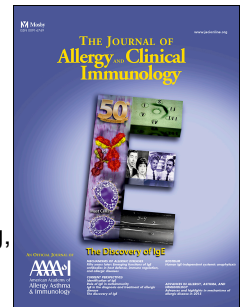


Accepted Manuscript

Early-onset childhood atopic dermatitis is related to *NLRP2* repression

Loreen Thürmann, PhD, Konrad Grützmann, PhD, Matthias Klös, MSc, Matthias Bieg, MSc, Marcus Winter, MSc, Tobias Polte, PhD, Tobias Bauer, PhD, Matthias Schick, Dipl-Ing, Melanie Bewerunge-Hudler, PhD, Stefan Roeder, PhD, Mario Bauer, MD, Dirk K. Wissenbach, PhD, Ulrich Sack, PhD, Dieter Weichenhan, PhD, Oliver Mücke, Christoph Plass, PhD, Michael Borte, MD, Martin von Bergen, PhD, Irina Lehmann, PhD, Roland Eils, PhD, Saskia Trump, PhD



PII: S0091-6749(17)31892-4

DOI: [10.1016/j.jaci.2017.11.018](https://doi.org/10.1016/j.jaci.2017.11.018)

Reference: YMAI 13158

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 5 August 2017

Revised Date: 17 November 2017

Accepted Date: 21 November 2017

Please cite this article as: Thürmann L, Grützmann K, Klös M, Bieg M, Winter M, Polte T, Bauer T, Schick M, Bewerunge-Hudler M, Roeder S, Bauer M, Wissenbach DK, Sack U, Weichenhan D, Mücke O, Plass C, Borte M, von Bergen M, Lehmann I, Eils R, Trump S, Early-onset childhood atopic dermatitis is related to *NLRP2* repression, *Journal of Allergy and Clinical Immunology* (2018), doi: 10.1016/j.jaci.2017.11.018.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Letter to the Editor**Early-onset childhood atopic dermatitis is related to *NLRP2* repression**

Loreen Thürmann, PhD^{1†}, Konrad Grützmann, PhD^{1,2†}, Matthias Klös, MSc¹, Matthias Bieg, MSc^{3,4}, Marc Winter, MSc¹, Tobias Polte, PhD¹, Tobias Bäcker, PhD³, Matthias Schick, Dipl-Ing⁵, Melanie Bewerunge-Heldler, PhD⁵, Stefan Roeder, PhD¹, Mario Bäcker, MD¹, Dirk K. Wissenbach, PhD^{6,7}, Ulrich Sack, PhD⁸, Dieter Weichenhan, PhD⁹, Oliver Mücke⁹, Christoph Plass, PhD⁹, Michael Borte, MD¹⁰, Martin von Bergen, PhD^{6,11}, Irina Lehmann, PhD^{1*†}, Roland Eils, PhD^{3,4,12,13†}, Saskia Trümp, PhD^{1,12*†}

- ¹ Department of Environmental Immunology, Helmholtz Centre for Environmental Research Leipzig - UFZ, Permoserstraße 15, Leipzig, 04318, Germany
- ² Center for Molecular Tumor Diagnostics (CMTD) at the National Center for Tumor Diseases (NCT), partner site Dresden, Schöbertstraße 15, Dresden, 01307, Germany
- ³ Division of Theoretical Bioinformatics, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, Heidelberg, 69120, Germany
- ⁴ Heidelberg Center for Personalized Oncology, DKFZ-HIPO, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 267, Heidelberg, 69120, Germany
- ⁵ Genomics and Proteomics Core Facility, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 580, Heidelberg, 69120, Germany
- ⁶ Department of Molecular Systems Biology, Helmholtz-Centre for Environmental Research - UFZ, Permoserstraße 15, Leipzig, 04318, Germany
- ⁷ Institute of Forensic Medicine, University Hospital Jena, Am Klinikum 1, Jena, 07747, Germany
- ⁸ Institute of Clinical Immunology, University Hospital Leipzig, Johannisallee 30, Leipzig, 04103, Germany
- ⁹ Division of Epigenomics and Cancer Risk Factors, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, Heidelberg, 69120, Germany
- ¹⁰ Municipal Hospital "St. Georg" Children's Hospital, Delitzscher Straße 141, Leipzig, 04129, Germany
- ¹¹ Department of Biochemistry, Faculty of Biosciences, Pharmacy and Psychology, University of Leipzig, Brüderstrasse 34, Leipzig, 04103, Germany
- ¹² Institute of Pharmacy and Molecular Biotechnology, and Bioquant Center, University of Heidelberg, Im Neuenheimer Feld 267, Heidelberg, 69120, Germany
- ¹³ Translational Lung Research Center Heidelberg (TLRC), German Center for Lung Research (DZL), University of Heidelberg, 69120, Germany

† equal contribution

***Corresponding authors:**

Dr. Saskia Trümp, Phone: +49341-235-1550, Fax: +49341-235-1887

saskia.truemp@ufz.de

Dr. Irina Lehmann, Phone: +49341-235-1265, Fax: +49341-235-1887,

irina.lehmann@ufz.de

Source of funding:

This work was supported by the Helmholtz Initiative on Personalized Medicine (iMed) and the Helmholtz Interdisciplinary Graduate School for Environmental Research (HIGRADE). S. Trümp was supported by the Federal Ministry of Education and Research grant 01ZX1402D.

Capsule summary

Early-onset atopic dermatitis (AD) related repression of the immune regulatory *NLRP2* is driven by promoter hypermethylation starting already at time of birth providing an early opportunity to modulate innate immunity to potentially mitigate AD development.

Keywords:

atopic dermatitis; environment; epidemiology; epigenetics; *NLRP2*; prenatal; tobacco smoke

1 To the Editor

2 Atopic dermatitis (AD) is a common skin disease in children. While genetic
3 predisposition and environmental factors are accepted mediators of AD development,
4 the role of the innate immune system is not yet completely elucidated. Likely mediators
5 of AD are nucleotide-binding oligomerization and pyrin domain containing-receptors
6 (NLRPs), which are involved in the innate immune response (1). So far, only few studies
7 investigated the role of NLRPs in adult AD, primarily focusing on single nucleotide
8 polymorphisms (SNPs), while only reporting transcriptional changes for *NLRP2* and
9 *NLRP3* (2, 3). To elucidate the relevance of NLRPs in childhood AD, this study focused on
10 these two NLRP family members.

11 We started by evaluating the transcription of *NLRP2* and *NLRP3* in whole blood of
12 children of the LINA cohort, showing AD symptoms or were diagnosed with AD by a
13 physician until the age of six years ("AD ever", Figure/Table E1). While *NLRP3*
14 expression was unaltered, *NLRP2* was decreased in one-year-old children with AD
15 compared to healthy controls (Figure 1A, Table E2).

16 Since there are different pheno- and endotypes of AD, supposedly based on different
17 molecular and cellular characteristics, we further separated the occurrence of AD in
18 early- and late-onset (Figure E2). Only for children with early-onset AD *NLRP2* was
19 differentially expressed in comparison to healthy controls (Figure 1B, Table E2). *NLRP3*
20 was neither differentially transcribed in early- nor late-onset AD compared to controls
21 (Table E2).

22 As DNA-methylation is as a potent epigenetic regulator of gene expression we next
23 investigated *NLRP2* promoter methylation. Using whole genome bisulfite sequencing
24 data from cord blood samples of a subset of children from our cohort, we observed
25 *NLRP2* promoter hypermethylation in children with early-onset AD compared to
26 controls (Figure 2A, Table E3). Validation of DNA-methylation (Figure E3, Table E4) in
27 the entire cohort confirmed an AD-related promoter hypermethylation at birth and at

age one, which was associated with an increased risk for the children to develop early-onset AD (Figure 2B). At both time points this hypermethylation was inversely correlated with *NLRP2* transcription (Figure E4) suggesting a functional translation of the methylation change to the transcriptional level.

Subsequently, we investigated whether the alterations in *NLRP2* expression and methylation arose from a specific blood cell population. Although monocytes, B cells, CD8+ and CD4+ T cells of children with AD showed a significant difference in *NLRP2* expression and methylation compared to controls, the pattern was similar in all populations suggesting that AD-associated differences in *NLRP2* are not related to changes in these specific cell types (Figure E5C).

Another level, by which variation in gene expression can be explained are expression quantitative trait loci (eQTLs). As the Genotype-Tissue-Expression portal describes rs514148 as the most significant eQTL for *NLRP2* in blood, we analyzed this SNP in our cohort. Both *NLRP2* transcription and promoter methylation were different between rs514148 genotypes (Figure E6), characterizing rs514148 also as a methylation QTL for *NLRP2*.

Reevaluating our AD-related regression analyses by including the rs514148 variant as a confounder retained the significant risk increase for AD development in relation to *NLRP2* repression and promoter hypermethylation (age one: transcription, adj. OR=0.75, 95% CI: 0.62-0.91; methylation, adj. OR=3.75, 95% CI: 1.26-11.18). Only recently released genotyping platforms cover rs514148 or other SNPs in linkage disequilibrium ($r^2 > 0.5$). As to our knowledge, none of these SNPs were included in prior AD-GWAS studies. Future studies will show, whether rs514148 will be confirmed as an AD risk locus.

