MANNOSE-BINDING LECTIN IN PREGNANCY COURSE AND OUTCOME

STUDIES IN HEALTHY WOMEN

AND RHEUMATOID ARTHRITIS PATIENTS

FLEUR E. VAN DE GEIJN

Mannose-binding Lectin in Pregnancy
Course and Outcome
Studies in healthy women and rheumatoid arthritis patients

Fleur Elisabeth van de Geijn

This research project was initated by the Department of Rheumatology, Erasmus MC University Medical Center Rotterdam, the Netherlands.

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Studies in healthy women and rheumatoid arthritis patients

Mannose-bindend lectine in het beloop van de zwangerschap en zwangerschapsuitkomst

Studies bij gezonden en patiënten met reumatoïde artritis

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Don’t go through life so fast that you forget to appreciate the beauty around you

Anonymous

Voor mijn ouders

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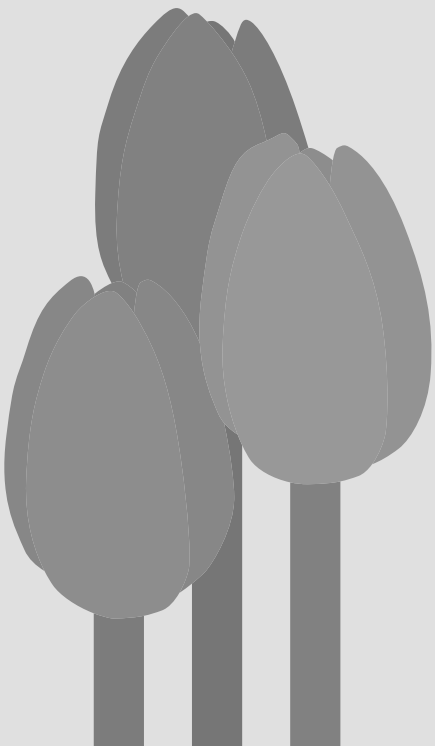
Chapter 1

General introduction



Introduction

Pregnancy is considered an immunological phenomenon since the pregnant mother needs to accommodate the fetal allograft. This requires profound changes of the maternal immune system. In this scope it has been hypothesized that the improvement of autoimmune diseases like rheumatoid arthritis (RA) is a result of adaptations of the immune system during pregnancy. The mechanism underlying this phenomenon and factors that can predict improvement or deterioration of RA are still largely unknown, but multiple hypotheses have been proposed [1-9]. It has been suggested that the adaptive immunity declines during pregnancy, enabling the pregnant body to tolerate the fetus as a semi-allograft. As a consequence, it can be hypothesized that innate immunity compensates during this state of reduced adaptive immunity. Mannose-binding lectin (MBL), a complement factor of innate immunity, has been hypothesized to play a role in the clearance of pathogenic autoantibodies from the circulation, in particular those autoantibodies that lack galactose sugar residues. An increase in MBL levels during pregnancy would therefore result in decreased levels of pathogenic autoantibodies and hence may be associated with decreased disease activity, and the reverse in the postpartum period. Furthermore, if the susceptibility to RA as well as to certain adverse pregnancy outcome measures are associated with low MBL levels, the increased incidence of RA or pre-eclampsia, preterm birth and other adverse pregnancy outcome measures could be the result of shared pathogenic factors.

The aim of the research described in this thesis is to determine whether MBL plays a role in healthy (pregnant) controls as well as in pregnancy-induced improvement of RA and deterioration postpartum. Moreover it is investigated whether MBL polymorphisms are a common pathogenic factor involved both in RA as well as in certain adverse pregnancy outcome measures.

The main objectives of this thesis are:

1. *To determine whether pregnancy has an effect on MBL serum levels and IgG galactosylation*
2. *To determine whether MBL in concert with IgG galactosylation is involved in the pathogenesis of RA in general and more specifically in the pregnancy-induced improvement of RA*
3. *To determine whether MBL is involved in pregnancy outcome in healthy controls and RA-patients*

Next the background, concepts and hypotheses of this thesis will be discussed in more detail.

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disorder characterized by inflammations of the joints, most notably of the hands and feet. However, also extra-articular manifestations are found in some patients, for example in the lungs, heart and vessels. RA has a great impact on patients, not only because of stiffness, loss of energy, impairment of movement and loss of work participation, but also because of the increasing treatment costs and income losses.

RA occurs in 0.5-1% of the world population. It is three times more common in women and it is often diagnosed during women's fertile years [10]. Prevalence increases with age and has an annual incidence of approximately 0.5 per 1000 in males and 0.9 per 1000 in females, resulting in about 150000 patients with RA in The Netherlands (in 2003) [11]. Large budgets on national healthcare costs are spent on management of rheumatic diseases, reflecting the serious implications of the disease.

RA is not a disease of recent times. Evidence of polyarthritis was found in archeological remains dating back to the beginning of our era. The first medical description of rheumatoid arthritis dates to the early 18th century. However, evidence of the existence of RA already appeared in early 17th century art, especially in the form of paintings by Flemish masters (Figure 1)**.** Garrod (1819-1907) was the first to define the disease as 'rheumatoid' arthritis, to distinguish it from rheumatoid fever and gout [12].

The classification criteria for RA are defined by the American College of Rheumatology (ACR) in 1987 [13] and comprise the fulfillment of at least four out of seven criteria that describe the disease. These criteria are mainly based on clinical parameters. Since these parameters are often only partly evident when the damaging effects of the inflammatory process has already initiated, the ACR criteria are not very suitable for the early diagnosis of RA. More indicative for early disease would be the presence of antibodies to cyclic citrullinated peptide (anti-CCP), which occur earlier in the disease course than rheumatoid factor (RF) and are more specific for RA [14, 15]. For prognosis and follow up of the patient the Disease Activity Score (DAS28), acute phase reactants like C-reactive protein (CRP), radiographic detection of erosive disease in the hand and feet joints and the Health Assessment Questionnaire (HAQ) are important.

Treatment

In 1950 major treatment options for RA became available after the discovery of Nobel Prize laureates Hench and Kendal that corticosteroids modify the disease course of RA patients. Nowadays, RA is treated with (a combination of) non-steroidal anti-inflammatory drugs, corticosteroids, disease-modifying antirheumatic drugs (DMARDs) and the relatively new biological agents. Only disease-modifying agents, biologicals, and to some

extent corticosteroids, can impede or halt the inflammatory and destructive processes [16, 17]. The most widely used DMARD is methotrexate (MTX), a cornerstone of most treatment regimens. In the last decade, the treatment of RA has improved considerably due to the widely implemented use of the combination of DMARDs and biologicals (like TNF inhibi-

Figure 1. The Three Graces by Peter Paul Rubens (1636-1638). Findings suggestive of the existence of rheumatoid arthritis appear in 17th century European art. Note swelling of the metacarpophalangeal and proximal interphalangeal joints of the right hand on the outer left figure. Copyright permission obtained by Prado Museum Madrid Spain.

tors). Aggressive early treatment with DMARDs and biologicals results in lower disease activity, prevention of erosions and preserved physical function in RA-patients.
During pregnancy, prednisone, sulfasalazine, hydroxychloroquine and gold injections, and in some cases ciclosporine and azathioprine, are the only RA drugs that can be prescribed safely [18]. Even before conception, all other RA drugs have to be discontinued and washed out to reduce the risk of teratogenicity. Infrequently anti-TNF biologicals are continued until the first trimester of pregnancy, but solid safety data are not yet available.

Pathogenesis of RA

The etiopathogenesis of RA remains largely unclear, but may result from infectious, environmental, genetic and hormonal factors [12, 19]. The geographic distribution of RA is worldwide, with a notably low prevalence in rural Africa and high prevalence in specific tribes of Native Americans [20]. There is no clear association between the prevalence of RA and socioeconomic status [20]. Recently smoking was identified as another causative agent to the development of anti-CCP-positive RA in HLA-DR shared epitope gene positive individuals [21]. Population- and twin studies have shown that genetic factors have some contribution to the pathogenesis of RA. There is excess disease concordance between monozygotic (15%) when compared with dizygotic (3.6%) twins in RA [22]. Genes encoding human leukocyte antigen (HLA-DRB1) shared epitope molecules also have an important contribution to this genetic risk, as well as the protein tyrosine phosphatase non-receptor 22 (PTPN22) gene, which is involved in multiple autoimmune diseases and plays an essential role in signal transduction and is integral in the T-cell antigen receptor signaling pathway [23]. The effect of other individual genes to RA is small, probably since the genetic contribution to the pathogenesis of RA is a result of the interplay of various genes altogether. Hormonal factors could also play a role in RA susceptibility since RA is most prevalent in women. Moreover, a reduced incidence is seen during pregnancy [24] and the onset of RA is sometimes triggered after pregnancy, when the protective immunosuppressive conditions of pregnancy subside [25, 26]. Also nulliparity, breastfeeding and delayed pregnancy have been suggested as risk factors for RA development [22-24]. This is in contrast to oral contraceptive use, which is thought to protect against disease onset and disease progression [25, 27-29].

Adaptive immunity of RA

The presence of autoantibodies produced by B-cells (plasma cells) and the presence of autoreactive T-cells in blood and synovial fluid, is one of the hallmarks of RA that distin-

guishes this disease from other inflammatory and degenerative joint diseases. In the RA-joints, proliferation of synovial cells and infiltration of the synovium by inflammatory cells takes place. The cell types that occupy the inflamed joint include T-cells, B-cells (plasma cells), neutrophils, fibroblast-like synoviocytes, monocytes, macrophages and mast cells [30]. Autoantibodies are already present in the earliest disease stages and may precede disease onset by several years [31]. Rheumatoid factors (RF), i.e. autoantibodies against the Fc moiety of IgG, are the oldest established marker-antibodies in RA and high serum titers of IgG-RF, IgM-RF and IgA-RF are moderately specific for RA. Likewise, the more recently described anti-citrullinated protein antibodies, commonly called ACPA, are even more specific for RA and present even earlier before the first disease manifestation and are abundant in synovial tissue [31]. Both diagnostic tools are associated with more severe and erosive disease and have prognostic value [22]. The somewhat disputed role of B-cells in the pathogenesis of RA is further supported by the fact that B-cell depletion therapy in the form of rituximab (Mabthera®) is quite successful.

Also T-cells are thought to play a role in the pathogenesis of RA. T-cells have several distinct functions in RA like antigen recognition, activation of other cell types through cell-cell interaction and cytokine production, which is also reported in the joints. At least two functional subsets of T-cells can be distinguished according to their cytokine secretion profiles: T helper 1 (Th1) cells, which produce interleukin-2 (IL-2), interferon-γ (IFN-γ) and lymphotoxin and some TNF-α, and T helper 2 (Th2) cells, which secrete IL-4, IL-5 and IL-10. In RA a shift toward T-lymphocytes with a Th1 cytokine secreting profile in the joints can be observed [32]. Pregnancy is associated with a more pronounced Th2 profile. Moreover, also the recently described so-called T helper 17 (Th17 cells), which secrete IL-17 are implicated with pathogenesis and severity in RA [33]. Blockade of cytokines like TNF-α is currently established as an effective therapy for RA [34].

Innate immunity - Complement system

Innate immunity, as opposed to adaptive immunity, provides immediate and early host defence. In innate immune defence all barriers and arms are already in place. This is in contrast to adaptive immune defence which needs several hours to days to recruit appropriate and specific immune cells to mediate an immune response. One of the components of innate immunity next to, for example toll-like receptors (TLRs), dendritic cells (DCs) and macrophages, is the complement system (Figure 2). Three distinct pathways of complement have been identified, i.e. the classical pathway, the alternative pathway and the lectin or mannose-binding lectin (MBL) pathway. The classical complement pathway is triggered by complement component C1q, which can bind to IgG or IgM antibodies that are bound to bacteria or other antigens forming antigen-antibody immune complexes. The alternative pathway is triggered

by direct recognition of microbial surface structures by C3 and properdin. This pathway is independent of immunoglobulins and C1q. Lastly, the lectin or MBL pathway is triggered by MBL bound to carbohydrates on microbial and antigen glycoproteins and glycolipids. In addition, the lectin pathway can be activated by a family of related proteins, the ficolins. These known as L-Ficolin, M-Ficolin and H-Ficolin, also called Ficolin-2, Ficolin-1 and Ficolin-3, respectively [35, 36]. Upon binding of MBL to its target surfaces, MBL-associated serine proteases (MASPs) become activated to serve the same function as C1r:C1s complex in the classical pathway and then activate the complement cascade by cleaving and activating C4 and C2. After C2-activation, the lectin complement pathway is further activated, finally resulting in the formation of the membrane attack complex C5b-9 (MAC). The MAC is the end product

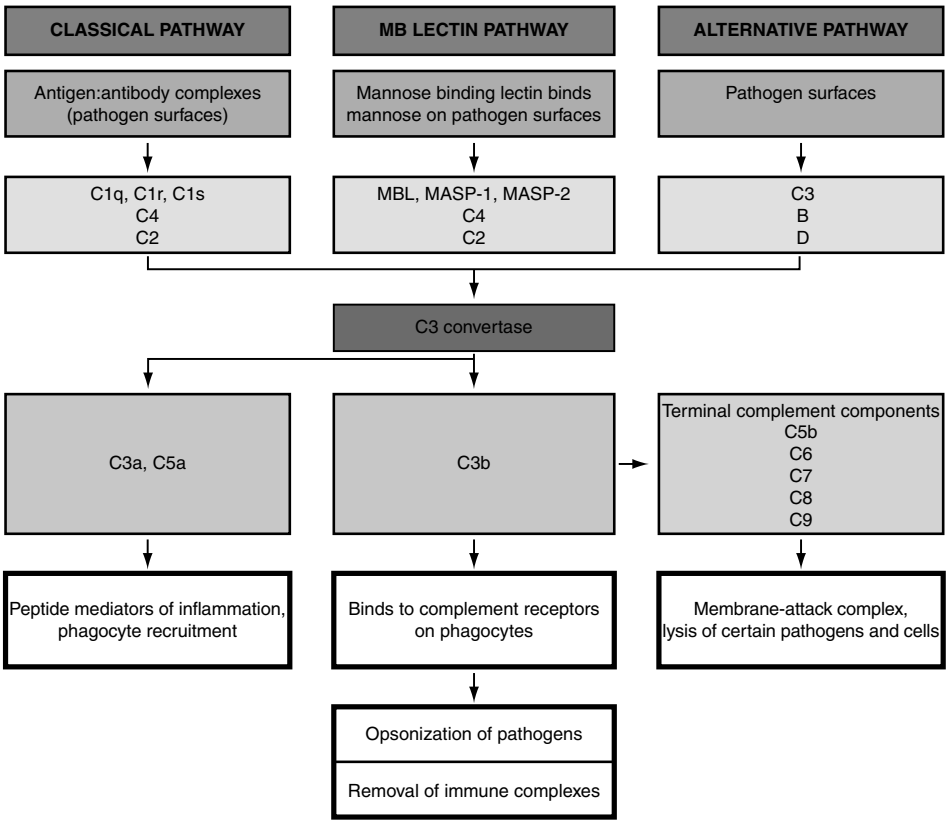


Figure 2. Schematic representation of the activation pathways of the complement system. The (mannose-binding) lectin pathway of complement is activated by MBL and ficolins. On binding to appropriate targets, MBL-MASP complexes cleave C4 and C2 to form C3 convertase. The classical and alternative pathways also generate C3 convertase enzymes, although via different intermediates. These ultimately cleave C3 into C3a and C3b, which have distinct anti-inflammatory properties. The lytic pathway (C5b-C9) is common to all three routes after C3 cleavage. Abbreviations: MBL, mannose-binding lectin; MASP, MBL-associated proteases. Adapted from Janeway 2006.

of the common terminal pathway of all three complement pathways and has many effector-functions including defence against microbes, opsonization of pathogens and the clearance of immune complexes, cellular debris and antigens. The cleavage of C5 results in C5a and C5b. C5b bound to C3b is involved in the common terminal pathway. C5a forms potent anaphylatoxins together with C3a. These anaphylatoxins are chemotactic for neutrophils and phagocytes and induce the release of inflammatory mediators from mast cells. The other splicing product of C3, next to C3a, is C3b that binds to complement receptors on phagocytes, which leads to opsonization of pathogens and also to the removal of (auto-)immune complexes.

Mannose-binding lectin (MBL)

MBL (mannose-binding lectin, but also referred to as mannan-binding lectin and mannose-binding protein) is one of the complement components that activates the lectin complement pathway. MBL is a calcium dependent (or C-type) lectin that is synthesized by the liver. Its production is increased 2- to 3-fold in response to infection and it is rapidly sequestered to sites of inflammation [37]. MBL has many functional and structural similarities to C1q, the comparative protein of the classical complement pathway. Both molecules form a trimeric structure resembling a bunch of tulips [36, 38] (Figure 3), which further oligomerize to form higher order multimers which are able to activate the complement cascade. The concentration of MBL in serum ranges 1000-fold; from 5 ng/ml to more than 5 µg/ml in different individuals, with a median of 1.5 µg/ml [36]. The cut-off level of MBL deficiency is not well established and used to be assay dependent, but generally MBL serum concentrations below 100 ng/ml (0.1 µg/ml) are considered deficient. Therefore approximately 10% of Caucasians are in this MBL deficient group. Additionally, individuals with intermediate MBL serum levels are also considered as having suboptimal MBL serum concentrations, namely approximately 30% of Caucasians, resulting in a large portion of the population being borderline MBL deficient (Table1).

The concentration of MBL in an individual is to a large degree determined by single nucleotide polymorphisms (SNPs) on chromosome 10 region 10q 21-24, in the promoter region and in exon 1 of the *MBL2* gene. The numbering of the gene is due to the existence of a pseudogene, *MBL1*, not giving rise to protein. The *MBL2* wildtype, common allele is the A-allele. The three SNPs of the first (of four) exon(s) are positioned on codon 52 (D-allele, rs5030737), codon 54 (B-allele, rs1800450) and codon 57 (C-allele, rs1800451). Individuals with the homozygous wildtype AA-genotype have the highest MBL serum concentrations. Individuals who are homozygous for the B, C or D alleles are MBL deficient. Individuals who are heterozygous, i.e. who have an A-allele together with a B, C or D-allele, have intermediate MBL serum concentrations. The O-allele represents the variant alleles B, C and D together, resulting in the AA-, AO- and OO-genotype. Basal MBL serum levels are also modified by the SNPs in the promoter region of the *MBL2* gene (H/L or X/Y) [39]. The two SNPs in the promoter

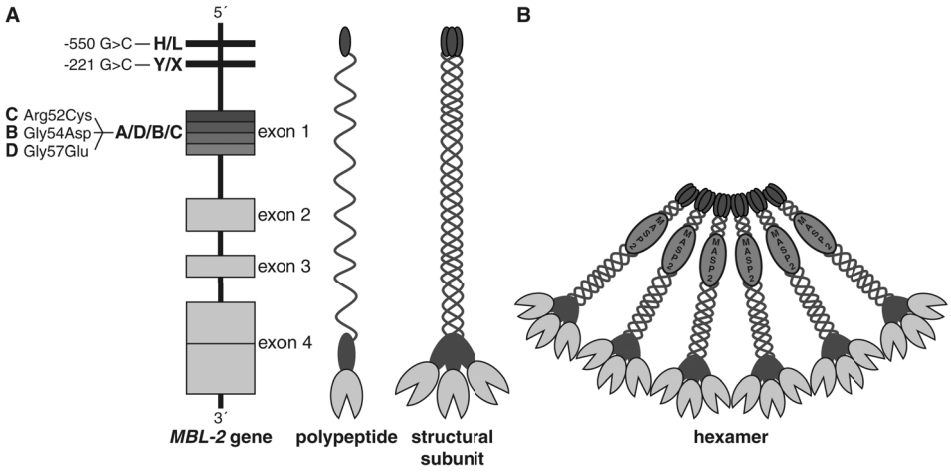


Figure 3. A) Structure of the human *MBL-2* gene and the encoded protein product. Positions of the exon 1 and promoter polymorphisms are shown, including the location of the SNPs described in this thesis. Different regions of the polypeptide are encoded by different exons of the *MBL-2* gene. Three idendical 32-kDa polypeptides form a structural subunit, based on the formation of a collagenous triple helix. In serum, MBL consists of oligomers ranging from dimers to hexamers in a bouquet-like structure. **B)**. Hexamer of human mannose-binding lectin structural subunits which resembles a bunch of tulips. The MBL subunits are cross-liked by disulphide bonds in the N-terminal regions. Each MASP molecule is believed to bind close to the hinge point of the collagenous region. Oligomerization (less structural subunits) results in increased clearance from the serum and reduced activity of the protein. Abbreviations: MBL, mannose-binding lectin; MASP, MBL-associated proteases; SNPs, single nucleotide polymorphisms. Copyright permission obtained from Blackwell Publishing United Kingdom.

region are positioned on codon -550 (H/L, rs11003125) and codon -221 (X/Y, rs7096206) of the *MBL2* gene. Linkage disequilibrium of the exon 1 mutations with the promoter polymorphisms results in six possible haplotypes (i.e. HYA, LYA, LXA, LYB, LYC and HYD) and every individual will express two out of these six haplotypes. The HYA and HYD haplotype induces high MBL concentrations, while the LXA haplotype cause reduced MBL concentrations.

Based upon the haplotypes individuals can be categorized into three groups that correlate best with MBL serum levels [40] and is therefore commonly used and accepted [41]: the high MBL production group A, (H or L)YA/(H or L)YA and (H or L)YA/LXA; the intermediate MBL production group B, LXA/LXA and (H or L)YA/O and the low or deficient MBL production group C, LXA/O and O/O (Table 1).

Table 1. Distribution of the MBL genotype groups in relation to the MBL genotypes

MBL genotype groups	MBL serum levels		Incidence	Genotypes
Group A	High	1.3 - 5.0 µg/ml	57.0%	(H or L) YA / (H or L) YA and (H or L) YA / LXA
Group B	Intermediate	0.6 - 1.3 µg/ml	27.5%	LXA / LXA and (H or L)YA / O
Group C	Low	0 – 0.6 µg/ml	15.5%	LXA / O and O / O

MBL and disease associations

MBL has been intensively studied on associations with multiple medical conditions (i.e. increased risk for disease or for a more complicated course of disease). These association studies may be divided into studies on associations between MBL and infectious diseases, MBL and autoimmune diseases and, lastly, MBL with other diseases or conditions, like pregnancy and pregnancy outcome.

Infections

Reduced MBL concentrations are associated with an increased frequency of infections of both viral and bacterial origin, particularly in children and patients with co-existing immune defects, including primary and secondary immunodeficiencies like after organ transplantation or chemotherapy [42-46]. Other studies have identified specific pathogens to which MBL-deficient individuals are more susceptible, like human immunodeficiency virus (HIV), *Plasmodium falciparum*, *Cryptosporidium parvum* and *Neisseria meningitidis*. However, there are also intracellular parasites (e.g. Mycobacteria, Leishmania and Legionella) to which MBL-deficient individuals may actually be protected [47].

Autoimmune diseases

In some autoimmune diseases such as systemic lupus erythematosus (SLE) cell debris derived from apoptotic cells might trigger an autoimmune response. Since MBL and the ficolins play a role in the clearance of apoptotic cell material removal, deficient serum concentrations of MBL or of the ficolins may lead to inefficient removal of apoptotic cells and therefore increased levels of pathogenic antigens [48]. In SLE MBL deficiency is associated with increased disease activity [49]. Additionally, in RA MBL gene polymorphisms that result in low MBL levels have been associated with disease susceptibility and also disease severity, but results have been conflicting [19, 50-53]. For RA it is not clear yet whether MBL polymorphisms predispose to the (early) development of severe disease since the patient cohorts investigated have been quite diverse and relatively small. Interestingly, it has been shown that MBL-deficient mice displayed defective apoptotic cell clearance, but did not develop autoimmune diseases [37].

Other conditions, pregnancy and pregnancy outcome

Lastly, MBL has also been studied in relation to other diseases and conditions. High levels of MBL are actually a risk factor to the rejection of a transplanted organ in the setting of ischemia and reperfusion [54]. MBL gene polymorphisms have also been studied in relation to outcome in pregnancy. Low MBL, demonstrated either by measuring the actual serum protein or by determining the relevant genotypes, has been associated with adverse pregnancy outcomes, like recurrent miscarriages, risk for chorioamnionitis, preterm

delivery and pre-eclampsia [55-62]. Pre-eclampsia is a common but severe pregnancy disorder. It is characterized by hypertension and *de novo* proteinuria [63] and is associated with high maternal and foetal morbidity and mortality. Abnormal formation of the placenta, placentation, is thought to play an important role in its pathophysiology [64, 65]. During early pregnancy apoptosis is an essential process in the continuous remodelling of the placenta. It has been speculated that MBL, as a scavenger of cellular debris and apoptotic material, might play a role in placentation. Deficient maternal MBL concentrations have also been described as being a risk factor for preterm birth and reduced birth weight [56]. One study showed an association of MBL gene polymorphisms with gestational diabetes mellitus, resulting in heavier infants [66].

To recapitulate, MBL can be regarded as immunomodulatory factor with pleiotropic functions that influences the disease course whether it is present in high or in low concentrations in the serum. It can play an inflammatory role by initiating the lectin pathway of complement, but it can also act anti-inflammatory in the clearance of (auto)immune complexes and cell debris from apoptosis. Finally, MBL may also directly suppress pro-inflammatory cytokine production by monocytes [67, 68], a function which is not yet clearly established.

Glycosylation of immunoglobulins and RA

MBL has been hypothesized to play a role in the clearance of pathogenic autoantibodies from the circulation in RA, in particular those autoantibodies that lack galactose sugar residues. An increase in MBL levels during pregnancy would therefore result in decreased levels of pathogenic autoantibodies and hence may be associated with decreased disease activity in RA-patients.

Autoantibodies, such as those found in autoimmune diseases like RA, belong to the family of immunoglobulins (Ig's), which are glycoproteins. The Ig's are divided into five isotypes based on the differences in the immunoglobulin heavy chains (IgG, IgM, IgA, IgE and IgD). The IgG molecule has two Fab moieties and one Fc moiety, which are built up from two heavy chains and two light chains. The Fab moieties comprise the antigen-binding site formed each by the variable regions of one heavy chain and one light chain. The Fc part in the constant region is formed by dimerization of part of the heavy chains and has the mechanistic ability to bind to complement and also to bind the Fc receptor on various cells.

Immunoglobulins carry variable sugar moieties on their heavy chains, which are associated with age, gender and disease(activity) [69, 70]. Immunoglobulin glycosylation variation has been best described in IgG, but also occurs in IgM and IgA [71, 72]. In IgG each of the heavy chains of the immunoglobulin carries a single N-glycan chain, which can show

variable glycosylation patterns [73] (Figure 4). This variable glycosylation pattern is a result of differences in the presence or absence of the core-linked fucose, the bisecting N-acetylglucosamine (GlcNAc) and/or galactose and sialic acid sugar residues on the outer arms. The outer arms of the N-glycan chains can contain 0, 1 or 2 galactose residues. These sets of oligosaccharides are commonly known as Gal-0 or agalactosyl IgG, Gal-1 or Gal-2, respectively, and the galactose molecules may be situated on either the α1,3 or α1,6 arm of the sugar molecule. On each galactose residue, one sialic acid residue can be present. If the α1,3 or α1,6 arm of the N-glycan does not contain a galactose residue, no sialic residue will be on that arm either.

Abnormal serum IgG glycosylation patterns are observed in RA and in a restricted number of other autoimmune diseases [69, 73-75] and tuberculosis [76]. Compared to healthy controls, higher levels of the agalactosyl IgG are found in RA-patients and these are associated with increased disease activity and a more progressive disease course [19, 73, 77]. These agalactosyl IgG's have also been shown to play a pathogenic role in a mouse model of arthritis [78]. As a result of the absence of galactose, agalactosyl IgG also lacks terminal sialic acid residues. It has been hypothesized that these sialic acid residues are involved in suppressing inflammation by influencing the balance between activating and inhibitory Fcγ-receptors [79]. Therefore, the pathogenicity of agalactosyl IgG may not primarily be due to a lack of galactose residues but due to the absence of sialic acid residues.

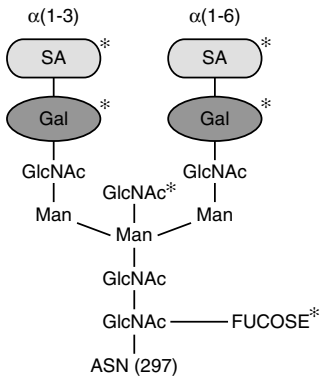


Figure 4. Schematic representation of the bi-antennary complex oligosaccharide structure attached to the asparagine 297 in the CH2 domain of each IgG Fragment crystalizable (Fc) portion. Differences in glycosylation are a result of the presence or absence of the sugar moieties marked with an asterisk (*). Abbreviations: SA, sialic acid; Gal, galactose; GlcNAc, N-acetylglucosamine; Man; mannose; ASN, asparagine. The GlcNAc between the two mannose structures is called the bisecting GlcNAc. Agalactosyl IgG or Gal-0 IgG refers to oligosaccharides completely lacking galactose (Gal).

Pregnancy-induced improvement of RA

In most RA-patients (53-90%) disease activity decreases spontaneously during pregnancy and increases early in the postpartum period [10]. Multiple studies have been undertaken in order to explain the beneficial effect of pregnancy on RA and many hypotheses have been postulated [1-9]. Nevertheless, the exact mechanisms are still unknown. Previously, physicians have tried to copy the favorable effect by treating patients with blood, placental products, or serum from pregnant women without much success [4]. Regarding hormone-based hypotheses, studies revealed that increased cortisol levels did not explain the improvement of RA because of the concomitant elevation of steroid binding globulin and the fact that the timing of gestational improvement does not coincide with the rise of the cortisol levels [80-82]. The elevated levels of sex hormones like estrogens, androgens and progesterone also could not satisfactorily explain the pregnancy-induced remission of RA. Treatment with female hormones exerts no or only a modest effect on disease activity in RA [83]. Prolactin, which is elevated during pregnancy and also during breastfeeding, could not explain pregnancy-induced improvement of RA as well [84].

Many other hormones, but also cytokine levels are increased during pregnancy. Changes in hormone levels have been associated with shifts in cytokine levels and other immunological (cellular) adaptations. Pregnancy is associated with decreased production of Th1 (T-helper 1 cell) associated cytokines (like IL-1, IL-2 and interferon-γ (IFN-γ)) and an increased production of Th2-associated cytokines (like IL-4, IL-5, and IL-10) and a decreased production of pro-inflammatory cytokines (like TNF-α and IL-12) [5]. Since RA is marked by a Th1 cytokine profile, a shift towards a Th2 cytokine profile is an attractive explanation of a pregnancy-induced remission of RA [6].

