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"Early lifestyle and environmental factors and their impact on infant allergy & obesity development"

Habilitationsschrift

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To my father.
I know you would have been proud.

Table of contents

Figure index

Table index

Abbreviations

AA	allergic asthma
AD	atopic dermatitis
ADIPOQ	adiponectin
AR	allergic rhinitis
BMI	body mass index
BPA	bisphenol A
BuP	butyl paraben
C/EBPα	co-activators CCAAT/enhancer binding protein α a
cDII	children's dietary inflammatory index
CFUs	colony forming units
CpG	cytosine-phosphate-guanine-dinucleotide
DNA	deoxyribonucleic acid
EDCs	endocrine disrupting chemicals
Eo/B	eosinophil/basophil progenitor sells
FA	food allergy
FASN	fatty acid synthase
GLUT4	the insulin-sensitive glucose transporter GLUT4,
IgE	immunoglobulin E
IL	interleukin
IQR	interquartile range
IRS1	insulin receptor substrate 1
LEP	leptin
LPL	lipoprotein lipase
MR	mean ratio
MSCs	mesenchymal stem cells
OR	odds ratio
OSB	oriented strand board
PPARγ	peroxisome proliferator-activated receptor- gamma
RCT	randomized control trail
SREBF1	sterol regulatory element binding transcription factor 1
Th1	T helper cell Type 1
Th2	T helper cell Type 2
VOCs	volatile organic compounds

1. Introduction and research question

The early bird catches the worm – a saying that describes nicely that it is of high importance to start acting as early as possible to achieve the optimal results. That this is also particularly true for the prevention of allergy & obesity development was shown by 12 original projects as part of this habilitation thesis. An overview of the entire topic is presented at page 7 and will be introduced in the following.

Chronic diseases such as allergies or obesity already start to establish in early childhood. Worldwide, approximately 15–30% of children live with allergic eczema [1] [2] and 4–10% of children suffer from food allergy [3] [4] [5]. According to the KIGGS Study, German children and toddlers (age 3-17 years) have a 12.8% lifetime **prevalence** for allergic eczema, 6% for asthma and 11% for rhinitis [6], 23.7% of these children have at least one of those 3 diseases until adulthood. In addition, 37,1% of the 3 to 17 year old have increased IgE levels against one of the eight most common airway allergens: Timothy (Lieschgras), rye (Roggen), birch (Birke), mug wort (Beifuß), cat/dog, house dust mite and mold [6]. In accordance to recent reports for the second outcome of interest, overweight and obesity affect up to one third of children in Europe and in Northern America [7] [8]. KIGGS outlined that 15.5% of all German 3 to 17 year old children will develop overweight (body mass index >90th percentile) - trends increasing. Assuming that chances are high that allergic or obese children will remain these pathological conditions until adulthood [9] [10] [11], they are facing a very unhealthy future when we think about associated comorbidities as well as personal, social and psychological consequences. Also the resulting long-term costs for the health system will be a considerable problem [12] [13] .

It is already known since more than a decade that chronic diseases (including allergies and obesity) start to develop very early in life and a combination of **genetic background** (determining about 30% of the disease risk) together **with environmental factors** (70% determination of disease risk) will prime the unborn fetus [14]. It is very unlikely that the genetic background of the human species has changed to such an extent over the past centuries that it could explore the high worldwide prevalence of allergies and obesity. Therefore, other factors like changes in lifestyle and environmental conditions seem to be key drivers in this context. Changes towards unhealthy diets, sedentary life styles, different microbial exposures

or absorption of the huge variety of environmental chemicals etc. are discussed to alter our immune, hormone or metabolic system - which may contribute to the development of allergies or obesity. In particular, the very **sensitive time window** before/during pregnancy and around birth is of highest importance when external environmental factors aim to modify individual physiological conditions. As outlined in the DOHaD hypothesis from 1990, exposure to environmental factors such as chemicals, drugs, stress or infections during specific sensitive periods of intrauterine fetal development or early childhood might predispose an organism to diseases in adult life [15] [16]. Later work proposed that **epigenetic modifications** might mediate some of these effects. Epigenetic modifications that can up- or down-regulate gene expression independent from the genetic code of the DNA (such as methylation of Cytosine-phosphate-Guanine (CpG) sites or modifications of histone molecules of the DNA) have been under intense investigations throughout the last years [17-19]. These epigenetic processes are most relevant in the early fetal life – in particular the first 1000 days from conception onwards have been suggested to determine individuals health into adulthood ("early priming") [20]. In line, fetal development is also very dependent on **stem cell physiology**. All types of stem cells are characterized by the ability to self-renew and to differentiate – processes mandatory for fetal tissue differentiation or organ development. It is known, that both maintenance of stemness and lineage commitment are tightly controlled by epigenetic mechanisms such as DNA methylation, or histone modifications [21]. Therefore, it seems a valuable approach to investigate epigenetic as well as stem cell modification to address early external factors with respect of priming long-term health.

In addition to investigating the relevant time window and associated processes where lifestyle and environmental factors can impact the unborn fetus, it is also important to consider how we are exposed to which substances. There are several individual substances/factors already known that can interfere with the normal physiologic development of the fetus when we consider their singular and independent exposure. However, according to the Horizon EU Research program [22], it is particularly important to also analyze **multiple exposures** or chemical mixtures; otherwise the risk to human health would be underestimated. This research focus is particularly based on the fact that nowadays the number of lifestyle and environmental chemicals is increasing rapidly so it seems mandatory to investigate not only

single parameters with respect to their impact on health outcome development but also consider their multiple interaction, for example via mixture modelling [23].

In general, many factors have been identified to determine individual health, either of the pregnant mother providing the growing environment for the fetus or of the newborn/infant itself. Based on that, several characteristics (see below) can be outlined for both allergy and obesity. However, there is still lack of knowledge of the causative mechanistic pathways in the disease development, so the prevention of infant allergy and obesity development as multifactorial diseases remains a global challenge.

Both diseases share several characteristics:

- Both have a multifactorial origin, so genetic background as well as lifestyle and environmental factors contribute to the disease development
- Both start to develop already in early childhood/infancy
- Both follow early priming processes in or even before pregnancy
- Both are directly associated with the individual's nutrition: Obviously, obesity is directly dependent on the amount and quality of nutrients/food consumed. In allergy patients, allergic episodes or inflammatory processes are often triggered by dietary allergens. Furthermore, diet associated environmental chemicals seem to affect both diseases.

Although sharing several characteristics, some disease specific aspects are provided in the following.

Per definition, an **allergy** is an abnormal adaptive immune response directed against non-infectious – and per se non-harmful - environmental substances (allergens) which results in diverse allergic symptoms. This overreaction of the immune system is seen in different allergic disorders, such as atopic dermatitis (eczema), some food allergies, allergic rhinitis (hay fever) and allergic asthma. The allergic immune response is characterized by the involvement of allergen-specific Immunoglobulin E (IgE) and T helper 2 (Th2) cells that recognize allergen-derived antigens [24] after their presentation via dendritic cells (Figure 1). These Th2 cells produce several allergy promoting Th2 cytokines that are mediating the inflammatory

reaction such as Interleukin (IL-) 4, IL-5 and IL-13. Usually, regulatory T cells (Treg) can balance the production of allergy driving Th2 cytokines supporting immune tolerance. Further, eosinophils and basophils as well as their precursor cells (hematopoietic stem cells) are important effector cells in human allergic diseases. Due to their release of pro-inflammatory mediators (such as histamine, leukotriene C4, major basic protein, eosinophil cationic protein, IL-4, and IL-13) they play a significant role in promoting allergic inflammation [25].

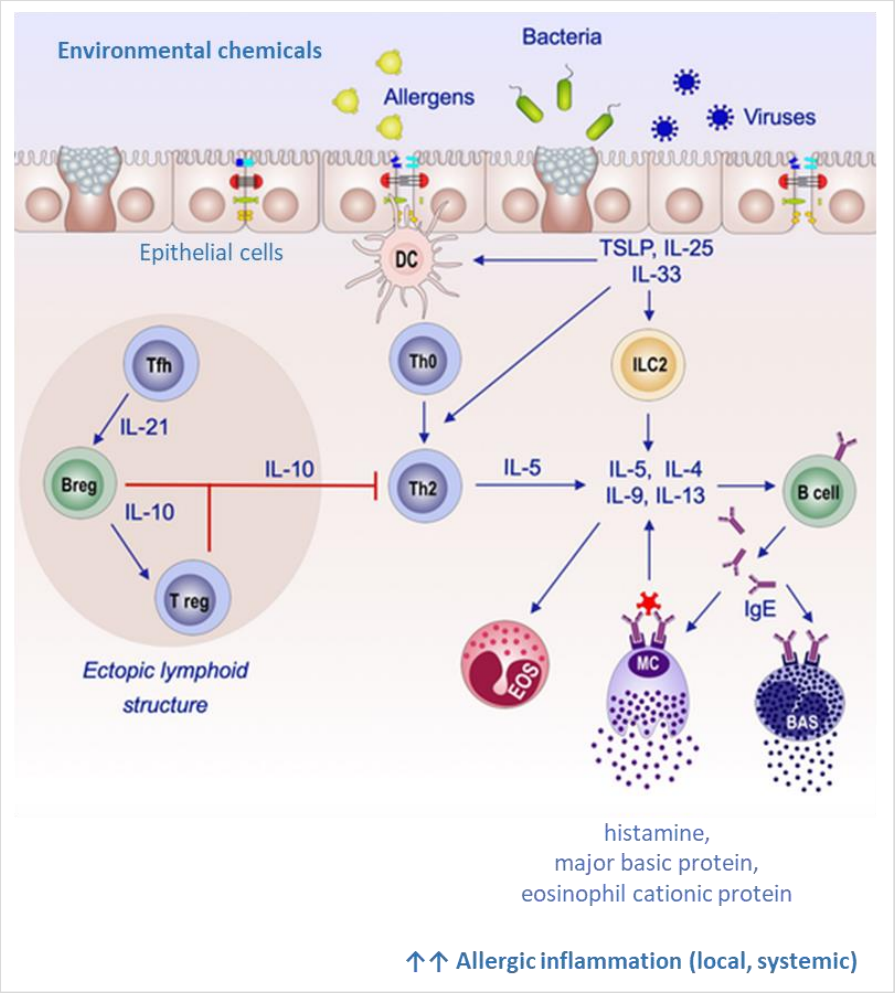

Figure 1: Mechanisms involved in allergic reaction: *This figure is reprinted and adapted from the original first publication in Meng et al., Allergy, 2019 [26], with permission from the applicable society / copyright owner.*
DC: dendritic cells, Th0: naïve T helper cells, Th1/Th2: T helper cells Type 1/2, Treg: regulatory T cells, Breg: regulatory B cells IL: Interleukin, TSLP: thymic stromal lymphopoietin, EOS: eosinophil granulocytes, MC: mast cells, BAS: basophil granulocytes

Several studies suggested that allergic diseases are following a time-based order throughout life: from atopic dermatitis and food allergy in infancy to gradual development into allergic asthma and allergic rhinitis in childhood [27]. This chronological development goes along with the development of the infant's anatomic structure, it follows the spatial evolution from skin to gastrointestinal and finally respiratory tract, a phenomenon defined as the "**atopic march**" [28]. In some children there are also some early precursor symptoms that might increase the risk for later development of the full disease; such as cradle cap for atopic dermatitis or wheezing for allergic asthma [29]. The process of sensitization usually results from contact of allergens with mucosal surfaces – therefore the **symptoms** of allergic manifestations are often

associated with the initial route of allergen exposure (for example: dermal route: itchy, red and dry skin lesions; oral route – gastrointestinal symptoms like vomiting, diarrhea or abdominal pain; or inhalation route: sneezing with runny nose, watering eyes, shortness of breath and cough). In principle, all symptoms are aimed on removing the allergen out of the body – however, the signal *that* a specific non-harmful substance has to be removed is falsely send in allergy patients.

Several **factors that contribute** to the individual's allergy development have been identified: genetic background (family history of atopy of either one or both parents), individual immune response (unchallenged early immune system, e.g. due to lower bacterial/pathogen contact in the western countries; "Hygiene hypothesis" [24]), barrier dysfunctions (increased entrance of allergens due to disturbed mucosal barrier), microbiome alterations (e.g. due to "farming effect", birth mode or breastfeeding duration), lifestyle behaviors (stress, nutrition, sun light exposure, vitamin D etc.) and environmental exposure (e.g. endocrine disruption chemicals; EDCs) are of importance. In particular, indoor air exposure is of high relevance, since the majority of the population is spending their time indoors facing exposure to several inhalation chemicals such as volatile organic compounds (VOCs) emitted from cigarette smoking, cleaning or renovation activities. However, the clear early causal pathogenic mechanisms in the context of allergy development still needs to be elucidated.

According to the World Health Organization (WHO) **overweight and obesity** are defined as an abnormal or excessive fat accumulation in the body that may impact individual's health [30]. The classification of being overweight or obese is based on the Body mass index (BMI), which is a simple index of weight-for-height (person's weight in kilograms divided by the square of his height in meters; kg/m^2). For adults, overweight is a BMI greater than or equal to 25; obesity a BMI greater than or equal to 30. For children, age and gender matched normalization needs to be considered when defining overweight and obesity. Therefore, international reference data are available from WHO (as z-scores from BMI values [30]) or from World obesity Task Force (WOTF; as age and gender matched BMI cut-offs [31]). In line with an increased BMI, blood lipid or body composition will most likely be negatively affected in overweight or obesity, as well as glucose and insulin metabolism (together with an increased risk of developing diabetes mellitus type II or metabolic syndrome). The most important regulator in the obesogenic phenotype is peroxisome proliferation active receptor gamma (PPAR γ; Figure 2A) [32]. His expression alone can successfully drive adipocyte differentiation and adipogenesis [33] from

mesenchymal stem/precursor cells, which results in an accumulation of more (hyperplasia) and/or bigger (hypertrophia) fat cells within the body (Figure 2B). Next to PPAR, other genes associated with adipogenesis are described to be altered in overweight/obesity (Figure 2).

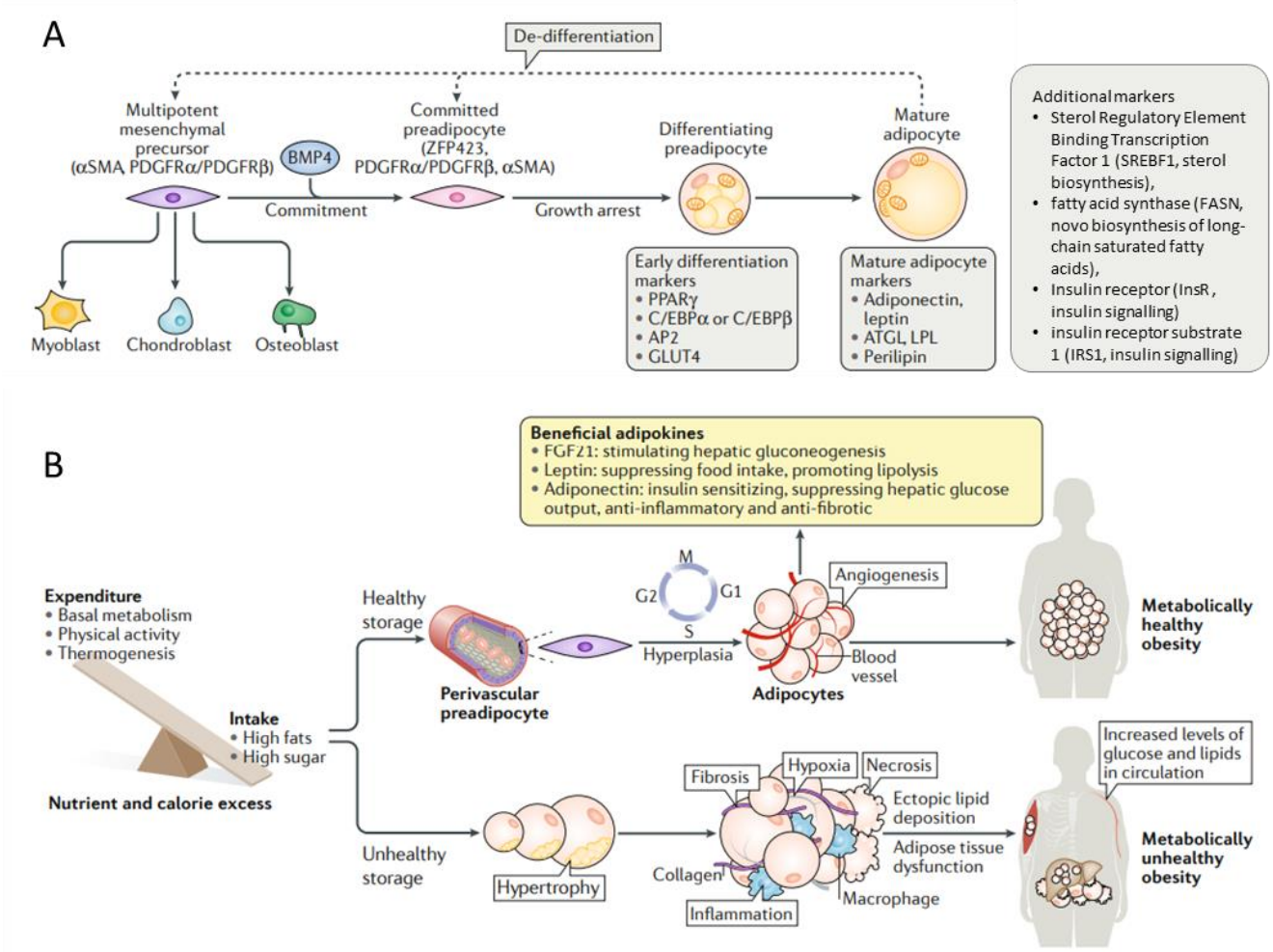

Figure 2: Mechanisms involved in adipogenesis: *This figure/legend is reprinted and adapted from the original first publication in Ghaben et al., Nat Rev Mol Cell Biol, 2019* [32] *with permission from the applicable society / copyright owner.* **(A)** Multipotent, fibroblast-like mesenchymal stem cells/precursors serve as adipocyte precursors (but would also be able to differentiate into myoblasts, osteoblasts and chondroblasts under specific growth conditions). Bone morphogenetic protein (BMP) signaling restricts these mesenchymal precursors to the adipocyte lineage ('commitment'). When the committed preadipocyte arrests its growth, it activates the master regulator of adipogenesis peroxisome proliferator-activated receptor-γ (PPARγ) and transcription co-activators CCAAT/enhancer binding protein α and β (C/EBPα and C/EBPβ). The following lipid accumulation drives the expression of the adipocyte fatty-acid binding protein (AP2) and the insulin-sensitive transporter GLUT4, marking adipocytes in early stages of differentiation. At the completion of differentiation, mature adipocytes express all the markers of early adipocyte differentiation as well as the peptide hormones adiponectin and leptin; the lipases adipose triglyceride lipase (ATGL) and lipoprotein lipase (LPL); and high levels of the lipid-droplet-associated protein perilipin 1. **(B)** When caloric excess exceeds energy expenditure, extra calories are stored in adipose tissue. Adipose tissue can primarily expand in two ways (1) through differentiation of resident tissue precursors to form new adipocytes (hyperplasia) or (2) through enlargement of existing adipocytes (hypertrophy). Hyperplasia is generally considered healthy, since the tissue is able to maintain proper vascularization and insulin-sensitizing, maintain the production of anti-inflammatory adiponectin and other metabolism-modulatory adipocytes. Hypertrophy is associated with an increase in hypoxia experienced by the cells because of their massively expanded size and the inability of the hypoxic tissue to induce vascularization. Instead, hypoxic adipose tissue increases the expression of pro-fibrotic genes and leads to tissue fibrosis. In some cases, these processes also go along with cell necrosis, leading to infiltration of immune cells causing tissue inflammation. All these processes decrease adipose tissue function, contribute to elevated blood levels (e.g. of lipids and glucose) and contribute to the development of metabolic disease with lipid deposition in other tissues, such as muscle and liver.

For overweight and obesity, both lifestyle factors such as a high caloric food intake and a predominantly sedentary behavior, as well as genetic predisposition contribute to the disease risk. However, these factors alone cannot explain the fast increase in obesity rates all over the world. Therefore, additional priming via lifestyle and environmental factors gained increasing attention in the scientific community [34] [35] to potentially find underlying mechanisms that might offer better prevention strategies.

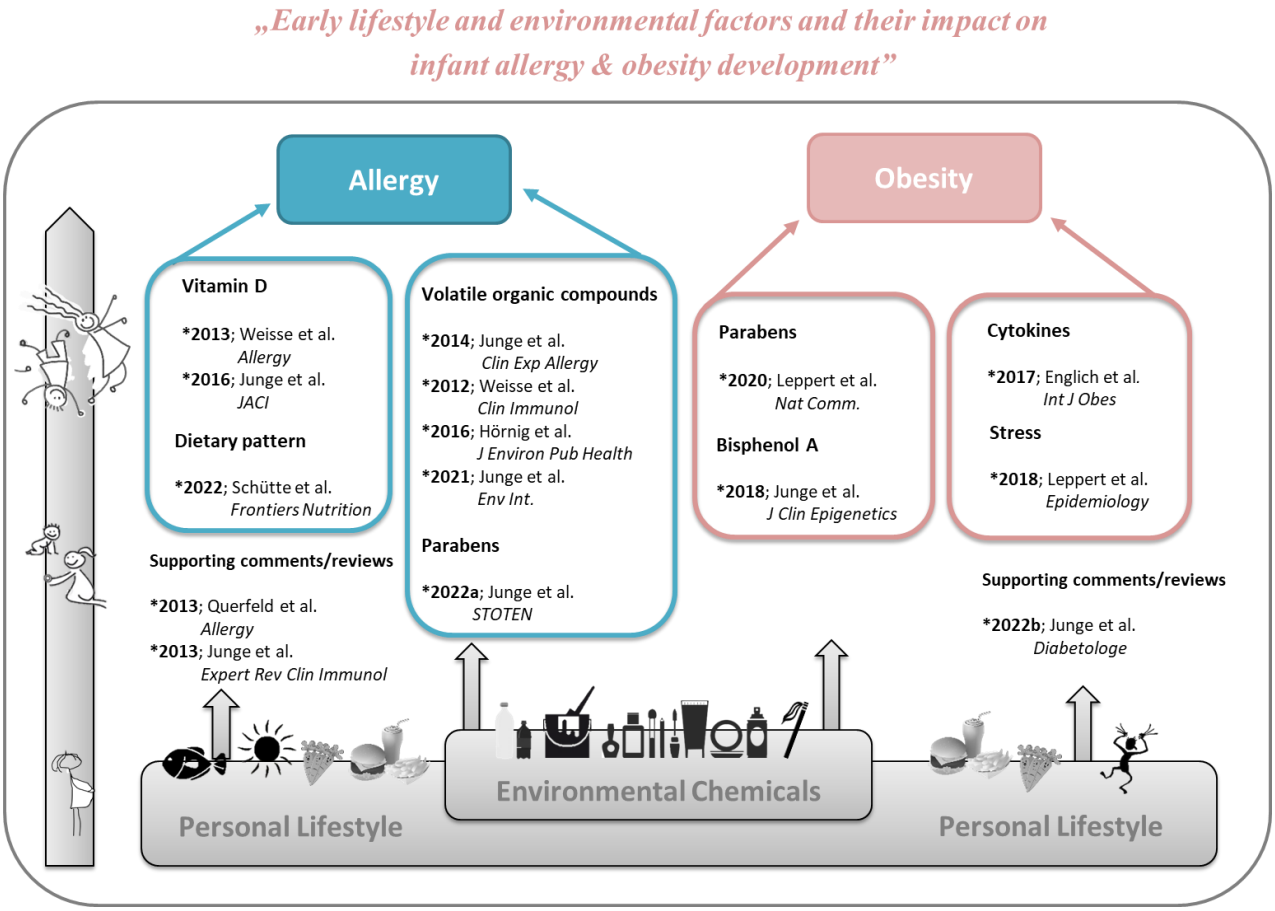

Figure 3: Overview of the investigated topic „Early lifestyle and environmental factors and their impact on infant allergy & obesity development" based on 12 original per reviewed research articles as first/senior author (in boxes: blue in the field of allergy, red in the field of obesity), supported by comments/reviews as first/senior author. Mechanistically, the main research of this thesis was based on epidemiological data from the mother child cohort LINA in combination with data from disease associated in vitro stem cell systems (hematopoietic as well as mesenchymal stem cells), and supported by epigenetic data. © Graphic designed by Kristin Junge

Overall, this work was aiming on the identification of early personal lifestyle or environmental factors the pregnant mother or the newborn/infant is exposed to which impact early infant allergies or obesity. This thesis is based on 12 original manuscripts as first/senior author (in blue or red boxes, see Figure 3) which addressed the following **research questions**:

- In terms of **allergies**, it was of particular interest if and how maternal or early infant **vitamin D** levels were associated with the disease development (Weisse et al., 2013), and if there were epigenetic regulations involved (Junge et al., 2016). Additionally, we were interested in the nutritional pattern of these allergic children later in life (Schütte et al., 2022).

- Further, it was under intensive investigation, how early indoor inhalation exposure to **volatile organic compounds** emitted by cigarette smoking, cleaning or renovation activities impact **allergy** development. Herein, particular focus was put on the potential mediating effect of allergy relevant hematopoietic stem cells (eosinophil/basophil progenitor cells) at different infant ages (birth: Junge et al. 2014, year one: Weisse et al., 2012, year two: Hörnig et al., 2016). Furthermore, VOCs emitted by wood products were under investigation with respect to asthma development (Junge et al. 2021). This project particularly focused on the potential mixture effect of a multiple daily life exposure to several of these wood emitted VOCs.

- Additionally, early exposure to **endocrine disrupting chemicals**, in particular different parabens, used as conservatives in cosmetics or food, were analyzed in the context of **allergy/asthma** development (Junge et al. 2022a). Additionally in this project, the potential mixture effect of a multiple daily life exposure to several of these parabens was addressed.

- Early exposure to **endocrine disrupting chemicals** has also been analyzed in the context of infant **obesity** development. Here, prenatal exposure to Bisphenol A (Junge et al, 2018) or different parabens (Leppert et al. 2020) was under investigation. Both projects were performed in a 3-dimensional setting, combining epidemiological cohort data with in vitro and in vivo analyses. Again, a deep focus was to investigate if epigenetic regulations were involved in the effect transmission from the mother to the offspring.

- In terms of **obesity**, also personal maternal health and lifestyle was of interest: It was analyzed how perinatal **maternal perceived stress** and prenatal maternal **cytokine milieu** were priming the weight development of the children later in life.

- In general, the research questions are mainly addressed in a translational setting combining data from a human epidemiological prospective cohort study (LINA) with mechanistic investigations via disease associated stem cell culture models (Allergy: eosinophil/basophil progenitor cell differentiation/hematopoietic stem cells, Obesity: adipocyte differentiation from mesenchymal stem cells) with particular focus on early priming via epigenetics.

  For mechanistically trans-generational in vivo analyses, several projects also included mouse experiments (Junge et al., 2022a, Junge et al. 2021, Leppert et al. 2020, Junge et al. 2018). These experiments have been performed in cooperation with / headed by PD Dr. Tobias Polte and the Helmholtz University Research Group 'Experimental Allergy and Immunology'.

2 Original work

The following articles are included in the present thesis; they are listed according to their appearance in the text. Original manuscripts **1-8** cover investigations on „Early lifestyle and environmental factors and their impact on infant allergy development" (blue color code in Figure 3), manuscripts **9-12** investigations on „Early lifestyle and environmental factors and their impact on infant obesity development" (red color code in Figure 3). All manuscripts are based on first or senior authorships (in part as shared authorship, marked with *). Manuscripts **a-c** are context related comments or reviews that have also been published as first or senior author. *Copyright: All work is reprinted from the original first publication listed below, with permission from APPLICABLE SOCIETY / COPYRIGHT OWNER.*

Vitamin D and allergy development:

1) **Weisse K**, Winkler S, Hirche F, Herberth G, Hinz D, Bauer M, Röder S, Rolle-Kampczyk U, von Bergen M, Olek S, Sack U, Richter T, Diez U, Borte M, Stangl GI, Lehmann I. Maternal and newborn vitamin D status and its impact on food allergy development in the German LINA cohort study (2013) Allergy 68(2):220-8

   Supporting information/supplements available at:
   https://onlinelibrary.wiley.com/doi/full/10.1111/all.12081

a) Querfeld U, Keil T, Beyer K, Stock P & Pilz S, März W & **Weisse K**, Lehmann I. Vitamin D in early life: good or bad for food allergies? (2013) Allergy. Aug;68(8):1081-3

b) **Junge KM**, Lehmann I & Borte M. Can vitamin D intake during pregnancy affect the risk of allergy in children? (2013) Expert Rev Clin Immunol 9 (8):699-701

2) **Junge KM,** Bauer T, Geissler S, Hirche F, Thürmann L, Bauer M, Trump S, Bieg M, Weichenhan D, Malm JP, Ishaque N, Mücke O, Röder S, Herberth G, Diez U, Borte M, Rippe K, Plass C, Hermann C, Stangl GI & Eils R & Lehmann I. Increased vitamin D levels at birth and in early infancy increase rather than reduce offspring allergy risk – evidence for involvement of epigenetic mechanisms (2016) JACI 137(2):610-3

   Supporting information/supplements available at:
   https://www.sciencedirect.com/science/article/abs/pii/S0091674915009410?via%3Dihub

3) Schütte O, Bachmann L, Shivappa N, Hebert JR, Felix JF, Röder S, Sack U, Borte M, Kiess W, Zenclussen AC, Stangl GI, Herberth G, **Junge KM**. Pro-inflammatory diet pictured in children with atopic dermatitis or food allergy: Nutritional data of the LINA cohort. Front Nutr. 2022 Apr 8;9:868872

Supporting information/supplements available at:
https://www.frontiersin.org/articles/10.3389/fnut.2022.868872/full#supplementary-material

Volatile organic compounds and allergy development:

4) **Junge KM**, Hörnig F, Herberth G, Röder S, Kohajda T, Rolle-Kampczyk U, von Bergen M, Borte M, Simon JC, Heroux D, Denburg JA, Lehmann I. The LINA Cohort: Cord Blood Eosinophil/Basophil Progenitors Predict Respiratory Outcomes in Early Infancy (2014) Clin Immunol 152 (1-2): 68-76

5) **Weisse K,** Lehmann I, Heroux D, Kohajda T, Herberth G, Röder S, von Bergen M, Borte M, Denburg J. The LINA cohort: indoor chemical exposure, circulating eosinophil/basophil (Eo/B) progenitors and early life skin manifestations (2012) Clin. Exp. Allergy 42 (9):1337 – 1346

Supporting information/supplements available at:
https://onlinelibrary.wiley.com/action/downloadSupplement?doi=10.1111%2Fj.1365-2222.2012.04024.x&file=cea4024-sup-0001-Table-Figure.doc

6) Hoernig F, Kohajda T, Roeder S, Herberth G, von Bergen M, Borte M, Diez U, Rolle-Kampczyk U, Simon JC, Denburg JA, Lehmann I, **Junge KM**. The LINA study: Higher sensitivity of infant compared to maternal Eo/B progenitor cells to indoor chemical exposures (2016) Env Publ Health: 2016:5293932.

Supporting information/supplements available at:
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4899584/5293932.f1.pdf

7) **Junge KM**, Buchenauer L, Elter E, Butter K, Kohajda T, Herberth G, Röder S, Borte M, Kiess W, von Bergen M, Simon JC, Rolle-Kampczyk UE, Lehmann I, Gminski R, Ohlmeyer M, Polte T. Wood emissions and asthma development: Results from an experimental mouse model and a prospective cohort study (2021). Environ Int. 2021 151:106449

Supporting information/supplements available at:
https://www.sciencedirect.com/science/article/pii/S016041202100074X?via%3Dihub#s0155

Endocrine disruptors (parabens) and allergy development:

8) **Junge KM**, Buchenauer L, Strunz S, Seiwert B, Thürmann L, Rolle-Kampczyk UE, Röder S, Borte M, Kiess W, von Bergen M, Simon JC, Zenclussen AC, Schöneberg T, Stangl GI, Herberth G, Lehmann I, Reemtsma T, Polte T. Effects of exposure to single and multiple parabens on asthma development in an experimental mouse model and a prospective cohort study (2022). Sci Total Environ. 2022 25;814:152676

Supporting information/supplements available at:
https://doi.org/10.1016/j.envint.2021.106449

Endocrine disruptors (parabens and bisphenol A) and obesity development:

9) Leppert B, Strunz S, Seiwert B, Schlittenbauer L,Schlichting R, Pfeiffer C, Röder S, Borte M, Stangl GI, Schöneberg T, Schulz A, Rolle-Kampczyk UE, Thürmann L, von Bergen M, Escher BI, **Junge KM*,** Reemtsma T*, Lehmann I*, Polte T*: Maternal paraben exposure triggers childhood overweight development (2020) Nat Comm 11, 561

Supporting information/supplements available at:
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7012887

10) **Junge KM**, Leppert B, Jahreis S, Wissenbach DK,Feltens R, Grützmann K, Thürmann L, Bauer T, Ishaque N, Schick, Bewerunge-Hudler M, Röder S, Bauer M, Schulz A,,Borte M, Landgraf K, Körner A, Kiess W, von Bergen M, Stangl GI, Trump S, Eils R & Polte T & Lehmann I. MEST mediates the impact of prenatal bisphenol A exposure on long-term body weight development (2018) Clin Epigenetics.2018 Apr 20;10:58

Supporting information/supplements available at:
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5910578

Cytokine and stress levels and obesity development:

11) Englich B, Herberth G, Rolle-Kampczyk U, Trump S, Röder S, Borte M, Stangl GI, von Bergen M, Lehmann I, **Junge KM**. Maternal cytokine status may prime the metabolic profile and increase risk for obesity in children (2017) Int J Obes. 2017 May 10. Doi: 10.1038/ijo.2017.113

Supporting information/supplements available at:
https://www.nature.com/articles/ijo2017113#Sec14

12) Leppert B*, **Junge KM***, Röder S, Borte M, Stangl GI,Wrigh RJ, Hilbert A, Lehmann I & Trump S. Longitudinal maternal stress assessment in LINA: Stress characteristics and impact on weight development in early infancy (2018) BMC Public Health

Supporting information/supplements available at:
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6208039

c) **Junge KM**, Zenclussen AC, Gernot Desoye. Themenheft „Fetale Programmierung und Diabetesprävention: (Über)Ernährung und Einfluss auf Funktion der Plazenta – Veränderungen im materno-fötalen Austausch bei Adipositas und Gestationsdiabetes Diabetologe. Springer Medizin Verlag GmbH (Accepted, to be published Dec.2022)

2.1 Vitamin D and allergy development

1) **Weisse K**, Winkler S, Hirche F, Herberth G, Hinz D, Bauer M, Röder S, Rolle-Kampczyk U, von Bergen M, Olek S, Sack U, Richter T, Diez U, Borte M, Stangl GI, Lehmann I. Maternal and newborn vitamin D status and its impact on food allergy development in the German LINA cohort study (2013) Allergy 68(2):220-8

a) Querfeld U, Keil T, Beyer K, Stock P, Pilz S, März W, **Weisse K**, Lehmann I. Vitamin D in early life: good or bad for food allergies? (2013) Allergy. Aug; 68(8):1081-3

b) **Junge KM**, Lehmann I & Borte M. Can vitamin D intake during pregnancy affect the risk of allergy in children? (2013) Expert Rev Clin Immunol 9 (8):699-701

2) **Junge KM,** Bauer T, Geissler S, Hirche F, Thürmann L, Bauer M, Trump S, Bieg M, Weichenhan D, Malm JP, Ishaque N, Mücke O, Röder S, Herberth G, Diez U, Borte M, Rippe K, Plass C, Hermann C, Stangl GI & Eils R & Lehmann I. Increased vitamin D levels at birth and in early infancy increase rather than reduce offspring allergy risk – evidence for involvement of epigenetic mechanisms (2016) JACI 137(2):610-3

3) Schütte O, Bachmann L, Shivappa N, Hebert JR, Felix JF, Röder S, Sack U, Borte M, Kiess W, Zenclussen AC, Stangl GI, Herberth G, **Junge KM**. Pro-inflammatory diet pictured in children with atopic dermatitis or food allergy: Nutritional data of the LiNA cohort. Front Nutr. 2022 Apr 8;9:868872

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ORIGINAL ARTICLE EPIDEMIOLOGY AND GENETICS

Maternal and newborn vitamin D status and its impact on food allergy development in the German LINA cohort study

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Abstract

Background: Vitamin D levels are known to be associated with atopic disease development; however, existing data are controversial. The aim of this study was to investigate whether corresponding maternal and cord blood vitamin D levels are associated with atopic outcomes in early infancy.

Methods: Within the LINA cohort study (Lifestyle and environmental factors and their Influence on Newborns Allergy risk), 25(OH)D was measured in blood samples of 378 mother–child pairs during pregnancy and at birth. Information about children's atopic manifestations during the first 2 years of life was obtained from questionnaires filled out by the parents during pregnancy and annually thereafter. Cord blood regulatory T cells (Treg) were detected by methylation-specific PCR using a Treg-specific demethylated region in the *FOXP3* gene.

Results: The median maternal $25(OH)D_3$ level was 22.19 ng/ml (IQR 14.40–31.19 ng/ml); the median cord blood $25(OH)D_3$ 10.95 ng/ml (6.99–17.39 ng/ml). A high correlation was seen between maternal and cord blood $25(OH)D_3$ levels, both showing a seasonal distribution. Maternal and cord blood $25(OH)D_3$ was positively associated with children's risk for food allergy within the first 2 years. Further, higher maternal $25(OH)D_3$ resulted in a higher risk for sensitization against food allergens at the age of two. Cord blood $25(OH)D_3$ levels were negatively correlated with regulatory T cell numbers.

Conclusion: Our study demonstrates that high vitamin D levels in pregnancy and at birth may contribute to a higher risk for food allergy and therefore argues against vitamin D supplement to protect against allergy.

In recent years, there has been a worldwide increase in the prevalence of allergic diseases, which greatly disrupts the lives of those affected. Many risk factors for the development of atopic diseases, including genetic background, individual immune response as well as lifestyle and environmental conditions, have been identified (1). One factor that also seems to interfere with the pathology of atopy, next to its osseous endocrine function, is vitamin D. The discussion about vitamin D as a risk factor for allergic diseases was raised following a publication, which showed an association between vitamin D supplementation due to rickets prevention with an increase in allergy prevalence throughout the 20th century (2). Thereafter, several birth cohort studies reported conflicting data on the risk and prevention of allergy following vitamin D exposure (3–5). On the one hand, *low* infantile vitamin D was associated with higher rates of atopic disease at the age

of 6 and 14 years (6) and enhanced eczema severity in children aged between 8 months and 12 years (7). Low maternal vitamin D levels during pregnancy were shown to increase the risk for infantile atopic dermatitis (8) and the severity of allergic rhinitis and asthma in the offspring (9). On the other hand, *high* vitamin D levels, especially as a consequence of vitamin D supplementation, were described to increase the risk of allergic disease (2, 10, 11). Children born to mothers with high (>30 ng/ml) vitamin D levels were more likely to develop offspring eczema compared to mothers with low (<10 ng/ml) vitamin D concentrations (12). Excessive vitamin D supplementation in early childhood has also been shown to increase the risk of sensitization to inhalant or food allergens, allergic rhinitis or asthma in children and adults (13–15).

To date, there are limited studies addressing the prenatal vitamin D status and its impact on the newborn immune status and later infantile atopic outcomes. Especially, the debate about the recommendation of a vitamin D supplementation during pregnancy or in early childhood to reduce the allergy risk of the child requires more and precise data on the role of vitamin D in the disease pathology. Therefore, the aim of this study was to investigate whether and how corresponding vitamin D levels of pregnant mothers and newborns are associated with the immune status at the time of birth and atopic outcomes in early infancy.

Methods

Study design and sample collection

Within the LINA cohort study (**L**ifestyle and environmental factors and their **I**nfluence on **N**ewborns **A**llergy risk), 629 mother–child pairs (622 mothers and 629 children; 7 twins) were recruited between May 2006 and December 2008 in Leipzig, Germany (51.4°N), to investigate the influence of lifestyle and environmental factors in the pre- and postnatal period on the immune system of the newborn and the child later in life, along with the risk of developing allergic disease. Mothers suffering from immune or infectious diseases during pregnancy were excluded from the study.

Blood samples were obtained from mothers at 34th week of pregnancy and cord blood at delivery. After birth, blood samples were collected annually from both the mother and child. During pregnancy and at children's first and second birthday, standardized questionnaires were recorded, collecting data about family history of atopy (FHA), housing and environmental conditions (first- or second-hand smoke, mould, traffic, noise, pets, renovation activities, personal lifestyle, etc.), as well as atopic outcomes of the children. All questionnaires were self-administered by the parents. Participation in the study was voluntary, and informed consent was obtained from all participants. The study was approved by the Ethics Committees of the University of Leipzig (046-2006, 160-2008).

Atopic outcomes

Atopic dermatitis was recorded as a parental report of a doctor-diagnosed dermatitis or of dermatitis symptoms during the last 12 months. Children were classified as having dermatitis symptoms when parents reported an intermittent itchy skin rash, which affected places other than the nappy area and lasted at least 2 weeks, or appeared repeatedly in the last 12 months. Food allergy was recorded as a parental report of a doctor diagnosis.

Analyses of serum 25(OH)D

In a LINA subgroup of 378 mother–child pairs (374 mothers and 378 children; 4 twins; Fig. 1), serum 25 hydroxy-vitamin D (25(OH)D) levels were measured. Cord and maternal vitamin D concentrations were determined by liquid chromatography tandem mass spectroscopy (HPLC-MS/MS) by the laboratory of the Institute of Agricultural and Nutrition Science, Martin-Luther-University Halle/Wittenberg (Halle/Saale, Germany) as previously described (16, 17). Briefly, 25 $(OH)D_3$ and $25(OH)D_2$ were obtained from standards, cord and maternal serum via protein precipitation using the HPLC-MS/MS *MassChrom* Reagent Kit (Chromsystems, Munich, Germany). Analysis was performed by Agilent 1100 HPLC (Agilent Technologies, Böblingen, Germany) with an API 2000 triple quadrupole mass spectrometer (Applied Biosystems, Darmstadt, Germany). A high-resolution analytical column was used for cord blood samples to eliminate the C3-Epimer of $25(OH)D_3$. Subsequent to chromatographic separation, $25(OH)D_3$ and $25(OH)D_2$ molecules were ionized in an atmospheric-pressure chemical-ionization source, followed by nitrogen collision–induced dissociation. The generated mass-specific ion fragments were detected with m/z 395/269

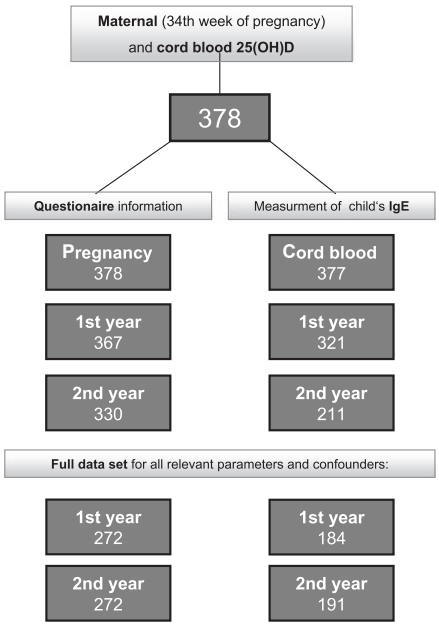

Fig. 1 Flow chart of the analysed LINA subcohort and resulting mother–child pairs after consideration of available measurements and questionnaire information.

for 25(OH)D_2, *m/z* 383/211 for 25(OH)D_3, *m/z* 383/211 for 3-Epi-25(OH)D_3 and *m/z* 389/211 for 25(OH)D_3-d_6 (internal standard).

The assay detection limit was defined as 6.7 ng/ml for maternal 25(OH)D_3 and 5.2 ng/ml for maternal 25(OH)D_2. Detection limit for cord blood 25(OH)D_3 and D_2 was 3 ng/ml. Samples with 25(OH)D values below detection limit are included in analyses using half of the defined detection limit (e.g. 1.5 ng/ml for cord blood 25(OH)D_3).

Analyses of IgE

Total IgE, as well as IgE specific for food allergens (fx5) or inhalative allergens (sx1), were determined at birth in the cord blood (only total IgE) and at one and two years of age by Phadia CAP System (Phadia Diagnostics, Freiburg, Germany). Total IgE levels > 0.7 kU/l in cord blood and > 3.8 kU/l at the age of one or two were classified as 'increased', as well as specific IgE (sx1 or fx5) > 0.35 kU/l. Values below the detection limit were included in analyses using half of the defined detection limit.

Analyses of regulatory T cells

Quantification of regulatory T cells was performed as described earlier (for details, see Hinz et al. (18)). Genomic DNA was isolated from whole blood using the DNA Blood Mini Kit (Qiagen, Hilden, Germany) and bisulphite-treated using the EpiTect 96 Bisulfite Kit (Qiagen, Hilden, Germany). Quantification of demethylation in Treg-specific demethylated region (TSDR) was performed by real-time PCR by Epiontis (Berlin, Germany). In the total LINA cohort (n = 629), Treg numbers were analysed in 426 cord bloods. Only samples with a copy number >10 were considered. As previously described, the number of Tregs in cord blood is presented as percentage corresponding to the measured amount of TSDR demethylation in the *FOXP3* gene (19, 20).

Statistical analyses

25(OH)D levels were not normally distributed; therefore, analyses were performed using nonparametric tests in general and are presented as medians with 25th-75th percentile. The chi-square test for cross-relationship was used to compare the analysed subcohort with the total cohort. Spearman's rank correlation test was performed to analyse the correlation between maternal and newborn 25(OH)D. Seasonal distribution of 25(OH)D concentration was calculated using ANOVA. The relationship between atopic outcomes and vitamin D levels (divided in quartiles) was addressed using the Mann–Whitney *U*-test. In addition, multivariate logistic regression models were used to consider possible confounding factors. Here, data are presented as odds ratios with 95% confidence interval adjusted for gender, number of siblings, increased cord blood IgE levels, FHA, cotinine levels during pregnancy, breast feeding, UV intensity at birth and vitamin D supplementation during the first year of life. The UV intensity at birth was classified according to the German Federal Office for Radiation Protection (Bundesamt für Strahlenschutz) in *low* (category 0–2, which is measured from October to March) and *high* UV intensity (category 3–7, which is measured from April to September) (21). Vitamin D supplementation was recorded as a parental report of vitamin D supplementation ever within the child's first year of life.

All P-values < 0.05 were considered to be significant. Adjustments due to multiple testing were not made because our analyses were based on *a priori* hypothesis (22). Statistical analyses were performed with STATISTICA for Windows, version 10 (Statsoft Inc. (Europe), Hamburg, Germany). For more details, see methods in the supplements.

Results

Characteristics of the analysed LINA subcohort

Characteristics of the analysed subcohort compared to the total LINA cohort are presented in Table 1. There were no differences between the analysed subcohort (n = 378) and the total cohort (n = 629) for all listed characteristics.

Vitamin D levels

Measured 25(OH)D_2 values were below the detection limit in each sample and were not included in further analyses. The median cord blood 25(OH)D_3 level was 10.95 ng/ml (interquartile range 6.99–17.39 ng/ml), and the median maternal blood 25(OH)D_3 concentration was 22.19 ng/ml (14.40–31.19 ng/ml; Table 2).

A high correlation was seen between maternal and cord blood 25(OH)D_3 levels (R = 0.812, P < 0.001; Fig. 2). Furthermore, both maternal and cord blood 25(OH)D_3 levels followed a significant seasonal distribution (P < 0.05, Fig. 3).

Of all pregnant mothers, 44.4% had deficient (<20 ng/ml), 25.7% had insufficient (20–29.9 ng/ml), and 29.9% had optimal (>30 ng/ml) 25(OH)D_3 levels (cut-offs according to (4)). 50.0% of the newborns had deficient 25(OH)D_3 levels (<11 ng/ml, cut-offs according to (23)). Only seven mothers obtained vitamin D supplements during pregnancy. There were no significant differences between the 25(OH)D_3 levels from mothers with (median 27.4 ng/ml) or without (median 22.2 ng/ml; Mann–Whitney *U*-test P = 0.411) vitamin D supplementation.

Infantile outcomes and IgE levels

Frequencies of atopic outcomes within the first 24 months are shown in Table 3(a). Within the analysed subcohort, 18.8% of the children were positive for symptoms of an atopic dermatitis within the first 24 months, 14.0% for physician-diagnosed atopic dermatitis and 5.5% for food allergy. Levels of children's IgE are shown in Table 3(b). Within the first 24 months, 87.5% of the children had increased total IgE, 22.3% had sensitizations against food allergens and 6.0% against inhalant allergens.

Table 1 Characteristics of the analysed subcohort compared to the total LINA cohort

	Analysed subcohort *N* = 378 *n* (%)	Entire LINA cohort *N* = 629 *n* (%)	*P*-value*
Gender of the child			
Male	196 (51.9)	327 (52.0)	0.967
Female	182 (48.1)	302 (48.0)	
Number of siblings			
>0	258 (68.3)	418 (66.5)	0.748
>1	92 (24.3)	156 (24.8)	
>2	24 (6.3)	45 (7.2)	
>3	2 (0.5)	8 (1.3)	
≥4	2 (0.5)	2 (0.3)	
Parental history of atopy			
None	129 (34.1)	210 (33.4)	0.980
Single	176 (46.6)	294 (46.7)	
Double	72 (19.4)	121 (19.2)	
Breast feeding			
≤3 months	64 (16.9)	112 (17.8)	0.712
≥4 months	270 (71.4)	443 (70.4)	
Total IgE in cord serum			
Increased†	65 (17.2)	84 (13.4)	0.684
Not increased	312 (82.5)	434 (69.0)	
UV intensity of month of birth‡			
Low	162 (42.9)	258 (40.4)	0.632
High	205 (54.2)	348 (54.5)	
Vitamin D supplementation during the first year of life§			
Yes	337 (91.8)	560 (92.4)	0.761
No	30 (8.2)	46 (7.6)	
Cotinine (μg/g) 34th week of pregnancy, log median (IQR)	0.65 (−0.22–1.67)	0.72 (−0.27–1.76)	0.391¶

*P-value from the chi-square test for cross-table relationship.
†IgE level > 0.7 kU/l.
‡According to the German Federal Office for Radiation Protection (www.bfs.de).
§23 mother–child pairs did not participate in the 1-year follow-up (entire LINA cohort *n* = 606, subcohort *n* = 367).
¶*P*-value from Student's *t*-test.

Table 2 Analysed maternal and cord blood 25(OH)D_3 concentrations

	Maternal 25(OH)D_3		Cord blood 25(OH)D_3	
	ng/ml (nM)	*N* = 374 *n* (%)	ng/ml (nM)	*N* = 378 *n* (%)
Median	22.2 (55.41)		10.95 (27.33)	
Quartiles				
1st Quartile	6.13–14.39 (15.3–35.92)	91 (24.3)	1.50–6.98 (3.74–17.42)	94 (24.9)
2nd Quartile	14.40–22.19 (35.94–55.39)	95 (25.4)	6.99–10.94 (17.45–27.30)	95 (25.1)
3rd Quartile	22.20–32.19 (55.40–80.35)	95 (25.4)	10.95–17.39 (27.33–43.40)	93 (24.6)
4th Quartile	32.20–60.80 (80.37–151.76)	93 (24.9)	17.40–40.10 (43.43–100.10)	96 (25.4)

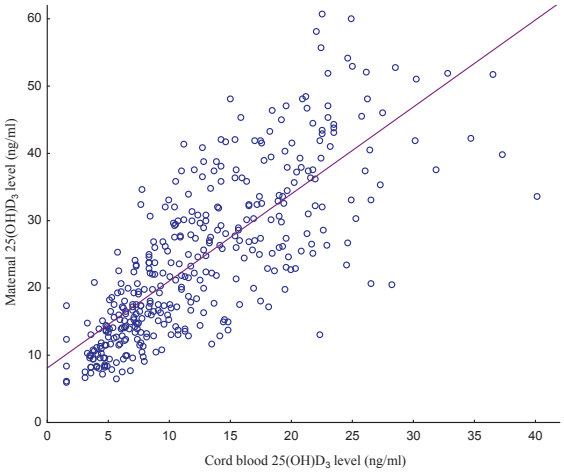

Fig. 2 Correlation between maternal and cord blood 25(OH)D_3 levels (ng/ml).

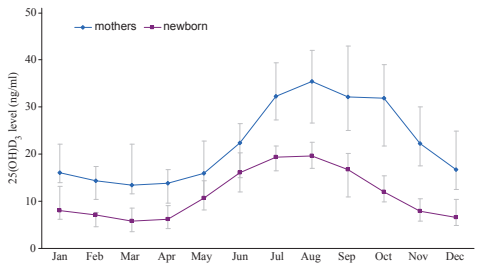

Fig. 3 Seasonal variation in 25(OH)D_3 levels (ng/ml) of mothers and newborns. Data are presented as medians with 25–75th percentile.

Vitamin D levels and outcomes

In a multivariate logistic regression model adjusted for known confounders (gender, number of siblings, increased cord blood total IgE levels, family atopy history, cotinine levels during pregnancy, breast feeding, UV intensity at birth and vitamin D supplementation within the first year of life), maternal 25(OH)D_3 levels were positively associated with questionnaire documented food allergy of the child (adj OR 3.66 [95% CI: 1.36–9.87] Table 4) within the second year of life or within the 2-year lifetime period (adj OR 1.91 [95% CI: 1.09–3.37]). Furthermore, maternal 25(OH)D_3 levels were positively associated with child's IgE specific to food allergens (fx5) at the age of two (adj OR 1.59 [95% CI: 1.04–2.45]). Neither atopic eczema, symptomatic or physician diagnosed, nor total IgE levels and IgE levels specific to inhalant

Table 3 Prevalence of (a) atopic outcomes and (b) increased IgE levels within the first or second year of life or 2-year lifetime period of the child

(a) Atopic outcomes	First year of life *N* = 272 % (*n*)	Second year of life *N* = 272 % (*n*)	Two-year lifetime period *N* = 272 % (*n*)
Atopic eczema (symptoms)			
Positive	11.4 (31)	11.8 (32)	18.8 (51)
Negative	88.6 (241)	88.2 (240)	81.2 (221)
Atopic eczema (diagnosed)			
Positive	9.9 (27)	8.8 (24)	14.0 (38)
Negative	90.1 (245)	91.2 (248)	86.0 (234)
Food allergy (diagnosed)			
Positive	3.3 (9)	2.9 (8)	5.5 (15)
Negative	96.7 (263)	97.1 (264)	94.5 (257)
(b) IgE levels	First year of life *N* = 184 % (*n*)	Second year of life *N* = 191 % (*n*)	Two-year lifetime period *N* = 184 % (*n*)
Total IgE*			
Increased	78.3 (144)	82.2 (157)	87.5 (161)
Not increased	21.7 (40)	17.8 (34)	12.5 (23)
Food allergens (fx5)†			
Increased	15.8 (29)	13.1 (25)	22.3 (41)
Not increased	84.2 (155)	86.9 (166)	77.7 (143)
Inhalative allergens (sx1)†			
Increased	3.8 (7)	4.7 (9)	6.0 (11)
Not increased	96.2 (177)	95.3 (182)	94.0 (173)

*IgE cut-off: 3.8 kU/l.
†IgE cut-off: 0.35 kU/l (CAP-class >0).

allergens (sx1) showed a significant association with maternal vitamin D.

Similarly, cord blood $25(OH)D_3$ was positively associated with questionnaire documented food allergy within the second year of life (adj OR 4.65 [95% CI: 1.50–14.48] Table 5). No association was found between cord blood vitamin D and infantile food allergy within the first year of life, as well as for atopic eczema, either symptomatic or physician diagnosed, total IgE or specific IgE (fx5 and sx1) levels at all times points.

Vitamin D level and cord blood regulatory T cells

Using samples from the LINA study, we have previously shown an association between the number of cord blood regulatory T cells and the occurrence of sensitizations against food allergens (fx5) later in life (18). We hypothesized that Treg may represent the mechanistic link between vitamin D and food allergy. Therefore, we have investigated in the present study whether maternal or cord blood $25(OH)D_3$ was associated with cord blood Treg numbers. We observed a negative correlation between cord blood vitamin D and cord blood Treg numbers ($R = -0.168$, $P = 0.031$; Fig. 4). For maternal vitamin D, this negative correlation was only seen in trend ($R = -0.143$, $P = 0.067$).

Discussion

In the present paper, we showed that maternal and cord blood $25(OH)D_3$ levels are positively associated with children's risk for food allergy or sensitization against food allergens within the first 2 years of life. Furthermore, we found a negative correlation between cord blood $25(OH)D_3$ levels and regulatory T cell numbers, which may be a contributing mechanism in the vitamin D-mediated allergy development.

In our study, the vitamin D status of pregnant mothers and their corresponding newborns was assessed in Leipzig, Germany. Majority of the pregnant mothers from our study had deficient (<20 ng/ml) or insufficient (20–29.9 ng/ml) 25 $(OH)D_3$ levels, according to the suggested cut-offs (4). Also half of the newborns had deficient $25(OH)D_3$ levels based on the cut-off level of <11 ng/ml for $25(OH)D_3$ (23). Comparable data have already been shown in other nonequatorial regions such as United States (23, 24), New Zealand (25) and Australia (26, 27). Even in areas near the equator, characterized by high UV intensity, vitamin D deficiencies were reported, for example in Costa Rica (18.6°N) where 28% of children, between 6 and 14 years old, were found to have insufficient or deficient vitamin D levels (28).

In the LINA study, we found a high correlation between $25(OH)D_3$ of pregnant mothers and their newborns, which was also observed in Pakistan (29) and Oakland (30) recently. This correlation is the result of the ability of 25 (OH)D and 24,25(OH)D to cross the placenta, thereby exposing the foetus to vitamin D during pregnancy (31). Next to the seasonal variations, it was also obvious that absolute vitamin D concentrations of newborns are consistently lower than maternal levels, which confirms the observations published before (31).

In the present analyses, we also showed that higher maternal or cord blood $25(OH)D_3$ levels were positively associated with a higher risk of developing food allergy or having increased food-specific IgE levels during the second year of life or within the 2-year lifetime period. Therefore, the risk for food allergy development resulting from cord blood 25 $(OH)D_3$ levels was higher compared to that resulting from maternal levels. Thus, it seems that cord blood vitamin D level is more relevant for later food allergy than maternal vitamin D level. However, this could not be confirmed for IgE specific for food allergens.

There is lack of consistent data addressing the association between vitamin D and food allergy or IgE. It was shown that vitamin D is able to both enhance (32) and inhibit (33, 34) the differentiation of plasma cells and IgE production. Nwaru et al. (35) demonstrated in a Finish cohort that dietary intake of vitamin D during pregnancy was inversely associated with IgE specific to food allergens. Liu et al. analysed the impact of certain risk genotypes involved in vitamin

Table 4 Association between maternal 25(OH)D_3 and atopic outcomes or IgE levels of the child

		Maternal 25(OH)D_3, quartiles							
		n (%)				OR (95% CI)			
	N	1st	2nd	3rd	4th	Unadjusted	*P*-value	Adjusted*	*P*-value
First year of life									
Atopic eczema (symptoms)	272	10 (15.2)	8 (11.0)	6 (9.5)	7 (10.0)	0.85 (0.60–1.19)	0.340	0.89 (0.63–1.32)	0.614
Atopic eczema (diagnosed)	272	4 (6.1)	9 (12.3)	7 (11.1)	7 (10.0)	1.12 (0.78–1.60)	0.538	1.16 (0.79–1.71)	0.451
Food allergy (diagnosed)	272	1 (1.5)	3 (4.1)	2 (3.2)	3 (4.3)	1.26 (0.69–2.30)	0.458	1.27 (0.67–2.40)	0.469
Increased total IgE†	184	35 (76.1)	34 (75.6)	41 (87.2)	34 (73.9)	1.03 (0.75–1.41)	0.846	1.06 (0.77–1.47)	0.715
Increased food allergens (fx5)‡	184	7 (15.2)	10 (22.2)	6 (12.8)	6 (13.0)	0.89 (0.62–1.27)	0.509	0.90 (0.61–1.32)	0.585
Second year of life									
Atopic eczema (symptoms)	272	6 (9.1)	10 (13.7)	10 (15.9)	6 (8.6)	1.00 (0.72–1.39)	0.984	1.02 (0.72–1.43)	0.928
Atopic eczema (diagnosed)	272	4 (6.1)	9 (12.3)	5 (7.9)	6 (8.6)	1.03 (0.71–1.50)	0.861	1.13 (0.74–1.72)	0.584
Food allergy (diagnosed)	272	0 (0.0)	0 (0.0)	4 (6.3)	4 (5.7)	**2.79 (1.14–6.82)**	**0.024**	**3.66 (1.36–9.87)**	**0.010**
Increased total IgE†	191	40 (81.6)	38 (82.6)	42 (84.0)	37 (80.4)	0.99 (0.71–1.38)	0.940	0.97 (0.69–1.36)	0.853
Increased food allergens (fx5)‡	191	4 (8.2)	4 (8.7)	8 (16.0)	9 (19.6)	1.46 (0.98–2.16)	0.063	**1.59 (1.04–2.45)**	**0.033**
Two-year lifetime period									
Atopic eczema (symptoms)	272	14 (21.1)	15 (20.5)	11 (17.5)	11 (15.7)	0.88 (0.67–1.16)	0.354	0.93 (0.70–1.24)	0.629
Atopic eczema (diagnosed)	272	6 (9.1)	14 (19.2)	8 (12.7)	10 (14.3)	1.07 (0.79–1.46)	0.655	1.14 (0.81–1.61)	0.444
Food allergy (diagnosed)	272	1 (1.5)	3 (4.1)	5 (7.9)	6 (8.6)	1.66 (1.00–2.76)	0.052	**1.91 (1.09–3.37)**	**0.025**
Increased total IgE†	184	39 (84.8)	38 (84.4)	43 (91.5)	41 (89.1)	1.20 (0.81–1.79)	0.358	1.19 (0.79–1.78)	0.401
Increased food allergens (fx5)‡	184	10 (21.7)	10 (22.2)	12 (25.5)	9 (19.6)	0.98 (0.72–1.34)	0.909	1.04 (0.75–1.45)	0.812

*confounders: gender of the child, number of siblings, cord blood total IgE, family history for atopy, log cotinine (34th week of pregnancy), breast feeding, UV intensity of month of birth and vitamin D supplementation during the first year of life.

†IgE level > 3.8 kU/l.

‡IgE level > 0.35 kU/l (CAP-class >0).

All *P*-values < 0.005, which is in bold, are significant.

D metabolism and IgE regulation on the association between vitamin D levels and food allergen–specific IgE. Comparable to the results from our LINA study, they showed that low-risk children (<2 risk genotypes) with deficient cord blood vitamin D levels (<11 ng/ml) had a lower food allergy frequency compared to children with higher cord blood levels (23). Another group showed a nonlinear (U-shaped) association between cord blood vitamin D and IgE levels through the age of 5 years. In that study, both high (>40 ng/ml) and low (<20 ng/ml) vitamin D levels were associated with high total IgE and IgE specific to certain inhalant allergens (36). Furthermore, they described an association between high, but not low, vitamin D levels and positive skin prick tests (36). A similar nonlinear relationship was also shown in adults (37). In our study, we could not reproduce this U-shaped relationship between IgE levels and vitamin D but confirmed the association between high vitamin D and an increased risk for food allergy up to the age of two.

Data from the present study provide a mechanistic understanding for the increased food allergy risk resulting from high cord blood vitamin D levels. We observed a correlation between high cord blood vitamin D levels and low numbers of cord blood Treg, which are responsible for maintaining immune tolerance. Within our LINA study, we already found that low cord blood Treg numbers are associated with a higher risk to develop atopic outcomes later in life (18). Because a deficient Treg response at birth consequently increases the risk for the development of an allergic immune response, our data suggest that vitamin D may modify the immune tolerance by affecting regulatory T cells. Our results are supported by an earlier birth cohort study that also showed cord blood vitamin D is negatively correlated with Treg numbers (38). Within the PASTURE study, a possible allergy-protective tolerance-inducing effect of vitamin D was suggested (39). Rochat et al. showed that prenatal vitamin D supplementation induces a tolerogenic phenotype of dendritic cells characterized by the expression of immunoglobulin-like transcripts (ILT)3 and ILT4. However, whether cord blood ILT3 and ILT4 mRNA levels are of relevance for the allergy risk later in life is unclear.

The strength of our study lies in the fact that the analysis of vitamin D was performed in corresponding mother–child pairs which are well characterized regarding immune parameters as well as for atopic outcomes and exposure variables. By coupling vitamin D measurements with immune parameters and disease outcomes, we were able to address the question of possible mechanisms responsible for the allergy-mediating effect of vitamin D. A weakness of the LINA study in general is the potential bias by high rates of participating atopic parents (65.9%) resulting in higher frequencies of children with atopic manifestations. We have considered this point by including family history of atopy as confounding variable in the regression models. One other limitation of the study is the low number of cases in certain

Table 5 Association between cord blood 25(OH)D_3 and atopic outcomes or IgE levels of the child

		Cord blood 25(OH)D_3, quartiles							
		n (%)				OR (95% CI)			
	N	1st	2nd	3rd	4th	Unadjusted	*P*-value	Adjusted*	*P*-value
First year of life									
Atopic eczema (symptoms)	272	12 (17.1)	6 (9.0)	5 (6.8)	8 (12.9)	0.85 (0.60–1.19)	0.346	0.94 (0.62–1.41)	0.756
Atopic eczema (diagnosed)	272	7 (10.0)	6 (9.0)	5 (6.8)	9 (14.5)	1.12 (0.78–1.61)	0.534	1.17 (0.76–1.81)	0.472
Food allergy (diagnosed)	272	3 (4.3)	1 (1.5)	3 (4.1)	2 (3.2)	0.98 (0.54–1.79)	0.950	0.92 (0.45–1.85)	0.812
Increased total IgE †	184	33 (73.3)	39 (84.8)	37 (77.1)	35 (77.8)	1.03 (0.75–1.42)	0.844	1.07 (0.74–1.54)	0.713
Increased food allergens (fx5) ‡	184	11 (24.4)	7 (15.2)	4 (8.3)	7 (15.6)	0.77 (0.54–1.11)	0.165	0.76 (0.49–1.18)	0.224
Second year of life									
Atopic eczema (symptoms)	272	7 (10.0)	11 (16.4)	6 (8.2)	8 (12.9)	1.00 (0.72–1.40)	0.992	0.98 (0.67–1.44)	0.926
Atopic eczema (diagnosed)	272	6 (8.6)	4 (6.0)	7 (9.6)	7 (11.3)	1.15 (0.79–1.69)	0.464	1.26 (0.78–2.02)	0.344
Food allergy (diagnosed)	272	0 (0.0)	1 (1.5)	3 (4.1)	4 (6.5)	**2.49 (1.08–5.75)**	**0.032**	**4.65 (1.50–14.48)**	**0.008**
Increased total IgE †	191	36 (76.6)	43 (87.8)	40 (80.0)	38 (84.4)	1.11 (0.79–1.56)	0.542	1.07 (0.73–1.58)	0.728
Increased food allergens (fx5) ‡	191	5 (10.6)	5 (10.2)	6 (12.0)	9 (20.0)	1.30 (0.88–1.92)	0.187	1.60 (0.99–2.57)	0.055
Two-year lifetime period									
Atopic eczema (symptoms)	272	17 (24.3)	14 (20.9)	8 (11.0)	12 (19.4)	0.84 (0.63–1.11)	0.217	0.91 (0.66–1.26)	0.569
Atopic eczema (diagnosed)	272	10 (14.3)	8 (11.9)	9 (12.3)	11 (17.7)	1.09 (0.80–1.48)	0.606	1.20 (0.81–1.76)	0.365
Food allergy (diagnosed)	272	3 (4.3)	2 (3.0)	5 (6.8)	5 (8.1)	1.34 (0.83–2.18)	0.234	1.70 (0.92–3.14)	0.089
Increased total IgE †	184	37 (82.2)	42 (91.3)	42 (87.5)	40 (88.9)	1.16 (0.78–1.72)	0.467	1.21 (0.77–1.91)	0.412
Increased food allergens (fx5) ‡	184	13 (28.9)	10 (21.7)	8 (16.7)	10 (22.2)	0.86 (0.63–1.18)	0.361	0.94 (0.65–1.37)	0.755

*Confounders: gender of the child, number of siblings, cord blood total IgE, family history for atopy, log cotinine (34th week of pregnancy), breast feeding, UV intensity of month of birth and vitamin D supplementation during the first year of life.

†IgE level > 3.8 kU/l.

‡IgE level > 0.35 kU/l.

All *P*-values < 0.005, which is in bold, are significant.

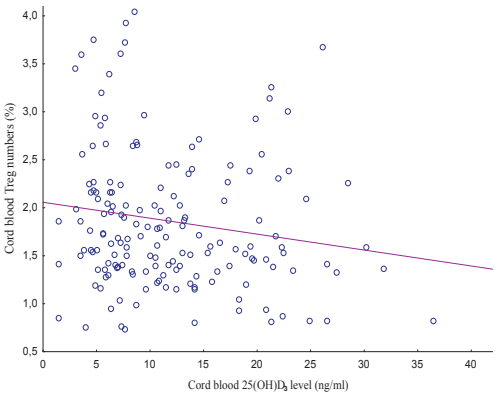

Fig. 4 Correlation between cord blood 25(OH)D_3 levels (ng/ml) and cord blood Treg numbers (%)

outcomes. Main results in this study were obtained regarding food allergy. For this outcome, we only use the questionnaire information regarding a physician-diagnosed food allergy without having data on provocation tests. This might reduce the strengths of the reported results. Therefore, the presented results have to be interpreted with caution and need further validation. However, by including immune and IgE data, we may overcome this limitation at least in part.

Conclusion

Many pregnant mothers worldwide were shown to have insufficient or deficient 25(OH)D concentrations, which subsequently result in insufficient or deficient vitamin D levels in the newborn. Currently, results addressing the impact of vitamin D deficiency on newborns' immune status and the risk of developing allergic diseases in early infancy have been controversial and lacking. The present analyses showed a positive association between maternal or cord blood vitamin D level and risk for food allergy in infants up to 2 years of age, suggesting that a high vitamin D level is a risk for the development of allergic diseases in early infancy. Furthermore, our data demonstrate that this association could be explained with an inhibition of regulatory T cell numbers at birth. As a clinical conclusion, we would not recommend to supplement with vitamin D to protect against allergy development because the results from our study indicate that low vitamin D levels are not necessarily associated with an increased risk for outcome development. Another fact pointing against vitamin D supplementation is its failing impact on blood 25(OH) D levels in the present analyses. In any case, it is of high relevance to clarify how vitamin D in pre- or neonatal period is

able to modify programming of future immune function and therefore atopic outcomes later on.

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Author contribution

Each named author has substantially contributed to this paper. Gunda Herberth, Mario Bauer, Stefan Röder, Michael Borte, Ulrike Diez and Irina Lehmann were involved in the development of the study design and the field work. Denise Hinz, Gunda Herberth, Mario Bauer and Sven Olek performed the *FOXP3* TSDR demethylation measurement. Frank Hirche, Sophie Winkler and Gabriele Stangl performed the vitamin D measurements, and Ulrike Rolle-Kampczyk and Martin von Bergen performed the cotinine analyses. Kristin Weisse, Sophie Winkler, Stefan Röder, Gunda Herberth and Irina Lehmann contributed to the statistical analysis. Ulrich Sack contributed with IgE measurements and discussion of the IgE data. Ulrike Diez, Thomas Richter, Frank Hirche and Gabriele Stangl contributed to the discussion of vitamin D levels in mothers and newborns. Kristin Weisse, Sophie Winkler and Irina Lehmann wrote the paper; all authors were involved in the revision of the final text.

Conflicts of interest

All authors have declared that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Methods.

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CORRESPONDENCE

Vitamin D in early life: good or bad for food allergies?

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There is controversial evidence for an influence of 25-Hydroxyvitamin D3 (25-OHD) levels and nutritional vitamin D supplementation, respectively, on the development of allergic diseases during childhood. We have read with great interest the paper by Weisse et al. who analyzed data of a subgroup of the prospective German LINA cohort study, including 378 mother–child pairs (1). The authors describe a positive association of 25-OH-D_3 levels, in both maternal and cord blood, with parent-reported doctor diagnosis of food allergy in the second year of life, but not in the first year. In addition, maternal 25-OH-D_3 levels were positively associated with the child's serum level of IgE specific for food allergens (but not inhalant allergens) at the age of two. We believe that unfortunately, this study suffers from some major limitations that do not allow the drawing of the published conclusions.

Most importantly, food allergy was diagnosed by parental report of a doctor diagnosis and not by an oral food challenge test. Although the authors have mentioned this limitation, we would like to point out that the prevalence of parent- or self-reported food allergy differs markedly when compared with objective measures. This has been shown in a meta analysis by Rona et al. and a systematic review by Zuidmeer et al. (2, 3). Therefore, a number of expert panels from various organizations have published consensus documents on the appropriate diagnosis of food allergies highlighting the importance of oral food challenges for diagnosing food allergy (4, 5). Thus, the authors used an outcome definition for food allergy that has not been validated. It does not allow the conclusion to be drawn that high vitamin D levels increase the risk of food allergy.

Furthermore, the authors had not prioritized their primary outcome and secondary outcomes. Multiple testing of 30 outcomes increases the finding of statistically significant associations by chance. This is acceptable if the results are clearly interpreted as explorative and are used as the basis for planning future studies. It is not acceptable for advice in favor of or against vitamin D supplements. Also, vitamin D deficiency is strongly associated with low birth weight (6). By not regarding birth weight as an important clinical parameter in this setting, the authors have missed the chance to clarify a potential association of birth weight and atopic disease.

Finally, the authors falsely concluded that cord blood vitamin D levels are correlated with regulatory T cells. This does not correspond to the scatter plot (Fig. 4) or the correlation coefficient of $r = -0.17$. It is a misinterpretation of their results based on only interpreting the *P*-value but ignoring the low correlation coefficient, which is close to zero (equal to no correlation) and far away from 1 (equal to best correlation). Therefore, the last sentence of the Results section in the abstract as well as the last two sentences of the Results in the main text could mislead the readers. Thus, the suggestion that Treg numbers in cord blood might mediate the effect of vitamin D levels on atopic outcomes remains hypothetical.

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We read with interest the article by Weisse et al. (1) who observed that higher 25-hydroxyvitamin D (25[OH]D) levels in maternal (34th week of gestation) and cord blood were associated with an increased risk of food allergy within the first 2 years of life. They hypothesized that this finding might be explained by an inhibition of regulatory T cells (Tregs) because they observed an inverse correlation between cord blood 25 (OH)D and numbers of Tregs (1). Weisse and colleagues finally concluded that their results would argue against vitamin D supplementation (1). This work is definitely of high interest but the conclusion may also cause confusion among physicians and parents on how to apply vitamin D supplementation during pregnancy and early life. We therefore wish to put the article of Weisse et al. into the perspective of existing information relating to vitamin D and food allergies (1, 2).

We want to guide particular attention to the work by Allen et al. who reported that in 1-year-old infants of Australian-born parents, vitamin D insufficiency, that is, 25(OH) D concentrations $\leq$ 20 ng/ml (50 nmol/l), was associated with significantly increased risk of peanut and egg allergies (3). In addition, these vitamin D insufficient infants were more likely to have multiple rather than single food allergies (3). These data were derived from a population-based sample and are based on the use of gold standard methods for food allergy status (3). By contrast, Weisse et al. (1) acknowledged as limitations of their study the potential bias by high rates of participating atopic parents and the lack of provocation tests. Another point to discuss is the inverse association between Tregs and 25(OH)D in the work by Weisse et al. (1). This stands in contrast to a recent pilot trial and a randomized controlled trial (RCT) showing that vitamin D supplementation significantly increases the numbers of Tregs in apparently healthy adults (4, 5). Taken together, the data by Weisse et al. on associations of 25(OH)D levels with food allergies and Tregs are thus contradictory to studies later in life (1–5). Is the relationship between vitamin D status and the immune system therefore completely different throughout the lifespan? To answer this question, we recommend further studies to address the relationship between vitamin D status and food allergies by measuring 25(OH)D repeatedly during different periods of life.

At present, we can only conclude that the existing evidence is insufficient to argue for or against vitamin D supplementation in pregnancy with regard to prevention of food allergies. This conclusion is also supported by the fact that Allen et al. (3) reported on no significant association between vitamin D supplement intake during pregnancy and food allergies. However, we also want to underline that according to data from available RCTs, vitamin D supplementation during pregnancy can significantly increase 25(OH)D levels and can be considered to be safe and probably beneficial with regard to maternal comorbidities of pregnancy, while other long-term health outcomes of vitamin D supplementation in pregnant women need to be addressed in further studies (6).

Author contributions

Stefan Pilz and Winfried März contributed equally to the conception, drafting, and revising the manuscript.

Conflicts of interest

None.

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REPLY

We appreciate the discussion on the role of vitamin D in allergy development and read with interest the letters by Pilz et al. and Querfeld et al., which give us the opportunity to further clarify some aspects of our study (1).

As highlighted by Pilz et al., a recently published Australian study (2) showed an association between vitamin D insufficiency and multiple food allergies based on data from one-year-old children. At first glance, the result of this study seems to be contradictory to our data. However, the Australian data were derived from one-year-old children, whereas we focused on maternal vitamin D during pregnancy and cord blood vitamin D at birth. The different result obtained from different time points could give evidence that in fact the relationship between the vitamin D status and the immune system might be different in the pre- and postnatal period. Furthermore, the result 'low vitamin D – high risk of food allergies' was only seen in infants whose both parents were born in Australia. Among all 481 participants as well as in infants with one or both parents born overseas, this result could not be confirmed, which even more is pointing out the heterogeneous results on vitamin D and allergy development.

We are aware of the limitation (as critically discussed in the article) that our food allergy outcome relies solely on the parental report of a doctor's diagnosis with the uncertainty that this might not have been verified by oral food challenge. However, it is general practice in epidemiological studies that food allergy is based on questionnaire information and not necessarily on additional provocation tests (3).

When we have studied the association between the vitamin D status during pregnancy and at birth with later atopic outcomes, we have also tested for a potential confounding effect of the birthweight. Comparing mothers having low vitamin D levels with mothers having the high vitamin D levels, we saw no differences regarding the birthweight of their children (3355 g and 3387 g for the 1st *vs*. 4th vitamin D quartile ($P = 0.629$), respectively). Moreover, including birthweight as a confounding factor in the regression model did not change the obtained results.

Regarding the comments on our Treg data, studies cited by Pilz et al. (4) showing that vitamin D supplementation increases %Treg were performed in healthy adults who are completely different in their immunological pathways compared with newborns. Within our LINA study, we also observed a positive association in the mothers: increasing maternal Treg numbers with higher maternal vitamin D levels (Spearman correlation, $R = 0.157$, $P = 0.043$). The inverse relationship we have found for cord blood Treg and cord blood vitamin D confirmed data from earlier studies (5) and may give further evidence that the vitamin D impact on immune regulation differs through life.

We agree that the observed correlation coefficient is rather low. However, this is not surprising because it was already shown that besides vitamin D, several factors may have an impact on Treg numbers including the maternal immune status, parental atopy history and maternal tobacco smoke exposure (6).

We are highly aware of the potential difficulties of presenting conflicting data on vitamin D. However, we saw these results in our study that do not support earlier data showing vitamin D as allergy preventive. We believe that it is our responsibility to communicate that vitamin D might also have – under special circumstances – inverse effects regarding allergy development, and this should be taken into account when recommendations for supplementations are drawn. Vitamin D undoubtedly has many beneficial effects but presumably acts via different mechanisms throughout the lifespan.

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Can vitamin D intake during pregnancy affect the risk of allergy in children?

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"Regarding asthma and allergy, available data are quite controversial, showing both predictive and adverse effects of vitamin D."

Vitamin D, formerly known as 'the bone vitamin', has become a subject of high interest in the last decade regarding its effects in different diseases such as cancer, diabetes, allergies or susceptibility to infections.

Especially in the field of allergy and asthma data are very heterogeneous and it is difficult to draw a clear picture about the impact of vitamin D in the disease development. In this context it is necessary to differentiate data on vitamin levels from different time points, because results suggest that the relationship between vitamin D status and the immune system might be different in the pre- and postnatal periods, in infancy or in adulthood. Here we would like to focus on the role of vitamin D during pregnancy or in cord blood (as a marker for the maternal vitamin supply) and its impact on later disease development of the child.

Regarding asthma and allergy, available data are quite controversial, showing both predictive and adverse effects of vitamin D.

Causing allergy

There are different studies showing that higher vitamin D levels in pregnancy or in cord blood are associated with an increased risk of getting food sensitization or food allergy [1], or atopic dermatitis [2] in early infancy. An inhibitory impact of maternal vitamin D on the development of the fetal adaptive immune response is discussed as a possible contributing mechanism. Maternal vitamin D levels were found to be associated with decreased amounts of 'allergy-preventive' regulatory T cells in the cord blood [1,3], which was shown to predict sensitization and atopic eczema later in children's life [4]. Addressing manifestations of the lungs, like wheezing (which might increase the risk developing asthma) or asthma, a variety of studies have also shown that higher vitamin D levels in pregnancy are associated with an increased risk of getting asthma [2,5] in early infancy.

Preventing allergy

On the other hand, there are also several studies that show higher vitamin D levels in pregnancy or in cord blood are associated with a decreased risk of getting food allergy [6,7], food sensitization [8], atopic dermatitis [9,10] or allergic rhinitis [11] in early infancy. An increased level of the 'immunosuppressive' cytokine IL-10 with an increased Il-10/IgE ratio in the summer months (and therefore higher vitamin D levels) is discussed in that context as a contributing mechanism [12]. Further studies showed that higher vitamin D levels in pregnancy or in cord blood were associated with a decreased risk of getting wheezing symptoms [10,13–15] or asthma [11]. The influence of vitamin D in lung development (such as fetal lung cell maturation and surfactant production) was discussed in that context as a contributing mechanism.

In addition to studies showing vitamin D as preventing or causing allergy, there are very interesting data from Rothers *et al.* that describe a U-shaped association; both

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low and high vitamin D levels increased the risk for an allergic sensitization [16]. Such U-shaped relationships were already found earlier in the context of vitamin D which was reviewed recently [17]. However, Rothers *et al*. could not show the effects of vitamin D for allergic rhinitis or asthma.

> "Sufficient vitamin D levels during pregnancy are doubtlessly beneficial; for example, to decrease the susceptibility for infectious diseases, to increase maternal health during pregnancy or to support fetal lung cell maturation."

When comparing data on vitamin D it needs to be considered that not all of them are based on the same vitamin D assessment and some of them might not reflect the true vitamin D status of the body. Only very few studies actually refer to objective 25-hydroxyvitamin D measurements, several are calculating vitamin D intake from patients' food questionnaires, others just use the season of birth as a marker for vitamin D levels. Notably, many of the studies showing allergy protective effects of vitamin D levels during pregnancy are not based on actual serum measurements.

In addition, different geographic regions and accordingly different individual genetic backgrounds might confound the overall results. Genetic variations in vitamin D metabolism and function such as in vitamin D binding protein, in vitamin D receptor or in catalyzing enzymes of vitamin D synthesis were found as potential underlying mechanisms (reviewed in [18]). Liu *et al*. analyzed the impact of certain risk genotypes involved in vitamin D metabolism and IgE regulation on the association between vitamin D levels and food allergen specific IgE. They showed that low-risk children (less than two risk genotypes) with deficient cord blood vitamin D levels (< 11 ng/ml) had a lower food allergy frequency compared with children with higher cord blood levels. However, when they considered more than two risk genotypes, this association was reversed [19].

It needs to be considered that allergy is not caused by a single condition such as vitamin D. The current literature is highlighting a huge variety of factors contributing to allergy development such as multifactorial environmental exposure, life style factors or differential microbial load. Also the fact that we are investigating a vitamin, which is physiologically absorbed via nutrition (even if this is not the only way of admission like in case of vitamin D) it needs to be mentioned that there could be different dietary confounders that also influence immunological pathways and therefore allergy development. The means of vitamin D consumption, either from fish, which is high in anti-inflammatory effective omega-3 fatty acids or for example from fortified milk, which is high in saturated fatty acids, is a further important issue [9].

However, when considering other outcomes than asthma and allergy, for example infectious manifestations, the results are more convincing. There are several studies showing that high vitamin D during pregnancy [20], at birth [15] or in infancy [21,22]), as a result of either supplementation [21,22]) or regular lifestyle [15,20] decrease the prevalence of infections. That was shown for example for infant's respiratory tract infections [15,20,21] or influenza [22], as well as for maternal bacterial vaginosis (reviewed in [23]). In a recently published randomized controlled trial of 247 Mongolian children with vitamin D deficiency during wintertime, vitamin D supplementation significantly reduced the risk of acute respiratory infections by about 50% [21].

> "…the available data are not clear enough to draw recommendations for a general vitamin D supplementation during pregnancy to protect against allergies."

