16.

Automated Blood Cell Analysis Impact on Neonatology
The Neonatal Immune System: Need for Automated Cell Analysis?

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Introduction

The world of neonatology is very special and although small is wonderfully wide. Yet it is stressful since decisions must be made rapidly and accurately. In no period of later life are patients so vulnerable as during those first 28 days. This report is structured into four parts. First our medical rationale is based on the neonatal specificities of the immune system. Secondly there is the impact of these specificities on diseases that occur during the neonatal period. Thirdly it is necessary to review the available parameters and the pitfalls associated with them. Finally the role of automated analysis will be discussed.

Neonatal specificities and conclusions

It is interesting to note that the two doors, the one that you step through into life and the one that leads out of life, are associated with physiological immunodeficiency. As neonatologists we are involved with that first door but we are not simply dealing with small children and small organs, of a couple of receptors here and a couple of cells and mediators there. Understanding the neonatal immune system involves processes that are of fundamental biological significance, e.g. maternofoetal rejection and tolerance of a semi-allogeneic foetus, apoptosis, and inflammation to name but a few facets which involve the neonatal immune system. Various aspects of the specific and nonspecific immune system are compromised during the neonatal period.

The functional competence of neonatal T cells is altered. T cell activating signals are reduced, CD8-cytotoxicity is impaired and T cell apoptosis is higher compared to adults. There are more CD_4 suppressor cells in neonatal blood and there is an altered CD_4:CD8 ratio with a predominance of a naïve phenotype ($CD_{45}RA$) and TH_2-dominance in cytokine production. The functional competence of the humoral immune system is reduced as well. Antibody production is lower; IgM predominates and the switch to IgG and IgA is delayed. Although antibodies from the mother should protect a mature newborn, this maternal-foetal antibody transport only starts at the 30th week of gestation, so very preterm neonates will not possess the sheltering antibodies. Several B cell functions are severely impaired including Ig synthesis, T cell help, and mechanisms in the interaction between CD_{40} and $CD_{40}L$. Complement activity is reduced.

Neonatal macrophages are phenotypically and functionally altered. Besides a reduced number, there is a shift towards a different type of macrophage. Phagocytosis and cytokine production are impaired. The ability to costimulate T cells and to present and process antigens via MHC class I and class II is reduced.

In contrast, the function to eliminate cells or apoptotic particles via antibody-dependent cell-mediated cytotoxicity is higher in macrophages from neonates than adults. If T cells form cord blood are incubated with neonatal macrophages, T cell proliferation is significantly reduced, as compared to co-incubation of neonatal T cells with adult macrophages, suggesting the important role of macrophages as accessory cells in the orchestration of neonatal T cell response.

It is therefore obvious that there are marked differences between the neonatal and adult scenes and that hypotheses regarding cell markers are not transferable between the two. The major differences are shown in **table 1**.

	Adults	Neonates
Survival	in vitro moderate	worse cell count
Costimulation	high	low T cell answer
HLA-DR	high	lower defence
Cytotoxicity	low	higher elimination

Table 1
Comparison between adult and neonatal macrophages.

Important granulocyte functions such as adherence, aggregation, chemotaxis, active migration, phagocytosis, intracellular killing and signal transduction are compromised compared to the adult. Both the proliferating pool of myelopoietic cells (where cell division occurs) and the storage pool (where no cell division occurs) are reduced to 25% of adult levels. With a turnover rate of 80% the whole bone marrow can therefore empty within a very few of hours.

In conclusion, the neonatal immune system shows a lowered ability to become activated; there is a tendency to immune paralysis; the kinetics of both cellular and humoral immunity are very much related to gestational age; and cytokine production and effects are altered in comparison to adults.

Impact on Diseases

Bacterial infection remains the main reason for neonatal morbidity and mortality (**Figure 1**). Bacterial infection continues to present diagnostic and therapeutic challenges for the neonatologist. Early onset bacterial infection (EOBI), usually defined as occurring up to 72 hours of postnatal life, is known to be associated with obstetric risk factors and high morbidity and mortality. Early antibiotic therapy is the only means to alter the disease outcome successfully. Since term, and particularly preterm neonates are born with features of immunologic immaturity, the differential diagnosis of EOBI is always present for the clinician, regardless of how minor or nonspecific the clinical features are. Besides the mainly nonspecific

clinical signs of EOBI, many well established biochemical and haematological parameters for its detection show weakness on closer scrutiny. No routinely used parameter alone or in combination (e.g., Immature:Total neutrophil ratio or CRP) possesses sufficient sensitivity to determine the need for antibiotic treatment. The incidence of EOBI varies from 2.1 per 1,000 full-term live births in Germany to 53 per 1000 full-term live births in India. For pre-term neonates the incidence is higher; so 19 pre-term infants per 1000 live births ‹ 1,500g will develop the EOBI (‹72 h) and 245 pre-term infants per 1000 live births ‹ 1,500g will develop the late onset (› 72 h) form.

