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Published in:
American Journal of Physiology - Lung Cellular and Molecular Physiology

DOI:
10.1152/ajplung.00293.2019

Am J Physiol Lung Cell Mol Physiol 318: L549–L561, 2020.
First published January 8, 2020; doi:10.1152/ajplung.00293.2019.

RESEARCH ARTICLE

Prenatal smoke effect on mouse offspring *Igf1* promoter methylation from fetal stage to adulthood is organ and sex specific

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Submitted 17 July 2019; accepted in final form 22 December 2019

Zeng Z, Meyer KF, Lkhagvadorj K, Kooistra W, Reinders-Luinge M, Xu X, Huo X, Song J, Plösch T, Hylkema MN. Prenatal smoke effect on mouse offspring *Igf1* promoter methylation from fetal stage to adulthood is organ and sex specific. *Am J Physiol Lung Cell Mol Physiol* 318: L549–L561, 2020. First published January 8, 2020; doi:10.1152/ajplung.00293.2019.—Prenatal smoke exposure (PSE) is associated with reduced birth weight, impaired fetal development, and increased risk for diseases later in life. Changes in DNA methylation may be involved, as multiple large-scale epigenome-wide association studies showed that PSE is robustly associated with DNA methylation changes in blood among offspring in early life. Insulin-like growth factor-1 (IGF1) is important in growth, differentiation, and repair processes after injury. However, no studies investigated the organ-specific persistence of PSE-induced methylation change of *Igf1* into adulthood. Based on our previous studies on the PSE effect on *Igf1* promoter methylation in fetal and neonatal mouse offspring, we now have extended our studies to adulthood. Our data show that basal *Igf1* promoter methylation generally increased in the lung but decreased in the liver (except for 2 persistent CpG sites in both organs) across three different developmental stages. PSE changed *Igf1* promoter methylation in all three developmental stages, which was organ and sex specific. The PSE effect was less pronounced in adult offspring compared with the fetal and neonatal stages. In addition, the PSE effect in the adult stage was more pronounced in the lung compared with the liver. For most CpG sites, an inverse correlation was found for promoter methylation and mRNA expression when the data of all three stages were combined. This was more prominent in the liver. Our findings provide additional evidence for sex- and organ-dependent prenatal programming, which supports the developmental origins of health and disease (DOHaD) hypothesis.

methylation persistence/reversibility; mouse liver and lung; prenatal smoke; pyrosequencing; three developmental stages

INTRODUCTION

Prenatal exposure to cigarette smoke during pregnancy is an environmental insult that has profound effects on DNA methylation patterns of the exposed fetus (42). Mounting evidence from population studies has identified prenatal smoke exposure (PSE)-associated alterations in global methylation in candidate gene and epigenome-wide association studies (EWAS) in children and adolescents (3, 4, 18, 24, 28, 39–41). Fetal exposure to maternal smoking in utero has been linked to adverse perinatal outcomes including low birth weight, elevated blood pressure, and obesity (25, 37). Furthermore, maternal smoking during pregnancy has been causally linked to the development of lung diseases, such as asthma and chronic obstructive pulmonary disease (COPD) (1, 12, 15). One initial study in a fetal rat model has demonstrated that PSE induced a smaller lung volume, lower number of saccules and septal crests, and decreased elastin fibers in the lung (9). It has been proposed that epigenetic modifications such as DNA methylation may mediate the adverse developmental consequences associated with smoking during pregnancy (21, 34). Of particular importance is the observation that maternal smoking during pregnancy is associated with changes in methylation in genes involved in fundamental developmental processes (7).

Previously, we reported the detrimental effects of PSE on promoter methylation of *Igf1* and *Igf1r*, which are involved in the regulation of pre- and postnatal development, using a fetal and neonatal mouse model (32, 33). The results from this study indicated that PSE contributes organ and sex specifically to the prenatal programming of methylation. Furthermore, the comparison between fetuses and neonates suggested the reversibility but also the persistence of PSE-induced differences in methylation patterns over time at the two time points (33). However, it is not clear whether the observed PSE-induced DNA methylation alterations persist throughout life or return to the baseline methylation levels existing in nonexposed animals. Moreover, it is unknown if the changes in PSE-induced DNA methylation are adaptive, proving to be beneficial later in life, or merely functionally neutral biological biomarkers, or perhaps even detrimental to the health of the affected offspring

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later on. Hence, in the current study, we aimed to investigate the specific CpG site-dependent reversibility and persistence of PSE-induced methylation patterns from fetal to adulthood and to address: first, the PSE effects on DNA methylation of *Igf1* in adult lung and liver tissues of male and female offspring; second, baseline DNA methylation patterns of *Igf1* across three developmental stages in normal lung and liver tissues of male and female offspring; third, the persistence/reversibility of PSE-induced DNA methylation across three different stages, comparing lung and liver tissues of male and female offspring; and finally, link PSE-induced changes of *Igf1* mRNA expression with promoter methylation, body weight and lung inflammation.

MATERIALS AND METHODS

Animals and cigarette smoke exposure. A total of 48 female and 48 male C57BL/6J mice were obtained from Harlan (Horst, The Netherlands) at 6 wk of age and housed under standard conditions with food and water provided ad libitum, with a 12-h light-dark cycle. The experimental setup was approved by the local committee on animal experimentation (DEC6589 B and C; University of Groningen, Groningen, The Netherlands) and under strict governmental and international guidelines on animal experimentation.

Mainstream cigarette smoke was generated by using Teague10 (Tobacco and Health Research Institute of the University of Kentucky, Lexington, KY). Over a period of 7 days, randomly selected primiparous female mice were adjusted to cigarette smoke by stepwise increasing the number of smoked cigarettes (3R4 cigarettes; 2.45 mg nicotine/cigarette) from two to five per smoking session. At adjustment day five after the end of the second smoking session, all female mice were injected with PMSG (1.25 IU) to stimulate ovulation and at day seven with hCG (1.25 IU) to induce ovulation and housed on a 1:1 mating ratio with males overnight. Mating was confirmed by the presence of vaginal plug the following morning.

Female mice were exposed to two air or whole body smoking sessions of 50 min with 3-h interval between both exposures per day, 7 days per week throughout gestation and housed in groups. After delivery, dams and their offspring were no longer exposed to cigarette smoke and were housed individually.

Each 12 male and 12 female fetuses of 5 smoke-exposed and 4 control dams were collected at embryonic stage 17.5 (E17.5). Their dams were euthanized under anesthesia. A total of 42 pups randomly selected from 9 smoke-exposed (11 male, 8 female) and 10 control (11 male, 12 female) dams were euthanized at postnatal day 3 (D3) for collection of lung and liver. Another 34 pups randomly selected from the same 9 smoke-exposed (6 male, 6 female) and 10 control (10 male, 12 female) dams were exposed to air in the Teague 10, from 8 wk of age for the following 12 wk (Adult) until euthanized for collection of lung and liver. Exposure to air in the Teaque 10 was necessary as these mice were part of a bigger study on pre- and or postnatal smoke exposure, aging, and COPD. The liver was immediately frozen in liquid nitrogen and stored at −80°C until further use. From the right lung, three fourth of the right lobes were immediately frozen and stored at −80°C, whereas the smallest lobe lung was fixed in 4% paraformaldehyde and embedded in paraffin for immunohistochemical analyses. The left lung was used for RNA and DNA isolation.

Isolation of DNA and RNA. Genomic DNA and total RNA were isolated using the AllPrep DNA/RNA Mini Kit (cat. no. 80204; Qiagen), according to the manufacturer's protocol.

Bisulfite pyrosequencing analysis. To assess the methylation level of *Igf1* gene promoter, bisulfite pyrosequencing was used. The selection of CpG sites located at the promoter region of *Igf1* was based on manual identification of CpG dinucleotides, using the ENSEMBL genome web browser. We focused on the mouse *Igf1* (ENSMUSG00000020053): transcript *Igf*-005 (ENSMUST00000122386) in this study. Extracted genomic DNA from lung and liver tissue was converted with sodium bisulfite following the manufacturer's instructions (catalog no. D5020, EZ DNA methylation-Direct Kit; Zymo Research). Pyrosequencing was used Pyromark PCR kit (cat. no. 978703; Qiagen) and performed on the PyroMarkQ24 (Qiagen) instrument. The analyses were performed as previously described (33).

mRNA expression analysis. cDNA was reversely transcripted by a Superscript II Reverse Transcriptase Kit. Quantitative real-time PCR (qRT-PCR) analysis for mRNA levels of *Igf1* (Mm00439560_m1), *Il1b* (Mm00434228_m1), *Il6* (Mm00446190_m1), *Tnfa* (Mm00443258_m1), and *Tgfb* (Mm01298616_m1) was performed using TaqMan Gene Expression Assays (Thermo Fisher Scientific, Carlsbad, CA) and normalized to the housekeeping gene *Gapdh* (Applied Biosystems, Mm99999915_g1). The analyses were performed as previously described (33).

Immunohistochemistry (IHC). Sections (3 μm) of formalin-fixed and paraffin-embedded lung tissue were used for double staining of MAC3 (macrophages, monoclonal rat anti-MAC3; BD Biosciences) and IRF5 (rabbit α-IRF5, ProteinTech Europe, Manchester UK), as well as MAC3 and YM1 (polyclonal goat anti-mouse eosinophil chemotactic factor (ECF-L, R&D Systems). To visualize MAC3, an immune alkaline phosphatase procedure was used with Fast Blue BB salt (Sigma Aldrich, Zwijndrecht, The Netherlands). IRF5 was visualized with ImmPACT NovaRED kit (Vector, Burlingame, CA). YM1 was visualized with 3-amino-9-ethylcarbazole (Sigma-Aldrich). The numbers of MAC3-positive/IRF5-positive and MAC3-positive/YM1-positive cells were counted manually in parenchymal lung tissue at ×20 magnification, and these numbers were corrected for the total area of lung tissue section as assessed by morphometric analysis using Aperio ImageScope viewing software 11.2.0.780 (Aperio, Vista, CA).