Another immunological hypothesis for the effect of pregnancy on disease activity in RA is the maternal-fetal disparity in HLA class II alloantigens [7]. The exposure of fetal (paternal) non-self HLA antigens, and in particular HLA-DR and HLA-DQ antigens, urges the maternal immune system to tolerate this semi-allograft in order to maintain pregnancy [7]. This tolerance may result in decreased disease activity. These findings would explain why not all patients would improve during pregnancy. Also the specific expression of HLA-G by the syncytiotrophoblast of the fetus may inhibit maternal NK cells and therefore reduce cytotoxicity by its binding to the killer-cell immunoglobulin-like receptors (KIRs) of the NK cells. This mechanism might play a role in pregnancy-induced improvement of RA as well [2, 7, 8, 73, 85, 86].

Also, during pregnancy, increased levels of α-2 pregnancy-associated protein [46] have been associated with decreased RA-disease activity as well and the reverse postpartum [85, 86]. An alternative hypothesis to explain pregnancy-induced improvement of RA is the action of regulatory T-cells (Tregs), which are induced during pregnancy. Regulatory T-cells may suppress activation and proliferation of conventional T cells and are able to

suppress autoimmunity by secretion of IL-10 [1, 9]. Moreover, in two small studies a decrease in pathogenic agalactosyl IgG has been associated with the pregnancy-induced improvement of RA [2, 3].
In conclusion, it is likely that multiple mechanisms work in concert to induce RA-improvement during pregnancy.

Pregnancy outcome and RA

Pregnancy outcome might be influenced by RA. An association has been described between several rheumatic diseases and unfavorable pregnancy outcome measures [87-91]. However, only a small number of studies examined pregnancy outcome specifically in RA. An increased risk of hypertensive disorders (gestational hypertension and pre-eclampsia) in women with RA has been demonstrated [92, 93], but not all studies support this finding [94-96]. A higher rate of prematurity [91, 95, 96], caesarean sections [93, 95, 96] and spontaneous abortions [97] is also reported, although results on the rate of spontaneous abortions are conflicting [95,98]. Newborns of women with RA were reported to have lower birth weight, especially newborns of women who have a high disease activity [94]. In contrast, the risk of low birth weight (less than 2500 gram) does not seem to be increased in women with RA [94, 95,98], but a higher rate of small for gestational age and intra-uterine growth retardation is reported [92, 93]. Possibly, prednisone use is also associated with lower birth weight [98,99]. Most previous studies were small, retrospective and did not specifically analyze RA-patients as a subgroup.

Aims of this thesis

Pregnancy is the only natural situation that results in spontaneous improvement of RA and a flare of the disease after delivery [1]. Insight into the underlying mechanisms may not only increase our knowledge on the phenomenon of pregnancy-induced remission in RA but may also contribute to a better understanding of pathogenic mechanisms underlying RA in general.

Until today it is unclear which mechanisms or what factors could induce this remission. It has been hypothesized that the complement factor mannose-binding lectin (MBL) might play a beneficial role that results from its binding to pathogenic agalactosyl IgG antibodies and may therefore function as a scavenger molecule involved in the efficient removal of pathogenic agalactosyl IgG containing immune complexes [19]. For that reason, if pregnancy causes a rise in MBL serum concentrations, this might provide an alternative hypothesis to explain the phenomenon of pregnancy-induced improvement of RA.

Furthermore MBL may be associated with pregnancy outcome. Therefore, if the susceptibility both for RA as well as certain adverse pregnancy outcome measures are associated with low MBL levels, the increased incidence in RA of pre-eclampsia, preterm birth and other pregnancy outcome measures could be the result of shared pathogenic factors.

The aim of this thesis is to determine whether MBL plays a role in healthy pregnancy and in pregnancy-induced improvement of RA and deterioration postpartum. Moreover it is investigated whether MBL polymorphisms are a common pathogenic factor involved both in RA as well as in certain adverse pregnancy outcome measures.

In the next chapter, **chapter 2,** the MBL serum concentrations and the lectin complement pathway activity during pregnancy and postpartum in healthy controls is described.

Since there are indications that MBL is associated with pregnancy outcome and is also associated with RA, first the role of MBL genotype groups in relation to pregnancy outcome in healthy controls is investigated in the next chapters; The **third chapter** considers a possible association with MBL genotype groups with pre-eclampsia in healthy controls and **chapter 4** reports a possible association between MBL genotype groups and preterm birth in healthy controls.

In **chapter 5** the question whether MBL genotype groups are associated with RA disease susceptibility and disease severity is addressed. In order to give a more definite answer to previous non-conclusive literature, two large cohorts are studied on the association between MBL genotype groups and RA disease susceptibility and RA disease severity, as defined by the need for anti-TNF therapy.

Chapter 6 reports a simultaneous increase in the glycosylation of IgG (in particular galactosylation and sialylation) and improvement of RA during pregnancy. After delivery the IgG glycosylation decreases and RA disease activity flares. Possible factors that might influence this glycosylation are studied.

In **chapter 7** the role of MBL during pregnancy and in the postpartum period is studied in RA. The possible association between maternal MBL genotypes on pregnancy-induced remission of RA is investigated, but also the possible association between MBL and IgG galactosylation changes during pregnancy and postpartum. Moreover, the role of MBL on pregnancy outcome in RA, like birth weight, gestational age, miscarriage and hypertensive disorders is described.

Chapter 8 is dedicated to the discussion of the main findings of this thesis in the light of the study objectives, followed by methodological considerations. New insights arising from this thesis into MBL, RA and pregnancy are described. Finally, recommendations for future studies are presented.

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Chapter 2

Mannose-binding lectin levels during pregnancy: a longitudinal study

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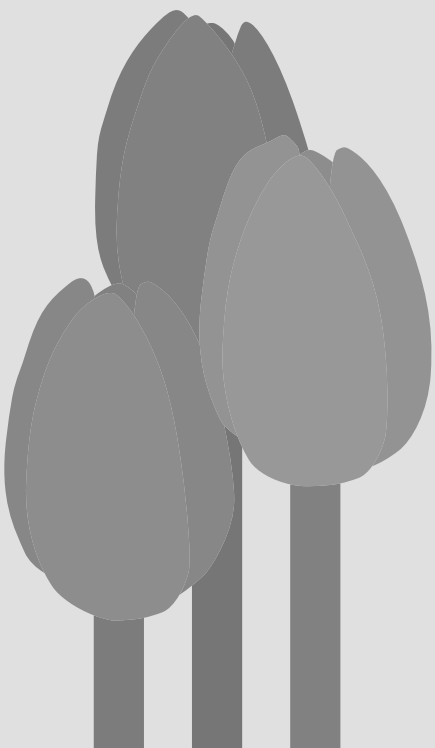


Abstract

Background: Pregnancy is associated with changes in the immune system. Although previous studies have focused mainly on adaptive immunity, there are indications that components of innate immunity, such as mannose-binding lectin (MBL), are associated with pregnancy outcome. Although this would suggest that pregnancy also involves adaptations in innate immunity, there are few studies in this area. Therefore, we aimed to determine whether MBL concentrations and the following steps in complement pathway activation are influenced by pregnancy.

Methods: MBL and Ficolin-2 concentrations, MBL–MBL-associated serine protease (MASP) complex activity, MBL pathway activity and classical complement pathway activity were determined by enzyme-linked immunosorbent assay (ELISA) in sera from pregnant women ($n = 32$) during each trimester and postpartum. MBL genotyping was performed by PCR.

Results: During pregnancy, MBL concentrations increased to 140% (interquartile range (IQR) 116–181%, $p < 0.0001$). This increase was already present at 12 weeks of pregnancy and was most pronounced in the high-production AA-genotype. Directly postpartum MBL concentrations dropped to 57% of baseline (IQR 44–66%, $p < 0.0001$). Variations in MBL levels were reflected by similar changes in the following steps of complement activation, ($r > 0.93$, $p < 0.01$). Ficolin-2 levels and classical complement pathway activity were not similarly influenced by pregnancy.

Conclusion: Pregnancy and the postpartum period profoundly influence MBL serum concentration and MBL complement pathway activity.

Introduction

Pregnancy is considered as an immunological phenomenon because the pregnant mother needs to accommodate the fetal allograft. This requires profound changes of the maternal immune system [1, 2]. Up to now, the majority of laboratory research has been focused on possible modifications in the adaptive immune system during pregnancy. In this scope, it has been hypothesized that autoimmune diseases such as rheumatoid arthritis and multiple sclerosis tend to improve during pregnancy due to adaptations of the immune system [3-6]. However, some clinical studies demonstrate that also certain components of innate immunity are associated with pregnancy outcome. This would suggest that innate immunity requires adaptations during pregnancy too. Mannose-binding lectin (MBL) is one of the components of the lectin pathway of complement that is associated with pregnancy outcome. The way in which innate immunity, and MBL in particular, changes during pregnancy has hardly been investigated [7].

MBL and Ficolin-2 are complement factors that are mainly produced in the liver. Both MBL and Ficolin-2 can activate the lectin pathway of complement. The lectin pathway can be activated by binding of MBL and Ficolin-2 to carbohydrate ligands followed by activation of MBL-associated serine proteases (MASPs) (MBL–MASP-2 complex). MASP-2 is the complement-activating enzyme that is complexed with MBL. This MBL–MASP-2 complex has the ability to activate complement factor C4. After C4 activation, the complement cascade is further activated resulting in the formation of the membrane attack complex (MAC) C5b-9. The MAC is the end product of the lectin pathway of complement and has many effector functions such as microbial defense. The classical and alternative pathways of complement also result in the formation of MAC, but by using a different mechanism.

Several reports suggest a role for MBL during pregnancy based on studies comparing MBL levels or MBL genotypes with pregnancy outcome. For example, low maternal serum levels of both MBL and Ficolin-2 have been found in women with recurrent miscarriages [8-11]. Deficient maternal MBL concentrations have also been described as a risk factor for preterm birth and reduced birth weight [12], although one report showed an association of MBL gene polymorphisms with gestational diabetes mellitus, resulting in heavier infants [13]. Chorioamnionitis is another measure for pregnancy outcome that has been associated with certain MBL gene polymorphisms which result in lower MBL serum concentrations [14].

These findings imply that high levels of MBL are beneficial for pregnancy outcome; therefore it is of great interest to determine the influence of pregnancy itself on circulating MBL levels. Up to now, this question has only been addressed in one small study by Kilpatrick [7] in which MBL levels were determined in the first trimester of pregnancy in eight women with a history of recurrent miscarriages. Little or no modulation of MBL

levels during the critical first trimester of pregnancy was found when compared with the pre-pregnant status. However, one healthy woman without an adverse medical history was studied during all trimesters of pregnancy and showed increased MBL levels in her third trimester [7].

The MBL serum concentration is strongly determined by its single nucleotide substitutions (SNPs) in the structural gene and in the promoter region [15, 16]. In exon 1 of the *MBL2* gene, the wildtype allele is referred to as A, whereas the O-alleles represent the variant alleles B, C and D together. Individuals with the AA wildtype genotype have higher MBL serum concentrations, whereas individuals with the AO- or OO-genotype show lower MBL serum concentrations. This is because of disturbed multimer formation, resulting in impaired function [17]. Moreover, basal MBL serum levels are also modified by the SNPs in the promoter region of the MBL gene [18]. Depending on genotype, the MBL serum concentration can therefore differ largely between individuals, but the basal level of MBL in each individual is reasonably stable throughout life [19]. Interestingly, about 30% of the general population with the AO- or OO-genotype has very low to deficient MBL levels. These individuals could be clinically considered as MBL deficient and at risk for infections. In the Ficolin-2 gene, 10 SNPs have been demonstrated recently [20], and a relationship between these SNPs and biological activity of Ficolin-2 has also been suggested [21].

The aim of the present study was to determine the influence of pregnancy on MBL serum concentrations and to investigate whether MBL exon 1 genotypes and promoter polymorphisms affect MBL levels during pregnancy. We verified whether changes in MBL concentrations during pregnancy result in comparable changes in the subsequent steps in the lectin pathway of complement activation. This was studied via measurements of the MBL–MASP complex activity and the MBL pathway activity. Finally, to investigate whether pregnancy-induced changes are specific for MBL, we determined the influence of pregnancy on Ficolin-2 levels and classical complement pathway activity as well. To address these questions, we used a prospective longitudinal study design of healthy pregnant women ($n = 32$) with uncomplicated pregnancies and determined SNPs in exon 1 and in the promoter region of the *MBL2* gene.

Patients and Methods

Human materials

Healthy pregnant Caucasian women without adverse obstetric history ($n = 32$) were recruited by a local midwifery practice and prospectively monitored.

Blood was taken during home visits three times during pregnancy (12, 20 and 30 weeks of gestation) and three times postpartum (6 and 12 weeks and between 6 and 9 months). The first five visit timepoints were all met within a deviation of 1 week. The last

timepoint at 6–9 months postpartum was set as baseline value. At that time, we considered the participants as being recovered from their pregnancies.
Serum was cooled, frozen at −80°C and thawed once for aliquotting. Genomic DNA of the mother was isolated from heparinized blood, and genomic DNA of the child was isolated from umbilical cord blood as described below. For three participants and four children, DNA was isolated from cells obtained from the buccal mucosa cells by brushing with a padded stick [22].
The women participated in the study after informed consent was given. The study was in accordance with the Helsinki II declaration and was approved by the local ethical committee.

Enzyme-linked immunosorbent assay protocol

For all enzyme-linked immunosorbent assays (ELISAs), Nunc Maxisorb plates (Nunc, Roskilde, Denmark) were coated using relevant antibodies in coating buffer (100 mM Na_2CO_3/$NaHCO_3$, pH 9.6) for 16 hrs at room temperature. After each step, plates were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20. Residual binding sites were blocked by incubation with PBS containing 1% bovine serum albumin (BSA). Unless otherwise indicated, all subsequent steps were incubated for 1 hr at 37°C in PBS containing 0.05% Tween 20 and 1% BSA. Detection antibodies were conjugated to digoxygenin (Dig) using Dig-3-*O*-methylcarbonyl-ε-aminocaproic acid-*N*-hydroxysuccinimide ester (Boehringer Mannheim, Germany), according to instructions provided by the manufacturer. Detection of binding of antibodies conjugated to Dig was performed by horseradish peroxidase (HRP)-conjugated sheep anti-Dig antibodies (Fab, Boehringer Mannheim). Enzyme activity of HRP was detected using 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (Sigma). The optical density (OD) at 415 nm was measured using a microplate biokinetics reader (EL312e; Biotek Instruments, Winooski, VT, USA). All incubation volumes were 100 µl/well.

All sera were analysed in duplicates in at least two dilutions. Concentration was expressed in ng/ml. Functional activity was expressed in U/ml on the basis of serial dilutions of a human pool serum used as a standard (set at 1000 U/ml).

MBL detection ELISA

Assessment of MBL concentration in serum was performed by sandwich ELISA, as described previously [23]. In brief, plates were coated with monoclonal antibody (mAb) 3E7 (anti-MBL mouse IgG1, kindly provided by T. Fujita, Fukushima Medical University School of Medicine, Fukushima, Japan) at a concentration of 5 µg/ml. Serum samples were incubated for 1 hr at 37°C, followed by detection with Dig-conjugated mAb 3E7. A calibration line was produced using pooled serum from healthy donors with a known concentration of MBL.

Measurement of Ficolin-2 serum concentration by ELISA

Plates were coated with mAb GN5 (mouse mAb anti-human Ficolin-2, kindly provided by T. Fujita, Fukushima Medical University School of Medicine, Fukushima, Japan) at a concentration of 5 µg/ml. Samples were incubated for 1 hr at 37°C, followed by incubation with Dig-conjugated mAb GN5. Detection of binding of antibodies was as described in the ELISA protocol. A calibration line was produced using pooled human serum from healthy donors with a known concentration of Ficolin-2 (kindly provided by Dr D.C. Kilpatrick, Scottish National Blood Transfusion Service Edinburgh, UK).

Assessment of MBL-MASP complex activity by ELISA

MBL-MASP complex activity was assessed, as described previously [24], based on a protocol developed by Petersen [25]. Activity of the MBL–MASP complex is determined by its ability to activate exogenously added complement factor C4. In brief, MBL–MASP complexes were allowed to bind to immobilized mannan (from *Saccharomyces cerevisiae*; Sigma (M7504)) overnight at 4°C in the presence of GVB++ buffer (Veronal-buffered saline containing 0.5 mM $MgCl_2$, 2 mM $CaCl_2$, 0.05% Tween 20 and 0.1% gelatin; pH 7.5) supplemented with 1 M NaCl. This was followed by the addition of purified haemolytically active C4 and the detection of deposited activated C4 binding using Dig-conjugated anti-C4 mAb. The detection limit of the MBL–MASP complex activity was 20 U/ml.

Assessment MBL pathway activity by ELISA

MBL pathway activity was assessed by ELISA as described by Roos *et al.* [16] with some modifications. Concentrations of the MAC C5b-9 are determined in the presence of anti-C1q antibodies to exclude activation by the classical complement pathway. In brief, plates were coated with mannan dissolved in PBS at a concentration of 10 µg/ml. Samples were diluted and pre-incubated in GVB++ buffer containing mAb85 (mAb anti-human C1q, kindly provided by Prof. E. Hack, Sanquin, Amsterdam, the Netherlands) at a concentration of 5 µg/ml. After this pre-incubation of 15 min at room temperature, the samples were added to the plate and incubated for 1 hr at 37°C. Complement activation was detected using Dig-conjugated mAb AE11 (mAb anti-human C5b-9, kindly provided by Dr T.E. Mollnes, Oslo, Norway). The detection limit of MBL pathway activity was 200 U/ml.

Assessment of classical complement pathway activity by ELISA

Classical complement pathway activity was also assessed by ELISA, as previously described [16]. In this assay, plates were coated with human IgM at a concentration of 2 µg/ml. Samples were diluted in GVB^{++} buffer and added directly to the plates to allow incubation for 1 hr at 37 °C. Complement binding was assessed using Dig-conjugated mAb directed against C5b-9.

DNA isolation

Heparinized whole blood or umbilical cord blood was lysed with lysis buffer NH_4Cl. Nuclei were washed in sterile MilliQ water. The pellet was resuspended in salt/EDTA (SE) buffer (75mM NaCl, 25mM EDTA, pH 8.0), and pronase (concentration 20 mg/ml) was added. After addition of sodium dodecyl sulfate (SDS) buffer, the extracted DNA was left overnight at room temperature. Then, proteins were precipitated by adding NaCl and ethanol and later dissolved in Tris/EDTA (TE) buffer (10 mM Tris/Cl, 1 mM EDTA, pH 8.0).

For three participants and four children, we isolated DNA from cells obtained from the buccal mucosa by brushing with a padded stick [22]. The sticks were returned to us by mail in a buffer of Salt/Tris/EDTA (STE), SDS and proteinase K. Upon arrival, proteinase K was added to the samples until a final concentration of 0.2 mg/ml. After the sticks had been spun, KAc and choloroform/isoamylalcohol were added. DNA was precipitated with ethanol and dissolved in TE buffer.

DNA concentrations were measured, and its purity was assessed on basis of UV absorption. Samples were frozen in –80° C until they were used.

MBL genotyping

The PCR and the melting curve profile were performed in LightCycler capillaries (Roche Diagnostics) with a final volume of 20 µl, containing 10 ng of genomic DNA, 0.5 µM of each primer, 0.15 µM of each hybridization probe, 1x LightCycler DNA Master Hybridisation Probes (Roche Molecular Biochemicals, Mannheim, Germany) and 2 mM MgCl2. The PCR amplification profile of both experiments consisted of 10 min at 95°C, followed by 45 cycles of 95°C for 3 sec, 60°C (63°C in case of detection of the MBL exon 1 polymorphism) for 15 sec and 72°C for 10 sec. Next, the melting curve profile was performed, which consisted of 1 cycle of 95°C for 3 min, 55°C for 1 min, 45°C for 30 sec and 40°C for 3 min, after which the temperature was slowly increased (0.1°C/s) to 80°C under continuous detection of the emitted light.

For the detection of the three exon 1 SNPs in codon 52, 54 and 57 of the structural gene, the forward primer 5′-TGAGTATGGTCAGCGTCTTA-3′ and the reverse primer 5′-TGGGCTGGCAAGACAACTATTAG-3′p were used for amplification. For genotyping, the 5′-LC Red 640-TTCTTCCTTGGTGCCATCACACCCA-3′-p detection probe and the fluorescein-labelled anchor probe 5′-CAGCCCAACACGTACCTGGTTCCCCCT-3′FLU were used (Tib-MolBiol, Berlin, Germany). For the detection of these SNPs, one set of hybridization probes was sufficient.

The detection of the (–550) H/L and (–221) X/Y promoter polymorphisms requires two sets of hybridization probes. The forward primer 5′-GCCAGAAAGTAGAGAGGTATTTAGC-3′ and the reverse primer 5′TGTGACATGCATGCGTGACTACTAGTAC-3′ were used to amplify a fragment spanning the two SNPs in the promoter region. For genotyping the H/L polymorphism at position –550, the fluorescein-labelled detection probe 5′-TTTTAGACAG-

GCAGGCTTGCCTGCCTGGGT-3′ FLU, which is complementary to the L allele, and the 5′-LC Red 705-AGCATTTTCTCTGGAAATTTCTTACTACGTTGG-3′-phosphorylated anchor probe were used. The 5′-CTATAAACATGCTTTCGGTGGCAGT-3′FLU detection probe and the LC Red 640-AACAAATGGGACCGTGCATTGCCA-3′-p anchor probe were used for genotyping the X/Y SNP. In each experiment, sequence-verified control donors for each genotype were used.

Statistical analysis

All analyses were carried out using two-tailed non-parametric tests, and results were considered statistically significant when *p*-values were <0.05.

The difference in MBL serum concentrations between the AA- and AO-/OO-genotype groups at all timepoints was evaluated using the Mann–Whitney *U*-test.

The medians of the pregnancy-induced increases in MBL serum concentration, MBL–MASP complex activity, MBL pathway activity and classical complement pathway activity were calculated as follows. First the mean value per participant was calculated of the three timepoint measurements during pregnancy; these means were used to calculate the median value of all participants together, categorized per genotype. The Wilcoxon signed rank test was used to compare the median value during pregnancy with the respective baseline values at 6 months postpartum.

The postpartum drop was calculated by dividing the value of the timepoint 6 weeks postpartum by the baseline value at 6 months postpartum and expressed in percentages. The Wilcoxon signed rank test was used to evaluate the difference between this timepoint and the baseline value.

Correlations were calculated using the Spearman rank test between the various measurements of MBL serum concentration, MBL–MASP complex activity and MBL pathway activity.

It has been indicated in the text for how many participants it has been possible to measure their baseline values.

Results

Description of cohort

The demographic and clinical characteristics of the study group ($n = 32$) are described in Table 1. Four women developed pregnancy complications: one woman developed pre-eclampsia defined according to the International Society for the Study of Hypertension in Pregnancy (ISSHP) criteria [26]; two women developed pregnancy-induced hypertension, for which no medication was prescribed. One woman delivered preterm (34 weeks gestation) due to premature rupture of membranes with unknown cause. Furthermore, four women developed

other (inflammatory) complications during pregnancy: monoarthritis of the knee, sinusitis and cystitis (twice). The mean gestational age at delivery was 283 days, range 238–294 days. All 32 neonates were born healthy without asphyxia. Two Caesarean sections were performed, one of which for maternal indication because of severe pre-eclampsia and one because of breech presentation.

MBL serum concentrations increase during pregnancy

MBL serum concentrations were measured at all six timepoints in 31 women. During pregnancy, the MBL serum concentration increased to 140% (median, interquartile range (IQR) 116-181%, $p < 0.0001$) compared with baseline, which is defined as the concentration at 6 months postpartum. The increase in MBL serum concentration was present in the first trimester of pregnancy (12 weeks) and did not significantly increase further during pregnancy. Directly postpartum (6 weeks) MBL concentrations dropped sharply to 57% of the baseline value (IQR 44-66%, $p < 0.0001$, Figure 1A).

Table 1. Clinical characteristics of the study group

Characteristics	Cases ($n = 32$)
Mean age at first trimester (years) (SD)	32 (4.4)
Number of nulliparous women (%)	14 (43.8)
Mean number of previous pregnancies (SD)	1.0 (1.1)
Number of previous miscarriages (%)	
0	27 (84.4)
1	4 (12.5)
2	1 (3.1)
Women who smoked during pregnancy (*n*) (%)	3 (9.4)
Mean gestational age at delivery (days) (range)	283 (238-294)
Mean birth weight (gram) (SD)	3492 (80)

Ficolin-2 serum concentrations are not influenced by pregnancy

Ficolin-2 serum concentrations were measured at all six timepoints in 29 women. Ficolin-2 serum concentrations did not show significant variations during pregnancy or postpartum (median Ficolin-2 serum concentration 1950 ng/ml, IQR 1429–2451 ng/ml, Figure 1B). Clearly, no decline in postpartum values could be appreciated.

Classical complement pathway activity changes only slightly during pregnancy

The classical complement pathway activity was measured at all six timepoints in 30 women. The classical complement pathway activity was influenced by pregnancy, but to a lesser extent than the MBL pathway activity, which is described later in the text. Pregnancy increased the classical pathway activity to 129% of the baseline value (median, IQR 116–138%, $p < 0.0001$). Postpartum (6 weeks), the activity of the classical comple-

ment pathway decreased to 115% of the baseline value (median, IQR 101–120%, $p < 0.01$, Figure 1C). Clearly, for the classical complement pathway activity as well, no significant postpartum drop could be appreciated.

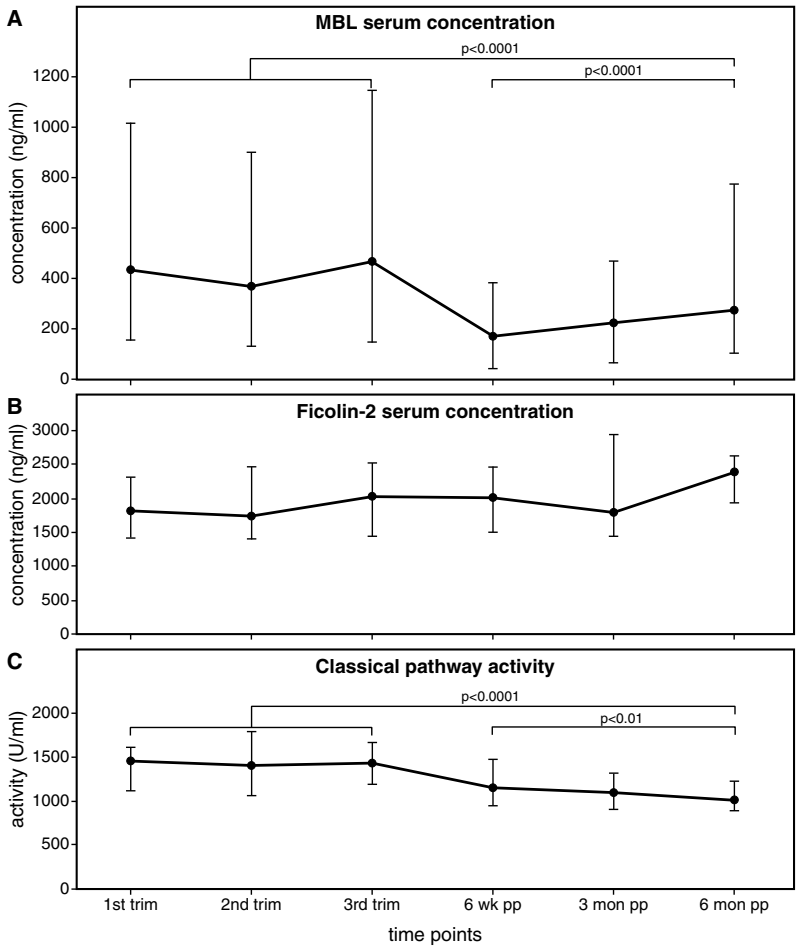


Figure 1. Median serum concentrations of mannose-binding lectin (MBL) and Ficolin-2 and complement activation via the classical pathway during pregnancy and postpartum. **A)** MBL serum concentrations were measured at indicated timepoints. Pregnancy increased MBL serum concentrations to 468 ng/ml (median, interquartile range (IQR) 143–1144 ng/ml, $p < 0.0001$). Directly postpartum (6 weeks) the MBL concentration dropped sharply to 168 ng/ml (median, IQR 42–381 ng/ml, $p < 0.0001$). **B)** Ficolin-2 serum concentrations did not show significant variations during pregnancy or postpartum. Clearly, no decline in postpartum values was observed. **C)** Pregnancy increased classical complement pathway activity to 1428 U/ml (median, IQR 1184–1668 ng/ml, $p < 0.0001$). Postpartum, the activity of the classical pathway gradually decreased. Data are presented as medians with IQRs.
Abbreviations: Trim, trimester; wk, weeks; mo, months; pp, postpartum.

Influence of the MBL genotype and promoter polymorphisms on basal MBL serum levels

As expected, MBL serum levels show a major inter-individual variation, but all showed the same pattern during pregnancy and postpartum (Figure 2). Therefore, we assessed the MBL genotypes of the subjects to examine whether the intraindividual fluctuations in MBL serum levels were dependent on the MBL genotypes. MBL genotypes were determined in 31 of the 32 women. MBL genotypes and median baseline values of complement pathway components are described in Table 2. The maternal MBL genotype and MBL promoter polymorphism frequencies summarized in Table 2 are in accordance with results from previous studies [15, 24]. As was expected, the median baseline MBL serum concentrations in the individuals with the AA-genotype were significantly higher than those with the AO- and OO-genotypes, $p < 0.0001$. Moreover, within the group of women with the AO-genotype, the AD subgroup showed higher baseline MBL serum concentrations than the AB subgroup, as described previously [15].

The effect of the various promoter polymorphisms on MBL serum concentrations was minimal within the group with the AA-genotype. Exceptions within this group were the two women who carried the LXA/LXA promoter polymorphism, who showed very low baseline concentrations of MBL (118 and 51 ng/ml). In women with the AO-genotype, the highest MBL levels were observed in women with the HYA/O-genotype, and women with the LXA/O-genotype showed the lowest MBL serum concentrations. The OO-genotype showed minimal MBL serum concentrations (83 ng/ml).

It can therefore be concluded that the AA-genotype shows the highest MBL levels, followed by the AO- and the OO-genotypes, supplemented with the effects of the promoter polymorphisms.

Increase in MBL serum concentration is related to maternal genotype

The increase in MBL serum concentrations during pregnancy was highest in the mothers with the AA-genotype (177% increase, IQR 124–212%, $p < 0.001$). Mothers with the AO-genotype showed a more moderate increase of 126% (IQR 91–144%, $p < 0.04$), as did the mother with the OO-genotype (117% increase), compared with their respective baseline values (Figure 3A).

The effect of the promoter polymorphisms could also be observed on the increased MBL levels during all trimesters of pregnancy. The frequency of the specific combinations of the different genotypes related to the various promoter polymorphisms was not sufficient to allow statistical analysis.