So finally, what is the answer to the question 'Can vitamin D intake during pregnancy affect the risk of allergy in children?' From our point of view and *just* considering the prevention of allergy or asthma, the available data are not clear enough to draw recommendations for a general vitamin D supplementation during pregnancy to protect against allergies. However, this should not be seen as a general statement against vitamin D. Sufficient vitamin D levels during pregnancy doubtlessly beneficial; for example, to decrease the susceptibility for infectious diseases, to increase maternal health during pregnancy or to support fetal lung cell maturation. Nevertheless, there are still a lot of open questions regarding the individual requirements which differ between age, race, lifestyle, genetic background or special disease conditions. Vitamin D supplementation during pregnancy should therefore not be a general choice for everyone. Anyway, adequate vitamin D levels, preferentially via diet or sun light exposure, should be achieved.

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Increased vitamin D levels at birth and in early infancy increase offspring allergy risk—evidence for involvement of epigenetic mechanisms

To the Editor:

Although a beneficial effect of vitamin D on health is widely accepted, its role in allergy development has been controversial. Both allergy-preventing and allergy-promoting effects have been reported. Thus, a deeper mechanistic understanding of how vitamin D is related to the regulation of immune reactivity and allergic inflammation is required. Vitamin D was shown to modify gene expression[1] through binding of the vitamin D receptor to vitamin D response elements. However, only 26% of the genes identified as regulated by vitamin D have a vitamin D response element in proximity to their transcription start site (TSS),[1] indicating that additional mechanisms are involved in the transcriptional control by vitamin D. As an additional mechanism, epigenetically mediated transcriptional deregulation through vitamin D–induced changes in DNA methylation was suggested.[2]

Here, we studied DNA-methylation pattern on a genomewide scale at base-pair resolution in healthy newborn children with high and low vitamin D levels to elucidate the role of vitamin D in epigenetic programming of an allergy-protective or allergy-promoting immune reactivity. Within the LINA (Lifestyle and environmental factors and their Influence on Newborns Allergy risk) mother-child cohort,[3] differential DNA methylation was assessed by using whole genome bisulfite sequencing in 6 cord blood samples comparing 3 children with high to 3 children with low 25-hydroxyvitamin D_3 (25[OH]D_3) levels. We assessed blood cell type composition on the basis of promoter methylation level from 5 lineage markers in each sample (see Methods in this article's Online Repository at www.jacionline.org). The analysis showed that the variation in high versus low vitamin D samples was in a nonsignificant range of 1% to 2% for all cell types analyzed in cord blood (see Table E2 in this article's Online Repository at www.jacionline.org). To omit differentially methylated regions (DMRs) solely caused by differences in cellular blood composition between low and high vitamin D samples, we used an additional threshold of 10% methylation difference for DMR calling. Differential methylation was validated in the entire cohort by target-specific methylation analysis. To decipher the regulatory role of 25(OH)D_3-induced DNA-methylation changes, we performed histone modification Chromatin Immuno Precipitation sequencing to segment the genome into distinct regulatory elements and linked differential DNA methylation to transcription (see this article's Online Repository). Furthermore, vitamin D levels and their link to allergic outcomes at different time points in early childhood were studied. Serum 25(OH)D_3 levels were measured in 378 newborns, 466 1-year-old children and 304 2-year-old children (see Fig E1 in this article's Online Repository at www.jacionline.org) by using high-performance liquid chromatography tandem mass spectrometry as described earlier.[4]

Median 25(OH)D_3 levels from birth until age 2 years, seasonal variation, correlations between different ages, and the impact of vitamin D supplementation are presented in Table E1 and Fig E2 (see this article's Online Repository at www.jacionline.org). As described earlier,[4,5] we found that cord blood 25(OH)D_3 level was positively associated with physician-diagnosed food allergy and atopic dermatitis until the children were 3 years old (Table I). In addition, an association was found between 25(OH)D_3 levels at age of 2 years and an enhanced risk of wheezing within the third year of life (Table I).

Next, we identified 508 significantly differentially methylated regions associated with 483 genes in children with high compared with low 25(OH)D_3 levels. Thereby, 25(OH)D_3-dependent methylation changes were predominantly associated with loss of DNA methylation (see Fig E3, *A*, and Table E3 in this article's Online Repository at www.jacionline.org), which is in line with earlier reported data.[2] Among those 508 DMRs, an intergenic DMR spanning 6 CpGs was identified, located 52,400 base pairs upstream of the TSS of group-specific component (vitamin D binding protein; Δmethylation, 17.3%; see Fig E3, *B*, and Table E3). However, validation in low versus high vitamin D cord blood samples in the entire LINA cohort using MassARRAY-based target-specific methylation analysis barely missed the significance level ($P = .063$; see Fig E5 in this article's Online Repository at www.jacionline.org). No DMRs were called for other known key regulators of vitamin D metabolism.

To further filter the 508 significant DMRs assessed by whole genome bisulfite sequencing, we used WEB-based GEne SeT AnaLysis Toolkit (WebGestalt) with all predicted target genes (see Table E3) for pathway analyses. As a preliminary result, 103 significantly affected pathways ($P < .01$) were identified (see Table E4 in this article's Online Repository at www.jacionline.org), including pathways with a potential link to immune system dysfunction or allergy development: "immune system disease," "lung disease/obstructive lung disease," and "skin and connective tissue disease." In total, 3 genes involved in all these 3 pathways were identified: thymic stromal lymphopoietin (*TSLP*), *IL17F*, and *MBL2*. *IL17F* and *MBL2* are involved in inflammation and infection, whereas for *TSLP* links have already been shown to both allergic diseases and vitamin D. *TSLP* was selected for further validation because of its known functional role in allergic diseases.

For *TSLP*, a DMR spanning 5 CpGs 113kbp upstream of the *TSLP* TSS was identified (Δmethylation, 24%; Fig E3, *C*, and Table E3). Further analysis of the region flanking this DMR revealed a broader, significantly regulated region that includes this DMR together with an annotated enhancer region located 100bp downstream (Chr5:110.292.001-110.293.600; $P = .0058$; Δmethylation, 12.2%). Although this broader region was at a remarkable distance from the *TSLP* TSS, chromatin interaction data indicate that this enhancer targets *TSLP* (Fig 1, *A*). We validated the loss of methylation in this region in relation to high cord blood 25(OH)D_3 levels by MassARRAY-based analysis in the entire LINA cohort (see Fig E4, *A*, in this article's Online Repository at www.jacionline.org). No overall correlation was found in all children or those with low 25(OH)D_3 levels. However, in children with high cord blood 25(OH)D_3 levels, the methylation level of this region correlated significantly with 25(OH)D_3 concentrations. This association was observed for 2 CpGs (Chr5:110.292.389-392) located in the *TSLP* enhancer ($P = .026$; $R = -0.236$;

TABLE I. Association between cord blood, year 1 and year 2 25(OH)D_3 levels, and atopic outcomes of the child in the months following the vitamin D measurement

		25(OH)D_3, quartiles							
		n (%)				OR (95% CI)			
	N	First	Second	Third	Fourth	Raw	*P* value	Adjusted	*P* value
Vitamin D birth → outcome month 0-36									
Atopic eczema (symptoms)	320	27 (8.4)	21 (6.6)	15 (4.7)	27 (8.4)	0.98 (0.79-1.22)	.885	1.05 (0.82-1.35)	.673
Atopic eczema (diagnosed)	305	15 (4.9)	13 (4.3)	13 (4.3)	20 (6.6)	1.17 (0.91-1.50)	.216	**1.34 (1.00-1.80)**	**.047**
Food allergy (diagnosed)	291	4 (1.4)	3 (1.0)	5 (1.7)	9 (3.1)	1.48 (0.97-2.25)	.066	**1.86 (1.08-3.20)**	**.023**
Wheezing ever	324	38 (11.7)	34 (10.5)	25 (7.7)	34 (10.5)	0.94 (0.77-1.15)	.542	0.97 (0.78-1.22)	.817
Wheezing recurrent	367	19 (5.2)	23 (6.3)	16 (4.4)	20 (5.4)	0.96 (0.77-1.20)	.701	1.07 (0.83-1.38)	.606
Vitamin D year 1 → outcome month 12-36									
Atopic eczema (symptoms)	409	25 (6.1)	17 (4.2)	30 (7.3)	19 (4.6)	0.97 (0.79-1.20)	.797	1.01 (0.98-1.04)	.690
Atopic eczema (diagnosed)	374	10 (2.7)	7 (1.9)	14 (3.7)	13 (3.5)	1.21 (0.91-1.62)	.191	1.04 (0.99-1.09)	.064
Food allergy (diagnosed)	370	3 (0.8)	5 (1.4)	5 (1.4)	7 (1.9)	1.33 (0.87-2.03)	.186	1.03 (0.97-1.09)	.291
Wheezing ever	409	25 (6.1)	37 (9.0)	33 (8.1)	30 (7.3)	1.06 (0.87-1.28)	.561	1.01 (0.99-1.04)	.312
Wheezing recurrent	408	13 (3.2)	17 (4.2)	21 (5.1)	22 (4.9)	1.25 (0.99-1.58)	.064	1.03 (0.99-1.06)	.097
Vitamin D year 2 → outcome month 24-36									
Atopic eczema (symptoms)	289	12 (4.2)	15 (5.2)	9 (3.1)	7 (2.4)	0.78 (0.58-1.05)	.101	0.92 (0.65-1.29)	.623
Atopic eczema (diagnosed)	289	4 (1.4)	5 (1.7)	7 (2.4)	6 (2.1)	1.16 (0.78-1.72)	.469	1.37 (0.87-2.14)	.168
Food allergy (diagnosed)	289	4 (1.4)	2 (0.7)	2 (0.7)	1 (0.4)	0.64 (0.34-1.22)	.170	0.95 (0.47-1.93)	.881
Wheezing ever	288	5 (1.7)	15 (5.2)	17 (5.9)	15 (5.2)	**1.35 (1.02-1.78)**	**.035**	**1.38 (1.01-1.90)**	**.044**
Wheezing recurrent	288	3 (1.0)	5 (1.7)	9 (3.1)	7 (2.4)	1.34 (0.91-1.97)	.142	1.50 (0.95-2.38)	.080

N = cases with questionnaire data and vitamin D measurement in the given combination. Odds ratios (ORs) with 95% CI and *P* value are shown either for raw data or adjusted for sex of the child, number of siblings, family history for atopy, maternal urine cotinine level (34th week of pregnancy), keeping of a cat, month of birth, and breast-feeding. Significant values are in boldface.

Fig 1, *B*) as well as for 1 further CpG (Chr5:110.292.306) located in the *TSLP* DMR ($P = .024$; $R = -0.233$; see Fig E6 in this article's Online Repository at www.jacionline.org). The methylation level of the TSLP enhancer region was stable from birth until age 3 years (Fig E4, *B* and *C*). On analyzing histone modifications in the *TSLP*-associated DMR and the adjacent enhancer, we found repressive marks (H3K9me3 and/or H3K27me3) in this region in blood cells of children with low 25(OH)D_3 levels whereas children with high 25(OH)D_3 levels were deprived of repressive histone marks. Neither the *TSLP* DMR nor the *TSLP* enhancer region has meQTL-single nucleotide polymorphisms in its neighborhood. Thus, we conclude that differences in *TSLP* methylation levels are not linked to genetic variation.

Altogether, the concomitant loss of DNA methylation and repressive chromatin marks strongly suggest a gain of expression of the target gene *TSLP*. In fact, a negative correlation between methylation in the enhancer region and *TSLP* mRNA expression was observed (Fig 1, *C*). The *TSLP* mRNA expression was significantly elevated at birth ($P = .048$) and age 1 year ($P = .003$) in the high vitamin D group (Fig 1, *D* and *E*). Finally, we found that children who suffered from wheezing symptoms later in life had significantly enhanced *TSLP* mRNA expression at age 1 year (Fig 1, *F*). Furthermore, a link between wheezing symptoms and reduced methylation at CpG Chr5:110.292.315 located in the *TSLP* DMR region could be shown (Δmethylation, 2%; $P = .045$; n = 309 controls vs n = 66 wheezing children).

TSLP plays a critical role in allergic diseases by inducing an inflammatory T_H2 response via conditioning dendritic cell maturation.[6,7] *TSLP* mRNA as well as protein levels were shown to correlate with asthma severity,[8] while treatment of patients with asthma with an anti-*TSLP* antibody reduced airway inflammation before and after allergen challenge.[9] Here, we demonstrated that high cord blood vitamin D levels were associated with epigenetic regulation of an enhancer region shown to interact with the *TSLP* promoter. Children with higher 25(OH)D_3 levels at birth showed a lower DNA-methylation level and a loss of repressive histone marks in this *TSLP* enhancer region, resulting in a higher *TSLP* mRNA expression. Furthermore, a link between enhanced *TSLP* expression and wheezing was found. Our result provides evidence that epigenetic deregulation of *TSLP* could be involved in the vitamin D–related programming for allergic diseases. However, this result does not exclude that vitamin D may act in comparable manner via other genes.

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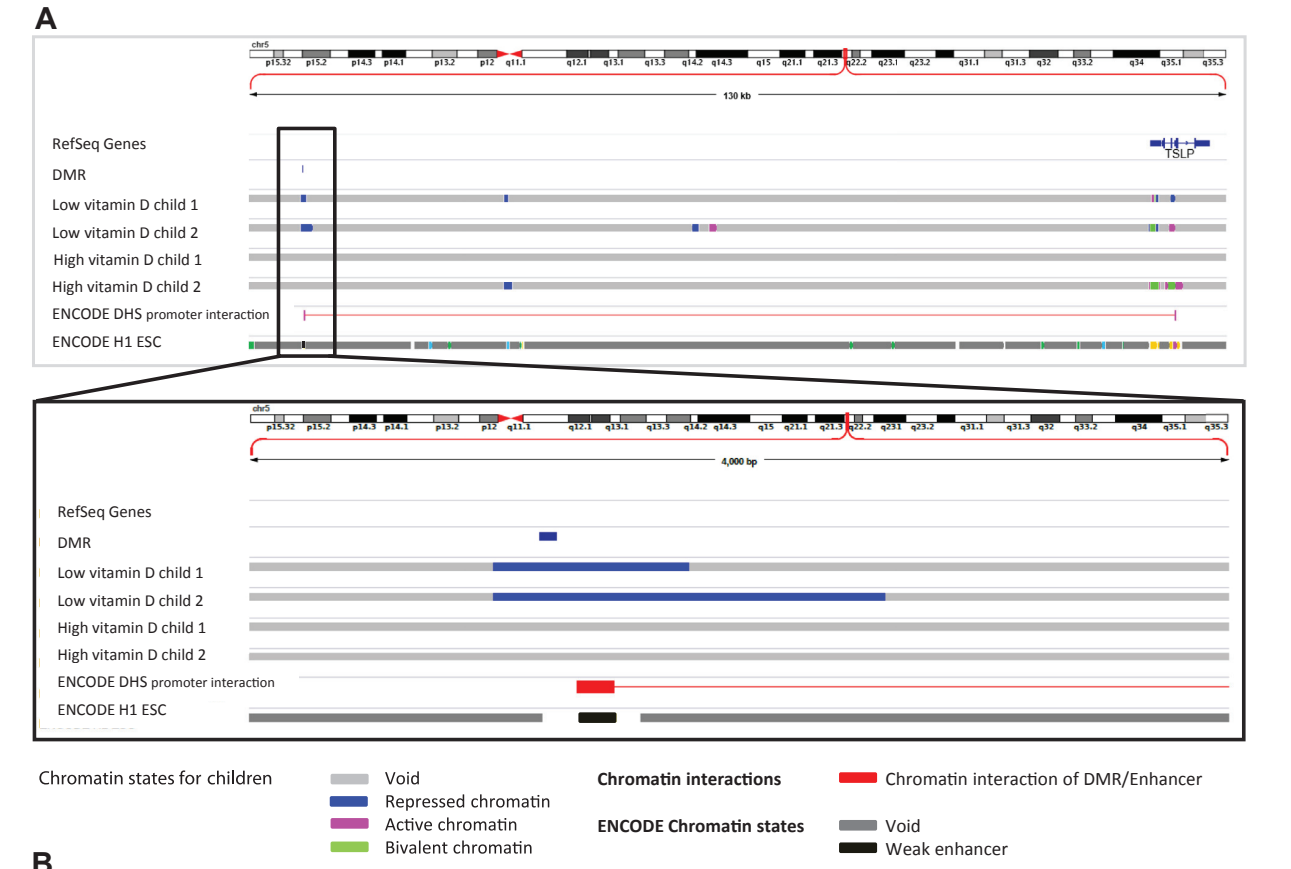
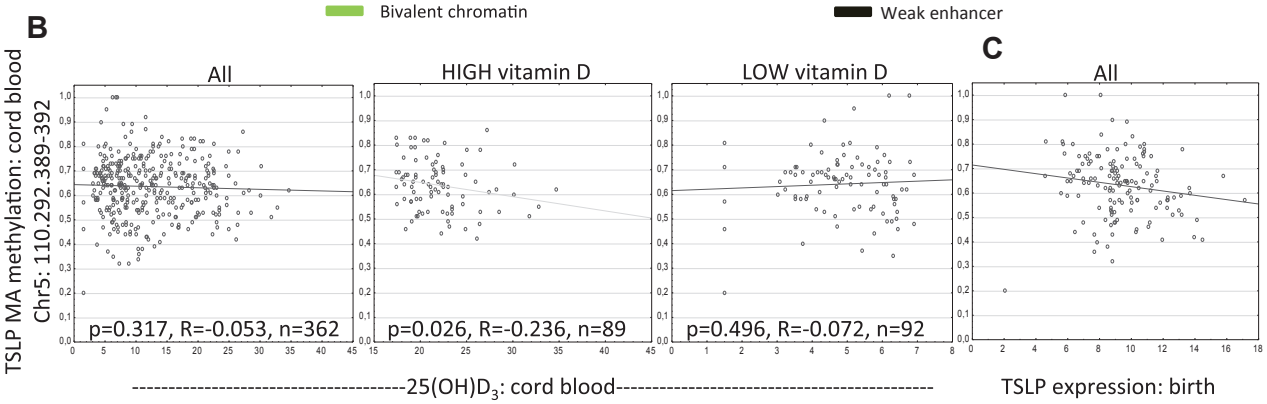
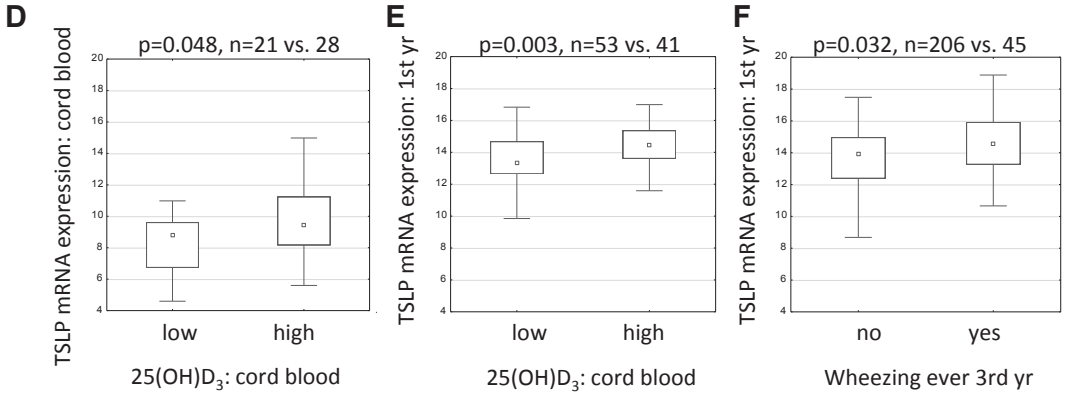

A
130 kb
RefSeq Genes
DMR
Low vitamin D child 1
Low vitamin D child 2
High vitamin D child 1
High vitamin D child 2
ENCODE DHS promoter interaction
ENCODE H1 ESC
TSLP
4,000 bp
Chromatin states for children
Void
Repressed chromatin
Active chromatin
Bivalent chromatin
Chromatin interactions
Chromatin interaction of DMR/Enhancer
ENCODE Chromatin states
Void
Weak enhancer


B
All
HIGH vitamin D
LOW vitamin D
TSLP MA methylation: cord blood
Chr5: 110.292.389-392
p=0.317, R=-0.053, n=362
p=0.026, R=-0.236, n=89
p=0.496, R=-0.072, n=92
C
All
25(OH)D3: cord blood
TSLP expression: birth


D
p=0.048, n=21 vs. 28
TSLP mRNA expression: cord blood
low
high
25(OH)D3: cord blood
E
p=0.003, n=53 vs. 41
TSLP mRNA expression: 1st yr
low
high
25(OH)D3: cord blood
F
p=0.032, n=206 vs. 45
TSLP mRNA expression: 1st yr
no
yes
Wheezing ever 3rd yr

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Effects of lidocaine on regulatory T cells in atopic dermatitis

To the Editor:

Atopic dermatitis (AD) is a chronic and relapsing skin disease characterized by inflammation and pruritus. In our previous hospital-based study of 1008 patients with AD, we found that the proportion of patients with severe AD was 10.7%.[1] Moreover, AD is a huge economic burden for families and society in general due to the long course of relapsed disease.

Regulatory T (Treg) cells control immune homeostasis and balance immune responses during inflammation. Treg cells suppress immune responses by interacting with effector T cells or antigen-presenting cells.[2] Recent clinical research has found that parents, particularly mothers, who have a history of atopy could have babies with less stable $FOXP3^+$ Treg cells in cord blood. These babies have the onset of AD less than 1 year after birth.[3] Therefore, it is likely that the abnormal numbers of Treg cells weaken the inhibition of T_H2 lymphocytes, thus resulting in AD inflammation.

Lidocaine is a widely used short-acting local anesthetic and antiarrhythmic agent. Previous studies demonstrated that lidocaine attenuated bronchoconstriction in patients with severe asthma, which enabled the dosage of oral corticosteroids to be reduced or eliminated in long-term treatment.[4] Because of the similarity of allergic diseases, lidocaine was used as a treatment for AD in China. Previous studies have shown that lidocaine dose-dependently inhibits the proliferative response and release of inflammatory factors from Staphylococcal enterotoxins A- and Staphylococcal enterotoxins B–stimulated PBMCs in patients with AD, contributing to clinical remission.[5]

In vivo, we sought to explore the effect of lidocaine on Treg cells and other key cytokines in patients with AD and murine AD models. Twenty patients were administered lidocaine (3 mg/kg per day) via a slow intravenous drip for 14 days. During

FIG 1. TSLP DMR, histone marks, and mRNA expression. **A,** Location of the *TSLP* DMR in relation to the *TSLP* gene *(upper part)*. The *TSLP* DMR shows a predicted chromosomal interaction with the *TSLP* promoter *(red bar)*. Furthermore, chromatin segmentation tracks generated by ChromHMM are displayed for 2 (high cord blood 25[OH]D_3) vs 2 (low cord blood 25[OH]D_3) samples over the region relating to the DMR and *TSLP* gene *(upper part)*, and a close-up of the DMR region *(lower part)*: Repressed chromatin states are observed in samples of low 25(OH)D_3 *(blue bars)* over the DMR region, and an ECNODE-predicted weak enhancer is found nearby (WE; *black bar*; *lower part* of the Fig 1, *A*). **B,** Association between *TSLP* enhancer methylation at birth (mean of 2 CpGs at Chr5:110.292.389-392, which marks the start of the enhancer region close to the *TSLP* DMR; analyzed via MassARRAY) and cord blood 25(OH)D_3; shown for all participants *(left column)*, only for those within the high vitamin D group (25[OH]D_3 >75th percentile, *middle column*), or for those within the low vitamin D group (25[OH]D_3 <25th percentile, *right column*). **C,** Association between methylation of the *TSLP* enhancer region (2 CpGs at Chr5:110.292.389-392) and *TSLP* mRNA expression, both analyzed in cord blood. **D,** Association between cord blood *TSLP* mRNA expression and 25(OH)D_3 concentrations. **E,** Association between *TSLP* mRNA expression at year 1 (first year) and cord blood 25(OH)D_3 concentration. **F,** *TSLP* mRNA expression at year 1 (first year) in children with (yes) or without (no) wheezing symptoms ever within the third year of life (*P* values from Mann-Whitney *U* test).

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ORIGINAL RESEARCH
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Pro-inflammatory Diet Pictured in Children With Atopic Dermatitis or Food Allergy: Nutritional Data of the LiNA Cohort

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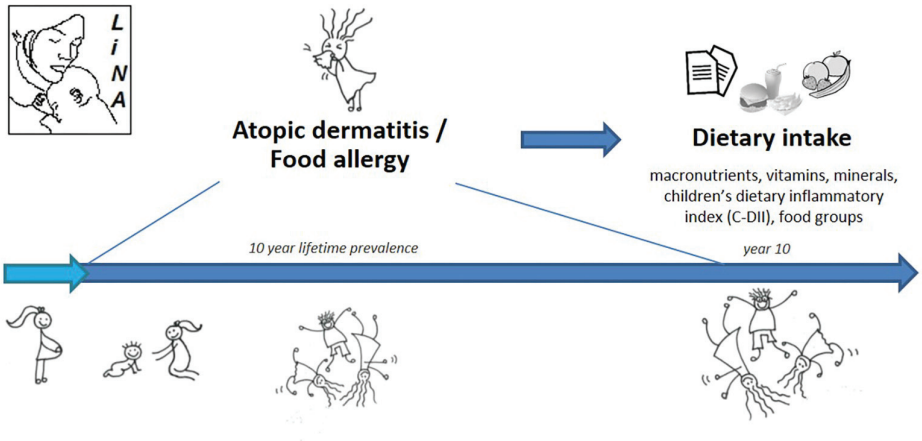
Background: Lifestyle and environmental factors are known to contribute to allergic disease development, especially very early in life. However, the link between diet composition and allergic outcomes remains unclear.

Methods: In the present population-based cohort study we evaluated the dietary intake of 10-year-old children and analyses were performed with particular focus on atopic dermatitis or food allergy, allergic diseases known to be affected by dietary allergens. Dietary intake was assessed *via* semi-quantitative food frequency questionnaires. Based on these data, individual nutrient intake as well as children's Dietary Inflammatory Index (C-DII^{TM}) scores were calculated. Information about atopic manifestations during the first 10 years of life and confounding factors were obtained from standardized questionnaires during pregnancy and annually thereafter.

Results: Analyses from confounder-adjusted logistic regression models (n = 211) revealed that having atopic outcomes was associated with having a pro-inflammatory pattern at the age of 10 years: OR = 2.22 (95% CI: 1.14–4.31) for children with atopic dermatitis and OR = 3.82 (95% CI: 1.47–9.93) for children with food allergy in the first 10 years of life.

Conclusion: A pro-inflammatory dietary pattern might worsen the atopic outcome and reduce the buffering capacity of the individual against harmful environmental exposures or triggers. For pediatricians it is recommended to test for the individual tolerance of allergenic foods and to increase the nutrient density of tolerable food items to avoid undesirable effects of eating a pro-inflammatory diet.

Keywords: atopic dermatitis (AD), food allergy (FA), food frequency questionnaires (FFQ), nutrients, food group consumption, C-DII, 10-year-old children



GRAPHICAL ABSTRACT | Aim and design of the analysed project within the prospective LiNA cohort.

INTRODUCTION

In Western countries the increasing prevalence of atopic diseases has become a major problem in human health. Because allergy onset and atopic march begin in infancy (1), early prevention is advisable. Worldwide, approximately 15%–30% of children live with dermatitis (2, 3) and 4–10% of children suffer from food allergy (4–6). Both atopic dermatitis and food allergy have the earliest onset within the atopic march, resulting in highest prevalence in children before school age (7). Many risk factors for the development of atopic diseases – acting independently or in multifactorial combination – have been identified (8, 9). These include genetic background, individual immune response, barrier dysfunctions, microbiome alterations, as well as lifestyle behaviors (10, 11) and environmental conditions (12).

Diet represents a source of components that could affect atopy in a number of ways. First, diet is a potential source of allergens. Diet also could provide substrate for components that interfere with the pathology of atopy. Finally, it is well-known that diet can modulate inflammatory and related immune responses that can ameliorate or exacerbate allergic or atopic reactions. However, the link between diet composition and the pathogenesis of allergies is complex and not well understood. Though the important role of breastfeeding and timing/manner of introducing solid food is well understood, there are few dietary factors consumed in early life that are described to alter the risk for allergic diseases [e.g., vitamin D, pro/prebiotics or omega(ω)-3 long-chain polyunsaturated fatty acids (13, 14)]. A position paper of the European Association of Asthma, Allergy and Clinical Immunology (EAACI) outlined that it is of high importance to understand how diet diversity modulates allergic outcomes (15). The task force also recommends to use indices in the future to better describe the allergic potential of foods or food patterns within the context of diet diversity. There are several dietary indices available that have been analyzed with respect to nutritional quality, adherence to dietary guidelines or recommendations as well as in association with specific outcomes, such as cardiovascular disease risk (16, 17). Because allergies are characterized by inflammatory processes (18, 19), it would be appropriate to apply an index representing the inflammatory potential of the individual diet in this context (20, 21).

In addition to the role of specific dietary factors contributing to the pathogenesis of allergic and atopic conditions, it also is important to consider that individuals suffering from food allergen-triggered symptoms develop a specific dietary pattern due to their mandatory avoidance of causative allergens (7, 22–25). Such dietary restrictions are, themselves, known to be associated with adverse health issues (22). Knowing the dietary intake of individuals affected by allergies, in particular of children, might offer possibilities to improve their immune response, reduce symptom severity or relapse frequency which

Abbreviations: AD atopic dermatitis, C-DII children's Dietary Inflammatory Index, FA food allergy, fx5 specific IgE for food allergen (mix), IL interleukin, sx1 specific IgE for respiratory allergen (mix), OR odds ratio.

is even more important in growing individuals. Therefore, the aim of the present study was to evaluate the nutritional pattern in a cohort of 10-year-old children with respect to their development of allergic diseases known to be directly affected by dietary allergens such as atopic dermatitis or food allergy. Because of the role of inflammation in these conditions, the children's Dietary Inflammatory Index (C-DII) was used to describe dietary exposure.

MATERIALS AND METHODS

Study Design

Within the population-based, prospective birth cohort study LiNA (Lifestyle and environmental factors and their Influence on Newborn's Allergy risk) 629 mother-child-pairs (622 mothers and 629 children; 7 twins) were recruited during regular appointments with their midwife during May 2006 and December 2008 in Leipzig, Germany. The aim of the study is to investigate how lifestyle and environmental factors in the pre and postnatal period influence the immune system of the newborn and the child later in life with consequences for future allergy risk. Mothers suffering from chronic immune or infectious diseases during pregnancy were excluded from the study, as well as mothers with non-German ancestry/non-Caucasian ethnicity. Further, only term ($\geq$ 37th week of pregnancy, $\geq$ 2,500 g birth weight) and healthy newborns (without postnatal infections that needed medical treatment) were included. General characteristics (such as sex of the child, mothers age at birth, birth mode, breastfeeding duration, presence of older siblings, parental school education, environmental tobacco smoke (ETS) exposure, pet keeping, family history of atopy, etc.) or outcome data were assessed during pregnancy and annually thereafter using questionnaires and in-person examinations. All questionnaires were self-administered by the parents (together with the children when they were old enough). Study participation was voluntary and written informed consent was obtained of all participants. The LiNA study was approved by the Institutional Review Board of the University of Leipzig and the Saxonian Board of Physicians (046-2006, 160-2008, 160b/2008, 144-10-31052010, 113-11-18042011, 206-12-02072012, 169/13-ff, 150/14-ff, EK-allg-28/14-1, 008/17-ek).

Dietary Assessment

Dietary intake was assessed at the age of 10 years using a semi-quantitative food frequency questionnaire (FFQ) asking for children's intake of foods in the past 12 months. The FFQ contained 106 food items from 14 different food/beverage groups (bread and rolls, spreads, cheese and cold sausage, cereals and cornflakes, milk (-products) and eggs, basic carbohydrates, meat, fish, vegetables, fruits, cake and desserts, (salty) sweets and nuts, fats and oils and beverages) with nine non-overlapping frequency categories (never to $\geq$4 times/d) as well as five relative portion size options (1/4, 1/2, 1, 2, 3) referring to an exemplified or pictured standard portion size (i.e., Equal to 1). Relevant data on the fat content and type of preparation were recorded (e.g., fat content of milk products/raw or cooked vegetables etc.). The data based on FFQ where then analyzed using DGExpert (version 1.9, based on codes by the German Food Code and Nutrient Database – BLS 3.02) which outlined the individual intake of 158 macro- and micronutrients for every child. A comparison to applicable reference values (D (Germany) –A (Austria) –CH (Switzerland) reference) is provided by DGExpert considering the children's personal data [age, sex, weight, height and Physical Activity Level (PAL)] to calculate individual energy- and nutrient requirements. Average PAL was considered to be 1.6 according to the German Society of Nutrition (DGE) and was further adopted according to children's activity on their way to school (how children went to school (walk/bike/car) and how long this took). In addition to the nutrient calculation, the consumption of specific food groups was analyzed according to the optimized mixed diet (OptimiX) recommendation (beverages [mL/d], bread and cereals [g/d], pasta, rice and potatoes [g/d], vegetables [g/d], fruits [g/d], milk and dairy products [g/d], meat and sausages [g/d], eggs [pieces/week], fish [g/week], fats [g/d] or tolerated food group [portions/d, sweets, snacks, soft drinks] (26).

Children's Dietary Inflammatory Index

Using data from the FFQ the inflammatory potential of the participant's diet was evaluated by calculating the children's Dietary Inflammatory Index (C-DII) for each child. The detailed C-DII methodology has been established and described earlier (27). Briefly, the Dietary Inflammatory Index (DII) classifies human dietary patterns on a continuous scale from anti-inflammatory (values <0) to pro-inflammatory (values >0) based on a broad literature database with respect to 45 foods or nutrients that were described to be associated with inflammatory markers such as interleukin (IL)-1b, IL-4, IL-6, IL-10, tumor necrosis factor (TNF)-a and C-reactive protein (CRP) (28). The DII was further adapted for children (C-DII) using 25 nutrients or food parameters (27). All parameters except selenium (missing software database information) were included for the LiNA C-DII; Anti-inflammatory parameters included: vitamin A, thiamine (vitamin B1), riboflavin (vitamin B2), niacin (vitamin B3), vitamin B6, folic acid (vitamin B9), vitamin D, vitamin C, vitamin E, beta carotene, fiber, mono-unsaturated fatty acid (MUFA), poly-unsaturated fatty acid (PUFA), magnesium (Mg) and zinc (Zn); Pro-inflammatory parameters included: vitamin B12, energy, carbohydrates, total fat, saturated fat, cholesterol, protein, alcohol and iron (Fe). Next to data on the inflammatory potential of the diet, dietary intakes from a wide range of diverse populations from different countries representing six continents were used to construct a consumption database that was referred to as Z-Scores (27).

Atopic Outcomes

Atopic dermatitis and food allergy were used as atopic outcomes in the present analyses. Atopic dermatitis was recorded annually *via* parental report of a doctor-diagnosed atopic dermatitis or as the diagnosis of the study physician at the annual LiNA medical examination. For food allergy the annual parental report of a doctor-diagnosed food allergy was used. Outcome prevalence was defined as at least one positive indication within the first 10 years of life.

IgE Measurements

Total immunoglobulin E (IgE), as well as IgE specific for food allergens (fx5) or inhalative allergens (sx1) were determined at children's age of 10 years by Phadia ImmunoCAP system (Thermo Fisher Scientific, Freiburg, Germany) from serum samples. Total IgE concentration >34.6 kU/l was classified as "increased," as well as specific IgE (sx1 or fx5) >0.35 U/l (29). Values below the detection limit were included in the analyses using half of the defined detection limit.

Statistical Analyses

After testing for normal distribution with Shapiro-Wilk test, descriptive analyses were performed using non-parametric tests for parameters found not to be distributed normally. Data are presented as medians with 25–75th percentile (1st to 3rd quartile) or as frequencies (%). χ^2-tests were used to compare characteristics in the analyzed sub-cohort at age 10 years with the total cohort recruited during pregnancy (sex of the child, mothers age at birth, birth mode, breastfeeding duration, presence of older siblings, parental school education (highest level), environmental tobacco smoke (ETS) exposure during pregnancy, pet keeping during pregnancy, family history of atopy and body mass index). Further, these characteristics were compared within the cohort for analysis with respect to children's anti-/pro-inflammatory C-DII. Characteristics known to be associated with atopic outcomes that were also associated with the C-DII were included as confounders in the regression models.

The relationship between atopic outcomes and nutrients/C-DII/food groups as well as the association between C-DII and food groups was addressed using the Mann–Whitney *U*-test. To adjust for confounders, multiple logistic regression models were used to calculate the risk of having a pro-inflammatory C-DII at the age of 10 years (dependent variable) with respect of the atopic outcome development within the first 10 years of life (independent variable) while adjusting for potential confounding factors (sex of the child, breastfeeding duration, parental school education, pet keeping during pregnancy and body mass index age 10). Data are presented as odds ratios with 95% confidence interval. All p-values <0.05 were considered to be significant. Statistical analyses were performed with STATISTICA for Windows, Version 13 (Statsoft Inc.), R (version 3.6.1; R development Core Team) or GraphPad Prism (Version 8.1.2.).

RESULTS

Characteristics of the Analyzed LiNA Sub-Cohort

From the total cohort (n = 629), 268 participated in the 10-year campaign and 211 of whom were available with complete FFQ as well as confounding data (**Supplementary Figure 1**). Drop outs resulted from loss to follow-up over 10 years (average annual drop out 8.95%). Reasons for drop out – if available – were for example family moving or less available time when kids entered school. General characteristics (sex of the child, mothers age at birth, birth mode, breastfeeding duration, presence of older siblings, parental school education (highest level), environmental tobacco smoke (ETS) exposure during pregnancy, pet keeping during pregnancy, family history of atopy and body mass index) of the analyzed sub-cohort compared to the total LiNA cohort are presented in **Table 1** with no differences seen between the two groups.

Atopic Outcomes

Within the analyzed sub-cohort prevalence of atopic dermatitis and food allergy during the first 10 years of life was 37.4 and 11.8%, respectively (**Supplementary Table 1**). From the 79 children diagnosed with atopic dermatitis, 76.2% had increased total IgE levels measured at the age of 10 years compared to the allergy-diagnostic reference value of 34.6 kU/l for 10-year-old children. Further, 25.4% of these children had increased food-allergen-specific fx5 levels as well as 61.2% increased airway-allergen-specific sx1 levels. In addition, from the 25 children with food allergy 90.5% had increased total IgE levels, 33.3% increased fx5 levels as well as 71.4% increased sx1 levels at the age of 10 years. For 16 children both atopic dermatitis and food allergy was reported.

General Dietary Intake

Because it was the first time that nutrients were assessed *via* FFQ and DGExpert in the LiNA cohort, a comparison of the final LiNA nutrient data set was performed with data from a study with similar design/geographical region as were available from EsKiMo, a nutritional module from the Robert Koch institute's KiGGs study (30). All analyzed macronutrients (% of total energy intake for fat, carbohydrates and proteins, absolute amounts of fatty acids, cholesterol, sugar and fibers) or absolute amounts of consumed minerals or vitamins were in a very similar range and thus comparable between LiNA and EsKiMo (31) for 10-year-old boys and girls (**Supplementary Table 2**; overall median difference between LiNA and EsKiMo was 8%).

For the following investigations, a representative subset of 35 nutrients (macronutrients, minerals, and fat/water soluble vitamins) was analyzed. In general, the overall intake of macro- and micronutrients of the LiNA participants was displayed as % of total energy intake (carbohydrates, fat, and proteins) or as absolute values; both compared to the D-A-CH-reference values which is shown in **Supplementary Figure 2** for all children and in **Supplementary Figure 3** for boys/girls separately. For macronutrients in all children, data exceeded the recommendation for total fat intake (30% of energy) and total protein intake (0.9 g/kg body weight; in LiNA adequate to an overall 6.6% of the total energy intake) as pictured in **Supplementary Figure 2A**, with the girls being significantly lower in protein intake than the boys (**Supplementary Figure 3A**). Children's minerals intake exceeded the D-A-CH reference for sodium (Na), chloride (Cl), magnesium (Mg), zinc (Zn), copper (Cu), and manganese (Mn), while calcium (Ca), phosphorus (P), iron (Fe) and iodine (I) and fluoride (F), in particular, were below D-A-CH reference values (**Supplementary Figure 2B**). According to sex differences, girls had a significant lower Na, Cl, K, Ca, P, Mg, Fe, I, F, and Cu intake than the boys (**Supplementary Figure 3B**).

TABLE 1 | Study characteristics.

	Analyzed sub-cohort Age 10 *N* = 211[a] n (%)	Entire LiNA cohort Pregnancy *N* = 629[a] n (%)	*p*-value χ^2 test
Sex of child			0.80
Male	107 (50.7)	330 (52.5)	
Female	104 (49.3)	299 (47.5)	
Mothers age at birth			0.74
≤25 years	16 (7.58)	66 (10.5)	
>25 – 30 years	72 (34.1)	239 (38.0)	
>30 – 35 years	77 (36.5)	214 (34.0)	
>35	46 (21.8)	110 (17.5)	
Birth mode			0.72
Spontaneous	152 (72.0)	471 (74.9)	
Cesarean section	56 (26.5)	132 (21.0)	
Others	2 (1.00)	7 (1.10)	
Breastfeeding duration			0.56
No	11 (5.20)	26 (4.10)	
3 months	27 (12.8)	112 (17.8)	
6 months	60 (28.4)	190 (30.2)	
12 months	107 (50.7)	254 (40.4)	
Presence of older siblings			0.79
Yes	74 (35.1)	208 (33.1)	
No	136 (64.5)	414 (65.8)	
Parental school education[b]			0.64
Low	2 (1.00)	16 (2.50)	
Medium	43 (20.4)	142 (22.6)	
High	165 (78.2)	464 (73.8)	
ETS[c] exposure pregnancy			0.34
No	168 (81.6)	464 (76.1)	
Yes	38 (18.4)	146 (23.9)	
Pet keeping pregnancy			0.62
No	128 (61.2)	358 (57.8)	
Yes	81 (38.8)	261 (42.2)	
Family history of atopy			0.97
None	69 (32.7)	212 (33.7)	
One	103 (48.8)	296 (47.1)	
Both	39 (18.5)	121 (19.2)	
Body mass index age 10[d]			
Underweight	25 (12.0)	–	
Normal weight	166 (79.4)	–	
Overweight/obese	18 (8.6)	–	

[a] *n may differ from 211/629 due to missing data.*
[b] *Low = 8 years school education; medium = 10 years school education; high = at least 12 years school education.*
[c] *Environmental tobacco smoke.*
[d] *Underweight (body mass index equivalent to <18,5 kg/m^2 at 18 years), normal weight (body mass index equivalent to 18,5 −<25 kg/m^2 at 18 years), overweight/obese (body mass index equivalent to ≥25 kg/m^2 at 18 years).*
General characteristics of the analyzed sub-cohort compared to the total LiNA cohort.

Potassium (K) intake was according to the recommendations. With respect to fat-soluble vitamins shown in **Supplementary Figure 2C** for the total sub-cohort, children were above (for vitamin A and K) and below (for vitamin E and in particular for vitamin D) the recommendation, with no differences between girls/boys (**Supplementary Figure 3C**). Water soluble vitamins (**Supplementary Figure 2D**) were all clearly on or above the recommended intake (for vitamin C, B1 (thiamine), B2 (riboflavin), B3 (niacin), B6, B7 (biotin), B9 (folate) and B12), with girls being significant lower in B5 (pantothenic acid), B7 and B12 intake than the boys (**Supplementary Figure 3D**). According to our data only vitamin B5 was consumed in amounts below the current recommendations (**Supplementary Figure 2D**), in particular by girls (**Supplementary Figure 3D**).

Dietary Intake With Respect to Atopic Diseases

The dietary intake assessed at the age of 10 years was analyzed with respect to children's development of atopic dermatitis or food allergy within the first 10 years of life (**Table 2**). Children with atopic dermatitis/food allergy consumed significantly lower amounts of fiber (in % of the total energy intake) than children without atopic dermatitis/food allergy. Sugar intake was lower in children with atopic dermatitis; however, overall sugar intake was above the recommendation of 10% of the total energy intake in all children. The intake of minerals was not different in children with or without atopic dermatitis/food allergy. Children with atopic dermatitis had a significant lower intake of vitamins C, E, and B7 compared to children who did not develop an atopic dermatitis within the first 10 years of life. However, both groups had either higher (for vitamin C and B7) or lower levels (for vitamin E) compared to the D-A-CH reference. Vitamin intake of children with food allergy was not different from those without food allergy within the first 10 years of life.

Children's Dietary Inflammatory Index

Children's dietary inflammatory index scores were calculated to quantify the inflammatory potential of the diet of LiNA children. In general, values above 0 indicate a more pro-inflammatory pattern, whereas values below 0 indicate an anti-inflammatory pattern. Overall, the LiNA children had a median C-DII of −0.97 (interquartile range (IQR): −2.06 to 0.26; n = 211), with girls being lower than boys (i.e., −1.22 (IQR: −2.17 to −0.24; n = 104 compared to −0.53 (IQR: −1.93 to 0.77; n = 107), respectively. Furthermore, general characteristics were compared between children who had a more anti-inflammatory (C-DII <0) and those who had a pro-inflammatory (C-DII >0) dietary pattern (**Table 3**). Data revealed that sex, breastfeeding duration, parental school education and children's body mass index at the age of 10 years differed between children with pro-inflammatory and those with anti-inflammatory C-DII score. Next, the C-DII was analyzed in the context of atopic dermatitis and food allergy. As shown in **Table 4**, there were no significant differences in C-DII between children with and those without atopic outcomes, although C-DII levels tended to be lower (indicating a more anti-inflammatory diet) in children without atopic outcomes. When C-DII was grouped into anti-inflammatory (<0) and pro-inflammatory (>0), regression models revealed that having atopic outcomes was associated with having a pro-inflammatory

TABLE 2 | Single nutrients and atopic outcomes.

	Atopic dermatitis within the first 10 years							Food allergy within the first 10 years						
	Without (*n* = 132)			With (*n* = 79)				Without (*n* = 186)			With (*n* = 25)			
	Median	Q 1st	Q 3rd	Median	Q 1st	Q 3rd	*p*-value	Median	Q 1st	Q 3rd	Median	Q 1st	Q 3rd	*p*-value #
Macronutrients														
Energy (kcal)	**2109**	1780	2590	**2152**	1715	2719	0.91	**2097**	1764	2608	**2464**	1826	2859	0.19
Fat (%E)	**35.5**	31.2	39.4	**36.4**	32.9	40.0	0.10	**35.8**	31.8	39.4	**36.3**	32.9	40.0	0.36
SFA (%E)	**15.3**	13.7	17.9	**16.0**	14.7	18.0	0.07	**15.7**	14.0	17.9	**15.8**	14.1	17.8	0.71
PUFAs (%E)	**4.7**	4.1	5.7	**4.7**	4.3	5.4	0.58	**4.7**	4.1	5.5	**4.6**	4.3	5.3	0.87
MUFAs (%E)	**12.3**	10.9	13.8	**12.6**	11.3	14.0	0.17	**12.3**	10.9	13.9	**12.8**	11.7	14.5	0.15
Chol (mg)	**286**	227	360	**293**	228	389	0.46	**283**	225	364	**330**	255	394	0.08
Carbs (%E)	**49.0**	45.5	53.5	**47.3**	44.3	51.6	0.09	**48.3**	44.5	53.1	**47.7**	44.4	52.0	0.53
Sugar (%E)	**21.9**	17.7	26.3	**19.5**	15.8	25.2	0.05	**20.6**	17.0	26.0	**20.6**	17.5	23.2	0.71
Fiber (%E)	**2.0**	1.7	2.5	**1.9**	1.5	2.2	0.04	**2.0**	1.6	2.4	**1.7**	1.5	2.0	0.02
Protein (%E)	**14.4**	13.2	15.8	**14.9**	13.3	16.2	0.23	**14.6**	13.2	16.1	**14.7**	13.8	15.5	0.91
Minerals*														
Na	**210.0**	166.4	280.5	**209.1**	162.7	298.2	0.67	**210.0**	165.5	286.4	**212.7**	186.4	300.9	0.39
K	**101.0**	79.0	121.0	**93.4**	73.8	117.2	0.23	**97.1**	75.9	120.7	**103.4**	84.8	111.0	0.75
Ca	**64.5**	53.6	89.5	**71.8**	50.9	91.8	0.92	**65.9**	51.8	88.2	**70.9**	55.5	91.8	0.64
Mg	**120.0**	96.5	151.3	**115.2**	90.0	154.8	0.36	**119.0**	95.7	151.3	**123.0**	96.0	153.2	0.69
P	**90.8**	74.4	116.8	**92.8**	72.8	119.2	0.91	**91.6**	72.8	115.2	**91.2**	77.6	124.0	0.48
Fe	**71.7**	56.0	88.9	**72.0**	54.2	94.2	0.90	**68.8**	55.0	90.0	**78.7**	56.0	97.5	0.33
Zn	**123.8**	94.8	144.3	**112.4**	93.7	155.0	0.71	**118.5**	93.7	147.0	**129.7**	102.4	144.6	0.83
J	**48.3**	38.6	60.3	**46.7**	38.3	61.1	0.48	**48.3**	38.3	60.0	**47.8**	38.3	66.7	0.96
Cl	**215.9**	173.8	293.2	**222.4**	161.2	305.3	0.89	**215.9**	170.6	288.8	**222.4**	183.5	315.9	0.42

(Continued)

TABLE 2 | (Continued)

	Atopic dermatitis within the first 10 years							Food allergy within the first 10 years						
	Without (*n* = 132)			With (*n* = 79)				Without (*n* = 186)			With (*n* = 25)			
	Median	**Q 1st**	**Q 3rd**	**Median**	**Q 1st**	**Q 3rd**	***p*-value**	**Median**	**Q 1st**	**Q 3rd**	**Median**	**Q 1st**	**Q 3rd**	***p*-value #**
Fl	**33.8**	27.8	42.8	**32.5**	26.5	44.0	0.66	**33.0**	27.0	42.5	**35.0**	30.0	45.5	0.30
Cu	**135.5**	109.5	165.0	**120.0**	98.0	180.0	0.20	**131.5**	106.0	168.0	**137.0**	109.0	169.0	0.85
Mn	**214.8**	163.3	286.3	**187.0**	152.5	281.5	0.21	**205.0**	157.5	284.5	**194.5**	161.5	281.0	0.84
Vitamins*														
A	**125.8**	93.8	172.9	**124.7**	95.7	176.2	0.94	**125.1**	94.0	175.4	**125.0**	98.1	161.9	0.92
C	**241.1**	164.8	327.8	**180.8**	125.7	344.3	0.02	**223.1**	145.4	327.5	**201.2**	135.2	352.5	0.84
D	**9.0**	7.0	13.0	**8.5**	5.5	13.5	0.59	**8.5**	6.5	13.0	**9.0**	5.5	13.5	0.66
E	**83.7**	68.8	114.3	**77.7**	60.0	103.6	0.03	**81.2**	63.6	108.2	**80.9**	66.2	110.0	0.94
K	**303.0**	210.4	417.4	**252.5**	175.3	400.5	0.14	**285.6**	197.8	411.5	**239.8**	182.3	396.5	0.68
B1	**146.9**	116.2	177.9	**140.0**	105.8	179.9	0.40	**143.8**	108.0	175.0	**156.0**	125.0	188.0	0.27
B2	**139.5**	109.5	169.5	**130.9**	101.8	169.1	0.43	**136.2**	106.0	167.0	**149.1**	120.9	178.2	0.41
B3	**240.5**	194.5	292.7	**230.9**	192.3	300.0	0.82	**236.7**	193.1	300.0	**260.8**	209.2	293.1	0.39
B5	**92.9**	74.8	116.8	**85.8**	66.8	109.4	0.11	**151.0**	118.0	194.0	**162.0**	131.0	203.0	0.48
B6	**157.0**	121.5	194.5	**150.0**	114.0	196.0	0.53	**223.5**	181.0	286.5	**252.5**	171.5	277.5	0.91
B7	**236.3**	188.3	285.3	**201.0**	158.5	285.5	0.047	**97.9**	75.8	135.0	**116.7**	72.9	133.3	0.85
B9	**105.6**	80.0	132.3	**88.8**	72.1	135.0	0.12	**216.0**	169.5	303.5	**270.5**	204.0	322.5	0.12
B12	**216.0**	162.3	301.8	**237.0**	177.5	319.5	0.24	**89.4**	73.2	113.6	**96.2**	76.0	108.4	0.67

**% of D-A-CH reference.*

 # p-values from Mann–Whitney U-test, for medians with first/third quartile (Q 1st/Q 3rd).

%E - percentage of energy intake.

Median daily nutritional intake of 10-year old children with or without atopic dermatitis/food allergy within the first 10 years of life.

All Median values are printed in bold.

TABLE 3 | C-DII and study characteristics.

	C-DII class 1 Anti-inflammatory (n = 151[a])		C-DII class 2 Pro-inflammatory (n = 60[a])		p-value
	n	%	n	%	χ^2 test
Sex of the child					
Male	70	46.4	37	61.7	0.02
Female	81	53.6	23	38.3	
Mothers age at birth					
≤25 years	13	8.6	3	5.0	0.45
>25 – 30 years	48	31.8	24	40.0	
>30 – 35 years	58	38.4	19	31.7	
>35	32	21.2	14	23.3	
Birth mode					
Spontaneous	108	72.0	44	73.3	0.52
C. section	40	26.7	16	26.7	
Others	2	1.3	0	0	
Breastfeeding duration					
no	10	6.8	0	0	0.047
3 month	18	12.2	9	15.8	
6 month	41	27.7	19	33.3	
12 months	79	53.4	28	49.1	
Presence of older siblings					
Yes	52	34.7	22	36.7	0.77
No	98	65.3	38	63.3	
Parental school education[b]					
Low	2	1.3	0	0	0.049
Medium	25	16.7	18	30	
High	123	82.0	42	70	
ETS exposure pregnancy					
No	122	83.6	46	76.7	0.22
Yes	24	16.4	14	23.3	
Pet keeping during pregnancy					
No	97	65.1	31	51.7	0.06
Yes	52	34.9	29	48.3	
Family history of atopy					
None	47	31.1	22	36.7	0.19
One	72	47.7	31	51.7	
Both	32	21.2	7	11.7	
Body mass index age 10[c]					
Under weight	17	11.4	8	13.3	0.02
Normal weight	115	77.2	51	85	
Overweight/obese	17	11.4	1	1.7	

[a] *n may differ from 151/60 due to missing data.*
[b] *Low = 8 years school education; medium = 10 years school education; high = at least 12 years school education.*
[c] *Underweight (body mass index equivalent to 18,5 kg/m^2 at 18 years), normal weight (body mass index equivalent to 18,5 −<25 kg/m^2 at 18 years), overweight/obese (body mass index equivalent to ≥25 kg/m^2 at 18 years).*
ETS - environmental tobacco smoke.
General characteristics of the analyzed sub-cohort with respect of having an anti-inflammatory (C-DII <0) or pro-inflammatory (C-DII >0) children's dietary inflammatory index at the age of 10 years.

pattern at the age of 10 years (**Table 5**; OR = 2.22, 95% CI: 1.14–4.31) for children with atopic dermatitis, OR = 3.82 (95% CI: 1.47–9.93) for children with food allergy in the first 10 years of life). These associations were independent of confounders. This more pro-inflammatory pattern in children with atopic outcomes was supported by analyses of specific consumed food groups: children that developed atopic dermatitis within the first 10 years of life consumed significantly less fruits and nuts, children with food allergy consumed significantly more of the tolerated food group including sweets/snacks etc. (**Figure 1**). Children with a pro-inflammatory diet (C-DII >0) consumed fewer vegetables, fruits and nuts, but more meat/sausages and more sweets/snacks (**Figure 2**).

DISCUSSION

In this project we assessed the dietary intake of 10-year-old children for the first time within the prospective birth cohort, LiNA. According to participants' overall dietary intake, we were able to show that they had an adequate intake of the majority of nutrients, with some even exceeding the recommendations (e.g., for total fat, SFA, protein, sugar, Na and Cl). In contrast, for some nutrients children did not even reach half of the recommendation (e.g., vitamin D, F, I). When compared to other studies, LiNA results on the intake of specific nutrients, as well as the consumed food groups [according to the OptiMix recommendation (26)] were very similar to other studies; for example, compared to the nutritional assessment within Robert-Koch-Institute's EsKiMo module (31). The overall median nutrient difference between children from LiNA and EsKiMo was 8%, supported by similar data on food consumption with respect to the recommendation as shown for 6–11 year-old children from EsKiMo: lower consumption of vegetables, fruits and carbohydrates (bread, cereals, pasta, rice and potatoes) as well as higher consumption of meat (meat, sausages, etc.) and sweets and snacks (31). Further, our data indicated that girls' intakes of minerals and water soluble vitamins was higher than boys' intakes. Sex-specific differences in food groups that provide these nutrients such as vegetables were also described previously (32–34).

In addition to the overall dietary pattern of the LiNA children, analyses on the intake of single nutrients and atopic outcomes were performed. We were not able to show a clear allergy-specific dietary pattern, although there were some changes in vitamins (B7, C, and E), sugar and fiber. Still, when interpreted according to the D-A-CH-references, these nutrients were lower (vitamin E) or higher (vitamin B7 and C and sugar) than the recommendation values independent of children's allergy development. However, children with atopic dermatitis/food allergy within the last 10 years of life were more likely to show a less anti-inflammatory/more pro-inflammatory dietary pattern at the age of 10 years as assessed *via* the C-DII. In line with this, we were also able to show that children who developed atopic dermatitis within the first 10 years of life consumed significantly less fruits and nuts – food groups which, next to others, provide mostly nutrients that would drive the C-DII toward an anti-inflammatory pattern. This was supported by the significantly lower intake of vitamin C, E, and B7 in LiNA children with atopic dermatitis within the first 10 years of life. In addition, children with food allergy within the first

10 years of life consumed significantly more of the tolerated food group [which emphasizes sweets/snacks etc., according to Kersting et al. (26)]. This food group provides mostly nutrients that drive the C-DII toward a pro-inflammatory pattern such as sugar or saturated fat (with the consequence that these energy-dense foods result in higher overall energy intake and greater inflammation). This was confirmed by showing that children's pro-inflammatory diet was associated with a lower intake of vegetables, fruits and nuts and a higher intake of meat products and sweets/snacks. We hypothesize that children might have developed this less-anti-inflammatory/more pro-inflammatory diet due to an avoidance of possible anti-inflammatory – but allergy triggering – food items. It was described earlier that therapeutic strategies in atopic dermatitis and food allergy often involve dietary exclusions, which may be seen as mandatory in children sensitive to food allergens for whom accidental and potentially life threatening anaphylactic reactions can occur (6). It was also described that these exclusions may impact diet quality, nutrient intake and nutrient demands. It was even shown that an unsupervised elimination diet in childhood might lead to malnutrition, growth retardation, vitamin deficiencies and associated health issues (22, 35). In the context of this study, a pro-inflammatory diet consumed by the children might worsen the atopic outcome itself (20) and furthermore reduce

TABLE 4 | C-DII and atopic outcomes.

	Atopic dermatitis within the first 10 years								
	Without disease outcome				With disease outcome				
	n	Median	1st quartile	3rd quartile	n	Median	1st quartile	3rd quartile	*p*-value[#]
All	132	**−1.19**	−2.13	−0.06	79	**−0.53**	−2.00	0.79	0.05
Boys	60	**−0.97**	−2.07	0.17	47	**−0.27**	−1.58	1.07	0.12
Girls	72	**−1.26**	−2.15	−0.32	32	**−1.19**	−2.18	0.40	0.57
	Food allergy within the first 10 years								
	Without disease outcome				With disease outcome				
	n	Median	1st quartile	3rd quartile	n	Median	1st quartile	3rd quartile	*p*-value
All	186	**−1.07**	−2.13	0.07	25	**−0.25**	−1.79	0.73	0.08
Boys	92	**−0.62**	−1.98	0.75	15	**−0.25**	−1.79	1.07	0.42
Girls	94	**−1.31**	−2.18	−0.35	10	**−0.03**	−2.06	0.73	0.12

 # p-values from Mann-Whitney U-test, for medians with first/third quartile.
Descriptive data on the children's dietary inflammatory index (C-DII) assessed at the age of 10 with respect to their development of atopic dermatitis or food allergy within the first 10 years of life. Shown among all 211 children from the LiNA-cohort, as well as separately for boys and girls, p-value from Mann-Whitney-U-test.

TABLE 5 | Pro-inflammatory C-DII and atopic outcomes.

	Pro-inflammatory diet age 10 (C-DII >0)											
AD	Crude						Adjusted*					
	n total	n C-DII >0	OR	(95% CI)		*p*-value	n total	n C-DII >0	OR	(95% CI)		*p*-value
All	211	60	1.89	1.02	3.49	0.04	201	57	2.22	1.14	4.31	0.02
Boys	107	37	1.87	0.83	4.23	0.13	102	36	2.65	1.04	6.72	0.04
Girls	104	23	1.62	0.61	4.31	0.33	99	21	2.52	0.86	7.36	0.09
	Pro-inflammatory diet age 10 (C-DII >0)											
FA	Crude						Adjusted*					
	n total	n C-DII >0	OR	(95% CI)		*p*-value	n total	n C-DII >0	OR	(95% CI)		*p*-value
All	211	60	2.65	1.13	6.24	0.03	201	57	3.82	1.47	9.93	0.01
Boys	107	37	1.81	0.59	5.53	0.29	102	36	4.57	1.17	17.9	0.03
Girls	104	23	4.22	1.09	16.4	0.04	99	21	7.24	1.51	34.8	0.01

**Logistic regression model adjusted for sex, breastfeeding duration, parental school education, pet keeping pregnancy and body mass index age 10; 10 missing cases on specific confounders, OR - odds ratio, CI - confidence interval.*
Logistic regression models – raw or adjusted for confounders - showing the risk for children consuming a pro-inflammatory diet at the age of 10 years (by having a C-DII >0) with respect to having developed atopic dermatitis (AD) or food allergy (FA) within the first 10 years of life.

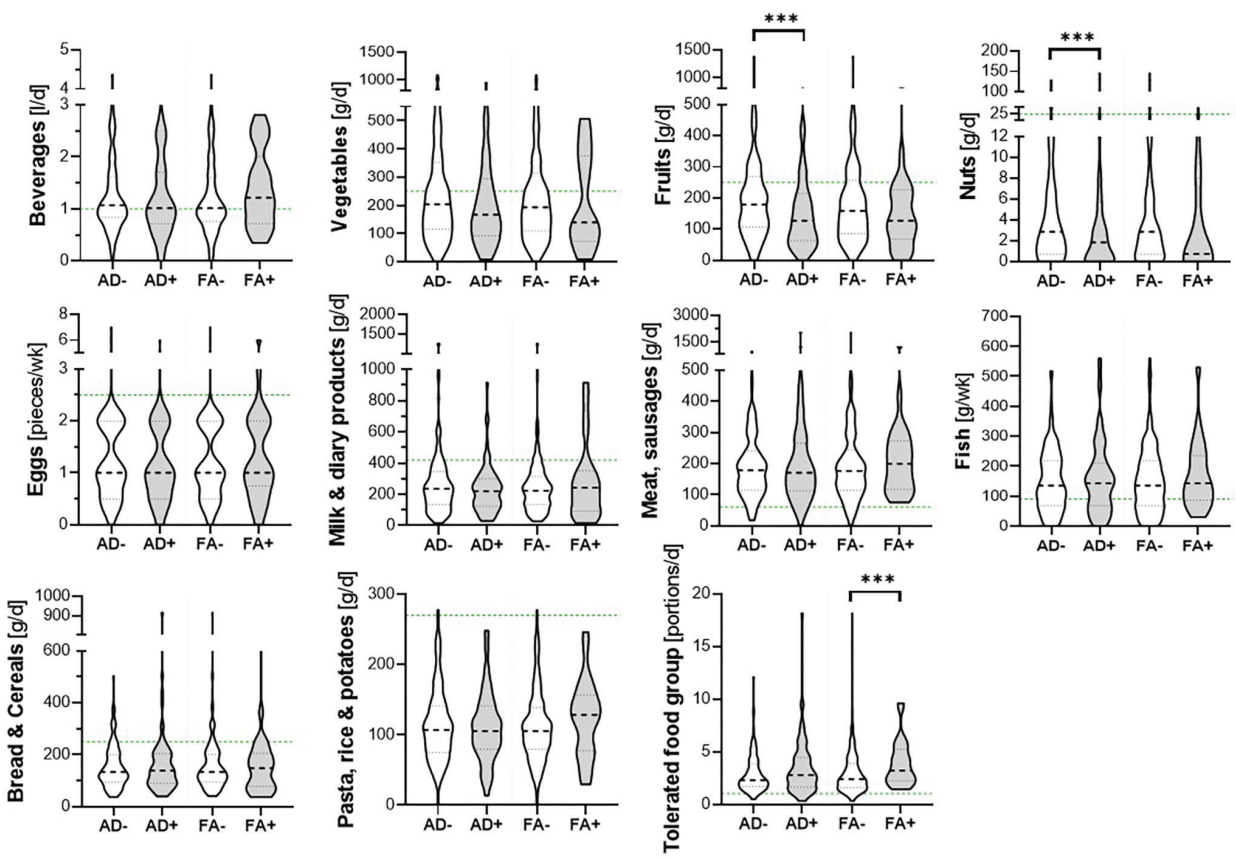


FIGURE 1 | Food groups and atopic outcomes. Children's consumed food groups at the age of 10 years according to having developed atopic dermatitis (AD +) or food allergy (FA +) within the first 10 years of life or not (AD- and FA-, respectively). Green line: OptiMix recommendations for 10–12 year old children. Data are presented as violin plots with median (bold dotted line) and 25 to 75th percentile (dotted line), $n = 211$. ***Significant difference ($p < 0.05$) between AD+/AD- or FA+/FA-, respectively (Mann–Whitney *U*-test).

their buffering capacity against harmful environmental exposures or triggers. An optimal nutritional status was described to be protective against both communicable and non-communicable diseases (36).

Because of the design of this study, reverse causality could not be ruled out. The diet as assessed at the age of 10 years could be a proxy for children's lifelong dietary pattern. The C-DII assessed in LiNA at the age of 10 was associated with markers of socio-economic status (SES) of their families assessed during pregnancy (such as parental school education). It has been shown previously that lower SES is associated with poorer nutrition (37). Studies also suggest that children begin to assimilate and mimic their parents' food choices at a very young age (38) and that this parent-child-transmission in dietary behaviors is dependent on SES (39). It was further shown that parental SES impacts childhood health issues (40) and that healthy lifestyle promotion alone might not substantially reduce the socioeconomic inequity in health (41). So, it also should be kept in mind that a pro-inflammatory diet consumed by the children on a daily basis throughout infancy also might have contributed to their allergy development. However, with the data available in our cohort, we are not able to examine the direction of temporal ordering of these effects in further detail.

To the best of our knowledge, this is the first use of the C-DII in association with atopic dermatitis and food allergy. Both atopic dermatitis and food allergy are characterized by inflammatory processes (18, 19), similar to other non-communicable diseases that are characterized by low-grade, chronic systemic inflammation. It was shown, for example, that in adults a pro-inflammatory diet (DII >0) was associated with an increased risk of certain cancers, cardiovascular disease, adverse mental health outcomes, and musculoskeletal disorders (21). It also is known that a pro-inflammatory diet is linked to greater all-cause mortality risk (42). The evidence for an association between DII and respiratory health, neurodevelopmental outcomes, metabolic syndrome, diabetes and obesity was described to be either conflicting or scarce (21). Furthermore, there are limited data in the context of DII and allergies, and the available data so far address mainly respiratory issues such as asthma or wheezing. For example, in children, a pro-inflammatory diet

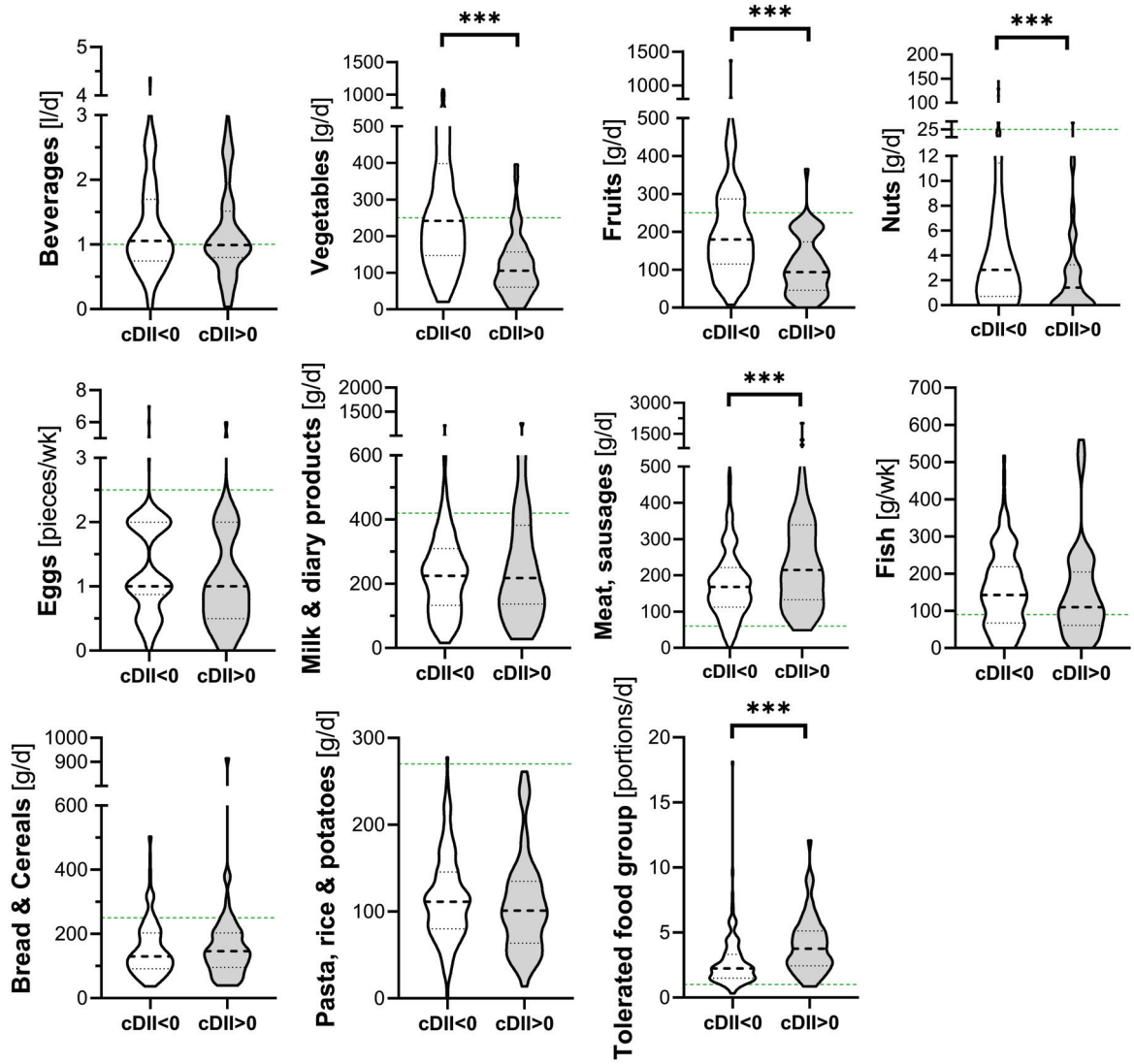


FIGURE 2 | Food groups and C-DII. Children's consumed food groups at the age of 10 years according to having a pro (C-DII >0) or anti-inflammatory (C-DII <0) diet at the age of 10 years. Green line: OptiMix recommendations for 10–12 year old children (26). Data are presented as violin plots with median (bold dotted line) and 25 to 75th percentile (dotted line), $n = 211$. ***Significant difference ($p < 0.05$) by Mann–Whitney *U*-test.

was not associated with current asthma or lung function, but in children with allergic airway inflammation, a higher DII score was associated with a 2.38 fold higher risk of wheezing (43). In addition, a pro-inflammatory diet was associated with asthma (20). Further, it was shown that higher inflammatory potential of the maternal diet was associated with increased odds of offspring asthma and/or wheeze by age 4 years, although results attenuated into non-significance after adjustment for confounders (44).

One strength of our study lies in the well-characterized participants regarding longitudinal atopic outcomes and exposure variable assessment, including diet. Therefore, a possible link between children's dietary intake of specific nutrients or specific indices such as the C-DII and allergy development could be investigated. The use of an index such as the C-DII offers an insight into the total dietary pattern compared to interpreting singular effects of specific nutrients. A limitation of the LiNA study in general is the potential bias by high rates of participating atopic parents (64.7%), limiting our ability to extrapolate findings to the general population (with approximately 30% prevalence of atopic outcomes). This fact is accumulating even more throughout the 10-year follow up, in detail 76% of the children positive for AD within our analyzed sub-cohort show a positive family history of atopy. This shift was also seen in the high rates of increased IgE levels in children negative for atopic dermatitis or food allergy. One further limitation of the study is the low number of cases in certain

outcomes, in particular when analyses are stratified for sex, which limited the power of the results. Furthermore, outcome data were obtained, in part, from parental questionnaire documented physician diagnosis of outcomes. This might reduce the strengths of the reported results. However, by including clinical allergy markers such as the IgE data we may overcome this limitation, at least in part. Further, the high rates of increased IgE levels at the age of 10 years in children positive of atopic dermatitis or food allergy at least once during their first 10 years outlines that these children have a persistent atopic phenotype also at the age of 10 years. Another major limitation is the missing questionnaire information on children's physical activity in general and in their leisure time in particular. Therefore, we probably have underestimated the energy expenditure in several children. However, our data are very similar to the study protocol from Eskimo (30) who also reported this limitation.

CONCLUSION

Children with atopic dermatitis/food allergy within their first 10 years of life were more likely to show a more pro-inflammatory dietary pattern assessed at the age of 10 years *via* the C-DII compared to children without allergic diseases. Because of their allergy history, these children may have developed a more pro-inflammatory dietary pattern due to avoidance of possible allergy triggers such as fruits or nuts for example. Overall, a pro-inflammatory dietary pattern might worsen the atopic outcome itself and reduce the buffering capacity of the individual against harmful environmental exposures or triggers. For pediatricians it is recommended to test affected children for their individual tolerance of allergenic foods to avoid a restrict elimination diet. Furthermore, an increased nutrient density of tolerable food items should be advised to omit undesirable effects of eating a pro-inflammatory diet.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because longitudinal LiNA datasets are not anonymized. Therefore, the raw cohort data cannot be provided as an open source file due to ethical declaration/data protection issues. Data can be requested in their analysed version from the corresponding author. Requests to access the datasets should be directed to KJ, kristin.junge@ufz.de.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of the University of Leipzig and the Saxonian Board of Physicians (046-2006, 160-2008, 160b/2008, 144-10-31052010, 113-11-18042011, 206-12-02072012, 169/13-ff, 150/14-ff, EK-allg-28/14-1, and 008/17-ek). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

KJ: conceptualization and project administration. OS, LB, NS, JH, and JF: methodology. OS, LB, and NS: software. OS, KJ, and SR: validation. OS and KJ: formal analysis, visualization, and writing – original draft preparation. AZ and GH: resources. MB, US, and WK: clinical resources. SR: data curation. GS, GH, JF, JH, AZ, OS, LB, SR, MB, US, NS, and WK: writing – review and editing. KJ and GS: supervision. GH: cohort PI. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.868872/full#supplementary-material

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Conflict of Interest: JH owns controlling interest in Connecting Health Innovations LLC (CHI), a company that has licensed the right to his invention of the dietary inflammatory index (DII®) from the University of South Carolina in order to develop computer and smart phone applications for patient counseling and dietary intervention in clinical settings. NS is an employee of CHI.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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2.2 Volatile organic compounds and allergy development

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The LINA cohort: Cord blood eosinophil/basophil progenitors predict respiratory outcomes in early infancy

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Abstract Rationale: Cord blood eosinophil/basophil progenitor cells (Eo/B) of high risk infants have been shown to predict respiratory illnesses in infancy. Here we investigated this association in a population-based cohort. Furthermore, we analysed whether newborns Th1/Th2 balance and prenatal environmental exposure impact Eo/B recruitment.
Methods: In a sub-cohort of the LINA study cord blood mononuclear cells were used for methylcellulose assays to assess Eo/B differentiation. Questionnaires were recorded during pregnancy and annually thereafter. Volatile organic compounds were measured during pregnancy and cord blood cytokines after ex vivo stimulation.

Abbreviations: Cb, cord blood; CFU, colony forming units; Eo/B, eosinophil/basophil; ETS, environmental tobacco smoke; FHA, family history of atopy; GM-CSF, granulocyte macrophage colony-stimulating factor; IL-3, IL-5, interleukin 3, interleukin 5; IL-5Rα, interleukin 5 receptor alpha sub-unit; VOC, volatile organic compounds; OR, odds ratio; Th2, T helper cell type 2.

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Results: Cord blood IL-4 and IL-13 positively correlated with Eo/B. Tobacco smoke related benzene was also positively associated with Eo/B. Enhanced Eo/B numbers increased the risk for wheezing within the first 24 months.
Conclusions: The association between cord blood Eo/B and respiratory illnesses is not restricted to high-risk children. Prenatal environmental exposure and a Th2 milieu at birth contribute to Eo/B recruitment.

1. Introduction

Pre- and early postnatal programming affects the structure and function of the fetal lung. A variety of immune parameters have been found to be involved in lung inflammation and to be affected by the intrauterine environment or environmental exposure during pregnancy. Thus, the immune status at the time of birth might be suitable to identify children at risk to develop respiratory diseases later in life. It has been shown for example that cord blood IL-8 was associated with the risk for wheeze in one year old children [1] or that the Th1/Th2 milieu at the time of birth is related to later asthma [2].

A cell population which is also discussed to predict respiratory and allergic outcomes are eosinophil/basophil progenitor cells in cord blood. It has been shown that there is a deficient maturation of cord blood eosinophil/basophil progenitors caused by a decreased expression of hemopoietic cytokine receptors in a selected group of newborns at risk of atopy [3]. It was also shown that in high risk infants cord blood hematopoietic progenitor cells predict the development of acute respiratory illnesses [4] or recurrent wheeze [5] during the first 12 months. However, it is still not clear, if the predictive value of these cord blood eosinophil/basophil progenitors in terms of respiratory outcomes is only restricted to high risk infants or might also be seen in a general study population. If so, that aspect might even better qualify these cells as a potential neonatal marker for later disease.

There is also literature showing that cord blood progenitors of high risk newborns are sensitive to lifestyle intrauterine modifications, such as n−3 fatty acid supplementation during pregnancy, which leads to an altered infant cord blood hematopoietic progenitor phenotype [5]. However, less is known whether – next to nutritional aspects – environmental triggers to which the newborn is indirectly exposed to *in utero* also impact on cord blood progenitor cell development. We showed in an earlier study that the direct exposure to tobacco smoke or tobacco smoke related volatile organic compounds (VOC) correlated with Eo/B progenitors in 1 year old children [6].

The aim of the present study was to investigate the association between cord blood Eo/B progenitors and respiratory outcomes in a general study population. We furthermore analysed whether environmental chemicals, such as tobacco smoke-related VOC alter cord blood Eo/B progenitor cell recruitment and whether associations between cord blood Eo/B progenitor cells and other immune parameters exist.

2. Materials and methods

2.1. Study design and sample collection

The LINA cohort study (*L*ifestyle and environmental factors and their *I*nfluence on *N*ewborns *A*llergy risk) recruited 629 mother–child-pairs between May 2006 and December 2008 in Leipzig, Germany, to investigate how environmental factors in the pre- and postnatal period influence immune system development and resulting disease risks [7,8]. Mothers suffering from immune or infectious diseases during pregnancy were excluded from the study.

606 mother–child-pairs participated in the one year, 546 in the two year follow-up. Cord blood was collected at delivery. Progenitor cell analyses were performed in a sub-cohort of 40 children. Standardized questionnaires were administered during pregnancy (at week 36) and annually thereafter, collecting information on family history of atopy (FHA) as well as housing and environmental conditions (first or second hand smoke, mould, traffic, noise, pets, renovation activities and personal lifestyle). At the age of one and two years, information about respiratory outcomes was collected. Information from both questionnaires were then summarised for the outcome prevalence within the first 24 months as used in the present paper. Wheezing was recorded as a parental report of wheezing symptoms either ever (wheezing ever) or in two or more than two periods within the last 12 months (recurrent wheezing). Asthma, bronchitis and obstructive bronchitis were recorded as physician diagnoses ('Has a doctor diagnosed your child with asthma/bronchitis/chronic obstructive pulmonary disease in the last 12 months?'). The control group didn't have any of the mentioned outcomes at all within the first 24 months of life. All questionnaires were self-administered by the parents. Participation in the study was voluntary and written informed consent was obtained from all participants. The study was approved by the Ethics Committee of the University of Leipzig (file ref # 046-2006, 160-2008).

2.2. Preparation of cord blood samples

Preparation of cord blood mononuclear cells was performed as described earlier [4,6]. Briefly, cord blood mononuclear cells were isolated from 2 to 3 ml fresh heparinised cord blood via Ficoll Paque density centrifugation within 6 h after blood collection. Cord blood mononuclear cells were cryopreserved in 1 ml of 90% fetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO) with 10×10^6 cells on average. Samples were stored in liquid nitrogen until shipping and analyses. Cells

were thawed by dropwise application of RPMI complete media. After one washing step cells were resuspended in McCoy 3+ media and incubated for 2 h in a plastic flak to eliminated adherent mononuclear cells [9]. Resulting non adherent mononuclear cells were used for methylcellulose assays. Viability of the cells after thawing averaged 82%. The investigator, who performed the progenitor assays and analyses, was blinded to identification of subjects and other data collected by protocol.

2.3. Methylcellulose assay

Methylcellulose assays were performed as described earlier [4,6,9]. In brief, duplicates of 2.5×10^5 non adherent mononuclear cells /35 mm × 10 mm culture dishes were incubated in 0.9% methylcellulose with Iscove's 2+ (modified Dulbecco's medium and 20% FCS supplemented with 1% penicillin–streptomycin and 5×10^{-5} mol/l beta-mercaptoethanol) in the presence of recombinant human cytokine IL-3 (1 ng/ml), IL-5 (1 ng/ml) or GM-CSF (10 ng/ml). After 14 days (37 °C and 5% CO_2) stage dependent progenitor cell differentiation was assessed by morphological distinguishable colony formation via inverted light microscopy. Colonies were defined as >40 cells.

2.4. Measurement of VOC concentrations

VOC concentrations at home were measured 4 weeks before birth as described earlier [6,10,11]. For VOC sampling during pregnancy passive samplers (3 M monitors, type OVM 3500; 3 M GmbH, Neuss, Germany) were placed in the room where mothers spent most of their time. VOC concentrations

Table 1 General study population characteristics.

	Analysed sub-cohort n (%), N = 40 [a]	Entire LINA cohort n (%), N = 629 [b]	χ^2-test [c]
Birth weight			
<3000 g	3 (7.5)	117 (18.6)	0.113
>3000–3500 g	19 (47.5)	241 (38.4)	
>3500–4000 g	14 (35.0)	198 (31.5)	
>4000 g	4 (10.0)	72 (11.5)	
Maternal age at delivery			
≤25	6 (15.0)	66 (10.5)	0.132
>25–30	19 (47.5)	239 (38.0)	
>30	15 (37.5)	323 (51.5)	
Gender of the child			
Female	20 (50.0)	302(48.1)	0.807
Male	20 (50.0)	327 (51.9)	
Siblings			
Yes	11 (27.5)	211 (33.5)	0.357
No	29 (72.5)	418 (66.5)	
Family history of atopy			
double positive	7 (17.5)	121 (19.2)	0.433
single positive	16 (40.0)	296 (47.1)	
negative	17 (42.5)	212 (33.7)	
Family history of asthma			
Positive	7 (18.4)	110 (18.2)	0.990
Negative	31 (81.6)	493 (81.8)	
Parental education [d]			
Low or Intermediate	11 (27.5)	160 (25.4)	0.736
High	29 (72.5)	469 (74.6)	
Keeping of cat			
Yes	6 (15.0)	113 (18.0)	0.568
No	34 (85.0)	516 (82.0)	
Mould in the dwelling			
Yes	15 (37.5)	243 (38.6)	0.873
No	25 (62.5)	386 (61.4)	
Cotinine (μg/g) 34th week of pregnancy (log)	0.55 (0.10–2.24)	0.72 (0.26–1.73)	0.525 [e]

[a] N may be different from 40 due to missing data.
[b] N may be different from 629 due to missing data.
[c] Calculated using the chi squared test for cross relationship.
[d] Low or intermediate = 10 yrs of schooling or less ('Mittlere Reife' or 'Hauptschulabschluss'); high = 12 yrs of schooling or more ('(Fach-)hochschulreife').
[e] p-Value from student's t-test.

were analysed using a coupled gas chromatograph/mass spectrometer (GC/MS) system and afterwards corrected for seasonal variations. Additional details regarding VOC sample collection analytical methods and seasonal variations are described elsewhere [12].