Eosinophils were determined by staining 4 μm cryosections of lung tissue for cyanide-resistant endogenous peroxidase activity with diaminobenzidine (Sigma-Aldrich). The number of eosinophils (4 random microscopic fields per lung section) was counted manually in a blinded manner at ×8 magnification and averaged. Neutrophils [glutathione-disulfide reductase (GR1)] and monoclonal rat anti GR1 antibody (BD Biosciences) were counted manually in a blinded manner at ×20 magnification, and numbers were corrected for the area that was counted (6 fields per section) by morphometric analysis using Aperio ImageScope viewing software 11.2.0.780 (Aperio, Vista, CA).

Calculations and statistical analysis. Relative gene expression was calculated using $2^{-\Delta Ct}$ method. Data of DNA methylation, mRNA levels, and numbers of positively stained cells are presented based on their distribution. The Kolmogorov-Smirnov test was used for normal distribution analyses of all data. The central tendency and spread of variables were described by the means ± SE and as the median (interquartile range) for skewed distribution. As only around half of the data set was normally distributed, we decided not to analyze upon factor interaction of the offspring's sex and the type of exposure but evaluate all analyzed parameters in the subgroups via a two-tailed Mann-Whitney *U* test. Comparisons of the methylation data at the three different stages were conducted using one-way analysis of variance (ANOVA). Correlation analysis of *Igf1* methylation data, mRNA levels, and body weight were assessed using nonparametric Spearman correlation test. Statistical significance was set as $P \leq 0.05$ for a two-tailed test. Statistical analyses were performed using IBM SPSS version 22 for Windows (Chicago, IL). Since our comparative analysis approach was hypothesis driven, and to present the reader all results, we did not adjust our significance levels for multiple testing, as suggested by McDonald (31).

RESULTS

PSE effect on DNA methylation in adult offspring was sex and organ specific. We conducted a comparative analysis of *Igf1* promoter methylation at eight different CpG sites in adult offspring between PSE and control mice, grouped by male and female, lung and liver. No PSE-induced methylation alterations were found in adult liver (Fig. 1, *A* and *B*). However, PSE male adult offspring had higher *Igf1* promoter methylation in the lung at CpG-1180 ($P < 0.05$) (Fig. 1, *C* and *E*), while PSE resulted in lower methylation of CpG-1254 in the adult lung of female offspring ($P < 0.05$) (Fig. 1, *D* and *F*).

No significant PSE effect was observed on *Igf1* mRNA expression levels in adult lung or liver (Fig. 1, *G* and *H*). In the adult control lung, methylation levels of two CpG sites of *Igf1* promoter correlated negatively with mRNA expression levels, respectively (CpG-1465, $r = -0.473$, $P = 0.035$; CpG-1357, $r = -0.463$, $P = 0.040$; Fig. 2, *A* and *B*). Female sex contributed most to the negative correlation between methylation of CpG-1465 and its mRNA expression ($r = -0.734$, $P = 0.010 < 0.05$). In adult mice liver tissue, no significant correlations were observed between the methylation levels at any of the *Igf1* CpG sites investigated and its mRNA levels.

Baseline Igf1 promoter methylation patterns over time in liver and lung from male and female offspring. The baseline DNA methylation pattern at eight CpG sites of the *Igf1* promoter was investigated across three stages (fetal stage, neonatal period, and adulthood) and comparisons were conducted between lung and liver, and male and female.

In the liver, *Igf1* methylation at six out of eight CpG sites was at the lowest level in adulthood, compared with the fetal and neonatal stages both in male and female offspring (CpG-1509, $P < 0.001$; CpG-1465, $P < 0.001$; CpG-1430, $P < 0.001$; CpG-1357, $P < 0.001$; CpG-1341, $P < 0.001$; CpG-1254, $P < 0.001$; Fig. 3, *A* and *a*). Methylation of CpG-1509 gradually declined from the fetal and neonatal stage to adulthood in both male and female offspring (Fig. 4, *A* and *a*, green symbols). However, for CpG-1465, these levels remained constant across the fetal to neonatal stage and then became

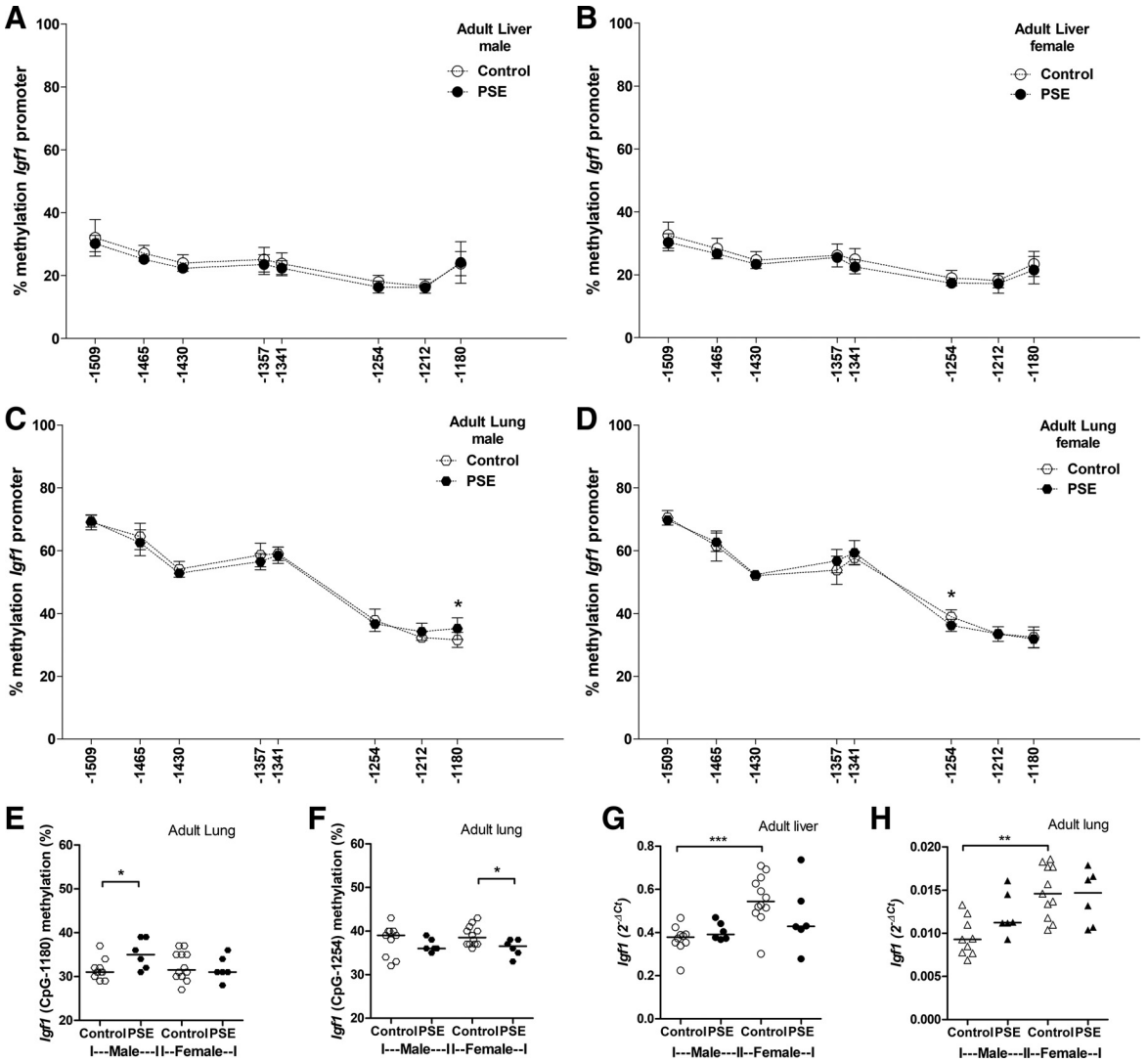

Fig. 1. Comparisons of prenatal smoke exposure (PSE) effect on *Igf1* promoter methylation and mRNA expression in adult males and females per organ (Mann-Whitney *U* test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). *A–D*: data are presented as means ± SE. *E–H*: data are presented as individual data points with the median as horizontal line. CpG site annotations relative to ATG start codon. Open symbols represent the control group; filled symbols represent the PSE group.

