Aalborg Universitet

human patients with allergic asthma following endobronchial allergen challenge,[E7] and genetic linkage studies showing an association between functional SNPs in the S1P receptor-1 and asthma.[E8] Thus, whether increased expression of ORMDL3 in hORMDL3$^{zp3-Cre}$ mice increases AHR in the absence of airway inflammation through reductions in sphingolipid levels is supported by some,[6] but not all, studies of sphingolipids.[7,8,E6,E7]

Interestingly, in Sptlc2$^{+/-}$ mice, the reduced synthesis of sphinganine and ceramide was associated with increases in AHR in the absence of inflammation,[6] a phenotype we have observed in hORMDL3$^{zp3-Cre}$ mice.[4] However, there are differences in the profile of sphingolipids reduced in hORMDL3$^{zp3-Cre}$ mice[4] (sphinganine, sphingosine, S1P, ceramide in serum; sphinganine in lung) compared with Sptlc2$^{+/-}$ mice (sphinganine, ceramide in lung). In addition, hORMDL3$^{zp3-Cre}$ mice[4] develop spontaneous airway remodeling and mucus expression not observed in Sptlc2$^{+/-}$ mice, suggesting that pathways to remodeling and mucus are independent of the ability of ORMDL3 to inhibit SPT and sphingolipid synthesis.

Studies have also examined the role of ceramide in mouse asthma models and demonstrated that allergen challenge increased lung levels of ceramide in WT mice,[E3] whereas pretreatment with pharmacologic inhibitors of ceramide reduced AHR, lung eosinophils, and T_H2 cytokines.[E3] These studies suggest that the reductions in serum ceramide levels we have noted in hORMDL3$^{zp3-Cre}$ mice should be associated with reduced AHR, rather than the increased AHR we have noted in hORMDL3$^{zp3-Cre}$ mice. Thus, the reduced serum ceramide levels in ORMDL3 TG mice, as well as the reduced lung ceramide levels in Sptlc2$^{+/-}$ mice, do not adequately explain the AHR changes we have noted in these mice.

In summary, in this study we have demonstrated that hORMDL3$^{zp3-Cre}$ mice had significantly reduced serum levels of the pathway of sphingolipids regulated by SPT (sphinganine, ceramide, sphingosine, and S1P), as well as reduced lung levels of sphinganine. In addition, we demonstrate that administration of S1P to naive hORMDL3$^{zp3-Cre}$ mice further increases their AHR associated with increased levels of peribronchial macrophages. These *in vivo* studies extend previous *in vitro* observations that ORMDL3 inhibits the generation of sphingolipids including ceramide and S1P.[3] Previous studies have also demonstrated that increased ORMDL3 activates the ATF6α pathway of the endoplasmic reticulum unfolded protein response and that this regulates levels of SERC2b, which can contribute to AHR.[4,9] In addition, ORMDL3 regulates levels of remodeling genes (TGF-β1, ADAM8) as well as CC and CXC chemokines implicated in asthma.[9] Further studies are needed to determine which ORMDL3-regulated pathway (eg, inhibition of sphingolipid synthesis, inhibition of ATF6α and SERCA2b, inhibition of remodeling genes, or other as yet unidentified pathways)[E9,E10] could contribute to increased AHR observed in hORMDL3$^{zp3-Cre}$ mice, and whether in patients with asthma SNPs associated with increased ORMDL3 expression result in reduced sphingolipid levels. Such insights are important in understanding why ORMDL3 on chromosome 17q21 is highly linked to human asthma.

Marina Miller, MD, PhD[a]
Peter Rosenthal, BS[a]
Andrew Beppu, BS[a]
Ruth Gordillo, PhD[b]
David H. Broide, MB, ChB[a]

REFERENCES

1. Moffatt MF, Kabesch M, Liang L, Dixon AL, Strachan D, Heath S, et al. Genetic variants regulating ORMDL3 expression contribute to the risk of childhood asthma. Nature 2007;448:470-3.
2. Verlaan DJ, Berlivet S, Hunninghake GM, Madore AM, Larivière M, Moussette S, et al. Allele-specific chromatin remodeling in the ZPBP2/GSDMB/ORMDL3 locus associated with the risk of asthma and autoimmune disease. Am J Hum Genet 2009;85:377-93.
3. Breslow DK, Collins SR, Bodenmiller B, Aebersold R, Simons K, Shevchenko A, et al. Orm family proteins mediate sphingolipid homeostasis. Nature 2010;463:1048-53.
4. Miller M, Rosenthal P, Beppu A, Mueller JL, Hoffman HM, Tam AB, et al. ORMDL3 transgenic mice have increased airway remodeling and airway responsiveness characteristic of asthma. J Immunol 2014;192:3475-87.
5. Warshauer JT, Lopez X, Gordillo R, Hicks J, Holland WL, Anuwe E, et al. Effect of pioglitazone on plasma ceramides in adults with metabolic syndrome. Diabetes Metab Res Rev 2015;31:734-44.
6. Worgall TS, Veerappan A, Sung B, Kim BI, Weiner E, Bholah R, et al. Impaired sphingolipid synthesis in the respiratory tract induces airway hyperreactivity. Sci Transl Med 2013;5:186ra67, Erratum in: Sci Transl Med 2013;5:192er7.
7. Roviezzo F, D'Agostino B, Brancaleone V, De Gruttola L, Bucci M, De dominicis G, et al. Systemic administration of sphingosine-1-phosphate increases bronchial hyperresponsiveness in the mouse. Am J Resp Cell Mol Biol 2010;42:572-7.
8. Price MM, Oskeritzian CA, Falanga YT, Harikumar KB, Allegood JC, Alvarez SE, et al. A specific sphingosine kinase 1 inhibitor attenuates airway hyperresponsiveness and inflammation in a mast cell-dependent murine model of allergic asthma. J Allergy Clin Immunol 2013;131:501-11.e1.
9. Miller M, Tam AB, Cho JY, Doherty TA, Pham A, Khorram N, et al. ORMDL3 is an inducible lung epithelial gene regulating metalloproteases, chemokines, OAS, and ATF6. Proc Natl Acad Sci USA 2012;109:16648-53.