Since the genetic background alone could not explain the link between *NLRP2* and AD, further factors, such as environmental exposure might have an additional impact on

NLRP2 regulation in AD. We assessed whether prenatal tobacco smoke exposure as a known risk factors for AD (4, 5) might play a role. Indeed, mediation analysis revealed that prenatal benzene exposure - a valid proxy for tobacco smoke exposure (Figure E7) - influenced AD development via changes in *NLRP2* promoter methylation and transcription (Figure E8).

Previously, in IFN γ stimulated hair follicle-derived keratinocytes (HFDKs) from AD patients - mimicking an inflammatory response - an increased *NLRP2* expression compared to healthy adults was observed (3). This is line with the immunosuppressive function of *NLRP2* to blunt excessive Nfkb-activation, as *NLRP2* is a known Nfkb inhibitor (1). Although, these results seem to contradict our findings, our observations were made in different cell types and at different time points (adulthood vs. early childhood). More importantly, the small number of studied HFDKs increases the likelihood that the genotype distribution determined the increased *NLRP2* expression in AD patients. The results presented here rather suggest that early dysregulation of *NLRP2* in unstimulated circulating immune cells constitutes a risk factor for the child to develop AD by diminishing the immunosuppressive potential of *NLRP2*.

As *NLRP2* inhibits Nfkb signaling, concomitant cytokine alterations might have an impact on AD. In fact, children with early-onset AD showed elevated IL-10 blood protein concentrations (Table E5) that were associated with both, *NLRP2* hypermethylation (adj. MR=1.15, 95% CI: 1.03-1.27) and transcriptional repression (adj. MR=0.87, 95% CI: 0.78-0.9). AD-related increase of IL-10 has been observed in skin lesions, but also in various blood cell types (6). Ricci *et al.* determined higher IL-10 serum concentrations in children with eczema (7), in line with our observation that elevated IL-10 increased the children's risk to develop early-onset AD (adj. OR: 2.81, 95% CI: 1.20-6.56). High concentrations of IL-10 have been implicated in the downregulation of the antimicrobial peptide human beta-defensin 2 (HBD-2) contributing to recurrent skin infections in AD (8). Notably, *NLRP2* seems to be a mediator of HBD expression as Ji *et al.*

observed a decrease in the bacterial-activation of HBDs by *NLRP2* silencing (9), suggesting an impaired host defense due to *NLRP2* repression. Susceptibility of the skin to adverse environmental factors might increase as a result of such underlying immune deviations. Therefore, infiltrating immune cells with an impaired *NLRP2* expression, as we have observed in children with early-onset AD, might not only contribute to IL-10 elevation in skin lesions of AD patients, but might also impair pathogen defense due to its negative effect on defensin expression.

This study broadens the view on how genetic variations in *NLR* genes might contribute to AD development. It describes a functional translation of the rs514148 genotype to *NLRP2* alterations, which increase the risk for early-onset AD. These early life changes in *NLRP2* might provide an opportunity to mitigate AD development by modulating innate immunity at a young age.

Loreen Thürmann, PhD^{1†},
 Konrad Grützmann, PhD^{1,2†},
 Matthias Klös, MSc¹,
 Matthias Bieg, MSc^{3,4},
 Marc's Winter, MSc¹,
 Tobias Polte, PhD¹,
 Tobias Bärer, PhD³,
 Matthias Schick, Dipl-Ing⁵,
 Melanie Bewerunge-Heldler, PhD⁵,
 Stefan Roeder, PhD¹,
 Mario Bärer, MD¹,
 Dirk K. Wissenbach, PhD^{6,7},
 Ulrich Sack, PhD⁸,
 Dieter Weichenhan, PhD⁹,
 Oliver Mücke⁹,
 Christoph Plass, PhD⁹,
 Michael Borte, MD¹⁰,
 Martin von Bergen, PhD^{6,11},
 Irina Lehmann, PhD^{1*†},

Roland Eils, PhD^{3,4,12,13†},

Saskia Trömp, PhD^{1*†}

Affiliation: (1) Department of Environmental Immunology, Helmholtz Centre for Environmental Research Leipzig - UFZ, Permoserstraße 15, Leipzig, 04318, Germany (2) Center for Molecular Tumor Diagnostics (CMTD) at the National Center for Tumor Diseases (NCT), partner site Dresden, Schöbertstraße 15, Dresden, 01307, Germany (3) Division of Theoretical Bioinformatics, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, Heidelberg, 69120, Germany (4) Heidelberg Center for Personalized Oncology, DKFZ-HIPO, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 267, Heidelberg, 69120, Germany, (5) Genomics and Proteomics Core Facility, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 580, Heidelberg, 69120, Germany (6) Department of Molecular Systems Biology, Helmholtz-Centre for Environmental Research - UFZ, Permoserstraße 15, Leipzig, 04318, Germany (7) Institute of Forensic Medicine, University Hospital Jena, Am Klinikum 1, Jena, 07747, Germany (8) Institute of Clinical Immunology, University Hospital Leipzig, Johannisallee 30, Leipzig, 04103, Germany (9) Division of Epigenomics and Cancer Risk Factors, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, Heidelberg, 69120, Germany, (10) Municipal Hospital "St. Georg" Children's Hospital, Delitzscher Straße 141, Leipzig, 04129, Germany (11) Department of Biochemistry, Faculty of Biosciences, Pharmacy and Psychology, University of Leipzig, Brüderstrasse 34, Leipzig, 04103, Germany (12) Institute of Pharmacy and Molecular Biotechnology, and Bioquant Center, University of Heidelberg, Im Neuenheimer Feld 267, Heidelberg, 69120, Germany (13) Translational Lung Research Center Heidelberg (TLRC), German Center for Lung Research (DZL), University of Heidelberg, 69120, Germany

† equal contribution

Acknowledgements

We would like to thank Marion Bähr and Monika Helf who provided support in MassARRAY validation and Kathrin Jäger from the Core Unit Fluorescence Technologies IZKF University of Leipzig for cell sorting of PBMC subpopulations. We are grateful to Dr. Gunda Herberth who provided information on cytokine concentrations in the LINA cohort. We cordially thank the participants of the LINA study as well as Sissanne Arnold, Beate Fink, Anne Hain, Anna Regis and Melanie Bansch for their excellent technical assistance and fieldwork.

This work was supported by the Helmholtz Initiative on Personalized Medicine (iMed) and the Helmholtz Interdisciplinary Graduate School for Environmental Research (HIGRADE). S. Trimp was supported by the Federal Ministry of Education and Research grant 01ZX1402D.

Author contributions

L.T., M.W., M.K., M.Ba., G.H., D.K.W., D.W., O.M., C.P., M.v.B., U.S., R.E., I.L., and S.T. performed and/or coordinated experimental work. K.G., L.T., M.Bi., T.B., S.R., M.Ba., M.S., M.BH., D.K.W., D.W., O.M., and S.T. performed data analysis. M.Bo., S.R., G.H., and I.L. collected data and provided proband materials. L.T., K.G., T.P., R.E., I.L., and S.T. prepared the initial manuscript and figures. I.L., R.E., and S.T. provided project leadership.

All authors contributed to the final manuscript.

Statement of conflicts

The authors declare no conflict of interest. Correspondence and further requests should be addressed to I.L. (irina.lehmann@fz.de) or S.T. (saskia.trimp@fz.de).

166 **References**

- 167 1. Fontalba A, G tierrez O, Fernandez-L na JL. NLRP2, an Inhibitor of the NF- B
168 Pathway, Is Transcriptionally Activated by NF- B and Exhibits a Nonfunctional
169 Allelic Variant. *The Journal of Immunology*. 2007;179(12):8519-24.
- 170 2. Nieb hr M, Ba mmer K, Heratizadeh A, Satzger I, Werfel T. Impaired NLRP3
171 inflammasome expression and function in atopic dermatitis due to Th2 milieu.
172 *Allergy*. 2014;69(8):1058-67.
- 173 3. Yoshikawa Y, Sasahara Y, Take chi K, Ts jimoto Y, Hashida-Okado T, Kitano Y, et al.
174 Transcriptional Analysis of Hair Follicle-Derived Keratinocytes from Donors with
175 Atopic Dermatitis Reveals Enhanced Induction of IL32 Gene by IFN-gamma. *Int J*
176 *Mol Sci*. 2013;14(2):3215-27.
- 177 4. Winter M, Th rmann L, G  Z, Sch rmann G, Herberth G, Hinz D, et al. The benzene
178 metabolite 1,4-benzoquinone reduces regulatory T-cell function: A potential
179 mechanism for tobacco smoke-associated atopic dermatitis. *Journal of Allergy and*
180 *Clinical Immunology*. 2017;140(2):603-5.
- 181 5. Sa lyte J, Reg eira C, Montes-Martinez A, Kh dyakov P, Takko che B. Active or
182 passive exposure to tobacco smoking and allergic rhinitis, allergic dermatitis, and
183 food allergy in adults and children: a systematic review and meta-analysis. *PLoS*
184 *Med*. 2014;11(3):e1001611.
- 185 6. Ohmen JD, Hanifin JM, Nickoloff BJ, Rea TH, Wyzykowski R, Kim J, et al.
186 Overexpression of IL-10 in atopic dermatitis. Contrasting cytokine patterns with
187 delayed-type hypersensitivity reactions. *J Immunol*. 1995;154(4):1956-63.
- 188 7. Ricci G, Patrizi A, Federica B, Calamelli E, Dell'Omo V, Bendani B, et al. Cytokines
189 Levels in Children Affected by Atopic and Nonatopic Eczema. *The Open*
190 *Dermatology Journal*. 2008;2:18-21.
- 191 8. Howell MD, Novak N, Bieber T, Pastore S, Girolomoni G, Bog niewicz M, et al.
192 Interleukin-10 downregulates anti-microbial peptide expression in atopic
193 dermatitis. *J Invest Dermatol*. 2005;125(4):738-45.
- 194 9. Ji S, Shin JE, Kim YS, Oh JE, Min BM, Choi Y. Toll-like receptor 2 and NALP2 mediate
195 induction of human beta-defensins by *Staphylococcus aureus* in gingival
196 epithelial cells. *Infect Immun*. 2009;77(3):1044-52.
- 197
- 198

Figure legends

Fig. 1: *NLRP2* and *NLRP3* transcription in childhood AD.