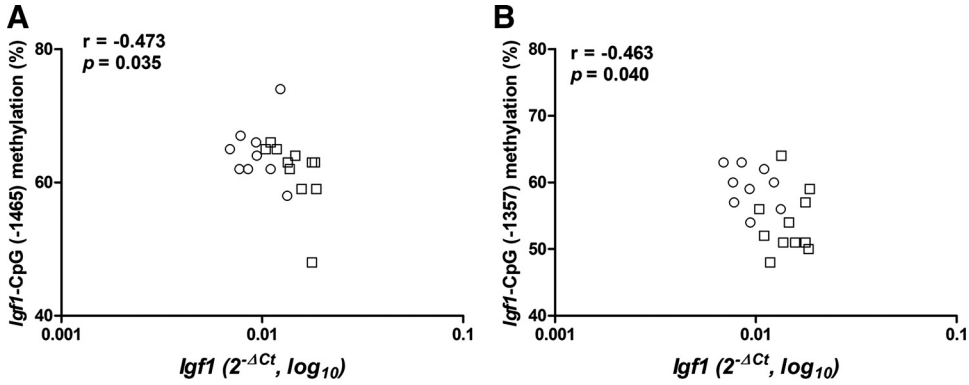

Fig. 2. Spearman correlation analysis, associating CpG (−1465, *A*) or CpG (−1357, *B*) promoter methylation with *Igf1* mRNA expression in adult lung of male (○, $n = 9$) and female (□, $n = 10$) controls.

hypomethylated in adulthood. This happened only in female offspring; in male offspring, the methylation pattern across the three stages remained the same as for CpG-1509 (Fig. 4, *B* and *b*, green symbols). Methylation of CpG-1430 in both male and female offspring increased from the fetal to the neonatal stage (contrary to CpG-1509), but reversed to the lowest levels in adulthood (Fig. 4, *C* and *c*, green symbols). Methylation patterns of CpG-1357 in male offspring, and CpG-1341 and CpG-1254 in both male and female offspring, across the three stages were the same as the CpG-1465 in female offspring (Fig. 4, *D* and *F*, and *e* and *f*, green symbols). However, the methylation pattern of CpG-1357 in female offspring was the same as the CpG-1509 in all offspring (Fig. 4*d*, green symbols). Interestingly, methylation of CpG sites CpG-1212 and CpG-1180 remained constant across the three stages in both male and female control offspring (Fig. 4, *G* and *H*, and *g* and *h*, green symbols).

As for the baseline DNA methylation patterns in the lung, six CpG sites were differentially methylated across the three stages in male offspring (CpG-1465, $P < 0.001$; CpG-1430, $P < 0.001$; CpG-1357, $P < 0.001$; CpG-1341, $P < 0.001$; CpG-1212, $P < 0.001$; CpG-1180, $P < 0.001$) and seven CpG sites in female offspring, showing additionally differential methylation at CpG-1254 ($P < 0.001$) (Fig. 3, *B* and *b*). Compared with the fetal and neonatal stages, all of these CpG sites were hypermethylated in adulthood, both in male and female offspring. Methylation of CpG-1465 decreased from the fetal to the neonatal stage while it increased substantially in adulthood, both in male and female offspring (Fig. 5, *B* and *b*, green symbols). However, methylation of CpG-1430 and CpG-1212 in female offspring persisted from the fetal to the neonatal stage and then became hypermethylated in adulthood. In male offspring, CpG-1212 showed the same methylation patterns as CpG-1465, whereas CpG-1430 gradually increased from the fetal stage to neonatal period and on to adulthood (Fig. 5, *C* and *G*, and *c* and *g*, green symbols). Methylation of CpG-1357, CpG-1341 and CpG-1180 in all offspring, showed the same patterns as CpG-1430 and CpG-1212 in female offspring across the three stages (Fig. 5, *D*, *E*, and *H*, and *d*, *e*, and *h*, green symbols). Baseline methylation patterns of CpG-1509 and CpG-1254, however, remained constant across all time points in both male and female offspring (Fig. 5, *a* and *f*, respectively, green symbols).

PSE effects on the persistence/reversibility of Igf1 promoter methylation over time in liver and lung tissue from male and female offspring. In the liver, PSE induced differential methylation across the three stages in CpG-1212 in both male and female offspring and in CpG-1180 only in female offspring (Fig. 3, *A* versus *C*, and Fig. 3, *a* versus *c*, and Fig. 4, *G* and *g*, and *H* and *h*). Comparing fetal and neonatal stages, PSE induced hypomethylation at CpG-1357, -1254, -1212, and -1180 while hypermethylation was only found at CpG-1180 (Fig. 4*D*, 4*F*, *G*, *g*, and *h*). Interestingly, for methylation of CpG-1509, we found a PSE-induced reversion to previous fetal status at the neonatal stage in female offspring. PSE-induced differences of CpG-1357 and CpG-1254 between the fetal and neonatal stages were found only in the male group, while the PSE effect on CpG-1180 methylation was found only in the female group. PSE-induced methylation differences of CpG-1212 were found in both sexes.

In the lung, PSE disrupted differential methylation across the three stages in CpG-1254 in female offspring (Fig. 3, *B* and *D*, and *b* and *d*, and Fig. 5*f*). In male offspring, PSE disrupted differential methylation across the fetal and the neonatal stages at CpG-1509, -1212 (from neonatal stage to adulthood) and whereas PSE induced hypermethylation between the fetal and neonatal stage at CpG-1430 in female offspring (Fig. 5, *A*, *f*, *G*, and *c*).

The possible (biological) relevance among the PSE-induced changes of body weight with Igf1 promoter methylation and its mRNA expression over time in liver and lung tissue. As we observed a drift in promoter methylation patterns of *Igf1*, and as IGF1 is a key modulator of growth, we further sought to investigate the biological relevance of the observed changes on body weight.

In Fig. 6, the PSE effect is shown on body weight of neonatal and adult offspring. In neonatal offspring, PSE downregulated body weight and *Igf1* mRNA levels ($P = 0.01$, data not shown). *Igf1* mRNA levels in neonatal liver were positively correlated to body weight, regardless of their sex or exposure ($r = 0.727$, $P < 0.0001$; Fig. 6, *A* and 6*B* and Table 1). In addition, neonatal body weight was associated with *Igf1* promoter methylation. Within the entire group of neonates, the correlation of body weight and CpG-site specific *Igf1* promoter methylation levels were strongest for *Igf1* CpG-1254 (Fig. 6*C* and Table 1). This was also seen when distinguishing between the offspring's sex or their exposure, and was most pronounced for male offspring (all male: $r = -0.60$, $P < 0.01$, Table 1). PSE did not affect body weight in adult offspring (Fig. 6*D*) or *Igf1* mRNA expression (data not shown). However, *Igf1* mRNA levels were negatively correlated with body weight ($r = -0.739$, $P < 0.0001$, Fig. 6*E* and Table 1). With respect to methylation, no significant correlations were found between methylation and body weight in all adult offspring (Fig. 6*F* and Table 1), albeit that

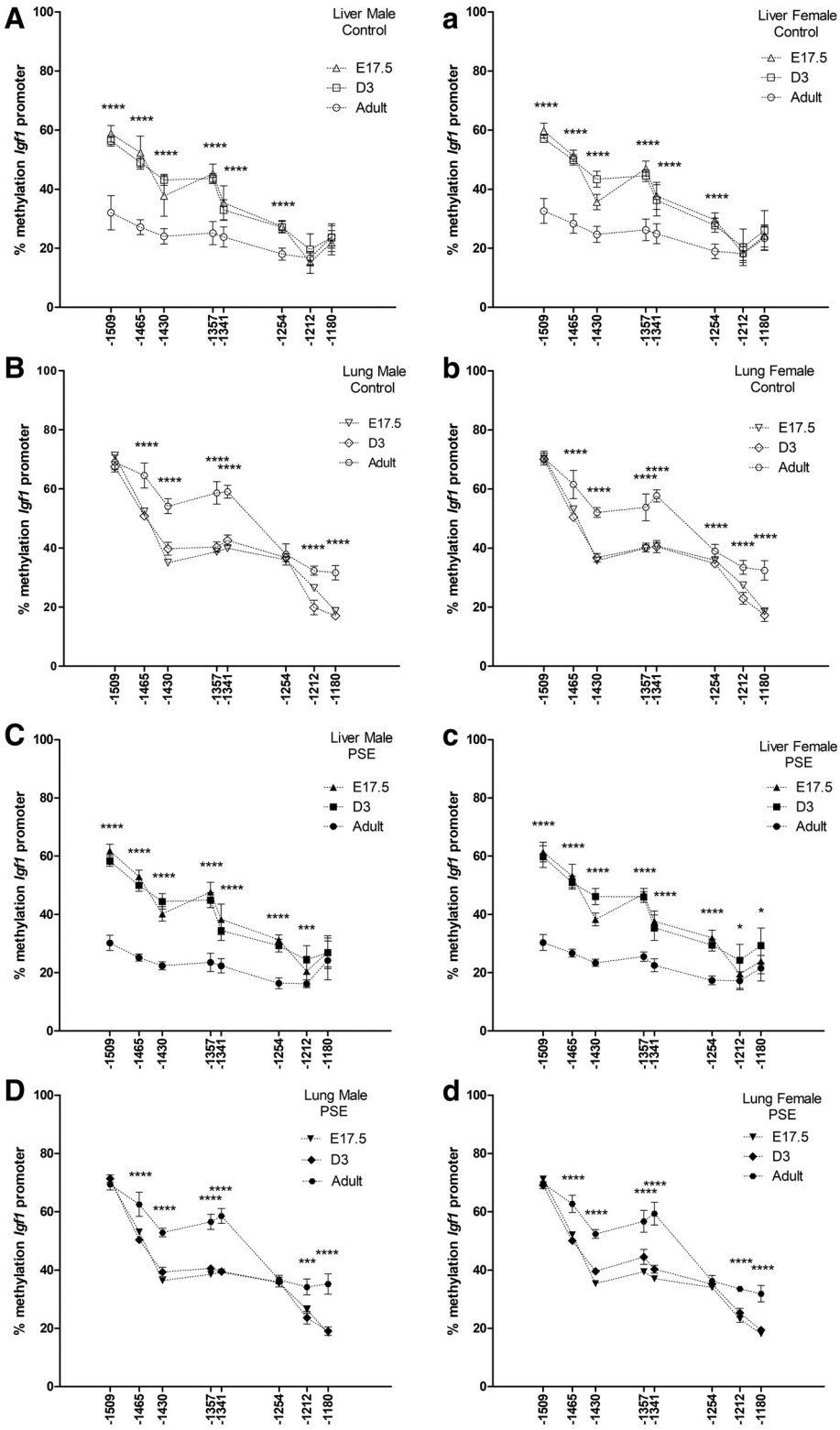

Fig. 3. Sex-dependent stage comparison of *Igf1* promoter methylation status per organ between controls and prenatal smoke exposure (PSE) group. *A* and *C*: liver. *B* and *D*: lung. ANOVA was used to do the comparison analysis among the fetal stage (embryonic day, E17.5), neonatal period (postnatal day 3, D3), and adulthood (Adult). $*P < 0.05$, $***P < 0.001$, $****P < 0.0001$. Data are presented as means ± SE; CpG site annotations relative to ATG start codon. Open symbols represent control group, and filled symbols are PSE group.