Prenatal phthalate exposure associates with low regulatory T-cell numbers and atopic dermatitis in early childhood: Results from the LINA mother-child study

To the Editor:

Phthalates serve as binders and plasticizers in everyday items, including cosmetics, household cleaners, food packaging, personal-care products, toys, and many other consumer products. Recent publications show that exposure to these chemicals may contribute to an increased risk of allergy development including asthma and atopic dermatitis.[1,2] An immune modulatory capacity of these compounds is assumed but not fully elucidated by now.[3] Epidemiological studies provide evidence that exposure to phthalates and their metabolites might be more critical in the prenatal period when the fetal immune system is developing.[4] According to the fact that regulatory T (Treg) cells are key players in the modulation of immune responses, we asked whether phthalates may affect the number of these cells leading to the observed increased risk to develop atopic dermatitis.[2] In a recent study,

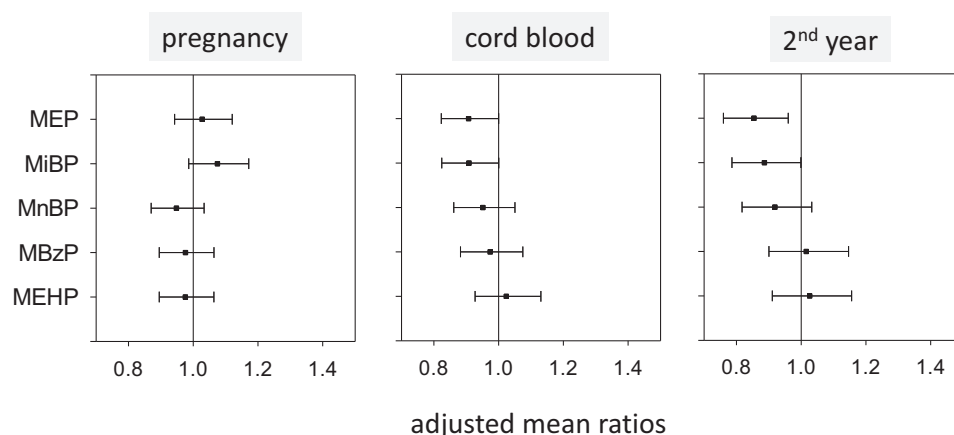## Prenatal phthalate exposure vs Treg numbers

FIG 1. Relationship between maternal urine phthalate metabolite concentrations and the number of Treg cells. Data represent samples with phthalate and Treg-cell measurements in pregnancy (n = 542) and with phthalate measurements in pregnancy and Treg cells in children in cord blood (n = 448) and at the age of 2 years (n = 331). Shown are the mean ratios with 95% CI. Mean ratios were adjusted for maternal atopic dermatitis, maternal smoking and/or ETS exposure at home during pregnancy, maternal education, cat ownership during pregnancy, and previous births. In addition, data from cord blood were adjusted for sex and those at the age of 2 years for sex and breast-feeding until 6 months.

TABLE I. Concentrations of phthalate metabolites in maternal urine (36th gestational week) in regard to the development of atopic dermatitis (physician diagnosed) in the first 3 years of children's life

	Atopic dermatitis 0-3rd year, median (IQR) (ng/mg)*		P
Phthalate metabolites	**Yes (n = 85)‡**	**No (n = 350)‡**	**value†**
MEP	60 (28.3-143.9)	50.5 (23.9-99.4)	.155
MiBP	**73.3 (53.2-102.9)§**	**62.4 (45.7-90.9)§**	**.036§**
MnBP	112.7 (78.8-169.7)	96.9 (66.7-143.9)	.057
MBzP	6.9 (3.7-11.7)	6.4 (3.7-10.6)	.646
MEHP	7.7 (5.5-11.5)	7.2 (5-10.8)	.438

IQR, Interquartile range.
*Concentrations were adjusted for creatinine (in ng per mg of creatinine) to control for urine dilution.
†*P* values from Mann-Whitney *U* test.
‡Cases with maternal phthalate measurements in maternal urine (36th gestational week).
§Significant values are marked in boldface (*P* < .05).

we demonstrated that low number of Treg cells at birth is associated with a higher risk to develop an atopic dermatitis within the first 3 years of life.[5] In the present investigation, we aimed to evaluate whether a high maternal phthalate burden impacts the number of Treg cells in pregnancy as well as in early childhood. Associations to children's atopic dermatitis were found with the primary metabolites of diethyl, di-n-butyl, butylbenzyl, or di-2-ethylhexyl phthalate.[2] Therefore, in our study, we measured the concentration of these metabolites (monoethyl phthalate [MEP], monoisobutyl phthalate [MiBP], mono-n-butyl phthalate [MnBP], monobenzyl phthalate [MBzP], mono-(2-ethylhexyl) phthalate [MEHP]) in maternal urine and assessed the relationship to Treg-cell numbers and the development of atopic dermatitis as well as to hay fever and allergic sensitization to food (fx5) and inhalant (sx1) allergens.

