Gene expression profiling at birth characterizing the preterm infant with early onset infection

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ABSTRACT

Objective: Early onset infection (EOI) in preterm infants < 32 weeks gestational age (GA) is associated with a high mortality rate and the development of severe acute and long term complications. The pathophysiology of EOI is not fully understood and clinical and laboratory signs of early onset infections in this patient cohort are often not conclusive. Thus, the aim of this study was to identify relevant pathomechanisms involved in EOI by using genome-wide gene expression (GWGE) analyses from cord blood in order to provide potential biomarkers enabling the early diagnosis of EOI.

Study design: This prospective cohort study was conducted in preterm infants < 32 weeks GA. GWGE analyses using Codelink human microarrays were performed from cord blood of preterm infants with and without EOI.

Results: GWGE analyses revealed differential expression of 292 genes in preterm infants with EOI as compared to infants without EOI. Infants with EOI could be further differentiated into two subclasses and were distinguished by the magnitude of the expression of genes involved in both neutrophil and T cell activation. A hallmark activity for both subclasses of EOI was a common suppression of genes involved in NK cell function, supported by NK cell numbers. Significant results were replicated in a second study cohort.

Conclusions: Gene expression profiles obtained at birth permit discrimination of preterm infants with and without EOI with impaired NK cell activation present in EOI as a potential future diagnostic tool to enable early diagnosis of EOI.
INTRODUCTION

Preterm birth (PTB) remains a major problem in perinatal medicine and accounts for 75% of the perinatal mortality and 50% of the perinatal morbidity [1]. Early onset infections (EOI) in PTB are associated with significant morbidity and a mortality rate of 15-50%, especially in very immature preterm infants [2, 3]. Although clinical and experimental studies continue to provide valuable insight into processes leading to EOI and its attendant complications [4, 5], biological pathways relevant to the complex pathophysiology of EOI remain poorly understood. This is reflected in the limitations of early diagnostic markers and the absence of targeted treatment approaches in this high-risk patient cohort. Clinical signs are ambiguous and difficult to interpret in the absence of reliable early biochemical markers [6-8]. The gold standard of blood culture-proven sepsis severely underestimates the rate of severe infections in newborns and especially preterm infants, as the diagnostic approach is complicated by maternal antibiotic therapy and hampered by small blood volumes [2, 9]. As outcome and prognosis of EOI mainly depend on early and efficient treatment, sensitive and specific indicators of EOI are crucial at the earliest stage of disease.

In this prospective cohort study, we applied gene expression profiling from cord blood of preterm infants < 32 weeks of gestational age to provide comprehensive biological information and identify biological pathways relevant for the development of EOI in order to enable the identification of early diagnostic markers.
MATERIAL AND METHODS

Patients

Newborn infants < 32 weeks gestational age (GA) were prospectively included in the study. Depending on the availability of diagnostic criteria at birth, infants were pro- or retrospectively excluded when one of the following diagnoses were present: Premature rupture of membranes \(\geq 3\) weeks prior to birth leading to oligo- or anhydramnios, severe congenital malformations, diagnosis of severe metabolic disorders, prepartum treatment of the mother with cytostatic or immunosuppressive medication other than for lung maturation, and postnatal treatment with corticosteroids in a dose \(\geq 1\) mg/kg body weight. Analysis of CRP, whole white blood count and microbiological examination of blood cultures, swabs, urine and stool samples were carried out in the first 72 hours of life. Patients were clinically re-evaluated in short intervals and continuously monitored for vital signs, i.e. heart rate, blood pressure, microcirculation and breathing pattern. Patients were allocated to one of the two following groups: (I) EOI and (II) non-EOI (no signs of infection in the first 72 h of life). EOI was diagnosed if the infant showed both, a pathologic ratio of immature to total granulocytes (IT-ratio \(\geq 0.2\) as determined by manual counts) and/or a pathologic white blood cell count (WBC) paralleled or followed by an increase in CRP \(\geq 6\) mg/l in the first 72 h of life [7, 10-15]. These laboratory signs had to be accompanied by at least three of the following clinical signs suggestive of bacterial infection in the new born infants: pallor, grey skin colour, capillary refill \(> 3\) sec, requiring volume resuscitation or substitution of any catecholamines, dyspnea, tachypnea, requiring respiratory support or supplemental oxygen, increased thermal instability, unexplained hypo- and hyperglycaemia, feeding difficulties, bilious reflux and abdominal distension, increasing incidence of apnoea and/or bradycardia, lethargy, irritability, and increased or decreased
muscle tone [2, 16]. All patients who did not meet the criteria for the EOI were included in the non-EOI cohort.

