Mannose-binding lectin levels during pregnancy: a longitudinal study

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BACKGROUND: Pregnancy is associated with changes in the immune system. Although previous studies have focussed 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 post-partum. 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 Post-partum 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. CONCLUSIONS: Pregnancy and the post-partum period profoundly influence MBL serum concentration and MBL complement pathway activity.

Key words: innate immunity/mannose-binding lectin/pregnancy/polymorphisms/lectin complement pathway

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 (Aluvihare *et al.*, 2005; Trowsdale and Betz, 2006). 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 (Birk and Rudick, 1986; Barrett *et al.*, 1999; Ostensen and Villiger, 2002; Hughes, 2004). However, some clinical studies demonstrate that also certain components of innate immunity are associated with pregnancy outcome (Christiansen *et al.*, 1999; Kilpatrick *et al.*, 1999a,b; Kruse *et al.*, 2002; Annells *et al.*, 2004, 2005). 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 complements that is associated with pregnancy outcome. The way in which innate immunity, and MBL in particular, changes during pregnancy has hardly been investigated (Kilpatrick, 2000).

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 (Christiansen et al., 1999; Kilpatrick et al., 1999a,b; Kruse et al., 2002). Deficient maternal MBL concentrations have also been described as a risk factor for preterm birth and reduced birthweight (Annells et al., 2004), although one report showed an association of MBL gene polymorphisms with gestational diabetes mellitus, resulting in heavier infants (Megia et al., 2004). Chorioamnionitis is another measure for pregnancy outcome that has been associated with certain MBL gene polymorphisms which result in lower MBL serum concentrations (Annells et al., 2005).

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 (2000) 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 (Kilpatrick, 2000).

The MBL serum concentration is strongly determined by its single-nucleotide substitutions (SNPs) in the structural gene and in the promoter region (Madsen et al., 1995; Roos et al., 2003). In exon 1 of the MBL2 gene, the wild-type allele is referred to as A, whereas the O-alleles represent the variant alleles B, C and D together. Individuals with the AA wild-type 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 (Barton et al., 2004). Moreover, basal MBL serum levels are also modified by the SNPs in the promoter region of the MBL gene (Madsen et al., 1998). Depending on the 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 (Ip et al., 2004). Interestingly, ~30% of the general population with the AO- or OO-genotypes have very low to deficient MBL levels. These individuals could be clinically considered as MBL deficient and at risk for infections. In the Ficolin gene, 10 SNPs have been demonstrated recently (Herpers et al., 2005), and a relationship between these SNPs and biological activity of Ficolin-2 has also been suggested (Hummelshoj et al., 2005).

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 post-partum (6 and 12 weeks and between 6 and 9 months). The first five visit time points were all met within a deviation of 1 week.

The last time point at 6–9 months post-partum was set as baseline value. At that time, we considered the participants as being completely 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 (Saftlas et al., 2004).

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 h 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 h 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 (Roos et al., 2001). 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 h 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 h 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 (Roos *et al.*, 2004), based on a protocol developed by Petersen (2001). 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.* (2003) 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 h 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 (Roos *et al.*, 2003). 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 h 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$_4$Cl. 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 sulphate (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 (Saftlas *et al.*, 2004). 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 the 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, 1× LightCycler DNA Master Hybridisation Probes (Roche Molecular Biochemicals, Mannheim, Germany) and 2 mM MgCl$_2$. The PCR amplification profile of both experiments consisted of 10 min at 95°C, followed by 45 cycles of 95°C for 3 s, 60°C (63°C in case of detection of the MBL exon 1 polymorphism) for 15 s and 72°C for 10 s. 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 s 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′-TGAGTATGGT-CAGCGTCTTA-3′ and the reverse primer 5′-TGGGCTGGCAA-GACAACTATTAG-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′-TTTTAGACAGGCAG-GCTTGCCTGCCTGGGT-3′ FLU, which is complementary to the L allele, and the 5′-LC Red 705-AGCATTTTCTCTGGAAATTTCT-TACTACGTTGG-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 time points 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 time

point 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 post-partum.