Postpartum drop in MBL concentration occurred equally in all genotypes

Although all women had detectable levels of MBL during pregnancy, a postpartum drop was observed in all women, independent of their MBL genotypes, that is, 55% in AA-

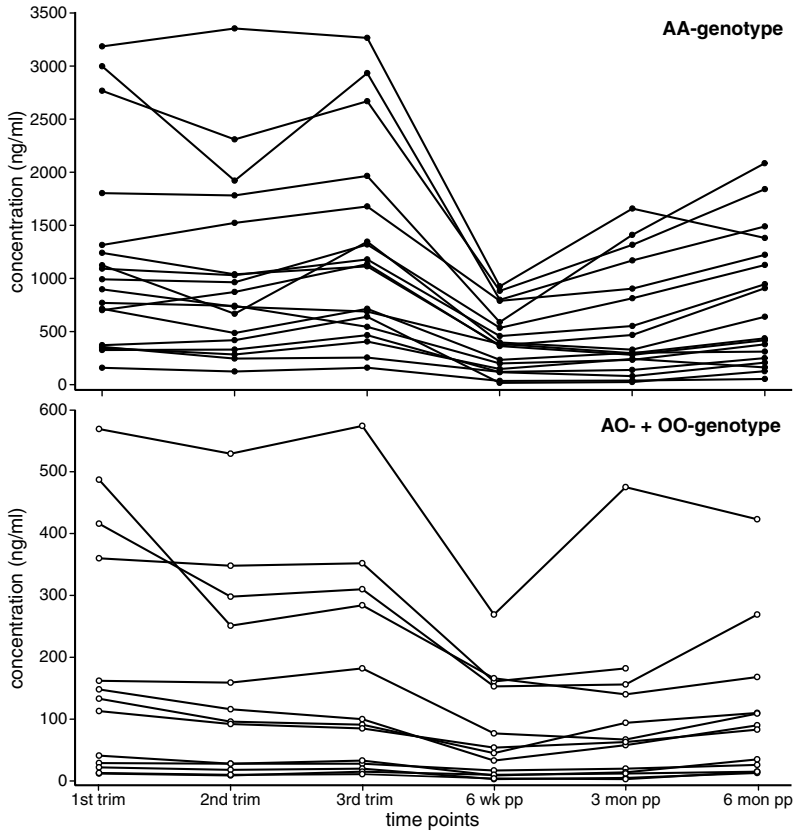


Figure 2. Mannose-binding lectin (MBL) serum concentration in healthy women during pregnancy and postpartum depicted by genotypes AA and AO + OO, separately. MBL serum levels show a major inter-individual variation, but MBL serum levels in all individual subjects showed the same pattern during pregnancy and postpartum, namely an increase during pregnancy (median to 140% of baseline value, interquartile range (IQR) 116–181%, $p < 0.0001$), and a significant decline postpartum (to 57% of baseline value, median, IQR 44–66%, $p < 0.0001$). MBL serum concentrations were measured at indicated timepoints. Each symbol represents one individual. The baseline value is defined as the MBL serum concentration at 6 months postpartum. Note that the Y-axes have different scales. Abbreviations: Trim, trimester; wk, weeks; mon, months; pp, postpartum.

genotype subjects ($p < 0.0001$), 60% in AO-genotype subjects ($p < 0.03$) and 65% in the OO-genotype subject. In some women, this drop in MBL serum concentrations resulted into MBL serum levels that would be clinically considered as MBL deficient.

For example, in one of the two women with the LXA/LXAgenotype, MBL levels remained very low (33, 36 and 51 ng/ml) at 6 weeks, 12 weeks and 6 months postpartum, respectively, in contrast to her higher MBL levels during pregnancy (156, 122 and 157 ng/ml in the first, second and third trimester, respectively).

Table 2. MBL genotype and median baseline values of complement pathway components

Genotypes				
MBL exon 1 genotype *n*, (%)	AA		AO	OO
	18 (58)		12 (39) AB 7 AC 1 AD 4	1 (3) DD 1
MBL promoter polymorphisms (*n*)				
HY/LY	8		2	-
LY/LY	1		2	-
LX/LY	2		5 3	-
HY/HY	3		-	1
LX/HY	2		-	-
LX/LX	2			-
Median baseline values				
MBL concentration (IQR) (ng/ml)	533 (259-1197)	AO	90 (21-139)	83
		AB	35 (13-110)[a]	
		AC	15	
		AD	269 (16-423)[a]	
MBL-MASP complex activity (IQR) (U/ml)	694 (432-2787)	AO	20 (20-1693)[a]	<20[b]
		AB	20 (20-238)[a]	
		AC	20	
		AD	585 (491-1693)[a]	
MBL pathway activity (IQR) (U/ml)	1031 (634-1665)	AO	200 (200-907)[a]	<200[b]
		AB	200 (200-453)[a]	
		AC	200	
		AD	577 (406-907)[a]	
Ficolin-2 (IQR) (ng/ml)	2401 (1936-2947)		2403 (2041-2767)	2398
Classical complement pathway activity (IQR) (U/ml)	1061 (908-1204)		941 (859-1245)	992

IQR, interquartile range.
a Owing to the low numbers not the IQR, but the range is given.
b These values are below detections limits.

The other woman with the LXA/LXA-genotype had the following MBL levels: 364, 411 and 632 ng/ml in the first, second and third trimester of pregnancy, respectively, and 8, 16 and 118 ng/ml at 6 weeks, 12 weeks and 6 months postpartum, respectively.

Fetal MBL genotype and maternal MBL serum concentrations

We hypothesized that fetal production of MBL might possibly contribute to the maternal MBL levels, involving feto–maternal transport via unknown passive or active mechanisms. By determining the genotype of the fetus and correlating it with the MBL concen-

tration in maternal serum, we wanted to investigate if the level of maternal MBL is related to the fetal MBL genotype.
The MBL exon 1 genotype and promoter polymorphisms were determined in the samples obtained from 30 children of the cohort. The fetal genotype did not show an additional contribution to the maternal MBL serum concentration or MBL activity levels during pregnancy or postpartum (data not shown).

Pregnancy-related changes in MBL concentrations are followed by similar changes in MBL-MASP complex activity

The MBL–MASP complex activity was measured at all six timepoints in 28 of the 32 women. In six women (AO- and OO-genotypes), all measurements were below detection limits, that is, 20 U/ml (Table 2). Statistical analyses were performed in the series in which the baseline values at 6 months postpartum were above detection limits ($n = 22$ women).

Changes in MBL serum concentrations were reflected by similar changes in the activity of the MBL–MASP complex. During pregnancy, there was an increase up to 172% in MBL–MASP complex activity (median of the cohort, IQR 144–238%, $p < 0.0001$) compared with baseline; directly postpartum this activity dropped to 70% of the baseline activity level (median, IQR 49–119%, $p < 0.0001$, Figure 3B). In some women, MBL–MASP complex activity decreased to undetectable levels postpartum.

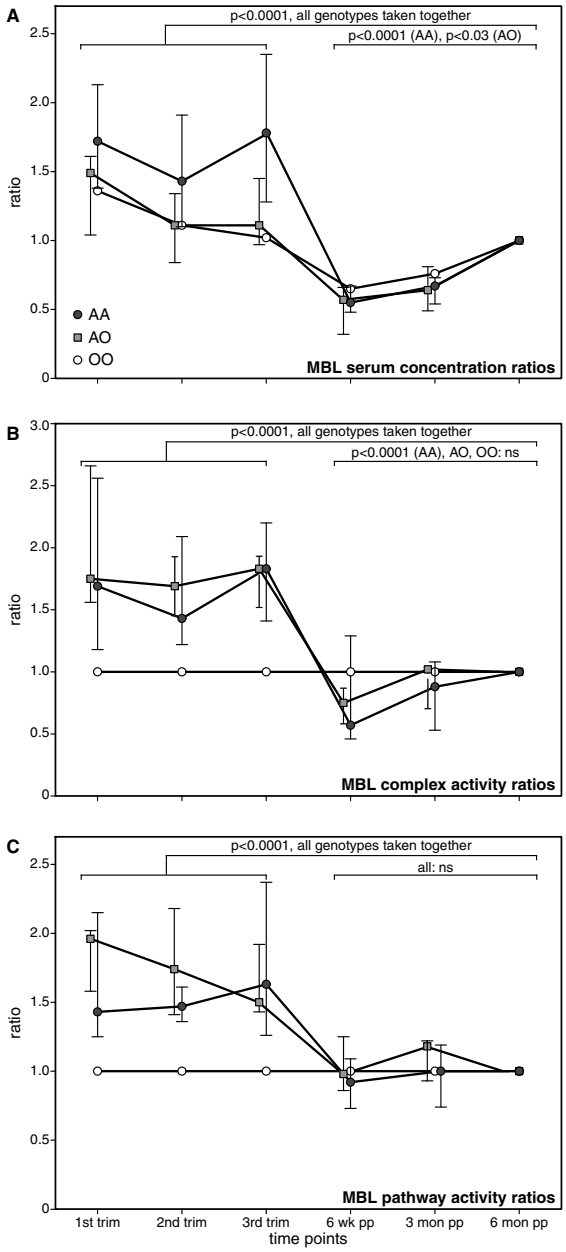
In women with the AA-genotype, the MBL–MASP complex activity increased to 158% during pregnancy (median, IQR 133–215%, $p < 0.001$) compared with their baseline level. The MBL–MASP complex activity of the AO-genotype subjects that was above detection limit showed an increase of 175% (IQR 146–224%, $p < 0.05$). The postpartum drop was 57% (AA-genotype) and 75% (AO-genotype) of the baseline level, which was significant in the AA-genotype ($p < 0.03$, Figure 3B).
At all timepoints, there was a very good correlation between MBL concentration and MBL-MASP complex-activity ($r > 0.93$, $p < 0.01$).

MBL pathway activity increases during pregnancy

The activity of the MBL pathway, assessed on the basis of formation of the C5b-9 complex, was measured at all six timepoints in 31 women. In six women (AO-genotype and OO-genotypes) all measurements were below detection limit, that is, 200 U/ml (Table 2). Statistical analyses were performed in the series in which the baseline values at 6 months postpartum were above detection limit ($n = 25$ women).

A highly significant increase in MBL pathway activity was observed during pregnancy (median 164% increase, compared with baseline level, IQR 139-215%, $p < 0.0001$, Figure 3C). The MBL pathway activity increased to 160% in the mothers with the AA-genotype (median, IQR 132-179%, $p < 0.0001$, compared with their baseline value). The MBL path-

Figure 3. Mannose-binding lectin (MBL) serum concentration, MBL–MASP complex activity and MBL pathway activity in healthy women during pregnancy and postpartum categorized per genotype. Ratios were calculated by dividing the concentration or activity at indicated timepoints by the baseline concentration or baseline activity. Baseline is defined as the value at 6 months postpartum. Data are presented as median ratios with interquartile ranges (IQRs).



A) MBL serum concentrations were significantly increased during pregnancy, $p < 0.0001$, all genotypes taken together. The AA-genotype increased to 177% (median, IQR 124–212%, $p < 0.001$), the AO-genotype to 126% (median, IQR 91–144%, $p < 0.04$) and OO-genotype to 117%, all compared with baseline value. The postpartum drop (6 weeks postpartum versus baseline) occurred equally in all genotypes (55, 60 and 65% of baseline concentration, AA-genotype ($p < 0.0001$), AO-genotype ($p < 0.03$) and OO-genotype, respectively). AA-genotype $n = 18$, AO-genotype $n = 11$, OO-genotype $n = 1$.

B) The MBL–MASP complex was activated significantly during pregnancy (to 172%, median, IQR 144–238%, $p < 0.0001$), all genotypes taken together, compared with baseline. The AA-genotype increased to 158% (median, IQR 133–215%, $p < 0.001$, compared with baseline), the AO-genotype to 175% (median, IQR 146–224, $p < 0.05$, compared with baseline). The postpartum drop was 57% (AA-genotype, $p < 0.03$) and 75% (AO-genotype, ns) of baseline value. AA-genotype $n = 15$, AO-genotype $n = 6$, OO-genotype $n = 1$.

C) The MBL pathway was activated significantly during pregnancy (to 164%, median, IQR 139–179%, $p < 0.0001$), all genotypes taken together. The AA-genotype increased to 163% (median, IQR 132–179%, $p < 0.0001$, compared with baseline). The AO-genotype increased to 171% (median, IQR 146–206%, $p < 0.03$, compared with baseline). There was no postpartum drop in MBL pathway activity 92% (AA-genotype, ns) and 98% (ns) of baseline value. AA-genotype ($n = 18$), AO-genotype ($n = 6$) and OO-genotype ($n = 1$).

Abbreviations: Trim, trimester; week, weeks; mon, months; pp, postpartum; ns: not significant.

way activity of the AO-genotype mothers that was above the detection limit showed an increase to 171% (IQR 146-206%, $p < 0.03$).

A significant decline in MBL pathway activity directly postpartum was not observed in any of the genotypes (92% and 98% of the baseline value, AA- and AO-genotypes, respectively, Figure 3C). However, in one woman (LXA/LXA-genotype), MBL pathway activity levels remained below detection limit postpartum in contrast to her well-detectable activity levels during pregnancy (735, 453 and 810 U/ml in the first, second and third trimesters, respectively).

There was a very strong correlation between MBL concentration, MBL–MASP complex activity and MBL pathway activity at all six timepoints ($r > 0.93$, $p < 0.01$).

No observed association between MBL genotype and pregnancy complications

The women who encountered complications during pregnancy, that is, pre-eclampsia ($n = 1$), premature birth ($n = 1$) and various infections ($n = 4$), all had different genotypes and sufficient MBL levels during pregnancy, comparable with the remainder of the study group. The number of women with pregnancy complications is too small to draw any conclusions on any association between MBL genotype and pregnancy complications.

Discussion

In this study, we show that MBL serum concentrations significantly increase during pregnancy from the first trimester onwards and decline at six weeks postpartum. Because Ficolin-2 levels and classical complement pathway activities are not influenced in a similar way during pregnancy and in the postpartum period, it can be concluded that pregnancy has a specific effect on MBL and its complement pathway.

During pregnancy, mothers with the wildtype AA-genotype showed higher MBL concentrations and higher MBL–MASP complex activity and MBL pathway activity than those with the AO- or OO-genotypes. There was no additional influence of the fetal MBL genotype on maternal MBL serum concentration. The fact that increased MBL concentrations result in clearly increased MBL pathway activity suggests a functional role of this molecule during pregnancy.

We have chosen the six months postpartum timepoint as our baseline value for all measurements. We are aware of the fact that our threshold point of six months postpartum as a baseline value may not be an accurate baseline for all participants. There might be some women who need more than six months to recover from their pregnancies. Taking into account that MBL values are subject to change in the postpartum period, we would suggest that MBL measurements in the context of research and clinical diagnostics should not be performed before a minimum of six months postpartum.

The observed increase in MBL during pregnancy in our study group of 32 women without adverse pregnancy history is in line with an observation of Kilpatrick [7], who described an increase in MBL during pregnancy in one woman with no obstetric history. The author described that in women with a history of recurrent spontaneous miscarriage ($n = 8$), no increase in MBL could be observed during the first trimester of pregnancy. It is therefore tempting to speculate that a rise in MBL contributes to normal placentation and ongoing pregnancy.

The functions of increased MBL levels during pregnancy are unknown. From what is known about the mode of actions of MBL, one can consider the following. It has been suggested that adaptive immunity declines during pregnancy, enabling the pregnant body to tolerate the fetus as a semi-allograft. As a consequence, the mother is more susceptible to infections, especially with viruses and (intracellular) microorganisms, such as Neisseria and Mycoplasma [27]. Therefore, it can be hypothesized that increased levels of MBL during pregnancy may be necessary to compensate for reduced T-cell function during a state of reduced adaptive immunity [28-30]. The increased MBL levels may therefore reflect a shift from adaptive to innate immunity during pregnancy. Another role of MBL during pregnancy could reside in its ability to bind to apoptotic cells to enhance their opsonization and subsequent removal [31-33]. At the fetal-maternal interface, apoptosis and degradation are normal physiological constituents of trophoblast turnover to establish normal placentation. This process of apoptosis is of major importance during placentation and subsequent growth and development of placenta and fetus. Therefore, an increase in MBL levels might be necessary to remove the increasing presence of apoptotic material during pregnancy.

It is interesting to note that in certain autoimmune diseases, such as rheumatoid arthritis and multiple sclerosis, higher MBL serum levels are associated with lower disease activity and more favorable disease outcome [34]. Because it is known that these diseases tend to improve during pregnancy [3-6], one could speculate that a higher level of MBL during pregnancy could be one of the factors responsible for the pregnancy-induced amelioration of these diseases.

The factors that are responsible for the increased production of MBL during pregnancy are not known. It has been shown that human growth hormone is able to induce MBL *in vitro* [35] and in non-pregnant humans [36-38]. In line hereupon, it can be hypothesized that placental growth hormone acts in a similar way during pregnancy. Placental growth hormone shows several similarities with human growth hormone and is produced in large quantities during pregnancy [39]. An alternative candidate responsible for the induction of MBL during pregnancy might be cortisol. Increased cortisol levels during human pregnancy have been described, and the corticosteroid dexamethasone has been shown to induce MBL mRNA in cultured hepatocytes [40]. One study shows no effect of the corti-

costeroid hydrocortisone on *in vitro* MBL production [35]. A pregnancy-induced increase in cortisol might consequently results in higher MBL levels [41].

Fetal production of MBL has been reported, as MBL could be detected in the amniotic fluid [42] and in umbilical cord blood [43, 44]. Therefore, the increase in MBL in the maternal circulation may also be a (partial) result of fetal production. We did not find an additional influence of the fetal MBL genotype on maternal MBL serum concentration in our study. Hence, a major contribution of fetal MBL production to maternal MBL serum concentrations during pregnancy can be ruled out. Furthermore, it is unknown if maternal–fetal transfer of MBL during pregnancy exists.

This study shows that MBL serum concentrations significantly increase during pregnancy from the first trimester onwards and drop sharply at six weeks postpartum. The fact that not only MBL concentrations are increased, but that this also results in increased activity of the entire MBL pathway, suggests an important immunological role for MBL during pregnancy. Sufficient MBL levels during (early) pregnancy might be an essential condition for maintaining a pregnancy and for protecting the mother against infections during pregnancy. Moreover, the pregnancy-induced increase in MBL concentrations might play a role in the improvement of autoimmune diseases during pregnancy, thereby providing conditions for a more favourable pregnancy outcome. These results offer unique opportunities to study the regulation of MBL and its role in the (patho)physiology of pregnancy in the future and the role of pregnancy in the amelioration of autoimmune diseases.

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Chapter 3

Mannose-binding lectin genotypes and pre-eclampsia: A case-control study

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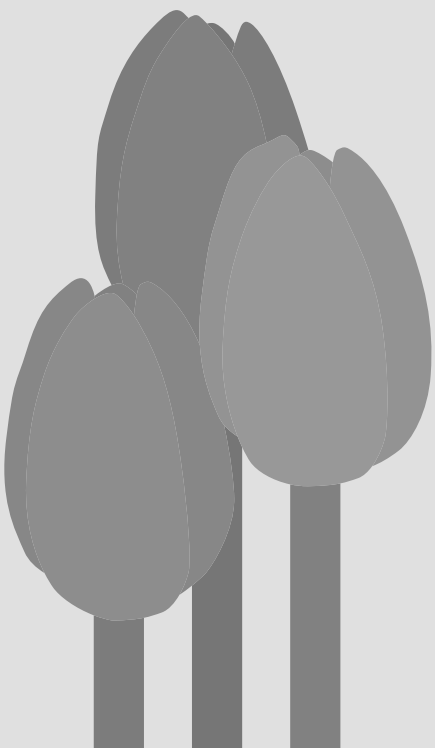

Abstract

Background: Both immunological and placental factors are involved in the pathogenesis of pre-eclampsia. The complement factor mannose-binding lectin (MBL) is associated with adverse pregnancy outcomes and has been suggested to play a role in abnormal placentation. We investigated whether MBL genotypes are associated with the systemic maternal syndrome pre-eclampsia.

Methods: *MBL2* gene polymorphisms were determined in a case-control study including 157 women with pre-eclampsia (case subjects) and 157 women with uncomplicated pregnancies (control subjects). Considering MBL polymorphisms, case and control subjects were categorized in groups of high (A), intermediate (B), and low (C) MBL production.

Results: No association was found between MBL genotypes and pre-eclampsia; adjusted odds ratios and 95% confidence intervals (95% CI) for group B were 0.97 (95% CI 0.46–2.07) and for group C were 1.44 (95% CI 0.52–3.94). A trend was found between MBL genotype groups B and C and *severe* pre-eclampsia or eclampsia.

Conclusions: MBL genotypes were not found to be associated with pre-eclampsia; however, low-MBL production genotypes might be considered as disease modifier. This suggests that MBL may play a role in modulating placental inflammation by facilitating clearance of apoptotic cells and cell debris.

Introduction

Pre-eclampsia is a common but severe pregnancy disorder that is unique to pregnancy and is associated with high maternal and fetal morbidity and mortality. Pre-eclampsia becomes clinically manifest in the second half of pregnancy and is characterized by hypertension and *de novo* proteinuria [1]. Abnormal formation of the placenta (placentation) is thought to play an important role in its pathophysiology [2, 3].

In uncomplicated pregnancies placentation involves trophoblast invasion and angiogenesis. Its success is dependent upon some degree of uterine inflammation, as has been shown by several studies both in humans and animals [4-6]. The importance of the immune system in placentation is further underscored by the detection of inflammatory cells [7] and mediators, such as cytokines [8, 9] and components of the complement system [10] in placentas of normal uncomplicated pregnancies. Because of the local presence of complement regulators, deposition of complement in the placenta usually does not lead to tissue damage [11]. However, if inflammation becomes too excessive, it might cause pregnancy complications such as miscarriage and pre-eclampsia [6, 12]. Uterine natural killer cells are the main regulators to balance the degree of inflammation at the feto-maternal interface [13]. Trophoblast debris, apoptotic cells, and progesterone have been proposed to regulate or stimulate inflammatory cytokines production by these uterine natural killer cells [6].

Mannose-binding lectin (MBL) is a component of innate immunity. It is produced in the liver and it is an oligo- or polymeric protein that circulates in serum. Its serum concentration is strongly determined by single nucleotide polymorphism (SNP) substitutions in the structural gene and in the promoter region [14]. In exon 1 of the structural region of the *MBL*2 gene, the wildtype allele is referred to as A, and the O-alleles represent the variant alleles B, C, and D together.

Individuals with the AA wildtype genotype have higher MBL serum concentrations, whereas individuals with the AO- and OO-genotype show lower MBL serum concentrations. This is caused by disturbed multimer formation, resulting in impaired function [15]. In addition basal MBL serum levels are influenced by the SNPs in the promoter region of the MBL gene [14].

MBL can cause activation of the lectin pathway of complement by binding to carbohydrate moieties on microorganisms and altered cells [16, 17]. In addition to complement activation, the protein has several distinct functions, including promotion of complement-independent opsonophagocytosis, modulation of inflammation, and promotion of apoptosis [18]. These properties make MBL an important mediator of immunity.

During normal healthy pregnancy, MBL concentrations are increased from the first trimester onwards until term and drop sharply directly after delivery [19]. This suggests a beneficial physiologic role for high MBL levels during normal pregnancy. It was shown

that this rise in MBL levels during pregnancy occurs in all pregnant women, but is most pronounced in mothers with the high-production MBL genotypes.

Considering the important role of inflammation in the pathophysiology of pre-eclampsia and the suggested beneficial role of high levels of MBL in normal healthy pregnancy, it was hypothesized that women with low-MBL production genotypes are more likely to develop pre-eclampsia. To elucidate the pathogenesis of pre-eclampsia, MBL polymorphisms both in the structural gene and in the promoter region were determined in women with pre-eclampsia and healthy pregnant control subjects using a case-control design.

Patients and Methods

Study group

The subjects for this case-control study have been described previously [20, 21] and are summarized in Table 1. Pre-eclampsia was defined by means of strict criteria: blood pressure ≥140 mm Hg systolic or diastolic blood pressure ≥90 mm Hg measured on at least two occasions in women who were normotensive before 20 weeks of gestation, and proteinuria (≥2+ [1 g/l]) on a voided specimen or (≥1+ [0.3 g/l] on a catheterized specimen, according to the criteria of the International Society for the Study of Hypertension and Pregnancy [22]. *Severe* pre-eclampsia was defined as an absolute diastolic blood pressure of ≥110 mm Hg and proteinuria (≥2+ [1 g/l]) on a catheterized specimen on admission. Eclampsia was clinically defined by pre-eclampsia complicated by seizures. Gestational age was calculated in days and weeks and preterm birth was defined as delivery at <37 weeks of gestation.

In brief, consecutive women who had pre-eclampsia during their first pregnancy were selected from a computer database and patient charts. Women were included who delivered on the obstetric service of the Leiden University Medical Center (n = 117) or at the St Joseph Hospital Veldhoven (n = 81), both in the Netherlands in the period from January 1, 1991, through December 31, 1996. Women (cases and control subjects) who had more than one pregnancy, multiple pregnancies, or had chronic hypertension, renal disease, diabetes, collagen vascular diseases, cancer, or thrombosis before their first pregnancy were excluded from the study [20]. None of the women with pre-eclampsia had venous thrombosis before (or during) the first pregnancy and thus none were excluded by this criterion.

We found 198 consecutive pre-eclampsia case subjects that were eligible for the study according to the criteria of the American College of Obstetrics and Gynaecology. Of these women, 163 (82%) were willing to participate in the study: 35 (18%) women with pre-eclampsia did not enter the study for various personal reasons (18 from the Leiden University Medical Center and 17 from the St. Joseph Hospital, Veldhoven): not

Table 1. Characteristics of study subjects

	Control subjects	Case patients
Number of subjects	157	157
Maternal age at delivery (years)	28.5 (3.8)	28.5 (4.9)
Gestational age (days)	278 (17.8)	238 (29.7)
Gestational age < 37 weeks	14 (8.9)	109 (69.4)
Gestational age < 34 weeks	5 (3.2)	73 (24.8)
Body Mass Index (kg/m^2)	23.6 (4.2)	24.6 (4.4)
Smoking (%)	28 (17.8)	16 (10.2)
Systolic blood pressure		
<20 weeks gestation (mm Hg)	118 (11)	127 (92)
>20 weeks gestation (mm Hg)	121 (10)	153 (16)
Diastolic blood pressure		
<20 weeks gestation (mm Hg)	69 (7)	72 (7)
>20 weeks gestation (mm Hg)	75 (7)	103 (9)
Proteinuria (mg/dl)	<30	30-100
Sex of child (male/female)	74/82*	83/71*
Race/ethnicity (% caucasian)	96	96

Data are represented as *n* (%) or mean (SD)

* The data of the gender of one child in the control group was missing and three children in the case patients group were missing.

willing to spend time (10), unknown address (6), not willing to have a venipuncture (3), expected insurance problems after DNA analysis (7), current pregnancy (3) language problems (2), or did not want to be remembered of the period of pre-eclampsia (4). The 163 patients who did participate all met the criteria of the American College of Obstetrics and Gynaecology. After the inclusion period several international studies advocated the International Study of Hypertension in Pregnancy criteria [22] as better and stricter criteria for selection and inclusion criteria for research studies. As a result women with only an increase in blood pressure values and not reaching the systolic 140 mm Hg and/or diastolic ≥ 90 mm Hg had to be excluded ($n = 6$). Therefore a total of $n = 157$ case subjects were included in our study.

Women with an uncomplicated pregnancy were used as control subjects and were consecutively selected from the same computer database according to the following criteria: first pregnancy, no rise in blood pressure, no hypertension or proteinuria, similar age (± 5 years), no biological relationship with cases or other controls, and a delivery date as close as possible to the delivery date of a case subject. Control subjects were not selected according to gestational age, as strictly speaking it is not a risk factor (or confounder) for pre-eclampsia. However to avoid an influence of preterm birth on the analyses, we also analyzed our data with the exclusion of preterm births of case and control subjects. In total 229 control subjects were asked to enter the study, 66 (29%) refused

to enter the study for various reasons: (46 from the Leiden University Medical Center and 20 from the St. Joseph Hospital, Veldhoven): not willing to spend time (19), unknown address (23), not willing to have a venipuncture (7), expected insurance problems after DNA analysis (7), current pregnancy (7) or a language problem (3).
In total, *n* = 157 case and *n* = 157 control subjects were included. A relatively higher proportion of women with *severe* pre-eclampsia and eclampsia were included, as both hospitals where the women delivered are tertiary referral centers.
Of the women included in the study, 96% were Caucasian. The Committee on Ethics in Human Research of both hospitals approved the study protocol. Data were analyzed anonymously.

Laboratory methods

Venous blood was collected into Monovette tubes (Sarstedt, Nümbrecht, Germany) containing 0.106 mol/L trisodium citrate. DNA was isolated from leukocytes and stored at -20°C.

MBL genotyping

Genotyping was performed using PCR LightCycler techniques as described previously [19]. Genotyping included the wildtype (A-allele) and the three SNPs of the first exon of the structural gene: codon 52 (D-allele), codon 54 (B-allele) and codon 57 (C-allele) and two of the polymorphisms in the promoter region at codons -550 (H/L) and -221 (X/Y) of the MBL2 gene. In each experiment, genotype matched and sequence-verified control donors were used. The researcher who performed the genotyping was blinded to case or control status. A 10% portion of the samples was reanalyzed at random, and the results were confirmed.

Categorization of the individuals upon MBL structural gene genotypes in conjunction with promoter genotypes

The three SNPs of the first exon of the structural gene are in linkage disequilibrium with the two promoter polymorphisms H/L and X/Y at codons -550 and –221, respectively. This results in six possible haplotypes: HYA, HYD, LYA, LYB, LYC, and LXA [14]. Each individual expresses two of these haplotypes. The LXA haplotype and the exon 1 polymorphisms (B-, C-, or D-allele, jointly the O-allele) cause deficient or reduced plasma concentrations [14].

Based on the genotypes the individuals can be categorized into three groups: group A ((H/L)YA/(H/L)YA and (H/L)YA/LXA), the high-MBL genotype group associated with high MBL serum levels; group B (LXA/LXA and (H/L)YA/O), the intermediate-MBL genotype group, associated with intermediate MBL serum levels; and group C (LXA/O and O/O), the low-MBL genotype group associated with MBL deficiency and hence the lowest MBL serum levels [23]. This genetic subdivision correlates best with MBL serum levels [14] and is therefore commonly used and accepted [23-25]. For that reason this subdivision is applied for all analyzes in this study.