Data were gained from our prospective birth cohort study LINA (Lifestyle and environmental factors and their Influence on Newborns Allergy risk). For this study, 629 mother-child pairs (622 mothers, 7 twin pairs) were recruited from March 2006 until December 2008 in Leipzig, Germany.[6] Annually, around the birthday of the child, follow-up investigations were performed with questionnaire evaluations for allergic outcomes (atopic dermatitis, hay fever, asthma) and confounders as well as clinical visits including blood collection. Early morning urine samples were collected during the third trimester (gestational age 36 weeks, n = 610) and analyzed for the primary phthalate metabolites (MEP, MiBP, MnBP, MBzP, and MEHP) using a multianalyte procedure as described by Feltens et al.[7] Absolute concentrations of phthalates were calculated on the basis of calibration curves and normalized to urinary creatinine concentrations. The number of Treg cells was determined by *FOXP3* methylation-specific real-time PCR in the Treg-cell–specific demethylated region in blood samples from pregnancy (34th gestational week, n = 607), cord blood (n = 448), and from the second year of children's life (n = 331) as described previously.[6] This method enables the identification of cells with stable Treg-cell phenotype and function.[8] Characteristics of the study population, detailed method description, and median values and interquartile ranges for all phthalate metabolites and Treg-cell numbers are given in this article's Online Repository (Tables E2 and E3 at www.jacionline.org).

Associations between maternal urine phthalate metabolite concentrations and the number of Treg cells were calculated using a linear regression model adjusted for maternal atopic dermatitis, maternal smoking and/or ETS exposure at home, maternal education, cat ownership, previous births (presence of older siblings), and in addition (only for children) for sex and breast-feeding until 6 months. Out of the analyzed phthalate metabolites, MEP and MiBP were associated with lower Treg-cell numbers in cord blood (adjusted mean ratios [aMR], 0.90, 95%

TABLE II. Relationship between phthalate metabolites (concentration in maternal urine 36th gestational week) exposure and the development of atopic dermatitis (physician diagnosed) in the first 3 years of children's life

Phthalate metabolites	Atopic dermatitis 0-3rd year; % (n/N), 19.5% (85/435)*			
	Crude OR (95% CI)	P value	Adjusted OR (95% CI)†	P value
MEP	1.6 (0.84-3.04)	.145	1.45 (0.75-2.83)	.268
MiBP	**2.15 (1.11-4.14)‡**	**.022‡**	**2.21 (1.1-4.45)‡**	**.026‡**
MnBP	1.79 (0.95-3.37)	.072	1.79 (0.91-3.52)	.090
MBzP	1.29 (0.68-2.47)	.427	1.28 (0.65-2.52)	.470
MEHP	1.47 (0.77-2.79)	.242	1.5 (0.76-2.98)	.238

*Cases with maternal phthalate measurements in maternal urine (36th gestational week).
†Analysis was performed using a logistic regression model with phthalate values categorized into quartiles; odds ratios (ORs) were adjusted for sex, maternal atopic dermatitis, maternal smoking, and/or ETS exposure at home, siblings, maternal education, cat ownership, and breast-feeding until 6 months.
‡Significant values are marked in boldface ($P < .05$).

CI, 0.82-1, $P = .046$ and aMR, 0.91, 95% CI, 0.82-1, $P = .047$, respectively) and in children aged 2 years (aMR, 0.85, 95% CI, 0.76-0.96, $P = .007$ and aMR, 0.88, 95% CI, 0.78-0.99, $P = .045$, respectively) but not in mothers during pregnancy (aMR, 1.02, 95% CI, 0.94-1.12, $P = .529$ and aMR, 1.07, 95% CI, 0.98-1.17, $P = .095$, respectively) (Fig 1). We repeated the analysis on the basis of a subgroup of 208 children with available data for maternal urine phthalate concentrations and Treg-cell numbers during pregnancy, as well as Treg-cell measurements in cord blood and at the age of 2 years and gained similar results. In addition here, at the age of 2 years, a reduced number of Treg cells was observed in association with high concentrations of MnBP during pregnancy (see Fig E1 in this article's Online Repository at www.jacionline.org). Although cord blood Treg-cell numbers are only affected by maternal exposure during pregnancy, we cannot exclude that phthalate exposure after birth also contributed to the observed low Treg-cell numbers at the age of 2 years.

Furthermore, the impact of high phthalate metabolite levels in pregnancy on the development of atopic dermatitis during the first 3 years in children's life was assessed by calculating odds ratios adjusted for sex, maternal atopic dermatitis, maternal smoking and/or ETS exposure at home, siblings, maternal education, cat ownership, and breast-feeding until 6 months. In our study, 19.5% of children developed an atopic dermatitis within the first 3 years of life. For children who developed an atopic dermatitis during this time, maternal urine MiBP concentrations were significantly higher compared with those in healthy controls (Table I). Furthermore, high maternal MiBP urine concentrations were associated with a higher risk for the child to develop an atopic dermatitis until the age of 3 years (adjusted odds ratio, 2.21; 95% CI, 1.1-4.45; $P = .026$; Table II). These results sustain earlier publications[2,9] presenting a relationship between prenatal exposure to phthalate metabolites and an increased risk for atopic dermatitis in early childhood. However, in our study, the metabolite MiBP, but not MBzP like in the mentioned publications, showed this association. This apparent discrepancy may reflect the fact that the studies have been conducted in different countries/continents, for example, New York (United States), Taiwan (Asia) versus our study, which is located in Leipzig (Germany, Europe), where the composition of plastics or consumer products may differ in the distribution of phthalates compared with that in the other continents. In addition, the consumer behavior in the different countries and continents may also impact the phthalate distribution. At least for MiBP it is obvious that in our study the concentrations found in urine are much higher (65.6 μg/g) compared with concentrations found in urine in Taiwan (15.2 μg/g), the United States (8.1 μg/g), or Mexico (8.4 μg/g).[7]

