BASIC SCIENCE ARTICLE

Mannose-binding lectin and mannose-binding protein-associated serine protease 2 levels and infection in very-low-birth-weight infants

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OBJECTIVE: The aim of this study was to explore the role of the lectin pathway in neonatal sepsis through the study of MBL and MASP2 levels and their relationship with infection in a cohort of very-low-birth-weight infants (VLBWI).
METHODS: MBL and MASP2 were measured in plasma samples of $n = 89$ VLBWI using ELISA and correlated with clinical parameters. MBL plasma levels were aligned with genotyping data of *mbl2* exon 1 polymorphisms, rs1800450, rs1800451, and rs5030737.
RESULTS: MBL levels were clearly determined by MBL genotype, i.e., AA individuals had tenfold higher MBL levels than AO individuals. MBL and MASP2 levels did not correlate with gestational age, apart from MASP2 levels on day 7. During the first 21 days of life, we noted a gradual increase in both MBL and MASP2 levels. On day 7 of life, MASP2 levels in infants developing late-onset sepsis measured before the onset of symptoms were found to be lower, as compared to non-LOS infants.
CONCLUSIONS: In our cohort of VLBWI, MBL levels were genetically determined, but not associated with gestational age or sepsis in the first 21 days of life. Lower MASP2 levels on day 7 may indicate increased risk for late-onset infection.

INTRODUCTION
Infections are a major threat to newborn infants, especially to those who are born highly preterm, with a gestational age (GA) lower or equal to 28 weeks and with a very low birth weight (VLBW), lower or equal to 1500 g. The infection risk profile of these children is mainly influenced by endogenous factors such as gestational age, birth weight, and immaturity of local barriers, but also by genetic background and by numerous interactions between biological characteristics, environment, and exposure to environmental procedures required for their survival. The preterm infants' defense against invasive pathogens mainly relies on the innate immune system, i.e., neutrophils, macrophages, but also soluble factors such as complement.[1] In our study, we hypothesized that low levels of the lectin pathway initiator molecules mannose-binding lectin (MBL) and MASP2 are related to an increased risk of infection in preterm infants. It is well known that the lectin pathway is predominantly activated by mannose-binding lectin (MBL), which binds to the membrane polysaccharides of bacteria and a variety of other pathogens.[2] The resulting complex then attaches to the mannose-binding protein-associated serine protease 2 (MASP2), which in turn activates the complement cascade by cleaving C4. MBL plasma levels are determined genetically, e.g. *mbl2* exon 1 polymorphisms, specifically rs1800450, rs1800451, and rs5030737.[2–4] In several cohorts, especially in children and immune-compromised individuals, reduced levels of lectin pathway proteins have been linked to a higher susceptibility for infection.[5–8] Studies including newborn infants, however, are scarce and have revealed contradicting data. Schlapbach et al.[9] found a correlation of increased MASP2 levels in cord blood and prospective development of necrotizing enterocolitis (NEC). Swierzko et al.[10] investigated the role of MASP2 levels for the susceptibility to infection and found no association. Some groups were able to show that MASP2 levels are associated with birth weight,[10–12] and that concentrations of MBL, as well as MASP2, correlate with gestational age.[10,11,13–15] Swierzko et al.,[12] however, did not find a correlation between MBL levels and gestational age. In addition to that, dynamic changes in MBL/MASP2 levels during the period of immunological adaptation and maturation have yet to be explored. It was the aim of this study to explore the association of MBL plasma levels with *mbl2* exon 1 polymorphisms in a cohort of very-low-birth-weight infants (VLBWI). Furthermore, we aimed to investigate a potential relation between plasma levels of MBL and MASP2 levels and inflammation in this cohort, particularly the risk of sepsis during the most vulnerable period of the first 21 days of life.[16]

MATERIALS AND METHODS
Design, setting, and subjects
The sample collection was performed within a prospective two-center study between January 2012 and March 2015 in the perinatal units of the University of Lübeck Children's Hospital and

