

Prenatal exposure to house dust mite allergen (Der p 1), cord blood T cell phenotype and cytokine production and atopic dermatitis during the first year of life

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This study investigated the influence of prenatal exposure to house dust mite (HDM, *D. pteronyssinus*) on interleukin (IL)-2, interferon-gamma (IFN- γ) and IL-4 producing CD4+ and CD8+ T lymphocytes in cord blood as well as on the development of sensitization and occurrence of atopic dermatitis (AD) as the first symptom of allergy during the first year of life. Dust samples ($n = 22$) were collected by vacuum cleaning the maternal mattress during early to mid-pregnancy. In these samples, the amount of the major HDM antigen (Der p 1) was assessed by a sensitive enzyme-linked immunosorbent assay technique (detection limit 0.004 $\mu\text{g/g}$ dust). Flow cytometry was used to determine cord blood lymphocyte subtypes and to quantify the intracellular amounts of IL-2, IFN- γ and IL-4 produced by cord blood CD4+ helper and CD8+ cytotoxic T lymphocytes, both spontaneously and after stimulation with phorbol-12-myristate-13-acetate and ionomycin. Children were followed for 1 yr for the presence of symptoms associated with allergy. In addition, at the age of 1 yr specific IgE to different classical inhalant and food allergens was measured. Higher prenatal exposure to Der p 1 ($> 0.2 \mu\text{g/g}$ dust) was associated with a significant lower percentage of IFN- γ producing stimulated CD4+ T lymphocytes, compared with lower prenatal Der p 1 exposure ($p = 0.03$). The presence of AD during the first year of life ($n = 9$) was associated with an increased number of naive CD4+ CD45RA+ lymphocytes ($p = 0.03$), with an increased spontaneous IL-4 production by CD8+ lymphocytes ($p = 0.04$) and with a decreased percentage of IFN- γ producing stimulated CD4+ lymphocytes ($p = 0.04$). Furthermore, exposure to HDM during pregnancy tended to be higher in mothers of children with AD during the first year of life when compared with those without AD ($p = 0.08$). This study shows that the level of prenatal exposure to Der p 1 influences the immune profile of cord blood T lymphocytes and the clinical outcome in early life. Therefore, the prenatal environment must be regarded as a possible early risk factors for allergic diseases in children.

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House dust mites (HDM) are one of the major sources of indoor allergens in temperate humid areas. Exposure to HDM is an important risk factor for sensitization and subsequent development of allergic disorders in genetic

susceptible patients (1–3). The process of sensitization is influenced by many factors, such as the dose and time of allergen exposure. For HDM allergens, there is a relationship between the dose of mite allergen exposure and development of

sensitization (4, 5). Furthermore, the time of exposure is probably of equal importance. Early infancy seems to be a critical period for primary sensitization (5). Additional data indicate that maternal exposure during pregnancy might influence foetal immune responses. The presence of lymphoproliferative responses to environmental allergens, such as HDM, on foetal and cord blood suggests that priming of the T cell system is initiated *in utero* and might be influenced by differences in seasonal exposure during pregnancy (6, 7). In addition, allergen-specific cytokine production by cord blood T lymphocytes has been demonstrated (8). It is not yet clear how relevant quantities of allergens could cross the placenta and sensitize the foetus, but recently the presence of Der p 1 in amniotic fluid and cord blood was shown (9, 10) and the transfer of inhalant and food allergens across the placenta was observed using an *in vitro* human perfusion model (11).

T lymphocytes play a pivotal role in the initiation and maintenance of the allergic inflammatory responses. Circulating T cells of individuals with allergy show a characteristic Th2-skewed profile in response to allergens such as HDM. Prescott et al. (12) showed that all neonates have a Th2-skewed cytokine profile at birth, comparable with the cytokine profile at the gestation-associated tissue in a successful pregnancy. In atopics, this Th2 cytokine profile is boosted during infancy, while in non-atopics, there is a shift towards a Th1 cytokine profile. Until now, it remains unclear if this process of prenatal T cell priming has an impact on these immunological changes during infancy and on later allergic status (13–15).