CpG-1254 methylation was strongly negatively correlated with body weight in PSE female offspring (Table 1).

To further investigate the relationship between *Igf1* promoter methylation and *Igf1* mRNA expression, data from the three developmental stages were combined. Table 2 shows that methylation of six CpG sites (CpG-1509, -1460, -1430, -1357, -1341, and -1254) was negatively correlated with *Igf1* mRNA expression in liver tissues ($P < 0.05$). However, no significant correlation was observed between methylation of CpG-1212 and CpG-1180 and its mRNA expression in any group in liver, except for a negative correlation at CpG-1180 in all mice ($r = -0.18$, $P = 0.05$). Negative correlations were also ob-

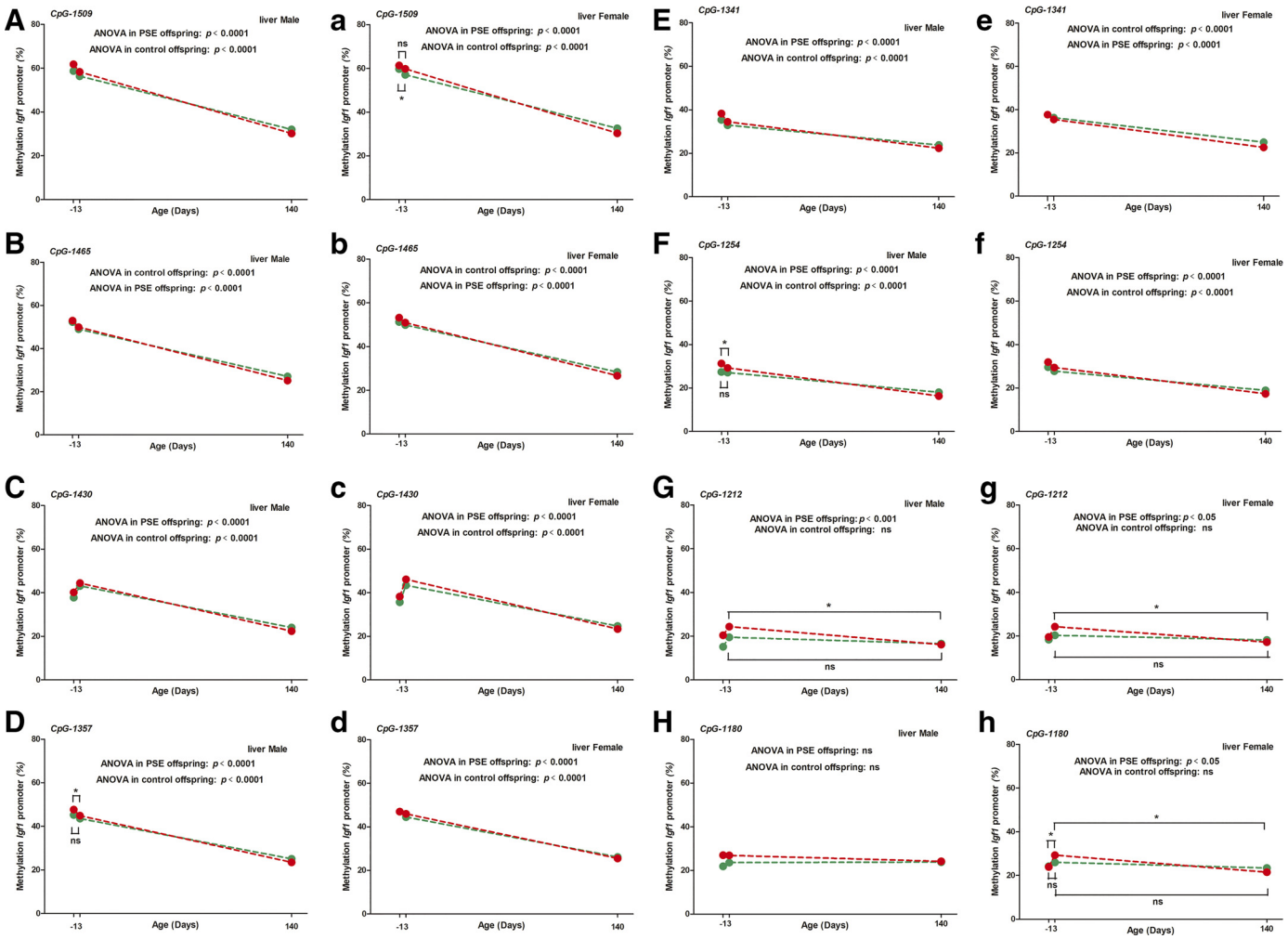

Fig. 4. Developmental stage comparisons of *Igf1* promoter methylation status per sex in liver tissues (male: *A–H*; female: *a–h*). ANOVA was used for comparisons among 3 different stages in control/prenatal smoke exposure (PSE) offspring. Mann-Whitney *U* test was used to test the comparisons between 2 time points in control/PSE offspring and only the PSE effect is displayed. $*P < 0.05$; ns, not significant. Data are presented as the mean. Number in *x*-axis: "−1" means E17.5, representing the fetal stage; "3" means 3 days after birth, representing the neonatal period; "140" means 140 days after birth, representing adulthood. Green symbols represent control offspring; red symbols represent PSE offspring.

served in lung tissues between methylation of CpG-1465, -1430, -1357, -1341, -1212, and -1180 and mRNA expression in any group, except for CpG-1465 and CpG-1430 in the male PSE group ($P < 0.05$). We found a positive correlation between *Igf1* mRNA expression and methylations of CpG-1509 in all animals. This was driven by PSE, especially in the female group ($r = 0.20$, $P = 0.04$; $r = 0.33$, $P = 0.01$; $r = 0.38$, $P = 0.04$). Methylation of CpG-1254 was negatively correlated with mRNA expression in all controls, which was most pronounced in female offspring ($r = -0.28$, $P = 0.03$; $r = -0.52$, $P = 0.00$). Meanwhile, we found it was negatively correlated with *Igf1* mRNA expression in all female animals whereas there was a positive correlation in male PSE group at this CpG site ($r = -0.31$, $P = 0.02$; $r = 0.40$, $P = 0.04$).

PSE effect on adult lung inflammation was sex specific. To get insight in the inflammatory response to PSE in the lung of adult offspring, the presence of M1 macrophages, M2 macrophages, eosinophils, and neutrophils was investigated, as well as mRNA expression levels of inflammatory cytokines including *Il1b*, *Il6*, *Tnfa* and *Tgfb*. PSE increased the numbers of M1 macrophages in female offspring, whereas no PSE effect was found in male counterparts (Fig. 7, *A* and *E*, $P = 0.0394$). PSE reduced the number of eosinophils, which again was only observed in female offspring (Fig. 7, *C* and *G*, $P = 0.0441$). PSE had no effect on the number of M2 macrophages and neutrophils in both sexes (Fig. 7, *B* and *F*, and *D* and *H*). PSE did also not affect mRNA expression of *Il1b*, *Il6*, *Tnfa* and *Tgfb*, albeit there was a trend for lower *Tgfb* mRNA expression, both in PSE male and female offspring (Table 3, $P = 0.07$, $P = 0.08$).

DISCUSSION

In this study we made three main observations. First, we found a CpG site-specific persistence, increase, or decrease of basal *Igf1* promoter methylation over time, which was organ specific. Second, PSE affected *Igf1* promoter methylation patterns, which was organ and sex dependent. Third, the PSE effect was less pronounced in adult offspring when compared with the fetal and neonatal stages.