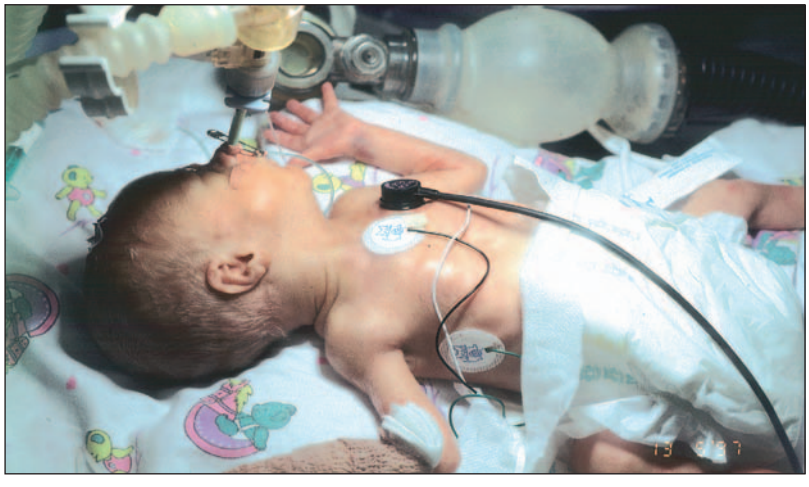

Figure 1
Neonatal bacterial sepsis is the main reason for morbidity and mortality.

The lung is the first site of the early onset of bacterial infection. Bacteria are present in the amniotic fluid and are aspirated by the neonate who goes on to develop (septic) pneumonia. The bacterial spectra (**table 2**) differ in the early and late onset bacterial infections (LOBI).

EOBI < 72h	LOBI > 72h
GB-Streptococcus	Staphylococcus (Coag. neg.)
Other streptococci	Staphylococcus aureus
Staphylococcus	E Coli
E coli	Enterococcus
Haemophilus	Enterobacter sp
Klebsiella	Klebsiella
Listeria	Pseudomonas
Others	Others

Table 2
Bacterial spectra for neonatal pneumonia. EOBI – early onset bacterial infection; LOBI = late onset bacterial infection.

The prenatal risk factors are:

- Premature rupture of the membranes (› 12 h before delivery)
- Foul smelling amniotic fluid
- Maternal temperature › 37.8°C (rectal)
- Maternal leukocytosis (› 15.0 x 10^9/L)
- C-reactive protein › 1.5 mg/dL
- Strep B-smear positive
- Cardiotocogram baseline 160/min, decelerations

In practice often there is no close correlation between the risk factors and patient outcome and we often see severely sick neonates with no risk factors in their case history.

The clinical signs are often uncharacteristic. There is an old saying in neonatology that 'the clinical signs are always ahead of the laboratory' but in the light of the new parameters this is not always true anymore. Besides, we have no reliable scoring system for neonatal bacterial infection. The clinical signs of bacterial infection are often minimal and nonspecific:

- Temperature instability (› 1°C)
- Tachypnoea/dyspnoea/respiratory decompensation
- Bradypnoea/apnoea
- Skin colour/capillary refill › 2 sec.
- Supraventricular/ventricular extrasystoles
- Bradycardia/tachycardia

- Lethargy
- Digestive problems
- Hepatosplenomegaly

Once a child develops a complication, which can happen very early in the course of disease, it is often too late to influence the outcome. Infectious complications also may lead to life-long disabilities. Complications include:

- Shock/systemic hypotension
- Renal failure, oliguria, anuria
- Respiratory failure
- Haemolysis
- Bleeding, petechiae
- Seizures
- Necrotising enterocolitis

Our neonatal diagnostic procedures include:

- Blood tests
- Blood culture (sensitivity in neonatology is very low)
- Bacterial smears (ear, throat, local)
- Chest X-ray
- Bladder puncture, lumbar puncture

The onset of an infection sometimes is characterized by a discrepancy between laboratory tests and clinical signs. The laboratory test is pathological indicating a (nonspecific) immunological response, whereas the clinician may still observe the patient in good health but enquires about the reliability of the laboratory monitoring. Since only early treatment saves the child's life, neonatologists have to be very fast to treat. The consequences of this scenario with an immune 'deficiency' and a rapid progression of infection may be rather inappropriate antibiotic prophylaxis. The results are negative: mother and neonate are separated; the neonate requires intravenous treatment with risk of side effects and the emergence of resistant bacteria. Not least is the cost of unnecessary intervention. To summarize: we urgently need sensitive and specific laboratory parameters from a minimum quantity of blood.

Parameters and Pitfalls

The pre-requisites and limitations of laboratory tests are: availability 24 hours a day, 7 days a week; results should be transmitted within 2 hours (e.g. the IL8 result is available in 40 minutes). Pre-analytical conditions are important; capillary blood should be allowed to flow out without squeezing; i.v. blood should be removed without haemolysing. Processing should be immediate.