In this study, we quantified Der p 1 exposure during early/mid-pregnancy and assessed the possible influences of Der p 1 on the cytokine profile of cord blood T lymphocytes as well as on the development of sensitization to allergens and the occurrence of atopic dermatitis (AD) during the first year of life. We hypothesize that the amount of Der p 1 exposure during pregnancy might influence the developing immune responses during foetal life and early infancy.

Methods

Study population

Data were collected during a Prospective study of newborns on the Influence of Perinatal factors On hypersensitivity (PIPO-study). Women were recruited during early pregnancy by gynaecologists in the region of Antwerp. During a home

visit, performed at early to mid-pregnancy, standardized questionnaires were used to collect information on family history and environmental characteristics, including presence of bedroom carpeting and furnishing and type of mattress. Concurrently, dust samples were collected by vacuum cleaning the maternal mattress (see below).

In 22 randomly selected neonates, cord blood samples were collected for intracellular cytokine assessment on T lymphocytes. The children were followed during their first year of life by questionnaires, focusing on the occurrence of AD and respiratory symptoms. Three months postpartum, a dust sample was collected by vacuum cleaning the mattress of the infant's bed (see below). At the age of 12 months, all children were clinically evaluated at our outpatient clinic. Furthermore, blood was drawn for determination of total and specific IgE to the following allergens: *Dermatophagoides pteronyssinus*, cat, dog, egg's white and cow's milk (CAP systems, Pharmacia Diagnostics, Brussels, Belgium).

The ethics committee of the University of Antwerp approved this study.

Collection of dust samples and quantification of Der p 1 allergens

Mattress dust collection. Dust was collected between 15 and 25 wk gestation from the maternal beds and 3 months postpartum from the baby beds by the same trained technician, using the same equipment and according standardized procedures. Dust was collected from the upper surface of the covered mattresses by vacuuming 1 m² for 2 min. A 1200 W vacuum cleaner provided with a pre-weighted filter (ALK, Hørsholm, Denmark), pore size 6 µm, was used. After vacuuming, the filter box was removed and stored in a plastic bag. After weighting the collected dust, the allergen was extracted in phosphate-buffered saline (PBS) with 0.05% NaN₃ and 25 mg% PMSF (100 ml/mg buffer). Extracts were stored at –20°C until analysis.

Measurement of Der p 1 in dust samples. Extracts were assayed for Der p 1 by using an amplified monoclonal antibody-based 'sandwich' enzyme-linked immunosorbent assay. Two amplification steps were used to optimize the detection limit: a biotinylated secondary antibody and biotinylated tyramine were added, which were both detected using streptavidin peroxidase.

Briefly, 96 well microplates (Corning-Costar, Cambridge, MA, USA) were coated with anti-Der p 1 monoclonal antibody (Clone 5H8, Indoor

Biotechnologies, Cardiff, UK) and blocked with bovine serum albumin (BSA; Sigma, St Louis MO, USA). Dilutions of Der p 1 standard or unknown sample were incubated for 1 h at room temperature (RT). The plates were washed and a second, biotinylated anti-Der p 1 monoclonal antibody (Clone 4C1, Indoor Biotechnologies) was added for 1 h at RT for detection. After a second wash, 100 µl/well streptavidin peroxidase (Sigma), 1/2000 in 1% BSA-PBS-T, was added and incubated for 15 min at RT, followed by washing. For the second amplification step, the plate was incubated with biotinylated tyramine in 10 ml of 50 mM Tris-HCl (pH = 8) and 0.03% H₂O₂ for 30 min at RT. The plates were washed and again incubated with 100 µl/well streptavidin peroxidase for 15 min at RT. After washing, 100 µl/well 3,3',5,5'-tetramethylbenzidine (TMB, Sigma) in citric acid-phosphate buffer (150 µl/10 ml) with H₂O₂ was added and incubated for 30 min at RT. The colorimetric reaction was stopped with H₂SO₄ and optical density was read at 450 nm.

The allergen concentration in dust samples was expressed as micrograms of Der p 1 per gram of dust. The detection limit of the Der p 1 assay was 0.004 µg/g dust. The intra-assay and the inter-assay coefficient of variation was 4% and 9%, respectively.