The comprehensive monitoring of the perinatal course is further defined in Supplemental Materials and Methods. The study has been approved by the legal ethical committee (File 79/01, University of Giessen, Germany).

**Blood sampling, RNA isolation and gene expression profiling**

Details of the microarray experiments and data analysis can be found in Supplemental Materials and Methods.

Briefly, blood was sampled from an indwelling umbilical artery catheter at birth. 250-300 µl of whole umbilical cord blood was obtained immediately after birth and directly transferred to 750-900 µl of the PAXgene Blood RNA System (PreAnalytiX, Heidelberg, Germany). RNA isolation was performed according to the manufacturer’s recommendations (PreAnalytiX). RNA was hybridized on CodeLink UniSet Human 10 K Bioarrays (GE Healthcare) using the CodeLink Expression Assay Kit (GE Healthcare) and samples processed using CodeLink Expression Software V4.1 (GE Healthcare). The dataset was normalized using quantiles normalization in R [17]. A rank statistics was used to identify differentially regulated genes between EOI and non-EOI [18]. Bioinformatic analyses were conducted using following software tools: “dChip” for hierarchical clustering, “DAVID” for gene ontology, “Pathway Explorer” and “Ingenuity Pathway Analysis” for pathway analyses following the software recommendations (Supplemental Materials and Methods).

Microarray results were validated within the patient cohort for microarrays and replicated in an independent second patient cohort by RT-PCR using TaqMan® technology (Applied Biosystems, Darmstadt, Germany) (Supplemental Materials and Methods). Complete data are available at the Gene Expression Omnibus (GEO) database under the accession number GSE5760.
**Microarray analysis**

*Principal component analysis (PCA)*

PCA is a mathematical vector space transformation which allows for reduction of multidimensional data sets to lower dimensions (principle components) accounting for variability in the data set [19]. PCA was conducted with 292 differentially regulated genes.

*Disease Load Index (DLI)*

To compare disease-dependent differences in the magnitude of gene expression in preterm infants, we used an aggregate measure designated Disease Load Index (DLI) as described previously [20]. The DLI is a unit-less measure representing the sum of the normalized expression values of defined differentially regulated genes in an individual. Here, the DLI for each infant of cluster 1 and 2 of Figure 1 was calculated, and the mean DLIs of each patient group (non-EOI; EOI) were compared. The significance of the difference between the DLIs of the patients groups was given as a p value deriving from Analysis of Variances (ANOVA) and pairwise Student’s t-test with Benjamini-Hochberg correction for group A genes and the non-parametric Kruskal-Wallis test and non-parametric pairwise Wilcoxon ranks sum test with Benjamini-Hochberg correction for group B genes.

**Measurement of NK cell number and activation**

Umbilical cord blood specimen for measurement of NK cell number were collected from a separate cohort of preterm infants (n=20) < 32 weeks of GA included and characterized exactly as described above. Hematopoietic cell staining was performed with a PeCy5.5-labeled mouse IgG1 anti-human CD45 antibody from Invitrogen (Carlsbad, CA, USA) and separation of leukocyte fractions by simultaneously using the following antibodies: PB-labeled mouse IgG1 anti-human CD3, Alx700-labeled mouse IgG1 anti-human CD19 and APC-labeled mouse IgG1 anti-human NKp46 from BD Biosciences (San Jose, CA, USA), APC-Alx750-labeled mouse IgG2a anti-human CD14 from Invitrogen, FITC-labeled mouse
IgG 1 anti-human CD15 from Miltenyi Biotec (Bergisch Gladbach, Germany) and corresponding isotype controls. Flow cytometry was performed on a LSRII Flow Cytometer (BD) using the proper controls to set gates and analyzed with FlowJo8.7.1-software. Dead cells were excluded using propidium iodide labelling and duplets by gating on single cells (FSC-H to FSC-W channel).