2.5. Measurement of Th2 cytokines

Cytokine measurements were performed following a whole blood stimulation as described earlier [11,13]. In brief, blood samples were diluted 1:1 with RPMI1640 medium without supplements and incubated for four hours at 37 °C with 50 μg/ml phytohemagglutinin (PHA). After centrifugation supernatant was collected and stored at −80 °C until analysis. Concentrations of the cytokines IL-4, IL-5, IL-13, TNFα IFNγ and IL-12 were measured in the supernatant by flow cytometry using a cytometric bead array (BD CBA Human Soluble Flex Set system, Becton Dickinson, Heidelberg, Germany) according to manufacturer's instructions; for more details see [14]. The detection limit was 3 pg/ml for all cytokines, except for IL5 with 2 pg/ml.

2.6. Analyses of urinary cotinine concentration

Cotinine was analysed in the urine of pregnant mothers (34th week). After dichloromethane extraction and chromatographic separation with a Chromolith Speed ROD column (Merck, Darmstadt, Germany) cotinine was determined using turbo ionspray ionization on the LC/MS/MS device API 4000 (Applied Biosystems, Darmstadt, Germany). Analysis of creatinine as a measure for individual urinary dilution was used for standardization of the metabolite.

2.7. Statistical analyses

Since the majority of parameters were not normally distributed, analyses were performed using non-parametric tests in general. To test the equal distribution of parameters in the analysed sub-cohort and the entire LINA cohort the chi squared test was performed. To address the relationship between atopic outcomes and Eo/B CFUs medians are compared using Mann–Whitney U-test. In addition, linear regression models were used to consider possible confounding factors. Data are presented as odds ratios with 95% confidence interval and were adjusted for cotinine levels during pregnancy, keeping of cat and mould in dwelling. In addition, we adjusted for family history of asthma (instead of family atopy history since we have focused our analyses on respiratory diseases).

Spearman's rank correlation test was applied to analyse the association between cytokine or VOC concentrations and numbers of Eo/B CFUs in cord blood. All p-values ≤ 0.05 were considered to be significant. Adjustments due to multiple testing were not performed since our analyses were based on an *a priori* hypothesis [15]. Statistical analyses were performed with STATISTICA for Windows, Version 10 (Statsoft Inc., USA).

Table 2 Prevalence of respiratory outcomes within the first 24 months of life.

	Analysed sub-cohort n	Entire LINA cohort n	χ^2-test[a]
No respiratory outcomes at all	15	224	
Wheezing ever	16	196	0.594
Wheezing recurrent	9	106	0.587
Bronchitis	15	231	0.935
Obstructive bronchitis	7	78	0.537
Asthma	0	7	0.494

[a] Calculated using the chi squared test for cross relationship.

3. Results

3.1. Study characteristics

General characteristics of the study participants (birth weight, maternal age at delivery, gender of the child, siblings, FHA, family history of asthma, parental education, keeping of a cat, mould in the dwelling as well as the exposure to ETS during pregnancy) are shown in Table 1. There were no differences between the analysed sub-cohort (n = 40) and the total LINA cohort (n = 629).

The prevalence of respiratory outcomes within the first 24 months is shown in Table 2. As well, no differences were seen between the analysed sub-cohort and the total LINA cohort within the first 24 months of life.

3.2. Association between cord blood Eo/B CFUs and general characteristics

It was further of interest if general newborn characteristics like weight of birth, maternal age at delivery, gender of the child or the number of siblings impact on newborns Eo/B CFUs (Table 3). No significant associations were seen for all of these parameters, however, boys seemed to have slightly more GM-CSF-stimulated Eo/B CFUs than girls (p = 0.053).

3.3. Association between cord blood Eo/B CFUs and outcomes

Numbers of Eo/B CFUs in association with respiratory outcomes are shown in Fig. 1. Children who ever developed wheezing symptoms (p = 0.033) or a physician diagnosed bronchitis (p = 0.044) had higher numbers of Eo/B CFUs, in particular in response to IL-5 stimulation in their cord blood. In addition, data were analysed in a linear regression model with and without considering confounding factors like parental history of asthma, cotinine levels during pregnancy, keeping of cat and mould in dwelling (Table 4). Newborns with higher numbers of cord blood IL-5 stimulated Eo/B CFUs had an increased risk of getting wheezing ever (adj OR: 1.57; IQR: 1.02–2.42; p = 0.042) or recurrent (adj OR: 1.65; IQR: 1.06–2.70; p = 0.048) within the first 24 months.

Table 3 Correlation between IL-3, IL-5 or GM-CSF stimulated cord blood Eo/B CFU and general characteristics.

	Eo/B CFU stimulated with					
	IL-3		IL-5		GM-CSF	
Spearman's rank correlation test	p	R	p	R	p	R
Birth weight	0.646	−0.078	0.467	−0.127	0.511	0.115
Maternal age at delivery	0.735	−0.058	0.703	0.067	0.804	0.043
Mann–Whitney U-test	p		p		p	
Gender of the child	0.298		0.163		0.053	
Siblings	0.390		0.406		0.553	

3.4. Association between cord blood Eo/B CFUs and VOC exposure during pregnancy

We demonstrated earlier within the LINA study that infants exposed to ETS or VOCs show higher numbers of Eo/B progenitor cells in their peripheral blood. To test whether ETS/VOC exposure in the home already during pregnancy contributes to the recruitment and differentiation of Eo/B progenitor cells, we analysed concentrations of single tobacco smoke related VOCs like benzene, toluene and xylene via

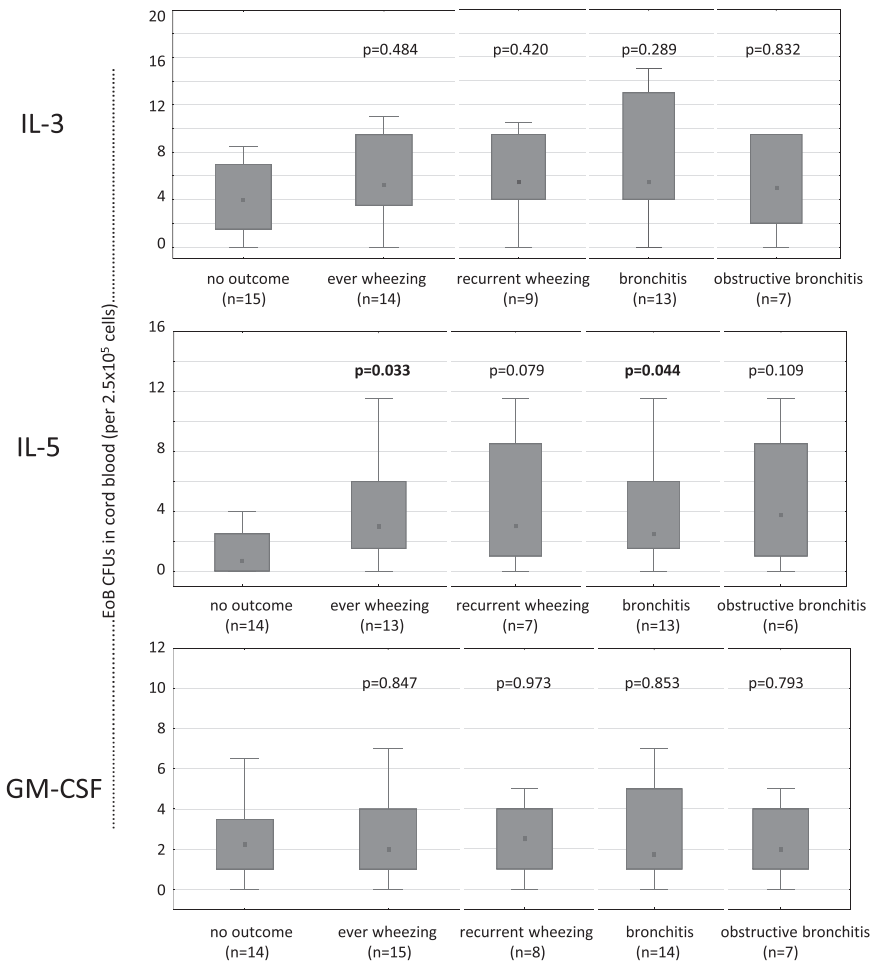

Figure 1 IL-3, IL-5 or GM-CSF stimulated Eo/B CFUs in cord blood of newborns with ever wheezing, recurrent wheezing, physician diagnosed bronchitis or obstructive bronchitis within the first 24 months compared to children without any respiratory outcome within the first 24 months. Data are presented as box plots with median and 25th to 75th percentile; p-levels are derived from Mann–Whitney U-test.

Table 4 Relationship between IL-3, IL-5 or GM-CSF stimulated cord blood Eo/B CFUs and later airway outcomes within the first 24 months of life. Data are presented as odds ratios (OR; either raw or adjusted for parental history of asthma, cotinine levels during pregnancy, keeping of cat, and mould in dwelling) with 95% confidence interval (CI). Analysed sub-cohort n = 40, significant results are presented in bold.

	Eo/B CFUs stimulated with					
	IL-3	IL-5	GM-CSF	IL-3	IL-5	GM-CSF
	OR (95% CI) raw			OR (95% CI) adjusted		
Wheezing (ever)	1.00 (0.93–1.07)	1.38 (0.97–1.97)	0.92 (0.69–1.24)	1.00 (0.91–1.09)	**1.57 (1.02–2.42)**	0.91 (0.60–1.38)
Wheezing (recurrent)	1.01 (0.93–1.09)	1.35 (0.94–1.92)	0.92 (0.63–1.34)	1.02 (0.92–1.13)	**1.65 (1.06–2.70)**	0.99 (0.60–1.62)
Bronchitis (diagnosed)	1.01 (0.94–1.09)	1.35 (0.94–1.92)	0.97 (0.74–1.27)	1.03 (0.94–1.14)	1.44 (0.96–2.17)	1.00 (0.64–1.57)
Obstructive bronchitis (diagnosed)	1.00 (0.93–1.09)	1.34 (0.95–1.91)	0.88 (0.58–1.35)	1.05 (0.94–1.17)	1.60 (0.97–2.65)	0.96 (0.51–1.78)

Spearman's rank correlation test (Table 5). The cord blood IL-5 and IL-3-stimulated colonies were positively correlated with benzene concentrations in the home of the study participants. No associations were seen for toluene and xylene.

3.5. Association between cord blood Eo/B CFUs and cord blood cytokines

Furthermore, we tested whether the immune- and in particular the Th1/Th2 cytokine-status of the newborn was related to the number of cord blood Eo/B colonies. After excluding one extreme value, we could show that cord blood IL-5 stimulated Eo/B CFUs were positively correlated with cord blood Th2 cytokines IL-4 ($R = 0.413$, $p = 0.019$) and IL-13 ($R = 0.369$, $p = 0.038$; Fig. 2). However, no correlation was seen for cord blood IL-5 and IL-5-stimulated colonies ($R = 0.042$, $p = 0.821$). For the Th1 cytokines TNFα, IFNγ and IL-12 no significant correlation was seen with Eo/B CFUs (data not shown).

4. Discussion

In this study we were able to show that cord blood Eo/B progenitor cells correlate with infant's later respiratory outcomes within the first 24 months in a general study population. Further, we could show that the neonatal Th2 milieu is associated with the progenitor cell differentiation. The fact that maternal exposure to tobacco smoke during pregnancy was associated with the number of cord blood Eo/B progenitor cells, gives further evidence for a prenatal programming of the immune system with consequences for the disease risk later in children's life.

It is well established that the triad of cytokines IL-3, IL-5 and GM-CSF significantly impact the differentiation of eosinophil/basophil progenitor cells [16,17] whereby IL-3 and GM-CSF favour early, and IL-5 the terminal cell development towards a mature eosinophil. Associations seen in the present study between outcomes and progenitor cells were all restricted to IL-5 stimulated Eo/B CFUs. Specifically, newborns that developed wheezing or bronchitis within the first two years of life had more terminal differentiated eosinophil progenitors in their cord blood. That was also seen in an earlier study, which showed that IL-5 responsive progenitors in cord blood of high risk infants were positively associated with recurrent wheezing (OR: 1.11, CI: 1.02–1.21) or atopic dermatitis (OR: 1.09, CI: 1.00–1.18) in the first year of life [5]. In that context it was discussed that the IL-5 responsiveness *in vitro* mimics the IL-5 stimulation *in vivo*. However, in the current analyses we could not show a correlation of cord blood IL-5 responsive Eo/B CFUs and cord blood IL-5 concentrations. One reason for that could be the very low IL-5 concentrations measured in cord blood of our study participants [13]. Nevertheless, we saw a significant correlation

Table 5 Correlation of VOC concentrations in the dwelling during pregnancy and cord blood IL-3-, IL-5- or GM-CSF- stimulated Eo/B CFUs. Data are presented as Spearman's rank correlations, significant p-values are printed in bold.

	Eo/B CFU stimulated with					
	IL-3		IL-5		GM-CSF	
	p	R	p	R	p	R
Benzene	**0.008**	**0.432**	**0.029**	**0.371**	0.714	0.064
Toluene	0.942	0.012	0.801	0.044	0.092	−0.290
m + *p*-Xylene	0.842	0.034	0.672	0.074	0.925	0.017
o-Xylene	0.766	0.051	0.743	0.058	0.863	−0.030

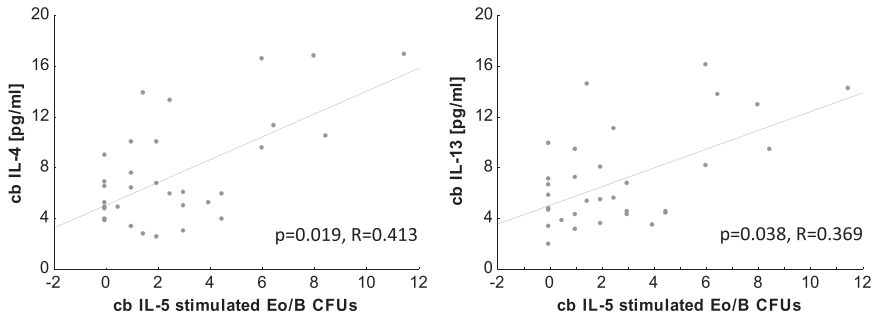

Figure 2 Correlation of cord blood (cb) Th2 cytokines IL-4 and IL-13 with the number of IL-5 stimulated cb Eo/B CFUs. P-levels and regression coefficients are derived from Spearman's rank correlations.

between the Th2 cytokines IL-4 as well as IL-13 and IL-5 responsive Eo/B CFUs. So children who had more terminally differentiated cord blood eosinophil progenitors (and in association with that a higher risk of wheezing or bronchitis later in life) also had a Th2 driven immune status at birth. It can be hypothesized that higher numbers of terminal differentiated cord blood progenitors and therefore assumed higher numbers of mature eosinophils (via *in situ* hematopoiesis [18]) have produced IL-4 and IL-13 themselves to enhance the Th2 milieu/inflammation [19]. Another explanation for this correlation might be that IL-4 and IL-13 were secreted by activated Th2 lymphocytes [20] which may have enforced early progenitor cell migrational response [21]. However, causal relationships remain speculative. Nevertheless, present data are in agreement with our data published on Eo/B CFUs of one year old children, where we also saw positive correlations of Eo/B CFUs and cord blood IL-4 or IL-13 (but not IL-5) in cradle cap positive children [6]. So a dominant Th2 milieu at birth seems to drive neonatal or postnatal eosinophil/basophil differentiation.

In contrast to these data demonstrating terminally differentiated eosinophil progenitor cells as outcome predictors, an analysis by Fernandes et al. showed positive associations for early differentiated IL-3 and GM-CSF stimulated Eo/B CFUs [4]. In detail, in cord blood of high risk infants IL-3 or GM-CSF stimulated Eo/B CFUs were predictive for acute respiratory illness with fever or wheeze within the first year of life. Since IL-3 and GM-CSF are not exclusively effective in the eosinophil lineage differentiation, it was discussed that these "early stage progenitors" might contribute to a multi-cellular instead of an eosinophilic inflammatory response [4].

One explanation for these different findings might be the diversity in the addressed outcomes. Although the majority of predictive associations are seen for respiratory outcomes, the difference in diagnosis assessment, in additional symptoms considered as well as in the specific pathophysiology of each disease might confound the obtained results.

In addition, predictive values of functionally assessed Eo/B progenitor cells shown in the past were all restricted to observations in high risk newborns/children according to their parents history of allergy/atopy or skin prick testing [4,5] and do therefore not allow to compare data one by one. It was discussed earlier that high risk infants have a delayed or altered immune response [13,22]. With that in mind and especially in consideration of a potential use of cord blood Eo/B progenitor cells as a marker correlating with later outcomes it is of high importance that associations are also visible in a general study population and not only in children at high atopy risk. However, more data are required to define for example a critical threshold for the number of colonies objectively identifying children at risk for later outcomes.

Another factor which was investigated in the present paper was the impact of prenatal environmental exposure on Eo/B progenitor cells in cord blood. As we have already shown recently for one year old cradle cap positive children, Eo/B CFUs seem to be sensitive to ETS or tobacco smoke related VOCs [6]. However, in contrast to the unborn fetus these one year old children are obviously directly exposed to indoor chemicals present in their homes. To our knowledge, less is known about how exposure to ETS or VOCs in the prenatal period impact cord blood Eo/B progenitor cells via *in utero* mechanisms. We could show a significant positive correlation between IL-5 and IL-3 stimulated Eo/B CFUs and benzene as a major component released by tobacco smoke [6,23,24]. Toluene and xylene can have various additional indoor sources, including building materials (paints, varnishes) or consumer products (fingernail polish, lacquers, adhesives, cleaning agents). The fact that only benzene correlated with progenitor cell numbers supports our hypothesis that tobacco smoke rather than other exposures might influence the proliferation and differentiation of cord blood Eo/B progenitor cells. Within an earlier study we have already reported that maternal exposure to VOCs appears to influence the fetal immune system development and the Th1/Th2 status at birth with a resulting Th2 skewed T-cell response in the cord blood [25]. These data suggest that VOC exposure in the prenatal period has an impact on the maturation of specific immune cells. This is in agreement with the hypothesis raised by the present paper that *in utero* exposure to environmental pollutants may contribute to increased numbers of Eo/B CFUs and might therefore drive later development of respiratory diseases. However, data needs to be interpreted with caution due to the low number of cases in the analysed sub-cohort. The size of the sub-cohort might also be the reason that we did not see effects for toluene or xylene. Also, questionnaire data on the individual smoking exposure were too less to be considered in the present analyses.

The strength of our study lies in the fact that the analyses of Eo/B progenitor cells were performed within the LINA cohort, where participants are well characterised regarding

their immune parameters, atopic outcomes and indoor air exposure to environmental chemicals. For example, individual ETS exposure was not only assessed by questionnaire data, but also by maternal urine cotinine levels and moreover the measurement of VOC concentrations in the homes. By coupling progenitor cell measurements with environmental exposures, cytokine measurements, and disease outcomes we were able to address the question of possible mechanisms responsible for the environmentally triggered increase in respiratory outcomes.

In contrast, high rates of participating atopic parents (about 65%) are a general weakness of the LINA study. This potential bias was addressed by including family history of asthma as a confounding variable in the present regression models on the respiratory outcomes. However, we don't have significant data on true allergic or atopic outcomes like asthma, since asthma prevalence at this age is comparably low. However, wheezing may favour asthma development later in life: It was shown for example that wheeze present in high-risk infants may be transient or remain persistent through childhood. It was also suggested that 15% of infants who wheeze progress to chronic asthma, mostly those associated with FHA [26].

Another limitation of the study is the low number of cases in certain outcomes and exposure scenarios due to the limited number of available samples on cord blood mononuclear cells in our study. This might reduce the strength of the reported results. Therefore, the presented results have to be interpreted with caution and need further validation.

5. Conclusion

In summary, in this study we could demonstrate the association between cord blood Eo/B progenitors and respiratory outcomes like wheezing or bronchitis within the first 24 months in a general study population. This aspect is particularly important if the potential predictive value of this cell population for respiratory outcomes is considered to be used in newborns or early infants. Furthermore, we suggest that a Th2 milieu at birth as well as exposure to environmental pollutants during pregnancy may have an impact on cord blood Eo/B progenitor cell recruitment which might, in turn, contribute to the disease development later in life.

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Conflict of interest

None of the authors has any potential financial conflict of interest related to this manuscript. Dr. Denburg reports that he is the CEO and Scientific Director of AllerGen NCE.

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ORIGINAL ARTICLE **Clinical Mechanisms in Allergic Disease**

The LINA cohort: indoor chemical exposure, circulating eosinophil/basophil (Eo/B) progenitors and early life skin manifestations

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Clinical & Experimental Allergy

Abstract

Background Hematopoietic progenitor cells, especially those committed to the Eo/B lineage, are known to contribute to allergic inflammation.

Objective The aim of the present study was to investigate whether environmental factors are associated with changes in numbers of circulating Eo/B progenitors at 1 year of age.

Methods Peripheral blood from 60 1-year-old children enrolled in the LINA (Lifestyle and environmental factors and their Influence on Newborns Allergy risk) birth cohort was assessed for Eo/B progenitor cells (Eo/B CFU) using standardized and validated methylcellulose assays. Frozen peripheral blood mononuclear cells (PBMC) were cultured in the presence of IL-3, IL-5 or GM-CSF, and Eo/B CFUs enumerated. Clinical outcomes and exposure to environmental tobacco smoke (ETS) were documented by standardized questionnaires, and indoor volatile organic compound (VOC) concentrations were assessed by passive sampling.

Results Children with skin manifestations (atopic dermatitis or cradle cap) within the first year of life had higher numbers of circulating IL-3-, IL-5- or GM-CSF-stimulated Eo/B CFUs ($P < 0.05$) at 1 year. In children with cradle cap, a positive correlation was found between Eo/B CFUs and exposure to ETS-related VOCs during pregnancy or at 1 year of age ($P < 0.05$).

Conclusions and Clinical Relevance This is the first demonstration that environmental exposures are positively associated with levels of circulating Eo/B progenitors. The recruitment and differentiation of Eo/B progenitors in response to environmental triggers may play a role in the development of skin manifestations during the first year of life.

Keywords atopic dermatitis, cradle cap, eosinophil/basophil progenitors, indoor chemical exposure, LINA, 1-year-old children, smoking, VOC

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Introduction

Many risk factors for the development of atopic disease, including genetic background, individual immune response and environmental conditions, have been identified [1]. Eosinophils and basophils are also well known to contribute to tissue allergic inflammation [2]. For example, mature eosinophils are increased in tissue, blood and bone marrow in asthma and atopic dermatitis, their numbers positively correlating with disease severity [2, 3]. In addition to mature cells, bone marrow Eo/B progenitors themselves can be released into the blood and migrate to tissues, under the influence of haemopoietic cytokines and chemokines, such as eotaxin (reviewed in [4, 5]). Several studies have also demonstrated that the number of Eo/B progenitors is increased in patients with allergic rhinitis, nasal polyposis, asthma or atopic dermatitis [6–9]. In addition, Eo/B progenitors can act as inflammatory effector cells producing a variety of pro-inflammatory cytokines and chemokines [10]. Altered phenotype and function of $CD34^+$ IL-$5R\alpha^+$ Eo/B progenitors have recently been demonstrated in several studies of cord blood of newborns at risk of atopy [11, 12] with progenitor profiles

predicting acute respiratory symptoms in 1-year-old infants [13].

Environmental exposures can further contribute to the development of allergic inflammation, especially exposure to indoor air pollutants [14–16]. A number of studies indicate that children exposed to environmental tobacco smoke (ETS) *in utero* or in infancy are at a higher risk of developing respiratory tract infections [17], wheezing or asthma [18]. In addition, ETS-exposure is associated with allergic skin manifestations, such as atopic dermatitis [19, 20]. Indoor exposure to chemical pollutants, such as volatile organic compounds (VOC), released from different sources including ETS [14, 21], can trigger allergic sensitization [15], allergic skin manifestations [22] and asthma [23, 24]. We hypothesized that a contributing mechanism underlying the association of indoor exposure with allergic manifestations was recruitment and differentiation of Eo/B progenitors. We therefore undertook an investigation of the association of pre- or post-natal environmental ETS or VOC exposure with numbers and function of peripheral blood Eo/B progenitors and the development of skin manifestations in children enrolled in the LINA birth cohort.

Methods

Study design and sample collection

The LINA cohort study (Lifestyle and environmental factors and their Influence on Newborns Allergy risk) recruited 622 mother-child pairs between May 2006 and December 2008 in Leipzig, Germany, to investigate how environmental factors in the pre- and post-natal period influence the immune system and whether they are determinants of increased allergy risk in the newborn and early childhood. A total of 606 mother-child pairs (599 mothers and 606 children) participated in the 1 year follow-up examination. Mothers suffering from immune or infectious diseases during pregnancy were excluded from the study.

Whole blood samples were obtained from mothers at the 34th week of pregnancy, and cord blood was collected at delivery. Whole blood samples were also collected from each mother and child at 1 year of age. Standardized questionnaires were administered during pregnancy (at week 34) and annually thereafter, collecting information on family history of atopy as well as housing and environmental conditions (active or passive smoking, mould, traffic, noise, pets, renovation activities) and personal lifestyle. At the age of 1 year information about clinical outcomes were collected. Atopic dermatitis was recorded as a parental report of a doctor-diagnosed dermatitis or of dermatitis symptoms. In detail, for a doctor-diagnosed dermatitis parents were asked whether a physician had diagnosed atopic dermatitis in the child in the last 12 months. Children were classified as having dermatitis symptoms when parents reported an intermittent itchy skin rash, which affected places other than the nappy area and lasted at least 2 weeks, or appeared repeatedly in the last 12 months. Cradle cap was recorded as a parental report of a doctor-diagnosed cradle cap. All questionnaires were self administered by the parents. In addition to the survey data from the questionnaires, individual chemical exposures to VOCs in the homes were assessed during pregnancy (passive sampling from 34th to 38th week) and at children's first birthday (passive sampling from 12th to 13th month of life). Participation in the study was voluntary and informed consent was obtained from all participants. The study was approved by the Ethics Committee of the University of Leipzig (046-2006).

Preparation of peripheral blood samples

In a subcohort of 60 randomly selected children progenitor analyses were performed. It was previously shown that a sample size in that range allows significant conclusions about Eo/B progenitor pathways [25]. As it was described in earlier experiments that family history of atopy (FHA) may influence numbers of Eo/B progenitors in peripheral blood, samples were chosen with similar numbers in groups of no, single or double FHA to avoid unbalanced changes in progenitor cell numbers. The investigator, who performed the progenitor assays and analyses, was blinded to identification of subjects and other data collected by protocol.

Frozen peripheral blood mononuclear cells (PBMCs) were isolated from 2 to 3 mL fresh heparinized peripheral blood via Ficoll Paque density centrifugation [26]. PBMCs were frozen in 1 mL aliquots of 90% fetal bovine serum and 10% dimethyl sulfoxide with 10–30×10^6 cells. The cell thawing protocol was according to Reece et al. [25]. Resulting non-adherent mononuclear cells (NAMNC) after 2 h incubation were used for methylcellulose assays. Viability in PBMCs after thawing and NAMNCs, after 2 h incubation averaged 90%.

Methylcellulose Assay

Methylcellulose assays were performed as described earlier [13, 25]. Each sample was analysed in duplicates and incubated in the presence of recombinant human cytokine IL-3, IL-5 or GM-CSF (R&D Systems Europe Ltd, Abingdon, Oxon, UK) over 14 days to assess stage dependent progenitor cell differentiation by morphological distinguishable colony formation (see Fig S1. in the supplementary information).

Measurement of VOC concentrations

Different VOC concentrations were measured for 4 weeks with passive samplers (3 M monitors, type OVM 3500; 3 M GmbH, Neuss, Germany) as described earlier [14, 27]. For measurements during pregnancy passive samplers were placed in the room where mothers spent most of their time, and for measurements at year one the sampler was placed in the child's bedroom. VOC concentrations were analysed by gas chromatography and afterwards corrected for seasonal variations. Additional details regarding VOC sample collection, analytical methods and seasonal variations are described elsewhere [28, 29].

Measurement of Th2 cytokines

For cytokine measurements heparinized blood was prepared as described earlier. In brief, concentrations of the phytohemagglutinin (PHA) stimulated cytokines IL-4, IL-5 and IL-13 were measured by flow cytometry using a cytometric bead array (BD CBA Human Soluble Flex Set system; Becton Dickinson, Heidelberg, Germany) according to manufacturer's instructions; for more details see Herberth et al. [30].

Statistical analyses

As the majority of parameters were not normally distributed, analyses were performed using non-parametric tests in general. To address the relationship between clinical outcomes and Eo/B CFUs, medians were compared using Mann–Whitney *U*-test. In addition, multiple logistic regression models were used to consider possible confounding factors. Data are presented as odds ratios with 95% confidence interval and were adjusted for maternal atopic dermatitis, maternal school education, cat ownership and smoking during pregnancy. Spearman's rank correlation test was applied to analyse the association between VOCs or cytokine concentrations and numbers of Eo/B CFUs, as well as between VOCs and the number of smoked cigarettes. Statistical power analyses for these correlation tests (highest estimated Rho 0.5, alpha 0.05 and beta 0.2) indicated a necessary sample size of 29 cases, suggesting that the sample size of 60 subjects, even when divided into subgroups ensure a sufficient statistical power. To verify obtained Spearman results, analyses were repeated after excluding outliers (below and above the 1.5-fold 25–75 percentile range). As no differences were seen after these exclusions data are presented as original data, all *P*-values < 0.05 were considered to be significant. Adjustments due to multiple testing were not performed as our analyses were based on an *a priori* hypothesis [31]. The χ^2-test for cross-relationship was used to compare the analysed subcohort with the total cohort. Statistical analyses were performed with STATISTICA for Windows, Version 7 (Statsoft Inc.).

Results

Characteristics of the analysed LINA subcohort

The frequencies of skin manifestations during the first year of life assessed via questionnaire are shown in Table 1. Within the analysed subcohort, 56.7% of the children were positive for cradle cap (CC) at 1 year. Atopic dermatitis (AD) was less prevalent, either when reported as a physician diagnosis (15.0%) or described by the parents as symptomatic atopic dermatitis (13.3%). For all skin manifestations, there were no mean differences between the analysed subcohort ($n = 60$) and the total cohort ($n = 606$, $P > 0.05$).

Eo/B CFUs and outcomes

One-year-old children positive for a skin manifestation had higher numbers of peripheral blood (pb) Eo/B CFUs (Fig 1). In detail, children positive for cradle cap had significantly more IL-5-stimulated Eo/B CFUs ($P = 0.012$), children positive for diagnosed atopic dermatitis had more IL-5- ($P = 0.002$) and GM-CSF- ($P = 0.044$) stimulated Eo/B CFUs and children positive for symptomatic atopic dermatitis had more IL-3- ($P = 0.045$), IL-5- ($P = 0.007$) and GM-CSF- ($P = 0.048$) stimulated Eo/B CFUs compared with children who were negative for these skin manifestations. This relationship was further calculated in a regression model adjusted for known confounding variables like maternal atopic dermatitis, maternal school education, cat ownership and smoking during pregnancy (Table 2). Here, children positive for cradle cap within the first year of life had higher amounts of IL-5-stimulated Eo/B CFUs (OR: 2.19, 95% CI: 1.10–4.35) and GM-CSF-stimulated Eo/B CFUs (OR:

Table 1. Prevalence of dermal outcomes within the first year of life

	Analysed subcohort ($n = 60$) *n* (%)	Total cohort ($n = 606$)* *n* (%)	χ^2-test *P*-value
Cradle cap (diagnosed)			
positive	34 (56.7)	354 (58.4)	0.808
negative	26 (43.3)	252 (41.6)	
Atopic dermatitis (diagnosed)			
positive	9 (15.0)	60 (9.9)	0.275
negative	51 (85.0)	546 (90.1)	
Atopic dermatitis (symptoms)			
positive	8 (13.3)	59 (9.7)	0.432
negative	52 (86.7)	544 (89.8)	

*total numbers may vary due to missing data ($n = 603$–606).

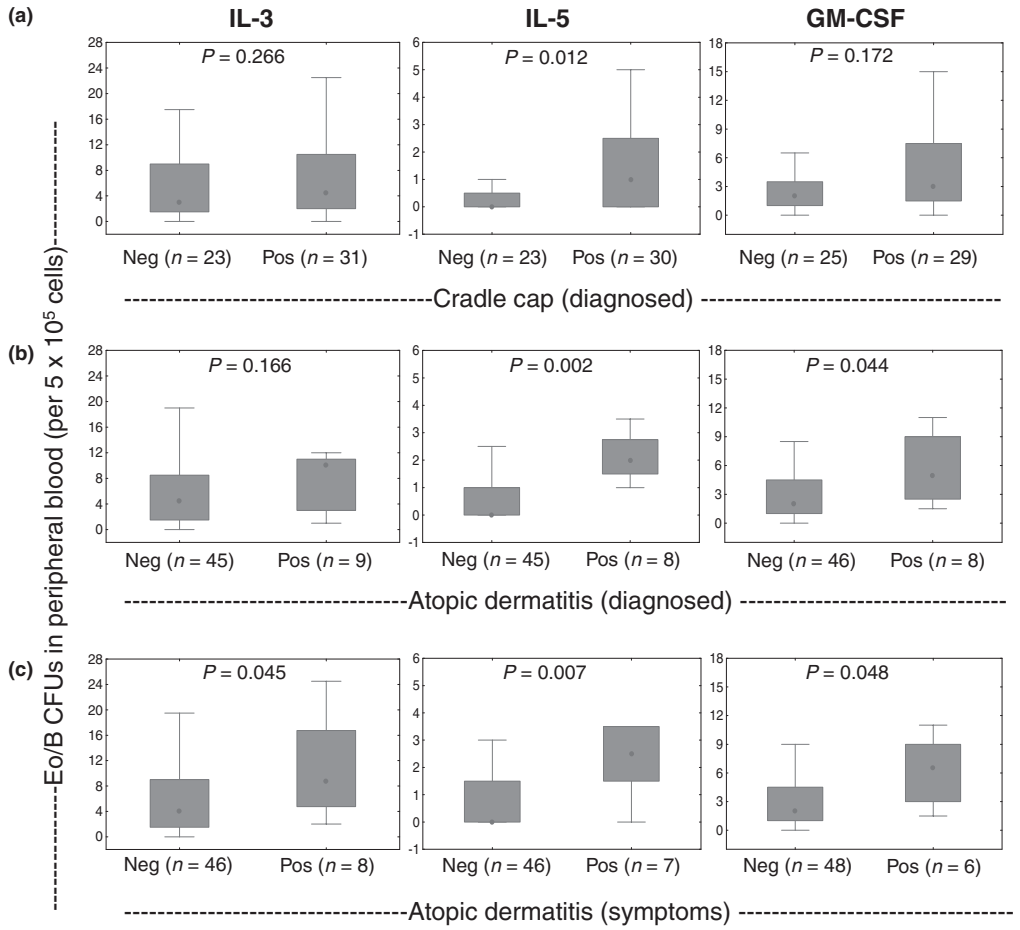

Fig. 1. IL-3, IL-5 or GM-CSF-stimulated Eo/B CFUs in peripheral blood of 1-year-old children with or without (a) cradle cap (diagnosed), (b) atopic dermatitis (diagnosed) or (c) atopic dermatitis (symptoms) within the first year of life. Data are presented as box plots with median and 25th to 75th percentile. *P*-values < 0.05 are considered to be significant (Mann–Whitney *U*-test).

1.25, CI: 1.01–1.55) at 1 year of age. Children positive for symptomatic atopic dermatitis within the first year of life had more IL-5-stimulated Eo/B CFUs (OR: 2.72, CI: 1.12–6.64).

Indoor chemical exposure: ETS and VOC

In the analysed subcohort, 35% of the pregnant women indicated their indoor exposure to ETS during pregnancy as '(almost) daily', 'once a week or more' or 'occasionally'. During the first year of life 18.3% of the children were exposed to ETS (Table 3). Questionnaire data revealed that 10 families gave up (indoor) smoking within the first year of life compared with their smoking behaviour during pregnancy.

A significant correlation was found between the number of smoked cigarettes per day and different VOC concentrations in the home either during pregnancy or during the first year of life (Table 4). Correlations were significant for single aromatic compounds like benzene, toluene and m,p-xylene, as well as for the total concentration of aromatic compounds and the total concentration of VOCs. Coincident with the higher prevalence of questionnaire-documented smoking practice during pregnancy compared with the first year of life, absolute concentrations of these VOCs were also higher during pregnancy compared with the first year of life (see Table S1. in the supplementary information).

Association between VOC/ETS and Eo/B CFUs

To investigate the association between smoking related VOCs and the numbers and differentiation of Eo/B progenitor cells, Spearman's rank correlation test was performed for the total subcohort ($n = 60$) and, in addition, separately for children positive or negative for skin manifestations. In detail, for the total subcohort there were no correlations between VOC concentrations

Table 2. Relationship between cradle cap or atopic dermatitis and IL-3, IL-5 or GM-CSF-stimulated Eo/B CFUs in peripheral blood of 1-year-old children. Data are presented as odds ratios (OR*) with 95% CI (analysed subcohort $n = 60$). Significant results are presented in bold.

	Cradle cap (diagnosed)	Atopic dermatitis (diagnosed)	Atopic dermatitis (symptoms)
IL-3-stimulated Eo/B CFUs	1.06 (0.96–1.18)	1.09 (0.97–1.24)	1.11 (0.97–1.26)
IL-5-stimulated Eo/B CFUs	**2.19 (1.10–4.35)**	1.14 (0.80–1.62)	**2.72 (1.12–6.64)**
GM-CSF-stimulated Eo/B CFUs	**1.25 (1.01–1.55)**	1.18 (0.98–1.42)	1.14 (0.95–1.37)

*OR are adjusted for maternal atopic dermatitis, maternal school education, cat ownership and smoking during pregnancy

Table 3. Questionnaire documented indoor exposures to ETS during pregnancy and during the first year of life (analysed subcohort $n = 60$)

During pregnancy	*n* (%)
Exposure to indoor ETS	
(almost) daily	15 (25.0)
once a week or more	1 (1.7)
occasionally	5 (8.3)
never	39 (65.0)
Number of smoked cigarettes/d*	
≥ 20	5 (8.8)
10–19	4 (7.0)
1–9	8 (14.0)
0	40 (70.2)
During first year of life	
Exposure to indoor ETS	
(almost) daily	8 (13.3)
once a week or more	0 (0.0)
occasionally	3 (5.0)
never	49 (81.7)
Number of smoked cigarettes/d	
≥ 20	2 (3.3)
10–19	4 (6.7)
1–9	4 (6.7)
0	50 (83.3)

ETS, environmental tobacco smoke (including smoking of the mother, father or others).
*Three missings.

and the number of Eo/B CFUs ($P > 0.05$). However, in children diagnosed with cradle cap within the first year of life, a positive correlation was found between IL-3- or GM-CSF-stimulated Eo/B CFUs at 1 year of age and exposure to VOC (single aromatic compounds benzene, toluene and m,p-xylene, total concentration of aromatics and total VOC concentration) during pregnancy or at 1 year of age (Table 5). In children with atopic dermatitis, either reported as a physician diagnosis or as AD symptoms, no correlation was seen between VOCs and Eo/B CFUs ($P > 0.05$).

Exposure to pre-natal ETS was associated with numbers of pb Eo/B CFUs and also specific for children positive for cradle cap within the first year of life (CC+). In detail, only in CC+ children there was a borderline correlation with the number of smoked cigarettes per day during pregnancy and GM-CSF-stimulated Eo/B CFUs ($P = 0.083$, $R = 0.333$, $n = 28$). In addition, when these CC+ children were subdivided into 'daily' exposure to ETS during pregnancy ($n = 4$) vs. 'never' ($n = 23$), 'daily' exposure was associated with 2.2-fold more GM-CSF-stimulated Eo/B CFUs compared with participants who documented their exposure to ETS as 'never' ($P = 0.049$). For exposure to post-natal ETS, no effect was seen on Eo/B CFU formation. In children with atopic dermatitis no correlation was found between ETS and Eo/B CFUs ($P > 0.05$).

Association between VOC/ETS and outcomes

To test whether exposure to VOCs during pregnancy or during the first year of life was directly associated with skin manifestations, a Mann–Whitney *U*-test was performed. No positive association was found between VOC exposure during pregnancy or during the first year of life and atopic dermatitis or cradle cap.

Exposure to ETS during pregnancy or during the first year of life did not show any association with skin manifestations.

Association between pre-natal VOC/ETS and cord blood cytokines

In CC+ children, a borderline significance was seen between VOC exposure during pregnancy (total concentration of aromatics ($P = 0.096$, $R = 0.340$, $n = 25$) or total concentration of VOCs ($P = 0.057$, $R = 0.386$, $n = 25$) and cord blood PHA-stimulated IL-13. For single VOCs as well as the Th2 cytokines IL-4 and IL-5 no correlation was found.

For exposure to ETS during pregnancy no effects were seen on CB Th2 cytokines IL-4, IL-5 or IL-13.

Association between cord blood cytokines and Eo/B CFUs

To test whether the immune status of the newborn was correlated with the number of pb Eo/B colonies, a Spearman's rank correlation test was performed. No significant effects were seen for the total subcohort. However, for CC+ children an association was seen between PHA-stimulated cord blood Th2 cytokines, IL-4 and

Table 4. Correlation of the number of smoked cigarettes per day with different VOC exposures, both either during pregnancy or during the first year of life. Data are presented as Spearman's rank correlations, *P*-values < 0.05 are considered to be significant and printed in bold (analysed subcohort $n = 60$)

	Benzene	Toluene	Xylene	Aromatics	Total VOCs
Number of smoked cigarettes/d during pregnancy vs. VOC exposure during pregnancy	**P = 0.000** **R = 0.556**	**P = 0.019** **R = 0.312**	*P* = 0.065 *R* = 0.248	**P = 0.013** **R = 0.329**	**P = 0.007** **R = 0.356**
Number of smoked cigarettes/d during the first year of life vs. VOC exposure during the first year of life	**P = 0.002** **R = 0.393**	*P* = 0.396 *R* = 0.114	**P = 0.014** **R = 0.321**	**P = 0.043** **R = 0.267**	*P* = 0.050 *R* = 0.258

Table 5. Correlation of different VOC exposures during pregnancy or during the first year of life with the number of IL-3-, IL-5- or GM-CSF-stimulated Eo/B CFUs in peripheral blood of 1-year-old children. Correlations are shown for cradle cap positive children within the first year of life ($n = 34$). Data are presented as Spearman's rank correlations, *P*-values < 0.05 are considered to be significant and printed in bold

CFUs	IL-3	IL-5	GM-CSF
VOC exposure during pregnancy			
Benzene	*P* = 0.184	*P* = 0.906	**P = 0.024**
	R = 0.245	*R* = −0.023	**R = 0.419**
Toluene	**P = 0.043**	*P* = 0.591	*P* = 0.253
	R = 0.366	*R* = −0.102	*R* = 0.219
Xylene	**P = 0.005**	*P* = 0.920	*P* = 0.855
	R = 0.487	*R* = 0.019	*R* = 0.036
Total concentration of aromatics	**P = 0.003**	*P* = 0.768	*P* = 0.681
	R = 0.511	*R* = 0.056	*R* = 0.078
Total concentration of VOCs	**P = 0.012**	*P* = 0.471	*P* = 0.063
	R = 0.447	*R* = 0.137	*R* = 0.350
VOC exposure during the first year of life			
Benzene	*P* = 0.621	*P* = 0.572	**P = 0.021**
	R = 0.096	*R* = 0.112	**R = 0.441**
Toluene	*P* = 0.482	*P* = 0.896	**P = 0.013**
	R = 0.136	*R* = 0.026	**R = 0.471**
Xylene	*P* = 0.111	*P* = 0.722	*P* = 0.081
	R = 0.302	*R* = 0.070	*R* = 0.342
Total concentration of aromatics	*P* = 0.150	*P* = 0.521	**P = 0.022**
	R = 0.274	*R* = 0.126	**R = 0.440**
Total concentration of VOCs	*P* = 0.075	*P* = 0.072	**P = 0.016**
	R = 0.335	*R* = 0.346	**R = 0.460**

IL-13 and the number of Eo/B CFUs at year one, with a trend for an association with IL-5 (Table 6). This association was not seen in children negative for cradle cap within the first year of life.

In children positive for atopic dermatitis (either diagnosed or symptoms) a significant positive correlation was found between CB PHA-stimulated IL-4 levels and IL-3- or GM-CSF-stimulated CFUs. No significant effect was seen in children negative for AD ($P > 0.05$).

All shown associations between environmental exposures, Eo/B CFUs and clinical outcomes are summarized in Fig. 2.

Table 6. Correlation of different PHA-stimulated Th2 cytokines in cord blood with the number of IL-3, IL-5 or GM-CSF-stimulated Eo/B CFUs in peripheral blood of 1-year-old children. Correlations are shown for cradle cap positive children within the first year of life ($n = 34$). Data are presented as Spearman's rank correlations, *P*-values < 0.05 are considered to be significant and printed in bold

CFU*s*	IL-3	IL-5	GM-CSF
Th2 cytokines in cord blood			
IL-4	**P = 0.045**	*P* = 0.782	**P = 0.024**
	R = 0.431	*R* = 0.064	**R = 0.480**
IL-5	*P* = 0.845	*P* = 0.078	*P* = 0.728
	R = −0.039	*R* = −0.394	*R* = 0.079
IL-13	*P* = 0.122	*P* = 0.993	**P = 0.036**
	R = 0.340	*R* = 0.002	**R = 0.450**

Discussion

In this study we were able to analyse for the first time the numbers and function of pb Eo/B progenitors of 1-year-old children in relation to skin manifestations and VOC exposure in the home.

Cradle cap (CC) is one of the most common dermatoses in early infancy [32, 33]. In the analysed LINA subcohort, CC was the most prevalent skin manifestation within the first year of life (57%), followed by AD (13-15%) which is comparable with previous cohort studies of dermal outcomes in early infancy (CC: 35.7-56.3% [32], AD: 8-10% [34, 35]). Although, there is no clear association between cradle cap and atopic dermatitis, it has been reported that a certain number of neonates with cradle cap develop an atopic dermatitis [36]. Possible links from the present analyses which could point out the possibility of an atopic component in cradle cap are mentioned later in the discussion.

It has been shown that numbers of pb Eo/B progenitors are elevated in atopic adult peripheral blood [6–8, 37]. Sehmi et al. demonstrated that numbers of $CD34^+$ cells in bone marrow are higher in atopic compared with non-atopic subjects and that this was accompanied by elevated numbers of Eo/B CFUs in peripheral blood [8]. Eo/B progenitors are also altered in neonates at high risk of atopy [12], and changes in CB Eo/B CFUs can predict acute respiratory symptoms in the first year of life [13]. In our LINA subcohort, we could demon-

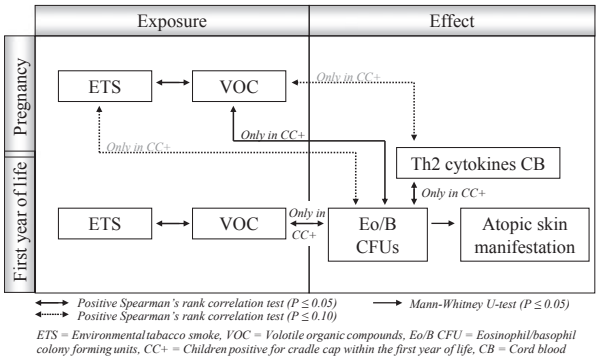

Fig. 2. Associations among exposure to environmental factors, Eo/B CFUs and clinical outcomes of 1-year-old children.

strate that 1-year-old children positive for CC or AD had significantly more IL-3-, IL-5- or GM-CSF-responsive pb Eo/B CFUs, compared with children who were negative for these skin manifestations. Although CC has to be distinguished from AD [38], the positive association of Eo/B progenitors with both skin manifestations, AD and CC, might suggest an atopic component for both outcomes. After adjusting for known confounders, the strongest elevation of CFUs in children positive for a skin manifestation was seen for IL-5-responsive cells. This IL-5 responsiveness indicates that children positive for AD or CC mainly had increased numbers of terminal differentiated circulating eosinophil progenitor cells. One possible mechanistic explanation of an elevated IL-5 responsiveness of pb Eo/B progenitors in relation to skin manifestations is an up-regulation in the expression of the IL-5 receptor alpha subunit, as previously shown for bone marrow $CD34^+$ cells in atopic asthmatics [39, 40], putatively in response to IL-5 itself [41] or to the two other cytokines (IL-3, GM-CSF) which bind to the common β subunit of the receptor [42]; however, we were not able to perform phenotyping (by flow cytometry or qPCR) to analyse differences in cell surface receptors.

In addition to stimulating terminal differentiation of Eo/B progenitors, IL-3, GM-CSF and IL-5 are essential in encouraging both early and terminal eosinophil development and differentiation respectively [4]. Therefore, Eo/B progenitors which are responsive to either one or more of these cytokines might give valuable information about overall eosinophil lineage commitment.

One factor known to be involved in eosinophil lineage commitment and differentiation in the context of allergic disease development is an altered Th2 response. Previous studies have shown that inhaled allergens induce a trafficking of IL-5 producing Th2 cells to the bone marrow where they promote eosinophilopoiesis [43]. Other groups have shown in mice that IL-4, which is recognized in allergy development, can direct bone marrow progenitor cell differentiation into Th2-cytokine-producing eosinophils [44]. Further data exist that eosinophils and basophils carry IL-4 and IL-13 transcripts during lineage differentiation, which enables rapid production of these Th2 cytokines [45]. These Th2 responses also seem to be involved, according to our present analyses, in the recruitment of Eo/B CFUs and subsequently in the following contribution to the development of skin manifestations. In our study, cord blood of children positive for CC within the first year of life revealed a significant positive correlation between electable CB Th2 cytokines (IL-13 and IL-4) and the number of IL-3- and GM-CSF-stimulated CFUs at 1 year. We hypothesize that these children were exposed to a stronger Th2 milieu at birth, or shortly thereafter, which played a role in shaping early Eo/B lineage commitment and skin inflammation later on. The observed association between a Th2 driven immune status at birth, the number of Eo/B progenitors and CC may further point to the possibility of an atopic component in CC.

In addition to Th2 milieu, another contributing factor to the development of allergic inflammation is environmental exposure. Environmental exposure during pregnancy or very early in life can have immunmodulatory effects [46] and affect clinical outcomes in infancy [17, 22, 47]. There are very limited data on the relationships among environmental factors, Eo/B progenitors and the development of atopy. Denburg et al. demonstrated that cord blood Eo/B progenitors can pre-natally be modified by maternal diet during pregnancy [11] and that children at 1 year of age were less likely to develop wheeze and/or atopic dermatitis after maternal fish oil supplementation during gestation. However, the precise mechanism underlying the effects of environmental/life style factors in this context remains unclear.

In our analysed LINA cohort we show from questionnaire data that indoor exposure to ETS is present during pregnancy and during the first year of life. However, data suggest that the prevalence of indoor smoking seems to be reduced after delivery. Different studies critically discuss that information about indoor exposure to ETS or other environmental pollutants received from questionnaires are dependent on individual honesty and compliance by the study participants; actual exposures are further dependent on differences in housing conditions or ventilation [22]. Accordingly, associations between questionnaire documented smoking and Eo/B CFUs or skin outcomes were less prominent than expected. When we analysed actual VOC concentrations within the homes by passive samplers which more directly reflect true exposures, we showed that exposure to specific VOCs significantly correlated with the number of smoked cigarettes/d, independent of the time

point when measurements were performed. Furthermore, the indoor VOC concentrations of benzene, toluene and xylene measured in our study were comparable to concentration shown in other cohorts [14, 28, 29, 48, 49].

In the present study we demonstrate that smoking-related VOC exposure, either during pregnancy or during the first year of life, correlates with Eo/B CFUs of CC+ 1-year-old children. These interactions occurred only related to IL-3- and GM-CSF-responsive progenitor cells but not to IL-5-responsive cells; most likely this is related to the power of our study as subject numbers for subanalyses were quite low. A more mechanistic possibility is that the cells associated with VOC exposure in CC+ children involve a less terminally differentiated Eo/B progenitor, as was observed in the Fernandes study [13]. This may hint to a developmental stage dependent effect of VOCs on the infant's immune response. The increased VOC responsiveness of immature Eo/B progenitor cells may result via *in situ haematopoiesis* [10] in higher numbers of terminal differentiated IL-5-responsive progenitor cells. Nonetheless, conversely, Denburg et al. demonstrated that fish oil supplementation during pregnancy resulted in significantly more IL-5- but not IL-3- and GM-CSF-stimulated CFUs in cord blood [11]. This suggests that the responsiveness of progenitor cells to environmental factors varies with the stage of their differentiation.

That we only observed a correlation between VOC exposure and Eo/B CFUs in cradle cap positive children, suggests that there might be genetic or gene-environment interactions at play in these children. Differing individual susceptibilities to environmental stimuli [50, 51], for example determined by polymorphisms in VOC detoxifying enzymes, could explain the observed result. Sun et al. hypothesized that deficient or altered phase I and II metabolic enzymes,such as the cytochrome P450 (CYP) enzymes CYP1A1, CYP1A2, CYP1B1 or glutathione-S-tranferases like GSTP1, which are involved for example in benzene metabolism, would affect individual susceptibility to benzene toxicity in bone marrow [52], which could, in turn, carry consequences for haematopoietic cell development even at low exposure concentrations. Consistent with that hypothesis, Wang et al. recently published data that genetic polymorphisms in GSTM1 and GSTP1 may be responsible for differences in the susceptibility to atopic dermatitis with regard to pre-natal smoke exposure [53].

In summary, in this study we were able to analyse for the first time the numbers and functional differentiation of pb Eo/B progenitors of 1-year-old children in relation to skin manifestations and ETS/VOC exposures. Our results suggest that ETS-exposure during pregnancy as well as ETS-related VOC exposure during pregnancy and the first year of life are associated with enhanced numbers of Eo/B CFUs at the age of 1 year. Furthermore, we found a relationship between a Th2 milieu at birth and the presence of Eo/B CFUs at the age of 1 year. Therefore, we hypothesize that pre-natal ETS/VOC exposure drives fetal T cell development towards Th2, and that this milieu contributes to downstream eosinophilic-basophilic differentiation and skin inflammation. In an earlier study, we were in fact able to show that VOC exposure during pregnancy correlates with higher amounts of Th2 or lower amounts of Th1 cells in cord blood [27]. Environmental exposures, such as ETS-associated VOC may contribute via a Th2 bias to the recruitment and differentiation of Eo/B progenitors which, in turn, could play a role in the development of skin manifestations during the first year of life.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Eosinophil/basophil colony forming unit (CFU; upper right corner) and granulocyte/macrophage colony forming unit (down left corner).

Table S1. VOC concentrations measured in the homes of the study participants during pregnancy or during first year of life. Data are presented as median with 25th and 75th percentile (analysed subcohort $n = 60$).

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Research Article

The LINA Study: Higher Sensitivity of Infant Compared to Maternal Eosinophil/Basophil Progenitors to Indoor Chemical Exposures

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Purpose. Enhanced eosinophil/basophil (Eo/B) progenitor cell levels are known to be associated with allergic inflammation and atopy risk. The aim of the present study was to investigate the influence of different indoor exposures on the recruitment and differentiation of Eo/B progenitors in mother-child pairs. *Methods*. In 68 mother-child pairs of the LINA study peripheral blood mononuclear cells were used to assess Eo/B colony forming units (CFUs). Information about disease outcomes and indoor exposures was obtained from questionnaires. Indoor concentrations of volatile organic compounds (VOCs) were measured by passive sampling. *Results*. Infant's Eo/B CFUs were positively associated with exposure to tobacco smoke, disinfectants, or VOCs. In contrast, for maternal Eo/B CFUs, only a few associations were seen. Higher numbers of infant Eo/B CFUs were observed in children with wheezing symptoms within the second year of life. *Conclusions*. We demonstrate that infant's hematopoietic cells seem to respond with more sensitivity to environmental exposure compared to maternal cells. At least in infants, an activation of these hematopoietic cells by environmental exposure could contribute to an enhanced risk for the development of respiratory outcomes.

1. Introduction

Eosinophils and basophils are typical effectors of allergic inflammation [1]. In asthmatic patients, the numbers of eosinophils and basophils were found to be increased in tissue, blood, and bone marrow and also correlated with disease severity [1–3]. Additionally, several studies showed that eosinophil/basophil progenitor cells, which develop from

bone marrow $CD34^+$ cells under the influence of specific chemokines and cytokines [4], are increased in the peripheral blood of atopic subjects with asthma [5, 6], allergic rhinitis [6–8], nasal polyposis [8], or atopic skin manifestations [7] which was also reviewed in Denburg and Keith [9] and Gauvreau and Denburg [10]. In children, cord blood Eo/B progenitors differ in phenotype and function among infants at risk for atopy [11]. Cord blood progenitors were further seen to be predictive for frequency and characteristics of acute respiratory illness in infants during the first year of life [12]. Next to these quantitative relations to disease outcomes, Denburg et al. demonstrated that maternal diet during pregnancy can interfere with the number and function of progenitor cells and subsequently with disease outcomes such as atopic dermatitis and wheeze at one year of age [13]. In agreement, our own group contributed data showing that Eo/B progenitor cells of one-year-old children with cradle cap were increased in association with environmental pollutants [14]. So far, data showing a responsiveness of Eo/B progenitors to lifestyle and environmental factors are limited to the infant hematopoietic system. Within the LINA study (Lifestyle and Environmental Factors and Their Influence on Newborns Allergy Risk) we already demonstrated that cord blood but not maternal Th1/Th2 cytokine levels depends on chemical exposure during pregnancy [15]. This result may point to an increased vulnerability of infant compared to adult T cells to environmental exposure. The aim of the present study was to investigate whether allergy relevant eosinophil/basophil progenitor cells from infants also respond with more sensitivity to environmental pollutants compared to progenitor cells from their mothers living in the same environment.

2. Materials and Methods

2.1. Study Design. The mother-child study LINA was designed to investigate how environmental factors in the pre- and postnatal period influence the immune system and whether they are determinants of increased allergy risk later in children's life. For this study, 629 mother-child pairs (including 7 twins) were recruited between May 2006 and December 2008 in Leipzig, Germany. Mothers suffering from immune or infectious diseases during pregnancy were excluded from the study. Six hundred six mother-child pairs participated in the one-year and 546 pairs in the two-year follow-up (one scheduled visit/year around the child's birthday). Blood samples from 397 mothers and 340 children were obtained at children's age of two (mean age: 2 years and 26 days, min–max: 1 year and 343 days–2 years and 161 days) as part of the scheduled visit. Sufficient peripheral blood mononuclear cells (PBMCs) for methylcellulose cultures were available for a subset of 68 corresponding mother-child pairs (66 mothers, 68 children; 2 sets of twins). All relevant *N*-numbers are shown in Figure A.1 in Supplementary Data available online at http://dx.doi.org/10.1155/2016/5293932. Participation in the LINA study was voluntary and written informed consent was obtained from all participants. The study was approved by the Ethics Committee of the University of Leipzig (file ref. # 046-2006, 160-2008).

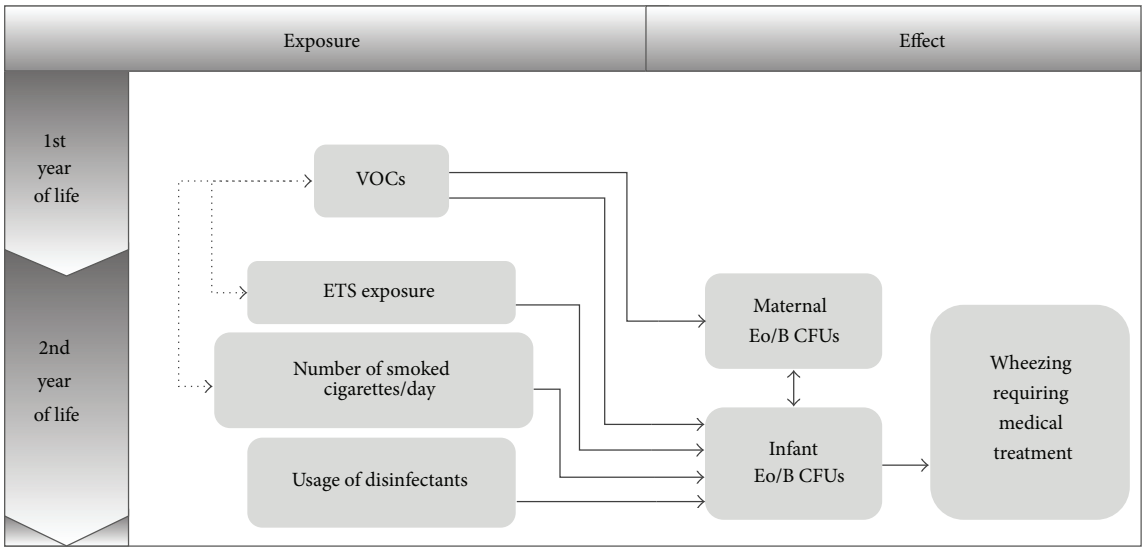
2.2. Questionnaire Data. Information concerning general aspects of life and environmental conditions during pregnancy was collected by detailed questionnaires during the 36th week of pregnancy. Further, information about respiratory outcomes of the child in the last 12 months as well as information about housing and environmental conditions (e.g., environmental tobacco smoke (ETS) exposure, and usage of cleaning agents) was obtained annually. All questionnaires were self-administered by the parents. For more detailed information, please see the methods section of Supplementary Data.

2.3. Preparation of Peripheral Blood Samples. PBMCs were isolated within six hours after collection of about 3 mL (child) to 5 mL (mother) fresh heparinised peripheral blood via Ficoll Paque density centrifugation. PBMCs were frozen in 1 mL aliquots of 90% fetal bovine serum and 10% dimethylsulfoxide with $10–30 \times 10^6$ cells. The cell thawing protocol and isolation of nonadherent mononuclear cells (NAMNCs) were performed according to Reece et al. [16]. Viability of PBMCs after thawing and NAMNCs after 2 h incubation averaged 93.4%.

2.4. Methylcellulose Cultures. In the present paper a well-established and prevalidated method of functional methylcellulose assay was used to assess Eo/B CFUs [12, 14, 16]. To assess eosinophil/basophil progenitor cell differentiation by colony formation 5×10^5 NAMNCs were incubated in duplicate in the presence of recombinant human cytokine IL-3 (1 ng/mL), IL-5 (1 ng/mL), or GM-CSF (10 ng/mL) (R&D Systems Europe Ltd., Abingdon, Oxon, UK) over 14 days. Eosinophil/basophil colony forming units (CFUs) were detected and enumerated by their characteristic morphology using a light inverted microscope. Details were described earlier [12, 14, 16]. The investigator, who performed the progenitor assays and analyses, was blinded to the identity of subjects and other data collected. Due to insufficient cell numbers after thawing, methylcellulose assays could not be performed for all three cytokines in certain samples. Case numbers for IL-3-, IL-5-, and GM-CSF-stimulated cultures resulted in a total of 67, 66, and 56 analysable samples for the children and in 63, 36, and 11 samples for the mothers, respectively.

2.5. Measurement of VOC and Cotinine Concentrations. To measure the individual exposure to volatile organic compounds (VOCs) in the homes, passive samplers (3M monitors, type OVM 3500; 3M GmbH, Neuss, Germany) were placed in the child's bedroom (or alternatively in the room where the child preferentially spent most of their time) around the first birthday of the child. Concentrations of VOCs were analysed as described earlier [17] and adjusted for seasonal variations as described in Schlink et al. [18]. For analyses of cotinine see the methods section of the Supplementary Data.

2.6. Statistical Analyses. Statistical tests were performed using STATISTICA for Windows Version 10.0 (StatSoft Inc. Europe, Hamburg, Germany). The chi squared test was



⟨···⟩ Positive Spearman's rank correlation test or Mann-Whitney U test ($p < 0.05$), entire LINA cohort
⟶ Positive Spearman's rank correlation test or Mann-Whitney U test ($p < 0.05$), subcohort with Eo/B progenitor analyses
VOCs: volatile organic compounds
ETS: environmental tobacco smoke
Eo/B CFUs: eosinophil/basophil colony forming units

Figure 1: Associations among indoor chemical exposures, Eo/B CFUs of mother-child pairs, and clinical outcomes of 2-year-old children.

performed to compare parameters of the analysed subcohort with the remaining LINA cohort (N: 546 − 68 = 478).

Analyses related to Eo/B CFUs are calculated within the described subcohort (N = 68 mother-child pairs, including two sets of twins). Calculations for general associations independent of Eo/B CFU analyses (e.g., ETS exposure versus VOC concentrations) were performed for the entire LINA cohort (N = 546). When statistical analyses required a division into subgroups (e.g., exposure to ETS: yes versus no) data were not presented for N-numbers <5.

It was tested whether the available sample size for Eo/B analyses has the power to detect expected effect of indoor pollutants (in particular VOCs). With a type one error rate (alpha) of 0.05, a power goal of 0.9, and an expected population correlation of 0.460 (according to our earlier paper [14] the Eo/B CFUs and the total sum of all VOCs correlated with R = 0.460), the required sample size for such analyses was 45.

Since the majority of parameters were not normally distributed, analyses were performed using nonparametric tests in general. To address the relationship between the numbers of Eo/B CFUs and atopic outcomes or exposure to indoor ETS or disinfectants, medians were compared using the Mann-Whitney U test. To verify these results, multiple logistic regression models were used to determine adjusted odds ratios (OR) with a 95% confidence interval. The following confounders were used: month of birth (for seasonal changes), gender of the child (for potential differences between boys and girls), parental school education (as a marker for the social status), and family history of atopy (to consider the individual genetic background), as well as exposure to indoor ETS during pregnancy, maternal cotinine level at the child's first birthday, the sum of all measured VOCs at the child's first birthday (both cotinine and VOCs as a marker for ETS exposure in the second year of life), dampness in the dwelling during second year of life (as a marker for early airway allergen exposure), and children with infections of the airways in the second year of life (to consider a potential nonallergic origin of wheezing). Spearman's rank correlation test was applied to analyse the association between maternal and infant number of Eo/B CFUs and between the number of smoked cigarettes and Eo/B CFUs as well as between VOC concentrations and the number of Eo/B CFUs. All p values <0.05 were considered to be significant. Adjustments due to multiple testing were not performed since our analyses were based on an *a priori* hypothesis [19].

3. Results

3.1. Characteristics of the Study Population. Characteristics of the study population are shown in Table 1(A). There were no differences in the distribution of considered parameters in the analysed subcohort (N = 68) compared with the remaining LINA cohort (N = 478).

In general, a positive correlation was found between corresponding maternal and infant IL-3- ($p < 0.001$, R = 0.447, N = 65) as well as GM-CSF- (p = 0.028, R = 0.686, N = 10) stimulated Eo/B CFUs (Table 2). All shown significant associations between Eo/B CFUs, environmental exposures, and clinical outcomes are summarized in Figure 1.

3.2. Indoor Chemical Exposures: Disinfectants, ETS, and VOCs. During the second year of life 61.5% of the families in

Table 1: Characteristics of the analysed subcohort and the remaining LINA cohort. (A) General characteristics assessed during the 36th week of pregnancy. (B) Indoor chemical exposures during the second year of life. (C) Infant's respiratory outcomes during the second year of life.

Parameters	Analysed subcohort n (%), $N = 68$[*]	Remaining cohort n (%), $N = 478$[†]	p value[‡]
(A) General characteristics			
Month of birth			
Nov–Feb	16 (23.5)	140 (29.3)	0.502
Mar-Apr, Sept-Oct	28 (41.2)	162 (33.9)	
May–Aug	24 (35.3)	176 (36.8)	
Gender of the child			
Female	29 (42.6)	235 (49.2)	0.349
Male	39 (57.4)	243 (50.8)	
Parental education[§]			
Low	0 (0)	6 (1.3)	0.294[**]
Intermediate	19 (27.9)	101 (21.1)	
High	49 (72.1)	371 (77.6)	
Family history of atopy[‖]			
Double positive	18 (26.5)	80 (16.7)	0.167
Single positive	26 (38.2)	234 (49.0)	
Negative	24 (35.3)	164 (34.3)	
Exposure to ETS in dwelling during pregnancy[¶]			
Yes	11 (16.2)	59 (12.3)	0.430
No	57 (83.8)	419 (87.7)	
Dampness in dwelling during the second year of life			
Yes	6 (9.2)	46 (10.9)	0.700
No	59 (90.2)	377 (89.1)	
(B) Indoor chemical exposure during the second year of life			
Exposure to ETS in dwelling[¶]			
Yes	7 (10.4)	27 (5.7)	0.222
No	60 (89.6)	445 (94.3)	
Number of smoked cigarettes/day in dwelling			
≥15	3 (4.5)	7 (1.5)	0.173[**]
1–14	4 (6.0)	10 (2.2)	
0	60 (89.5)	447 (96.3)	
Usage of disinfectants			
Yes	40 (61.5)	294 (65.0)	0.608
No	25 (38.5)	158 (35.0)	
(C) Respiratory outcomes during second year of life			
Wheezing ever			
Positive	15 (22.7)	101 (21.8)	0.878
Negative	51 (77.3)	362 (78.2)	
Recurrent wheezing			
Positive	12 (19.0)	54 (12.5)	0.207
Negative	51 (81.0)	377 (87.5)	
Wheezing requiring medical treatment			
Positive	7 (10.3)	26 (5.7)	0.231
Negative	61 (89.7)	432 (94.3)	
Bronchitis			
Positive	18 (28.6)	125 (27.7)	0.887
Negative	45 (71.4)	326 (72.3)	

Table 1: Continued.

Parameters	Analysed subcohort n (%), $N = 68$*	Remaining cohort n (%), $N = 478$†	p value‡
Obstructive bronchitis			
Positive	6 (9.4)	42 (9.6)	0.962
Negative	58 (90.6)	396 (90.4)	

*N may be different from 68 due to missing data.
†N may be different from 478 due to missing data.
‡Calculated using the chi squared test for cross relationship.
§Low = 9 yrs of schooling or less "Hauptschulabschluss"; intermediate = 10 yrs of schooling "Mittlere Reife"; high = 12 yrs of schooling or more "(Fach-)hochschulreife."
‖Family history of atopy is defined as occurrence of asthma, atopic dermatitis, hay fever, or food allergy.
¶Yes = (almost) daily, once a week or more, or occasionally; no = never.
**Calculated although n numbers are <5 in some subgroups.

Table 2: Numbers of maternal and infant IL-3-, IL-5-, or GM-CSF-stimulated eosinophil/basophil (Eo/B) colony forming units (CFUs), presented as median and interquartile range (IQR). Correlation between maternal and infant Eo/B CFUs was calculated using Spearman's rank correlation test; p values < 0.05 are considered to be significant and printed in bold.

Eo/B CFUs	Mother	Child	p	R
	Median (IQR)			
IL-3	8.5 (3.5–17.5)	9.0 (4.0–15.0)	<**0.001**	0.447
IL-5	0.5 (0.3–1.5)	0.5 (0–1.5)	0.565	0.101
GM-CSF	5.5 (1.0–7.0)	2.5 (1.0–8.3)	**0.028**	0.686

the analysed subcohort declared their usage of disinfectants in the household as "once a week or more," "once a month or more," or "occasionally," with no differences when compared to the remaining LINA cohort ($p = 0.608$, Table 1(B)).

Within the analysed subcohort, 10.4% of the participants were exposed to indoor ETS "(almost) daily," "once a week or more," or "occasionally," with no differences when compared to the remaining LINA cohort ($p = 0.222$, Table 1(B)). ETS exposed mothers had higher urine cotinine levels (median: 42.15 μg/g, IQR: 2.02–308.30) compared to mothers without ETS exposure (median: 1.22 μg/g, IQR: 0.43–3.92; $p < 0.001$). Further, a positive correlation between maternal urine cotinine levels and the number of smoked cigarettes per day in the dwellings was observed ($p < 0.001$, $R = 0.171$). Smoking at home was related to enhanced indoor concentrations of the aromatic VOCs benzene ($p = 0.002$) and m + p-xylene ($p = 0.048$). In addition the number of smoked cigarettes per day was correlated with indoor benzene concentrations ($p = 0.008$, $R = 0.117$).

3.3. Association between Eo/B CFUs and Indoor Chemical Exposure. Two-year-old children exposed to ETS at home had significant higher numbers of IL-3- ($p = 0.010$) and GM-CSF-stimulated Eo/B CFUs ($p = 0.014$, Table 3 and Supplementary Table A.1) compared to children without ETS exposure. Coincident with these findings, children's IL-3- and GM-CSF-stimulated Eo/B CFUs correlated positively with the number of smoked cigarettes per day (Table 4). In contrast, maternal IL-3-stimulated Eo/B CFUs showed no association either with ETS exposure at home or with the number of smoked cigarettes per day.

With respect to VOCs, positive correlations were found between children's IL-3-, IL-5-, or GM-CSF-stimulated Eo/B CFUs and the sum of all measured VOCs as well as for the single smoking related VOCs benzene, m + p-xylene, and o-xylene ($p < 0.05$, Table 5). In mothers, only one correlation was seen for IL-3-stimulated Eo/B CFUs and benzene.

The usage of disinfectants was found to be associated with increased numbers of GM-CSF-stimulated Eo/B CFUs among infants ($p = 0.031$, Table 3), while maternal Eo/B CFUs did not vary significantly.

3.4. Respiratory Outcomes. Within the analysed subcohort, 22.7% of the children were positive for wheezing ever, 19.0% for recurrent wheezing, and 10.3% for wheezing requiring medical treatment during the second year of life. Furthermore a physician-diagnosed bronchitis was seen in 28.6% of the children and obstructive bronchitis in 9.4%. There were no differences in the distribution of considered respiratory outcomes in the analysed subcohort ($N = 68$) compared to the remaining cohort ($N = 478$, $p > 0.05$, Table 1(C)).

3.5. Association between Infant Eo/B CFUs and Respiratory Outcomes during the Second Year of Life. Children who suffered from wheezing requiring medical treatment during the second year of life had significantly more IL-3- ($p = 0.015$) and GM-CSF-stimulated Eo/B CFUs ($p = 0.023$, Figure 2) at the age of two. The association between IL-3-stimulated Eo/B CFUs and wheezing with medical treatment remains stable after adjustment for possible confounding factors (month of birth, gender of the child, parental school education, family history of atopy, and exposure to indoor ETS during pregnancy as well as maternal cotinine level, the sum of all measured VOCs, dampness, and infections of the airways in the second year of life). We considered airway infections as an additional factor since wheezing episodes can also occur together with airway (and in particular virus) infection. No significant association was found between Eo/B CFUs and the occurrence of wheezing symptoms ever (IL-3: $p = 0.926$; IL-5: $p = 0.379$; GM-CSF: $p = 0.943$), recurrent wheezing (IL-3: $p = 0.574$; IL-5: $p = 0.415$; GM-CSF: $p = 0.909$),

Table 3: Association between indoor chemical exposures and the number of IL-3-, IL-5-, or GM-CSF-stimulated eosinophil/basophil (Eo/B) colony forming units (CFUs) of two-year-old children and their mothers. Data are shown as median and interquartile range (IQR); analyses were performed using Mann-Whitney U test; p values < 0.05 are considered to be significant and printed in bold.

Indoor exposures	Exposure to ETS in dwelling			Usage of disinfectants in the household		
	Yes	No	p	Yes	No	p
Eo/B CFUs	Median (IQR)			Median (IQR)		
Mother						
IL-3	18.8 (5.0–55.5)	8.5 (3.8–17.0)	0.116	10.5 (4.0–16.5)	7.8 (4.0–17.5)	0.790
IL-5			*	0.5 (0.3–1.3)	1.0 (0.5–1.5)	0.597
GM-CSF			*	7.0 (5.5–7.0)	4.8 (1.0–5.5)	0.312
Child						
IL-3	23.0 (9.5–32.0)	8.0 (3.5–14.0)	**0.010**	9.8 (4.8–19.8)	6.0 (3.0–12.5)	0.067
IL-5	1.0 (0.5–4.5)	0.5 (0–1.5)	0.100	0.5 (0–1.8)	0.5 (0–1.5)	0.930
GM-CSF	12.0 (3.0–16.0)	2.5 (1.0–6.5)	**0.014**	3.8 (2.0–12.3)	1.8 (1.0–5.5)	**0.031**

* Reduced number of cases; see Table A.1 of the Supplementary Data.
ETS: environmental tobacco smoke.

Table 4: Correlation of indoor smoked cigarettes per day and IL-3-, IL-5-, or GM-CSF-stimulated eosinophil/basophil (Eo/B) colony forming units (CFUs) in peripheral blood of two-year-old children and their mothers. Data are shown as Spearman's rank correlations; p values < 0.05 are considered to be significant and printed in bold.

	Eo/B CFUs mother						Eo/B CFUs child					
	IL-3		IL-5		GM-CSF		IL-3		IL-5		GM-CSF	
	p	R	p	R	p	R	p	R	p	R	p	R
Number of smoked cigarettes/day in dwelling	0.131	0.194	*	*	*	*	**0.007**	**0.330**	0.096	0.208	**0.011**	**0.339**

* Reduced number of cases; see Table A.1 of the Supplementary Data.

bronchitis (IL-3: $p = 0.281$; IL-5: $p = 0.067$; GM-CSF: $p = 0.095$), or obstructive bronchitis (IL-3: $p = 0.308$; IL-5: $p = 0.495$; GM-CSF: $p = 0.663$).

4. Discussion

Within earlier studies, we showed that there are increases in blood eosinophil/basophil progenitor cells in one-year-old children in association with exposure to environmental chemicals [14]. In the current work we wanted to clarify whether this progenitor cell responsiveness is specific to the infant hematopoietic system or can also be seen in adults. Therefore, we analysed mother-child pairs living under the same environmental conditions for their differentiation of Eo/B progenitor cells.

To our knowledge absolute Eo/B colony numbers of mothers and their infants have not been compared before. It is well known that numbers of progenitor cells, except in bone marrow, are highest in cord blood and decrease in peripheral blood later in life [20]. Our data suggest that the absolute number of eosinophil/basophil progenitor cells in peripheral blood of two-year-old children is already comparable to adult levels, either when compared with their own mothers or with peripheral blood samples from former studies [21].

According to our hypothesis we could demonstrate with the present data that infant's progenitor cells seem to respond with more sensitivity to environmental pollutants (ETS, VOCs, and disinfectants) compared to maternal progenitor cells. This is in agreement with results shown earlier within the LINA study: VOCs emitted due to renovation activities were observed to influence the child's but not the mother's immune response. In cord blood but not in peripheral blood of the mothers increased IL-4 and IL-5 serum levels [15] were seen in relation to chemical exposure due to renovation activities during pregnancy. The current study provides further evidence that under similar exposure scenarios the infant's immune system is more susceptible to the influence of environmental exposure compared to the maternal immune system. We hypothesize that this increased sensitivity goes back to the still not fully mature infant's immune system. Compensation mechanisms which might lower/negate the adverse effects of environmental exposure in adults might not yet be fully developed in young children.

In addition, our data support results showing that enhanced numbers of Eo/B progenitor cells as a consequence of environmental pollutants may increase the risk for wheeze and skin manifestations in early infancy [13, 14]. However, these earlier studies based their findings of lifestyle- or environment-dependent differentiation of Eo/B progenitors on a selected high-risk study population [13, 14]. In the present paper we provide strong evidence that the impact of environmental pollutants on stem cell differentiation is also seen in the general population.

Table 5: Correlation of indoor volatile organic compound (VOC) concentrations and IL-3-, IL-5-, or GM-CSF-stimulated eosinophil/basophil (Eo/B) colony forming units (CFUs) in peripheral blood of two-year-old children and their mothers. Shown are aromatic VOCs as well as the sum of all analysed VOCs. Data are presented as Spearman's rank correlations; p values < 0.05 are considered to be significant and printed in bold.

VOCs	Eo/B CFUs mother						Eo/B CFUs child					
	IL-3		IL-5		GM-CSF		IL-3		IL-5		GM-CSF	
	p	R	p	R	p	R	p	R	p	R	p	R
Benzene	**0.029**	**0.275**	0.076	0.299	0.544	0.206	0.101	0.202	**0.036**	**0.258**	**0.036**	**0.281**
Toluene	0.899	0.016	0.834	0.036	0.779	0.096	0.750	0.040	0.314	0.126	0.433	0.107
m + p-Xylene	0.872	0.021	0.957	0.009	0.447	0.256	0.418	0.101	**0.006**	**0.332**	**0.017**	**0.318**
o-Xylene	0.483	0.090	0.882	−0.026	0.472	0.243	0.174	0.168	**0.005**	**0.345**	**0.002**	**0.407**
Styrene	0.777	0.037	0.282	−0.184	0.728	0.119	0.830	0.027	0.507	0.083	0.807	0.033
Sum of all VOCs	0.093	0.213	0.848	0.033	0.148	0.467	**0.045**	**0.246**	**0.013**	**0.303**	**0.047**	**0.267**

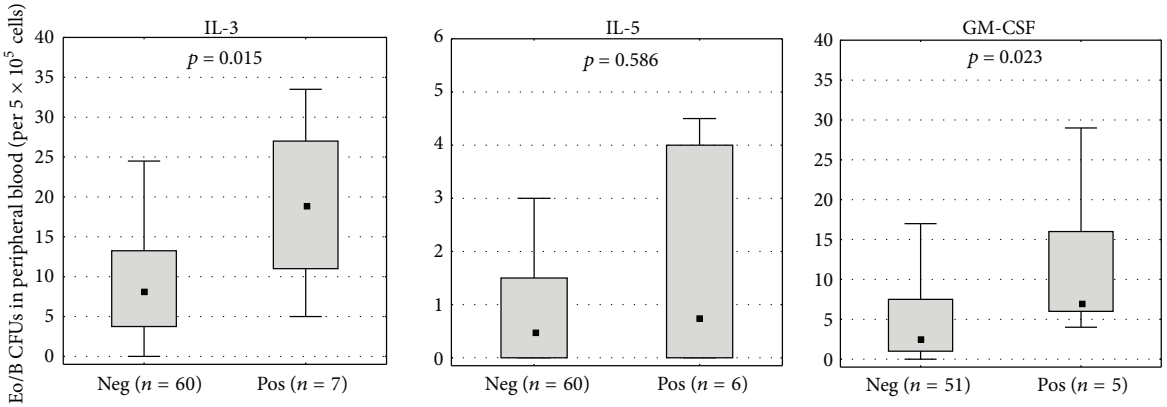


Figure 2: IL-3-, IL-5-, or GM-CSF-stimulated Eo/B CFUs in peripheral blood of two-year-old children with (pos) or without (neg) wheezing requiring medical treatment during the second year of life. Data are shown as box plots with median and 25th to 75th percentile. p values < 0.05 are considered to be significant (Mann-Whitney U test).

The fact that exposure to tobacco smoke/VOCs seems to influence the development of respiratory diseases has been shown before. There are several epidemiological studies demonstrating that exposure to prenatal smoke or passive tobacco smoke early in life is associated with an increased risk of wheezing in early infancy [22–26]. For example, passive household smoke exposure enhanced the risk for wheeze in children ≤2 years of age (OR = 1.35) [22]. Similarly, Pattenden et al. [25] demonstrated that smoking exposure within the first 2 years of life was associated with increased risk for wheeze (OR: 1.17).

There is furthermore evidence that exposure to tobacco smoke/VOCs provokes an immunological imbalance. Newborn children from smoking mothers were reported to have fewer cord blood regulatory T cell numbers [27] and an enhanced susceptibility to microbial infections through alterations of toll-like receptor- (TLR-) signalling [28] as well as a weak Th1 stimulation capacity [29]. As demonstrated in an earlier study by our group, exposure to VOCs may also direct the child's immune system towards a Th2 phenotype [17, 30]. A Th2 milieu caused by external stimuli has been shown to induce trafficking of IL-5-producing Th2 lymphocytes to the bone marrow where they promote eosinophilopoiesis through IL-5R signalling [11, 31]. In addition, the Th2 cytokines like IL-4 and IL-13 were described to regulate the transmigration of eosinophils from bone marrow to the tissues [32]. In children with skin manifestations a correlation between IL-4 blood levels and stimulated Eo/B progenitor cells was reported [14]. Thus, considering the fact that environmental pollutants such as tobacco smoke or VOCs induce a Th2 response in the child, we hypothesize that this may favour Eo/B progenitor cell differentiation, which could in turn contribute to an enhanced development of respiratory outcomes.

Finally, the present data demonstrate that Eo/B progenitors of two-year-old children which correlated positively with wheezing in early childhood were progenitors stimulated by IL-3 and GM-CSF: cytokines which are known to influence early eosinophil/basophil lineage differentiation [12]. Fernandes et al. [12] also showed that IL-3- and GM-CSF-stimulated Eo/B CFUs in cord blood are predictive for acute respiratory illnesses with fever or wheeze in the first year of life. This group and others discussed the hypothesis that immature progenitors are key determinants of atopic risk [10, 12]. Our data confirm this hypothesis by showing a correlation between severe wheeze (requiring medical treatment) and

early-stage Eo/B CFUs in two-year-old infants. Furthermore, within the LINA study, we have also found a positive association between Eo/B progenitor cells in cord blood and respiratory outcomes during the first two years [33]. We could not include data on asthma in our paper, since asthma prevalence at this age is comparably low. However, wheezing may favour asthma development later in life: it was shown, for example, that wheeze present in high-risk infants may be transient or remain persistent through childhood. It was also suggested that 15% of infants who wheeze progress to chronic asthma, mostly those associated with family history of atopy [34].