A) Gene expression of *NLRP2* (age one: n=247 ctr vs. 153 AD, adj. odds ratio (OR)=0.83, 95% CI: 0.72-0.95; age four: n=97 ctr vs. 83 AD) and *NLRP3* (age one: n=246 vs. 150, age four: n=97 vs. 84) in children developing AD in the first six years of their life ("AD ever"). **B)** *NLRP2* transcription in early- (blue; age one: adj. OR=0.76, 95% CI: 0.65-0.89; age four: adj. OR=0.71, 95% CI: 0.52-0.95) or late-onset AD (red). Logistic regressions were adjusted for gender, maternal atopic dermatitis, parental education, smoking during pregnancy, breastfeeding, cat keeping (n (age one)= 247 control/ 53 late/100 early, n (age four)= 97 control/33 late/50 early). Please also refer to Table E2B.

Fig. 2: *NLRP2* promoter methylation in children with early-onset AD.

A) Whole genome bisulfite sequencing data of cord blood DNA showed *NLRP2* promoter hypermethylation in children developing early-onset AD (n=7) compared to healthy controls (n=6, two-way-ANOVA: p -value $<1e-17$, total of 57 CpGs, also see Table E3). The promoter region was defined based on ENCODE ChromHMM data (orange bar, black bar: sufficient coverage thereof). The gray bar depicts the region of validation (two-way ANOVA: p -value $<0.0005^{***}$, mean, $\pm$ SEM). **B)** *NLRP2* promoter methylation of children with early-onset AD (n=90) compared to healthy controls at the time of birth (n=227) and age one (n=248 controls/100 early, Mann-Whitney U-test: p -value $<0.0005^{***}$, box plots: median and upper/lower quartiles, whiskers represent non-outlier range). Logistic regression for the association of promoter methylation and early-onset AD at birth (adj. OR=4.45, 95% CI: 1.96-10.11, age one: adj. OR=3.74, 95% CI: 1.80-7.75).

Figure 1

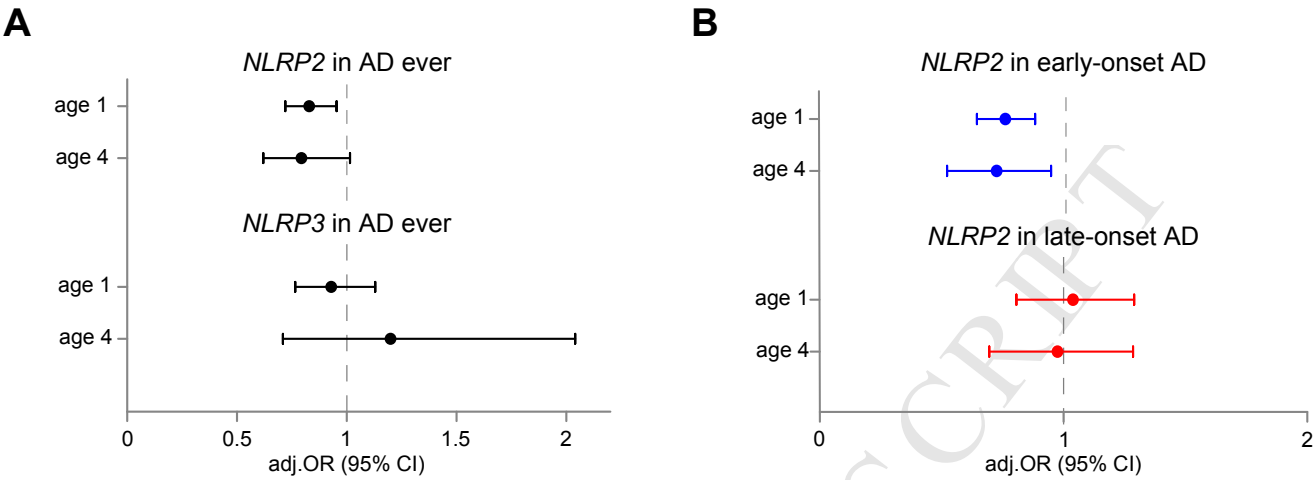
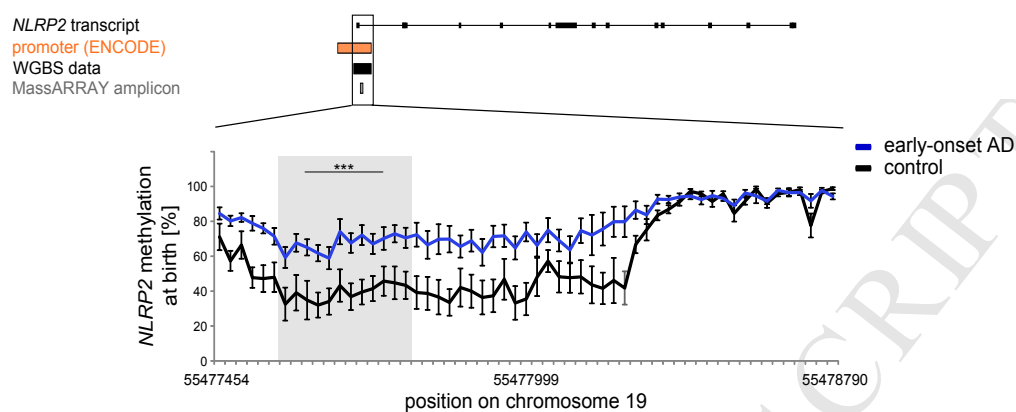


Figure 2

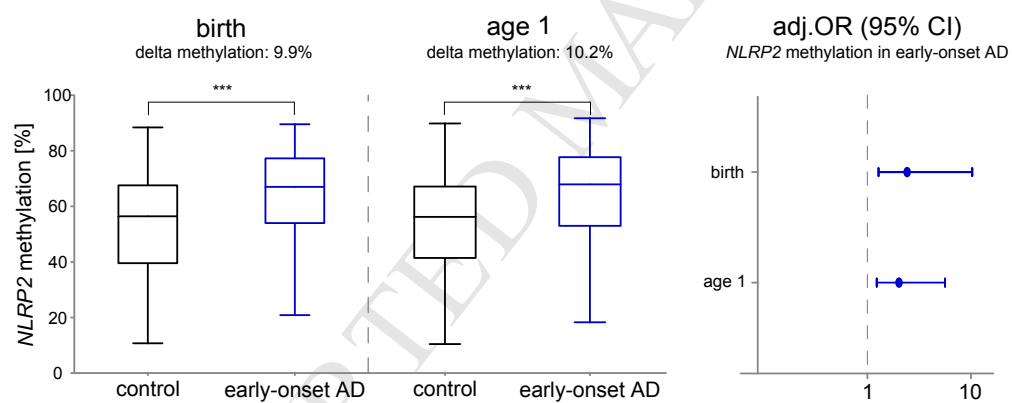
A

discovery cohort



B

entire cohort



Online Repository

Extended Methods

Study cohort and sample selection

This study is based on the prospective birth cohort LINA (Lifestyle and Environmental Factors and Their Influence on Newborn's Allergy Risk), which recruited 629 mother-child pairs from March 2006 until December 2008 in Leipzig, Germany. Standardized questionnaires (self-administered by the parents) on lifestyle/environmental exposures and atopic outcomes of the children were recorded annually. Blood samples of children were obtained at the time of birth and each following year at scheduled clinical visits.

Early-onset AD was defined as disease occurrence either reported as physician-diagnosed or as symptoms according to Hanifin and Rajka (1) criteria until the age of two (2) and late-onset AD as diagnosis after age two until the age of six. Controls were neither diagnosed with AD nor did they ever show any symptoms of AD. Participation in LINA was voluntary and approved by the Institutional Review Board of the University of Leipzig (046-2006, 160-2008, 160-2008, 160b/2008, 144-10-31052010, 113-11-18042011). Informed written consent was given by the parents.

This study is based on a subcohort of the entire LINA cohort considering all children, for whom blood samples at birth and one year after were available together with AD information up to the age of six. For cohort characteristics see Table E1. An overview of biosamples is given in Figure E1.

