

Letter to the Editor

Endogenous metabolites and inflammasome activity in early childhood and links to respiratory diseases

To the Editor:

The NLR family, pyrin domain containing 3 (NLRP3) inflammasome is a pivotal host platform for sensing of endogenous and exogenous danger signals and for the subsequent orchestration of inflammatory responses. Beside pathogenic agents, endogenous dietary metabolites such as fatty acids can trigger the assembly of NLRP3 inflammasomes, cytosolic protein complexes that activate caspase-1 (CASP1), leading to the cleavage of pro-IL-1 β and pro-IL-18.¹ NLRP3 and the expression of pro forms of the IL-1 β cytokine family are transcriptionally induced by Toll like receptor (TLR) activation. Although the link between glucose or fatty acids and the activation of NLRP3 inflammasome is well established in mice,² comparable data for humans were reported only for adipose tissue.³ Studies for children are completely missing so far. Therefore, in the present study, our aim was to investigate whether the metabolic state is associated with the immature immune system already in early childhood. By performing a broad metabolic profiling, we explored the involvement of endogenous metabolites in the expression of TLR and NLRP3 inflammasome components in children at birth and at age 1 year using samples from the prospective birth cohort LINA (Lifestyle and environmental factors and their Influence on Newborns Allergy risk).⁴

In the LINA study, 629 mother-child pairs (622 mothers and 7 twin pairs) were recruited from March 2006 until December 2008 in Leipzig, Germany.⁴ Annually, around the birthday of the child, follow-up investigations were performed with questionnaire evaluations for respiratory (wheezing, airway infections, bronchitis, asthma) and allergic (atopic dermatitis, hay fever, allergic sensitization) outcomes and clinical visits including blood collection. In children's blood samples at birth (cord blood, $n = 471$) and at age 1 year ($n = 513$), mRNA expression for NLRP3, CASP1, MyD88, TLR1, TLR2, TLR5, TLR6, TLR7, TLR9, and TLR10 as well as the concentration of the effector cytokines IL-1 β (protein and mRNA), IL-18 (mRNA), and specific IgE was measured. Metabolomic analysis was performed in sera of 495 newborns (cord blood) and 449 1-year-old children by using the AbsoluteIDQ p180 Kit (Biocrates Life Science AG, Innsbruck, Austria). Method details, characteristics of the study population, median values, and interquartile ranges for all metabolites and immune parameters are given in Tables E2-E4 in this article's Online Repository at www.jacionline.org.

At birth as well as in the first year of life, high hexose levels correlated with increased expression of TLRs and CASP1 and in part with NLRP3. Contrarily, a reduced expression of TLRs and inflammasome components was measured in the presence of high concentrations of amino acids and lysophosphatidylcholines (lysoPCs). To control for multitesting, we applied a Bonferroni correction (significance level $P < 4.2 \times 10^{-4}$) and could show that the observed associations between metabolites and immune/inflammasome parameters remained significant (data are presented as heat maps of Spearman correlation

coefficients) (Fig 1). Furthermore, by calculating mean ratios adjusted for the possible confounding factors season of birth, mode of delivery, cat keeping, parental history of atopy, sex, and parental education, the entirely detected relationships remained significant (see Figs E2 and E3 in this article's Online Repository at www.jacionline.org). Although the concentration of metabolites and inflammatory parameters at birth did not correlate with concentrations in the first year of life (see Tables E3 and E4), the relationship between metabolites and inflammatory parameters at each time point was nearly identical (Fig 1). These findings indicate that there is a valid relationship between sugars, lysoPCs, amino acids, and TLRs/inflammasome components that is dynamically modulated by the metabolic state.

It is obvious from *in vitro* and *in vivo* animal studies that high glucose levels provide a priming signal for IL-1 β by activation of thioredoxin-interactin protein, which subsequently binds to NLRP3, inducing IL-1 β secretion in islet cells.² In addition, high concentrations of glucose induce the generation of reactive oxygen species, which is a sufficient second signal for promoting inflammasome activation.² Our epidemiological data show that at least in cord blood, the NLRP3 expression is associated with high hexoses levels. Furthermore, the expression of the NLRP3 downstream target CASP1 was strongly correlated with high hexose levels at birth as well as in the first year. We validated these findings by performing *in vitro* assays using PBMCs from healthy adult donors and demonstrated that glucose induces a higher expression of NLRP3, CASP1, as well as IL1B (see Fig E7 in this article's Online Repository at www.jacionline.org). *In vitro* experiments with human monocytes already demonstrated that high glucose induces TLR mRNA expression,⁵ which is in line with our results showing that elevated blood concentration of hexoses is related to increased mRNA expression of TLRs and the adaptor molecule MyD88 at both analyzed time points. Our *in vitro* data again support this result by showing a glucose-induced activation of MYD88 (Fig E7). Earlier experimental studies show that the *Nlrp3* promoter is activated by TLR/MyD88-mediated signaling.⁶ Our data support these results; we found that increased expression of TLRs/MyD88 correlated with increased expression of NLRP3 and CASP1 (see Figs E4 and E5 in this article's Online Repository at www.jacionline.org). We could furthermore show that both NLRP3 and CASP1 were related to the expression of the effector cytokines IL-1 β (mRNA and protein) and IL-18 (mRNA) (see Fig E6 in this article's Online Repository at www.jacionline.org). Thus, our results may provide the first evidence that the association between hyperglycemia, inflammasome activation, and IL-1 β production reported so far only in animal models² is also present in humans.

As further metabolites linked to inflammasome activity we have identified amino acids and lysoPCs. Our results may point to an anti-inflammatory effect of both groups showing a strong negative association with TLR and inflammasome component expression. These data are in line with previous reports showing that lysoPCs may act as attenuators of sepsis and as immune suppressors^{7,8} whereas dietary supplementation with certain amino acids (eg, valine, histidine, and arginine) was reported to alleviate tissue and systemic inflammation.^{9,10} Our

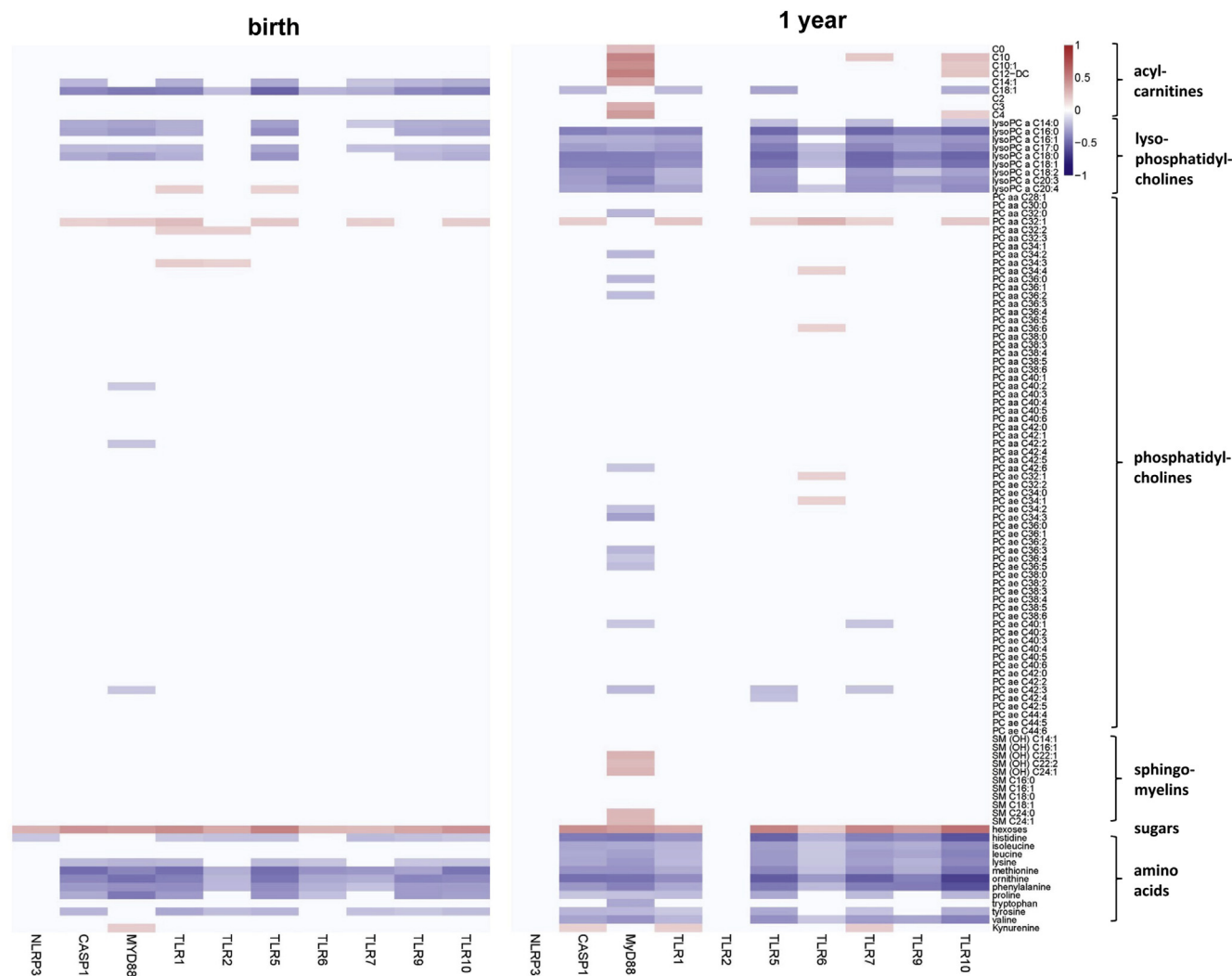


FIG 1. Heat maps present the correlation between blood metabolite concentrations and expression of TLRs/MyD88 and inflammasome components (NLRP3/CASP1) at birth (cord blood) and in the first year of life (1 year). The color of each cell in the heat map represents the correlation coefficient (R) from Spearman correlation. Only significant values after Bonferroni correction ($P < 4.2 \times 10^{-4}$) are presented.

