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The Role of Immature Granulocyte Count and Immature Myeloid Information in the Diagnosis of Neonatal Sepsis

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1. Introduction

Although diagnostic and therapeutic approaches to neonatal sepsis considerably progressed over the last decades, distinguishing infected from non-infected patients still remains a major challenge, especially in the early phase of disease when symptoms are often subtle and unspecific. Development and application of potent antibiotic medication and the advances in neonatal care could improve treatment but incidence of neonatal sepsis is still high. Compared to an incidence rate ranging from 1.5 to 3.5 per 1000 for neonatal early onset sepsis (EOS) and up to 6 per 1000 live births for late onset sepsis (LOS) in developed countries, the reported incidence of neonatal sepsis varies from 7.1 to 38 per 1000 live births in Asia and from 6.5 to 23 per 1000 live births in Africa (Vergnano et al., 2005). The physiologic immature state of the immune system and reduced levels of preformed maternal antibodies in preterm infants together with organ immaturity and a lower expression of major histocompatibility complex (MHC) class II antigens on monocytes contribute to a disturbed equilibrium of pro- and anti-inflammatory factors resulting in a reduced immune defence making the preterm infant more susceptible for sepsis and its short and long term complications (Azizia et al., 2012; Stoll et al., 2004). Prospective data collection of 16 participating centers of the National Institute of Child Health and Human Development Neonatal Research Network revealed a declining incidence of blood culture proven EOS from 19.3 per 1000 in 1991-93 to 15.4 per 1000 live births in 1998-2000 among very low birth weight (VLBW) infants, whereas the incidence of late-onset septicemia was 22% and remained essentially unchanged over the observed period of time. However, the potentially life threatening character of EOS is reflected by high mortality rates reaching between 1.6% in nonblack term infants and 37% in preterm infants with VLBW (Fanaroff et al., 2007; Weston et al., 2011). Infants in this study without EOS showed a significantly reduced mortality risk of 13%.

Considering these data it is comprehensible that many neonatologists hazard the consequences of possibly unnecessary exposure to antimicrobial agents in neonates suspected for sepsis with unspecific symptoms and uncertain infectious state to avoid fatal outcome caused by a delay in treatment. The above-mentioned declined incidence of EOS was mainly caused by a change in the pathogen distribution showing a decline in group B streptococcus (GBS) sepsis but an increase in *Escherichia coli* sepsis with a rate of 85% of ampicillin resistance (Fanaroff et al., 2007). As the use of broad-spectrum antimicrobial agents like the combination of ampicillin and an aminoglycoside is considered as the optimal treatment of infants with suspected EOS (Polin, 2012), the progressively increasing burden of antimicrobial resistance would actually require a more targeted drug therapy in the future to confine human and economic costs. The often unspecific early symptoms and the potential rapid deterioration necessitate early identification of patients at risk. This should help to avoid a delay in treatment and could prevent further complications. On the other hand, overtreatment of newborn infants with maternal risk factors and uncertain infectious status suspected for sepsis could also be reduced helping to avoid prescription of unnecessary prophylactic broad-spectrum antibiotic medication, to restrain the development of antimicrobial resistance, exposing the patients to possible severe adverse effects and help to increase cost effectiveness.

1.1. Diagnostic approaches to neonatal sepsis

Despite a myriad number of scientific studies evaluating the performance of laboratory markers, risk scores, and clinical features in neonatal sepsis, the search for a perfect diagnostic test with high accuracy and reliability still seems to be a quest for the holy grail (Briggs et al., 2000). Still, no single laboratory parameter and none of the newly created clinical risk scores are generally accepted to define the diagnosis of sepsis in its early course with 100% accuracy and confidence (Fowlie & Schmidt, 1998; Rodwell et al., 1988). A systematic review of the literature of 194 studies reporting on different diagnostic tests to predict the presence or absence of bacterial infection in infants up to 90 days of age generally described a poor methodological quality (Fowlie & Schmidt, 1998). Even in rigorous studies the accuracy of the tests showed enormous variation and the diagnostic value was considered as limited in this population (Fowlie & Schmidt, 1998). Although blood culture is considered as the gold standard to confirm the diagnosis of sepsis, this method has its limitations in a neonatal - especially in a preterm - population (Chiesa et al., 2004; Fowlie & Schmidt, 1998). In a study to determine the minimum required blood volume to detect bacteremia Schelonka found that a 0.5 mL blood sample –as commonly obtained in neonatal intensive care units (NICU) - is insufficient to obtain sensitive results when the colony count is less than 4/mL. This is of special interest as it has been shown that low-level bacteremia is common in infants less than two months of age accounting up to 68% (Kellogg et al., 1997; Schelonka et al., 1996). Furthermore false negative results can be obtained due to the presence of antimicrobial agents in the blood because of an early onset of treatment based on empirically decision making representing a regular practice in clinical routine (Fowlie & Schmidt, 1998). Because blood culture bottles require sufficient incubation time,

results are delayed and can normally be expected within three days, whereas in infants up to an age of 72 hours blood cultures require a longer incubation time. In a retrospective observational study comprising more than 2900 neonates the median time to positivity of blood cultures was significantly shorter in Gram negative (11.2 hours; 8.5-15.7) compared to Gram positive organisms (23.6 hours; 15.3-4.6). These findings could have important clinical implications to optimize antimicrobial therapy. The authors suggest targeting only for Gram positive germs when the blood culture is still negative after 48 hours and to cease treatment in well-being infants without clinical and laboratory signs of infection after 72 hours when blood culture remains sterile (Guerti et al., 2011).