RNA isolation and qPCR

RNA isolation and cDNA generation was performed as previously described (3). Briefly, cDNA was used as templates for gene expression measurements on the 96.96 Dynamic Array (Fluidigm, San Francisco, CA, USA) of *NLRP2* (forward primer: 5'-

acggaagaacgaccacct -3', reverse primer: 5'- gatgacatttcctttcgtttctg -3', UPL #67) and *NLRP3* (forward primer: 5'- gtgtcctccaagctcctct -3', reverse primer: 5'- aagtgaggtggctgttcacc -3', UPL #27) using *GAPD* (forward primer: 5'- gctctctgctcctcctgttc-3', reverse primer: 5'-acgaccaaaccgttgactc-3', UPL (Roche Universal Probe Library) #60) and *GUSB* (forward primer: 5'- cgccctgcctatctgtattc -3', reverse primer: 5'- tccccacagggagtgtgtag -3', UPL #57) as reference genes. All reactions were performed in triplicates. Gene expression values were determined by $2^{-\Delta\Delta CT}$ method and normalized to the lowest measured value.

DNA-methylation analyses by WGBS

Available cord blood raw DNA-methylation values of a subset of LINA samples with available whole-genome bisulfite sequencing data (WGBS, European Genome-Phenome Archive under accession number EGAS00001000455, n=13, bisulfite conversion efficiency per sample > 99.8% (3, 4)) were used for a preliminary assessment of *NLRP2* promoter DNA-methylation differences in relation to AD development. Based on ENCODE ChromHMM definitions 57 CpGs (with a WGBS coverage <5) in the promoter were analyzed.

DNA-methylation analysis by MassARRAY

Genomic DNA of whole blood samples of time of birth, age one and age four was isolated using the QIAmp DNA Blood Mini Kit (Qiagen, Hilden, Germany), followed by bisulfite conversion using the EZ methylation kit (Zymo Research, Freiburg, Germany) according to the manufacturer's instructions (refer to Figure E1 for sample overview). As recommended by Suchiman *et al.* and Bock *et al.* (5, 6) the quality of the bisulfite treated DNA was controlled by test PCRs yielding amplicons with increasing length. The bisulfite conversion efficiency was assessed based on the "MassArray" R package version 1.28.0 (7).

Using the amplicon “*TMEM241*” (refer to (3) and Table E4) quality scores of conversion efficiency were determined to be < 2 , as recommended by Suchiman *et al.* (5). PCR primers (Table E4) for the targeted DNA-methylation analysis of the *NLRP2* promoter by MassARRAY in the entire LINA cohort - covering 12 CpGs - were designed according to the DNA-methylation changes observed in the WGBS data (Table E3). Bisulfite treated DNA was PCR amplified using HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany) with the following cycling program: 95°C for 15 min, followed by 45 cycles of 94°C for 30 sec, 52°C for 30 sec, 72°C for 1 min and a final elongation step at 72°C for 5 min on a T1 Thermocycler (Biometra, Göttingen, Germany). The PCR product was *in vitro* transcribed and enzymatically cleaved using the EpiTyper T Complete Reagent Set (Agena Bioscience, Hamburg, Germany) and subjected to mass spectrometry analysis. The validity of the derived DNA-methylation values was confirmed by correlation of expected and measured DNA-methylation values (*NLRP2*: $R^2=0.96$, Figure E3) using DNA-methylation standards (0%, 20%, 40%, 60%, 80%, and 100% methylated genomic DNA).

Cell separation and analysis

For analysis of *NLRP2/3* transcription and *NLRP2* promoter DNA-methylation in monocytes, B cells, CD4+ and CD8+ T cells, PBMCs of seven one-year-old healthy children and seven age matched children diagnosed with early-onset AD and showing AD symptoms according to Hanifin and Rajka (1) criteria were used. The genotype distribution of rs514148 was not different between the control (n(AA)= 2, n(AG)= 3, n(GG)=2) and the early-onset AD group (n(AA)= 3, n(AG)= 2, n(GG)=2; Fisher’s exact test for cross relationship: p -value=1.000).

First, cells were stained with Fixable Viability Dye eFluor 450 (#65-0863-14 eBioscience, Frankfurt, Germany) for 30 min at 4°C. Subsequently, cells were washed with PBS containing 1% FCS and incubated with Cyto-Stat Coulter Clone CD3-PC5 liquid

murine monoclonal antibody (PN 6607010, Beckman-Coulter, Krefeld, Germany), CD4 Antibody Qdot 605 conjugate, (Molecular Probes #Q10008, Thermo Fisher Scientific, Darmstadt, Germany), CD8-PE (#0452, Beckman-Coulter), CD14-FITC (PN IM0645, Beckman-Coulter) and allophycocyanin Mouse Anti-Human CD19 (Clone: HIB19: #561742, BD Bioscience) for 20 min at 4°C. Washed and stained cells were sorted on a BD FACS Aria III, SORP (BD Bioscience, Heidelberg, Germany).

Total RNA was extracted using peqGold RNA Pure (peqlab, Erlangen, Germany) following manufacturer's instructions and, except for monocyte cell fraction, genomic DNA was isolated QIAmp DNA Blood Mini Kit (Qiagen, Hilden, Germany).

Gene expression in PBMC subpopulations was assessed on a LightCycler 480 (Roche Applied Science, Mannheim, Germany) with the following settings: 5 min at 95°C, 45 cycles at 95°C for 15 s, followed by 1 min at 60°C and 30 s at 72°C using the FastStart Universal Probe Master Mix (Rox) (Roche Applied Science). A semi-quantitative approach based on external standard curves and in-run calibrator samples was used to determine gene expression normalized to the calibrator and *GAPD* as reference gene.

Genotype assessment of rs514148

To determine the genotype of the SNP rs514148 (chr19:55,474,502, GRCh37) in the subcohort with available DNA-methylation information at birth and age one (n=365), PCR products (forward primer: 5'-ttcaagacctcagacaggtt-3', reverse primer: 5'-cttactgaaggctacaggc-3', sequencing primer: 5'-aatgggtaggatcatggc-3') obtained from whole blood genomic DNA were analyzed by Sanger sequencing (GATC Biotech AG, Köln, Germany). Genotype distribution and allele frequency were tested for Hardy-Weinberg equilibrium (HWE, goodness-of-fit Chi-square, $p=0.12$, n (AA)=118, n(AG)=166, n(GG)=81).

Cytokine measurements

Concentrations of ten different cytokines (see Table E5) were determined in the supernatants of whole blood samples of one-year-old children as previously described (8).

Environmental tobacco smoke exposure

Parental smoking behavior including the number of cigarettes smoked in the homes of study participants during pregnancy was assessed by questionnaire. Indoor benzene concentrations were measured between the 34th-36th week of gestation using passive samplers (9).

Statistical analysis

Chi-square was used to test for equal parameter distribution in the subcohort and the entire LINA cohort. As non-parametric tests either rank sum analysis by Mann-Whitney U (MWU) or Spearman correlations were applied.

Logistic regression models were applied to determine odds ratios (OR) and 95% confidence intervals (CI) adjusting for possible confounders of AD (gender, maternal atopic dermatitis, parental educational level, smoking during pregnancy, breastfeeding and cat keeping). Transcription and DNA-methylation models were additionally adjusted for gestational age and mode of delivery. Multiple regression models were applied for the association analysis of continuous variables, results are given as mean ratios (MR) with 95% CIs. All continuous variables were ln-transformed.

Two-way analysis of variance (ANOVA) was applied to assess differential methylated CpGs in the *NLRP2* promoter. To evaluate the relationship of indoor benzene concentrations and tobacco smoke exposure one-way ANOVA was applied. Genotype differences in *NLRP2* transcription and promoter DNA-methylation were assessed by Kruskal-Wallis test.

To evaluate the relationship of prenatal benzene exposure, *NLRP2* DNA-methylation, -

transcription and AD development a mediation analysis was conducted (PROCESS macro v2.16 for SPSS, www.processmacro.org) with a bootstrap approach using 10000 samples to estimate the statistical significance of the indirect effect as bias-corrected CIs (10).

P -values ≤ 0.05 were considered significant. Analyses were performed in either STATISTICA 13.0 for Windows (Dell Inc., USA), SigmaPlot version 12.0 (Systat Software GmbH, Erkrath, Germany), IBM SPSS Statistics version 22 (IBM Corps., USA) or R 3.0.0 (11).

References

1. Hanifin JM, Rajka G. Diagnostic features of atopic eczema. *Acta Derm Venereol.* 1980;92:44-7.
2. Carlsten C, Dimich-Ward H, Ferguson A, Watson W, Rousseau R, Dybuncio A, et al. Atopic dermatitis in a high-risk cohort: natural history, associated allergic outcomes, and risk factors. *Ann Allergy Asthma Immunol.* 2013;110(1):24-8.
3. Bauer T, Trump S, Ishaque N, Thurmann L, Gu L, Bauer M, et al. Environment-induced epigenetic reprogramming in genomic regulatory elements in smoking mothers and their children. *Mol Syst Biol.* 2016;12(3):861.
4. Trump S, Bieg M, Gu Z, Thurmann L, Bauer T, Bauer M, et al. Prenatal maternal stress and wheeze in children: novel insights into epigenetic regulation. *Sci Rep.* 2016;6:28616.
5. Suchiman HE, Slieker RC, Kremer D, Slagboom PE, Heijmans BT, Tobi EW. Design, measurement and processing of region-specific DNA methylation assays: the mass spectrometry-based method EpiTYPER. *Front Genet.* 2015;6:287.
6. Bock C, Halbritter F, Carmona FJ, Tierling S, Datlinger P, Assenov Y, et al. Quantitative comparison of DNA methylation assays for biomarker development and clinical applications. *Nat Biotechnol.* 2016;34(7):726-+.
7. Thompson RF, Suzuki M, Lau KW, Grealley JM. A pipeline for the quantitative analysis of CG dinucleotide methylation using mass spectrometry. *Bioinformatics.* 2009;25(17):2164-70.
8. Englich B, Herberth G, Rolle-Kampczyk U, Trump S, Röder S, Borte M, et al. Maternal cytokine status may prime the metabolic profile and increase risk for obesity in children. *International Journal of Obesity.* 2017.
9. Winter M, Thürmann L, Gu Z, Schüürmann G, Herberth G, Hinz D, et al. The benzene metabolite 1,4-benzoquinone reduces Treg function - a potential mechanism for tobacco smoke-associated atopic dermatitis. *Journal of Allergy and Clinical Immunology.* 2017.
10. Hayes AF. *Introduction to Mediation, Moderation, and Conditional Process Analysis: A Regression-Based Approach*: Guilford Press; 2013. 507 p.
11. Team RC. *R: A language and environment for statistical computing*. R Foundation for Statistical Computing Vienna, Austria 2013 [Available from: <http://www.R-project.org/>].

