype YA/YA there was a significant correlation between the two parameters (Figure 3b; Spearman's rho 0.41, p=0.01).
The correlation between the Fine-score and MBL levels at day 1 could be analyzed in all 201 included patients. MBL levels at day 1 were not correlated with the Fine-score when all genotypes were included in the analysis (n=201; Pearson correlation coefficient -0.14, p=0.85). However, when only patients with genotype YA/YA were analyzed, a non-significant, negative trend was found between the Fine-score and MBL levels in the acute phase (n=56; Pearson correlation coefficient -0.241, p=0.073). Acute-phase responsiveness, either positive or negative, was not correlated with the Fine-score in the patient group as a whole (n=143), nor in those with genotype YA/YA (n=43)(Spearman's rho, p>0.09 for all analyses).

Discussion

Our results show that acute-phase responsiveness of MBL in patients with CAP was highly dependent upon the *MBL2* genotype. In general, acute-phase responsiveness was

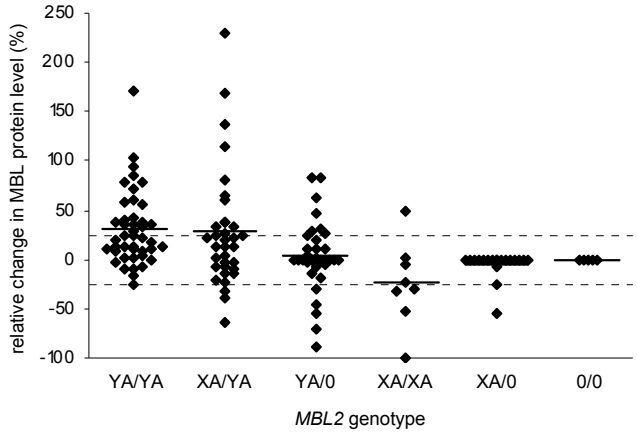

Figure 2 | ***Dynamics of MBL protein levels in CAP according to genotype***
The relative change (percent) of the MBL level in the acute phase of disease compared to the convalescent level is plotted, stratified by MBL2 *genotype. The cut-off for positive (+25%) and negative (-25%) acute-phase responses are plotted (dotted lines). Positive acute-phase responses were associated with the presence of the YA haplotype and negative responses with its absence. (♦ = individual patient, ▬ = mean)*

observed more frequently in patients with MBL-sufficient genotypes than in patients with MBL-deficient genotypes. In patients with MBL-deficient genotypes, MBL levels were low in both the acute and convalescent phase of disease. In patients with wild-type exon 1 genotype A/A, acute-phase responsiveness of MBL was influenced by the promoter X/Y polymorphism. Positive acute-phase responsiveness was associated with the presence of the YA haplotype, while negative acute-phase responsiveness was associated with its absence. MBL and CRP levels at day 1 were not correlated in the study population as a whole. However, there was a significant correlation between them in patients with wild-type genotype YA/YA.

The current definition of acute-phase responsiveness based on relative changes in the protein level does not take into account whether absolute MBL levels are considered deficient. In this study, we considered patients with MBL levels below 0.2 µg/ml at both the acute and convalescent stage of pneumonia to be MBL-deficient at all time points and therefore classified them as not having an acute-phase response of MBL.

The acute-phase response comprises a large number of systemic changes accompanying inflammation that can be distant from the site of inflammation and can involve many organ systems.[18] The concentration of many plasma proteins is changed during the acute-phase response, including several complement components.[18] Whether MBL exhibits an acute-phase response has been an issue of extensive debate since this was first postulated,[7] as MBL responses have shown a large degree of heterogeneity. Monitoring of MBL serum levels after surgery has shown conflicting results.[8-10] When the *MBL2* exon 1 genotype was considered, postoperatively increased MBL levels were found only in patients carrying wild-type exon 1 A/A alleles.[19] In patients with severe infection, no clear acute-phase response was evident from mean MBL levels, even when stratified by exon 1 genotypes (A/A versus A/0 and 0/0).[11] However, 58.6% of the patients did show an acute-

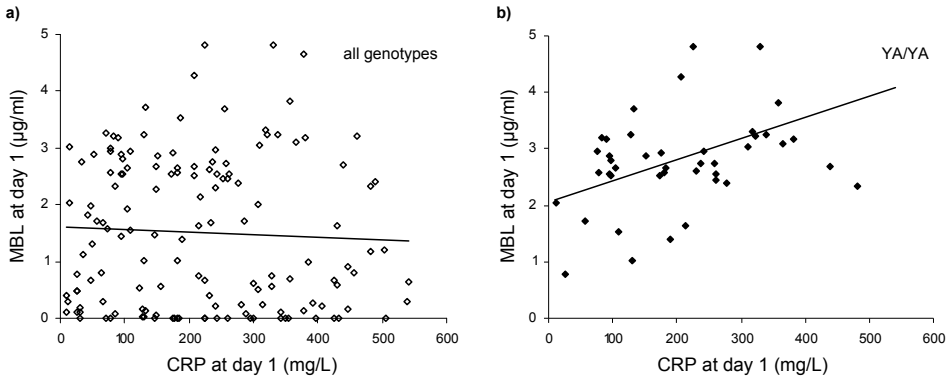

Figure 2 | ***Correlation between MBL protein levels and CRP in CAP***

The correlation between MBL protein levels and CRP in CAP at day 1 of admission are plotted. (a) There was no correlation between MBL and CRP in overall analysis of all genotypes. (b) However, MBL and CRP were correlated in patients with MBL2 *genotype YA/YA (p=0.01). (◇/◆ = individual patient, ▬ = regression line).*

phase response either by increased (31.4%) or decreased (27.3%) MBL levels in the acute phase. It was found also that in community acquired pneumococcal pneumonia, MBL did not act uniformly as an acute-phase reactant in all patients and no correlation with CRP levels was found.[12]

Our results show that besides exon 1 SNPs, the promoter X/Y polymorphism can influence individual MBL acute-phase responsiveness, too. The deficient genotypes, XA/0 and 0/0, cannot display an acute-phase response, as MBL activity is absent at all phases. Positive and negative acute-phase responses in all other genotypes are reflected by the balance of upregulating transcription of the wild-type A allele and the consumption of MBL. The promoter X SNP is thought to hamper this upregulation. The influence of the X/Y promoter SNP on the capability of mounting an acute-phase response could be an explanation for the heterogeneity in MBL acute-phase responsiveness described in the studies above.[10-12]

Our results are in accordance with a previous study showing that the capacity to increase MBL levels from day 1 to day 5 of febrile neutropenia in pediatric oncology patients was associated with the HY promoter haplotype.[13] In contrast to our results, increasing MBL levels were also found in the three pediatric patients with genotype XA/XA. This difference may be due to a difference in study design, as the MBL levels were followed longitudinally during the acute phase rather than comparing acute-phase and convalescent levels. Secondly, no cut-off was used to define whether the change in MBL concentration was considered an acute-phase reaction.

In the current study, a significant correlation between MBL and a known acute-phase reactant, CRP, could be demonstrated when genotypes were taken into account. CRP is capable of activating the classical complement pathway.[20] Interestingly, CRP has also been described to inhibit MBL-mediated complement activation via inhibition of the alternative pathway amplification loop, suggesting a coordinated role for these proteins in complement activation during the acute-phase response.[21]

The effect of the X/Y promoter SNP on acute-phase responsiveness was also reflected in the different responses of genotypes XA/XA and YA/0. Both genotypes displayed

comparable intermediate MBL levels in convalescence. In the acute phase, however, patients with genotype XA/XA displayed negative acute-phase responsiveness of MBL more often. This suggests that genotype XA/XA was less able to upregulate production to compensate for the consumption of MBL in the acute phase than genotype YA/0. Therefore, it could be argued that if certain patient groups were to be supplemented with MBL, this supplementation should not be restricted to MBL-deficient genotypes but should include genotype XA/XA as well.

No apparent relationship between MBL levels or acute-phase responsiveness at day 1 and the Fine-score were found when *MBL2* genotypes were not taken into account. In patients with genotype YA/YA, however, a negative trend was found between the Fine-score and MBL levels in the acute phase. This observation suggests that in severe CAP, the consumption of MBL exceeds the capacity of upregulating production, even in patients with genotype YA/YA.

In conclusion, our data show that MBL acute-phase responsiveness is highly dependent upon the *MBL2* genotype, where the X/Y promoter SNP determines the capability of mounting positive acute-phase responses in individuals with exon 1 wild-type genotype A/A. These data suggest that heterogeneity in protein responses should always be viewed in the light of possible influences of genetic differences in both the structural and the regulatory parts of the gene.

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7

L-ficolin acts as an acute phase reactant in community-acquired pneumonia

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Submitted

L-ficolin acts as an acute phase reactant in community-acquired pneumonia

Abstract

L-ficolin is an activator of the lectin pathway of the complement system and resembles MBL in structure and function. Previously, MBL was shown to act as an acute phase reactant in community-acquired pneumonia which was highly dependent upon *MBL2* single nucleotide polymorphisms (SNPs). This study investigated the acute phase responsiveness (APR) of L-ficolin and the influence of two promoter SNPs (-986G>A and -4A>G) on L-ficolin dynamics in 126 patients with community acquired pneumonia.