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the Children's Hospital Aschaffenburg-Alzenau. Inclusion criteria: After informed consent of parents or legal representatives, peripheral whole-blood (lithium-heparin) was taken from VLBWI infants born without lethal malformations. During the time period, 181 VLBWI infants were eligible, five infants died within the first 72 h, in 87 infants parents were not approached for participation. In the enrolled infants, blood withdrawal was in line with the required laboratory checks at different time points after birth (day 1, $n = 89$; day 7 ± 2 days, $n = 87$; day 21 ± 2 days, $n = 60$) and in line with the pediatric guidelines of the European Medical Agency, with regard to maximum blood volume withdrawal for research purposes. To evaluate MBL and MASP2 levels according to the diagnosis of sepsis and its time point, we included those protein levels that were assessed in closest proximity before the onset of sepsis symptoms (pre-event levels; day 1 of life: $n = 5$, day 7 of life: $n = 17$, day 21 of life: $n = 5$).

Genotyping of mbl 2 exon 1 polymorphisms
DNA extraction was performed using a commercial DNA purification kit (Quiagen, Hilden, Germany). The DNA was washed twice and eluted. Genotypes were determined with the TaqMan 5′ nuclease assay (Applied Biosystems, Foster City, CA) and the 7900HT Real-Time PCR System. Context sequences were as follows:

rs1800450: 5′-TGGTTCCCCCTTTTCTCCCTTGGTG[C/T]CATCACGCCCATCTTTGCCTGGGAA-3′

rs1800451: 5′-ACACGTACCTGGTTCCCCCTTTTCT[C/T]CCTTGGTGCCATCACGCCCATCTTT-3′

rs5030737: 5′-CCCTTTTCTCCCTTGGTGCCATCAC[A/G]CCCATCTTTGCCTGGGAAGCCGTTG-3′

The distribution of genotypes was appropriated to allele frequencies, as determined by the Hardy–Weinberg equilibrium, seen in the Caucasian population.[4,17] The statistical analyses were limited to individuals with complete genotype characterization for all the three MBL genotypes ($n = 83$).

Definitions
Sepsis: Sepsis was considered as a condition with at least two signs of systemic inflammatory response (temperature >38 °C or <36.5 °C, tachycardia >200/min, new onset or increased frequency of bradycardias or apneas, hyperglycemia >140 mg/dL, base excess < −10 mval/L, changed skin color, and increased oxygen requirements), one laboratory sign (C-reactive protein >2 mg/dL, immature/neutrophil ratio >0.2, white blood cell count <5/nL), and neonatologist's decision to treat with antibiotics for at least 5 days, regardless of the proof of causative agent in blood culture.

Blood-culture-proven sepsis: Blood-culture-proven sepsis was defined as sepsis criteria (see above) plus detection of a causative pathogen in the blood culture.

The definition was based on the criteria of the Neo-Kiss surveillance system.[18,19]

Early-onset sepsis: Early-onset sepsis was registered in cases of sepsis before 72 h of life.

Late-onset sepsis: Late-onset sepsis was defined as sepsis with onset after 72 h of life.

ELISA
ELISA analysis of MBL and MASP2 plasma levels was performed using commercial kits (Hycult Biotec, Uden, the Netherlands). Frozen plasma was thawed and diluted at 1:100 for MBL-ELISA and 1:8 for MASP2-ELISA (readouts in ng/mL).

Statistical analysis
Data analysis was performed using the SPSS 22.0 data analysis package (IBM, Munich, Germany). Hypotheses were evaluated in univariate analyses with Fisher's exact test and Mann–Whitney U test. Correlations were estimated with the Spearman's rho test. A p value <0.05 was considered as statistically significant for single tests.

Ethics
The study parts were approved by the local committee on research in human subjects of the University of Lübeck and the ethical committee of the Bavarian Medical Board (Landesärztekammer Bayern).