TABLE I. Relationship between IL-1 β protein and IL-1 β mRNA concentrations at birth (cord blood), 1-year blood, and the development of childhood diseases in the first (1 y) and second year (2 y) of life, respectively*

	IL-1 β protein blood concentrations		IL-1 β mRNA blood expression	
	Birth	1 y	Birth	1 y
	aOR (95% CI) [†]			
Outcome at age	1 y	2 y	1 y	2 y
Atopic dermatitis	0.97 (0.74-1.28)	1.26 (0.97-1.63)	0.98 (0.74-1.31)	0.81 (0.58-1.15)
Sensitization:				
to food allergens [‡]	1.13 (0.88-1.46)	1.04 (0.80-1.35)	0.99 (0.76-1.28)	0.86 (0.62-1.19)
to inhalant allergens [‡]	1.33 (0.75-2.38)	0.92 (0.63-1.34)	1.02 (0.56-1.87)	0.84 (0.52-1.36)
Bronchitis	1.25 (1.01-1.54) [§]	1.23 (1.05-1.44) [§]	0.91 (0.73-1.13)	1.23 (1.00-1.50) [§]
Wheezing	1.15 (0.93-1.42)	1.11 (0.93-1.32)	1.26 (1.00-1.54) [§]	1.27 (1.01-1.59) [§]
Airway infections	1.62 (0.88-2.99)	1.09 (0.90-1.32)	1.02 (0.83-1.24)	1.07 (0.84-1.36)

aOR, Adjusted odds ratio.

*Analysis was performed using a logistic regression model with IL-1 β values categorized in quartiles.

[†]aOR for parental educational level, month of birth, mode of delivery, cat keeping during pregnancy, siblings, sex, and smoking during pregnancy. Atopic dermatitis and allergic sensitization were additionally adjusted for parental history of atopy.

[‡]According to the Phadia CAP System, concentrations of more than 0.35 kU/L were regarded as positive.

[§]Significant numbers are in boldface ($P < .05$).

in vitro data sustain these findings at least for amino acid (valine) treatment where we observed a diminished expression of CASP1, IL1B, and MyD88 but not of NLRP3 and the analyzed TLRs.

On the basis of our data and findings from other groups, it is reasonable that an imbalance between endogenous metabolites (eg, elevated glucose levels and reduced amino acid levels) may lead to an increased inflammatory status. Indeed, in our study, molecules capable of sensing metabolites (TLRs and NLRP3/CASP1) were associated with increased concentrations of the effector cytokines IL-1 β and IL-18. The role of these cytokines for the development of childhood diseases is poorly investigated. Here, we found that cord blood IL-1 β (Table I) but not IL-18 (Table E5) concentrations were predictive for the development of bronchitis and wheezing by age 2 years. Thus, the balance between endogenous metabolites seems to be important for the regulation of an inflammatory immune response independently of invading pathogens. Because we could show that the inflammasome effector cytokine IL-1 β was linked to a higher risk of respiratory diseases during the first 2 years of life, our results may give evidence that the metabolome is of importance for respiratory diseases in early childhood.

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Online Repository

Supporting information for

Endogenous metabolites and inflammasome activity in early childhood and links to respiratory diseases

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METHODS

Study design

Six-hundred and twenty-nine mother-child pairs (622 mothers, 7 twin 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, for details see ^{E1, E2}. The LINA study was designed to include only healthy mothers. Mothers suffering from immune or infectious diseases during pregnancy were excluded from the study. Annually, around the birthday of the child, follow-up investigations were performed with questionnaire evaluations and clinical visits including blood collection. Participation was voluntary and informed consent was given by the parents. This study was approved by the Ethics Committee of the University of Leipzig, Germany (file reference 046-2006, 160-2008).

Disease outcomes

Information on respiratory and allergic outcomes was collected using questionnaires self-administered by the parents. Wheezing was recorded as parental report of wheezing symptoms ever within the last 12 months. Airway infections, bronchitis, asthma and atopic dermatitis were recorded as physician diagnoses ('Has a doctor diagnosed your child with atopic dermatitis/asthma/bronchitis/airway infections in the last 12 months?'). Airway infections included common cold, snuffle, sore throat and were classified into no, rare (one to seven/year) and frequent (more than seven/year). For each disease outcome a control group was defined including children having never developed symptoms or being diagnosed for this disease until the age of two. Allergic sensitisation was assessed by measuring specific IgE levels for inhalant (sx1) and food allergens (fx5).

IgE measurement

The concentrations of specific IgE against food (fx5) and inhalant allergens (sx1) in sera of one- and two-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 dander. Samples with a specific IgE concentration >0.35 kU/l were regarded as positive.

Cytometric bead array

Concentration of IL-1 β was measured in plasma samples by flow cytometry using a cytometric bead array (BD CBA Human Soluble Flex Set system, Becton Dickinson, Heidelberg, Germany) and a protocol described before^{E2}. In brief, cytokine specific antibody coated beads were incubated for 1 hour with 25 μ l of diluted plasma samples or standard solution. Thereafter, samples were incubated with the corresponding PE labelled detection

antibodies for 2 hours. After one washing step samples were measured by flow cytometry. Analysis of data and quantification of cytokines was performed using the FCAP ArrayTM software (Becton Dickinson, Heidelberg, Germany) on the basis of corresponding standard curves. Finally, plasma dilution factor was accounted. Detection limit were 2.3 pg/ml and cytokine concentrations below the limit of detection were assigned to a value of one half of the detection limit.

Targeted metabolome profiling

Metabolomic analysis was performed in sera of 495 newborns (cord blood) and 449 one year old children. Measurement of samples (10 µl) was performed using the AbsoluteIDQ[®] p180 Kit (Biocrates Life Science AG, Innsbruck, Austria) as described in ^{E3}. This allowed the simultaneous quantification of 186 metabolites using liquid chromatography and flow injection analysis mass spectrometry. The measurement was carried out on an ABI Sciex API5500Q-TRAP mass spectrometer equipped with an Acquity UPLC system (Waters GmbH, Eschborn, Germany). An electrospray ionisation (ESI) source was used in both positive and negative modes. MS/MS analysis was run in Multiple Reaction Monitoring (MRM) mode. The quantification of metabolites was achieved by reference to appropriate internal standards. After flow injection mass spectrometry measurement, raw data processing, peak integration, isotope correction, calibration and quality control assessment were performed by the MetIQTM software package which is an integral part of the Absolute IDQTM kit.

To determine the stability of results we analysed 3 technical replicates of an in house pool serum (PS) as quality control in seven independent experiments. PS was generated from 5 healthy donors, mixed in a ratio 1:1, aliquoted and stored at -80°C. Metabolites were classified as stable if all measurements of PS were inside a range of +/- 50 % of the median. In total, 108 metabolites passed this quality control. Out of these, the metabolites covered a

biologically relevant panel including the group of sugars (which is the sum of hexoses, n=1), amino acids (n=11), acylcarnitines (n=9), lysophosphatidylcholines (n=9), sphingomyelins (n=11), phosphatidylcholines (n=66), biogenic amines (n=1). Concentrations are given in absolute μM concentrations.

RNA Extraction, cDNA Synthesis and qPCR for whole blood

Total RNA was prepared from whole blood samples by using peqGold RNA Pure (peqlab, Erlangen, Germany) according to manufacturer's instruction. The cDNA synthesis was carried out with 5 μg of RNA by using ImProm-IITM Reverse Transcription System (Promega, Mannheim, Germany).

Gene expression was measured using the 96.96 Dynamic Array (Fluidigm, San Francisco, CA, USA). Intron-spanning primers for NLRP3, CASP1, IL-1B, IL-18, TLR1, TLR2, TLR5, TLR6, TLR7, TLR9, TLR10 and the TLR adaptor molecule MYD88 were designed and UPL probes were selected by the Universal Probe Library Assay Design Center (<http://qpcr.probefinder.com/organism.jsp>, Table EI). A preamplification reaction was performed by pooling all primers (final concentration, 50 nM), 5 μl of cDNA and 2x PreAmp Master Mix (Applied Biosystems/Life Technologies GmbH, Darmstadt, Germany). The cycling program consisted of 95°C for 10 min, followed by 14 cycles of 95°C for 15 sec and 60°C for 4 min on a LightCycler 480 (Roche Applied Science, Mannheim, Germany). The qPCRs of 1:5 diluted with TE buffer preamplified templates were performed following manufacture's instruction for UPL (Roche Applied Science, Mannheim, Germany) assays. Briefly, for each individual assay, a 10X Assay Mix that contained 2 μM of each forward and reverse primer, 1 μM UPL probe and 0.025% Tween-20 was prepared, and 5 μl of the mix was loaded into the assay inlets of the array. Into the sample inlets, 5 μl of the following solution was dispensed: 2.5 μl of PreAmp sample in 1.1X of FastStart Universal Probe Master Mix (Roche Applied Science, Mannheim, Germany). The cycling program consisted of 2 min

at 50°C, 10 min at 95°C, followed by 35 cycles of 95°C for 15 sec, 70°C for 5 sec, and 1 min at 60°C. All reactions were performed in triplicates. Gene expression values were determined by using the 2- $\Delta\Delta$ CT method with GAPDH and GUSB, as reference genes normalized to the lowest measured value.