L-ficolin levels were increased significantly during the acute phase of pneumonia (+76.2%±104.0%, mean±SD). L-ficolin acted as an acute phase protein in 97 of 126 patients (77.0%), of whom 91 (93.8%) showed a positive APR and 6 (6.2%) a negative APR. L-ficolin levels were positively correlated with levels of C-reactive protein. The relative change in L-ficolin levels or APR did not differ between the respective promoter genotype groups. However, absolute L-ficolin concentrations were increased in patients with the -986G>A promoter SNP and, to a lesser extend, in those with the -4A>G SNP in both the acute and convalescent phase.

In conclusion, L-ficolin acted as an acute phase reactant in CAP independent of the *FCN2* promoter genotype. The relative change in protein level and the frequency of positive APR were even higher than observed for MBL in the same cohort. This suggests that for L-ficolin, either the production is more upregulated, or the consumption is less pronounced than with MBL during the acute phase of community-acquired pneumonia.

Introduction

L-ficolin is a pattern recognition receptor that initiates the lectin pathway of complement (recently reviewed by Matsushita).[1] It binds to a variety of acetylated moieties such as N-acetyl glucosamine (GlcNac), N-acetylmannosamine, N-acetyl galactosamine, N-acetylgalactosamine and N-acetylcysteine and neutral carbohydrates such as β-(1→3)-D-glucan.[2,3] These ligands can be found on the surface of many different bacteria and binding of L-ficolin has been demonstrated to *Salmonella typhimurium* TV119 (a Ra chemotype strain expressing a lipopolysaccharide with a GlcNAc residue at the non-reducing terminus), *Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*.[3-6]

Both in structure and in functionality, L-ficolin is very similar to mannose-binding lectin (MBL), the first discovered initiator of the lectin pathway. Both proteins have a multimeric structure, bind to bacterial surfaces through high avidity binding with multiple globular heads, act as opsonins and activate complement through associating with MASP-2 at the

collagenous region.[7] The main difference is in the nature of the ligand-binding domain, which is a carbohydrate-recognition domain in MBL and a fibrinogen-like domain in L-ficolin. H-ficolin and M-ficolin are two other human ficolins which have the same structural characteristics as L-ficolin.[1]

Recently, we demonstrated that MBL shows acute phase responsiveness during community-acquired pneumonia (CAP), a characteristic which was highly dependent upon structural and promoter polymorphisms of *MBL2*.[8] Structural and promoter SNPs have also been described in *FCN2* and they are known to influence basal L-ficolin levels.[9-11] No data on acute phase responsiveness of L-ficolin and the effect of *FCN2* SNPs have been published to date.

We determined two *FCN2* promoter SNPs (-986G>A and -4A>G) and L-ficolin levels at the acute and convalescent phase in patients with community-acquired pneumonia, and analyzed whether acute-phase responsiveness was associated with the observed genotypes.

Materials and Methods

Patients

All adult patients (>18 years) presenting with CAP in our general 600-bed teaching hospital during the period from October 2004 to August 2006 were included in this prospective study as described before.[8,12] Patients with a history of recent hospitalization (<30 days) or a congenital or acquired immunodeficiency (including the use of prednisone 20 mg/day for more than three days) were excluded.

Whole blood samples were taken at day 1 of admission for DNA extraction. Serum samples were drawn at the acute phase (day 1), during infection (day 2, 3, 5, 10) and during convalescence (day 30 or later) and stored at -80 °C for further analysis. Data on clinical parameters on the day of admission were collected and used to calculate the pneumonia severity index (i.e., Fine-score) as described before, stratifying patients in categories with low (Fine-score ≤90; 0.0%-2.8% risk), medium (91-130; 8.2-12.5%) and high (>130; >25%) risk of mortality.[12,13] The study was approved by the local Medical Ethics Committee and written informed consent was obtained from each patient.

Genotyping of FCN2 promoter SNPs

FCN2 genotypes for the -4A>G SNP was determined using a previously described DGGE assay.[10] The -986G>A promoter SNP was determined by PCR, using two SNP-specific PCR assays with different forward primers (GCA TCC CGA TGG CA and GCA TCC CGA TGG CG) and the same reverse primer (ATG AGA GTA GGT GAA TTT CAC TCC T). PCR was performed in reaction mixtures of 10x SuperTaq Buffer (HT Biotechnology LTD Cambridge, England), 5 U/µl SuperTaq polymerase (HT Biotechnology LTD Cambridge, England), deoxynucleoside triphosphates with dUTP (Roche Applied Science, Mannheim, Germany), 200 nM of each primer (Isogen Bioscience BV, Maarssen, The Netherlands), 400 µg/ml of bovine serum albumin (New England BioLabs, Ipswich, England), and approximately 100 ng of genomic template DNA in a total volume of 25 µl. Forty cycles of denaturation (30 s; 95°C), attachment (30 s; 62°C) and elongation (30 s; 72°C) were passed and PCR products were assessed on agarose gel. The genotype was deduced from the presence or absence of the two products.

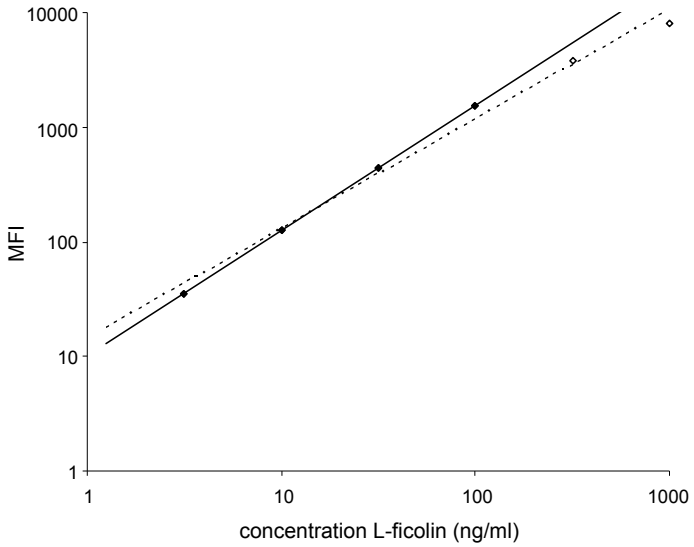

Figure 1 | ***L-ficolin standard curve in the fluorescent-bead-based flow cytometric immunoassay***
The correlation coefficient (R^2) was 0.99 over the complete concentration range tested (◊, dashed line). Linearity was best between 3.16 ng/ml and 100 ng/ml (R^2=1.0; ♦, solid line). (MFI = median fluorescence intensity; representative of 5 experiments)

Determination of L-ficolin levels

A fluorescent-bead-based flow cytometric immunoassay was developed to determine L-ficolin levels, using an ELISA-validated antibody pair as catching and detection antibody, GN5 and GN4-biotin, respectively (kindly provided by Hycult Biotechnologies, Uden, The Netherlands) .[14] A Bio-Plex immunoassay was developed according to the manufacturers instructions and using the manufacturers buffers (Bio-Rad Laboratories, Hercules, CA). The catching antibody GN5 was covalently attached to fluorescent carboxylated beads (Bio-Rad Laboratories, Hercules, CA) and stored in storage buffer. For each assay, the beads were resuspended in assay buffer and 1000 beads were added per well in a 96-well MV Multiscreen filter plate (Millipore, MA) and washed 3 times with washing buffer using a vacuum manifold. Serum samples were diluted 1:250 in serum diluent buffer. An L-ficolin standard (kindly provided by Hycult Biotechnologies, Uden, The Netherlands) was serially diluted in serum diluent buffer to a concentration range of 1000 ng/ml to 3.16 ng/ml (dilution factor $10^{0.5}$). Of each serum sample, the L-ficolin standards (in duplicate) and the negative control (serum diluent buffer), 50 µl was added to 1000 beads per well and incubated for 60 mins on a plate shaker at 300 rpm at room temperature in the dark. The plate was washed 3 times with washing buffer and 25 µl of biotinylated GN4 antibody (10 µg/ml in detection antibody diluent) was added and incubated for 30 mins under the same conditions. After washing, 50 µl of phycoerythrin-coupled streptavidin diluted in assay buffer from 100x stock solution (Bio-Rad Laboratories, Hercules, CA) was added and incubated for 10 mins. The plate was washed 3 times and the beads were resuspended in 100 µl assay buffer for analysis using a Bio-Plex 100 system in combination with the Bio-Plex Manager software, version 4.1.1 (Bio-Rad Laboratories, Hercules, CA). The median fluorescence intensity (MFI) was converted to µg/ml by interpolation from the standard curve.