The post-partum drop was calculated by dividing the value of the time point 6 weeks post-partum by the baseline value at 6 months post-partum and expressed in percentages. The Wilcoxon signed rank test was used to evaluate the difference between this time point 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 I. 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 (Brown *et al.*, 2001); 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 time points 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 post-partum. 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 post-partum (6 weeks) MBL concentrations

Table I. Demographic and 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 (%)	3 (9.4)
Mean gestational age at delivery (days, range)	283 (238–294)
Mean birthweight (gram) (SE)	3492 (±80)

dropped sharply to 57% of the baseline value (IQR 44–66%, $P < 0.0001$, Figure 1A).

Ficolin-2 serum concentrations are not influenced by pregnancy

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

Classical complement pathway activity changes only slightly during pregnancy

The classical complement pathway activity was measured at all six time points 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%, $P < 0.0001$). Post-partum (6 weeks), the activity of the classical complement 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 post-partum drop could be appreciated.
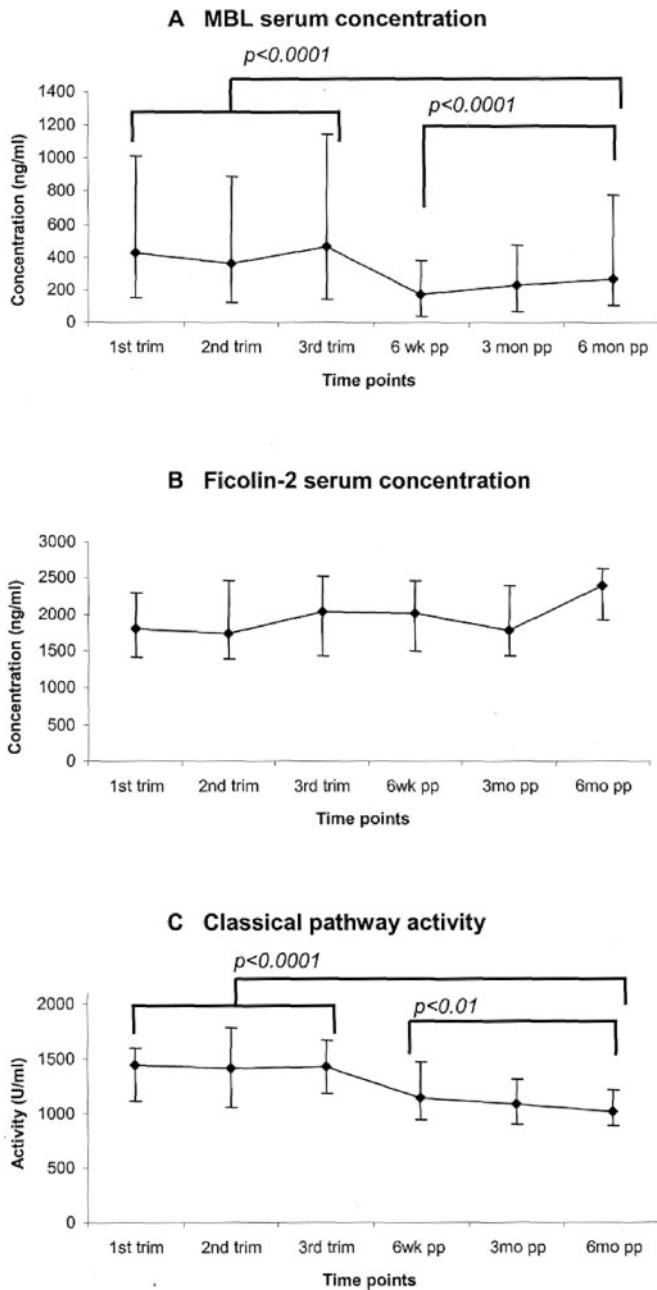
Influence of the MBL genotype and promoter polymorphisms on basal MBL serum levels

As expected, MBL serum levels show a major inter-individual variation, but all showed the same pattern during pregnancy and post-partum (Figure 2). Therefore, we assessed the MBL genotypes of the subjects to examine whether the intra-individual 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 II. The maternal MBL genotype and MBL promoter polymorphism frequencies summarized in Table II are in accordance with results from previous studies (Madsen *et al.*, 1995; Roos *et al.*, 2004). 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 (Madsen *et al.*, 1995).