Statistical analysis

Statistical analysis was performed using SPSS 12.0.01 for Windows (SPSS Inc, Chicago, IL). To test the hypothesis that women who develop pre-eclampsia have a higher probability of belonging to one of the two lower MBL genotype groups C or B compared with normal pregnant women, a χ^2 analysis or contingency table was used. In this case-control design it was calculated that (based on the MBL genotype group C frequency of 14.8% and on the MBL genotype group B frequency of 34.1% [25] with a power 80% and α value of 0.05, an odds ratio (OR) of 2.35 could be detected for differences in expression between MBL genotype groups A and C; when both groups B and C are taken together, an OR of 2.00 could be detected for differences in expression compared with the MBL genotype group A. We used logistic regression analysis with the risk of pre-eclampsia, *severe* pre-eclampsia or eclampsia as dependent variable and the different MBL genotype groups as independent variables. Based on literature we considered the following variables *a priori* to be possible confounders for the prevalence of pre-eclampsia, *severe* pre-eclampsia or eclampsia: gestational age (analyzed in weeks), maternal smoking during pregnancy, maternal body mass index, maternal age at delivery, and gender of the child. Simple regression analyses were performed to determine which confounders had to be included in the multiple regression analyses.

Results

We found no differences of the SNPs in the *MBL2* structural gene, taken together as the O-allele, in women with pre-eclampsia and control group (O-allele 0.22 and 0.21 respectively, OR 1.02, 95% confidence interval (CI) 0.69 –1.49).

Association of MBL genotype groups and pre-eclampsia

Table 2 shows the crude and adjusted ORs and CIs for the association between MBL genotype groups and women with pre-eclampsia (n = 157). The crude and adjusted ORs did not show a statistically significant association between MBL genotype groups and women with pre-eclampsia.

We did not see a different effect when the intermediate- and low-MBL genotype groups B and C were grouped together in all analyses (data not shown). Moreover we did not find a significant additional effect in the ORs after adjustment for all possible confounders together (*i.e.*, maternal smoking during pregnancy, maternal body mass index, maternal age at delivery, and gender of the child).

Table 2. Frequencies of the mannose-binding lectin (MBL) genotype groups and their association with pre-eclampsia

		MBL group			
		A	B	C	Missing
Control subjects (*n* = 157)	*n* (%)	88 (56.1)	47 (29.3)	21 (13.4)	1 (0.64)
Women with pre-eclampsia (*n* = 157)	*n* (%)	86 (55.8)	46 (29.3)	22 (14.0)	3 (1.91)
	Crude OR (95% CI)[a]	1	1.00 (0.61-1.66)	1.07 (0.55-2.09)	
	Adjusted OR (95% CI)[b]	1	0.97 (0.46-2.07)	1.44 (0.52-3.94)	

The MBL genotypes are categorized as MBL genotype group A, B and C. High-MBL genotype group A consists of YA/YA- and YA/XA-genotypes. Intermediate-MBL genotype group B consists of XA/XA- and YA/O-genotypes. The low-MBL genotype group C consists of XA/O- and O/O-genotypes.
OR = Odds Ratio; CI = confidence interval
a Crude OR, without any adjustment for possible confounders.
b Adjusted OR, after adjustment for all possible confounders: gestational age (weeks), maternal smoking during pregnancy, maternal Body Mass Index, maternal age (years) at delivery, and gender of child.

Association of MBL genotype groups and more severe disease (severe pre-eclampsia and eclampsia)

A trend in the association toward more severe disease (*severe* pre-eclampsia or eclampsia) could be appreciated. The simple regression analysis showed that gestational age is related to MBL genotype groups and pre-eclampsia. After correction for gestational age we found a stronger trend in the association between the low MBL genotype group C and *severe* pre-eclampsia and eclampsia itself. This association even became significant after correction for all possible confounders (described below as adjusted OR).

Crude ORs and 95% CIs for *severe* pre-eclampsia for group B were as follows: OR = 1.12, CI = 0.51–2.50; for group C: OR = 0.84, CI = 0.26–2.71. Adjusted ORs for *severe* pre-eclampsia for group B were: OR = 2.11, CI = 0.49–9.15; for group C: OR = 3.38, CI = 0.47–24.23. Crude ORs and 95% CIs for eclampsia for group B were: OR = 3.12, CI = 0.71–13.6; for group C: OR = 4.19, CI = 0.79–22.3. Adjusted ORs for eclampsia for group B were: OR = 9.90, CI = 0.86–114.03; for group C: OR = 14.70, CI = 1.18–182.76.

Discussion

In the present study MBL genotypes were not associated with pre-eclampsia, indicating that MBL is not a risk factor for this syndrome.

Because low-MBL production genotypes have been associated with preterm birth [26], one can speculate that a proper analysis is only possible by exclusion of preterm births in this study. Even when preterm births were excluded (among case and control subjects), we did not find an association between MBL genotypes and pre-eclampsia (data not shown). As expected, the women with pre-eclampsia more frequently delivered preterm (preterm deliveries in pre-eclampsia group, *n* = 109/157, 69.4%, *severe* pre-eclampsia group, *n* = 31/36, 83.8%; eclampsia group, *n* = 9/11, 81.8%).

Both case and control subjects were included over a large period of time. Therefore it is important to know that pregnancy surveillance protocols of the Dutch Society of Obstetrics and Gynaecology did not change significantly during that time. Moreover genotypes have been studied, and they would not have evolved during this period either. Finally, control subjects were selected as close to the delivery date of the case subjects, thereby avoiding selection bias in this inclusion time of 6 years.

Previously MBL has been studied in pregnancy. Low MBL, demonstrated either by measuring the actual serum protein or by determining the relevant genotypes, has been associated with adverse pregnancy outcomes, including recurrent miscarriages, risk for chorioamnionitis, and preterm delivery [26-30]. Kilpatrick studied MBL serum levels in relation to pre-eclampsia previously [31]; he did not find an association between MBL serum levels and pre-eclampsia, but he did find lower MBL serum levels in a group with recurrent pre-eclampsia (*n* = 12). However the data in this study are difficult to interpret, as the blood samples taken to measure MBL were drawn either around delivery or in the postpartum period. It has been shown that MBL levels vary greatly peri- and postpartum and therefore are not indicative of MBL levels before or during pregnancy[19].

In contrast to our data Sziller *et al.* [32] published data demonstrating that the MBL codon 54 gene polymorphism, compatible with low MBL production, protects against the development of pre-eclampsia. Differences can be explained by differences in study design. Sziller *et al*. used a limited number of patients: the number of patients expressing the MBL codon 54 gene polymorphism in that study is very low (*n* = 7), thereby bringing into question the power of that study. Furthermore these investigators examined the influence of only one polymorphism in the structural gene; the other polymorphisms in the structural gene and in the promoter region were not examined. Finally, in our study only women with first pregnancies were included, whereas Sziller *et al*. included a high percentage of multigravida (43.2%), and studying multigravida introduces covariates including obesity, previous pre-eclampsia, and chronic hypertension.

It is known that MBL is not only involved in predisposition for several diseases but that it may also act as a disease modifier [33-36]. Therefore it was investigated whether MBL genotypes are associated with the most severe spectrum of pre-eclampsia, namely *severe* pre-eclampsia and eclampsia. We observed a trend for the association of the low (C) and intermediate (B) MBL genotype groups with *severe* pre-eclampsia and eclampsia. Although the subgroups with *severe* pre-eclampsia (n = 36) and eclampsia (n = 11) are small for subanalysis, the association even became significant after correction for all possible confounders.

To explain the finding of an association of low- and intermediate-MBL genotype groups with *severe* pre-eclampsia and eclampsia, the following rationalization can be postulated. For normal placentation, a low degree of inflammation is beneficial at the fetal–maternal interface [6]. Several disorders of placentation, including pre-eclampsia, are characterized by excess inflammation at the fetal-maternal junction, resulting in tissue damage and inadequate formation of the placenta [6, 12]. In regard to pregnancy it is of particular interest that MBL facilitates the clearance of apoptotic cells and cell debris by phagocytes [37-39]. This process of apoptosis is vital for adequate placentation in early pregnancy, for example, during the extravillous trophoblast invasion when cell debris and apoptotic cells have to be cleared [20, 40-42]. Defects in these apoptotic mechanisms have been associated with pre-eclampsia [43, 44], as unrecovered cell debris can cause an influx of immunological cells and thereby excess inflammatory reactions, resulting in abnormal placentation. Because several inhibitors of complement are expressed in the placenta, to prevent complement-mediated tissue injury at the fetal-maternal junction [11], one can speculate that in the placenta the anti-inflammatory properties of MBL that do not require complement activation, predominate over the proinflammatory properties that depend on activation of the complement system. High levels of MBL could thereby protect against *severe* pre-eclampsia and eclampsia by downregulating excess inflammation in the placenta.

In summary, we found no association between MBL genotype polymorphisms and pre-eclampsia. However a trend in the association between the low- and intermediate-MBL genotype group C and B and the prevalence of *severe* pre-eclampsia and eclampsia was observed, which might implicate a role of MBL in modulating placental inflammation by facilitating clearance of apoptotic cells and cell debris. However this latter finding must be reconfirmed in studies incorporating larger numbers of patients with *severe* pre-eclampsia and eclampsia.

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Chapter 4

Mannose-binding lectin genotypes are associated with shorter gestational age.

An evolutionary advantage of low-MBL production genotypes?

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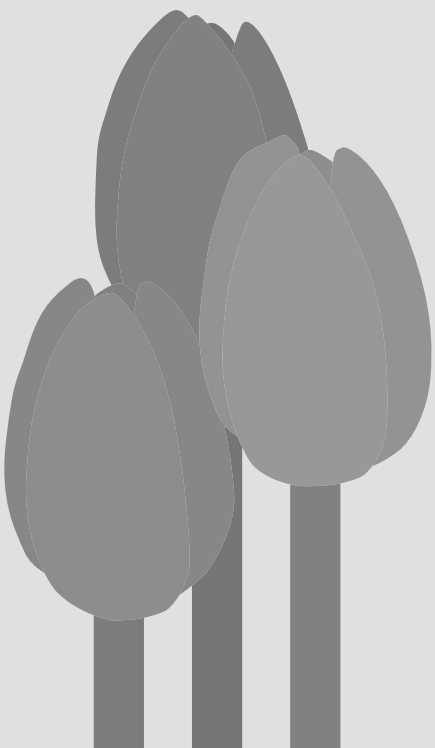

Abstract

Background: The complement factor mannose-binding lectin (MBL) is associated with adverse pregnancy outcome. MBL serum concentrations are increased from early pregnancy onwards and depend upon several gene polymorphisms. We investigated whether MBL polymorphisms are associated with term and preterm birth, since preterm birth is the leading cause of neonatal morbidity and mortality.

Methods: *MBL2* gene polymorphisms were determined in 157 nulliparous women. Considering MBL polymorphisms cases were categorized in groups of high (A), intermediate (B) and low (C) MBL production. Kaplan–Meier survival and multiple linear regression analysis were performed.

Results: Women with high MBL genotype group A had a shorter gestational age (274 days ± SD 21) than the women with the intermediate MBL genotype group B (283 days ± SD 12) and the low MBL genotype group C (284 days ± SD 9). This difference in mean gestational age is almost totally attributable to premature births in group A, since 12 of the 14 preterm births were from women with the high MBL genotype group A and only two from the intermediate MBL genotype group B.

Conclusions: We found an association between the maternal high MBL genotype group A and premature birth, suggesting that during pregnancy MBL-associated inflammation caused by higher MBL activity may contribute to earlier delivery. Furthermore, this finding might explain why so many individuals are MBL deficient in the general population.

Introduction

Despite many medical and social advances of the last decades, the incidence of preterm birth has remained almost constant (about 9%) in the modern world [1]. Besides concerns including prevention, treatment and management of preterm birth, the etiology of gestational age in human parturition needs attention, because of the high neonatal morbidity and mortality associated with preterm birth [2]. Many factors are suggested to play a role in gestational age at delivery including infectious, immunological, genetic and environmental factors (such as stress, smoking, obesity and heavy work) [3].

The hypothesis that inflammation might play a role in preterm labor and thus shorter gestational age is supported by studies demonstrating that conditions involving increased inflammation, like infections, e.g. periodontitis [4] and systemic autoimmune diseases, e.g. rheumatoid arthritis [5], are associated with shorter gestational age. In line with these clinical findings, polymorphisms in inflammatory response genes contribute to inflammation and result in shorter gestational age. Further evidence in support of a genetic role in the pathogenesis of premature birth comes from the observation of clustering and recurrence of spontaneous preterm delivery in individual women, families and ethnic groups [2, 3, 6].

Mannose-binding lectin (MBL) is a factor of innate immunity and initiates and activates the lectin pathway of complement. It can therefore be considered as a pro-inflammatory protein. MBL serum concentrations are strongly determined by single nucleotide polymorphisms (SNPs) in the structural gene (exon 1 of the *MBL2* gene: AA-, AO- and OO- genotype. The wildtype allele is referred to as A, while the O-allele represents the variant alleles B, C, and D together. Individuals with the AA wildtype genotype have the highest MBL serum concentrations, and individuals with the OO-genotype the lowest, because of disturbed multimer formation, resulting in impaired function [7, 8]. Moreover, basal MBL serum levels are modified by the SNPs in the promoter region of the *MBL2* gene (H/L or X/Y) [9].

Maternal MBL serum levels are increased from the first trimester of pregnancy and onwards, suggesting a role of MBL in nidation, placentation and maintenance of pregnancy [10]. This rise in MBL levels during pregnancy is strictly related to the maternal genotype. Multiple studies have described low maternal MBL levels (or low-MBL production genotypes) in association with adverse pregnancy outcome [11-16].

We investigated whether MBL polymorphisms are involved in human parturition and gestational age.

Materials and Methods

The women in our study have been described previously as the control group in a case (pre-eclampsia)-cohort study [17, 18]. Their characteristics are described in Table 1. All women were selected from a computer database and patient charts. Nulliparous women (n = 157) were selected according to the following criteria: first pregnancy, no rise in blood pressure, no hypertension or proteinuria, no multipara, no multiple pregnancies nor suffered from chronic hypertension, renal disease, diabetes, collagen vascular diseases or cancer, nor encountered thrombosis before their first pregnancy [17]. Controls were not selected according to gestational age. All women who delivered before 37 0/7 weeks of gestation were not induced for labor.

Table 1. Study group characteristics

Characteristics	Cases (n = 157)
MBL genotype group A	88 (56.1)
MBL genotype group B	47 (29.9)
MBL genotype group C	21 (13.4)
Missing (not categorizable)	1 (0.64)
Maternal age at delivery (years)	28.5 (3.8)
Maternal body mass index (kg/m^2)	23.6 (4.2)
Maternal smoking	28 (17.8)
Caucasian race (%)	96
Gestational age (days)	278 (17.8)
Birth weight of child (grams)	3282 (575)
Gender of child (male/female)	74/75

Data are represented as mean (SD) or n (%)

Gestational age is defined as the time measured from the first day of the woman's last menstrual cycle or if uncertain by ultrasound before 12 week's gestation to delivery. Normal gestational age is between 37 and 42 weeks (259-294 days) of pregnancy. Premature birth is defined as parturition before 37 0/7 weeks of gestation.
The local Ethics Review Board approved the study protocol.

MBL genotyping and division in genotype groups

Genotyping was performed using PCR LightCycler techniques as described previously [10]. Based upon the genotypes, individuals were categorized into three groups: the high-MBL genotype group A associated with high MBL serum levels ((H/L)YA/(H/L)YA and (H/L)YA/LXA); the intermediate-MBL genotype group B associated with intermediate MBL serum levels (LXA/LXA and (H/L)YA/O); and the low-MBL genotype group C associated with MBL deficiency and hence the lowest MBL serum levels (LXA/O and O/O) [19]. This subdivision

correlates best with MBL serum levels [7] and is therefore commonly used and accepted [19, 20] and therefore also applied for all analyzes in this study.

Statistical analysis

We used the Kaplan-Meier method for evaluating the gestational age in weeks and its correlation with the three MBL genotype groups. Differences between MBL genotype groups were assessed by the log-rank test. Furthermore we used multiple linear regression analysis to compare gestational age as the dependent variable with the different MBL genotype groups as independent variables with adjustment for all selected candidate confounders. Based on literature we considered the following variables as possible confounders: maternal smoking during pregnancy, maternal body mass index and maternal age at delivery. None of the possible confounders had a significant effect on the difference between the MBL genotype groups.

Results

Women with high-MBL genotype group A had a shorter gestational age (274 days ± SD 21 = 39 1/7 weeks ± SD 3 0/7) than the women with the intermediate-MBL genotype group B (283 days ± SD 12 = 40 3/7 weeks ± SD 1 5/7) and the low-MBL genotype group C (284 days ± SD 9 = 40 4/7 weeks ± SD 1 6/7). Since 12 of the 14 preterm births (86%) were from women with the high-MBL genotype group A and only two in the intermediate-MBL genotype group B, the difference in gestational age could almost totally be attributed to a higher frequency of premature births in MBL genotype group A (13.6% preterm births) versus MBL genotype group B and C together (2.9%). Conversely, no significant difference in gestational age between the different MBL genotype groups was found when the analysis was restricted to only women that delivered at term, i.e. 37 0/7 weeks of gestation or more. All neonates (n = 5) that were born before 34 weeks of gestation were from women with MBL genotype group A.

The relationship between MBL genotype groups and gestational age in weeks was assessed using Kaplan-Meier survival analysis. The cumulative probability of delivery and the gestational age in relation to the different MBL genotype groups is shown in Figure 1. MBL genotype groups were significantly associated with the length of gestational age (log-rank test, $p = 0.01$). In further analysis, MBL genotype group A showed a significantly shorter gestational age than MBL group B and C together (log-rank test, $p = 0.002$). However, the difference between the gestational age between MBL genotype group B and C was not significant (log-rank test, $p = 0.40$).

We performed multiple regression analysis as well, and found a significant overall MBL genotype group effect on gestational age in days (F-test, $p = 0.009$), indicating a sig-

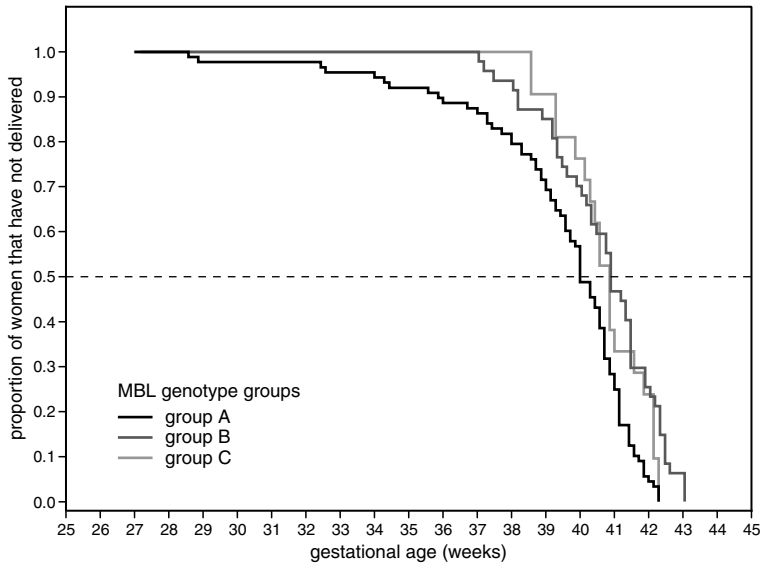

Figure 1. Kaplan-Meier survival curve showing gestational age in weeks between the different MBL genotype groups. MBL genotype group A is associated with high MBL serum levels; MBL genotype group B with intermediate MBL serum levels and MBL genotype group C with MBL deficiency and hence the lowest MBL serum levels.

nificant difference between any of the MBL genotype groups altogether. Further analysis indicated a significant difference between MBL genotype groups A and B (p = 0.009, confidence interval (CI) 2.1-14.7 days) and also between MBL genotype groups A and C (p = 0.02, CI 1.5-18.7 days). There was no significant difference between MBL genotype groups B and C. None of the possible confounders had a significant effect on the difference between the MBL genotype groups.

As expected, this shorter gestational age resulted in lower birth weight (high-MBL group A 3163 ± SD 621 grams, intermediate-MBL group B 3423 ± SD 526 g and low-MBL group C 3454 ± SD 352 grams, p = 0.01 and p = 0.04 vs. A, respectively).

The frequency of premature birth in our study population (about 9%) is the same as that found for nulliparous women in the general Dutch population. [21].

Discussion

We found that the high-MBL production genotype group A is associated with preterm birth compared to the intermediate-MBL production group B and the low-MBL production group C. The difference in gestational age between MBL genotype group A versus MBL genotype groups B and C together can be almost totally attributed to a higher percentage of premature births in women with MBL genotype group A. Since preterm birth

is the leading cause of neonatal morbidity and mortality, this finding might have clinical implications.

Previously, low-MBL production maternal genotypes or low MBL serum levels have been associated with adverse pregnancy outcomes, including recurrent miscarriages, recurrent pre-eclampsia, risk for chorioamnionitis and preterm delivery [11-16]. Up until now only Annells *et al.* [11] published data on preterm delivery and MBL. They demonstrated that the MBL codon 54 (B-allele, AO- or OO-genotype) gene polymorphism, compatible with low or intermediate MBL production, is associated with preterm delivery in a stratified group of women with preterm birth of < 29 weeks of gestation. The difference with our study outcome may be explained by differences in study design. We studied women that delivered preterm, defined as a gestational age before 37 0/7 weeks of gestation, whereas Annells *et al.* studied a stratified preterm birth group of less than 29 weeks of gestation. Also, they investigated only the influence of the polymorphisms in the structural gene; the polymorphisms in the promoter region were not examined. Moreover, in our study only women with first pregnancies were included whereas Annells *et al.* included multi- as well as nulliparous women. This might introduce a selection bias of recurrent preterm delivery in multiparous women. In addition, we did not select on preterm delivery per se, but included healthy nulliparous women. Finally, confounders were different in both studies: smoking, which may play a role in placentation and gestational age, was more frequent in women described by Annells *et al.* (35%) compared to our study group (18%).

The mechanism through which high MBL levels could cause shorter gestational age might be explained as follows: medical conditions that are associated with local or systemic inflammation predispose to shorter gestational age/premature delivery [3]. For example, it has been shown that local reproductive tract infections like chorioamnionitis, or periodontitis significantly increase the risk of subsequent preterm birth [22-24]. Rheumatological diseases, associated with autoimmune inflammation of the joints, have also been associated with increased preterm delivery and reduced birth weight [5, 25, 26] as well as infections like pyelonephritis, pneumonia, syphilis, and malaria [24]. For that reason chronic higher levels of MBL may predispose to premature delivery by aggravating those local or systemic inflammatory medical conditions that are associated with premature delivery. Aside from the association between overt inflammation during pregnancy and shorter gestational age with the role that polymorphisms in inflammatory response genes play herein, it should also be acknowledged that pregnancy itself can be considered as a state of increased inflammation [27]. Therefore, gene polymorphisms that increase the magnitude or duration of inflammation can be associated with preterm birth during uncomplicated pregnancies [28]. In line with this also for polymorphisms associated with higher levels of the pro-inflammatory molecule MBL a similar role can be considered.

In view of the association between MBL deficiency and the increased risk for infections [29], it has puzzled many scientists why so still so many individuals in the general (Caucasian) population are MBL deficient; up to almost 50% when MBL genotype groups B and C are taken together [20]. This suggests that MBL deficiency also might have an evolutionary advantage. The association of MBL genotype groups B and C with longer gestational age could be advantageous since women with this genotype have pregnancies of longer duration and hence give birth to children with a better start in life.

In summary, we found an association between the maternal high-MBL genotype group A and premature birth, suggesting that during pregnancy MBL-associated inflammation caused by higher MBL activity may contribute to earlier delivery.
Furthermore, this finding might explain why so many individuals are MBL deficient in the general population.

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Chapter 5

Mannose-binding lectin polymorphisms are not associated with rheumatoid arthritis — confirmation in two large cohorts

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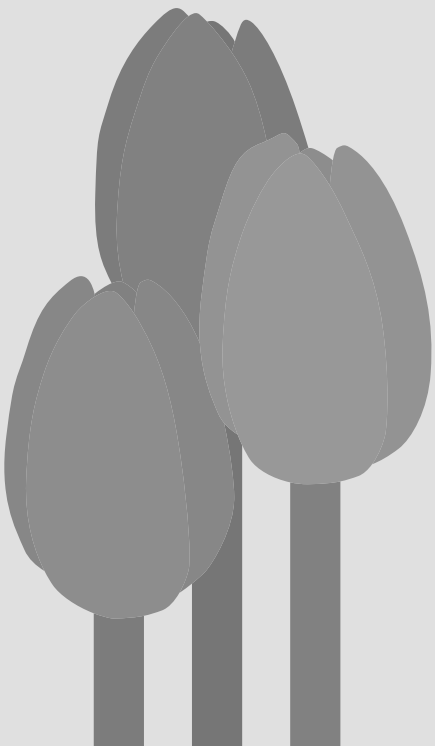

Abstract

Background: In RA, conflicting results have been described on the association between genotypes of the complement factor mannose-binding lectin (MBL) and disease susceptibility and severity. This might be due to underpowerment of previous research work and the fact that no confirmation cohorts were used. Therefore a different approach is warranted.

Methods: *MBL2* gene polymorphisms were determined in two RA cohorts (378 and 261 cases) and 648 controls. Considering MBL polymorphisms, cases and controls were categorized in groups of high, intermediate and low MBL production. The total sample size allows detection of a potential association between RA susceptibility and MBL groups with an odds ratio of 1.37 ($\alpha < 0.05$; $1-\beta > 0.8$). Disease severity as defined by the need for anti-TNF therapy was also analyzed for possible associations with MBL groups.

Results: There was no difference in the frequencies between MBL genotypes of RA cases and controls that are associated with high (cases 54.4%, controls 57.0%), intermediate (cases 28.9%, controls 27.5%) or low (cases 16.7%, controls 15.5%) MBL production. Furthermore, there was no association between MBL groups and disease severity.

Conclusions: MBL genotype groups are not associated with RA disease susceptibility or severity in this large study including a confirmation cohort. Compared with previous smaller studies these results add to more definite conclusions.

Introduction

Rheumatoid arthritis (RA) susceptibility has been associated with defects in innate immunity. Mannose-binding lectin (MBL) deficiency is one of the defects in innate immunity with the highest prevalence. MBL is a complement component that activates the lectin complement pathway. Its serum concentration is strongly determined by single nucleotide polymorphisms (SNPs) in the structural gene and the promoter region [1]. MBL gene polymorphisms have been associated with outcome in multiple diseases [2-4]. The role of MBL in RA susceptibility and severity is still controversial [4-13].

Contradictory results are a general point of concern in genetic studies. To overcome this problem, recently criteria for genetic association studies have been suggested within the National Cancer Institute and National Human Genome Research Institute (NCI-NHGRI) Working Group on Replication in Association Studies [14]. The Working Group pleaded for large sample size genotype-phenotype studies that replicate both positive as well as negative findings in multiple well-described cohorts with enough power and clear statistics and also pleaded for publishing the so-called negative studies. Until now none of the research performed on RA and MBL was in accordance with all criteria, especially most studies were underpowered and in none of the studies confirmation cohorts were used. Therefore, the association of MBL and RA remains unclear.

Therefore, we aimed to investigate whether MBL polymorphisms are associated with susceptibility for RA using a study design in accordance with the NCI-NHGRI criteria. Second, we aimed to determine whether MBL polymorphisms are associated with disease severity and clinical characteristics.

Methods

Cases and controls

DNA samples were obtained from *n* = 378 consecutive RA-patients followed in an outpatient clinic (Cohort 1). They fulfilled the ACR 1987 revised criteria for RA and to be even more certain of a diagnosis of RA, additionally had at least rheumatoid factor (RF) positivity, positivity for cyclic citrullinated peptide antibody (anti-CCP) or proof of joint erosions. DNA samples from another group of RA-patients (*n* = 261, Cohort 2) were obtained from women who were followed as part of the PARA-study, a nationwide prospective cohort study in which women with RA (according to ACR 1987 criteria) are studied during pregnancy and postpartum [15].

All controls (*n* = 648) have been described previously [2, 16-18]. Genomic DNA of *n* = 461 healthy voluntary donors was provided by the Sanquin Bloodbank, Rotterdam, The Netherlands [2, 16] and *n* = 187 controls were healthy pregnant females [17, 18].

All cases and controls included in this study are unrelated Dutch Caucasians. This study is in compliance with the Helsinki declaration and the local Ethics Review Boards approved all study protocols. Data were analyzed anonymously. The characteristics of cases and controls are described in Table 1.

Table 1. Cohort characteristics

	Controls (*n* = 637)	Cases Cohort 1 (*n* = 375)	Cases Cohort 2 (*n* = 259)
Mean age (years)	36.7 (8.23)*	59.3 (13.6)	31.9 (3.88)
Female gender	414 (64.9)**	278 (74.1)	259 (100)
Disease duration (years)		8.0 (0-53)	4.9 (0.1-28.6)
Anti-CCP positive	-	104/125 (83.2)***	162/258 (62.8)
Rheumatoid Factor (IgM) positive	-	339/369 (91.8)	184/257 (71.6)
Joint erosions	-	291/357 (81.5)	164/253 (64.8)
Median number of DMARDs (min-max) current or past use	-	3 (1-13)	2 (0-9)
Current or past use of anti-TNF therapy	-	120/375 (32.0)	35/259 (13.5)

Data are represented as *n* (%) or mean (SD or range)

* Controls versus cases Cohort 1 or cases Cohort 2, both $p < 0.0001$

** Controls versus cases Cohort 1, $p < 0.002$; Controls versus cases Cohort 2, $p < 0.0001$

*** anti-CCP was not routinely analyzed in cases Cohort 1

Determination of disease severity markers and MBL serum concentrations

Current or past use of anti-TNF therapy was used as a marker of disease severity in both RA-cohorts (*n* = 120 and *n* = 35 RA-cases from Cohorts 1 and 2, respectively). Anti-TNF therapy was chosen since its prescription is strictly regulated in The Netherlands. Its costs are only reimbursed for RA patients that have therapy-resistant disease, which is defined as failure on at least two disease-modifying anti-rheumatic drugs (DMARDs) including methotrexate (MTX) and still active disease (disease activity score (DAS28) > 3.2), despite MTX therapy at 25 mg weekly or at maximum tolerated doses.

Clinical characteristics were determined in a subgroup from RA Cohort 2, which was also seen before pregnancy. At that timepoint disease activity was scored using DAS28 (*n* = 129) and serum was obtained to determine the titers of anti-CCP (*n* = 129, EliATM CCP test, Phamarcia Diagnostics, Freiburg, Germany), IgA-, IgM- and IgG-Rheumatoid Factor (*n* = 129, Biognost, Heule, Belgium) and MBL protein concentrations (*n* = 132, sandwich ELISA as described previously [18]).