Genotype		n	Age (years ± SD)	Sex (M:F)	Fine-score
Total		126	61.4 ± 16.8	1.7:1	82.2 ± 31.2
-986G>A	G/G	29	65.2 ± 14.0	1.9:1	89.6 ± 29.2
	G/A	68	59.3 ± 17.6	1.3:1	78.8 ± 30.6
	A/A	29	62.3 ± 17.3	2.6:1	82.8 ± 34.1
-4A>G	A/A	67	65.2 ± 14.5[†]	2.0:1	90.5 ± 29.3[‡]
	A/G	52	58.0 ± 18.3[†]	1.5:1	74.4 ± 31.1[‡]
	G/G	7	50.0 ± 18.1[†]	0.8:1	60.0 ± 27.4[‡]

Table 1 | ***Characteristics of patients with CAP by* FCN2 *promoter genotype***
Patient characteristics are given for each genotype group of the -986G>A and -4A>G promoter SNPs. (n = number of patients, M = male, F = female)
[†] *Welch test, p=0.03*
[‡] *F-test, p<0.01*

Statistical analysis

To determine the potential influence of genotype on protein levels, data on *FCN2* genotypes and L-ficolin protein levels were analyzed in the convalescent sera. The difference in L-ficolin level between the acute and convalescent phase sera was analyzed to determine whether L-ficolin acts as an acute phase reactant in CAP. Next, the dynamics of protein levels were analyzed by genotype to measure the influence of the *FCN2* SNPs on the acute-phase responsiveness of L-ficolin. A decrease or increase of the L-ficolin level by at least 25% in the acute phase compared to the convalescent phase was defined as an acute-phase reaction.[15,16]

Genotype groups were compared first by means of univariate analysis (ANOVA; F-test, or Welch test in case of unequal variances across groups based on Levene tests; Bonferroni post-hoc tests). Unpaired continuous variables were analyzed with a Student's t-test after correction for inequality of variances (based on Levene tests). Normality of the difference between two paired variables was analyzed with use of a Kolmogorov-Smirnov procedure. For paired continuous variables with normally distributed differences, a paired Student's t-test was used. Paired continuous variables without normally distributed differences were analyzed using a Wilcoxon signed ranks test. Categorical variables were analyzed with a Pearson's chi-squared test or Fisher's exact test.

To adjust for confounders, multivariate logistical regression models using backward stepwise elimination by likelihood ratio tests were used. Since the Fine-score incorporates both patient (i.e., age and gender) and clinical characteristics, this score was used as extra single (composite) covariate.

To establish the correlation between parameters, the Spearman's rho correlation coefficient was calculated. Data were analyzed with SPSS software version 15.0 (SPSS, Chicago, USA).

Results

In figure 1, the correlation between the L-ficolin concentration in the bead-based immunoassay and the median fluorescence intensity (MFI) is shown. While the correlation

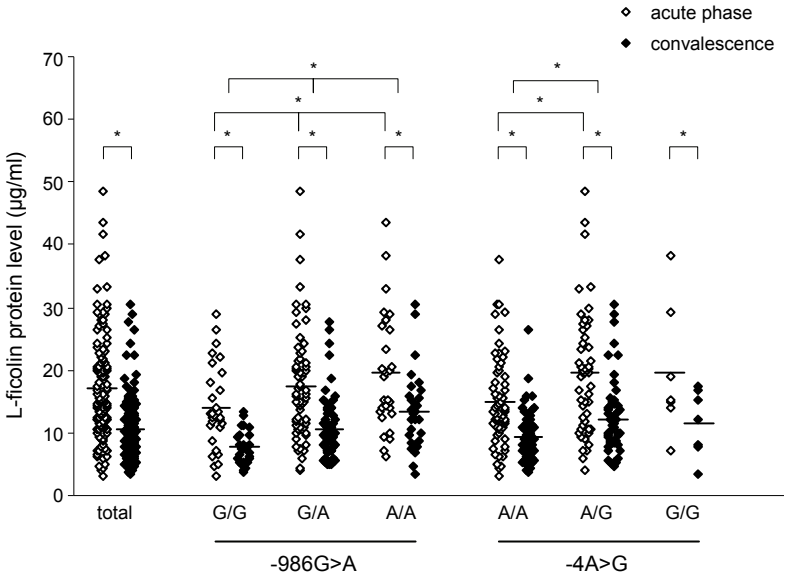

Figure 2 | ***L-ficolin levels in community-acquired pneumonia by* FCN2 *promoter genotype***
*L-ficolin levels were measured at the acute phase of CAP and in convalescence. Mean L-ficolin levels were significantly increased at the acute phase compared to convalescence, independent of the promoter genotypes. The -986G>A promoter SNP significantly increased L-ficolin levels in both the acute and convalescent phase. The -4A>G SNP was associated with increased protein levels, but only between wild-type genotype A/A and heterozygous variant A/G. (◇/◆= L-ficolin level of individual patients at the acute phase/convalescence, ▬ = mean, * = p<0.05)*

coefficient (R^2) over the complete range was 0.99, linearity was best between 3.16 ng/ml and 100 ng/ml (R^2=1.0) due to the high MFI values at the upper concentration range. Serum samples were therefore diluted 1:250 in order to give the most reliable estimate of the L-ficolin concentration.

For 126 of the 201 included patients, whole blood samples and both acute and convalescent phase serum samples were available for analysis of the dynamics of L-ficolin levels. Acute-phase responsiveness could not be analyzed in the remaining 75 patients because convalescent phase serum samples were not available (58 patients) or DNA samples were not available (17 patients). These patients did not differ from the remaining study group in L-ficolin levels on day 1 of admission (17.3 ± 10.4 µg/ml vs. 17.1 ± 8.6 µg/ml; Student's t-test, p=0.93) or age (65.8 ± 18.2 years vs. 61.4 ± 10.8 years; mean ± SD; Student's t-test, p=0.09), but they did show a higher mortality rate (9/75 vs. 1/126; Fisher's exact test, p=0.001).

Patient characteristics are shown in table 1 per genotype for the -986G>A and -4A>G promoter SNPs. For the -986G>A SNP, the genotype groups did not differ in age (ANOVA F-test, p=0.27), sex (χ^2 test, p=0.35), or Fine-score (ANOVA F-test, p=0.30). For the -4A>G SNP, a significant difference was found in age (Welch test, p=0.03) and Fine-score (ANOVA F-test, p<0.01), but not in sex (χ^2 test, p=0.38).

L-ficolin levels in the acute phase were significantly higher than in convalescence (Figure 2; 17.1 ± 8.6 µg/ml vs. 10.6 ± 5.2 µg/ml; Wilcoxon signed ranks test, p<0.001). This significant

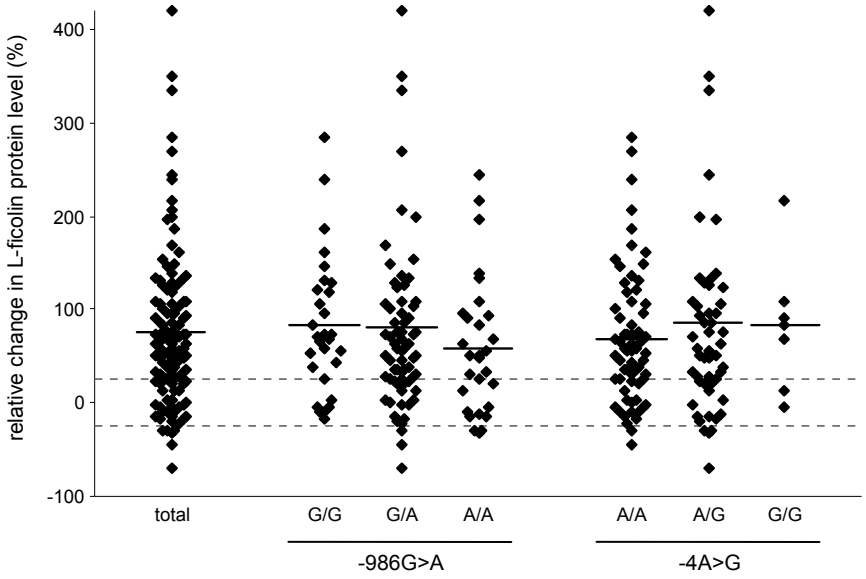

Figure 3 | ***Dynamics of L-ficolin protein levels in CAP by* FCN2 *promoter genotype***
The relative change (percent) of the L-ficolin level in the acute phase of disease compared to the convalescent level is plotted, stratified by FCN2 *promoter genotypes. The cut-off for positive (+25%) and negative (-25%) acute-phase responses is plotted (dashed lines). No effect of the promoter genotypes was found on the mean relative difference in L-ficolin levels. (♦ = individual patients, ▬ = mean)*

increase of L-ficolin levels in the acute phase was found in all genotype groups of the -986G>A and -4A>G SNPs (Figure 2; paired T tests and Wilcoxon signed ranks tests of the acute and convalescent phase within a genotype group, $p<0.05$). When analyzing between genotype groups, a significant effect of the -986G>A SNP was found on the L-ficolin level, both in the acute phase (Figure 2; G/G 14.0 ± 6.7 µg/ml, G/A 17.4 ± 8.8 µg/ml, A/A 19.5 ± 9.2 µg/ml; ANOVA F-test, $p=0.048$) and in convalescence (Figure 2; G/G 7.7 ± 2.5 µg/ml, G/A 10.7 ± 4.9 µg/ml, A/A 13.5 ± 6.3 µg/ml; Welch test, $p<0.001$), with a gradual increase in protein levels from the homozygous wild-type genotype G/G, to the homozygous variant A/A. Also for the -4A>G SNP, a significant difference between genotype groups was found in L-ficolin level in the acute phase (Figure 2; A/A 14.9 ± 7.0 µg/ml, A/G 19.5 ± 9.6 µg/ml, G/G 19.7 ± 10.6 µg/ml; ANOVA F-test, $p=0.01$) and convalescent phase (Figure 2; A/A 9.2 ± 3.8 µg/ml, A/G 12.3 ± 6.2 µg/ml, G/G 11.6 ± 5.3 µg/ml; Welch test, $p=0.02$). However, only the difference between the lower protein levels of genotype A/A compared to levels in genotype A/G was significant in Bonferroni post-hoc analysis ($p=0.004$) and no gradual effect was found.