Generally, blood culture-proven EOS has been described as quite uncommon in a large multicenter investigation of neonates with VLBW occurring in only 1.9%. Whereas almost 50% of the study population was characterized for sepsis because of clinical signs, in 98% blood culture reports revealed negative results, but antibiotic treatment was continued fearing false negative results possibly due to maternal antibiotic medication (Stoll et al., 1996). A high rate of failed detection of bacterial growth in blood cultures of VLBW neonates between 27% and 92% could possibly be explained by a transient or intermittent bacteremia as sepsis is known to be a dynamic process (Haque, 2010). Beyond that, interpreting results when organisms are of low or questionable virulence as pathogen or possible contamination- frequently occurring during blood collection (Pourcyrus et al., 1993)- is often difficult.

1.1.1. The White blood cell count (WBC) as primary diagnostic tool in neonatal sepsis

Although several studies have shown a poor predictive value performing a single WBC as a screening method in asymptomatic neonates with infectious risk factors or later on culture proven sepsis (Ottolini et al., 2003; Rozycki et al., 1987), the assessment of a complete blood cell count (CBC) is usually performed as a routine method to evaluate newborns at risk. The recent introduction of several new parameters to the routine CBC with white blood differential performed by automated hematology analyzer have enabled quantification of cells which were previously solely classified as abnormal flags (Briggs et al., 2003). This refers mainly to the compartment of immature neutrophil granulocytes (IG) which have been detected in various conditions including later stage of pregnancy, steroid therapy, cancer, trauma, or myeloproliferative diseases (Briggs, 2009). They have been considered as helpful early indicators of infectious conditions (Buttarelli & Plebani, 2008; Rodwell et al., 1988) and have a long clinical tradition in the diagnosis of bacterial sepsis in neonates (Akenzua et al., 1974).

A commonly used index to comprise the fraction of IG in the clinical practice is the IT (immature-to-total-neutrophil)-ratio which is defined as the proportion of the number of immature cells including blasts, band cells, myelocytes, and metamyelocytes to the number of mature neutrophil cells. It is a manual count usually determined by a peripheral blood smear. Already more than three decades ago elevation of IT-ratio was considered to be a useful aid in the diagnosis of neonatal bacterial sepsis. The authors suggested that the

higher the degree of elevated IT-ratio was, the higher was the risk of bone marrow depletion and death from sepsis (Christensen et al., 1981).

In a retrospective multicenter cohort analysis including 166092 neonates with suspected EOS admitted to 293 NICUs in the United States low WBC counts, low absolute neutrophil counts, and high IT-ratios were associated with increasing odds of infection. Elevated IT-ratios of >0.2 , >0.25 , and >0.5 had low sensitivities (54.6%, 47.9%, 21.9%, respectively), but were associated with relatively high specificities (73.7%, 81.7%, 95.7%, respectively) and negative predictive values (NPV) (99.2%, 99.2%, 99.0%, respectively), whereas positive predictive values (PPV) were low (2.5%, 3.2%, 6.0%, respectively). The authors concluded that due to the low sensitivity these CBC-derived indices do not represent reliable diagnostic markers to rule out EOS in neonates (Hornik et al., 2012). The very high negative predictive accuracy of more than 90% is in contrast to high rates of elevated IT-ratios between 25% and 50% in non-infected infants (Polin, 2012).

In a large historical cohort study comprising more than 3100 neonates, patients were evaluated for EOS. In this study a normal WBC was defined as an IT-ratio of less than 0.2 and a total WBC between 6000 and 30000/ μL . Two serial normal WBCs with normal IT-ratios performed 8 to 12 hours apart and a negative blood culture at 24 hours were predictive of healthy newborns in the evaluation of EOS in the first 24 hours after birth and showed a negative predictive value of 100%. The sensitivity of 2 normal WBCs and a negative blood culture at 24 hours was 100%, as was NPV. The specificity was 51%, and the PPV was 8.8% (Murphy & Weiner, 2012). These results suggest that combinations of parameters and repeated performance of diagnostic tests are likely to increase accuracy.

In a review article Cornbleet reported a wide range of sensitivity and specificity for the IT-ratio and predicted a possible replacement by the measurement of newly created markers for infection such as inflammatory factors, adhesion molecules, cytokines, neutrophil surface antigens, and bacterial DNA (Cornbleet, 2002). Recent advances in basic science of predicting and diagnosing neonatal sepsis are developing towards more and more sophisticated approaches like the determination of proteomic inflammatory biomarkers in amniotic fluid (Buhimschi et al., 2009). Regarding these new techniques, the diagnostic value of traditional laboratory methods has to be critically analysed. However, comparing these new methods for the detection of neonatal sepsis with the measurement of WBCs including the assessment of the IG count (IGC) as well as the IT-ratio, the additional sample volumes, delayed availability of results, and considerably higher labour and laboratory costs should be taken into account.

1.1.2. Potential confounders in the manual assessment of IG by microscopic view of a manual smear

Anyhow, the detection of IGs by microscopic count necessitates experienced laboratory staff; furthermore morphological classification of IGs are subject to a considerable reader bias and interpretative errors; especially in neonates where leukocytosis occurs frequently in the first days of life this method seems to provide only limited reproducibility (Chiesa et al.,

2004; Schelonka et al., 1995). Contrariwise in performing a standard 100-cell manual differential small numbers of IGs are often underestimated as they can often be overlooked in samples of leukopenic patients. Another study highlighted the wide range of inter- and intraobserver variance in microscopic band cell identification: A smear of a blood sample from a septic patient was prepared, stained and a PowerPoint presentation was made twice of 100 random cells and sent to 157 different hospital laboratories in the Netherlands for a leukocyte differential. In the first survey neutrophils were differentiated in segmented and band neutrophils whereas in the second survey no discrimination was made between segmented and band neutrophils. Albeit the morphologic characteristics of a band cell are well defined, this study showed an enormous intervariability of enumeration of band cells so that the authors recommended to cease quantitative reporting of counted band cells especially in regard to other diagnostic tools like C-reactive protein (CRP), procalcitonin, and cytokines (van der Meer et al., 2006). Hence, several authors consider the manual count as inappropriate as a reference method for detection of IGs (Fernandes & Hamaguchi, 2007; Senthilnayagam et al., 2012).