[Table E1]

Statistical analysis

Statistical analyses were performed using Statistica for Windows Version 10.0 (StatSoft Inc. (Europe), Hamburg, Germany) and R version 3.1.0 (2014_04_10) "Spring Dance"/package pheatmap.

The chi-square test was used to test the relationship between the analysed cohort at birth and at one year (1 yr). All *P* values < 0.05 were considered to be significant.

Spearman rank test was used to calculate whether concentrations of measured parameters at birth (cord blood) correlated with concentrations at the age of one. A Bonferroni-correction was performed giving a significance level lower than $0.05/216 = 0.0002$ for metabolites and $0.05/26 = 0.002$ for immune parameters. The relationship between metabolite concentrations in sera and inflammasome components (NLRP3, CASP1) as well as TLR expression in blood cells was calculated by Spearman rank test for birth and 1 yr data. Heat maps were generated with the correlation coefficients (*R*) after Bonferroni-correction for multiple testing. Bonferroni-corrected significance level was lower than $0.05/118 = 4.2 \times 10^{-4}$. Scatterplots represent the association between TLRs/MyD88 and NLRP3 expression in the corresponding period of life.

To further substantiate the association between metabolites, inflammasome components and TLR expression, we used a linear regression model adjusted for covariates known to influence an inflammatory immune response (season of birth, mode of delivery, cat keeping during

pregnancy and parental education). Because measured metabolite concentrations were not normally distributed a logarithmic transformation was performed. Data are presented as mean ratios (MR) which are the back-transformed effects from the regression model of the logarithmically transformed outcome. Mean ratios were also calculated for the association between inflammasome components (NLRP3, CASP1) and the level of effector cytokines IL-1 β (protein and mRNA) as well as IL-18 (mRNA). Cytokine concentrations were not normally distributed and did not reach normal distribution after log-transformation; therefore a categorisation into quartiles was performed.

For the relation between the effector cytokines IL-1 β (protein, mRNA), IL-18 (mRNA) and disease outcomes we calculated odds ratios adjusted for season of birth, mode of delivery, cat keeping during pregnancy, parental education, siblings, gender and smoking during pregnancy. For the association between cytokines and allergic outcomes the parental history of atopy was added as an additional covariate. Cord blood cytokines were used to predict outcomes in the first year of life (1 yr) whereas cytokine concentrations from 1 yr samples were used to predict disease outcomes in the second year of life (2 yr).

In-Vitro Model

Preparation of glucose-, amino acid and lysophosphatidylcholine solutions

A 20% glucose stock solution (1100 mM, life technologies, gibco, Carlsbad, CA, USA) was prepared with MilliQ-H₂O and sterile filtered. Valine solutions (Genaxxon BioScience GmbH, Ulm, Germany) were freshly prepared before every experiment and dissolved in cell culture medium to produce a 300 mM stock solution. A Lysophosphatidylcholine (LPC) mix was purchased from Sigma-Aldrich (L4129, St. Louis, MO, USA) and dissolved in chloroform, required amounts were vacuum-dried and re-suspended in sterile PBS (pH 7.4) to

prepare a 1mM stock solution that was stored at 4°C. All stock solutions were used for cell culture experiments (see below).

Human PBMC isolation and cell culture

Peripheral blood mononuclear cells (PBMCs) were purified from buffy coats of healthy human donors (Institute of Transfusion Medicine, University of Leipzig, Germany) via Ficoll density gradient centrifugation (Ficoll Paque PLUS, GE Healthcare Europe GmbH, Freiburg, Germany). PBMCs were cultured in RPMI1640 medium without glucose (life technologies, gibco, Carlsbad, CA, USA) and 10% dialyzed fetal bovine serum (PAN Biotech, Aidenbach, Germany). Glucose (5.5 mM and 15 mM) was added at indicated concentrations. Cells were additionally stimulated with 100 ng/mL lipopolysaccharid (LPS, *E. coli* 026:B6, Sigma-Aldrich, Germany). After six hours of incubation cells were harvested for RNA isolation.

For LPC and Valine experiments PBMCs were cultured in RPMI1640 (Biochrom, Germany) and 10% fetal bovine serum (Biochrom, Germany) for 24 hours. After addition of 25 µM LPC or 5 mM Valine cells were cultured for additional 24 hours. 100 ng/mL LPS were added for the last four hours of incubation. Finally PBMCs were harvested for RNA isolation.

RNA isolation and real-time PCR

RNA from PBMCs was isolated using peqGOLD TriFast (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. 200 ng of RNA were transcribed into cDNA with ImProm-II-Reverse Transcriptase (Promega, Mannheim, Germany). Real-time PCR was performed with EvaGreen (Biotium, Hayward, CA, USA) and BIOTAQ DNA Polymerase (Bioline, Berlin, Germany) on the LightCycler 480 System (Roche Diagnostics GmbH, Mannheim, Germany). After denaturation for 5 min at 95°C PCR was performed for 45 cycles, each consisting of denaturation for 15 sec at 95°C, annealing for 30 sec at 60°C and elongation for 30 sec at 72°C. Sequences of the used primer pairs are listed in Table EI.

Relative gene expression was calculated with the $2^{-\Delta\Delta CT}$ method and GAPDH as a reference gene.

Statistical analysis

The numbers of independent experiments are shown in figure legends respectively. All graphs show mean values with standard error of the mean (SEM). Statistical significance was calculated using the unpaired Student's *t*-test (Statistica for Windows Version 10.0 (StatSoft Inc. (Europe), Hamburg, Germany)).

RESULTS

[Tables E2, E3, E4, E5]

In vitro Results

To validate our main findings (positive association between hexoses and negative between lysoPCs/amino acids and inflammasome components/TLRs) we performed *in vitro* assays using PBMCs from healthy adult donors. We incubated PBMCs in the presence of LPS (to simulate signal 1 for inflammasome activation) with and without glucose, lysoPCs (LPC), or the amino acid valine. Glucose treatment significantly increased the expression of NLRP3, CASP1, MYD88 and IL1B (Fig E7). For TLR9 and TLR10 a trend for increased expression was observed without reaching significant levels. The amino acid valine significantly reduced the expression of CASP1, MYD88 and IL1B whereas the mix of lysoPCs (LPC) did not have any effect on the expression of all markers.

TABLE EI. Primer pairs for real-time PCR analysis.

Gene	UPL probe	Forward primer	Reverse primer
CASP1	52	gcgaagcatactttcagtttca	tctccttcaggaccttgctg
GAPD	60	gctctctgctcctcctgttc	acgaccaaaccgttgactc
GUSB	57	cgccctgcctatctgtattc	tccccacaggagtggtgtag
IL18	46	gcttcctctcgcaaaaact	tgatgcaattgtcttactggtt
IL1B	78	tacctgtcctgcgtgttgaa	tcttgggtaattttgggatct
MYD88	33	tgctcgagctgcttaccaa	cctgctgctgcttcaagata
NLRP3	27	gtgtcctccaagctcctct	aagtgaggtggctgttcacc
TLR1	79	cctagcagttatcacaagctcaaa	tctttccttgggccattc
TLR2	56	ctctcgggtgcggaatgic	aggatcagcaggaacagagc
TLR5	16	tgacacaatctcggctgact	tgtcaggaacatgaacatcaatc
TLR6	45	tggctttgggtcagtgta	tttatatgccgtagctgatctaatttt
TLR7	81	ttaaccaattgcttccgtgtc	gggtgccacactcaatctg
TLR9	56	tgtgaagcatccttcctgtga	gagagacagcgggtgcag
TLR10	34	cttttgcaccaacctgaa	ccctctctcacatctccttttg

TABLE II. Characteristics of the analyzed LINA cohort at birth (n = 629 newborns) and at the age of one (1 yr, n = 606 children).

Parameters	newborns	1 yr	P value [#]
	n (%), N = 629	n (%), N=606	
Gender of the child			
male	327 (52.0)	311 (51.3)	0.921
female	302 (48.0)	295 (48.7)	
Season of birth			
Summer (april-september)	358 (56.9)	348 (57.4)	0.943
Winter (october-march)	271 (43.1)	258 (42.6)	
Mode of delivery			
spontaneous	478 (78.1)	465 (77.9)	0.973
caesarean section	134 (21.9)	132 (22.1)	
Parental history of atopy[†]			
negative	212 (33.8)	207 (34.2)	0.985
single positive	295 (47.0)	287 (47.4)	
double positive	121 (19.3)	111 (18.3)	
Parental education*			
low	16 (2.5)	14 (2.3)	0.996
intermediate	144 (22.9)	139 (22.9)	
high	469 (74.6)	453 (74.8)	
Cat keeping during pregnancy			
yes	113 (18.0)	109 (18.0)	1.000
no	515 (82.0)	496 (82.0)	
Childhood diseases			
	1 yr	2 yr	
	n (%), N = 606	n (%), N = 546	
Atopic dermatitis			
yes	60 (9.5)	40 (6.4)	0.418
no	546 (90.5)	468 (93.6)	
Specific IgE[†]			
sensitisation to food allergens (fx5) [‡]			
yes	79 (15.4)	41 (12.1)	0.498
no	435 (84.6)	298 (87.9)	
sensitisation to inhalant allergens (sx1) [‡]			
yes	17 (3.4)	18 (5.6)	0.453
no	499 (96.6)	321 (94.4)	
Bronchitis			
yes	138 (22.8)	143 (27.8)	0.485
no	468 (77.2)	371 (72.2)	
Wheezing			
yes	122 (20.1)	102 (18.7)	0.802
no	484 (79.9)	444 (81.3)	
Airway infections			
no	162 (26.7)	92 (16.8)	0.011
rare	431 (71.1)	391 (71.5)	
frequent	13 (2.1)	64 (11.7)	

[#]P value from chi squared test for cross relationship

[†]History of atopy is defined as: occurrence of asthma or atopic dermatitis or hay fever.

