Immune Responsiveness to LPS Determines Risk of Childhood Wheeze and Asthma in 17q21 Risk Allele Carriers

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Running head: Immune response to LPS and wheeze/asthma in 17q21 genotype

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At a Glance Commentary:

Scientific Knowledge on the Subject

Asthma risk in children is strongly conferred by a specific genotype, namely variants at the chromosome 17q21 locus which may interact with various environmental exposures. On the other hand, animal models have shown that specific microbe-rich environments may provide protection against asthma by engaging and shaping the innate immune response. In line, growing up on a traditional farm is associated with less wheeze and asthma in children, indicating a crucial role for a microbe-rich environment in early life.

What This Study Adds to the Field

Findings from the rural PASTURE birth cohort indicate that asthma development in 17q21 risk allele carriers is associated with an impaired immune responsiveness to the potent innate stimulus LPS. In contrast, risk allele carriers with an activated Th1/Th2/Th17 immune response after innate stimulation by the age of 1 year were at no

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increased risk of wheeze and asthma up to the age of 6 years. Oral rather than inhaled exposures and the resulting changes in the compositional structure of the early gut microbiome restored responsiveness to microbial LPS. These findings suggest that within the 17q21 genotype, asthma risk might be mitigated by restoring Th1/Th2/Th17 activation after microbial stimulation.

Online data supplement: This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org.

Abbreviations:

95%-CI	95%-confidence interval
DL	detection level
GEE	general estimation equations
IFN	interferon
IgG	Immunoglobulin G
IL	interleukin
LCA	latent class analysis
LPS	lipopolysaccharide
OR	odds ratio
PBMC	peripheral blood mononuclear cell
PCoA	principal coordinate analysis
SCFA	short chain fatty acids
SNP	single nucleotide polymorphism
TGF	Transforming growth factor
TNF	tumor necrosis factor

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ABSTRACT

Background: In murine models microbial exposures induce protection from experimental allergic asthma through innate immunity. Our aim was to assess the association of early life innate immunity with the development of asthma in children at risk.

Methods: In the PASTURE farm birth cohort innate, Th2, Th1 and Th17 cytokine expression at age 1 year was measured after stimulation of PBMCs with lipopolysaccharide (LPS) in N=445 children. Children at risk of asthma were defined based on single-nucleotide polymorphisms at the 17q21 asthma gene locus. Specifically, we used the SNP *rs7216389* in the GSDMB gene. Wheeze in the 1st year of life was assessed by weekly diaries and asthma by questionnaire at age 6 years.

Results: Not all cytokines were detectable in all children after LPS-stimulation. When classifying detectability of cytokines by latent class analysis, carrying the 17q21 risk allele *rs7216389* was associated with risk of wheeze only in the class with the lowest level of LPS-induced activation, odds ratio (OR)=1.89, 95%-CI 1.13-3.16, p=0.015. In contrast, in children with high cytokine activation after LPS-stimulation no association of the 17q21 risk allele with wheeze (OR=0.63, 95%-CI 0.29-1.40, p=0.258, p=0.034 for interaction) or school age asthma was observed. In these children consumption of unprocessed cow's milk was associated with higher cytokine activation (OR=3.37, 95%-CI 1.56-7.30, p=0.002), which was in part mediated by the gut microbiome.

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Conclusions: These findings suggest that within the 17q21 genotype asthma risk can be mitigated by activated immune responses after innate stimulation, which is partly mediated by a gut-immune axis.

Abstract word count: 250

Key Words: innate immune response, children, 17q21 genotype, farm environment, unprocessed cow's milk, gut microbiome.

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INTRODUCTION

A multitude of studies has shown that growing up on a traditional farm is associated with less wheeze and asthma in children, indicating a crucial role for a microbe-rich environment in early life.(1-4) The underlying mechanisms are, however, still not yet clear. Animal models have shown that specific microbe-rich environments may provide protection against asthma by engaging and shaping the innate immune response.(5)

On the other hand, asthma risk in children is strongly conferred by a specific genotype, namely variants at the chromosome 17q21 locus.(6-10) Symptomatic risk allele carriers constitute a phenotype associated with early episodes of viral wheeze(11), repeated exacerbations(12, 13) and an increased risk for persistent wheeze and asthma at school age.(14) The 17q21 locus interacts with various environmental exposures and may thus constitute a switch towards risk when interacting with higher number of siblings and passive smoke exposure(14, 15) or towards protection when interacting with furred pets and farm animal sheds(14, 16, 17). The underlying immunomodulatory mechanisms are not yet clear.(18)

We hypothesized that increased expression of peripheral blood cytokines after stimulation with a potent innate stimulus, which we used as a marker for an activated early innate immune response, could counteract the increased risk for wheeze and asthma in 17q21 risk allele carriers. Moreover, we hypothesized that a microbe-rich environment in early life shapes a child's early immune response and may thus play a major role in this complex interaction, specifically within the setting of a rural study with traditional farm and non-farm environments. Some of the results of this study have been previously reported in the form of an abstract.(19)

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METHODS (cf. Supplemental Methods)

Study population

PASTURE is a large prospective birth cohort study conducted in rural areas of 5 European countries: Austria, Finland, France, Germany, and Switzerland. The study design has been described earlier.(20) Briefly, pregnant women were recruited during the last trimester of pregnancy between 2002 and 2005. Women living on an actively run farm where livestock was held were considered farming women. Pregnant women living in the same rural area but not occupationally involved in farming activities were assigned to the non-farm reference group. In all, 1,133 women were included in the study (530 farming and 603 non-farming women). Study population for the current analyses were all children with available cytokine measurements at 1 year. The study was approved by local research ethics committees in each country, and written informed consent was obtained from the children's parents.

Questionnaires

Extensive questionnaires were administered in the third trimester of pregnancy and repeatedly after birth until 6 years. Furthermore, at age 8-53 weeks parents were asked to complete weekly and monthly diaries. All questionnaires and diaries assessed illnesses as well as farm related and other environmental exposures. Wheeze in the 1st year of life was defined as any wheeze during the previous 7 days as registered by the weekly diaries. Asthma was defined as a physician's diagnosis of asthma or recurrent obstructive bronchitis established until 6 years.

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Blood sampling

At birth, cord blood samples were taken for genotyping; venous blood samples were collected at age 1 year.

Cytokine production after stimulation with innate stimulus

Cytokine levels of IL-1β, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17A, IFN-γ, and TNF-α were measured after stimulation of whole blood with lipopolysaccharide (LPS 0.1µg/ml; kindly provided by Profs. Holst and Brade, Borstel) for 24 hours at age 1 year in all study centers except France. Detection limits of cytokines, percent non-detects and basic descriptions of cytokine levels are given in Table E1.

Genotyping of single-nucleotide polymorphism (SNP) at 17q21

Genotyping was performed at the Centre National de Génotypage, Evry, France, using the iPLEX Gold technology and single-nucleotide polymorphisms (SNPs) at the 17q21 asthma gene locus were genotyped. For the current analyses, we used the SNP *rs7216389* in the GSDMB gene, which was coded for its risk allele (T). This SNP within the 17q21 locus has been associated with childhood asthma(6, 9-11, 21) and has been used in previous analyses of the PASTURE cohort.(14, 22) Of all 939 children with available DNA samples from cord blood, quality of DNA and genotyping was sufficient for 896 children.

Fecal sampling

Fecal samples were collected from 720 children at 1 year and DNA was extracted to determine bacterial communities defined by 16S rRNA gene analyses and

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preprocessed as recently described.(23) Microbial variables included in statistical analyses comprised aggregated variables derived from the alpha-diversity (richness, i.e. the number of amplicon sequence variants, and Shannon index) and principal coordinate analysis (PCoA). Furthermore, estimated microbiome age was calculated and two butyrate variables were created: a butyrate score based on bacterial taxa predicting butyrate production and a gene assay based on the relative abundance of the gene encoding the main enzyme of bacterial butyrate metabolism.(23)

Statistical analyses

For all analyses on the 17q21-SNP *rs7216389* we combined the heterozygous (CT) and homozygous (TT) as “risk allele carriers”. For the analysis of dichotomous or categorical variables we used the χ^2-test.

Since levels of cytokines after LPS-stimulation were below detection level in a large proportion of children, all cytokine measurements were dichotomized at detection level (Table E1). For the grouping of children according to cytokine profiles we then conducted a latent class analysis (LCA) on the dichotomized measurements of cytokines with a detectability rate <99%. As a sensitivity analysis, we conducted the same latent class analysis including all cytokines irrespective of the detectability rate.