MBL genotyping and categorization of the individuals upon MBL genotypes

Genotyping was performed using PCR LightCycler techniques as described previously [18]. Genotyping included the wildtype (A-allele) and the three single nucleotide polymor-

phisms (SNPs) of the first exon of the structural gene: codon 52 (D-allele, rs5030737), codon 54 (B-allele, rs1800450) and codon 57 (C-allele, rs1800451) and two of the SNPs in the promoter region codon -550 (H/L, rs11003125) and codon -221 (X/Y, rs7096206) of the *MBL2* gene. The B-, C-, and D-allele are jointly referred to as 'O'. Linkage disequilibrium of the structural exon 1 mutations with the promoter polymorphisms results in six possible haplotypes (i.e. HYA, LYA, LXA, LYB, LYC and HYD). Every individual will express two out of these six haplotypes. The HY haplotype induces high MBL concentrations, while exon 1 mutations (O variant) and the LX haplotype cause reduced MBL concentrations.

Therefore, based upon the haplotypes, individuals can be categorized into three groups: the high-MBL production group A, (H or L)YA/(H or L)YA and (H or L)YA/LXA; the intermediate-MBL production group B, LXA/LXA and (H or L)YA/O and the low- or deficient MBL production group C, LXA/O and O/O. This genetic subdivision correlates best with MBL serum levels [1] and is therefore commonly used and accepted [3] and applied to all analyses in this study.

In each experiment, genotype-matched and sequence-verified control donors were used.

Statistical analysis

SPSS 12.0.1 was used for all statistical analyses. Pearson $\chi 2$ tests were performed to test Hardy-Weinberg equilibrium and to analyze possible associations between MBL genotype groups and RA. The Spearman rank and Kruskal–Wallis test was performed for comparison between MBL genotype groups and MBL serum levels, DAS28 and anti-CCP or RF titers. Logistic regression analysis was used for adjustment for gender and age. Differences between continuous variables were analyzed by unpaired *t*-tests. A significance level of $p < 0.05$ was used for all analyses.

Power analysis

Power analyses were performed based on published frequencies of the MBL genotype groups in Caucasians; MBL genotype group C frequency of 14.8% and MBL genotype group B frequency of 34.1% [3]. With a power of 80% and $\alpha = 0.05$ in RA Cohort 1 an odds ratio (OR) of 1.60 for MBL genotype group C alone versus MBL genotype group A and an OR of 1.44 for MBL genotype group B and C together versus MBL genotype group A could be detected for the association of RA susceptibility and MBL genotype groups. For RA Cohort 2 an OR of 1.69 for group C versus group A and an OR of 1.51 of group B plus C versus A could be detected. When the two groups were taken together, an OR of 1.51 for group C versus group A and an OR of 1.37 for group B plus C versus A could be detected for the association of RA susceptibility and MBL genotype groups.

Results

Accuracy genotyping procedure

Ten percent of the samples were randomly re-analyzed with identical results. The genotype distribution was in Hardy-Weinberg equilibrium for all SNPs tested in cases and controls. In five cases and nine controls, the exon 1 SNPs could not be determined, and in two controls the promoter SNPs could not be determined. Therefore, these cases (n = 5, <1%) and controls (n = 11, 1.7%) could not be grouped in one of the MBL genotype groups and were excluded from analysis, resulting in a total of n = 634 cases and n = 637 controls to be analyzed.

As expected, there was a good correlation between MBL genotype groups and MBL concentrations, Spearman ρ = 0.82; Group A median 607.5 ng/ml, Group B median 192.1 ng/ml, Group C median 50.7 ng/ml, n = 132, p < 0.0001.

Association of MBL genotype groups and disease susceptibility

Cohort characteristics are summarized in Table 1. There was no difference in the frequencies between MBL genotypes of RA-cases and controls that are associated with high A (cases 54.4%, controls 57.0%), intermediate B (cases 28.9%, controls 27.5%) or low C (cases 16.7%, controls 15.5%) MBL production (Table 2), indicating that there is no association between MBL genotype groups and RA susceptibility.

Table 2. Frequencies of MBL2 genotype groups and their association with disease susceptibility and disease severity

	MBL Group					
	A *n* (%)	B *n* (%)	C *n* (%)	Pearson's *p*-value	A *vs* C OR (95% CI)	A *vs* B+C OR (95% CI)
Disease susceptibility						
Cohort 1 cases (*n* = 375)	204 (54.4)	112 (29.9)	59 (15.7)	0.682*	1.06 (0.74-1.53)	1.11 (0.86-1.44)
Cohort 2 cases (*n* = 259)	141 (54.4)	71 (27.4)	47 (18.1)	0.613*	1.22 (0.82-1.82)	1.11 (0.83-1.48)
All cases (*n* = 634)	345 (54.4)	183 (28.9)	106 (16.7)	0.648*	1.13 (0.83-1.54)	1.11 (0.89-1.39)
Controls (*n* = 637)	363 (57.0)	175 (27.5)	99 (15.5)	-	-	-
Subgroup analysis for disease severity (need for anti-TNF therapy)						
Cohort 1 anti-TNF (*n*=120)	64 (53.3)	35 (29.2)	21 (17.5)	0.812**	1.21 (0.66-2.22)	1.07 (0.69-1.65)
Cohort 1 others (*n* = 255)	140 (54.9)	77 (30.2)	38 (14.9)	-	-	-
Cohort 2 anti-TNF (*n* = 35)	21 (60.0)	6 (17.1)	8 (22.9)	0.320**	1.17 (0.41-2.86)	0.77 (0.37-1.59)
Cohort 2 others (*n* = 224)	120 (53.6)	65 (29.0)	39 (17.4)	-	-	-
All cases anti-TNF (*n* =155)	85 (54.8)	41 (26.5)	29 (18.7)	0.636**	1.15 (0.70-1.89)	0.98 (0.68-1.41)
All cases others (*n* = 479)	260 (54.3)	142 (29.6)	77 (16.1)	-	-	-

*Cases versus controls, OR = odds ratio, CI = confidence interval

** RA-cases needing anti-TNF therapy (current or past use) versus RA-cases using conventional DMARDS and never used anti-TNF therapy

Association of MBL genotype groups and disease severity and disease characteristics

No association was found between MBL genotype groups and the current or past use of anti-TNF therapy, as marker for disease severity (Table 2). Furthermore, no associations were found between MBL genotype groups or serum concentration and the autoantibody titers of anti-CCP, IgA-, IgM- and IgG-RF and DAS28 (data not shown).

Discussion

MBL genotype groups are not associated with disease susceptibility, severity and disease characteristics in this large cohort of RA-patients.

We are aware that cases and controls differed regarding age and gender. Therefore, all analyses were repeated with correction for these factors, but still no differences could be observed. It was also not likely that these could have induced bias, since the MBL genotype distribution or serum levels are not influenced by age or gender [19]. It can be reasoned that our conclusions may only be applicable to Caucasian populations since in non-Caucasian populations the distribution and frequencies of MBL polymorphisms are different [8, 13].

One could argue that in complex genetic diseases like RA ORs between 1.1 and 1.3, for disease susceptibility and disease severity, could still be of interest and that therefore the present study still might be somewhat underpowered. Based upon the MBL genotype group frequencies of this study, one could calculate that the entire study population, i.e. cases and controls together, should include at least between 1900 and 14000 subjects to obtain significant ORs between 1.1 and 1.3. Besides the fact that it is generally not acceptable to add additional cases to a representative sample once analyses are performed, it is not expected that including more cases to our study would result in different conclusions, since not even a trend towards statistical significance was observed.

Another possibility to increase the power is to perform a meta-analysis on all existing data. A prerequisite for a meta-analysis is that MBL genotype groups are defined identically in all studies. It has been shown that defining MBL genotype groups can be most accurately done by combining SNPs in the structural gene with the SNPs in the promoter region. Therefore, this approach was chosen in this study. We found only one study on MBL genotypes and RA susceptibility in which MBL genotype groups could be defined identically [4]. Unfortunately, the frequencies of the control group were not described in detail, therefore making subdivision impossible for the controls. If we combined the RA patients of Graudal's study [4], (n = 140) with the cases in our study (n = 639) and compare them to the healthy controls in our study (n = 646) still similar non-statistical significant results were obtained; OR 1.16; 95% CI 0.70-1.93 for MBL group A versus group C and OR 1.12; 95% CI 0.77-1.61 for MBL genotype group A versus MBL genotype group B + MBL

genotype group C. Performing a meta-analysis on studies that investigate disease severity was not possible for the additional reason that previous studies determined disease severity differently. Furthermore, some studies used markers of disease outcome (erosions) and others disease activity as determinant of disease severity. Since both depend upon treatment strategies, which have changed markedly in recent years, recent and older literature cannot be combined in one meta-analysis.

Previous studies on MBL in relation to RA susceptibility describe ambiguous results. An association with low MBL and RA has been detected in four studies [5-8], two of which were performed in Asian populations that are known to have marked differences in MBL frequencies [6, 8]. The other two studies were performed in Caucasians, but did not study all promoter polymorphisms next to the exon 1 polymorphisms. No association was found in five studies [4, 9-12]. However, no definitive conclusions can be drawn from these studies, since they lack sufficient power and not all known MBL variant alleles were analyzed. Furthermore, none of the studies confirmed its results in an independent second cohort. MBL in relation to disease severity and disease characteristics like erosions [4-7, 12, 13], autoantibody titers [7, 11, 12] and disease activity [7, 8] has been studied in several studies [4-8, 11-13]. Their results are equivocal and several studies are hampered by a lack of power [4, 11, 12]. Also in none of these studies confirmation cohorts were included.

In conclusion, no association between RA disease susceptibility and MBL genotype groups was found. Since the results of this study were obtained in two separate cohorts of patients with enough power to demonstrate even small differences, a more definite conclusion can be drawn that MBL genotype groups are not a risk factor for the development of RA or disease severity.

Key Messages

- MBL genotype groups are not associated with susceptibility for RA.
- No association between MBL genotype groups and disease severity of RA was found.

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Chapter 6

IgG galactosylation and sialylation are associated with pregnancy-induced improvement of RA and the postpartum flare: results from a large prospective cohort study

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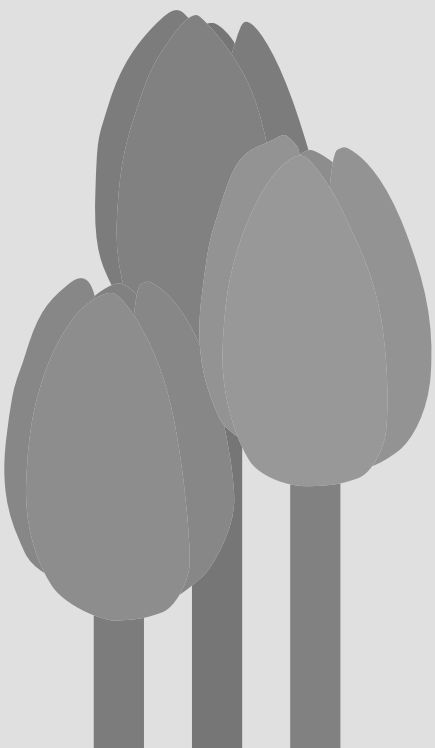
Submitted



Abstract

Background: Improvement of rheumatoid arthritis (RA) during pregnancy has been causatively associated with increased galactosylation of immunoglobulin G (IgG) N-glycans. Since previous studies were small, did not include the postpartum flare and did not study sialylation, these issues were addressed in the present study.

Methods: Serum from 148 RA-cases and 32 healthy controls was collected at several timepoints before, during and after pregnancy. Improvement during pregnancy and postpartum flare were determined according to the EULAR response criteria. Galactosylation and sialylation of IgG and the presence of bisecting N-acetylglucosamine (GlcNAc) were analyzed by MALDI-TOF-MS.

Results: IgG1 and IgG2 galactosylation of the cases and controls increased during pregnancy with a maximum in the third trimester. Galactosylation decreased directly postpartum. IgG galactosylation of controls was at a higher level than cases ($p < 0.001$ at all timepoints). A similar pattern was observed for sialylation. Moreover, there was a good correlation between galactosylation and sialylation. The increase in galactosylation was significantly more pronounced in cases with improvement than in cases without improvement during pregnancy. The reverse was true for deteriorators and non-deteriorators postpartum. The presence of bisecting GlcNAc was not significantly influenced by pregnancy or postpartum for cases and controls.

Conclusions: This large cohort study demonstrates the association of changes in galactosylation with both pregnancy-induced improvement and postpartum flare in RA-patients. The good correlation between galactosylation and sialylation implicates that the effect of galactosylation might be mediated through sialylation.

Introduction

Profound changes of the maternal immune system are required to accommodate the fetus during pregnancy. In this scope it has been hypothesized that the improvement of autoimmune diseases like rheumatoid arthritis (RA) during pregnancy is a result of these adaptations of the immune system. Conversely a flare up of the disease postpartum can be observed in RA. Although many hypotheses have been proposed [1-7], the mechanisms underlying this phenomenon are still largely unknown [8]. In addition, the pathogenesis of RA is not fully understood. Evidence is accumulating that autoantibodies are important in the pathogenesis of RA [9-11]. Autoantibodies belong to the family of immunoglobulins, which are glycoproteins. The glycosylation pattern of immunoglobulin G (IgG) has been studied most extensively [12]. Many different IgG glycoforms can be identified due to the presence of a single N-glycan chain attached to the asparagine 297 on the CH2 domain of each IgG Fragment crystallizable (Fc) portion [13]. This N-glycan shows heterogeneity due to the presence or absence of the core-linked fucose, the bisecting N-acetylglucosamine (GlcNAc), and galactose residues as well as sialic acid on the N-glycan antennae (Figure 1) [6, 14]. The presence or absence of fucose has not been associated with RA or pregnancy [15] and the presence or absence of the 'bisecting' GlcNAc has not yet been studied during pregnancy or in RA-patients. Regarding galactosylation of the outer arms of the N-glycan chains (the α1,3 or the α1,6 arm), three subfamilies called IgG-G0 or Gal-0 (agalactosyl

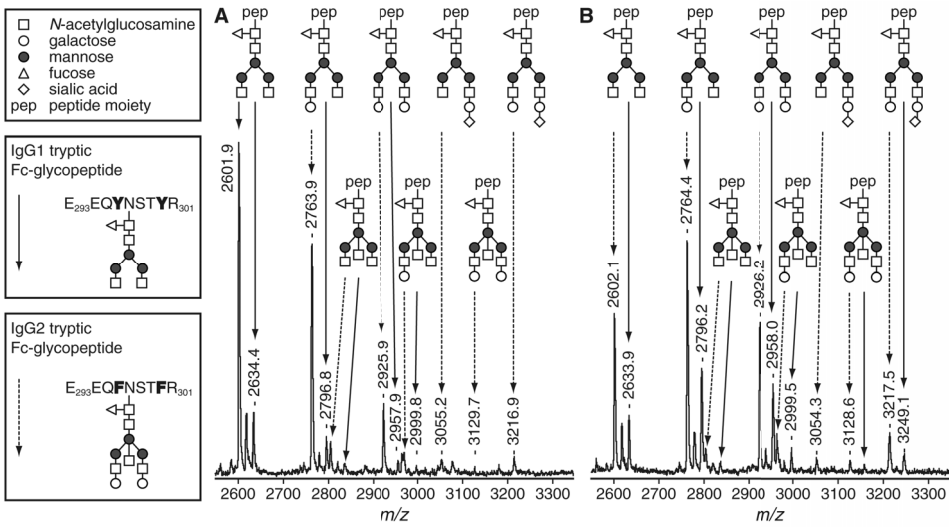


Figure 1. MALDI-TOF-MS analysis of tryptic glycopeptides of IgG1 and IgG2. Glycopeptides derived from IgG1 and IgG2 were analyzed for galactosylation and sialylation in the linear positive mode. A representative sample of an RA-patient before pregnancy (**A**) and in the third trimester (**B**) is shown.

IgG, no galactose), IgG-G1 or Gal-1 (galactose on one arm) and IgG-G2 or Gal-2 (galactose on both arms) have been defined [16]. In the Gal-1 and Gal-2 situation one terminal sialic acid residue can be present on each galactose residue.

Compared to healthy controls, higher levels of the agalactosyl IgG are found in RA-patients and these are associated with increased disease activity and a more progressive disease course [6, 17]. Moreover, in two small studies, increased galactosylation during pregnancy has been associated with the pregnancy-induced improvement of RA [7, 18]. Recently, animal studies provided evidence that antibody effector functions are not merely dependent upon the level of galactosylation but are related to the terminal sialic acid residues that can be present on the galactose molecules. (Figure 1) [19]. If the same hold true for humans, this could provide an alternative explanation for the pregnancy-induced improvement of RA.

We investigated the changes in galactosylation (Gal-0, Gal-1, Gal-2) of IgG in a large cohort of RA-patients together with controls from pre-pregnancy onwards until six months postpartum. This enabled us to study not only the association between galactosylation and improvement of RA during pregnancy, but also its association with the postpartum flare. Also factors that influence IgG galactosylation were analyzed. The MALDI-TOF-MS was applied allowing more accurate detection of N-glycan glycosylation. Furthermore, we investigated whether IgG galactosylation levels are associated with sialylation levels during pregnancy and in the postpartum period. Finally, we studied whether the presence of a bisecting GlcNAc on IgG is associated with pregnancy-improved disease activity or the flare postpartum in RA-patients.

Materials and methods

Study population

The present study is part of the PARA-study (Pregnancy-induced Amelioration of Rheumatoid Arthritis). This is an ongoing prospective cohort study in which women with RA are followed preferably from preconception until six months postpartum. The outline of this study has been described in detail [20]. Data of the first 148 Caucasian RA-patients (cases) are included in this study. Healthy pregnant Caucasian volunteers without adverse obstetric history (controls, $n = 32$) were followed from first trimester of pregnancy to six months postpartum. The study is in compliance with the Helsinki Declaration and approved by the Ethics Review Board at the Erasmus MC University Medical Center, Rotterdam, The Netherlands.

Data collection

N = 57 cases were followed from pre-pregnancy, *n* = 65 cases from the first trimester, *n* = 14 cases from the second trimester and *n* = 12 cases from the third trimester and onwards.

Disease activity was scored using a disease activity score (DAS28) with three variables (swollen joint count, tender joint count and a C-reactive protein (CRP) level) [8, 21].

Categorization of disease activity and clinical response

In accordance with the EULAR criteria remission of RA was defined as DAS28<2.6 and intermediate and high disease activity as DAS28>3.2 [22]. Improvement of disease activity during pregnancy was defined according to the EULAR criteria as 'good', 'moderate' (combined to 'responders') or 'non-responders' [22]. In line with the EULAR criteria, the response criteria can only be applied to those patients with an initial DAS28>3.2 at first trimester (*n* = 75). Deterioration of disease activity after delivery was defined according to so called 'reversed' EULAR criteria [20, 22]. Since there is no baseline DAS28 requirement for these criteria, this classification was applied to all cases. An early flare was defined when deterioration began between six weeks and three months postpartum and a late flare with disease deterioration between three to six months postpartum.

IgG glycosylation analysis

IgG was purified from serum using Protein A-Sepharose beads and treated with trypsin. These beads bind IgG1, IgG2 and IgG4, but not IgG3 [14]. Glycopeptides were desalted and purified from IgG tryptic digests by reverse phase-SPE. Therefore, each well of a Supelco DSC-18 plate SPE-96 (25 mg) was pre-treated with 1 ml of 80% acetonitrile (AcN), 0.1% trifluoroacetic acid (TFA), then 1 ml of 50% AcN, 0.1% TFA, and finally 1 ml of 0.1% TFA. The total digest (40 µl) was added to the wells, followed by three washes with 1 ml of 0.1% TFA. Glycopeptide samples were eluted into a V-bottom 96-well microtiter plate with 300 µl of 18% AcN containing 0.1% TFA. Glycopeptide samples were dried by vacuum centrifugation and dissolved in 100 µl water.

Galactosylation and the incidence of bisecting GlcNAc were analyzed for all samples: aliquots (1 µl) of the glycopeptide samples after reverse phase purification were spotted on a polished steel 384-positions MALDI-TOF-MS target plate and allowed to dry. Sample spots were overlaid with 1 µl α-cyanocinnamic acid matrix (5 mg/ml in 50% AcN) and allowed to dry at room temperature, resulting in a microcristalline sample preparation. Glycopeptides were analyzed in the reflectron positive mode on an Ultraflex II MALDI-TOF-MS (Massaspectrometer, Bruker Daltonics, Bremen, Germany). The laser power was kept constant throughout the measurements, which were performed in the automatic mode. *N* = 100 shots were acquired per position, and spectra were acquired from *n* = 30

different positions per spot, resulting in a sum spectrum obtained by accumulation from 3000 spectra per sample spot.

For the analysis of sialylation, 1 µl aliquots of the glycopeptide samples were spotted on a MTP AnchorChip 600/384 plate (Bruker Daltonics) and allowed to dry. Sample spots were overlaid with 1 µl of 2,5-dihydroxybenzoic acid matrix (5 mg/ml in 50% AcN with 0.1% TFA) and allowed to dry at room temperature, resulting in a macrocristalline sample preparation. Glycopeptides were analyzed by MALDI-TOF-MS in the linear positive mode. Per sample spot 2000 spectra were accumulated. These analyses were performed in three subgroups: first, n = 10 cases and n = 10 controls randomly selected from our cohort at every timepoint before (cases), during and after pregnancy. Second, sialylation was determined in n = 15 responders and n = 15 non-responders selected upon the most pronounced and the least-pronounced changes in disease activity during pregnancy. Third, sialylation was determined in n = 15 cases with a flare early postpartum and n = 15 cases without a flare selected upon the most pronounced and the least-pronounced changes in disease activity postpartum.

Mass spectra were processed in FlexAnalysis (Bruker Daltonics) with baseline subtraction and peak detection of the IgG glycopeptide signals. Peak lists were imported into Excel.

From the MALDI-TOF-MS measurements in the linear positive mode, IgG1 and IgG2 signals for six glycoforms were analyzed: Gal-0 without bisecting GlcNAc, Gal-1 without bisecting GlcNAc, Gal-2 without bisecting GlcNAc and Gal-0+bisecting GlcNAc (G0+N), Gal-1+bisecting GlcNAc (G1+N), Gal-2+bisecting GlcNAc (G2+N).

The levels of galactosylation of IgG1 without bisecting GlcNAc was expressed by available antenna position using the following term:

(0.5x IgG1-G1 + IgG1-G2) / (IgG1-G0 + IgG1-G1 + IgG1-G2) x 100%

The incidence of bisecting GlcNAc on IgG1 was analyzed using the following term:

(IgG1-G0+N + IgG1-G1+N + IgG1-G2+N) / (IgG1-G0+N + IgG1-G1+N + IgG1-G2+N + IgG1-G0 + IgG1-G1 + IgG1-G2) x 100%

Based on the MALDI-TOF-MS measurements in the linear positive mode, the incidence of sialic acid (SA) per galactose moiety on IgG1 was determined using the following term:

(IgG1-G1-SA + IgG1-G2-SA) / (IgG1-G1 + IgG1-G1-SA + 2x IgG1-G2 + 2x IgG1-G2-SA)

The terms shown for IgG1 are similar for IgG2.

Statistical analysis

Statistical analysis was performed using SPSS version 15.0 for Windows and SAS 9.1. A two-sided *p*-value ≤ 0.05 was considered statistically significant.

The IgG galactosylation and sialylation profiles in cases and controls during pregnancy and postpartum were estimated using a Linear Mixed Model (LMM) with unstructured residual correlation. Using this model we tested for differences in the galactosylation and sialylation levels between cases and controls at each timepoint and for changes on the time intervals from pre-pregnancy to pregnancy, within pregnancy and within the postpartum period.

LMM was also applied to compare differences in IgG galactosylation and sialylation between responders, non-responders, flare and no-flare postpartum. A possible association between galactosylation and sialylation was analyzed by Spearman rank-test.

A multivariate analysis, conditional on the timepoint of visit, was performed to investigate which covariates determine the level of galactosylation. It was assumed that their effects are constant in time. A two-step selection procedure was used. First each covariate was tested separately by univariate analysis. Secondly, the covariates that had a *p*-value lower than 0.2 where combined in a multivariate analysis. The following covariates were tested for their effect on IgG galactosylation levels: medication use ((dichotomous; yes/no): salazopyrine, prednisone, methotrexate or biological(s) use), DAS28, presence of erosions, rheumatoid factor positivity and anti-CCP positivity, whether individuals breastfed postpartum and age of the mother at time of delivery.

Finally, to determine whether changes in IgG galactosylation precede changes in disease activity during pregnancy or in the postpartum period, for every timepoint interval the change in IgG galactosylation was divided by the total change in galactosylation. The change in disease activity was also calculated per interval and divided by the total change in disease activity. This resulted in a percentage of change per timepoint interval. A paired sample *t*-test tested for equality of the changes in galactosylation and disease activity. All analyzes were performed for all time intervals mentioned above.

Results

Description of study cohort

All cases *(n = 148)* fulfilled the ACR 1987 revised criteria for RA (Table 1).

MALDI-TOF-MS measurements and accuracy and reproducibility

The intraday and interday variability for the analyzed glycopeptides of IgG1 and IgG2 was below 4% and 6%, respectively. The IgG4 glycopeptides were not analyzed due to their low abundance.

Table 1. Cohort characteristics

	Cases (*n* = 148)	Controls (*n* = 32)
Mean age at delivery, years (SD)	32.3 (3.8)	32.0 (4.4)
Median disease duration at delivery, years (range)	8.0 (0.7-29.7)	-
Number of nulliparous women	70/147 (47.6)	14/32 (43.8)
Mean gestational age at delivery, weeks (range)	39.3 (31.4-42.1)	40.4 (34.0-42.0)
Breastfeeding (6 weeks postpartum), *n* (%)	62/148 (41.9)	27/32 (84.4)
Anti-CCP positive, *n* (%)	93/147 (63.3)	-
Rheumatoid Factor (IgM) positive, *n* (%)	108/148 (73.0)	-
Erosive disease, *n* (%)	43/147 (70.7)	
DAS28-CRP3 >3.2 in first trimester, *n* (%)	75 (61.5)	-
Classification of disease activity during pregnancy, *n* (%)		
- Good response / moderate response	37/75 (49.3)	-
- No response	38/75 (50.7)	-
Classification of disease activity during postpartum period (*early flare*), *n* (%)		
- Severe deterioration / moderate deterioration,	35/141* (24.8)	-
- No deterioration	106/141* (75.2)	-
Classification of disease activity during postpartum period (*late flare*), *n* (%)		
- Severe deterioration / moderate deterioration	29/141* (20.6)	-
- No deterioration	112/141* (79.4)	-
Median number of DMARDs** (incl prednisone) prior to conceive (min-max)	2 (0-7)	-
No DMARD** use prior to conceive, *n* (%)	7/147 (4.8)	
Use of methotrexate prior to conceive, *n* (%)	75/147 (51.0)	-

Data are represented as *n* (%) or mean (SD or range)

*n = 7 cases are missing, since a small proportion of DAS scores are missing

**DMARDs: disease modifying anti-rheumatic drugs

IgG galactosylation profiles during pregnancy and postpartum of cases and controls

For the cases, already in the first trimester the incidence of IgG1 galactosylation was increased significantly compared to preconception levels (mean 43.4% (SD 8.3%) to 48.4% (SD 8.4%)). This increase continued until the third trimester (mean 53.7% (SD 8.3%), $p < 0.0001$). At 6 weeks postpartum this incidence was significantly decreased to mean 46.9% (SD 7.2%). At 6 months postpartum the galactosylation levels further decreased (mean 44.9% (SD 7.7%), $p < 0.0001$, Figure 2a). IgG2 galactosylation profiles show a very similar pattern as IgG1 (Figure 2b).

In the controls, IgG1 and IgG2 galactosylation profiles were at a significantly higher level than cases ($p < 0.001$), and changes were less pronounced than in the cases (Figure 2).

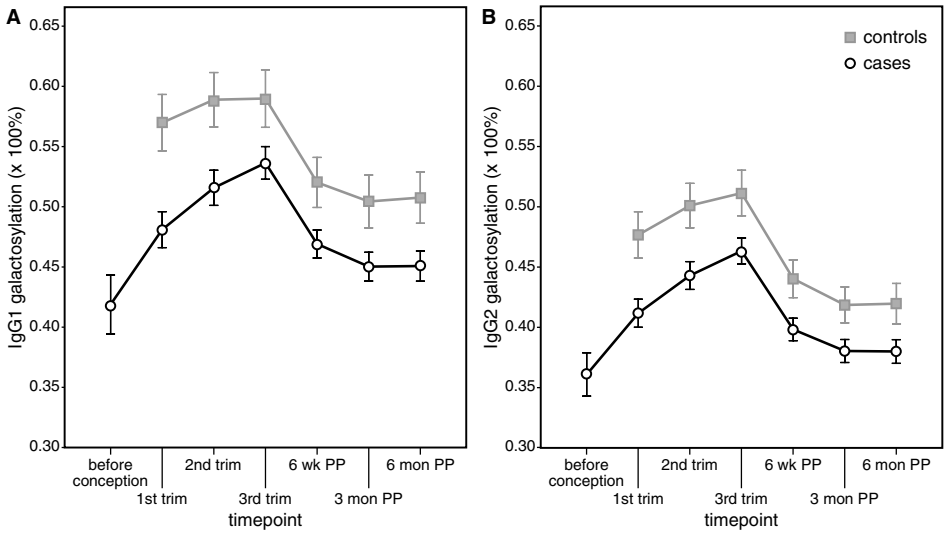


Figure 2. Mean galactosylation of IgG1 (**A**) and IgG2 (**B**) (in percentages) in cases and controls during pregnancy and postpartum. IgG1 and IgG2 galactosylation levels increase during pregnancy and decline postpartum. IgG1 and IgG2 galactosylation profiles of controls are at a significantly higher level than cases ($p < 0.001$, Linear Mixed Model at all timepoints). The vertical bars illustrate the 95% confidence intervals.
Abbreviations: trim, trimester of pregnancy; wk, weeks; PP, postpartum; mon, months.

IgG galactosylation and disease activity levels

IgG1 galactosylation levels are associated with disease activity at every timepoint (Pearson correlation R between 0.35 and 0.49, $p < 0.005$). Lower disease activity levels show higher galactosylation levels, and resemble more the levels of the controls. The IgG1 galactosylation level which is associated with disease remission (DAS28<2.6) is dependent on the timepoint of measurement (Figure 3). Similar data were observed for IgG2 (data not shown).

Changes in IgG galactosylation are associated with improvement of disease activity in responders and non-responders during pregnancy

The change in galactosylation from the first to the third trimester was significantly different between responders ($n = 37$) and non-responders ($n = 38$) for IgG1 (6.8% (SD 0.80%) versus 4.2% (SD 0.79%), respectively, $p < 0.02$), whereas for IgG2 a trend could be observed (5.6% (SD 0.51%) versus 4.5% (SD 0.50%), respectively, $p = 0.11$, Figure 4a).