The strength of our study derives from the fact that the analyses of Eo/B progenitor cells were performed in mother-child pairs which are well characterised regarding their immune parameters, atopic outcomes, and indoor air exposure to environmental chemicals. For example, individual ETS exposure was assessed not only by questionnaire data, which are always dependent on honesty and compliance of the participants [35], but in addition by analysis of maternal urine cotinine levels and moreover the measurement of VOC concentrations in the homes. All of these parameters highly support each other and represent an objective ETS exposure scenario, which shows a consistent positive association with infant Eo/B CFUs. By coupling progenitor cell measurements with environmental exposures and disease outcomes we were able to address the question of possible mechanisms responsible for the environmentally triggered increase in allergic outcomes.

A weakness of the LINA study in general is the potential bias by high rates of participating atopic parents (about 65%). We addressed this point by including family history of atopy as a confounding variable in the regression models. The high prevalence of atopic parents (who are already aware of their atopic disease and probably avoid potential hazards more than others) might also be one reason for the quite low prevalence of children exposed to ETS during the second year of life (10% versus 18.7% shown earlier for Germany [25]). One other limitation of the LINA study is that measurements of VOC and cotinine concentrations are only available at the child's first birthday due to missing home visits in the second year of life. However, we could demonstrate an almost consistent smoking behaviour of the study participants within the first and second year of life (85.7%). Therefore, we assume that VOC and cotinine levels measured at the child's first birthday also represent the child's exposure around the second birthday. This was confirmed by significant associations between ETS exposure within the second year of life and VOC or cotinine concentrations measured at the child's first birthday. Another limitation of the study is the restricted number of cases with stem cell analyses due to the very high experimental effort resulting in low numbers of children in certain outcomes. However, to our knowledge, the number of Eo/B progenitor cell analyses included in the present paper (in total almost 140) is higher than in any other earlier published study focused on health effects in relation to Eo/B progenitor cell function. Also some conditions of stimulated Eo/B progenitor cells (especially maternal IL-5 and GM-CSF CFU) resulted in small numbers of cases due to low number of available PBMCs. This might reduce the strengths of the reported results. Therefore, the presented results have to be interpreted with caution and need further validation.

5. Conclusions

In the present study we could confirm our earlier published data [14] showing that infant Eo/B progenitor cell differentiation is associated with indoor chemical exposure. Therefore, at least in infants, an increase of these hematopoietic cells by environmental exposure could contribute to an enhanced risk of the development of respiratory outcomes. The association of indoor chemical exposure and the differentiation of Eo/B progenitors appears to be mainly restricted to the infant's hematopoietic system. This is consistent with earlier results from the LINA study showing that cord blood but not maternal Th1/Th2 cytokine levels depends on chemical exposure during pregnancy [15]. Taken together, we can state that children's immune and hematopoietic cells seem to be more sensitive to environmental exposure compared to maternal cells. These results further support the hypothesis of a highly vulnerable and exposure sensitive time window in the perinatal period with consequences for children's disease risk. Protection against harmful environmental exposure and lifestyle conditions is therefore of much higher relevance for young children compared to adults. However, data needs to be confirmed in a larger cohort to verify the results based on the present small sample size.

Abbreviations

CI:	Confidence interval
CFU:	Colony forming unit
Eo/B:	Eosinophil/basophil
ETS:	Environmental tobacco smoke
GM-CSF:	Granulocyte macrophage colony-stimulating factor
IL:	Interleukin
IQR:	Interquartile range
LINA:	Lifestyle and Environmental Factors and Their Influence on Newborns Allergy Risk
NAMNC:	Nonadherent mononuclear cell
OR:	Odds ratio
PBMC:	Peripheral blood mononuclear cell
R:	Spearman's rank correlation coefficient
VOC:	Volatile organic compound.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

Authors' Contributions

Irina Lehmann and Kristin M. Junge contributed equally to this work.

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Wood emissions and asthma development: Results from an experimental mouse model and a prospective cohort study

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ABSTRACT

Background: Increased use of renewable resources like sustainably produced wood in construction or for all sorts of long-lived products is considered to contribute to reducing society's carbon footprint. However, as a natural, biological material, wood and wood products emit specific volatile organic compounds (VOCs). Therefore, the evaluation of possible health effects due to wood emissions is of major interest.
Objectives: We investigated the effects of an exposure to multiple wood-related VOCs on asthma development.
Methods: A murine asthma model was used to evaluate possible allergic and inflammatory effects on the lung after short- or long-term and perinatal exposure to pinewood or oriented strand board (OSB). In addition, wood-related VOCs were measured within the German prospective mother–child cohort LINA and their joint effect on early wheezing or asthma development in children until the age of 10 was estimated by Bayesian kernel machine regression (BKMR) stratifying also for family history of atopy (FHA).
Results: Our experimental data show that neither pinewood nor OSB emissions even at high total VOC levels and a long-lasting exposure period induce significant inflammatory or asthma-promoting effects in sensitized or non-sensitized mice. Moreover, an exposure during the vulnerable time window around birth was also without effect. Consistently, in our mother–child cohort LINA, an exposure to multiple wood-related VOCs during pregnancy or the first year of life was not associated with early wheezing or asthma development in children independent from their FHA.
Conclusion: Our findings indicate that emissions from wood and wood products at levels commonly occurring in the living environment do not exert adverse effects concerning wheezing or asthma development.

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1. Introduction

Innovative and sustainably produced wood products, when coupled with sustainable forest management, have been considered to contribute substantially to climate change mitigation (Sathre and Gustavsson 2009; Sathre and O'Connor 2010). Compared with other "non-wood" materials like concrete, steel or plastic, wood products usually have a lower quantity of greenhouse gas (GHG) emissions during product manufacturing (Bergman et al. 2014; Hildebrandt et al. 2017). Furthermore, carbon remains fixed in wood products throughout their service life (Kalt 2018). The GHG saving potential of an increased use of wood in construction but also a growing application of wood for interior works, furniture, doors and countless other long-living products could expand anthropogenic carbon stocks and reduce society's carbon footprint (Hildebrandt et al. 2017; Kalt 2018). However, as a natural, biological material, wood emits specific volatile organic compounds (VOCs). Considering that many people in industrialized nations spend most of their time indoors (Leech et al. 2002), the question arises which effects wood and wood products might have on human health and wellbeing. Widely used wood products in construction but also for flooring and furniture are softwoods like pine and engineered wood like oriented strand board (OSB). Softwoods release the highest concentration of wood VOC because of their emission of terpenes such as α-pinene and 3-carene (Pohleven et al. 2019). Wood materials like OSB emit predominantly terpenes as well as aldehydes (Makowski et al. 2005).

Several epidemiological studies indicate an association between exposure to indoor VOCs after redecoration of dwellings with painting or floor covering and adverse respiratory symptoms as well as an increased risk for allergic manifestations, wheeze or obstructive bronchitis (Diez et al. 2000; Ernstgard et al. 2007; Franck et al. 2014; Wieslander et al. 1997). In contrast, epidemiological studies on health effects of VOCs emitted by wood and wood products are scarce. The few data available are mainly from studies investigating the impact of occupational VOC exposure on respiratory symptoms or irritations on mucous membranes showing no conclusive evidence of an association between terpene or aldehyde exposure and adverse health effects (Cakmak et al. 2014; Glas et al. 2015; Salonen et al. 2009). Studies with healthy adult volunteers who were short-term exposed to different levels of VOCs from pinewood or OSB revealed no sensory irritation of the airways or eyes and no negative effects on pulmonary function either (Gminski et al. 2011a; Gminski et al. 2011b). However, indoor VOC exposure has been shown to impact infant's immune system development (Lehmann et al. 2001; Hörnig et al., 2016; Junge et al., 2014), and respiratory diseases in particular in early childhood even at very low exposure concentrations. Importantly, this harmful impact on children s health might result from prenatal exposure already (Lehmann et al., 2002; Diez et al., 2003; Franck et al. 2014). Unfortunately, there is no information from epidemiological studies so far as to whether exposure to wood-related VOC during pregnancy or in the very first years of life may have an effect on children's asthma risk later in life, in particular if these children were at high risk due to a family history of atopy (FHA). This is important, as pregnancy is not only a period that is particularly susceptible to ambient air pollution (Gruzieva et al. 2019) but at the same time frequently goes along with increased renovation activities and the purchase of new furniture. Moreover, there are no experimental in vivo studies on healthy or allergen-sensitized mice investigating neither the effect of long-term exposure to single VOCs nor to the whole mixture of VOCs emitted by wood and wood products. Therefore, the present study focussed on effects of short- and long-term as well as perinatal exposure to pinewood or OSB on airway inflammation using a murine asthma model. To confirm, that the results on perinatal exposure to wood products and offspring's asthma risk obtained with the murine model are relevant for humans, comparable analyses were performed in the prospective mother–child cohort LINA.

2. Materials and methods

2.1. Mice

Female BALB/cByJ mice were obtained from the Elevage Janvier Laboratory (Le Genest St Isle, France). Mice were purchased with a specified 7-wk age upon delivery followed by a 7-d adaptation period. Mice were bred and maintained in the animal facility at the University of Leipzig (Germany). The exposed mice and the control animals were housed in different ventilated cabinets with 23 °C room temperature, 60% humidity, and 12 h day/night rhythm. Cages were bedded with Vermiculite bedding material. Mice received conventional mouse feed (Altromin, Lage, Germany) and water *ad libitum*. All animal experiments involved groups of 4 mice/cage and were performed at least 3 times according to institutional and state guidelines. The cross-generational experiments were performed two times with 3 dams (each with 2–5 pups). Male Balb/cByJ (8 weeks of age) for mating were also obtained from the Elevage Janvier Laboratory. The Committee on Animal Welfare of Saxony approved animal protocols used in this study (TVV 23/16).

2.2. Exposure of mice to pinewood and OSB

The wooden products were installed at the top of the cages to prevent skin contact with the mice. As the emission rates generally decrease over time, the samples were replaced weekly to maintain a similar range of total VOC (TVOC) levels over the whole exposure period. To minimize other VOC sources different bedding materials had been tested *beforehand*. Silica and vermiculite based bedding emitted least but silica partly adsorbed the unsaturated aldehydes (data not shown). Therefore, vermiculite was consequently used. Solid pine timber boards (*Pinus sylvestris* L., purchased form sawmill) contained mainly heartwood. To provide comparable material, the samples were composed from different boards. Each sample consisted of several pieces of wood (2–3 cm × 12.5–15.0 cm × 2.5 cm). The full size of the samples varied in order to obtain similar VOC level in the cages even with wood samples that have different area specific emission rates. Two different batches of OSB were obtained (industrial mill and hardware store). All were made of Scots pine (*Pinus sylvestris* L.) and bonded with pMDI (poly-methylene diphenyl diisocyanate) resin. High exposure: During the first week of animal exposure, these fresh samples (total surface area: 2.049 cm^2 made of 11 pieces) were used. Therefore, OSB samples were stored in a freezer directly after production until the start of the exposure. For the remaining exposure period from week two on, the OSB samples were stored for one day in a climate chamber for artificial aging in order to change the VOC composition towards a higher aldehyde proportion. For the lower exposure OSB from the hardware store have been used (total surface area: 1.239 cm^2 made of 7 pieces). To provide comparable material, the samples were composed from different boards and panels, respectively. With the emission test chamber method according to DIN EN ISO 16000-9:2008 (Standards-committee 2008) the area specific emission rates (in $\mu g\ m^{-2}\ h^1$) of these samples were determined. Since the climatic conditions (humidity, temperature, and air exchange rate) are fixed in the cages, the defined TVOC levels were obtained by adjusting either the emitting surface sample area, the area specific emission rate, or both. The samples were wrapped in aluminum foil and stored in freezer until start of exposure. Three different exposure protocols were used (A - acute, B - chronic, C - perinatal) as displayed in Supplementary Fig. 1.

2.3. VOC monitoring and analysis in the mouse model

The determination of VOC was performed according to DIN ISO 16000–6:2012 (Standardization. 2012). The air in the cages was monitored several times per week and sampled on stainless steel sampling tubes filled with Tenax TA® sorbent (200 mg, 35/60 mesh). Each tube was spiked with 200 ng toluene-d_8 as an internal standard. The tubes

were thermal desorbed (TD: Ultra Series 2 50:50 and Unity Series 2, Markes International Ltd., USA). Identification and quantification were carried out with an Agilent 7890A gas chromatograph (GC) coupled with an Agilent 5975C mass spectrometer (MS) (Agilent Technologies Inc., USA). For separation a VF1701ms capillary column (CP9151, Agilent Technologies Inc., USA, length: 30 m, inner diameter: 0.25 mm, film thickness: 0.25 μm) with helium as carrier gas was used. The GC temperature program include the following steps: 32 °C hold for 3 min, increase by 6 °C min^{-1} to 90 °C, 90 °C hold for 4 min, increase by 8 °C min^{-1} to 200 °C, increase by 12 °C min^{-1} to 240 °C, 240 °C hold for 2 min. The MS was operated in scan mode with 5 scans s^{-1} in the mass range of 22 – 300 u. The results are presented as concentration of VOC in chamber or cage air (in $\mu g/m^3$). Only a defined number of VOC is considered to characterize the wood-related emission behaviour of the tested materials and the sampled air in the cages. The selection is based on the main representatives of the substantial compound classes (terpenes: α-pinene, β-pinene, 3-carene, limonene, phellandrene, terpinene, terpinolene, tricyclene, camphene, myrcene, cymol; (un)saturated aliphatic and aromatic aldehydes: pentanal, hexanal, heptanal, 2-heptenal, octanal, 2-octenal, nonanal, benzoic aldehyde; organic acids: acetic acid, propionic acid, hexanoic acid and some others: 2-butanone, pentanol, toluene). For most of these VOCs the compound-specific response factors were determined with a multi-point calibration with reference compounds. Substances without reference compounds were quantified using the response factor of compounds with similar chemical structure or the internal standard. In this context the total volatile organic compounds (TVOC) is the sum of the concentrations of the selected VOC. This correspond to at least 95% of all the detected VOC.

2.4. HDM-induced acute and chronic asthma model

To investigate possible adverse effects of pinewood and OSB emissions we induced different asthma phenotypes in mice. For an acute airway inflammation Balb/c mice were sensitized via the airways with 5 μg house dust mite extract (HDM, *D. pteronyssinus 1,* endotoxin: 1.273 EU/ml, Greer Laboratories, USA) in 40 μl saline on day 1 followed by 2.5 μg HDM given intranasally (i.n.) on days 7 to 11 and 14 to 16. Control mice received normal saline i.n. Airway hyperreactivity (AHR) was measured on day 17 and mice were sacrificed on day 18. To induce a chronic asthma-like phenotype, Balb/c mice were immunized with HDM (5 μg) on day 1 and challenged with HDM (2.5 μg) twice per week for 11 weeks (Supplementary Fig. 1). AHR was assessed on day 76 and mice were sacrificed on day 77.

2.5. Measurement of airway responsiveness

Lung resistance was measured by using invasive plethysmography in response to inhaled methacholine, as described previously (Jahreis et al. 2018; Polte et al. 2015). Briefly, to measure lung resistance (RL) mice were anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine, Bayer, Leverkusen, Germany), intubated, and mechanically ventilated at a tidal volume of 0.2 ml and a frequency of 150 breath/min. Baseline RL and responses to aerosolized saline (0.9% NaCl) were measured first, followed by responses to increasing doses (2.5 to 40 mg/ml) of aerosolized methacholine.

2.6. Collection of bronchoalveolar lavage (BAL) fluid

The assessment of cells in the lavage fluid and BAL cell differentials were determined as described previously (Petzold et al. 2014; Polte et al. 2015). Briefly, the trachea was cannulated and the right lung was lavaged three times with 400 μl NaCl 0.9%. Cells in the lavage fluid were counted using a hemocytometer, and BAL cell differentials were determined on slide preparations stained with Diffquick® (Medion Diagnostics AG, Düdingen, CH) on blinded samples by an independent investigator. At least 100 cells were differentiated into eosinophils, macrophages, lymphocytes and neutrophils by light microscopy based on conventional morphologic criteria.

2.7. Lung histology

Left lung was fixed in 10% formalin and stained with Haematoxylin Eosin (H E, MERCK, Darmstadt, Germany). For quantification and objective evaluation of the degree of histological inflammation, whole lung sections were scanned with a digital camera (Zeiss, 5 shots per lung) and analyzed with HistoClick-Software based on morphometric image analysis (Petzold et al. 2014; Polte et al. 2015). The degree of inflammation is expressed by the number of pixels, which correlate to the stained cells of interest. Total matrix deposition was assessed on Martius Scarlet Blue (MSB)-stained sections (Polte et al. 2009). Lung sections were also immunostained with anti-α-smooth muscle actin (SMA) primary Ab to distinguish smooth muscle cells. The primary Ab was detected with a HRP-labeled secondary Ab (both Abcam, USA). Digital photographs of four bronchioles per tissue section were taken and analyzed with HistoClick-Software (Polte et al. 2009).

2.8. HDM-specific IgE assay

HDM-specific IgE serum levels were measured by sandwich ELISA according to a standard protocol. Briefly, 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated overnight with 25 μg/ml HDM. After washing and blocking plates, serum was added and incubated at 4 °C overnight. Subsequently, 96-well plates were washed and 100 μl of biotin-anti-mouse IgE (BioLegend, CA, USA) was added and incubated for 1 hr. The wells were washed with PBS followed incubation with avidin-horse radish peroxidase (Biolegend) for 30 min. TMB substrate solution (100 μl) was added and incubated in the dark for 30 min. The OD was determined at 450 nm using a BioTek microplate reader (BioTek Instruments, Bad Friedrichshall, Germany).

2.9. Cytokine production

Splenocytes or mediastinal lymphnode cells (5×10^6 cells/ml per well) were isolated and re-stimulated in vitro with 100 μg/ml HDM in culture medium (RPMI medium supplemented with 10% FCS, 100 U/ml Penicillin, 100 μg/ml Streptomycin) one day after airway function test. After three days cytokines were measured in supernatants from re-stimulated spleen cells or from lung tissues using DuoSet® ELISA kits (R D Systems, Minneapolis, USA) according to the manufacturer's instructions.

2.10. Collagen analysis

Collagen content was measured in lung tissue homogenates by a biochemical assay according to the manufacturer's instructions (Sircol collagen assay, Biocolor, Ireland) as described previously (Rothemund et al. 2013; Schutze et al. 2010).

2.11. Measurement of 8-isoprostane

Lung tissues were homogenized using lysing matrix A (FastPrep®24 homogenizer, MP Biomedicals, LLC, Eschwege, Germany) and aliquots of the obtained supernatants were hydrolyzed (15% KOH) and deproteinized (ethanol containing 0.01% BHT). 8-isoprostane concentration was determined by specific immunoassay according to manufacturer's instructions (Cayman Chemical Company, Ann Arbor, MI, USA) as described previously (Bönisch et al. 2012; Schutze et al. 2010).

2.12. LINA study design and sample collection

The German prospective mother–child cohort LINA (Lifestyle and environmental factors and their Influence on Newborns Allergy risk)

recruited 622 mothers (629 children) at 34 weeks of gestation between May 2006 and December 2008 in Leipzig, Germany. Mothers with severe immune or infectious diseases during pregnancy were excluded from the study. Standardised self-administered questionnaires were collected annually starting in pregnancy, assessing general information about personal lifestyle, housing and environmental conditions as well as family history of atopy (FHA) and disease state.

Study participation was voluntary and written informed consent was obtained by all participants. The study was approved by the Ethics Committee of the University of Leipzig (file ref # 046-2006, 160-2008, 160b/2008, 144-10-31052010, 113-11-18042011, 206-12-02072012, #169/13-ff, #150/14-ff, 008/17-ek).

2.12.1. Wheezing/Asthma prevalence of children

Respiratory health of children was assessed by questionnaires asking parents annually for physician diagnosed respiratory diseases like asthma, obstructive bronchitis, wheezing etc. From that information, the prevalence for early wheezing (children from whom at least one wheezing episode within the first 2 years of life was reported) as well as the 10-year lifetime prevalence of asthma was used for the present BKMR analyses. As controls, only children who had never experienced any respiratory diseases like asthma, obstructive bronchitis or wheezing within the first 10 years were included. Furthermore, all BKMR analyses were also stratified for family history of atopy (at least one parent positive).

2.12.2. Redecoration activities

Information about redecoration activities of study participants were used from the pregnancy questionnaire at 36th week of pregnancy. Participants were asked if they got new furniture within the last 12 months, and if so, from what material (solid wood, particleboard, others). Further, participants were asked if new flooring was performed in their homes during the last 12 months, and if so, from what material (parquet, laminate, carpet, others).

2.12.3. Assessment of wood-related VOC in LINA

To measure the individual exposure to volatile organic compounds (VOCs) in the homes, passive samplers (3 M monitors, type OVM 3500; 3 M GmbH, Neuss, Germany) were placed in the middle of the room at 1.5–2 m height during pregnancy (sampling from 34th to 38th week) and at the end of children's year one (sampling from 12th to 13th month of life). During pregnancy, the passive samplers were placed in the sleeping room or the living room of the mother. In the first year of life, the measurement was carried out in the child's bedroom or in the living room of the parents depending on the preferred location of the child. Concentrations of VOCs were analysed as described previously (Lehmann et al. 2001).

2.13. Statistical analysis

Experimental data sets from in vivo mouse studies were processed and analysed in GraphPad PRISM 7.02 for windows (GraphPad Software, Inc.). All p-values of less than 0.05 were considered significant using ANOVA and Kruskal-Wallis multiple comparison tests.

With respect to the epidemiological data, analyses were performed using non-parametric tests in general since the majority of parameters were not normally distributed. To address the relationship between wood related VOCs and their indoor source, medians were compared using Mann–Whitney *U* test. To flexibly model the individual and joint effects of exposure to mixtures of wood-related VOCs on early wheeze (n = 483) or asthma prevalence within the first 10 years (n = 282) of life Bayesian kernel machine regression (BKMR) was performed with 50,000 iterations according to Preston et al. (2020) and Bobb et al. (2018). Wood related VOCs were ln-transformed and z-scored. As some VOCs were highly correlated (see Spearman correlation matrix shown in Supplementary Fig. 2A, B) exposures were categorized into non-overlapping groups (group 1: α-pinene, β-pinene, 3-carene, limonene; group 2: pentanal, hexanal, heptanal, octanal, nonanal). Hierarchal variable selection was implemented thus incorporating this information about the structure of the mixture into the model. The following confounders were included in the BKMR model: family history of atopy (except for the analyses stratified for FHA), smoking during pregnancy, maternal age at delivery, gender of the child, parity, parental school education, keeping of pets, delivery mode, breastfeeding duration and overweight development in infancy (being ever overweight from 2 to 10 years of age, classification according to IOTF (Leppert et al. 2020)). The Chi^2-test for cross-relationship was used to compare the analysed BKMR sub-cohort until the age of 10 years with the total cohort according general study characteristics/confounders. Statistical analyses were performed with STATISTICA for Windows, Version 13 (Statsoft Inc.) and R (version 3.6.1; R development Core Team) for the *bkmr* package (Bobb et al. 2018).

3. Results

3.1. Short-term exposure to pinewood/OSB in mice

To investigate the role of VOCs emitted by pinewood or OSB we exposed mice directly to wood samples installed on the top of the cage as described in methods in more detail. The mice were exposed to a TVOC concentration range between 2.1 and 5.6 mg/m^3 (pinewood, Fig. 1A) or between 1.8 and 4.3 mg/m^3 (OSB, Fig. 2A) starting before sensitization until the end of the asthma protocol (Supplementary Fig. 1). VOCs emitted from pinewood were mainly terpenes (>90%, Fig. 1B) and here in particular α-pinene and 3-carene (together > 90%, Supplementary Fig. 3A). Newly purchased OSB emits VOCs with a high portion of terpenes only in the first days, while aldehydes occur later when terpene concentration decrease (Makowski et al. 2005). To mimic this pattern, we therefore started the exposure with fresh OSB samples but used artificial aged samples with lower terpene and higher aldehyde emissions (Fig. 2B) subsequently. Short-term exposure to pinewood for a period of 20 days had no effect on the number of eosinophils or other immune cells in the BAL fluid in HDM-sensitized and non-sensitized mice compared to un-exposed control animals (Fig. 1C, Supplementary Fig. 3B). Furthermore, we did not find significant effects on AHR (Fig. 1D), airway inflammation in the lung as demonstrated by H E stained lung sections (Fig. 1E) and verified by objective, investigator-independent computer analysis (Fig. 1F). In addition, the HDM-specific IgE levels and the release of Th2 cytokines in cell culture supernatants of HDM-re-stimulated splenocytes (Fig. 1G, H) or lymphnode cells (Supplementary Fig. 3C) were comparable in pinewood exposed and unexposed mice. The same applies for the Th17 cytokine IL-17 and the Th1 cytokine IFN-γ (Fig. 1H).

Unexpectedly, in HDM-sensitized mice short-term exposure to VOCs emitted by OSB even reduced total cell number (Supplementary Fig. 4A) and the number of eosinophils in the BAL fluid (Fig. 2C) and decreased lung inflammation (Fig. 2E and Supplementary Fig. 4B). Moreover, HDM-specific IgE levels and the production of the Th2 cytokines IL-5 and IL-13 but also IL-17 and IFN-γ in re-stimulated splenocytes as well as IL-13 in mediastinal lymphnodes were diminished (Fig. 2F-G and Supplementary Fig. 4C). In contrast, OSB-derived VOCs had no impact on lung resistance (Fig. 2D). Exposure of non-sensitized animals was without any effect on the measured parameters (Fig. 2C-G).

As a positive control, exposure of HDM-sensitized mice to diesel exhaust particles (DEP), which are known to induce adjuvant effects in the lung (Takano et al. 1997) exerted an increased allergic airway inflammation and an impaired lung function (Fig. 2C-G).

3.2. Long-term exposure to pinewood/OSB in mice

To elucidate the effect of long-term exposure to pinewood we used differently stored samples to get a low and a high TVOC concentration

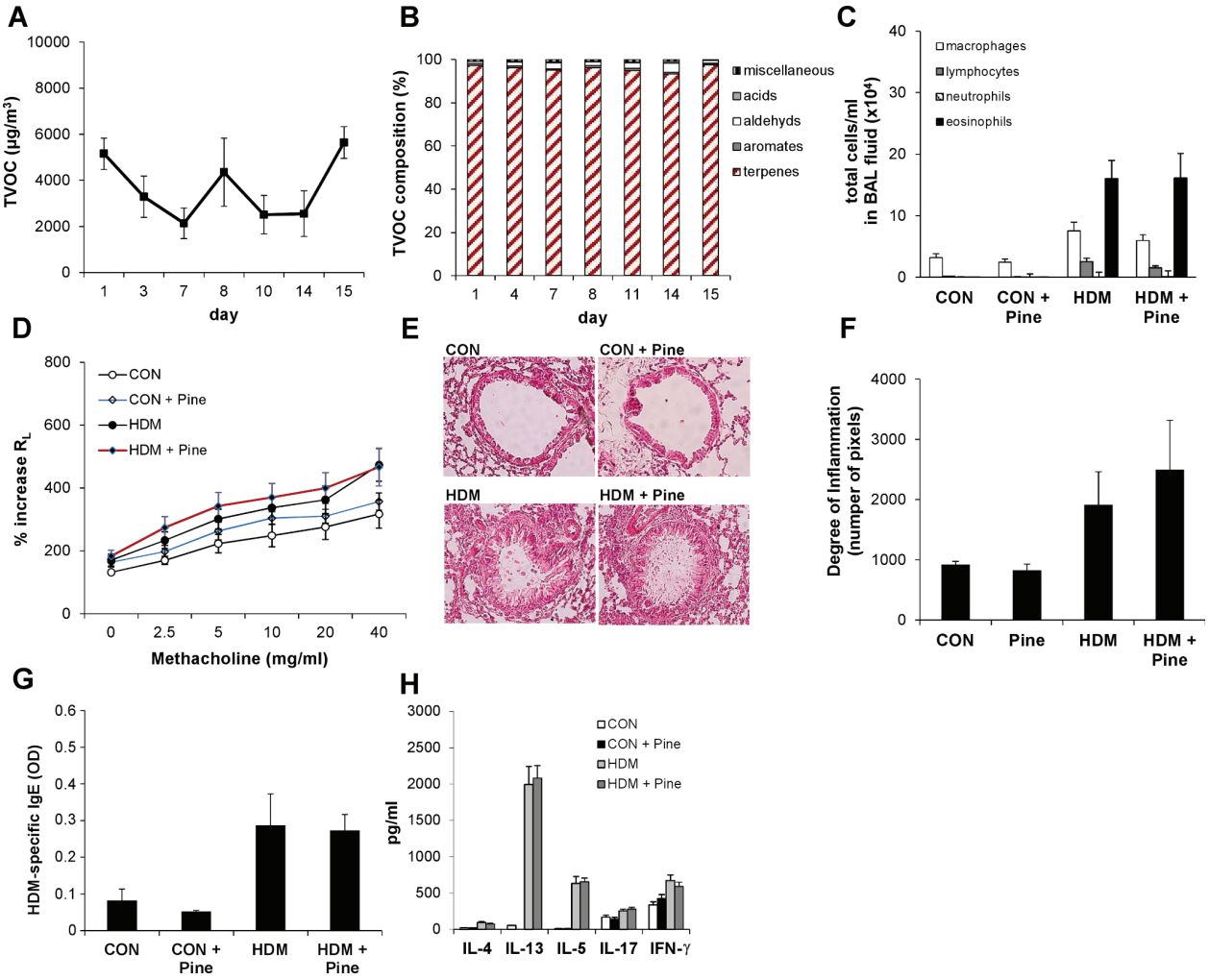

Fig. 1. Effect of short-term exposure to pinewood on asthma development in HDM-sensitized mice. (A) TVOC concentrations and (B) TVOC composition for the whole exposure period are shown. (C) Total cell number in BAL fluid, (D) lung resistance, (E) airway inflammation examined by lung histology (H E, x100), (F) quantified by an investigator-independent computer-based analysis, (G) HDM-specific IgE serum levels and (H) cytokine production of re-stimulated splenocytes were examined in HDM-sensitized and control mice. Data are expressed as mean ± SEM, n = 3 (A, B), n ≥ 9 (C – H).

range. Pinewood with TVOC emission between 1.5 and 6.3 mg/m^3 over a 12-week period (Fig. 3A, low exposure) was without any effects on eosinophilic inflammation in the BAL fluid, AHR and lung inflammation measured by analysing H E stained lung sections (Fig. 3B-D, Supplementary Fig. 5A). In parallel, HDM-specific IgE levels (Fig. 4A) and the cytokine release (Supplementary Fig. 5B, C) were unaffected by long-term low exposure to pinewood in HDM-sensitized and non-sensitized animals. Direct exposure of allergen-sensitized or non-sensitized mice to high TVOC concentrations (3 – 18 mg/m^3, Fig. 3A, high exposure) for 12 weeks had also no significant effect on airway inflammation (Fig. 3B, D, Supplementary Fig. 5A), lung function (Fig. 3C), IgE (Fig. 4A) and Th2 cytokine as well as IL-17 and IFN-γ levels (Supplementary Fig. 5B, C).

Furthermore, in this long-lasting asthma model we investigated the effect of continuous exposure to pinewood on airway remodelling. Increased subepithelial deposition of extracellular matrix (ECM) proteins, specifically collagen, and increased smooth muscle mass are prominent features of airway remodelling. We examined matrix deposition (collagen and fibrin) in lung sections stained with Martius scarlet blue and measured the amount of total lung collagen. Long-term exposure to both, low and high TVOC concentrations did not increase matrix deposition and lung collagen in sensitized and non-sensitized mice compared to their respective controls (Fig. 4B, C). Moreover, pinewood exposure at both concentration ranges had no significant effect on smooth muscle cells in the lung (Fig. 4D). To evaluate whether pinewood VOCs may induce oxidative stress we measured 8-isoprostane levels as marker for lipid peroxidation in lung homogenates (Alessandrini et al. 2009). Again, there were no significant differences detectable between pinewood-exposed sensitized or non-sensitized mice compared to the unexposed control animals (Supplementary Fig. 5D).

To simulate long-term exposure to OSB, HDM-sensitized and non-sensitized control mice were exposed over a 12-weeks period to a lower (0.8 – 1.7 mg/m^3) and a higher (1.2 and 4.3 mg/m^3) range of OSB-emitted VOCs (Fig. 5A), whereby the higher concentration range emitted similar TVOC levels as in the acute asthma model. As already reported for short-term exposure, long-term exposure of HDM-sensitized mice to the higher concentration range revealed a significantly decrease in serum IgE (Fig. 6A) and Th2 cytokine levels (Supplementary Fig. 6B). The total cell number (Supplementary Fig. 6A) as well as the number of eosinophils in the BAL fluid (Fig. 5B), IL-17 or IFN-γ levels in HDM-re-stimulated splenocytes and IL-13, IL-5 and IFN-γ production in re-stimulated lymphnode cells were slightly but not significantly reduced (Supplementary Fig. 6B, C). Furthermore, there was again no significant effect on AHR, lung inflammation (Fig. 5C, D), and airway remodelling

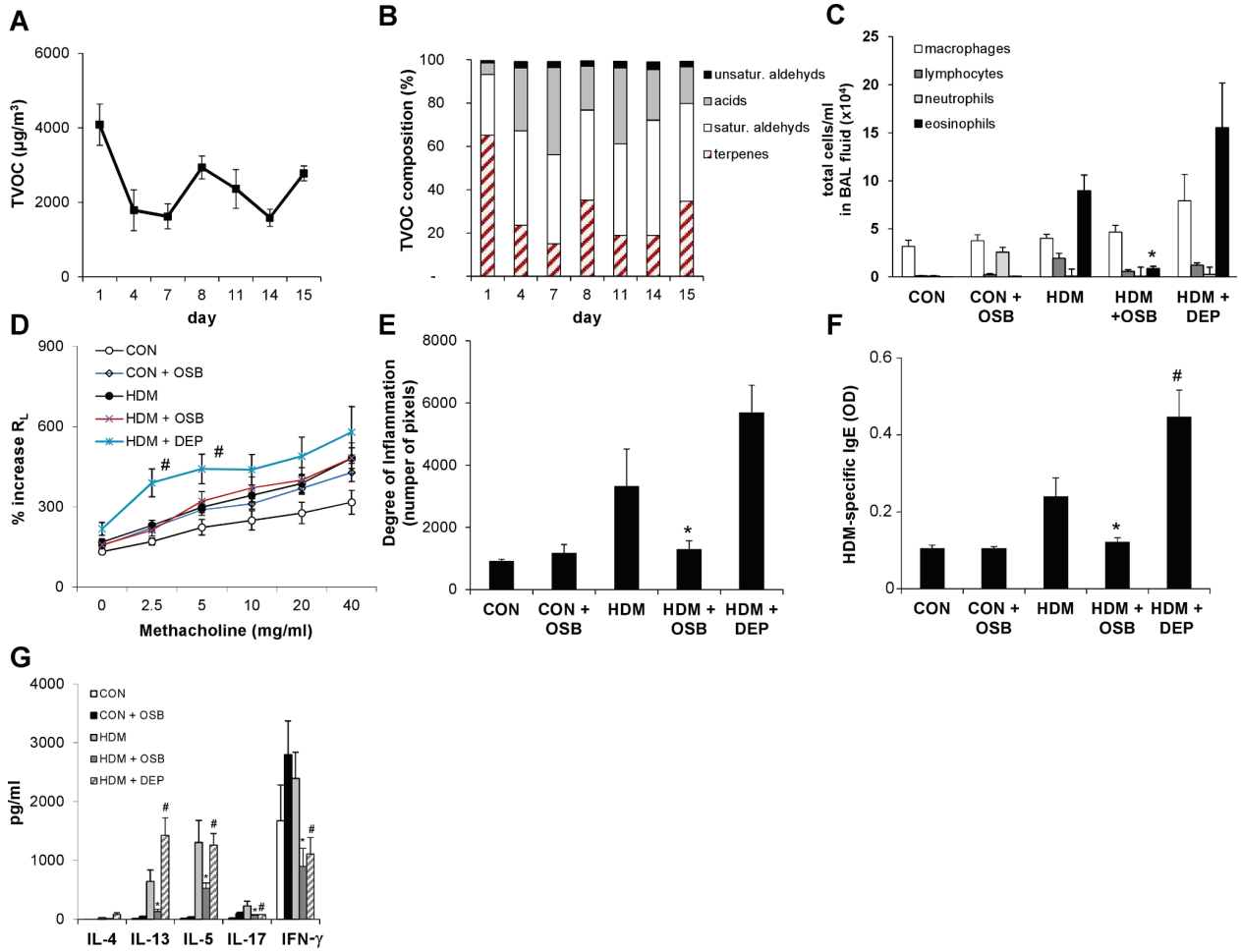

Fig. 2. Effect of short-term exposure to OSB on asthma development in HDM-sensitized mice. (A) TVOC concentrations and (B) TVOC composition for the whole exposure period are shown. (C) Total cell number in BAL fluid, (D) lung resistance, (E) airway inflammation examined by lung histology (H E, x100), (F) quantified by an investigator-independent computer-based analysis, (G) HDM-specific IgE serum levels and (H) cytokine production of re-stimulated splenocytes were examined in HDM-sensitized and control mice. Mice exposed to diesel exhaust particles (DEP) served as positive control. Data are expressed as mean ± SEM, n = 3 (A, B), n = 6 (DEP, C – G), n ≥ 9 (OSB, C – G). $^{*}P < 0.5$ HDM *vs*. HDM + OSB, $^{\#}P < 0.05$ HDM *vs*. DEP.

(Fig. 6B-D).

Exposure to OSB emitting VOCs at the lower concentration range had no significant effects on the measured parameters in HDM-sensitized mice (Fig. 5B-D, Fig. 6A-D, Supplementary Fig. 6A-C). Both concentration ranges did not affect non-sensitized animals. Furthermore, there were also no signs of oxidative stress detectable (Supplementary Fig. 6D).

3.3. Perinatal exposure to pinewood/OSB in mice

To analyse the effects of an early life exposure on asthma development in the offspring, dams were exposed to wood products during pregnancy and breastfeeding (perinatal). Grown-up offspring were then subjected to antigen sensitization without being further exposed to pinewood or OSB.

Maternal exposure to pinewood (concentration range between 2.2 and 11 mg/m^3, Fig. 7A) had no adverse effect on total cell number or number of eosinophils in the BAL fluid while exposure to OSB (range between 0.9 and 4 mg/m^3, Fig. 7B) significantly reduced the eosinophilic inflammation in the BAL fluid (Fig. 7C, Supplementary Fig. 7A). In contrast, pinewood and OSB both did not affect AHR (Fig. 7D), airway inflammation in the lung as demonstrated by H E stained lung sections (Supplementary Fig. 7B) and verified by objective, investigator-independent computer analysis (Fig. 7E) in HDM-sensitized offspring compared to HDM-sensitized mice from unexposed controls. In addition, the HDM-specific IgE levels and the release of Th2 cytokines as well as IL-17 in cell culture supernatants of HDM-re-stimulated splenocytes were comparable to those from offspring from pinewood- or OSB-exposed and unexposed dams while IFN-γ production was significantly increased in splenocytes but not in lymphnode cells (Fig. 7F, G, and Supplementary Fig. 7C).

3.4. Sources of VOC emissions in LINA

General study characteristics and factors known to individually influence children's asthma development as well as the early wheezing within the first 2 years and asthma prevalence within the first 10 years of life are shown in Supplementary Table 1. Case numbers are displayed for the total LINA cohort (n = 629), for the sub-cohort used to analyse the VOC sources (information available for new flooring and new furniture of the study participants during pregnancy as well as VOC measurements during pregnancy; n = 598) and for the final sub-cohorts when BKMR analyses were performed (available information about all used confounders, VOC measurements during pregnancy as well as wheezing within the first 2 years; n = 483 or asthma within the first 10 years of life; n = 282). For BKMR analyses at year one, there were 7 (wheezing

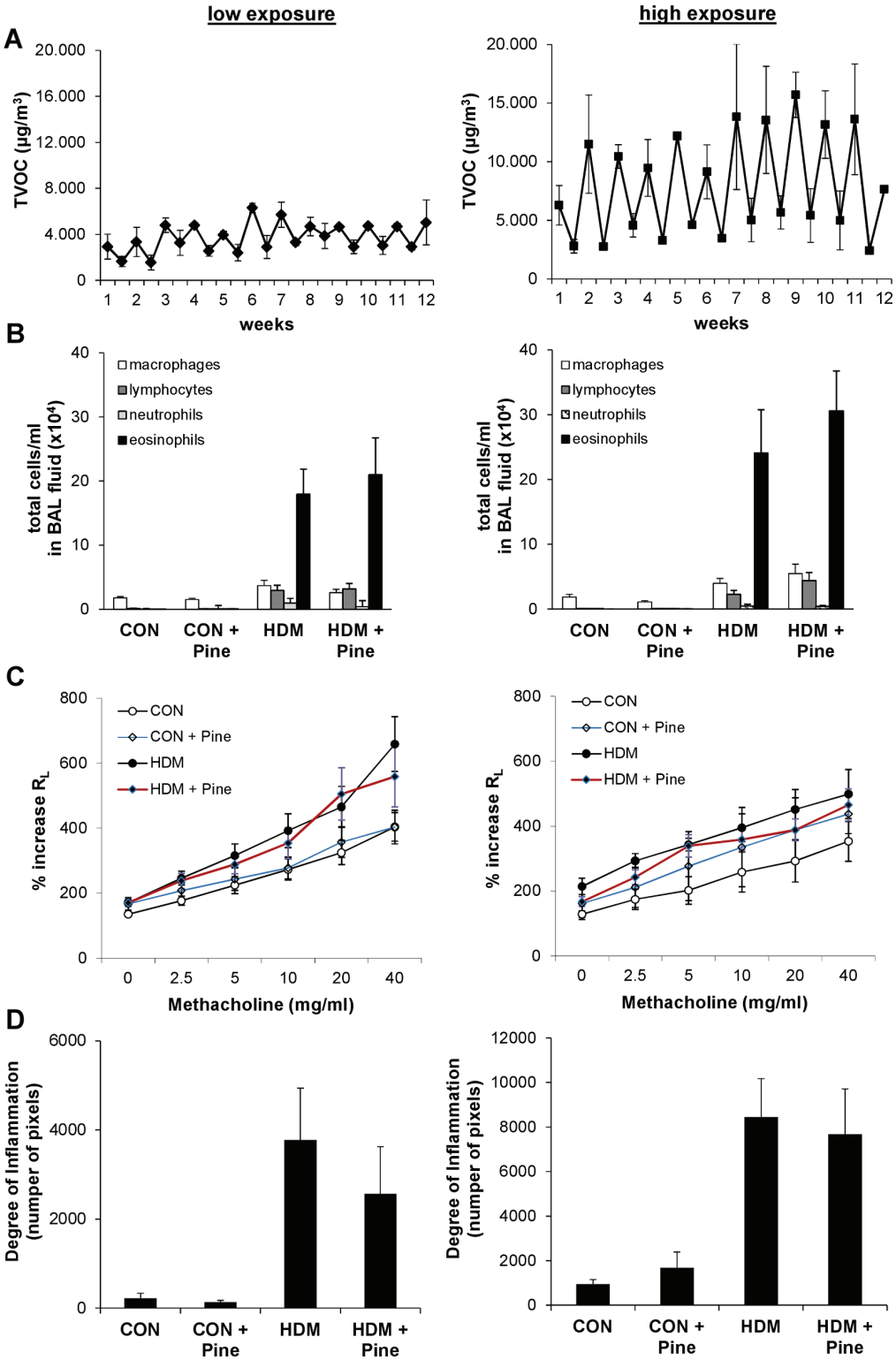

Fig. 3. Effect of long-term exposure to pinewood on airway inflammation and lung function in HDM-sensitized mice. (A) TVOC concentrations for low and high exposure are shown. (B) Total cell number in BAL fluid, (C) lung resistance and (D) airway inflammation were examined in HDM-sensitized and control mice. Data are expressed as mean ± SEM, n = 3 (A), n ≥ 9 (B – D).

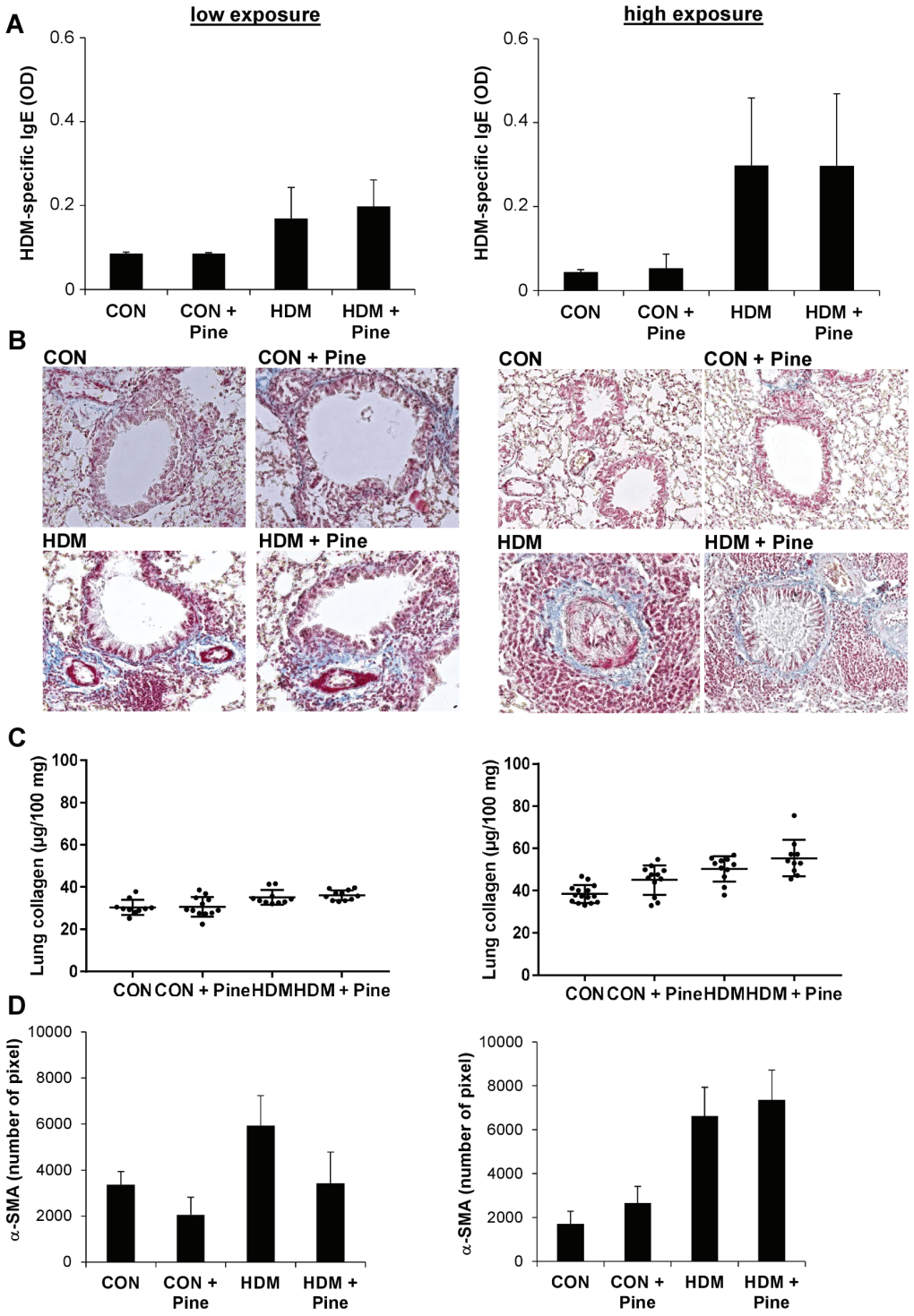

Fig. 4. Effect of long-term exposure to pinewood on IgE level and airway remodeling in HDM-sensitized mice. (A) HDM-specific IgE serum levels, (B) MSB-stained lung tissues (x100), (C) total lung collagen, and (D) proliferation of airway smooth muscle cells as quantified by an investigator-independent computer-based analysis were examined in HDM-sensitized and control mice. Data are expressed as mean ± SEM, n ≥ 9.

model) or 3 (asthma model) missing VOC measurements compared to VOC measurements during pregnancy. There were no differences in the general distribution of study characteristics/confounders in the final BKMR sub-cohorts for wheezing/asthma compared to the entire LINA cohort.

VOCs shown to be emitted by softwood and related products such as terpenes (α-pinene, β-pinene, 3-carene, and limonene) and aldehydes (pentanal, hexanal, heptanal, octanal and nonanal) were present in the homes of the study participants in the highly sensitive perinatal time period. Single wood-related VOC concentrations measured during pregnancy and around children's first birthday are shown in Supplementary Table 2, their total sum being similar for both time points

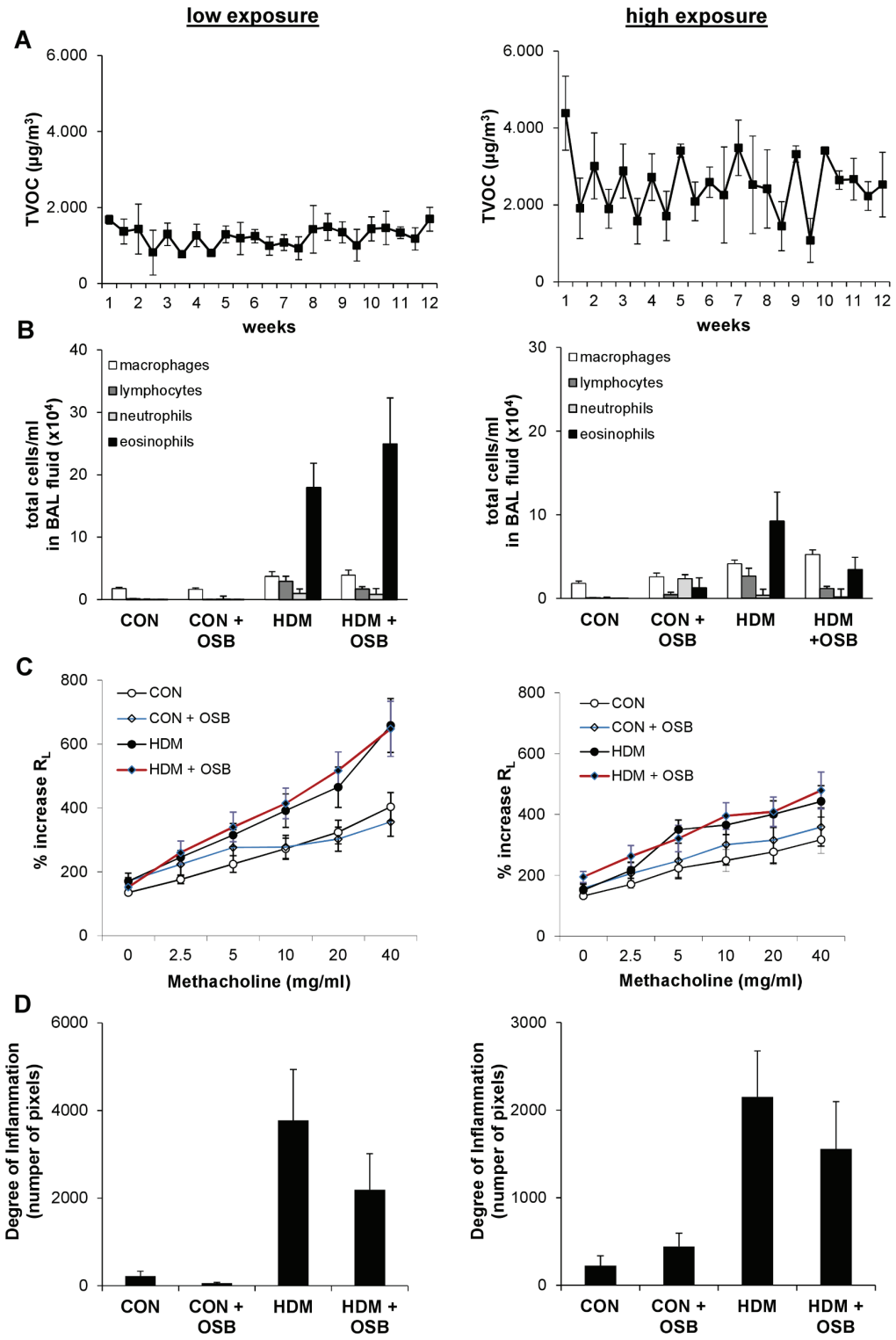

Fig. 5. Effect of long-term exposure to OSB on airway inflammation and lung function in HDM-sensitized mice. (A) TVOC concentrations for low and high exposure are shown. (B) Total cell number in BAL fluid, (C) lung resistance and (D) airway inflammation were examined in HDM-sensitized and control mice. Data are expressed as mean ± SEM, n = 3 (A), n ≥ 9 (B – D).

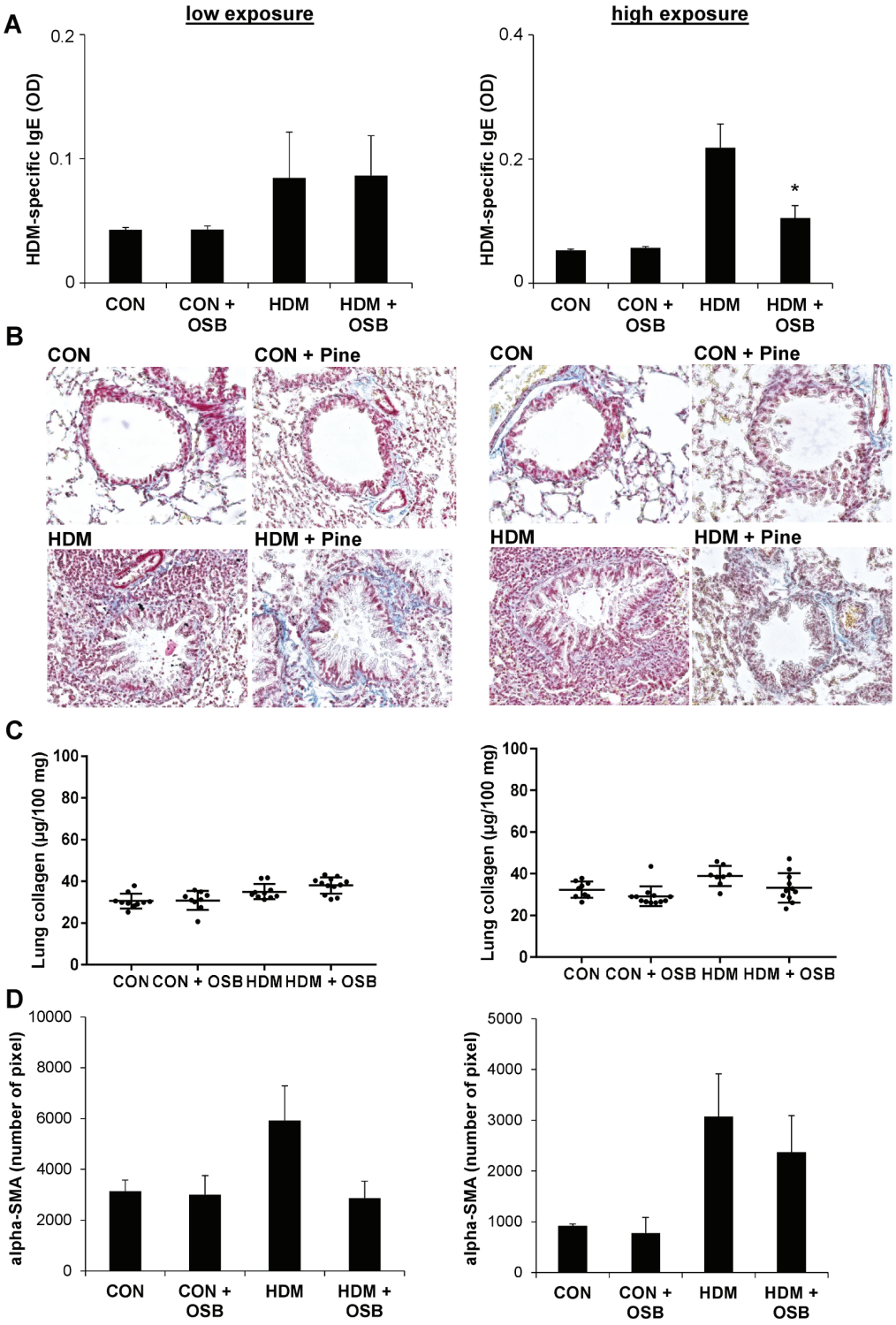

Fig. 6. Effect of long-term exposure to OSB on IgE level and airway remodeling in HDM-sensitized mice. (A) HDM-specific IgE serum levels, (B) MSB-stained lung tissues (x100), (C) total lung collagen and (D) proliferation of airway smooth muscle cells as quantified by an investigator-independent computer-based analysis were examined in HDM-sensitized and control mice. Data are expressed as mean ± SEM, n ≥ 9, *$P < 0.5$ HDM *vs.* HDM + OSB.

(pregnancy: 44.9 µg/m^3; year one: 47.5 µg/m^3).

To further display potential indoor sources of wood-related VOCs, questionnaires were analysed addressing if study participants had purchased new furniture, and if so from what material. Further, information on renovation activities in particular new flooring was considered. Exemplary shown for the time point of pregnancy, participants who documented new furniture from solid wood had a significant higher concentration of wood-related VOCs in their homes compared to participants without new solid wood furniture (Fig. 8A, Supplementary Table 3). In addition, documented parquet flooring significantly

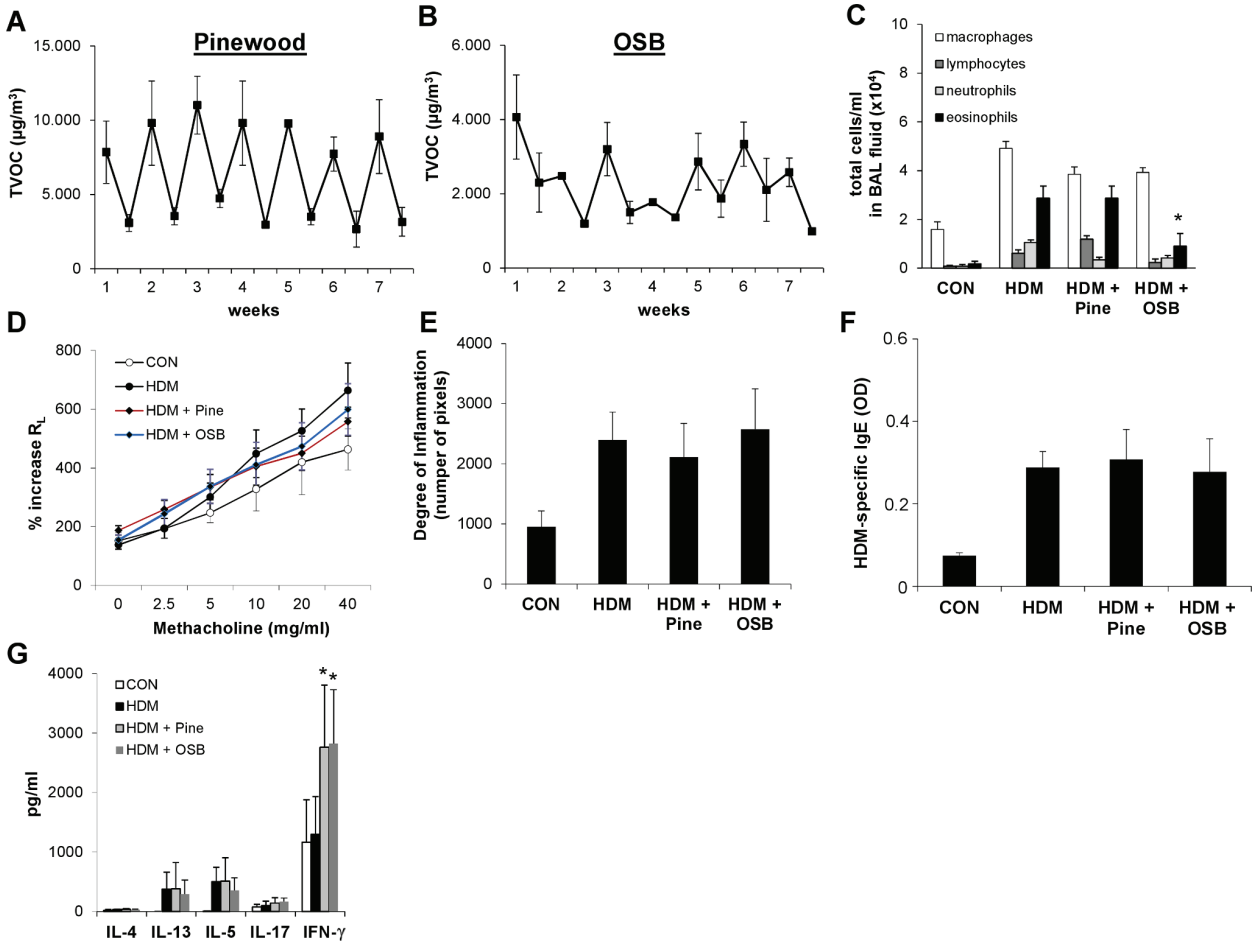

Fig. 7. Effect of perinatal exposure to pinewood or OSB on asthma development in the offspring. TVOC concentrations for the whole exposure period for (A) pinewood and (B) OSB are shown. (C) Total cell number in BAL fluid, (D) lung resistance, (E) airway inflammation examined by lung histology (H E, x100), (F) quantified by an investigator-independent computer-based analysis, (G) HDM-specific IgE serum levels and (H) cytokine production of re-stimulated splenocytes were examined in HDM-sensitized and control mice. Data are expressed as mean ± SEM, n = 2 (A, B), n ≥ 9 (C – H), *$P < 0.5$ HDM *vs.* HDM + Pine or HDM + OSB.

enhanced the wood-related VOC concentration compared to no parquet flooring. In contrast, particleboard furniture as well as other types of flooring did not influence wood-related VOCs.

3.5. Wood-related VOC exposure and wheezing/asthma in LINA

Since our study participants are realistically rather exposed to a complex mixture of wood-related VOCs instead of single VOCs, an epidemiological mixture analyses via BKMR was applied for the LINA data. Within the BKMR subcohort, 141 children (29.2%) developed an early wheeze within the first 2 years of life, 29 children (10.3%) developed asthma within the first 10 years of life, respectively.

Taken together, mixture analyses revealed no effects on early wheeze or asthma risk, neither when exposure to wood-related VOCs was during pregnancy (P) or year one (Y1) nor when only high risk children with a positive FHA were considered. In detail, BKMR offered different calculations to mimic different possible mixture scenarios. The *univariate exposure–response function* pictures the full range of one mixture component when simultaneously all other mixture components are held at their median concentrations (early wheeze: Figure S8A, C (P), Figure S9A, C (Y1); asthma: Fig. 8B, D (P), Fig. 9A, C (Y1)). The *cumulative effect* displays the comparison when all of the mixture components were fixed at their median value, with when all of the mixture components are at a particular (same) percentile (early wheeze: Figure S8B, D (P), Figure S9B, D (Y1); asthma: Fig. 8C, E (P), Fig. 9B, D (Y1)).

Taken together, we were able to show consistent results from experimental mouse and cohort data revealing no harmful effect of wood-related VOCs on respiratory outcomes, independent from an allergic risk/sensitization pattern, and from duration or time point of exposure.

4. Discussion

An increased use of sustainably produced wood in construction or for all sorts of long-lived products might be able to contribute to reducing society's carbon footprint (Hildebrandt et al. 2017; Kalt 2018). In this context, the evaluation of possible health effects by wood emissions is of considerable interest.

In regard to indoor air quality, TVOC levels below 1 mg/m^3 are declared as good air quality by the German Environment Agency while TVOC levels between 3 and 10 mg/m^3 are classified as hygienically critical and TVOC levels higher than 10 mg/m^3 as unacceptable condition (Umweltbundesamt 2007). Therefore, in the present study we exposed mice to wood-generated TVOC concentrations above 3 mg/m^3 up to TVOC levels of more than 10 µg/m^3. In contrast to earlier mouse studies investigating the effect of exposure to single VOCs (Bönisch et al. 2012; Li et al. 2017; Nielsen et al. 2005) we confronted the animals directly to pinewood or OSB and therefore to the whole emission

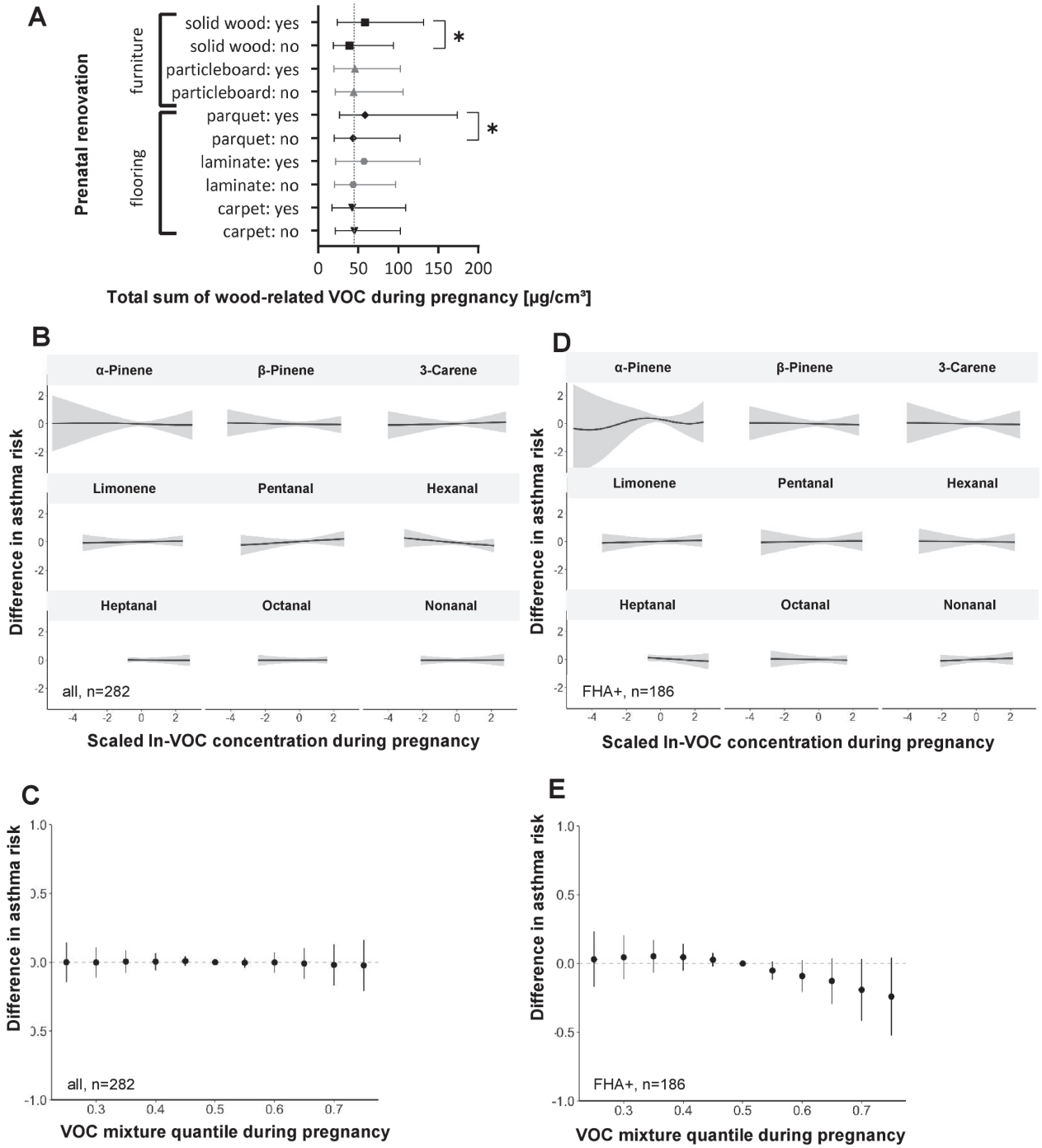

Fig. 8. Wood-related VOCs during pregnancy. (A) Indoor sources of wood-related VOCs: Total sum of wood-related VOCs was calculated from terpenes (α-pinene, β-pinene, 3-carene, limonene) and aldehydes (pentanal, hexanal, heptanal, octanal and nonanal), measured at 36th week of pregnancy. Shown are median with interquartile range; *$p < 0.05$, Mann-Whitney *U* test; vertical line at overall median (44.91 µg/m^3, n = 598); for individual case numbers and concentrations see Supplementary Table 3. (B - C) VOC exposure during pregnancy and asthma risk within the first 10 years of life in all children and (D - E) stratified by their family history of atopy (FHA). Data are estimated by Bayesian Kernel Machine Regression (BKMR) and by considering the following covariates: family history of atopy (except for D, E), smoking during pregnancy, maternal age at delivery, gender of the child, parity, parental school education, keeping of pets, delivery mode, breastfeeding duration and overweight development in infancy (VOCs are ln-transformed, scored and grouped by correlating exposures: α-pinene, β-pinene, 3-carene, limonene and pentanal, hexanal, heptanal, octanal, nonanal). (B/D) Univariate exposure–response function and 95% confidence bands; pictures the full range of one mixture component when simultaneously all other mixture components are held at their median concentrations (C/E) Overall/Cumulative effect; pictures the comparison when all of the mixture components were fixed at their median value, with when all of the mixture components are at a particular (same) percentile, shown are estimated differences in asthma risk and 95% credible intervals.

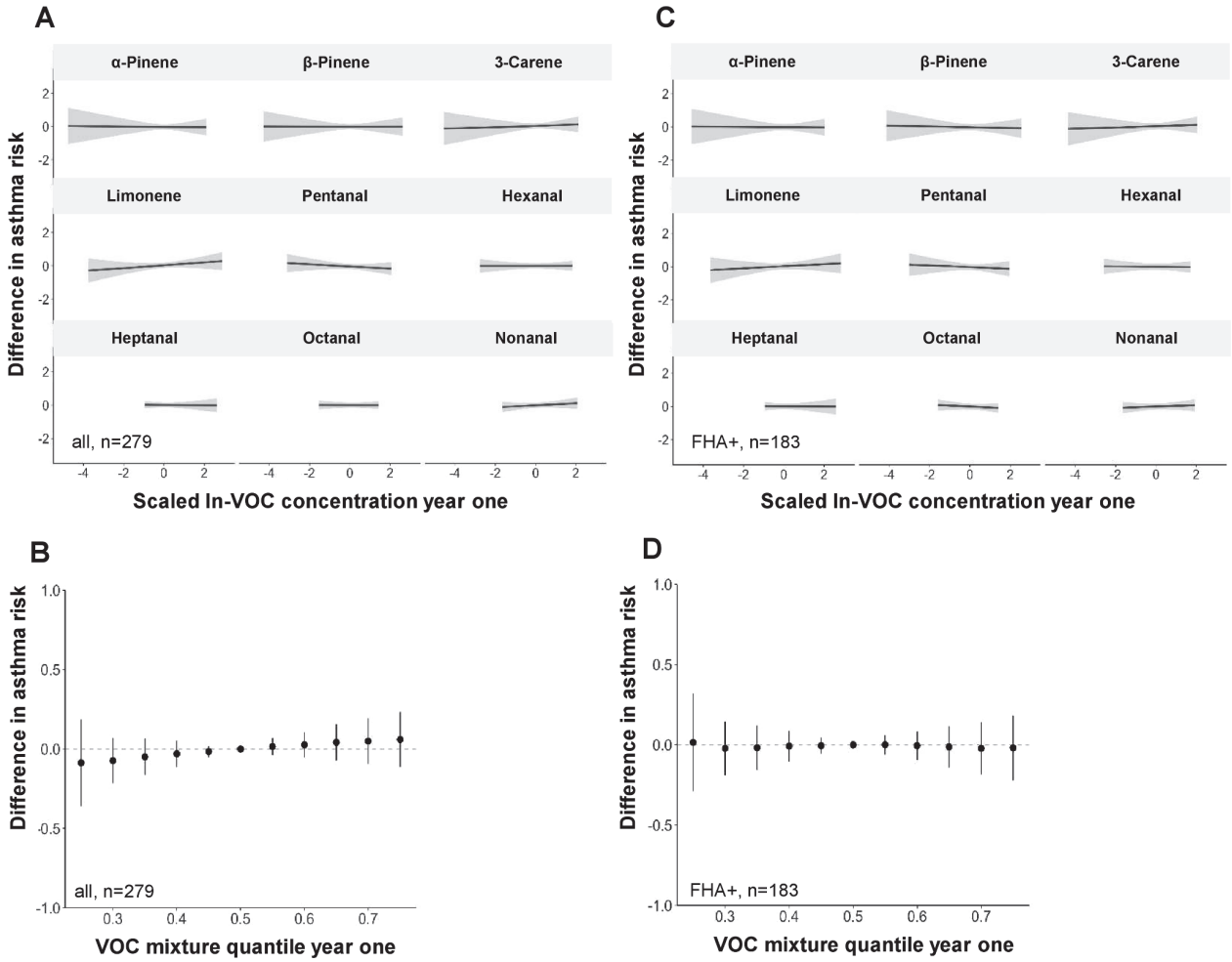

Fig. 9. Wood-related VOCs year one. (A - B) VOC exposure at year one and asthma risk within the first 10 years of life in all children and (C - D) stratified by their family history of atopy (FHA). Data are estimated by Bayesian Kernel Machine Regression (BKMR) and by considering the following covariates: family history of atopy (except for C, D), smoking during pregnancy, maternal age at delivery, gender of the child, parity, parental school education, keeping of pets, delivery mode, breastfeeding duration and overweight development in infancy (VOCs are ln-transformed, scored and grouped by correlating exposures: α-pinene, β-pinene, 3-carene, limonene and pentanal, hexanal, heptanal, octanal, nonanal). (A/C) Univariate exposure–response function and 95% confidence bands; pictures the full range of one mixture component when simultaneously all other mixture components are held at their median concentrations (B/D) Overall/Cumulative effect; pictures the comparison when all of the mixture components were fixed at their median value, with when all of the mixture components are at a particular (same) percentile, shown are estimated differences in asthma risk and 95% credible intervals.

spectrum of the products. This approach also allowed us to mimic long-term exposures about twelve weeks to examine the impact of continuously inhalation of an indoor-relevant VOC mixture on the airways, something that has not been examined so far. Moreover, within the long-lasting asthma model we could also evaluate possible effects on airway remodelling, the structural alterations that occur as result of chronic asthma development (Fehrenbach et al. 2017). Overall, our data demonstrate that neither pinewood nor OSB emissions even at high TVOC levels and a long-lasting exposure period induce significant inflammatory or acute/chronic asthma-promoting effects in sensitized or non-sensitized mice. At most, one could indicate a slight trend of an increased lung resistance and elevated lung collagen and isoprostane levels in non-sensitized mice after long-term exposure to high-emitting pinewood. In that respect, there are a few human studies investigating health effects of occupational VOC exposure in sawmills or joineries with high terpene levels. The data show no association between terpene exposure (9 – 214 mg/m^3) and respiratory symptoms of participants (Eriksson et al. 1997; Eriksson et al. 1996) but also an impaired lung function of workers exposed to an average terpene concentration of 258 mg/m^3 (Hedenstierna et al. 1983). These different findings might be due to the different terpene levels, but it also needs to be considered that other factors occurring in sawmills like dust particles or mould might trigger the observed respiratory symptoms (Hedenstierna et al. 1983). Experimental studies with human participants exposed to wood-related VOCs under controlled conditions using a specific exposure chamber revealed no irritation of the airways or effects on lung function at TVOC levels of pinewood up to 13 mg/m^3 (Gminski et al. 2011b), of OSB up to 9 mg/m^3 (Gminski et al. 2011a) or hexanal concentrations of 42 mg/m^3 (Ernstgard et al. 2006). In contrast, exposure to a VOC mixture (α-pinene, β-pinene, 3-carene) of 450 mg/m^3 induced a mild airway inflammation but had no effect on pulmonary function (Johard et al. 1993). Either way, the few observed respiratory effects only occur at very high VOC levels that do not normally occur in the general living environment.

A more surprising result from our murine asthma model was the significant reduction of allergic airway inflammation after short-term exposure to OSB emitting, beside terpenes, higher levels of aldehydes. In vitro studies indicate that aldehydes might rather have a more

adverse impact than terpenes (Gminski et al. 2010) making it difficult to interpret these findings. It is also interesting, that OSB exposure not only diminished the asthma-relevant Th2 cytokines IL-13 and IL-5 but also the Th17 cytokine IL-17 and the Th1 cytokine IFN-γ suggesting a general immune suppressive function. However, considering that the asthma-reducing effect was observed using a protocol for a mild asthma phenotype the findings should not be overestimated.

Concerning chemical-induced health effects, it became evident that the prenatal and early postnatal period is a critical time window to environmental exposures (Martino and Prescott 2011; Rubin and Soto 2009). In recent studies we could demonstrate that low-dose exposure of adult mice to endocrine disrupting chemicals such as phthalates or parabens had no effect on asthma development or metabolic parameters but led to an increased airway inflammation or weight gain in the offspring after exposure during pregnancy and the lactational period (Jahreis et al. 2018; Leppert et al. 2020). Therefore, we additionally examined the effect of maternal exposure over 7 weeks to pinewood or OSB on asthma development in the offspring. Here as well, both wood products did not decisively affect the severity of the asthma phenotype in the next generation. Next, we evaluated the effect of early-life exposure to wood-related VOC on children's asthma risk in our mother–child cohort LINA. While several epidemiological studies have investigated the impact of terpenes or aldehydes on the disease risk in adults (Cakmak et al. 2014; Glas et al. 2015; Salonen et al. 2009), there are no data so far addressing the effect of prenatal or early-life exposure to wood VOCs on asthma development in children. Within our LINA study, we could confirm wood products as a principal source for wood-specific emission by linking the exposure to new wooden furniture or flooring with increased levels of typical wood VOCs. To investigate the possible impact of prenatal and early postnatal exposure to wood-related VOCs on children's early wheezing until the age of 2 / asthma risk until age 10 we used a mixture model to assess combined effects of the measured VOCs (Bobb et al. 2018; Preston et al., 2020). Individuals are ubiquitously exposed to multiple wood-related VOCs and therefore the understanding of a possible joint VOC effect on wheezing/asthma risk is critically important. Taken together, epidemiological BKMR data did not show any harmful effect of the perinatal exposure to wood-related VOCs measured in the homes of study participants, independent from the time point of exposure (pregnancy/year one) or their individual atopy risk. However, indoor measurements were performed with different time delays after redecoration activities. Thus, the measured concentrations might just be proxies for experienced higher exposure concentrations. Of course, also VOCs from other sources such as tobacco smoke were shown to impact respiratory disease risk, in particular in the highly sensitive perinatal period (Franck et al. 2014; Junge et al., 2014); however this aspect was adjusted for – next to others - by including exposure to environmental tobacco smoke in the BKMR as a confounding factor. The use of BKMR allowed us to examine the joint and individual effects of exposure to multiple wood-related VOCs on children's early wheezing/asthma risk within the first 10 years of life by evaluating potential non-linear exposure–response functions and interactions among wood-related VOCs. With this approach we were able to verify the experimental mouse work showing wood products as non-critical with respect to early wheezing/asthma development in childhood.

A general limitation of the LINA study is the potential bias by high rates of participating atopic parents who may have had special interest in the topic of the study. We have considered this point by always including the parental atopy history as confounding variable in the overall BKMR models. In addition, wheezing and asthma outcome diagnosis is a parental reported doctor's diagnosis that may carry some minor inaccuracies. With respect to the VOC analyses and their potential sources, until now the decay of VOC concentrations after renovation activity is not well described. Moreover, indoor measurements were performed with different time delays after redecoration activities. Thus, the measured concentrations in the homes of the study participants were just proxies for supposedly experienced higher exposure concentrations. However, we were able to overcome this point by a direct exposure scenario within the experimental mouse analyses.

5. Conclusions

We investigated the impact of exposure to VOCs emitted by pinewood and OSB on the airways of allergen-sensitized and non-sensitized mice using an acute, chronic and cross-generational asthma model. We found that neither short-term, long-term nor perinatal exposure to these wood products had pro-inflammatory or asthma-promoting effects in healthy or sensitized animals. In our mother–child cohort LINA, we further examined the outcome of exposure to multiple wood-related VOCs during pregnancy or the first year of life on children's wheezing/asthma risk. Using the BKMR approach, we clearly demonstrated that the mixture of wood-related VOCs has no impact on early wheezing/asthma development in children until age 10. To our knowledge, this is one of the first studies assessing the effects of the whole spectrum of VOC emission from wood products using an in vivo model and BKMR to analyse joint effects of multiple wood-related VOCs on children's wheezing/asthma risk in a mother–child cohort. Our findings indicate that emissions from wood and wood products at levels usually occurring in the living environment do not induce adverse effects in the respiratory system.

Author contribution

T.P. designed and conducted the experimental study; E.E. performed the mouse experiments; K.J. and L.B. analysed the cohort data; K.B. and M.O. measured VOCs in the mouse study; T.K., U.E.R.K and M.v.B. were involved in the VOC analytic in the cohort; I.L., G.H., S.R., M.B. and W.K. developed the LINA study design or performed cohort clinical visits; T. P., K.J., L.B., R.G., J.C.S. and M.O. discussed the data with substantial contributions from I.L.; T.P., K.J., and L.B. wrote the paper with substantial inputs from K.B. and I.L.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2021.106449.

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2.3. Endocrine disrupting chemicals (parabens) and allergy development

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Effects of exposure to single and multiple parabens on asthma development in an experimental mouse model and a prospective cohort study

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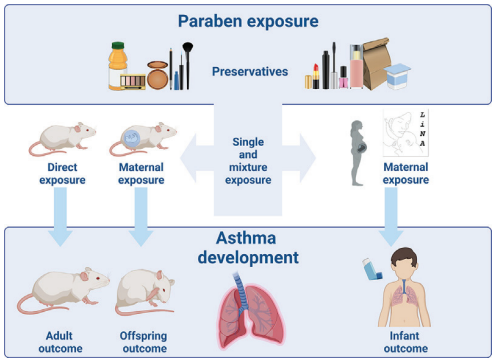
HIGHLIGHTS

- Effects of single and multiple parabens on asthma were assessed in mice and humans.
- Adverse and preventive effects of single parabens on asthma were found in mice.
- Paraben mixtures revealed no effects in mouse or in a mother-child cohort.
- Mixtures should be considered in evaluation of individual specific disease risks.

GRAPHICAL ABSTRACT



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ABSTRACT

Parabens are widely used preservatives present in consumer products like cosmetics and food. Although several epidemiological studies suggest that early-life exposure to parabens might alter the immune response and allergy risk in childhood, the evidence with respect to asthma is not clear. Therefore, we investigated the effect of paraben exposure on asthma development in mice and humans. Using a murine asthma model the experimental data show both, an

Abbreviations: AHR, airway hyperreactivity; BAL, bronchoalveolar lavage; BKMR, Bayesian kernel machine regression; CI, confidence interval; CON, control; EtP, ethyl paraben; FHA, family history of atopy; HDM, house dust mite; H&E, haematoxylin and eosin staining; iBuP, i-butyl paraben; IFN, Interferon; IgE, immunoglobulin E; IL, interleukin; MeP, methyl paraben; nBuP, n-butyl paraben; OR, odds ratio; PrP, propyl paraben; Th1/Th2, T helper cells type 1/2.

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asthma-reducing effect after direct exposure of adult mice to n-butyl paraben (nBuP) as well as an asthma-promoting effect after maternal exposure to ethyl paraben (EtP) in the female offspring. Interestingly, exposure of mice to a mixture of EtP and nBuP starting prenatally until the end of asthma induction in the adult offspring was without effect on allergic airway inflammation. In addition, parabens were determined within the German prospective mother-child cohort LINA and their single and mixture effect on asthma development in children within the first 10 years of life was estimated by logistic and Bayesian kernel machine regression (BKMR). Both approaches revealed no adverse effects of parabens on children's asthma development, neither when stratified for being at risk due to a positive family history of atopy nor when analysed separately for sex specificity. Therefore, we conclude that although single parabens might differentially impact asthma development, an adverse effect could not be seen in a multiple paraben exposure setting. Consequently, not only the time point of exposure but also multiple exposure scenarios to parabens should be considered in the evaluation of individuals' specific disease risk.

1. Introduction

Parabens are alkyl esters with antimicrobial activity that are extensively used as preservatives in consumer product like cosmetics and food (Halla et al., 2018) and can also be found in the human environment (Wei et al., 2021). A ubiquitous exposure to single parabens was described in several studies, however it seems to be even more realistic that individuals are facing a multiple exposure scenario (Berger et al., 2020).

Parabens are considered to have endocrine disrupting activity and are therefore indicated as chemicals of emerging concern that exert adverse human health effects. Exposure to these chemicals has been linked to breast cancer (Darbre et al., 2004), reproductive malfunctions (Nishihama et al., 2016), and childhood overweight development (Leppert et al., 2020). Furthermore, several studies associated the use of paraben-containing products with allergic immunoreaction, causing contact dermatitis (Yim et al., 2014) and an increasing risk for atopic eczema (Lowe et al., 2019; Mitsui-Iwama et al., 2019; Thurmann et al., 2021).