Prenatal exposure to Der p 1. Prenatal exposure to HDM was defined as low when the amount of Der p 1 in the dust samples was below 0.2 µg/g dust. This cut-off point was based on the highest amount of Der p 1 found in dust samples of paediatric hospital beds (n = 10), which are generally considered as containing low levels of HDM (16).

Collection of cord blood

Cord blood was collected at birth in EDTA tubes (Becton Dickinson, Meylan, France) by needle aspiration from the placental end of the umbilical vein. All children were born at term (≥37 wk gestation) after uncomplicated pregnancies and normal deliveries. All cord blood samples were processed within 4 h after birth, as cytokine production diminishes when the delay between blood sampling and start of cell separation increases (17).

Flow cytometric analysis of lymphocyte subsets on cord blood

For cell surface marker analysis, 50 µl whole cord blood was incubated for 15 min in the dark at 4°C with 10 µl of three-colour monoclonal

antibody panels (CD3-PerCP + CD4-FITC + CD45RO-PE; CD3-PerCP + CD4-PE + CD45RA-FITC; CD3-PerCP + CD4-FITC + CD8-PE; CD45-PerCP + CD3-FITC + CD19-PE; Becton Dickinson Immunocytometry Systems, Erembodegem, Belgium). Subsequently, the red blood cells were lysed with 2 ml of a lysing solution (Becton Dickinson Immunocytometry Systems) for 20 min at RT. The remaining cells were pelleted at 400 g for 10 min, fixed with 1% paraformaldehyde in PBS and analysed by FACScan flow cytometry (Becton Dickinson) within 24 h. By gating on sideward scatter and CD3+ or CD45+ cells, the different lymphocyte subsets were determined.

Intracellular cytokine staining of cord blood lymphocytes

Interleukin-2, IFN-γ and IL-4 producing cells were quantified as previously described (17) (Fig. 1). Isolated cord blood mononuclear cells were cultured in AIM-V culture medium (Life Technologies, Merelbeke, Belgium) for 6 h at 37°C and 5% CO₂. One part of the cells was stimulated with 50 ng/ml phorbol-12-mirystate-13-acetate (PMA, Sigma), 1 µg/ml ionomycin (Sigma) and 2 µM monensin (Sigma). The other part of the cells remained unstimulated, in the presence of 2 µM monensin. After incubation, cells were labelled with monoclonal antibodies against CD3 and CD8 surface antigens (anti-CD3-PerCP, anti-CD8-FITC, Becton Dickinson Immunocytometry Systems) and subsequently fixed with 4% paraformaldehyde in PBS (Sigma). To identify T helper lymphocytes, the CD4 surface antigen was not used because surface CD4 is internalized after stimulation with PMA and ionomycin (18, 19). Cell membranes were permeabilized with 0.3% saponin (Sigma) in PBS and incubated with anti-cytokine antibodies (mouse anti-Hu IL-2-PE, mouse anti-Hu IL-4-PE, mouse anti-IFN-PE, Fast Immune, Becton Dickinson Immunocytometry Systems).

Ten thousand cells were analysed on an FACScan flow cytometer (Becton Dickinson Immunocytometry Systems) and processed with WinMDI 2.7 software. T helper cells were defined as CD3+CD8- and T cytotoxic cells as CD3+CD8+. According to PerCP and FITC emission, CD3+CD8- and CD3+CD8+ lymphocyte populations were gated out in a compensated fluorescence plot. Within this gate, intracellular cytokine production (PE emission) was measured in CD3+CD8- and CD3+CD8+ lymphocytes. For this purpose, markers were set on the 99th percentile of an isotype matched irrelevant control antibodies (PE-IgG1