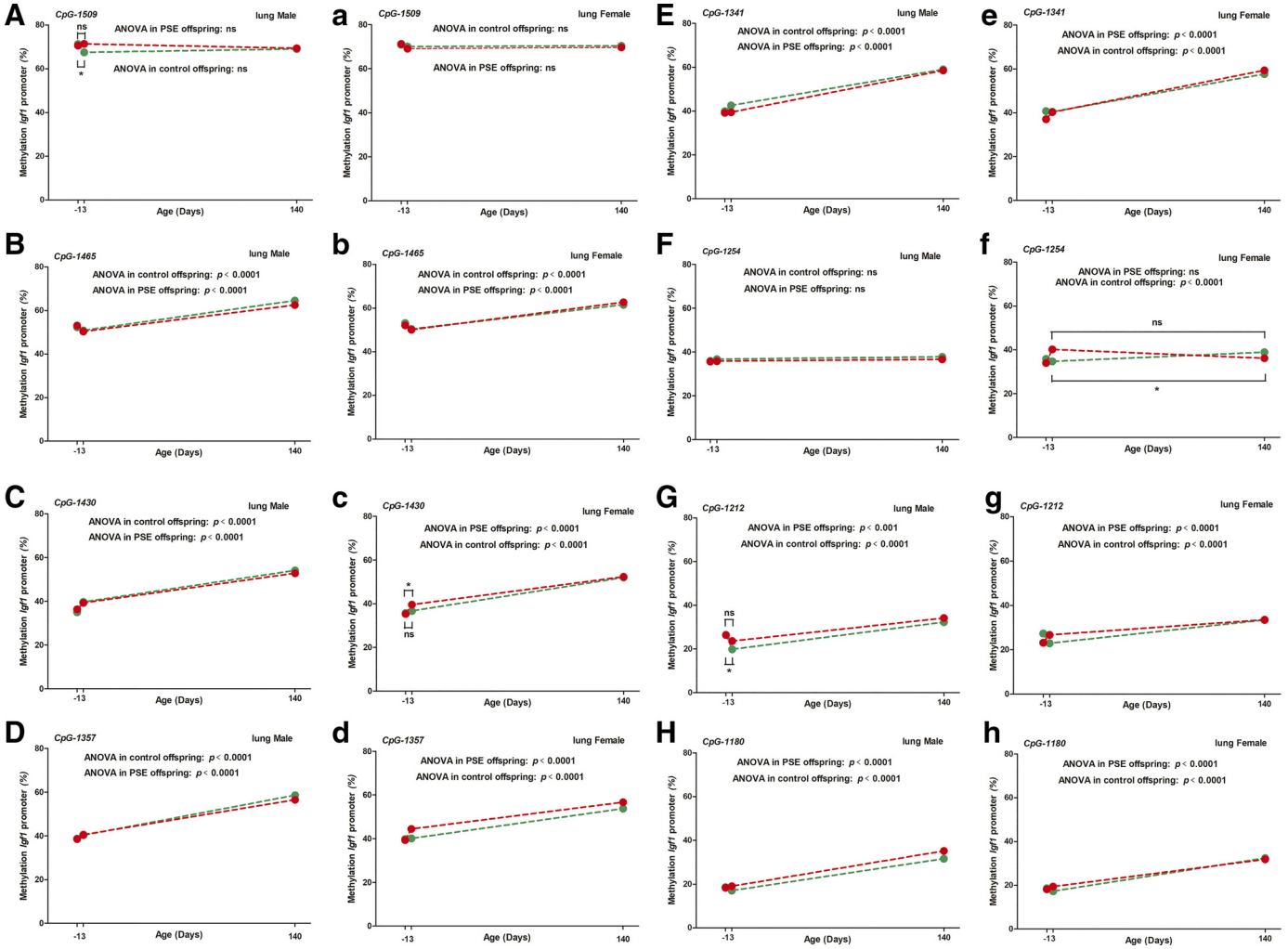

Fig. 5. Developmental stage comparisons of *Igf1* promoter methylation status per sex in lung tissues (male: *A–H*; female: *a–h*). ANOVA was used for comparisons among 3 different stages in control/PSE offspring. Mann-Whitney *U* test was used to test the comparisons between 2 time points in control/prenatal smoke exposure (PSE) offspring and only the PSE effect is displayed. $*P < 0.05$; ns, not significant. Data are presented as the mean. Number in *x*-axis: " −1" means E17.5, representing the fetal stage; "3" means 3 days after birth, representing the neonatal period; "140" means 140 days after birth, representing adulthood. Green symbols represent control offspring; red symbols represent PSE offspring.

Regarding the observation that persistence or change of basal methylation over time is CpG site dependent, our results confirm previous studies in humans showing similar patterns for a large variety of genes and CpG sites, which were organ dependent (28, 39). In particular, this has been described for aryl hydrocarbon receptor repressor (*AHRR*), where in peripheral blood, for some of the CpG sites, these patterns were affected by PSE, but for some others they were not. Interestingly, CpG sites of which methylation persisted across the three different developmental stages did not correlate with mRNA expression and were also not affected by PSE. This was the case for both the lung and liver. This may indicate that methylation of these CpG sites alone does not significantly drive specific gene expression (36). Another interesting observation was that in the lung, methylation for most CpG sites increased in adulthood, but for liver it was the opposite. It has been demonstrated that in general, sites showing overall low DNA methylation tend to increase methylation with age while those with high DNA methylation tend to lose methylation with age (14). Methylation in the liver was strongly and inversely correlated with mRNA expression at CpG sites further away from the transcription start site, whereas in the lung the negative correlation coefficients were smaller and most pronounced with CpG sites around the transcription start site. DNA methylation levels at a promoter-associated CpG island are generally negatively associated with gene expression (17). Furthermore, loss of (global) methylation is very much linked with aging (43), although our 4-mo-old adult mice cannot be considered to be old mice. The observation that methylation profiles were very different in the liver compared with lung also confirms previous studies in humans, showing that as the patterns of gene expression differ across organs, so do the patterns of DNA methylation (6, 44). In fact, the organ of location drives the primary difference in DNA methylation profiles, even if they originate from the same individuals (10, 16, 45). Given that DNA methylation profiles are highly divergent in different organs, comparing DNA methylation across organs presents huge challenges in population studies. Our study provides a relatively ideal mouse model to investigate such profiles. Generally, our research not only supports

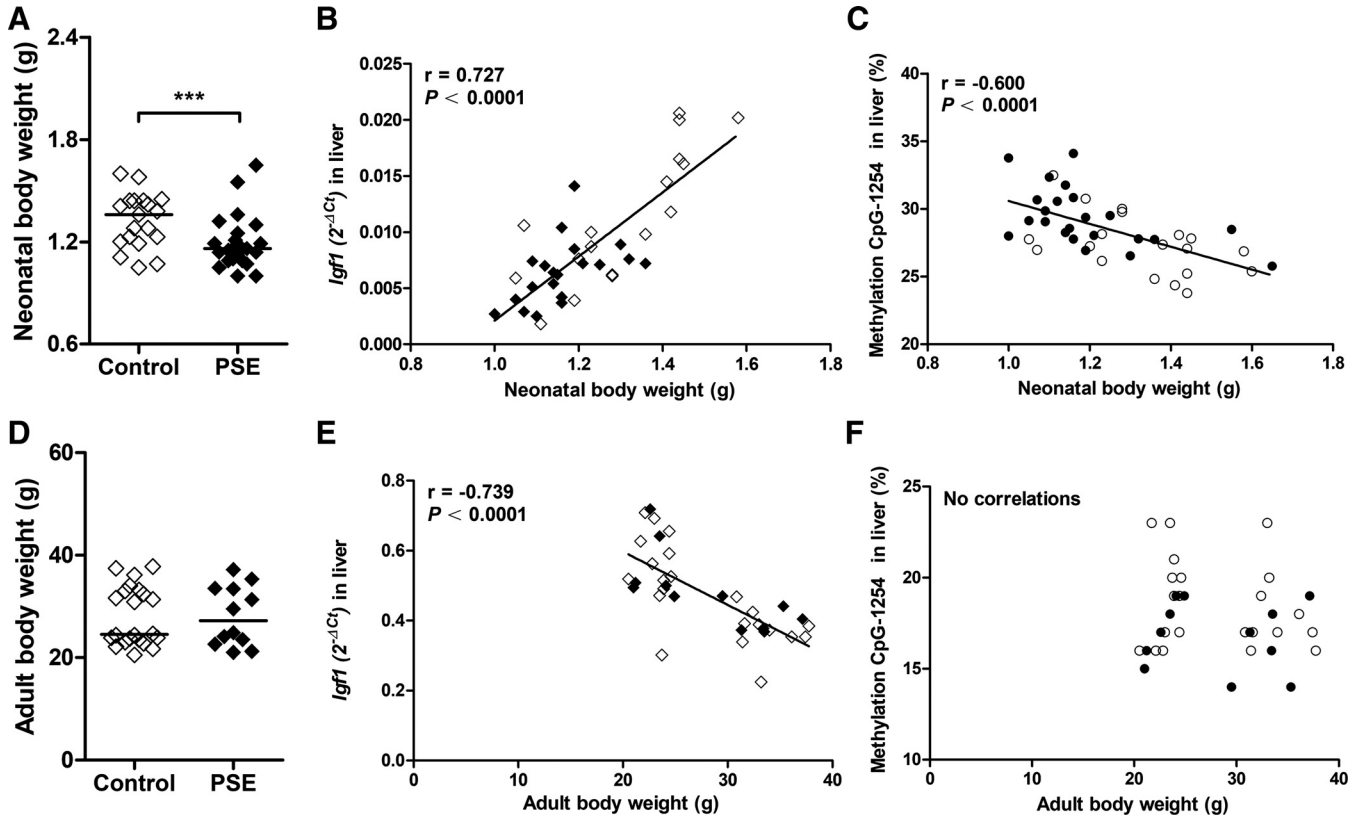

Fig. 6. Prenatal smoke exposure (PSE) effect on body weight, as well as the association of body weight with hepatic *Igf1* mRNA expression and CpG-1254 methylation in neonatal (*A*–*C*) and adult (*D*–*F*) offspring. Mann-Whitney *U* test was used to compare the body weight between the control offspring and the PSE offspring at 2 developmental stages, respectively. Data in *A* and *D* are presented as individual data points with the median as horizontal line. ***$P < 0.001$. Nonparametric Spearman correlation test was used to analyze the correlations of body weight with hepatic *Igf1* mRNA levels, methylation of *Igf1* CpG-1254 at 2 developmental stages, respectively. Data in *B*, *C*, *E*, and *F* are presented as individual data points. Open symbols represent control offspring; filled symbols represent PSE offspring.

results obtained by human studies but also establishes different profiles of DNA methylation through the whole life course across tissues.