2. Automated detection of immature granulocytes- Clinical applicability

Automated measurement of IGC could represent a reliable and utile method in the prediction of bacterial infection in neonates. In a study evaluating 106 samples from patients with an absolute neutrophil count (ANC) less than $2.0 \times 10^9/L$ measured with an automated 5-part differential hematology instrument the IGC showed a very good precision and accuracy when compared with a flow cytometric neutrophil count using monoclonal antibodies for cell classification (Amundsen et al., 2012). In another investigation of 200 febrile patients suspected to have infection the performance characteristics of automated IGCs in predicting blood culture results and their clinical utility were assessed. The study population included adults, children, infants, and neonates. Measurements were performed using the Coulter Act Diff 5 counter which can perform a 5-part differential leucocyte count and can also numerate the percentage and absolute number of IG using a technology that combines cytochemistry, focused flow impedance, and light absorbance. The means of IGC and the percentage of IG (IG%) between culture positive and negative groups were statistically significant suggesting that they are potential markers for bacteremia. Among the 51 culture positive cases, 49 had an IT-ratio $> 0.65\%$ giving a sensitivity of 96.1%. IGC of $0.03 \times 10^3/\mu L$ and IG% of 0.5% offered a sensitivity of 86.3% and 92.2%, respectively. Higher values of IGC > 0.3 and IG% > 3 had a specificity greater than 90%, although the values were infrequent. Receiver operating characteristic (ROC) curves showed that IGC was a better predictor of infection than WBC and ANC in adults and the ratios IGC/WBC and IGC/ANC did not improve the prediction outcome (Senthilnayagam et al., 2012). Another study reported an in parallel increase of IG values to an increase of the ANC and an inverse relation to the lymphocyte count (Bernstein & Rucinski, 2011).

In an adult study population including patients suspected for sepsis higher percentages of IGs have been observed in infected than in non-infected patients and in patients with positive than patients with negative blood cultures (Ansari-Lari et al., 2003). Also in preterm

hospitalized infants elevations of IGs were associated with positive blood culture results. In this study, values exceeding 0.5% showed a more than three-fold increased likelihood of a positive blood culture (Nigro et al., 2005).

In several studies comparing the manual microscopic method and the automated method for IG% and IGC significant correlation coefficients between 83% and 87% have been demonstrated (Field et al., 2006; Senthilnayagam et al., 2012). Compared with a flow cytometric reference count with monoclonal antibodies the correlation coefficient was even higher and amounted to 96% (Briggs et al., 2003). It has been shown that an increased percentage of more than 2% of IGs can be useful in identifying infection even when the neutrophil count is within the normal range and infection is not suspected. Conversely, in patients with a high IGC sample rates were positive for CRP and the erythrocyte sedimentation rate in 84% and 95%, respectively. Furthermore, elevated IGC showed a correlation with other inflammation markers such as CD 64 expression on polymorphonuclear cells and interleukin 6 concentration (Briggs et al., 2003).

The detection of IGs using automated hematology analyzers represents a fast, accurate, and less-labor intensive method and could improve screening and monitoring for neonatal septicaemia (Briggs et al., 2000; Fernandes & Hamaguchi, 2007; Nigro et al., 2005). The detection limit of IGs has been described to be 0.1% which is considerably lower than in a manual smear. The automated simultaneous enumeration of IGs in the course of performing a routine CBC provides additional information without the need of further costs and blood sampling, which might be of special importance in preterm babies. This new technology of automated measurement of IGs offers additional information reflecting the increase in bone marrow activity as an indicator of a left-shift of neutrophil cells in a more sensitive and specific way than the manual examination of a peripheral blood smear differential count.

Detection of IGs comprises the amount of metamyelocytes and myelocytes, but not band neutrophils and therefore reflects early stages of maturation of granulocytes. As the band cell is defined as a cell in the transitional state of granulopoietic maturation after the differentiation of metamyelocytes and myelocytes, the band count itself has been described as nonspecific, imprecise, and inaccurate as laboratory marker for the early detection of sepsis (Bernstein & Rucinski, 2011; Cornbleet, 2002). Hence, determination of IGs in contrast to the more mature band neutrophils, which arise later on, could be advantageous at the onset of moderate to severe inflammation (Cornbleet, 2002). Moreover, it has already been shown that the measurement of granulocyte maturation correlates to the identification of sepsis (Bernstein & Rucinski, 2011).

2.1. Neutrophil positional parameters as a new tool in the prediction of sepsis

Morphologic and physical properties of cells including cell volume, internal composition, and cytoplasmic granularity and nuclear structure assessed via cell conductivity and cell scatter are described as positional parameters. In contrast to more mature cells IGs and band cells for instance are known to be larger, whereas nuclear composition is less complex. Based upon these facts positional parameters are used to classify different types of white

blood cells, but recently this method has also been applied as a screening tool for neonatal sepsis to detect morphologic changes within the same blood cell population (i.e. in reactive neutrophil cells occurring during acute bacterial infection). Chaves et al. tried to assess and quantify these parameters as indicators of acute infection. In retrospective studies of adult septic patients and controls, the mean neutrophil volume (MNV) and its standard deviation, the neutrophil volume distribution width (NDW), reflecting the neutrophil size variability, showed high specificities (Chaves et al., 2005; 2006). In the control group the neutrophil population presented more homogenous than in bacteremic patients and the individual cell size varied less. Furthermore, a correlation of NDW respectively MNV and positive blood culture results, higher percentages of neutrophils and higher WBCs has been shown, whereas increased values were also present in patients without leukocytosis or neutrophilia possibly representing an important early diagnostic parameter in this subgroup of patients (Chaves et al., 2006). Another study using this technology showed good performance characteristics of MNV in detecting LOS in VLBW neonates with a NPV of 98.9%. Because of a considerably lower PPV the authors emphasize the possible combination with CRP-values in the prediction of sepsis. Interestingly, in contrast to an adult population the NDW did not reveal any clinical significance in a neonatal population. The authors suggested that this might be due to an originally more heterogeneous morphology of neutrophil cells in newborn infants (Raimondi et al., 2010; Raimondi et al., 2011).