In order to assess the association of the 17q21-SNP, which we used as indicator for an increased asthma risk, with the outcome of wheeze in the 1st year of life, general estimation equations (GEE) were conducted, stratified for the latent classes of cytokine detectability. Furthermore, multinomial logistic regression models with the latent cytokine class after LPS-stimulation as categorical outcome were performed in risk

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allele carriers, the low cytokine class being used as reference category. All exposures with a p-value ≤0.1 were then included in a stepwise variable selection procedure. Similarly, the microbial variables as well as relevant single taxa were included in multinomial logistic regression models.

In addition, we conducted a mediation analysis to assess whether the effect of the relevant environmental exposures on high cytokine class were mediated by the fecal microbiome in the 17q21 risk allele population. All above models were adjusted for study center, GEE models with repeated (weekly) outcomes of wheeze in the 1st year of life were additionally adjusted for age in weeks and the final stepwise and mediation models were adjusted for farming and study center. Effect estimates are presented as adjusted odds ratios (OR), a p-value of 0.05 was considered significant. In the mediation analysis, all effects are given as farm- and center-adjusted regression parameters (ß). Due to the exploratory character of the analyses, corrections for multiple testing were not performed. Statistical analyses were performed with SAS 9.4 (The SAS Institute, Cary, NC, USA), Mplus 8.1 (Muthén & Muthén, Los Angeles, CA, USA) and R 3.41 (www.r-project.org).

RESULTS

Study population

In total, 445 children had measurements of all assessed cytokines after LPS-stimulation at 1 year (Figure E1). The children with LPS-stimulated cytokines and the total PASTURE study population did not differ significantly with respect to relevant exposures or confounders (farming status, gender, parental atopy, older siblings, maternal smoking

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in pregnancy, maternal education, early farm milk consumption, wheeze, asthma, Table E2). Of all 445 children, 377 had information on the 17q21 risk allele, with 257 (68.2%) being a risk allele carrier (185 heterozygous, 72 homozygous).

Cytokine responsiveness and latent classes of detectable cytokines after LPS-stimulation

LPS-stimulation of PBMCs yielded many undetectable measurements of assessed cytokines (Figure 1): while IL-1β, IL-6, IL-10 and TNF-α were detectable in all or nearly all children, a different pattern was observed for the other cytokines, with detectability varying between 3.1% for IL-5 and 80.4% for IFN-γ.

To classify children into distinct patterns of cytokine detectability after the innate stimulus LPS, we dichotomized all cytokines into detectable vs. non-detectable and included those with a detectability <99% in a latent class analysis.

The solution yielding best results with respect to objective criteria was a 3-class solution (Table 1) yielding patterns of selective responsiveness. The largest latent class (N=231) comprised mostly children with a low percentage of detectable IL-4, IL-5, IL-13, IL-17, IL-12p70 and IFN-y, i.e. of low Th2/lowTh1/low Th17 cytokine detectability. For example, IL-12p70 was detectable in 46.5% of the children in total, but only in 1.3% of the children in this class. We thus labelled this class “low class”. The intermediate class (N=119) showed very high detectability of IL-12p70 and IFN-y with intermediate detectability of IL-13 and IL-17 which we labelled “intermediate class”. In turn, the smallest latent class (N=95) comprised children with high detectability of almost all cytokines after LPS-stimulation, which we thus labelled “high class”. Of note, in all three

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resulting classes IL-1β, IL-6, IL-10 and TNF-α were detectable in all or nearly all children. Inclusion of these cytokines with a detectability ≥99% in the LCA yielded almost identical classes, with only 7 (1.6%) children being assigned to a different class.

17q21 risk allele carriers were similarly distributed over cytokine classes after LPS-stimulation (69.0% in low, 65.6% in intermediate and 69.2% in high cytokine class, p=0.620). *Vice versa*, cytokine classes after LPS-stimulation were similarly distributed in 17q21 risk allele carriers (54.9% low, 24.5% intermediate, 21.0% high, Table E3) and non-carriers (52.5%, 27.5%, 20.0%, respectively, p=0.824).

17q21 risk allele is associated with more wheeze in the first year of life and more asthma at 6 years – but not in children with activated response

In analyses stratified for the three cytokine classes, we assessed the effect of the 17q21 risk SNP on wheezing in early life using generalized equation estimations (GEE, Figure 2). In the total population, 17q21 risk allele carriers were at increased risk of having wheezed in the 1st year, though not significantly so (OR=1.40, 95%-CI 0.97-2.01, p=0.071). If, however, the child was in the lowest cytokine class, the risk of wheeze in the 1st year was significantly increased in 17q21 risk allele carriers as compared to children with no risk allele (OR=1.89, 95%-confidence interval (95%-CI) 1.13-3.16, p=0.015). In contrast, in the highest cytokine class no such effect was observed (OR=0.63, 95%-CI 0.29-1.40, p=0.258) indicating a significant interaction between 17q21 risk allele and cytokine class on wheeze (p=0.034 for interaction).

Furthermore, children with a 17q21 risk allele were at significantly increased risk of having asthma at 6 years compared to children with no risk allele (OR=3.66, 95%-CI

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1.25-10.72, p=0.018). This genotype-dependent risk was however only present in children in the low cytokine class (OR=3.93, 95%-CI 0.87-17.71, p=0.075), though not significantly so potentially, due to low numbers (16/124, 12.9% vs. 2/55, 3.6%). In contrast, in the group of children in the high cytokine class, the 17q21 risk allele exerted no effect on the outcome of asthma at 6 years (OR=1.35, 95%-CI 0.24-7.60, p=0.733).

Environmental exposures are associated with high cytokine class

As shown above, 17q21 risk allele carriers were only at increased risk of wheeze in the 1st year of life and of asthma at 6 years if the child was in the lowest cytokine class after LPS-stimulation. Thus the question arose, which factors contribute to a higher cytokine class within the group of 17q21 risk allele carriers and might thus potentially counterbalance the genetically increased risk of wheezing and asthma.

Of all assessed environmental exposures, only farm-related exposures were significantly associated with the high cytokine class in center-adjusted analyses in 17q21 risk allele carriers (Table 2, similar effects were seen in the total population Table E4). Among these were exposures both in pregnancy and in the 1st year of life such as consumption of farm milk and exposure to stables and farm animals. It is noteworthy that the effect of boiled and unboiled farm milk was of similar magnitude (data not shown). However, consumption of farm milk was not the only diet-related exposure: introduction of a high variety of foods was also associated with the high cytokine class, though not significantly so. Other factors such as sex, birth weight, gestational age, parental atopy, older siblings, maternal smoking, maternal infections in pregnancy, infections in the 1st year of life defined as the number of weeks with respiratory tract infections, fever or otitis, breastfeeding, regular stay in daycare and pet keeping in the

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house showed no significant effect on cytokine class in 17q21 risk allele carriers (data not shown). In order to disentangle the various factors associated with the high cytokine class we conducted a stepwise multivariate multinomial logistic regression including all relevant factors from bivariate analyses. The only variable, however, selected into the final model was consumption of farm milk in the 1st year of life (OR=3.37, 95%-CI 1.56-7.30, p=0.002 for high vs. low cytokine class). Adjusting for living on a farm did not change the magnitude of the effect (OR=3.56, 95%-CI 1.34-9.48, p=0.011).

Gut microbiome, cytokine classes and wheeze

Multivariate stepwise analyses differentiated between exposure to farm milk and exposure to barns or stables, i.e. between oral and inhaled exposures, with only consumption of farm milk remaining in the final model. This lead us to hypothesize that the gut microbiome at 1 year might influence both cytokine classes and the association with wheeze in the 1st year of life in children at risk of asthma and might thus act as a mediator of the farm milk effect. Therefore, we assessed various dimensions of the compositional structure of the early gut microbiome and assessed their association with cytokine classes.