With respect to PSE-induced *Igf1* promoter methylation effects across the three stages, both organ- and sex-specific effects were found in this study. It is well documented that most in utero and in general, smoke exposure are associated with changes in the (neonatal) epigenetic profile. When smoke-induced modifications of DNA methylation in the exposed human fetus take place during the early phases of embryogenesis, they were shown to be maintained into postnatal life (27). A longitudinal model has provided evidence that prenatal exposure to smoking has persistent effects on the DNA methylation of offspring, at least until adolescence (28). However, most of these results are based mainly on cross-sectional population studies and show only correlations. A prospective study in children combining serial blood sampling at multiple time points has shown powerful evidence of the persistence of *AHRR* methylation changes induced in utero (36). However, this could be shown only for blood cells. In our mouse model, PSE caused differences across the three stages at two CpG sites in the liver and one CpG site in the lung, compared with controls. Furthermore, PSE-induced alterations of DNA methylation either in fetal stage or neonatal stage could persist in adulthood. These observations from our mouse model are consistent with the human results on persistent (*AHRR*, *MYO1G*, *CYP1A1*, and *CNTNAP2*) or reversible (*GFI1*, *KLF13* and *ATP9A*) methylation alterations in the smoke-exposed children, 17 yr after prenatal exposure, as shown by Richmond et al. (40). Recently, a similar mouse study reported that perinatal smoke exposure induces persistent hypermethylation in both global DNA and promoters of *IFN-γ* and *Thy-1* in lung tissue of adult offspring at 10–12 and 20 wk of age (8), while in our lung data PSE-induced methylation alterations of one CpG site in *Igf1* promoter showed an opposite pattern of hypomethylation. Also, our model further showed a different PSE-induced effect on promoter methylation of a single gene in different tissues. Remarkably, the methylation levels after PSE in lung tissue of female offspring reversed to status at the fetal stage and became persistent over the life course, but they persisted through all stages in male offspring. This observation is not consistent with many epidemiological studies supporting a sex bias, as females seem to being preferentially protected from early lung environmental irritants (35). Some PSE effects on methylation alterations of other CpG sites occurred only in males, regardless of lung or liver tissues, and were therefore in line with human studies. To some extent, the common patterns observed here in male and female offspring might also be explained by the shared prenatal exposure to maternal smoke, which confers a high degree of epigenetic similarity between neonatal tissues, whereas subsequent variation in the postnatal

Table 1. *Spearman correlation analysis of body weight with Igf1 mRNA expression and promoter methylation in neonatal and adult liver tissues, respectively*

Weight	All All	All Male	All Female	All Control	All PSE	Male Control	Male PSE	Female Control	Female PSE
					Neonatal liver tissue				
Igf1 ($2^{-\Delta Ct}$)									
r	**0.73**	**0.75**	**0.83**	**0.82**	**0.51**	**0.87**	0.45	**0.85**	**0.74**
P	**0.00**	**0.00**	**0.00**	**0.00**	**0.02**	**0.00**	ns	**0.01**	**0.00**
Igf1 promoter methylation, %									
CpG-1509									
r	−0.25	−0.15	−0.25	0.11	0.20	0.56	−0.23	−0.22	−0.18
P	ns	ns	ns	ns	ns	0.07	ns	ns	ns
CpG-1465									
r	−0.30	**−0.44**	−0.12	0.21	**−0.50**	−0.21	−0.49	**0.72**	−0.49
P	0.06	**0.04**	ns	ns	**0.02**	ns	ns	**0.04**	ns
CpG-1430									
r	**−0.42**	**−0.50**	−0.31	−0.37	−0.29	−0.58	−0.35	−0.24	−0.23
P	**0.01**	**0.02**	ns	ns	ns	0.06	ns	ns	ns
CpG-1357									
r	**−0.44**	**−0.47**	−0.36	−0.22	**−0.45**	−0.27	−0.47	−0.09	−0.46
P	**0.00**	**0.03**	ns	ns	**0.03**	ns	ns	ns	ns
CpG-1341									
r	−0.04	−0.05	0.05	0.00	−0.01	0.16	0.01	0.08	−0.01
P	ns	ns	ns	ns	ns	ns	ns	ns	ns
CpG-1254									
r	**−0.60**	**−0.60**	**−0.60**	−0.51	**−0.53**	0.56	−0.40	−0.46	**−0.65**
P	**0.00**	**0.00**	**0.01**	0.02	**0.01**	0.07	ns	ns	**0.02**
CpG-1212									
r	−0.20	−0.10	−0.27	−0.15	0.01	0.06	0.38	−0.20	−0.22
P	ns	ns	ns	ns	ns	ns	ns	ns	ns
CpG-1180									
r	**−0.03**	−0.15	−0.40	−0.35	−0.15	0.18	0.21	−0.39	−0.33
P	**0.03**	ns	ns	ns	ns	ns	ns	ns	ns
					Adult liver tissue				
Igf1 ($2^{-\Delta Ct}$)									
r	**−0.74**	−0.30	−0.21	**−0.71**	**−0.79**	−0.40	−0.09	−0.18	−0.26
P	**0.00**	ns	ns	**0.00**	**0.00**	ns	ns	ns	ns
Igf1 promoter methylation, %									
CpG-1509									
r	−0.14	−0.36	0.21	−0.21	0.04	−0.47	0.06	0.17	0.15
P	ns	ns	ns	ns	ns	ns	ns	ns	ns
CpG-1465									
r	−0.31	−0.25	−0.10	−0.22	**−0.60**	−0.26	−0.52	−0.08	−0.25
P	ns	ns	ns	ns	**0.04**	ns	ns	ns	ns
CpG-1430									
r	−0.27	−0.46	0.11	−0.23	−0.37	−0.53	−0.24	0.04	0.03
P	ns	ns	ns	ns	ns	ns	ns	ns	ns
CpG-1357									
r	0.00	0.28	0.29	−0.03	0.03	0.21	0.52	0.04	**0.88**
P	ns	ns	ns	ns	ns	ns	ns	ns	**0.02**
CpG-1341									
r	−0.05	0.29	0.08	−0.10	0.19	0.28	0.75	0.08	0.12
P	ns	ns	ns	ns	ns	ns	ns	ns	ns
CpG-1254									
r	0.02	0.10	**0.54**	−0.03	0.11	−0.03	0.49	0.29	**0.99**
P	ns	ns	**0.02**	ns	ns	ns	ns	ns	**0.00**
CpG-1212									
r	0.22	0.17	−0.11	−0.30	0.05	0.03	0.06	−0.24	0.03
P	ns	ns	ns	ns	ns	ns	ns	ns	ns
CpG-1180									
r	0.00	−0.10	−0.17	−0.09	0.03	−0.43	0.00	−0.18	−0.23
P	ns	ns	ns	ns	ns	ns	ns	ns	ns

PSE, prenatal smoke exposure; ns, not significant ($P > 0.05$). Significant values are shown in boldface. 0.00, $P < 0.01$.

environment is expected to result in epigenetic divergence after birth.

Regarding PSE-induced methylation alterations in adult offspring, these were limited and only observed in the lung. Comparing this to PSE effects in fetal and neonatal offspring, as described previously (32, 33), most methylation alterations seem therefore to be lost in adulthood. Furthermore, PSE-induced differences in methylation at novel CpG sites in the adult lung were sex dependent, a phenomenon also found in our previously described studies in fetal and neonatal offspring. Methylation levels at all measured *Igf1* CpG sites in the adult liver were much lower than in lung tissue, with the

Table 2. *Spearman correlation analysis between Igf1 mRNA expression and promoter methylation by combining the data of all three different stages in liver and lung tissues*

Igf1 ($2^{-\Delta Ct}$)	All All	All Male	All Female	All Control	All PSE	Male Control	Male PSE	Female Control	Female PSE
					Liver				
Igf1 promoter methylation, %									
CpG-1509									
r	−0.82	−0.82	−0.81	−0.83	−0.77	−0.77	−0.81	−0.87	−0.72
P	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CpG-1465									
r	−0.74	−0.78	−0.72	−0.74	−0.72	−0.74	−0.77	−0.73	−0.67
P	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CpG-1430									
r	−0.51	−0.54	−0.50	−0.60	−0.34	−0.58	−0.41	−0.64	−0.27
P	0.00	0.00	0.00	0.00	0.01	0.00	0.03	0.00	ns
CpG-1357									
r	−0.76	−0.75	−0.79	−0.81	−0.63	−0.76	−0.64	−0.84	−0.70
P	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CpG-1341									
r	−0.70	−0.69	−0.72	−0.72	−0.65	−0.66	−0.66	−0.77	−0.65
P	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CpG-1254									
r	−0.81	−0.82	−0.81	−0.83	−0.76	−0.75	−0.82	−0.90	−0.68
P	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CpG-1212									
r	−0.18	−0.20	−0.17	−0.05	−0.17	0.08	−0.25	−0.26	−0.12
P	0.05	ns	ns	ns	ns	ns	ns	ns	ns
CpG-1180									
r	−0.18	−0.18	−0.18	−0.08	−0.18	0.11	−0.30	−0.23	−0.09
P	0.05	ns	ns	ns	ns	ns	ns	ns	ns
					Lung				
CpG-1509									
r	0.20	0.21	0.16	0.08	0.33	0.14	0.32	−0.07	0.38
P	0.04	ns	ns	ns	0.01	ns	ns	ns	0.04
CpG-1465									
r	−0.55	−0.45	−0.62	−0.70	−0.40	−0.60	−0.29	−0.79	−0.50
P	0.00	0.00	0.00	0.00	0.00	0.00	ns	0.00	0.01
CpG-1430									
r	−0.44	−0.35	−0.51	−0.47	−0.41	−0.38	−0.30	−0.50	−0.52
P	0.00	0.01	0.00	0.00	0.00	0.04	ns	0.00	0.00
CpG-1357									
r	−0.54	−0.58	−0.50	−0.67	−0.43	−0.64	−0.56	−0.68	−0.36
P	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	ns
CpG-1341									
r	−0.55	−0.46	−0.61	−0.61	−0.55	−0.48	−0.52	−0.68	−0.55
P	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00
CpG-1254									
r	−0.08	0.11	−0.31	−0.28	0.18	−0.07	0.40	−0.52	−0.05
P	ns	ns	0.02	0.03	ns	ns	0.04	0.00	ns
CpG-1212									
r	−0.62	−0.54	−0.69	−0.74	−0.48	−0.63	−0.39	−0.78	−0.59
P	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00
CpG-1180									
r	−0.59	−0.57	−0.60	−0.61	−0.55	−0.58	−0.57	−0.61	−0.56
P	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

PSE, prenatal smoke exposure; ns, not significant ($P > 0.05$); 0.00, $P < 0.01$.

majority usually unaffected, but sometimes enhanced, by PSE. Interestingly, *Igf1* mRNA expression levels in adult livers were 40 times higher than mRNA levels in the lung, which was contrary to the results we observed in fetal and neonatal offspring. However, as the liver is the main organ for secretion of IGF1, higher *Igf1* mRNA expression could be expected in adulthood (5, 20). mRNA was inversely correlated with methylation at two different sites, but only in the adult lung. When all methylation and mRNA data were combined across the three different stages, *Igf1* mRNA expression was inversely and strongly correlated with promoter methylation, which was more prominent in the liver. These correlations were not affected by PSE. The phenomena showed here in adult offspring confirms the downregulatory role of promoter-associated methylation in the general regulation of gene expression.

