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Maternal autoimmune thyroid disease (ATD) and the fetal immune system

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Abstract

Objective: Several studies indicate that *in utero* exposure to maternal autoimmune diseases and transplacental passage of autoantibodies affect the risk of autoimmunity in the offspring, e.g. maternally derived GAD65 autoantibody correlates with decreased risk of type 1 diabetes, whereas thyroid peroxidase autoantibody (TPOAb) positivity at birth is associated with increased incidence of autoimmune thyroid disease (ATD) later in life. The aim of this study was to identify immunological changes in children born to mothers with thyroid autoimmunity that may be related to *in utero* exposure to autoantibodies.

Design and method: Open label prospective analysis of cord blood lymphocytes and serum cytokines by Flow Cytometry in children born to mothers with ATD (n=31) and to healthy mothers (n=76) and titers of thyroid autoantibodies were determined in cord blood and in maternal peripheral blood at delivery.

Results: We found an increase (almost 30%) in the frequency of cord blood natural killer (NK) cells ($p=0,0016$) and a minor increase in the subset of T cells expressing NK markers ($p=0,028$), in children born to ATD mothers. There were no detectable differences in the phenotype or frequency of cord blood memory/activated T cells, including $CD4^+CD25^+$ T cells, between the two groups. The levels of pro-inflammatory cytokines $TNF-\alpha$, IL-10, IL-12p70, IFN- γ and IL-1 β were significantly decreased in offspring of ATD mothers as compared to healthy controls.

Conclusions: Maternal thyroid autoimmunity and transplacental passage of autoantibodies against thyroid antigens may affect the generation or expansion of cells with NK activity and the secretion of inflammatory cytokines.

Introduction

During the last years, the role of maternally derived antibodies in neonatal immunity has been extensively studied (Linder et al., 1998, Linder et al., 1999, Hanson et al., 2003). Less is however known regarding the impact of maternal autoimmunity and transplacental passage of autoantibodies on the perinatal immune system and its influence on the risk of developing autoimmune diseases later in life. ATD affect 5-15% of women and 1-5% of men in the general population, and are characterized by lymphocyte infiltration of the thyroid and thyroid autoantibody production. Genetic and environmental factors are involved in disease etiology (Brawerman 2000, Caturegli et al., 2007). Hyperthyroidism occurs in approximately 1% of the infants of mothers with Graves' disease due to transplacental transmission of stimulatory anti-thyroid stimulating hormone (TSH) receptor antibodies. The disease typically resolves with loss of maternal antibodies in the first four months of life. However, if untreated, fetal and neonatal hyperthyroidism may lead to death (Luton et al., 2005, Polak 1998). In a previous study we found that children who develop ATD later in life have high cord blood titers of TPOAb compared to healthy controls, suggesting that maternally transferred anti-thyroid antibodies also may play a role in the actual immunological priming of ATD (Svensson et al., 2006). More direct indication of autoantibody driven priming of autoimmunity was suggested by a study in the non-obese diabetic (NOD) mouse, where the elimination of maternally transferred beta cell specific autoantibodies was found to decrease the prevalence of diabetes in the offspring (Greely et al 2002). In clear contrast to these findings, epidemiological studies in humans have revealed a correlation of maternal T1D and transferred GADA with decreased risk of T1D in the offspring (Dahlqvist et al., 1991, Warram et al., 1991, Koczwara et al., 2004), suggesting that transferred diabetes specific autoantibodies may actually actively promote tolerance. Previous observations of ours suggest that maternal T1D indeed is associated with specific fetal immunological priming, e.g. increased cord blood levels of proinflammatory cytokines and activation and expansion of CD4⁺CD25⁺ regulatory T cells.

The aim of this study was to identify potential changes/deviations in the fetal immune system of children born to mothers with ATD in an attempt to clarify whether exposure to maternal autoimmunity may constitute an environmental trigger of autoimmunity in the offspring.