Of the first 3 axes from principal coordinates analysis (PCoA) of the gut microbiome at 1 year only the 1st PCoA-axis – which correlated positively with the relative abundance of *Rikenellaceae*, *Ruminococcaceae*, *Faecalibacterium* and *Roseburia* (Figure E2) – was significantly associated with the high cytokine class in 17q21 risk allele carriers (Table 3, no effects were seen in the total population Table E5). Furthermore, the microbial diversity, i.e. Shannon index and species richness, showed a significant elevated effect on the high cytokine class. When defining “high-risk” not as ≥1 17q21

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risk alleles but as homozygous risk allele carriers, the microbial effects became stronger, with the effect of the butyrate score reaching statistical significance for the high cytokine class (Table E6). In contrast, the butyrate gene assay showed no effect, irrespective of genotype, potentially due to small numbers (N=77, data not shown). Interestingly, when testing for associations of single taxa with cytokine class in risk allele carriers, the same taxa that played a role in butyrate production and bacterial maturation, i.e. *Coprococcus* and *Roseburia*, were relevant (data not shown).(23)

Piecing the puzzle together – mediation analyses

To further disentangle the associations of farm milk consumption, fecal microbiome and cytokine class, we conducted a mediation analysis in risk allele carriers to assess whether the effect of farm milk consumption on cytokine class was mediated by the fecal microbiome (Figure 3). Indeed, the indirect effect of farm milk depicted by the path from exposure through fecal microbiome to high cytokine class was significant ($p=0.042$), indicating that the effect of farm milk on cytokine class is partially mediated by the fecal microbiome with 23% of the total effect of farm milk being mediated by the 1st PCoA-axis of the fecal microbiome.

DISCUSSION

Data from the rural PASTURE birth cohort suggest a complex interaction of genetics, diet, the gut microbiome, early immune responses after innate stimulation and disease, indicating that a multitude of dimensions matter for wheeze and asthma development. In this exploratory analysis of our high dimensional data, the 17q21 risk allele was no longer associated with an increased risk of early wheezing or asthma at school age

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among children with an early-activated immune response after innate LPS-stimulation. The activation of the early immune response was associated with an oral but not inhaled farm exposure, i.e. consumption of unprocessed cow's milk (farm milk). This beneficial effect was in turn partially mediated by the gut microbiome.

Children at risk of asthma

We defined children at risk of asthma based on the 17q21 gene locus, which has repeatedly been related to childhood asthma, particularly early in life.(6-10) A cluster of SNPs in this region is associated with childhood onset asthma and the SNP used here is a good representative of this cluster, as recent data suggest that GSDMB may play a major role.(8, 9, 14, 24) Thus, as a marker for elevated asthma risk in children in the current analyses we used SNP *rs7216389* in the GSDMB gene, which was in strong linkage disequilibrium with other SNPs in this region. Indeed, in our population children with at least 1 risk allele of the *rs7216389* SNP were at almost 4-times the risk of being asthmatic at age 6 years as compared to children with no risk allele. Not surprisingly, the association with wheeze in the 1st year of life was much weaker, as most of the early wheezers in the PASTURE cohort were classified as transient or intermediate phenotypes, with no, or almost no symptoms at age 6 years.(22)

Deficient responsiveness to innate stimuli

In the PASTURE birth cohort, we investigated the early immune response based on cytokine measurements after stimulation with the potent innate microbial trigger LPS. Only those at-risk children without activation of Th1/Th2/Th17 cytokines after LPS were significantly at risk of early wheeze or asthma by the age of 6 years. In other words, a

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strengthened innate-induced activation in the 1st year of life protected from asthma development in carriers of the 17q21 risk alleles. The cytokines represented in the three latent classes are related to both innate and adaptive immunity. In fact, all 'innate' cytokines (IL-1ß, IL-6, TNF-α) were detectable in almost all children after LPS-stimulation and were thus excluded from latent class analysis. The intermediate class was characterized by cytokines related to Th1 (IL-12p70, IFN-γ) and to a lesser extent to Th17 (IL-17) responses. Interestingly in this class, although levelling out the risk of wheeze to some extent in the at-risk population, the effect was not as pronounced as in the highest cytokine class, suggesting that the Th1/Th2 paradigm does not explain our results. The highest class in turn included cytokines related to Th1, Th2 and Th17 immune responses. A sensitivity analysis including the highly detectable innate cytokines in analysis resulted in almost identical allocation of the children to the 3 latent classes. Furthermore, when using higher than detection level cut-offs for either the highly-detectable cytokines or all cytokines, e.g. lowest decile, quartile or median, the resulting latent classes showed no comparable interaction with asthma risk and were not associated with any environmental exposures (data not shown). These findings indicate that responsiveness to the potent innate stimulus LPS at the interface to adaptive immunity, rather than levels of individual innate or Th1/Th2/Th17-associated cytokines matter. Low Th1/Th2/TH17 responses after innate stimulation may reflect an inefficient innate-adaptive crosstalk in the 1st year of life which in turn confers risk of school age asthma in 17q21 risk allele carriers. In contrast, more robust immune responses to LPS may be relevant for two reasons: First, a propensity of early life immune activation via TLR-signaling can counterbalance potentially harmful immune

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responses genetically set through a risk genotype. Evidence for this hypothesis of robust immune responses to LPS is provided by some recent publications: We have shown that *ex vivo* farm dust or LPS-stimulation can restore TNFAIP3 expression, an anti-inflammatory protein within the NF-KB signaling pathway, to healthy levels. This was feasible even in manifest asthmatics and shifted NF-κB signaling associated gene expression towards an anti-inflammatory state.(25) Furthermore, not only NF-kB signaling was stimulated by LPS or farm-dust stimulation but also the MAPK-signaling pathway. In this context, farm-dust stimulation was associated with increased DUSP1 expression, a negative regulator of the MAPK-signaling pathway for inflammation, reaching healthy levels and downregulated inflammatory MAPKs.(26) Both reports show that potent LPS- (or farm dust) induced immune response can effectively counteract pro-inflammatory responses. Whether our observations are generalizable from LPS to other innate stimuli must however be further investigated.

Innate immunity in farming environments

Innate stimulation from environments rich in microbial exposures has been shown in a number of previous cross-sectional farm studies. For example, increased gene expression of TLR downstream signaling molecules such as IRAK-1, IRAK-2, and RIPK1 as well as HLA-DRA, and SOCS-4 was found among farm children, whereby the expression of IRAK-1, IRAK-2, and RIPK1 partially mediated the protective farm effect on asthma.(27) These data suggest that activation of innate immunity is associated with both farm exposure and reduced asthma risk. In the Amish farm population a strong reduction in the prevalence of asthma and atopy was found. Furthermore, the protective effect of Amish environmental exposure on experimental allergic airway disease

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disappeared in MYD88/TRIF knock out mice suggesting that childhood asthma is associated with deficiencies in the innate immune response and that specific environments may provide protection against asthma by innate immune activation.(5). Our findings refine this concept suggesting that the interaction between innate and adaptive responses after microbial (LPS) stimulation may particularly matter for asthma development in 17q21 risk allele carriers.

It is noteworthy that in our analyses, even though almost all farm-related exposures were significantly associated with an innate-induced activated immune response in univariate analyses, only the consumption of unprocessed cows milk remained in the final farm-adjusted multivariate model. This potentially indicates that not inhaled exposures – such as stable contact – are essential for activation of circulating immune cells after LPS, but rather dietary features and associated characteristics of the early gut microbiome. These results support the notion of a gut-immune axis in very young children. In contrast, the previously reported protective effect of inhaled exposure to stables in 17q21 risk allele carriers on wheeze is presumably transmitted by mucosal mechanisms rather than by a systemic immune response.(14) These results underline the multitude of mechanisms by which farm exposures early in life may protect from asthma: both stable exposure and farm milk consumption are independent protective factors for the development of disease. However, only 23% of the milk effect was mediated by the gut microbiome, indicating additional protective mechanisms. Potentially epigenetic mechanism like DNA methylation, histone modification or microRNAs contribute to the effects induced by unprocessed milk.(28) It remains, however, unclear which ingredients in farm milk exert the observed beneficial effect.

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The observation that boiling did not alter the effect suggests that heat stabile compounds such as fatty acids, in particular ω-3 polyunsaturated fatty acids which are precursors of anti-inflammatory mediators,(29) and oligosaccharides acting as prebiotics may alter the compositional structure of the gut microbiome and thereby boost the early immune response. Other milk components like bovine IgG or TGF-ß might also play a role: The former can bind to bacterial and viral pathogens, enhance phagocytosis and may neutralize pathogens, whereas the latter promotes epithelial barrier functioning and might favor the differentiation of Tregs that can reduce inflammation locally.(30) A recent review on the impact of raw milk on the immune system in early life summarized that the industrial processing of milk and dairy products, needed to ensure microbiological safety, typically results in denatured milk proteins which lose their functional activity, suggesting that preserving milk proteins and preventing glycation may be important innovations to help prevent future disease.(28)

The immuno-modulatory role of the gut microbiome

Alterations in the compositional structure of the gut microbiome such as decreased diversity, inadequate maturation and lower abundance of taxa producing short chain fatty acids have all been incriminated as conferring asthma risk(31-33) and bacterial metabolites have often been found to be associated with health effects.(34) Data from animal models further point to the importance of bacterial metabolites transmitting signals from the gut to the bone marrow thereby shaping immune responses.(35) These experimental studies also demonstrate that early life represents a critical window during which the gut microbiome can shape systemic immune function later in life.(36)

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We have recently shown for the PASTURE cohort that accelerated maturation of the gut microbiome in the 1st year of life was directly associated with decreased asthma risk at 6 years.(23). Here we report that the compositional structure of the gut microbiome by the age of 1 year, but not the microbiome maturation, indirectly impacts on asthma development in risk allele carriers via activated immune responses after innate stimulation. Thus, different facets of the gut microbiome may have an impact on the gut-lung and the gut-immune axis, respectively, all eventually directly and indirectly contributing to decreased asthma risk. Common to both pathways may be the microbial capacity for production of short chain fatty acids such as butyrate.