In general, insulin-like growth factor-1 (IGF1) drives growth, whereas the absence of IGF1 signaling results in severe growth retardation (22). In this study, PSE-reduced hepatic *Igf1* mRNA and hepatic *Igf1* mRNA was positively correlated with body weight in neonatal offspring and negatively correlated with body weight in adult offspring. As hepatic IGF1 seemed to drive body weight in the neonatal stage, body weight was negatively correlated with the methylation status of *Igf1* (especially CpG-1254). Therefore, a regu-

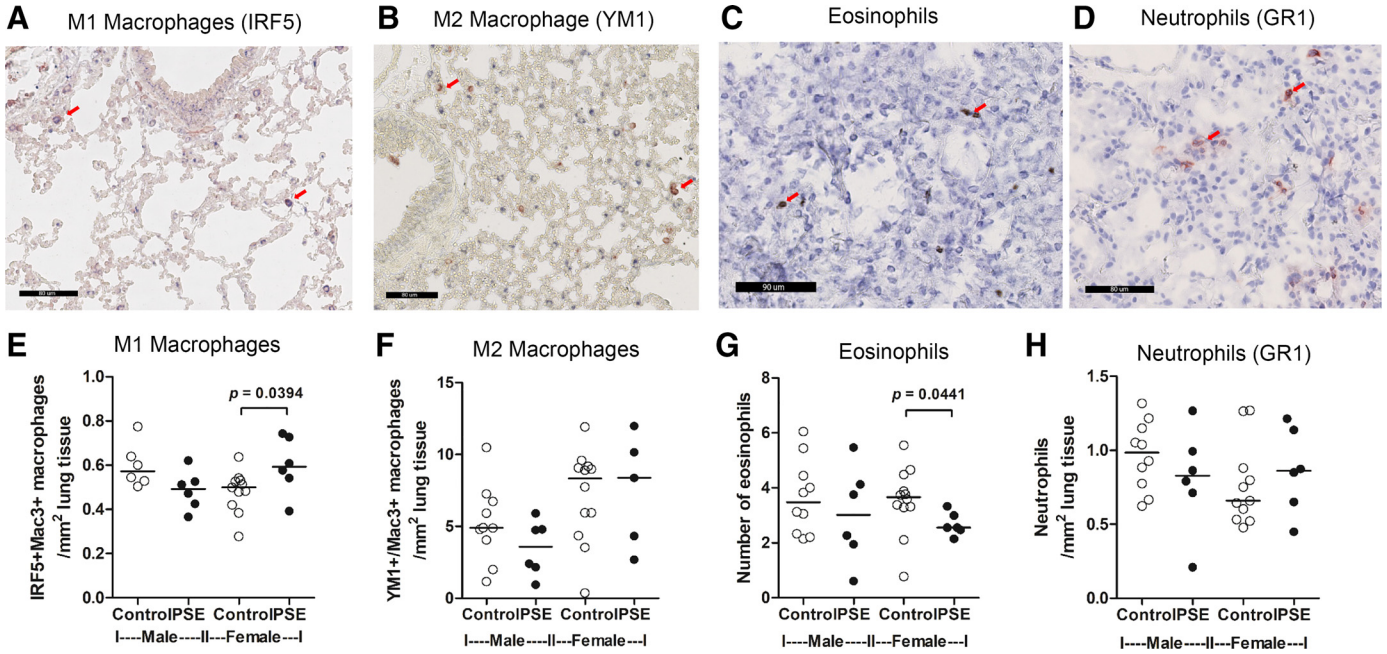

Fig. 7. Inflammatory cell phenotypes in lung tissue from the adult prenatal smoke exposure (PSE) mice. Representative pictures (red arrows point to positive cells) and numbers of M1 macrophages (IRF5/MAC3) (*A* and *E*), M2 dominant macrophages (YM1/MAC3) (*B* and *F*), eosinophils (endogenous peroxidase activity) (*C* and *G*), and neutrophils (GR1) (*D* and *H*). Original magnifications were ×40 (*A–D*). Mann-Whitney *U* test was used to compare the inflammatory cell numbers between the control offspring and the PSE offspring. Data are presented as individual data points with the median of cell numbers as horizontal line (*E–H*). Open symbols represent control offspring; filled symbols represent PSE offspring.

latory role for the methylation status of *Igf1* (especially CpG-1254) could be assumed in early life. The observed negative correlation of *Igf1* mRNA with body weight in adults can be explained by a lower body weight in female offspring, which express more *Igf1* mRNA than males. The role of IGF1 in adulthood may be entirely different than in the early developmental stages and more related to the initiation and progression of different diseases such as asthma (26), obesity, cardiovascular disease, and cancer (2), which most probably also depends on environment and presence of the IGF1R and the free IGF1 bioavailability. PSE is linked with both asthma and COPD later in life (1, 15, 30) and of interest is that the PSE effect on lung inflammation in adult offspring was sex specific. In PSE females, more M1 macrophages and less eosinophils were observed, while M2 macrophages, neutrophils, and inflammatory cytokines were not affected. The observed PSE-induced increase in M1 macrophages supports the higher risk for COPD as M1 macrophages have the classical properties of proinflammation and increased numbers are found in smokers and COPD patients (19, 23, 29). Additionally, eosinophils are traditionally linked with allergic asthma (11), whereas eosinophils probably play a minor role in COPD, albeit that their presence has been reported in proximal airways during viral-induced exacerbations (38).

In summary, our data show that PSE affects *Igf1* promoter methylation patterns in the adult lung, rather than the adult liver. Furthermore, the PSE-affected *Igf1* promoter methylation changes across three developmental stages was organ and sex specific. For most CpG sites, an inverse correlation was found for promoter methylation and mRNA expression when the three stages were combined. This was more prominent in the liver. To our knowledge, this is the first study to investigate the effects of in utero smoke exposure on promoter methylation persistence/reversibility from the fetal

Table 3. *mRNA expression levels of the inflammatory cytokines in lung tissues of adult PSE offspring and adult control offspring*

		Adult PSE Offspring			Adult Control Offspring			
Cytokine ($2^{-\Delta Ct}$)	Sex	*n*	Median (P_{25}, P_{75})	Range	*n*	Median (P_{25}, P_{75})	Range	*P* Value
IL-1β (10^{-3})	Male	6	3.05 (2.00, 4.25)	1.70–5.60	10	3.90 (3.63, 5.03)	2.50–5.50	0.14
	Female	6	4.45 (3.23, 5.28)	3.00–6.70	12	3.80 (2.68, 4.35)	2.00–7.50	0.28
IL-6 (10^{-4})	Male	6	1.10 (0.92, 1.56)	0.75–1.74	9	1.33 (0.87, 1.63)	0.58–1.96	0.86
	Female	6	1.69 (0.77, 2.07)	0.52–2.09	11	1.03 (0.78, 1.78)	0.67–2.66	0.58
TNF-α (10^{-4})	Male	6	3.67 (2.97, 6.63)	2.89–8.42	9	6.87 (3.73, 8.90)	3.15–13.04	0.18
	Female	6	4.17 (3.38, 8.03)	3.18–9.92	12	4.78 (3.50, 5.83)	1.26–11.37	0.96
TGF-β (10^{-2})	Male	6	2.79 (2.53, 3.39)	2.44–4.75	10	4.09 (3.70, 4.53)	2.54–5.57	0.07
	Female	6	3.58 (3.17, 3.85)	2.85–3.91	12	2.65 (2.28, 3.61)	0.93–4.26	0.08

Comparisons between the prenatal smoke exposure (PSE) offspring and the control offspring. All *P* values of the inflammatory cytokines were >0.05, using Mann-Whitney *U* test.

stage to adulthood of a specific gene in two organs. These findings reveal sex- and organ-dependent adverse effects of PSE on promoter methylation, which supports the observation that the intrauterine environment has the capability to "program" the fetus by inducing subtle changes in organ functions, which might predispose to disease in adulthood (13).

ACKNOWLEDGMENTS

We thank Dr. Nick Webber for constructive comments and English language editing.