The relative change in L-ficolin levels in the acute phase compared to convalescence, and hence the acute-phase responsiveness, was not influenced by promoter genotypes (Figure 3 and Table 2). The mean relative increase in L-ficolin levels in the acute phase was 76.2% ± 104.0 and acute-phase responsiveness of L-ficolin was observed in 97 of 126 (77.0%) patients with CAP. Of these 97 patients, 91 (93.8%) showed a positive acute-phase response and 6 (6.2%) showed a negative response. The relative changes in protein levels

Genotype	n	Change in L-ficolin level	Acute phase response (APR) (n, (%))			
		(mean ± SD)	No APR	Total APR	Positive APR	Negative APR
Total	126	76.2% ± 104.0	29 (23.0%)	97 (77.0%)	91 (72.2%)	6 (4.8%)
-986G>A						
G/G	29	82.2% ± 73.9	7 (24.1%)	22 (75.9%)	22 (75.9%)	0 (0.0%)
G/A	68	80.9% ± 124.4	15 (22.1%)	53 (77.9%)	50 (73.5%)	3 (4.4%)
A/A	29	59.1% ± 73.6	7 (24.1%)	22 (75.9%)	19 (65.5%)	3 (10.3%)
-4A>G						
A/A	67	68.6% ± 70.3	16 (23.9%)	51 (76.1%)	45 (73.4%)	2 (3.0%)
A/G	52	85.2% ± 139.1	11 (21.2%)	41 (78.8%)	37 (71.2%)	4 (7.7%)
G/G	7	82.2% ± 72.0	2 (28.6%)	5 (71.4%)	5 (71.4%)	0 (0.0%)

Table 1 | ***Relative change in L-ficolin levels and acute phase responsiveness of L-ficolin in CAP***
The occurrence of acute phase responsiveness (APR) is given for all patients and shown in detail for the promoter genotype groups. An increase (positive APR) or decrease (negative APR) of the MBL level by at least 25% in the acute phase compared to the convalescent phase was considered an acute-phase reaction. No differences were found between the genotype groups in univariate and multivariate analysis.

as well as frequencies of acute phase responsiveness were found to be similar in all the different genotype groups of the two promoter SNPs (Figure 3 and Table 2): no differences were found in univariate analysis between the genotype groups of the -986G>A SNP (ANOVA F-test, p=0.6 and χ^2 test, p=0.96 for the relative change and the acute phase responsiveness, respectively) and the -4A>G SNP (ANOVA, F-test; p=0.7 and χ^2 test, p=0.88). Also when corrected for possible confounding by differences in severity of disease as expressed by the Fine-score in the multivariate logistic regression model, both promoter SNPs were not correlated with acute phase responsiveness (-986G>A: OR 0.9 [95%CI 0.4-1.9] and -4A>G: OR 0.8 [0.4-1.6]).
There was a significant positive correlation between L-ficolin and CRP levels at day 1 of admission in overall analysis (Figure 4; Spearman's rho correlation coefficient 0.46, p<0.001).

Discussion

This study shows that L-ficolin acts as an acute phase protein in community-acquired pneumonia, independent of the -986G>A and -4A>G promoter SNPs. In the acute phase, mean L-ficolin levels were significantly increased by 76.2% compared to convalescence. The majority of patients showed a positive acute phase response and L-ficolin levels were positively correlated with the acute phase reactant CRP. Negative acute phase responses were uncommon. The relative increase in protein level and the high frequency of positive acute phase reactivity was observed to the same extend in all genotype groups of the two promoter SNPs. However, absolute levels of L-ficolin were increased in the presence of the

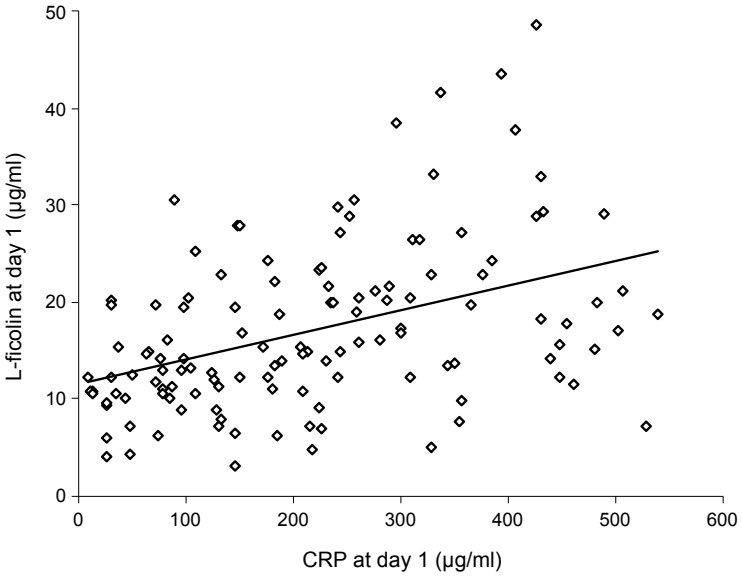

Figure 4 | ***Correlation between L-ficolin protein levels and CRP in CAP***
A positive correlation was found between L-ficolin levels and CRP in CAP at day 1 of admission (R^2 0.46, p<0.001). (◇ = individual patients, ▬ = regression line)

-986G>A and -4A>G promoter polymorphisms in both the acute and convalescent phase.
For this study, we adapted an existing L-ficolin ELISA to the format of a fluorescent-bead-based flow cytometric immunoassay. In this assay, the read-out of the detection antibody was also fluorescence based, with a high discriminatory potential in the range of 1 to 1000 ng/ml. Therefore, serum samples could be diluted to a higher extend than in the conventional ELISA, thus requiring a 15-fold lower volume of serum.[14] Furthermore, a fluorescent-bead-based assay can be used to measure multiple targets simultaneously in a multiplex setup. We set out to combine the L-ficolin assay with an assay for H-ficolin, using a previously described anti-H-ficolin antibody 4H5 (Hycult Biotechnologies, Uden, The Netherlands).[17] However, this anti-H-ficolin antibody cross-reacted with L-ficolin bound on the GN5 coated bead (data not shown), making it unsuitable for a multiplex setup. Since the anti-H-ficolin antibody is used as both a catching and detection antibody in the previously described ELISA, interference of the measurement of H-ficolin by L-ficolin cross-reactivity is a potential risk in this ELISA assay.[17] Future development of multiplex bead-based ficolin assays will depend on availability of new panels of suitable antibodies.
In a previous study, we described the acute phase responsiveness of MBL and its dependence on *MBL2* promoter and exon 1 polymorphisms in the same cohort of CAP patients.[8] In this cohort, L-ficolin showed a higher relative increase in protein level than MBL, also when compared to wildtype *MBL2* genotype YA/YA (mean increase 76.2% vs. 14.6% and 30.8%, respectively). Also, the frequency of acute phase responsiveness was higher for L-ficolin than for MBL (77.0% vs. 38.5% and 46.5%, respectively). The difference between L-ficolin and MBL can be explained by the more dramatic effect of the combined promoter and exon 1 *MBL2* haplotypes on MBL levels compared to the effect of *FCN2* promoter SNPs. The latter do influence the absolute levels of L-ficolin, but not to the

extend as to prevent upregulation in the acute phase of infection. This would also explain the lower frequency of negative acute phase responses in L-ficolin compared to MBL (4.8% vs. 10.5%). However, negative acute phase responsiveness was as uncommon in the MBL wildtype YA/YA as in L-ficolin. Thus, the higher increase of L-ficolin compared to the increase of MBL in the YA/YA genotype suggests that L-ficolin is more upregulated during the acute phase than MBL. Alternatively, our data could also be explained by assuming that less L-ficolin is consumed during the acute phase. Unlike our findings with MBL, we found no effect of structural SNPs in the coding regions of *FCN2* (c.707C > T and c.772G > T) on acute phase responsiveness (data not shown).