2.2. The Sysmex XE-2100

The Sysmex XE-2100 (Sysmex Corporation, 2005), a multiparameter automated hematology analyzer offers the possibility to detect IGs including metamyelocytes, myelocytes, and promyelocytes by the measurement of white blood cell differential counts by flow cytometry in the DIFF-channel. Besides the quantification of IGs, physical properties of immature cells and reactivated neutrophils are provided. Therefore blood samples are incubated with Stromatolyser-IM, a fluorescent dye and a proprietary reagent, selectively leaking the membrane of mature leukocytes. Immature myeloid cells are not modified in their size, structure and integrity, because the IG has a lower cholesterol content than the mature granulocyte, and its phospholipid composition has a relatively higher ratio of phosphatidylcholine and a lower ratio of sphingomyelin (Gottfried, 1967). Depending on the dispersion angle when the cell passes the beam of a semiconductor laser, information about the volume, inner structure and complexity, and DNA/RNA content of each cell is obtained by a combination of forward-scattered light, lateral-scattered light, and lateral fluorescent light. The light is received by a photodiode respectively a photomultiplier tube and is then converted into electrical pulses. The higher content of RNA and DNA in IGs compared with segmented neutrophils is reflected in an increased fluorescence emission after excitation with the laser beam. The XE-2100 is equipped with an additional immature information (IMI) channel, where not only IGs, but also bands, blasts, and hematopoietic progenitor cells are detected. Detection of cell size, information about the nuclei and composition of cytoplasm is generated by direct current and radio frequency resistance when cells pass an aperture in the IMI-channel. The direct current (DC) pulse height is equivalent to cell volume. The radio frequency (RF) measurement provides information on the internal

composition of the cell (nucleus, granules). Differences in RF resistance detected as electrical pulses are plotted in a two dimensional scattergram reflecting the distribution of cell and nucleus size (Sysmex Corporation, 2005).

The IMI-channel determines the total number of myeloid precursor cells by distinguishing selectively immature myeloid cells from mature leukocytes. The reaction principle of the IMI-channel is based on differences in membrane composition between mature and immature cells. It has been shown that the flow cytometric IGC performed by the Sysmex XE-2100 is superior to the manual morphology count as a reference method for IG counting and that the percentage of IGs is a better predictor of infection than the WBC (Ansari-Lari et al., 2003; Fernandes & Hamaguchi, 2007).

3. Reference values in an adult and pediatric population

Generally, normal values of laboratory parameters in a neonatal population are difficult to define, because removal of blood is usually not performed in healthy neonates and reference ranges are composed by assessing patients with minor illness (Christensen et al., 2009). To our knowledge, the first published reference values for neutrophil cells in neonates including the total neutrophil count, the absolute number of immature neutrophils, and the IT-ratio during the first 28 days of life refers to a study by Manroe in 1979 (Manroe et al., 1979). About 15 years later the same study group found that in VLBW these reference values are of limited applicability because a wider range of distribution was found in this subgroup of patients compared to larger or older counterparts (Mouzinho et al., 1994). These new data comprised a wider range of the absolute total neutrophil count and a considerable decreased lower limit in the first 60 hours after birth, whereas reference ranges for the immature neutrophil count and IT values remain unchanged. It has been assumed that low neutrophil counts soon after birth might be caused by a placental factor inhibiting neutrophil production. Clearance of this factor within the first week could lead to the observed increase in immature neutrophil cells. Anyhow, the capability of the bone marrow to rapidly produce immature as well as mature neutrophil forms by the second week is well documented. Neutropenia occurred rarely in infants at an age of more than 7 days, but neutrophilia occurred frequently in association with stress conditions inducing an adrenergic increase in cyclic adenosine monophosphate (cAMP) leading to a release of neutrophil cells (Mouzinho et al., 1994). Hence, neutropenia has been described as a better predictor for neonatal sepsis than an elevated neutrophil count because besides accelerated utilization in case of infection there are fewer factors (i.e. hemolytic disease, asphyxia, maternal hypertension) causing a decrease of neutrophil granulocytes. Lower levels of normal for neutrophil values have been set at $1800/\text{mm}^3$ at birth and $< 7800/\text{mm}^3$ 12-14 hours after birth in term and late preterm infants (Manroe et al., 1979).

A large trial evaluating more than 30000 samples from infants born at 23 to 42 weeks of gestational age reinvestigated the previously published reference ranges using an automated blood cell counter (Schmutz et al., 2008). In this study lower limits of normal for the neutrophil count were determined as follows (Table 1):

Gestational age	Neutrophil count at birth	Neutrophil count 6-8h pn
> 36 weeks	3500/ μ L	7500/ μ L
28-36 weeks	1000/ μ L	3500/ μ L
< 28 weeks	500/ μ L	1500/ μ L

Table 1. Neutrophil count at birth and 6-8 hours postnatally (pn) comparing groups of different gestational age (Polin, 2012; Schmutz et al., 2008).

The notable difference in altitude between the two studies might have influenced the results. The dynamic process of granulopoiesis after birth is reflected by a rapid increase of neutrophil cells reaching peak levels at 6 to 8 hours postnatally (Polin, 2012; Schmutz et al., 2008). Allowing sufficient reaction time to inflammatory stimuli alterations in mature and immature granulocytes are more likely to occur between 6 to 12 hours after birth. This should be taken into account when planning blood sampling (Polin, 2012).