The kinetics of neonatal bacterial infection follows a set sequence illustrated in **figure 2**. This sequence can be translated into laboratory tests (**figure 3**). The parameters can be divided into soluble (serum/plasma) and cellular (EDTA/heparin) and there exist a large number of evaluated tests in neonatology. The parameters consist of cytokines, adhesion molecules, soluble receptors, carrier molecules, acute phase proteins, cell counts, surface molecules, cytokine receptors and cell functions.

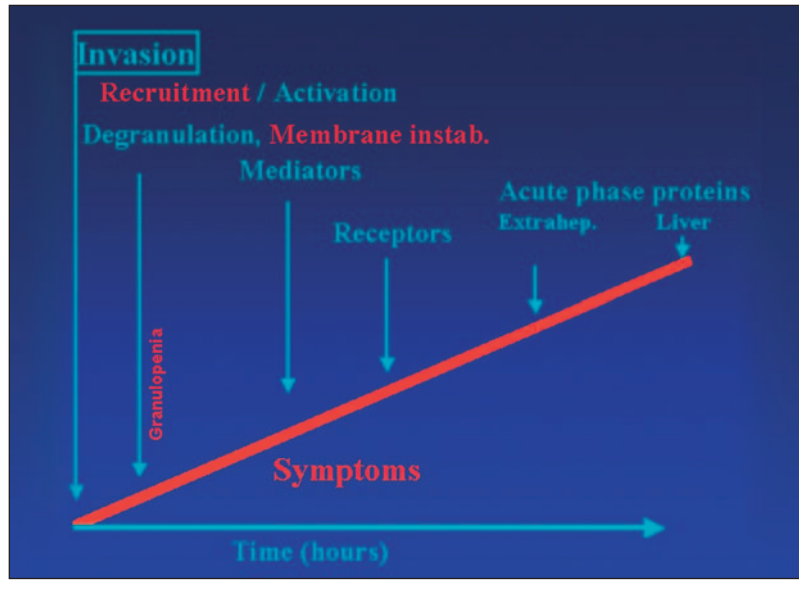

Figure 2
The kinetics of neonatal infection.

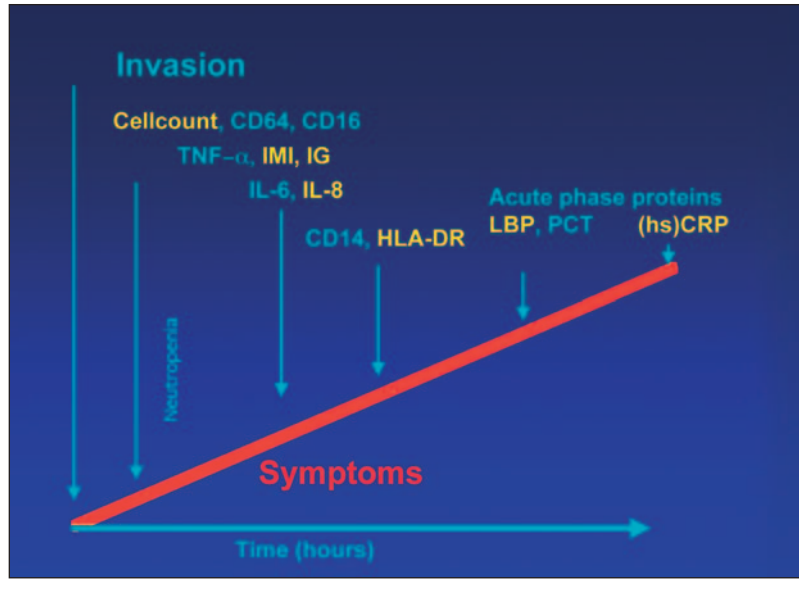

Figure 3
Neonatal bacterial infection – kinetics translated into laboratory tests.

Soluble Parameters: The soluble parameters are listed in **table 3**. From all listed parameters, IL8, IL6 and CRP are frequently used together in neonatology. Other pro- and anti-inflammatory cytokines, adhesion molecules, soluble receptors and carrier molecules are currently under investigation.

Soluble Parameter	Tests
Cytokines – Proinflammatory Inhibitory	IL-8, IL-6, TNF-α (IL-12, IL-18 ...) IL-10, IL-4, TGF-β
Adhesion molecules	sCD54, sCD11b, sCD18
Soluble receptors	sCD14
Carrier molecules	LBP, PCT
Acute phase proteins	CRP, hsCRP test, Fibrinogen

Table 3
Soluble parameters in neonatal infection. LBP = lipopolysaccharide binding protein; PCT = Procalcitonin; CRP = C-reactive protein; hsCRP test = High Sensitivity C-reactive protein.

Pitfalls arise from sampling techniques. IL-8 concentrations become (falsely) elevated when there is haemolysis. Good sampling technique is therefore mandatory to avoid unnecessary therapeutic intervention. When counting leukocytes by an unautomated counter, it is very important to correct for the presence of nucleated red blood cells.