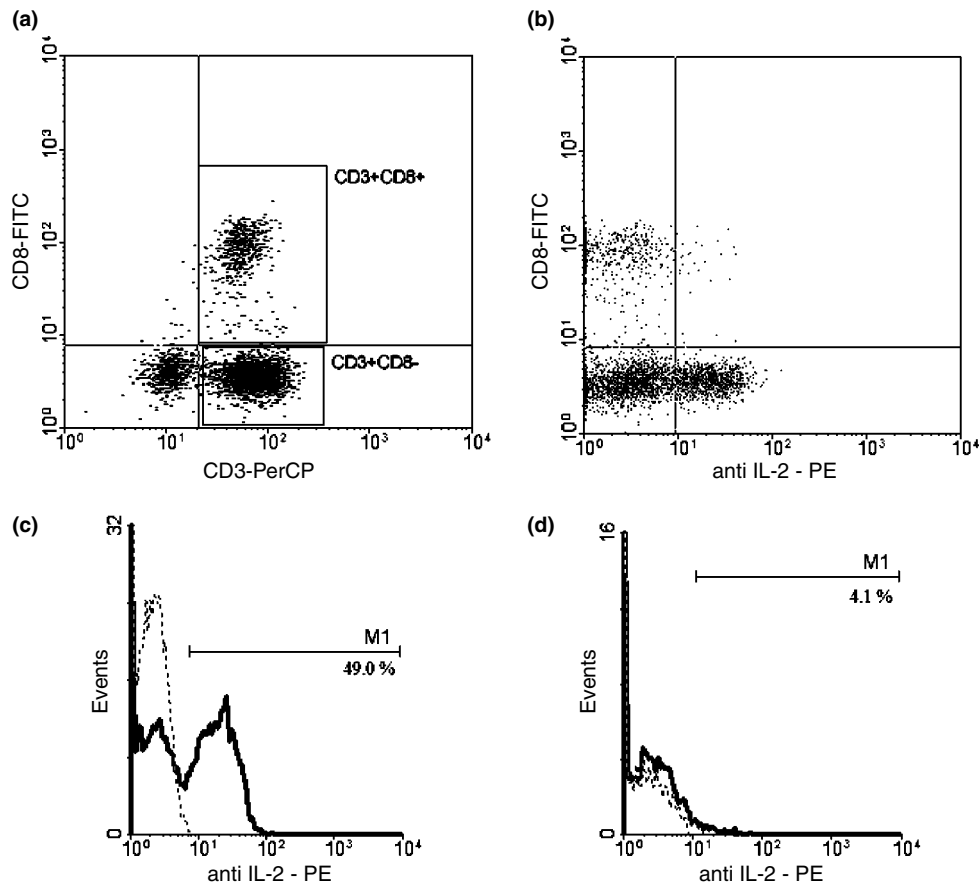


Fig. 1. Intracellular IL-2 production by cord blood T lymphocytes. Analysis gate was set on lymphocytes depending on forward and sideward scatter properties. According to PerCP and FITC emission, T helper lymphocytes were defined as CD3+CD8- and T cytotoxic cells as CD3+CD8+ cells and were gated out. (a) Intracellular IL-2 production (PE emission) is measured in CD3+CD8- and CD3+CD8+ lymphocytes. (b) Within the gate of CD3+CD8- (c) and CD3+CD8+ (d) lymphocytes, intracellular IL-2 producing cells are shown on a histogram mode (bold line). Markers (M1) are set on the 99th percentile, using isotype-matched irrelevant antibodies as a reference (dotted line). Results are expressed as the percentage of cytokine-producing CD3+CD8- and CD3+CD8+ lymphocytes.

mouse antibodies; Serotec, Oxford, UK) as a reference. Results were expressed as the percentage of cytokine-producing cells.

Statistics

Results are expressed as median and range. Differences between groups were analysed using chi-square and Mann-Whitney *U*-test. Correlations were evaluated with the Spearman rank correlation coefficient. A difference of $p < 0.05$ was considered significant.

Results

Der p 1 levels in maternal mattress dust samples in relation to parental, neonatal and housing characteristics

The concentration of Der p 1 in dust samples from the mother's mattresses showed a wide range from below detection limit up to 25.2 µg/g

dust, with a median value of 1.6 µg/g dust. These levels were not related to the presence of carpeting in the bedroom, the frequency of mattress vacuum cleaning or the age of the mattresses.

The levels of Der p 1 in mattress dust samples from mother's beds correlated well with the levels measured in baby beds ($r = 0.67$; $p = 0.001$).