In addition to the above-described link between MiBP and atopic dermatitis, we could show that exposure to high concentrations of MEP and MnBP during the fetal period was associated with an increased risk to sensitization to food allergens (fx5) at the age of 2 years and in trend also at the age of 1 year (see Table E4 in this article's Online Repository at www.jacionline.org). We furthermore could demonstrate that a high number of Treg cells at birth is protective against sensitization to food allergens at the age of 1 year and a high number of Treg cells at the age of 2 years was associated with a lower risk for sensitization to inhalant allergens at this age (see Table E5 in this article's Online Repository at www.jacionline.org). As Treg cells are keeping immune responses in balance, lower numbers of these cells may facilitate the development of allergic diseases, as we could show earlier for atopic dermatitis.[5,6] Interestingly, maternal Treg-cell numbers in pregnancy were not associated with phthalate metabolite concentrations, pointing out that the immature immune system of the fetus and toddler is more vulnerable to these chemicals. Although we are not able to provide a mechanistic explanation for the impact of MEP and MiBP on Treg-cell numbers (which might implicate the differentiation and/or proliferation of these cells), our epidemiological findings may encourage for the performance of *in vitro* assays in this direction in future studies. Taken together, our data suggest that MiBP being associated with reduced numbers of Treg cells may facilitate the development of an atopic dermatitis in early childhood. Because DiBP, the parent compound from which MiBP originates, is widely used in food packages, adhesives, and cosmetics, the usage of these products especially during pregnancy should be avoided.

Gunda Herberth, PhD[a]*
Arkadiusz Pierzchalski, PhD[b]*
Ralph Feltens, PhD[b]
Mario Bauer, PhD[a]
Stefan Röder, PhD[c]
Sven Olek, PhD[d]
Denise Hinz, PhD[a,e]
Michael Borte, PhD[f]
Martin von Bergen, PhD[b,g,h]
Irina Lehmann, PhD[a]
for the LINA Study Group

From the Departments of [a]Environmental Immunology and [b]Molecular Systems
Biology, UFZ - Helmholtz Centre for Environmental Research Leipzig, Leipzig,
Germany; [c]UFZ - Helmholtz Centre for Environmental Research Leipzig,
Core Facility Studies, Leipzig, Germany; [d]Ivana Tuerbachova Laboratory for Epige-
netics, Epiontis GmbH, Berlin, Germany; [e]the Division of Vaccine Discovery, La
Jolla Institute for Allergy & Immunology, La Jolla, Calif; [f]Children's Hospital,
Municipal Hospital "St Georg," Academic Teaching Hospital of the University of
Leipzig, Leipzig, Germany; [g]the Institute of Biochemistry, University of Leipzig,
Leipzig, Germany; and [h]the Department of Chemistry and Bioscience, Aalborg
University, Aalborg, Denmark. E-mail: gunda.herberth@ufz.de. Or: irina.
lehmann@ufz.de.

REFERENCES

1. Gascon M, Casas M, Morales E, Valvi D, Ballesteros-Gomez A, Luque N, et al.
   Prenatal exposure to bisphenol A and phthalates and childhood respiratory tract
   infections and allergy. J Allergy Clin Immunol 2015;135:370-8.
2. Just AC, Whyatt RM, Perzanowski MS, Calafat AM, Perera FP, Goldstein IF, et al.
   Prenatal exposure to butylbenzyl phthalate and early eczema in an urban cohort.
   Environ Health Perspect 2012;120:1475-80.
3. Rogers JA, Metz L, Yong VW. Review: endocrine disrupting chemicals and
   immune responses: a focus on bisphenol-A and its potential mechanisms. Mol
   Immunol 2013;53:421-30.
4. Whyatt RM, Rundle AG, Perzanowski MS, Just AC, Donohue KM, Calafat AM,
   et al. Prenatal phthalate and early childhood bisphenol A exposures
   increase asthma risk in inner-city children. J Allergy Clin Immunol 2014;134:
   1195-7.e2.
5. Herberth G, Bauer M, Gasch M, Hinz D, Roder S, Olek S, et al. Maternal and cord
   blood miR-223 expression associates with prenatal tobacco smoke exposure and
   low regulatory T-cell numbers. J Allergy Clin Immunol 2014;133:543-50.
6. Hinz D, Bauer M, Roder S, Olek S, Huehn J, Sack U, et al. Cord blood Tregs with
   stable FOXP3 expression are influenced by prenatal environment and associated
   with atopic dermatitis at the age of one year. Allergy 2012;67:380-9.
7. Feltens R, Roeder S, Otto W, Borte M, Lehmann I, von Bergen M, et al. Evaluation
   of population and individual variances of urinary phthalate metabolites in terms of
   epidemiological studies. J Chromatogr Sep Tech 2015;6:290.
8. Wieczorek G, Asemissen A, Model F, Turbachova I, Floess S, Liebenberg V, et al.
   Quantitative DNA methylation analysis of FOXP3 as a new method for counting
   regulatory T cells in peripheral blood and solid tissue. Cancer Res 2009;69:
   599-608.
9. Wang IJ, Lin CC, Lin YJ, Hsieh WS, Chen PC. Early life phthalate exposure and atopic
   disorders in children: a prospective birth cohort study. Environ Int 2014;62:48-54.

Dysregulation of lipidomic profile and antiviral immunity in response to hyaluronan in patients with severe asthma

To the Editor:

Features of patients with severe asthma include a greater frequency and severity of hospitalizations caused by pneumonia, severe influenza, and sinopulmonary infections.[1] Viral infections are frequent triggers of asthma exacerbations.[1] Impaired antiviral responses in asthmatic patients have been noted.[2] However, the mechanisms of this phenomenon are not well understood.

The asthmatic airway wall undergoes many alterations, including increased and changed deposition of extracellular matrix. Hyaluronan (HA), a major component of extracellular matrix, accumulates in the lung and serum of asthmatic patients and correlates with disease severity.[3] Low-molecular-weight (LMW) forms of HA generated during tissue injury or

inflammation have been linked to asthma,[3,4] but the mechanisms of that link are not well understood.