Changes in IgG galactosylation are associated with early and late disease flare postpartum

Cases with a late flare may also have experienced an early flare ($n = 9$). The change in galactosylation from six weeks to three months postpartum was significantly different

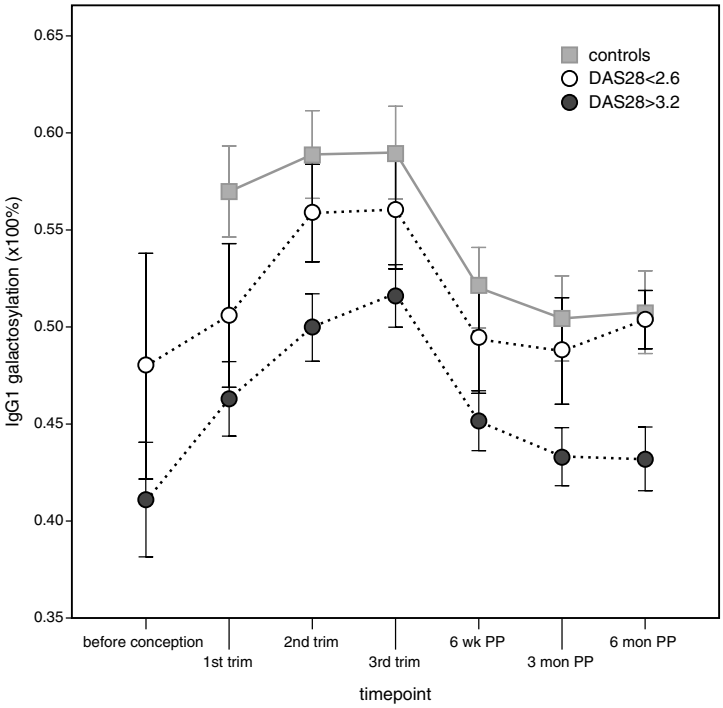


Figure 3. Mean IgG1 galactosylation levels in relation to RA disease activity levels. For this purpose at every timepoint all cases were divided in two categories; i.e. those with a DAS28>3.2 or DAS28<2.6. Please note that each timepoint may include different RA-cases. For comparison controls are added to the graph. The IgG1 galactosylation level which is associated with disease remission (DAS28<2.6) is dependent on the timepoint of measurement. Similar data were observed for IgG2 (data not shown). The vertical bars illustrate the 95% confidence intervals. Abbreviations: DAS28, disease activity score; trim, trimester of pregnancy; wk, weeks; PP, postpartum; mon, months.

between the cases with an early flare (n = 35) and without flare (n = 106), namely -3.3% (SD 0.58%) versus -1.3% (SD 0.33%), respectively for IgG1, $p < 0.004$; and for IgG2: -2.7% (SD 0.42%) versus -1.3% (SD 0.24%), respectively, $p < 0.004$, Figure 4b. The change in IgG galactosylation between three and six months postpartum was significantly different between the cases with a late flare (n = 29) and without late flare (n = 112), namely -1.2% (SD 0.49%) versus +0.58% (SD 0.33%), respectively for IgG1, $p < 0.0001$; and for IgG2 -1.1% (SD 0.30%) versus +0.16% (SD 0.20%), respectively, $p < 0.0004$, Figure 4c.

Galactosylation changes do not precede disease activity changes

When changes in IgG galactosylation and changes in RA disease activity were tested for equality, this hypothesis could not be rejected, indicating that IgG galactosylation and DAS28 may change synchronically in time.

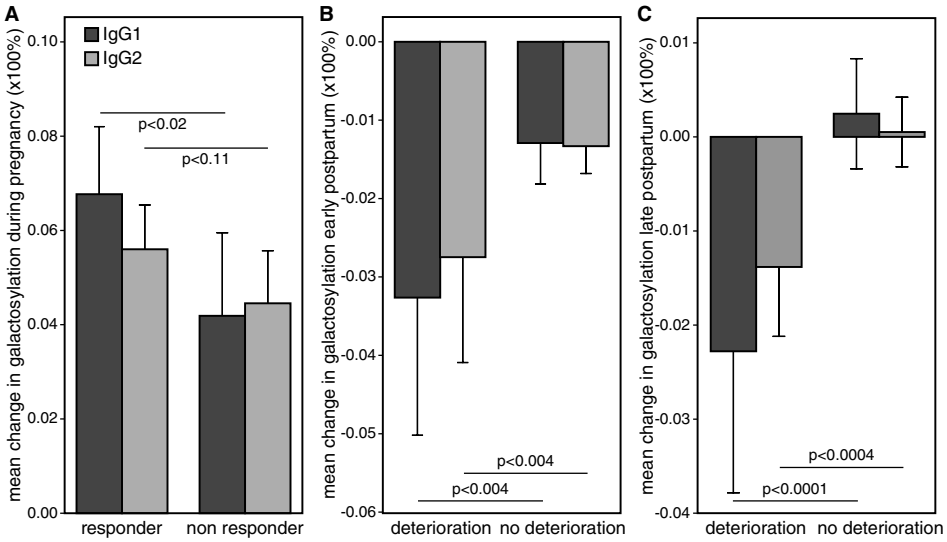


Figure 4. Mean change in IgG1 and IgG2 galactosylation (x100%) for responders and non-responders during pregnancy and early or late flare postpartum. **A**) Mean change in IgG1 and IgG2 galactosylation (x100%) during pregnancy in (good and moderate) responders according to the EULAR response criteria ($n = 37$) and non-responders ($n = 38$). The change in IgG galactosylation was significantly different between responders and non-responders for IgG1 ($p < 0.02$), whereas for IgG2 a trend towards significance could be observed ($p = 0.11$). **B**) Mean change in IgG1 and IgG2 galactosylation (x100%) in the postpartum period in cases with an early flare between 6 weeks and 3 months postpartum (deterioration, $n = 35$) and cases without an early flare (no deterioration, $n = 106$). The change in galactosylation was significantly different between early flare and no early flare for IgG1 and IgG2 ($p < 0.004$). **C**) Mean change in IgG1 and IgG2 galactosylation (x100%) in the postpartum period in cases with a late flare from 3 to 6 months postpartum (deterioration, $n = 29$) and cases without a late flare (no deterioration, $n = 112$). The change in galactosylation was significantly different between late flare and no late flare for IgG1 and IgG2 ($p < 0.0001$ and $p < 0.0004$, respectively).
The vertical bars illustrate the 95% confidence intervals.

IgG sialylation during pregnancy and postpartum

IgG sialylation was, like galactosylation, determined as total percentage of sialic acid (SA) residues per N-glycan. The presence of SA on IgG1 and IgG2 is low in the serum for cases and controls (all measurements taken together: mean 5.8% (SD 2.3%) SA per N-glycan for IgG1 for cases (controls 7.1%, SD 2.7%) and 6.6% (SD 2.6%) per N-glycan for IgG2 for cases (controls 7.9%, SD 2.5%)).

Also the sialylation as percentage of SA per galactose sugar moiety was calculated: mean 6.0% (SD 2.2%) SA per galactose for IgG1 for cases (controls 6.2%, SD 1.4%) and 7.9% (SD 2.3%) per galactose for IgG2 for cases (controls 8.2%, SD 1.8%).

In RA-cases N-glycan sialylation levels and IgG galactosylation were significantly correlated (Spearman ρ 0.57 and 0.69 for IgG1 and IgG2, respectively, both $p = 0.0001$). In

controls, the correlation between sialylation and IgG galactosylation was 0.77 and 0.72 for IgG1 and IgG2, respectively, both $p = 0.0001$).

The mean sialylation levels per N-glycan increased during pregnancy and decreased postpartum for cases and controls, following a similar pattern as the IgG galactosylation levels. The controls showed a higher level of sialylation than the cases (data not shown).

The increase in N-glycan sialylation was larger in responders than in non-responders during pregnancy (within responders +1.8%, SD 0.42%, $p = 0.0007$; within non-responders +0.3%, SD 0.42%, not significant). The results for IgG2 were similar (data not shown). In the postpartum period for IgG1 N-glycan sialylation no significant changes were observed between cases with an early flare and cases without flare. However, for IgG2 sialylation a statistically significant decrease in N-glycan sialylation could be observed in cases with an early flare (-0.95%, SD 0.40%, $p = 0.024$).

Dependent variables of galactosylation

To investigate which factors determine the level of galactosylation multivariate analyses were performed. Univariate analyses revealed the following covariates with a significant negative effect on IgG galactosylation: sulfasalazine use, prednisone use, DAS28, RF positivity, anti-CCP positivity and erosions for IgG1 and sulfasalazine use, prednisone use, DAS28, RF positivity, anti-CCP positivity for IgG2. In the multivariate analyses only DAS28 and sulfasalazine ($p = 0.06$) for IgG1 and DAS28 and prednisone use ($p < 0.05$) for IgG2 still had a significant negative effect on IgG galactosylation.

Interestingly, when a constant effect in time of the covariates was not assumed, we found that the effect of breastfeeding postpartum varied over time in the multivariate analyses. This effect varied from a slightly positive, but not statistically significant effect at six weeks postpartum (+0.34%, $p = 0.58$) to a statistically significant negative effect thereafter (-1.27% at three months and -2.32% at six months postpartum, $p = 0.046$ and $p = 0.041$, respectively). The overall average (negative) effect of breastfeeding on IgG galactosylation was not significant in the multivariate analyses ($p = 0.68$). All other covariates had a constant negative effect over all timepoints.

Presence of bisecting GlcNAc and its influence on galactosylation

The presence of IgG with a bisecting GlcNAc is low in the serum (first trimester mean 13.7%, SD 2.8% for IgG1 (both cases and controls) and mean 13.3%, SD 3.2% or mean 13.5%, SD 3.5% (cases and controls, respectively) for IgG2. The presence of bisecting GlcNAc was not influenced by pregnancy or postpartum and was similar in cases (range min-max IgG1 13.7-14.7%, range min-max IgG2 13.2-14.3%) and controls (range min-max IgG1 13.7-14.4%, range min-max IgG2 13.0-14.0%). Moreover uni- and multivariate analyses did not reveal any effect of the previously mentioned covariates on the presence of bisecting GlcNAc.

The presence of the bisecting GlcNAc is related to IgG galactosylation. The levels of galactosylation of IgG1 or IgG2 with bisecting GlcNAc were at a significant lower level than IgG1 or IgG2 without bisecting GlcNAc (range min-max IgG1 with bisecting GlcNAc 38.2-44.8%, range min-max IgG2 with bisecting GlcNAc 31.8-38.9 %) at every timepoint ($p < 0.0001$), but showed a similar pattern in time.

Discussion

This study demonstrates the association between changes in IgG galactosylation and changes in RA-disease activity during pregnancy and also postpartum. The most prominent increase in IgG galactosylation was observed in RA-patients that spontaneously improved during pregnancy, whereas the reverse was observed for the flare postpartum. IgG galactosylation changes synchronically in time with DAS28 disease activity. A good correlation between IgG N-glycan galactosylation and sialylation was demonstrated. This could indicate that the association of IgG galactosylation with pregnancy-induced improvement of RA and the postpartum flare may be mediated by N-glycan sialylation.

IgG galactosylation and RA in relation to pregnancy has been studied previously. Our results are in line with a previous study in which galactosylation levels and clinical outcome in RA are described during pregnancy using the lectin analysis method [18]. The application of the MALDI-TOF-MS allowed us to analyze both IgG1 and IgG2 and to analyze specifically the Fc-fragment glycosylation (and galactosylation). This is in contrast to the lectin method, which cannot distinguish between IgGs and determines a combined value for the Fc and Fab fragment glycosylation. Compared to previous literature, we studied a larger cohort with a longer follow-up time postpartum. This enabled the description of the postpartum flares and the identification of factors that influence galactosylation. Moreover a control group was added and internationally recognized outcome measures like DAS28 and EULAR response criteria were applied [18]. Based upon studies in one patient it had been suggested that pregnancy-induced remission is associated with a fixed galactosylation level [7]. In contrast we demonstrated that the level of clinical remission was associated with a different level of galactosylation per timepoint during pregnancy and postpartum (Figure 3).

We have shown that the IgG galactosylation changes take place simultaneously with the changes in RA disease activity. For that reason one could argue that changes in IgG galactosylation are a mere epiphenomenon accompanying changes in disease activity. However, the strongest argument that galactosylation of IgG, and in particular agalactosyl IgG itself, indeed plays a pathogenic role, is derived from animal studies. In these studies arthritis could only be transferred by infusion of agalactosyl IgG [23].

The pro-inflammatory role of agalactosyl IgG may be explained in multiple ways: first, IgG can act as an auto-antigen itself in RA. Since RF preferentially binds to agalactosyl IgG, this would result in more pronounced RF-agalactosyl IgG interaction and hence more immune complex deposition and inflammation [24, 25].

Secondly, the pathogenicity of agalactosyl IgG in RA-patients is thought to be associated with its ability to activate the complement pathway via binding to mannose-binding lectin (MBL) [26]. However, this hypothesis has been questioned recently based upon studies in MBL-deficient mice [13].

As a result of the absence of galactose, agalactosyl IgG antibodies also lack terminal sialic acid residues. These terminal sialic acid residues have recently been implicated in suppressing inflammation via the induction of inhibitory FcγRIIb expression in mice [19, 27]. Our analysis revealed a good correlation between IgG N-glycan galactosylation and sialylation. Moreover, similar profiles of IgG galactosylation levels and N-glycan sialylation levels are shown during pregnancy and postpartum, both for IgG1 and IgG2. This implicates that also in humans the effect of galactosylation could be mediated through the presence of increased N-glycan sialylation of IgG. Although more pronounced, the changes in N-glycan sialylation in responders and non-responders during pregnancy and cases with an early flare and cases without a flare postpartum were not statistically significant, which is in contrast to the statistically significant IgG galactosylation changes (data not shown). This may be since N-glycan sialylation is only present in small percentages in the serum and that therefore measurements have large standard deviations from the mean. Therefore, for clinical association studies it can still be more practical to determine the level of IgG galactosylation instead of sialylation.

Since we have shown that changes in IgG galactosylation levels and subsequently in sialylation levels are associated with improvement of RA during pregnancy and the flare postpartum, identification of factors that influence galactosylation might give insight into pathogenic mechanisms underlying RA and might be a lead for the development of future therapies. For this purpose multivariate analyses were performed. These revealed that only disease activity and timepoint in pregnancy remained as an explanatory parameter for IgG galactosylation. Interestingly, when the effect of breastfeeding was analyzed, with the assumption that its effect could vary over time, it was found that its effect on IgG galactosylation varied from a slightly positive, but not statistically significant, effect at six weeks postpartum to a statistically significant negative effect thereafter. The average (negative) effect of breastfeeding postpartum was not significant. We do not have an explanation for this phenomenon, however, breastfeeding has been associated with increased RA-disease activity in earlier studies [28].

Pregnancy-induced changes in cytokine or hormonal levels could be a possible explanation for the changes in IgG galactosylation during pregnancy and postpartum. It has been suggested that IL-6 [29] or pregnancy-associated hormones like estrogen [25] or prolac-

tin [30] could induce altered glycosyltransferase (or other (iso)enzyme) activity in B-cells that could result in immunoglobulins with different glycoforms. Prolactin levels are more than ten-fold increased during pregnancy and stay at a high level during breastfeeding postpartum and could therefore play a role in galactosylation changes [30].

For the first time it has been shown that the levels of bisecting GlcNAc are not influenced by pregnancy. Pekelharing *et al* found no changes in the presence of GlcNAc during pregnancy using gas-liquid chromatography [15], not distinguishing between antenna GlcNAc and the bisecting GlcNAc on other positions. The clinical relevance of bisecting GlcNAc is still unknown. Interestingly, the levels of IgG galactosylation with bisecting GlcNAc were significantly lower than the levels of IgG without bisecting GlcNAc. One can speculate that steric hindrance by the presence of a bisecting GlcNAc on the binding of galactose moieties plays a role. Otherwise yet-unidentified factors of the local microenvironment of immunoglobulin-secreting plasma cells that might result in increased presence of bisecting GlcNAc on immunoglobulins and on the same time decreased IgG galactosylation could also play a role.

In conclusion, this large prospective cohort study demonstrates the association between IgG galactosylation changes with pregnancy-induced improvement and postpartum flare in RA-patients. Since IgG galactosylation was associated with sialylation, also increased sialylation during pregnancy may provide an explanation for the pregnancy-induced improvement of RA. The levels of IgG galactosylation largely depend on the trimester of pregnancy or the timepoint of visit postpartum and disease activity, even after correction for medication use.

Future studies should focus on unraveling the exact mechanism behind the changes in IgG galactosylation and sialylation and on the consequences of these changes on the function of IgG itself during pregnancy and postpartum. In particular it should be studied whether its ability to interact with Fcγ receptors is changed during pregnancy and postpartum.

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Chapter 7

Can mannose-binding lectin explain course and outcome of pregnancy in rheumatoid arthritis?

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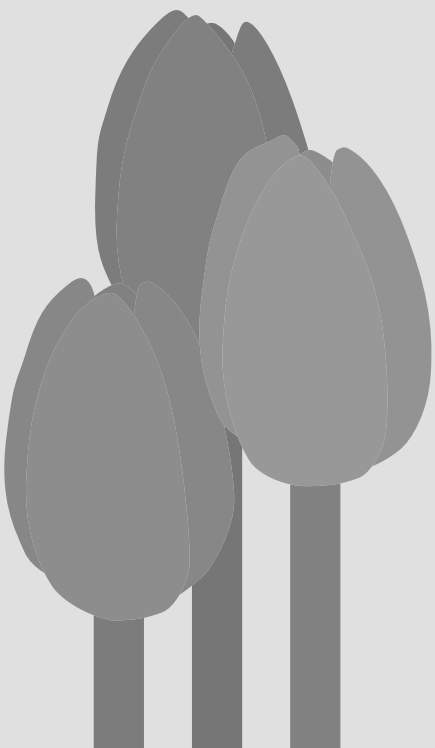
Submitted



Abstract

Background: Rheumatoid arthritis (RA) improves during pregnancy and flares after delivery. It has been hypothesized that high levels of the complement factor mannose-binding lectin (MBL) are associated with a favourable disease course of RA by facilitating the clearance of pathogenic IgG lacking galactose sugar moieties. During pregnancy increased galactosylation of IgG can be observed, whereas simultaneously MBL levels increase. The latter being strictly related to maternal MBL genotypes. Therefore, increased MBL levels in concert with increased IgG galactosylation may be associated with pregnancy-induced improvement of RA.

Objectives: To investigate whether MBL genotypes are associated with changes in RA-disease activity and with changes in IgG galactosylation during pregnancy and postpartum. Also the association between MBL genotypes and pregnancy outcome in RA is studied.

Methods: Serum from 216 RA-cases and 31 healthy controls of the Pregnancy-induced Amelioration of RA (PARA)-study was collected before, during and after pregnancy. IgG galactosylation was determined by MALDI-TOF-MS. Disease activity was determined using DAS28. MBL genotypes were determined. Pregnancy outcome measures studied were gestational age, birth weight, miscarriage and hypertensive disorders.

Results: No association was found between MBL genotype groups and changes in RA-disease activity ($p = 0.89$) and changes in IgG galactosylation (cases $p = 0.75$ and controls $p = 0.54$) during pregnancy and postpartum. Furthermore, MBL genotype groups are not related to the studied pregnancy outcome measures.

Conclusion: This study does not provide evidence for a role for MBL in the improvement of RA during pregnancy, nor for a role in pregnancy outcome.

Introduction

Pregnancy is the only natural situation that results in spontaneous improvement of rheumatoid arthritis (RA) and a flare of the disease after delivery in a substantial number of patients. Insight into the mechanism may therefore not only enlarge our knowledge on the phenomenon of pregnancy-induced remission in RA, but may also contribute to a better understanding of pathogenic mechanisms underlying RA in general. It has been hypothesized that high levels of the complement factor mannose-binding lectin (MBL) are associated with a favorable disease course of RA by binding to and hence facilitating the clearance of pathogenic immunoglobulin G, which lacks galactose sugar moieties (called agalactosyl IgG) [1].

MBL is the initiator of the innate immunity lectin pathway of complement and its serum levels are highly variable between individuals due to the presence of single nucleotide polymorphisms (SNPs) in the promoter region and in exon 1 of the *MBL2* gene. It has been shown that during pregnancy MBL levels are markedly increased and that this increase is strictly related to the high maternal MBL production genotypes [2].

It has been shown *in vitro* that MBL can bind to pathogenic agalactosyl IgG [3]. In RA-patients levels of agalactosyl IgG decline during pregnancy and IgG galactosylation increases [4, 5]. Therefore, it has been suggested that during pregnancy the MBL protein could play a role in the clearance of pathogenic (agalactosyl IgG)-immune complexes by serving as a scavenger molecule [1]. This would decrease RA-disease activity during pregnancy and consequently the low levels of MBL postpartum could be responsible for the postpartum flare [2].

Apart from the role of MBL in RA, MBL has also been, in healthy individuals, associated with pregnancy outcome like gestational age, birth weight, recurrent miscarriages, risk for chorioamnionitis and severe (or recurrent) pre-eclampsia [6-9]. Whether the same holds true for RA is unknown.

We therefore not only aim to provide evidence for a role for MBL in the improvement of RA during pregnancy, but also for a role in the pathogenesis of RA in general, by investigating whether high MBL production genotypes are associated with improvement of RA disease activity and with the changes in IgG galactosylation during pregnancy and the postpartum flare. Moreover, the possible association between MBL genotypes and pregnancy outcome in RA is studied.

Materials and methods

Study population

The current study is embedded in the PARA-study, a prospective cohort study on pregnancy and RA [10]. In this study RA-patients are visited preferably before pregnancy, three times during pregnancy and three times postpartum. Disease activity of RA was scored using the internationally recognized disease activity score (DAS28) with three variables (swollen joint count, tender joint count and C-reactive protein (CRP) level) since this variant of the DAS28 is most reliable during pregnancy [11]. Some cases were analyzed during more than one pregnancy. Controls were followed from first trimester of pregnancy and onwards. Data of in total $n = 216$ Caucasian RA-patients (cases) and $n = 31$ healthy pregnant Caucasian volunteers without adverse obstetric history (controls) are included. The study is in compliance with the Helsinki Declaration and approved by the Ethics Review Board at the Erasmus MC University Medical Center, Rotterdam, The Netherlands.

Data collection

Available data of cases differed per research question. To investigate whether MBL genotypes are associated with changes in RA-disease activity during pregnancy and postpartum, data of maximal $n = 181$ cases were available. Cases that experienced a miscarriage were excluded. To investigate whether MBL genotypes are associated with changes in IgG galactosylation data of maximal $n = 145$ cases were available. The association between MBL genotypes and pregnancy outcome including miscarriages could be studied in $n = 214$ cases. For the analyses of the pregnancy outcome measures birth weight and gestational age non-Caucasians, twin pregnancies and pregnancies that resulted in a child with a malformation were excluded, resulting in $n = 184$ cases to be studied. Data were analyzed with and without the cases that participated twice or more.

Categorization of disease activity and clinical response

In accordance with the EULAR criteria remission of RA was defined as DAS28<2.6 and intermediate and high disease activity as DAS28>3.2. Improvement of disease activity during pregnancy was defined according to the EULAR criteria as 'good', 'moderate' (combined to 'responders') or 'non-responders'. In line with the EULAR criteria, the response criteria can only be applied to those patients with an initial DAS28>3.2 at first trimester ($n = 84$). Deterioration of disease activity after delivery was defined according to so called 'reversed' EULAR criteria [10]. Since there is no baseline DAS28 requirement for these criteria, this classification was applied to all cases. An 'early flare' was defined when deterioration began between six weeks and three months postpartum and a 'late flare' with disease deterioration between three to six months postpartum.

MBL genotyping

Genotyping was performed using PCR LightCycler techniques as described previously [2]. Genotyping included the wildtype (A-allele) and the three SNPs of the first exon of the structural gene: codon 52 (D-allele, rs5030737), codon 54 (B-allele, rs1800450) and codon 57 (C-allele, rs1800451) and two of the SNPs in the promoter region codon -550 (H/L, rs11003125) and codon -221 (X/Y, rs7096206) of the *MBL2* gene. Based upon the haplotypes individuals can be categorized into three groups that correlate best with MBL serum levels [12]: the high-MBL production group A, the intermediate-MBL production group B and the low- or deficient MBL production group C.

IgG galactosylation analysis

IgG was purified from serum of cases and controls as described before [13]. Next, IgG galactosylation was analyzed by MALDI-TOF mass spectrometry (MS) of tryptic glycopeptides and massspectra of IgG1 and IgG2 were processed in FlexAnalysis (Bruker Daltonics).

Pregnancy outcome definitions

Preterm birth was defined as gestational age of <37 weeks of gestation and low birth weight was defined as a birth weight of <2500 grams. As described before, the birth weights analyzed were corrected for gestational age and gender of the child by using the birth weight standard deviation (SD) score. Birth weight SD scores as well as uncorrected birth weights are added to the analyses [14]. Hypertensive disorders were scored according to the criteria of the International Society for the Study of Hypertension and Pregnancy (ISSHP) [7]. Miscarriage was scored in case of a spontaneous loss of a pregnancy before the 20th week.

Statistical analysis

Statistical analysis was performed using SPSS 15.0 and SAS 9.1. A two-sided p-value $\leq$ 0.05 was considered statistically significant.

The disease activity (DAS28, cases) and galactosylation profile (cases and controls) was estimated using a Linear Mixed Model (LMM). Using this model possible associations between the MBL genotype groups and DAS28 or IgG galactosylation were investigated.

A chi-squared analysis was performed to compare MBL genotype groups of responders versus non-responders, of cases with a flare postpartum versus no flare, of cases which had a miscarriage versus no miscarriage, of cases which had a preterm birth versus no preterm birth, and of cases with a child with low birth weight versus child with no low birth weight. Logistic regression analysis was performed for dichotomous variables and linear regression was performed for linear data. Based on literature we considered the following variables *a priori* to be possible confounders (when applicable): gestational age, maternal smoking during pregnancy, maternal age at delivery, gender of the child, prednisone use in first

trimester, parity, disease activity in first trimester (DAS28). First, simple regression analyses were performed to determine which confounders had to be included in the multiple regression analyses.

Table 1. Cohort characteristics

	Cases (*n* = 214)	Controls (*n* = 31)
MBL genotype group A	114 (53.3)	16 (51.6)
MBL genotype group B	59 (27.6)	8 (25.8)
MBL genotype group C	41 (19.2)	7 (22.6)
Number of Caucasians	207 (96.7)	31 (100)
Number of nulliparous women	113/214 (52.8)	14/31 (45.2)
Mean age at delivery, years, (SD) (range)	32.5, 3.7 (21.9 – 40.6)	32.1, 4.5 (24.2 - 40.1)
Mean gestational age at delivery, weeks (range)	39.4 (31.4-42.1)	40.0 (34.7-42.0)
Smoking during pregnancy	6/206 (2.9)	3/31 (9.7)
Miscarriage	23 (10.7)	-
Hypertension	25/210 (11.7)	2 (6.5)
Pre-eclampsia	4/210 (1.9)	1 (3.2)
Anti-CCP positive	134/213 (62.9)	-
Rheumatoid Factor (IgM) positive	161 (75.1)	-
Erosive disease	136/210 (64.8)	
Median disease duration at delivery, years (range)	7.9 (0.7-29.0)	-
Use of prednisone in first trimester	60/164 (36.6)	
Median number of DMARDs** (incl prednisone) prior to conceive (min-max)	2.3 (0-6)	-
Use of methotrexate prior to conceive	120/212 (56.6)	-
DAS28-CRP3 >3.2 in first trimester	94/155 (60.6)	-
Classification of disease activity during pregnancy *n* (%)		
- Good response or moderate response	40/84 (47.6)	-
- No response	44/84 (52.4)	-
Classification of disease activity during postpartum period (early flare) *n* (%)		
- Severe deterioration or moderate deterioration	39/167* (23.4)	-
- No deterioration	128/167* (76.6)	-
Classification of disease activity during postpartum period (late flare) *n* (%)		
- Severe deterioration or moderate deterioration	28/152* (18.4)	-
- No deterioration	124/152* (81.6)	-

Data are represented as *n* (%) or mean (SD or range)

* cases are missing, since in a proportion of DAS scores are missing

**DMARDs: disease modifying anti-rheumatic drugs including prednisone

Results

Clinical characteristics of the study group

The characteristics of cases and controls are described in Table 1.

Accuracy genotyping procedure

MBL genotypes were determined in $n = 216$ cases and $n = 31$ controls. In two cases the promoter SNPs could not be determined. Therefore these cases could not be grouped in one of the MBL genotype groups and were excluded form analyses, resulting in a total of $n = 214$ cases and $n = 31$ controls to be analyzed.

No association of MBL genotype groups and RA disease activity

No significant difference in DAS28 levels is observed between MBL genotype group A, B and C at all timepoints during pregnancy and postpartum ($p = 0.899$, figure 1A). Also, when cases were categorized in responder and non-responders-status during pregnancy

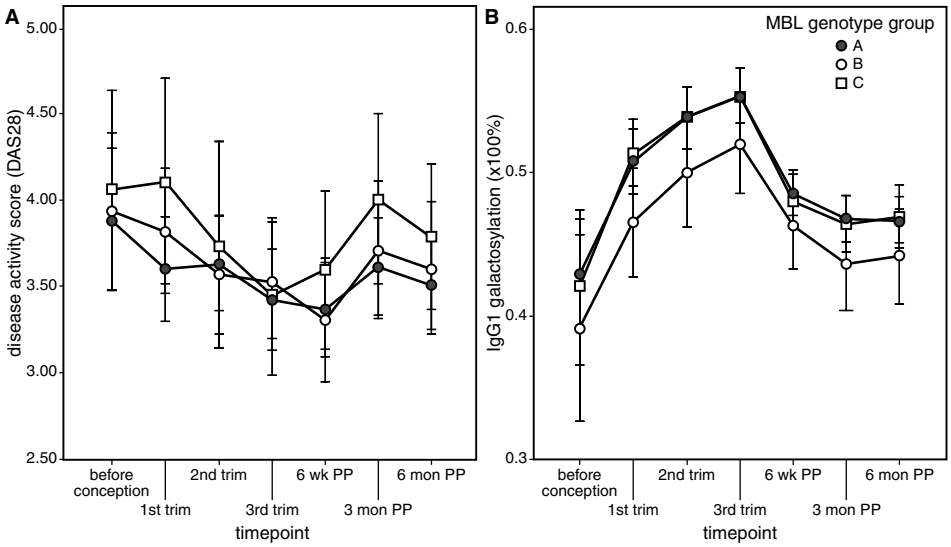


Figure 1. Mean RA disease activity score (DAS28) and mean IgG1 galactosylation in relation to MBL production genotype groups during pregnancy and postpartum in RA-cases. **A**) Mean RA disease activity score (DAS28) during pregnancy and postpartum in relation to MBL production genotype groups A (high), B (intermediate) and C (low). No significant difference in DAS28 levels is observed between MBL genotype groups A, B and C at all timepoints during pregnancy and postpartum ($p = 0.899$). **B**) Mean IgG1 galactosylation (x100%) of RA-cases during pregnancy and postpartum per MBL production genotype group. No significant difference in IgG galactosylation levels is observed between MBL genotype groups A, B and C at all timepoints during pregnancy and postpartum ($p = 0.75$). Data for IgG2 galactosylation and for the controls show similar results (data not shown).
The vertical bars illustrate the 95% confidence intervals.
Abbreviations: trim, trimester of pregnancy; wk, weeks; PP, postpartum; mon, months.

or cases that had an early or late flare postpartum and cases that did not flare postpartum, no differences were observed between the MBL genotype groups (responders/non responders: MBL genotype group A versus B+C, Odds Ratio (OR) 0.91, 95% Confidence Interval (CI) 0.58-1.42; early flare/no flare: MBL genotype group A versus B+C, OR 0.69, CI 0.39-1.25; late flare/no flare: MBL genotype group A versus B+C, OR 1.00, CI 0.51-1.98). Similar results were found when groups A, B and C were analyzed separately (data not shown) and when cases that participated twice or more were excluded.