Strength and limitations

The major strength of the PASTURE study is its unique nature as a birth cohort within a rural farming environment, an environment that has already changed since the beginning of the study in 2002. Furthermore, the multitude of measurements assessed in early life, such as genetics, cytokines and fecal microbiome enable a quite comprehensive analysis of the early origins of childhood asthma. Nevertheless, the number of children with all measurements available at age 1 year and with a follow-up at age 6 years was fairly small, leading to small numbers for several analyses, even more so as the asthma prevalence in our rural population was comparatively low. Moreover, our analyses are of exploratory character and would benefit from a confirmation in a replication cohort.

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Conclusion

Our findings from the rural PASTURE birth cohort indicate that asthma development in 17q21 risk allele carriers is associated with an impaired immune responsiveness to the potent innate stimulus LPS. In contrast, risk allele carriers with an activated Th1/Th2/Th17 immune response after innate stimulation by 1 year were at no increased risk of wheeze and asthma up to age 6 years. Oral rather than inhaled exposures and the resulting changes in the compositional structure of the early gut microbiome restored responsiveness to microbial LPS. Based on these results from our exploratory analyses, we speculate that within the 17q21 genotype asthma risk might be mitigated by restoring Th1/Th2/Th17 activation after microbial stimulation.

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FIGURE LEGENDS

Figure 1. Proportion of samples with detectable LPS-stimulated cytokines at 1 year (percent above detection level), N=445.

Figure 2. Effect of the 17q21 risk allele on wheeze in the 1. year of life: adjusted* odds ratios from GEE analyses stratified for cytokine class. OR = odds ratio; 95%-CI = 95%-confidence interval. * Adjusted for age and center. † Data shown are adjusted for age only: due to small numbers, when adjusting for center the generalized Hessian matrix is not positive definite, hence no confidence intervals can be calculated. Effect size however remains unchanged.

Figure 3. Mediation of the effect of farm milk consumption on cytokine class by the fecal microbiome in children at risk of asthma (17q21 risk allele, N=201). The total effect of farm milk consumption on high cytokine class (vs. intermediate or low class) at age 1 year in children at risk of asthma is divided into a direct and an indirect path (estimates and p-values of each effect are indicated separately). The 1st axis from principal coordinate analysis (PCoA) of the fecal microbiome at age 1 year is shown as a possible indirect link between farm milk exposure and high cytokine class. All effects are adjusted for farming and center. Direct paths are shown in blue, indirect paths are shown in red. Statistically significant estimates and p-values are given in **bold**.

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Table 1. Detectable LPS-stimulated cytokines at age 1 year: allocation to latent classes (**A.** Cytokines included in latent class analysis with detectability <99%; **B.** cytokines excluded from latent class analysis with detectability ≥99%).

				Latent cytokine classes							
		Total		LC1 Low class		LC2 Intermediate class		LC3 High class			
		N=445		N=231 (51.9%)		N=119 (26.7%)		N=95 (21.4%)		p-value	
		n	%	n	%	n	%	n	%	Chi2	trend
A.	IL-4	54	12.1	1	0.4	8	6.7	45	47.4	<0.001	<0.001
	IL-5	14	3.1	0	0.0	3	2.5	11	11.6	<0.001	<0.001
	IL-12p70	207	46.5	3	1.3	112	94.1	92	96.8	<0.001	<0.001
	IL-13	152	34.2	23	10.0	34	28.6	95	100.0	<0.001	<0.001
	IL-17A	136	30.6	17	7.4	30	25.2	89	93.7	<0.001	<0.001
	IFN-γ	358	80.4	145	62.8	118	99.2	95	100.0	<0.001	<0.001
B.	IL-1β	443	99.5	231	100.0	118	99.2	94	98.9	0.329	0.154
	IL-6	442	99.3	231	100.0	118	99.2	93	97.9	0.104	0.035
	IL-10	445	100.0	231	100.0	119	100.0	95	100.0	–	–
	TNF-α	445	100.0	231	100.0	119	100.0	95	100.0	–	–

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Table 2. Factors associated with cytokine classes in 17q21 risk allele carriers.*

Exposure variable		N	Cytokine class	OR	95%-CI	p-value
Characteristics at birth						
Farm child		126/257	Intermediate vs. low	0.91	0.49-1.69	0.774
			High vs. low	1.53	0.77-3.03	0.223
Maternal farm exposures in pregnancy						
Regular stay in stables		130/246	Intermediate vs. low	1.06	0.57-1.97	0.855
			High vs. low	2.70	1.28-5.67	**0.009**
Regular stay in barn		96/245	Intermediate vs. low	1.13	0.59-2.18	0.714
			High vs. low	2.96	1.43-6.15	**0.004**
# farm animal species:	0 (ref.)	104/255	Intermediate vs. low	1.00	–	–
			High vs. low	1.00	–	–
	1	87/255	Intermediate vs. low	1.36	0.67-2.76	0.391
			High vs. low	1.10	0.47-2.57	0.825
	≥2	64/255	Intermediate vs. low	1.66	0.72-3.83	0.235
			High vs. low	2.37	1.01-5.54	**0.046**
Any farm milk consumption		111/257	Intermediate vs. low	1.28	0.69-2.36	0.440
			High vs. low	2.22	1.12-4.41	**0.022**
Farm exposures 1. year of life						
Regular stay in stables		50/248	Intermediate vs. low	1.24	0.55-2.81	0.608
			High vs. low	2.26	1.00-5.14	**0.050**
Regular stay in barn		50/248	Intermediate vs. low	0.89	0.37-2.15	0.798
			High vs. low	2.04	0.86-4.82	0.105
Contact with hay		22/251	Intermediate vs. low	0.73	0.18-2.86	0.647
			High vs. low	2.94	1.05-8.26	**0.041**
Any farm milk consumption		76/248	Intermediate vs. low	1.18	0.59-2.39	0.637
			High vs. low	3.06	1.45-6.44	**0.003**
Analysis in milk drinkers only:						
Milk consumption:	Any UHT milk (ref.)	31/165	Intermediate vs. low	1.00	–	–
			High vs. low	1.00	–	–
	Any pasteurized milk	58/165	Intermediate vs. low	1.33	0.43-4.13	0.624
			High vs. low	1.71	0.38-7.72	0.485
	Any farm milk	76/165	Intermediate vs. low	1.36	0.48-3.87	0.566
			High vs. low	3.75	1.06-13.25	**0.040**
Other exposures 1. year of life						
Food diversity score:	0-8 items (ref.)	51/248	Intermediate vs. low	1.00	–	–
			High vs. low	1.00	–	–
	9-11 items	91/248	Intermediate vs. low	1.13	0.49-2.58	0.781
			High vs. low	1.32	0.47-3.67	0.596
	12-15 items	106/248	Intermediate vs. low	1.24	0.54-2.85	0.612
			High vs. low	2.54	0.95-6.77	0.063

* Results from multinomial logistic regression models with cytokine class as 3-categorical outcome and low cytokine class as reference category; results are adjusted for center;

Statistically significant p-values are given in **bold**;

OR = odds ratio; 95%-CI = 95% confidence interval.

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Table 3. Effect of fecal microbiome at age 1 year on cytokine classes in 17q21 risk allele carriers (N=209).*

Fecal microbiome (z-scores)		Cytokine class	OR	95%-CI	p-value
Principal coordinate analysis:	1st axis	Intermediate vs. low	1.04	0.72-1.50	0.834
		High vs. low	1.90	1.22-2.95	**0.004**
	2nd axis	Intermediate vs. low	0.86	0.59-1.26	0.443
		High vs. low	0.85	0.55-1.30	0.455
	3rd axis	Intermediate vs. low	1.11	0.76-1.63	0.583
		High vs. low	1.00	0.65-1.54	0.990
Shannon diversity index		Intermediate vs. low	1.11	0.76-1.63	0.594
		High vs. low	1.58	1.03-2.43	**0.035**
Species richness		Intermediate vs. low	1.10	0.72-1.67	0.657
		High vs. low	1.63	1.04-2.54	**0.033**
Estimated microbiome age		Intermediate vs. low	1.06	0.74-1.52	0.734
		High vs. low	1.47	0.92-2.33	0.103
Butyrate score		Intermediate vs. low	0.72	0.50-1.05	0.088
		High vs. low	1.33	0.95-1.86	0.094

* Results from multinomial logistic regression models with cytokine class as 3-categorical outcome and low cytokine class as reference category; results are adjusted for center;

Statistically significant p-values are given in **bold**;

OR = odds ratio; 95%-CI = 95% confidence interval.