Validation and replication of the microarray results by TaqMan RT-PCR

To validate the microarray data, TaqMan quantitative real-time (RT)-PCR was performed for 10 human genes deriving from the microarray results (ANXA1, CD163, GNLY, HIF1A, KLRC2, KLRD1, MPO, PGLYRP1, TNFRSF10A, CD177) and three housekeeping genes G6PD (glucose-6-phosphate dehydrogenase), SDHA (succinate dehydrogenase complex, subunit A, flavoprotein (Fp)) and PGK1 (phosphoglycerate kinase 1) as internal controls for normalization. The details are given in Supplemental Materials and Methods.

To test whether the microarray results could be replicated in a second patient cohort, the gene expression of the same 10 genes was investigated in 43 new preterm infants by RT-PCR as described in Supplemental Materials and Methods. The patient characteristics of the second patient cohort (replication cohort: 15 patients with EOI, 28 non-EOI) are given in Supplemental Table 2.
RESULTS

Thirty very preterm infants were prospectively enrolled in the study. 24 of 30 samples met the high RNA quality criteria and were further processed for microarray analyses; of these, 16 were retrospectively allocated to the EOI cohort based on the presence of clinical parameters for EOI in the first 72 hours of life. Eight infants without EOI were assigned to the control group (non-EOI). The characteristics of the patient cohorts are given in Table 1.

**Gene expression analysis of cord blood reveals differential gene expression profiles in preterm infants with EOI at birth**

Comparison of gene expression of infants with and without EOI at birth revealed 292 differentially regulated genes (FDR<0.1). Of these, 219 genes had significantly higher gene expression levels (up regulated genes) in infants with EOI, while 73 genes had significantly lower expression levels (down regulated genes) (Suppl. Tables 1a and b). The up regulated genes were involved in biological categories including immune response, carbohydrate metabolism and cell motility while the down regulated genes were mainly involved in immune response, cell adhesion, development and regulation of transcription.

The differentially regulated genes were mapped to pathways involved in natural killer cell mediated cytotoxicity, T cell receptor signaling pathway, leukocyte transendothelial migration, Antigen processing and presentation, MAPK signaling, VEGF-/Wnt-/Jak-STAT-signaling, glycolysis and HIF- and GATA3-signaling (Table 2).

**Gene expression profiling reveals two groups of preterm infants with EOI**

Hierarchical clustering of the differentially regulated genes (FDR<0.1) separated the cohort of preterm infants into cluster 1 and 2 (Fig. 1). Cluster 1 contained preterm infants from both groups, EOI (EOI*) as well as non-EOI while cluster 2 contained infants with EOI (EOI**) with one exception. Thus, two subclasses of EOI were identified, designated as EOI*, occurring mainly in cluster 1, and EOI** in cluster 2. The two subclasses of EOI were also 9
identified by principal component analysis (PCA) which provided a high degree of separation between the two subclasses EOI* and EOI** (Fig. 2).

The two subclasses EOI* and EOI** were distinguished by the expression of two groups of genes, namely group A and B genes (Fig. 1): Group A genes were over expressed in EOI** as compared to EOI* and non-EOI and were involved in *neutrophil activation*, *T cell proliferation*, *hypoxia induced signalling*, and *carbohydrate metabolism*. Group B genes were down regulated in both EOI* and EOI** as compared to non-EOI and were involved in *NK cell activation*. To compare for differences in the magnitude of the gene expression of group A and B genes, the aggregative Disease Load Indices (DLIs) of group A and B genes in EOI* and EOI** were determined (Fig. 3): EOI** had a significantly higher DLI for group A genes than both EOI* (p=0.0143) and non-EOI (p=1e-05). Hence, the subclass EOI** activated genes involved in neutrophil activation, T cell proliferation, hypoxia induced signaling and carbohydrate metabolism on a higher level than subclass EOI*. For group B genes, no significant differences occurred in the expression level between the subclasses EOI* and EOI** (Fig. 3, p=0.8292). But both EOI* and EOI** showed a significantly lower DLI for group B genes compared to non-EOI (p=0.02797 and p=0.00357, respectively) indicating decreased activation of genes involved in NK cell activation.