The significant correlation between CRP and L-ficolin levels at the acute phase of CAP is comparable with the correlation of CRP and MBL in subjects with *MBL2* genotype YA/YA.[8] Besides an activating role in the classical pathway of complement, an inhibiting role is described for CRP in the MBL mediated lectin pathway.[18,19] Although an influence of CRP on L-ficolin mediated lectin pathway activation has not been described, a similar effect as described with MBL can be expected, as CRP exerts its effect on the lectin pathway via inhibition of the alternative pathway amplification loop and C4b binding protein.[19-21]

Promoter polymorphisms in *FCN2* are known to influence basal L-ficolin levels. In healthy individuals, the -986G>A and -4A>G SNPs were reported to be associated with increased protein levels, comparable with the effect observed in our cohort in the convalescent phase.[9,11] In a recent study, a significant increase in mean L-ficolin levels was found in the acute phase of severe malaria compared to convalescence, but individual relative changes were not further investigated.[22] In the same study, a similar effect of the -986G>A *FCN2* promoter SNP was observed on acute phase L-ficolin levels in mild malaria, although the A/A genotype group was too small to observe the “dose-dependent” effect of the SNP as observed in our cohort and the patients with mild malaria showed no increased, but a decreased L-ficolin level during the acute phase. By analyzing the relative change of L-ficolin levels in individual patients rather than mean absolute levels, our study showed that L-ficolin levels are indeed upregulated in the majority of patients. Furthermore, we showed that the -986G>A and -4A>G SNPs are not only associated with increased protein levels compared to wildtype in the convalescent phase, but also when the protein is upregulated during the acute phase of infection.

In conclusion, this study showed that L-ficolin acts as an acute phase reactant in CAP independent of the *FCN2* promoter genotype. However, promoter polymorphisms -986G>A and -4A>G were associated with a higher absolute concentration of L-ficolin, both in the acute and convalescent phase of infection. The relative change in protein level and the frequency of positive acute phase responsiveness were higher than observed for MBL in the same cohort, even in patients with wild-type MBL. This suggests that for L-ficolin, either the production is more upregulated, or the consumption is less pronounced than with MBL during the acute phase of community-acquired pneumonia.

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8
Discussion

Discussion

Mannose-binding lectin and L-ficolin: Similar, yet not the same

Intriguing advances have been made in recent years in identifying the mechanisms and consequences of immune defects of pattern recognition receptors in innate immunity.[1] The subject of this thesis is the interplay between the gene, protein and disease for two of these pattern recognition receptors: mannose-binding lectin (MBL) and L-ficolin. MBL is a multimeric protein that activates complement after binding sugar moieties by its carbohydrate recognition domain (CRD). L-ficolin is a complement-activating, multimeric protein as well. It differs from MBL by its ligand binding domain: a fibrinogen-like domain (FBG), binding acetylated ligands.

MBL and L-ficolin share many similarities in structure, resulting in similar functionality of the two proteins. The bouquet-like, multimeric structure, with stem-like collagenous regions and multiple ligand-binding domains, enables high-avidity binding to repeating structures which may be present on the surface of micro-organisms or on host cells. The collagenous region of both proteins interacts with serine proteases to activate downstream complement. Both proteins act as acute phase proteins and the genes of both proteins harbour multiple polymorphisms that affect their plasma level and amino acid sequence. These similarities, reflecting the redundancy of the innate immune system, pose problems when investigating these proteins in diagnostic procedures or in epidemiological studies.

Although MBL and L-ficolin share this many similarities, they are not the same. The differences in the nature of the ligand-binding domains and in the effect of the promoter and structural polymorphisms are reflected in a different interplay between gene, protein and disease. This points towards differences in biological function.

Pitfalls of MBL and L-ficolin assays: Immunoassays

Since MBL deficiency is associated with infection and autoimmunity, diagnostic procedures to determine MBL levels, lectin pathway activity and *MBL2* genotypes are widely performed as part of disease association studies and immune status assessment in clinical settings. It is well known that when determining MBL levels in these settings, one should use antibodies recognizing only the multimeric form of MBL, as this is the functional form of circulating MBL. Indeed, currently used MBL ELISA's assess functional MBL levels by measuring multimeric MBL bound to mannose-coated surfaces with monoclonal antibodies, as polyclonal detection can give rise to falsely elevated test results by detection of monomers of variant MBL peptides, resulting from SNPs in exon 1.[2]

As far as the ficolins are concerned, this problem does not seem to exist, as no polymorphisms have been identified to date that lead to non-functional monomers. Indeed, a frequently used sandwich ELISA for H-ficolin employs one and the same monoclonal antibody as catching as well as detection antibody, recognizing the fibrinogen-like binding domain without the ability - or need - to distinguish monomers from multimers. However, other problems are faced with immunoassays for the ficolins. As functional polymorphisms in exon 8 affect the ligand-binding properties of L-ficolin, clinical

association studies cannot be performed merely by measuring plasma L-ficolin levels. In such studies, at least there is a need for correcting for exon 8 genotypes, as L-ficolin levels do not solely reflect L-ficolin functionality. An immunoassay, measuring L-ficolin bound to a ligand-coated surface, similar to current MBL assays would be the preferred method, as it would correct for differences in ligand-binding properties. The difficulties with such an assay design for L-ficolin are that no specific ligand is known to date and the antigenic similarities between the different ficolins can lead to cross-reactivity of the presently available antibodies. We found out that setting up a particle-based multiplex flow cytometric immunoassay, using fluorescent distinct beads as a carrier for different ligands to enable the detection of multiple analytes in a single small volume sample, was hampered by the competitive binding of different ficolins to acetylated ligands and the cross-reactivity of the anti-L-ficolin antibody with H-ficolin (data not shown). Recently, a similar cross-reactivity was described for anti-M-ficolin antibodies binding to L-ficolin.[3]

Functional MBL assays, assessing MBL-mediated lectin pathway activity by measuring down-stream components like C4, the membrane attack complex or complement-mediated cell lysis, can be influenced by classical pathway interference.[4] Such interference could lead to false positive test results. Addition of anti-C1q antibodies has shown to inhibit classical pathway interference in functional MBL assays to prevent that MBL deficiency is overlooked and patients are misdiagnosed.[4,5] The alternative pathway of complement normally does not pose this problem of interference, as the dilutions of the test plasma used in the MBL assays do not facilitate alternative pathway activity.[6] The possible interference of ficolins in such assays has not been described, but also was not investigated in detail yet. Although ficolins do not bind to mannan used as the activating surface ligand, competitive binding of MASP-2 may interfere with the downstream activation of complement factors.

Recently, an attempt was made to set up a functional assay for measuring lectin pathway activity as a whole, independent of the activating pattern recognition receptor.[7] N-acetylglucosamine-pentamer conjugated to dipalmitoylphosphatidylethanolamine was used as a ligand to bind MBL and the ficolins. Unfortunately, only MBL and L-ficolin were able to bind and activate complement.

Pitfalls of MBL and L-ficolin assays: Genotyping assays

Since sequencing of the *MBL2* gene revealed 87 polymorphic sites, genotyping of *MBL2* is complicated by the question which SNPs to determine.[8] Although earlier studies sometimes determined only the B allele in exon 1, most recent studies assess all three exon 1 variants, commonly (but not always) combined with genotyping of one or more promoter SNPs. By studying acute phase responsiveness of MBL, we have shown that analyzing combined haplotypes of the X/Y promoter and exon 1 SNPs is crucial for understanding the role of genetic polymorphisms on MBL protein levels.[9]

Ideally, haplotypes should be determined directly, by assays that unequivocally determine the *cis* or *trans* location of multiple SNPs. An example of such an approach is described in chapter 4, where a SNP PCR targeting the X/Y polymorphism of the *MBL2* promoter is combined with a denaturing gradient gel electrophoresis (DGGE) assay to genotype the three exon 1 SNPs.[10] In fact, all DGGE assays accomplish to determine *cis/trans* positioning, as the DGGE pattern is dependent on the sequence of the individual

amplicons of the target gene on both chromosomes in a way that allows single nucleotide changes to be distinguished. A sequencing assay targeting a polymorphic site at the start of the amplicon also can determine *cis/trans* positioning.[11] These techniques cannot be used in all cases, as the distance between the SNPs of interest can exceed the (current) maximum possible amplicon length.

Instead of directly determining *cis/trans* positioning, haplotypes are often inferred from data of different single SNP assays. Based on the frequency and co-occurrence of SNPs, haplotype frequencies are estimated and assigned to individual patients.[12] The accuracy is highly dependent on the size of the study population and infrequent haplotypes may be missed. In our cohort of patients with community acquired pneumonia, the haplotyping software predicted haplotypes for *FCN2* of three SNPs near exon 3 that could be falsified by the DGGE results determining the SNPs on one amplicon (data not shown). Multivariate analysis can also be used to correct for possible confounding by linkage of SNPs. Although multivariate analysis does not distinguish *cis* or *trans* positioning, it does not use falsely assumed haplotypes either.