Interestingly, with regard to allergic asthma most of the epidemiological studies did not find a correlation between exposure to parabens and the prevalence for asthma (Lee-Sarwar et al., 2018; Spanier et al., 2014; Vindenes et al., 2021). One study showed a positive association of children's urinary paraben concentrations and their asthma-related emergency department visits between 6 and 19 years (Quiros-Alcala et al., 2019), while, in the same survey, no association was found between their paraben concentrations and asthma prevalence until the age of 18 years (Spanier et al., 2014). So far, there is only one study indicating a correlation between in-utero exposure to ethyl paraben (EtP) and an increased asthma risk in children, while other parabens or their sum were without effect (Vernet et al., 2017). Another study showed even decreased odds of probable asthma in association with propyl paraben (PrP) levels (Berger et al., 2018). Thus, although several epidemiological studies suggest that exposure to parabens has the potential to alter immune functions and the risk of allergic diseases in childhood, the association to asthma is not well studied and inconsistent.

In this context, data from animal experiments could provide additional information to the sparse epidemiological data but, so far, there are no experimental in vivo studies investigating the effect of paraben exposure on asthma to clarify the inhomogeneous picture. Therefore, the current study aims to characterize the impact of paraben exposure on asthma using an experimental mouse model and epidemiological analyses from a well-characterized mother-child cohort by outlining different exposure time points and exposure scenarios.

2. Material and methods

2.1. Animals

2.1.1. Mice

Female BALB/cByJ mice (6 – 8 weeks of age) were obtained from the Elevage Janvier Laboratory (Le Genest St Isle, France). Mice were bred and maintained in the animal facility at the University of Leipzig (Germany) under conventional conditions with 23 °C room temperature, 60% humidity, and 12 h day/night rhythm. All mice received phytoestrogen-free diet (C1000 from Altromin, Lage, Germany) and water ad libitum. Experiments involving induction of allergic airway inflammation included groups of 4 – 6 mice/cage and were performed at least two times according to institutional and state guidelines. Animal protocols used in this study were approved by the Committee on Animal Welfare of Saxony/Leipzig (Permit Number: TVV01/15, 14/18).

2.1.2. Exposure protocols to parabens

To evaluate the effect of parabens on asthma development, we exposed Balb/c mice to a representative of a short-chain (ethyl paraben, EtP) as well as a long-chain (n-butyl paraben, nBuP) paraben using different exposure protocols in an established mouse asthma model (Jahreis et al., 2018; Petzold et al., 2014; Polte et al., 2015). Both parabens used were already shown to have the most disease-affecting impact compared to other parabens of a comparable chain length (Leppert et al., 2020; Thurmann et al., 2021; Vernet et al., 2017). Doses used in the mouse model were estimated from an acceptable daily intake in humans based on No Observed Adverse Effect Levels of 1000 mg kg^{-1} bodyweight (EtP) and 2 mg kg^{-1} bodyweight (nBuP), respectively (EFSA, 2004; Fisher et al., 1999).

First, 6 to 8-week-old female mice were directly exposed to EtP (10 mg kg^{-1} bodyweight/day) and nBuP (20 μg kg^{-1} bodyweight/day) starting one week prior house dust mite (HDM) sensitization until the end of the asthma protocol (Supplementary Fig. E1A). Application was performed by subcutaneous (s.c.) injection of 0.875 mg EtP or 1.75 μg nBuP (Sigma-Aldrich Chemie GmbH, Munich, Germany) in 100 μl corn oil twice per week. Control dams received only the vehicle. In a second protocol and to study the effects of maternal exposure on the progeny, mice were exposed to EtP or nBuP starting before mating until weaning when pups were 3 weeks old (perinatal exposure, Supplementary Fig. E1B). Third, and based on the results seen in the first and second protocol, the effect of a paraben mixture of EtP and nBuP was evaluated while mice were exposed to the parabens starting one week before mating until the end of the asthma protocol induced in the offspring at 6 – 8 weeks (Supplementary Fig. E1C). Each exposure protocol was performed at least two times with at least 3 dams per group/condition resulting in ≥7 pups per group/condition (each female or male, with a maximum of 3 pups per sex per dam).

2.1.3. Asthma induction

To induce an asthma-like phenotype with airway hyperresponsiveness, eosinophilic inflammation, elevated allergen-specific IgE levels and an increased production of the T helper 2 (Th2) cytokines IL-4, IL-5 and IL-13 Balb/c mice were sensitized via the airways with 25 μg house dust mite extract (HDM, endotoxin: 1.273 EU/ml, Greer Laboratories, USA) in 40 μl saline on day 1. This was followed by 5 μg HDM given intranasally (i.n.) on days 7 to 11 and 14 to 16. Control mice received normal saline i.n. Airway hyperreactivity (AHR) was measured on day 17 and mice were sacrificed on day 18 (Supplementary Fig. 1).

2.1.4. Measurement of airway responsiveness

To measure lung resistance (R_L) mice were anesthetized (100 mg kg^{-1} ketamine and 10 mg kg^{-1} xylazine, Bayer, Leverkusen, Germany), intubated, and mechanically ventilated at a tidal volume of 0.2 ml and a frequency of 150 breath/min. Baseline R_L and responses to aerosolized saline

(0.9% NaCl) were measured first, followed by responses to increasing doses (2.5 to 40 mg/ml) of aerosolized methacholine.

2.1.5. Collection of bronchoalveolar lavage (BAL) fluid

To assess eosinophilic inflammation in the BAL all cells within the lavage fluid were counted using a hemocytometer. Diffquick® (Medion Diagnostics AG, Düdingen, CH) stained cytospins were differentiated into eosinophils, macrophages, lymphocytes and neutrophils according to morphological criteria as described previously (Bickert et al., 2009; Polte et al., 2008).

2.1.6. Lung histology

Left lung was fixed in 10% formalin and stained with Haematoxylin & Eosin (H&E, MERCK, Darmstadt, Germany) to analyze inflammatory infiltrates in the airways (Petzold et al., 2014; Polte et al., 2015).

2.1.7. HDM-specific IgE assay

HDM-specific IgE serum levels were measured by ELISA according to a standard protocol. Briefly, 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated overnight with 25 μg/ml HDM. After washing and blocking plates, serum was added and incubated at 4 °C overnight. Subsequently, 96-well plates were washed and 100 μl of biotin-anti-mouse IgE (BioLegend, CA, USA) was added and incubated for 1 h. The wells were washed with PBS followed incubation with avidin-horse radish peroxidase (Biolegend) for 30 min. TMB substrate solution (100 μl) was added and incubated in the dark for 30 min. The OD was determined at 450 nm using a BioTek microplate reader (BioTek Instruments, Bad Friedrichshall, Germany).

2.1.8. Cytokine production

One day after airway function test splenocytes or mediastinal lymph node cells (1×10^7 cells/ml per well) were isolated and re-stimulated in vitro with 200 μg/ml OVA in culture medium (RPMI medium supplemented with 10% FBS, 100 U/ml Penicillin, 100 μg/ml Streptomycin). After three days of culture cytokines were measured in supernatant using DuoSet® ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.2. Human cohort

2.2.1. LINA study design and sample collection

To further address the paraben-asthma relationship with epidemiological data we used the German prospective mother-child cohort LINA (Lifestyle and environmental factors and their Influence on Newborns Allergy risk). LINA recruited 622 mothers (629 children) at 34 weeks of gestation between May 2006 and December 2008 in Leipzig, Germany (Hinz et al., 2010; Weisse et al., 2013). Mothers with severe immune or infectious diseases during pregnancy were excluded from the study. Standardised self-administered questionnaires were collected annually starting in pregnancy, assessing general information, personal lifestyle, housing and environmental conditions as well as family history of atopy (FHA) and disease state (see Supplementary Table E1).

Study participation was voluntary and written informed consent was obtained by all participants. The study was approved by the Ethics Committee of the University of Leipzig (file ref # 046-2006, 160-2008, 160b/2008, 144-10-31052010, 113-11-18042011, 206-12-02072012, #169/13-ff, #150/14-ff, 008/17-ek).

2.2.2. Analysis of urinary paraben concentrations in human samples

Urinary concentrations of eight paraben species were determined in 504 maternal urine samples of the 34th week of gestation. The samples were prepared and analysed as described and validated by Schlittenbauer et al. (2016). In brief, urine samples, quality controls, and solvent blanks were thawed at room temperature, vortexed, and centrifuged. Aliquots were spiked with internal standard solution and a deconjugation standard. Hydrolysis was achieved by adding enzyme buffer solution and incubation in an ultrasonic bath. The enzyme was removed by centrifugation/filtration using Amicon® Ultra-0.5 filters. LC-MS analysis was performed on a UPLC™ system (ACQUITY I-Class, Waters Cooperation Milford, MA, USA) coupled to a triple quadrupole mass spectrometer (Xevo TQ-S, Waters Cooperation, Manchester, UK) equipped with an electrospray ionization (ESI) source. For each analyte (MeP, EtP, nPrP, iBuP, nBuP), a quantification (Q) and a confirmation (q) MRM transition was selected. Quality criteria for positive confirmation of peaks were (i) presence of both MRM transitions, (ii) a retention time within 0.03 min, and (iii) a relative ion ratio within 50 to 150% compared to a standard. The limits of quantification (LOQ) were 0.5 μg/l for MeP and 0.1 μg/l for the others parabens (EtP, nPrP, iBuP, nBuP). Levels below the LOQ were included in the model analyses as half of the detection limit. For 498 participants valid paraben concentrations above the detection limit were obtained, for 274 of them also information about asthma prevalence until the age of 10 years as well as confounding factors were available (cases and paraben concentrations used within asthma analyses are shown in Supplementary Table E2). For iPrP, sBuP and BzP more than 70% of samples had concentrations below the LOQ and, therefore, were excluded from further analysis. For paraben analysis of mouse samples 20 μl urine and 180 μl milliQ water was mixed and the same protocol as described above was applied.

2.2.3. Asthma prevalence of children

Respiratory health of children was assessed by questionnaires asking parents annually for physician diagnosed respiratory diseases like asthma, obstructive bronchitis, wheezing etc. From that information, the prevalence for the 10-year lifetime prevalence of asthma was used for the present regression and BKMR analyses. As controls, only children who had never experienced any respiratory diseases like asthma, obstructive bronchitis or wheezing within the first 10 years were included. All model analyses with respect to paraben exposure and asthma risk were performed in all children of the sub-cohort, sex-specific as well as in children at risk for atopic diseases due to a positive family history of atopy (at least one parent positive). Analyses could not be performed separately in children without a family history of atopy (FHA) due to low case numbers of asthma positive children in that group (n = 4).

2.3. Statistical analysis

Experimental data sets from in vivo mouse studies were processed and analysed in GraphPad PRISM 7.02 for windows (GraphPad Soft-ware, Inc.). Data were expressed as mean ± SEM and *P* values of less than 0.05 were considered significant by *t*-test and Wilcoxon-Mann-Whitney test. With respect to the epidemiological data, only cases with complete data on prenatal urine paraben concentration, asthma prevalence within the first 10 years of life and relevant confounding factors were included in the statistical analyses (n = 274). The Chi2-test (categorical parameters) or Mann-Whitney *U* test (continuous parameters) was used to compare the analysed sub-cohort (n = 274) until the age of 10 years with the total cohort (n = 629) according general study characteristics/confounders. Logistic regression and Bayesian kernel machine regression (BKMR) models were performed with STATISTICA for Windows, Version 13 (Statsoft Inc.) and R (version 3.6.1; R development Core Team) for the bkmr package (Bobb et al., 2018), each considering the following confounders: smoking during pregnancy (as maternal log urine cotinine levels), maternal age at delivery, sex of the child (except for the analyses stratified for sex), family history of atopy (except for the analyses stratified for FHA), number of siblings, parental school education (low/medium/high) and urine creatinine levels. BKMR was performed with 50,000 iterations according to Bobb et al. (2018) and Preston et al. (2020) while paraben concentrations were ln-transformed and z-scored. BMKR analyses with respect to the asthma risk within the first 10 years of life revealed the following aspects: (A) overall/cumulative effect (pictures the comparison when all of the mixture components/all parabens were fixed at their median value, with when all of the mixture components/all parabens are at a particular (same) percentile), (B) the univariate exposure-response function (which pictures the full

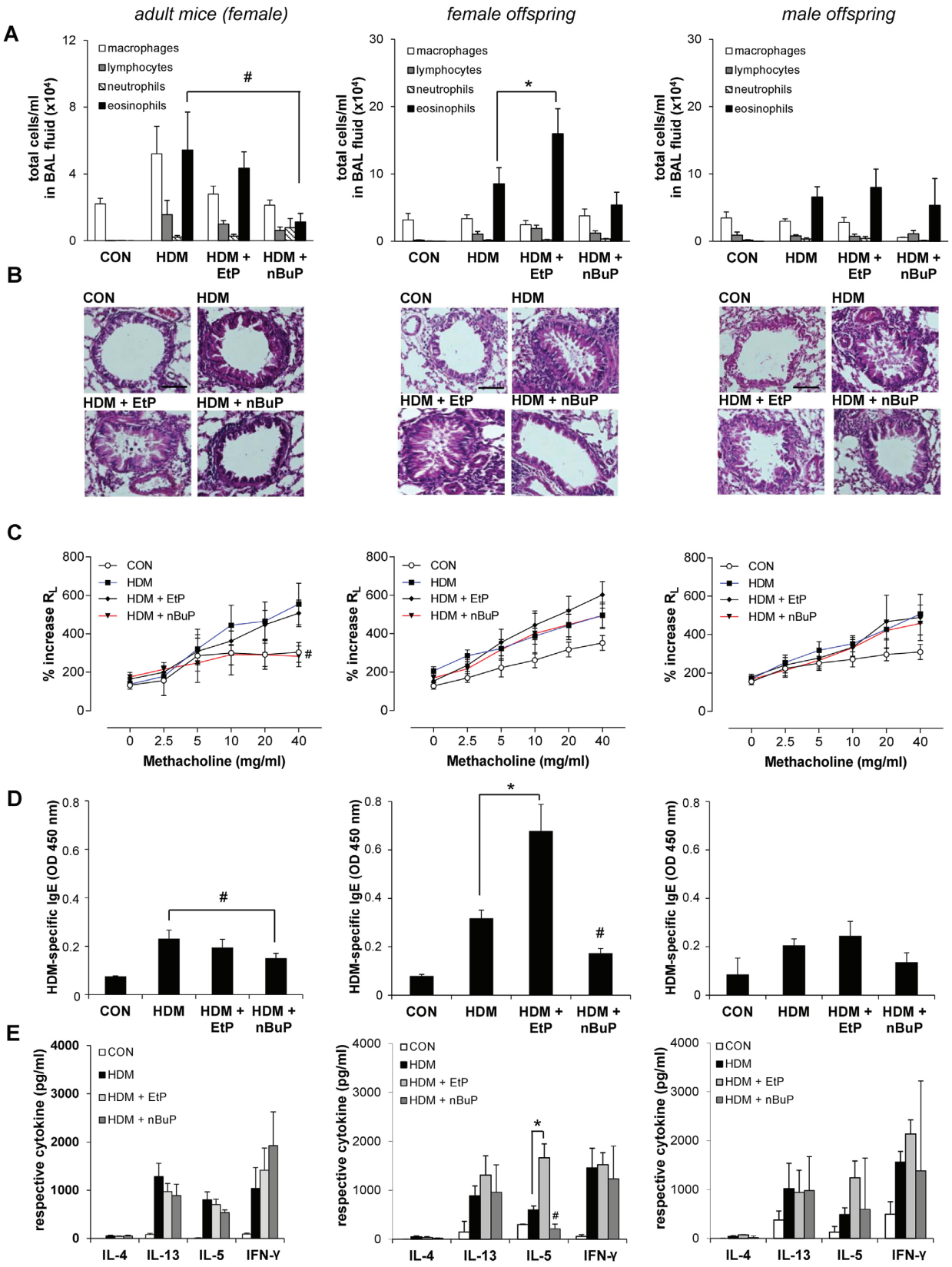

Fig. 1. Exposure to EtP or nBuP has different effects on allergic airway inflammation in a mouse asthma model. Cell number in BAL fluid (A), airway inflammation (B, H&E, ×100), lung resistance (C), HDM-specific IgE (D), and cytokine (E) levels were examined in offspring from paraben-exposed dams or directly exposed adult mice. Mean ± SEM, n ≥ 7 mice/group, $P < 0.05$, *HDM vs. EtP, #HDM vs. nBuP; BAL – bronchoalveolar lavage, HDM – house dust mite, CON - control, scale bar - 100 μm.

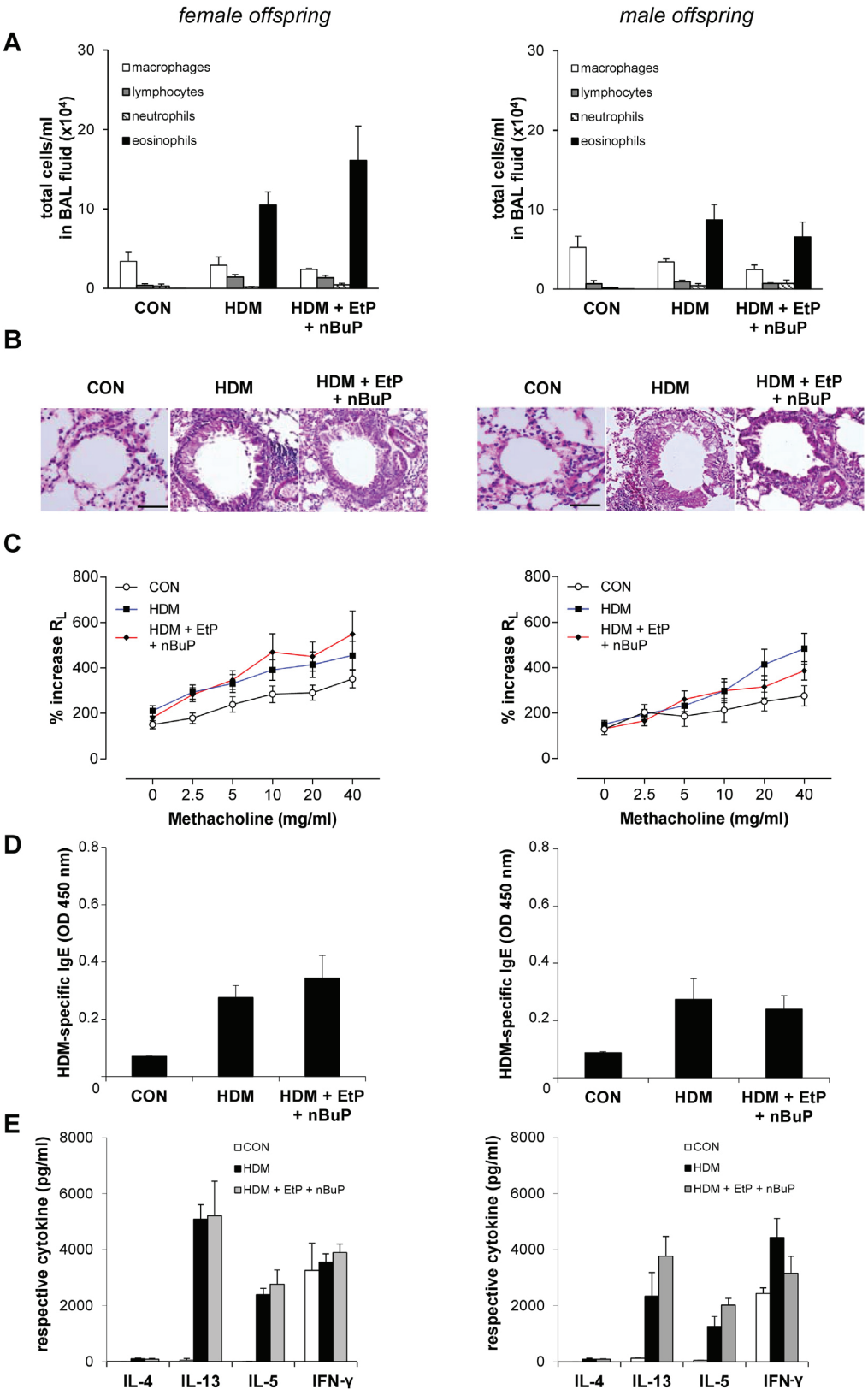

Fig. 2. Exposure to a mixture of EtP and nBuP has no effects on allergic airway inflammation in a mouse asthma model. Total cell number in BAL fluid (A), airway inflammation (B, H&E, ×100), lung resistance (C), HDM-specific IgE (D), and cytokine (E) levels were examined in offspring from paraben-exposed dams. Mean ± SEM, n ≥ 7 mice/group, scale bar - 100 μm.

range of one mixture component/one paraben when simultaneously all other mixture components/all other parabens are held at their median concentrations) and (C) the interaction analyses, that pictures the difference in asthma risk when one mixture component/one paraben changes from its 25th to 75th percentile while all other mixture components/all other parabens are fixed at their 25th percentile, as compared to when all other mixture components/parabens are fixed at their 75th percentile.

3. Results

3.1. Effect of single exposure to EtP or nBuP on allergic asthma in a HDM-induced murine asthma model

First, adult exposure to nBuP diminished eosinophilic inflammation in the bronchoalveolar lavage (BAL) fluid (Fig. 1A) and airway inflammation in the lung as demonstrated by H&E-stained lung sections (Fig. 1B) and verified by an objective, investigator-independent computer analysis (Supplementary Fig. E2). Furthermore, nBuP exposure significantly decreased airway hyperreactivity (AHR, Fig. 1C) and HDM-specific IgE levels (Fig. 1D) and slightly reduced the production of the Th2 cyrokines IL-13 and IL-5 (Fig. 1E), while exposure to EtP was without significant effect on the asthma phenotype (Fig. 1A–E).

Second, maternal paraben exposure on allergic airway inflammation in the progeny was assessed (Supplementary Fig. E1B). Thereby, exposed dams showed urine paraben levels in a comparable range to those detected in highly exposed mothers of our cohort (highest quintile urine concentration: EtP 18.1 μg/l, nBuP 5.9 μg/l; mean urine concentration in exposed mice: EtP 25.9 μg/l, nBuP 19.6 μg/l). Maternal exposure to EtP significantly increased the number of eosinophils within the BAL fluid in female offspring (Fig. 1A) and led to substantially more inflammatory infiltrates in the airways compared to female offspring from non-exposed dams (Fig. 1B). Furthermore, HDM-specific IgE levels were elevated in maternally exposed female offspring compared to control (Fig. 1D), while no significant effect on AHR was observed (Fig. 1C). Moreover, HDM-restimulated splenocytes from female offspring of EtP-exposed dams produced significantly more of the Th2 cytokine IL-5 and in trend of IL-13, while there was no significant effect on the Th1 cytokine IFN-γ (Fig. 1E). Interestingly, maternal exposure to nBuP had no significant effect on the asthma-like phenotype in female offspring (Fig. 1A–E). In addition, male offspring were not affected by any paraben (Fig. 1A–E). Taken together, within our mouse studies we observed varying effects of a single paraben exposure on asthma development depending on the applied paraben, sex and the time point of exposure.

3.2. Effect of exposure to a mixture of EtP and nBuP on allergic asthma in a murine asthma model

To further explore these contradictory effects of single EtP or nBuP at different time points, we exposed mice to a mixture of both parabens by also combining the exposure time points (Supplementary Fig. E1C). Interestingly, under these conditions we did not observe any effect of the paraben mixture on eosinophilic inflammation, AHR, antigen-specific IgE or Th2 cytokine levels in HDM-sensitized mice independent of the sex (Fig. 2A–E, Supplementary Fig. E3).

3.3. Association between prenatal exposure to single parabens and asthma development in children within the first 10 years

The epidemiological analysis was done in a subgroup of 274 LINA children with available maternal exposure data and disease outcome information until the age of 10 years. There were no differences in general characteristics comparing this sub-cohort compared to the total LINA cohort (Supplementary Table E1). Within the analysed sub-cohort, 26 children (9.5%) developed asthma within the first 10 years of life. Our logistic regression models - mimicking exposure to a single paraben - revealed neither effects in the analysed total sub-cohort nor when stratified for children being at risk due to a positive family history of atopy or when analysed separately for boys and girls (Table 1A–D). Corresponding paraben concentrations within these analysed groups are outlined in Supplementary Table E2.

Table 1
Association of prenatal exposure to different parabens [μg/l] and asthma development within the first 10 years of life within the LINA cohort. Displayed are the effects for all children (A) and for those with a positive family history of atopy (FHA+; B), as well as separately for boys (C) and girls (D).

(A)

	All (n = 274)							
	Crude				Adjusted[a]			
	OR	95% CI		*p*-Value	OR	95% CI		*p*-Value
MeP	1.00	0.999	1.002	0.477	1.00	0.999	1.001	0.936
EtP	1.01	0.996	1.016	0.264	1.00	0.993	1.015	0.474
nPrP	1.00	0.999	1.005	0.289	1.00	0.997	1.004	0.649
iBuP	1.03	0.964	1.096	0.403	1.02	0.955	1.096	0.513
nBuP	1.00	0.970	1.023	0.768	0.99	0.970	1.020	0.679

(B)

	FHA+ (n = 191)							
	Crude				Adjusted[a]			
	OR	95% CI		*p*-Value	OR	95% CI		*p*-Value
MeP	1.00	0.999	1.002	0.741	1.00	0.999	1.001	0.988
EtP	1.01	0.995	1.016	0.272	1.01	0.994	1.018	0.317
nPrP	1.00	0.997	1.004	0.615	1.00	0.997	1.004	0.772
iBuP	1.03	0.970	1.103	0.300	1.03	0.959	1.101	0.432
nBuP	1.00	0.974	1.021	0.809	1.00	0.974	1.020	0.784

(C)

	Boys (n = 134)							
	Crude				Adjusted[a]			
	OR	95% CI		*p*-Value	OR	95% CI		*p*-Value
MeP	1.00	0.999	1.002	0.335	1.00	0.999	1.002	0.693
EtP	1.01	0.996	1.018	0.219	1.01	0.995	1.019	0.281
nPrP	1.00	0.998	1.005	0.312	1.00	0.997	1.004	0.646
iBuP	1.04	0.974	1.109	0.242	1.05	0.975	1.134	0.191
nBuP	1.00	0.975	1.026	0.996	1.00	0.978	1.019	0.864

(D)

	Girls (n = 140)							
	Crude				Adjusted[a]			
	OR	95% CI		*p*-Value	OR	95% CI		*p*-Value
MeP	1.00	0.990	1.004	0.406	1.00	0.989	1.003	0.287
EtP	1.00	0.973	1.025	0.916	0.99	0.954	1.019	0.399
nPrP	1.00	0.990	1.010	0.978	0.99	0.983	1.007	0.387
iBuP	0.27	0.031	2.396	0.237	0.33	0.038	2.803	0.302
nBuP	0.96	0.844	1.081	0.468	0.95	0.843	1.077	0.435

CI – confidence interval, OR – odds ratio.
[a] Logistic regression model adjusted for smoking during pregnancy (as urine cotinine levels), urine creatinine levels during pregnancy, number of siblings, maternal age at delivery, parental school education, family history of atopy (except B) and sex (except C and D). For specific case numbers and paraben concentrations see Supplementary Table E2.

3.4. Effects of multiple paraben exposure on asthma development in children within the first 10 years

Coherent with the murine experiments, we also considered a multiple exposure situation within the epidemiological data via Bayesian kernel machine regression (BKMR; (Bobb et al., 2018; Junge et al., 2021)). Interestingly, also the BKMR model did not outline an adverse effect of exposure to multiple parabens and the asthma risk in children (shown for the total sub-cohort/in children with FHA+, Fig. 3), and separately for both sexes

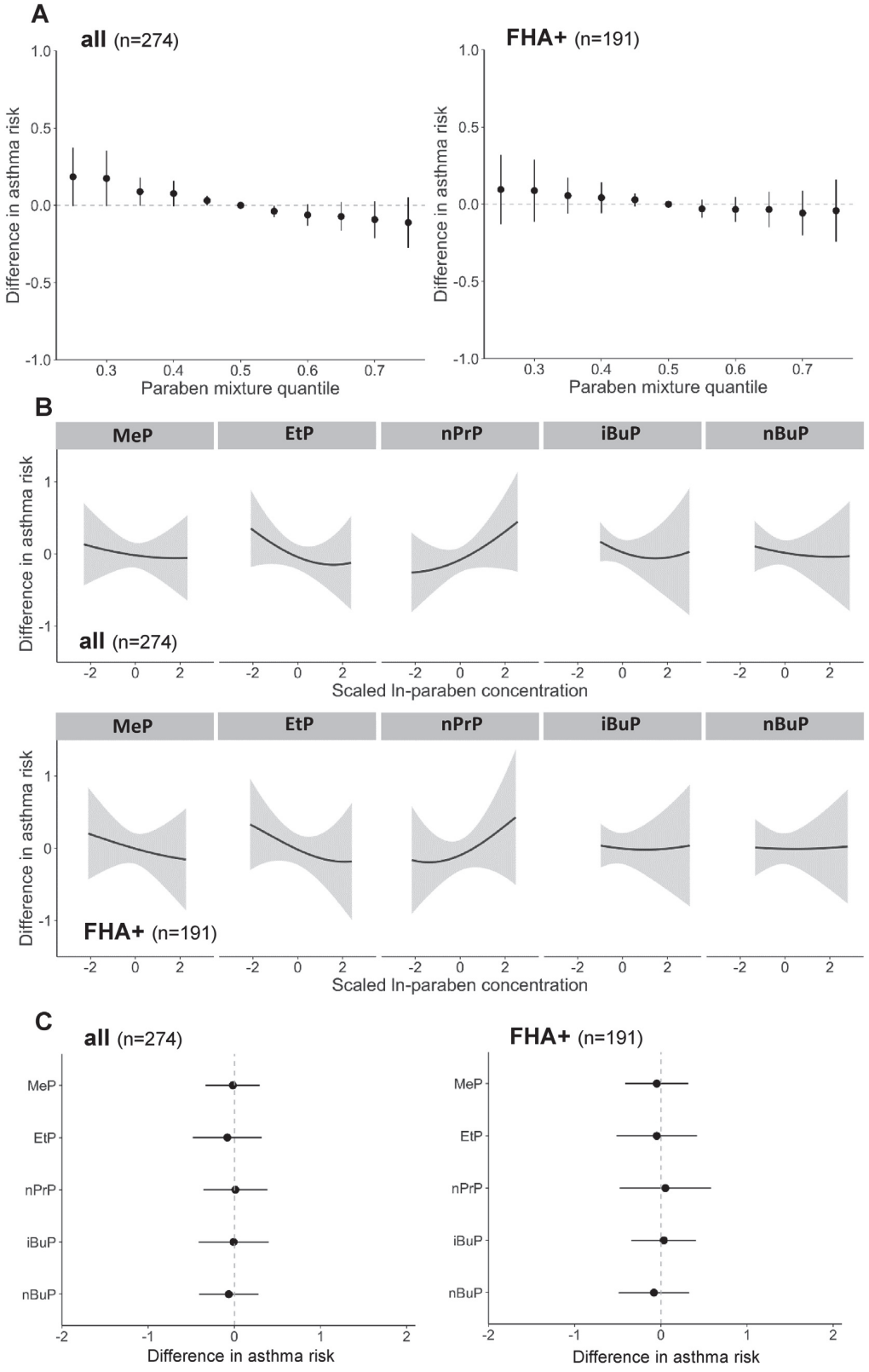

Fig. 3. Mixture analyses of prenatal paraben exposure and children's asthma risk within the first 10 years of life. Displayed are the effects for all children and for those with positive family history of atopy (FHA+) analysed by BKMR and adjusted for sex, urine cotinine during pregnancy, number of siblings, parental school education, maternal age at delivery, urine creatinine during pregnancy and FHA (except for the FHA+ sub-cohort). Shown are estimated differences in asthma risk and 95% credible intervals via (A) overall/cumulative analyses, (B) univariate exposure-response function, (C) interaction analyses.

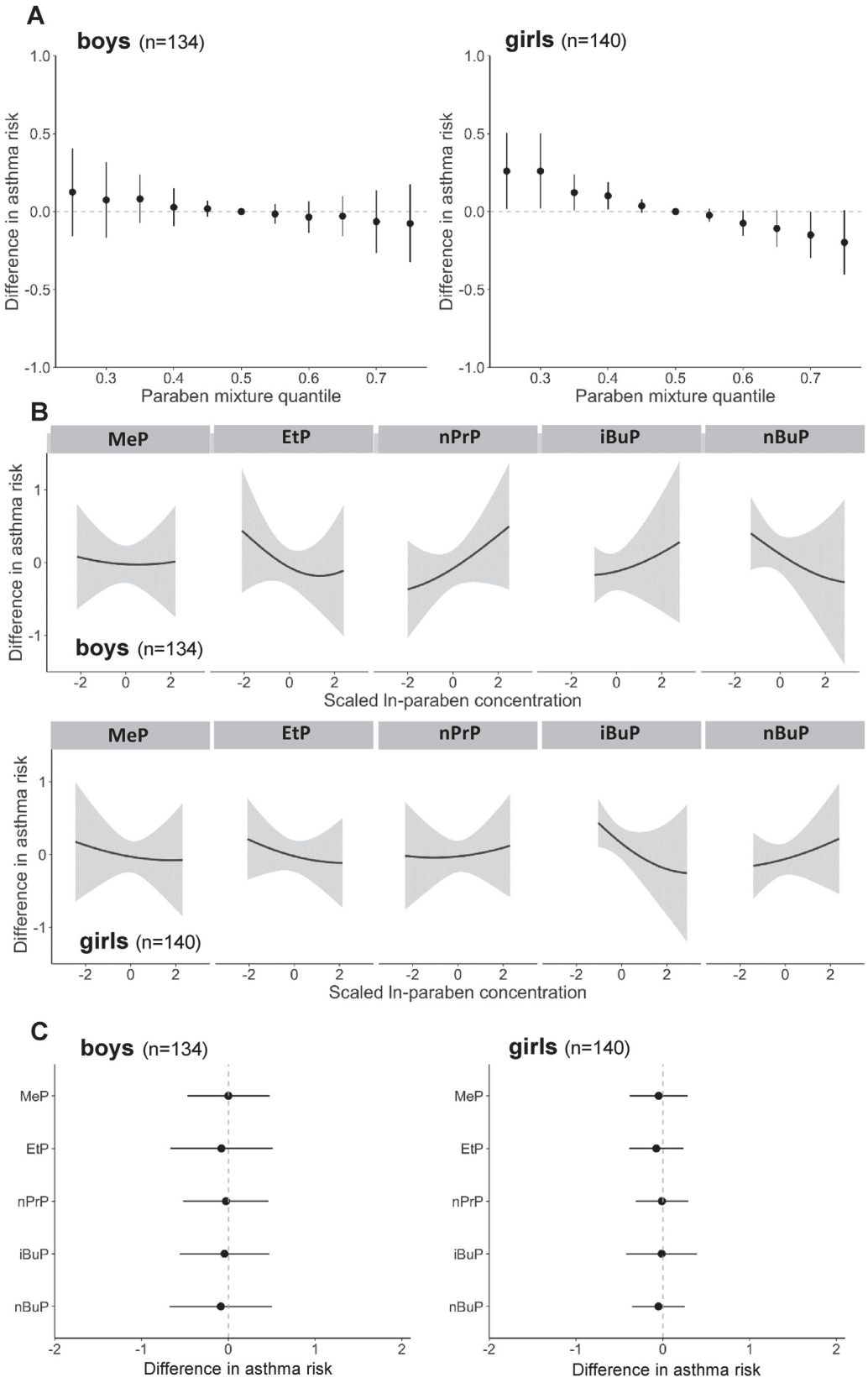

Fig. 4. Sex-specific analyses of prenatal paraben exposure and children's asthma risk within the first 10 years of life. Displayed are effects for boys/girls analysed by BKMR adjusted for urine cotinine during pregnancy, number of siblings, parental school education, and maternal age at delivery, urine creatinine during pregnancy, and FHA. Shown are estimated differences in asthma risk and 95% credible intervals via (A) overall/cumulative analyses, (B) univariate exposure-response function, (C) interaction analyses.

(Fig. 4). Furthermore, in girls this association was rather inverse when interpreting the cumulative simulation (Fig. 4A).

4. Discussion

Within this current study, experimental mouse and cohort data were assessed regarding single and multiple paraben exposure and asthma development which has not been performed earlier neither in a mouse nor in a translational setting. First, we investigated the impact of parabens in a mouse asthma model using different exposure protocols. Exposure of mice to EtP and nBuP that was estimated from an acceptable daily intake in humans based on NOAEL led to urine paraben levels in a comparable range to those detected in highly exposed mothers of our cohort. Still, it needs to be considered that humans are facing additional exposure routes (e.g. via ingestion or the lung) compared to the solely exposure via skin in our mouse model that could lead to differences in toxicokinetics (Prah et al., 2004).

Overall, our murine experimental data for single paraben exposure show both, an asthma-reducing effect after direct exposure of adult mice to nBuP as well as an asthma-promoting effect after maternal exposure to EtP in the female offspring. This effect was not observed in the male offspring demonstrating sex-specific health effects of parabens that we (Leppert et al., 2020) and others have shown previously for chemicals with endocrine disrupting activity (Chang et al., 2022; Palanza et al., 2021). The asthma-promoting effect in the offspring after maternal exposure to EtP was in contrast to most of the recent epidemiological studies that demonstrated no considerable correlation between any single paraben exposure and asthma risk (Lee-Sarwar et al., 2018; Spanier et al., 2014; Vindenes et al., 2021). However, one study showed a specific association between maternal single EtP levels and an increased asthma rate in 5-year-old children (Vernet et al., 2017) while no effects were seen for other parabens. Another study demonstrated a decreased asthma risk in association with PrP levels (Berger et al., 2018), similar to the result we observed for nBuP in our mouse asthma model. These contradictory findings of parabens might be explained by their chemical structure and different chain length. In this context, it was shown before that PrP and nBuP have a higher estrogenic activity in comparison to EtP (Routledge et al., 1998). However, the underlying mechanisms why parabens can induce different health effects have to be further explored.

Based on our observations we hypothesized that the diverse effects of EtP and nBuP might be different in a setting more comparable to the real life situation with multiple and long lasting or chronic exposure scenarios. It was outlined recently that it is of high importance also to consider a multiple exposure situation, since individuals are certainly exposed to a mixture of parabens in daily life (Huhn et al., 2021). In our experimental study exposing mice over a long period (dams and their progeny into adulthood) as well as to a mixture of both parabens showed no effect on the adult offspring asthma phenotype. This finding might suggest that in the mixed application EtP and nBuP “neutralize” each other at the biological effect level, in particular when exposure is long lasting.

With respect to our epidemiological data our findings were comparable to other cohort studies (Lee-Sarwar et al., 2018; Spanier et al., 2014; Vindenes et al., 2021): our logistic regression models – mimicking prenatal exposure to a single paraben - revealed neither effects in the analysed total sub-cohort nor when stratified for children being at risk due to a positive family history of atopy or when analysed separately for boys and girls. Furthermore, the associations in Leppert et al. (Leppert et al., 2020) showed that pregnant mothers indeed are facing a multiple paraben exposure. To address this multiple exposure scenario also in the current context, we have used a mixture approach within our epidemiological data. Bayesian kernel machine regression (BKMR) was applied for the LINA data as described earlier (Bobb et al., 2018; Junge et al., 2021) because classical regression models do not consider potential dynamic and kinetic interactions between several parabens (Bobb et al., 2018). This statistic modelling approach adds important information on just considering raw sum associations via logistic regression models (Vernet et al., 2017) by mimicking different interacting mixture scenarios. Herein, paraben combinations did not indicate an adverse effect of prenatal paraben exposure on children's asthma risk. Moreover, in girls this association was rather inverse when interpreting the cumulative simulation. Still, this was based only on 10 female asthma cases and, therefore, should be deeper addressed in further studies. This potential mixture effect could explain why there is only little evidence from existing human epidemiological studies linking single paraben exposure and asthma risk. However, the exposure situation in cohorts can vary widely depending on the individual lifestyle but also on population- or country-specific habits (WHO, 2009) making it difficult to transfer our findings to different human environmental conditions. In addition, the recently shown adverse effect of paraben exposure on eczema risk (Thurmann et al., 2021) needs to be mentioned. Nevertheless, eczema occurs earlier in life than asthma with a potentially stronger impact of prenatal exposure. For asthma, the period after birth may play a more important role. A different or longer usage of paraben-containing products in older children may have caused different disease effects depending on the parabens exposed.

A general limitation of the LINA study is the potential bias by high rates of participating atopic parents who may have had special interest in the topic of the study. We have considered this point by always including the parental history of atopy as a confounding variable in the overall regression and BKMR models. In addition, the outcome diagnosis (asthma, as well as obstructive bronchitis or wheezing in controls) is a parental reported doctor's diagnosis that may carry some minor inaccuracies. With respect to the paraben analyses, it needs to be outlined that there are only one-time spot urine samples available in LINA. However, it was shown earlier within this cohort, that high maternal one-time urine concentrations of the analysed parabens were related to mothers usage of leave-on cosmetic products during the entire pregnancy (Leppert et al., 2020). This shows on one hand a potential source of the preservatives, and on the other hand that the measure of parabens at only one single time point could be a valid surrogate for an overall paraben exposure during pregnancy.

5. Conclusion

We investigated the effect of parabens on allergic airway inflammation after exposure at different time points using a mouse asthma model. We found an asthma-reducing effect after direct exposure of mice to nBuP and an asthma-promoting effect in the female offspring after maternal exposure to EtP. In our mother-child cohort LINA, we further examined single and mixture effects on asthma development in children within the first 10 years of life by logistic and Bayesian kernel machine regression. Both approaches revealed no adverse effects of parabens on children's asthma development. Interestingly, exposure of mice to a mixture of EtP and nBuP was without effect on allergic airway inflammation. To our best knowledge, this is the first study assessing the effects of paraben exposure using an experimental asthma model and in addition, combining this with BKMR to analyze joint effects of multiple parabens on children's asthma risk in a mother-child cohort. Our results clearly highlight the importance of the time point of exposure but also the role of mixtures that may not only implicate additive but also opposed effects, which should be considered in the evaluation of individuals' specific disease risk.

CRediT authorship contribution statement

Kristin Junge: Conceptualization, Methodology, Analysis, Writing - Original draft preparation, Visualization.

Lisa Buchenauer: Methodology, Analysis, Investigation, Visualization, Writing - Reviewing and Editing, Funding acquisition.

Sandra Strunz: Analysis, Investigation, Visualization, Writing- Reviewing and Editing.

Bettina Seiwert: Methodology, Analysis, Writing- Reviewing and Editing.

Loreen Thürmann: Analysis, Writing- Reviewing and Editing.

Ulrike Rolle-Kampczyk: Methodology, Writing- Reviewing and Editing.

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Tobias Polte: Conceptualization, Methodology, Analysis, Investigation, Writing- Original draft preparation, Visualization, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.152676.

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Maternal paraben exposure triggers childhood overweight development

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Parabens are preservatives widely used in consumer products including cosmetics and food. Whether low-dose paraben exposure may cause adverse health effects has been discussed controversially in recent years. Here we investigate the effect of prenatal paraben exposure on childhood overweight by combining epidemiological data from a mother–child cohort with experimental approaches. Mothers reporting the use of paraben-containing cosmetic products have elevated urinary paraben concentrations. For butyl paraben (BuP) a positive association is observed to overweight within the first eight years of life with a stronger trend in girls. Consistently, maternal BuP exposure of mice induces a higher food intake and weight gain in female offspring. The effect is accompanied by an epigenetic modification in the neuronal Pro-opiomelanocortin (POMC) enhancer 1 leading to a reduced hypothalamic POMC expression. Here we report that maternal paraben exposure may contribute to childhood overweight development by altered POMC-mediated neuronal appetite regulation.

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Childhood obesity has reached epidemic dimension in most of the developed countries and continues to increase globally. In accordance to recent reports overweight and obesity affect up to one third of children in Europe and in Northern America[1,2]. Both, life style factors such as a high caloric food intake and a predominantly sedentary behaviour, as well as genetic predisposition contribute to the risk for overweight and obesity. However, both factors alone cannot explain the fast increase in obesity rates all over the world[3]. Additional priming for overweight development by environmental factors gained increasing attention in the scientific community[4,5].

In critical time windows such as the foetal development perturbations of the physiological endocrine and metabolic signalling can result in long lasting health effects[6–8]. Synthetic chemicals interfering with the endocrine system (endocrine disrupting chemicals, EDC) represent a classical example for environmental factors contributing to programming towards overweight and obesity, in particular in the perinatal period[9]. EDCs are added to products as preservatives or plasticizers, for antimicrobial and antifungal function or as flame retardants[10,11]. Thus, exposure to EDCs is ubiquitous. These chemicals can enter the body via food and water intake, skin absorption, and inhalation[5,12]. Many of them are able to cross the blood-placenta barrier with the risk to exert their harmful properties already during prenatal development[13–15]. A group of EDCs that is suspected to interfere with ontogenesis and exert potential endocrine disrupting properties but has not yet been extensively studied in the context of overweight development are parabens.

Parabens are alkyl esters of p-hydroxybenzoic acid (PHBA) with antimicrobial and antifungal properties frequently used as preservatives in cosmetic products, toiletries, food (E214-219), and pharmaceuticals. They are found in the majority of leave-on cosmetics and in rinse-off products[16]. Parabens enter the human body mainly through ingestion or skin absorption and can commonly be detected in urine, blood and breast milk[17,18]. Paraben level vary among the different parabens, tissue and time after topical application with previously reported concentration of 0.5 $\mu g\,l^{-1}$ butyl paraben to 43.9 $\mu g\,l^{-1}$ methyl paraben in urine samples and about 4-times lower serum level[18]. The fast metabolization of parabens in the liver (half-life in humans less than 24 h) and direct urinary clearance of conjugated metabolites may explain the higher urine concentration compared to serum. Thus, as the limit of detection is comparable in the two matrices, it is more likely to detect representative paraben concentrations in urine, rather than in serum.

There is increasing evidence from epidemiological studies that parabens might be associated with breast cancer[19] and allergy development[20]. With regard to development of overweight and obesity there is only one study showing elevated urinary levels of 3,4-dihydroxybenzoic acid (3,4-DHB), a common paraben metabolite, in obese children[21]. However, the findings are not explicit because there are other sources of 3,4-DHB like plants and many fruits[21]. Furthermore, there is no study evaluating maternal exposure to parabens during pregnancy as potential risk factor for childhood overweight development.

In this study we show evidence for a positive association between maternal urinary concentrations of butyl paraben (BuP) and childhood overweight within the first eight years of life in our prospective mother-child cohort LINA. In an in vivo mouse model, we demonstrate that maternal exposure to nBuP induces an increased food intake and weight gain in female offspring. Our data provide evidence that a neuronal dysregulation of satiety could contribute to the observed gain in body weight mediated by an epigenetic silencing and reduced hypothalamic expression of the gene *proopiomelanocortin (pomc)* well-known to be involved in appetite regulation[22].

Results

Cosmetic products as source of paraben exposure. Within the LINA mother-child study 629 mother-child pairs were recruited between 2006 and 2008. General characteristics of the study participants are shown in Supplementary Table 1 with no differences compared to the analysed sub-cohort for longitudinal BMI development (year 2–8) and paraben exposure; $n = 223$. Maternal paraben exposure was assessed by urine measurements showing high exposure levels for methyl paraben and lower for butyl parabens (Supplementary Table 2). As a potential source of paraben exposure the usage of cosmetic products during pregnancy was assessed by questionnaires. Indicated cosmetic products were searched for their paraben content with the TOXFOX app (described in Methods) and categorised in leave-on and rinse-off products. Since exposure time is obviously very short for rinse-off products and TOXFOX indicated only 31 out of 414 rinse-off products as paraben containing (Supplementary Fig. 1), only leave-on products with a high exposure time and high body area coverage were considered for comparison with urine measurements. Valid data on the application of paraben containing or paraben-free leave-on products during pregnancy was available from 414 participants. Indeed, 26% used at least one cosmetic leave-on product that contained parabens, whereas 56% used paraben-free leave-on products only (Supplementary Fig. 1).

When comparing measured urinary paraben concentrations, mothers that used paraben containing leave-on products had up to 3.0-fold higher concentrations for methyl paraben (MeP), ethyl paraben (EtP), n-propyl paraben (nPrP), and n-butyl paraben (nBuP) compared to mothers using paraben free leave-on products, as shown in Table 1. Thus, the usage of paraben containing leave-on products can be considered as one potential source for maternal paraben exposure.

Prenatal paraben exposure and weight development in LINA. Further, we defined overweight children using age and sex specific cut-offs from the International Obesity Task Force (IOTF).

Table 1 Maternal urinary paraben concentrations [µg/l] in regard to cosmetic product application (A) usage of leave-on products during pregnancy without parabens ($n = 276$) or (B) usage of leave-on products during pregnancy containing parabens ($n = 138$).

	(A)	Median	<25%	>75%	(B)	Median	<25%	>75%	*P*-value
MeP		28.05	6.62	133.20		68.80	17.00	167.20	0.0018
EtP		1.89	0.51	9.88		2.90	0.79	18.10	0.0466
nPrP		3.20	0.72	14.60		7.40	1.50	25.80	0.0025
iBuP		0.10	0.05	0.70		0.20	0.05	0.94	0.1875
nBuP		0.41	0.10	2.70		1.24	0.30	4.55	0.0004

Shown are median, as well as first (<25%) and fourth quartile (>75%), *P*-values are derived from Mann–Whitney-*U*-test

Table 2 Prenatal paraben exposure and weight development of children.

	birth	OR	95% CI	*P*-value	2–8 years	OR	95% CI	*P*-value
MeP		1.18	0.63–2.23	0.606		1.15	0.58–2.28	0.691
EtP		1.39	0.67–2.88	0.382		1.00	0.49–2.03	0.991
nPrP		0.86	0.44–1.69	0.671		1.04	0.53–2.03	0.920
iBuP		1.61	0.81–3.18	0.175		2.40	1.16–4.98	0.018
nBuP		1.45	0.73–2.89	0.293		2.17	1.06–4.47	0.035

Shown are odds ratios (OR) for macrosomia at birth ($n = 496$) and ever overweight development in early to mid childhood (age 2–8 years, $n = 223$) with 95% confidence intervals (95% CI) derived from logistic regression models adjusted for the sex of the child, smoking during pregnancy, parental school education, gestational week at delivery, existence of siblings, breast-feeding duration (not for models with only birth weight as outcome) and age of the mother at birth. OR are shown for high (3rd tertile) paraben exposure as compared to low (1st tertile) exposure in pregnancy

Table 3 Longitudinal effect of prenatal paraben exposure on weight development in childhood (1–8 years).

	Tertile	All: beta	95% CI	*P*-value	Female: beta	95% CI	*P*-value	Male: beta	95% CI	*P*-value
MeP	2	−0.10	−0.36, 0.16	0.436	−0.06	−0.44, 0.33	0.77	−0.19	−0.53, 0.15	0.265
	3	−0.04	−0.30, 0.22	0.758	−0.03	−0.42, 0.35	0.87	−0.02	−0.35, 0.31	0.901
EtP	2	0.18	−0.08, 0.44	0.183	0.17	−0.26, 0.56	0.404	0.21	−0.14, 0.56	0.246
	3	−0.01	−0.27, 0.25	0.944	0.05	−0.36, 0.46	0.852	−0.02	−0.34, 0.31	0.924
nPrP	2	0.08	−0.18, 0.34	0.555	0.24	−0.14, 0.62	0.216	−0.11	−0.46, 0.25	0.555
	3	−0.04	−0.30, 0.21	0.729	0.08	−0.21, 0.47	0.682	−0.11	−0.43, 0.22	0.515
iBuP	2	0.35	0.08, 0.62	0.011	0.46	0.07, 0.85	0.021	0.26	−0.10, 0.62	0.162
	3	0.26	0.02, 0.05	0.035	0.53	0.16, 0.89	0.005	0.05	−0.26, 0.36	0.752
nBuP	2	0.13	−0.12, 0.39	0.342	0.21	−0.17, 0.59	0.275	0.07	−0.28, 0.42	0.706
	3	0.21	−0.04, 0.47	0.095	0.36	−0.03, 0.74	0.069	0.07	−0.26, 0.40	0.672

Shown are changes in the intercept as betas with 95% confidence intervals (CI) for a change of BMI per tertile increase in paraben exposure for all children ($n = 392$), female only ($n = 193$) and male only ($n = 199$) derived from an adjusted GEE model with fitted cubic splines at ages 12, 25, 38, 61, 98 moths and exchangeable correlation matrix. Models have been adjusted for sex of the child, smoking during pregnancy, parental school education, gestational week at delivery, existence of siblings, breast-feeding duration and age of the mother at birth

Adjusted logistic regression models were applied to study a potential risk increase resulting from prenatal paraben exposure comparing weight of children at birth (>4000 g or macrosomia vs. <4000 g) and afterwards (overweight vs. non-overweight children; Table 2). Since reference data are not available for one-year-old children, only the time window between age two and eight could be considered for this analysis. While high exposure to parabens did not affect the risk for macrosomia at birth, there was evidence for the long chain parabens iBuP and nBuP to increase the risk for ever overweight in early to mid-childhood (Table 2).

To investigate the impact of prenatal paraben exposure on longitudinal children's weight development we applied GEE analysis on BMI-data from age 1–8 years. We found evidence for an early manifestation of differences between prenatally low (1st tertile) and high (3rd tertile) exposed children for iBuP and nBuP (intercept: 0.26 kg m^{-2}, 95% CI: 0.02,0.05; 0.21 kg m^{-2}, 95% CI: −0.04,0.47, respectively) and no evidence for MeP, EtP and nPrP (Table 3). Thereby, the BuP (both iBuP and nBuP) related BMI increase was more evident in girls compared to boys and effects hold true after adjustment for confounders. There was no evidence for a change of the slope between exposure groups over time.

Impact of paraben exposure on adipocyte development in vitro. Parabens have been shown to promote adipocyte differentiation in mouse fibroblasts[23]. To evaluate a potential direct effect of parabens on human adipocyte differentiation an established human mesenchymal stem cell differentiation assay was applied[9]. Adipocyte differentiation, assessed by the cell index values of impedance-based real-time monitoring and by the amount of triglyceride storage, was not affected by nBuP exposure (Fig. 1a–c) nor by the other parabens measured in LINA (Supplementary Fig. 2A, B). Furthermore, gene expression of the transcription factor *PPARG* showed no differences in nBuP-treated cells compared to control (Fig. 1d). Looking closer into PPARγ regulation, we found no evidence for PPARγ activation by nBuP in an artificial reporter gene assay (Supplementary Table 3). Moreover, nBuP exposure did not activate the androgen, progesterone and glucocorticoid receptor but exerted a strong impact on oestrogen receptor-α (ER-α) activity (Supplementary Table 3). Interestingly and in contrast to the other results, *leptin (LEP)* expression in adipocytes was downregulated by nBuP with a significant effect even at 0.5 µM. For validation of these findings the secretion of adiponectin and leptin into the cell culture supernatant was assessed. Also decreased levels of secreted leptin were approved after exposure to nBuP, with a significantly effect at 10 µM. Secreted adiponectin levels significantly increased after exposure to nBuP in a concentration-dependent manner (Fig. 1e). Paraben exposure at the used concentrations had no effect on cell viability (Supplementary Fig. 2C).

Furthermore, using adipocytes differentiated from primary mouse mesenchymal stem cells we observed comparable results to the human in vitro analyses showing no direct effect of nBuP exposure on adipogenesis (Supplementary Fig. 3A–C).

Maternal nBuP increased weight in offspring of female mice. To further investigate a potential causal relationship between an increased risk for childhood overweight and maternal exposure to certain parabens, we exposed Balb/c mice during gravity and the breast-feeding period to BuP and measured offspring's body weight and several metabolic parameters. Due to its higher human exposure (see Supplementary Table 2) nBuP (1.75 µg twice per week s.c.) was used in the experimental setup. nBuP urine concentration was measured in exposed mice and was found to be in a comparable range as observed in the LINA study (highest tertile urine concentration LINA mothers: 18.5 µg l^{-1}; mean urine concentration in exposed mice: 19.6 µg l^{-1}).

Female offspring from perinatally nBuP-exposed dams showed a significantly higher weight than control animals over the entire observation period with a weight increase of 20 to 45% compared to 10% in the controls (GEE estimate: 3 g higher body weight in the exposed group; SE: 0.5 g, $p = 5.3 \times 10^{-9}$, Fig. 2a). The elevated body

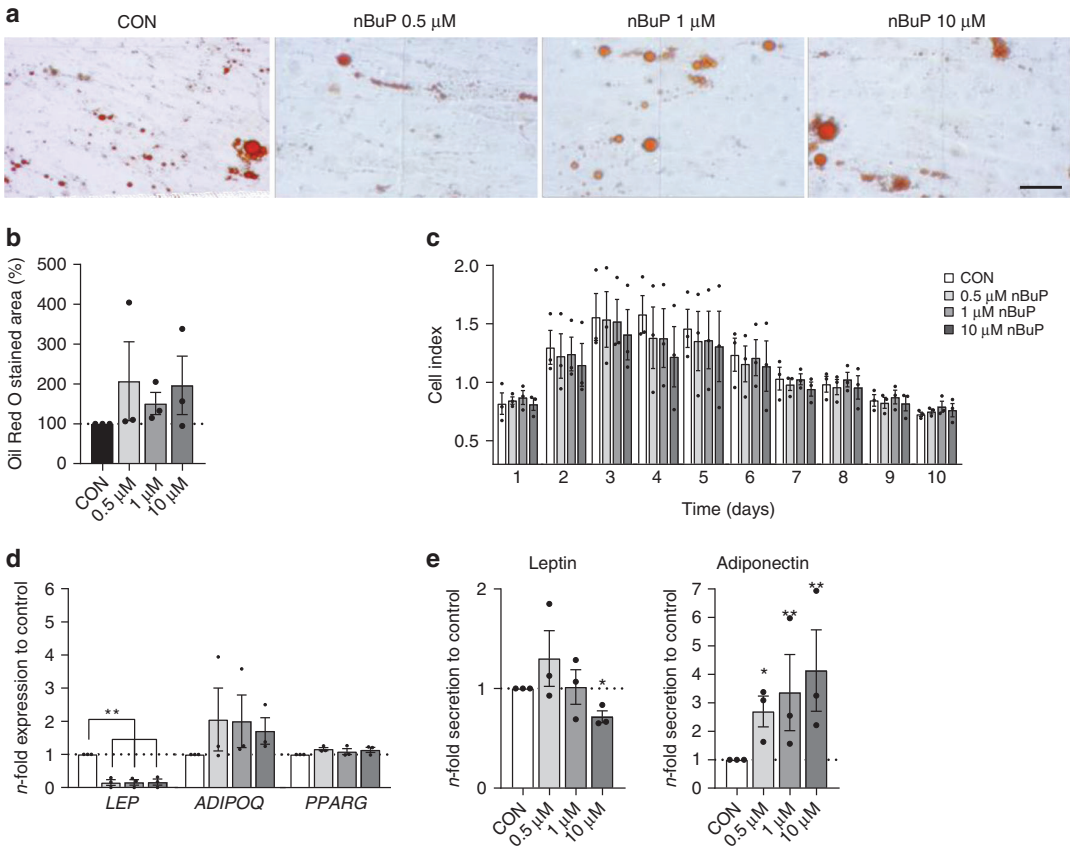

Fig. 1 Effect of nBuP exposure on adipocyte differentiation. In vitro adipocyte differentiation from human MSCs in the presence of nBuP. a Representative Oil Red O stained pictures after differentiation (scale bar: 100 µm). **b** Triglyceride storage of adipocytes assessed via Oil Red O staining. **c** Real-time monitoring of cell differentiation (xCELLigence: normalised cell index) over a 17-day period. **d** Gene expression of *leptin (LEP), adiponectin (ADIPOQ)* and the transcription factors *peroxisome proliferator-activated receptor gamma (PPARG)*. **e** Leptin and adiponectin levels in cell culture supernatant. Data are expressed as mean ± SEM of $n = 3$ experiments. Significant differences were derived by ANOVA with $^{*}P < 0.05$ and $^{**}P < 0.01$. Source data are provided as a Source Data file.

weight became manifest shortly after birth and was linked to a higher fat and a lower lean mass as measured by whole body composition analysis using nuclear magnetic resonance technology (Fig. 2b). In contrast, weight development of male offspring was not affected by perinatal nBuP exposure (GEE estimate: 0.5 g higher body weight in the exposed group, SE: 0.6 g, $p = 0.34$, Fig. 2a, b). Furthermore, female mice from nBuP-exposed dams showed an increase in weekly food intake over the observation period compared to control mice (GEE estimate: 0.5 g additional food intake per week in the exposure group; SE: 0.2 g, $p = 0.006$; Fig. 2c). In addition, while the fasting serum glucose levels were elevated in the female offspring of nBuP-exposed dams, we did not detect impaired glucose and insulin tolerances (Fig. 2d, e). The male progeny showed no differences in glucose and insulin levels compared to the control mice from unexposed dams (Fig. 2d, e). Further, nBuP exposure to female adult mice had no effect on weight, food intake and leptin serum levels (Supplementary Fig. 4).

Within the female F1 generation, elevated body weight of mice was associated with an increased size of adipocytes (Fig. 3a, b). Gene expression analysis revealed no changes for the *glycose transporter 4 (glut4)*, the *insulin receptor (insr)* and *pparg* in adipose tissue of female offspring from nBuP-exposed dams compared to control animals (Fig. 3c). Furthermore, while serum leptin levels were elevated in the offspring from nBuP-exposed dams, the concentrations of adiponectin, resistin, ghrelin, and insulin were not affected compared to control animals (Fig. 3d). In addition, maternal nBuP exposure did not influence 17β estradiol levels in female and male offspring (Supplementary Fig. 5).

Maternal nBuP reduced POMC expression via nPE1 methylation. The results from the human adipocyte differentiation assay revealed no conclusive impact of nBuP exposure on adipogenesis. Moreover, the female offspring of nBuP-exposed dams released leptin as expected as a result of a higher weight gain. Accordingly, we next focused on gene analyses important for the central regulation of food intake in the hypothalamus. Expression of the *leptin receptor (lepr)* mRNA was downregulated in female offspring of nBuP-exposed dams (Fig. 4a) suggesting a potentially impaired leptin signalling. This finding was supported by a very low expression of *pomc* mRNA in female offspring compared to the progeny from non-exposed mice (Fig. 4a). The mRNAs of the *melanocortin type 4 receptor (mc4r)*, a target for the POMC cleavage product alpha-melanocyte-stimulating hormone (α-MSH), the *agouti-related neuropeptide gene (agrp)* and the *insulin receptor X (insr)* were unaffected in the overweight mice (Fig. 4a).

Because environmental factors influencing ontogenesis that may affect the disease risk later in life have been shown to exert their effects via the induction of epigenetic changes such as DNA methylation[24,25], we investigated whether the paraben-driven *pomc* downregulation in female offspring is due to nBuP-induced alterations in DNA methylation of regulatory regions (nPE1,

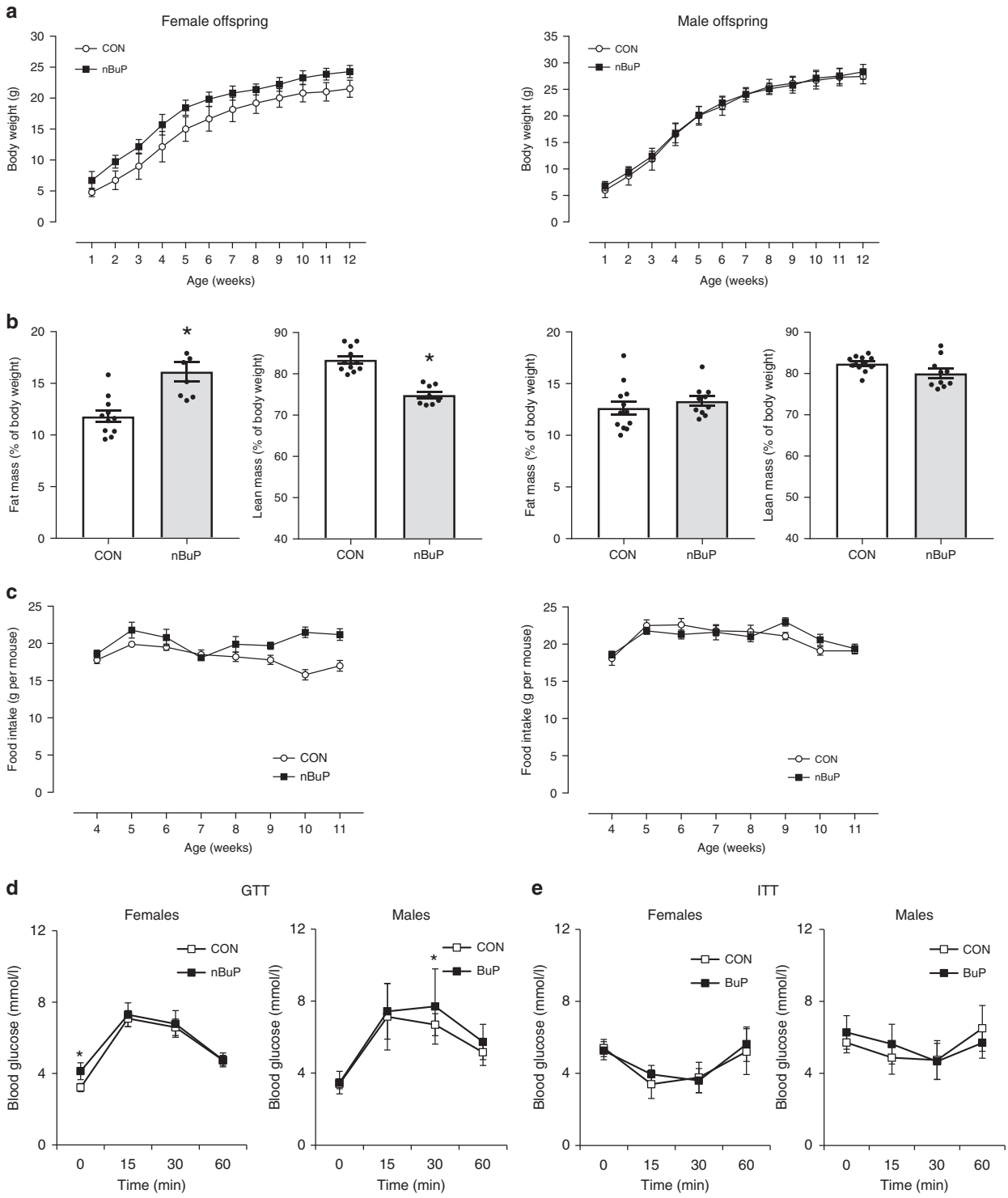

Fig. 2 Perinatal exposure to nBuP and weight development, food intake and glycose metabolism in the offspring. a Bodyweight development, (CON: $n = 18$, nBuP: $n = 16$), **b** body composition (CON: $n = 11$, nBuP: $n = 9$) and **c** food intake (CON: $n = 10$, nBuP: $n = 6$) are shown from female (left) and male offspring (right) from nBuP-exposed dams, **d** Glucose tolerance test (GTT, CON: $n = 18$, nBuP: $n = 10$) and **e** insulin tolerance test (ITT, CON: $n = 15$, nBuP: $n = 10$) were performed in 9-weeks old offspring. Data are expressed as mean ± SEM, $^*P < 0.05$, unpaired t-test. For longitudinal analysis GEE models were applied (**a** female mice $\beta = 3$ g, SE = 0.5 g, $p = 5.3 \times 10^{-9}$, male mice $\beta = 0.5$ g, SE: 0.6 g, $p = 0.34$; **c** female mice $\beta = 0.5$ g per week SE: 0.2 g, $p = 0.006$, male mice $\beta = 0.25$ g per week, SE = 0.18, $p = 0.17$). Source data are provided as a Source Data file.

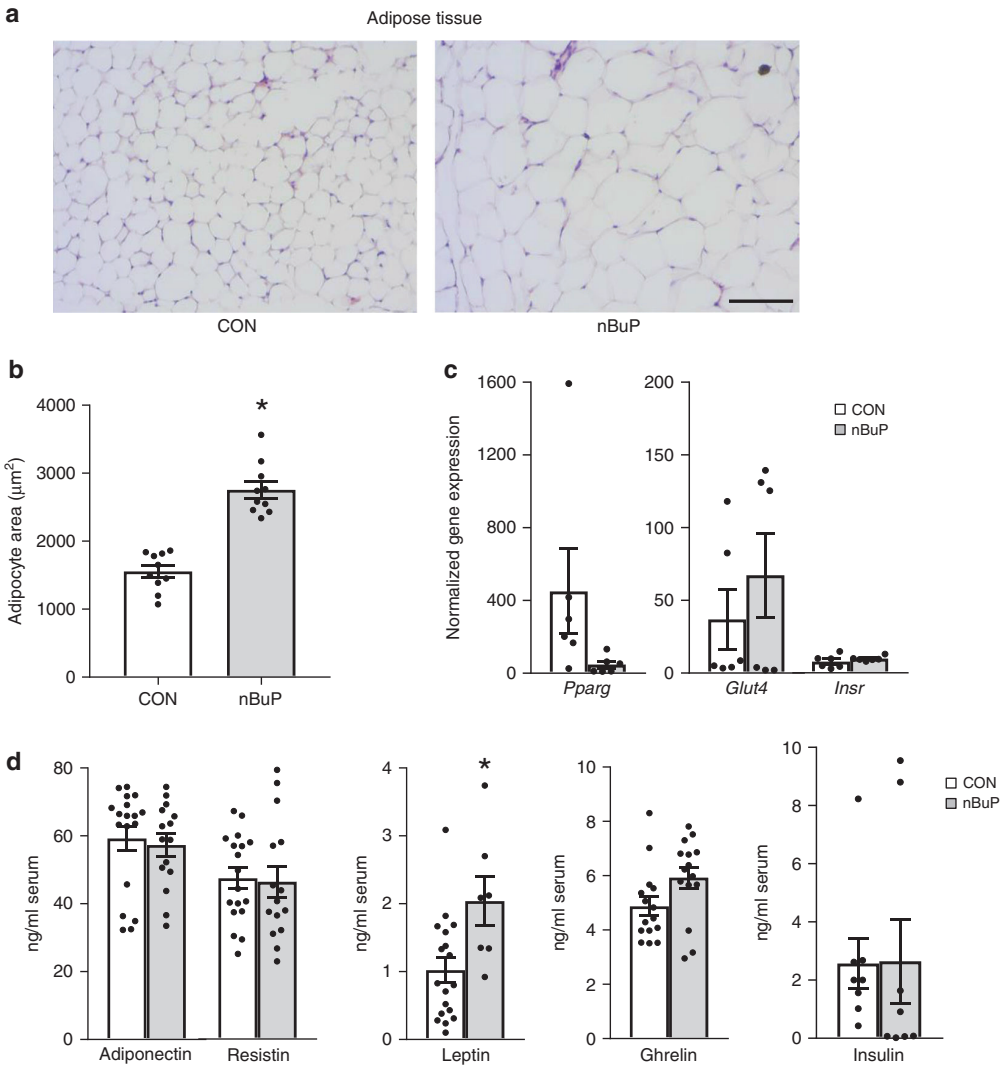

Fig. 3 Perinatal nBuP exposure, adipocyte area and gene and protein expression of key genes in the offspring. a Representative picture of stained slices (H&E, ×20, scale bar: 100 µm) of visceral adipose tissue and (**b**) the illustration of the adipocyte area from female offspring of nBuP-exposed dams ($n = 10$), (**c**) The mRNA expression levels of selected target genes investigated in adipose tissue of female offspring ($n = 6$), (**d**) Levels of adiponectin and resistin (CON: $n = 18$, nBuP: $n = 15$), leptin (CON: $n = 18$, nBuP: $n = 7$), ghrelin ($n = 15$) and insulin ($n = 8$) measured in serum of female offspring. Data are expressed as mean ± SEM, $^*P < 0.05$, unpaired *t*-test. Source data are provided as a Source Data file.

nPE2, Supplementary Fig. 6) of the *pomc* gene. We detected an increased DNA methylation of nPE1 (Fig. 4b) while we did not observe any methylation changes in promoter and nPE2 regions (Supplementary Fig. 7). Furthermore, the hypermethylated nPE1 and reduced *pomc* mRNA expression was already detectable in the offspring from nBuP-exposed dams directly after weaning (Fig. 4c).

To evaluate whether the nBuP-induced hypermethylation is linked to overweight development in the offspring, one-week-old pups from nBuP-exposed dams were treated with the DNA methyltransferase inhibitor 5-Aza-2'-deoxycytidine (Aza) for two weeks until weaning[26]. Treatment of the offspring with Aza reduced the body weight and the food intake caused by maternal nBuP exposure (Fig. 4d), as well as adipocyte area, and leptin serum levels and restored *lepr* expression in the hypothalamus (Supplementary Fig. 8). Moreover, the paraben-induced nPE1 hypermethylation and the diminished hypothalamic *pomc* expression in the offspring from nBuP-exposed dams were reversed in the presence of Aza (Fig. 4e, f), while nPE1 methylation and *pomc* mRNA expression in the control group were reduced.

Discussion

In this study a complex translational research design was applied to study the potential impact of maternal paraben exposure on children's weight development. Findings from the prospective birth cohort study LINA were used to establish hypothesis driven mechanistic studies in human and mouse in vitro models. Based on these results a mouse in vivo experiment provided the final proof-of-concept and deeper mechanistic insight (Fig. 5).

Evidence from our LINA cohort study indicates that maternal exposure to butyl paraben (BuP) may trigger overweight development in early childhood. Using a mouse model, we verified these epidemiological data and provide a mechanistic explanation by demonstrating that prenatal exposure to nBuP induced an

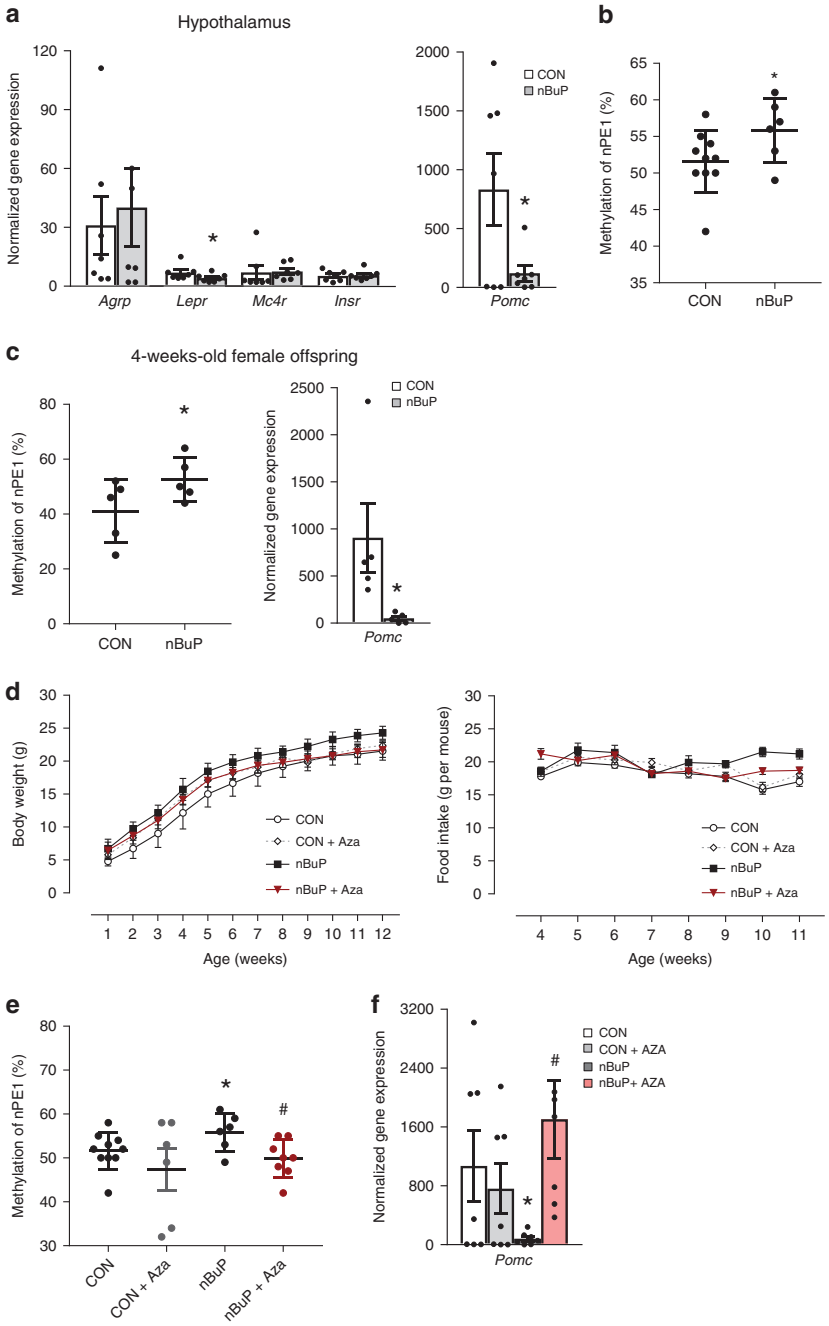

Fig. 4 Perinatal nBuP exposure reduced *pro-opiomelanocortin (pomc)* expression and induced a DNA hypermethylation of nPE1. a Expression levels of genes important for the neuronal regulation of satiety and hunger ($n = 7$) and (**b**) DNA methylation of the neuronal POMC enhancer nPE1 (chr12:3941750-3942350, CON: $n = 10$, nBuP: $n = 6$) were analysed in the hypothalamus of the 12-week-old female offspring. Values for DNA methylation in both groups are pictured for CpG1 (cg3942264). **c** DNA Methylation of nPE1 and *pomc* gene expression from the 4-weeks-old female offspring of nBuP-exposed dams are shown ($n = 5$). **d** After treatment of F1 mice with the DNA methyl-transferase inhibitor Aza body weight development (CON: $n = 18$, CON + Aza: $n = 12$, nBuP: $n = 16$, nBuP + Aza: $n = 8$) and food intake (CON: $n = 10$, CON + Aza: $n = 9$, nBuP: $n = 6$, nBuP + Aza: $n = 8$) were evaluated. **e** nPE1 methylation (CON: $n = 10$, CON + Aza: $n = 6$, nBuP: $n = 6$, nBuP + Aza: $n = 8$) and (**f**) *pomc* gene expression ($n = 7$) was measured in Aza-treated female offspring from nBuP-exposed dams. Data are expressed as mean ± SEM, $^{*}P < 0.05$ for nBuP vs. CON, $^{\#}P < 0.05$ for nBuP + Aza vs. nBuP, Student's unpaired *t*-test. For longitudinal analysis (**d**) GEE models were applied (see Supplementary Table 5). Source data are provided as a Source Data file.

increased food intake and weight gain in female offspring. Our data indicate that a neuronal dysregulation of food intake could contribute to the observed effect since we could show an epigenetic silencing and reduced hypothalamic expression of the gene *proopiomelanocortin (pomc)* known to be strongly associated with food intake regulation.

Maternal urine samples at 34th week of gestation contained detectable paraben concentrations, supporting the hypothesis of

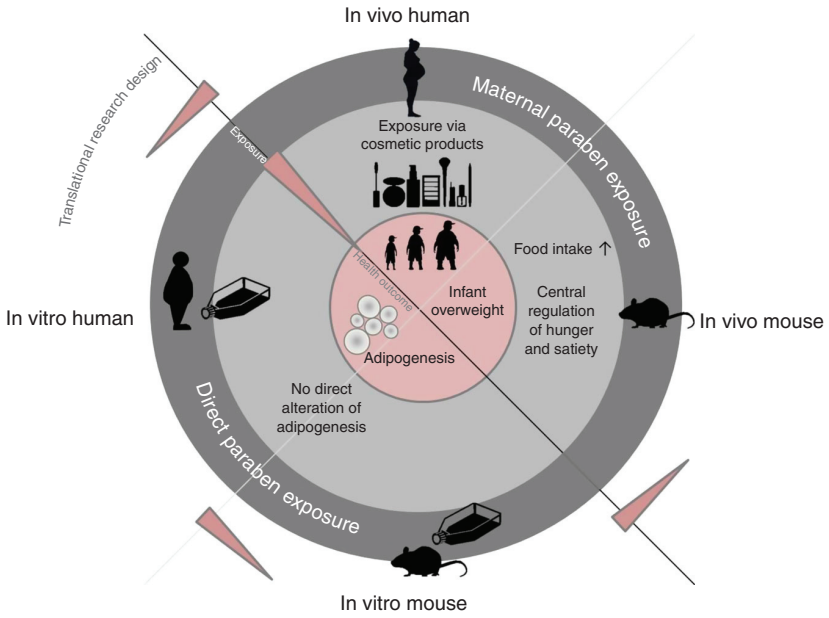

Fig. 5 Translational research design. Summary scheme of the present investigations describing the complex translational research design of the study. Findings from the prospective birth cohort study LINA were used to establish hypothesis driven approaches in human and mouse in vitro studies. Based on these results overall mechanistic analyses were addressed in the final mouse in vivo settings.

ubiquitous human exposure. As parabens are frequently used in cosmetic products, it seems reasonable that cosmetics are a principal source for human paraben exposure. This assumption could be supported by linking the questionnaire data on the usage of cosmetics of the mothers with urine paraben measurements within the LINA cohort. Mothers that used paraben-containing cosmetic leave-on products on a daily basis had significantly higher urinary paraben concentrations, although the actual number of paraben-containing products used during pregnancy might be underestimated due to limited data on cosmetic product composition from the LINA recruitment period between 2006 and 2008. However, despite the significant link between the usage of leave-on cosmetics and urinary paraben concentrations, we cannot exclude that further sources in addition to cosmetics have contributed to increased maternal urine concentrations. For the actual exposure situation it has to be considered that e.g., the Commission of the European Union has directed a maximum concentration for particular preservatives in cosmetic products in 2014[27]. Thus, current exposure levels might be lower compared to the LINA recruitment period.

Data from the LINA study provide some evidence that high prenatal paraben butyl paraben exposure can contribute to an increased risk of being overweight in early to mid-childhood. Stratified for sex, this effect seems to be stronger in girls compared to boys. Interestingly, effects for short chain parabens could not be confirmed in association with children's weight development.

To verify a potentially direct effect of maternal nBuP exposure on weight development and metabolism we applied an appropriate mouse model[9]. The dose of paraben exposure were estimated from a daily intake of BuP in humans of 20 µg kg^{-1} body weight based on a No Observed Adverse Effect Level (NOAEL, 2 mg kg^{-1} body weight) reported in a previous study[28,29]. This dose corresponds to a weekly BuP uptake of 3.5 µg in our mice experiments. The resulting urinary BuP levels in the mouse model were similar to those observed in highly exposed mothers of the LINA cohort suggesting that BuP concentrations investigated in the mouse model are representative for the real exposure situation in humans. Our results demonstrated that female but not male offspring of exposed dams showed higher weight over the entire observation period.

To identify the underlying mechanisms of the nBuP-induced weight gain we investigated a possible effect on adipocyte differentiation using a human and a murine mesenchymal stem cell assay. We have successful applied this model earlier to test adipogenic effects of bisphenol A[9]. Here, we chose paraben concentrations based on previous publications[23] and the assumption that 0.5 µM (the lowest concentration used) are approximately equivalent to the highest paraben concentrations found in LINA for MeP. Interestingly, our results did not indicate that nBuP up to a concentration of 10 µM has a direct impact on adipogenesis. This was in contrast to previous in vitro studies demonstrating an increasing effect of nBuP exposure on adipocyte differentiation as revealed by adipocyte morphology and lipid accumulation of mouse fibroblast cells with increasing effect size from MeP to nBuP (MeP < EtP < …..nBuP)[23,30,31]. Moreover, we did not detect an activation of PPARγ by nBuP, although other studies using mouse cell lines reported an activation of this receptor which is known to be crucial in adipogenesis[23].

Although in vitro adipocyte differentiation was unaffected in our human stem cell differentiation assay, we found adiponectin and leptin expression and secretion to be altered. In vivo, leptin activates POMC neurons to induce satiety in the hypothalamic food intake control region via α-MSH release[22]. Hence, reduced leptin signalling may lead to an inappropriate satiety signalling and increased food intake, which could then promote overweight development in children.

In our mouse model we showed that the weight gain in the offspring of exposed animals was accompanied by an increased food intake, fat mass and increased adipocyte size but no differences in glucose and insulin tolerances. In vivo, leptin is expressed in adipose tissue proportionally with fat mass[32]. As expected, an increase of serum leptin level was observed in mice with a higher fat to lean mass ratio. In accordance with our in vitro analysis there was no effect on adipocyte differentiation and gene expression of *pparg* in adipose tissue. These findings

indicate that maternal nBuP exposure may not directly affect adipogenesis leading to the observed weight gain. Therefore, looking more closely on hypothalamic food intake regulation, we found prominent genes to be differentially expressed between maternally exposed and non-exposed offspring. Gene expression of the *leptin receptor* (*lepr)* and *pomc* was downregulated due to nBuP exposure. POMC is involved in anorexic signalling pathways to suppress food intake. After stimulation, α-MSH is released from POMC by proteolysis and binds to MCR4 to induce satiety[33,34]. To shed some further light on a potential mechanism, we found a DNA hypermethylation of the neuronal enhancer nPE1 but no methylation changes in the POMC promoter and nPE2 regions. It has been recently shown that nPE1 is primarily responsible for the POMC transcriptional regulation leading to a higher body weight and food intake only after deletion of this particular enhancer e.g., in comparison to nPE2[35]. Treating pups with the DNA methyltransferase inhibitor Aza reduced the nBuP-induced increased body weight, food intake and *pomc* expression suggesting a direct link between DNA hypermethylation and the observed phenotype. Results of Aza treatment experiments have always to be interpreted with caution since the Aza treatment affects the entire genome. However, comparing the Aza effect in controls and nBuP exposed animals there is some evidence for an involvement of DNA hypermethylation in phenotype development. Anyhow, further investigations are needed to clarify the epigenetic alterations more specifically. We also cannot exclude that additional pathways might be involved in mediating the nBuP-induced increased weight.