**Increased activation of neutrophils in preterm infants with EOI**

Neutrophils play a pivotal role in the innate immune response, as they migrate to the site of infection, and help limit microbial infections. Increased activity of neutrophils in infants with EOI, especially in subclass EOI**, is indicated by over expression of group A genes involved in phagocytotic activity, granula secretion and respiratory burst of neutrophils as depicted in the interaction network in Fig. 4. The increased activation is given by over expression of phospholipid scramblase 1 (PLSCR1), an enzyme involved in hematopoietic proliferation and differentiation of neutrophils (Table 3). Over expression of proteinase-3 (PRTN3) and the proinflammatory calgranulins A (S100A8), B (S100A9) and C (S100A12) suggest enhanced
neutrophil chemotaxis, adhesion and migration in infants with EOI. Furthermore, increased expression of the formyl peptide receptor 1 (FPR1) and CD177, both receptors on the surface of neutrophils, indicated enhanced transmigration. An increase in interleukin 8 (IL8) expression further suggests increased recruitment and activation of neutrophils. Strong activation of the neutrophils was also reflected by the over expression of myeloperoxidase (MPO), neutrophil cytosolic factor 2 (NCF2), lactoferrin (LTF), azurocidin 1 (AZU1), peptidoglycan recognition protein 1 (PGLYRP1) as well as cathepsin D (CTSD).

**Decreased activation of Natural Killer (NK) cells in preterm infants with EOI**

NK cells constitute a component of the innate immune system in combating intracellular pathogens and activating and modulating the adaptive immune response. Activation of NK cells is regulated by the expression of a variety of receptors.

In EOI, the NK cell activating Killer cell lectin-like receptors (KLRs) such as KLR subfamily B, member 1 (KLRB1), KLR subfamily C, member 2 (KLRC2) and KLR subfamily D member 1 (KLRD1) as well as C-Type lectin domain family 1, member B (CLEC1B) were down regulated while the inhibiting leukocyte immunoglobulin-like receptor, subfamily B, member 3 (LILRB3) was up regulated (Table 3). Granulysin (GNLY), which is downstream of the KLRB1, KLRC2 and KLRD1 signalling, is an antimicrobial, cytolytic protein in the granules of NK cells and was also down regulated in EOI.

Two transcription factors known to regulate the expression of KLRs on NK cells were down regulated as well: GATA binding protein 3 (GATA3) and CREB binding protein (CREBBP). GATA3 is a transcription factor preferentially expressed in NK and T cells that plays an important role in the early phase of NK cell development. Its activity is crucial for the diversification of the NK cell receptor repertoire and the interferon γ (IFNG) production and thus pivotal for an effective NK cell response against viruses and bacteria. The down regulation of GATA3 and CREBBP in infants with EOI could explain the reduced gene
expression of NK cell receptors in these patients as depicted in the interaction network in Fig.
5.

The measurement of NK cell counts in a cohort of preterm infants with and without EOI showed no significant difference in NK cell number between the two groups (non-EOI 7.8±5.3 cells/µl vs. EOI 5.8±5.3 cells/µl; mean and standard deviation (SD) each). This result suggested that the down regulation of NK cell activating genes as seen in EOI was not related to the NK cell count.

Validation of microarray results

To validate the microarray data, TaqMan quantitative real-time (RT)-PCR was performed on 10 human target genes involved in EOI (ANXA1, CD163, GNLY, HIF1A, KLRC2, KLRD1, MPO, PGLYRP1, TNFRSF10A, CD177).

The overall correspondence between gene expression levels by microarrays and by RT-PCR analyses was high as indicated by the coefficient of determination $R^2 = 0.88$ (Suppl. Fig. 1).