A quite similar time course has been shown for the absolute immature neutrophil count: Maximal values increase from 1100/ μ L soon after birth to a peak of 1500/ μ L at 12 hours postnatally. In contrast to that, maximum normal values for the IT-ratio have been observed directly after birth followed by a decline with increasing age (Polin, 2012; Schmutz et al., 2008). In the most immature infants between 24 and 26 weeks of gestational age, an elevation of ANC has been shown during the first month of life. In the first three weeks of life a weekly decrease of ANC to values between 2000/ μ L and 4000/ μ L has been observed. As the prevalence of both neutropenia as well as neutrophilia decreased with maturity, it can be concluded that granulopoietic function stabilizes with higher gestational age enabling adequate reactions to infectious or stress stimuli. Deviations from the normal range of neutrophil granulocytes without additional signs of clinical symptoms or conditions occurred frequently even in a hospitalized population (Juul et al., 2004). In the face of these data more interest should be attracted on considering the gestational age as well as the time point of blood sampling when interpreting CBC results (Polin, 2012). The influence of birth weight on CBC in healthy term infants was examined in a study performed by Ozyürek and co-workers. Their data revealed a clear difference in several CBC parameters comparing healthy, term infants with intrauterine growth retardation to appropriate for gestational age (AGA) counterparts showing neutropenia in 21% as well as higher IT-ratios in small for gestational age (SGA) newborns. Beyond these findings, a higher rate in immature neutrophil cells, namely in the absolute number of metamyelocytes, was observed in the SGA babies. The authors suggested that this elevation might be interpreted as a reaction of the bone marrow to compensate for the initially frequent low neutrophil count (Ozyurek et al., 2006).

Some authors have considered the method of automated measurement of IGC as not sensitive enough to be used as a sole screening assay for the prediction of infection. However, it has been demonstrated that a high percentage of IG (> 3%) is a very specific predictor (> 90%) of sepsis (Ansari-Lari et al., 2003) and that IG values less than 0.5% are associated with a high negative predictive value. These findings might be of use in a clinical context (Nigro et al., 2005). Recently published reference values have defined a median of $0.63 \times 10^3/\mu$ L (0.1–2.4; 2.5%–97.5% confidence interval) for IG number (IG#) and a cut-off

value of 3.2% for IG% as optimal for a normal adult population. Using a cut-off in a range between 4% and 5% of total WBC would result in a too high rate of missed cases (Bernstein & Rucinski, 2011). In a large outpatient pediatric population comprising more than 2400 samples, age dependent upper limits for reference ranges for the automated enumeration of IG were defined as 0.30% and 40/ μ L for IG% and IG#, respectively for children aged below 10 years (Roehrl et al., 2011). Above the age of 10 years, an upper limit of 0.90% and 70.0/ μ L for relative and absolute IG count was recommended (Roehrl et al., 2011). In this study blood samples were analyzed using the Sysmex XT-1800i instrument (Sysmex, Kobe, Japan). The defined upper limits showed no differences dependent on the patient sex. As expected the cause of elevated IGC differed between both groups. While respiratory or gastrointestinal infections were common associations with elevated IGC in the group < 10 years, the older children showed hematologic malignancies, drug therapy (glucocorticoids, chemotherapy), severe infections, and pregnancy (young females). In a subgroup analysis of patients < 1 year this study revealed age-stratified nonparametric estimates of upper limits of normal (95th percentiles) and associated 90% confidence intervals (CI) for IG# and IG% of 40/ μ L (30.0–50.0) and 0.30% (0.20–0.40). In addition, this study described an important observation: Even the most abnormal IGCs in the younger age group were quite low compared with abnormal IGCs in the older individuals. This fact highlights the importance of particular reference values appropriate for different age groups. Otherwise especially younger children with associated disease and with only small elevations of IGCs could be overlooked (Roehrl et al., 2011). As neonates represent a highly particular and often vulnerable patient population we aimed at investigate a possible correlation between IGC and sepsis.

3.1. “The predictive value of immature granulocyte count and immature myeloid information in the diagnosis of neonatal sepsis”- own experience and study results

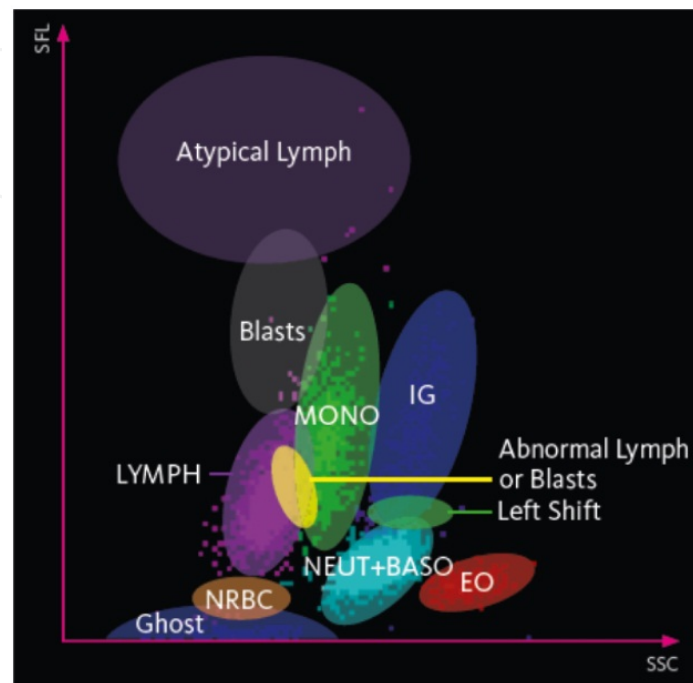
Our study group tried to determine the predictive value of the IG# and the immature myeloid information (IMI) in neonatal early onset sepsis performing a historical cohort study (Cimenti et al., 2012).