*low, 9 yr of schooling or less “Hauptschulabschluss”; intermediate, 10 yrs of schooling “Mittlere Reife”; high, 12 yrs of schooling or more “(Fach-)hochschulreife”

[‡]According to the Phadia CAP System concentrations >0.35 kU/l were regarded as positive.

[†]Due to missing blood samples case numbers are lower.

TABLE EIII. Concentration of and correlation between endogenous metabolites in sera of children at birth (cord blood) and at the age of one from the prospective birth cohort LINA.

	Metabolite concentrations [μM]. Median (IQR)		Spearman correlation between metabolites from birth and 1 yr blood samples	
	birth n=495	1 year n=449	Spearman (R)	p value*
Acylcarnitines				
C0	15.5 (13.2-17.7)	41.2 (35.5-47.5)	0.052	0.333
C10	0.12 (0.11-0.14)	0.36 (0.29-0.5)	-0.157	0.003
C10:1	0.07 (0.06-0.08)	0.17 (0.13-0.23)	-0.130	0.014
C12-DC	0.19 (0.18-0.21)	0.39 (0.28-0.49)	-0.291	< 0.0001
C14:1	0.03 (0.03-0.04)	0.08 (0.06-0.11)	-0.028	0.600
C18:1	0.11 (0.08-0.15)	0.17 (0.14-0.26)	0.017	0.752
C2	3.57 (2.91-4.48)	6.26 (5.14-7.83)	0.020	0.702
C3	0.24 (0.2-0.3)	0.58 (0.47-0.75)	0.070	0.189
C4	0.13 (0.11-0.16)	0.39 (0.31-0.55)	0.073	0.170
Lysophosphatidylcholines				
lysoPC a C14:0	2.22 (2.05-2.45)	2.11 (1.79-2.51)	0.089	0.096
lysoPC a C16:0	63.4 (53.2-76.6)	73.9 (58.5-96.1)	0.125	0.019
lysoPC a C16:1	3.63 (3.02-4.27)	1.66 (1.34-2.13)	0.106	0.047
lysoPC a C17:0	0.74 (0.6-0.94)	1.11 (0.83-1.58)	0.151	0.004
lysoPC a C18:0	14.4 (12-17.7)	24.5 (18.5-31.9)	0.104	0.051
lysoPC a C18:1	11.7 (9.93-13.5)	16.4 (13.4-21.2)	0.057	0.283
lysoPC a C18:2	9.17 (7.36-11)	24.9 (19.7-33.1)	0.042	0.429
lysoPC a C20:3	3.32 (2.74-4.02)	1.61 (1.28-2.06)	0.051	0.338
lysoPC a C20:4	12.6 (10.2-14.9)	4.57 (3.56-5.89)	0.098	0.066
Phosphatidylcholines				
PC aa C28:1	0.88 (0.76-1.06)	2.16 (1.79-2.64)	0.098	0.066
PC aa C30:0	2.95 (2.53-3.44)	2.99 (2.39-3.84)	0.007	0.896
PC aa C32:0	15.3 (12.7-18.3)	13.6 (11.8-15.7)	0.136	0.010
PC aa C32:1	12.1 (9.51-15.8)	10.1 (7.46-13.5)	-0.045	0.397
PC aa C32:2	0.66 (0.48-0.92)	2.34 (1.65-3.14)	0.078	0.142
PC aa C32:3	0.23 (0.19-0.29)	0.38 (0.31-0.45)	0.151	0.004
PC aa C34:1	105 (91.8-119)	173 (145-199)	0.085	0.111
PC aa C34:2	84.2 (71.2-101)	315 (269-369)	0.103	0.053
PC aa C34:3	2.8 (2.33-3.53)	11 (8.46-13.9)	0.094	0.078
PC aa C34:4	0.52 (0.43-0.62)	0.82 (0.63-1.14)	0.131	0.014
PC aa C36:0	1.55 (1.27-1.89)	1.38 (1.03-1.84)	0.023	0.660
PC aa C36:1	19.8 (16.9-22.8)	40.80 (33.8-47.9)	0.044	0.406
PC aa C36:2	44.5 (37.6-53.3)	212 (183-244)	0.075	0.158
PC aa C36:3	63.7 (53.6-73.5)	106 (89.5-120)	0.099	0.062

PC aa C36:4	135 (118-151)	130 (111-150)	0.181	0.001
PC aa C36:5	5.2 (4.18-6.71)	8.39 (6.57-11.5)	0.076	0.156
PC aa C36:6	0.31 (0.26-0.37)	0.4 (0.3-0.54)	0.129	0.016
PC aa C38:0	1.46 (1.21-1.76)	2.21 (1.85-2.71)	0.093	0.081
PC aa C38:3	36.1 (31-41.9)	32.8 (26.5-38.5)	0.020	0.702
PC aa C38:4	90.4 (78.8-104)	76.6 (65.9-90.8)	0.165	0.002
PC aa C38:5	21.8 (18.8-25.2)	36.2 (28.8-42.9)	0.182	0.001
PC aa C38:6	60.6 (50.1-72.9)	49.1 (37.8-60.8)	0.069	0.199
PC aa C40:1	0.35 (0.3-0.42)	0.39 (0.33-0.45)	0.063	0.238
PC aa C40:2	0.25 (0.2-0.37)	0.29 (0.25-0.35)	0.009	0.860
PC aa C40:3	0.54 (0.46-0.64)	0.49 (0.43-0.58)	0.090	0.090
PC aa C40:4	2.59 (2.16-3.19)	3 (2.41-3.61)	0.195	< 0.0002
PC aa C40:5	4.13 (3.29-5.27)	7.6 (5.96-9.28)	0.093	0.081
PC aa C40:6	22.6 (17.7-28.7)	17.4 (13.60-21.8)	0.074	0.164
PC aa C42:0	0.45 (0.36-0.54)	0.55 (0.47-0.65)	0.211	< 0.0001
PC aa C42:1	0.28 (0.22-0.34)	0.27 (0.22-0.33)	0.142	0.008
PC aa C42:2	0.19 (0.16-0.25)	0.2 (0.17-0.24)	-0.002	0.970
PC aa C42:4	0.24 (0.2-0.32)	0.2 (0.17-0.23)	0.082	0.126
PC aa C42:5	0.30 (0.25-0.36)	0.39 (0.32-0.47)	0.084	0.116
PC aa C42:6	0.44 (0.39-0.5)	0.61 (0.51-0.71)	0.082	0.122
PC ae C32:1	2.61 (2.15-3.14)	2.67 (2.26-3.29)	0.143	0.007
PC ae C32:2	0.58 (0.48-0.73)	0.54 (0.44-0.68)	0.098	0.066
PC ae C34:0	1 (0.81-1.24)	0.99 (0.77-1.36)	0.098	0.065
PC ae C34:1	4.7 (3.93-5.61)	8.24 (7.04-9.88)	0.125	0.019
PC ae C34:2	3.12 (2.61-3.68)	8.43 (6.87-10.1)	0.161	0.002
PC ae C34:3	1.26 (1.02-1.56)	5.44 (4.33-6.82)	0.121	0.023
PC ae C36:0	0.75 (0.64-0.9)	0.73 (0.59-0.88)	0.147	0.006
PC ae C36:1	2.53 (2.07-3.07)	5.39 (4.33-6.84)	0.145	0.006
PC ae C36:2	2.22 (1.84-2.69)	10.3 (8.09-12.5)	0.090	0.093
PC ae C36:3	1.79 (1.48-2.16)	5.43 (4.50-6.33)	0.100	0.059
PC ae C36:4	9.16 (7.66-10.7)	13.3 (11.1-15.9)	0.116	0.029
PC ae C36:5	5.99 (4.85-7.19)	7.98 (6.32-9.9)	0.156	0.003
PC ae C38:0	1.28 (1.08-1.54)	1.23 (1-1.45)	0.097	0.068
PC ae C38:2	0.8 (0.58-1.28)	1.84 (1.48-2.3)	0.069	0.197
PC ae C38:3	2.74 (2.05-4.36)	2.7 (2.2-3.27)	0.133	0.012
PC ae C38:4	7.47 (6.09-8.57)	9.69 (7.87-11.9)	0.114	0.032
PC ae C38:5	6.76 (5.6-7.91)	14.4 (12.6-17)	0.054	0.307
PC ae C38:6	2.95 (2.42-3.51)	4.5 (3.72-5.45)	0.081	0.128
PC ae C40:1	0.89 (0.71-1.19)	0.87 (0.69-1.15)	0.028	0.598
PC ae C40:2	0.87 (0.7-1.15)	1.13 (0.92-1.37)	0.189	0.000
PC ae C40:3	1.32 (0.89-2.45)	0.82 (0.7-0.95)	0.030	0.576
PC ae C40:4	1.82 (1.47-2.29)	1.85 (1.54-2.19)	0.206	< 0.0001
PC ae C40:5	2.12 (1.58-3.28)	2.83 (2.4-3.36)	0.188	0.000
PC ae C40:6	2.01 (1.68-2.46)	3.07 (2.53-3.68)	0.148	0.005
PC ae C42:0	0.47 (0.42-0.54)	0.56 (0.48-0.67)	0.004	0.940
PC ae C42:2	0.27 (0.22-0.37)	0.41 (0.34-0.5)	0.025	0.643
PC ae C42:3	0.47 (0.37-0.63)	0.6 (0.50-0.72)	0.077	0.147