There was no relation between Der p 1 levels in maternal mattress dust samples and parental characteristics, such as the presence of allergic symptoms and specific IgE to inhalant allergens. However, in families with at least one other child, Der p 1 levels in maternal mattress dust samples were significantly higher, compared with families without children [median value and range (µg/g dust): 4.5 (0.2–25.2) vs. 0.5 (<0.004–2.1); $p = 0.03$].

In six neonates, prenatal exposure to Der p 1 was defined as low (<0.2 µg/g dust), as opposed to 16 neonates with higher prenatal Der p 1 exposure. The two study groups were compar-

Table 1. Characteristics of the study population according to the level of prenatal Der p 1 exposure

Median value (range)	Low prenatal HDM (n = 6)	High prenatal HDM (n = 16)
Birth weight (g)	3165 (3090–4070)	3550 (3050–4075)
Sex (m/f)	4/2	8/8
Der p 1 ($\mu\text{g/g}$ dust)		
Maternal bed	0.02 (<0.004–0.04)	2.03 (0.25–25.17)
Infant bed	0.20 (<0.004–2.03)	2.01 (0.01–9.0)
Age mattress mother (months)	48 (2–120)	60 (24–132)
Total IgE (kU/l)	7 (1–18)	13 (1–597)
Exposure to cigarette smoke	0/6	2/16
Exposure to animals	3/6	6/16
Presence of siblings	0/6	10/16
Parental history of atopy	2/6	9/16

able considering birth weight, sex ratio, the age of the mattress, exposure to cigarette smoke and animals, number of siblings, the duration of breastfeeding, parental history for atopy and total IgE (Table 1). None of the children exposed to low Der p 1 levels had siblings, whereas the majority of children with higher prenatal Der p 1 exposure had at least one sibling.

Influence of the level of prenatal exposure to Der p 1 on intracellular cytokine production by cord blood T lymphocytes and on lymphocyte subsets in cord blood

Spontaneous production of IL-2, IL-4 and IFN- γ by cord blood T lymphocytes, determined intracellularly, was not significantly different in newborns with low or higher prenatal exposure to HDM. However, higher prenatal exposure to Der p 1 was associated with a significantly lower percentage of IFN- γ producing stimulated CD4+ T lymphocytes when compared with lower prenatal Der p 1 exposure [median value and range (%): 2.9 (0.9–12.4) vs. 6.0 (2.5–9.8); $p = 0.03$]. No differences were detectable in CD8+ T cells. The percentage of IL-2 and IL-4 producing stimulated CD4+ and CD8+ lymphocytes was unrelated to amount of prenatal exposure to Der p 1.

The lymphocyte subsets on cord blood (CD19+ lymphocytes, CD3+ lymphocytes, CD3+CD4+ lymphocytes, CD3+CD8+ lymphocytes, CD3+CD4+CD45RO+ lymphocytes, CD3+CD4+CD45RA+ lymphocytes) were not influenced by the level of prenatal exposure to Der p 1.

Atopic dermatitis as main clinical outcome parameter

As AD can be considered as the initial clinical manifestation of the 'atopic march' (20), the study population was subdivided into two

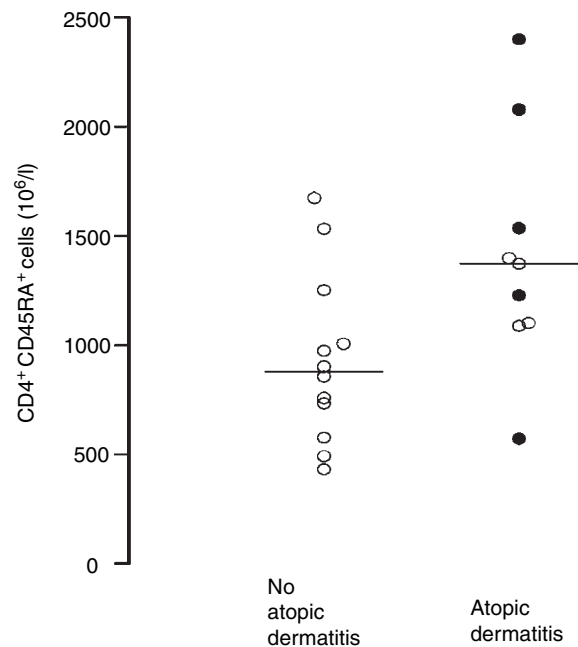


Fig. 2. Number of cord blood CD4+CD45RA+ cells in relation to the presence of atopic dermatitis during the first year of life. Horizontal bars show median values. Filled circles indicate presence of specific IgE to food and/or inhalant allergens at the age of 1.