The consequences of not determining haplotypes are different for *MBL2* and *FCN2*, as the effect of the polymorphisms differ for the two genes. With MBL, exon 1 SNPs encode variant oligopeptide chains that corrupt multimerization. The ratio of wild-type and variant chains eventually determines the amount of functional multimers. Since promoter SNPs influence the amount of transcription of the downstream gene and therefore the ratio of wild-type and variant chains, it is necessary to determine haplotypes of promoter and exon 1 SNPs in heterozygotes. No structural variants interfering with multimerization are known for L-ficolin. The effect of promoter SNPs on L-ficolin levels therefore can be analyzed without constructing haplotypes with structural SNPs. However, when studying the effect on L-ficolin function, combined promoter and exon 8 haplotypes become important, as they determine the ratio between peptide chains with average (wildtype), weaker (Thr236Met) or stronger (Ala258Ser) ligand binding properties and therefore the overall avidity of the multimers.

Interplay between the MBL gene, protein and disease

Besides the static effect of gene polymorphisms on functional MBL levels and the effect of MBL levels on disease susceptibility, disease itself exerts an effect on MBL levels and lectin pathway function. Direct effects of disease on MBL activity are observed in systemic lupus erythematosus with autoantibodies against MBL and in hypothyreoidy reducing MBL production.[13,14]

Indirect effects of disease on lectin pathway functionality are suggested by data from two studies on *Legionella pneumophila* pneumonia. In our study of an outbreak of legionellosis, we found deficient MBL-mediated complement activation despite MBL-sufficient genotypes.[10] In an Australian outbreak study, MBL-mediated lectin pathway activity was impaired in patients compared to healthy controls, while MBL levels did not differ between the groups.[15] Together these data suggest that in legionellosis, MBL-mediated complement activity is inhibited downstream of MBL itself.

MBL levels are further influenced by disease through the combined effect of MBL consumption on the one hand and the increased production during an acute phase response on the other. In CAP, we have shown that these effects are highly dependent of

the *MBL2* promoter genotype, as low-producing promoter alleles could not compensate for MBL consumption, while high-producing alleles could compensate and show net increased MBL levels in the acute phase.[9]

Interplay between the L-ficolin gene, protein and disease

Genetic variants in the *FCN2* gene determine the plasma level and ligand binding avidity of L-ficolin.[16-18] Since L-ficolin is a pattern recognition receptor in early innate immune defence, L-ficolin levels and genetic polymorphisms have been associated with susceptibility to and outcome of infectious diseases, like respiratory infections, streptococcal infections and leprosy.[19-22]

Like with MBL, disease influences the level of L-ficolin. In chapter 6 it is shown that L-ficolin acts as an acute phase protein, since L-ficolin levels are raised significantly during the acute phase of community-acquired pneumonia. A similar increase in L-ficolin levels was observed in the acute phase of severe malaria.[16] In both studies, the L-ficolin levels in the acute and convalescent phase were also influenced by *FCN2* promoter polymorphisms. However unlike with MBL, acute phase responsiveness itself was not determined by promoter polymorphisms (chapter 6). Apparently, low-producing promoter genotypes do not hamper upregulation of transcription and can compensate for L-ficolin consumption during the acute phase of infection, while the *MBL2* promoter variant cannot. This is probably due to the fact that with L-ficolin, even in homozygosity for a low-producing promoter, still both transcripts lead to production of oligopeptides contributing to the formation of functional multimers.

Function of MBL and L-ficolin in inflammation

Both MBL and L-ficolin were shown to act as acute phase proteins (chapters 6 and 7). With an average 1.2-fold increase for MBL (in wildtype heterozygotes) and 1.8-fold increase for L-ficolin in our cohort of community-acquired pneumonia, both proteins exceeded the defined 25% threshold for acute phase responsiveness in the majority of patients (Chapters 5 and 6). It could be questioned how relevant this increase is as compared to the typical >100-fold increase of CRP, the prototype of an acute-phase reactant. The decreased levels of MBL in patients with the low-producing allele XA/0 may hint to a biological relevant function during the acute phase of infection. Apparently, MBL is consumed during the acute phase to an extend that needs swift upregulation of transcription. Secondly, both MBL and L-ficolin levels are positively correlated with CRP. The higher the CRP response, the more MBL and L-ficolin are upregulated. Thirdly, MBL and L-ficolin are initiators of the lectin pathway, that encompasses a self-amplifying cascade. Therefore, even a small increase in the level of these proteins may lead to a much larger increase of the effector proteins downstream.

The positive correlation between MBL, L-ficolin and CRP levels seems contradictory to the ability of CRP to inhibit complement activation via the alternative and lectin pathways. CRP interacts with factor H and C4b-binding protein leading to a decrease in C3- and C5-convertase activity of the alternative pathway, an inhibition of the alternative pathway amplification loop and a reduction in C3b deposition and target lysis via the lectin

pathway.[23,24] The complement-inhibiting properties of CRP are restricted to an activated form of CRP following subunit dissociation, while the native form of CRP is capable of activating the upstream part of the classical pathway by interacting with the globular head of C1q.[25,26] These properties point to a dual role of CRP in inflammation, where native CRP first promotes complement activation, compatible with the co-occurrence of a rise in MBL and L-ficolin levels. After a conformational change on its target sites, CRP limits excessive complement activation, also arising from the increased lectin pathway activity, to prevent collateral damage to the host tissues at the site of an infection.

MBL and L-ficolin in current and future health care

Diagnostic procedures to identify MBL deficiency are widely performed as part of immune status assessment in regular health care settings. MBL deficiency can be diagnosed by determining *MBL2* genotypes, MBL levels or MBL-mediated lectin pathway activity. Different commercial kits are available to easily determine all three parameters. As disease itself may lower MBL levels and/or functionality in patients despite MBL-sufficient genotypes (Chapters 3 and 5), the diagnosis of MBL deficiency should be confirmed by *MBL2* genotyping when decreased MBL levels are found and follow-up samples should be examined to confirm low MBL levels in convalescent samples.

At the moment, procedures to identify MBL deficiency are performed particularly in patients with recurrent infections. As new disease association studies and animal models reveal the involvement of MBL in the pathophysiology of other, non-infectious diseases, MBL diagnostics could expand to other clinical areas as well. MBL deficiency was associated with recurrent infective exacerbation in patients chronic obstructive pulmonary disease (COPD) and with decreased life expectancy in cystic fibrosis.[27,28] In patients on haemodialysis, MBL deficiency was an independent risk factor for all-cause mortality, including infections and cardiovascular disease.[29] In neutropenic patients, conflicting results have been found for MBL-deficiency as a risk factor for infections, possibly due to differences in the depth of bone-marrow suppression and consequent residual phagocytic function necessary for MBL-mediated opsonophagocytis.[30-34] Further studies should reveal whether certain patient groups can be identified in which MBL is of importance during neutropenia. As a protective effect of MBL-deficiency, decreased reperfusion-induced tissue injury after ischaemia was demonstrated in stroke, in therapeutic coronary intervention after myocardial infarction and in renal ischaemia.[35-37] Furthermore, MBL deficiency influences long-term outcome after solid organ transplantation, increasing the risk of infectious complications, but reducing the risk of rejection.[38] In all these settings, measurement of MBL levels and genotypes could have a prognostic value. The association of MBL-deficiency with recurrent meningococcal and pneumococcal infections may warrant vaccination against these pathogens in MBL-deficient individuals.[39,40]

For the ficolins, diagnostic procedures are not yet performed on a large scale in regular health care. The relevance of ficolin variants is not yet clear, and immunoassays and genotyping assays are still in-house assays not ready for large scale testing. Furthermore, no test is currently available to determine ficolin-mediated complement activity. Further studies must reveal whether determining ficolin variants and the development of easily performed diagnostic assays in a clinical setting is desirable. As with MBL, this can be done as part of immune status assessment, but a recent study showing an association between

L-ficolin structural variants and risk of catheter infection in patients on continuous ambulatory peritoneal dialysis seems to promise that a prognostic value of L-ficolin diagnostics may be found eventually.[41]

In the case where MBL-deficiency is a prognostic factor, intervention in the lectin pathway could be of therapeutical value. MBL substitution therapy was safe in the first phase I and II trials in adults and children.[42-44] MBL-deficient neutropenic patients, neonates with decreased MBL levels due to prematurity and MBL-deficient cystic fibrosis patients are considered eligible for further studies on MBL substitution therapy. In case of complement-mediated damage to host tissues, inhibition of the lectin pathway may be beneficial, for example in acute management of stroke or myocardial infarction or in transplant rejection. To this end, specific inhibitors of complement like the staphylococcal lectin pathway inhibitor could be used, although safety issues remain relevant when using bacterial products therapeutically.[45] Another approach might be to inhibit MBL-mediated complement activity by competitive binding of MASP-2, the first enzyme to set of the complement cascade. Although complete ficolin molecules could be used to this end, recombinant collagen-like domains of MBL or ficolins seem safer as they are not expected to have a biological function.