3.1.1. Patients and methods

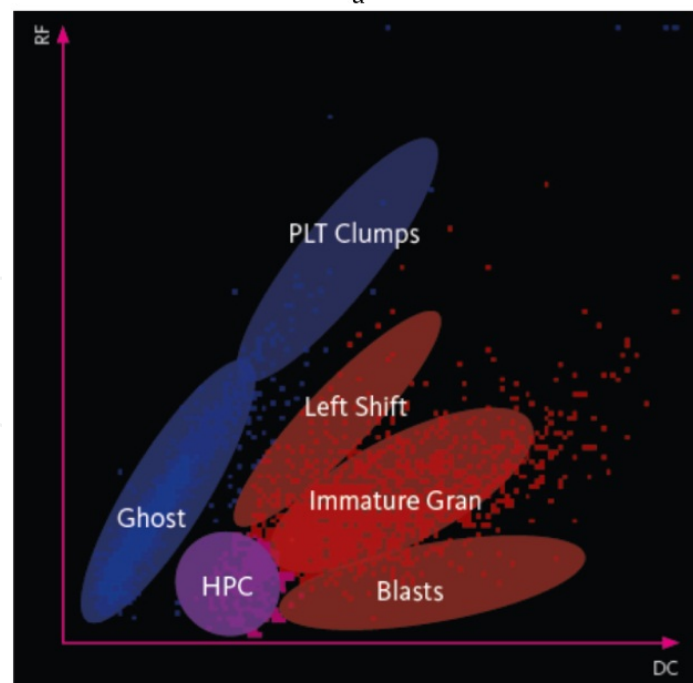
We collected 133 blood samples of neonates admitted to the NICU of the Pediatric Department of the Medical University Graz, a tertiary care center. Based on their admission diagnosis and their clinical course patients were divided in two groups. The first group consisted of patients with blood culture verified bacteremia, clinically strongly suspected sepsis, or elevated inflammatory parameters, a history of risk factors, and antibiotic treatment ≥ 7 d. Patients in the second group were asymptomatic, healthy children without any infectious risk factors constituting the control group. They were admitted to the NICU because of low birth weight, delayed postnatal transition or prematurity.

Blood sampling was routinely performed in all neonates and repeated depending on their clinical course. Blood samples were collected into microvette tubes (Sarstedt, Nümbrecht, Germany) and analyzed using the Sysmex XE-2100 (13). In cases of suspected bacterial

infection, blood samples were always taken before the initiation of antibiotic therapy. ROC curves were used for comparison of infectious indices by plotting the test sensitivity (equivalent to the true positive rate) on the y-axis and 1-specificity (equivalent to the false positive rate) on the x-axis for all possible cut off values of the diagnostic test (see Figure 1).



a



b

Figure 1. a and b: Diff- and IMI scattergram showing graphic output of WBC differential results performed with the Sysmex XE 2100. By courtesy of © Sysmex Europe GmbH, Norderstedt, Germany.

The Youden’s index (sensitivity in %)/100 + (specificity in % - 1)/100 - 1 was used for determination of optimal cut-off values. The area under the curve (AUC) was calculated using the binormal approach by McClish. ROC curve are used to assess the diagnostic accuracy of a test. The ROC curve allows analyses of the trade-offs between sensitivity and specificity at all possible cut-off points and is often used to determine optimal cut-off values and to compare the usefulness of two more diagnostic tests. The area under the curve (AUC) is another useful tool describing the discriminative ability of a test across the full range of cut-offs. A test with an AUC greater than 0.9 has high accuracy, while 0.7–0.9 describes moderate accuracy, 0.5–0.7 implies low accuracy and 0.5 displays a chance result (Akobeng, 2007; Fischer et al., 2003).

Of 133 blood samples of patients admitted to our neonatal intensive care unit 21 neonates were suspected and treated for sepsis (mean gestational age 34.1 weeks, mean birth weight 2287 g, 9 male, 12 female, 12 patients had a history of premature rupture of membranes (PROM)). In the control group 112 healthy neonates were analyzed (mean gestational age 34.2 weeks, mean birth weight 2128 g, 59 male, 53 female, 31 patients with a history of PROM).

3.1.2. Results

The number of IMI classified cells (IMI#) was significantly elevated in patients with sepsis compared to the control group (639/ μ L (144; 2029) vs. 89/ μ L (40; 133), $p=0.000065$). The number of IMI/ total leucocyte count (IMI%) in patients with sepsis was significantly elevated compared to the control group (4.5 (1.3; 9.5) vs. 0.7 (0.5; 1.1), values expressed in %, $p=0.000076$). IG# was significantly elevated in neonates with sepsis compared to the control group (0.28×10^3 / μ L (0.03; 0.56) vs. 0.05×10^3 / μ L (0.05-0.09), $p=0.049$). The percentage of IG% was significantly elevated in septic neonates vs. infants in the control group (1.3 (0.5; 4.5) vs. 0.5 (0.4; 0.7), values expressed in %, $p=0.022$) (Cimenti et al., 2012). The AUC for the IMI# was 0.76 and 0.70 for IG% and IMI%, respectively. The positive and negative predictive value, sensitivity, specificity, and the Youden’s index at different cut off values are listed in Table 2 (Cimenti et al., 2012).

Parameter	Cut-off	PPV	NPV	sensitivity	specificity	Youden's index
IG#	0.24	0.60	0.31	0.60	0.88	0.48
IG%	1.3	0.67	0.27	0.67	0.88	0.55
IMI#	262	0.80	0.09	0.80	0.72	0.52
IMI%	0.02	0.70	0.14	0.70	0.76	0.46
IT ratio	0.06	0.75	0.20	0.75	0.94	0.69

Table 2. Positive predictive value, negative predictive value, sensitivity, and specificity of IG#, IG%, IMI#, IMI% and IT ratio for optimal cut off values determined by ROC analysis using the Youden’s index in 21 neonates with sepsis compared to 112 neonates with negative infectious status (Cimenti et al., 2012).