Further problems may arise from combined use of IL-8 and CRP. **Figure 4A** illustrates the kinetics of IL-8 based on neonates with bacterial infection compared with a group of healthy control subjects. IL-8 plasma levels are significantly higher in the infected group than the controls during the first 12 hours and later decrease, so that the significance disappears.

In the infected group, CRP concentrations are significantly elevated after 24 hours (**figure 4 B**). The positive predictive value of the CRP is relatively low, but as the IL-8 concentration falls, the CRP level rises. The different kinetics of the parameters between neonates and adults are schematically depicted in **figure 5**. The yellow line denotes the behaviour in neonates. The half-life of IL-8 is 3 hours. The IL-8 concentration level has dropped below its cut-off value before the CRP level exceeds its cut-off value. There is a clear gap where neither parameter indicates bacterial infection. The blue line represents behaviour of these parameters in the adult. The gap often does not occur.

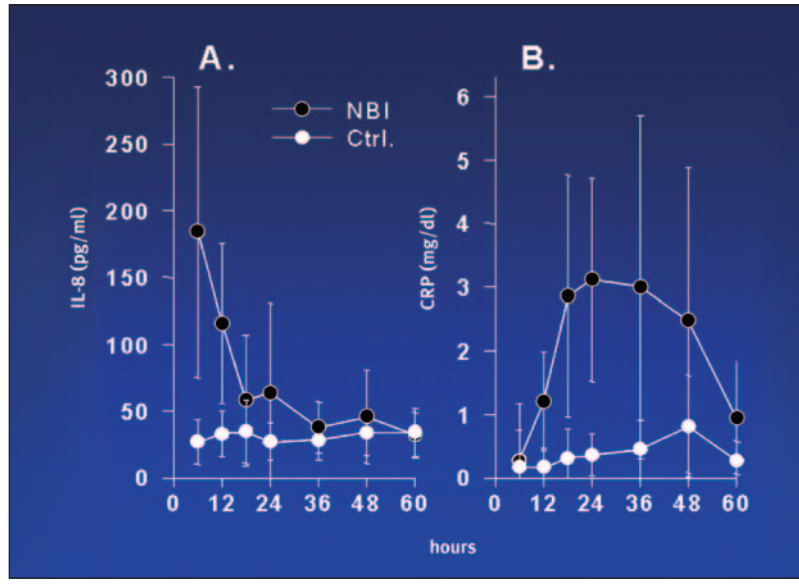

Figure 4
Neonatal(A) IL-8 plasma levels over time and (B) CRP levels over time in bacterial infection.

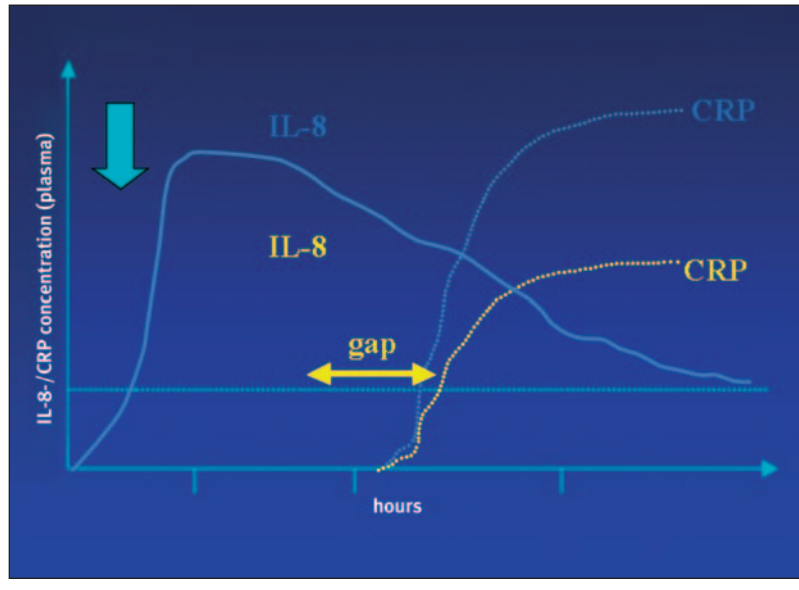

Figure 5
Kinetics of IL-8 and CRP: bacterial infection in adults (blue) and neonates (yellow).

Other problems are the determinants of the measured parameters, for example, the dependency of cytokine production on the mode of delivery. Significant differences occur in IL-8 concentrations. **Figure 6** shows three series of deliveries: Spontan = spontaneous vertex delivery; VE= vacuum extraction delivery; CS = delivery by Caesarian Section. Figure 6A depicts IL-8 plasma plasma concentrations; Fig. 6B IL-8 concentrations in lysed whole blood.