RESULTS
Clinical characteristics
We analyzed the data of $n = 89$ preterm infants with a mean gestational age of 27.9 ± 2.6 weeks (range 23+0 to 33+6 weeks; clinical characteristics, Table 1). First, we assessed the association of MBL and MASP2 levels with genetic and clinical factors, specifically:

mbl2 genotype and MBL levels. As outlined in Fig. 1, AA individuals ($n = 49$) had tenfold higher MBL levels than AO individuals ($n = 32$) on day 1, day 7, and day 21 of life.

Table 1. Clinical characteristics of the study cohort

Parameter	
Number of infants	89
Gestational age (mean ± SD, weeks)	27.9 (2.6)
Birth weight (mean ± SD, g)	1002 (323)
Female gender (%)	51.7
Small for gestational age (%)	15.7
Maternal background (%)	
Caucasian	90
Turkey, Middle East	10
Clinical sepsis (%)	32.6
Early-onset sepsis	5.6
Late-onset sepsis	27.5
Cause of preterm birth (%)	
Preterm labor	26.1
Amniotic infection syndrome	30.7
Pathological Doppler, IUGR	25.0
HELLP, EPH gestosis	14.8
Placental abruption, uterus rupture	3.4
rs1800450 exon 1 -54 G → D, $n = 82$ (%)	
wt	70.7
Heterozygous	28.0
Homozygous	1.2
rs1800451 exon 1 -57 G → E, $n = 78$ (%)	
wt	93.6
Heterozygous	6.4
rs5030737 exon 1 -52 R → C, $n = 78$ (%)	
wt	91.0
Heterozygous	9.0
MBL genotype (%)	
AA	59.8
AO	39.0
OO	1.2

SD standard deviation, *IUGR* intrauterine growth restriction, *HELLP* highly elevated liver enzymes and low platelets, *EPH* edema proteinuria hypertension, *wt* wild-type, *MBL* mannose-binding lectin
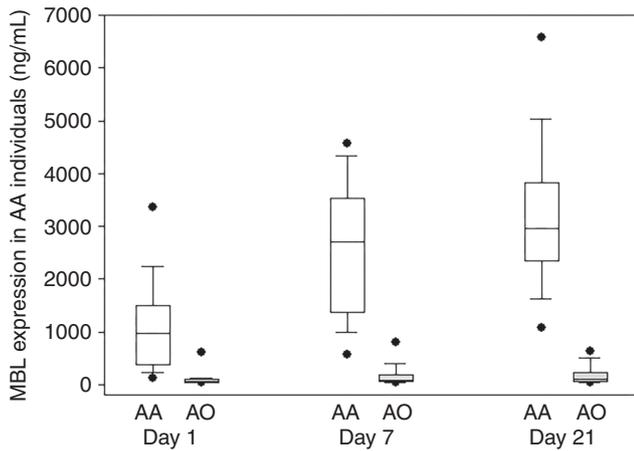
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Fig. 1 Genetically determined MBL levels during the early weeks of life. MBL levels were determined in plasma samples of VLBWI on day-of-life 1 ($n = 82$, $n = 50$ AA and $n = 32$ AO), d7 ± 2 ($n = 80$, $n = 48$ AA, $n = 32$ AO), and d21 ± 2 ($n = 55$, $n = 34$ AA, $n = 21$ AO), and stratified according to MBL genotype. AA individuals had tenfold higher MBL levels than AO individuals throughout the first 21 days of life, while MBL levels were undetectable in a single individual with OO genotype. Whisker-Boxplots indicate median, interquartile ranges, 95% CI and outliers as individual dots

Fig. 2 MASP2 levels during the early weeks of life. MBL levels were determined in plasma samples of VLBWI during the early weeks of life; day 1 $n = 89$, d7 ± 2 $n = 87$; d21 ± 2 $n = 60$. Whisker-Boxplots indicate median, interquartile ranges, 95% CI and outliers as individual dots