Figure legends

Fig. E1: Sample overview of the analyzed subcohort.

Given are the numbers of available blood samples used for cytokine measurements in children with AD and the corresponding controls of the LINA cohort. In addition, available samples for DNA-methylation, transcription and genotype analyses at the time of birth, at the age of one and four are shown.

Fig. E2: AD incidence.

Incidences of AD in the LINA cohort up to the age of six are shown separately for children with early- or late-onset AD. The percentage was calculated as the number of new AD cases related to the number of valid questionnaires per year.

Fig. E3: Quality control of the MassARRAY amplicon used for *NLRP2* promoter DNA-methylation assessment.

The graph shows DNA-methylation values derived by MassARRAY measurements of standard samples (0%, 20%, 40%, 60%, 80%, and 100% methylated genomic DNA) representing the mean DNA-methylation of all 12 CpGs within the MassARRAY amplicon (given are mean DNA-methylation values of four independent measurements, +/- SD).

Fig. E4: Relationship between *NLRP2* DNA-methylation and transcription.

A) *NLRP2* promoter DNA-methylation is significantly negatively correlated with *NLRP2* transcription at the time of birth (n=326) and age one (n=386, *p*-value from Spearman correlation). **B)** This relationship is sustained in the adjusted regression model at the time of birth and age one (birth: adj. MR=0.57, 95% CI: 0.63-0.71; age one: adj. MR=0.57, 95% CI: 0.63-0.69; MRs calculated by multiple regression, adjusted for AD confounders, mode of delivery and gestational age).

Fig. E5: *NLRP2* and *NLRP3* transcription in PBMC subpopulations of children with early-onset AD.

A) *NLRP2* transcription is decreased in children with early-onset AD in comparison to healthy controls in the different PBMC cell subtypes investigated (two-way ANOVA: *p*-value=0.0003). **B)** *NLRP2* promoter hypermethylation observed in children with early-onset AD compared to healthy controls in the different PBMC subpopulations. DNA methylation was determined by MassARRAY. Numbers in the black bars indicate the DNA methylation difference between healthy controls and children suffering from early-onset AD (two-way ANOVA: *p*-value=0.009). **C)** Normalized *NLRP3* expression in the different PBMC subtypes showed no difference comparing children with early-onset AD to healthy controls. (**p*-value< 0.05 and ****p*-value< 0.0005 from two-way ANOVA, mean +/- SEM, n=7 AD, n= 7 controls; n= 6 AD and n= 5 controls for *NLRP2* in monocytes; n= 6 AD and n= 5 controls for *NLRP2* methylation in CD8⁺ T-cells)

Fig. E6: *NLRP2* regulation is influenced by the genotype.

NLRP2 transcription and promoter methylation is dependent on the genotype of rs514148 (chr19:55474502, human genome assembly GRCh37) located upstream of the *NLRP2* promoter (line represents the median, *p*-value from Kruskal-Wallis test, n(eQTL)=111(AA)/ 158(AG)/ 78(GG) and n(meQTL)= 118(AA)/ 166(AG)/ 81(GG)).

Fig. E7: Indoor benzene concentrations as a proxy for smoking behavior.

Shown are ln-transformed indoor benzene concentrations measured in the homes of study participants during pregnancy and their relationship to **A)** the frequency of indoor smoking behavior (n=378 never-, n=27 occasional-, n=25 almost daily smokers), and **B)** the total number of cigarettes smoked in the homes of the study participants (no cigarettes: n=374, 1-10 cigarettes per day: n=31, >10 cigarettes per day: n= 14). Graphs depict mean +/- SEM.

Fig. E8: Effect of prenatal tobacco smoke exposure on early-onset AD is mediated by *NLRP2*.

The mediation analysis suggests that prenatal exposure to high benzene concentrations influences the development of early-onset AD via changes in *NLRP2* promoter methylation and -transcription. The table summarizes unstandardized effect sizes (n=292, adjusted for AD confounders and rs514148 genotype, significance determined by bias-corrected 90% CI of 10000 bootstrapped samples, n.a.: no *p*-values are calculated for this model type by the PROCESS macro, effects are significant if confidence intervals do not contain 0).

Table E1: Characteristics of the entire LINA cohort and the investigated AD subcohort.

	entire cohort n= 629 (%)*	subcohort n=430 (%)*	p-value[#]
gender of the child			
male	330 (52.5)	234 (54.4)	0.573
female	299 (47.5)	196 (45.6)	
mode of delivery			
spontaneous	469 (74.6)	339 (78.9)	0.665
caesarean section	126 (20.0)	84 (19.5)	
gestational age			
33 rd -38 th week	124 (19.7)	83 (19.3)	0.723
39 th -41 st week	427 (67.9)	287 (66.7)	
42 nd - 43 rd week	77 (12.2)	60 (14.0)	
tobacco smoke exposure during pregnancy**			
(almost) daily	48 (7.6)	25 (5.8)	0.366
occasionally	47 (7.5)	27 (6.3)	
never	534 (84.9)	378 (87.9)	
breast-feeding (until 6th month)			
yes	442 (70.3)	313 (72.8)	0.719
no	139 (22.1)	105 (24.4)	
parental education level***			
low	16 (2.5)	9 (2.1)	0.736
intermediate	144 (22.9)	106 (24.7)	
high	469 (74.6)	315 (73.3)	
cat keeping			
yes	95 (15.1)	66 (15.3)	0.955
no	511 (81.2)	364 (84.7)	
maternal history of atopic dermatitis			
positive	112 (17.8)	74 (17.2)	0.866
negative	517 (82.2)	356 (82.8)	
atopic dermatitis			
never	378 (60.1)	264 (61.4)	0.929
ever (1 st to 6 th year)	228 (36.2)	166 (38.6)	
early-onset (1 st or 2 nd year)	138 (21.9)	105 (24.4)	
late-onset (> 2 nd year)	90 (14.3)	61 (14.2)	

* numbers may be different from total sum due to missing data

[#] p-value from chi-squared test for cross relationship^{**} answers based on question "Did you or anybody else smoke inside your home during the last 12 months?"^{***} low, 9 years of schooling or less "Hauptschulabschluss"; intermediate, 10 years of schooling "Mittlere Reife"; high, 12 years of schooling or more "(Fach-)hochschulreife"

Table E2: (A) ORs for risk increase of AD development in association to transcription of *NLRP2* and *NLRP3*. **(B)** Mean and median of normalized expression values of *NLRP2* for controls (n=247, age one/ 97, age four) and children who develop AD ever (n=153, age one/ 83, age four) or early- (n=100, age one/ 50, age four) or late-onset AD (n=53, age one/ 33, age four) up to the age of six.

(A)

		raw model			adjusted model		
gene	age	n (control/AD)	OR (CI 95%)	p-value	n (control/AD)	adj.*OR (CI 95%)	p-value
AD ever							
NLRP2	1	253/ 159	0.82 (0.72-0.94)	0.004	247/ 153	0.83 (0.72-0.95)	0.009
NLRP2	4	99/ 87	0.81 (0.65-1.01)	0.064	97/ 83	0.79 (0.62-1.02)	0.064
NLRP3	1	252/ 156	0.91 (0.76-1.10)	0.319	246/ 150	0.93 (0.77-1.13)	0.456
NLRP3	4	99/ 88	1.13 (0.69-1.86)	0.619	97/ 84	1.20 (0.71-2.04)	0.490
early-onset AD							
NLRP2	1	253/ 102	0.76 (0.65-0.88)	0.0002	247/ 100	0.76 (0.65-0.89)	0.0005
NLRP2	4	99/ 52	0.71 (0.55-0.93)	0.011	97/ 50	0.71 (0.52-0.95)	0.021
NLRP3	1	252/ 99	0.89 (0.72-1.11)	0.311	246/ 97	0.49 (0.75-1.18)	0.610
NLRP3	4	99/ 53	1.15 (0.65-2.03)	0.620	97/ 51	1.24 (0.66-2.34)	0.497
late-onset AD							
NLRP2	1	253/ 57	1.01 (0.82-1.25)	0.897	247/ 53	1.02 (0.81-1.29)	0.856
NLRP2	4	99/ 35	0.98 (0.74-1.82)	0.874	97/ 33	0.95 (0.70-1.29)	0.720
NLRP3	1	252/ 57	0.94 (0.72-1.22)	0.633	246/ 53	0.90 (0.68-1.02)	0.481
NLRP3	4	99/ 35	1.10 (0.55-2.22)	0.780	97/ 33	1.15 (0.53-2.50)	0.717