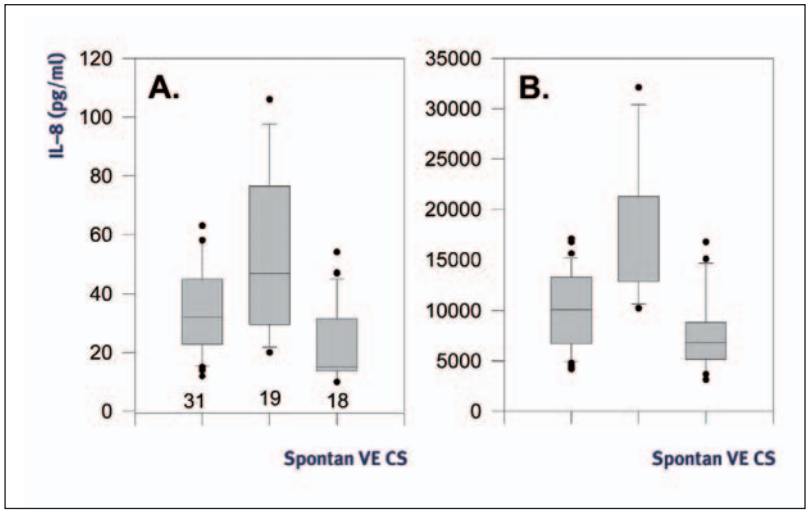

Figure 6
IL-8 concentration and mode of delivery.
Spontan = spontaneous vertex delivery;
VE = vacuum extraction delivery;
CS = Caesarian section delivery.
(A.) plasma concentrations;
(B.) concentrations in lysed EDTA-blood.

Cytokine effects depend on concentrations and the signal cascade when they are released. Therefore, from the mere measurement of concentration it is difficult to deduce its function.

C-reactive protein (CRP) can be used to exclude an infection or monitor its course. A CRP level less than 1.0 mg/dl 48 hours after IL-8 elevation occurs excludes infection. But, once again, the CRP concentrations are dependent on the age of the neonate. The kinetics show a dependency on hours of extra-uterine life. To detect CRP with high sensitivity we can show preliminary results (**Figure 7**).

The role of Lipopolysaccharide Binding Protein (LBP) is currently under discussion. LBP has a key function in sepsis but once more there are the problems in neonates of gestational age-dependency and also age-dependency. A specific reference range for neonates is therefore necessary and this also holds true for Procalcitonin (PCT).

In summary because of the different kinetics compared to adults, specific neonatal reference values are required for the soluble parameters. These assays require skilled personnel and they are expensive.

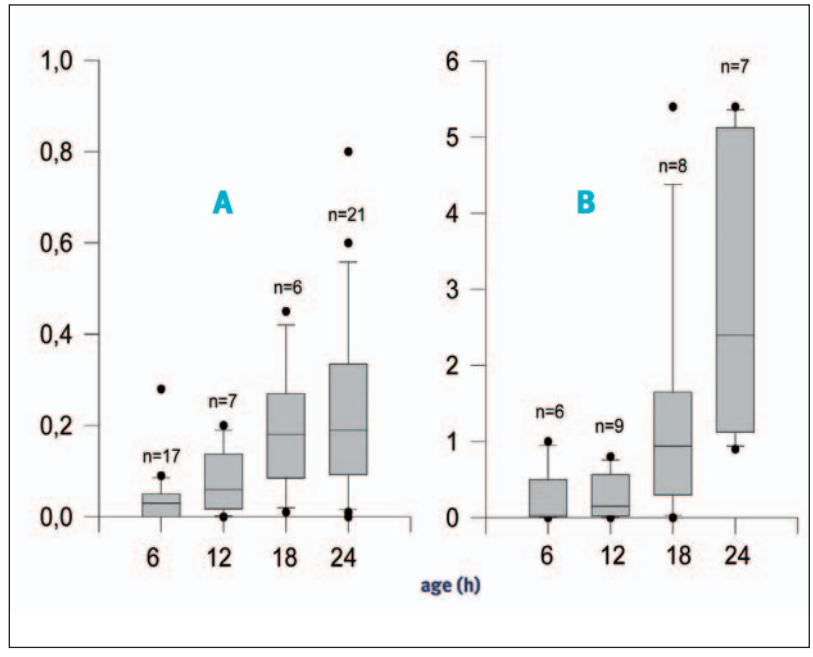

Figure 7
High sensitive CRP (hsCRP) mg/dL in term neonates (A) without and (B) with bacterial infection.

Cellular Parameters: (**Table 4**).
HLA-DR expression on monocytes is significantly lower than in adults; the kinetics are age-dependent; it is not yet automated and external standards do not exist. A predictive value, as we observe in adult septic patients, is not available in neonatology.

Cellular Parameter	Subtype
Cell count	Granulocytes, lymphocytes, monocytes
Surface molecules	CD16, CD64, HLA-DR
Subpopulations	Myeloid precursors, CD14/CD16, CD4/CD8
Cytokine receptors	TNF-R, IL-1β -R
Cell functions	Phagocytosis, apoptosis, TNF-release

Table 4
Cellular parameters in neonatal infection.