Replication of the results in an independent second patient cohort

To test the reproducibility of the obtained results, 10 selected genes were analyzed by RT-PCR in a second patient cohort (replication cohort, n=43) (Suppl. Table 2). The results show an overall good correlation ($R^2=0.74$) of the gene expression between the first and the second cohort of preterm infants indicating reproducibility of the results (Suppl. Fig. 2).

The RT-PCR results in the second patient cohort confirmed significant up regulation of ANXA1, CD163, MPO, PGLYRP1, HIF1A, TNFRSF10A and CD177 in the group of infants with EOI. In contrast, genes involved in NK cell activation, i.e. KLRC2, KLRD1 and GNLY were found to be significantly down regulated (Fig. 6).
DISCUSSION AND CONCLUSIONS

We performed transcriptional profiling from cord blood samples to obtain insights into the pathways involved in early EOI development in very premature infants. Comparison of the gene expression profiles of infants with EOI and without EOI revealed NK cell inactivation to be a hallmark of EOI discriminating EOI and non-EOI neonates. (Fig. 1 and 2).

The impairment of NK cell function plays a critical role in the host response to infectious challenges in preterm infants with EOI, although these central regulators of the cellular innate immune response and host defense have thus far not received appropriate attention in this cohort I [21]. The results indicate, that decreased activation rather than lowered cell numbers account for the impairment of NK cell function. Additional evidence for the decreased activation of NK cells in infants with EOI is indicated by the down regulation of GATA3 and CREBBP which regulate the expression of the activating KLRs.

NK cell interactions with other immune cells are regulating a wide range of immune responses [22]: Not only does NK cell and macrophage interaction during bacterial sepsis impact bacterial clearance [23], the interaction between these cells and neutrophils has been shown to determine clearance of listeria infections. The decrease in NK cell activation may contribute to impaired clearance of pathogens leading to overwhelming systemic infections, frequent in the premature infant. Insufficient elimination of pathogens due to impaired orchestration by NK cells could also explain the excessive neutrophil response in patients with EOI, consistent with findings from studies in infants with fetal inflammatory response syndrome (FIRS) [24].

Recent studies in infants up to three years of age revealed lowered adaptive immune system responses and specifically inhibition of NK cell activation in septic shock as described in this study [25, 26]. This is consistent with the findings of El-Sameea et al. [27] and Georgeson et
al. [28], who showed a positive correlation between reduced NK cell activity and the presence, severity and outcome of neonatal sepsis in term newborns. Although these findings have never been applied to the cohort of preterm infants, our results suggest that the analysis of NK cell activation/inactivation in preterm infants at birth provides valuable information indicating disease development and better understanding its pathophysiology.

To conclude, the results of this study indicate that (1) transcriptome patterns from cord blood enable discrimination of preterm infants with and without EOI and that (2) EOI can be divided in two subclasses which show a common phenomenon of dysregulation of NK cell activation, but differ in the activation level of neutrophils.

We propose that the addition of NK cell activity into the standard diagnostic repertoire for critically ill neonates could be a useful complement to current laboratory diagnostics to improve early diagnosis of EOI.

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REFERENCES

1. Slattery MM, Morrison JJ. Preterm delivery. *Lancet* 2002; *360*: 1489-97.

2. Stoll BJ, Gordon T, Korones SB et al. Early-onset sepsis in very low birth weight neonates: a report from the National Institute of Child Health and Human Development Neonatal Research Network. *J Pediatr* 1996; *129*: 72-80.

3. Stoll BJ, Hansen NI, Sanchez PJ et al. Early onset neonatal sepsis: the burden of group B Streptococcal and E. coli disease continues. *Pediatrics* 2011; *127*: 817-26.

4. Jobe AH. Antenatal factors and the development of bronchopulmonary dysplasia. *Semin Neonatol* 2003; *8*: 9-17.

5. Leviton A, Kuban K, O'Shea TM et al. The relationship between early concentrations of 25 blood proteins and cerebral white matter injury in preterm newborns: the ELGAN study. *J Pediatr* 2011; *158*: 897-903 e1-5.

6. Laborada G, Rego M, Jain A et al. Diagnostic value of cytokines and C-reactive protein in the first 24 hours of neonatal sepsis. *Am J Perinatol* 2003; *20*: 491-501.