* logistic regression adjusted for gender, breast-feeding, maternal history of atopic eczema, parental educational level, cat keeping and prenatal tobacco smoke exposure

(B)

gene (age)	control		AD ever		early-onset AD		late-onset AD	
	mean (SEM)	median (IQR)	mean (SEM)	median (IQR)	mean (SEM)	median (IQR)	mean (SEM)	median (IQR)
<i>NLRP2</i> (1)	6.62 (0.09)	6.83 (5.82-7.57)	6.17 (0.14)	6.46 (5.47-7.31)	5.89 (0.19)	6.38 (5.01-7.18)	6.68 (0.17)	6.73 (5.82-7.47)
<i>NLRP2</i> (4)	5.93 (0.13)	6.18 (5.26-6.89)	5.51 (0.14)	5.59 (4.73-6.37)	5.26 (0.16)	5.29 (4.54-6.29)	5.89 (0.26)	6.13 (5.18-6.82)

Table E3: *NLRP2* promoter DNA-methylation differences between children with early-onset AD (n=7) and controls (n=6) based on WGBS data. CpGs considered in the MassARRAY analysis are highlighted with a gray background. Shown are Bonferroni-corrected *p*-values from two-way ANOVA.

genomic position of CpG*	mean DNA- methylation control (SE) [%]	mean DNA- methylation AD (SE) [%]	delta DNA- methylation [%]	Bonferroni corrected <i>p</i> -value
chr19:55477454	71,1 (7,6)	84,5 (3,5)	13,4	0.367
chr19:55477470	57,4 (5,7)	80,3 (3)	22,9	0.099
chr19:55477474	66,5 (7,9)	82,2 (2,5)	15,7	0.261
chr19:55477499	47,8 (5,9)	78,9 (4,2)	31,1	0.014
chr19:55477557	47,2 (7,7)	76 (2,9)	28,8	0.015
chr19:55477561	48 (8,5)	71,4 (4,8)	23,4	0.031
chr19:55477633	32,6 (9,4)	59,3 (6)	26,7	<0.001
chr19:55477653	39,1 (10,2)	67,7 (5,1)	28,6	0.002
chr19:55477670	34,9 (11,1)	65,1 (4,7)	30,2	0.017
chr19:55477697	32 (7,2)	61,8 (4,6)	29,8	<0.001
chr19:55477723	34,1 (7,5)	59 (6,4)	24,9	0.007
chr19:55477729	43,2 (9,3)	74,1 (7,2)	30,9	0.004
chr19:55477734	36,9 (7,6)	67,6 (5,6)	30,8	<0.001
chr19:55477755	39,5 (7,1)	72,2 (5,7)	32,7	0.002
chr19:55477759	41,5 (7,2)	67,1 (5,8)	25,6	0.011
chr19:55477807	45,8 (8,4)	70,2 (6,5)	24,5	0.021
chr19:55477810	44,8 (9,4)	72,9 (4,9)	28,1	0.003
chr19:55477813	43,4 (7,8)	70,6 (5,8)	27,2	0.01
chr19:55477849	39,2 (9,4)	72,4 (6,9)	33,2	<0.001
chr19:55477936	38,7 (9,5)	66,6 (8)	27,8	<0.001
chr19:55477944	36,6 (9,9)	69,8 (8,6)	33,2	<0.001
chr19:55477952	33,5 (7,6)	69,8 (7,6)	36,3	<0.001
chr19:55477968	42,1 (10,9)	65,6 (6,3)	23,5	0.202
chr19:55477974	40 (9,5)	68,9 (6,3)	28,9	0.107
chr19:55477976	36,4 (9,7)	62,3 (7,7)	25,8	0.107
chr19:55477982	37,4 (9,4)	71,3 (6,2)	34,0	<0.001
chr19:55477999	46,8 (11,7)	71,7 (6,4)	25,0	0.002
chr19:55478006	33,3 (9,6)	64,8 (6,6)	31,6	<0.001
chr19:55478038	35,5 (9,3)	73,9 (5,4)	38,4	<0.001
chr19:55478104	48,1 (10,9)	66,3 (6,4)	18,2	0.027
chr19:55478140	57,3 (6,3)	75 (6,9)	17,7	0.224
chr19:55478145	48,2 (9)	69,1 (6,3)	20,8	0.058
chr19:55478161	47,8 (8,7)	63,7 (7,8)	15,9	0.141
chr19:55478173	48,2 (9,6)	74,7 (7,1)	26,5	0.017
chr19:55478183	43,6 (10,6)	72,2 (11,6)	28,5	0.007
chr19:55478185	41,6 (9)	75,6 (9,4)	34,0	0.002
chr19:55478262	46,1 (13)	79,8 (8,6)	33,7	0.063
chr19:55478278	41,9 (9,5)	79,8 (8,8)	37,9	<0.001
chr19:55478321	66,8 (5)	86,5 (5)	19,7	0.222
chr19:55478348	75,2 (6,2)	83,7 (5,3)	8,5	0.609
chr19:55478442	83,5 (2,8)	92,7 (2,6)	9,2	0.626
chr19:55478472	86,9 (2,3)	92,6 (2,1)	5,6	0.772
chr19:55478524	91,2 (2,2)	93,9 (2,1)	2,8	0.89
chr19:55478526	97,2 (1,4)	94,6 (1,9)	-2,6	0.897
chr19:55478530	95,8 (1,4)	92,5 (2,8)	-3,2	0.865
chr19:55478588	91,4 (3,1)	94,8 (2,7)	3,4	0.867
chr19:55478598	95,8 (1,4)	93,1 (1,4)	-2,7	0.893
chr19:55478603	84,6 (4,7)	89,1 (3,4)	4,6	0.793
chr19:55478616	91,7 (2,1)	96,3 (1,8)	4,6	0.82
chr19:55478621	98,6 (1,4)	94,6 (4,2)	-4,0	0.826
chr19:55478640	90,3 (2,6)	91,6 (1,7)	1,2	0.946
chr19:55478648	95,9 (1,5)	97,8 (1,1)	1,9	0.927
chr19:55478710	96,6 (2,4)	96,4 (1,8)	-0,2	0.995
chr19:55478718	98,2 (1,3)	96,5 (1,5)	-1,7	0.935
chr19:55478774	77,7 (6,9)	91,8 (3,9)	14,1	0.401
chr19:55478781	97,1 (1)	97,7 (1,5)	0,7	0.976
chr19:55478790	98,8 (0,8)	94,3 (1,7)	-4,5	0.827

* human genome assembly GRCh37

Table E4: MassArray primer

assay name	amplicon coordinate*	forward primer (5'→3')	reverse primer(5'→3')
<i>TMEM241</i>	chr18:21002501-21002763	x-ttatgtttggtttatagtattag	y- tatactaccctacactatac
<i>NLRP2</i>	chr19: 55477611-55477835	x-tggaggattataggtggag	y- tcccaacacctaaccctac

x=aggaagagag, y=cagtaatacgactcactataggagaaggct

* human genome assembly GRCh37

Table E5: Cytokine protein concentrations in sera of one-year-old children: controls (n= 262), diagnosed with early-onset AD (n=62).

cytokine	mean control (SE) [pg/ml]	mean early-onset AD (SE) [pg/ml]	median control (IQR) [pg/ml]	median early-onset AD (IQR) [pg/ml]	p-value [#]	Bonferroni corrected p-value <0.05
IL-4	8.4 (0.3)	8.0 (0.5)	7.3 (5.8-9.3)	7.1 (5.8-9)	0.7056	no
IL-5	4 (0.1)	4.2 (0.3)	3.8 (3.3-4.4)	3.8 (3.5-4.3)	0.5887	no
IL-6	71.2 (18.2)	94.4 (32.1)	24.1 (13.1-46.5)	32.4 (15.3-71.6)	0.1295	no
IL-8	442.7 (33.4)	751.3 (139.7)	223.2 (106.2-557)	402.1 (105.3-745.7)	0.1506	no
IL-10	9.4 (0.2)	11.0 (0.8)	8.5 (7.1-10.5)	9.8 (8.2-11.8)	0.0001	0.001
IL-12	6.4 (0.3)	6.5 (0.4)	5.6 (4.6-7.1)	6.0 (4.6-7.9)	0.2962	no
IL-13	8.1 (0.3)	8.4 (0.5)	7.5 (4.7-10.2)	8.1 (6.2-10.5)	0.2667	no
IFN γ	10.4 (0.4)	10.8 (1.7)	10.2 (5.1-14.1)	9.7 (2.8-13.4)	0.2525	no
MCP	352.1 (22.9)	465 (64.7)	232.9 (154.9-416.4)	275.5 (154.5-549)	0.4357	no
TNF α	44.8 (12.5)	62.0 (22.4)	19.2 (12.1-34.9)	21.6 (13.6-34.3)	0.3741	no