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).
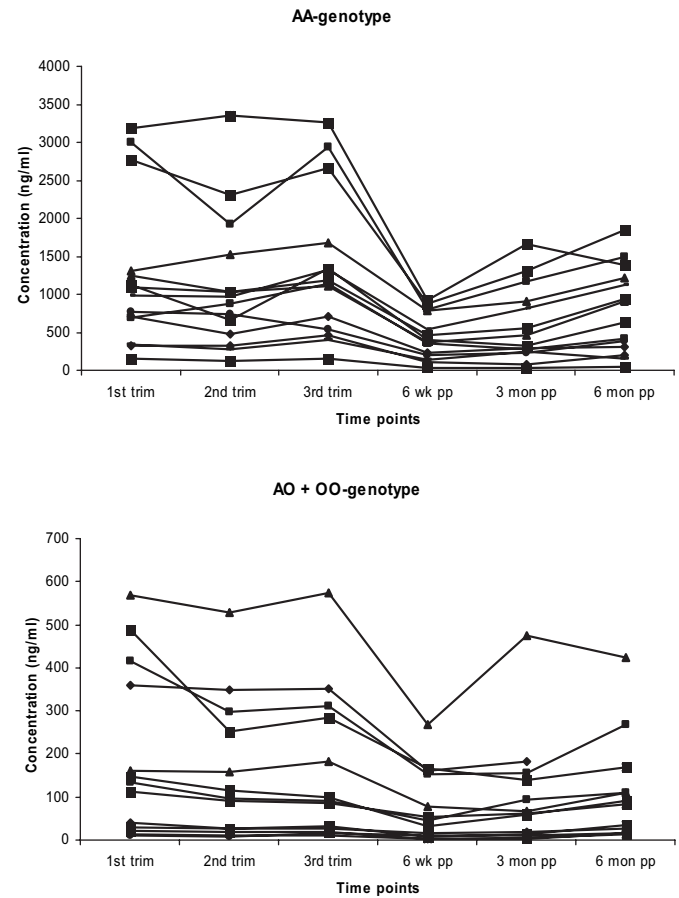
A MBL serum concentration

B Ficolin-2 serum concentration

C Classical pathway activity

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

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.

AA-genotype

AO + OO-genotype

Figure 2. Mannose-binding lectin (MBL) serum concentration in healthy women during pregnancy and post-partum 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 post-partum, namely an increase during pregnancy [median to 140% of baseline value, interquartile range (IQR) 116–181%, p<0.0001], and a significant decline post-partum (to 57% of baseline value, median, IQR 44–66%, $P < 0.0001$). MBL serum concentrations were measured at indicated time points. Each symbol represents one individual. The baseline value is defined as the MBL serum concentration at 6 months post-partum. Note that the *Y*-axes have different scales. Trim, trimester; wk, weeks; mon, months; pp, post-partum.

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.

Table II. MBL genotypes and median baseline values of complement pathway components

Genotype	AA		AO	OO
MBL exon 1 genotype (n) (%)	AA		AO	OO
	18 (58)		12 (39)	1 (3)
			AB 7	DD 1
			AC 1	
			AD 4	
MBL promoter polymorphisms (n)				
HY/LY	8		2	—
LY/LY	1		2	—
LX/LY	2		5	—
HY/HY	3		3	1
LX/HY	2		—	—
LX/LX	2		—	—
Median baseline values				
MBL concentration (ng/ml) (IQR)	533 (259–1197)	AO	90 (21–139)	83
		AB	35 (13–110)[a]	
		AC	15	
		AD	269 (16–423)[a]	
MBL–MASP complex activity (U/ml) (IQR)	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 (U/ml) (IQR)	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 detection limits.