groups, depending on the occurrence of chronic and/or recurrent episodes of AD. Follow-up data during the first year of life showed that nine of 22 children suffered from AD (AD group). In five of nine children with AD, specific IgE was detectable to one ($n = 2$) or two ($n = 3$) of the allergens tested: four children had specific IgE to cow's milk and/or egg white, one child showed specific IgE to HDM and egg white. In the non-AD-group, none of the children showed an elevation of specific IgE to the allergens tested.

In children with AD, the absolute number of naive T helper lymphocytes (CD3+CD4+CD45RA+) on cord blood was significantly higher compared with the non-AD group [median values and range ($10^6/\text{l}$): 1373 (573–2401) vs. 880 (432–1674); $p = 0.03$] (Fig. 2). The absolute number of cord blood lymphocytes and other lymphocyte subsets analysed (CD19+ lymphocytes, CD3+ lymphocytes, CD3+CD4+ lymphocytes, CD3+CD8+ lymphocytes, CD3+CD4+CD45RO+ lymphocytes) was comparable between the two study groups.

Spontaneous IL-2 and IFN- γ production was very low and was observed in less than 1% of cord blood T lymphocytes. However, basal IL-4 production by cord blood CD8+ T lymphocytes was significantly higher in children without AD as opposed to children with AD (median values and range (%): 1.3 (0.7–2.0) vs. 0.7 (0.0–1.6);

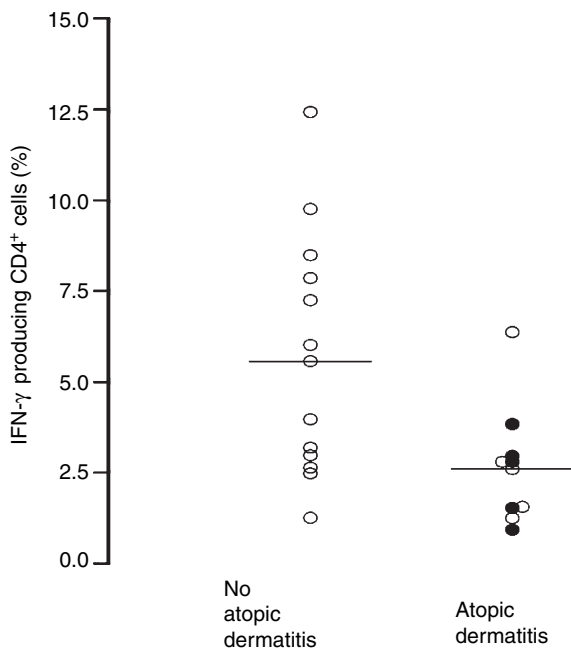


Fig. 3. Percentage of IFN- γ -producing cord blood CD4 $^{+}$ cells, after stimulation with PMA and ionomycin, in relation to the presence of atopic dermatitis during the first year of life. Horizontal bars show median values. Filled circles indicate presence of specific IgE to food and/or inhalant allergens at the age of 1.

$p = 0.02$). Furthermore, the group of children with AD showed a significant lower percentage of IFN- γ producing stimulated cord blood CD4 $^{+}$ T lymphocytes when compared with children without AD [median values and range (%): 2.6 (0.9–6.4) vs. 5.6 (1.3–12.4); $p = 0.04$] (Fig. 3). For cord blood CD8 $^{+}$ T lymphocytes, the percentage of IFN- γ producing stimulated cells was not related to the presence of AD. Furthermore, the percentage of IL-2 and IL-4 producing cord blood CD4 $^{+}$ as well as CD8 $^{+}$ stimulated lymphocytes was unrelated to AD.

The development of AD tended to be associated with higher prenatal Der p 1 exposure [median value and range ($\mu\text{g/g}$ dust): 1.0 (<0.004–25.2) vs. 2.4 (0.004–17.1); $p = 0.08$].