Gestational age and birth weight. In our setting, MBL and MASP2 levels did not correlate with gestational age, apart from MASP2 levels on day 7 ($R^2 = 0.19$, $p < 0.001$). Birth weight did not correlate with MBL and MASP2 levels. In small-for-gestational age (SGA, birth weight <10th percentile) infants, we noted higher MASP2 levels on day 1 (median/IQR in ng/mL; 10/15 vs. 7/10 ng/mL, $p = 0.05$) and day 7 (75/119 vs. 19/48 ng/mL, $p = 0.02$), as compared to infants with a birth weight appropriate for gestational age. No gender differences were observed.

Cause of preterm birth. We evaluated whether infants born due to potential infection at the feto–maternal interface (suspected or proven amniotic infection syndrome (AIS), preterm labor; $n = 50$) differed in their MBL and MASP2 levels from infants born due to non-infectious reasons, i.e. placental abruption or intrauterine growth restriction/pathological Doppler ($n = 39$). On day 1 of life, no significant differences in MBL and MASP2 levels were noted. The same observation was made in a subgroup analysis excluding infants born due to preterm labor without suspected/proven AIS. Likewise, both groups did not differ in MBL levels on day 7 and day 21 of life. On day 7, reduced MASP2 levels were noted in infants with infectious cause of preterm birth (median/IQR; 19/27 vs. 30/46 ng/mL, $p = 0.03$), which was not persistent on day 21 of life.

MBL and MASP2 levels, and infection in VLBW infants
Longitudinal expression of MBL and MASP2 levels. During the first 21 days of life, we noted a gradual increase in expression levels of MBL and MASP2 (Figs. 1 and 2). MBL levels on day 1 correlated with MBL levels on day 7 ($R = 0.89$, $p < 0.001$) and day 21 ($R = 0.78$, $p < 0.001$). Likewise, MASP2 levels on day 1 correlated with MASP2 levels on day 7 ($R = 0.7$, $p < 0.001$) and day 21 ($R = 0.53$, $p < 0.001$). On day 21, a weak inverse correlation between MBL and MASP2 levels was found ($R = -0.43$, $p = 0.001$), while no correlations between both the biomarkers were observed on days 1 and 7.

Early-onset sepsis. In our cohort, five infants developed clinical early-onset sepsis (EOS). On day 1 of life, neither MBL levels nor MASP2 levels differed between EOS and non-EOS infants.
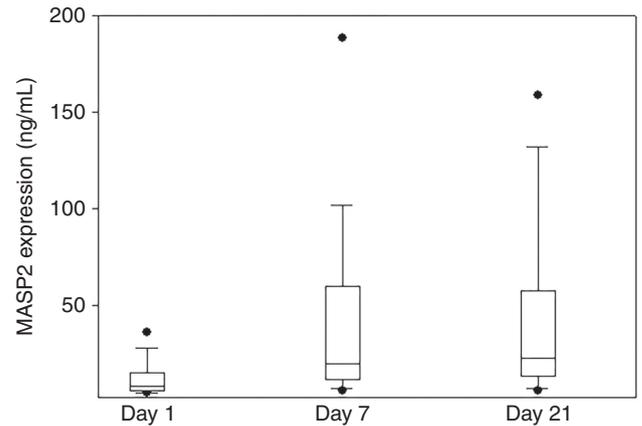
Late-onset sepsis. Twenty-five out of the 89 infants developed late-onset sepsis (LOS; after mean/median 12/11 days), two infants had two episodes of LOS. With regard to MBL levels measured before the onset of sepsis symptoms, no differences between LOS and non-LOS infants were observed (Fig. 3a). Pre-event MASP2 levels were lower in LOS patients, as compared to non-LOS infants, on day 7 of life (Fig. 3b). Among sepsis patients, no difference in pre-event levels was noted between clinical sepsis and blood-culture-proven sepsis (median/IQR, ng/ml; MBL: 700/2409 vs. 302/1660, $p = 0.9$; MASP2: 15/37 vs. 19/17, $p = 0.9$). Table 2 describes the dynamic expressions of MBL and MASP2 levels in unaffected VLBWI, clinical sepsis patients, and blood-culture-confirmed sepsis patients, irrespective of the time point of sepsis. Blood-culture-confirmed sepsis patients had significantly lower MASP2 levels on day 7, as compared to unaffected controls, while no other differences were noted.