No association of MBL genotype groups and IgG galactosylation changes

No significant difference in IgG1 galactosylation levels is observed between MBL genotype group A, B and C at all timepoints during pregnancy and postpartum as shown in figure 1B ($p = 0.75$, cases). Similar non-significant results were obtained for IgG2 as well as in controls (data not shown).

Uni- and multivariate analysis revealed that MBL genotype groups do not affect IgG galactosylation levels, even after correction for possible confounders, like medication use, disease activity and clinical characteristics.

No association of MBL genotype groups and pregnancy outcome in RA

In RA the gestational age or birth weight did not differ significantly between the MBL genotype groups ($p = 0.78$ and $p = 0.95$, respectively). Accordingly, there was a similar distribution of preterm birth and low birth weight infants among MBL genotype groups ($p = 0.75$ and $p = 0.68$, respectively).

The distribution of miscarriages (23 of 201 cases) was also not significantly different between the MBL genotype groups, $p = 0.81$.

All logistic regression and all linear regression analyses could not show an association between MBL genotype groups and the pregnancy outcome measures preterm birth, low birth weight, hypertensive disorders, or miscarriage and gestational age, birth weight SD score or birth weight (Table 2), even after correction for multiple possible confounders as described above. Subgroup analysis for nulliparous women, as well as for cases that did not use prednisone in the first trimester of pregnancy did not reveal any effect of MBL on gestational age, birth weight or birth weight SD score (data not shown). Grouping of the intermediate and low MBL genotype groups B and C did not reveal a different effect in all linear and logistic analyses (data not shown).

Discussion

In this study no association was found between MBL genotype groups and improvement of RA during pregnancy, nor with levels of IgG galactosylation and changes thereof,

Table 2. Regression analysis MBL genotype groups and pregnancy outcome measures in RA-patients. The pregancy outcome measures studied are gestational age, birth weight, birth weight SD score, miscarriage, hypertension, short gestational age and low birth weight

MBL genotype groups ABC (3 strata)				**MBL genotype group A *vs* B plus C (dichotomous)**		
Variable stratified	**beta-coefficient**	***p*-value**	***n***	**beta-coefficient**	***p*-value**	***n***
Continuous variables						
Gestational age (weeks)						
No correction	-0.062	0.739	156	-0.085	0.767	156
Correction for all confounders	-0.27	0.199	126	-0.363	0.260	126
Birth weight (grams)						
No correction	-25.69	0.672	157	-6.16	0.947	157
Correction for all confounders	-81.76	0.205	127	-69.56	0.480	127
Birth weight SD score						
No correction	-0.015	0.896	156	0.03	0.865	156
Correction for all confounders	-0.052	0.671	126	0.004	0.983	126
Dichotomous variables						
Variable stratified	**OR**	**95% CI**	***n***	**OR**	**95% CI**	***n***
Miscarriage						
No correction	1.13	0.64 - 1.99	21/205	1.31	0.53 - 3.23	21/205
Correction for all confounders	0.99	0.53 - 1.83	20/178	1,12	0.43 - 2.87	20/178
Hypertension						
No correction	0.93	0,53 - 1,66	23/174	0.72	0.30 - 1.78	23/174
Correction for all confounders	0.76	0.36 - 1.61	16/127	0.58	0.18 - 1.83	16/127
Gestational age <37 wks						
No correction	1.30	0.68 - 2.58	14/157	1.20	0.40 - 3.60	14/157
Correction for all confounders	2.12	0.80 - 5.60	13/127	2.38	0.47 - 12.1	13/127
Low birth weight <2500g						
No correction	0.93	0.40 - 2.28	10/158	0.76	0.21 - 2.82	10/158
Correction for all confounders	0.92	0.30 - 2.90	9/127	0.87	0.14 - 5.38	9/127

95% CI = 95% confidence interval
OR = adds ratio

thereby questioning a role for MBL not only in the pregnancy-induced improvement of RA in particular, but also for a more general role of MBL in the pathogenesis of RA. Moreover, MBL genotype groups did not show statistically significant associations with gestational age, birth weight, miscarriage and hypertensive disorders in RA-pregnancies.

Previously, high MBL levels have been associated with less severe disease in RA [1]. It has been suggested that the beneficial effect of MBL results from its binding to the pathogenic agalactosyl IgG antibodies, and MBL may therefore function as a scavenger molecule involved in the efficient removal of pathogenic agalactosyl IgG containing immune complexes [1]. Because pharmaceutical induction of MBL is not yet possible, this hypothesis cannot be properly tested *in vivo*. However, during pregnancy MBL levels increase and RA disease activity improves along with a decrease in the levels of pathogenic agalactosyl IgG. This all makes pregnancy in RA the ideal 'experiment of nature' to gain support for the aforementioned hypothesis. Nevertheless, even this ideal setting did not support a role for MBL in the pathogenesis of RA.

Alternative hypotheses have been proposed to explain the pregnancy-induced improvement of RA, like the induction of regulatory T-cells, immunomodulatory properties of pregnancy hormones, a shift towards a Th2 associated cytokine profile and immunosuppression as a result of increased fetal-maternal HLA disparity [15]. It is likely that multiple mechanisms may work in concert to induce RA-improvement during pregnancy.

Finally, the possible association between MBL genotypes and pregnancy outcome was investigated. Previous literature demonstrated in healthy individuals that MBL is associated with pregnancy outcome like preterm birth, low birth weight, recurrent miscarriages, risk for chorioamnionitis and more severe or recurrent pre-eclampsia [6-9]. Our study in RA-patients showed no significant association between MBL genotype groups and the pregnancy outcome measures gestational age, birth weight, miscarriage and hypertensive disorders. In line with a previous study on the effect of MBL on gestational age in healthy women [6], an association was found between preterm birth and the maternal high-MBL production genotype group A, although in the present study on RA-patients it did not reach statistical significance (OR 2.38, 95% CI 0.47-12.1). For the other pregnancy outcome measures, like pre-eclampsia, the present study might lack power.

In conclusion, this study does not suggest a role for MBL in the phenomenon of pregnancy-induced improvement of RA, nor in the pathogenesis of RA in general. Future studies should focus on other mechanisms to explain the pregnancy-induced remission of RA and the flare postpartum.

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We would like to acknowledge Dr. C.J.M. de Groot from the Department of Obstetrics and Gynaecology from Medical Center Haaglanden, The Hague, The Netherlands for advice.

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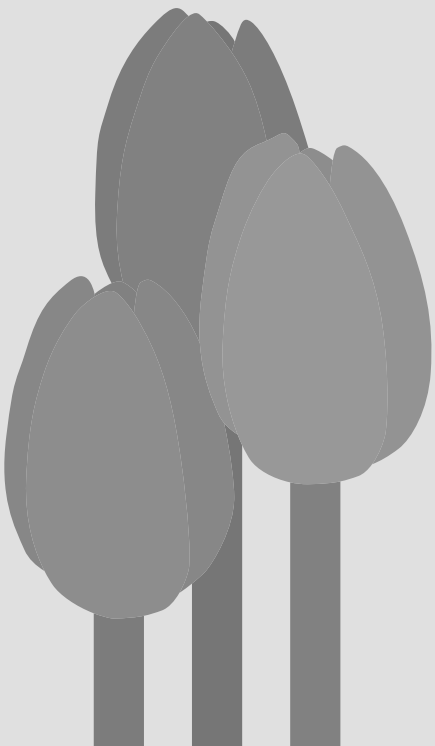
Chapter 8

General discussion



1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease that is favorably influenced by pregnancy but relapses after delivery [1]. Insight into this mechanism may not only increase our knowledge on the phenomenon of pregnancy-induced remission in RA but may also contribute to a better understanding of pathogenic mechanisms underlying RA in general.

Until today it is unclear which mechanism or what factor could induce this remission. It has been hypothesized that the complement factor mannose-binding lectin (MBL) might play a beneficial role that results from its binding to pathogenic agalactosyl IgG antibodies and may therefore function as a scavenger molecule involved in the efficient removal of pathogenic agalactosyl IgG containing immune complexes [2].

This thesis describes the role and function of mannose-binding lectin during pregnancy and postpartum in healthy individuals and in RA-patients. The main objectives of this thesis were:

1. To determine whether pregnancy has an effect on MBL serum levels and IgG galactosylation
2. To determine whether MBL in concert with IgG galactosylation is involved in the pathogenesis of RA in general and more specifically in the pregnancy-induced improvement of RA
3. To determine whether MBL is involved in pregnancy outcome in healthy controls and RA-patients

This chapter presents the main findings of this thesis in a broader perspective (paragraph 2), discusses methodological considerations (paragraph 3), presents the new insights obtained from the findings (paragraph 4) and offers recommendations for future research (paragraph 5).

2. Main findings

Objective 1. To determine whether pregnancy has an effect on MBL serum levels and IgG galactosylation MBL measurements during pregnancy and postpartum in healthy women showed that MBL concentrations were increased from the first trimester onwards, followed by a sharp decline postpartum, after which levels recovered. These findings were unique for MBL, since Ficolin-2, another initiator of the lectin complement pathway was not influenced similarly. Also, the classical complement pathway was hardly influenced by pregnancy and in the postpartum period. Moreover, it was shown that the increase in MBL levels was strictly related to the maternal MBL genotype (Chapter 2).

Determination of IgG galactosylation during pregnancy and postpartum in healthy women and in RA patients showed an increase in IgG galactosylation during pregnancy and a decrease in the postpartum period. These galactosylation changes were also associated with disease activity changes in RA during pregnancy and postpartum. Namely, cases that experience improved disease activity during pregnancy (responders) experienced a larger increase in galactosylation than non-responders. The reverse was true for cases that experienced a postpartum flare of the disease; these cases experienced a larger decrease in IgG galactosylation. Multivariate analysis revealed that both RA disease activity and timepoint of measurement during pregnancy or postpartum had the largest influence on IgG galactosylation levels, and not medication use or breast feeding postpartum. Moreover it was shown that at all timepoints during and after pregnancy there was a good correlation between galactosylation and sialylation of IgG. This is noteworthy since recent literature suggests that the effector functions of IgG are mainly a result of changes in sialylation and not of galactosylation [3] (Chapter 6).

Objective 2. To determine whether MBL in concert with IgG galactosylation is involved in the pathogenesis of RA in general and more specifically in the pregnancy-induced improvement of RA

First, the association between RA disease susceptibility and disease severity and MBL genotypes was studied. In RA conflicting studies have been described on the association between MBL genotypes and disease susceptibility and severity. By studying two large cohorts of $n = 375$ and $n = 259$ non-pregnant RA-patients, it was shown that MBL genotype groups are not associated to RA disease susceptibility and disease severity (as defined by the need for anti-TNF therapy). MBL genotype groups were neither associated with RA disease characteristics like (positive) autoantibody titers of anti-CCP, IgA-, IgM- and IgG-RF (Chapter 5).

Next, the possible association between improvement of RA disease activity during pregnancy and MBL genotypes was studied. It was shown that MBL groups are not associated with improvement of RA disease activity during pregnancy or with the postpartum flare. Since literature showed that MBL binds to pathogenic agalactosyl IgG *in vitro* and therefore could possibly act as scavenger of pathogenic IgG during pregnancy, this hypothesis was also tested. However, it was shown that MBL genotypes are not associated with the changes in IgG galactosylation during pregnancy and postpartum in RA-cases and controls (Chapter 7).

Objective 3. To determine whether MBL is involved in pregnancy outcome in healthy controls and RA-patients

It was shown that MBL levels are increased during healthy pregnancy and that this was most pronounced in women belonging to the high-MBL production genotype group. This finding might suggest a physiological role for MBL in pregnancy. Therefore, the association of MBL genotype groups and pregnancy outcome (birth weight, gestational age and pre-eclampsia) was studied in healthy women as well as in RA-patients. First, pre-eclampsia was studied in relation to MBL genotypes in the cohort of healthy nulliparous women. It was demonstrated that low-MBL production genotypes might predispose to *severe* pre-eclampsia or eclampsia. MBL genotypes were not associated with pre-eclampsia itself. Hypertensive disorders during pregnancy are thought to result from inadequate formation of the placenta in the beginning of pregnancy. Adequate formation of the placenta requires continuous tissue remodeling and apoptosis. Since MBL is thought to be involved in the clearance of apoptotic cells and cell debris, the association of low-MBL production genotypes with severe pre-eclampsia might be explained by the fact that when MBL levels are low, these apoptotic cells are cleared less efficiently. This could induce excess inflammation resulting in inadequate formation of the placenta (Chapter 3).

Moreover, in contrast to our hypothesis, in the same cohort of healthy nulliparous women, the high-MBL production genotype group is associated with shorter gestational age and subsequently lower birth weight (Chapter 4).

The two main findings of chapter three and four, namely a trend in the association between low-MBL production genotype groups and *severe* pre-eclampsia and eclampsia and the association of high-level MBL genotype groups and preterm birth, could be reconciled by acknowledging the fact that placentation in early pregnancy and delivery at the end of pregnancy are two distinct immunological processes. In these different processes the immune system, and MBL in particular, may play different roles (that is an anti-inflammatory and a pro-inflammatory role), which clearly have to stay in balance during the different physiological processes in pregnancy. An anti-inflammatory role for MBL is proposed from early pregnancy onwards in balancing apoptosis and inflammation. Previously, healthy pregnancy has been proposed as being dependent on an adequate level of inflammation [4]; excessive inflammation seems to be associated with pre-eclampsia [5]. The level of inflammation may be dependent on apoptotic syncytiotrophoblast debris shed into the circulation from the placenta: if the shedding of debris becomes excessive or if the clearance becomes deficient, too much inflammation may result. One function of MBL may be clearance of apoptotic cell material in the circulation [6-8] and it is thus possible that pregnant women with low MBL may be at higher risk of excessive inflammatory responses due to failure of this clearance. On the other hand, towards delivery MBL may play a pro-inflammatory role in the initiation of parturition, since high-MBL production genotypes are associated with earlier birth. This pro-inflammatory effect is probably due to MBL's role as initiator of the lectin complement pathway.

Since MBL was associated with the pregnancy outcome measures gestational age and pre-eclampsia in healthy women, this hypothesis was also tested in RA-patients from

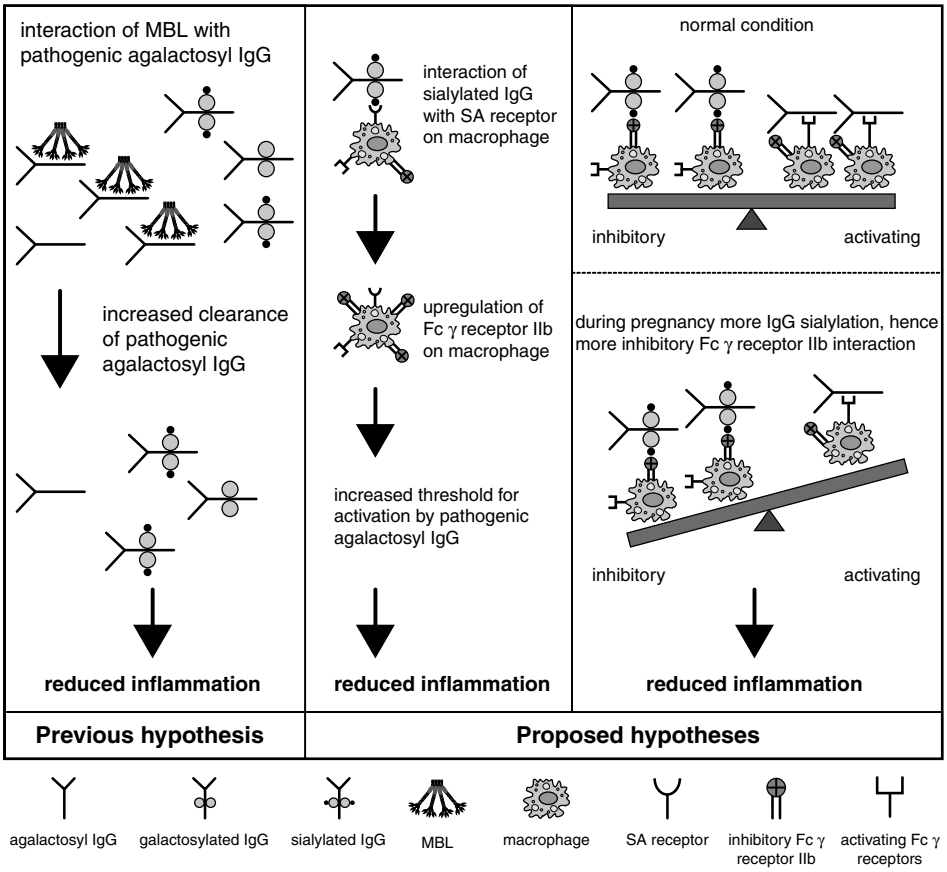


Figure 1. Previous and proposed mechanisms of improvement of RA during pregnancy.
A) MBL binds to (pathogenic) agalactosyl IgG, but not to normally galactosylated IgG. It is hypothesized that the agalactosyl IgG-MBL complex can therefore be more efficiently cleared. Galactosylated IgG is not cleared, since there is no binding to MBL. Clearance of pathogenic agalactosyl IgG leads to reduced inflammation. **B**) It is hypothesized that sialylated IgG Fc protein interacts with macrophages via a specific receptor. As a result the macrophages upregulate the expression of the inhibitory Fc gamma receptor IIb on their surface which in turn raises the threshold required for pathogenic agalactosyl IgG to engage with activating Fc receptors and to trigger the inflammatory process. Hence, this leads to reduced inflammation. **C**) Agalactosyl IgG preferentially binds to activating Fc receptors expressed by macrophages leading to macrophage activation (right side of the balance), whereas sialylated IgG preferentially binds to inhibitory Fc gamma receptors on the macrophages leading to suppression of macrophage function (left side of the balance). During pregnancy, when the ratio of sialylated IgG is increased, the balance is tipped towards more interaction with inhibitory Fc gamma receptor IIB, leading to reduced inflammation.
Abbreviations: FcγRIIb: Fragment crystallizable gamma receptor IIb; MBL: mannose-binding lectin; SA: sialic acid
Adapted from Kaveri 2008[10] and Anthony 2008[9]

the PARA-cohort (Pregnancy-induced Amelioration of Rheumatoid Arthritis-cohort). However, in contrast to the findings in healthy individuals, MBL genotype groups are not related to gestational age, birth weight, miscarriage, and hypertensive disorders within the PARA-cohort. These findings might be explained by the fact that the PARA-cohort is a quite heterogeneous group of nulli- and multiparous women with different levels of disease activity, which can influence pregnancy outcome, although correction for these confounders still did not reveal a significant correlation (Chapter 7).

When the results of this thesis are taken together, it can be concluded that, despite the main hypothesis, MBL does not play a role in RA and pregnancy-induced remission of RA and the flare postpartum. MBL does play a role in pregnancy outcome in healthy controls, but not evidently in RA-patients. It was shown that improvement of RA during pregnancy was associated with increased galactosylation of IgG. This increased galactosylation also resulted in increased sialylation during pregnancy. These sialic acid sugar moieties can influence the balance between activating and inhibitory Fc γ receptors towards the inhibitory receptors IIb by preferential binding and therefore lead to a reduced inflammatory profile [9]. Moreover enhanced expression of the inhibitory Fc γ IIb receptor on macrophages after interaction with sialylated IgG can raise the threshold required to trigger an inflammatory response leading to reduced inflammation[10]. These mechanisms could provide alternative hypotheses to explain the pregnancy-induced improvement of RA and the postpartum flare (Figure 1).

3. Methodological considerations

This paragraph describes the strengths and limitations of the research described in this thesis, divided into specified subjects.

3.1 MBL genotype groups

MBL can be studied by measuring its protein level in serum or by determining its genotype. Chapter 2 shows that MBL serum levels are influenced by pregnancy and the postpartum period and that MBL levels during pregnancy are dependent on the maternal and not the fetal MBL genotypes. Both chapter 2 and 5 show an excellent correlation between MBL serum levels and MBL genotypes. Therefore in subsequent studies MBL genotypes were categorized into three groups that are associated with high, intermediate or low MBL protein concentrations in the serum, which correlate best with MBL levels in the serum, as was also shown by other researchers previously [11, 12]. The advantage of combining and hence reducing all known SNPs in the MBL gene into three MBL genotype groups, is that in statistical analyses multiple testing can be avoided. The disadvantage is

that it is not always possible to compare the results with previous literature that used a different categorization, as discussed in chapter 5.

The majority of the participants of the PARA-study is Caucasian (96%), indicating that the results of the studies may only be generalized to Caucasian populations, since the frequency of MBL polymorphisms differs between populations. It should be noted that the frequencies of MBL polymorphisms are not related to gender or age, and that therefore the results of chapter 5 can be applied to the general Caucasian population of all gender and age.

3.2 Candidate gene approach

In this thesis a candidate gene approach was chosen to unravel the role of MBL in RA disease susceptibility and severity and various pregnancy outcome measures in healthy controls as well as in RA-patients. By choosing one promising protein and studying its effect clear conclusions can be drawn from research. This is also a strong point of our studies. This is in contrast to the increasingly prevalent genome wide association studies in which identification of multiple genetic risk alleles without prior knowledge of function is performed because of technological advances in high-throughput genotyping [13]. This approach sometimes leads to multiple candidate genes with unknown functions, the risk of false positive results and of yet unknown importance, demanding further studies.

3.3 Study cohorts

3.3.1 The PARA-study cohort

Size

The PARA-study cohort (Pregnancy-induced Amelioration of Rheumatoid Arthritis) is an unique cohort that prospectively followed n = 350 RA-patients from pre-conception, during pregnancy and up to six months postpartum with not only clinical data, but also blood and urine samples. This has never been done before. It was possible to compose this large(st) cohort of RA-patients with a pregnancy-wish or an early pregnancy due to a committed network of national rheumatologists that work closely together and the good infrastructure in the Netherlands that enabled us to visit all participants at home.

Dedication by the participants of the PARA-study was overwhelming, since the drop-out rate was less than 1% and some women even participated more than one pregnancy. This was even more noteworthy since all women underwent six or seven home visits, while filling in questionnaires and donating blood- and urine samples each time. This dedication might be explained by the severe impact of the disease on daily life. The need for more information on RA and pregnancy is great; there are still many questions that need answers, not only for RA-patients, but also for rheumatologists and others.

Validated instruments to determine disease activity

One of the strengths of our studies is that within the PARA-study cohort internationally recognized outcome measures like DAS28 and EULAR response criteria were used. The variant of the DAS28 used in these studies was also validated for use during pregnancy [14].

Responders and non-responders

Since a large number of RA-cases could be studied, it was feasible to divide the cohort (using internationally recognized criteria) into cases that have reduced disease activity during pregnancy (named responders) and cases that do not improve during pregnancy (named non-responders). Conversely, in the postpartum period also cases could be identified as experiencing a flare (early or late) postpartum and cases that do not experience an (early or late) flare postpartum. Thus far it had never been possible to create this subdivision. It is important to make this subdivision since it enables us to determine whether the observed changes in the immune system during pregnancy were merely a result of pregnancy or clearly associated with the phenomenon of pregnancy-induced improvement of RA.

Reference group

By comparing our data with a control group of 32 healthy pregnant women within the PARA-study, which were followed from the first trimester and onwards, it was feasible to differentiate which pregnancy-induced changes were unique to pregnancy and which were distinctive in RA. This strengthened the results found compared to previous literature.

Recovery from pregnancy

The PARA-study cohort had a longer follow-up time in the postpartum period than earlier studies, which enabled the description of the early and late postpartum flares. However, it became apparent that at the last visit at six months postpartum the RA-cases and controls might not have fully recovered from their pregnancies. Therefore it could be questioned whether this would be the best endpoint of measurement in the study. This should be taken into consideration when interpreting the results.

Heterogeneity and generalizability

The PARA-study cohort is a heterogeneous cohort, since nulli- and multiparous women were included with different RA-disease durations, using different medications resulting in different RA-disease activities. Since all RA-patients were included provided they fulfilled the ACR 1987 criteria, the conclusions based on this cohort can consequently be extrapolated to a large population.

3.3.2 Cohorts to study the association between MBL and RA disease susceptibility and severity

Replication cohort

In chapter 5 the possible association between MBL and RA disease susceptibility and severity was studied. To allow confirmation of the (negative) results found, also a large replication cohort was recruited. Including a replication cohort is in line with the recent recommendations of the National Cancer Institute and National Human Genome Research Institute (NCI-NHGRI) Working Group on Replication in Association Studies [15]. The Working Group pleaded for large sample size genotype-phenotype studies that replicate both positive as well as negative findings in multiple well-described cohorts with enough power and clear statistics and also pleaded for publishing the so-called negative studies.

3.3.3 The pre-eclampsia and healthy nulliparous controls cohort

Homogeneity

The last cohort studied in this thesis, the retrospective cohort of 157 nulliparous cases with pre-eclampsia and 157 healthy nulliparous controls, had a very homogeneous composition due to strict inclusion criteria, which increased the strength of power and enabling to make strong conclusions. However no replication cohort was available to confirm the results found.

4. New insights

The studies in this thesis have contributed to multiple new insights into MBL, RA and pregnancy.

First, it has been shown that MBL protein levels increase from the first trimester of pregnancy onwards and decrease sharply postpartum. It was also apparent that it took at least six months after delivery for MBL levels to reach the levels from before conception. Therefore, taking into account that MBL levels are subject to change in the postpartum period, it would be recommended that MBL measurements in the context of research and clinical diagnosis should not be performed before a minimum of six months postpartum.

Second, the early rise in MBL levels during early healthy pregnancy may indicate that MBL contributes to normal placentation and ongoing pregnancy, as is underscored by our findings of a trend in the association between low-MBL production genotype groups and *severe* pre-eclampsia. Normal placentation requires apoptosis and turnover of trophoblasts. One of the roles of MBL could be to bind to apoptotic cells to enhance their opsonization and subsequent removal.

Third, it was shown that the maternal high MBL genotype group predisposes to premature birth in healthy individuals, suggesting that during pregnancy MBL-associated inflammation caused by higher MBL activity may contribute to earlier delivery and consequently to lower birth weight. Individuals with MBL deficiency are more prone to infections, however the high prevalence of MBL deficiency in the general population (up to 50%) suggests an evolutionary advantage of MBL-deficiency as well [12]. The finding of this study that women with MBL deficiency have pregnancies of longer duration and hence give birth to children with a better start in life could be such an advantage.

Next, the increase in MBL during pregnancy, as a central component of innate immunity, may also reflect a shift from adaptive to innate immunity during pregnancy, to compensate for reduced T-cell function during a state of reduced adaptive immunity as pregnancy is.

Also, it was shown for the first time that maternal, and not fetal, MBL genotypes are associated with MBL protein levels during pregnancy, making maternal-fetal transfer of MBL across the placenta less likely.

Another new insight is the fact that MBL genotype groups are not associated with RA susceptibility and severity and that those MBL genotypes that result in the most prominent rise in MBL levels are not associated with improvement of RA disease activity during pregnancy. Therefore modulation of MBL serum levels will not be the lead for the development of new therapeutics in RA.

Moreover, the finding that not only IgG galactosylation but also IgG sialylation changes are associated with RA disease activity during pregnancy and in the postpartum period is a new insight. IgG galactosylation has a good correlation with IgG sialylation. One could speculate that when the factors that actually influence IgG galactosylation as well as sialylation are known, that this could have clinical consequences for RA-patients. In other auto-immune diseases like Guillain-Barré syndrome, immune-mediated thrombocytopenia or Kawasaki disease it has recently been shown that increased sialylation of intravenous immunoglobulin (IVIG) significantly enhanced the therapeutic effect of IVIG by reducing inflammation [3, 10, 16, 17].

5. Future directions

This chapter will elaborate on some of the opportunities for future research on the following topics: 1) the PARA-study: RA and pregnancy; 2) elucidation of pregnancy-induced improvement of RA; 3) IgG galactosylation and sialylation and their anti-inflammatory effects; and 4) MBL and pregnancy outcome.

PARA-study: RA and pregnancy

The PARA-study cohort provides extensive clinical and serological data. Still many relevant research questions on RA and pregnancy can be answered with the information not yet analyzed. For example or it could be determined whether disease activity or medication use plays a role in fecundity or fertility within RA-patients. In the population of women that participated twice in the PARA-study, it can be investigated whether women experience the same change in disease activity during pregnancy and postpartum in subsequent pregnancies and what could be possible influential factors.

Elucidation of pregnancy-induced improvement of RA

Other theories on pregnancy-induced improvement and RA are also worthwhile to test, like pregnancy-induced hormones, regulatory T-cells, Th1/Th2 cytokine balances, the role of IL-17 and IgG sialylation. It can be hypothesized that differential levels of pregnancy-induced hormones between responders and non-responders of the PARA-study could provide directions to study the pregnancy-induced remission of RA. Regulatory T-cells (Treg) are induced during pregnancy and secrete IL-10, which provides an enhanced suppression of pro-inflammatory cytokines produced by effector T cells [18]. Whether Tregs are differentially expressed in responders and non-responders is not yet known. The Th1/Th2 cytokine balance, with the associated T-cell derived cytokines during pregnancy and in the postpartum period can be studied in the materials available. Additionally, the role of the promising interleukin-17, which may play an inflammatory role in RA [19] could be studied in a state of reduced inflammation like during pregnancy and conversely postpartum.

IgG galactosylation and sialylation and their anti-inflammatory effects

This thesis proposes a new hypothesis that IgG galactosylation and consequently IgG sialylation might play a role in pregnancy-induced improvement of RA (Figure 1). It would be interesting to further study factors that may influence IgG galactosylation or sialylation. The enzymatic activity responsible for glycosylation (galactosylation and sialylation) within these B-cells during pregnancy could be studied *in vitro*, by culturing the cells with pregnancy-associated hormones or cytokines. Identification of the mechanism behind sialylation during pregnancy would enable us to pharmaceutically induce sialylation *in vivo*. It could be hypothesized that factors that increase sialylation would be more abundant in responders than in non-responders during pregnancy, and the reverse postpartum.