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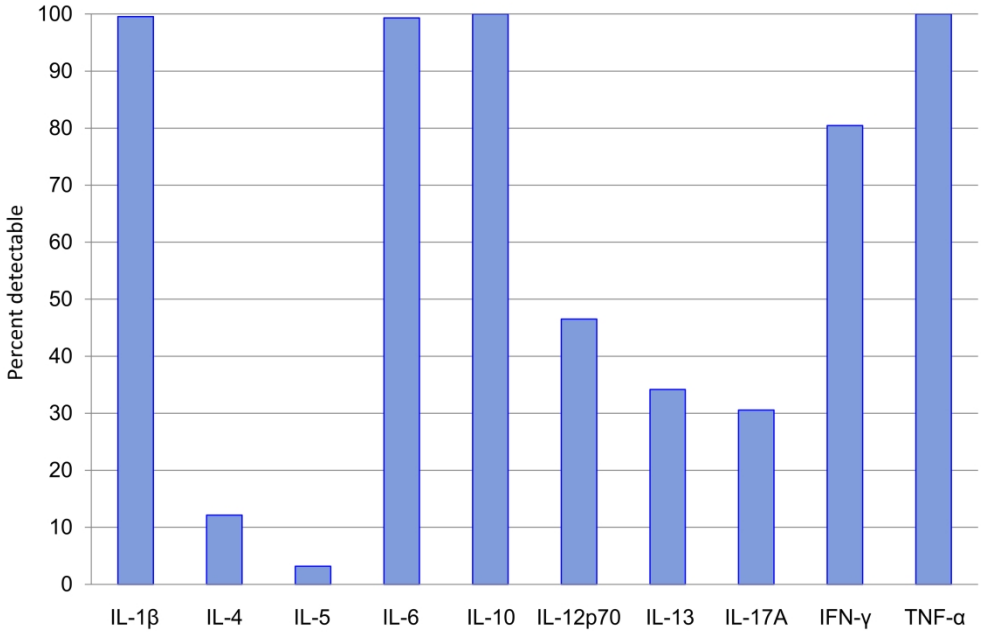

Figure 1. Proportion of samples with detectable LPS-stimulated cytokines at 1 year (percent above detection level), N=445.

262x166mm (300 x 300 DPI)

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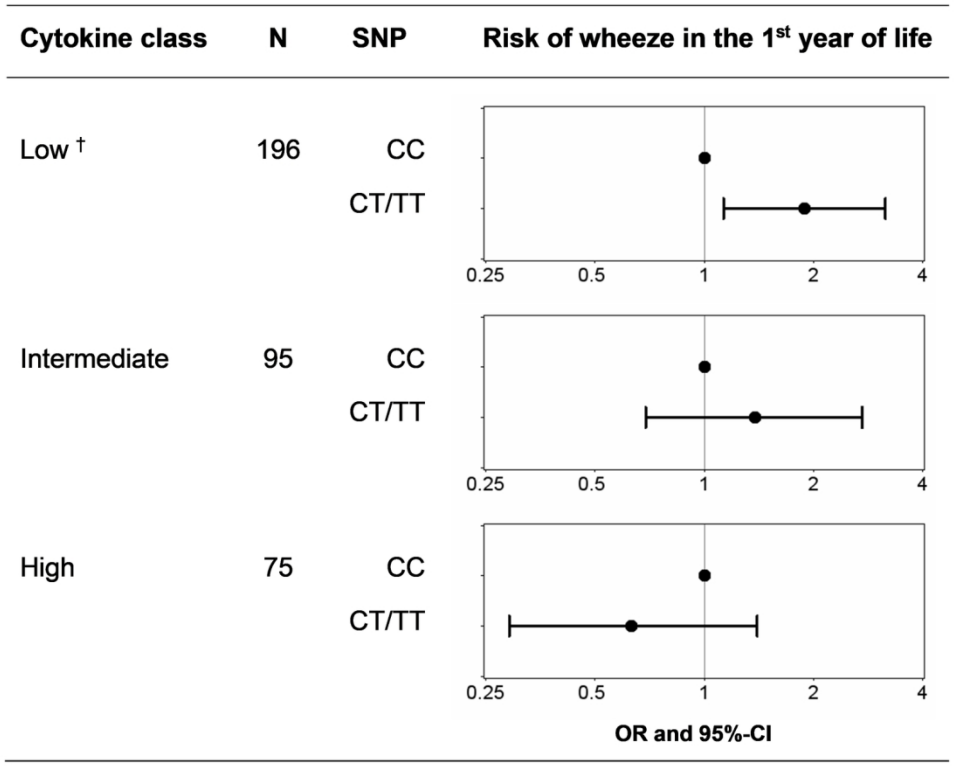

Figure 2. Effect of the 17q21 risk allele on wheeze in the 1. year of life: adjusted* odds ratios from GEE analyses stratified for cytokine class. OR = odds ratio; 95%-CI = 95%-confidence interval. * Adjusted for age and center. † Data shown are adjusted for age only: due to small numbers, when adjusting for center the generalized Hessian matrix is not positive definite, hence no confidence intervals can be calculated. Effect size however remains unchanged.

144x112mm (300 x 300 DPI)

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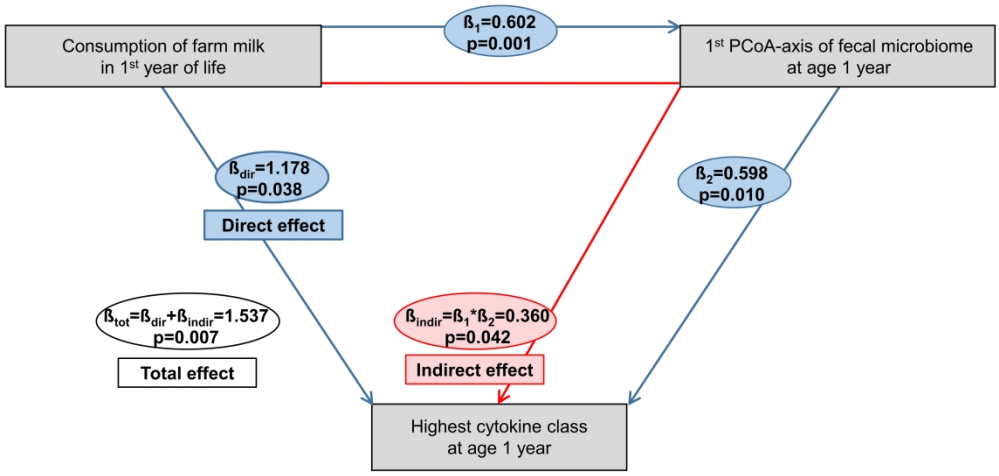

Figure 3. Mediation of the effect of farm milk consumption on cytokine class by the fecal microbiome in children at risk of asthma (17q21 risk allele, N=201). The total effect of farm milk consumption on high cytokine class (vs. intermediate or low class) at age 1 year in children at risk of asthma is divided into a direct and an indirect path (estimates and p-values of each effect are indicated separately). The 1st axis from principal coordinate analysis (PCoA) of the fecal microbiome at age 1 year is shown as a possible indirect link between farm milk exposure and high cytokine class. All effects are adjusted for farming and center. Direct paths are shown in blue, indirect paths are shown in red. Statistically significant estimates and p-values are given in bold.