Historically the Immature:Total (IT) granulocyte ratio has been important in the prediction of neonatal bacterial infections. The reference range in health is < 0.2. However, there are many problems regarding its relevance in neonatology including methodology, statistics, cytological expertise and labour intensity. Therefore, as was observed in our prospective study, the accuracy of a 'left shift' in a manual differential is questionable.

Automated Markers

Our study group has undertaken investigations using the automated counts from the XE-2100 mainly the IMI channel counts. This channel is described in detail in previous presentations in the Symposium Proceedings.

First we have looked at the total leukocyte counts, the immature granulocyte counts from the IMI channel and the microscopy counts for the first 120 hours of life in a group of healthy term infants (**figure 8**). A good correlation exists between the IMI channel and the microscopy differentials. The kinetics of immature granulocytes, showing a rapid drop in health, suggest that limits can be defined to permit the detection of bacterial infection (**figure 9**). We examined children with clinical and serological early-onset bacterial infection and compared IMI channel counts with manual differentiation (**figure 10**). Initially there is a good correlation but this worsens during the course of infection. It is presumed that the latter is due to increasing cell membrane instability.

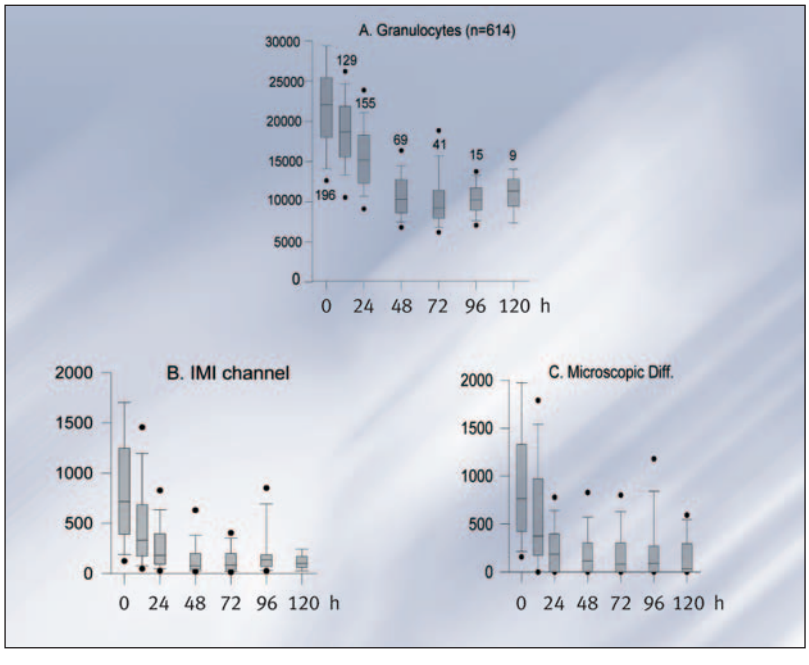

Figure 8
Blood cell counts:
A = automated XE-2100 granulocyte count;
B = automated XE-2100 IMI channel count;
C = microscopy granulocyte count. All counts expressed/mm³.

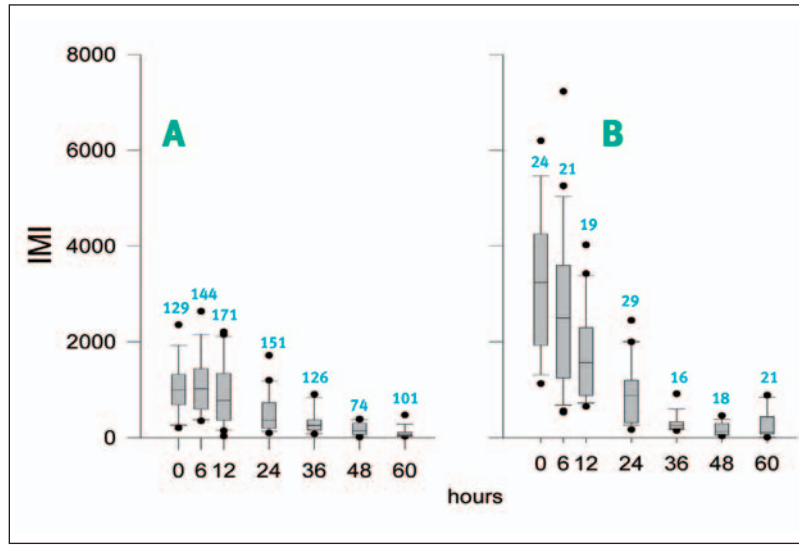

Figure 9
IMI counts in healthy term neonates (A) and neonates with bacterial infection (B). All counts expressed/mm³.