Materials and Methods

Subjects

Cord blood samples were collected from children born to healthy mothers (n=76) or to mothers with ATD (n=31) for analysis of lymphocyte subsets and cytokine profile. Autoantibody titers were determined in both cord blood and maternal peripheral blood collected at delivery. Mothers with emergency delivery or with pathological pregnancies were excluded from the study. All caesarean sections were elective and were not due to pathological causes of fetal distress. Cord blood samples and peripheral maternal blood was collected, into EDTA tubes immediately after delivery by a dedicated and trained nurse, at Malmö University Hospital, Lund University and the hospitals in Ystad, Helsingborg and Kristianstad, and was processed within 24 h. Healthy mothers were randomly selected and mothers with ATD were referred from the Department of Gynecology and Obstetrics primary care unit or were recruited at the Department of Endocrinology, Malmö University Hospital. In the latter group patients were previously instructed to contact the clinic as far as pregnancy was verified. All patients with ATD were positive for antibodies against thyroid peroxidase at diagnosis and the duration of disease varied between 9 and 248 months before pregnancy. The study subjects were not included in other studies and did not have other autoimmune diseases. The first visit was within 1-2 weeks after remittance or contact. During pregnancy patients were followed every 4 to 8 weeks at the outpatient endocrine clinic and before each visit serum concentrations of triiodothyronine (T3), free thyroxine (fT4) and thyrotropin (TSH) were determined. In patients on thyroxine substitution the dose was adjusted as needed, usually with 25-50 micrograms, to maintain the TSH concentration within the reference range (0.4-4.0 mIU/L) and during the first two trimesters below 2mIU/L. A low TSH-value was accepted in the first trimester without changing the thyroxine dose.

The study was approved by the Research Ethics Committee of the Medical Faculty at Lund University and written informed consent was obtained from participating mothers.

Quantitative analysis of plasma cytokines

Cord blood plasma cytokine levels were analysed in 31 children born to ATD mothers and 76 children born to healthy mothers. Tumour necrosis factor (TNF)- α , interleukin (IL)-2, IL-4, IL-8, IL-10, IL-12p70, IL-1 β , interferon (IFN)- γ , were measured using a cytometric bead array (CBA; Becton Dickinson, Franklin Lakes, NJ, USA), according to the manufacturer's instructions. Samples were analysed with a FACSCalibur flow cytometer using CBA software (both from Becton Dickinson). Briefly, 50 μ l of mixed beads coated with cytokine specific capture antibodies were added to 50 μ l of patient plasma and incubated for 1,5 h at room temperature. After washing, 50 μ l of phycoerythrin-conjugated (PE) antibodies were added. Simultaneously, 50 μ l of standards for each cytokine (0-5000 pg/ml) were treated similarly to generate a standard curve. Two-colour flow cytometry was performed using a FACSCalibur flow cytometer (Becton Dickinson). Data was acquired and analysed using Becton Dickinson CBA software. Forward and side scatter gating was employed to exclude any sample particles other than the 7,5 μ m polystyrene beads. A single operator performed flow cytometry analyses and sample cytokine concentrations were determined based on the standard curves using the CBA software. The lower limit of detection for the various cytokines evaluated ranged from 2 to 10 pg/ml depending on the cytokine. For results above the upper limit of detection, serial dilutions of samples were performed to determine cytokine levels accurately. Although the levels of several of the cytokines analysed were found to be low, especially in the ATD group, with values below the detection limit, all data points were considered for the statistical evaluations.

Flow cytometry of cord blood lymphocytes

Lymphocyte subset analysis was performed on cord blood from 28 children born to ATD mothers and from 50 children born to healthy mothers. Whole blood was stained with fluorochrome-conjugated

antibodies directed against the following markers; CD3 (PE- or fluorescein isothiocyanate (FITC)-conjugated), CD4 (peridin chlorophyll (PerCp)-conjugated), CD8 (PE- or PerCp-conjugated), CD25 (FITC-conjugated), CD62L (APC-conjugated), CD45RA (PE-conjugated), CD45RO (APC-conjugated), CD19 (PerCp-conjugated), CCR4 (PE-conjugated) and isotype controls, IgG2a (FITC-conjugated) and IgG1 (PE- and APC-conjugated), all from Becton Dickinson. After 20 min incubation with antibody mixture at room temperature, erythrocytes were lysed, using lysis buffer (Becton Dickinson), according to the manufacturer's instructions, centrifuged at 400g for 10 min, washed in FACS Buffer (0,5 % bovine serum albumine (BSA) and 2mM EDTA in phosphate buffered saline (PBS), centrifuged and diluted in appropriate volume of FACS buffer. Samples were acquired on a four-colour FACSCalibur flow cytometer (Becton Dickinson) and analysed using CellQuest software (Becton Dickinson).