354x170mm (300 x 300 DPI)

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Immune Responsiveness to LPS Determines Risk of Childhood Wheeze and Asthma in 17q21 Risk Allele Carriers

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METHODS

Questionnaires

Extensive questionnaires were administered by means of interview to the parents of the child. The first questionnaire within the third trimester of pregnancy assessed relevant maternal exposures during pregnancy, e.g. maternal regular contact with a stable or barn (stay in stable or barn during pregnancy at least 15 minutes per week in at least 1 trimester), regular contact with a number of farm animals (several times per month to cows, pigs, poultry, or sheep), any smoking, any farm milk consumption, infections during pregnancy and older siblings defined as ≥2. Furthermore, parental history of a doctor's diagnosis of asthma, atopic dermatitis or hay fever was assessed. Data on potential confounders, i.e. sex, birth weight and gestational age, as well as the presence of animals in the house were assessed at the age of 2 months. After birth, weekly and monthly diaries between 8 and 53 weeks of life assessed the occurrence of wheeze, infections and fever episodes on a weekly basis. Furthermore, farm related exposures such as stay in stables or barns and consumption of farm milk were assessed in these diaries on a weekly basis, as well as breast feeding, feeding practices and daycare, defined as having spent time with other children not including siblings. In total 1,031 individuals, i.e. 91% of the originally included 1,133 children, contributed to this analysis with a minimum of 20 weekly diaries in the first year of life. Wheeze in the 1st year of life was defined as any wheeze during the previous 7 days as registered by the weekly diaries. Regular stay in stables was defined as a positive answer in at least 17 weekly diaries, regular stay in barns as a positive answer in at least 4 weekly diaries, and regular exposure to daycare as a positive answer in at least 26 weekly diaries, all

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corresponding to the respective 3rd quartile of the weekly frequency in the total population. Presence of infections was also registered by the diaries on a weekly basis. For analysis we defined the total number of weeks with infections as the sum of weeks with either respiratory tract infections (any occurrence of rhinitis or cough in the absence of wheeze) or fever (≥38.5°C) or otitis. Based on parental reporting of feeding practices in the monthly diaries a diversity score was calculated, including the major 15 food items, which were defined as the ones introduced in the first year of life to approximately 80% of the children or more.(E1) Furthermore, at 1, 4, and 6 years of age questionnaires assessed both the exposure of the children to various environmental factors, especially farm related exposures, as well as respiratory and other health issues of the children throughout childhood. Questions of these extensive questionnaires were based on previously published studies.(E2-E4) At the 6 year follow-up, 863 children provided data on asthma. Asthma at age 6 years was defined as a parent-reported physician's diagnosis of asthma at least once per lifetime or spastic, obstructive, or asthmatic bronchitis at least twice.

Blood sampling

At birth, cord blood samples were taken for genotyping and venous blood samples were collected from study children at the age of 1 year.

Stimulated cytokine production

Immune responsiveness *ex vivo* was assessed by measuring cytokine production in stimulated whole blood cell cultures at the age of 1 year. Directly after blood sampling, diluted blood samples (1:8) were incubated for 24 h with the innate stimulus

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lipopolysaccharide (LPS, 0.1 µg/ml; kindly provided by Profs. Holst and Brade, Borstel). Stimulated supernatants were frozen (-80°C) and later analyzed in a batch for the following cytokines: interleukin (IL)-1β, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17A, IFN-γ, and TNF-α. Concentrations of all cytokines were measured using multiplexed cytometric bead array CBAFlex system according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA) in Marburg, Germany. Cytokine measurements were available in all study centers except France. Detection limits of cytokines, percent non-detects and basic descriptions of cytokine levels are given in Table E1.

Genotyping of single-nucleotide polymorphism (SNP) at 17q21

Genotyping was performed using the iPLEX® Gold technology, a MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry) system from SEQUENOM at the Centre National de Génotypage, Evry, France. Technical errors were minimized by comparing genotype frequencies with the expected allelic population equilibrium based on the Hardy-Weinberg equilibrium test. cDNA was amplified in duplicate using an iCycler (Bio-Rad Laboratories, Hercules, Calif) and 18S as reference gene. Of all 939 children with available DNA samples from cord blood, quality of DNA and genotyping was sufficient for 896 children.

Single-nucleotide polymorphisms (SNPs) at the 17q21 asthma gene locus were genotyped. Selection of SNPs for the candidate approach was based on previous reports from large consortium-based studies.(E5, E6) For the current analyses, we used the SNP *rs7216389* in the GSDMB gene, which was coded for its risk allele (T). This SNP within the 17q21 locus has been associated with childhood asthma(E5, E7-E10) and has been used in previous analyses of the PASTURE cohort.(E11, E12) The given

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SNP was in high linkage-disequilibrium with the other assessed 17q21-SNPs from the candidate approach (*rs8076131* in the ORMDL3 gene and *rs2290400* in the GSDMB gene).

Fecal sampling

Fecal samples were collected from 720 children at age 1 year, of these, 618 children also had fecal samples collected at age 2 months. In all fecal samples DNA was extracted to determine bacterial communities defined by 16S rRNA gene analyses and preprocessed as recently described.(E13) Briefly, the V4 region of the 16S rRNA was amplified and sequenced by the University of California Davis Genome Center on an Illumina MiSeq instrument. Raw sequencing data from each run were demultiplexed using Sabre, quality-filtered by QIIME2-2018,(E14) and denoised using DADA2.(E15) Taxonomy was assigned based on the 99% GreenGenes database.(E16) Furthermore, fecal samples were processed as previously described to measure metabolite levels of short-chain fatty acids (SCFA) in 209 children of the PASTURE study at the age of 1 year.(E17)

Microbial variables included in statistical analyses comprised aggregated variables derived from the alpha-diversity (richness, i.e. the number of species, and the Shannon index) and principal coordinate analysis (PCoA), which was done on the unweighted Unifrac distance, calculated on the level of amplicon sequence variants in the rarefied sample using GuniFrac.(E18) The top 3 principal axes from PCoA were standardized and included in analyses. Furthermore, two butyrate variables were created. Firstly, a butyrate score was created by modeling SCFA-levels available in 209 children at age 1 year on the relative abundances of all bacterial genera with random forest models using

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R-package ranger.(E19) These prediction models were then applied to the entire study population, thus predicting SCFA production for all children. Secondly, by qPCR the relative abundance of the gene encoding the main enzyme in the bacterial butyrate metabolism, i.e. butyryl-CoA:acetate CoA-transferase, was determined in a subsample of 138 children. This gene assay targets the main pathway of the bacterial butyrate metabolism, i.e. conversion of butyryl-CoA to butyrate.(E20) In addition, estimated microbiome age was defined.(E13) For this, the exact age of fecal sampling was modeled by a random forest of the composition of bacterial genera at age 2 months and 1 year in 133 healthy individuals with fecal samples available at both time points. Model building was restricted to healthy individuals, i.e. without diarrhea between age 2 months and 1 year and never affected by wheeze or asthma, since the aim was to estimate the biological age of the healthy microbiome. This prediction model was then applied to the entire population at 1 year.

Statistical analyses

For all analyses on the 17q21-SNP *rs7216389* we combined the heterozygous (CT) and homozygous (TT) as “risk allele carriers”. For the analysis of dichotomous or categorical variables we used the χ^2 test.

Since levels of cytokines after LPS-stimulation were below detection level in a large proportion of children, all cytokine measurements were dichotomized at detection level (Table E1). For the grouping of children according to cytokine profiles we then conducted a latent class analysis (LCA) on the dichotomized measurements of cytokines with a detectability rate <99%, with individuals assigned to the class for which they had the highest posterior probability of belonging. The Bayesian Information

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Criterion (BIC) was used for determining the optimal number of classes. As a sensitivity analysis, we conducted the same latent class analysis including all cytokines irrespective of the detectability rate <99%.

In order to assess the association of the 17q21-SNP, which we used as indicator for an increased asthma risk, with the outcome of wheeze in the first year of life, general estimation equations (GEE) with an autoregressive correlation structure for within-group correlation, a binomial distribution, a log link function and model-based standard errors were conducted, stratified for the latent classes of cytokine detectability. Furthermore, multinomial logistic regression models with the latent cytokine class after LPS-stimulation (low, intermediate, high) as a categorical 3-stage outcome were performed in risk allele carriers to assess the association of cytokine class with various environmental exposures. The low cytokine class was used as reference category. All independent factors with a p-value ≤0.1 were then included in a stepwise variable selection procedure. Similarly, specific variables calculated from the 16S rRNA of the fecal microbiome and the metabolite levels of short-chain fatty acids were included in multinomial logistic regression models with the latent cytokine class as categorical outcome. To test for associations of single taxa with the latent cytokine class we first tested for differences in relative abundance by Wilcoxon test, main associations ($p<0.1$) were then confirmed in multinomial logistic regression models with center-log-ratio-transformed variables.