These prospects of diagnostic and therapeutic applications of MBL and ficolins point the way for future research in this field. Further clinical association studies and animal models will identify new patient groups that may benefit from lectin pathway assessment as part of therapeutic management protocols. Because of redundancy in innate immunity, these studies should consider not only a single gene, but look at the molecular network in which the target gene is involved. This can be done by studying haplotypes or by combined analysis of multiple polymorphic sites, or with phenotypical characterization of different aspects of the molecular network involved. One strategy would be to start with measuring lectin pathway activity, and subsequently unravel the details of a found variety with more specific assays. For studying the possible therapeutical use of substitution or inhibition, animal models need to be further developed. However, co-evolution of man and his microbiota may have led to host-specificity of the studied compound, complicating the development of models in other species. For the ficolins, specific functional assays still remain to be developed. The specific ligands needed for these assays could be identified by affinity or inhibition assays.

MBL and L-ficolin: Similar, yet not the same

It remains an intriguing question why genetically derived deficiency does exist for MBL, but not for L-ficolin. Both genes display multiple SNPs that influence the protein function of the respective proteins in a similar way. Three *MBL2* exon 1 SNPs all lead to impaired multimerization, while two *FCN2* exon 8 SNPs alter the ligand binding properties. Apparently, evolution has targeted these genes on multiple occasions, all leading to variation at the same “level” of protein function and preserved the resulting phenotypes. With MBL, the population apparently benefitted with the existence of completely deficient individuals, with possible survival advantages under certain circumstances for those with deficient or intermediate phenotypes. The genetically determined variation in L-ficolin functionality leads to relative small differences in ligand binding affinity. Nevertheless, these differences are preserved in the population by two different balanced

polymorphisms. Whether complete or near-complete deficiency of L-ficolin is associated with a dramatically increased susceptibility to infections is unknown at the present.

Although MBL and L-ficolin share a great number of functional properties in innate immunity, perhaps the functional differences in the setting of infection could explain the difference in the nature of the genetic variation. The protective effect of MBL deficiency has been described for many parasitic infections, including malaria, which could be a driving force behind *MBL2* diversity.[11] Recently, it was demonstrated that L-ficolin levels are not correlated to the risk of malaria, excluding this disease as a shaping factor in *FCN2* diversity.[16] A possible difference in the etiological role in parasitic infections could explain the "population need" for MBL-deficiency, without the need for L-ficolin deficiency. The exclusive binding of mannose by MBL, but not by L-ficolin, could be a molecular basis for this proposed difference in pathogen driven evolution.

Differences in the recognition of (altered) self antigens could be another explanation. Both MBL and L-ficolin are thought to play a role in apoptosis and necrosis by binding late apoptotic and necrotic cells and DNA, favouring phagocytosis and clearance.[46-49] Both molecules interact with the coagulation cascade via MASP-1 and MASP-2.[50,51] However, because of its binding specificity for acetyl groups, L-ficolin can bind to a wide variety of acetylated molecules, including host proteins, other than those bound by MBL.[52] This difference could implicate different functions in for example apoptosis or coagulation and this may have driven evolution of the two molecules in different directions.

As a third hypothesis, the nature of the genetic variation between MBL and L-ficolin may only differ in the mechanism, yet leading to the same outcome. MBL and L-ficolin are engaged in a variety of physiological systems like innate immunity, apoptosis and coagulation. The effect of L-ficolin structural variants on ligand binding has only been demonstrated for acetylated ligands, showing a decrease or increase in binding avidity.[17,18] Perhaps the effect could be much larger with other types of ligands, leading to total loss of binding capability of L-ficolin, and thus mimicking functional deficiency of MBL.

L-ficolin shares its structural and functional properties with human H-ficolin and M-ficolin (summarized in Chapter 1).[53] All three multimeric proteins harbour a fibrinogen-like domain (FBG) responsible for ligand binding and a collagenous region capable of binding MASPs and activating complement. At the amino-acid level, the homology between L-ficolin and M-ficolin is 80%, while the homology with H-ficolin is only 48%. Since the genes for L-ficolin and M-ficolin are both located on chromosome 9, they have probably arisen from gene duplication, as have the *MBL1P1* and *MBL2* genes.[54]

The human ficolins are differentially expressed in tissues, suggesting that they are involved in distinct processes in different anatomical compartments.[55] L-ficolin is produced in hepatocytes and present in plasma, acting as an opsonins and a complement activating pattern recognition receptor. H-ficolin is mainly expressed in the liver and lung, acting in plasma similar to L-ficolin, but also as an opsonin in the alveolar space. M-ficolin is mainly produced in leukocytes, bone marrow and spleen and is found on the surface and in secretory granules of monocytes and only at very low levels in plasma, with a suggested role as phagocytic receptor.[56-58]

The ligand binding specificities also vary between the three ficolins, probably because the FBG domain of L-ficolin harbours four binding pockets, while M-ficolin and M-ficolin have only one (see Chapter 1). The biological relevance of this difference remains to be elucidated. Hypothetically, the different ligand specificities could have been shaped by ligands differences in the respective anatomical compartments where the ficolins are

expressed.

The human ficolin genes all are polymorphic, with SNPs found in the promoter regions, introns and exons.[18,55] For H-ficolin, a frame-shift deletion has initially been described in a patient with recurrent infections, leading to a truncated, non-functional protein.[59] The *FCN2* gene showed the most genetic diversity, especially in the FBG domain. For two of these SNPs it was shown that they alter the affinity of L-ficolin for GlucNac.[18] Interestingly, the ficolin polymorphisms showed a distinct geographical pattern.[55] Since the effect of all of these different SNPs on protein function has not yet been elucidated, it cannot be distinguished whether they have arisen in different populations under the same evolutionary pressure, resulting in similar effects, or that they have been shaped towards different functionality by different challenges specific for each population.

At the first glance MBL and ficolins look very much alike, with similar protein domains, multimeric structure, pattern recognition and complement activating properties. Yet the small differences in exactly these characteristics may give rise to essential differences in biological function, and this might have driven the different evolutionary strategies in shaping polymorphisms of the genes. In their similarities and differences, MBL and ficolins are an excellent example of one of nature's successful strategies: creating necessary redundancy, without unnecessary abundance.

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9

Summary
Samenvatting

Summary

Mannose-binding lectin and L-ficolin: Similar, yet not the same

Intriguing advances have been made in recent years in identifying the mechanisms and consequences of genetic diversity and immune defects of pattern recognition receptors in innate immunity. The subject of this thesis is the interplay between the gene, protein and disease for two of these receptors: mannose-binding lectin (MBL) and L-ficolin. By studying genotypes, protein levels and functionality in a cohort of hospitalized patients with community-acquired pneumonia and in an outbreak of legionellosis, we aimed to obtain more insight into the reciprocal effect of these proteins and pneumonia on each other, in the light of the genetic background of the patients.

Both MBL and L-ficolin are part of the complement system of innate immunity, sharing similarities in structure and function. Both proteins have trimeric collagen-like subunits assembled into higher order oligomers, held together by disulfide bridges at the cysteine-rich N-terminal domain. The multimeric structure exposes multiple ligand-binding C-terminal heads: carbohydrate-recognition domains for MBL and fibrinogen-like domains for L-ficolin. This enables the binding of repeating molecular patterns on pathogens with high avidity, resulting in complement activation and in opsonization for phagocytosis.

Multiple genetic polymorphisms in the gene encoding MBL lead to impaired oligomerization, resulting in deficiency of functional MBL. The gene encoding L-ficolin revealed to be highly polymorphic as well, with two single nucleotide polymorphisms (SNPs) leading to an altered substrate binding affinity of the protein. Both genes harbor promoter SNPs that influence plasma protein levels.

MBL deficiency has been associated with an increased risk in infection. Conflicting results have been published on the effect of MBL deficiency on pneumococcal infections. In our cohort of community-acquired pneumonia, we did not find an association between MBL genotypes and pneumococcal infection. MBL-sufficient genotypes were more frequently found in a small group of patients with atypical pneumonia. In a large outbreak of legionellosis at a Dutch flower show, MBL genotypes were not a risk factor for legionellosis. However, patients with legionellosis displayed deficient MBL-mediated complement activation even with MBL-sufficient genotypes. Together, these genotypical and functional data suggest that the observed deficiency of lectin pathway activation is an effect of legionellosis rather than a risk factor for acquiring it.

MBL and L-ficolin both revealed to act as acute-phase reactants in community-acquired pneumonia. However, the effect of genetic variants on this response differed between the two proteins. With MBL, the acute phase responsiveness was highly dependent upon the genotype. With L-ficolin, the genotype only determined variations in the protein levels, not in the acute-phase responsiveness itself.

At the first glance MBL and L-ficolin look very much alike, with similar protein domains, multimeric structure, pattern recognition and complement activating properties. Yet the small differences in exactly these characteristics may give rise to essential differences in biological function, and this might have driven the different evolutionary strategies in shaping polymorphisms of the genes. In their similarities and differences, MBL and L-ficolin are an excellent example of one of nature's successful strategies: creating necessary redundancy, without unnecessary abundance.