With our mouse experiment, we demonstrated that the foetal development seems to be a sensitive time window for nBuP exposure with respect to body weight regulation while exposure of the adult animals did not show an effect on weight gain, food intake and serum leptin levels.

The fact that the weight gain was preferential seen in girls or the female offspring in our experimental model and that nBuP exposure affects the ER-α activity might suggest a role of the different oestrogen levels between males and females. Although oestrogen is rather associated with an increased risk to develop visceral obesity due to its reduced production in woman after menopause[36], other studies have shown that oestrogen treatment at critical early periods of development induced an obesogenic effect which was maintained until adulthood[37,38]. Nevertheless, the research is currently lacking comprehensible mechanistic explanations for the observed associations of prenatal exposure to oestrogenic compounds and an increased risk for childhood overweight.

In summary, our study results strongly suggest that prenatal exposure to nBuP increases overweight development in the offspring. The effect is mediated by an epigenetic enhancer modification leading to an altered expression of *pomc*, which plays a crucial role in controlling the neuronal regulation of food intake. Our findings do not implicate to disregard the importance of a balanced diet or sufficient exercise for weight management but call attention to the great significance of environmental exposures during pregnancy for the disease susceptibility in later life.

Methods

LiNA study design and sample collection. The German prospective birth cohort LINA (Lifestyle and environmental factors and their Influence on Newborns Allergy risk) recruited 622 mothers (629 children) at 34 weeks of gestation between May 2006 and December 2008 in Leipzig, Germany. Mothers with severe immune or infectious diseases during pregnancy were excluded from the study. Standardised self-administered questionnaires were collected annually starting in pregnancy, assessing general information about personal lifestyle, housing and environmental conditions and disease state. Study participation was voluntary and written informed consent was obtained by all participants. The study was approved by the Ethics Committee of the University of Leipzig (file ref # 046-2006, 160-2008, 160b/2008, 144-10-31052010, 113-11-18042011, 206-12-02072012, #169/13-ff, #150/14-ff). The LINA study is registered in birthcohorts.net.

Analysis of urinary paraben concentrations in human samples. Urinary concentrations of eight paraben species were determined in 504 maternal urine samples of the 34th week of gestation (reduced sample size due to available urine samples). The samples were prepared and analysed as described and validated by Schlittenbauer et al.[39]. In brief, urine samples, quality controls, and solvent blanks were thawed at room temperature, vortexed, and centrifuged. Aliquots were spiked with internal standard solution and a deconjugation standard. Hydrolysis was achieved by adding enzyme buffer solution and incubation in an ultrasonic bath. The enzyme was removed by centrifugation/filtration using Amicon® Ultra-0.5 filters. LC-MS analysis was performed on a UPLC™ system (ACQUITY I-Class, Waters Cooperation Milford, MA, USA) coupled to a triple quadrupole mass spectrometer (Xevo TQ-S, Waters Cooperation, Manchester, UK) equipped with an electrospray ionisation (ESI) source. For each analyte (MeP, EtP, iPrP, nPrP, sBuP, iBuP, nBuP, BzP), a quantification (Q) and a confirmation (q) MRM transition was selected. Quality criteria for positive confirmation of peaks were (i) presence of both MRM transitions, (ii) a retention time within 0.03 min, and (iii) a relative ion ratio within 50 to 150% compared to a standard. The limits of quantification (LOQ) were 0.5 $\mu g\,l^{-1}$ for MeP and 0.1 $\mu g\,l^{-1}$ for the others parabens (EtP, iPrP, nPrP, sBuP, iBuP, nBuP, BzP). For 498 participants valid paraben concentrations above the detection limit were obtained (cases used for weight analyses are shown in Supplementary Table 2). For iPrP, sBuP and BzP more than 70% of samples had concentrations below the LOQ and were therefore excluded from further analysis. For paraben analysis of mouse samples 20 µl urine and 180 µl milliQ water was mixed and the same protocol as described above was applied.

Assessment of cosmetic product application. The usage of cosmetic products during pregnancy was assessed within the recruitment period via questionnaires at the 34th week of gestation. Participants could name up to 6 cosmetic products that were used on a daily basis. These cosmetics were categorised in leave-on products (crèmes, body lotion, make-up or leave-on facial cleansings) and rinse-off products (toothpaste, rinse-off facial cleansings, hairstyling, perfume and others). The content of parabens in the named cosmetic products was assessed between April and July 2016 via the TOXFOX app for iOS from the Friends of the earth Germany (Bund für Umwelt und Naturschutz Deutschland), which provides information about the content of endocrine disrupting chemicals like parabens in an online data base of cosmetic products. Insufficiently indicated cosmetic products that could not be assigned specifically to a particular brand/product were excluded from further analysis.

Anthropometric data collection. Children's body weight and height were assessed annually during clinical visits or were obtained from regular preventive medical check-ups asked for in the LINA questionnaires. At birth and the one-year follow-up children's length was measured horizontally; afterwards standing height was measured to the nearest 0.1 cm. Body weight was measured to the nearest 0.1 kg. To adjust for child's age and sex, BMI was classified according to the Extended International (IOTF) Body Mass Index Cut-Offs for Thinness, Overweight and Obesity in Children[40] on a detailed monthly basis for children from age 2–8 years. Children with BMI equivalent to an adult BMI ≥ 25 (for example: 36-month-old girl: 17.64 kg m^{-2}, 36-month-old boy 17.85 kg m^{-2}) were classified as overweight (OW), children with BMI equivalent to an adult BMI < 25 as non-overweight (NOW). Further, we have classified weight at birth as ≥4000 g (or macrosomia; considering gestational age as a confounding factor in our model analyses) or as birth weight <4000 g ($n = 496$). Longitudinal BMI information was available for 396 children from year 1–8. Classification in NOW and OW was performed in early to mid-childhood (year 2–8; $n = 223$). Due to missing reference data the first year could not be included in this analysis. This should not be of relevance for the paraben effect since Körner et al.[41] described a longitudinal overweight stabilising effect only after the age of 3 years. We were able to verify the OW classification based on a Bioelectrical Impedance Analysis (BIA) performed at children's age of eight. Children classified as OW had about 30% higher body fat mass percentage at 8 years of age compared to normal weight children (fat mass overweight = 19.3%, $n = 58$; fat mass normal weight = 14.9%, $n = 138$, $P < 0.001$).

In vitro adipocyte differentiation. Human adipose-derived mesenchymal stem cells (MSC) derived by a female donor (ATCC, LGC Standards (Wesel, Germany), PCS-500-011; LOT #59753760) and appropriate culture media were purchased from ATCC (LGC Standards,Wesel, Germany, PCS-500-030, PCS-500-040 and PCS-500-050). Cells were cultured under normal conditions at 37 °C, 5% CO_2 and 95% humidity according to the manufacturer's instructions[9]. In brief, for adipocyte differentiation MSCs passage 3–5 were seeded at 9.000 cells per cm^2 in a 96-well plate and grown to 70% confluence. For initiation of differentiation cells were fed with Adipocyte Differentiation Initiation Medium (ADIM; ATCC Adipocyte Differentiation Toolkit PCS-500-050). ADIM was changed to Adipocyte Maintenance Medium (ADMM; ATCC Adipocyte Differentiation Toolkit PCS-500-050) after 4 days and changed regularly. Cells were treated with parabens at different concentrations (working solution in 0.05% ethanol) during the entire differentiation

period. Butyl paraben exposure was represented in the experimental setup by nBuP due to its higher human exposure (see Supplementary Table 1) compared to iBuP. Proliferation and differentiation were monitored in real-time by the impedance-based xCELLigence MP System (ACEA Biosciences Inc., San Diego, USA) on a microelectrode 96 well E-View-Plate (ACEA Biosciences Inc.). A cell index was assessed every 10 min by normalising an electrical impedance measurement to a bland value for each well. Measurements were paused for media changes. After a total of 16 days, cells were stained with Oil Red O for triglyceride depots and harvested for qPCR analysis. Oil Red O staining was quantified via absorbance measurement at 510 nm. qPCR was carried out in a Roche Light Cycler 480 system with primer sequences being listed in the Supplementary Table 4. Human adiponectin and leptin protein concentrations were assessed in cell culture supernatants of human MSC derived adipocytes by commercially available ELISA kits (Adiponectin ELISA Kit, Human (KHP0041); Leptin ELISA Kit, Human (KAC2281) from Invitrogen/Thermo Fischer, Ulm, Germany) according to the manufacturer's instructions. Undiluted samples were measured in triplicates, using a 1× secondary antibody and compared to 8 standards, ranging from 0−1000 pg ml^{-1} for leptin and 0–23 ng ml^{-1} for adiponectin. Cytotoxicity of exposed chemicals was assessed prior to and after adipocyte differentiation via a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid) assay.

To follow our translational research design, a validation of the human adipose-derived MSC experiment was performed using adipocyte derived murine MSCs (Cyagen, MUBMD-01001, Strain C57BL/6 Mouse Adipose-Derived Mesenchymal Stem Cells) according to manufacturer's instructions. In brief, for adipogenic differentiation mMSC were passaged onto 12-well plates at a concentration of 20,000 cells per cm^2 and were grown to at least 100% confluence in Basal Media (Cyagen, MUXMX-90011). After that the cells underwent adipogenic differention according to manufacturer's instructions (using Cyagen GUXMX-90031) following a maximum of 5 cycles of 3 day induction and 1 day maintenance period. With each cycle of induction, cells were exposed to Ethanol control (0.05%), 0.5 µM, 1 µM, or 10 µM nBuP. Rosiglitazone provided by Cyagen was only used as positive control. After the cells formed big and round lipid droplets they were harvested and processed for further analyses (see human MSCs for Oil Red O staining and qPCR analyses).

Reporter gene assay. Butyl paraben was tested in five reporter gene cell lines[42] based on the human nuclear receptors peroxisome proliferator activated receptor PPARγ (CellSensor PPARγ-UAS-BLA293-H, Thermo Fisher Scientific), androgen receptor AR (CellSensor AR UAS BLA GRIPTITE), oestrogen receptor ER (CellSensor ER a UAS BLA GRIPTITE), progesterone receptor PR (CellSensor PR-UAS-BLA HEK293T), and glucocorticoid receptor GR (CellSensor GR-UAS-BLA HEK293T). The experiments were performed as described previously[43,44] using in parallel reference compounds for quality control and dosing the parabens in methanol assuring that the final methanol was <0.1%. 4000 cells (PPARγ), 3500 cells (ER), or 4500 cells (AR, PR, GR) were seeded per well in 384-well plates with 30 µl of medium and incubated for 24 h at 37 °C and 5% CO_2. The methanolic stock solutions of the paraben were diluted with assay medium and a 10-step serial dilution-series was prepared in a 96-well dosing plate and 10 µl of each dilution were added to the 384-well cell plates. The dosed cells were incubated another 22 h at 37 °C and 5% CO_2. Activation of nuclear receptors PPAR, AR, ER, PR, GR was detected after adding 8 µl of FRET detection including ToxBlazer mixture per well followed by 2 h incubation at room temperature. Blue (activated) and green signals (inactive) were detected using an excitation filter of 409 nm and emission filters of 460 nm and 530 nm, respectively. Induction of the transcription factor or, more precisely, expression of the reporter gene β-lactamase was measured by FRET fluorescence. For the cytotoxicity, an excitation filter of 600 nm and an emission filter of 665 nm were used. The activation of ARE was detected by quantification of the luciferase reporter product using luciferin/ATP reagent as described previously[43,45]. Concentration-response curves were plotted for cytotoxicity and activation of the reporters. The inhibitory concentration for 10% of cytotoxicity IC_{10} was deduced from a log-sigmoidal fit of the concentration-cytotoxicity curve[43]. Only concentrations below IC_{10} were further processed for activation. Linear concentration-response curves up to 30% effect level or an induction ratio of 4 for the AREc32 assay were plotted and effect concentration triggering 10% of the maximum effect of a positive control, EC10, or causing an induction ratio IR of 1.5, $EC_{IR1.5}$ were derived[46].

Mice. BALB/cByJ mice (6–8 weeks of age) were obtained from the Elevage Janvier Laboratory (Le Genest St Isle, France). Mice were bred and maintained in the animal facility at the University of Leipzig (Germany) under conventional conditions with 23 °C room temperature, 60% humidity, and 12 h day/night rhythm. Control and nBuP-exposed dams and pups were housed in polyphenylsulfone (PPS) cages and bedded with LIGNOCEL® bedding material. All mice received phytoestrogen-free diet (C1000 from Altromin, Lage, Germany) and water ad libitum. Experiments included groups of 4-6 mice per cage and were performed at least two times according to institutional and state guidelines. Animal protocols used in this study were approved by the Committee on Animal Welfare of Saxony/Leipzig (Permit Number: TVV01/15).

Exposure to nBuP and Aza treatment. To investigate the impact of an intrauterine exposure on weight development in the offspring we exposed pregnant mice to nBuP via subcutaneous (s.c.) injection of 1.75 µg nBuP (Sigma-Aldrich Chemie GmbH, Munich, Germany) in 100 µl corn oil twice per week until weaning when pups were 4 weeks old (perinatal exposure). Control dams received only the vehicle. To investigate a possible involvement of epigenetic alterations offspring were treated i.p. with 160 µg kg^{-1} body weight Aza (Sigma-Aldrich) solved in PBS 3 times per week starting 1 week after birth until weaning[26]. Each exposure protocol was performed at least two times from at least 3 dams (each with 2–5 pups with not more than 3 pups per sex).

Assessment of weight and metabolic parameters. Body weight of the pups was measured twice a week and a mean weight per week was calculated for each mouse. At the end of the observation period (12 weeks) whole body composition (fat mass and lean mass) was determined in awake mice based on nuclear magnetic resonance technology using an EchoMRI700™ instrument (Echo Medical Systems, Houston, TX, USA) in the offspring of control and nBuP exposed dams. Food intake was monitored after weaning until week 11. For determination of the adipocyte area visceral adipose tissue was removed and stored into in 4% paraformaldehyde for more than 24 h to complete fixation. Afterwards the tissues were dehydrated, embedded in paraffin and sectioned into 3 µm sections. After a deparaffinization, tissue sections were stained with hematoxylin and eosin (H&E) for the adipocyte area assessment. Insulin tolerance test (ITT) was performed in the offspring 9 weeks after birth. Insulin (0.75 U kg^{-1} body weight) was injected intraperitoneally. For glucose measurements blood of tail vein was taken at four time points at 0, 15, 30 and 60 min after insulin injection. For the glucose tolerance test (GTT) glucose (2 g kg^{-1} body weight) was injected i.p. and the measurement was performed equally. Adiponectin, Leptin, Resistin, Insulin, and acetylated Ghrelin serum concentrations were determined by ELISA using mouse standards according to the manufacturer's guidelines (Mouse Adiponectin, Leptin, Resistin ELISA; R&D Systems/bio-techne, Minneapolis, USA, Mouse/Rat/Human 17-beta Estradiol ELISA; Abcam, Cambrigde, UK, Mouse/Rat Insulin ELISA; bertin-pharma, Montigny-le-Bretonneux, France and Mouse/Rat acylated Ghrelin ELISA; BioVendor, Karasek, Czech Republic).

RNA extraction, cDNA synthesis and qPCR. Dissection of the hypothalamus was conducted from the ventral side of the brain. The optic chiasm was removed away from the anterior portion of the hypothalamus. The mammillary nuclei were dissected from the posterior of the hypothalamus. The entire hypothalamus was prepared including the arcuate, ventromedial, dorsomedial, and paraventricular nuclei. Total RNA was extracted from adipocytes of humans, visceral adipose tissue and hypothalamus of mice by using QIAzol Lysis Reagent (QIAGEN, Hilden, Germany) and RNAeasy Plus Mini Kit (QIAGEN) following manufactures instructions. Two hundred nanogram were used for cDNA synthesis by RevertAid™ H Minus Reverse Transcriptase (Thermo Fisher Scientific, MA, USA). qPCR was performed with BIOTAQ™ DNA polymerase (Bioline GmbH, Luckenwalde, Germany) and SYBRgreen I nucleic acid gel stain (Thermo Fisher Scientific) on a LightCycler 480 (Roche Applied Sciences, Penzberg, Deutschland) with the following cycling programme: 10 min at 95 °C, followed by 45 cycles of 95 °C for 10 s, 20 s at 68 °C and 72 °C for 20 s. All reactions were performed in duplicates. Primers were designed exon spanning. Primer sequences of target genes are listed in Supplementary Table 6. Expression values of target genes were evaluated using 2-ΔΔCT method with *U6 small nucleolar RNA* (*u6*), *beta-actin* (*actb*), the *beta-glucuronidase* (*gusb, GUSB*) and *phosphoglycerate kinase 1 (PGK1)* as reference genes and normalised to the lowest measured value.

Pyrosequencing. For DNA methylation analysis of POMC promoter and enhancers genomic DNA (gDNA) of the hypothalamus was isolated using the DNeasy Blood and Tissue Kit (QIAGEN). Two hundred nanogram gDNA was bisulfite converted using the EZ DNA Methylation™ Kit (ZymoResearch, CA, USA). The pyrosequencing assay for DNA-methylation analysis of enhancers was designed on the forward strand and on the reverse complement-strand for POMC promoter using the PyroMark Assay Design version 2.0.1.15 software (Qiagen, POMC enhancer nPE1: forward primer 5'-GTGGGTAAGTTTGAGTTTTGAA TG-3', reverse primer 5′-biotin-ACCCTTCCTCAAAAATACAAAATTC-3', sequencing primer 5'-AGTTTGAGTTTTGAATGT-3', amplicon coordinates: chr12: 3942231-3942372; POMC enhancer nPE2: forward primer 5'-TTTTTT TGTTTTGTGGGGTATGTAGT-3', reverse primer 5′-biotin-AAAAACCCTAA TAAAAAACCCCTTAA-3', sequencing primer: 5'-AATATATGTATTAGTGGA TGAAA-3', amplicon coordinates: chr12: 3944766-3944876; POMC promoter: forward primer 5'-GTTGGGTGGGTGAGTTTT-3', reverse primer 5′-biotin-CAC CATTCTTAATTAAATTCTTCCTAACC-3', sequencing primer: 5'-GTGGGTGA GTTTTGGA-3', amplicon coordinates: chr12:3954708-3954911). The genomic position of the amplicons for DNA-methylation of the POMC promoter and enhancers nPE1/nPE2 were determined based on Rubinstein et al.[33], Drouin et al.[47], and Langlais et al.[48]. (see Supplementary Fig. 7).

Bisulfite treated gDNA was amplified with the HotStar Taq DNA Polymerase Kit (QIAGEN) with the following cycling programme: 15 min at 95 °C, followed by 45 cycles of 94 °C for 30 s, 30 s at 56 °C and 72 °C for 50 s, and a final elongation for 10 min at 72 °C. DNA methylation was assessed by pyrosequencing on a PyroMark Q48 (QIAGEN) following manufacture's instruction.

Statistical analysis. LINA study data were evaluated by STATISTICA for Windows, Version 12 (Statsoft Inc., USA) and STATA version 15.1 (StataCorp LLC, USA). A GEE model with exchangeable correlation matrix and cubic splines with knot points at 12, 25, 38.2, 61 and 98,2 months was applied to test for longitudinal differences in BMI between low (1st tertile, reference), medium (2nd tertile) and high (3rd tertile) paraben exposure groups. Logistic regression models were performed to analyse the impact of prenatal paraben exposure on infants' risk for macrosomia at birth and overweight development afterwards (year 2–8). Both models were adjusted for the sex of the child, smoking during pregnancy, parental school education, gestational week at delivery, existence of siblings, breast feeding duration (not for models with only birth weight as outcome) and age of the mother at birth. All confounders were chosen according to their potential weight association after a literature review. To test the equal distribution of parameters in the analysed sub-cohort and the entire LINA cohort the chi squared test was performed. Mann–Whitney-*U*-test was used for the comparison of median paraben levels of mothers that used paraben containing vs. paraben free cosmetic products. Experimental data sets from in vivo and in vitro studies were processed and analysed in GraphPad PRISM 7.02 for windows (GraphPad Software, Inc.) and R version 3.5.1. All *p*-values are derived by GEE models with exchangeable correlation matrix for longitudinal mouse data, ANOVA or unpaired *t*-test.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All relevant data supporting the findings of the study are available in this article, its Supplementary Information file, the Source Data file (Figs. 1B–E; 2A–E; 3B–D; 4A–F and Supplementary Figs. 2A–C; 3B–C; 4A–C; 5; 7A–B; 8A–C), or from the corresponding authors on request. However, epidemiological cohort data cannot be provided as an open source file due to ethical declaration issues and can be requested in their analysed version from the corresponding author.

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Author contributions

K.J., I.L. and T.P. designed and conducted the study and experiments; B.L., K.J. and C.P. analysed the cohort data; B.L., S.S and I.K. performed the cell culture and mouse experiments; B.S., L.S. and T.R. established and performed the paraben analytic; R.S. and B.I.E. performed the reporter gene assays; M.Bo., S.R. and I.L. developed the LINA study design, A.S. assessed the whole body composition; M.Ba. conducted the PCR analysis, U. E.R-K. and M.v.B. prepared the human urine; S.S. and L.T. performed the pyrosequencing, T.P., B.L., K.J., I.L., G.I.S. T.S. and B.I.E. discussed the data with substantial contributions from T.R. and M.v.B.; K.J. and B.L. designed Fig. 5. B.L. and T.P. wrote the paper with substantial inputs from G.I.S., T.S., I.L., KJ, B.I.E., M.v.B. and T.R.

Competing interests

The authors declare no competing interests.

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MEST mediates the impact of prenatal bisphenol A exposure on long-term body weight development

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Abstract

Background: Exposure to endocrine-disrupting chemicals can alter normal physiology and increase susceptibility to non-communicable diseases like obesity. Especially the prenatal and early postnatal period is highly vulnerable to adverse effects by environmental exposure, promoting developmental reprogramming by epigenetic alterations. To obtain a deeper insight into the role of prenatal bisphenol A (BPA) exposure in children's overweight development, we combine epidemiological data with experimental models and BPA-dependent DNA methylation changes.

Methods: BPA concentrations were measured in maternal urine samples of the LINA mother-child-study obtained during pregnancy ($n = 552$), and BPA-associated changes in cord blood DNA methylation were analyzed by Illumina Infinium HumanMethylation450 BeadChip arrays ($n = 472$). Methylation changes were verified by targeted MassARRAY analyses, assessed for their functional translation by qPCR and correlated with children's body mass index (BMI) *z* scores at the age of 1 and 6 years. Further, female BALB/c mice were exposed to BPA from 1 week before mating until delivery, and weight development of their pups was monitored ($n \geq 8$/group). Additionally, human adipose-derived mesenchymal stem cells were treated with BPA during the adipocyte differentiation period and assessed for exposure-related epigenetic, transcriptional and morphological changes ($n = 4$).

Results: In prenatally BPA-exposed children two CpG sites with deviating cord blood DNA-methylation profiles were identified, among them a hypo-methylated CpG in the promoter of the obesity-associated mesoderm-specific transcript (*MEST*). A mediator analysis suggested that prenatal BPA exposure was connected to cord blood *MEST* promoter methylation and *MEST* expression as well as BMI *z* scores in early infancy. This effect could be confirmed in mice in which prenatal BPA exposure altered *Mest* promoter methylation and transcription with a concomitant increase in the body weight of the juvenile offspring. An experimental model of in vitro differentiated human mesenchymal stem cells also revealed an epigenetically induced *MEST* expression and enhanced adipogenesis following BPA exposure.

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Conclusions: Our study provides evidence that *MEST* mediates the impact of prenatal BPA exposure on long-term body weight development in offspring by triggering adipocyte differentiation.

Keywords: EDC, Prenatal exposure, Infants, Obesity, LINA, Mice, Mesenchymal stem cells, Epigenetics, DNA methylation, Adipogenesis,

Background

Exposure to endocrine-disrupting chemicals (EDCs) during critical windows in development can permanently alter normal physiology and increase susceptibility to diseases like obesity, asthma, or cancer later in life [1]. Especially the prenatal and early postnatal period is highly vulnerable to EDC exposure as it is the time of developmental programming important for organogenesis and tissue differentiation [2]. The growing knowledge about the human epigenome emphasized the importance of environmental exposure-related epigenetic modifications predisposing an individual to the development of disease. Understanding the underlying effects leading to a disruption in epigenetic programming by EDCs during fetal development is important and might aid future prevention strategies for such diseases.

One EDC with a well-described impact on the human epigenome during development is bisphenol A (BPA). BPA is a chemical used in the manufacturing of polycarbonate plastics and epoxy resins contained in a variety of consumer products. It is readily released to the environment leading to extensive human exposure in industrialized countries [3, 4]. BPA has been detected in human blood, urine, adipose tissue, breastmilk, and also in placental tissue and amniotic fluid [3], suggesting that exposure already starts during the sensitive prenatal phase. BPA is classified as an endocrine disruptor because of its ability to mimic hormone activity, for example, through estrogen-, and peroxisome proliferator-activated receptor gamma (PPARγ) signaling [5, 6]. After oral administration BPA is rapidly biotransformed to glucuronidated BPA in the liver via UDP-glucuronosyltransferase (UGT) and is eliminated by urinary excretion within 24 h [7, 8]. Complementary, studies in rats suggest that BPA metabolism might change during pregnancy due to alterations in UGT isoforms and expression level [9]. In addition, decreased UGT levels in fetal liver can lower the excretion capacity for BPA, making the fetus even more vulnerable to environmental pollutant EDC exposure [10–12]. So far, data on human BPA metabolism during pregnancy or early childhood are missing, but it seems reasonable to assume that also in pregnant women and in the developing fetus a lower excretion capacity might increase their vulnerability to BPA exposure with potential consequences for children's later disease development.

In this context, BPA is highly discussed in terms of increasing the risk for obesity pathology but only few controversial studies on human prenatal BPA exposure exist so far [13–15]. Although animal studies are available to a greater extent, derived results are inconsistent and mechanistic investigations, for example, regarding underlying BPA-related epigenetic changes, are lacking. Epigenetic alterations related to BPA exposure have previously been associated with an increased risk of carcinogenesis [16–18] in rodent models of hepatic and prostate cancer. So far, no data on BPA-induced epigenetic modifications leading to overweight development exist.

Therefore, the aim of the present study was to analyze epigenetic alterations in the cord blood of prenatally exposed children and their potential link to overweight development as part of the German prospective LINA mother-child cohort. Findings from our epidemiological study were validated by applying an experimental mouse model for prenatal BPA exposure and an in vitro stem cell differentiation model demonstrating the impact of BPA exposure on adipocyte development.

Methods

LINA study design and sample collection

The LINA cohort study (Lifestyle and environmental factors and their Influence on Newborns Allergy risk) recruited 622 pregnant mothers (629 mother-child-pairs) between May 2006 and December 2008 in Leipzig, Germany, to investigate how environmental factors in the pre- and postnatal period influence disease risks later in children's life [19–21]. Mothers suffering from immune or infectious diseases during pregnancy were excluded from the study.

Six hundred six mother-child-pairs participated in the year 1, 420 in the year 6 follow-up. Standardized questionnaires were administered during pregnancy (34th week of gestation) and annually thereafter, collecting general information about study participants, about housing and environmental conditions as well as about personal lifestyle. At the age of 1 and 6 years, height and body weight of the children were assessed during clinical visits. BMI z score were calculated according to the WHO references [22]. All questionnaires were self-administered by the parents. Participation in the study was voluntary and written informed consent was obtained from all participants. The study was approved by the Ethics Committee of the

University of Leipzig (file ref. # 046–2006, #206–12-02072012).

Analyses of urinary bisphenol A concentration in human samples

BPA quantification was carried out for 552 maternal urine samples (34th week of gestation) using a multianalyte procedure as described by Feltens et al. [23] and in more detail in the supplementary material. Absolute concentrations of BPA were calculated based on calibration curves and normalized to urinary creatinine concentrations as previously described [24].

In vivo mouse model

BALB/c mice (6–8 weeks of age) were obtained from the Elevage Janvier Laboratory (Le Genest St Isle, France). Mice were bred and maintained in the animal facility at the University of Leipzig (Germany) and housed under conventional conditions with 23 °C room temperature, 60% humidity, and 12 h day/night rhythm. Cages were bedded with LIGNOCEL® bedding material (fine particles < 200 μm 0.2%). Mice received phytoestrogen-free diet (C1000 from Altromin, Lage, Germany) and water ad libitum from custom-built glass bottles to avoid contamination with BPA. All animal experiments involved groups of 4–6 mice/cage and were performed according to institutional and state guidelines. The Committee on Animal Welfare of Saxony approved animal protocols used in this study.

Dams were exposed to 5 μg/ml BPA (Sigma Aldrich, Munich, Germany) via the drinking water 1 week before mating until delivery of the offspring. For each exposure group (control or BPA), the exposure protocol was performed at least two times in at least three dams (each with 2–5 pups). Serum was collected from dams at the end of the BPA exposure. 1 week after delivery, pups were weighed two times per week and a mean weight per week was calculated for each mouse. At the end of the observation period (10 weeks), whole body composition (fat mass and lean mass) was determined in awake mice based on nuclear magnetic resonance technology using an EchoMRI700™ instrument (Echo Medical Systems, Houston, TX, USA) in the offspring of control and BPA exposed dams. Further, DNA-methylation analysis (MassARRAY) as well as gene expression analysis was performed in visceral fat tissue as described below in 10-week-old offspring. For measurement of fat mass/lean mass, MassARRAY and gene expression analysis, we used at least four mice per group from two to four dams (to avoid litter effects), but in any case with an equal number of male and female mice.

Murine BPA ELISA

BPA concentration in serum was detected with BPA Assay Kit (Immuno-Biological Laboratories, Hamburg, Germany). Serum samples, enzyme-labeled BPA and anti-BPA serum were added to a pre-coated microtiter plate with anti-rabbit IgG and incubated for 1 h at room temperature. After washing, TMB was added as substrate and color reaction was detected at 450 nm. BPA serum concentration was calculated from a standard curve with a detection range from 0.3 to 100 ng/ml. Measured BPA serum levels in adult mice reached 19 ng/ml.

In vitro adipocytes model

Human adipose-derived mesenchymal stem cells (MSC; ATCC®, PCS-500-011; #59753760) and culture media were purchased from LGC Standards (Wesel, Germany). For adipocyte differentiation MSCs at passage 1–3 were seeded at 9600 cells/cm^2 and were cultured with Adipocyte Differentiation Initiation Medium (ADIM; ATCC Adipocyte Differentiation Toolkit PCS-500-050) for 4 days. Thereafter, Adipocyte Maintenance Medium (ADMM; ATCC® Adipocyte Differentiation Toolkit PCS-500-050) was applied for the subsequent 11 days. Media was changed every 2 to 4 days according to the manufacturer's instructions. During the entire differentiation period, cells were exposed to 10 or 50 μM BPA (Sigma Aldrich, Munich, Germany) or a solvent control (0.05% ethanol); freshly added after every medium change. The differentiation process was monitored in real-time by the impedance-based xCELLigence SP System (ACEA Biosciences Inc., San Diego, USA) on a microelectrode 96 well E-View-Plate (ACEA Biosciences Inc.). The growth rate was monitored every 10 min by electrical impedance measurements that were paused for media changes and a Cell Index was calculated, by normalization to a blank value for each well. After differentiation, cells were stained with Oil Red O for 45 min for triglyceride depots and mRNA was extracted (see supplementary material).

A MTT assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid) was applied to a BPA concentration series to identify appropriate non-toxic concentrations for the in vitro assay. For details, see supplementary material.

DNA methylation analysis via 450 K array

Genomic DNA was isolated from cord blood samples using the QIAmp DNA Blood Mini Kit (Qiagen, Hilden, Germany) followed by bisulfite conversion using the EZ-96 DNA Methylation Kit (Zymo Research Corporation, Orange, USA) according to the manufacturer's recommendations. All samples subsequently subjected to DNA methylation analyses passed the initial quality control check ($n = 472$).

A genome-wide DNA methylation screen was performed based on the Infinium HumanMethylation450 BeadChip (Illumina, San Diego, USA) array (see supplementary material). Data were normalized using the SWAN (subset-

quantile within array normalization) method of the minfi R package [25]. DNA methylation values, described as beta values (β), were recorded for each locus in each sample. For statistical analyses β values were logit transformed to *M* values [26].

To account for potential differences in cell composition, we used publically available FACS data of sorted cord blood cells [27] implemented in the R package *FlowSorted.CordBloodNorway.450 k: Illumina HumanMethylation data on sorted cord blood cell populations* (version 1.4.0) [28] and the estimateCellCounts-function of the minfi R package. The resulting information on CD4+ T cells, CD8+ T cells, natural killer cells, B cells, granulocytes, and monocyte proportions were used as confounders in the subsequent regression analysis. In addition, previously identified factors with an impact on cord blood methylation were considered as confounders including the maternal vitamin D level [29], prenatal benzene exposure, maternal smoking [30, 31], and maternal stress during pregnancy [32]. Differentially methylated CpGs were determined by applying logistic regression models on methylation *M* values [33] adjusted for the confounders mentioned above. A Bonferroni correction was applied on obtained *p* values resulting in a significance level of $p < 2.37E\text{-}7$. For details, see supplementary material.

MassARRAY validation of DNA methylation

A quantitative DNA methylation analysis of the human *MEST* promoter was performed in cord blood samples of the LINA cohort and in in vitro adipocytes using Sequenom's MassARRAY platform as described previously [31]. Briefly, a PCR amplicon was designed on the reverse strand covering chr7: 130,132,068-130,132,287 including cg17580798 (Fig. 1, *MEST* forward primer: aggaagagagTTTAGAGGTAGTTTTAGTTYGG, reverse primer: cagtaatacgactcactatagggagaaggctCCRCTACTAA CCAACTCTAC with an annealing temperature of 52 °C). A total of 24 CpGs was covered by the amplicon. For analysis, all high mass, duplicate, and silent peaks were excluded from the analysis retaining 14 CpGs, which were averaged and used for further analysis.

gDNA extracted from murine adipose tissue F1 ($n \geq 3$, per treatment group) was bisulfite converted using the EZ DNA Methylation kit (Zymo Research, Freiburg, Germany) and subjected to MassARRAY analysis. Genome coordinates of the human *MEST* promoter were lifted over to the mouse genome assembly mm10 and corresponding primer pairs on the forward strand were designed (*Mest* forward primer: aggaagagagAGGAGGTTTGTGTTTTTAATG, reverse: cagtaatacgactcactatagggagaaggctCACCCACTT CTTTTCTACC, annealing temperature: 60 °C, amplicon coordinates: chr6: 30,737,347-30,737,692).

Gene expression analysis

Gene expression analysis in samples of the LINA cohort was performed as described earlier [31] and in more detail in the supplementary material. Briefly, intron-spanning primers were designed, and UPL probes were selected by the Universal Probe Library Assay Design Center. After a preamplification step qPCRs were conducted on 96.96 Dynamic Array (Fluidigm, San Francisco, CA, USA). Gene expression values were determined with *glycerinaldehyd-3-phosphat-dehydrogenase (GAPD)* and *glucuronidase beta (GUSB)* as reference genes and normalized to the lowest measured value. The following primer pairs were used for *MEST* (primer-for 5′- atcgtggaagcgcttttg, -rev 5′-gaccagatcgattctgcttgta, UPL50) and the reference genes *GAPD* and *GUSB* (primer-for 5′-gctctctgctcctcctgttc, -rev 5′-acgaccaaatccgttgactc, UPL 60; -for 5′-cgccctgcctatctgt attc, -rev 5′-tccccacagggagtgtgtag, UPL 57, respectively).

Mest expression in murine fat tissue was assessed by qPCR. Expression values were determined by applying the 2-ΔΔCT method and normalized to *Gapdh and UBC (–for 5′*-gtctgctgtgtgaggactgc, *rev 5′-* cctccagggtga tggtctta *UPL 77).*

Furthermore, gene expression of *peroxisome proliferator-activated receptor gamma* (*PPARG), sterol regulatory element-binding factor 1 (SREBF1), lipoprotein lipase (LPL), leptin (LEP), fatty acid synthase (FASN), mesoderm specific transcript (MEST*), *estrogen receptor alpha* (*ESR1),* and

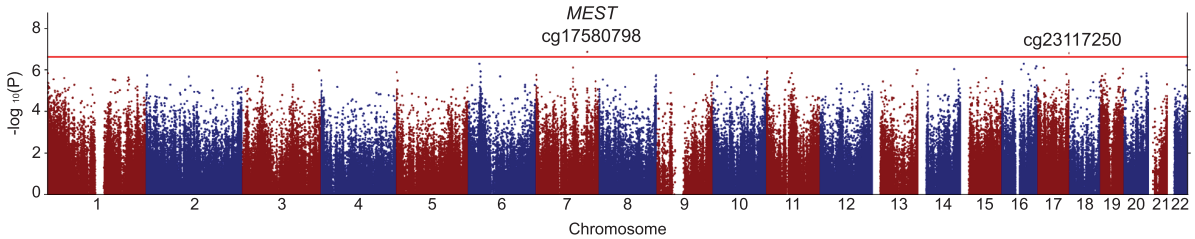

Fig. 1 Epigenome wide analysis and *MEST* methylation assessment. Manhattan-Plot from 450 K array comparing children prenatally exposed to high vs. low BPA. Shown are significant CpGs observed in cord blood that passed threshold for Bonferroni correction (red threshold line, $p < 2.37E\text{-}7$)

insulin receptor substrate 2 (*IRS2)* was assessed by qPCR of human in vitro derived adipocytes.

All used primer pairs are listed in Additional file 1: Table S1. All primers were designed as intron spanning assays to assure specificity.

Statistical analyses

To test the equal distribution of parameters in the analyzed sub-cohort and the entire LINA cohort, the chi-squared test was performed. LINA study data were evaluated by STATISTICA for Windows, Version 12 (Statsoft Inc., USA). 450 k data were analyzed and processed using the R packages minfi and qqman (R version 3.3.1, R Foundation for Statistical Computing).

BPA concentrations and DNA methylation levels were log transformed for further statistical analyses. To assess longitudinal associations of gene expression and weight development, a generalized estimating equation (GEE) model was applied. Mediator models for the connection of prenatal BPA exposure with the methylation status and children's BMI *z* scores were analyzed using the PROCESS macro v2.16.3 in IBM SPSS Statistics version 22 (IBM Corps., USA) [34]. All models were adjusted for the gender of the child, smoking during pregnancy, parental school education, solid food introduction, gestational week at delivery, number of household members, and early delivery (< 37 weeks of gestation). Weight-related confounders were chosen according to a literature review.

Experimental data sets from murine and in vitro studies were processed and analyzed in GraphPad PRISM 7.02 for windows (GraphPad Software, Inc.). All p values ≤ 0.05 were considered to be significant.

Results

General study characteristics

Our analyzed sub-cohort was comprised of the 408 children for whom data on prenatal BPA exposure and the cord blood methylation status were available. General characteristics of the study participants (gender, birth weight, gestational week at delivery, smoking during pregnancy, parental school education, household members, breastfeeding, and introduction to solid food) of the sub-cohort (n = 408) were not different from the total LINA cohort (n = 629) as shown in Table 1. Median urinary BPA concentrations at pregnancy were 12.7 ng/mg creatinine. Low BPA exposure was defined as < 7.6 ng/mg creatinine (< 25%; 1st or lower quartile) and high BPA exposure as > 15.9 ng/mg creatinine (> 75%, 4th or upper quartile). BMI z scores at year 1 ranged from − 3.5 to 2.9 with a median of − 0.2, BMI *z* scores at year 6 ranged from − 2.2 to 4.2 with a median of 0.0.

Table 1 General study population characteristics

	Entire LINA cohort *n* (%), *n* = 629[a]	Analyzed sub-cohort *n* (%), *n* = 408	χ^2 test[b]
Gender of the child			0.966
Female	302(48.0)	197(48.3)	
Male	327(52.0)	211(51.7)	
Birth weight			0.941
≤ 3000 g	123(19.6)	68(16.7)	
> 3000–3500 g	242(38.5)	157(38.5)	
> 3500–4000 g	192(30.6)	129(31.6)	
> 4000 g	71(11.3)	54(13.2)	
Gestational week at delivery			0.834
< 37 weeks	25(4.0)	10(2.5)	
37–40 weeks	389(62.0)	255(62.5)	
> 40 weeks	214(34.0)	143(35.0)	
Smoking during pregnancy			0.833
Never	534(84.9)	358(87.7)	
Occasionally	47(7.4)	23(5.6)	
Daily	48(7.6)	27(6.6)	
Parental school education[c]			0.969
Low	16(2.5)	8(2.0)	
Intermediate	144(22.9)	96(23.5)	
High	469(74.6)	304(74.5)	
Household members			0.932
2	33(5.2)	20(4.9)	
3	365(58.0)	257(63.0)	
> 4	203(32.3)	129(31.6)	
Breastfeeding exclusive			0.968
1–3 months	112(17.8)	69(16.9)	
1–6 months	190(30.2)	121(29.7)	
1–12 months	254(40.4)	172(42.2)	
Introduction to solid food			0.897
1–3 months	23(3.7)	11(2.7)	
4–6 months	251(39.9)	156(38.2)	
7–12 months	305(48.5)	205(50.2)	
Urinary BPA concentration during pregnancy			0.263[d]
Median [ng/mg creatinine]	12.7	12.7	
IQR[e] [ng/mg creatinine]	7.5–16.0	7.6–15.9	
BMI *z* score at year 1	*n* = 564	*n* = 366	
Median	− 0.24	− 0.16	
IQR	− 0.90–0.35	− 0.79-0.43	
BMI *z* score at year 6	*n* = 303	*n* = 192	
Median	− 0.02	0.05	
IQR	− 0.67-0.50	− 0.51-0.54	

[a] *n* may be different from 629 due to missing data
[b] Calculated using the chi-squared test for cross relationship
[c] Low = 8 years of schooling ('Hauptschulabschluss`); intermediate = 10 years of schooling (`Mittlere Reife`); high = 12 years of schooling or more (`(Fach-)hochschulreife')
[d] *p*-value derived by Student's *t* test between group means
[e] IQR: inter quartile range (25th to 75th percentile)

Prenatal BPA exposure and cord blood DNA methylation of MEST

As there is growing evidence that epigenetic mechanisms such as DNA methylation changes can contribute to prenatal programming of diseases, the potential impact of BPA on children's DNA-methylation pattern was assessed. Using bisulfite converted gDNA from cord blood, genome-wide changes in DNA methylation were evaluated by applying Illumina Infinium HumanMethylation450 BeadChip arrays. Differentially methylated CpG sites were computed using a regression model for high (fourth quartile) versus low (first quartile) prenatal BPA exposure. Two CpGs passed the threshold for Bonferroni correction (Fig. 1a and Table 2), including a hypomethylated CpG (cg17580798) in the *MEST* promoter (chr7:130132199, $p = 1.35E-07$) and cg23117250 (chr17: 80649886, intronic, $p = 1.55E-07$) that is located in an intron of *RAB40B*. *RAB40B* encodes for a poorly characterized protein proposed to be involved in vesicle transport [35] and cancer progression [36].

Thus, we focused further analyses on cg17580798 since *MEST*, as a member of the alpha/beta hydrolase superfamily, is reported to control the initial phase of early adipose tissue expansion by regulating adipocyte size [37]. Although cg17580798 is located in the first intron of *MEST*, ENCODE histone modification data suggest that it is a promoter region. That indeed this region is potentially transcriptionally regulating is supported by its overlap with a DNase I hypersensitivity cluster.

MEST promoter methylation around cg17580798 was validated by MassARRAY (see Additional file 2: Figure S1). The MassARRAY amplicon included 24 CpG sites of which 14 CpG sites passed quality control and were averaged as "total promoter methylation." BPA exposure was associated with total promoter methylation (adj.MR: 0.88, 95% CI (0.80, 0.97), $p = 0.010$), as well as methylation of the CpG corresponding to cg17580798 only (adj.MR: 0.90, 95% CI (0.82, 0.99), $p = 0.033$). The methylation difference between low and high BPA exposure was 2.6 and 2.3%, respectively.

BPA associates with MEST promoter methylation and MEST expression in cord blood

MEST expression was measured in 408 cord blood samples of the LINA cohort. Complete information of *MEST* methylation status, *MEST* expression and prenatal BPA exposure was available for 361 children. High prenatal BPA exposure (> 75%, upper quartile (UQ)) was associated with a decrease in *MEST* promoter methylation at birth as determined by MassARRAY (Fig. 2a). Further, this *MEST* promoter hypomethylation was associated with an increase in *MEST* RNA expression, which was not observed in lowly exposed children (Fig. 2b). There was no direct effect of prenatal BPA exposure on cord blood *MEST* expression. However, applying a mediation model using PROCESS in SPSS, prenatal BPA exposure was linked indirectly to *MEST* expression by *MEST* promoter methylation (ab = 0.47, 95% CI (0.07, 1.24); Fig. 2c and Additional file 1: Table S2) at time of birth. Furthermore, *MEST* expression at birth was positively correlated with BMI *z* scores (adj.MR: 1.13, 95% CI (1.02, 1.26), $p = 0.024$).

BPA increases risk for childhood overweight development via MEST methylation

In addition, we were interested whether the changes in *MEST* promoter methylation that were associated with BPA exposure have relevance for the later weight development of the child. Therefore, we applied a mediator analysis, adjusted for weight-related confounders, to assess the impact of prenatal BPA exposure on children's BMI *z* scores at year 1, which might be mediated by neonatal *MEST* promoter methylation. Indeed the mediation analysis indicates that the effect of prenatal BPA exposure on BMI *z* scores is mediated by *MEST* promoter methylation in cord blood (ab = 0.29, 95% CI (0.03, 1.09), Fig. 3a and Additional file 1: Table S3). Furthermore, the impact of cord blood *MEST* promoter methylation on BMI *z* scores at year 6 was mediated by the BMI *z* scores at year 1 (ab = − 0.18, 95% CI (− 0.51, − 0.06), Fig. 3b and Additional file 1: Table S4).

MEST expression is associated with longitudinal weight development

The longitudinal impact of altered *MEST* expression at birth due to prenatal BPA exposure was calculated using a generalized estimating equation (GEE) model including BMI *z* scores and *MEST* expression at birth and year 6 as well as weight related confounders. We found that a longitudinally higher *MEST* expression at birth and year 6 was positively correlated with longitudinal weight development at birth and year 6 (adj.RR: 1.03, 95% CI (1.00, 1.07), $p = 0.021$).

Table 2 Epigenome-wide association study (EWAS) comparing children prenatally exposed to high vs. low BPA. Shown are significant CpGs observed in cord blood that passed Bonferroni correction

CpG	Chromosome	Position	Region	Host gene	*p* value [a]	Δ β[b]
cg17580798	7	130,132,199	Promoter	*MEST*	1.35E-07	−1.8%
cg23117250	17	80,649,886	Intron	*RAB40B*	1.55E-07	−2.0%

[a] *p* values are derived from a regression model with prenatal vitamin D level, prenatal benzene exposure, maternal smoking, maternal stress, and cord blood cell composition as confounding parameters

[b] Methylation differences are shown as Δ methylation values (β)

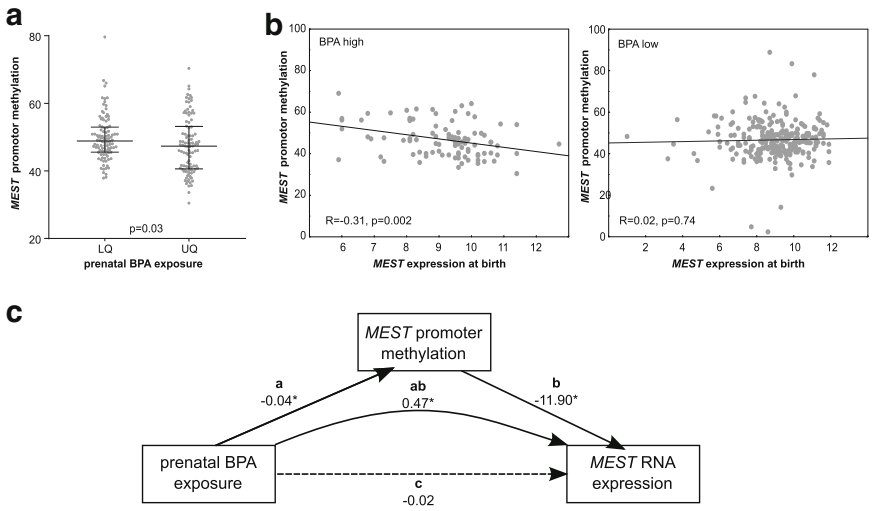

Fig. 2 Association of BPA with *MEST* promoter methylation and *MEST* expression in cord blood **a** *MEST* promoter methylation (=mean of MassARRAY amplicon) in cord blood of low (< 25%; lower quartile (LQ), $n = 102$) and high (> 75%, upper quartile (UQ), $n = 101$) BPA-exposed children. p value from MWU-test. **b** *MEST* promoter methylation and expression in cord blood are correlated in children with high prenatal exposure to BPA (UQ, $n = 94$), while *MEST* expression is not correlated with *MEST* promoter methylation in lowly exposed children (remaining 75%, $n = 267$). R and p values from Spearman correlation. **c** Mediator model for the association of prenatal BPA exposure, cord blood *MEST* promoter methylation and expression. Models were adjusted for gender of the child, smoking during pregnancy, parental school education, solid food introduction, week of gestation at delivery, number of household members, and early delivery. Shown are effect sizes with $*p < 0.05$

In vivo mouse model: impact of prenatal BPA exposure on weight development

To validate our findings from the LINA cohort and add further information on *Mest* methylation and expression in fat tissue, we applied a mouse model under standardized conditions. Mice offspring of BPA-exposed mothers were followed up until 10 weeks after delivery. Weight was assessed twice a week, beginning 1 week after delivery, and compared to unexposed control animals (Fig. 4a). Prenatally BPA exposed mice had a significantly higher weight over the entire observation period compared to unexposed control mice ($p = 0.004$, p value derived by ANOVA) with a mean difference of 0.85 g (week 2) to 3.04 g (weeks 3, 8, and 9). There was no gender difference in BPA-dependent weight development (Additional file 3: Figure S2). Furthermore, lean mass and fat mass were assessed at 10 weeks, with BPA-exposed mice showing a 53% higher fat mass than control mice ($p = 0.013$, Fig. 4b). *Mest* methylation and expression was assessed at 10 weeks in fat tissue samples. *Mest* methylation was reduced by 7% in BPA exposed mice ($p < 0.001$, Fig. 4c) with a corresponding increase in *Mest* expression by 2.1-fold in BPA exposed mice ($p = 0.022$, Fig. 4d).

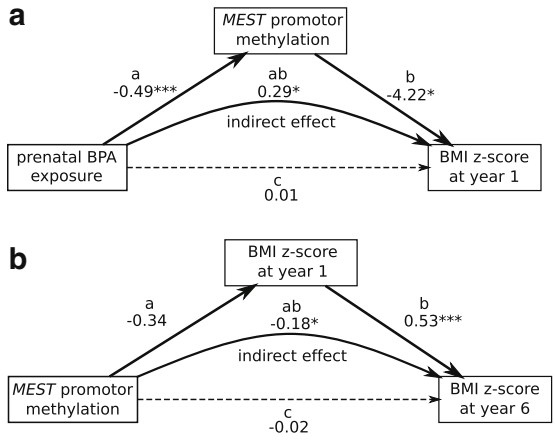

Fig. 3 Mediator models **a** for the association of prenatal BPA exposure with *MEST* promoter methylation and children's BMI z scores at year 1, **b** for the association of cord blood *MEST* methylation with children's BMI z score at years 1 and 6. Models were adjusted for gender of the child, smoking during pregnancy, parental school education, solid food introduction, week of gestation at delivery, number of household members, and early delivery. Shown are effect sizes with $*p < 0.05$; $***p < 0.001$

In vitro model: impact of BPA exposure on adipocyte differentiation

Differentiation of human MSC to adipocytes was monitored in real time using the impedance-based xCELLigence System. 10 or 50 μM BPA were applied during the entire differentiation period and compared to a solvent control (EtOH, 0.05%). BPA caused a dose-dependent decrease in cell index values after the differentiation initiation period compared to unexposed controls (Fig. 5a). Significance was reached from day 3 on for 50 μM and

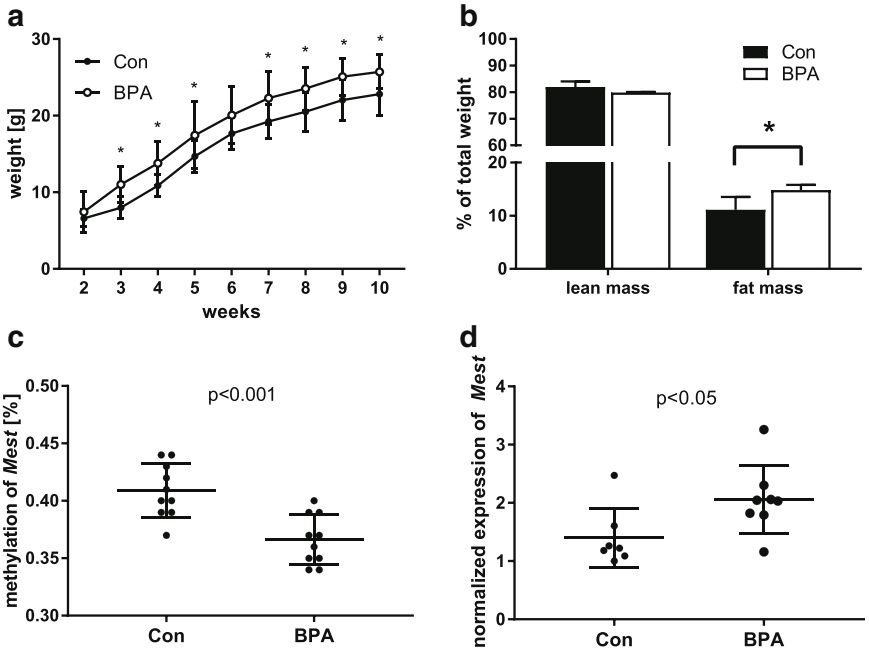

Fig. 4 BPA effects in a murine in vivo model. **a** Impact of prenatal BPA exposure on weight development in the offspring. Shown are means and standard deviations from $n \geq 8$/group and p values are derived from ANOVA. **b** Differentiation of offspring weight at 10 weeks in lean and fat mass. **c** Targeted MassARRAY *Mest* methylation analysis in fat tissue of 10-week-old offspring after prenatal BPA exposure compared to controls. **d** *Mest* expression analysis in visceral fat tissue of 10-week-old offspring after prenatal BPA exposure compared to controls. For **b**, **c**, and **d** data of $n \geq 4$, mice is presented with $^{*}p < 0.05$ and $^{***}p < 0.001$

from day 5 on for 10 μM BPA until the end of the observational period. Oil Red O staining of lipid droplets showed significantly more droplets for 50 μM BPA ($p < 0.001$; Fig. 5b, c) but not for 10 μM BPA compared to unexposed control cells.

mRNA analysis of adipocyte-specific genes after 17 days of differentiation in the presence of 50 μM BPA revealed a significant upregulation of *PPARγ* (2.2 ± 1.15-fold, $p = 0.005$), its target gene *LPL* (4.4 ± 2.6-fold, $p = 0.029$); *SREBF1* (1.8 ± 0.4-fold, $p = 0.005$), its target gene *IRS2* (1.9 ± 0.6-fold, $p = 0.015$; Fig. 2b), and *ESR1* (8.4 ± 3.8-fold, $p = 0.006$). For 10 μM BPA, a significant increase in gene expression was detected for *LPL* (1.9 ± 0.3-fold, $p = 0.002$), *SREBF1* (1.5 ± 0.1-fold, $p < 0.001$) and *FASN* (1.9 ± 0.7-fold, $p = 0.046$, Fig. 5d).

MEST methylation and expression was measured in differentiated adipocytes as shown in Fig. 5e, f. *MEST* promoter methylation (total and cg17580798) was decreased by 28% after exposure to 50 μM BPA compared to the control. In accordance, *MEST* expression was significantly increased in adipocytes exposed to 50 μM BPA (1.6 ± 0.4-fold, $p = 0.027$, Fig. 5e, f).

Our in vitro results are not influenced by any cytotoxic effects of BPA, as can be seen from the performed MTT assay (Additional file 4: Figure S3). There was no change in cell viability up to 50 μM BPA, although cells exposed to 100 μM BPA showed a slight but significantly lower cell viability after 48 h ($p = 0.027$).

Discussion

Our study provides first evidence that prenatal BPA exposure causes epigenetic changes in the *MEST* promoter potentially contributing to overweight development in children with longitudinal effects until the age of 6 years (Additional file 5: Figure S4). Results from our experimental models support these epidemiological findings: prenatally BPA exposed mice showed hypo-methylation of the *MEST* promoter region and developed a significantly higher body weight compared to controls. Furthermore, a stimulating impact of BPA on adipocyte differentiation from human MSC was observed. Although these experimental data have to be interpreted with caution, since the applied exposure concentrations were quite different compared to the real human exposure situation, there is some evidence for an involvement of *MEST* in BPA-induced adipogenesis.

Results from this study may provide a first mechanistic explanation how prenatal BPA potentially exposure contributes to overweight development in the children. We identified two differentially methylated CpG sites in cord blood in association to prenatal BPA exposure, among them one hypo-methylated CpG in the *MEST* promoter. *MEST* is a paternally imprinted gene that encodes a

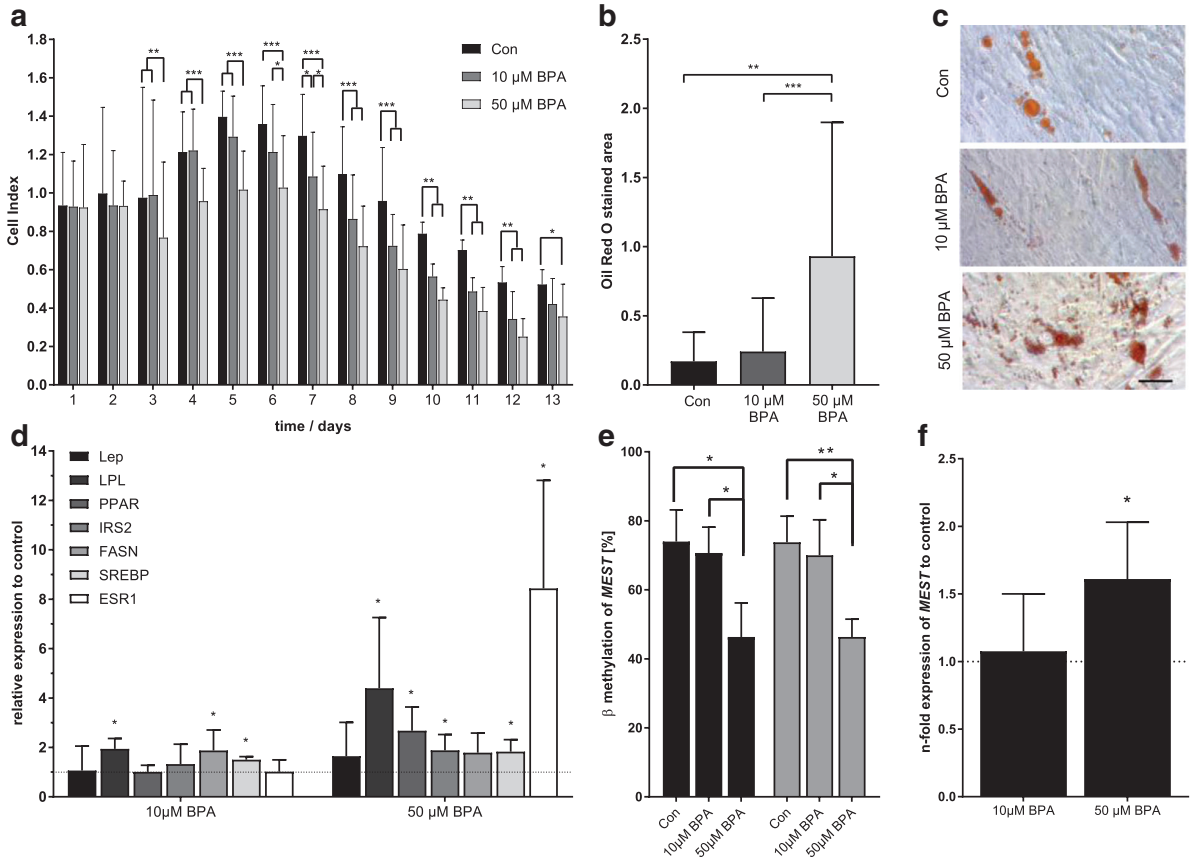

Fig. 5 In vitro adipocyte differentiation from human MSCs: exposure to BPA (10 µM, 50 µM) compared to solvent control (EtOH 0.05%). **a** Real-time monitoring of cell differentiation (xCELLigence: normalized cell index) over a 17-day period (mean ± SD, $n = 4$). **b** Quantification of Oil Red O stained area (mean ± SD, $n \geq 20$ from one experiment). **c** Exemplary histological Oil Red O staining of adipocytes (black bar = 100 µm). **d** qPCR data of genes involved in adipogenesis ($n \geq 3$) normalized to EtOH control (*Lep* = leptin, *LPL* = lipoprotein lipase, *PPARγ* = peroxisome proliferator activated receptor gamma, *IRS2* = insulin receptor substrate 2, *FASN* = fatty acid synthase, *SREBF1* = sterol receptor element binding factor 1, *ESR1* = estrogen receptor alpha). **e** Targeted MassARRAY analysis of *MEST* promoter methylation, shown are the measurement of the single CpG cg17580798 covered by the amplicon (gray bars, $n = 3$) and the mean of the MassARRAY amplicon (black bars). **f** qPCR data of *MEST* ($n \geq 3$, relative to EtOH control); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ from Student's *t* test/ANOVA

member of the α/β hydrolase fold family, and its expression has been described to be associated with obesity [38–40], adipocyte size [37], and preadipocyte proliferation [41] in mouse and human studies. *MEST* knock-out mice showed reduced body weight and less obesity. *Mest* expression has been associated with variable obesity in mice and is attenuated by a positive energy balance [42]. High *Mest* expression was found in high-gainers even at only 1 week of high fat diet and may therefore be able to foreshadow food metabolism capacity in mice [43–45].

Recently, a link between prenatal BPA exposure and an epigenetic modification in the imprinted *Mest* gene was observed in a murine study. Trapphoff et al. reported hypomethylation of the *Mest* promoter due to BPA exposure in murine oocytes [46]. Perinatal BPA exposure interferes with DNA methyltransferase 3a/3b (DNMT3A/DNMT3B) expression in mice, specifically affecting the de novo methylation of imprinted genes [47], which might be a contributing factor to the observed hypo-methylation. In this study, we show for the first time that also in humans prenatal BPA exposure is related to DNA methylation changes in the *MEST* promoter. It was already suggested that *MEST* methylation levels are associated with obesity risk in humans [48, 49]. Thus, our observed hypo-methylation in the *MEST* promoter may link prenatal BPA exposure to the overweight development in the offspring. In line with this hypothesis, we showed that *MEST* expression was associated with BMI increase on a longitudinal scale.

Although the observed methylation difference in the *MEST* promoter between BPA high and low exposed children in cord blood samples was only 1%, we nevertheless believe that this very small difference in the

methylation status could be of biological relevance. *MEST* is expressed in mesenchymal tissue and also in MSCs, which are the source of adipose tissue, but not in blood cells. Since cord blood contains a sizeable number of MSCs, we suppose that the observed BPA-related hypo-methylation in the cord blood samples of our study relates to an expansion in the cord blood MSC fraction and *MEST* activation. Unfortunately, we were not able to test this hypothesis within our LINA study due to limited cell availability. However, data from an earlier study may support the idea that changes in specific cell populations in response to environmental exposure might be the cause of small DNA methylation differences observed in whole blood samples and, moreover, might be also of biological relevance if this particular cell population is involved in pathophysiology [30].

Since we were not able to isolate and test MSCs from our study participants, we applied an in vitro model to analyze the impact of BPA on MSCs. In adipocytes differentiated from BPA-exposed human MSCs, we showed a hypo-methylation of the *MEST* promoter region and an enhanced *MEST* expression. Although the applied BPA concentrations in this experimental model were much higher compared to the real exposure situation in humans, these data nevertheless may support the hypothesis that BPA induces *MEST* activation in human MSCs, which further corroborates a role of *MEST* in BPA-induced adipogenesis.

A limitation of this study is the missing information about maternal weight before and during pregnancy as a potential confounding factor. Further, BPA concentrations were measured in spot urine samples. BPA concentrations vary widely throughout the day and spot urine BPA concentrations only reflect exposure of the last 4–6 h [7]. Moreover, we cannot exclude the possibility of BPA contaminations introduced by tubing or reaction tubes during the storage and analytical procedure as pointed out to be critical by recent publications [50, 51]. However, samples were all stored in the same tubes and were analyzed at the same time, suggesting rather a systematic overestimation of the BPA concentration than a random contamination effect. The strength of our study is the combination of epidemiological data with in vivo and in vitro experimental models. For the first time, we performed a genome-wide DNA-methylation analysis in the cord blood of prenatally BPA-exposed children and found an epigenetic link between BPA exposure and overweight development.

Conclusion

In conclusion, our study demonstrates that prenatal BPA exposure seems to be a contributing factor in the development of an early overweight phenotype by implicating epigenetic changes in the obesity-related gene *MEST*.

Additional files

Additional file 1: Table S1. Primer for gene expression analysis. **Table S2.** Mediator model for the association of prenatal BPA exposure with cord blood *MEST* DNA methylation and expression (according to Fig. 1c). **Table S3.** Mediator model for the association of prenatal BPA exposure with cord blood *MEST* DNA methylation and children's BMI *z* scores at year 1 (according to Fig. 3a). **Table S4.** Mediator model for the association of cord blood *MEST* DNA methylation and children's BMI *z* scores at years 1 and 6 (according to Fig. 3b). (DOCX 45 kb)

Additional file 2: Figure S1. Shown are the location of the *MEST* gene on chromosome 7 (upper part), the 450 K array CpG in the *MEST* promoter (middle part) and the region covered by the MassARRAY amplicon within the promoter region (CpG sites are depicted in red). (PDF 32 kb)

Additional file 3: Figure S2. BPA effect on weight development assessed in a murine in vivo model stratified for gender. Shown are means and standard deviation from $n \geq 8$ mice/group for all, female and male mice separately. *p* values are derived from ANOVA. (TIFF 1362 kb)

Additional file 4: Figure S3. MTT assay: MTT test for cell viability after exposure to BPA and the solvent control EtOH (0.05%), normalized to unexposed control, Student's *t* test $*p < 0.05$, mean ± SD, $n = 3$. (JPEG 105 kb)

Additional file 5: Figure S4. Summary scheme: results overview and hypothesis indicating the influence of prenatal BPA exposure on *MEST* methylation and expression that is associated with adipocyte differentiation and overweight development in infant offspring. (PNG 19 kb)

Abbreviations

95% CI: 95% confidence interval; BMI: Body mass index; BPA: Bisphenol A; Con: Control; DNMT: DNA methyltransferase; EDC: Endocrine-disrupting chemicals; *ESR1*: *Estrogen receptor alpha*; EtOH: Ethanol; *FASN*: *Fatty acid synthase*; *GAPDH*: *Glycerinaldehyd-3-phosphat-dehydrogenase*; gDNA: Genomic DNA; GEE: Generalized estimating equation; *GUSB*: *Glucuronidase beta*; *IRS2*: *Insulin receptor substrate 2*; *LEP*: *Leptin*; *LPL*: *Lipoprotein lipase*; *MEST*: *Mesoderm specific transcript*; MR: Mean ratio; MSC: Mesenchymal stem cells; MWU: Mann-Whitney *U* test; *PPARγ*: *Peroxisome proliferator-activated receptor gamma*; RR: Risk ratio; *SREBF1*: *Sterol regulatory element-binding factor 1*; UGT: UDP-glucuronosyltransferase; WHO: World Health Organization

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Availability of data and materials

The datasets regarding the LINA cohort (human data) generated and/or analyzed during the current study are not publicly available due to limited consent of the study participants but are available from the corresponding author on reasonable request. The datasets regarding in vivo mouse work as well as in vitro analysis used and/or analyzed during the current study are available from senior author Tobias Polte on reasonable request.

Authors' contributions

BL, KJ, LT, KL, and AK performed and/or coordinated the experimental work. LT, KG, BL, TB, NI, MS, MBH, and RE performed the epigenetic analysis. DW, RF, and MvB conducted the BPA measurement in LINA. SJ, AS, and TP planned and performed the mouse experiments. SR, MB, and IL collected the data and provided the proband material. KJ, BL, LT, ST, TP, and IL prepared the initial manuscript and figures. IL, RE, KJ, ST, GS, WK, and MvB provided project leadership. All authors contributed to the final manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The human study was approved by the Ethics Committee of the University of Leipzig (file ref. # 046-2006, #206-12-02072012). Participation in the human study was voluntary and written informed consent was obtained from the parents of the participating children.
The Committee on Animal Welfare of Saxony approved animal protocols used in this study.

Consent for publication

Not applicable—no individual patient data is reported.

Competing interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

Maternal cytokine status may prime the metabolic profile and increase risk of obesity in children

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BACKGROUND: The maternal inflammation status during pregnancy has been associated with metabolic imprinting and obesity development in the child. However, the influence of the maternal Th2 cytokines, interleukin-4 (IL4), IL5 and IL13, has not been studied so far.

METHODS: We investigated the relationship between maternal innate (IL6, IL8, IL10 and tumor necrosis factor-α (TNFa)) and adaptive (interferon-γ, IL4, IL5 and IL13) blood cytokine levels at 34 weeks of gestation and children's overweight development until the age of 3 years in 407 children of the German longitudinal LINA (Lifestyle and Environmental Factors and their Influence on Newborns Allergy risk) cohort. Children's body weight and height were measured during the annual clinical visits or acquired from questionnaires. Body mass index (BMI) Z-scores were calculated according to the WHO reference data to adjust for child's age and gender. Cytokine secretion was stimulated with phytohemagglutinin or lipopolysaccharide and measured by cytometric bead assay. Furthermore, we assessed metabolic parameter in blood of 318 children at age 1 using the AbsoluteIDQ p180 Kit (Biocrates LIFE Science AG).

RESULTS: Applying logistic regression models, we found that an increase of maternal IL4 and IL13 was associated with a decreased risk for overweight development in 1- and 2-year-old children. This effect was consistent up to the age of 3 years for IL13 and mainly concerns children without maternal history of atopy. Children's acylcarnitine concentrations at 1 year were positively correlated with maternal IL13 levels and inversely associated with the BMI Z-score at age 1.

CONCLUSIONS: We were able to show for the first time that the maternal Th2 status may be linked inversely to early childhood overweight development accompanied by an altered metabolic profile of the fetus. However, our data do not support a direct mediating role of acylcarnitines on maternal IL13-induced weight development.

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INTRODUCTION

There is evidence that many early childhood diseases are already primed in the highly sensitive prenatal time window by the *in utero* environment. In particular, maternal inflammatory states during pregnancy have been associated with the onset of civilization diseases like asthma or allergies.[1–3] But also for the high prevalence of childhood obesity the maternal immune status during pregnancy has been discussed as a contributing factor.[4–6] However, data on the involvement of the innate (interleukin-6 (IL6), IL8, IL10 or tumor necrosis factor-α (TNFα)) or adaptive (interferon-γ (IFNγ), IL4, IL5 or IL13) immune status is inconsistent or even missing out some important associations in general: For example, Dahlgren *et al.*[7] found that increased maternal IL6 was associated with overweight development in rat offspring, whereas Danielsen *et al.*[8] observed no association between maternal inflammatory markers like TNFα, IL1β and IL6 and overweight development in their 20-year-old children. Interestingly, whether the maternal Th2 cytokines, IL4, IL5 and IL13, during pregnancy may have a role in children's obesity development has not been studied so far.[2]

The hypothesis that these cytokines (IL4, IL5 and IL13) may act in the context of obesity/adipogenesis at all, was derived from results of mechanistic studies. Obesity is characterized by a chronic inflammatory state of the adipose tissue with impact also on the systemic inflammation status. In adipose tissue of lean subjects, all of the three cytokines have been described to function as anti-inflammatory. In detail, IL4 was able to stabilize local immune response, maintain insulin sensitivity or limit further adipogenesis.[9,10] Next to IL4, IL13 production has been shown to limit diet-induced inflammation and insulin resistance in mice.[11]

In addition to a potential weight-related effect of the maternal cytokines, obesity has been described to be associated with other early life systemic parameters. It was shown that obesity-related changes of the metabolite profile in adults may also be primed during fetal and early childhood development. The metabolite profile comprises information on metabolic processes including for example disease-triggering changes of lipid metabolism in obesity and may thereby help to understand underlying pathophysiological mechanisms in the onset of the disease. Not surprisingly, owing to fatty acid metabolism within the mitochondria, an impaired mitochondrial function has been associated with obesity and type-2 diabetes mellitus. As a consequence thereof

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altered carnitine concentrations, as main transporters of fatty acids into the mitochondria, have been found. Increased levels of short and intermediate carnitines indicate impaired mitochondrial function.[12,13] Consequently, increased plasma carnitine levels have been associated with obesity and type-2 diabetes mellitus in adults.[14] However, few studies exist on metabolite alterations in childhood obesity[15–18] and to our knowledge there is no study that examines the relationship between prenatal cytokine exposure and metabolome priming in children.

Therefore, our aim was to investigate the influence of maternal innate (IL6, IL8, IL10 and TNFα) and adaptive (IFNγ, IL4, IL5 and IL13) cytokine blood concentrations on the weight development of their children up to the age of 3 in the German prospective birth cohort LINA (Lifestyle and Environmental Factors and their Influence on Newborns Allergy risk). Further, we wanted to examine the potential association between maternal cytokine concentrations and the early infant metabolic profile at 1 year of age.

MATERIALS AND METHODS

Study characteristics

The prospective birth cohort LINA recruited 622 pregnant women at 34th weeks of gestation (7 twin-pairs, finally 629 mother–child pairs) between May 2006 and December 2008 in Leipzig, Germany.[19] Annually thereafter questionnaires about life style factors and health outcomes of children were collected, clinical parameters assessed, as well as blood samples collected during clinical visits. All questionnaires were self-administered by the parents. Participation in the study was voluntary and written informed consent was obtained by all participants. The study was approved by the Ethics Committee of the University of Leipzig (file ref # 046–2006, 160–2008, 160b/2008).

Anthropometric data

Children's body weight and height were measured during the annual clinical visits or were obtained from regular preventive medical check-ups asked for in the questionnaire. Child length was measured horizontally at birth and at year 1 follow-up. Standing height was measured from year 2 onward to the nearest 0.1 cm. Body weight was also measured to the nearest 0.1 kg, with body mass index (BMI) Z-scores calculated according to the WHO reference data to adjust for child's age and gender. Children with BMI Z-score < -1 were classified as underweight ($<$ 5th pecentile or corresponding adult BMI of < 19 kg m^{-2}), children with a BMI Z-score $\geqslant -1$ and < 1 were classified normal weight (corresponding adult BMI of < 25 kg m^{-2}) and children with BMI Z-scores $\geqslant 1$ were classified as overweight ($\geqslant$85th percentile or corresponding adult BMI of $\geqslant 25$ kg m^{-2}).

Cytokine measurement in cord blood

At the 34th week of gestation, maternal blood samples were collected. For cytokine concentration measurements, whole blood samples (500 µl) were incubated for 4 h at 37 °C with either phytohemagglutinin (50 µg ml^{-1}; Sigma Aldrich, Hamburg, Germany) for IL4, IL5, IL13 (Th2) and IFNγ (Th1) assessment or lipopolysaccharide (1 µg ml^{-1}, *E. coli* 026:B6; Sigma Aldrich) for IL6, IL10, IL8 and TNFα (inflammatory status) assessment. Cytokine concentrations were measured by flow cytometry using a cytometric bead array (BD CBA Human Soluble Flex Set system, Becton Dickinson, Heidelberg, Germany) as described before.[1]

Metabolome analysis

The metabolic profile was assessed in blood sera of 1-year-old children using the AbsoluteIDQ p180 Kit (Biocrates LIFE Science AG, Innsbruck, Austria) as described in more detail elsewhere.[20] Metabolome measurements were available from 318 children.

Statistical analysis

A LINA subcohort was defined with complete data for all cytokine and body weight measurements and follow-up questionnaires up to age 3 (including data regarding confounding factors) and maternal cytokine analyses during pregnancy ($n = 407$). A χ^2 test was applied to determine differences in the distribution of general study characteristics distribution between the entire LINA cohort and the analyzed subcohort. Owing to the fact that measured cytokine and metabolic parameters were not normally distributed, tests for non-normally distributed samples were performed (Mann–Whitney *U*-test, Spearman correlation). A Student's *t*-test was applied for comparison of normal distributed BMI Z-scores. To assess the effect of maternal cytokines on children's overweight development, adjusted logistic regression models were applied. Confounding parameters (gender, birth weight, birth season, early delivery, smoking during pregnancy, parental school education, number of household members,

Table 1. LINA study characteristics

	Entire LINA cohort n *(%),* n = *629*[a]	*Analyzed subcohort* n *(%),* n = *407*	χ^2-*test*[b]
Gender of the child			0.832
Female	302 (48.0)	203 (49.9)	
Male	327 (52.0)	204 (50.1)	
Birth weight			0.990
$\leqslant$3000 g	123 (19.6)	75 (18.4)	
>3000–3500 g	242 (38.5)	162 (39.8)	
>3500–4000 g	192 (30.6)	128 (31.4)	
>4000 g	71 (11.3)	42 (10.3)	
Birth season			0.806
Summer	346 (55.0)	222 (54.5)	
Winter	269 (42.8)	185 (45.5)	
Week of gestation at delivery			0.997
<37 weeks	25 (4.0)	17 (4.2)	
37–40 weeks	389 (62.0)	252 (61.9)	
>40 weeks	214 (34.0)	138 (33.9)	
Smoking during pregnancy			0.374
Never	534 (84.9)	369 (90.7)	
Occasionally	47 (7.4)	24 (5.9)	
Daily	48 (7.6)	14 (3.4)	
Parental school education[c]			0.661
Low	16 (2.5)	5 (1.2)	
Intermediate	144 (22.9)	80 (19.7)	
High	469 (74.6)	322 (79.1)	
Household members			0.985
2	33 (5.5)	23 (5.7)	
3	365 (60.7)	251 (61.7)	
>4	203 (33.8)	133 (32.7)	
Breastfeeding exclusive			0.679
1–3 months	87 (13.8)	58 (14.2)	
1–6 months	155 (24.6)	115 (28.3)	
1–12 months	253 (40.2)	234 (57.5)	
Introduction of solid food			0.728
1–3 months	23 (3.9)	12 (2.9)	
4–6 months	251 (43.5)	159 (39.1)	
7–12 months	305 (52.7)	236 (58.0)	
Pregnancy IgE level			0.964
<20 kU l^{-1}	268 (42.6)	169 (41.5)	
20–100 kU l^{-1}	238 (37.8)	152 (37.3)	
>100 kU l^{-1}	123 (19.6)	86 (21.1)	

Abbreviation: LINA, Lifestyle and Environmental Factors and their Influence on Newborns Allergy risk. [a]*n* may be different from 629 because of missing data. [b]Calculated using the χ^2 test for cross relationship. [c]Low = 8 years of schooling ('Hauptschulabschluss'); intermediate = 10 years of schooling ('Mittlere Reife'); high = 12 years of schooling or more ('(Fach-) hochschulreife').

breastfeeding, solid food introduction and pregnancy IgE levels) were selected after a literature review according to their influence on weight development and maternal cytokine status. Parameters that were originally not normally distributed were log-transformed for inclusion in the logistic regression model (cytokines, pregnancy IgE). For the analysis of metabolome data, a Bonferroni post-hoc correction of the *P*-value was applied ($P < 4.63 \times 10^{-4}$). Mediator analyses were performed with PROCESS macro (version 2.16.3, www.processmacro.org) for IBM SPSS (version 22, IBM Corps., Armok, NY, USA), using model 4 with 1000 bootstrap samples to determine bias corrected bootstrap confidence intervals and adjusted for the confounders used in the logistic regression models (as described in Hayes[21]).

RESULTS

General study characteristics

General study characteristics are given in Table 1. For 407 children, complete data on maternal cytokine status during pregnancy, body weight development after birth and confounding variables were available. There were no differences regarding gender, birth weight, birth season, week of gestation at delivery, parental school education, breastfeeding, introduction to solid food, smoking during pregnancy and pregnancy IgE level between the total LINA cohort ($n = 629$) and the analyzed subcohort ($n = 407$), therefore all following analyses were based only on this subcohort. Children's BMI Z-scores from year 1 to year 3 are shown in Table 2. Overall >69% of children were normal weight and 17% were overweight within the first 3 years of age, with a peak of 26.3% overweight children at 2 years of age. Under weight children were not considered for this analysis and therefore only results comparing normal weight and overweight are presented.

Maternal cytokines and offspring BMI

Range and distribution of maternal cytokine levels in normal and overweight children are shown in Table 3. Median values of IL4 and IL13 were significantly lower in the overweight group at year 1 and year 2, whereas IL13 and IFNγ levels were significantly lower in the overweight group at year 3. Adjusted logistic regression models were applied to test for the association between maternal cytokines and body weight of the child (Figure 1a and Supplementary Table S1). Models were adjusted to confounding parameters that have been associated with overweight development in earlier studies (birth weight, early delivery, smoking during pregnancy, parental school education, household members, breastfeeding and introduction of solid food) and maternal cytokine status (birth season and pregnancy IgE level). High maternal IL4 levels were associated with a reduced risk for overweight development at year 1 (adjusted odds ratio (adjOR) 0.52 (0.28, 0.97)) and year 2 (adjOR: 0.63 (0.42, 0.97)). High maternal IL13 levels reduced the risk for overweight development persistently from year 1 to year 3 (adjOR year 1: 0.57 (0.33, 0.97), adjOR year 2: 0.68 (0.47, 0.98),

Table 2. Body weight distribution in the analyzed LINA subcohort

BMI Z-score		*Year 1 Sub-cohort*[a] n = *407 (%)*	*Year 2 Sub-cohort*[a] n = *407 (%)*	*Year 3 Sub-cohort*[a] n = *407 (%)*
< −1	Under weight	94 (23.1)	26 (6.4)	41 (10.1)
−1 to <1	Normal weight	278 (68.3)	274 (67.3)	298 (73.2)
> 1	Overweight	35 (8.6)	107 (26.3)	68 (16.7)

Abbreviations: BMI, body mass index; LINA, Lifestyle and Environmental Factors and their Influence on Newborns Allergy risk. [a]No differences in comparison with the entire LINA cohort were observed using the χ^2 test for cross relationship.

Table 3. Distribution of maternal blood cytokine levels in normal and overweight children

	Year 1		*Year 2*		*Year 3*	
	Normal weight (n = *278*)	*Overweight* (n = *35*)	*Normal weight* (n = *274*)	*Overweight* (n = *107*)	*Normal weight* (n = *298*)	*Overweight* (n = *68*)
PHA stimulated (adaptive)						
IFNγ	428.0 (228.8, 928.9)	334.8 (160.3, 661.1)	432.0 (231.1, 872.5)	366.6 (195.3, 941.0)	**432.0 (231.1, 883.6)**	**258.9 (171.5, 804.3)***
IL4	**15.1 (10.7, 22.1)**	**12.3 (9.4, 14.7)****	**15.6 (11.2, 21.7)**	**13.3 (9.4, 19.0)***	15.0 (10.7, 21.6)	13.1 (10.2, 17.7)
IL5	3.2 (1.5, 4.0)	2.9 (1.5, 3.7)	3.2 (2.1, 4.0)	3.3 (1.5, 4.0)	3.2 (1.5, 4.0)	3.4 (1.5, 3.9)
IL13	**22.1 (14.8, 34.3)**	**16.3 (13.9, 22.7)***	**21.8 (15.4, 34.3)**	**18.6 (13.3, 27.4)***	**21.8 (14.8, 34.6)**	**18.0 (13.6, 25.4)****
LPS stimulated (innate)						
TNFα	1729 (1296, 2653)	2474 (1331, 3454)	1761 (1235, 2784)	1865 (1409, 3142)	1760 (1250, 2809)	1952 (1400, 2937)
IL6	9809 (6937, 12 675)	8693 (6451, 145 957)	10 023 (6771, 12 955)	9159 (7458, 12 907)	10 023 (6937, 12 821)	8400 (66 676, 12 284)
IL8	2912 (2250, 4259)	3184 (2057, 5387)	2857 (2223, 4259)	3030 (2144, 4184)	2882 (2223, 4150)	3072 (2162, 4850)
IL10	12.7 (6.7, 24.3)	13.8 (7.9, 33.5)	12.4 (6.5, 22.8)	11.6 (7.6, 30.7)	12.0 (6.8, 23.8)	15.1 (7.8, 33.5)

Abbreviations: IFNγ, interferon-γ; IL, interleukin; LPS, lipopolysaccharide; PHA, phytohemagglutinin; TNFα, tumor necrosis factor-α. Significantly different values from Mann–Whitney *U*-test are presented in bold with $*P < 0.05$ and $**P < 0.01$. Pregnancy cytokine concentrations (pg l^{-1}) are presented as median values with interquartile range in brackets for normal weight and overweight children.