In addition, we conducted a mediation analysis to assess whether the effect of the relevant environmental exposures on high cytokine class were mediated by the fecal microbiome in the 17q21 risk allele population. All above models were adjusted for

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study center. Due to repeated assessment of wheeze based on weekly diaries from age 8 to 53 weeks, all GEE models were additionally adjusted for age in weeks. The final stepwise and mediation models were adjusted for farming and study center. Effect estimates are presented as adjusted odds ratios (OR), a p-value of 0.05 was considered significant. In the mediation analysis, all effects are given as farm- and center-adjusted regression parameters (ß). Furthermore, the percent of the total effect that is mediated is calculated based on regression parameters. Due to the exploratory character of the analyses, corrections for multiple testing were not performed. Statistical analyses were performed with SAS 9.4 (The SAS Institute, Cary, NC, USA), Mplus 8.1 (Muthén & Muthén, Los Angeles, CA, USA) and R 3.41 (www.r-project.org).

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SUPPLEMENTARY FIGURE LEGENDS

Figure E1. Study population and sample sizes.

Figure E2. Association of the 1st axis from principle coordinate analysis (PCoA) with bacterial genera.

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Table E1. Description of LPS-cytokines at age 1 year, N=445.

	Detection	Detectable		Distribution (pg/ml)		
Cytokine	**Level (pg/ml)**	**N**	**%**	**1st Quartile**	**Median**	**3rd Quartile**
IL-1β	6.50	443	99.6	673.06	1082.08	1709.20
IL-4	4.34	54	12.1	<DL	<DL	<DL
IL-5	3.14	14	3.2	<DL	<DL	<DL
IL-6	4.89	442	99.3	10921.80	16538.50	23393.10
IL-10	5.71	445	100.0	239.06	387.92	551.88
IL-12p70	13.04	207	46.5	<DL	<DL	1.94
IL-13	4.45	152	34.2	<DL	<DL	0.81
IL-17A	20.98	136	30.6	<DL	<DL	0.46
IFN-γ	15.19	358	80.5	3.22	9.41	26.66
TNF-α	10.17	445	100.0	381.80	688.30	1205.10

DL = detection level

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Table E2. Population characteristics.

		Total PASTURE N=1133		LPS-stimulated cytokine data available at age 1yr * N=445	
		n/N	%	n/N	%
Farm child		530/1133	46.8	219/445	49.2
Female gender		530/1090	48.6	222/444	50.0
Maternal atopy		280/1130	24.8	123/443	27.8
Paternal atopy		225/1078	20.9	93/436	21.3
≥2 older siblings		379/1133	33.4	161/445	36.2
Smoking in pregnancy		155/1126	13.8	57/445	12.8
Maternal education	Low	202/1133	17.8	72/445	16.2
	Medium	487/1133	43.0	204/445	45.8
	High	444/1133	39.2	169/445	38.0
Any farm milk consumption (1st year of life)		311/1031	30.2	139/431	32.2
Any wheezing episode in 1st year of life		359/1031	34.8	141/431	32.7
Diagnosis asthma ever at 6yrs		78/928	8.4	38/395	9.6

* No significant differences compared to children with no cytokine data.

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Table E3. Detectable LPS-stimulated cytokines at age 1 year: allocation to latent classes in 17q21 risk allele carriers and non-carriers (**A.** Cytokines included in latent class analysis with detectability <99%; **B.** cytokines excluded from latent class analysis with detectability ≥99%).

				Latent cytokine classes							
		Total		LC1 Low class		LC2 Intermediate class		LC3 High class		p-value	
SNP	**Cytokine**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**Chi2**	**trend**
CT/TT		N=257		N=140 (54.5%)		N=63 (24.5%)		N=54 (21.0%)			
A.	IL-4	28	10.9	1	0.7	4	6.3	23	42.6	<0.001	<0.001
	IL-5	7	2.7	0	0.0	1	1.6	6	11.1	<0.001	<0.001
	IL-12p70	113	44.0	2	1.4	60	95.2	51	94.4	<0.001	<0.001
	IL-13	83	32.3	13	9.3	16	25.4	54	100.0	<0.001	<0.001
	IL-17A	78	30.4	9	6.4	18	28.6	51	94.4	<0.001	<0.001
	IFN-γ	210	81.7	94	67.1	62	98.4	54	100.0	<0.001	<0.001
B.	IL-1β	256	99.6	140	100.0	62	98.4	54	100.0	0.213	0.676
	IL-6	255	99.2	140	100.0	62	98.4	53	98.1	0.295	0.140
	IL-10	257	100.0	140	100.0	63	100.0	54	100.0	–	–
	TNF-α	257	100.0	140	100.0	63	100.0	54	100.0	–	–
CC		N=120		N=63 (52.5%)		N=33 (27.5%)		N=24 (20.0%)			
A.	IL-4	18	15.0	0	0.0	3	9.1	15	62.5	<0.001	<0.001
	IL-5	5	4.2	0	0.0	2	6.1	3	12.5	<0.001	<0.001
	IL-12p70	55	45.8	1	1.6	30	90.9	24	100.0	<0.001	<0.001
	IL-13	41	34.2	8	12.7	9	27.3	24	100.0	<0.001	<0.001
	IL-17A	37	30.8	5	7.9	9	27.3	23	95.8	<0.001	<0.001
	IFN-γ	90	75.0	33	52.4	33	100.0	24	100.0	<0.001	<0.001
B.	IL-1β	119	99.2	63	100.0	33	100.0	23	95.8	0.133	0.092
	IL-6	119	99.2	63	100.0	33	100.0	23	95.8	0.133	0.092
	IL-10	120	100.0	63	100.0	33	100.0	24	100.0	–	–
	TNF-α	120	100.0	63	100.0	33	100.0	24	100.0	–	–

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Table E4. Factors associated with cytokine classes in total cytokine population.*

Exposure variable		N	Cytokine class	OR	95%-CI	p-value
Characteristics at birth						
Farm child		219/445	Intermediate vs. low	1.35	0.86-2.12	0.196
			High vs. low	1.80	1.07-3.05	**0.027**
Maternal farm exposures in pregnancy						
Regular stay in stables		228/423	Intermediate vs. low	1.50	0.95-2.39	0.084
			High vs. low	2.63	1.50-4.62	**<0.001**
Regular stay in barn		172/421	Intermediate vs. low	1.58	0.98-2.55	0.060
			High vs. low	2.20	1.27-3.80	**0.005**
# farm animal species:	0 (ref.)	180/443	Intermediate vs. low	1.00	–	–
			High vs. low	1.00	–	–
	1	145/443	Intermediate vs. low	1.17	0.69-1.99	0.565
			High vs. low	1.21	0.63-2.30	0.567
	≥2	118/443	Intermediate vs. low	2.01	1.09-3.71	**0.025**
			High vs. low	1.95	1.01-3.75	**0.045**
Any farm milk consumption		193/445	Intermediate vs. low	1.29	0.81-2.04	0.277
			High vs. low	2.02	1.19-3.41	**0.009**
Farm exposures 1. year of life						
Regular stay in stables		105/431	Intermediate vs. low	2.07	1.19-3.58	**0.010**
			High vs. low	1.65	0.89-3.03	0.109
Regular stay in barn		93/431	Intermediate vs. low	1.14	0.62-2.12	0.673
			High vs. low	1.75	0.93-3.30	0.083
Contact with hay		48/435	Intermediate vs. low	1.54	0.70-3.41	0.284
			High vs. low	2.21	1.03-4.74	**0.042**
Any farm milk consumption		139/431	Intermediate vs. low	1.98	1.20-3.25	**0.007**
			High vs. low	2.84	1.62-5.00	**<0.001**
Analysis in milk drinkers only:						
Milk consumption:	Any UHT milk (ref.)	48/280	Intermediate vs. low	1.00	–	–
			High vs. low	1.00	–	–
	Any pasteurized milk	93/280	Intermediate vs. low	0.91	0.39-2.13	0.834
			High vs. low	1.75	0.53-5.79	0.356
	Any farm milk	139/280	Intermediate vs. low	1.74	0.80-3.77	0.162
			High vs. low	3.70	1.30-10.55	**0.014**
Other exposures 1. year of life						
Food diversity score:	0-8 items (ref.)	100/431	Intermediate vs. low	1.00	–	–
			High vs. low	1.00	–	–
	9-11 items	156/431	Intermediate vs. low	1.16	0.63-2.14	0.633
			High vs. low	1.35	0.65-2.81	0.417
	12-15 items	175/431	Intermediate vs. low	1.43	0.79-2.61	0.240
			High vs. low	2.01	1.00-4.07	0.051

* Results from multinomial logistic regression models with cytokine class as 3-categorical outcome and low cytokine class as reference category; results are adjusted for center;

Statistically significant p-values are given in **bold**;

OR = odds ratio; 95%-CI = 95% confidence interval.