Autoantibody measurement

Autoantibodies against thyroid peroxidase (TPOAb) and thyroglobulin (TgAb) were determined in plasma from children and their mothers with ATD. Autoantibodies were detected using a solid phase, enzyme-labelled, chemiluminescent sequential immunometric assay, based on the binding of autoantibody to highly purified Tg or TPO coated beads, and detection in a second step by monoclonal murine anti-human IgG antibodies conjugated to alkaline phosphatase, using the Anti-TGAb and Anti-TPOAb kits and the Immulite 2000 Analyzer (Immulite 2000, DPC, Los Angeles, CA). The assay was performed according to the instructions given in the Immulite 2000 Operator's Manual. Antibody concentrations are expressed in IU/ml, and are determined by a master curve generated by running a set of standard concentrations spanning the reportable range of the assay (up to 3000 IU/ml) and two adjustors (included in the Immulite 2000 kit), containing low or high concentration of Tg or TPO antibodies. Analytical sensitivity for TgAb and TPOAb was 2.2 and 5.0 IU/ml, respectively.

Statistics

CBA and FACS data points were non-normally distributed and median, minimum/maximum and non-parametric Mann-Whitney tests were used. Correlation analysis between cord blood lymphocyte

subsets, cytokine levels and plasma autoantibody titers were performed only in children where both data were available (n=22). Correlation analysis was performed using Pearson's *r*-test. Graphs and analyses were performed using PRISM 4 (GraphPad Software, San Diego, Ca, USA). Unless stated otherwise, P-values below 0,05 were considered significant.

Results

TPOAb and TgAb in cord blood of children born to mothers with ATD

We have previously reported a correlation between increased risk of ATD in offspring with cord blood autoantibody positivity (Svensson et al., 2006). In this study we analysed TPOAb and TgAb titers in cord blood and maternal peripheral blood at delivery. All children born to mothers with ATD (n=22) were positive for TPOAb and TgAb and there was a linear positive correlation in autoantibody titers between mother and child (Figure 1), confirming previous results showing that mothers with ATD transfer thyroid-specific autoantibodies to their children during pregnancy (Kvetny and Poulsen 2006).

Changes in cord blood lymphocyte subsets in children born to mothers with ATD

To further determine whether exposure to maternal ATD could induce changes in the fetal immune system that might be associated with autoimmune priming we analysed the distribution of cord blood lymphocyte subsets in children born to mothers with ATD and to healthy mothers. The frequency of NK cells (CD3⁻CD56⁺CD16⁺) and T cells expressing NK markers (CD3⁻CD16⁺CD56⁺) was significantly increased in children born to ATD mothers (n=28) as compared to healthy controls (n=50) (*P* = 0,0016 and 0,028) (Figure 2). There was, however, no differences in the frequencies of B or T cells (Figure 2), the CD4/CD8 ratio, or in the fraction of activated/memory T cells and CD4⁺CD25⁺ regulatory T cells, as determined by the expression of CD62L, CD44, CD45RA/RO and CD25, T cell surface markers (data not shown).

Decreased inflammatory cytokines in cord blood of children born to mothers with ATD

To define whether maternal autoimmunity and transplacental passage of thyroid autoantibodies may be associated with inflammatory responses in the newborn, we next analysed cord blood levels of

common pro-inflammatory and regulatory cytokines by flow cytometry using a multiplex assay (CBA). TNF- α , IL-12p70, IFN- γ , IL-1 β and IL-10 were significantly decreased in children born to ATD mothers (n=31) compared to healthy controls (n=76) ($P=0,0001$), whereas the levels of IL-2, IL-4 and IL-8 did not differ between healthy and ATD cord blood (Table 1 and Figure 3). Cord blood autoantibody titers did not correlate with any of the immunological parameters analysed.

Discussion

Potential pathogenic mechanisms for maternal transfer of autoantibodies in neonatal autoimmunity have been described (Lee 2005, Tincani et al., 2005, Tincani et al., 2006). However, the immunological consequences of transplacental passage of autoantibodies without a direct pathogenic effect have not been investigated in ATD.