Recently, we described the mechanism by which LMW HA can activate cytosolic phospholipase $A_2\alpha$ (cPLA$_2\alpha$) and arachidonic acid (AA) production.[5] Previously, we reported increased expression of cPLA$_2\alpha$ in PBMCs of patients with severe asthma.[6] cPLA$_2\alpha$ is a rate-limiting enzyme in eicosanoid production, which is responsible for liberation of AA from cellular membranes.[5] AA is the precursor of leukotrienes, prostaglandins (PGs), hydroxyeicosatetranoic acids (HETEs), thromboxanes, lipoxins, and epoxides, many of which are involved in asthma pathogenesis,[7] and they are altered in patients with viral infections.[8]

A thorough analysis of LMW HA's effects on the lipidomic profile or global gene expression in asthmatic patients has never been performed, and its possible influence on the disease progression has not been noted. Therefore we performed a systemic analysis of LMW HA signaling in PBMCs of patients with mild-to-moderate and severe asthma by using liquid chromatography and mass spectrometry combined with microarray, real-time PCR, and multiplex protein analyses. Details of methodology are provided in the Methods section in this article's Online Repository at www.jacionline.org.

Thirteen asthmatic patients and six control subjects were enrolled under a National Heart, Lung, and Blood Institute review board–approved protocol (99-H-0076). Severe asthma was defined according to European Respiratory Society/American Thoracic Society guidelines.[9] Participants' demographic and phenotypic characteristics are presented in Tables E1 and E2 in this article's Online Repository at www.jacionline.org.

Of the 151 lipid species profiled, we detected 68 before or after LMW HA stimulation. At baseline, fewer metabolites were generated through the COX pathway compared with the combined lipoxygenase, CYP450, and nonenzymatic pathways in each group (Fig 1, *A*, and see Fig E1, *A*, in this article's Online Repository at www.jacionline.org). The relative percentage of all COX metabolites at baseline was significantly lower in patients with severe asthma than in control subjects (Fig 1, *A*, and see Fig E1, *A*). However, after LMW HA stimulation, there was a significant increase in the relative percentage of COX-generated mediators and a corresponding decrease of metabolites derived from the other pathways in all groups (Fig 1, *A*, and see Fig E1, *A*).

Analysis of individual lipids revealed that 22 were significantly upregulated by using LMW HA (Fig E1, *B*). Although at baseline several lipid species tended to have lower concentrations in patients with severe asthma, the increase in 9 metabolites after LMW HA treatment was significantly more pronounced in those patients compared with control subjects (Fig 1, *B*). These included the COX metabolites thromboxane B_2 (TXB$_2$), prostaglandin E_2, D_2 and B_2 (PGE$_2$, PGD$_2$, and PGB$_2$) and metabolites from other pathways, including 15-hydroxyeicosatetraenoic acid (15-HETE) (through lipoxygenase), 11,12-epoxyeicosatrienoic acid (11,12-EET), and 14,15-EET (through CYP450), and non-AA metabolites, such as 13-hydroxyoctadecatrienoic acid (13-HOTrE(y)) and (16(17)-epoxydocosapentaenoic acid 16(17)-EpDPE). Only in patients with severe asthma did the levels of most of these metabolites reach the range of activation of their cognate receptors, although 15-HETE, 14,15-EET, and 16(17)-EpDPE were significantly upregulated also in patients with mild-to-moderate asthma. Treatment with a specific cPLA$_2\alpha$

METHODS
Study design

Six-hundred twenty-nine mother-child pairs were recruited within the prospective birth cohort study LINA (Lifestyle and environmental factors and their Influence on Newborns Allergy risk) from March 2006 until December 2008 in Leipzig, Germany. Blood samples were obtained during pregnancy (mother, 34th week of gestation), at birth (venous umbilical cord blood), and annually around the child's birthday. Early morning urine samples were obtained from mothers in the 36th week of gestation. Data on confounding variables, prenatal exposure, lifestyle factors, and children's disease outcomes were gained from questionnaires filled in by the parents 4 weeks before birth and annually thereafter. The present investigation comprises of mothers having urine sample analyses and measurement of Treg cells in blood samples as well as children with Treg-cell measurements in cord blood and at the age of 2 years.

Participation was voluntary and informed consent was given by the parents. This study was approved by the Ethics Committees of the University of Leipzig (file references 046-2006, 160-2008).

Quantification of Treg cells

Isolation of genomic DNA. Genomic DNA (gDNA) was isolated from whole blood using the DNA Blood Mini Kit according to manufacturer's instructions (Qiagen, Hilden, Germany). Briefly, 200 μL whole blood was treated with 20 μL Proteinase K (activity, 600 mAU/mL) and incubated with Buffer AL in a ratio of 1:1 (10 minutes, 56°C, 350 rpm). Cell lysis was stopped by adding 200 μL ethanol. The complete sample volume was transferred onto a QIAamp Mini spin column and centrifuged (12,000 rpm). DNA bound to the QIAamp membrane was washed twice using Buffers AW1 and AW2 (12,000 rpm, 1 minute; 13,000 rpm, 5 minutes). DNA was eluted by adding Buffer AE and stored at 4°C until subsequent analysis, applying the mean of the 2 reference genes for normalizing.

Bisulfite conversion of gDNA and quantification of Treg cells by FOXP3 methylation-specific real-time PCR. Bisulfite treatment of gDNA was conducted using EpiTect 96 Bisulfite Kit (Qiagen, Hilden, Germany) as described by the manufacturer. Briefly, 1 μg of gDNA, 85 μL of the Bisulfite Mix, and 35 μL of the DNA Protect Buffer were mixed in a 96-well EpiTect Conversion Plate, and bisulphite conversion was performed in a thermal cycler according to manufacturer's instruction. Converted gDNA was stored at −20°C until further analysis.