7. Mathers NJ, Pohlandt F. Diagnostic audit of C-reactive protein in neonatal infection. *Eur J Pediatr* 1987; *146*: 147-51.

8. Ottolini MC, Lundgren K, Mirkinson LJ et al. Utility of complete blood count and blood culture screening to diagnose neonatal sepsis in the asymptomatic at risk newborn. *Pediatr Infect Dis J* 2003; *22*: 430-4.

9. Connell TG, Rele M, Cowley D et al. How reliable is a negative blood culture result? Volume of blood submitted for culture in routine practice in a children's hospital. *Pediatrics* 2007; *119*: 891-6.

10. Berger C, Uehlinger J, Ghelfi D et al. Comparison of C-reactive protein and white blood cell count with differential in neonates at risk for septicaemia. *Eur J Pediatr* 1995; *154*: 138-44.
11. Rodwell RL, Taylor KM, Tudehope DI, Gray PH. Hematologic scoring system in early diagnosis of sepsis in neutropenic newborns. *Pediatr Infect Dis J* 1993; **12**: 372-6.

12. Squire E, Favara B, Todd J. Diagnosis of neonatal bacterial infection: hematologic and pathologic findings in fatal and nonfatal cases. *Pediatrics* 1979; **64**: 60-4.

13. Xanthou M. Leucocyte blood picture in healthy full-term and premature babies during neonatal period. *Arch Dis Child* 1970; **45**: 242-9.

14. Xanthou M. Leucocyte blood picture in ill newborn babies. *Arch Dis Child* 1972; **47**: 741-6.

15. Zhou Z, Kozlowski J, Schuster DP. Physiologic, biochemical, and imaging characterization of acute lung injury in mice. *Am J Respir Crit Care Med* 2005; **172**: 344-51. Epub 2005 May 13.

16. Gerdes JS. Clinicopathologic approach to the diagnosis of neonatal sepsis. *Clin Perinatol* 1991; **18**: 361-81.

17. Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* 2003; **19**: 185-93.

18. Breitling R, Armengaud P, Amtmann A, Herzyk P. Rank products: a simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments. *FEBS Lett* 2004; **573**: 83-92.

19. Daffertshofer A, Lamoth CJ, Meijer OG, Beek PJ. PCA in studying coordination and variability: a tutorial. *Clin Biomech (Bristol, Avon)* 2004; **19**: 415-28.

20. Porter JD, Merriam AP, Leahy P et al. Dissection of temporal gene expression signatures of affected and spared muscle groups in dystrophin-deficient (mdx) mice. *Hum Mol Genet* 2003; **12**: 1813-21.
21. Marodi L. Neonatal innate immunity to infectious agents. Infect Immun 2006; 74: 1999-2006.

22. Zhang C, Zhang J, Tian Z. The regulatory effect of natural killer cells: do "NK-reg cells" exist? Cell Mol Immunol 2006; 3: 241-54.

23. Scott MJ, Hoth JJ, Gardner SA et al. Natural killer cell activation primes macrophages to clear bacterial infection. Am Surg 2003; 69: 679-86; discussion 86-7.

24. Madsen-Bouterse SA, Romero R, Tarca AL et al. The transcriptome of the fetal inflammatory response syndrome. Am J Reprod Immunol 2010; 63: 73-92.

25. Adkins B, Leclerc C, Marshall-Cla rke S. Neonatal adaptive immunity comes of age. Nat Rev Immunol 2004; 4: 553-64.

26. Ridge JP, Fuchs EJ, Matzinger P. Neonatal tolerance revisited: turning on newborn T cells with dendritic cells. Science 1996; 271: 1723-6.

27. el-Sameea ER, Metwally SS, Mashhour E et al. Evaluation of natural killer cells as diagnostic markers of early onset neonatal sepsis: comparison with C-reactive protein and interleukin-8. Egypt J Immunol 2004; 11: 91-102.