Samenvatting

Mannose-bindend lectine en L-ficolin: Gelijkend, maar niet gelijk

Het begrip over de aard en de effecten van genetische variaties en immuundeficiënties van patroonherkennende receptoren van het aangeboren immuunsysteem wordt steeds groter. Het onderwerp van dit proefschrift is de interactie tussen genetische variatie, eiwit en ziekte van twee van dit soort receptoren: mannose-bindend lectine (MBL) en L-ficolin. Door het genotype, de eiwitspiegels en de functionaliteit te bestuderen bij patiënten die opgenomen werden met een thuis opgelopen longontsteking en bij een uitbraak van veteranenziekte, is getracht meer inzicht te verkrijgen in de wederzijdse effecten van deze eiwitten en longontsteking op elkaar, bezien vanuit de genetische achtergrond.

MBL en L-ficolin maken deel uit van het complementsysteem van de aangeboren afweer, en lijken in opbouw en functionaliteit erg op elkaar. Beide eiwitten zijn opgebouwd uit collageen-achtige trimeren, die samen een oligomere eiwitstructuur vormen, bij elkaar gehouden door zwavelbruggen in het N-terminale deel. Door de multimere structuur heeft het eiwit meerdere ligand-bindende delen: koolhydraat-herkennende domeinen bij MBL en fibrinogeen-achtige domeinen bij L-ficolin. Dit maakt het mogelijk om repeterende moleculaire patronen op ziekteverwekkers met een hoge aviditeit te binden, hetgeen leidt tot complement activering en opsonisatie voor fagocytose.

Het MBL gen kent meerdere genetische variaties (polymorfismen) die oligomerisatie verhinderen en zo leiden tot deficiëntie van functioneel MBL. Het L-ficolin gen bleek ook meerdere polymorfismen te bevatten, waarvan twee leiden tot een verandering in de bindingseigenschappen van het eiwit. Beide genen bevatten variaties in de promoter regio, die invloed hebben op de plasmaspiegels van de eiwitten.

MBL deficiëntie is geassocieerd met een verhoogd infectierisico. Tegenstrijdige resultaten zijn beschreven over het effect van MBL deficiëntie op een infectie met pneumokokken. Wij vonden geen associatie tussen MBL genotypes en pneumokokkeninfecties. Genetische MBL deficiëntie werd wel vaker gezien in een kleine groep patiënten met atypische longontsteking. Bij een grote Nederlandse uitbraak van legionellose bleek er geen verband te bestaan tussen MBL genotypes en infectierisico. De patiënten hadden echter geen MBL-gemedieerde complement activering ondanks MBL-sufficiënte genotypes. Deze gegevens over genotype en functionaliteit suggereren dat de afwezigheid van lectine route activiteit eerder een effect dan een oorzaak van legionellose is.

Zowel MBL als L-ficolin blijken zich tijdens een longontsteking te gedragen als acute fase eiwitten. Het effect van genetische polymorfismen op deze acute fase reactie verschilt echter tussen de twee. De acute fase reactiviteit van MBL is sterk afhankelijk van het genotype, terwijl polymorfismen van L-ficolin wel in het algemeen een effect hebben op de plasmaspiegel, maar niet op de acute fase reactiviteit zelf.

Op het eerste gezicht lijken MBL en L-ficolin sterk op elkaar door hun vergelijkbare eiwitdomeinen, multimere structuur, patroonherkenning en complementactiverende eigenschappen. Maar de kleine verschillen in precies deze kenmerken kunnen aanleiding geven tot wezenlijke verschillen in de biologische functie, waardoor beide genen op een verschillende manier zijn geëvolueerd. In al hun overeenkomsten en verschillen zijn MBL en L-ficolin daarom “gelijkend, maar niet gelijk”.

10
Curriculum vitae

Curriculum vitae

Bjorn Lars Herpers was born on February 16th 1974 in Schaesberg. In 1992 he graduated summa cum laude at Gymnasium Rolduc in Kerkrade, and started to study medical biology at the University of Utrecht. After three years, he started to study medicine as well. He graduated cum laude in medical biology in 1999 and obtained his medical degree in 2001. After one year of residency in internal medicine at Gooi-Noord Hospital under supervision of prof.dr. D.W. Erkelens† and dr. P. Niermeier, he switched to a residency in medical microbiology at the University Medical Center Utrecht and the St. Antonius Hospital Nieuwegein under supervision of prof.dr. J. Verhoef and dr. B.M. de Jongh. During his residency, he started to work on his thesis on genetic polymorphisms in MBL and L-ficolin, two complement-activating pattern recognition receptors. In 2009 he became a medical microbiologist and joined the staff at the Regional Public Health Laboratory Kennemerland in Haarlem.
Bjorn is married to Sonja Herpers-Versluijs and they live together with their children Felix and Lois.

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Dankwoord

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De totstandkoming van dit proefschrift is onlosmakelijk verbonden met de opleiding tot arts-microbioloog in de regio Utrecht. De vrijheid, de middelen en de aansporing die tijdens de opleiding zijn gegeven om dit promotieonderzoek te doen, zijn het gevolg van de visie en de samenwerking van de verschillende opleiders in het Universitair Medisch Centrum Utrecht en het St. Antonius Ziekenhuis Nieuwegein. Jan, Bartelt en Annemarie, bedankt voor het creëren van deze stimulerende opleidingsomgeving. Op beide locaties is er aandacht en ruimte voor de wetenschappelijke onderzoekskant van het vak. Jos en Ger, ook jullie dank ik voor de begeleiding op dit terrein.

De combinatie van opleiding en promotieonderzoek brengt echter ook een praktisch probleem met zich mee: naast de consultentelefoon kan immers niet (altijd) gepipetteerd worden. Gelukkig kon ik hierbij rekenen op de steun van een zeer ervaren researchanalist. Ben, zonder jouw enthousiasme en werklust had dit promotieonderzoek twee maal zo lang geduurd. Dank voor je toewijding en gezelligheid in al die jaren.

In het begin van mijn opleiding in het St. Antonius Ziekenhuis was er een wens om MBL diagnostiek in het laboratorium in te voeren. Bij het zien van de overeenkomsten tussen twee plaatjes van MBL en L-ficolin werd een onderzoeksidee geboren. Heleen, bedankt dat je dit idee hebt omarmd en telkens weer hebt bijgeslepen, in de werkbespreking en in de prettige gesprekken op je kamer. Erik, je idee voor het gebruik van DGGE was niet alleen het begin van de praktische experimenten, maar ook van mijn opleiding in de moleculaire biologie, waarvoor dank. Marie-Monique, bedankt voor je enthousiaste inbreng tijdens je stage. Het was een zeer productieve en leerzame periode.

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Dit proefschrift gaat over kleine verschillen in moleculen die al heel lang bestaan in heel veel diersoorten. De duizelingwekkende schaal in afmeting, tijd en consequenties waarop de processen die bij dit onderzoek aan de orde zijn geweest zich voltrekken, heeft mij er nog meer van overtuigd: homo mensura *ipsi*

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List of publications

List of publications

Microinjection of catalase cDNA prevents hydrogen peroxide-induced motoneuron death
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*Neuroreport, 1999:**10**(12): 2647-50*

Lentivirus-mediated gene transfer in primary T cells is enhanced by a central DNA flap
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*Gene Ther, 2001:**8**(3): 190-8*

IL-7 differentially regulates cell cycle progression and HIV-1-based vector infection in neonatal and adult CD4+ T cells
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*Proc Natl Acad Sci U S A, 2001:**98**(16): 9277-82*

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Herpers BL, de Jongh BM, van der ZK, van Hannen EJ
*J Clin Microbiol, 2003:**41**(10): 4815-6*

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Herpers BL, Immink MM, de Jong BA, van Velzen-Blad H, de Jongh BM, van Hannen EJ
*Mol Immunol, 2006:**43**(7): 851-5*

A patient with *Legionella pneumophila* serogroup-3 pneumonia, detected by PCR
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*Ned Tijdschr Geneeskd, 2005:**149**(36): 2009-12*

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Wiertsema SP, Herpers BL, Veenhoven RH, Salimans MM, Ruven HJ, Sanders EA, Rijkers GT
*J Allergy Clin Immunol, 2006:**117**(6): 1344-50*

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Herpers BL, IJzerman EP, de Jong BA, Bruin JP, Lettinga KD, Kuipers S, Den Boer JW, van Hannen EJ, Rijkers GT, van Velzen-Blad H, de Jongh BM
*Hum Immunol, 2009:**70**(2): 125-9*

Mannose binding lectin plays a crucial role in innate immunity against yeast by enhanced complement activation and enhanced uptake of polymorphonuclear cells
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*BMC Microbiol, 2008:**8**229*

Hemolytic assay for the measurement of functional human mannose-binding lectin: a modification to avoid interference from classical pathway activation
Herpers BL, de Jong BA, Dekker B, Aerts PC, van DH, Rijkers GT, van Velzen-Blad H
*J Immunol Methods, 2009:**343**(1): 61-3*

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Herpers BL, Vlaminckx B, Burkhardt O, Blom H, Biemond-Moeniralam HS, Hornef M, Welte T, Kuijper EJ
*Clin Infect Dis, 2009:**48**(12): 1732-5*

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*Clin Exp Immunol, 2009:**156**(3): 488-94*

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