DISCUSSION
Our results suggest, that in our VLBWI cohort, MBL levels seem higher in the group of patients with genotype AA than in patients who are carriers of single-nucleotide polymorphisms, while we have noted no relationship with gestational age and the risk for sepsis in the first 21 days of life. In babies with LOS, we found lower pre-sepsis MASP2 levels, compared with controls.

Consistent with previous studies,[20,21] we were able to show a clear effect of the SNPs in *mbl2* exon 1 on MBL plasma levels, with strongly reduced levels in heterozygous genotypes compared to wild-type individuals. MBL plasma levels derived from longitudinal samples on days 1, 7, and 21 of life indicated an increase over time in infants carrying AA, while MBL levels remained significantly lower in AO individuals. This is in line with findings of Frakking et al. who also described that MBL levels in AA individuals increase with age, while those in AO or OO individuals remain low or undetectable.[13] We also found a high inter-individual variation in MBL levels in AA and AO carriers. It should be noted that we only examined three exon 1 SNPs, while there are a number of genetic modifiers that may also affect the MBL levels, e.g. in the promoter region.[21] In the entire cohort of infants with a birth weight <1500 g, we did not observe major influences of gestational age on MBL and MASP2 levels. This is certainly in contrast to previous studies showing an association of MBL[11,13–15] and MASP2[10,15] with gestational age, but may reflect an effect of different inclusion criteria such as different average gestational age and the comparison with more mature or even term infants.

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Table 2. Dynamic expression of MBL and MASP2 levels in sepsis patients

Late-onset sepsis (LOS)	MBL day 1	MBL day 7	MBL day 21	MASP2 day 1	MASP2 day 7	MASP2 day 21
No LOS, $n = 64$	305/1074/39–5186	1118/3089/40–5303	1593/3115/40–6785	9/11/4–91	29/66/5–698	41/83/5–1036
Clinical LOS, $n = 12$	196/1014/40–1467	885/2123/40–3231	2052/3473/74–4255	7/10/4–27	15/51/4–115	23/42/5–303
Culture confirmed LOS, $n = 13$	307/938/54–2104	1196/2010/66–3932	2694/3364/53–5775	7/7/4–21	13/14/4–81[a]	20/22/6–172