28. Georgeson GD, Szony BJ, Streitman K et al. Natural killer cell cytotoxicity is deficient in newborns with sepsis and recurrent infections. Eur J Pediatr 2001; 160: 478-82.
FIGURE LEGENDS

Figure 1. Transcriptional profiles of preterm using hierarchical clustering of differentially expressed genes based on 292 differentially regulated genes.

Hierarchical Clustering of differentially expressed genes of infants with and without EOI resulted in two main clusters (cluster 1 and 2). Group A genes are up regulated genes in infants with EOI and involved in neutrophil activation, T cell proliferation, hypoxia induced signaling and carbohydrate metabolism. Group B genes are down regulated in infants with EOI and mainly involved in NK cell activation. The group of infants with EOI could be further differentiated in a group with low expression of Group A genes (EOI*) and EOI with high expression of Group A genes (EOI**).

Figure 2. Three-dimensional principal component analysis (PCA) based on 292 differentially regulated genes.

Individual patients are plotted based on their respective positions along the three axes derived from PCA (mean centering and scaling). Patient sub-classifications are indicated by color. PCA provides a high degree of separation between the two subclasses EOI* and EOI**, and non-EOI.

Figure 3. Mean DLIs of differentially regulated group A and B genes in preterm infants with and without EOI.

Comparison of Disease Load Indices (DLIs) of non-EOI, EOI with low expression of Group A genes (EOI*) and EOI with high expression of Group A genes (EOI**). Group A genes show significantly higher DLI of group A genes in EOI** compared to EOI* and non-EOI. Group B genes show significantly lower DLI in both EOI* and EOI** compared to non-EOI. The significance of the difference between the DLIs of the patients groups was given as a p value deriving from pairwise Student’s t-test with Benjamini-Hochberg correction for group A genes and from non-parametric Kruskal-Wallis test and non-parametric pairwise Wilcoxon ranks sum test with Benjamini-Hochberg correction for group B genes.
**Figure 4. Regulation of neutrophil activation.**

The gene interaction network *regulation of neutrophil activation* of the differentially regulated genes in EOI shows the interaction between calgranulins (S100A8, S100A9, S100A12) and genes involved in phagocytotic activity, granula secretion and respiratory burst of neutrophils (e.g. MPO, AZU1, LTF, NCF2). Up regulated genes are depicted in red, down regulated genes in green. P-value and fold change are given beneath each gene symbol. Genes with an unknown regulation are depicted in white. Relationships and interactions between molecules are abbreviated as following: A: activation; B: binding; C: causes/leads to; CC: chemical-chemical interactions; CP: chemical-protein interactions; E: expression (includes metabolism/synthesis for chemicals); EC: enzyme catalysis; I: inhibition; L: proteolysis (includes degradation for chemicals); LO: localization; M: biochemical modification; MB: group/complex membership; P: phosphorylation/dephosphorylation; PD: protein-DNA interactions; PP: protein-protein interactions; PR: protein-RNA interactions; RB: regulation of binding; RE: reaction; RR: RNA-RNA interactions; T: transcription; TR: translocation..

**Figure 5. Influence of GATA3 and IL10 on regulation of NK cells and lymphocytes.**

The gene interaction network *influence of GATA3 and IL10 on regulation of NK cells and lymphocytes* of the differentially regulated genes in EOI shows the interaction of GATA3, IL10 and CREBBP with KLRs. Relationships and interactions between molecules are given in the figure legends of Figure 4.

**Figure 6. Comparison of gene expression between EOI and non-EOI for selected genes in a second patient cohort (replication cohort).**

TaqMan quantitative RT-PCR results of 10 selected genes were compared in infants with and without EOI within the replication cohort: RT-PCR results confirmed a significant over expression of ANXA1, CD163, MPO, PGlyRP1, HIF1A, TNFRSF10A and CD177 and a
significant down regulation of genes involved in NK cell activation, i.e. KLRC2, KLRD1 and GNLY in the group of infants with EOI.

P values are given for ANXA1, PGLYRP1, TNFRSF10A using the Welch test, for CD163, HIF1A, GNLY using the parametric Kruskal-Wallis rank sum test and for KLRC2, KLRD1, MPO, CD177 using the Student’s t-test.