Here we report data suggesting that thyroid autoimmunity, in children born to mothers with ATD, may be initiated already during fetal life. We found a strong positive correlation between maternal and cord blood autoantibody titers, reflecting a high degree of transplacental antibody transfer. The cord blood cytokine profile in ATD was significantly altered, interestingly with decreased levels of IL-12p70, IFN- γ , IL-1 β , TNF- α and IL-10. Flow cytometry analysis of lymphocyte subsets revealed a substantial, almost 30%, increase of the NK cell population, and a slight, yet significant, increase in the fraction of T cells expressing NK markers. Altered NK cell activity and/or frequency in peripheral blood of ATD subjects, as reported by others (Wenzel et al., 1998, Solerte et al., 2005, Hidaka et al., 1992), is suggestive of a role of NK cells in thyroid autoimmunity.

The fact that there was no correlation between immunological phenotypes and autoantibody titers suggests either that the specific immune profile in ATD offspring is established independently of the levels of transferred autoantibodies or alternatively as a result of additional ATD associated factors acting in conjunction with autoantibody transfer.

Autoantibodies, especially in complex with apoptotic cell material (e.g. RNA and DNA) or antiviral antibodies, are known to stimulate plasmacytoid DC (pDC) activity through Fc receptor binding (Balmelli et al., 2005, Lovgren et al., 2006). Previous data of ours suggest that maternal enteroviral infection during pregnancy might be a trigger of ATD in childhood (Svensson et al., 2004). Whether maternally transmitted autoantibodies with concomitant viral activation of pDCs promote fetal NK cell expansion remains to be addressed. However, since IFN- α , secreted by activated pDCs, is a potent stimulator of both NK cell proliferation (Gerosa et al., 2005) and plasma cell differentiation (Jego et al., 2003), and also drives autoimmune thyroid disease (Caturegli et al., 20075), increased levels of IFN- α and altered pDC activation/number may be associated with the observed NK cell phenotype. Further, pDCs are potent modulators of myeloid DC (mDC) activity and downstream T cell effector function (Fonteneau et al., 2004, Zou et al., 2001, Meyers et al., 2006). The unexpected general decrease in cord blood cytokine levels in children born to ATD mothers might be a result, either of an altered balance between pDC and mDC activity, and/or a shift in activating *versus* inhibitory Fc receptor signalling.

In clear contrast to ATD, intra uterine exposure to maternal autoimmune diabetes (T1D) and transplacental passage of pancreas specific autoantibodies seems to decrease the risk of autoimmunity in the offspring (Dahlqvist et al., 1991, Warram et al., 1991, Koczwara et al., 2004). In an attempt to identify factors involved in this transferred protection we have previously demonstrated an increased frequency and an activated phenotype of the CD4⁺CD25⁺ Treg population (Holm et al., 2008). Importantly, our current analysis of ATD cord blood revealed no changes in CD4⁺CD25⁺ Treg population. In this context it is worth noting that human CD4⁺CD25⁺ Treg have been reported to downregulate pDC mediated NK cell activation (Romagnani et al., 2005). To suggest that this is a mechanism active in this setting is premature, but based on our previous data on T1D, and those presented here, we propose that increased risk *versus* protection from autoimmunity in the offspring may be associated with distinct fetal immune priming. Indeed, it is tempting to speculate that increased inflammatory responses associated with transfer of diabetes-specific autoantibodies to newborns of mothers with T1D (Holm et al., 2006) might prime the fetal immune system towards

tolerance while cellular changes associated with transfer of thyroid autoantibodies, not accompanied by detectable inflammatory responses, might rather predispose to autoimmunity. In the future gene expression profiling of ATD and T1D cord blood immune cells, including dendritic cell and Treg populations, may reveal specific immunological signatures, which could further explain and eventually predict the risk of developing autoimmune disease later in life. Because of the prospective nature of this study, clinical follow-up of these children may reveal whether the decreased levels of proinflammatory cytokines or the increased frequency of cord blood cells with NK activity could play a role in the future development of thyroid autoimmunity.

Declaration of interest

The authors have no financial or other relationships which may lead to a conflict of interest that could be perceived as prejudicing the impartiality of the research reported

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Figure legends

Figure 1 Correlation in autoantibody titers between cord blood and maternal peripheral blood at partus.

Figure 2 Increased frequencies of NK cells and T cells expressing NK markers in ATD cord blood compared to healthy controls. Summary of frequencies of natural killer (NK) cells (defined by the expression of CD16 and CD56, e.g. including both $CD56^{\text{dim}}CD16^{+}$ and $CD56^{\text{bright}}CD16^{-}$ NK cells), T cells ($CD3^{+}$), B cells ($CD19^{+}$) and T cells expressing NK markers (defined by the expression of CD3, CD16 and CD56) as determined by FACS analysis, in cord blood of children born to healthy

mothers (control, triangles), and mothers with ATD (ATD, circles). Median values for each patient group is marked with a horizontal line.