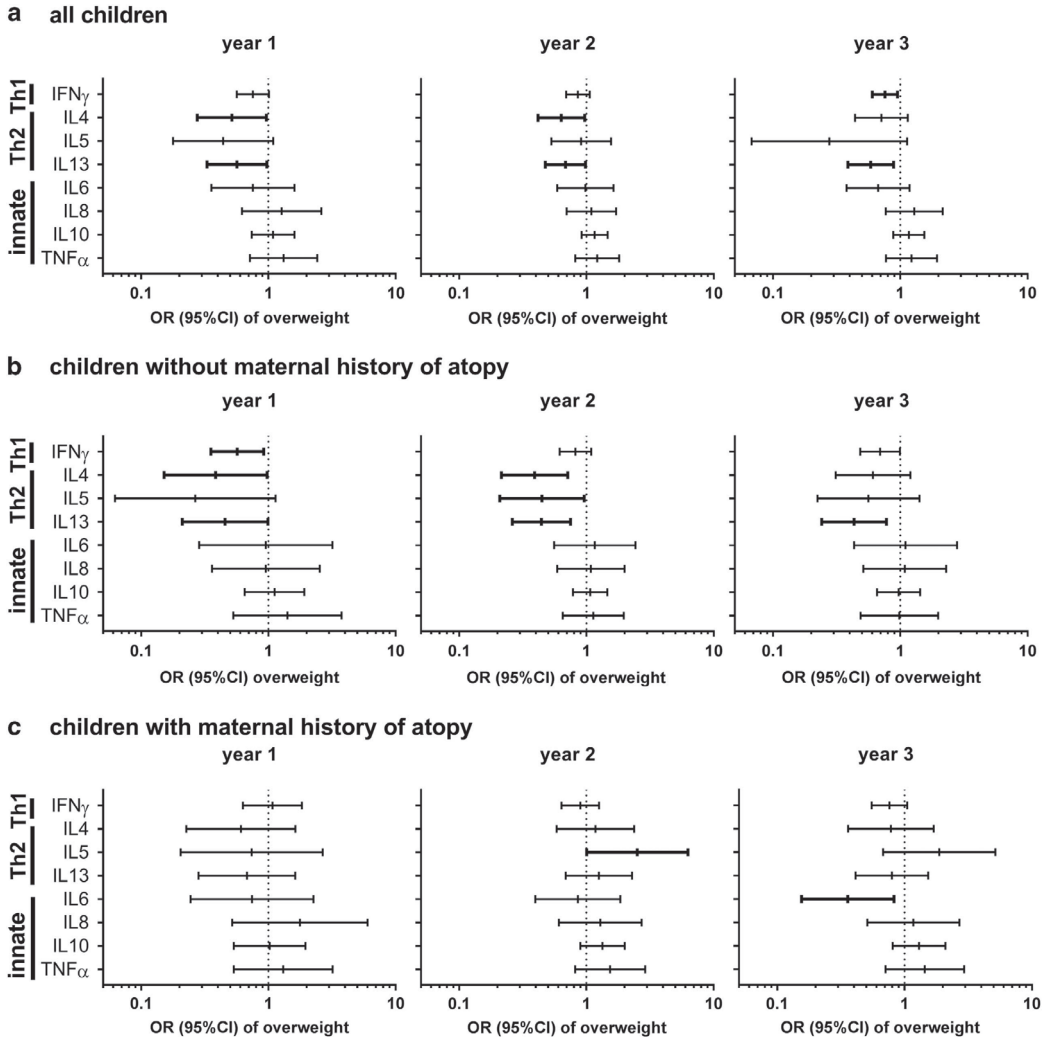

Figure 1. Effect of maternal cytokine concentrations during pregnancy on weight development in children. (**a**) Influence of prenatal cytokine exposure on overweight development until the age of 3 years. Shown are odds ratios with 95% confidence interval (OR, 95% CI) in a logistic regression model adjusted for gender, birth weight, birth season, early delivery, smoking during pregnancy, parental school education, household members, breastfeeding, introduction of solid food and pregnancy IgE level. Significant associations are presented in bold ($P < 0.05$). (**b**) Logistic regression model only considering children without maternal history of atopy. (**c**) Logistic regression model only considering children with maternal history of atopy.

adjOR year 3: 0.58 (0.30, 0.88)) and high IFNγ levels in pregnancy were associated with reduced risk for overweight development at year 3 only (adjOR: 0.76 (0.60, 0.95)). Importantly, this association was only found in children without maternal history of atopy (Figure 1b and Supplementary Table S1) with the most persistent effect for high maternal IL13 level. High pregnancy IL13 level in non-atopic mothers reduced the risk for overweight development in their children up to the age of 3 years (adjOR year 1: 0.46 (0.21, 0.99), adjOR year 2: 0.44 (0.26, 0.75), adjOR year 3: 0.43 (0.24, 0.78)).

Children of atopic mothers had an increased risk for being overweight at 2 and 3 years of age when exposed to higher maternal IL5 (adjOR: 2.52 (1.00, 6.3)) and lower IL6 level (adjOR: 0.36 (0.16, 0.82)), respectively (Figure 1c and Supplementary Table S1).

As the most dominant and long-term stable association was seen for IL13, we calculated the mean BMI Z-score for children exposed to low (first quartile) or high (fourth quartile) maternal

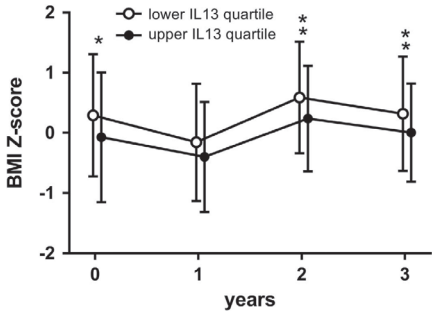

Figure 2. Weight distribution of children exposed to high (fourth quartile) and low (first quartile) prenatal IL13 levels from birth to age 3. Shown are BMI Z-scores as mean ± s.d. with $*P < 0.05$, $**P < 0.01$.

Table 4. Correlation of maternal cytokine level during pregnancy and acylcarnitines of children at year 1

	PHA stimulated (adaptive)							
	IFNγ		*IL4*		*IL5*		*IL13*	
(A) Acylcarnitines	*R*	P-*value*	*R*	P-*value*	*R*	P-*value*	*R*	*P-value*
C0	0.10	6.34×10^{-2}	0.15	8.85×10^{-3}	−0.10	7.33×10^{-2}	0.18	1.63×10^{-3}
C10	0.15	9.13×10^{-3}	0.14	1.16×10^{-2}	−0.16	3.75×10^{-3}	0.23	$\mathbf{4.51\times10^{-5}}$
C10:1	0.11	5.10×10^{-2}	0.15	7.28×10^{-3}	−0.17	3.14×10^{-3}	0.22	$\mathbf{9.36\times10^{-5}}$
C12-DC	0.11	4.73×10^{-2}	0.18	1.23×10^{-3}	−0.17	2.48×10^{-3}	0.29	$\mathbf{2.38\times10^{-7}}$
C14:1	0.10	7.01×10^{-2}	0.03	6.06×10^{-1}	−0.15	8.88×10^{-3}	0.12	3.75×10^{-2}
C18:1	0.10	8.88×10^{-2}	0.15	7.19×10^{-3}	0.01	8.24×10^{-1}	0.19	6.19×10^{-4}
C2	0.15	6.22×10^{-3}	0.11	5.84×10^{-2}	0.04	4.57×10^{-1}	0.10	7.47×10^{-2}
C3	0.08	1.58×10^{-1}	0.16	3.98×10^{-3}	−0.07	2.20×10^{-1}	0.22	$\mathbf{1.10\times10^{-4}}$
C4	0.10	6.78×10^{-2}	0.17	2.39×10^{-3}	−0.13	1.76×10^{-2}	0.24	$\mathbf{1.18\times10^{-5}}$
	LPS stimulated (innate)							
	IL6		*IL8*		*IL10*		*TNFα*	
(B) Acylcarnitines	*R*	P-*value*	*R*	P-*value*	*R*	P-*value*	*R*	P-*value*
C0	0.10	6.34×10^{-2}	−0.01	8.90×10^{-1}	−0.23	$\mathbf{2.74\times10^{-5}}$	−0.14	1.35×10^{-2}
C10	0.15	9.13×10^{-3}	0.04	4.54×10^{-1}	−0.19	5.87×10^{-4}	−0.01	9.04×10^{-1}
C10:1	0.11	5.10×10^{-2}	0.04	4.40×10^{-1}	−0.18	1.38×10^{-3}	−0.05	3.75×10^{-1}
C12-DC	0.11	4.73×10^{-2}	−0.01	8.17×10^{-1}	−0.29	$\mathbf{1.12\times10^{-7}}$	−0.07	2.21×10^{-1}
C14:1	0.10	7.01×10^{-2}	0.03	5.48×10^{-1}	−0.11	5.68×10^{-2}	0.04	5.04×10^{-1}
C18:1	0.10	8.88×10^{-2}	−0.09	1.21×10^{-1}	−0.23	$\mathbf{3.01\times10^{-5}}$	−0.11	4.19×10^{-2}
C2	0.15	6.22×10^{-3}	0.11	5.20×10^{-2}	0.00	9.37×10^{-1}	−0.10	8.28×10^{-2}
C3	0.08	1.58×10^{-1}	−0.02	7.15×10^{-1}	−0.22	$\mathbf{7.95\times10^{-5}}$	−0.19	8.85×10^{-4}
C4	0.10	6.78×10^{-2}	0.01	9.25×10^{-1}	−0.24	$\mathbf{1.88\times10^{-5}}$	−0.12	3.53×10^{-2}

Abbreviations: IFNγ, interferon-γ; IL, interleukin; LPS, lipopolysaccharide; PHA, phytohemagglutinin; TNFα, tumor necrosis factor-α. Significantly different values are presented in bold with $P < 4.63\text{x}10^{-4}$. Shown are Spearman correlation coefficients (R) and *P*-values.

IL13 levels and found significantly lower BMI Z-scores from birth until the age of 3 years for children exposed to high maternal IL13 levels with $P = 0.015$ (birth), $P = 0.007$ (24 months) and $P = 0.012$ (36 months), respectively (Figure 2).

Maternal cytokines and offspring metabolic profile

Furthermore, we assessed the metabolic profile of the 1-year-old children with regard to maternal cytokine production. Following correction for multi-testing ($P < 4.63\times10^{-4}$), we could show a positive association between maternal IL13 and acylcarnitine levels of C10, C10:1, C12-DC, C3 and C4, whereas maternal IL10 negatively correlated with acylcarnitines (C0, C10, C12-DC, C18:1, C3, C4) (Table 4). Other metabolic parameters like amino acids, lyso phosphatidyl cholines, phosphatidyl cholines, sphingolipids and sugars were not correlated to maternal cytokine levels (Supplementary Figure S1).

Offspring BMI and metabolic profile

Increased BMI Z-score at year 1 were associated with low levels of acylcarnitines at the same age (C10:1, $P = 0.0472$; C14:1, $P = 0.0021$; C18:1, $P = 0.0005$; C2, $P = 0.0217$). Acylcarnitines C0 and C2 at year 1 were also negatively correlated with the BMI Z-score at year 2 (C0 $P = 0.005$, C2 $P = 0.020$). There was no association of acylcarnitines with the BMI Z-score at year 3.

Further, we wanted to address a potential mediating effect of acylcarnitines on overweight development in response to low maternal IL13 levels in the prenatal period. When acylcarnitines were introduced in the logistic regression model as a confounding factor, the association between low maternal IL13 and overweight development remained significant with no significant effect of the acylcarnitines. Applying a mediator analysis (bootstrapping mediator analysis with the PROCESS tool in SPSS), a mediating effect of acylcarnitines (C10:1 CI (−0.197, 0.107), C14:1 CI (−0.140, 0.064), C18:1 CI (−0.272, 0.011) and C2 CI (−0.114, 0.010)) could not be found.

DISCUSSION

The early prenatal time period is described as a highly sensitive time window in terms of priming the unborn fetus for later disease development. Either changes in external/environmental[3,22–24] or internal/immune factors[1,2,25] were shown to be associated with an altered risk for the child to develop civilization diseases like allergies or obesity. However, little is known about the influence of the maternal immune status during pregnancy on the weight development of their children, especially for the contribution of Th2 cytokines. Therefore, our aim was to investigate the influence of maternal Th1 and Th2 cytokine blood concentrations on the weight development of their children up to 3 years in the German prospective birth cohort LINA. Here we report for the first time an association between the maternal pregnancy Th2 cytokine level, overweight development and metabolic alterations in children. Especially low maternal level of IL4 and IL13 were related to an increased risk of overweight development in children up to year 3. Both cytokines have an anti-inflammatory function in adipose tissue and are representative for the lean Th2 phenotype of adipose tissue in adults. Furthermore, IL4 has been directly linked to adipogenesis, with IL4 inhibiting terminal differentiation of pre-adipocytes *in vitro* via a signal transducer and activator of transcription 6 (STAT6)-mediated pathway.[9] However, as there is evidence from mouse experiments that both IL4 and IL13 may not be able to pass the placental barrier,[26] a direct influence of maternal IL4 and IL13 on adipogenesis seems to be questionable. Nevertheless, it has been shown that cytokines are able to influence the nutrition release from the placenta by binding to

specific receptors at the placental blood barrier,[27] thereby changing the prenatal environment and having an indirect influence on the development of the fetus.[28]

As overweight development and obesity are related to changes of the metabolite profile we have also assessed the potential priming effect of maternal cytokine concentrations on early infant metabolic programming. We found positive associations of maternal Th2 cytokines, in particular IL13, and acylcarnitines in children and an inverse association of acylcarnitine levels and BMI Z-scores. Although higher acylcarnitine levels in adults with obesity have been reported, studies on metabolic profiles in overweight children under 5 years are sparse. Acylcarnitines transport fatty acids into the mitochondria for β-oxidation, a process that is often impaired in patients with obesity and accompanied by a reduced oxidatively mitochondrial capacity[29] and increased intracellular acylcarnitine levels. In order to prevent intracellular carnitine-related cytotoxicity, carnitines are secreted from muscle or adipose tissue into the blood, leading to increased blood carnitine levels in patients with obesity.[13] Nevertheless, infantile mitochondria are much more flexible and can compensate for increased fatty acid metabolism, potentially resulting in overcompensation with decreased acylcarnitine levels observed in overweight children.[30] Although we found an association between maternal IL13 and children's acylcarnitine levels, our data do not support a mediating role of acylcarnitines on maternal IL13-induced weight development. One explanation could be that acylcarnitine levels could be altered as a secondary marker because of a different lipid metabolism in patients with obesity and just picture children's overweight state with no causal direct effect via IL13.

Furthermore, we observed that children without maternal history of atopy are more prone to overweight development when exposed *in utero* to low levels of IL4 and IL13 compared to children with maternal history of atopy. We hypothesized that atopic mothers have higher intrinsic IL4 and IL13 levels because of their allergic disease status and therefore do not reach the low overweight associated cytokine levels, but this could not be approved in our data.

The strength of our study is the combination of epidemiological data focusing on a qualitative immune status assessment in the highly vulnerable prenatal period, children's postnatal body weight development, as well as early infant metabolome analyses. This offers the possibility to decipher a potential priming effect of the maternal immune status during pregnancy on children's longitudinal body weight development, as well as weight-related metabolomic alterations. A limitation of this study is the low case number of children with obesity. Therefore, we have grouped children with BMI Z-scores $\geqslant 1$ (≥85th percentile) and children with a BMI Z-score $\geqslant 2$ (≥97th percentile) together in our analyses as 'overweight'. Another limitation in our LINA study is the missing information about maternal weight before and during pregnancy, which could act as a potential confounding factor representing family overweight/obesity predisposition. Therefore, we cannot exclude the possibility that altered pregnancy cytokine levels and children's metabolic state are subsequent to maternal obesity.

Taken together, we were able to show a potential priming effect of the maternal immune status during pregnancy on children's longitudinal body weight development, as well as weight-related metabolomic alterations in acylcarnitines.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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RESEARCH ARTICLE Open Access

Early maternal perceived stress and children's BMI: longitudinal impact and influencing factors

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Abstract

Background: Maternal perceived stress has been discussed to contribute to the development of childhood overweight. Our aim was to investigate the longitudinal relationship of early maternal perceived stress and BMI z-scores in preschool children (≤ five years).

Methods: A longitudinal analysis was conducted in 498 mother-child pairs of the German prospective birth cohort LINA with information on maternal perceived stress during pregnancy, one and two years after birth. BMI z-scores were based on annual measurements of children's weight/height and calculated based on WHO reference data. General estimation equations were applied to evaluate the impact of maternal stress on children's longitudinal BMI z-scores. Potential stressors contributing to the perceived stress of the mother were assessed by linear regression models. Using mediation analyses we evaluated the relationship between stressors, maternal perceived stress, and children's BMI z-score development.

Results: Postnatal maternal stress during the first year after birth had a positive longitudinal relationship with children's BMI z-scores up to the age of five years. Gender-stratified analyses revealed that only girls showed this positive association while boy's BMI z-scores were unaffected by maternal stress. We identified three neighborhood strains and two socio-demographic factors, which contributed to the maternal perceived stress level. Stressors themselves did not directly affect girl's BMI z-scores but rather mediated their effect through the perceived stress level.

Conclusions: While different stressors contribute to maternal stress, the perceived stress level - rather than the stressors themselves - is strongly positively associated with BMI z-score development in girls.

Keywords: Stress dimensions, Perceived stress, Weight development, Stressor, Infant, Preschool children

Background

Overweight and obesity prevalence, especially among preschool children, has risen dramatically world-wide over the last decades affecting 6.1% of children under five years of age in 2016 [1]. Recent data from the KiGGS study shows that in Germany this fraction is even higher with 9.5% of children age two-six being overweight, of which 2.8% are classified as obese [2]. This is particularly concerning as most of these children will remain overweight in adolescence and adulthood, increasing their risk for co-morbidities like cardiovascular diseases or type 2 diabetes mellitus [3, 4].

Unhealthy diet and physical inactivity have been described as the main risk factors contributing to obesity development [5]. Consequently, child obesity intervention and prevention studies are mainly focusing on implementing changes in eating behavior and physical activity. However, most of these studies failed to reach long-term effects [6, 7], suggesting that other factors such as the living environment and parental behavior play an important role in the context of children's overweight development. Among others, psychological aspects like early infant parental distress [8] as well as

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maternal depression [9] emerged as potential factors promoting children's overweight.

Accumulating research shows the wide-ranging consequences of maternal stress on children's health, which among others include an increased risk for behavioral problems, asthma, reduced birth weight, and an increased risk for becoming overweight [10–12]. Perceived stress is the individual perception about the stressfulness of life and the ability to handle such stress, which can be influenced by a variety of sources. These so-called stressors include socioeconomic disadvantages as well as recent life events like divorce/separation that can ultimately lead to stress-related physiological dysregulations [13, 14]. In this context maternal stress during pregnancy is known to alter signaling in the hypothalamic-pituitary-axis (HPA) exposing the developing fetus to an excess of glucocorticoids [15], one of the mechanisms discussed to contribute to prenatal growth restriction [16, 17] and an accelerated catch-up-growth increasing the risk for obesity in children's later life [18–20]. While in the prenatal phase the physiological stress response of the mother can directly affect the child, in the postnatal period - as assessed here in the first two years after birth - the impact of maternal stress on parenting behavior and mother-child interactions become important. There is evidence that changes in feeding styles and practices [21] due to parental or maternal stress can have a significant impact on children's food composition and energy intake [22]. Especially maternal stress becomes important as mothers often spend significantly more time in direct interaction with the child compared to the fathers. For example higher infant energy intake and increased consumption of breads and cereals during the first six month after birth have been described in association with maternal stress or depression [23], as has children's reduced consumption of fruits and vegetables [24, 25]. Moreover, stress perceived by children themselves seems to alter their energy intake and food selection with a preference for sweet and high fat foods [25].

The majority of studies conducted so far focused on prenatal or postnatal stress exclusively, while longitudinal maternal stress assessments in relation to children's weight development are rare. As recently reviewed by *Tate et al.* [12] and *O'Connor et al.* [26] most of these studies only used either longitudinal information on maternal stress or children's overweight development. Therefore, our aim was to investigate the association between maternal stress and body mass index (BMI) trajectories in children in a longitudinal manner, including maternal stress evaluations from pregnancy until children's age of two years and annual weight assessments of the children up to the age of five years. We complement our study by analyzing which stressors might contribute to the perceived maternal stress level and therefore might have a potential impact also on children's weight development throughout the years. It has been discussed that the living environment including noise exposure and an unsecure living environment influence weight development in adults [27–29]. Since data on the effects of such stressors on children is sparse [30] we included not only the socioeconomic status of the study participants, but also factors characterizing their living environment such as traffic or residential noise in our analysis.

We hypothesize that with our longitudinal qualitative stress assessment we will be able to identify a time window, in which the child is particularly vulnerable to maternal stress and that such stress exposure experienced in this time window might have a long-lasting effect on the development of overweight in the child.

Methods

Study characteristics

The German prospective birth cohort LINA (Lifestyle and Environmental Factors and their Influence on Newborns Allergy risk) recruited 629 mother-child pairs at pregnancy (36th week of gestation) during May 2006 and December 2008, as has been described in more detail elsewhere[31–33]. Lifestyle, housing, and environmental factors were assessed by questionnaires during pregnancy and annually thereafter. Stress questionnaires from three time points (pregnancy, age 1, age 2) together with information on gender, gestational week at delivery, mode of delivery, breastfeeding and prenatal environmental tobacco smoke exposure (ETS) were available for 498 mother-child pairs, which we defined as our analyzed sub-cohort (Table 1). All questionnaires were self-administered by the parents and participation in the study was voluntary. Written informed consent was obtained from all individual participants included in the study. The study was approved by the Ethics Committee of the University of Leipzig (file ref # 046-2006, 160-2008, 160b/2008, 144-10-31052010, 113-11-18042011).

Perceived maternal stress assessment

Maternal stress levels were assessed at 36th weeks of gestation and at the one- and two-year follow-up using the 20-item reduced Perceived Stress Questionnaire (PSQ), a validated instrument by *Fliege et al.* [34, 35]. The PSQ is comprised of 5 items for each of the different stress dimensions "demands", "tension", "worries", and "lack of joy". All items were scored on a four-point scale according to the frequency of perception with one (hardly ever) to four (usually). The total stress score was derived as the mean of all 20 scored questions. The scores for each dimension were derived accordingly from the 5 dimension-specific questions [36]. Higher scores indicate higher stress levels.

Table 1 General study characteristics of the LINA cohort

	entire LINA cohort n (%), $n = 629$ [a]	analyzed sub-cohort n (%), $n = 498$ [a]	χ^2-test
Gender			0.810
Male	330 (52.5)	253 (50.8)	
Female	299 (47.5)	245 (49.2)	
Week of gestation at birth			0.951
<37 weeks	25 (4.0)	16 (3.2)	
37-40 weeks	389 (61.8)	308 (61.8)	
> 40 weeks	214 (34.0)	174 (34.9)	
Mode of delivery			0.979
Spontaneous	471 (74.9)	387 (77.7)	
C-section	132 (21.0)	104 (20.9)	
Others	7 (1.1)	7 (1.4)	
Birth weight			0.996
< 3000g	123 (19.6)	92 (18.5)	
≥ 3000g – 3500g	242 (38.5)	195 (39.2)	
≥ 3500g – 4000g	192 (30.5)	151 (30.3)	
≥ 4000g	71 (11.3)	60 (12.0)	
Household members			0.887
2	33 (5.2)	26 (5.2)	
3	365 (56.6)	300 (60.2)	
≥4	203 (32.3)	196 (39.4)	
Breastfeeding			0.515
1.-3. month	112 (17.8)	87 (17.5)	
1.-6. month	268 (42.6)	166 (33.3)	
1.-12. month	254 (40.4)	226 (45.4)	
Parental education [b]			0.697
Low	16 (2.5)	6 (1.2)	
Medium	144 (22.9)	101 (20.3)	
High	468 (74.4)	391 (78.5)	
Household income / month			0.648
< 2000€	240 (38.2)	172 (34.5)	
2000€ - 4000€	308 (49.0)	171 (34.3)	
> 4000€	42 (6.7)	35 (7.0)	
Separation/divorce [c]			0.973
Yes	25 (4.0)	23 (4.6)	
No	169 (26.9)	158 (31.7)	
Prenatal ETS exposure [d]			0.243[e]
Median [μg/g creatinine]	2.0	1.85	
< 25% , > 75%	0.8,5.6	0.75,4.95	

[a] n may be different from total n due to missing data
[b] Low = 8 yrs of schooling ('Hauptschulabschluss`); medium = 10 yrs of schooling (`Mittlere Reife`); high = 12 yrs of schooling or more (`(Fach-)hochschulreife')
[c] Parental separation/divorce in the last 3 years from children's age 2 years
[d] *ETS* environmental tobacco smoke (urinary cotinine level at pregnancy)
[e] *p*-value derived from Student's *T* test between group means

Anthropometric data

Children's body weight and height up to the age of five years were obtained from annual clinical visits or from questionnaires of well-child exams ("U examination"). Child length was measured horizontally at birth and at the year 1 follow-up using an infantometer ("Dr. Keller II"). From year two onwards standing height was measured without shoes to the nearest 0.1 cm ("Dr. Keller I"). Body weight was measured to the nearest 0.1 kg, and BMI z-scores were calculated according to the WHO reference data [37] to adjust for child's age and gender. The use of z-scores is recommended for several reasons. First, z-scores are calculated based on the distribution of the reference population (both the mean and the SD); thus, they reflect the reference distribution. Second, as standardized measures, BMI z-scores are comparable across age and sex. Third, a group of z-scores can be subject to summary statistics such as mean and SD and - even more importantly in our case - can be studied as a continuous variable. Children with BMI z-score < -1 were classified as underweight, children with a BMI z-score of -1 to <1 were classified as normal weight, and children with BMI z-scores ≥1 were classified as overweight.

Assessment of stressors

We assessed the impact of several stressors evaluated by questionnaires on maternal stress perceived during the first year after birth. The stressors evaluated considered the neighborhood quality (living conditions, exposure to traffic or residential noise) and the socio-demographic factors (household income, parental educational level, number of household members, the age of the mother at birth, divorce/separation).

Information about the household income, the parental educational level and the number of household members (all children and adults in the household), were recorded once during pregnancy. Neighborhood quality was assessed each year from pregnancy onwards using the questions summarized in Additional file 1: Table S1. The information on divorce/separation was based on a retrospective assessment at year two with respect to the preceding three years, the exact time point of divorce/separation was not inquired.

Statistical analysis

In all analyses except where explicitly stated otherwise, a p-values ≤ 0.05 was considered to be significant. Whenever Bonferroni-correction was applied the adjusted significant levels are indicated.

To test for potential differences in study characteristics of the entire cohort compared to the analyzed sub-cohort and to test for equal distribution of parameters in the gender-specific analyses chi-square tests were conducted. The maternal perceived stress scores were assessed by Spearman correlations and a repeated measurement analysis of variance (RANOVA) was performed to assess time-dependent changes.

Generalized estimating equation (GEE) models with unstructured correlation matrices were applied to assess the effect of maternal stress on children's longitudinal BMI z-scores (birth to age five or age one to age five respectively). These models were calculated with BMI z-scores and maternal perceived stress scores as continuous variables and were adjusted for weight-related confounding parameters based on a literature review[38–40], namely: gestational week at delivery, mode of delivery, breastfeeding duration, exposure to environmental tobacco smoke (urinary cotinine level during pregnancy, as described in Table 1), and for gender if applicable. To test for gender differences in response to maternal perceived stress the GEE models were stratified accordingly.

A principal factor analysis with oblique rotation was conducted on 10 questionnaire items assessing neighborhood strains to extract potential underlying scales (Additional file 1: Table S1). Factors with an eigenvalue greater than 1 were chosen following Kaiser's criterion. Scales were composed of variables with factor loadings greater 0.4. These extracted scales together with different socio-demographic-factors (household income, parental educational level, number of household members, age of the mother at birth, divorce/separation) were assessed by linear regression for their impact on maternal perceived stress and the different dimensions thereof.

Mediation analysis based on the PROCESS SPSS macro (release 2.16.3 [41]) was applied to test the hypothesis whether the stressors identified to contribute to the perceived maternal stress level affect children's BMI z-score development directly or indirectly. For this purpose, children were categorized in being overweight (BMI z-score ≥ 1) ever in the first five years of life and compared to children never being overweight (BMI-z--scores <1) in this time window. Perceived stress scores and stressors were considered as continuous variables in these analyses. Confidence intervals were computed based on 5000 bootstrap samples.

General statistical analyses, regression- and GEE models were conducted using STATISTICA 12.0 for Windows (Dell Inc., USA) or IBM SPSS Statistics version 22 (IBM Corps., USA) respectively.

Results

Study characteristics

Our analyses were based on the sub-cohort of 498 mother-child pairs for which a complete PSQ assessment for pregnancy, year 1 and year 2 including all weight-related confounders were available. General study characteristics of the analyzed sub-cohort (n=498) and the entire LINA cohort (n=629) were equally distributed as shown in Table 1.

In the analyzed sub-cohort the majority of children (68 %) started daycare in their second year of life.

Perceived maternal stress assessment

Pre-and postnatal maternal stress levels were highly correlated with each other (Spearman correlation, birth vs. age 1: R= 0.60, $p<10^{-13}$; age 1 vs. age 2: R= 0.69, $p<10^{-13}$; birth vs. age 2: R= 0. 58 $p<10^{-13}$). There was a statistically significant increase in the maternal perceived stress scores over time as determined by RANOVA (F = 3772, $p<0.0005$, partial eta-squared = 0.94). An overview of median, minimum, maximum, lower and upper quartiles of maternal stress scores over time is given in Table 2A.

Anthropometric data

On average, about 15.5 % of the analyzed LINA children became overweight during the first five years of life, reaching highest percentages at year two and three. A summary of categorized BMI z-scores of preschool children up to the age of five years is given in Table 2B.

Longitudinal association of maternal perceived stress and children's BMI z-scores

The adjusted GEE model showed a significant longitudinal effect on children's BMI z-scores until the age of 5 years only for maternal perceived stress assessed at year one (adj. β: 0.23, 95% CI (0.08-0.37), p = 0.002) (Table 3A, Additional file 1: Table S2). There was no effect of maternal stress during pregnancy or year two on children's BMI z-scores. Neither weight- nor height- z-scores were affected by maternal stress (data not shown).

Stratifying the GEE model for gender revealed a positive association of BMI z-scores with maternal stress during the first year after birth only for of girl's (adj. β: 0.30, 95% CI (0.11-0.49), p = 0.002; Table 3B, Additional file 1: Table S3), whereas no association was seen for boys. This gender-specific effect was not based on different study characteristics between boys and girls, as can be seen from Additional file 1: Table S4. As only maternal perceived stress during the first year after birth had an effect on BMI z-scores, we focused our further analyses on this early postnatal period.

Influence of different stress dimensions on BMI z-score development

Median, minimum, maximum, lower (<25%) and upper quartiles (>75%) of the four maternal stress dimensions assessed by the PSQ ("demands", "tension", "worries" and "lack of joy") are given in Additional file 1: Table S5. GEE models were applied to evaluate their association with children's longitudinal BMI z-scores. Results of the adjusted models are summarized in Table 4. After Bonferroni-correction, the stress dimensions "tension", "lack of joy", and "demands" showed a significantly positive association with children's BMI z-scores. Similar to the total stress score the different dimensions of maternal stress only showed an association to girl's BMI z-scores. "Worries", "lack of joy", and "demands" were significantly associated with higher BMI z-scores in girls, "lack of joy" showed the best model fit (QIC).

Influence of maternal stress on early maternal feeding behavior

The strong association of maternal perceived stress on BMI z-scores during the first year of life suggested a possible involvement of breastfeeding duration or the time of solid food introduction. However, both parameters, evaluated in three-month-intervals during the first year of life, were not related to pre- and postnatal maternal perceived stress in our analyses (Additional file 1: Table S6).

Multiple stressors contribute to maternal stress perception

Based on a 10-items questionnaire assessing the neighborhood quality (Additional file 1: Table S1), three factors, which in combination explained 58.1% of the variance (Additional file 1: Table S7), were extracted. The items that clustered on the same factor loadings suggest that the factors represent "poor living conditions", burdens due to "traffic", and exposure to "residential noise",

Table 2 Descriptive statistics of (A) maternal perceived stress scores. Given are median, min, max, and quartile boundaries (n=498). (B) BMI z-score categories within the analyzed sub-cohort[a]

A - Stress score		median	min	max	<25 %	>75%
pregnancy		1.9	1.0	3.6	1.6	2.3
year 1		2.0	1.1	3.9	1.7	2.4
year 2		2.2	1.0	3.9	1.9	2.5
B - BMI z-score[a]		Year 1 n = 487(%)	Year 2 n = 456(%)	Year 3 n = 428(%)	Year 4 n = 394(%)	Year 5 n = 352(%)
< 0	Underweight	112 (23.0)	25 (5.5)	43 (10.0)	40 (10.2)	45 (12.8)
0 < 1	Normal weight	336 (69)	307 (67.3)	309 (72.2)	303 (76.9)	266 (75.6)
≥ 1	Overweight	39 (8.0)	124 (27.2)	76 (17.8)	51 (12.9)	41 (11.6)

[a]Categorization based on WHO-reference data

Table 3 Impact of maternal perceived stress levels on longitudinal BMI z-score development in preschool children (birth-age 5)

		β estimate[a]	95% CI	*p*-value
A - Maternal perceived stress at				
Pregnancy	(*n* = 498)	0.06	-0.07 – 0.20	0.372
Year 1	(*n* = 491)	**0.23**	**0.08 – 0.37**	**0.002**
Year 2	(*n* = 473)	0.09	-1.47 – 1.64	0.283
Pregnancy to year 2	(*n* = 473)	0.06	-0.01 – 0.12	0.078
B - Sex-stratified effect of maternal perceived stress at year 1				
Girls only	(*n* = 241)	**0.30**	**0.11 – 0.49**	**0.002**
Boys only	(*n* = 250)	0.10	-0.11 – 0.31	0.333

(A) Effects of maternal perceived stress during pregnancy, year 1 and year 2
(B) Sex disparity in susceptibility to maternal perceived stress at year 1.
Significant associations are presented in bold ($p \leq 0.05$)
[a]Estimates derived from general estimation equations *GEE* for BMI z-scores (birth to age 5) as dependent variable, adjusted for gestational week at delivery, mode of delivery, pregnancy cotinine levels and breastfeeding duration (not for pregnancy stress levels)

respectively (Additional file 1: Table S7). For each of these three factors corresponding variables were created including items with a factor loading >0.4. The variable "poor living conditions" considered the occurrence of vandalism, graffities, dirty streets and attempted break-ins in the living environment. The impairment due to "traffic" was summarized by items asking for disturbance due to traffic noise or related odors or exhausts. The variable "Noise" took into account noise from pedestrians and neighbors.

Table 4 Effect of the different stress dimensions on the longitudinal BMI z-score development in preschool children

	β estimate[a]	95% CI	*p*-value[b]	QIC[c]
Entire cohort (*n* = 491)				
Worries	0.14	0.01 – 0.27	0.039	2051.99
Tension	0.19	0.07 – 0.31	0.003	2041.97
Lack of Joy	0.15	0.04 – 0.27	0.009	2045.80
Demands	0.19	0.05 – 0.33	0.006	2048.45
Girls only (*n* = 241)				
Worries	0.24	0.06 – 0.41	0.009	1005.93
Tension	0.22	0.05 – 0.39	0.014	1013.26
Lack of Joy	0.23	0.08 – 0.38	0.002	1003.31
Demands	0.24	0.05 – 0.43	0.012	1012.31
Boys only (*n* = 250)				
Worries	-0.01	-0.18 – 0.18	0.963	1056.08
Tension	0.13	-0.03 – 0.29	0.108	1048.00
Lack of Joy	0.06	-0.11 – 0.23	0.503	1054.36
Demands	0.11	-0.08 – 0.31	0.247	1053.93

[a]Estimates derived from general estimation equations (GEE) for BMI z-scores (birth to age 5) as dependent variable, adjusted for gestational week at delivery, mode of delivery, pregnancy cotinine levels and breastfeeding duration
[b]Bonferroni adjusted significance level, $p \leq 0.0125$
[c]Quasi- Akaike Information Criterion *QIC* for model selection

All three of these variables showed a significant association to the maternal stress level at year one (Fig. 1).

Of the socio-demographic factors analyzed, only low household income contributed significantly to the overall stress perceived by the mothers (Fig. 1, Additional file 1: Table S8). Parental separation or divorce during the first three years (available from 191 participants) significantly increased the total maternal stress level at year 1 and three of the four different dimensions thereof (Additional file 1: Table S9).

Associations of different stressors on stress dimensions

While "worries", "tension", and "demands" were similarly positively associated with the factors of poor neighborhood quality, "lack of joy" was not affected (Additional file 1: Figure S1A). However, "lack of joy" was positively associated with a "low household income", as was an increase in "worries". A low educational level and the number of household members, which were not associated to the overall maternal stress level, were significantly associated with the stress dimensions "lack of joy" and "demands".

Impact of stressors on BMI z-score development

To further elucidate how the stressors, which showed a significant association with the maternal perceived stress level at year 1 (see Additional file 1: Table S8), namely noise, traffic, poor living environment) and a low household income affect the BMI z-score development we assessed their relationship by mediation analyses. These stressors did not affect girl's BMI z-score development directly. However, they had an indirect effect on BMI z-scores mediated by maternal perceived stress (Additional file 1: Figure S1B). Due to the small case number the effect of divorce/separation could not be further evaluated in the mediation analysis.

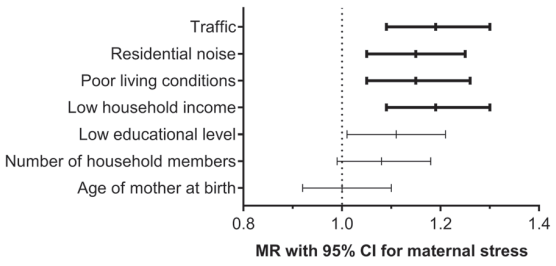

Fig.1 Effects of different stressors on maternal perceived stress. Mean ratios and 95 % confidence intervals for the effect of shown stressors on maternal perceived stress levels at year 1 were calculated from multiple regression models. Significant associations after Bonferroni correction are depicted in bold, $p \leq 0.007$

Discussion

Obesity is a multifactorial disease with an often protracted onset in childhood and adolescence. Despite the appreciation that high caloric intake and sedentary behavior contribute to overweight development, less attention has been given to the effects of pre- and postnatal perceived maternal stress on weight development in early childhood. Given the few longitudinal studies evaluating this relationship in children [8, 39, 42, 43] our aim was to investigate whether and how maternal stress assessed at different time points in the highly vulnerable early pre- and postnatal period is related to overweight development in preschool children.

Previous studies have suggested that dramatic life events during the prenatal phase, such as the occurrence of a natural disaster or the death of a close relative, can contribute to overweight development in preschool children or in young teenagers [44–46]. In our study we did not observe a longitudinal association of prenatal stress on BMI z-scores but rather identified the perceived stressfulness for the mother during the first year of life as an important factor for an increase in BMI z-scores. This observation is in line with a recent meta-analysis by *Tate et al.,* suggesting that toddlers (1-3 years old) are more vulnerable to maternal stress than infants (<one year) [12]. In addition, as the type of stress evaluated in our study is most likely not comparable in its impact to a natural disaster, the missing association between prenatal perceived stress and BMI z-scores might also at least in part be related to the difference in the type of stress evaluated.

As only maternal stress during the first year after birth had an effect on BMI trajectories we hypothesized that duration of breast feeding, which can be negatively affected by maternal stress, might play a role [47]. However, we did not observe significant differences in breastfeeding duration or the time of solid food introduction in relation to the maternal stress level. This does not exclude the possibility that maternal feeding styles and attitudes may play a role [48, 49] as earlier studies indicate that the parent-created environment can foster obesity-promoting feeding styles and attitudes, which shape the child's food preferences [50, 51]. In older children however, other social influences and food environments experienced in daycare or preschool may dilute the maternal stress effect. Although this is in line with our observation that only maternal perceived stress during the first year but not thereafter had a persistent effect on BMI z-scores, the design of our study did not allow further evaluation of parental feeding styles and attitudes.

Although high maternal stress levels were associated with higher BMI z-scores in the total LINA cohort, this effect was only present in girls, whereas boys were not affected. Similar observations were made by *Suglia et al.* [39], who described a higher risk of being obese for five-year-old girls, who had experienced high cumulative stress (including food insecurity, housing insecurity, maternal depressive symptoms, and maternal substance abuse) compared to girls without this experience, a similar effect was missing in boys [39].

In adults and adolescents gender disparity in stress perception and processing has previously been associated to differences in coping mechanisms [52–54]. Children seem to respond in a similar way as in particular girls have been described to respond by impulsive eating, emotional binge eating, and by requesting sweet and high fat foods [25, 39, 55]. In light of these previous findings, our results suggest that already at a very young age changes in eating behavior might play a role in the gender-disparity of BMI-development related to the experience of stress.

In accordance with what we saw for the overall maternal perceived stress, "demands", "worries" and "lack of joy" had a strong positive association with BMI z-scores in girls only. There are several studies suggesting that maternal depression can promote overweight development in children [56, 57], with indications that this might also be a gender-specific effect [9], e.g. *Hernandez et al.* reported that maternal depression placed females but not males at a higher risk for obesity at age 18 [58].

On a last scale of our analyses we aimed to characterize potential stressors, which add to the perceived maternal stress level throughout the years. We were able to identify three potential stressors, which were all related to the quality of the living environment (burden due to traffic, residential noise, and poor living conditions) contributing to the overall maternal perceived stress.

We show in this study that different sources of noise including "Residential Noise" - noise from neighbors and pedestrians - and "Traffic" summarizing traffic noise and exhaust, can affect the maternal stress level. Noise exposure is a well-described example of a potentially obesogenic factor, which has already been studied for its impact on prenatal/postnatal growth [59–61] and in association to adiposity and metabolic outcomes in adults [27, 29, 62]. Noise during pregnancy and childhood increased the risk of overweight at age 7, although no association to BMI z-scores was found in this study [63]. Also, an unsecure living environment has been suggested to contribute to overweight development. While *Mathis et al.* observed that adults who perceive their neighborhood as unsecure were 12 times more likely to be overweight [64], in children neighborhood crime was associated with an increase in weight and a limited outdoor activity [65]. Next to the quality of the living environment the only other stressor with a significant impact on overall perceived maternal stress was a low household income, These stressors are likely related to a low socioeconomic status of the families, which is

known to be strongly associated with obesity in the Western world [66, 67].

Interestingly, the stressors themselves did not have a direct effect on girl's BMI z-scores but rather mediated their effect through their impact on the maternal overall perceived stress level. As the maternal perceived stress is most likely a cumulative account of these stressors and potentially also covers others stress-related factors, single stressors might not have a sufficient predictive power. This is in line with the observation that a combination of adverse effects within a family can increase the obesity risk, whereas unfavorable social factors in isolation often do not [68, 69].

Conclusion

In summary, with our longitudinal qualitative stress assessment we were able to provide evidence for the idea that childhood BMI trajectories develop early and that maternal stress during the first year after birth is a persistent positive predictor of BMI z-scores in girls up to the age of five years.

To reduce the risk for childhood obesity – in particular in girls - behavioral interventions to reduce the mental stress in mothers should be considered in the future.

Additional file

Additional file 1: Table S1. Questionnaire for the assessment of the living environment. **Table S2.** Impact of maternal perceived stress levels during pregnancy, year 1 and year 2 on longitudinal BMI z-score development in preschool children (birth-age5). **Table S3.** Gender disparity in susceptibility to maternal stress-related BMI development in preschool children age 1-5 years. **Table S4.** Comparison of gender-related study characteristics of the analyzed sub-cohort. **Table S5.** Characteristics of maternal perceived stress scores of the four different stress dimensions at year 1. **Table S6.** Influence of maternal stress during the first year after birth on breastfeeding duration and introduction of solid food. **Table S7.** Summary of exploratory factor analysis results of questionnaire items assessing the living environment. **Table S8.** Association of different stressors with the maternal stress levels at year 1. **Table S9.** Contribution of separation or divorce on perceived maternal stress at year 1. **Figure S1.** (A) Associations of different stressors and the four different stress dimensions at year 1. (B) Summary of mediation analysis. (DOCX 241 kb)

Abbreviations

BMI: Body mass index; GEE: Generalized estimating equation; PSQ: Perceived Stress Questionnaire; WHO: World Health Organization

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Availability of data and materials

The datasets regarding the LINA cohort generated and/or analyzed during the current study are not publicly available due to limited consent of the study participants but are available from the corresponding author on reasonable request.

Authors' contributions

BL and ST performed the statistical analyses. BL, ST and KJ wrote the initial manuscript. MBo, SR, KJ, and IL collected data and provided proband materials. IL, ST, GS, AH, RW provided project leadership. All authors contributed to and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from all individual participants included in the study and participation in the study was voluntary. The study was approved by the Ethics Committee of the University of Leipzig (file ref # 046-2006, 160-2008, 160b/2008, 144-10-31052010, 113-11-18042011). All administrative permissions were obtained to access and use the data obtained in the LINA cohort.

Consent for publication

Not applicable.

Competing interest

The authors declare that they have no competing interests.

Publisher's Note

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(Über)Ernährung und Einfluss auf die Funktion der Plazenta

Veränderungen im materno-fetalen Austausch bei Adipositas und Gestationsdiabetes

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Zusammenfassung

Für eine optimale Entwicklung des Feten ist ein adäquater Austausch von Nährstoffen über die Plazenta unabdingbar. Die Schwangerschaft stellt damit ein höchst sensitives Zeitfenster dar, in dem Fehlernährung, Übergewicht, sowie einhergehende Stoffwechselveränderungen wie Diabetes mellitus die fetale und kindliche Gesundheit lebenslang prägen können. Eine entscheidende Rolle in diesem Kontext übernimmt die Plazenta, welche als erstes materno-fetales Kontaktorgan zum einen die durch das mütterliche Übergewicht und/oder Gestationsdiabetes veränderten Nährstoffe an den Feten weitergibt, zum anderen auf in diesem Zusammenhang veränderte Nährstoff-Transportmechanismen zurückgreifen muss. Der kindliche Organismus wird durch diese unphysiologischen Bedingungen geprägt. Eine solche Fehlprogrammierung der kindlichen Organfunktionen und Stoffwechselregulationen stellt die Basis dar für sich später entwickelnde chronische Krankheiten wie Adipositas, Diabetes mellitus oder kardiovaskuläre Erkrankungen bei den Nachkommen. Dieser Review gibt einen aktuellen Überblick über Ernährungsempfehlungen in der Schwangerschaft, Funktion und Nährstofftransport in der Plazenta, materno-fetale Transportprozesse sowie Aspekte der plazentaren immunologischen Prozesse – stets mit speziellem Fokus auf Veränderungen bei Gestationsdiabetes und Adipositas.

Trailer

Laut dem Institut für Qualitätssicherung und Transparenz im Gesundheitswesen (IOTiG) sind ca. 16,4 % der schwangeren Frauen zu Beginn der Schwangerschaft von Adipositas betroffen, bei 9,5 % der schwangeren Frauen wurde ein Gestationsdiabetes (GDM) festgestellt – Tendenz steigend [29]. Der für eine optimale Entwicklung des Feten wichtige physiologische Austausch von Nährstoffen über die Plazenta ist bei diesen Erkrankungen gestört, wodurch das noch ungeborene Kind bereits im Hinblick auf spätere ungünstige Gesundheitseffekte geprägt werden kann. Die zugrunde liegenden Prozesse bieten eine Möglichkeit für frühe Interventions- oder Präventionsstrategien. Dieser Review gibt einen aktuellen Einblick in dieses Themenfeld.

Fazit für die Praxis

- Physiologischer Austausch von Nährstoffen über die Plazenta ist für eine gesunde Entwicklung des Kindes unabdingbar
- Mütterliche Adipositas und GDM verändern u.a. die Nährstoffverfügbarkeit, zugrunde liegende Transportmechanismen in der Plazenta, den materno-fetalen Austausch sowie die plazentare Immunantwort
- Mütterliche Adipositas und GDM führen zu einer unphysiologischen frühen Prägung des Kindes; das Risiko für spätere Erkrankungen ist erhöht
- Präventionsorientierte Konzepte können bereits während der Schwangerschaft einen wichtigen Beitrag zur Primärprävention leisten, insbesondere, wenn sie im oder noch vor dem ersten Trimester begonnen werden

(Over)Nutrition and its Impact on Placenta Function

Changes in Maternal-Fetal Exchange in Obesity and Gestational Diabetes

Abstract

An adequate exchange of nutrients via the placenta is of particular importance for an optimal growth of the unborn fetus. Therefore, pregnancy pictures a highly sensitive time window where an inadequate or unbalanced diet together with overweight or related metabolic conditions such as gestational diabetes can prime infant health for life. Of particular importance is the placenta as the first fetal-maternal contact organ. Both nutrient availability and placental nutrient transporters are characterized by the obesogenic/diabetic phenotype of the mother and impact fetal development. Disturbed early priming of the newborn's organ function and metabolic processes will increase the risk for later development of chronic diseases such as obesity, diabetes type 2 or cardiovascular disease. This review provides a recent overview about dietary guidelines during pregnancy, function and nutrient transport of the placenta, fetal-maternal exchange as well as involved placental immunological pathways. All aspects are presented with a particular focus on changes induced by obesity and/or gestational diabetes.

Key Words

Pregnancy, nutrient transport, early priming, metabolism, civilisation diseases

Einleitung

Für eine optimale Entwicklung des Feten ist ein adäquater Austausch von Nährstoffen über die Plazenta - der physiologischen Schaltstelle zwischen Mutter und Feten – unabdingbar. Maßgeblich hierfür sind eine bedarfsgerechte mütterliche Nahrungsaufnahme, ihre physiologische Nährstoffmetabolisierung sowie die uneingeschränkte Funktion der Plazenta. Die Schwangerschaft stellt damit ein höchst sensitives Zeitfenster dar, in dem Fehlernährung, Übergewicht, sowie einhergehende Stoffwechselveränderungen wie Diabetes mellitus die fetale und kindliche Gesundheit lebenslang prägen können. Zudem können eine Vielzahl von exogenen Lebensstil- und Umweltfaktoren während der Schwangerschaft die kindliche Entwicklung nachhaltig beeinflussen [38] [39, 44] [43]. So spielt z.B. eine Aufnahme von mit Umweltchemikalien belasteten Lebensmitteln, beispielsweise mit endokrinen Disruptoren, eine entscheidende Rolle. Von diversen Untersuchungen weiß man, dass hier insbesondere die frühe Schwangerschaft besonders suszeptibel ist („pränatale Programmierung“).

Die Schwangerschaft stellt eine anabole Stoffwechsellage dar, in dem der mütterliche Organismus Reserven anlegt, um den Feten über die Plazenta, sowie das Neugeborene über die Muttermilch adäquat mit den notwenigen Nährstoffen zu versorgen. Es sinkt die Insulinsensitivität in den Geweben der Mutter, zudem unterstützen Glukoneogenese und Glykogenolyse die Zuckerbereitstellung für den kindlichen Organismus. Diese Veränderungen können – unkompensiert - bei einigen Frauen zum GDM führen. Physiologischerweise steigen neben der Verfügbarkeit von Glukose zudem die Konzentrationen an Triglyceriden, Cholesterin und im späteren Verlauf der Schwangerschaft an freien Fettsäuren im Blut der Mutter an. Diese Nährstoffe gelangen dann über die Plazenta zum Feten.

Prinzipiell trennt die strukturelle Anatomie der Plazenta den direkten Kontakt von mütterlichem und kindlichem Blut. Dennoch ermöglichen Transportproteine, elektro-chemische Gradienten und Diffusionskanäle einen Stoffaustausch zwischen beiden Individuen. Im Speziellen gibt es für Glukose, Aminosäuren und Fettsäuren spezifische plazentare Transportmechanismen (Abb.1), welche jedoch in pathologischen Stoffwechselzuständen wie Adipositas oder Diabetes mellitus entsprechend verändert sein können.

Laut dem Institut für Qualitätssicherung und Transparenz im Gesundheitswesen (IOTiG) sind ca. 16,4% der schwangeren Frauen zu Beginn der Schwangerschaft von Adipositas betroffen, bei 9,5 % der schwangeren Frauen wurde ein Gestationsdiabetes (GDM) festgestellt [29]. Beide Erkrankungen stellen deutliche Risikofaktoren für unerwünschte Schwangerschaftskomplikationen wie Präeklampsie, Kaiserschnittgeburten, zu schwer geborene Feten (large-for-gestational-age; kindliches Geburtsgewicht >90.Perzentile) sowie intensiv-medizinische Betreuung nach der Geburt dar. Zudem ist das Risiko für das Kind deutlich erhört, im Verlaufe seines Lebens selbst Adipositas oder einen Diabetes mellitus sowie einhergehende Komorbiditäten zu entwickeln.

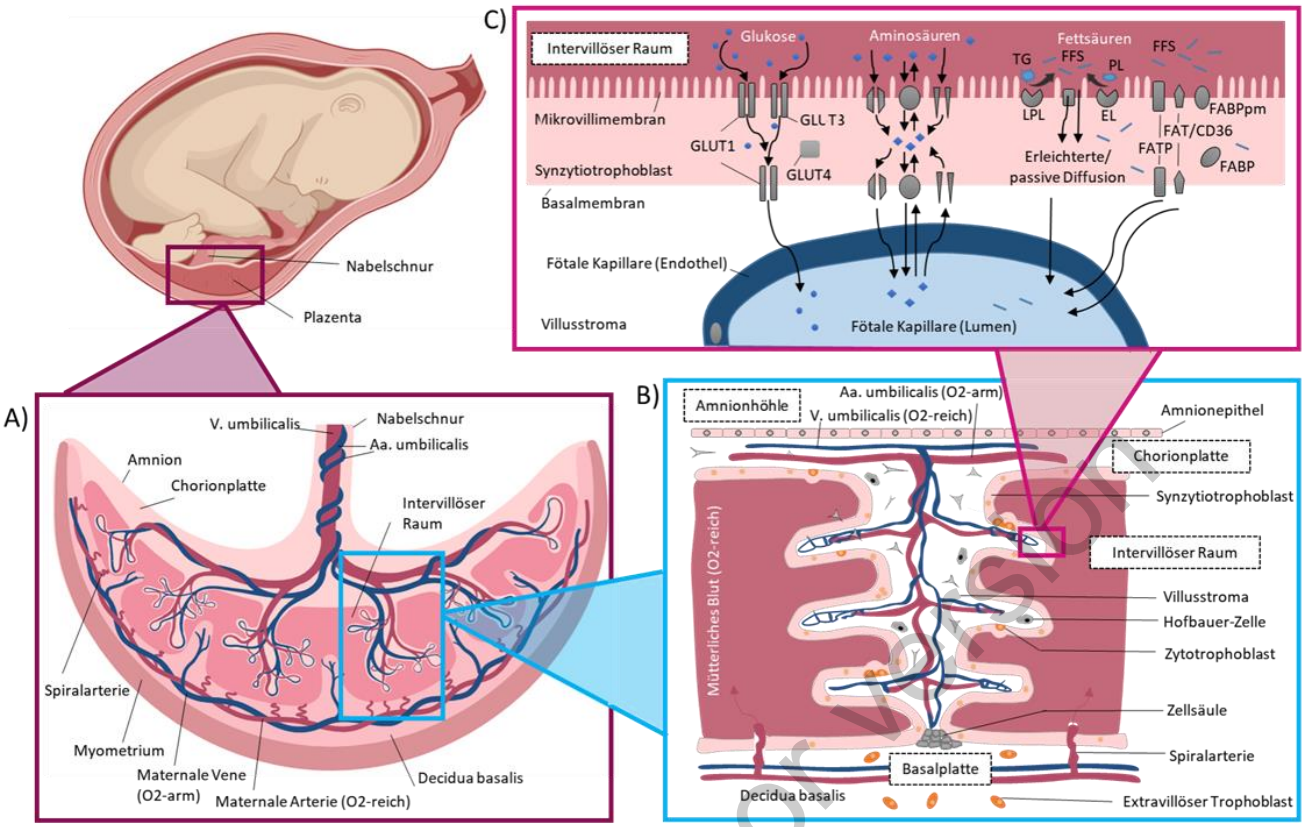

Abbildung 1: Schematische Darstellung (A) vom Aufbau der reifen Plazenta (B) des Zottenbaums sowie (C) der Transport-Mechanismen der Nährstoffe; adaptiert nach [7]

Ernährung und Überernährung in der Schwangerschaft

Da eine Unter- oder Überversorgung des ungeborenen Kindes mit Nährstoffen dessen gesunde Entwicklung im Kindes- und Erwachsenenalter negativ beeinflusst, sollte ein normales Körpergewicht, eine ausgewogene Ernährung, sowie eine adäquate Gewichtszunahme in der Schwangerschaft im Fokus für werdende Mütter stehen.

Generelle Ernährungsempfehlungen der D-A-CH (Deutsch-Schweiz-Österreichische Gesellschaft für Ernährung) geben in der Schwangerschaft einen zusätzlichen Energiebedarf für das 2. (+250 kcal/d) und 3. Trimester (+500 kcal/d) an [23]. Aufgrund der maternalen Neubildung von Geweben sowie des kindlichen Wachstums erhöht sich der physiologische Proteinbedarf Erwachsener von 0,8 g/kg Körpergewicht pro Tag bei einer Schwangeren im 2. und 3. Trimester um 7 g/d, bzw. 21 g/d, sprich auf 0,9 bzw. 1,0 g/kg pro Tag [23]. Die Zufuhrempfehlungen für Fett (30-35% der täglichen Energiezufuhr) und essentielle Fettsäuren (Linolsäure (n-6): 2,5 % der täglichen Energiezufuhr, α-Linolensäure (n-3): 0,5% der täglichen Energiezufuhr) orientieren sich an den regulären Richtwerten für Erwachsene [23].

Frauen mit einem niedrigen Ausgangs-Body-Mass-Index (BMI <18,5 kg/m²) sollten laut Empfehlung des *Institute of Medicine* (IOM) etwa 12,5-18 kg zunehmen, Frauen mit Normalgewicht (BMI 18,5-24,9 kg/m²) etwa 11,5-16 kg, Frauen mit Übergewicht (BMI 25-30 kg/m²) etwa 7-11,5 kg und Frauen mit Adipositas (BMI >30 kg/m²) etwa 5-9 kg [54]. Eine übermäßige Gewichtszunahme sowohl vor als auch während der Schwangerschaft ist mit einem erhöhten Risiko für einen GDM verbunden [54]. Dies erhöht wiederrum das Risiko für einen manifesten Typ 2 Diabetes der Mutter nach der Schwangerschaft als auch das Risiko des Kindes für kardiovaskuläre Erkrankungen / Übergewicht im späteren Leben.

Strategien zur Prävention von Überernährung und insbesondere GDM sind von besonderer Bedeutung, insbesondere da sich Behandlung und Management bei Schwangeren von der bei Nicht-Schwangeren unterscheiden muss. So ist z.B. eine medikamentöse Einstellung des GDM als auch eine drastische Gewichtsreduktion im Hinblick der ungünstigen Konsequenzen für den Feten nicht uneingeschränkt anwendbar. Spezifische Maßnahmen sind daher nötig, um die mütterliche Gewichtsentwicklung bzw. den GDM zu kontrollieren sowie die spätere Progression eines GDM in einen manifesten Typ 2 Diabetes der Mutter zu vermeiden. Die Änderung des Lebensstils gilt dabei als essentiell: das Zusammenspiel von Ernährungstherapie, Gewichtsmanagement und sportlicher Betätigung bessert die Kontrolle der Blutglukosespiegel deutlich [14] [48] [55]. Zusammenfassend sollte die Nahrung genug Makro- und Mikronährstoffe enthalten, um eine optimale Entwicklung des Feten zu gewährleisten. Gleichzeitig sollte sie so konzipiert sein, dass der postprandiale Glukoseanstieg limitiert ist und die empfohlene Gewichtszunahme (s.o.) eingehalten wird [55].

Die Blutglukosespiegel sowie hyperglykämische Episoden sind primär abhängig von der Kohlenhydrataufnahme – daher sollten vor allem die Art der Kohlenhydrate (komplexe sind gegenüber einfachen Kohlenhydraten zu bevorzugen), als auch die Aufnahmemenge und eine gleichmäßige Aufnahmeverteilung über den Tag im Fokus stehen [55]. Die exakte Aufnahmemenge an Kohlenhydraten sollte individual abgestimmt werden. Die Aufnahmeempfehlungen für Kohlenhydrate bei Schwangeren mit GDM liegen laut IOM bei 46–65 % der täglichen Energiezufuhr [54], wobei ein Minimum von 175 g/d sichergestellt werden soll [14] - bevorzugt über stärkehaltige Kohlenhydratquellen und Vollkornprodukte. Die empfohlene totale Proteinaufnahme bei GDM liegt bei 10–35% der täglichen Energiezufuhr, minimal 71 g/d - bevorzugt gedeckt über pflanzliche Proteinquellen, mageres Fleisch und Fisch. Die Empfehlung für Fett liegt bei 20–40 % der täglichen Energiezufuhr, mit maximal 10 % der täglichen Energiezufuhr aus gesättigten Fetten, 10–20 % aus einfach ungesättigten Fetten (MUFAs), und 5–10 % aus mehrfach ungesättigten Fetten (PUFAs). Eine übermäßige Gewichtszunahme sollte vermieden werden. Eine Reduktion der Gesamtenergiezufuhr um 30-33% wird empfohlen für Schwangere mit Übergewicht bzw. für jene, die ihre empfohlene Gewichtszunahme bereits erreicht haben, um eine Überschreitung der Empfehlungen zu vermeiden.

Auch wird u.a. diskutiert, dass eine günstige Beeinflussung des intestinalen mütterlichen Mikrobioms einen präventiven Charakter für GDM haben könnte, da die intestinale Bakterienflora seinerseits den Glukose- und Fettstoffwechsel des Wirts/der Schwangeren beeinflussen kann. Dennoch gibt es wenig Beweiskraft, dass eine Gabe von Probiotika den Stoffwechsel der Schwangeren sowie deren Risiko für GDM bedeutend verändert [15].

Plazentastruktur und -funktion

Die **Bildung der Plazenta** beginnt mit der Implantation, dem Zeitpunkt, an dem sich die Blastozyste, bestehend aus Embryoblast und Trophoblast, in die Gebärmutterwand einnistet. In diesem Zeitfenster (Tag 5-8 post conceptionem, p.c.) haben mütterliches Endometrium und Blastozyste optimale uterine Bedingungen geschaffen, die eine Entwicklung des Embryos ermöglichen. Insbesondere schützt eine feto-maternale Immuntoleranz vor dem Abstoßen des „väterlichen Antigen-tragenden“ Feten. Der Implantationsprozess ist durch die Prozesse „Apposition“ (Initialkontakt zwischen Blastozyste und uterinem Endometrium), „Adhäsion“ (irreversible Anheftung der Blastozyste an das maternale Epithel) sowie „Invasion“ (Eindringen der Blastozyste in das Endometrium) gekennzeichnet. Die Trophoblasten differenzieren sich hierbei in Synzytiotrophoblasten, villöse und extravillöse Zytotrophoblasten. Die umgebenden Stroma-Zellen des Endometriums differenzieren sich in dieser Phase in spezialisierte Sekretzellen („Dezidualisierung“). Bis zum Ende der 3. Woche p.c. entwickelt sich aus Trophoblast und extraembryonalem Bindegewebe ein vaskularisierter Zottenapparat (Chorion), der im Stroma des Endometriums (Dezidua) verankert ist. Das Chorion, der mit maternalem Blut aus den Spiralarterien gefüllte Raum zwischen den Chorionzotten (intervillöser Raum, Abb. 1A), und die Dezidua sind die wesentlichen Teile der Plazenta. Die Veränderung der Uterusschleimhaut hängt von der Stimulation der durch Ovar, Plazenta und Trophoblasten ausgeschütteten Hormone ab (u.a. humanes Choriongonadrotropin (hCG), Plazentalaktogen, Östrogene sowie Progesteron). Die feto-plazentare Zirkulation beginnt etwa in der 3. Woche p.c., wenn die fetalen Gefäße die Plazenta mit den Geweben des Embryonalkörpers verbinden. Im weiteren Verlauf der Schwangerschaft passt sich die Plazenta in ihrer Entwicklung den steigenden Nährstoffanforderungen des Feten an. Mit der Entwicklung der Nabelschnur aus dem Haftstiel ist

die endgültige Verbindung zwischen Mutter und Feten geschaffen. Über sie gelangen fortan die von der Mutter aufgenommenen Substrate an das Kind.

Basierend auf der strukturellen Anatomie, gibt es zwei Schichten, welche die in den intervillösen Raum sezernierten Substrate, Gase und Wasser passieren müssen, um von der mütterlichen Zirkulation zur kindlichen zu gelangen („**Plazentaschranke**“ Abb. 1B). Die erste Schicht bildet der Synzytiotrophoblast (SCTB). Dieser besteht aus 2 polarisierten Membranen, einer der mütterlichen Seite zugewandten Mikrovilli-Membran (MVM) sowie einer der kindlichen Zirkulation zugewandten Basalmembran (BM). Beide Membranen verfügen über spezifische Transporter zur Nährstoffaufnahme und –abgabe (Abb. 1C). Die zweite zu passierende Schicht bilden die Epithelien der kindlichen Blutgefäße.

Eine Vielzahl an Faktoren beeinflusst den **Stofftransfer** zwischen der mütterlichen und kindlichen Zirkulation: der Blutfluss im Uterus/in der Plazenta sowie in der Nabelschnur, der Konzentrationsgradient der Nährstoffe zwischen Mutter und Fetus sowie die Dicke, Oberfläche und metabolische Aktivität der Plazenta [27]. Der Transport von Molekülen, welche die Membranen der Plazenta über einfache Diffusion passieren können (u.a. Sauerstoff und CO2, evtl. Fettsäuren) bzw. deren plazentare Transportkapazität sehr hoch ist (z.B. Glukose), ist stark abhängig vom Konzentrationsgradienten, dem Blutfluss und der Plazentamorphologie. Substanzen, die aufgrund von Ladung, Löslichkeitsverhalten oder Molekulargewicht weniger leicht die Plazentamembran passieren können, folgen Mechanismen der erleichterten Diffusion über spezifische Transportproteine (z.B. *glucose transporter proteins* (GLUTs) für Glukose). Proteine (in Form von Aminosäuren) und Lipide (in Form von Fettsäuren) müssen hingegen aktiv, also unter Aufwendung von Energie, zum Feten transportiert werden.

Für **Glukose** existieren verschiedene GLUTs, welche in beiden Membranen des SCTB exprimiert werden. Die mütterliche Expression ist dabei zur Sicherung der kindlichen Glukose-Versorgung höher, als die des Feten. Primär plazenta-relevant ist GLUT-1, abhängig vom Schwangerschaftsalter aber auch GLUT-3, 8 und 12 sowie der insulinsensitive GLUT-4 (*Abb. 1C*) [35].

Für den Transfer von **Aminosäuren** (AS) gibt es ebenso in beiden Membranen des SCTB Transportproteine, etwa 20 verschiedene wurden bereits identifiziert. In den Membranen gibt es u.a. akkumulative Transporter zur Aufnahme von AS in den SCTB im Ko-transport mit Na+ (System A, *Abb. 1C*), sowie austauschende Transporter, welche an der MVM Aminosäuren aus dem mütterlichen Blut gegen Aminosäuren aus dem SCTB tauschen (System L). Über die Weitergabe der AS an der BM der fötalen Seite ist weniger bekannt, sie erfolgt über vermutlich selbige Transportmechanismen und unterstützend über erleichterte Diffusion [65].

Für die Versorgung des Feten mit **Fetten** gibt es ebenfalls spezifische Prozesse im SCTB: die im Laufe der Schwangerschaft in der Plazenta unterschiedlich stark vorhandenen Lipasen (Lipoproteinlipase (LPL) und Endothellipase (EL) [30]) spalten die Triglyceride (TG) bzw. Phospholipide (PL) aus dem mütterlichen Blut in freie Fettsäuren (FFA), welche ihrerseits in den SCTB aufgenommen werden (*Abb. 1C)*. Die Mechanismen, durch welche die FFA in den SCTB aufgenommen bzw. über die BM an die fetale Zirkulation weiter gegeben werden, sind zum Teil [34], jedoch bislang nicht endgültig bekannt [33]. So können sie u.a. durch einfache/erleichterte Diffusion aufgenommen werden, unterstützt durch die in der MVM lokalisiertem *placenta membrane fatty acid transport proteins* (pmFATP), der *Fatty acid translokase* (FAT/CD36) und des *fatty acid binding protein*s (FABP). Die Weitergabe der FFA im Zytosol erfolgt u.a. über Bindung an das FABP.

Plazentafunktion bei GDM und Adipositas

Insbesondere in den frühen Phasen der Schwangerschaft können Dysfunktionen durch endogene oder exogene Einflussgrößen zu einer abnormalen Plazentabildung mit veränderter Größe, Form und Mikroanatomie führen. Dies kann u.a. zu langfristigen Funktionseinschränkungen sowie maternalen oder fetalen Komplikationen wie Präeklampsie oder intrauteriner Wachstumsretardierung führen. Veränderungen der Plazenta im Zusammenhang mit Übergewicht und Diabetes haben ebenfalls weitreichende Konsequenzen wie z.B. ein übernormales fetales Wachstum (Makrosomie) mit einer Anreicherung von Fettdepots [11]. Dennoch ist der Beitrag der Plazenta in der Vermittlung der langfristigen kindlichen Symptome noch nicht vollständig verstanden, über verschiedene Aspekte herrscht aber allgemeiner Konsens.

Bei Schwangeren mit Adipositas sind viele mütterliche Metabolite, Hormone, Wachstumsfaktoren und Zytokine im Vergleich zu normalgewichtigen Schwangeren verändert,

welche die Plazenta beeinflussen können [40]. Schwangere mit Adipositas zeigen u.a. eine periphere Insulinresistenz, Hyperinsulinämie und höhere Spiegel an Leptin, Insulin-like-growth factor 1 (IGF-1), Blutfetten und pro-inflammatorischen Zytokinen sowie niedrige Adiponektinlevel. Zudem liegt ein erhöhtes Nährstoffangebot vor [40]. Die Hyperinsulinämie der Mutter aktiviert plazentare Glukosetransporter (insbesondere GLUT-1 in der BM) und sorgt so für eine erhöhte Glukoseverfügbarkeit für den Feten. Dies steigert seinerseits die Bildung fetaler Fettdepots [19]. Trotz der peripheren Insulinresistenz in der Schwangeren mit Adipositas scheint die Insulinempfindlichkeit in der Plazenta nicht beeinflusst. Jedoch zeigte sich die plazentare Aufnahme an Aminosäuren bei Schwangeren mit Adipositas verändert: Die Aktivität des System A, nicht aber des System L, an der MVM korrelierte positiv mit dem kindlichen Geburtsgewicht, möglicherweise vermittelt über Insulin/IGF-I und mTOR Signalwege [37]. Ebenso wird eine veränderte Expression des plazentaren FAT/CD36 und FABP beschrieben, welches den Feten vermehrt mit Fettsäuren versorgt [62]. All diese Prozesse können zu einer Überversorgung des Feten mit Nährstoffen und damit zu seiner übernormalen Gewichtszunahme, einhergehend mit einer Übergewichtsentwicklung im Kindesalter, beitragen. Daten aus *omics*-Analysen der Plazenta zeigen diesbezüglich veränderte Muster in Transkriptom, Proteom und Metabolom im Hinblick auf Lipidmetabolismus und Entzündungsgeschehen/Immunantwort von Schwangeren mit im Vergleich zu Schwangeren ohne Adipositas (reviewed in [40]). Es werden Strategien diskutiert, dass die Verwendung eines Adiponektinrezeptor-Agonisten zur Vermeidung der kindlichen Prägung durch die maternalen Adipositas-Signalmoleküle beitragen könnte [40].

Da Schwangere mit GDM sehr oft auch adipös sind, ähneln die Veränderungen an der Plazenta sehr oft denen von Schwangeren mit Adipositas ohne GDM. Bei Frauen mit Diabetes zeigen sich gehäuft strukturelle Veränderungen im Hinblick auf ein größeres Plazentavolumen sowie eine veränderte Zottenvaskularisation und –reife [18].

Materno-fetaler Transport

Am Beginn der Schwangerschaft, nach der Implantation der Blastozyste und deren späterer Differenzierung in Embryo und Plazenta (s.o.), wird die entstandene embryo-plazentare Einheit durch Sekretion der uterinen Drüsen mit Nährstoffen versorgt (histiotrophe Ernährung [8]). Zwar ist die genaue Zusammensetzung dieser Sekrete unbekannt, aber sie enthalten unter anderem Glykogen, Glukose, Aminosäuren sowie auch Peptide und Proteine. Sie werden vom Synzytiotrophoblasten aufgenommen. Dies ist das Plazentaepithel, welches vom mütterlichen Blut im intervillösen Raum umspült wird (Abb.1).

Die Glukosekonzentrationen im mütterlichen Serum sind am Anfang des ersten Trimesters höher als am Ende [1], während die Konzentrationen von Fettsäuren im ersten Trimester relativ konstant bleiben [2]. Über mütterliche Aminosäurekonzentrationen ist in dieser frühen Schwangerschaftsphase nichts bekannt. Das Metabolom wurde untersucht und zeigt einige Veränderungen gegenüber späteren Schwangerschaftsperioden [32] [45].

Die weiteren Schritte, die involviert sind, um diese Nährstoffe in das entstehende fetale Gefäßsystem zu transportieren, sind zu diesem frühen Zeitpunkt völlig unbekannt. Einige Transportermoleküle wurden in der frühen Plazenta identifiziert, und deren Lokalisation, hauptsächlich am Synzytiotrophoblasten, legt eine Rolle bei der Regulation der Aufnahme in den Synzytiotrophoblasten nahe. Deren Zusammenspiel mit dem plazentaren Metabolismus und wie sie zur Versorgung des Feten beitragen, ist unbekannt.

Am Ende der Schwangerschaft werden Glukose und Fettsäuren als Makronährstoffe entlang eines Konzentrationsgradienten von der Mutter zum Feten transportiert. Die Konzentrationen der Aminosäuren sind in der fetalen Zirkulation höher als in der mütterlichen [21]. Daher muss die Plazenta Energie aufwenden für die fetale Versorgung mit Aminosäuren. Der Konzentrationsgradient der Glukose zwischen Mutter und Feten wird sowohl von mütterlichen also auch fetalen Konzentrationen bestimmt. Da der Fetus Insulin sezerniert, welches die Glukoseaufnahme in fetale Gewebe stimuliert, beeinflusst fetales Insulin die fetale Glukosekonzentration, und damit sowohl den Gradienten als auch die Glukosemenge, die durch die Plazenta transportiert wird. Ob ähnliche Prinzipien auch den Transport von Fettsäuren bestimmen, ist unbekannt, aber vorstellbar. Grundsätzlich werden nur bis zu 3% der mütterlichen Fettsäuren durch die Plazenta transportiert, die meisten fetalen Fettsäuren, mit Ausnahme der essentiellen v.a. α-Linolensäure und Linolsäure, werden aus Glukose synthetisiert. Der Aminosäuretransport wird vermutlich vor allem durch die Menge an entsprechenden Transportermolekülen im Synzytiotrophoblasten

bestimmt, wodurch die Plazenta über die Regulation von Menge und Aktivität dieser Transporter einen Einfluss bekommt. Eine Besonderheit sind die Aminosäurezyklen, die vor allem Stickstoff in die fetale Leber bringen [3].

Materno-fetaler Transport bei GDM und Adipositas

Über die Ernährung des Embryos und der Plazenta bei Diabetes oder Adipositas in der Frühphase der Schwangerschaft ist kaum etwas bekannt, ebenso wenig, ab wann sich der Stoffwechsel der Mutter ändert und damit die Konzentrationen mütterlicher Nährstoffe. Bei Adipositas sind die Konzentrationen an Leucin, Isoleucin und Arginin in den uterinen Sekretionen erhöht [41]. Das ist interessant, weil diese Aminosäuren stimulierende Wirkung auf das fetale Pankreas haben und damit die Insulinsekretion regulieren, zeitlich bevor Glukose einen Effekt hat. Für mütterliche Nährstoffkonzentrationen im ersten Trimester und wie sich diese durch mütterliche Überernährung verändern, liegen wenige Daten vor. Glukosekonzentrationen sind trotz Insulinresistenz durch Adipositas nicht beeinflusst, wohl als Ergebnis erhöhter Insulin- bzw. C-Peptidkonzentrationen [1]. Auch die Konzentrationen an Fettsäuren sind in dieser Frühphase der Schwangerschaft gleich wie bei normalgewichtigen Schwangeren. Interessant ist jedoch, dass n-3 vielfach ungesättigte Fettsäuren zwar nicht vom mütterlichen BMI beeinflusst werden, aber von der Insulinresistenz. Je höher diese sind, parallel auch je höher die C-Peptid Konzentrationen sind, desto niedriger sind die Konzentrationen dieser Fettsäuren. Für Docosahexadiensäure sind diese Abhängigkeiten auch vom fetalen Geschlecht beeinflusst [2]. Ob und wie sich diese Konzentrationsveränderungen auf die Versorgung des Feten auswirken, ist völlig unbekannt.

Mit mütterlicher Überernährung und Stress ist oftmals Insulinresistenz verbunden. Diese bzw. die Insulinopenie bei Typ 1 Diabetes gehen einher mit Hyperglykämie der Mutter. Diese führt zur Hyperglykämie beim Feten. Zwar sind diese metabolischen Veränderungen der Mutter auch mit Änderungen in Menge und Aktivität der plazentaren Glukosetransporter, vor allem von GLUT1, verbunden, dies hat jedoch keine Auswirkungen auf den transplazentaren Glukosefluss, welcher vor allem durch den Konzentrationsgradienten zwischen Mutter und Fetus bestimmt wird. Die mit der Hyperglykämie verbundene Hyperinsulinämie des Feten führt zur Verstärkung des Gradienten und damit zu einem kontinuierlich verstärkten Glukosefluss zum Feten. Der Gradient wird damit auch vom Feten mitbestimmt, der unabhängig von mütterlichen Glukosekonzentrationen Glukose durch die Plazenta ‚zieht' (‚Glucose steal phenomeon' [20]). Das erklärt auch das Auftreten von fetaler Adipositas trotz gut eingestelltem Diabetes.
Es gibt einige Hinweise darauf, dass zumindest bei mütterlicher Adipositas, auch manche Fettsäuren vermehrt durch die Plazenta transportiert werden. Ob das wesentlich zur fetalen Adipositas beiträgt, ist unklar [34].

Die Hyperinsulinämie des Feten ist besonders ausgeprägt zu Beginn des dritten Trimesters [64]. Sie stimuliert den fetalen aeroben Metabolismus und damit den Sauerstoffbedarf. Die Plazenta reagiert darauf mit vielfältigen Adaptationen: 1) Sie erhöht die Transportoberfläche für Sauerstoff durch Verstärkung der Vaskularisierung; 2) Sie trägt zur Verminderung des Risikos der Bildung von prä-atherosklerotischen Läsionen im feto-plazentaren Gefäßbett bei, welche den Blutfluss und damit die Sauerstoffversorgung des Feten einschränken könnten, und 3) sie verbessert die Transportfähigkeit für Eisen. Dieses wird in der fetalen Leber vor allem zur vermehrten Hämoglobinsynthese benötigt, was wiederum eine verstärkte Erythropoese begünstigt [17]. Auch andere Veränderungen der Plazenta tragen zum Schutz des Feten bei. Diese protektiven Funktionen haben aber eine limitierte Kapazität. Wenn diese erschöpft ist, kann das nachteilige Folgen für Plazentafunktion und fetale Entwicklung haben [22]. Die unmittelbaren Folgen können vielfältig sein, wie eine Beeinträchtigung des feto-plazentaren Blutflusses und damit einhergehend der fetalen Sauerstoffversorgung. Der Fetus reagiert darauf mit einer Einschränkung des fetalen Wachstums, um den Sauerstoffbedarf zu reduzieren. Falls das noch nicht ausreicht um Wachstum und letztendlich Überleben sicherzustellen, kann das den intrauterinen Fruchttod nach sich ziehen. Dies manifestiert sich in erhöhten Risiko für intrauterinen Fruchttod bei Schwangerschaften mit gestörtem mütterlichen Metabolismus, wie z.B. bei Diabetes [17].

Rolle von Makrophagen/ Hofbauerzellen

Die Plazenta enthält eine Vielzahl an Makrophagen an zwei verschiedenen Positionen: in der Dezidua und im Stroma der villösen Zotten. Diese Lokalisation bestimmt auch ihre speziellen Funktionen. In der Dezidua als Kontaktstelle

zwischen Mutter und Plazenta sind sie vor allem involviert in die Anpassung der mütterlichen Immuntoleranz an den semi-allogenen Allograft, den die Plazenta darstellt [51] [24].

Zottenmakrophagen haben spezielle, anti-inflammatorische Funktionen, die bisher wenig verstanden sind [69]. Innerhalb der Zotten liegen sie nahe zu fetalen Blutgefäßen bzw. Kapillaren sowie zu den Trophoblasten. Dies erklärt auch ihre vermutete Rolle in der parakrinen Regulation der Vaskularisierung der Zotten. Man kann sie schon im ersten Trimester finden, was auch eine Bedeutung in der Entwicklung der frühen Plazenta nahelegt. Sie sind eine heterogene, nicht proliferierende Population vom anti-inflammatorischen M2-Phänotyp mit M2a-, M2b- und M2c-Charakteristika, und können sich mit ihrer ausgeprägten Plastizität an die metabolischen, inflammatorischen und oxidativen Stressbedingungen der Umgebung anpassen. Einzelne Effekte der Makrophagen auf Trophoblasten wurden identifiziert [12], wie z.B. Stimulierung der Proteinsynthese, verstärkte Sekretion von hCG und reduzierte Sekretion von Angiotensin II and 6-keto-Prostaglandin-F1alpha [12]. Details des parakrinen Wechselspiels der Zellpopulationen in den Zotten mit den Makrophagen sind noch unerforscht.

Makrophagen bei GDM und Adipositas

Es ist unklar, ob sich die Gesamtzahl der Makrophagen in den Zotten bei mütterlicher Adipositas ändert, was vermutlich mit Änderungen der Subphänotypen zusammenhängt. Trotzdem bleiben anti-inflammatorische Eigenschaften bei GDM erhalten und tragen somit zum Schutz der Plazenta und des Feten vor der pro-inflammatorischen Umgebung bei GDM und mütterlicher Adipositas bei [59]. Bei mütterlicher Adipositas produzieren die Zottenmakrophagen mehr IL-1β, TNF-α und IL-6 [13]. Der Effekt auf IL-1 ist zum Teil induziert durch Palmitinsäure, eine gesättigte Fettsäure, deren Konzentration in der mütterlichen Zirkulation bei Adipositas und GDM erhöht ist. Palmitinsäure aktiviert auch das Inflammasom der Makrophagen, welches letztendlich zu deren Apoptose führen kann [56]. Die anti-oxidative Eigenschaft der Makrophagen ist bei GDM und mütterlicher Adipositas verstärkt, vor allem durch Insulin, Leptin und TNF-α, deren Konzentrationen in der fetalen Zirkulation bei diesen Bedingungen erhöht sind. Anti-inflammatorische Zytokine wie IL-4 und IL-13 haben keinen Effekt [58]. Die bisher bekannten Aktivitäten der Makrophagen in den Zotten tragen durch ihre Anpassungsfähigkeit zu einer protektiven plazentaren Funktion bei GDM und mütterlicher Adipositas bei [16] [22].

Adaptive Immunantwort der Schwangerschaft

Bereits in den 50er Jahren stellte Medawar seine Hypothese vor, dass die Schwangerschaft mit einem Allotransplantat vergleichbar ist, welches jedoch aus 3 Gründen nicht abgestoßen wird: a) Unreife des Feten, b) Anergie des mütterlichen Immunsystems, definiert als das Fehlen einer Immunantwort auf Antigene, und c) Trennung zwischen mütterlichen und fetalen Strukturen [47]. Heute gilt diese Theorie als komplett falsch [49]. Mütterliches Blut und darin enthaltene Immunzellen sind aufgrund der Zottenstruktur der Plazenta im permanenten Kontakt mit fetalem Gewebe. Außerdem war es bereits seit 1893 durch die Pionierarbeit von Georg Schmorl bekannt, dass fetale Zellen die maternale Zirkulation sowie sämtliche mütterliche Organe erreichen [60]. Dieser Prozess ist als Mikrochimerismus bekannt. Die sezernierten fetalen Zellen sind bereits in der 8. Woche der Schwangerschaft im mütterlichen Blut in einer für die genetische Diagnostik von Trisomien ausreichenden Menge vorhanden [5].