Quantification of demethylation in Treg-cell–specific demethylated region was performed by real-time PCR by Epiontis (Berlin, Germany; for details, see data in this article's Online Repository at www.jacionline.org). As described previously, the number of Treg cells in blood is presented as percentage corresponding to the measured amount of Treg-cell–specific demethylated region demethylation in the *FOXP3* gene.[E1]

Phthalate metabolite measurement in urinary samples

Phthalate quantification was carried out for 610 early morning maternal urine samples. In the present study, data for 542 mothers, the subset with Treg-cell measurements in their blood, are presented. Urine samples were collected at 36th weeks' gestation and stored in polypropylene tubes at −80°C until further analysis. Primary phthalate metabolite (MEP, MiBP, MnBP, MBzP, MEHP) quantification was carried out for all samples using a multianalyte procedure as described by Feltens et al.[E2] Absolute concentrations of phthalate metabolites were calculated on the basis of calibration curves and normalized to urinary creatinine concentrations as previously described.[E3] Concentrations are given in ng per mg creatinine.

Allergic outcomes

Information on allergic outcomes was collected using questionnaires self-administered by the parents. Atopic dermatitis and hay fever were recorded as physician diagnoses ("Has a doctor diagnosed your child with atopic dermatitis in the last 12 months?"; "Has a doctor diagnosed your child with hay fever in the last 12 months?"). The lifetime prevalence within the first 3 years of life was determined by adding the information of having doctor's-diagnosed atopic dermatitis or hay fever from each year. The control group was defined including children having never developed symptoms or being diagnosed for this disease until the age of 3 years.

Allergic sensitization was assessed by the measurement of specific IgE against food allergens (fx5) and inhalant allergens (sx1). Samples were categorized as positive by the cutoff value of more than 0.35 kU/L.

IgE measurement

The concentrations of specific IgE against food (fx5) and inhalant allergens (sx1) in sera of 1-year-old children were determined by the Phadia CAP System (Phadia GmbH, Freiburg, Germany). The allergen multipanel fx5 consists of hen's egg, cow's milk, wheat, fish, peanut, and soy. Sx1 includes timothy, rye, mugwort, birch, house dust mite (*Dermatophagoides pteronyssinus*), cat, and dog. Samples with a specific IgE concentration of more than 0.35 kU/L were regarded as positive.

Confounding variables

Information on maternal history of atopy and prenatal exposure was assessed by detailed questionnaires answered by parents during the third trimester of pregnancy and annually after delivery. To address maternal atopy history, we included asthma, hay fever, and atopic dermatitis ("Did you ever suffer from asthma, allergic rhinitis, atopic dermatitis?"). Smoking or exposure to environmental tobacco smoke at home during pregnancy was recorded with the question "Did you or anybody else smoke inside your dwelling during the last twelve months?" If yes; occasionally, once per week, or daily. Maternal education was assessed by asking "Which is the highest education level you have?" "low, 9 yr of schooling or less" "Hauptschulabschluss"; "intermediate, 10 yrs of schooling" "Mittlere Reife"; high, 12 yrs of schooling or more "(Fach-)hochschulreife." Furthermore, the number of present siblings, the presence of pets (including cats) during pregnancy, and the duration of breast-feeding were recorded.

Statistical analysis

Statistical analyses were performed using Statistica for Windows Version 10.0 (StatSoft Inc [Europe], Hamburg, Germany). The chi-square test for cross-relationship was performed to ensure the equal distribution of parameters in the analyzed subgroups and the entire LINA cohort. All P values of less than .05 were considered to be significant. Because measured phthalate metabolite concentrations and Treg-cell numbers were not normally distributed, a logarithmic transformation was performed. Furthermore, the data were categorized into quartiles and used in the regression models. To analyze the relationship between maternal urine phthalate metabolite concentrations from pregnancy and the number of Treg cells in blood samples from pregnancy, cord blood, and at children's age of 2 years, linear regression models were used. The linear regression models were adjusted for the possible confounding factors maternal atopic dermatitis, maternal smoking and/or ETS exposure at home, maternal education, cat ownership, previous births (presence of older siblings), and also (only for children) for sex and breast-feeding until 6 months. Data are presented as mean ratios, which are the back-transformed effects from the regression model of the logarithmically transformed outcome. Odds ratios were calculated to show the relationship between prenatal phthalate concentrations in maternal urine and the development of atopic dermatitis (physician diagnosed) within the first 3 years of life. The logistic regression models were adjusted for sex, maternal atopic dermatitis, maternal smoking and/or ETS exposure at home, presence of older siblings, maternal education, cat ownership, and breast-feeding until 6 months.

RESULTS
Study population characteristics

Characteristics of the study population are listed in Table E1. There were no differences in the distribution of considered

parameters in the analyzed subgroups compared with the entire LINA cohort. Out of the 622 mothers participating in the study, 542 had urinary samples with phthalate metabolite measurements as well as Treg-cell measurements in blood. Out of the 470 children with cord blood samples, in 448 the number of Treg cells was assessed whereas at the age of 2 years, Treg-cell numbers were measured in 331 out of 339 blood samples. In the analyzed subgroup of children with prenatal maternal urine phthalate measurements, 85 (19.5%) developed an atopic dermatitis until the age of 3 years compared with 92 (18.5%) children from the entire LINA cohort.

REFERENCES

E1. Wieczorek G, Asemissen A, Model F, Turbachova I, Floess S, Liebenberg V, et al. Quantitative DNA methylation analysis of FOXP3 as a new method for counting regulatory T cells in peripheral blood and solid tissue. Cancer Res 2009;69:599-608.

E2. Feltens R, Roeder S, Otto W, Borte M, Lehmann I, von Bergen M, et al. Evaluation of population and individual variances of urinary phthalate metabolites in terms of epidemiological studies. J Chromatogr Sep Tech 2015;6:290.