Figure 3 Levels of proinflammatory cytokines in ATD cord blood compared to healthy controls. Median values for each patient group is marked with a horizontal line and the limit of detection of each cytokine is indicated by a full horizontal line

Table 1 Summary of secreted cytokines in cord blood plasma as determined by cytometric bead array (CBA). The results are displayed in median pg/ml (minimum/maximum) values. *** $P < 0,0001$ Mann-Whitney non-parametric test.

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Figure 1

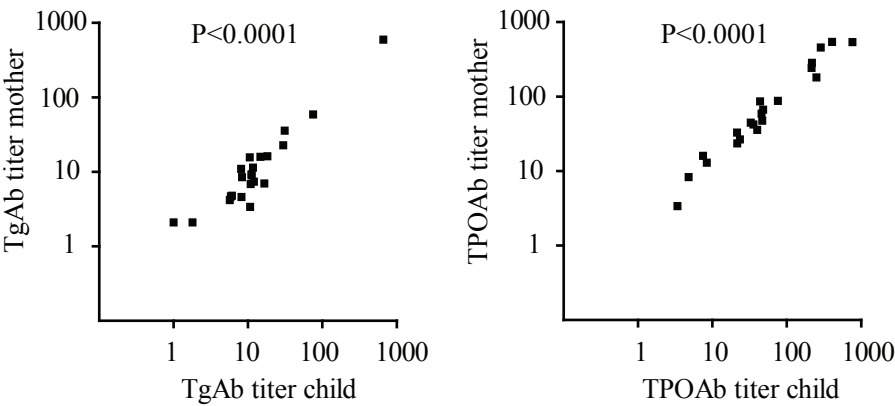


Figure 2

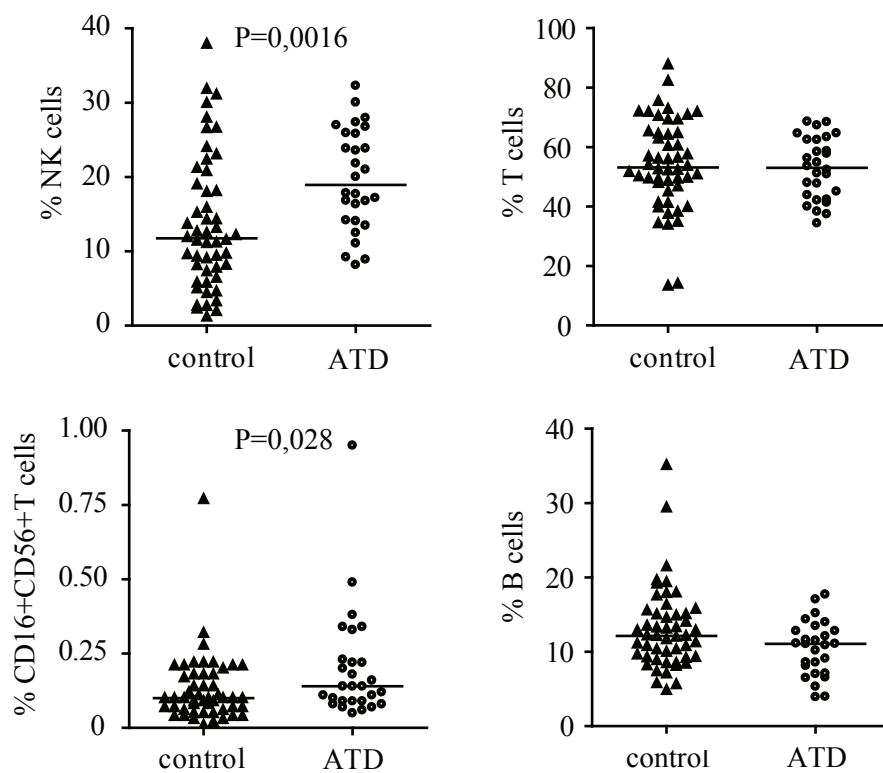


Figure 3