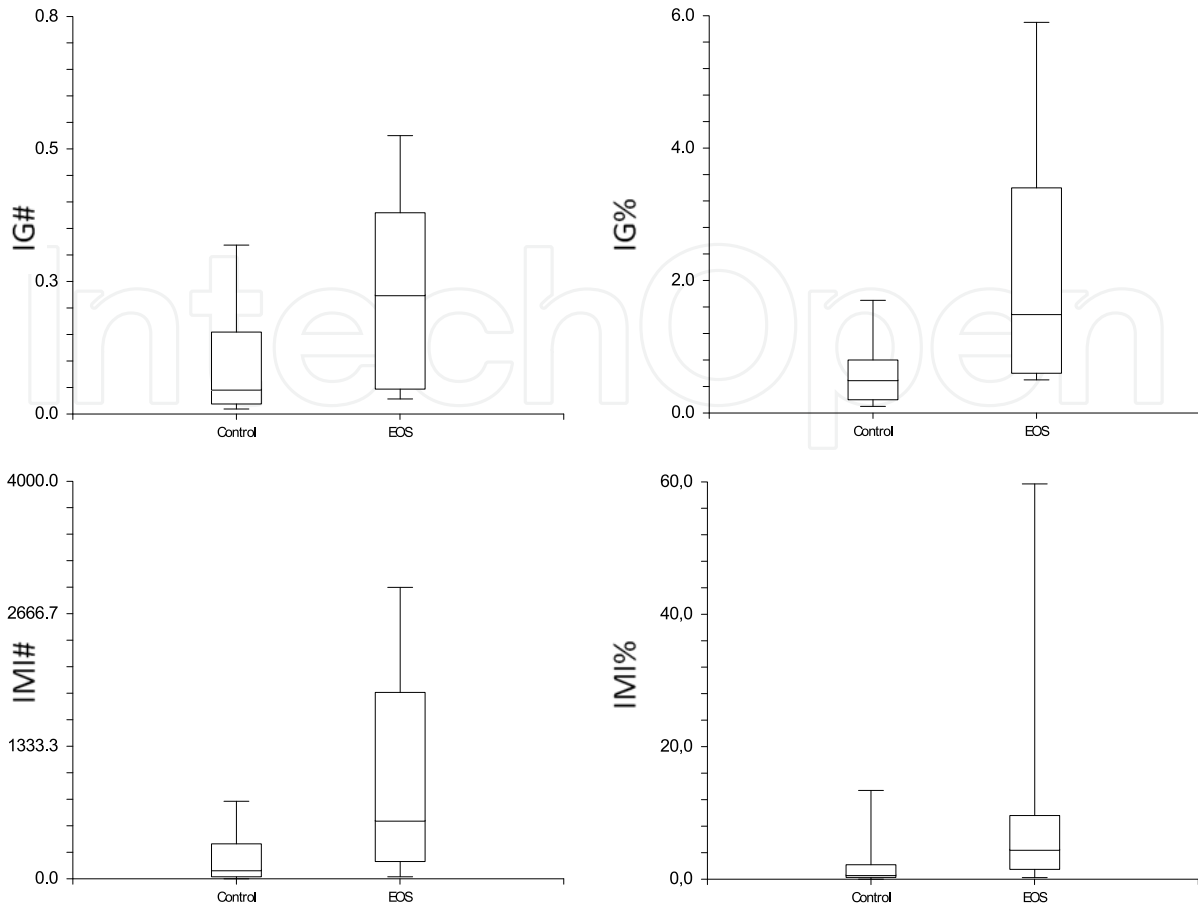


Figure 2. Boxplot diagram showing the distribution of IG#, IG%, IMI#, and IMI% values in neonates with sepsis compared to the control group. The top and the bottom of the box represent the 25th and 75th percentile; the line in the box indicates the median. The whiskers display the largest data less than or equal to the 75th percentile plus 1.5 times interquartile range and the lowest data greater than or equal to the 25th percentile minus 1.5 times interquartile range (Cimenti et al., 2012).

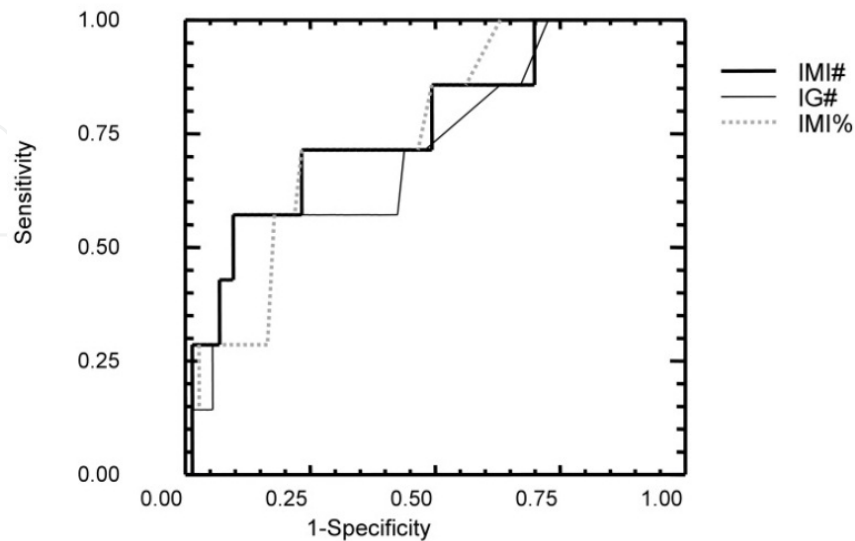


Figure 3. Receiver operating characteristic (ROC) curves of IMI# (thick line), IG# (thin line) and IMI% (dotted line).