PC ae C42:4	0.43 (0.34-0.56)	0.65 (0.54-0.76)	0.128	0.016
PC ae C42:5	1.18 (0.95-1.63)	1.6 (1.39-1.91)	0.108	0.042
PC ae C44:4	0.25 (0.22-0.3)	0.4 (0.33-0.47)	0.151	0.005
PC ae C44:5	0.66 (0.55-0.79)	1.3 (1.07-1.61)	0.180	0.001
PC ae C44:6	0.6 (0.5-0.74)	0.94 (0.73-1.16)	0.157	0.003
Sphingomyelins				
SM (OH) C14:1	2.44 (2.07-2.93)	4.96 (3.94-6.13)	0.190	0.000
SM (OH) C16:1	1.93 (1.63-2.35)	3.01 (2.33-3.81)	0.136	0.010
SM (OH) C22:1	2.88 (2.39-3.51)	11.3 (9.01-13.9)	0.219	< 0.0001
SM (OH) C22:2	2.65 (2.17-3.24)	8.54 (6.75-10.1)	0.124	0.020
SM (OH) C24:1	0.84 (0.71-1.06)	1.1 (0.84-1.41)	0.089	0.095
SM C16:0	69.9 (61-79.4)	121 (101-139)	0.127	0.017
SM C16:1	11.6 (9.74-13.5)	13 (10.6-15)	0.133	0.012
SM C18:0	22.3 (18.6-25.8)	25.1 (21-30.9)	0.118	0.027
SM C18:1	14.2 (12-16.9)	9.78 (8.08-12.1)	0.163	0.002
SM C24:0	14.4 (12.1-16.6)	19.3 (16.2-23.5)	0.184	0.001
SM C24:1	25.5 (21.5-29.7)	45.5 (39.2-57)	0.183	0.001
Sugars				
hexoses	2231 (1100-3855)	3818 (2982-4406)	-0.056	0.294
Amino acids				
histidine	124 (104-150)	106 (91.7-124)	0.081	0.124
isoleucine	83.1 (72.1-96.5)	92.1 (71.4-116)	0.104	0.049
leucine	156 (134-183)	168 (134-216)	0.108	0.041
lysine	441 (384-516)	238 (192-295)	-0.015	0.771
methionine	35.8 (28.90-44.2)	28.90 (21.60-37.6)	0.091	0.084
ornithine	185 (144-222)	124 (90.4-189)	-0.015	0.780
phenylalanine	108 (96.80-124)	93.6 (75.8-114)	0.030	0.570
proline	211 (184-247)	256 (207-310)	0.032	0.548
tryptophan	87.4 (75.6-98.6)	73.9 (63-87)	-0.090	0.088
tyrosine	81.9 (69.8-94)	84.2 (67.5-109)	0.127	0.016
valine	238 (206-272)	248 (206-304)	0.056	0.288
Biogenic amines				
kynurenine	5.89 (4.89-7.05)	4.13 (3.44-4.91)	0.080	0.127

* Significance level after Bonferroni-correction $P < 0.0002$

TABLE EIV. mRNA expression of immune parameters in whole blood and IL-1 β protein plasma concentration and correlation between samples at birth (cord blood) and children aged one year from the prospective birth cohort LINA.

parameter	Expression§/concentration [#] of immune parameters		Spearman correlation between inflammasome components and TLRs from birth and 1 yr blood samples	
	birth	1 year		
	Median (IQR)	Median (IQR)	Spearman (R)	p value*
NLRP3	7.69 (6.78-8.55)	6.42 (5.80-7.08)	0.007	0.900
CASP1	7.15 (5.82-8.22)	7.43 (6.56-8.21)	0.027	0.608
MYD88	8.09 (6.77-9.50)	7.60 (6.04-9.10)	-0.269	< 0.0001
TLR1	4.97 (3.89-5.96)	5.37 (4.72-6.00)	-0.022	0.674
TLR2	5.04 (4.11-5.91)	4.37 (3.81-4.96)	0.080	0.131
TLR5	9.81 (7.38-12.01)	10.24 (7.82-11.45)	0.002	0.969
TLR6	4.04 (3.14-5.01)	5.85 (5.27-6.54)	-0.061	0.248
TLR7	4.89 (3.75-6.24)	8.84 (7.47-10.02)	-0.019	0.725
TLR9	8.68 (6.58-10.36)	1.76 (9.78-12.2)	0.024	0.653
TLR10	8.63 (7.26-9.92)	8.18 (6.66-9.66)	0.065	0.221
IL-1 β protein#	2.57 (1.15-4.85)	1.15 (1.15-3.81)	0.033	0.523
IL-1 β mRNA	16.25 (14.92-17.86)	13.53 (11.61-15.28)	0.128	0.016
IL-18 mRNA	15.88 (14.93-16.81)	13.95 (12.61-15.08)	-0.065	0.220

* Significance level after Bonferroni-correction $P < 0.002$

§ relative mRNA expression

pg/ml

TABLE EV. Relationship between IL-18 mRNA concentrations in cord and 1 yr blood and the development of childhood diseases in the first and second year of life respectively. [#]

IL-18 mRNA blood expression		
	birth	1 year
aOR (95% CI)*		
Outcome at age	1 yr	2 yr
Atopic dermatitis	0.91 (0.68-1.22)	1.08 (0.77-1.52)
Sensitisation		
to food allergens [†]	0.99 (0.76-1.28)	0.77 (0.55-1.06)
to inhalant allergens [†]	1.28 (0.70-2.32)	0.70 (0.43-1.14)
Bronchitis	0.97 (0.79-1.21)	0.95 (0.78-1.16)
Wheezing	1.03 (0.83-1.29)	0.94 (0.75-1.17)
Airway infections	0.54 (0.26-1.11)	0.90 (0.64-1.26)

[#] Analysis was performed using a logistic regression model with IL-18 values categorized in quartiles; values in boldface are significant ($P < 0.05$).

*Adjusted odd ratios (aOR) for parental educational level, month of birth, mode of delivery and cat keeping during pregnancy, siblings, gender, smoking during pregnancy. Atopic dermatitis and allergic sensitisation were additionally adjusted for parental history of atopy.

[†] According to the Phadia CAP System concentrations >0.35 kU/l were regarded as positive.

References

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- E2. Herberth G, Hinz D, Roder S, Schlink U, Sack U, Diez U, et al. Maternal immune status in pregnancy is related to offspring's immune responses and atopy risk. *Allergy* 2011; 66:1065-74.
- E3. Markle JG, Frank DN, Mortin-Toth S, Robertson CE, Feazel LM, Rolle-Kampczyk U, et al. Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. *Science* 2013; 339:1084-8.

Figure Legends

FIGURE E1

Flow diagram illustrating the analyzed samples in the LINA study.

FIGURE E2

Associations between metabolite blood concentrations (hexoses, amino acids) and expression of TLRs and inflammasome components (NLRP3/CASP1) at birth (cord blood) and in the first year of life (1 year). Linear regression models (shown are mean ratios and 95% confidence intervals) adjusted for season of birth, mode of delivery, cat keeping during pregnancy, parental history of atopy, gender and parental education.

FIGURE E3

Associations between metabolite blood concentrations (lysoPCs) and expression of TLRs and inflammasome components (NLRP3/CASP1) at birth (cord blood) and in the first year of life (1 year). Linear regression models (shown are mean ratios and 95% confidence intervals) adjusted for season of birth, mode of delivery, cat keeping during pregnancy, parental history of atopy, gender and parental education.

FIGURE E4

Scatterplots represent the association between expression of TLRs and the inflammasome components NLRP3 and CASP1 at birth (cord blood). Correlation coefficient and P values from Spearman correlation are shown.

FIGURE E5

Scatterplots represent the association between expression of TLRs and the inflammasome components NLRP3 and CASP1 at the first year of life. Correlation coefficient and P values from Spearman correlation are shown.

FIGURE E6

Relationship between expression of inflammasome components NLRP3/CASP1 and the effector cytokines IL-1 β (protein, mRNA) and IL-18 (mRNA) at birth (cord blood) and in the first year of life. Data are presented as mean ratios with 95% confidence intervals.

FIGURE E7

In vitro analysis showing the impact of glucose (glc) on NLRP3, CASP1, MYD88, IL1B, TLR1, TLR9 and TLR10 mRNA expression. Isolated PBMCs (n=4 healthy donors (n=3 for TLR1)) were incubated for 6 hours in glucose free RPMI1640 + 10% dialyzed FBS, 100 ng/ml of LPS and different glucose concentrations (0 nM, 5.5 mM, 15 mM). Total RNA was extracted, transcribed into cDNA and analyzed via real-time PCR. Shown are mean values with standard error (SEM) of relative mRNA expression from at least 3 independent experiments. P value significance levels: * p < 0,05; ** p < 0,01; *** p < 0,001.