Das adaptive Immunsystem der Schwangeren muss zum einen aktiv gegenüber möglichen Erregern sein und zum anderen eine tolerante Immunantwort gegenüber dem Feten entwickeln. Diese aktive Toleranz ist sehr spezifisch und zeitlich begrenzt [61]. Wichtige Akteure in der Erzeugung und Aufrechterhaltung von Toleranz sind regulatorische T-Zellen (Treg) [68]. Interessanterweise sezerniert die Plazenta lösliche Substanzen, beispielsweise humanes Chorion-Gonadotropin (hCG), die die Konversion von naiven T-Zellen in Treg begünstigen [52]. Somit unterstützt der Fetus seine eigene Toleranz. Vor allem während des zweiten Trimesters der Schwangerschaft herrscht ein anti-inflammatorisches Milieu in der feto-maternalen Grenzzone, aber auch Zellen aus der Peripherie akquirieren während der Schwangerschaft ein nicht-inflammatorisches Profil [6]. Treg und Th17-Zellen sind in der Lage zu interkonvertieren, und dies scheint in der Schwangerschaft ein wichtiger Regulationsmechanismus zu sein [26]. Welche Faktoren hierzu wichtig sind, ist noch nicht ganz verstanden, wobei Hormone wie Vitamin D eine wichtige Rolle zugeschrieben wird. Neben T-Zellen spielen auch B-Zellen eine wichtige Rolle in der Toleranzerzeugung und -aufrechterhaltung in der Schwangerschaft. So ist es bekannt, dass B-Zellen in der Schwangerschaft sogenannte asymmetrische

Antikörper produzieren, die schwangerschaftsprotektiv sind [46] [25]. Darüber hinaus expandiert während der Schwangerschaft eine Population von B-Zellen, die in der Lage sind, IL-10 zu sezernieren und somit eine Reihe von anti-inflammatorischen Prozessen zu begünstigen [57] [10]. Auch spielen diese Zellen eine wichtige Rolle im Falle einer Infektion [9].

Adaptive Immunantwort bei GDM und Adipositas

Ergebnisse aus Mausstudien und humanen Kohorten legen nahe, dass mit Überernährung assoziierte Erkrankungen wie Adipositas und Diabetes einen Zustand der chronischen Inflammation hervorrufen, der mit Risiken für die Schwangere und das Ungeborene einhergeht. Hierzu gehören neben Frühgeburten und Präeklampsie sowohl eine Makrosomie als auch eine intrauterine Wachstumsretardierung. Die Hintergründe hierfür sind komplex, jedoch ist eine Verschiebung des adaptiven Immunsystems zugunsten eines pro-inflammatorischen Profils eine Konstante. Mäuse, deren Mütter in der Schwangerschaft eine fettreiche Diät erhalten haben, zeigten ein höheres Risiko für Asthmaentwicklung durch aktivierte inflammatorische Antworten in der Peripherie und in der Lunge als Mäuse deren Mütter eine normale Diät bekommen hatten [53]. Auch neigten sie dazu, selbst Adipositas zu entwickeln [36].

Nicht nur das Gleichgewicht Inflammation/Anti-Inflammation verschiebt sich in der Schwangerschaft, sondern auch die Zusammensetzung der Mikrobiota [28], welche einen großen Einfluss auf die Entwicklung des neonatalen Immunsystems hat [4] [67]. Die Mikrobiota wiederum ist abhängig von der mütterlichen Ernährung und kann von externen Faktoren beeinflusst werden, beispielsweise Umweltchemikalien [42]. Die Stoffwechselprodukte der Mikrobiota können in die Plazenta und zum Feten gelangen und dementsprechend die Plazentafunktionalität sowie das fetale Wohlbefinden und Wachstum beeinflussen. Nach ballaststoffreicher Diät während der Schwangerschaft hatten Mütter erhöhte Mengen mikrobiell produzierter kurzkettiger Fettsäuren, wie Essig-, Butter- und Propionsäure im Stuhl [50]. Ihre Neugeborenen zeigten erhöhte Essigsäure- und Propionsäure-Werte im Blut, die nicht von der eigenen Mikrobiota stammen konnten, weil diese sich erst später entwickelt. Darüber hinaus hatten sie mehr Treg in Thymus und Milz [50] [31]. Eine ausgewogene Ernährung während der Schwangerschaft ist daher essentiell für die Funktionalität der Plazenta und für das Gleichgewicht von Immunabwehr und Immuntoleranz. Dabei hat das mütterliche Mikrobiom einen großen Einfluss auf dieses Wechselspiel.

Fazit

Die Prävalenzen an mütterlichem Übergewicht und GDM steigen weltweit, deren Nachkommen tragen daher von Beginn an ein erhöhtes Risiko für gesundheitliche Probleme im weiteren Leben. Hierbei ist ein ungünstiger genetischer Hintergrund ebenso maßgeblich, wie Ernährung, Lebensstil und Umweltfaktoren. Eine entscheidende Rolle in diesem Kontext übernimmt die Plazenta, die als erstes materno-fetales Kontaktorgan die durch das mütterliche Übergewicht und/oder GDM veränderten Nährstoffe über veränderte Transportmechanismen an den Feten weitergibt, wodurch dieser unter diesen Dysbalancen bzw. unphysiologischen Bedingungen geprägt wird. Diese „Fehlprogrammierung“ der kindlichen Organfunktionen und Stoffwechselregulationen stellt die Basis für sich später entwickelnde chronische Krankheiten wie Adipositas, Diabetes mellitus oder kardiovaskuläre Erkrankungen dar. Präventionsorientierte Konzepte können daher bereits während der Schwangerschaft einen wesentlichen Beitrag zur Primärprävention zahlreicher Erkrankungen im späteren Leben des Kindes leisten, insbesondere, wenn sie im oder noch vor dem ersten Trimester begonnen wird [63] [66].

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3. Discussion

Within the following sections an overall interpretation and discussion of data and results seen in the 12 original (and 3 associated) manuscripts will be presented. In particular, when research was performed in earlier years, results will be imbedded in the recent literature available. For detailed description of individual theoretical background, materials/methods, results as well as individual discussion please see the original manuscript shown right before. Overall, the investigated early exposures to lifestyle and environmental factors (Figure 3) are discussed according to their route of exposure (diet/diet associated exposure, airway exposure, etc.) and the addressed disease development in childhood (allergies or obesity). Furthermore, the potential role of epigenetic modifications as well as a future outlook will be presented.

3.1 Diet/diet-associated exposure and allergy development

Via our mandatory daily nutrition, we are facing a huge variety of food and nutrients (macronutrients such as carbohydrates, fat or protein as well as micronutrients such as vitamins or minerals) that can - beneficially or not - impact our health. However, we also get in contact with several co-products that have been enriched in food or food products via cultivation or production (e.g. pesticides or herbicides, such as glyphosate), food processing (e.g. parabens, added as food conservatives), packaging (e.g. plasticizer such as phthalates or BPA), or even preparation (e.g. perfluoroalkyl and polyfluoroalkyl substances (PFAS), they keep food from sticking to cookware etc.). Overall, several of these substances are discussed to alter immune regulation and associated pathways and might therefore contribute to the disease development – in particular, if exposure occurs within the highly sensitive pre or postnatal period.

As part of this thesis we epidemiologically investigated in this context the food nutrient vitamin D (Weisse et al., 2013, Junge et al., 2016) as well as one class of food associated environmental chemicals named parabens (Junge et al., 2022) with respect to their impact on allergy development. In addition, we were also interested to see consequences in the dietary pattern of children that developed allergies later in infancy (Schütte et. 2022). A summary of the results discussed within this sub-topic 3.1. is shown in Figure 4.

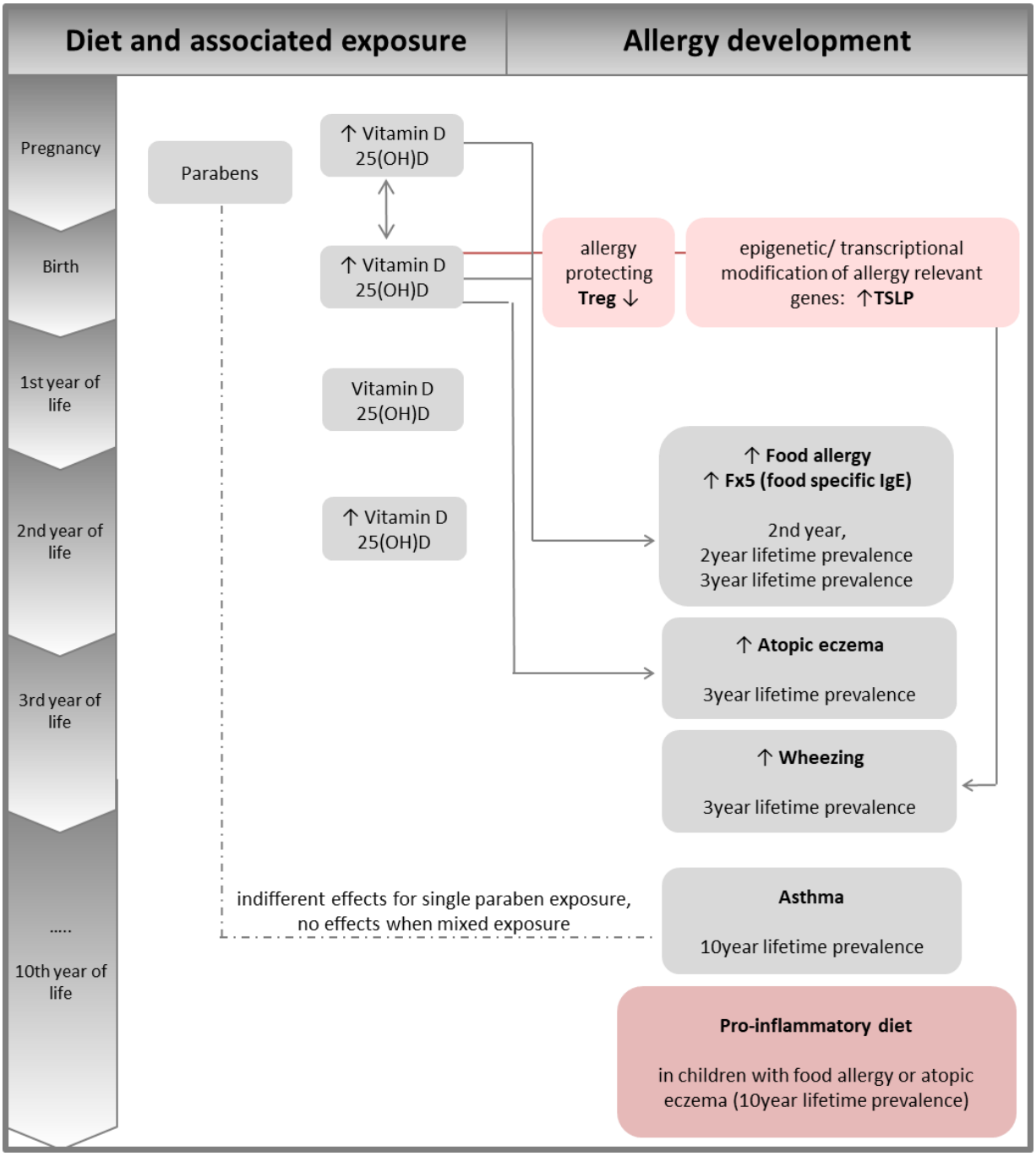

Figure 4: Results summary scheme of different diet/diet associated exposure conditions (vitamin D, parabens) and allergy development: Main focus in this field was to unravel the potential mechanistic contribution of epigenetic modifications on allergy relevant immune parameters (regulatory T cells (Treg), or thymic stromal lymphopoietin (TSLP), light pink boxes; Weisse et al. 2013, Junge et al. 2016). Further, single vs. mixed paraben exposure (preservatives used for example in foods or cosmetics) was addressed (Junge et al. 2022a). In addition, consequences in the dietary pattern of allergic children were evaluated (Schütte et al. 2022). All pictured parameters were addressed within the LINA cohort. © Graphic designed by Kristin Junge

Vitamin D can be obtained from diet, but also from supplements, or seasonal exposure via skin (solar ultraviolet irradiation via photochemical and thermal conversion of the cholesterol precursor 7-dehydrocholesterol). Next to its established function in bone metabolism, there literally has been an explosion in investigations about the role of vitamin D in the regulation

of immunity in the last decades. It was shown that on the molecular level, the hormonal form of vitamin D ($1,25(OH)_2D$) signals through the nuclear vitamin D receptor (VDR), a ligand-regulated transcription factor. The VDR and enzymes for vitamin D metabolism are widely expressed within innate and adaptive immune cells or mediators of the immune system [36]. Since the body's vitamin D level is 90% depended on ultraviolet radiation, increasing time spend indoors together with increasing use of sun protecting skin care is leading to decreased median vitamin D levels in the population worldwide, with several potential consequences for the immune system [37]. With respect to the involvement of vitamin D in allergy development there has been a very controversial field of literature from 1999 onwards [38] where an increased allergy prevalence has been described after broad infant vitamin D supplementation due to rickets prevention. However, next to "allergy promoting" associations, allergy preventing associations with 25(OH)D have also been shown widely. Data from the LINA cohort showed a negative association, meaning higher maternal/cord blood 25(OH)D levels were associated with a higher risk of the children to develop food allergy at the age of two years (Weisse et al. 2013 [39]) and within the 3 year lifetime prevalence (Junge et al. 2016 [40]). This data gained a lot of interest both in the public but also in the scientific community (published "Correspondence" in the Journal of Allergy – see Querfeld et al., 2013 [41]; invited "Editorial" in Expert Reviews in Clinical Immunology – see Junge et al., 2013 [42], as well as 160 international paper citations up to 08/2022). Interestingly, almost 10 years after the paper based on the LINA data has been published, the field is still not showing a clear picture. A most recent literature review from Rueter et al. 2022 [37] entitled "Pre- and Postnatal Vitamin D Status and Allergy Outcomes in Early Childhood", outlined 11 observational studies available until now in the context of early vitamin D and allergy development (Table 1). Shown herein, several studies from different latitudes (from 52°N to 32°S), with different mean cord blood vitamin D levels (from 15.8 nmol/l to 64 nmol/l) and with different allergic endpoints in focus (atopic eczema, food allergy, wheezing, asthma or allergic rhinitis) outlined preventing, promoting or no effects of higher vitamin D levels on allergy development in childhood. However, observational studies still carry some confounders that might affect results. Rueter et al. also reviewed 3 available interventional prospective studies (Randomized Control Trails, RCTs), assuming a better option to unravel a causative link of an early vitamin D supplementation during pregnancy and later allergy development. Interestingly, in a British study maternal supplementation with 800 IU vitamin D during pregnancy lead to a - non

significant - adjusted odds ratio for food allergy in 3 year old children of 4.53 (CI 0.52–39.33; p = 0.17) compared to placebo [43], while wheeze, atopic eczema and rhinitis showed an – also non-significant – opposite picture (OR for all of latter outcomes about 0.6 [43]). This was very similar to the results seen within LINA (aOR for cord blood vitamin D: 4.65 (CI: 1.5 – 14.48), p=0.008; Weisse et al., 2013 [39]), however, without reaching the significance level in the British study (most likely due to limited power since study arms just included n=50 vs. n=56 cases). Also 2 other RCTs on maternal vitamin D supplementation and allergic outcomes did not show any significant associations for wheezing [44 45] and asthma [46 47]. In line, Tarenke et al. reported from a systematic review and meta-analysis of randomized controlled trials, there is no clear evidence to recommend vitamin D as allergy preventive with respect to respiratory outcomes [48]. Future studies should therefore be harmonized according to different methods of evaluating sensitization and outcomes, intervention products (ergocalciferol versus cholecalciferol), doses of vitamin D and intervention periods.

Table 1: Prospective birth cohort studies of 25(OH)D cord blood (CB) levels and allergy outcome in childhood (from Rueter et al. 2022 [37])

Reference and Year Latitude	Study Population	25(OH)D Level	Main Results	Higher 25(OH) D Level
Camargo et al. (2011) Wellington and Christchurch, New Zealand Latitude 41–43 πS	922 mother–child pairs General population	CB Median = 44 nmol/L (IQR 29–78)	Lower CB 25(OH)D (<10 ng/mL) levels were at higher risk for wheezing at 15 mo, 3 and 5 years than higher CB levels (>30 ng/mL). No association with asthma at 5 years of age	W↓ A←→
Rothers et al. (2011) Tuscon, Arizona Latitude 32.22 πN	219 mother–child pairs General population	CB Median = 64 nmol/L (IQR 49–81)	Lower (<50 ng/mL) and higher (≥100 ng/mL) levels of CB 25(OH)D were associated with increased total IgE and aeroallergen (sIgE) sensitization at 1, 2, 3, and 5 years of age. No association with AR or asthma at 5 years	S U A←→ AR←→
Jones et al. (2012) Perth, Australia Latitude 31.95 πS	231 mother–child pairs High-risk population	CB Mean = 58 nmol/L (SD ± 24.1)	Lower CB level is associated with a higher risk for eczema at 12 months. No association with wheeze, allergen sensitization or IgE-mediated food allergies	E↓ W←→ S←→ FA←→
Weisse et al. (2013) Leipzig, Germany Latitude 51.4 πN	378 mother–child pairs General population	CB median = 27 nmol/L (IQR: 17–43)	Positive association between CB 25(OH)D levels with food allergy within 2nd year of life. No association with eczema and sensitization	FA↑ S←→ E←→
Baiz et al. (2014) Poitiers and Nancy, France Latitude 46–48 πN	239 mother–child pairs General population	CB Mean = 44 nmol/L (IQR: 38 nmol/L)	Inverse association between CB 25(OH)D levels with early transient wheezing and eczema by age 1, 3, and 5 years. No association with asthma and AR at 5 years of age	W↓ E↓ A←→ AR←→

…to be continued on the next page.

Stelmach et al. (2015) Lodz, Poland Latitude 51.76 πN	240 mother–child pairs General population	CB median 15.8 nmol/mL (IQR 10.4–21.3)	Inverse association between CB 25(OH)D levels wheezing in first 2 years of life. No association with food allergy and eczema	W↓ E←→ FA←→
Palmer et al. (2015) Adelaide, Australia Latitude 34.92 πS	270 mother–child pairs High-risk population	CB mean 57.0 nmol/L (SD ± 24.1)	Inverse association between CB 25(OH)D and eczema to 3 years, stronger association at 1 year of age. IgE-mediated food allergies at 1 year of age in 4/260 (1.5%) children. The risk of IgE-mediated food allergies at 1 year of age (but not at 3 years of age) decreased as CB 25(OH)D concentration increased. No association with asthma, allergic rhinitis or sensitization	E↓ FA↓ A←→ AR←→
Visness et al. (2015) Baltimore, Boston, New York and St Louis, USA Latitude 38–42 πN and Madison, Wisconsin Latitude 43.1 πN	435 mother–child pairs and 258 mother–child pairs Both high-risk populations	CB median 50.3 nmol/mL (range 10.5–109.2) and CB median 52.8 nmol/mL (range 10.0–194.3)	No association between CB 25(OH)D and any wheeze in first year or recurrent wheeze at 3 years. No association with food allergy or food or aeroallergen sensitization to 5 years old. No association with wheeze in the first 3 years, of life, food or aeroallergen sensitization to 5 years old and asthma at 6 years of age	W←→ FA←→ S←→ and W←→ S←→
Gazibara et al.(2015) Rotterdam, The Netherlands Latitude 51.91 πN	2407 mother–child pairs General population	CB median 40.2 nmol/L (range 11–144.9)	No association between CB 25(OH)D levels divided into tertiles (lowest tertile (30.5 nmol/L), middle tertile (≥30.5–49), and highest tertile (≥49.0) and wheeze within the first 6 years of age	W←→
Blomberg et al. (2017) Massachusetts, USA Latitude 42.41 πN	1418 mother–child pairs General population	CB mean or median level not given	No association between CB 25(OH)D levels of sufficiency, deficiency and insufficiency (divided into 6 categories) and eczema outcome within the first 7 to 8 years of life	E←→
Hennessy et al. (2018) Cork, Ireland Latitude 51.89 πN	1050 mother–child pairs General population	CB mean 35.5 nmol/L (SD ± 18.2)	No association between CB 25(OH)D levels and: Persistent eczema in the first 2 years, Food allergy and food and aeroallergen sensitization at 2 years of age, Asthma at 5 years of age	E←→ FA←→ S←→ A←→

↓: inverse association; ←→: no association; ↑: positive association; U = U-shaped association; E: eczema; FA: food allergy; W: wheeze; A: asthma; AR: allergic rhinitis; S: sensitization

Although it seems difficult to draw a clear picture within epidemiological studies, processes on how vitamin D is affecting immune cells and immune parameters that are involved in allergic reactions (see Figure 1) are better understood. It was shown for example, that vitamin D, in particular the active form $1,25(OH)_2D_3$ effect Th1 as well as Th2 differentiation in human cord blood T cells [49], cells that are directly involved in the allergic pathogenesis. Furthermore, potential immunomodulatory functions of vitamin D on regulatory T cells were described [50]. Within the LINA cohort we were also able to show that low cord blood Treg numbers (detected via the demethylation in the Treg-specific de-methylated region (TSDR) are associated with an increased risk for allergic diseases later in life [51]. In line, we also saw a vitamin D - Treg association in the LINA cohort (Weisse et al. 2013 [39]) supporting the link between higher vitamin D levels and an increased risk to develop food allergy at the age of two years by having lower cord blood Treg numbers. So, it seems that this cell population might have contributed to the observed allergic manifestation. Next to a potential effect of vitamin D on T helper and T regulatory cells, there is also evidence that vitamin D might have an impact on antigen-presenting cells (APCs), in particular dendritic cells [52]. APCs also express the vitamin D receptor. It has been suggested that vitamin D programs DCs for tolerance by reducing their capability to activate and generate T cells, and increasing their potential to upregulate Treg (review in [37] [52]). Within this field, thymic stromal lymphopoietin (TSLP, see Figure 1) is an important player since it was shown to be critical in the context of allergy pathogenesis by inducing an inflammatory Th2 response via conditioning dendritic cell maturation [53]. Within Junge et al., 2016 [40], where extended the LINA analyses longitudinally (vitamin D analyses up to the age of two years and outcome development until the age of three) we were also able address this potential vitamin D – DC interplay. With extensive epigenetic analyses we were able to see that high cord blood vitamin D was associated with an epigenetic modification of the TSLP enhancer. In detail, children with the 25% highest vitamin D levels had a lower DNA-methylation together with a loss of repressive histone marks that resulted in a higher TSLP mRNA expression. This higher TSLP expression was also shown to be associated with (pre-) allergic outcomes like wheezing. Taken together, although our data suggest rather an allergy promoting than an allergy protecting effect of vitamin D, we were able to support these results mechanistically on several arms of investigations (Treg, TSLP). Based on our results a random supplementation with vitamin D, in particular in the very early time window in pregnancy and around birth should be seen with caution. As a minimum, baseline vitamin status, required

vitamin D dose (IU) as well as the individual allergic predisposition should be considered to check before the lipid soluble and therefore body accumulating vitamin D supplement is taken.

Via our daily nutrition we are not just up taking diet specific nutrients (such as vitamin D) but also several co-products that have been enriched in the food or food product consciously or unconsciously. One class of chemicals that are added as preservatives to foods as well as cosmetics - are the parabens. According to the US food and drug administration, parabens are added to prevent the growth of harmful bacteria and mold in order to protect both the product and consumer. Parabens are classified as endocrine disrupting chemicals (EDC) that act hormone like within the human body (according to their very similar chemical structure compared to the physiological hormone). Hereby, the endocrine properties increase with the chain length of the individual parabens. Although several epidemiological studies suggest that exposure to parabens has the potential to alter immune functions and the risk of allergic diseases in childhood [54], the association to asthma is not well studied and inconsistent. Therefore, in Junge et al. 2022a [55] we were interested to see the impact of paraben exposure on asthma development using an experimental mouse model and epidemiological analyses from the LINA cohort by outlining different exposure time points and exposure scenarios. One particular focus in this project was to address single vs mixed chemical exposure. Therefore, we applied Bayesian kernel machine regression (BKMR). While we saw both adverse and preventive effects of single parabens on asthma development in mice, paraben mixtures neither revealed effects in mice (via multiple exposure) nor in our mother-child cohort (via BKMR modelling), either when stratified for being at risk due to a positive family history of atopy or when analyzed separately for sex specificity. In conclusion, although single parabens might differentially impact asthma development, an adverse effect could not be seen in a multiple - daily life - paraben exposure setting. Consequently, not only the time point of exposure but also multiple exposure scenarios to parabens should be considered in the evaluation of individuals' specific disease risk [55].

Based on the results we saw for prenatal diet (vitamin D) or diet-associated exposure (parabens) and allergy development up to the age of ten years, we were also interested about the dietary consequences/dietary behavior in these allergic children later in life. Interestingly,

in Schütte et al. 2022 [56] we found that the children who develop food allergy or atopic eczema in their first 10 years of life were more likely to have a pro-inflammatory dietary pattern (assessed via the dietary inflammatory index (DII) [57 58]) at the age of 10 years. As we have described in Schütte et al. [56], the DII classifies human dietary patterns on a continuous scale from anti-inflammatory (values <0) to pro-inflammatory (values >0) based on a broad literature database with respect to 45 foods or nutrients that were described to be associated with inflammatory markers such as interleukin (IL)-1b, IL-4, IL-6, IL-10, tumor necrosis factor (TNF)-a and C-reactive protein (CRP) [57]. Later, this index was adapted to the nutrition of children (C-DII) [58]. To the best of our knowledge, in Schütte et al. [56] it was the first use of the C-DII in association with atopic dermatitis and food allergy. Based on our results we concluded that due to their allergy history, these children may have developed a more pro-inflammatory dietary pattern due to avoidance of possible allergy triggers such as fruits or nuts for example (which would have driven their dietary pattern more into an anti-inflammatory direction). Overall, a pro-inflammatory dietary pattern might worsen the atopic outcome itself and reduce the buffering capacity of the individual against harmful environmental exposures or triggers. Based on that data pediatricians should test affected children for their individual tolerance of allergenic foods to avoid a restrict elimination diet. Furthermore, an increased nutrient density of tolerable food items should be advised to omit undesirable effects of eating a pro-inflammatory diet.

These long lasting effects on child health and associated nutritional patterns are even more pointing out that it is mandatory to start allergy prevention earliest possible (e.g. by avoiding exposure to risk factors already during pregnancy) to protect the children walking through the atopic march with all consequences in later life.

3.2 Airway exposure and allergy development

Next to environmental exposures via the diet (such as specific nutrients or diet associated co-products) as described above, individuals are also facing other routes of exposure - such as the airways. It seems obvious, that in particular airway related (pre-)allergic outcomes such as wheezing [29] or asthma might be effected by substances that enter the body via the lung. As seen in Figure 1 and discussed within 3.1, cells of the adaptive immune system (such as T helper cells or T regulatory cells) contribute to the allergic phenotype. Moreover, cells of the innate immune system, such as eosinophil or basophil granulocytes (also illustrated in Figure

1) contribute to this non-physiologic condition. Eosinophils for example produce several cytotoxic proteins (such as Major Basic Protein (MBP), Eosinophilic Cationic Protein (ECP) or Eosinophilic Peroxidase (EPO)) which physiologically provide defense strategies against parasites, bacteria and viruses etc. However, these proteins also promote the allergic reaction: MBP for example induces the release of histamine from mast cells. Histamine on one hand triggers the occurrence of rash, with swelling, redness and itchiness of the skin or –on the other hand – vasoconstriction of the airways. Not surprisingly, it was shown that mature eosinophils are increased in tissue, blood and bone marrow in patients with asthma and atopic dermatitis, with numbers positively correlating with disease severity [59] [60]. Mature eosinophils differentiate from eosinophil-lineage committed progenitor cells (EoPs), which arise from bone marrow-derived CD34+ hematopoietic progenitor cells (HPCs). In addition to mature cells, also these bone marrow progenitor cells can be released into the blood, migrate to allergic tissues, differentiate in situ and contribute themselves to the allergic phenotype [61] [62] [63] [64]. However, whether and how also environmental chemicals – in particular when exposure occurs early in life - can influence these allergy relevant progenitor cells (Eo/B CFUs) and therefore contribute to the diseases pathology was not clear and therefor aim of several projects within this thesis (see Figure 5 for a summary). In detail, we investigated specific volatile organic compounds (VOCs) which are one of the most common and important classes of indoor air pollutants, in particular with the rapid urbanization, economic growth [65] and the increasing time spend indoors in recent years [66]. VOCs have different sources and specific classes of VOC can individually be related to them. Indoors, VOCs are mainly emitted from various building materials such as wood, furniture, paint or floor covering, personal care products (such as disinfectants) and tobacco smoking [65].

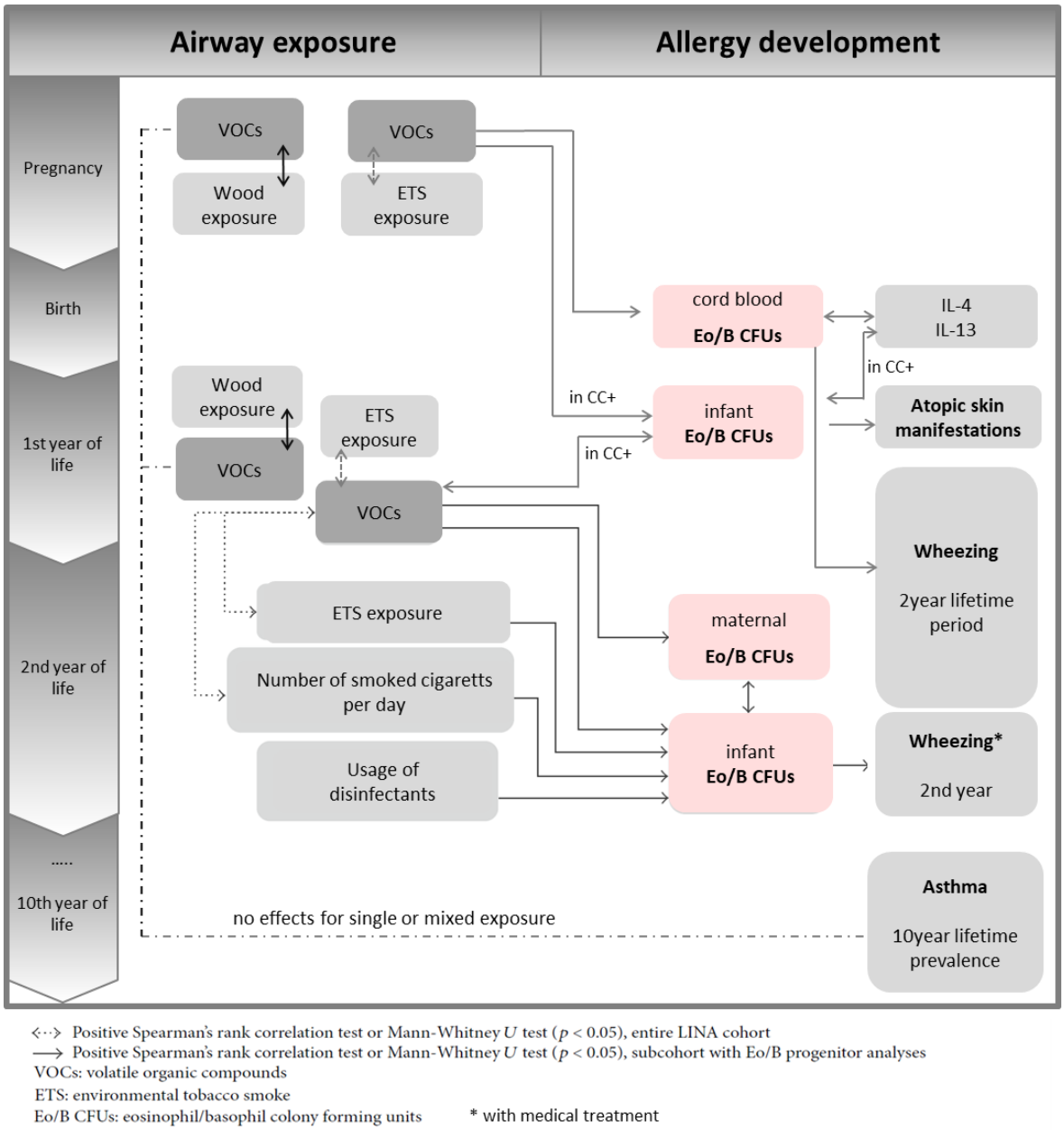

Figure 5: Results summary scheme of different airway exposure conditions (different volatile organic compounds; VOCs) and allergy development: Main focus in this field was to unravel the potential mechanistic contribution of allergy relevant hematopoietic stem cells (Eo/B CFUs, pink boxes). These cells were addressed within the LINA cohort in a longitudinal manner – at birth, as well as at children's age of one and two years. Also the maternal status of Eo/B progenitors was assessed at children's age of two years (Weisse et al. 2012, Junge et al. 2014, Hörnig et al. 2016). In addition, single vs. mixed VOC exposure was addressed in the LINA cohort as well as in a transgenerational mouse model (Junge et al. 2021). © Graphic designed by Kristin Junge

It was already shown that renovation activities (so the usage of VOC emitting flake board, wall-to-wall carpets and PVC) during pregnancy induce an Th2 shift in fetal but not in maternal immune system [67]. Next to others, this data shows that there is a difference in the succeptibilty of the immature immune system of fetus compared to the immune system of the mother. In addition, this Th1/Th2 imbalance already seen at birth was related to the allergic sensitization of the LINA children at the age of one [67]. That the Th2 cytokines IL-4 and IL-13 differentially

effect also Eo/B maturation has been shown before [68]. Within LINA, we also saw that cord blood Eo/B progenitor cells were associated with a Th2 cytocine mileau (Il-4 and Il-13) at birth (Junge et al., 2014) [69]. With respect to our hypothesis, we were able to show that VOC emitted from environmental tobacco smoke (ETS) increase the number of these allergy relevant progenitor cells at birth / in cord blood. In addition, children with higher numbers of Eo/B CFUs at birth had an increased risk to develop wheezing symptoms during the first two years of life (Junge et al., 2014) [69]. Until than, this was the first manuscript showing that the number of these pregenitor cells in cord blood might be usable as an early meassureable predictor for allergy conditions later in life [69].

Furthermore, there is evidence that the formation of these Eo/B progenitor cells is significantly promoted by TSLP [70]. As already discussed in 3.1., we also saw an epigenetically modified higher TSLP expression in the LINA children with wheezing symptoms in early childhood in dependence on their vitamin D concentrations in cord blood. Most interestingly, also maternal and cord blood vitamin D concentration were clearly correlated with the number of cord blood Eo/B CFUs (unpublished data: *maternal vitamin D* during pregnancy vs IL-3 stimulated Eo/B CFUs in cord blood: n= 25, R=0,379, p=0,062 as well as GM-CSF stimulated Eo/B CFUs: n=23, R=0,519, p=0,011; *cord blood vitamin D* vs IL-3 stimulated Eo/B CFUs in cord blood: n= 25, R=0,435, p=0,030 as well as GM-CSF stimulated Eo/B CFUs: n=23, R=0,432, p=0,039. There were no effects seen for IL-5 stimulated (the more terminal differentiated) Eo/B CFUs and vitamin D during pregnancy or cord blood. Although a causality cannot be drawn, there might indeed be a pro-allergic component of vitamin D within the LINA study population – potencially via a TSLP triggered in situ hematopoiesis of eosinophilic progenitor cells, that might additionally be increased by VOC exposure .

Next to the analysis of the eosinophil progenitors in cord blood, we also analyzed the cells at children's age of one (Weiße et al., 2012 [71]) and two years (Hoernig et al., 2016 [72]). In line, VOCs associated with exposure to environmental tobacco smoke during the first year of life were related with increased numbers of allergy relevant Eo/B progenitor cells and consequences for (pre-)allergic outcomes (Weiße et al., 2012 [71]) such as cradle cap or atopic dermatitis. Although on the first glance, there might not be a direct link between airway exposure and allergic skin manifestations, there is data from most recent investigations also showing that VOCs are possitively associated with atopic dermatitis [73]. Finally, we were also interested, if these eosinophil progenitors also show a difference in their sensitivity in infants

compared adults. Therefore, we analysed these cells in two year old children and their moms at the time when children had their second birthday. Both individuals were obviously sharing the same environmental exposure in their homes. Indeed, we saw a stonger sensitivity of the infant compared to the maternal eosinophil progenitor cells with respect to VOC exposure from environmental tobacco smoke or the usage of disinfectants. Also, higher numbers of eosinophil progenitor cells of two year old children were associated with an increased risk of developing wheezing within the second year (Hoernig et al. 2016 [72]). As show more recently by a comprehensive multiscale network analysis of transcriptomic and epigenetic data in asthmatic 7 to 15 year old children, also other environmental chemicals such as B[a]P promote eosinophil recruitment (via increased eosinophil marker genes like IL5RA) [74] [75]. So taken together in a longitudinal view, prenatal and early infant environmental VOC exposure seems to increase allergy relevant eosinophil progenitor cell in the children via a Th2 driven background. Further, this progenitor cell increase was associated with different atopic outcomes in a timeline manner: while skin manifestations were associated very early in life (within first 12 months), an increased risk for wheezing was observed in the second year. This is in line with the occurrence of allergic manifestations within the atopic march [76] starting from atopic dermatitis, while manifestations of the airways and lung usually follow. Therfore, we were also interested in the potencial impact of VOCs on asthma development – an allergic condition that usually starts to develop later in life around school age. For that we used VOC analysed in pregnancy and the first year of life, and here in particular VOCs emitted from wood products. This is important, as pregnancy is not only a period that is particularly susceptible to ambient air pollution [77] but at the same time frequently goes along with increased renovation activities and the purchase of new furniture (Junge et al., 2021 [78]). Interestingly, an exposure to single or multiple wood-related VOCs during pregnancy or the first year of life was not associated with early wheezing or asthma development in the LINA children, which was also independent from their family history of atopy. These effects were supported by intense invstigations in a murine transgenerational asthma model. VOCs emitted from pinewood or/and OSB (put up in the murine cages) were analysed with respect to their potencial airway modification in allergen-sensitized and non-sensitized mice using an acute, chronic and cross-generational asthma model. We found that neither short-term, long-term nor perinatal exposure to these wood products had pro-inflammatory or asthma-promoting effects in healthy or sensitized animals. This overall “no effect” result of wood related VOCs

such as terpenes (α-pinene, β-pinene, 3-carene, limonene) and aldehydes (pentanal, hexanal, heptanal, octanal and nonanal) was well received since innovative and sustainably produced wood products, when coupled with sustainable forest management, have been considered to contribute substantially to climate change mitigation and are therefore recommended as alternative construction or daily life materials [79] [80].

So taken together, our analyses demonstrated that the effects of VOCs canot be lumped together: An early exposure to aromatic VOCs that were associated with environmental tobacco smoke indeed seem to promote the allergy development by modifying several different immunological parameters (discussed above) whereas VOCs from natural, biological material (wood and wood products) were shown not to adversly effect the respiratory system.

To overall sum up the generated data within the field of allergies, we were able to provide manysided answers for the research questions outlined in the beginning of this thesis. From personal lifestyle perspective, we were clearly able to see within the LINA cohort that maternal and early infant vitamin D levels were possitively associated with later infant allergy development, potencially transmitted via an epigenetic regulation of TSLP. Additionally, we were able to see that the nutritional pattern of these allergic children later in life was dependent on their allergic outcomes, with a more pro-inflammatory diet seen in children that developed an allergic condition. From the environmental perspective, we were able to see that a prenatal exposure to conservatives (parabens) was differentially associated with allergies of the children / offspring. While single parabens might differentially impact asthma development, an adverse effect could not be seen in a multiple paraben exposure setting. When we applied the same single vs. mixture approach for VOC emitted from wood or wood products, we saw no increase in allergy risk of the children later in life. On the other hand, aromatic VOCs emitted for example from tobacco smoke enhanced the risk for allergies in the children later in life. One mechanistic explanation might be an increasd number of allergy relevant eosinophil/basophil progenitor cells due to aromatic VOC exposure.

3.3 Oral/dermal exposure via endocrine disrupting chemicals and obesity development

In the following part of this discussion, we are focusing the manuscripts that have been published on the second outcome of interest: obesity. As introduced already, overweight or

obesity are characterized by an increased accumaulation of fat tissue within the body which is a process directly dependent on the energy content of nutrients/foods consumed. However, also diet associated chemicals are considered to have an impact within obesity development [81]. These chemicals were named “obesogens” [82], one particular class as endocrine-disrupting chemicals (EDCs) [83]. According to their very similar hormone-like chemical structure, EDCs can act directly on hormone receptors (as hormone mimics or antagonists) or modify proteins that control the delivery of a certain hormone to its physiological target cell or tissue [84] [85] and therfore impact obesity development [86] as well as a variety of other diseases [87]. In particular during critical time windows in development, EDCs can permanently alter normal physiology and increase susceptibility to diseases like obesity later in life [88]. In this context, the prenatal and early postnatal period is highly vulnerable to EDC exposure as it is the time of developmental programming important for organogenesis and tissue differentiation [89] [90]. The group of chemicals that has been identified as EDcs is highly heterogeneous and includes synthetic chemicals used as industrial solvents/lubricants as well as their byproducts [polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), dioxins], plastics [bisphenol A (BPA)], plasticizers (phthalates), pesticides [methoxychlor, chlorpyrifos, dichlorodiphenyltrichloroethane (DDT)], fungicides (vinclozolin), pharmaceutical agents [diethylstilbestrol (DES)] [84] or preservatives (parabens) [91]. However, also, natural chemicals found in human and animal food (e.g., phytoestrogens, including genistein and coumestrol) can act as endocrine disruptors [84].

Within this thesis, two classes of EDCs were adresses in the context of obesity development: Bisphenol A (e.g. within plastic materials) and the parabens (e.g. within food or cosmetics). The route of exposure to these chemicals is mainly oral or dermal – although not exclusivly. Both project were performed in a translational research design, combining data from the prospective mother-child cohort study LINA, from cross-generational mouse experiments (F0 and F1 generation) and from *in vitro* mesenchymal stem cell culture. Both project also had a particular focus on early priming via epigenetic modifications [90] [92]. The results that are discussed in this section are summarized in Figure 6.

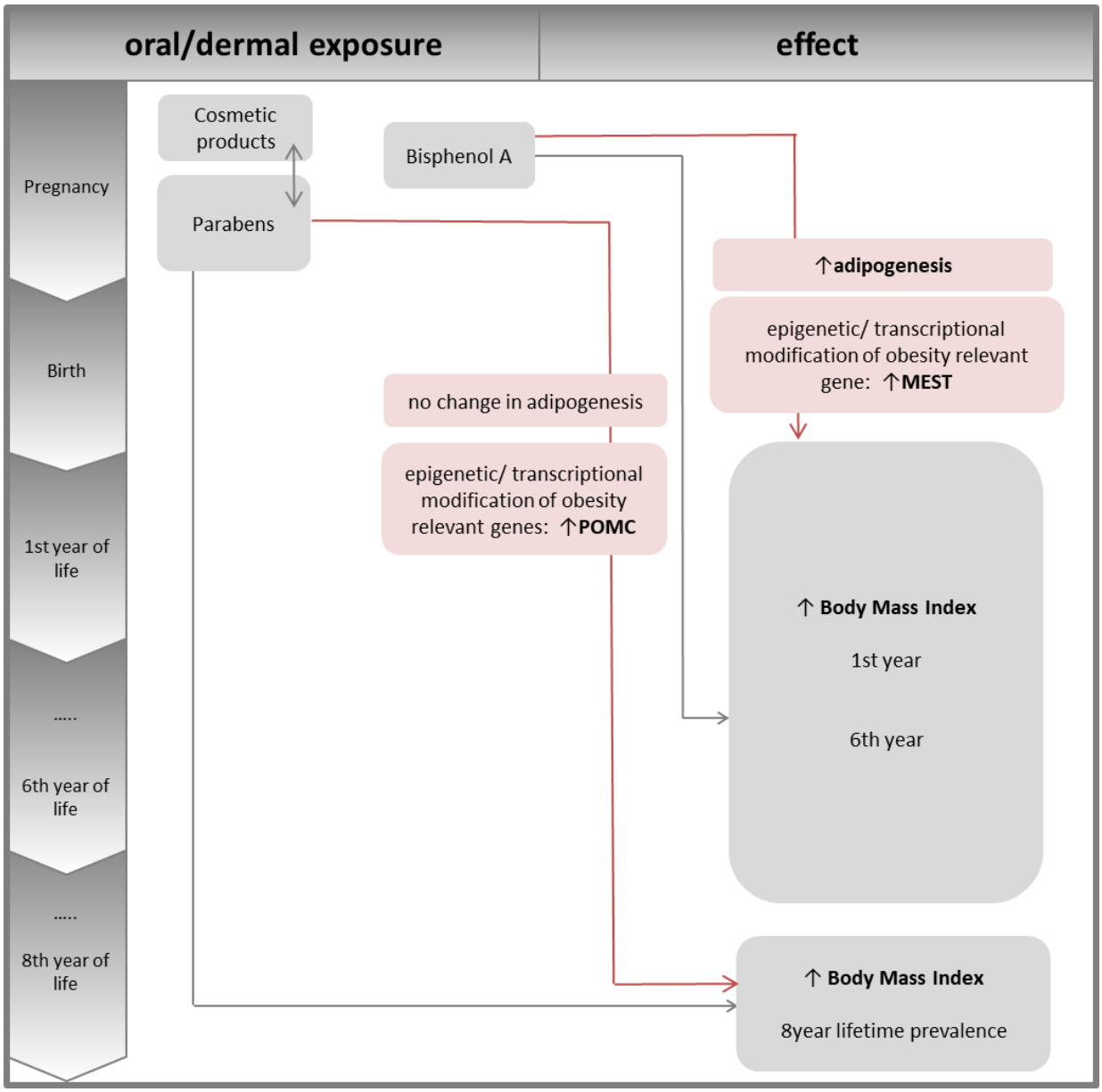

Figure 6: Results summary scheme of different oral/dermal exposure conditions (different parabens or Bisphenol A) and obesity development: All projects herein were performed in a three-dimensional setting combining data from the LINA cohort with data from transgenerational mouse and cell culture models. Main focus in this field was to unravel the potential mechanistic contribution of an altered adipogenesis via obesity relevant mesenchymal stem cells (pink boxes; Junge et al. 2018, Leppert et al. 2020). © Graphic designed by Kristin Junge

Interestingly, maternal expsore to BPA (Junge et al. 2018) [90] and butyl paraben (BuP; Leppert et al. 2020) [92] showed similar "obesogenic" effects in the corresponding infants/offspring with increasing levels of EDC exposure being associated with higher infant body weight. However, the mechanistic regulation interestingly was different. On one hand, Junge et al. [90] epidemiologically provided first evidence that prenatal BPA exposure causes epigenetic changes in the MEST promoter which was potentially contributing to the overweight development in children until the age of 6 years via an increased adipogenesis. Independent

from chemical exposure, the MEST gene has been described in association with obesity [93] [94] [95], adipocyte size [96], and preadipocyte proliferation [97] in mouse and human studies before. The epidemiological LINA results were also supported by experimental mouse models: prenatally BPA exposed mice showed a hypo-methylation of the MEST promoter region and developed a significantly higher body weight compared to controls. Furthermore, a direct stimulating impact of BPA on adipocyte differentiation from human MSC was observed (from Junge et al. 2018 [90]). On the other hand, in Leppert et al. 2020 [92] the effect of prenatal paraben exposure on offspring/infant weight development was analysed. Although prenatal BuP exposure was also shown to be associated with an increased risk for later overweight/obesity, there was no direct effect seen within the adipocyte differentiation from human MSC after BuP exposure. So the underlaying obesigenic mechanism of BuP was obviosly different compared to BPA. Interestingly, in Leppert et al. it was observed that maternal BuP exposure of mice induces a higher food intake of the animals and consequently provoked the weight gain in female offspring. This effect was accompanied by an epigenetic modification in the neuronal Pro-opiomelanocortin (POMC) enhancer 1 leading to a reduced hypothalamic POMC expression. POMC is involved in anorexic signalling pathways to suppress food intake. After stimulation, α-MSH is released from POMC by proteolysis and binds to MCR4 to induce satiety [98] [99]. So taken together, we reported that maternal paraben exposure may contribute to childhood overweight development by altered POMC-mediated neuronal appetite regulation. Overall our data support, that an early exposure to EDCs, in particular BPA as well as BuP, seem to have early priming effects on the fetus with respect to an increasing risk for overweight development later in life – however, the pathways of regulation differed between chemicals.

Although there is a variety of information on EDCs already available in the context of prenatal exposure and infant weight development [100], there is still a lot to be clarified in this context in the future. Next to our's - it was seen in several projects that prenatal exposure to EDCs might cause epigenetic modifications in the offspring [101] with consequences for later overweight development [102], however, there is still a "black box" on the molecular initial event and how the chemicals/EDCs mechanistically induce this change of the DNA state. On one hand, the prenatal EDC exposure might primarily cause an adverse effect on the mother itself and resulting mediators are than be passed further to the offspring and affect it's health indirectly. Levels susceptible for EDC triggered changes might be for example the maternal immune

system [103] (e.g. via cytokine levels), the maternal metabolism [104] or the maternal microbiome [105] etc. Resulting unfavourable mediators might then be transferred via the maternal-fetal interface/the placenta to the fetus and promote it's disease develoment. For example, it was shown that paraben exposure was positively associated with gestational weight gain (GWG) rate during pregnancy, especially during the first trimester [106]. That gestational overweight is a major risk factor for obesity in childhood was shown several times [107]. On the other hand, EDC exposure – in low dose daily life exposure situations - might be tolerable for the maternal organism (with even no changes in maternal mediators), but the chemicals might reach the unborn fetus and induce an adverse effect directly in the highly susceptible and unprotected child (immature detoxification capacities, immature immune system, etc.). Furthermore, EDCs might also act on the placenta morphology/physiology itself, or impact the variety of nutrient transport via the placenta (see Figure 7; from Junge et al. 2022b, *accepted* [108]). That EDcs have the potencial to alter placentation and related pathways was shown before [109] [110], however data on an EDC driven altered nutrient transport in the placenta is scarce. Of course, it might also be a combination of both a maternal and fetal effect. Future studies are needed in that context to address the molecular initial event of a prenatal obesogenic EDC effect in order to offer possible intervention strategies [111].

Another aspect, which should also experience more future research is the fact, that the human individual (as well as animals, wildlife, etc.) is not only facing exposure to one specific chemical but rather a combination/multiple exposure of several compounds in daily life. As already discussed in the field of allergies earlier, there is also data that multiple exposure to several daily life chemicals should be considered in the context of obesity. As published most recently, a mixture of EDCs was able to induce adipogenesis and DNA methylation changes in human mesenchymal stem cells [112]. Other groups also used the BKMR approach [113] that was applied also in Junge et al. 2021 [78] and Junge et al. 2022a [55] in the context of a potential mixture effect of EDCS in obesity.

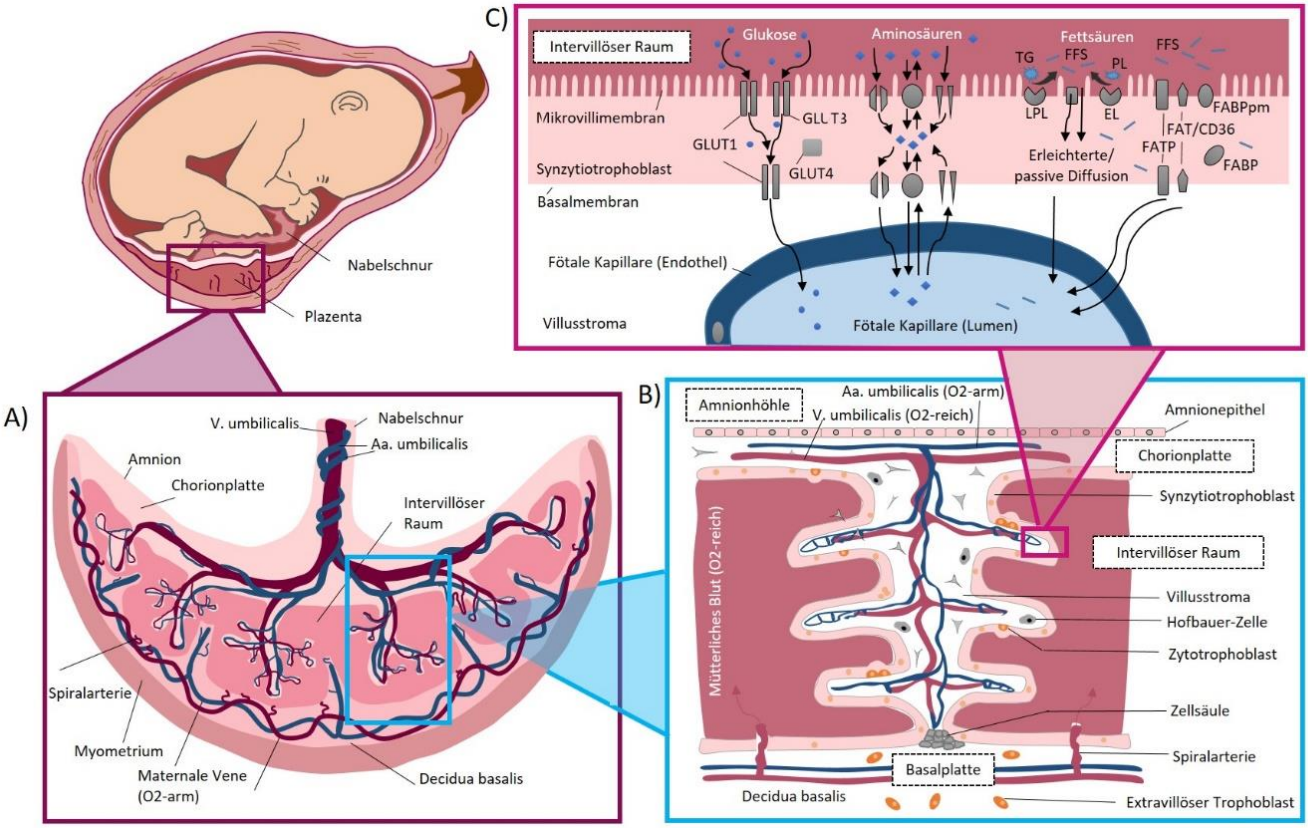

Figure 7: Scheme of the maternal-fetal interface (A) structure of the term Plazenta (B) the villous tree as well as (C) transport mechanisms of nutrients, adapted from [114], accepted to be published in Junge et al. 2022b [108] © Graphic designed by Kristin Junge

So taken together, as part of this thesis we were able to show that a prenatal exposure to EDCs seem to be a potent trigger in the obesity development, by inducing different epigenetic modifications that might contribute to the disease pathogenesis. However, even in any given environment, there is considerable individual variation in body weight and fat mass, suggesting that obesity is affected by complex interactions between genetic, developmental, behavioral, and environmental influences [115].

3.4 Stress & cytokine levels and obesity development:

As just outlined above, next to prenatal external obesogenic chemicals from the environment, there is also a variety of internal mediators of the pregnant mother that might increase the risk for childhood obesity development. Despite the appreciation that high caloric intake and sedentary behavior contribute to overweight development, less attention has been given to the effects of pre- and postnatal perceived maternal stress or the maternal Th2 cytokine level on weight development in early childhood. In this last part of the discussion, we are therefore

addressing maternal stress (Leppert et al. 2018 [116]) as well as the maternal cytokine profile (Englich et al. 2017 [117]) as proxies' of the personal lifestyle and health. A summary of the discussed results of this section is shown in Figure 8.

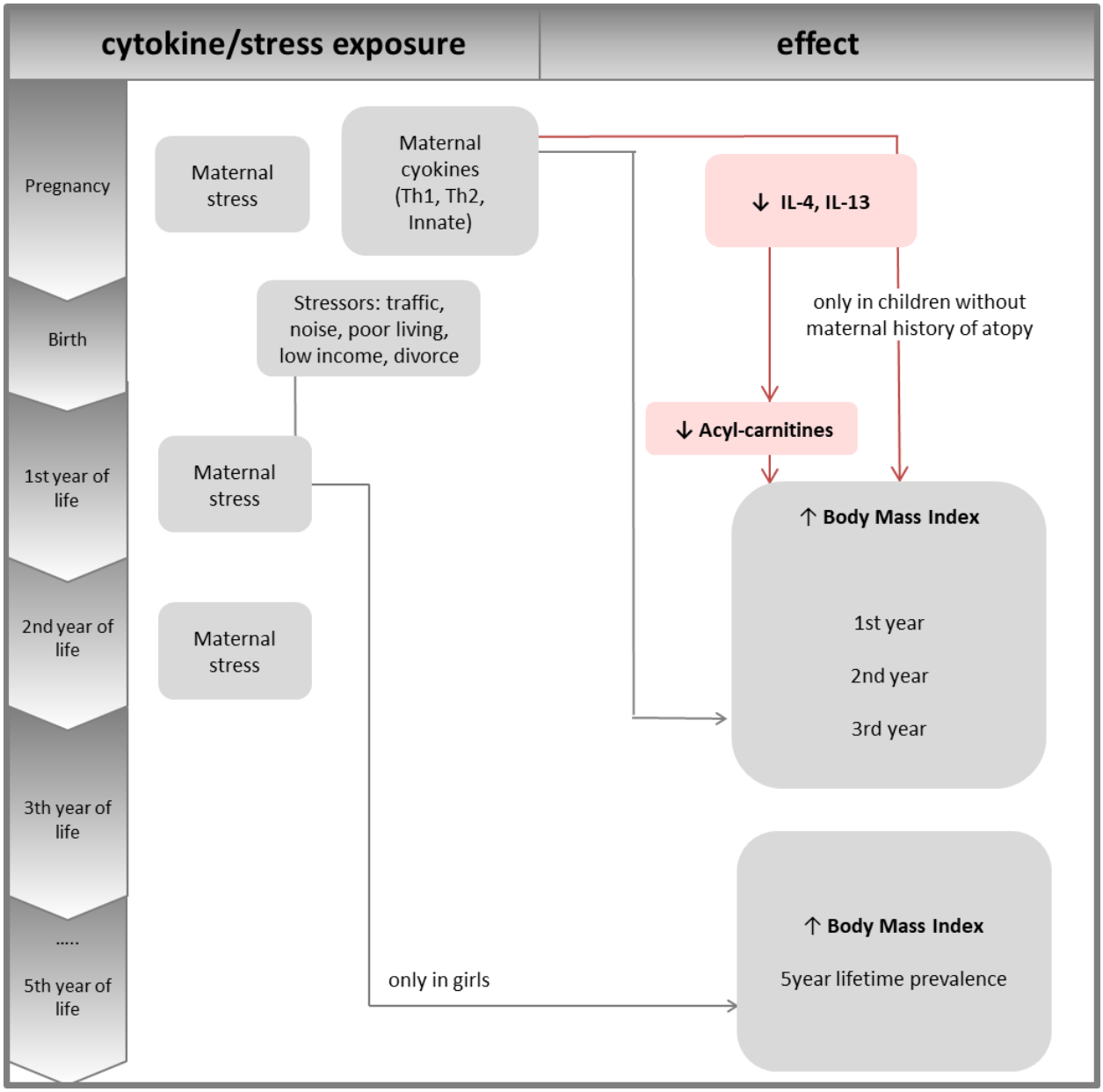

Figure 8: Results summary scheme of maternal cytokine/stress exposure and obesity development: All projects herein were performed within the LINA cohort. Main focus in this field was to unravel how individual maternal health conditions / lifestyle can prime obesity risk in the offspring (Englich et al. 2017, Leppert et al. 2018). © Graphic designed by Kristin Junge

On one hand, our aim was to investigate the longitudinal relationship of early maternal perceived stress (so during pregnancy as well as during the first and second year of life) and BMI development in children up to five years of age. Indeed, psychological aspects like early infant parental distress [118] as well as maternal depression [119] have been introduced as potential factors promoting children's overweight [120], however have not been addressed in

this broad and longitudinal setting. With our data we were able to provide evidence that childhood BMI trajectories develop early and that in particular postnatal maternal stress during the first year after birth is a persistent positive predictor of BMI z-scores up to the age of five years - while no obesogenic effects were related to the perceived stress during pregnancy or the second year of life. We hypothesized that the duration of breast feeding might have been negatively affected by maternal stress [121] during the first year with consequences for later weight development [122]. However, we were not able to show any differences in the breastfeeding duration or the time of solid food introduction in relation to the maternal stress level of our cohort population. So since the maternal stress during the first year was not translated to the child via the nutrition, there are obviously other routes of stress-transmission involved – such as the direct stress experience of the child via the mother. Interestingly, we saw the stress related to higher BMI only in girls. Indeed, it was shown before that girls respond to perceived stress by excessive eating behaviors, and by requesting sweet and high fat foods [123] [124] [125]. So maybe already at that very young age, the girls of the LINA cohort might have developed a different dietary behavior due to the perceived stress of the mother during the first year of life. We further suggested in Leppert et al. [116] that the perceived maternal stress may be experienced less intensely or may be better compensated by boys, which was also seen in other studies [124]. Based on our data we draw the recommendation that midwives, gynecologists, pediatricians and general practitioners should be particularly attentive to signs of maternal stress in the first year following the child's birth to reduce the risk for childhood obesity – in particular in girls. If mothers are helped early on or are offered support, we may be able to "kill two birds with one stone": To improve maternal well-being and also prevent their children becoming overweight.

Next to a potentially altered dietary behavior of maternal-stress experienced children, the question was also raised if and how perceived stressful events of the pregnant mother can mechanistically be translated to the fetus and impact its health development. Indeed, there are several pathways / levels of modifications that seem to play a role. One major level of regulation due to perinatal stress were shown to be epigenetic modifications [126]. Also, in the LINA cohort it was shown that prenatal stress was associated with epigenetic driven changes of immune system relevant outcomes [127]. Furthermore, it has been described that pregnant women suffering from self-perceived stress, anxiety, and depression show dysfunctions of their immune system - which may be responsible for fetal and/or newborn disorders via

compromised placental gene regulation [128]. "Deprogramming" of the immune system due to perceived stress of the mother might therefore contribute to an unfavorable health development of the child.

Since immune cells have also been highlighted as important factors contributing to the pathogenesis of childhood obesity, we were also interested to assess the maternal immune system with respect to their cytokine profile (innate (IL6, IL8, IL10 and TNFα) and adaptive (IFNγ, IL4, IL5 and IL13) cytokine blood concentrations; Englich et al. 2017 [117]) and the associated risk for obesity in their children later in life. In particular, the Th2 cytokines (IL4, IL5 and IL13) had not been studied in that context before. Based on the generated data we were able to show for the first time that the maternal Th2 status may be linked inversely to early childhood overweight development. In contrast to the fact that Th2 cytokines usually drive the allergic reaction / inflammation, these cytokine were indeed shown to have anti-inflammatory properties in adipose tissue in several mechanistic studies [129] [130] [131]. Most dominantly, this inverse Th2 cytokine association with the BMI was seen in mother-child pairs when the mother was free from any history of atopy. As overweight development and obesity are related to changes of the metabolite profile we have also assessed the potential priming effect of maternal cytokine concentrations on early infant metabolic programming [117]. Indeed, we found positive associations of maternal Th2 cytokines, in particular IL13, and acylcarnitines in children and an inverse association of acylcarnitine levels and BMI Z-scores. Taken together, we were able to show a potential priming effect of the maternal immune status during pregnancy on children's longitudinal body weight development, as well as weight-related metabolomic alterations in acylcarnitines. However, our data do not support a mediating role of acylcarnitines on maternal IL13-induced weight development [117].

So taken together, within the field of obesity, we were able to provide extensive answers for the research questions outlined in the beginning of this thesis. From the environmental perspective, we were able to see that an exposure to EDCs during pregnancy, in particular to bisphenol A and butyl paraben carries an obesogenic risk for the children later in life. Mechanistically, we were able to show that one option of effect translation from the mother to the child might be via epigenetic mechanisms. However, the pathways affected via EDC driven epigenetic modifications were different for the two EDCS addressed: We saw an altered adipogenesis due to BPA vs. an altered hunger-satiety regulation in the brain after BuP

exposure. From the personal perspective, we were additionally able to provide knowledge that high maternal perceived stress, particularly in the first year of the child, and well as a reduced Th2 milieu during pregnancy also seem to play a role when the overall obesogenic risk of a child is calculated.

3.5 Outlook

The prevalence of allergies and obesity has increased tremendously in the last years. Both diseases share the combination of genetic, physiological, environmental and behavioral factors in their pathology. Once present, allergies and obesity can be a significant burden on individuals, communities and economic resources. As for other non-communicable diseases (NCDs), the best option for reducing the disease burden is their prevention. Since the risk for such diseases can be established very early in life in the maternal womb, it is important to consider specific risk factors already before or from conception onwards tackle an obesogenic and/or allergenic environment [132].

The generated data from 12 original and 3 supporting articles presented within this thesis provided knowledge that indeed might help shaping a healthier environment for the expecting mother and the developing fetus. The World Health Organization (WHO) 2019 named five key risk factors that need to be focused on during pregnancy or childhood with respect to the disease **prevention**: unhealthy diet, tobacco use, harmful use of alcohol, physical inactivity, and air pollution [133]. Based on the results of this thesis (and additional broad literature available), this recommendation should be extended on the exposure to environmental chemicals, outlining that – next to an inhalative exposure – also their oral or dermal absorption route carries an unfavorable health risk. Therefore, on one hand, future strategies should limit environmental exposure (meaning lowering their industrial usage) to an absolute tolerable minimum - enforced via stakeholder recommendations and policy makers. On the other hand, supportive environments and communities are fundamental in shaping people's health awareness and health oriented choices. Furthermore, midwives, gynecologists, pediatricians and general practitioners should be particularly attentive to the maternal health in or even before pregnancy, raising aweness and therefore providing optimal possible growth and developmental conditions for the fetus and child. One option to empower the pregnant mother itself reducing their exposure to harmful environmental chemicals might be the usage of already available Apps, like TOXFOX for example [92] - just scan the product to buy and get

the information on harmful ingredients. However, the underlying databases do not particularly focus on scientific data in the pre/perinatal time period. There are projects ongoing at UFZ that aim to close this gap.

However, to be able to significantly reduce disease prevalence, there is still a lot that needs to be considered in further research.

As already discussed earlier - it was seen in several projects that prenatal exposure to EDCs might cause **epigenetic modifications** in the offspring [101] with consequences for later overweight development [102], however, there is still a "black box" on the molecular initial event and how the chemicals/EDCs mechanistically induce this change of the DNA state. Deeper knowledge in that field would be the basis offering possible intervention strategies [111]. According to Cavalli et al. it is important in this context to distinguish driver from passenger roles of epigenetic alterations – which will make it possible to identify diseases in which epigenetics might affect diagnosis, prognosis and therapy. Dissecting the interplay between epigenetic components and other disease pathways will also allow the development of combinatorial intervention approaches [16]. Herein, nutritional influences on epigenetic programming have also been discussed with respect to asthma, allergy, and obesity development [134]. Nutritional programming is also a recognized term in the literature [135].

Another topic that has increased in its importance in the last years is the involvement of the human **microbiota** in individual health. Also our group has shown very recently that prenatal glyphosate exposure – a herbicide used in the production of plants – might alter the gut microbiota of the offspring with consequences for their later immune response [136], or even disease development. There are several most recent reviews available about the role of the microbiome in the pathogenesis of obesity [137]. In detail, some animal and human studies have shown that produced metabolites of gut microbiota are correlated with host obesity, energy metabolism, and inflammation [138]. It needs to be elucidated in future projects which microbial pattern might help prevent or might even promote disease development such as allergies or obesity. Since both diseases and the microbiota itself share the same determining factors such as term and mode of delivery, exposure to antibiotics, maternal diet, presence of siblings and family members, pets, genetics, local environment, and geographical location [139], it seems indeed reasonable that these tiny microorganisms might have a larger impact on our health

than formerly expected. It is already under investigation if and how this microbial community can be shaped during early nutrition [139] [140].

Overall, it is a highly considerable path for the future to deeper identify and promote daily life **nutrition** as an enormous chance to **increase resilience** against harmful substances of our obesogenic/allergenic environment. As discussed above, mechanistic levels susceptible for nutritional modifications could be epigenetic programming, the individuals' immune response or even the microbial community, if not all. In particular, the early life interplay between external preventive (e.g. nutritional compounds) and triggering compounds (e.g. unavoidable chemicals) and the identification of potential coping effects/mechanisms is of high relevance with respect of drawing prevention or intervention strategies for early infant disease development. There is indeed work ongoing in that field: It was shown for example that Mediterranean diet and vegetable-based dietary patterns were positively associated with higher psychological resilience (stress coping capacity), whereas western-type diets were not [141]. Future work should aim to identify nutritional compounds/patterns that could act as resilience factors by offering higher coping capacity for chemical induced stress or adverse health outcomes in infancy. One most recent project is awaiting results as investigating omega-3 fatty acids to prevent neonatal tobacco-related outcomes (INFANTS) in a double-blind, randomized, placebo-controlled parallel clinical trial in pregnant smokers **[142].** The research aspect of nutritional resilience (meaning the interpretation of effects from a **multiple parallel exposure** to nutrients as well as chemicals etc.) follows the Horizon EU Research program [22]: herein it was outlined that it is particularly important to also analyze multiple exposures or chemical mixtures; otherwise the risk to human health would be falsely interpreted. **Mixture modelling** [23] (as applied in several projects of this thesis) would also help in this context to identify possible agonistic or antagonistic effects as a consequence of the multiple daily life exposure. Or in other words, to identify how a beneficial nutritional pattern might help to compensate harmful exposure effects of environmental chemicals.

One final aspect that might also be received by future investigation is the potential **interplay** between the two diseases addressed within this thesis. It was described for example that **obesity** is a major risk factor and disease modifier for asthma in children and adults [143]. It was also described that obese asthmatic patients have more symptoms, more frequent and severe exacerbations, reduced response to several asthma medications, and decreased quality of life

compared to non-obese asthmatic patients [143]. Also other **allergic outcomes** such as allergic rhinitis, allergic conjunctivitis, atopic dermatitis or food allergy were – in part – shown to be associated with obesity [144]. It was hypothesized that an imbalance in the obesity shaped immune responses may support or facilitate a hypersensitive reaction of the immune system or even decrease the body's immunological tolerance, ultimately leading to allergic diseases [145]. However, more studies are needed to deeper characterize the immunological effects of obesity and its possible impact on allergic diseases. Nevertheless, there is growing evidence that weight loss interventions – next to a reduction of obesity - also help improve asthma outcomes [143]. The same is true for minimizing harmful environmental exposures: reducing the obesogenic triggers during pregnancy will have beneficial effects on the obesity development of the child later in life and in the same line reduce the chance of an obesity driven allergic outcome development.

4. Summary

The prevalence of allergies and obesity are a major human health burden. Both diseases share the combination of genetic, physiological, environmental and behavioral factors in their pathology. Once present, allergies and obesity can be a significant burden on individuals, communities and economic resources. As for other non-communicable diseases (NCDs), the best option for reducing the disease burden is their prevention. Since the risk for such diseases can be established very early in life already in the maternal womb, it is important to consider specific risk factors already before or from conception onwards. As part of this habilitation thesis, in 12 original and 3 supporting manuscripts (all as first or senior author) we provided scientific knowledge on several early lifestyle and environmental factors that seem to impact the disease development. The research questions are mainly addressed within the human epidemiological prospective cohort study (LINA), supported by mechanistic investigations via disease associated stem cell culture models and with particular focus on early priming via epigenetics.

In terms of **allergies**, we were able to show that maternal or early infant **vitamin D** levels were positively associated with the disease development (Weisse et al., 2013), and that epigenetic regulations in the TSLP gene seem to be involved (Junge et al., 2016). Additionally, we were able to see that children who developed an allergy later in life showed an altered – unfavorable - **nutritional pattern** as a consequence of their avoidance of allergen-containing foods (Schütte et al., 2022). Furthermore, via our daily nutrition we are not just up taking diet specific nutrients (such as vitamin D) but also several co-products. Therefore we also analyzed an early life exposure of a class of preservatives – the **parabens** – with respect to childhood allergy development. Here, we were able to show that although single parabens might differentially impact asthma development, an adverse effect could not be seen in a multiple - daily life - paraben exposure setting (Junge et al. 2022b).

Further, we showed that also other routes of exposure matter with respect to **allergy** development. For example, early indoor inhalative exposure to **volatile organic compounds** (VOCs) emitted by cigarette smoking, cleaning or renovation activities negatively impact allergy development. Herein, a potential mediating effect of allergy relevant hematopoietic stem cells (eosinophil/basophil progenitor cells) at different infant ages was detected (Birth:

Junge et al. 2014, year one: Weisse et al., 2012, year two: Hörnig et al., 2016). Interestingly, VOCs emitted from wood products did not show harmful effects with respect to asthma development – either singularly or in multiple daily life exposure to several of these emitted VOCs (Junge et al. 2021).

As the second outcome of interest, the effects of different lifestyle and environmental factors on **obesity** development were investigated. Herein, early exposure to **endocrine disrupting chemicals** such as **Bisphenol A** (Junge et al, 2018) or **Butyl-paraben** (Leppert et al. 2020) were shown to increase the risk for childhood overweight/obesity. Both projects were performed in a 3-dimensional setting, combining epidemiological cohort data with in vitro and in vivo analyses, with a deep focus on epigenetic regulations. Interestingly, although both chemicals had obesogenic effects, the the pathways of regulation differed between chemicals (induced adipogenesis vs. altered hunger/satiety regulation in the brain).

In terms of the risk of developing **obesity**, personal health and lifestyle were analyzed. Here, it was shown that perinatal **maternal perceived stress** during the first year of life (Leppert et al. 2018) as well as the prenatal maternal **cytokine milieu** (Englich et al. 2017) were priming towards an increased weight development of the children later in life.

Taken together, the knowledge created within these habilitation projects will help to (1) minimize or reduce the individual exposure against substances shown to be harmful with respect to the disease development (e.g. by recommending using Smartphone Apps that provide a huge data base on daily life products and containing chemicals) (2) increase awareness of stakeholders and policy makers to create a non-obesogenic and non-allergenic public environment, in particular for the pregnant mothers (e.g. via general recommendations or restriction guidelines) (3) increase awareness of midwives, gynecologists, pediatricians and general practitioners to be particularly attentive to the maternal health and her environment in or even before pregnancy providing optimal possible growth and developmental conditions for the fetus and child (4) identify further research aspects such as (a) to detect the deep underlying molecular initial event of certain exposures in the mother and/or fetus, (b) detect chemical driven changes in the nutrient transport at the fetal – maternal interface (Junge et al. 2022b) or (c) detect nutrients/nutritional patterns that might increase the individuals resilience via harmful environmental exposures. This knowledge will help to develop potential

therapeutic/interventional options in cases and situations where a harmful early life exposure is unavoidable.

Zusammenfassung

Die hohe Prävalenz von Allergien und Übergewicht stellt ein massives Problem für die menschliche Gesundheit dar. Beide Erkrankungen determinieren sich als Kombination aus sowohl genetischen, physiologischen als auch aus umwelt- und verhaltensassoziierten Faktoren. Einmal entwickelt, geht mit ihnen eine individuelle, gesellschaftliche und ökonomische Belastung einher. Wie für andere nicht-übertragbare Krankheiten gilt auch für Allergien und Übergewicht: die beste Möglichkeit zur Reduktion der krankheitsbedingten Belastung ist deren Prävention. Die Risikofaktoren für beide Erkrankungen können sich bereits sehr frühzeitig manifestieren, nämlich bereits im Mutterleib. Daher ist es sehr bedeutsam, mögliche Risiken bereits ab oder sogar noch vor der Empfängnis zu kennen und zu berücksichtigen. Im Rahmen dieser Habilitationsschrift wurde in zwölf Originalarbeiten sowie drei begleitenden Reviews/Kommentaren gezeigt, wie verschiedene frühe Lebensstil- und Umweltfaktoren die Entwicklung von Allergien oder Übergewicht beeinflussen können. Die Forschungsfragestellungen wurden hauptsächlich im Rahmen der prospektiven Mutter-Kind-Studie LINA adressiert, unterstützt durch mechanistische Untersuchungen an krankheits-relevanten Stammzellkulturmodellen. Ein Hauptfokus lag dabei auf der möglichen frühen Prägung durch epigenetische Regulationsprozesse.

Im Kontext von **Allergien** konnten wir zeigen, dass die **Vitamin D**-Spiegel der Schwangeren und des Neugeborenen positiv mit der Entwicklung von Nahrungsmittelallergien assoziiert waren (Weisse et al. 2013). Zudem schien die epigenetische Regulation des immunsystem-relevanten Genes TSLP an diesem Effekt beteiligt zu sein (Junge et al. 2016). Weiter konnten wir zeigen, dass Kinder, die Allergien entwickelt hatten, später im Leben ein ungünstigeres – eher pro-inflammatorisches - **Ernährungsmuster** aufwiesen (Schütte et al. 2022).
Neben spezifischen Nährstoffen nehmen wir über die tägliche Ernährung auch verschiedene Begleitsubstanzen auf – wie zum Beispiel **Parabene** (eingesetzt u.a. als Konservierungsmittel). Daher haben wir ebenfalls untersucht, wie sich eine Parabenbelastung in der Schwangerschaft auf die Allergieentstehung im Kindesalter auswirkt. Hierbei zeigte sich, dass einzelne Parabene das Asthmageschehen hemmen oder fördern können, wohingegen eine multiple Exposition gegenüber mehreren Parabenen keinen Einfluss auf das Erkrankungsrisiko hatte (Junge et al. 2022a).

Neben dem oralen Aufnahmepfad spielen natürlich auch inhalativ aufgenommene Substanzen eine Rolle im Kontext der Allergieentstehung. Hier sahen wir, dass eine frühzeitige Belastung gegenüber **flüchtigen organischen Verbindungen (VOCs)**, welche im Innenraum u.a. über Zigarettenrauch, bei Reinigungs- oder Renovierungsaktivitäten freigesetzt werden, einen negativen Einfluss auf die Allergieentstehung im Kinderalter hat. Insbesondere scheinen hier allergierelevante hämatopoetische Stammzellen (eosinophile/basophile Vorläuferzellen) zu verschiedenen Zeitpunkten in der frühen Kindheit eine effekttragende Rolle zu spielen (analysiert zur Geburt: Junge et al. 2014, im ersten Lebensjahr: Weisse et al., 2012, im zweiten Lebensjahr: Hörnig et al., 2016). Interessanterweise zeigten VOCs, welche aus Holz- oder Holzprodukten freigesetzt wurden, keine ungünstigen Effekte auf die Asthmaentstehung im Kindesalter, sowohl in singulärer Betrachtung als auch in einer multiplen Mischungsexposition (Junge et al., 2021).

Auch im Kontext des zweiten im Fokus stehenden Endpunktes, kindlichem **Übergewicht,** wurde der Einfluss von Lebensstil- und Umweltfaktoren auf die Krankheitsentstehung untersucht. Hierbei konnte gezeigt werden, dass die pränatale Exposition gegenüber endokrinen Disruptoren wie **Bisphenol A** (Junge et al., 2018) oder **Butyl-Paraben** (Leppert et al., 2020) das Risiko erhöht, im Kindesalter ein Übergewicht zu entwickeln. Beide Projekte wurden in einem dreidimensionalen Versuchssetting durchgeführt, wobei epidemiologische Erhebungen mechanistisch mittels Zellkulturexperimenten als auch mittels in-vivo Analysen verifiziert wurden. Dabei konnten wir interessanterweise zeigen, dass der beschriebene obesogene Effekt der beiden Chemikalien auf unterschiedlichen Regulationsmechanismen beruhte (gesteigerte Adipogenese durch Exposition mit BPA vs. veränderte Hunger-/ Sättigungsregulation im Gehirn bei Parabenbelastung).

Neben der Chemikalienexposition wurde auch der persönliche Gesundheits- und Lebensstil der Schwangeren im Hinblick auf eine kindliche Übergewichtsentstehung untersucht. Hierbei konnten wir zeigen, dass ein veränderter mütterlicher **Immunstatus** in der Schwangerschaft (Englich et al., 2017) als auch der mütterlich **empfundene Stress** im ersten Lebensjahr (Leppert et. al, 2018) das Risiko erhöht, dass das Kind später im Leben ein Übergewicht entwickelt.

Zusammenfassend konnte durch das im Rahmen dieser Habilitationsschrift generierte Wissen dazu beigetragen werden, dass (1) die individuelle Exposition einer Schwangeren gegenüber schädlich wirksamen Substanzen minimiert oder reduziert werden kann (zum Beispiel durch die Nutzungsempfehlung verschiedener Smartphone-Apps, welche eine große Datenbank zu bedenklichen Substanzen aus Produkten des täglichen Lebens bereit stellen), (2) das Bewusstsein von Interessenvertretern und Politik erhöht wird, ein risikoarmes Lebensumfeld zu schaffen, was insbesondere die Schwangere vor risikobehafteten Substanzen im Kontext der Allergie- und Übergewichtsentstehung schützt (z.B. über generelle Empfehlungen oder Verbote von zu verwendender Substanzen), (3) das Bewusstsein von Hebammen, Gynäkologen, sowie Kinder- und Allgemeinärzten erhöht wird, die mütterliche Gesundheit, und insbesondere auch die mütterliche Umwelt vor und während der Schwangerschaft im Blick zu haben, um eine optimale Entwicklung des Fötus und Kindes zu ermöglichen. Zudem konnten weitere zukünftige Forschungsansätze aufgezeigt werden, die zu einem tieferen Verständnis der Erkrankungsentstehung und demnach zu besseren Präventions- oder Interventionsmöglichkeit beitragen: (a) Es sollte weiter adressiert werden, welche zugrunde liegenden molekularen Mechanismen (molekulares initiierendes Event) bei einer krankheitsrelevanten Chemikalienexposition bei Mutter und/oder Fötus induziert werden. (b) Ebenso von Interesse ist es, welche möglichen chemikaliengetriebenen Veränderungen es im Nährstoff-/Substrattransport an der materno-fötalen Schnittstelle/Plazenta (Junge et al. 2022b) gibt oder (c) ob, und wenn ja, welche Ernährungsmuster/Nährstoffe es gibt, die die individuelle Resilienz/Stresstoleranz gegenüber ungünstigen Umweltexpositionen erhöhen können. Damit könnte man proaktiv dazu beitragen, mögliche ungünstige Effekte umweltassoziierter Substanzen aus dem täglichen Leben abzupuffern, wenn ein generelles Vermeiden nicht sichergestellt werden kann.

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Appendix

Curriculum vitae

Declaration

Acknowledgements

Curriculum vitae

Personal details

Name:	Dr. Kristin M. Junge (birth name: Kristin M. Weiße)
Nationality:	German
Place of birth:	Leipzig, Germany

Professional career

12/2022	**Professor for Nutritional Science** AKAD University Stuttgart, School of Health & Social Sciences
06 / 2009 – 11 / 2022	**Scientist** Helmholtz Centre for Environmental Research – UFZ Department of Environmental Immunology
Since 07 / 2020	C**oordinator of research theme "Nutritional Immunology and Metabolism"** Helmholtz Centre for Environmental Research – UFZ Department of Environmental Immunology (Prof. Ana Zenclussen)
Since 10 / 2018	Teaching/Lecturer activity at Martin-Luther-University Halle-Wittenberg: Nutritional Sciences
07 / 2010 – 06 / 2020	**Head of research group "Stem cells in allergy and obesity"** Helmholtz Centre for Environmental Research – UFZ Department of Environmental Immunology (Prof. Irina Lehmann)
01 / 2010 – 06 / 2010	Postdoctoral fellow McMaster University, Hamilton, ON, Canada Department of Medicine Division of Clinical Immunology and Allergy (Prof. Judah Denburg)
06 / 2006 – 05/ 2009	**PhD-student** Martin-Luther-University Halle-Wittenberg Department of Human Nutrition PhD thesis ***"Influence of lupin protein (L. angustifolius) on lipid metabolism and atherogenesis"***

Education

2001/2006	Student of Nutritional Sciences Martin-Luther-University Halle-Wittenberg Faculty of Natural Sciences III Institute for Agricultural and Nutritional Sciences *Diploma thesis:* ***"Homocysteine thiolactone-induced hyperhomocysteinemia does not alter concentrations of cholesterol and SREBP-2 target gene mRNAs in rats"***
1989/2001	Primary/ Secondary school (*Gymnasium*) and Abitur in Leipzig

Halle,

Dr. Kristin M. Junge

Declaration

I declare that this habilitation thesis **„Early lifestyle and environmental factors and their impact on infant allergy & obesity development"** has been composed solely by myself and has not been submitted, in whole or in part, in any previous application for a degree at this or any other university. Except where stated and indexed otherwise by reference citation or acknowledgments, the work presented is my own.

Halle,

Dr. Kristin M. Junge

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