The levels of Der p 1 in baby beds were comparable in children with or without AD.

Discussion

It has been shown that postnatal exposure to HDM predisposes for sensitization and allergic disease (5). On the contrary, the data on the influence of prenatal allergen exposure are controversial. The presence of HDM-specific proliferative responses by cord blood mononuclear cells are not generally accepted as a firm proof for *in utero* priming (21, 22). However, additional

data, such as the demonstration of cord blood-derived HDM-specific T-cell clones (8) and the presence of Der p 1 in amniotic fluid and cord blood (9, 10) indicate that exposure to environmental aeroallergens might happen *in utero*. However, the exact mechanism and potential clinical relevance of this intrauterine exposure remain unknown.

In this study, the influence of prenatal HDM exposure on cord blood T lymphocyte parameters (subtypes, intracellular IL-2, IL-4 and IFN- γ production) and on the development of sensitization and allergic symptoms during the first year of life was evaluated.

For this purpose, dust samples were collected during early to mid-pregnancy, which can be considered as a crucial period in the development of foetal immune responses (6, 7). The level of Der p 1 was assessed in dust samples from the maternal mattresses and is generally considered as a reliable index of natural exposure (23).

Der p 1 allergen levels assessed in this study are low compared with studies performed in other countries, such as United Kingdom and Australia (13), but are comparable with measurements in the Netherlands (24). The fact that the levels of Der p 1 are low in this study cannot be considered as a disadvantage in interpreting the data, as it has been extensively shown that even low levels of mite allergen exposure may be sufficient for sensitization (2–5). However, the presence of other siblings in the household during pregnancy was related to higher Der p 1 levels, as already reported by Wickens et al. (25).

In this study, a relatively higher prenatal exposure to Der p 1 was associated with a significantly lower percentage of IFN- γ producing stimulated cord blood CD4 $^{+}$ T lymphocytes. This is in accordance with studies that focus on the enzymatic cysteine protease activity of Der p 1, which may contribute to its allergenicity. Due to its proteolytic activity, Der p 1 cleaves CD25, the α subunit of the receptor for the Th1 cytokine IL-2, from the surface of T cells. As a result, these cells show markedly diminished production of IFN- γ in response to anti-CD3 stimulation (26, 27). These lower levels of the Th2 antagonist IFN γ , assessed in cord blood, might compromise the postnatal immune deviation process and therefore favour the boosting of the Th2 cytokine profile (12).

Early postnatal exposure to HDM is a risk factor for the development of AD (1). Our data suggest that it might be necessary to extend the vulnerable period for sensitization to the prenatal period, as the development of AD during the first

year of life also tended to be associated with higher prenatal Der p 1 exposure.

In addition, the presence of AD, which can be considered as the first manifestation of allergic diseases in early life (20), is associated with an increased number of naive CD4+ T lymphocytes in cord blood, suggesting a less mature status of the T helper cells. Furthermore, the lower percentage of IFN- γ producing stimulated cord blood CD4+ T lymphocytes in neonates with subsequent AD confirms, at the intracellular level, previous data on extracellularly secreted INF- γ by cord blood mononuclear cells (12, 28, 29). This decreased IFN- γ production by cord blood cells can be considered as an 'intrinsic' characteristic associated with AD, as cytokine evaluation in later life confirms these data in children and adults with AD (30). The decreased spontaneous IL-4 production by cord blood T lymphocytes in children with AD are in line with the described lower Th2 responses in cord blood of neonates with subsequent atopy (12).

Previous studies on the effect of prenatal HDM exposure on cord blood parameters, such as HDM-specific proliferative responses and extracellular cytokine secretion by mononuclear cells, could not detect any association (13–15, 31, 32). However, some methodological differences may have contributed to the different results of the studies, such as the timing of prenatal sampling, the assessment of cord blood characteristics, the composition of the study group and the design of the study (interventional or non-interventional).

Our data suggest that prenatal HDM exposure might influence developing immune responses on cord blood and clinical outcome in early life. The prenatal environment must therefore be considered as one of the possible early risk factors for allergic diseases in children. The real extent of the importance of prenatal allergen exposure must be further explored in future studies.

Acknowledgments

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