FIGURE E8

In vitro analysis showing the impact of lysophosphatidylcholines (LPC) and the amino acid Valine (Val) on NLRP3, CASP1, MYD88, IL1B, TLR1, TLR9 and TLR10 mRNA expression. Isolated PBMCs (n=4 healthy donors) were incubated for 24 hours in RPMI1640 + 10% FBS, 100 ng/ml of LPS (Ctrl) and with or without 25 μ M LPC or 5 mM Val. Total RNA was extracted, transcribed into cDNA and analyzed via real-time PCR. Shown are mean values with standard errors (SEM) of relative mRNA expression from 4 independent experiments. P value significance levels: * p < 0,05; *** p < 0,001.

medical consultation

n=629

n=606

birth

year 1

cytokines

n=471

n=513

metabolites

n=495

n=449

mRNA analysis

n=451

n=495

Specific IgE
Sx1/Fx5

n=516

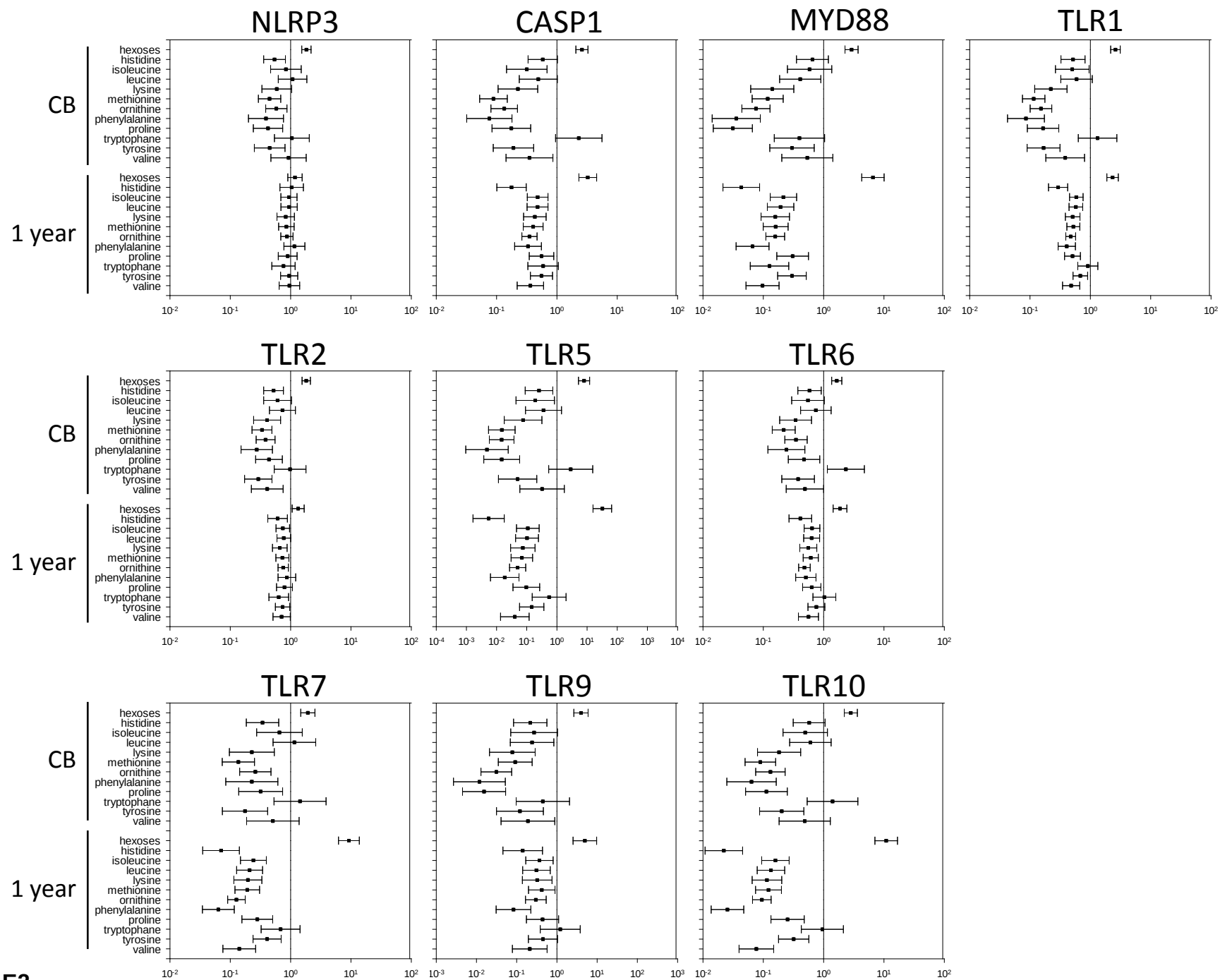
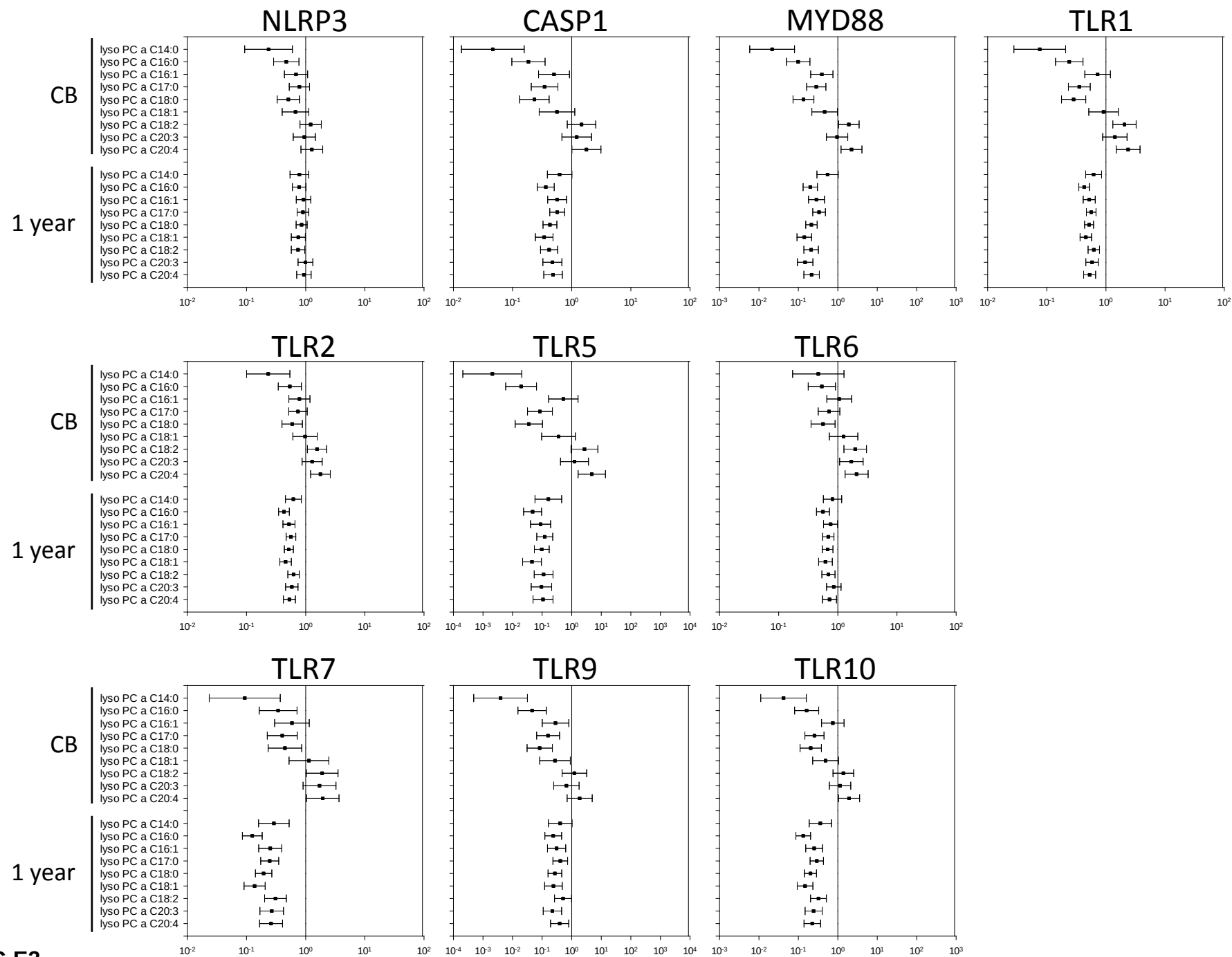
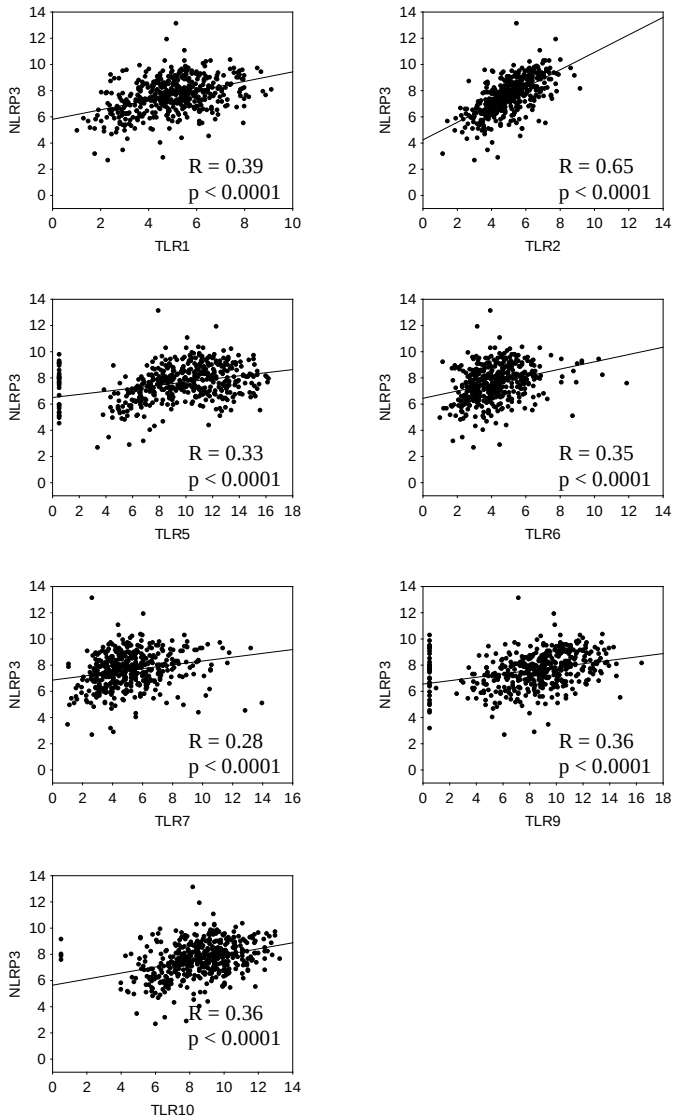