MBL and pregnancy outcome

It has been shown that MBL may play a role in pregnancy outcome, like gestational age, birth weight and pre-eclampsia. It could be studied further whether there are other pregnancy outcomes with which MBL is associated.

In conclusion, future studies should focus on unraveling the mechanism behind improvement of RA during pregnancy and the flare postpartum. It may be that changes in IgG galactosylation and sialylation play a role during pregnancy and postpartum. The role of MBL in RA has been studied in detail and may need no further elaboration. It should be noted though, that it is still unsure whether there is actually one factor to be identified that induces remission of RA during pregnancy. It might be more likely that multiple factors work in concert to influence disease activity.

6. Final remarks

The studies performed in this thesis do not fully elucidate the phenomenon of pregnancy-induced improvement of RA and the flare postpartum. It has been shown that MBL levels rise during pregnancy and decrease postpartum, but MBL did not induce improvement of RA during pregnancy and a flare of the disease after delivery. Moreover, MBL is not a risk factor for the susceptibility of RA and disease severity in RA.

Another hypothesis for the pregnancy-induced improvement of RA and the flare postpartum was therefore studied. It has been shown that the changes in IgG galactosylation and sialylation during pregnancy and postpartum were associated with improvement and deterioration of RA disease activity, respectively. It is hypothesized that the sialic acid sugar moieties can influence the balance between activating and inhibitory Fc-gamma receptors towards the inhibitory receptors by preferential binding and therefore lead to a reduced inflammatory profile. Moreover enhanced expression of inhibitory Fc-gamma receptor on macrophages after interaction with sialylated IgG can raise the threshold required to trigger an inflammatory response.

MBL genotype groups are associated with pregnancy outcome measures, showing an anti-inflammatory role for MBL in early pregnancy and a pro-inflammatory role towards delivery. Healthy women with low-MBL production genotypes seem to have an increased risk for *severe* pre-eclampsia or eclampsia, but have a longer duration of pregnancy. This effect was not reproduced in the RA-patients of the PARA-cohort, probably because of the heterogeneity of this cohort.

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Summary

This thesis aimed to describe the role of mannose-binding lectin (MBL) during pregnancy and postpartum in healthy individuals and in RA-patients (**Chapter 1**). The main objectives of this thesis were: (1) To determine whether pregnancy has an effect on MBL serum levels and IgG galactosylation, (2) To determine whether MBL in concert with IgG galactosylation is involved in the pathogenesis of RA in general and more specifically in the pregnancy-induced improvement of RA, and (3) To determine whether MBL is involved in pregnancy outcome in healthy controls and RA-patients.
The first objective is addressed in Chapter 2 and 6, the second objective is addressed in chapters 5 and 7, and the last objective in Chapter 3, 4 and 7.

Chapter 2 describes MBL levels during healthy pregnancy. In healthy women MBL concentrations were increased from the first trimester of pregnancy onwards. In the AA-genotype MBL serum concentrations increased to 177% (median, interquartile range (IQR) 124–212%, $p < 0.001$), the AO-genotype increased to 126% (median, IQR 91–144%, $p < 0.04$) and the OO-genotype to 117%, all compared with baseline value. After parturition MBL levels declined sharply. In the AA-genotype was a decline of 55% of baseline concentration ($p < 0.0001$); in the AO-genotype 60% ($p < 0.03$) and in the OO-genotype 65% of baseline concentration. After the decline the MBL levels normalized again. It was shown that levels of MBL were strictly related to the maternal, and not the fetal MBL genotype.
The serum concentrations of Ficolin-2, a second initiator of the lectin complement pathway, as well as the classical complement pathway activity, were hardly influenced by pregnancy or the postpartum period, indicating a unique effect of pregnancy on MBL. Moreover, the variations in MBL levels were reflected by similar changes in the following steps of MBL complement pathway activation. It could be concluded that pregnancy and the postpartum period profoundly influence MBL serum concentration and MBL complement pathway activity.

In **chapter 3** the first of two studies on the association between MBL genotype groups and pregnancy outcome in healthy women is described. Since it was demonstrated that MBL levels were increased during healthy pregnancy, the role of MBL genotypes in pregnancy outcome (birth weight, gestational age and pre-eclampsia) was studied. In a cohort of nulliparous women ($n = 157$ with pre-eclampsia and $n = 157$ with uncomplicated pregnancies) a trend was shown in the association between low-MBL production genotypes and *severe* pre-eclampsia (odds ratio (OR) 3.38, 95% CI 0.47 – 24.23) or eclampsia (OR 14.70, 95% CI 1.18 – 182.76), but not with pre-eclampsia itself. The association of low-MBL production genotypes with *severe* pre-eclampsia might be explained by the following. Hypertensive disorders such as pre-eclampsia during pregnancy are thought to result from inadequate formation of the placenta in the beginning of pregnancy. Ad-

equate formation of the placenta requires continuous tissue remodelling and apoptosis. MBL is thought to be involved in the clearance of apoptotic cells and cell debris. When MBL levels are low, these apoptotic cells are cleared less efficiently. The remaining cell debris could induce excess inflammation resulting in inadequate formation of the placenta and risk of pre-eclampsia.

Chapter 4 presents the second study on the association between MBL genotype groups and pregnancy outcome in $n = 157$ healthy nulliparous women, namely the outcome measures gestational age and birth weight. The high-MBL production genotype group was associated with shorter gestational age ($p = 0.002$) and subsequently lower birth weight. Twelve of the 14 preterm births (87.5%) were from women with the high-MBL genotype group, while this group forms only 56% of the whole cohort.

Chapter 5 deals with the previous conflicting results on the association between MBL genotypes and RA disease susceptibility and severity. In two large cohorts of $n = 375$ and $n = 259$ RA-patients it was shown that MBL genotype groups are not associated with RA susceptibility and disease severity (as defined by the need for anti-TNF therapy), unrelated to pregnancy. MBL genotype groups were neither associated with RA disease characteristics like positive autoantibody titers of anti-CCP, IgA-, IgM- and IgG-rheumatoid factor. The strength of this study was the fact that all outcomes were confirmed by a second RA-cohort. Compared with previous smaller studies these results add to more definite conclusions on the absence of an association between MBL and RA disease susceptibility and severity.

Chapter 6 studies galactosylation and sialylation of IgG during pregnancy and in the postpartum period, as an alternative hypothesis for pregnancy-induced improvement of RA. By applying the MALDI-TOF mass spectrometer it was shown that IgG1 and IgG2 galactosylation was increased during pregnancy and was decreased in the postpartum period, albeit at a lower level than controls ($n = 148$ RA-patients and $n = 32$ controls, $p < 0.001$). These IgG galactosylation changes were also associated with disease activity changes (DAS28) in RA during pregnancy and postpartum (R between 0.35 and 0.49, $p < 0.005$, dependent on timepoint of measurement). Cases that had improved disease activity during pregnancy (responders) experienced a larger increase in IgG galactosylation than non-responders ($p < 0.02$). The reverse was true for cases that had a postpartum flare of the disease; these cases experienced a larger decrease in galactosylation ($p < 0.0001$). Additionally, it was shown that the presence of the sialic acid sugar moiety is highly associated with IgG galactosylation (R between 0.57 and 0.69, $p < 0.0001$). Multivariate analysis revealed that both RA disease activity and timepoint of measurement during pregnancy or postpartum had the largest influence on IgG galactosylation levels, and not medication use or breast feeding postpartum.

Chapter 7 aimed to investigate whether increased MBL levels (or high-MBL production genotypes) in concert with increased IgG galactosylation are associated with preg-

nancy-induced improvement of RA. No association was found between MBL genotype groups and changes in RA-disease activity ($p = 0.89$) and changes in IgG galactosylation (cases $p = 0.75$ and controls $p = 0.54$) during pregnancy and postpartum. Therefore, it can be concluded that MBL does not function as a scavenger molecule involved in the removal of pathogenic agalactosyl IgG. Furthermore, in RA MBL genotype groups are not related to the pregnancy outcome measures gestational age, birth weight, miscarriage, and hypertensive disorders. It can therefore be concluded that future studies should focus on other mechanisms to explain the pregnancy-induced remission of RA and the flare postpartum.

Chapter 8, the general discussion, starts with presenting the main findings in the light of the study objectives, followed by methodological considerations. New insights arisen from this thesis into MBL, RA and pregnancy are described. Finally, recommendations for future studies are presented, with an emphasis on the possible anti-inflammatory effects of IgG galactosylation and sialylation.

Samenvatting

Dit proefschrift beschrijft de rol van het complement eiwit mannose-bindend lectine (MBL) gedurende de zwangerschap en na de bevalling (postpartum) bij gezonde vrouwen en bij vrouwen met reumatoïde artritis (RA).

RA is een chronische inflammatoire autoimmuunziekte van de gewrichten waarbij de ziekteactiviteit vaak afneemt tijdens de zwangerschap. Na de bevalling vlamt de ziekte dikwijls weer op. MBL is een eiwit dat onderdeel is van het aangeboren immuunsysteem en zou een rol spelen bij het verwijderen van pathogene autoantistoffen (bijvoorbeeld immunoglobulinen, zoals IgG), die ook bij RA bekend zijn. Deze autoantistoffen worden deels verantwoordelijk gehouden voor de ziekteactiviteit bij RA. De beste MBL-binding zou zijn met die immunoglobulinen, die galactose suikermoleculen missen. Juist deze antilichamen zijn pathogeen, in die zin dat ze ziekte-bevorderend werken in RA. De stijging van MBL in het bloed tijdens de zwangerschap zou ervoor kunnen zorgen dat er minder pathogene immunoglobulinen circuleren, omdat MBL deze zou 'wegvangen', waardoor er een lagere ziekteactiviteit zou ontstaan tijdens de zwangerschap. Omgekeerd kan er ook een hogere ziekteactiviteit postpartum ontstaan, als de MBL spiegels zijn afgenomen en minder pathogene immunoglobulinen worden weggevangen.
Als lage MBL serum concentraties vaker gemeten worden bij patiënten met RA of vrouwen die een vroeggeboorte of miskraam kregen, zou dit relatieve tekort aan MBL een oorzakelijke factor kunnen zijn.

De volgende vraagstellingen staan centraal in dit proefschrift: (1) Heeft zwangerschap invloed op MBL serum concentraties en IgG galactosylering; (2) Zijn MBL en IgG galactosylering betrokken bij de pathogenese van RA in het algemeen en meer specifiek bij de zwangerschapgeïnduceerde verbetering van RA; (3) Is MBL betrokken bij zwangerschapsuitkomsten zoals vroeggeboorte, miskramen en laag geboortegewicht bij gezonde vrouwen of bij vrouwen met RA. Hierbij wordt onderzocht of bepaalde genotype groepen van MBL, die zorgen voor verschillende serumconcentraties van MBL buiten de zwangerschap, geassocieerd zijn met vooraf gedefinieerde zwangerschapsuitkomsten. De eerste doelstelling wordt onderzocht in hoofdstuk 2 en 6, de tweede doelstelling in hoofdstuk 5 en 7 en de laatste doelstelling wordt beschreven in hoofdstuk 3, 4 en 7.

Hoofdstuk 1 geeft een algemene introductie over de onderwerpen beschreven in dit proefschrift en plaatst de doelstellingen in een breder kader. **Hoofdstuk 2** beschrijft de MBL concentraties tijdens de zwangerschap en de postpartum periode van gezonde vrouwen. Bij gezonde vrouwen waren de MBL concentraties gedurende de gehele zwangerschap verhoogd. Dit begon al in het eerste trimester. Bij het wildtype AA-genotype steeg de MBL concentratie tot 177% (mediaan, interquartile range (IQR) 124-212%, $p < 0.001$), het AO-genotype steeg tot 126% (mediaan, IQR 91–144%, $p < 0.04$) en het OO-genotype tot 117%, allen vergeleken met de concentratie buiten de zwangerschap. Na

de bevalling was er een scherpe daling van MBL spiegels. Voor het AA-genotype was er een daling van 55% ($p < 0.0001$), voor het AO-genotype was dit 60% ($p < 0.03$) en voor het OO-genotype een daling van 65% ten opzichte van de concentratie buiten de zwangerschap. Hierna normaliseerden de concentraties weer. De hoogte van de MBL serum concentraties werd uitsluitend verklaard door het maternale, en niet door het foetale genotype.

De concentraties van ficoline-2, een tweede initiator van de lectine complement cascade, werden nauwelijks beïnvloed door zwangerschap of de postpartum periode. Dit benadrukt het unieke effect van zwangerschap op de MBL concentraties. Tevens werd gevonden dat de verhoging en verlaging van de MBL spiegel gevolgen had voor de activiteit van de gehele lectine complement cascade. De activiteit van de lectine complement cascade nam toe met het stijgen van de MBL spiegels tijdens de zwangerschap en nam af met het dalen van de spiegels in de postpartum periode. Hieruit kan geconcludeerd worden dat zwangerschap en de postpartum periode een belangrijke invloed hebben op MBL concentraties en lectine complement cascade activiteit.

In **hoofdstuk 3** wordt de eerste van twee studies gepresenteerd die een mogelijke associatie tussen MBL genotype groepen en zwangerschapsuitkomst in gezonde vrouwen onderzoeken. Aangezien was aangetoond dat MBL concentraties stegen tijdens de zwangerschap van gezonde vrouwen werd besloten de associatie van MBL genotype groepen met zwangerschapsuitkomsten (geboortegewicht, zwangerschapsduur en zwangerschapsvergiftiging) te onderzoeken. Zwangerschapsvergiftiging (pre-eclampsie) is een ernstig ziektebeeld dat zich kenmerkt door een hoge bloeddruk (hypertensie) en eiwitten in de urine (proteïnurie) tijdens de zwangerschap. Deze eerste studie onderzoekt de mogelijke relatie tussen MBL genotype groepen en pre-eclampsie. In een onderzoekscohort van vrouwen die nog nooit bevallen waren (nullipara, $n = 157$ met pre-eclampsie en $n = 157$ met ongecompliceerde zwangerschappen) werd een trend gevonden in de associatie tussen de lage-MBL productie genotype groep en *ernstige* pre-eclampsie (een gevorderde vorm van pre-eclampsie, odds ratio (OR) 3.38, 95% betrouwbaarheidsinterval (BI) 0.47-24.23) en eclampsie (*ernstige* pre-eclampsie met epileptische insulten, OR 14.70, 95% BI 1.18 – 182.76), maar niet met pre-eclampsie. De associatie tussen de lage-MBL productie genotype groep en *ernstige* pre-eclampsie/eclampsie zou als volgt verklaard kunnen worden. Normale gezonde aanleg van de placenta in het begin van de zwangerschap wordt gekenmerkt door een continue proces van ombouwen van placentaweefsel, waarbij geprogrammeerde celdood (apoptose) een belangrijke rol speelt. MBL is betrokken bij de klaring van deze apoptotische cellen en celpuin (débris). Hypertensieve aandoeningen tijdens de zwangerschap, zoals pre-eclampsie, zouden veroorzaakt worden door gebrekkige aanleg van de placenta. Als er lage MBL concentraties zijn, kunnen de apoptotische cellen minder efficiënt weggeruimd worden. Het achterblijven van

celdébris zou dan een ontstekingsreactie kunnen veroorzaken en als gevolg daarvan een gebrekkige aanleg van de placenta en verhoogd risico op pre-eclampsie.

Hoofdstuk 4 beschrijft de tweede studie die een mogelijke associatie tussen MBL genotype groepen en zwangerschapsuitkomst in $n = 157$ gezonde nullipara onderzocht. Deze studie analyseert de mogelijke relatie tussen MBL genotype groepen en zwangerschapsduur en/of geboortegewicht. De hoge-MBL productie genotype groep was geassocieerd met een kortere zwangerschapsduur ($p = 0.002$) en daardoor ook een lager geboortegewicht. Twaalf van de 14 vroeggeboorten (85.7%, zwangerschapsduur korter dan 37 weken) waren van vrouwen die de hoge-MBL genotype groep bezaten, terwijl deze groep slechts 56% van het gehele cohort vormt.

In **hoofdstuk 5** wordt een definitiever antwoord gegeven op de vraag of er nu wel of geen associatie is tussen MBL genotypen en het ontstaan van RA en ziekte ernst van RA. In twee grote onderzoekscohorten van $n = 375$ en $n = 259$ RA-patiënten werd aangetoond dat MBL genotype groepen niet geassocieerd zijn met het ontstaan van RA en RA ziekte ernst (gedefinieerd als de noodzaak om anti-TNF therapie te gebruiken), onafhankelijk van zwangerschap. MBL genotype groepen waren ook niet geassocieerd met RA ziektekenmerken zoals positieve autoantistoffen titers van anti-CCP en subklassen van reumafactor (RF) IgA-RF, IgM-RF en IgG-RF. De kracht van de studie lag in het feit dat alle resultaten werden bevestigd in een tweede RA-onderzoekscohort. Vergeleken met eerdere kleinere studies draagt dit onderzoek bij aan een meer definitieve conclusie dat er geen associatie is tussen MBL en het ontstaan van RA en ziekte ernst.

In **hoofdstuk 6** is het verloop van de galactosylering en sialylering van IgG tijdens de zwangerschap en in de postpartum periode onderwerp van studie. Een toename van de galactosylering en sialylering van IgG zou een alternatieve hypothese kunnen vormen als oorzaak van zwangerschapsgeïnduceerde verbetering van RA. Het is namelijk bekend dat een afname van de galactosylering geassocieerd is met hogere ziekteactiviteit. Met behulp van de MALDI-TOF massaspectrometer werd aangetoond dat bij de $n = 148$ bestudeerde RA-patiënten IgG1 en IgG2 galactosylering was toegenomen tijdens de zwangerschap en af nam na de bevalling. Dit profiel werd ook waargenomen bij de gezonde zwangeren, maar de galactosylering ligt dan op een hoger niveau ($p < 0.001$). Deze veranderingen in IgG galactosylering waren ook geassocieerd met veranderingen in RA ziekteactiviteit (DAS28) tijdens de zwangerschap en in de postpartum periode (R tussen 0.35 en 0.49, $p < 0.005$, afhankelijk van het tijdstip). Bij de vrouwen wier ziekteactiviteit afnam tijdens de zwangerschap (gedefinieerd volgens de criteria van de European League Against Rheumatism (EULAR), responders), nam de IgG galactosylering juist meer toe dan bij vrouwen wier ziekteactiviteit niet verbeterde tijdens de zwangerschap (non-responders, $p < 0.02$). Omgekeerd daalde IgG galactosylering meer bij vrouwen die een flare in de ziekteactiviteit postpartum doormaakten, dan bij vrouwen die geen flare doormaakten, $p < 0.0001$. Tevens werd aangetoond dat de aanwezigheid van de

siaalzuur suikergroep (sialylering) sterk gecorreleerd is met de aanwezigheid van IgG galactose groepen (galactosylering, R tussen 0.57 en 0.69, $p < 0.0001$). Tevens werd onderzocht welke variabelen IgG galactosylering beïnvloeden. Multivariate analyse wees uit dat zowel RA ziekteactiviteit als het meetmoment tijdens de zwangerschap of in de postpartum periode de grootste invloed hebben op IgG galactosylering en niet het eventuele medicatie gebruik of het borstvoeding geven postpartum.

Hoofdstuk 7 onderzoekt of hoge MBL spiegels (of hoge-MBL productie genotype groepen) samen met toegenomen IgG galactosylering tijdens de zwangerschap geassocieerd zijn met zwangerschapsgeïnduceerde verbetering van RA. Er werd geen associatie gevonden tussen MBL genotype groepen en veranderingen in RA ziekteactiviteit ($p = 0.89$) en veranderingen in IgG galactosylering (RA-patiënten $p = 0.75$ en gezonde controles $p = 0.54$) gedurende de zwangerschap en in de postpartum periode. MBL functioneert dus niet als 'opruimer' van het pathogene agalactosyl IgG. Bovendien waren MBL genotype groepen bij RA-patiënten niet geassocieerd met zwangerschapsuitkomsten zoals zwangerschapsduur, geboortegewicht, miskramen en hypertensieve aandoeningen. Er kan daarom geconcludeerd worden dat toekomstige studies zich waarschijnlijk eerder op andere mechanismen dan die van MBL zouden moeten richten om de zwangerschapgeïnduceerde verbetering van RA en de flare postpartum te verklaren.

Hoofdstuk 8, de algemene discussie, begint met het presenteren van de belangrijkste bevindingen in het licht van de onderzoeksvragen, gevolgd door de methodologische beperkingen van de studies die van belang zijn bij de interpretatie van de bevindingen. Nieuw verkregen inzichten in de rol van MBL, RA en zwangerschap worden beschreven. Hoofdstuk 8 eindigt met aanbevelingen voor toekomstig onderzoek naar zwangerschapgeïnduceerde verbetering van RA, waarbij de belofte vooral ligt bij de mogelijk anti-inflammatoire effecten van IgG galactosylering en sialylering, dan wel hormoon- of cytokine interacties.

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Fleur van de Geijn, 2009

About the author

Fleur Elisabeth van de Geijn, the author of this thesis, was born on September 8th 1977 in Leiden, The Netherlands. She grew up in The Hague and Wassenaar. She attended secondary school at the Rijnlands Lyceum Wassenaar. In 1995 she moved to Utrecht to study Pharmacy at the University of Utrecht. After two years of Pharmacy (propedeuse obtained) she could start her medical studies.

In 2001 she was inspired to continuing research by a research internship at the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins School of Medicine, Baltimore, USA, performing prostate cancer research under supervision of Mrs. Prof.dr. E. van der Wall, Prof.dr. M.A. Carducci and Dr. R. Pili. In 2002 the fruits of this research were awarded with the Talma Eijkman Award and the Nijbakker Morra Award for outstanding research and research report for students.

During her practical training as a doctor she performed clinical internships at the Kent & Sussex Hospital in Tunbridge Wells, UK (Ophtalmology) and at the Tygerberg Hospital near Cape Town, South Africa (Pediatrics and Dermatology).

In April 2004 she obtained her medical degree and moved to Rotterdam. Subsequently she started working on the PhD project described in this thesis with Mrs. Prof.dr. J.M.W. Hazes, Prof.dr J.D. Laman and Dr. R.J.E.M. Dolhain at the departments of Rheumatology and Immunology at the Erasmus MC University Medical Center Rotterdam.

In July 2008 she started her residency in Internal Medicine at the Erasmus MC University Medical Center Rotterdam en from July 2009 onwards at the Sint Franciscus Gasthuis Rotterdam (supervisors Prof.dr. J.L.C.M. van Saase and Dr. A.P. Rietveld, respectively).

List of publications

Van de Geijn FE, De Man YA, Wuhrer M, Willemsen SP, Deelder AM, Hazes JM, Dohain RJ. Can mannose-binding lectin explain course and outcome of pregnancy in rheumatoid arthritis? Submitted 2009

Van de Geijn FE, Wuhrer M, Selman HJ, Willemsen SP, De Man YA, Deelder AM, Hazes JM, Dolhain RJ. IgG galactosylation and sialylation are associated with pregnancy-induced improvement of RA and the postpartum flare: results from a large prospective cohort study. Submitted 2009

Van der Bol JM, De Jong FA, Van Schaik RH, Sparreboom A, Van Fessem MA, *Van de Geijn FE*, Van Daele PL, Verweij J, Sleijfer S, Mathijssen RH. Effects of mannose-binding lectin polymorphisms on Irinotecan-induced febrile neutropenia. Submitted 2009

Van de Geijn FE, Hazes JM, Geleijns K, Emonts M, Jacobs BC, Dufour-van den Goorbergh BC, Dolhain RJ. Mannose-binding lectin polymorphisms are not associated with rheumatoid arthritis - confirmation in two large cohorts. Rheumatology. 2008;47(8):1168-71

Van de Geijn FE, Dolhain RJ, van Rijs W, Willemsen SP, Hazes JM, de Groot CJ. Mannose-binding lectin genotypes are associated with shorter gestational age. An evolutionary advantage of low MBL production genotypes? Mol Immunol. 2008;45(5):1514-8

De Man YA, Dolhain RJ, *Van de Geijn FE*, Willemsen SP, Hazes JM. Disease activity of rheumatoid arthritis during pregnancy: results from a nationwide prospective study. Arthritis Rheum. 2008;59(9):1241-8

Van de Geijn FE, Dolhain RJ, van Rijs W, Hazes JM, de Groot CJ. Mannose-binding lectin genotypes and preeclampsia: a case-control study. Hum Immunol. 2007;68(11):888-93

Wuhrer M, Stam JC, *Van de Geijn FE*, Koeleman CA, Verrips CT, Dolhain RJ, Hokke CH, Deelder AM. Glycosylation profiling of immunoglobulin G (IgG) subclasses from human serum. Proteomics. 2007;7(22):4070-81

De Man YA, Hazes JM, *Van de Geijn FE*, Krommenhoek C, Dolhain RJ. Measuring disease activity and functionality during pregnancy in patients with rheumatoid arthritis. Arthritis Rheum. 2007;57(5):716-22

Van de Geijn FE, Roos A, de Man YA, Laman JD, de Groot CJ, Daha MR, Hazes JM, Dolhain RJ. Mannose-binding lectin levels during pregnancy: a longitudinal study. Hum Reprod. 2007;22(2):362-71

De Man YA, Hazes JM, *Van de Geijn FE*, Gasthuis E, Krommenhoek C, Dolhain RJ. PARA-studie: Pregnancy-induced Amelioration of Rheumatoid Arhtritis Stand van zaken 2002-2007: het eerste lustrum. Ned Tijdschr Reumatologie 2007;10:5-8

Qian DZ, Ren M, Wei Y, Wang X, *Van de Geijn FE*, Rasmussen C, Nakanishi O, Sacchi N, Pili R. In vivo imaging of retinoic acid receptor beta2 transcriptional activation by the histone deacetylase inhibitor MS-275 in retinoid-resistant prostate cancer cells. Prostate 2005;64: 20–8

Van de Geijn FE, Morris SR, Sacchi N, Carducci MA, Pili R. Modulation of prostate tumor response to retinoids by histone deacetylase inhibitors. Proceedings AACR 2002;43:92.

PhD Portfolio

Mannose-binding lectin in pregnancy course and outcome.
Studies in healthy women and rheumatoid arthrtitis patients

Fleur van de Geijn, December 17th, 2009

Department of Rheumatology, Erasmus MC
Molecular Medicine Research School
June 2004 - June 2008

PhD training	Year	ETCS
General academic skills and research skills		
Biomedical English Writing and Communication	2005	3
Ethics and Scientific Integrity	2006	1.5
Statistics: Classical Methods for Data-analysis, NIHES [1]	2006	6
Working with SPSS [2] for Windows, NIHES	2006	0.5
Laboratory animal science (Article 9)	2007	5
In-depth courses		
Biomedical Research Techniques by Molecular Medicine Postgraduate School	2004	0.5
Medical Immunology Course Avans Hogeschool Utrecht	2004	2
Molecular Immunology Course by Molecular Medicine Postgraduate School	2005	4
(Lab)trainings, classes and journalclub meetings in order to comply with the requirements for the registration for SMBWO [3] Immunologist	2004-2008	20

Symposia and conferences		
Najaarsdagen NVR [4] Annual scientific meeting, Veldhoven and Arnhem	2004+2008+2009	1
ALIFI [5] Symposium Amsterdam	2004	0.5
Molecular Medicine Day, Rotterdam	2004 – 2008	2
NVVI [6] Immunology course, Lunteren	2005 + 2006	2
'AIOS [7] wetenschapsdagen' Erasmus MC, Rotterdam	2005 – 2008	1
Annual Meeting EWRR [8], Crete, Greece	2006	1
Annual Meeting EULAR [9], Amsterdam	2006	1.5
Annual meeting NVVI [6], Noordwijkerhout	2007	1
'Wetenschapsdagen Interne Geneeskunde' Dept of Internal Medicine Erasmus MC Rotterdam	2005 + 2008 + 2009	2
Annual Meeting EWRR [8], Toulouse, France	2008	1
Reumatoloog in opleiding (RIO) dagen, Egmond aan zee	2009	0,5

Teaching		
Tutoring 1st year medical students	2004	1.5
Coaching 1st year medical students 'Experiences in medical research' Dept of Immunology Erasmus MC Rotterdam	2004 + 2005 + 2007	1
Lectures immunological and medical case histories 2nd year medical students Dept of Immunology, Erasmus MC Rotterdam	2006 + 2007	2
Coaching 2nd year medical students 'How to write a review' Dept of Immunology, Erasmus MC Rotterdam	2007	1
Supervising and teaching MSc students, (Co-assistenten), Erasmus MC and Sint Franciscus Gasthuis Rotterdam	2008 - current	

Oral presentations

Molecular Medicine Day Rotterdam	2004	
Minisymposium Molecular Mechanisms of Auto-Immune Disease Dept of Immunology Erasmus MC Rotterdam	2005	
Seminar Dept of Nephrology Leiden University Medical Center	2005	
Seminar Dept of Immunology Erasmus MC Rotterdam	2005 + 2007	
Seminar Dept of Rheumatology Erasmus MC Rotterdam	2008	
Najaarsdagen Nederlandse Vereniging voor Reumatologie (NVR), Arnhem	2008 + 2009	

International poster presentations

European Workshop on Rheumatology Research (EWRR) Crete, Greece	2006	
European League Against Rheumatism (EULAR) Amsterdam	2006	
European Workshop on Rheumatology Research (EWRR) Toulouse, France	2008	
European League Against Rheumatism (EULAR) Copenhagen, Denmark	2009	

Other

Meet-the-expert breakfasts and lunches	2004 – 2008	0.5
Member PhD committee Dept of Immunology, Erasmus MC Rotterdam	2005	1.5
Referee activities for international scientific journals (Arthritis and Rheumatism)	2007 - current	

Residency Internal Medicine, Erasmus MC and Sint Franciscus Gasthuis, Rotterdam	2008-current

Travel awards

NVVI travel grant	2006
Reumafonds (Dutch Arthritis Association) meeting grant	2008
EULAR travel grant	2009

[1] Netherlands Institute for Health Sciences (NIHES)
[2] Statistical Package for the Social Sciences (SPSS)
[3] Biomedical scientist, Stichting ter bevordering van instelling en instandhouding van een stelsel van opleidingen tot Medisch-Biologisch Wetenschappelijk Onderzoeker (SMBWO)
[4] Dutch Society for Rheumatology, Nederlandse Vereniging voor Reumatologie (NVR)
[5] Amsterdam-Leiden Institute for Immunology (ALIFI)
[6] Dutch Society for Immunology, Nederlandse Vereniging voor Immunologie (NVVI)
[7] Resident, Assistent-In-Opleiding-tot-Specialist (AIOS)
[8] European Workshop on Rheumatology Research (EWRR)
[9] European League Against Rheumatism (EULAR)