E3. Remane D, Grunwald S, Hoeke H, Mueller A, Roeder S, von Bergen M, et al. Validation of a multi-analyte HPLC-DAD method for determination of uric acid, creatinine, homovanillic acid, niacinamide, hippuric acid, indole-3-acetic acid, and 2-methylhippuric acid in human urine. J Chromatogr B Analyt Technol Biomed Life Sci 2015;998-9:40-4.

Prenatal phthalate exposure vs Treg numbers
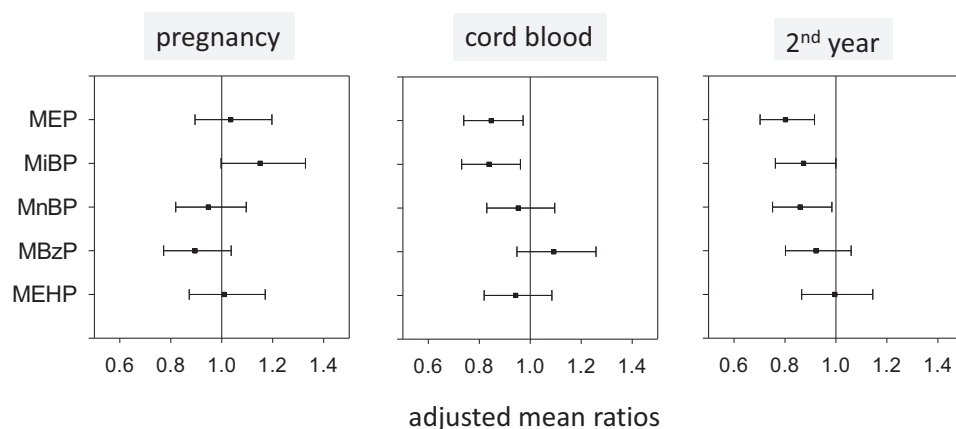
FIG E1. Relationship between maternal urine phthalate metabolite concentrations and the number of Treg cells. Results based on the subgroup of children with available data for maternal urine phthalate concentrations in pregnancy as well as measurements of Treg cells in pregnancy, in children's cord blood, and at the age of 2 years (n = 208). Shown are the mean ratios with 95% CI. Mean ratios were adjusted for maternal atopic dermatitis, maternal smoking and/or ETS exposure at home during pregnancy, maternal education, cat ownership during pregnancy, and previous births. In addition, data from cord blood were adjusted for sex and those at the age of 2 years for sex and breast-feeding until 6 months.

TABLE E1. Characteristics of the analyzed subgroups and the entire LINA cohort

		Analyzed subgroups			
Parameters	Entire LINA cohort, n (%), N = 629	Maternal urine samples in pregnancy, n (%), N = 542†	Cord blood samples at birth, n (%), N = 448‡	Blood samples at children's age of 2 y, n (%), N = 331§	P value*
Sex of the child					
Male	327 (52)	282 (52)	211 (47.1)	165 (49.8)	.885
Female	302 (48.0)	260 (48)	237 (52.9)	166 (50.2)	
Maternal history of atopy‖					
No	330 (53.1)	280 (51.7)	242 (54)	166 (50.2)	.954
Yes	292 (46.9)	262 (48.3)	206 (46)	165 (49.8)	
Maternal atopic dermatitis					
No	517 (82.2)	443 (81.7)	373 (83.3)	265 (80.1)	.947
Yes	111 (17.6)	99 (18.3)	75 (16.7)	66 (19.9)	
Maternal education¶					
Low	24 (3.8)	20 (3.7)	13 (2.9)	6 (1.8)	.98
Intermediate	194 (30.8)	165 (30.4)	143 (31.9)	113 (34.1)	
High	411 (65.3)	357 (65.9)	292 (65.2)	212 (64)	
Breast-feeding until 6 mo					
No	139 (23.92)	112 (20.7)	102 (22.8)	77 (23.3)	.987
Yes	442 (76.07)	386 (71.2)	311 (69.4)	242 (73.1)	
Cat ownership during pregnancy					
No	516 (82)	443 (81.7)	364 (81.2)	262 (79.2)	.957
Yes	113 (18)	99 (18.2)	84 (18.8)	69 (20.8)	
Presence of older siblings					
No	420 (66.8)	359 (66.2)	298 (66.5)	218 (65.9)	.999
Yes	209 (33.2)	183 (33.8)	150 (33.5)	113 (34.1)	
Smoking during pregnancy#					
Never	527 (84.7)	465 (85.8)	383 (85.5)	287 (86.7)	1
Occasionally	43 (6.9)	37 (6.8)	23 (5.8)	20 (6)	
Once per week	4 (0.7)	4 (0.7)	3 (0.7)	1 (0.3)	
Daily	48 (7.7)	36 (6.6)	36 (8)	23 (6.9)	

Because of missing data, case number may vary for some variables.

*P value from chi-square test for cross-relationship.

†Mothers with phthalate and Treg-cell measurements in pregnancy.

‡Children with Treg-cell measurements in cord blood.

§Children with Treg-cell measurements at the age of 2 years.

‖History of atopy is defined as occurrence of asthma or atopic dermatitis or hay fever.

¶Low, 9 years of schooling or less "Hauptschulabschluss"; intermediate, 10 years of schooling "Mittlere Reife"; high, 12 years of schooling or more "(Fach-)hochschulreife."

#Maternal smoking and/or ETS exposure during pregnancy at home.

TABLE E2. Maternal urine phthalate metabolite concentrations (36th gestational week, n = 542, samples with Treg-cell measurements)

Phthalate metabolite	Median (IQR) (ng/mg)*
MEP	50 (24.8-102.3)
MiBP	66.6 (48.9-99.8)
MnBP	104.9 (69.9-146.8)
MBzP	6.7 (3.8-11.6)
MEHP	7.3 (5.1-11.4)

IQR, Interquartile range.