Data are expressed as median/IQR/min–max in ng/mL
[a]On day 7 of life infants with blood-culture-confirmed sepsis have lower MASP2 levels than infants without sepsis ($p = 0.008$, Mann–Whitney U-test)
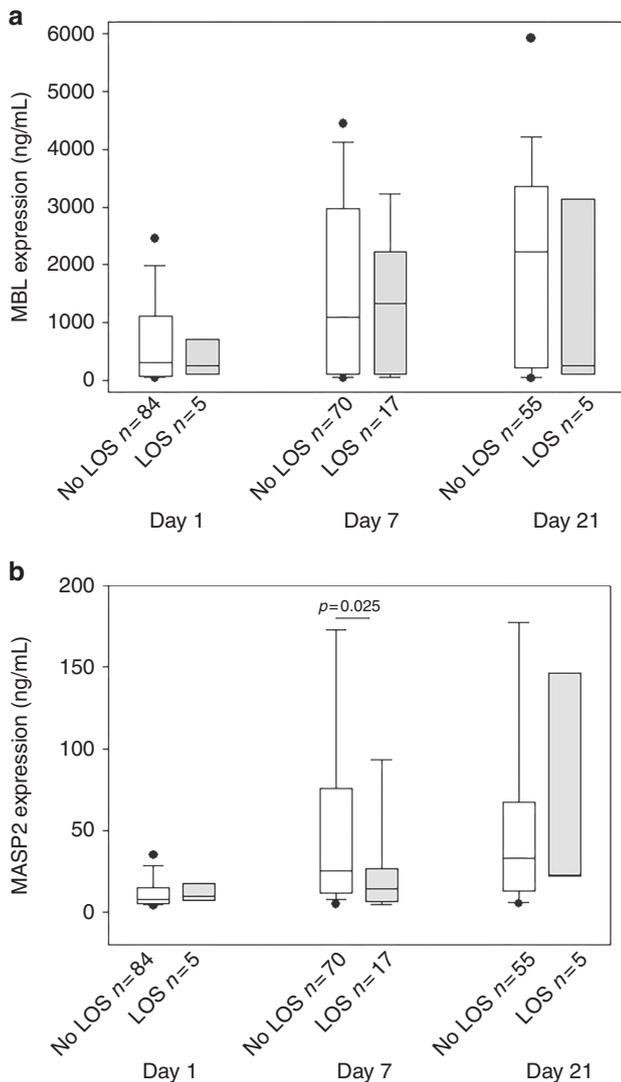
Fig. 3 MBL and MASP2 levels before the onset of late-onset sepsis. MBL (**a**) and MASP2 (**b**) levels were determined in the plasma samples of preterm infants developing late-onset sepsis before the onset of clinical symptoms (gray plots), as compared to the unaffected controls at the corresponding time point of closest proximity before sepsis in cases (white plots). Whisker-boxplots indicate median, interquartile ranges, 95% CI and outliers as individual dots. A p value of <0.05 was regarded as significant (Mann–Whitney U-test)

Based on previously noted differences[22] in the immune repertoire between SGA infants and those being appropriate for gestational age (AGA), we hypothesized that SGA infants might have lower levels of lectin pathway molecules. Surprisingly, SGA did express higher MASP2 levels on day 1 and day 7, a finding that needs to be confirmed in future studies. Reduced plasma levels of MBL have been related to an increased susceptibility to infection in neonates,[11,23,24] however, we were not able to confirm this association in our cohort. In addition, infectious complications at the feto–maternal interface do not affect the MBL plasma levels, and MBL/MASP2 levels do not predict the risk for early-onset sepsis. We noted a gradual increase of both MBL and MASP2 levels over time. In contrast to other studies,[25,26] MBL levels in our analysis were not different between infants developing LOS and unaffected infants. This may be due to our focus on a more premature cohort, in which infants are expected to have low MBL levels by default. As we found in a previous study, differences in the infection risk based on genetically determined MBL levels do not seem to arise until children are older[27] MASP2 levels, on the other hand, were reduced on day 7 in infants who subsequently developed LOS. Though future large-scale studies are needed to determine the predictive value of MASP2, this indicates MASP2 as a potential biomarker. The finding is supported by previous studies that reported reduced MASP2 levels in pediatric cancer patients who developed fever and neutropenia,[28] as well as the development of CMV after kidney transplantation.[29]

In our prospective study, we examined a well-defined cohort at the risk for infections, which is cared for under standardized conditions in tertiary-level NICUs. To our knowledge, this is the first study to investigate the dynamic changes of lectin pathway molecule expression in the period of highest vulnerability after birth, in line with genetic determinants (*mbl2* carrier status). Our approach has limitations. The study cohort is relatively small, and longitudinal sampling was not achievable in all enrolled infants. Moreover, the study design is descriptive and hypothesis-generating. Given the urgent need for significant biomarkers to differentiate sepsis patients from unaffected controls and to guide anti-infective therapy in exposed preterm infants, MASP2 is a promising candidate, which needs further exploration in both mechanistic models and large-scale clinical cohorts.

ADDITIONAL INFORMATION
Competing interests: The authors declare no competing interests.

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