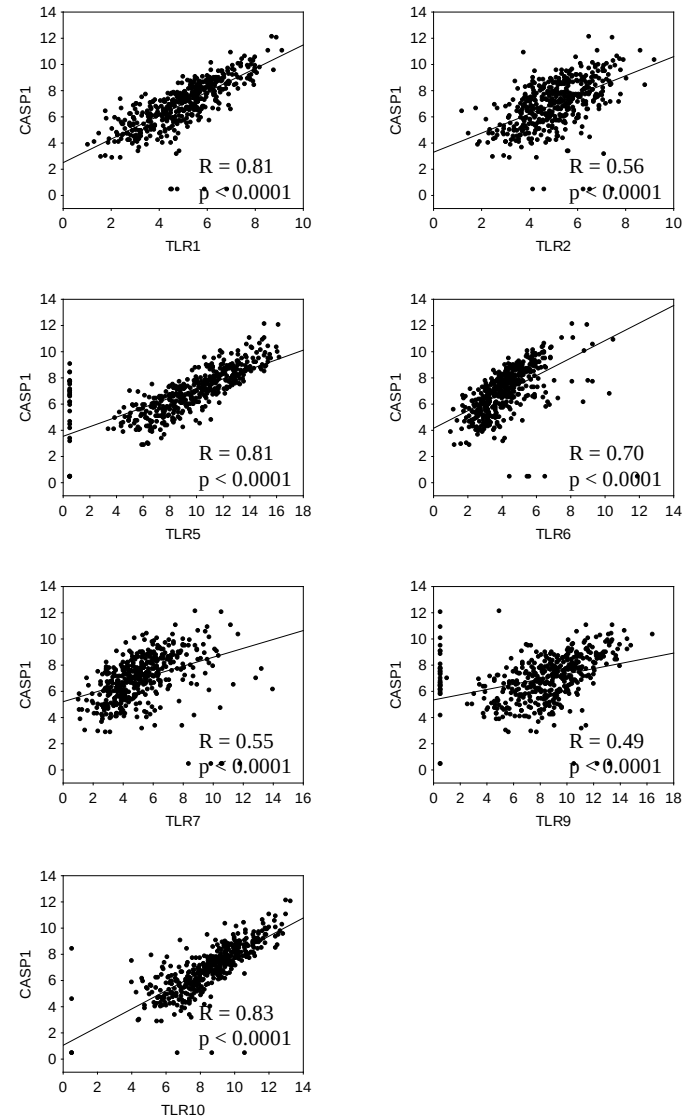
FIG E2



TLRs vs NLRP3

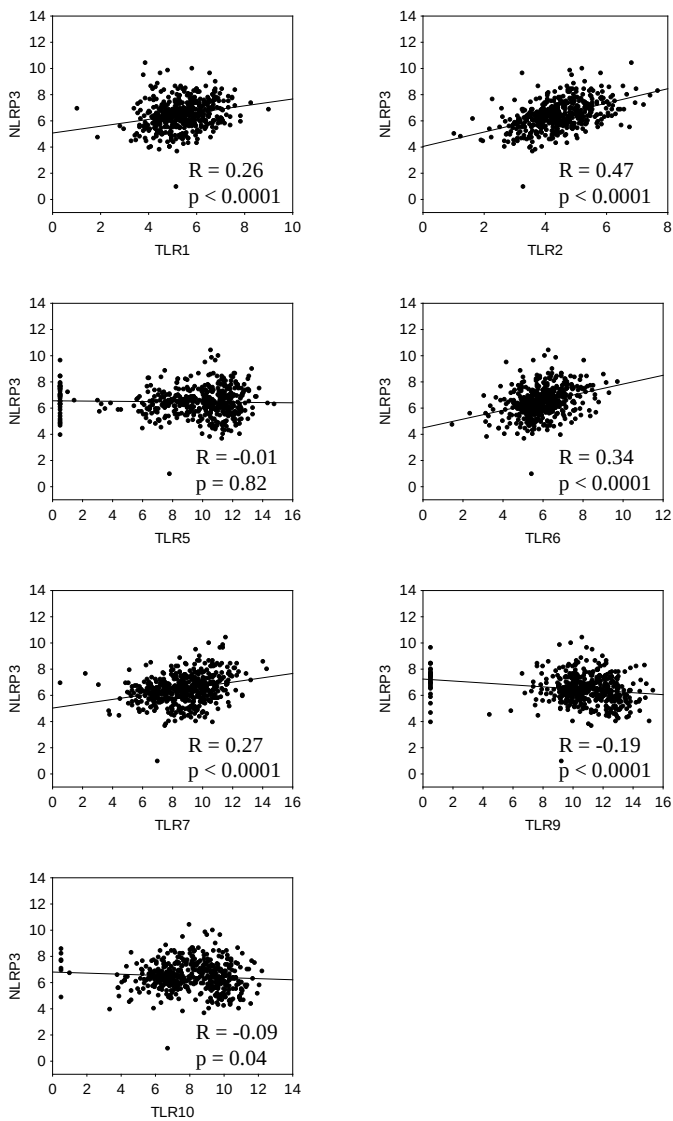


TLRs vs CASP1

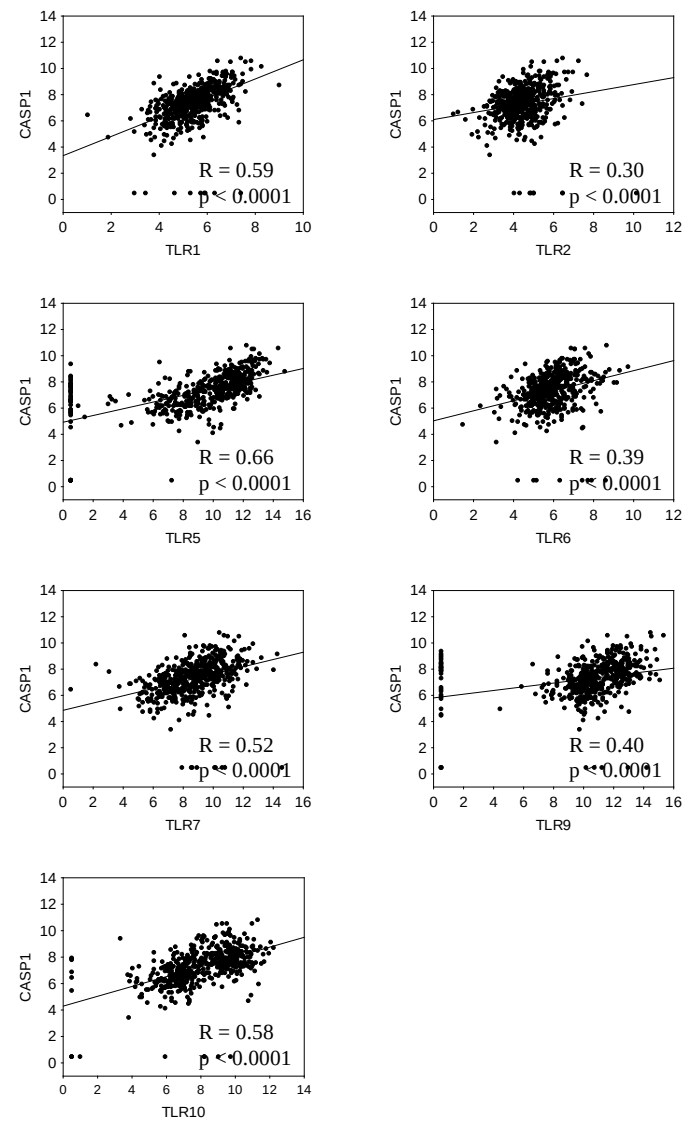


1 year

TLRs vs NLRP3