*Concentrations were adjusted for creatinine (in ng per mg of creatinine) to control for urine dilution.

TABLE E3. Treg-cell numbers in samples with phthalate measurements as detected by means of demethylation in the FOXP3 gene

Treg cell	Median (IQR) (%)*
Maternal blood (34th gestational week) n = 542	0.91 (0.56-1.34)
Birth (cord blood) n = 448	1.18 (0.75-1.71)
Children aged 2 y n = 331	3.31 (2.49-4.39)

IQR, Interquartile range; *TSDR*, Treg-cell–specific demethylated region.
*The number of Treg cells is presented as percentage of Treg cells in whole blood corresponding to the measured amount of TSDR demethylation in the FOXP3 gene. The detection limit of the *FOXP3* TSDR demethylation assay is 0.03%; all values were above this limit.

TABLE E4. Relationship between maternal urine phthalate metabolite concentrations (36th gestational week) and allergic outcomes in the first 3 years of children's life

	Allergic outcomes, adjusted OR (95% CI)*						
	Hay fever	Sensitization to food allergens (fx5)†			Sensitization to inhalant allergens (sx1)†		
Phthalate metabolites	0-3rd year, 3.6% (17 of 475)	First year, 15.3% (79 of 515)	Second year, 12.1% (41 of 340)	Third year, 13.8% (40 of 289)	First year, 3.3% (17 of 515)	Second year, 5.3% (18 of 340)	Third year, 12.1% (35 of 289)
MEP	1.05 (0.62-1.76) $P = .857$	1.23 (0.97-1.57) $P = .091$	**1.49 (1.05-2.13)‡** $P = \mathbf{.024}$	1.23 (0.88-1.72) $P = .217$	0.95 (0.59-1.53) $P = .856$	1.14 (0.69-1.85) $P = .602$	1.06 (0.75-1.51) $P = .729$
MiBP	1.09 (0.66-1.80) $P = .734$	0.97 (0.77-1.23) $P = .835$	1.08 (0.77-1.52) $P = .630$	0.95 (0.69-1.32) $P = .775$	1.16 (0.74-1.83) $P = .512$	1.16 (0.71-1.88) $P = .551$	1.17 (0.82-1.66) $P = .370$
MnBP	1.01 (0.61-1.66) $P = .972$	1.24 (0.98-1.58) $P = .065$	**1.47 (1.05-2.07)‡** $P = \mathbf{.025}$	1.16 (0.84-1.58) $P = .357$	1.07 (0.68-1.69) $P = .771$	0.94 (0.59-1.50) $P = .803$	1.02 (0.73-1.44) $P = .879$
MBzP	0.98 (0.58-1.67) $P = .943$	0.83 (0.65-1.05) $P = .127$	0.95 (0.68-1.33) $P = .780$	0.92 (0.66-1.27) $P = .607$	1.03 (0.65-1.62) $P = .907$	0.97 (0.59-1.58) $P = .902$	0.94 (0.66-1.33) $P = .731$
MEHP	1.34 (0.79-2.26) $P = .267$	0.83 (0.65-1.05) $P = .604$	1.23 (0.88-1.72) $P = .212$	1.18 (0.85-1.64) $P = .327$	1.12 (0.72-1.76) $P = .599$	1.29 (0.78-2.11) $P = .314$	1.32 (0.92-1.88) $P = .127$

Note the reduced case numbers for allergic sensitization because blood samples were not available for all children participating in the follow-up investigation.

*Analysis was performed using a logistic regression model with phthalate values categorized into quartiles; odds ratios (ORs) were adjusted for sex, parental history of atopy, maternal smoking and/or ETS exposure at home, siblings, maternal education, cat ownership, and breast-feeding until 6 months.

†According to the Pharmacia CAP System, concentrations of >0.35 kU/L were regarded as positive.

‡Significant values are marked in boldface ($P < .05$).

TABLE E5. Relationship between Treg-cell numbers and allergic outcomes in the first 3 years of children's life

| | Allergic outcomes, adjusted OR (95% CI)* | | | | | | |
| | Hay fever | Sensitization to food allergens (fx5)† | | | Sensitization to inhalant allergens (sx1)† | | |
Treg cells	0-3rd year, 3.6% (17 of 475)	First year, 15.3% (79 of 515)	Second year, 12.1% (41 of 340)	Third year, 13.8% (40 of 289)	First year, 3.3% (17 of 515)	Second year, 5.3% (18 of 340)	Third year, 12.1% (35 of 289)
Treg-cell cord blood	1.59 (0.72-3.54) $P = .248$	**0.72 (0.54-0.96)‡** $P = .024$	0.97 (0.67-1.41) $P = .882$	0.83 (0.55-1.21) $P = .361$	0.97 (0.54-1.73) $P = .926$	0.71 (0.37-1.39) $P = .321$	1.02 (0.68-1.53) $P = .904$
Treg-cell second year	0.89 (0.51-1.57) $P = .695$	NA	1.02 (0.75-1.39) $P = .869$	0.84 (0.61-1.16) $P = .292$	NA	0.71 (0.43-1.15) $P = .169$	**0.68 (0.46-0.99)‡** $P = .046$

NA, Not applicable.

*Analysis was performed using a logistic regression model with Treg-cell values categorized into quartiles; odds ratios (ORs) were adjusted for sex, parental history of atopy, maternal smoking and/or ETS exposure at home, siblings, maternal education, cat ownership, and breast-feeding until 6 months.

†According to the Pharmacia CAP System, concentrations of >0.35 kU/L were regarded as positive.

‡Significant values are marked in boldface ($P < .05$).