Post-partum drop in MBL concentration occurred equally in all genotypes

Although all women had detectable levels of MBL during pregnancy, a post-partum drop was observed in all women, independent of their MBL genotypes, that is, 55% in AA-subjects ($P < 0.0001$), 60% in AO-subjects ($P < 0.03$) and 65% in the OO-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/LXA-genotype, MBL levels remained very low (33, 36 and 51 ng/ml) at 6 weeks, 12 weeks and 6 months post-partum, respectively, in contrast to her higher MBL levels during pregnancy (156, 122 and 157 ng/ml in the first, second and third trimester, respectively).

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 post-partum, 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 concentration 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 post-partum (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 time points 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 II). Statistical analyses were performed in the series in which the baseline values at 6 months post-partum 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 post-partum 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 post-partum.

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 post-partum 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).
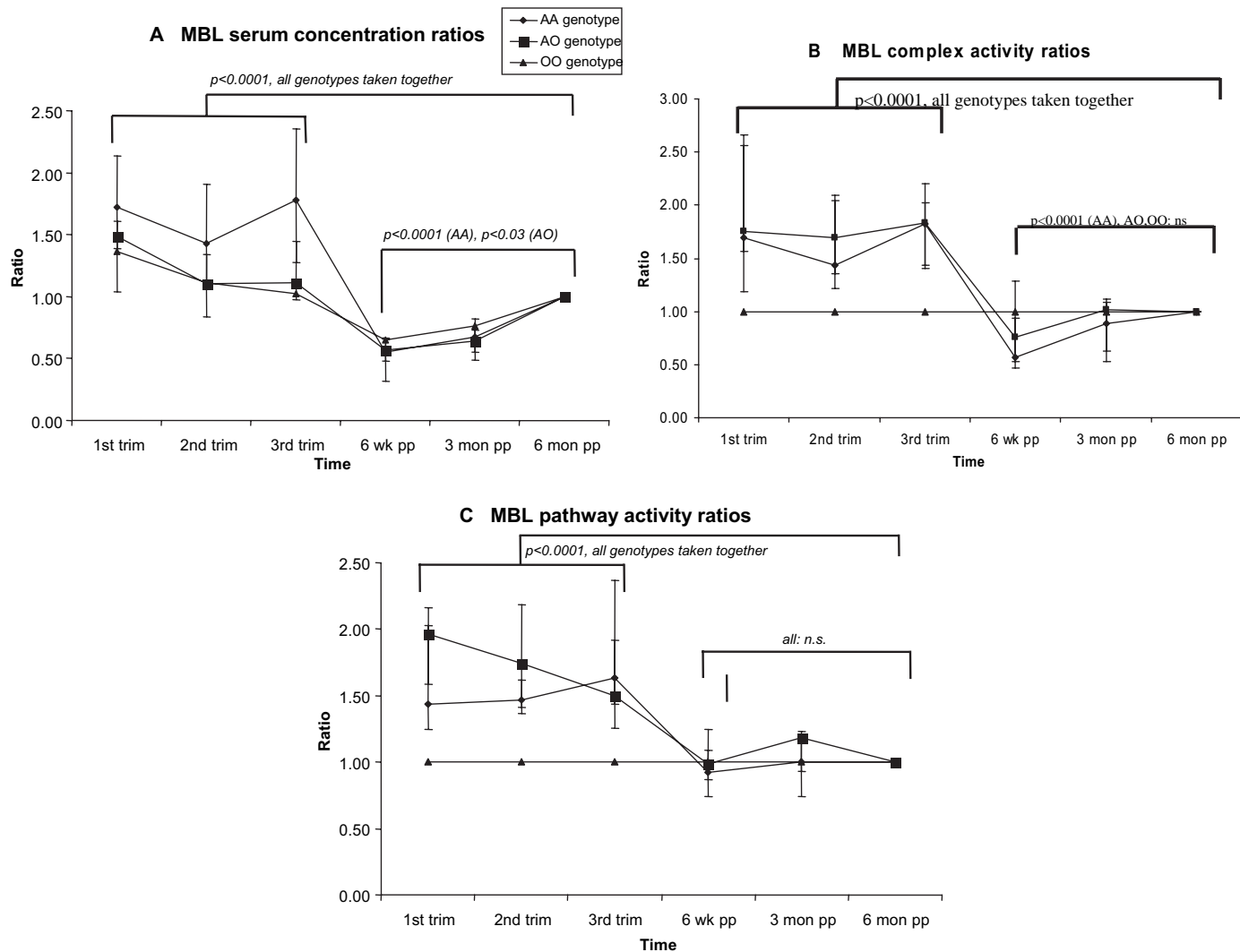
Figure 3. Mannose-binding lectin (MBL) serum concentration, MBL–MASP complex activity and MBL pathway activity in healthy women during pregnancy and post-partum categorized per genotype. Ratios were calculated by dividing the concentration or activity at indicated time points by the baseline concentration or baseline activity. Baseline is defined as the value at 6 months post-partum. 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 post-partum drop (6 weeks post-partum 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 post-partum 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 post-partum 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 ($n = 1$). Trim, trimester; week, weeks; mon, months; pp, post-partum; ns: not significant.