[#] p-value from Mann-Whitney U-test

ACCEPTED MANUSCRIPT

Figure E1

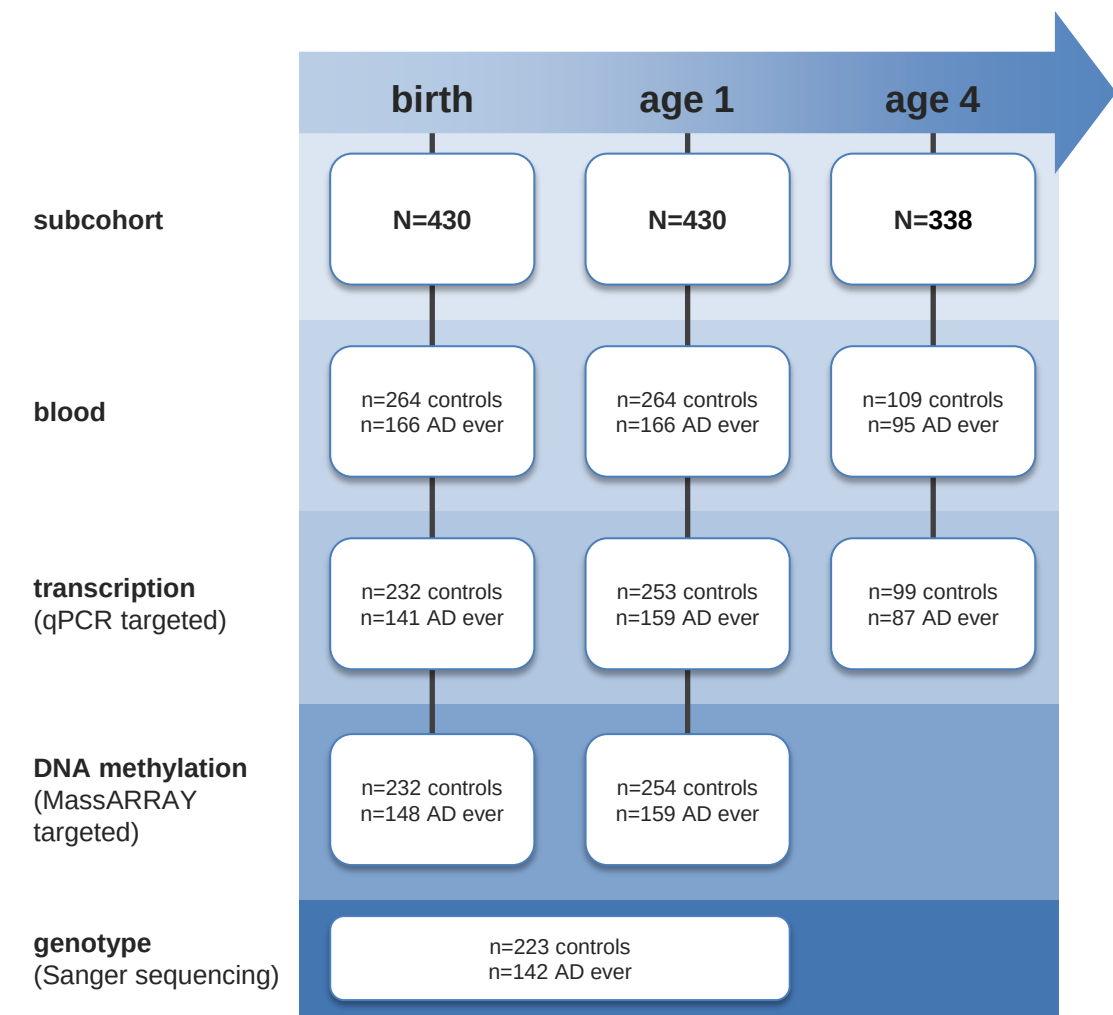


Figure E2

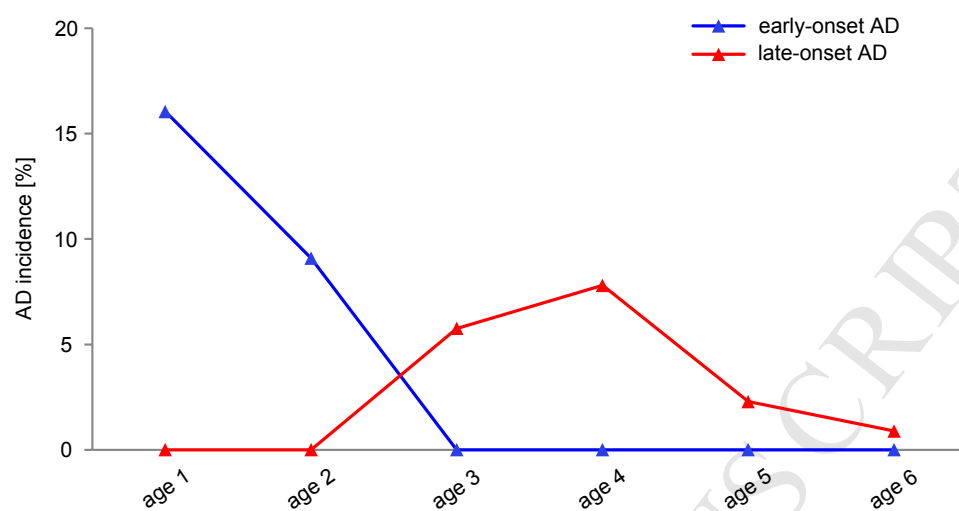


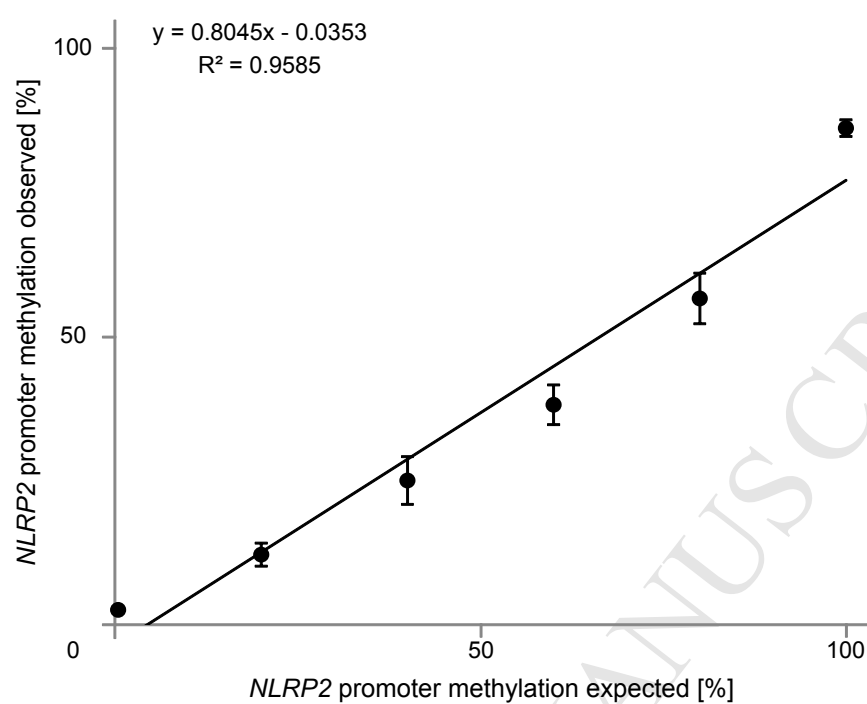
Figure E3

Figure E4

ACCEPTED MANUSCRIPT

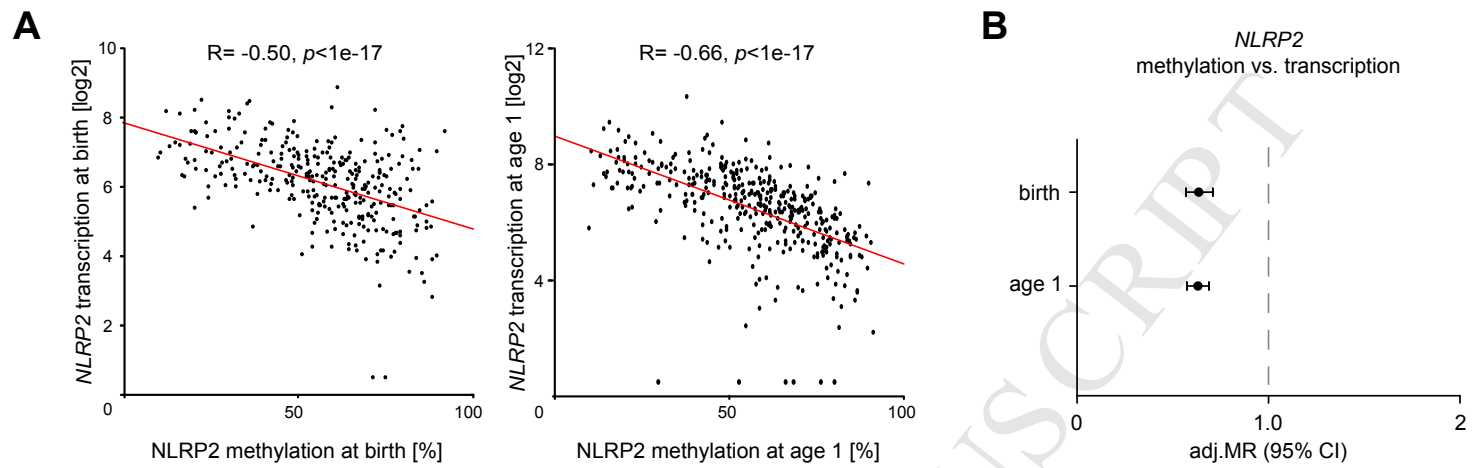


Figure E5

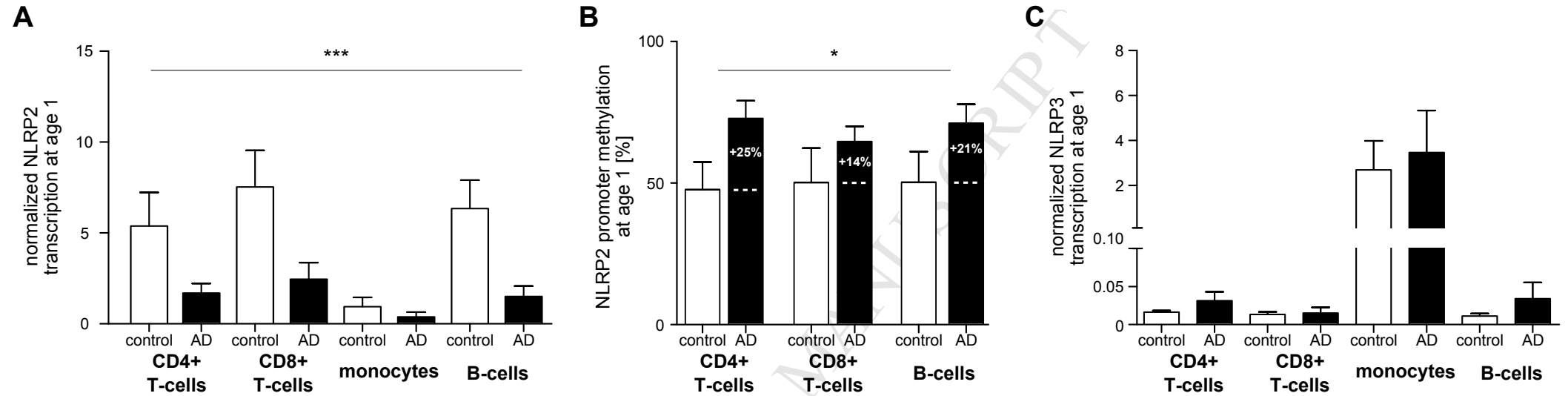


Figure E6

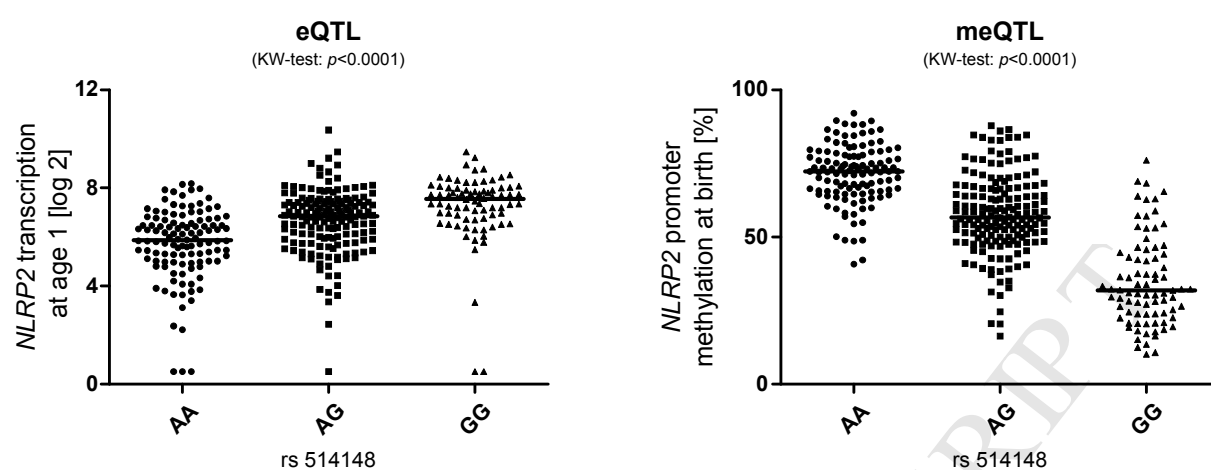
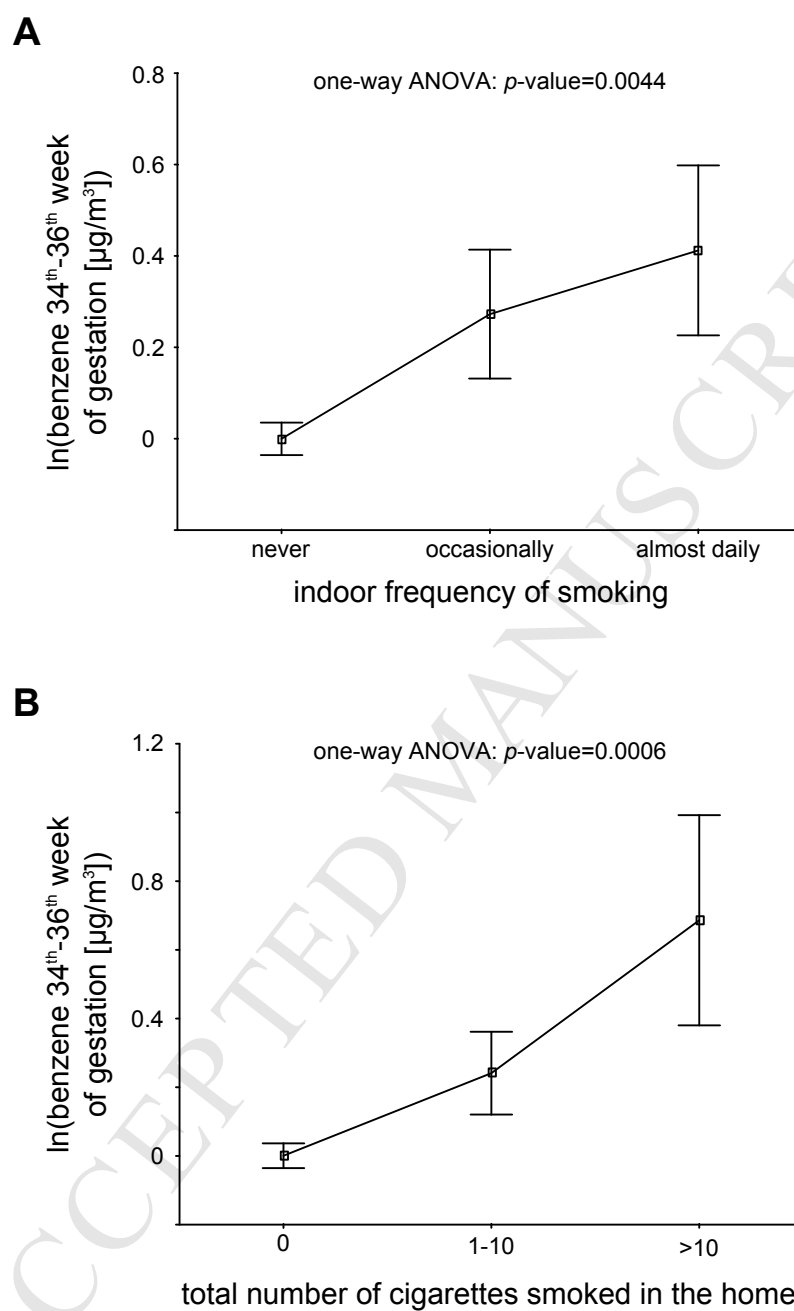