TLRs vs CASP1



NLRP3/CASP-1 vs effector cytokines

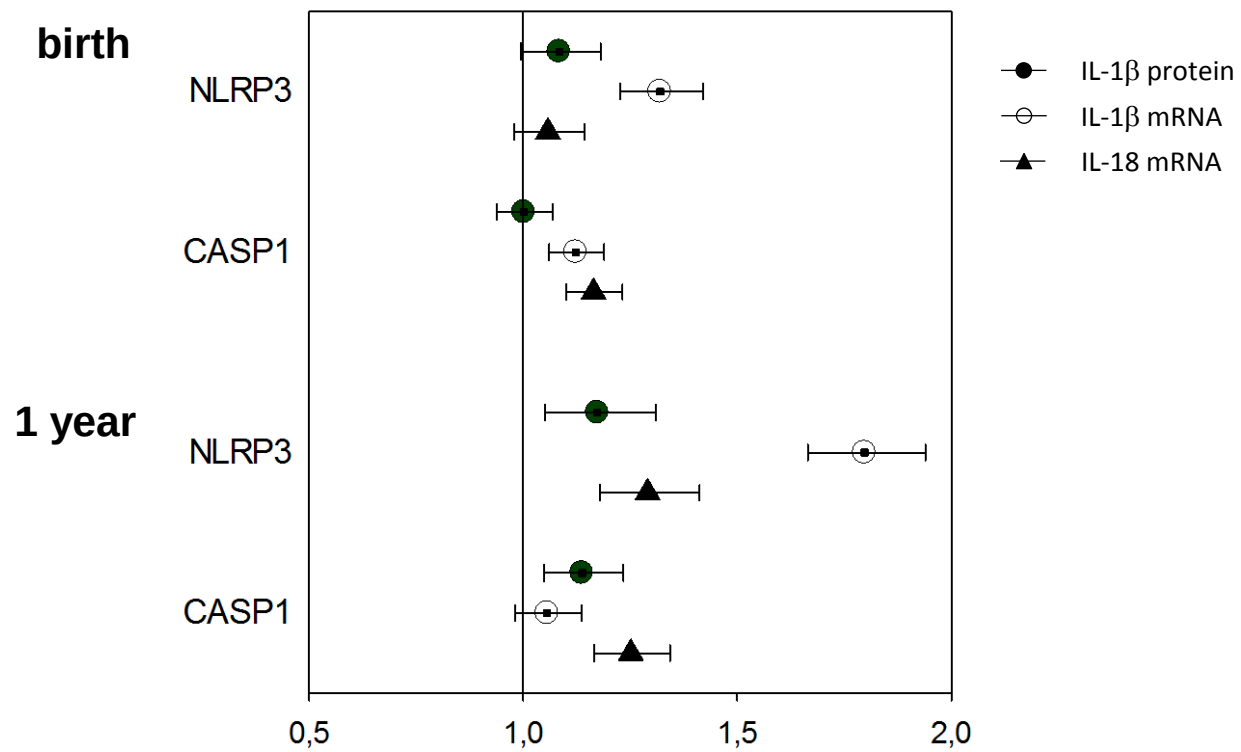


FIG E6

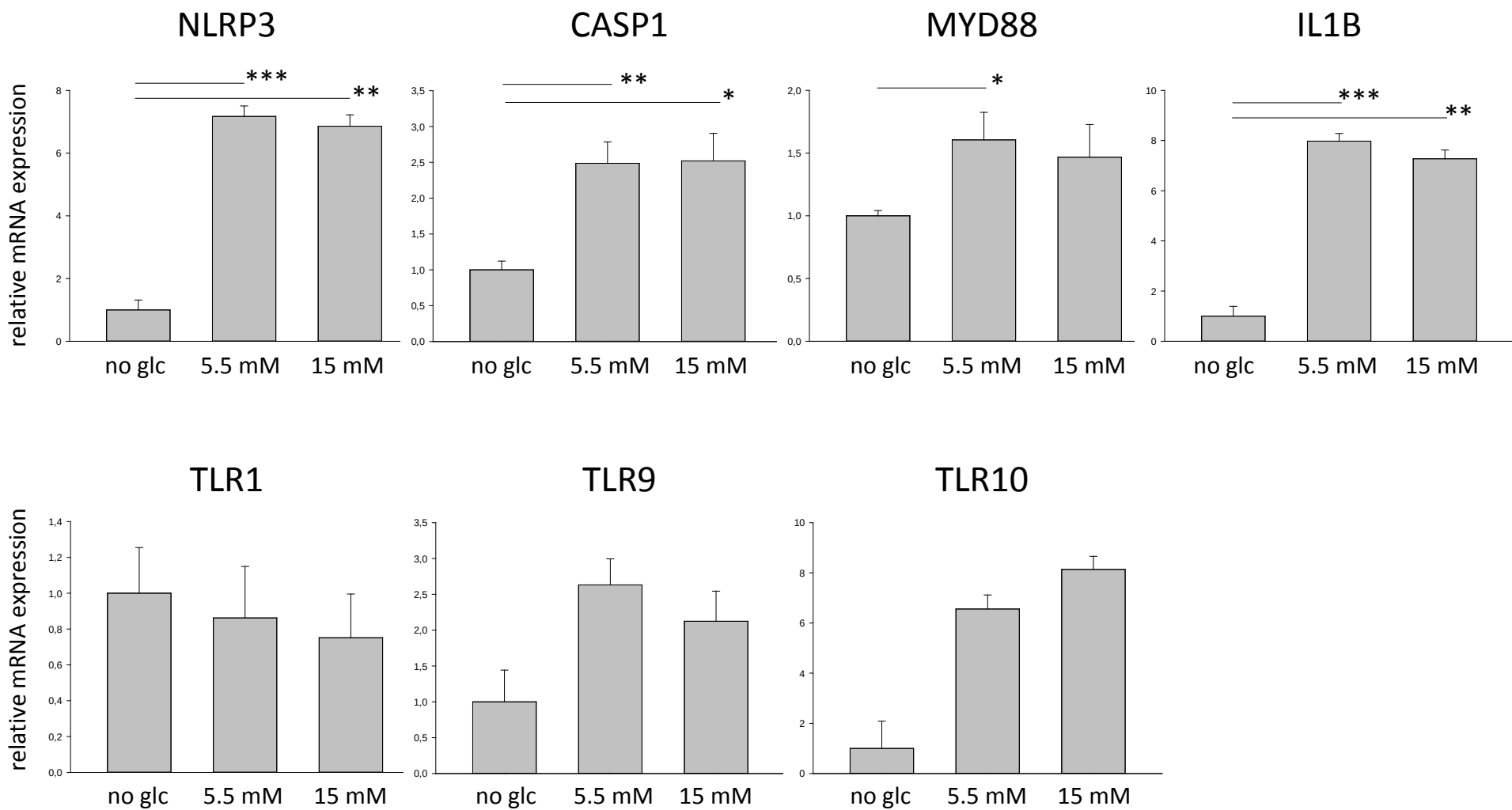


FIG E7

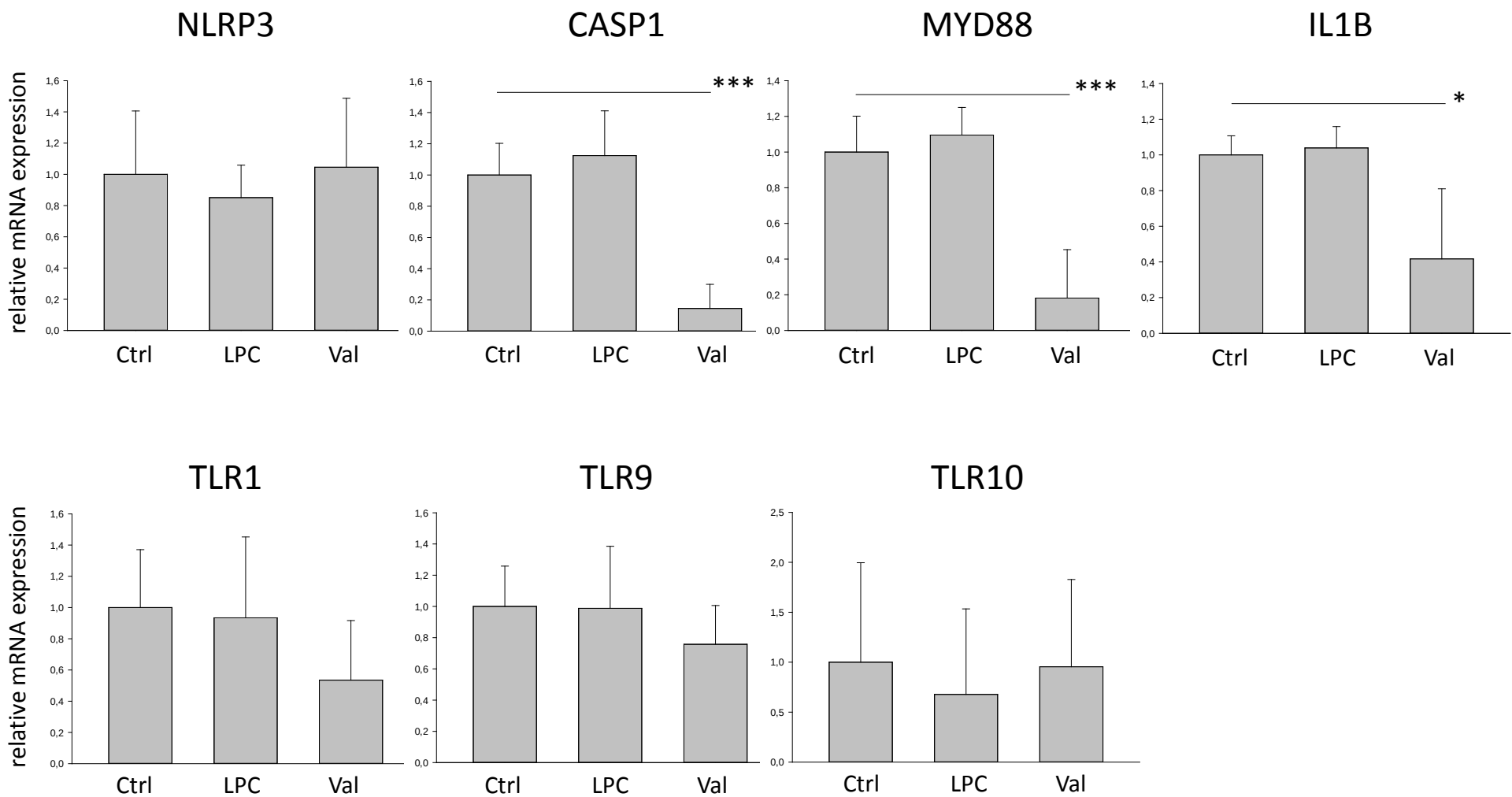


FIG E8