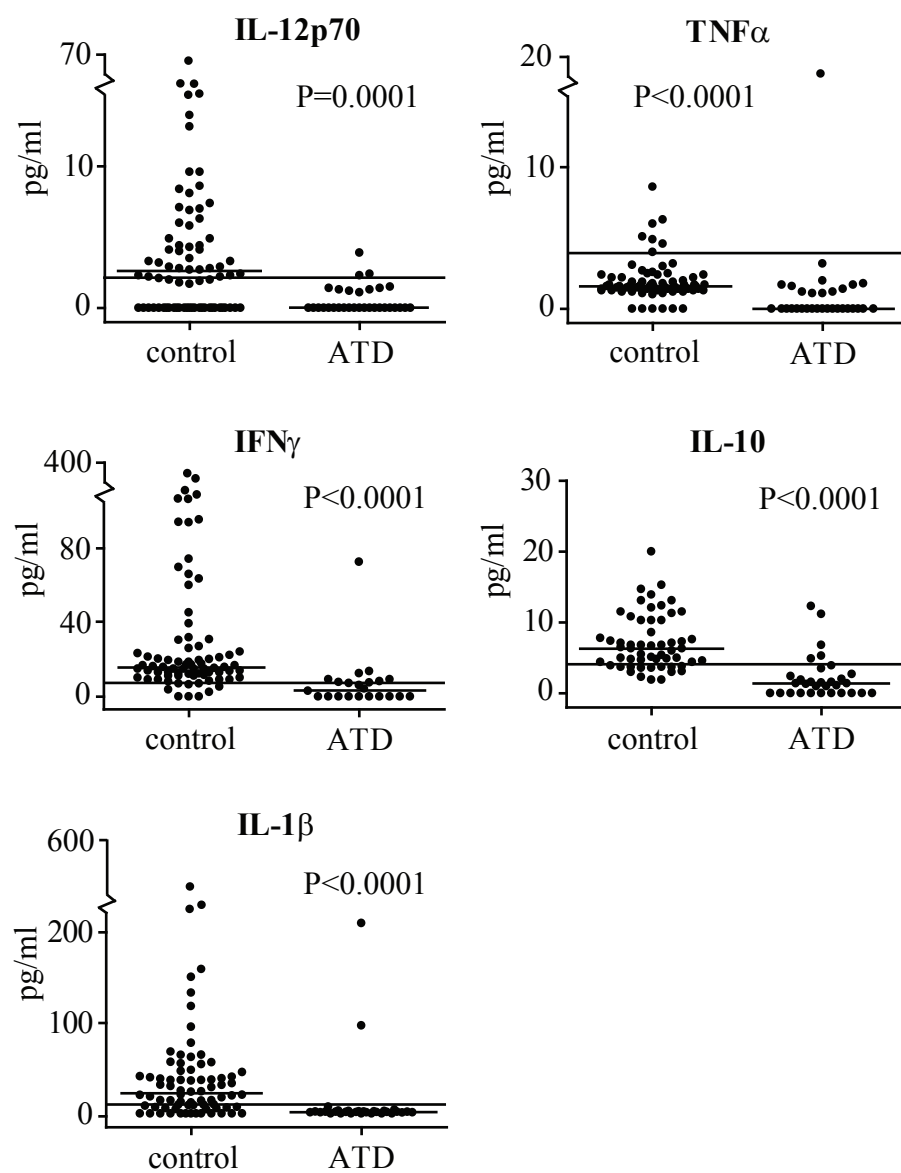


Table 1

	TNF α	IL-1 β	IL-8	IL-12p70	IL-2	IL-4	IL-6	IL-10	IFN γ
Healthy	1,6 (0; 8,6)	22,5 (0; 518)	45,8 (3,4; 2541)	2,4 (0; 68)	1,9 (0; 18,1)	2,6 (0; 11,6)	4,1 (0; 2871)	5,7 (1,7; 165)	14,3 (0; 372)
ATD	0*** (0; 17,8)	1,8*** (0; 210)	92,9 (6,9; 5314)	0*** (0; 3,9)	1,4 (0; 9,2)	2,8 (0; 14,2)	2,7 (0; 1661)	1,4*** (0; 12,3)	3,3*** (0; 65,4)

Summary of secreted cytokines in cord blood plasma as determined by cytometric bead array (CBA). The results are displayed in median pg/ml (minimum/maximum) values.*** P< 0,0001 Mann-Whitney non-parametric test. IFN: interferon; IL: interleukin; TNF: tumour necrosis factor.