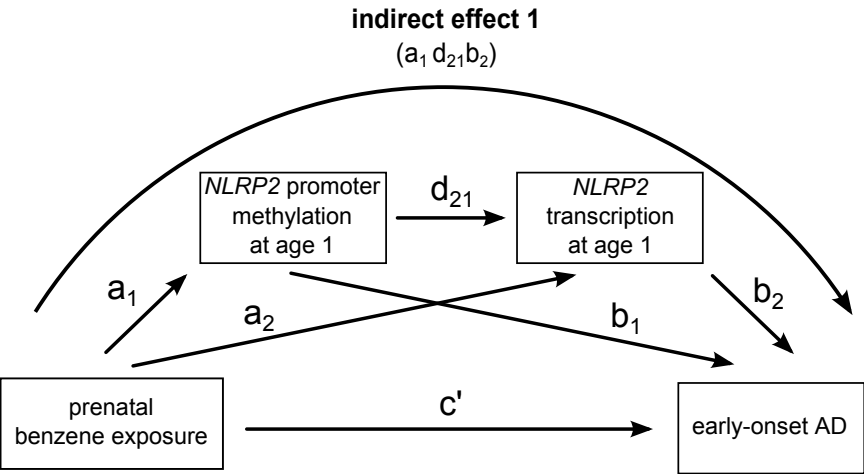


Figure E7



age 1	p-value	lower CI	higher CI	effect size
a_1	0.0347	0.0109	0.0871	0.0490
a_2	0.6108	-0.2337	0.1234	-0.0551
b_1	0.0316	0.3083	2.3199	1.3141
b_2	0.0461	-0.3705	-0.0356	-0.2031
d_{21}	<0.0000	-2.0217	-1.1095	-1.5656
c'	0.7984	-0.2973	0.4066	0.0546
indirect effect 1 ($a_1 d_{21} b_2$)	n.a.	0.0112	0.1730	0.0644
indirect effect 2 ($a_1 b_1$)	n.a.	0.0014	0.0463	0.0156
indirect effect 3 ($a_2 b_2$)	n.a.	-0.0113	0.0652	0.0112