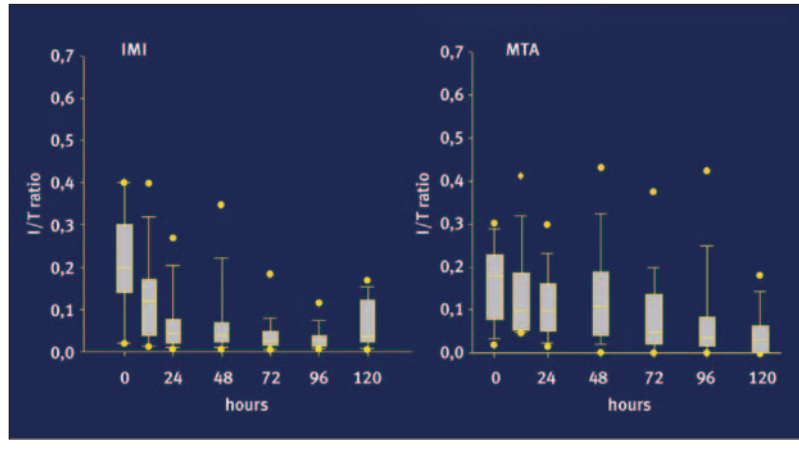

Figure 10
Comparison of IMI counts with manual counts in children with EOBI.

The IMI channel is also proving helpful in the recognition of pre-term neonates with nosocomial infection. **Figure 11** shows preliminary results of pre-term neonates with infection compared with age-matched controls.

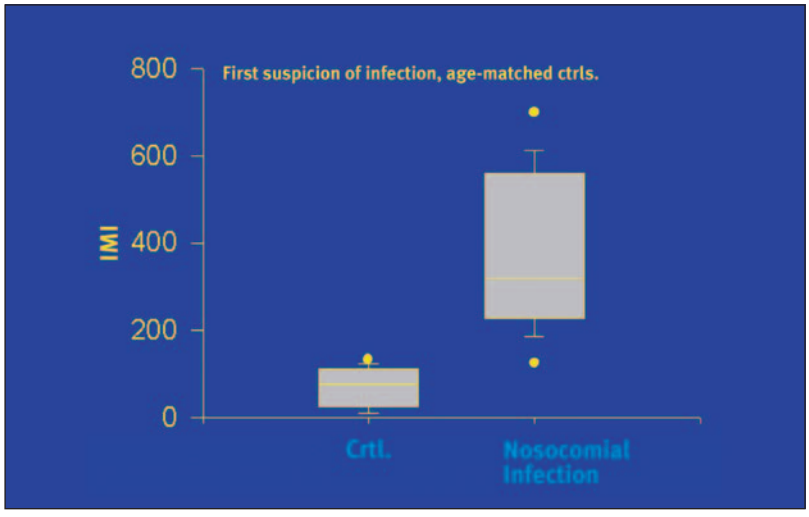

Figure 11
IMI counts in preterm neonates (32 – 36 gestational weeks) and neonates with nosocomial infection (n = 17).

The IMI count is significantly higher in patients with early-onset bacterial infections than in healthy term neonates. This is clearly illustrated in **figure 12** showing WBC, I/T, CRP, IL-8 and IMI counts at 6 hours, 18 hours, 27 and 60 hours. These are the routine parameters that we use. All the parameters marked in red are considered pathological. At 6 hours there are no symptoms and the IL-8 is elevated. It is important to observe this child very closely but not treat. After 18 hours, the child develops symptoms and there is an increase in the IMI channel count above the healthy reference range. The IL-8 is almost down to the cut-off; the CRP is not yet elevated; there is no leukocytosis and the microscopy I/T ratio is not yet pathological. At this point treatment is commenced. By 27 hours the child develops a leukocytosis; the I/T ratio is abnormal; the CRP is elevated and the IMI count remains elevated. By 60 hours the child has fully recovered.

An elevated IMI channel count, as well as a microscopy left shift in neonatology is not specific for infection but also occurs in acidosis and asphyxia.

Non-specific stimulation of the bone marrow leads to a proliferation of nucleated red blood cells (NRBC) in neonatal bacterial infection. NRBC are normally found in low numbers during the first 120 hours of life in the term neonate. The number and duration of presence of NRBC in the peripheral blood depends on gestational age (**figure 13**) and on associated pathology. The NRBC may prove to be a very useful indicator for intrauterine stress (**figure 14**) particularly when the precipitating event, e.g. placental insufficiency, has occurred early in pregnancy rather than an event occurring late in pregnancy, e.g. strangulation by the umbilical cord. Low concentrations of NRBC, not detectable microscopically, are often the first indication of nosocomial infection. Although the presence of NRBC is non-specific it serves as a valuable alert sign.