At all time points, 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 time

points in 31 women. In six women (AO- and OO-genotypes), all measurements were below detection limit, that is, 200 U/ml (Table II). Statistical analyses were performed in the series in which the baseline values at 6 months post-partum 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 pathway 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 post-partum 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 post-partum 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 time points ($r > 0.93$, $P < 0.01$).

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 6 weeks post-partum. Because Ficolin-2 levels and classical complement pathway activities are not influenced in a similar way during pregnancy and in the post-partum period, it can be concluded that pregnancy has a specific effect on MBL and its complement pathway.

During pregnancy, mothers with the wild-type 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 6 months post-partum time point as our baseline value for all measurements. We are aware of the fact that our threshold point of 6 months post-partum as a baseline value may not be an accurate baseline for all participants. There might be some women who need >6 months to recover from their pregnancies. Taking into account that MBL values are subject to change in the post-partum period, we would suggest that MBL measurements in the context of research and clinical diagnostics should not be performed before a minimum of 6 months post-partum.

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 (2000), 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* (Beagley and Gockel, 2003). 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 (Malan Borel *et al*., 1997; Raghupathy, 1997; Szekeres-Bartho *et al*., 1997). 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 (Ogden *et al*., 2001; Nauta *et al*., 2004; Stuart *et al*., 2005). 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 favourable disease outcome (Graudal *et al*., 1998). Because it is known that these diseases tend to improve during pregnancy (Birk and Rudick, 1986; Barrett *et al*., 1999; Ostensen and Villiger, 2002; Hughes, 2004), 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* (Sorensen *et al*., 2006) and in non-pregnant humans (Hansen *et al*., 2001; Hansen, 2003; Gravholt *et al*., 2004). 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 (Lacroix *et al*., 2002). 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 (Arai *et al*., 1993). One study shows no effect of the corticosteroid hydrocortisone on *in vitro* MBL production (Sorensen *et al*., 2006). A pregnancy-induced increase in cortisol might consequently result in higher MBL levels (Marnach *et al*., 2003).

Fetal production of MBL has been reported, as MBL can be detected in the amniotic fluid (Malhotra *et al.*, 1994) and in umbilical cord blood (Kielgast *et al.*, 2003; Maruyama *et al.*, 2003). 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 6 weeks post-partum. 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.

Acknowledgements

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