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Table E5. Effect of fecal microbiome at age 1 year on cytokine classes in in total cytokine population (N=358).*

Fecal microbiome (z-scores)		Cytokine class	OR	95%-CI	p-value
Principal coordinate analysis:	1st axis	Intermediate vs. low	0.96	0.72-1.27	0.775
		High vs. low	1.38	1.00-1.90	0.052
	2nd axis	Intermediate vs. low	0.83	0.62-1.11	0.200
		High vs. low	0.87	0.63-1.22	0.423
	3rd axis	Intermediate vs. low	1.08	0.83-1.40	0.576
		High vs. low	1.09	0.81-1.47	0.572
Shannon diversity index		Intermediate vs. low	1.04	0.78-1.38	0.795
		High vs. low	1.18	0.86-1.62	0.316
Species richness		Intermediate vs. low	0.95	0.70-1.27	0.706
		High vs. low	1.13	0.81-1.56	0.474
Estimated microbiome age		Intermediate vs. low	1.13	0.83-1.54	0.436
		High vs. low	1.19	0.84-1.68	0.324
Butyrate score		Intermediate vs. low	0.87	0.67-1.13	0.295
		High vs. low	1.20	0.92-1.57	0.184

* Results from multinomial logistic regression models with cytokine class as 3-categorical outcome and low cytokine class as reference category; results are adjusted for center;

Statistically significant p-values are given in **bold**;

OR = odds ratio; 95%-CI = 95% confidence interval.

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Table E6. Effect of fecal microbiome at age 1 year on cytokine classes in homozygous 17q21 risk allele carriers (N=61).*

Fecal microbiome (z-scores)		Cytokine class	OR	95%-CI	p-value
Principal coordinate analysis:	1st axis	Intermediate vs. low	1.31	0.64-2.71	0.463
		High vs. low	4.83	1.58-14.75	**0.006**
	2nd axis	Intermediate vs. low	1.18	0.57-2.44	0.655
		High vs. low	0.92	0.40-2.10	0.841
	3rd axis	Intermediate vs. low	0.96	0.49-1.89	0.908
		High vs. low	0.92	0.43-1.97	0.828
Shannon diversity index		Intermediate vs. low	0.80	0.34-1.88	0.611
		High vs. low	3.17	1.31-7.64	**0.010**
Species richness		Intermediate vs. low	0.78	0.31-1.99	0.608
		High vs. low	2.97	1.20-7.33	**0.018**
Estimated microbiome age		Intermediate vs. low	0.88	0.46-1.66	0.687
		High vs. low	1.81	0.71-4.61	0.214
Butyrate score		Intermediate vs. low	0.59	0.27-1.31	0.195
		High vs. low	3.08	1.33-7.14	**0.009**

* Results from multinomial logistic regression models with cytokine class as 3-categorical outcome and low cytokine class as reference category; results are adjusted for center;

Statistically significant p-values are given in **bold**;

OR = odds ratio; 95%-CI = 95% confidence interval.

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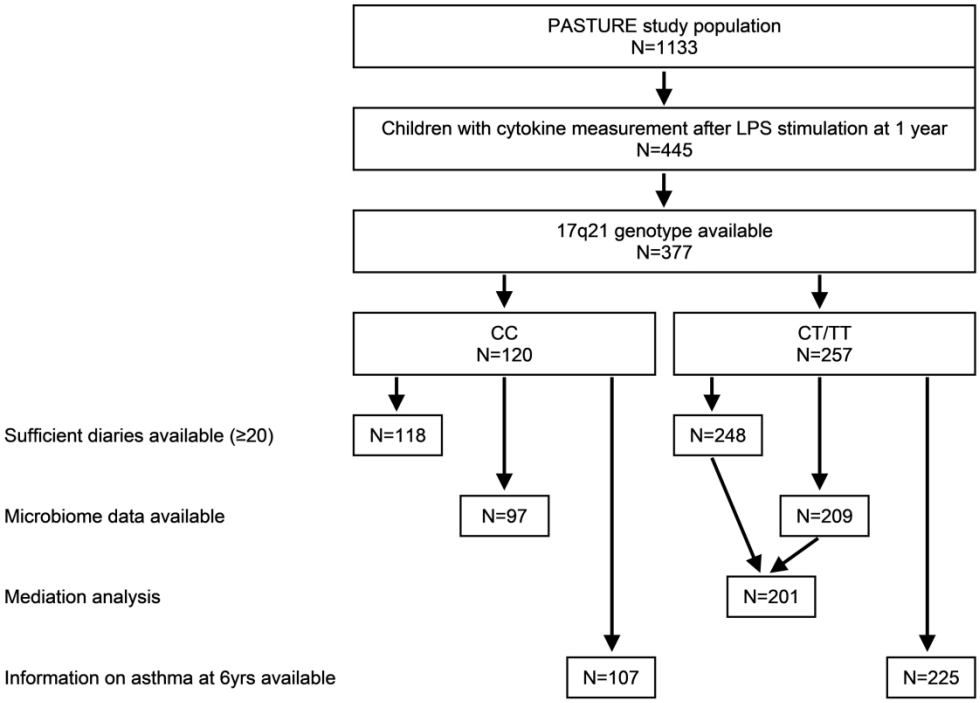

Figure E1. Study population and sample sizes.

260x186mm (300 x 300 DPI)

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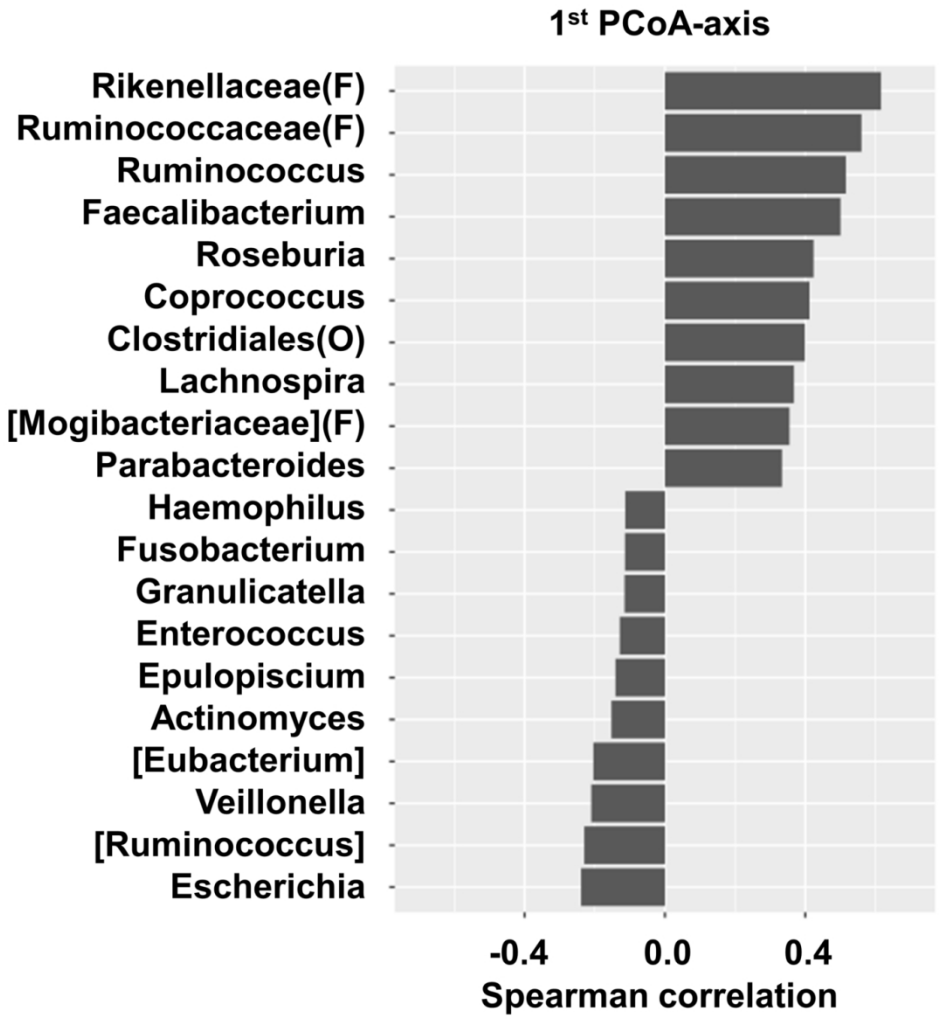

Figure E2. Association of the 1st axis from principle coordinate analysis (PCoA) with bacterial genera.

124x129mm (300 x 300 DPI)

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