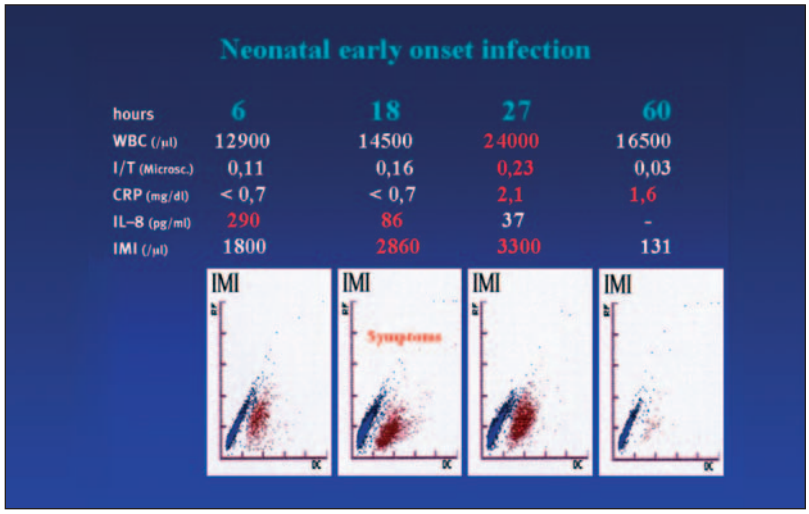

Figure 12
Neonatal early onset infection: WBC, I/T ratio, CRP, IL-8 and IMI counts at 6, 18, 27 and 60 hours. Figures in red considered abnormal.

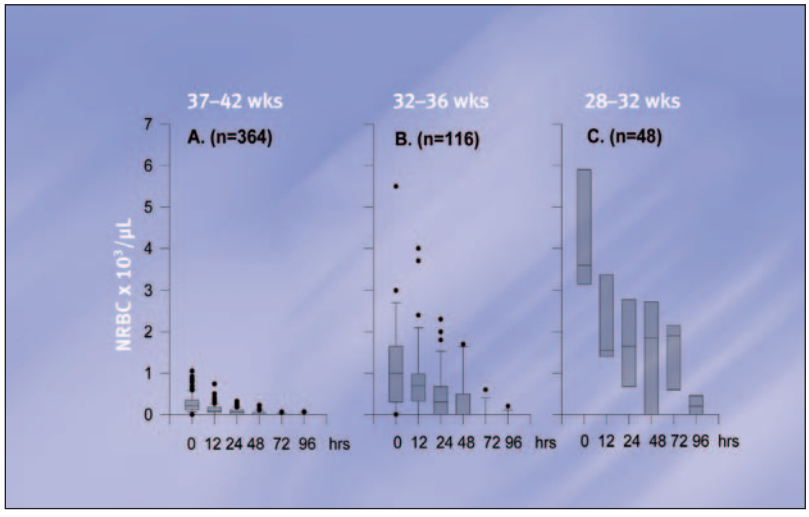

Figure 13
NRBC count depends on gestational age.

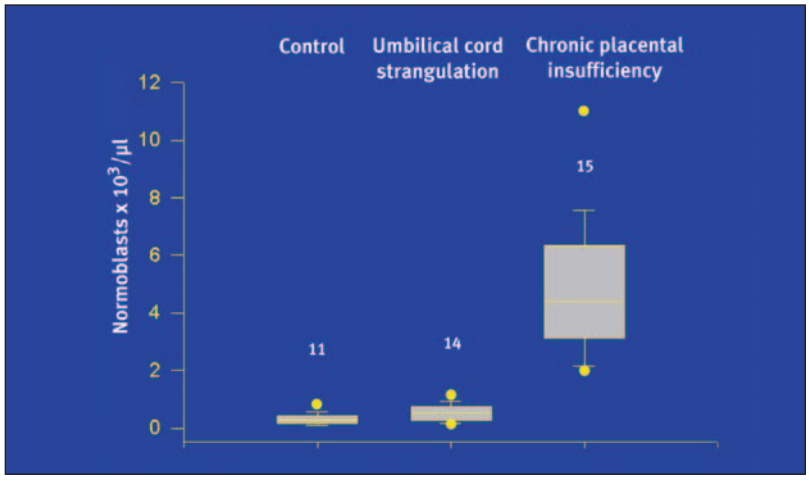

Figure 14
NRBC count as an indicator of intra-uterine stress.

Conclusions

The impact of automated cell analysis on neonatology is potentially considerable. There is vastly improved precision and accuracy of counts and speed of performance. Our IMI study has already resulted in a reduction of 30% in manual differential counts. One criticism is the required sample volume, of 150 µL, which is large for a pre-term neonate weighing 500 or 600 gm. Cells, rather than cytokines, are the effectors and should be the future laboratory tests for neonatology. The need to identify and count subpopulations and to automatically correct the differential leukocyte count, so important in neonatology are now reality. The IMI and NRBC counts offer valuable alerts for a variety of important neonatal diseases where early recognition can be life saving. On the other side, epiphenomena, related to the IMI channel, may yet emerge. Time alone will tell.

The results of provisional studies are reported in this article. We are embarking on a prospective study of IMI, IG, NRBC plus IL-8 and CRP in the prediction and monitoring of neonatal bacterial infection.

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