3.1.3. Reference values for a neonatal study population based on ROC analysis

Preliminary data revealed a cut-off value for IG% of 1.3% based on calculations using the Youden's index and a median of $0.05 \times 10^3/\mu\text{L}$ (0.05-0.09; 2.5%–97.5% confidence interval) for IG# in our control group of asymptomatic, healthy subjects. According to our data the measurement of IMI# compared to IG# seems to be more favourable as determined by ROC analysis as there seems to be a tendency that the IM# has a higher predictive value than the IG# (Figure 3). Setting a cut off value of 262/ μL the measurement of IMI# revealed a positive predictive value of 0.80.

3.2. Possible limitations of the use of IG

White blood cell differential counts are not only influenced by infection. The gestational as well as the infant's age in hours at the time of blood collection, the method of blood sampling and the infant's gender might affect the results as well as the method of delivery, or maternal risk factors like hypertension (Chirico et al., 1999; Christensen et al., 2009; Escobar, 2003; Kayiran et al., 2003; Newman et al., 2010; Schelonka et al., 1995; Schmutz et al., 2008). Even the influence of the sea level on ANC in neonates has been described leading to a wider range of reference values with isolated elevated counts of absolute neutrophil granulocytes but normal IT-ratios in healthy term babies (Lambert et al., 2009).

A prospective longitudinal study observed higher cord blood cortisol levels, as a sensitive marker of intrauterine stress, in infants born by vaginal delivery compared to elective cesarean section in term neonates and a significant positive correlation between total leukocyte and neutrophil counts. After 12 hours of life no differences in the variation of leukocyte counts remained. Although a significant increase of immature neutrophil counts in vaginally delivered infants or after long labour has been previously described (Hasan et al., 1993; Schelonka et al., 1994), no significant differences in the IT-ratio were detected between the two groups (Chirico et al., 1999). In a prospective observational study including 60 preterm infants with a gestational age < 32 weeks prenatal growth retardation has been shown to be an independent factor for significantly lower counts of leukocytes, total neutrophil and immature neutrophil counts in very immature preterm infants immediately after birth when compared with AGA counterparts. It has been assumed that these low numbers of circulating white blood cells might reflect the reduced bone marrow reserves (Wirbelauer et al., 2010). As the median granulocyte count in the SGA group was with a count of $1.058/\mu\text{L}$ near to absolute granulopenia one could consider this as a risk factor for sepsis, as previous studies had reported an association between early onset neutropenia and sepsis (Christensen et al., 2006). But low numbers of inflammatory cells could also represent a possible protection mechanism for pulmonary and central-nervous disease by reducing inflammatory events postnatally (Wirbelauer et al., 2010). Among extremely low birth weight (ELBW) neonates low neutrophil counts (< $1000/\mu\text{L}$) have been observed with a rate of five times higher than reported in the general NICU population. Most cases were present in the first days of life and represented a transient phenomenon. SGA or maternal pregnancy induced hypertension were common causes for these alterations, whereas in over

one third of cases no cause had been detected. Except in proven early onset bacterial infection, the presence, severity and duration of low counts showed no relationship with mortality rate, whereas neutropenia within the first 3 days of life showed an association with necrotizing enterocolitis (NEC) or nosocomial infection (Christensen et al., 2006). Using IGC in a clinical context should incorporate these factors as well as the likelihood of infection in every individual patient.

As for the blood cell count it has been shown that the performance characteristics in distinguishing between infants with and without infections improve significantly during the first 4 hours after birth. The AUC of the WBC, ANC and IT-ratio showed an increase from 0 hours of 0.52, 0.55 and 0.73, respectively to 0.87, 0.85 and 0.82, respectively 4 hours after birth (Newman et al., 2010). In a review article on new technologies for a diagnostic approach in neonatal sepsis Srinivasan and Harris considered the future development of computerized algorithms including these variables as possibly useful to estimate the probability of sepsis (Srinivasan & Harris, 2012). Taking this fact into account it might be advisable to perform serial IGCs, especially in cases where sepsis had to be ruled out and the result of the test will have a therapeutic consequence (i.e. discontinuing of antibiotic treatment).

The clinical applicability of automated IG counting might be limited by the relatively poor sensitivity of this method (Ansari-Lari et al., 2003; Nigro et al., 2005). Considering the satisfactory specificity and the high NPV this parameter could represent a valuable additional aid in combination with other laboratory markers or diagnostic algorithms. However, as higher values of IG% of more than 3% have been shown to predict blood culture positive results (Ansari-Lari et al., 2003), this subgroup of patients should probably be reevaluated for potential infection even in the absence of specific symptoms.

4. Conclusion

As clinical signs in preterm and term infants with severe bacterial infection are often non-specific and scarce, automated detection of IGs and IMI seems to act as a useful adjunctive tool in the diagnosis of neonatal sepsis. Technical development of automated hematology analyzers has led to a precise, fast, accurate, and reproducible determination of IGs. The detection of all immature cells including blasts in a separate channel might be advantageous at an early stage of sepsis, when these cells are released from bone marrow and the peripheral neutrophil count can still be in the normal range.

Although automated determination of IGs is currently carried out in the area of research, evidence exists that this method seems to be worth to be implemented in clinical practice especially as an adjunctive tool in determining early phase of bacterial sepsis. The fact that measurement of this parameter in the course of routine determination of a white blood differential count does not necessitate any additional sample volume, personal effort, or costs, might be a valuable additional argument. Further well designed prospective trials are mandatory to validate the performance characteristics of these new parameters as diagnostic tool in neonatal sepsis. In this context, the availability of an internal quality control and the

development and implementation of external quality assessment schemes to evaluate analytical performance, compare different laboratories and methods as well as the definition of standards represent indispensable conditions for a reasonable use in clinical routine (Briggs, 2009).

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