GRANTS

This work is funded by Lung Foundation Netherlands (Longfonds) Grant 3.2.11.013 (to M. N. Hylkema).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Z.Z. and M.N.H. conceived and designed research; Z.Z., K.F.M. and K.L. performed experiments; W.K. and M.R.L assisted to conduct experiments; Z.Z. and K.F.M. interpreted results of experiments; Z.Z. and M.N.H. analyzed data; Z.Z. prepared figures and tables; Z.Z. drafted manuscript; K.F.M., K.L., X.X., X.H., J.S., T.P. and M.N.H. edited and revised manuscript; K.F.M., K.L., W.K., M.R.L., X.X., X.H., J.S., T.P. and M.N.H. approved final version of manuscript.

REFERENCES

1. **Alati R, Al Mamun A, O'Callaghan M, Najman J, Williams G.** In utero and postnatal maternal smoking and asthma in adolescence. *Epidemiology* 17: 138–144, 2006. doi:10.1097/01.ede.0000198148.02347.33.
2. **AsghariHanjani N, Vafa M.** The role of IGF-1 in obesity, cardiovascular disease, and cancer. *Med J Islam Repub Iran* 33: 56, 2019. doi:10.34171/mjiri.33.56.
3. **Bauer T, Trump S, Ishaque N, Thürmann L, Gu L, Bauer M, Bieg M, Gu Z, Weichenhan D, Mallm JP, Röder S, Herberth G, Takada E, Mücke O, Winter M, Junge KM, Grützmann K, Rolle-Kampczyk U, Wang Q, Lawerenz C, Borte M, Polte T, Schlesner M, Schanne M, Wiemann S, Geörg C, Stunnenberg HG, Plass C, Rippe K, Mizuguchi J, Herrmann C, Eils R, Lehmann I.** Environment-induced epigenetic reprogramming in genomic regulatory elements in smoking mothers and their children. *Mol Syst Biol* 12: 861, 2016. doi:10.15252/msb.20156520.
4. **Breton CV, Byun HM, Wenten M, Pan F, Yang A, Gilliland FD.** Prenatal tobacco smoke exposure affects global and gene-specific DNA methylation. *Am J Respir Crit Care Med* 180: 462–467, 2009. doi:10.1164/rccm.200901-0135OC.
5. **Butler AA, LeRoith D.** Minireview: tissue-specific versus generalized gene targeting of the igf1 and igf1r genes and their roles in insulin-like growth factor physiology. *Endocrinology* 142: 1685–1688, 2001. doi:10.1210/endo.142.5.8148.
6. **Byun HM, Siegmund KD, Pan F, Weisenberger DJ, Kanel G, Laird PW, Yang AS.** Epigenetic profiling of somatic tissues from human autopsy specimens identifies tissue- and individual-specific DNA methylation patterns. *Hum Mol Genet* 18: 4808–4817, 2009. doi:10.1093/hmg/ddp445.
7. **Chhabra D, Sharma S, Kho AT, Gaedigk R, Vyhlidal CA, Leeder JS, Morrow J, Carey VJ, Weiss ST, Tantisira KG, DeMeo DL.** Fetal lung and placental methylation is associated with in utero nicotine exposure. *Epigenetics* 9: 1473–1484, 2014. doi:10.4161/15592294.2014.971593.
8. **Cole E, Brown TA, Pinkerton KE, Postma B, Malany K, Yang M, Kim YJ, Hamilton RF Jr, Holian A, Cho YH.** Perinatal exposure to environmental tobacco smoke is associated with changes in DNA methylation that precede the adult onset of lung disease in a mouse model. *Inhal Toxicol* 29: 435–442, 2017. doi:10.1080/08958378.2017.1392655.
9. **Collins MH, Moessinger AC, Kleinerman J, Bassi J, Rosso P, Collins AM, James LS, Blanc WA.** Fetal lung hypoplasia associated with maternal smoking: a morphometric analysis. *Pediatr Res* 19: 408–412, 1985. doi:10.1203/00006450-198519040-00018.
10. **Davies MN, Volta M, Pidsley R, Lunnon K, Dixit A, Lovestone S, Coarfa C, Harris RA, Milosavljevic A, Troakes C, Al-Sarraj S, Dobson R, Schalkwyk LC, Mill J.** Functional annotation of the human brain methylome identifies tissue-specific epigenetic variation across brain and blood. *Genome Biol* 13: R43, 2012. doi:10.1186/gb-2012-13-6-r43.
11. **Eapen MS, Myers S, Walters EH, Sohal SS.** Airway inflammation in chronic obstructive pulmonary disease (COPD): a true paradox. *Expert Rev Respir Med* 11: 827–839, 2017. doi:10.1080/17476348.2017.1360769.
12. **Foreman MG, Zhang L, Murphy J, Hansel NN, Make B, Hokanson JE, Washko G, Regan EA, Crapo JD, Silverman EK, DeMeo DL; COPDGene Investigators.** Early-onset chronic obstructive pulmonary disease is associated with female sex, maternal factors, and African American race in the COPDGene Study. *Am J Respir Crit Care Med* 184: 414–420, 2011. doi:10.1164/rccm.201011-1928OC.
13. **Ganu RS, Harris RA, Collins K, Aagaard KM.** Early origins of adult disease: approaches for investigating the programmable epigenome in humans, nonhuman primates, and rodents. *ILAR J* 53: 306–321, 2012. doi:10.1093/ilar.53.3-4.306.
14. **Gutierrez-Arcelus M, Lappalainen T, Montgomery SB, Buil A, Ongen H, Yurovsky A, Bryois J, Giger T, Romano L, Planchon A, Falconnet E, Bielser D, Gagnebin M, Padioleau I, Borel C, Letourneau A, Makrythanasis P, Guipponi M, Gehrig C, Antonarakis SE, Dermitzakis ET.** Passive and active DNA methylation and the interplay with genetic variation in gene regulation. *eLife* 2: e00523, 2013. [Erratum in *eLife* 2: e01045, 2013.] doi:10.7554/eLife.00523.
15. **Hylkema MN, Blacquière MJ.** Intrauterine effects of maternal smoking on sensitization, asthma, and chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 6: 660–662, 2009. doi:10.1513/pats.200907-065DP.
16. **Jiang R, Jones MJ, Chen E, Neumann SM, Fraser HB, Miller GE, Kobor MS.** Discordance of DNA methylation variance between two accessible human tissues. *Sci Rep* 5: 8257, 2015. doi:10.1038/srep08257.
17. **Jones MJ, Goodman SJ, Kobor MS.** DNA methylation and healthy human aging. *Aging Cell* 14: 924–932, 2015. doi:10.1111/acel.12349.
18. **Joubert BR, Felix JF, Yousefi P, Bakulski KM, Just AC, Breton C, Reese SE, Markunas CA, Richmond RC, Xu CJ, Küpers LK, Oh SS, Hoyo C, Gruzieva O, Söderhäll C, Salas LA, Baïz N, Zhang H, Lepeule J, Ruiz C, Ligthart S, Wang T, Taylor JA, Duijts L, Sharp GC, Jankipersadsing SA, Nilsen RM, Vaez A, Fallin MD, Hu D.** DNA methylation in newborns and maternal smoking in pregnancy: genome-wide consortium meta-analysis. *Am J Hum Genet* 98: 680–696, 2016. doi:10.1016/j.ajhg.2016.02.019.
19. **Kaku Y, Imaoka H, Morimatsu Y, Komohara Y, Ohnishi K, Oda H, Takenaka S, Matsuoka M, Kawayama T, Takeya M, Hoshino T.** Overexpression of CD163, CD204 and CD206 on alveolar macrophages in the lungs of patients with severe chronic obstructive pulmonary disease. *PLoS One* 9: e87400, 2014. doi:10.1371/journal.pone.0087400.
20. **Kikuchi K, Bichell DP, Rotwein P.** Chromatin changes accompany the developmental activation of insulin-like growth factor I gene transcription. *J Biol Chem* 267: 21505–21511, 1992.
21. **Knopik VS, Maccani MA, Francazio S, McGeary JE.** The epigenetics of maternal cigarette smoking during pregnancy and effects on child development. *Dev Psychopathol* 24: 1377–1390, 2012. doi:10.1017/S0954579412000776.
22. **Kruis T, Klammt J, Galli-Tsinopoulou A, Wallborn T, Schlicke M, Müller E, Kratzsch J, Körner A, Odeh R, Kiess W, Pfäffle R.** Heterozygous mutation within a kinase-conserved motif of the insulin-like growth factor I receptor causes intrauterine and postnatal growth retardation. *J Clin Endocrinol Metab* 95: 1137–1142, 2010. doi:10.1210/jc.2009-1433.
23. **Kuschner WG, D'Alessandro A, Wong H, Blanc PD.** Dose-dependent cigarette smoking-related inflammatory responses in healthy adults. *Eur Respir J* 9: 1989–1994, 1996. doi:10.1183/09031936.96.09101989.
24. **Ladd-Acosta C, Shu C, Lee BK, Gidaya N, Singer A, Schieve LA, Schendel DE, Jones N, Daniels JL, Windham GC, Newschaffer CJ, Croen LA, Feinberg AP, Daniele Fallin M.** Presence of an epigenetic signature of prenatal cigarette smoke exposure in childhood. *Environ Res* 144: 139–148, 2016. doi:10.1016/j.envres.2015.11.014.
25. **Lawlor DA, Najman JM, Sterne J, Williams GM, Ebrahim S, Davey Smith G.** Associations of parental, birth, and early life characteristics with systolic blood pressure at 5 years of age: findings from the Mater-University study of pregnancy and its outcomes. *Circulation* 110: 2417–2423, 2004. doi:10.1161/01.CIR.0000145165.80130.B5.
26. **Lee H, Kim SR, Oh Y, Cho SH, Schleimer RP, Lee YC.** Targeting insulin-like growth factor-I and insulin-like growth factor-binding pro-

tein-3 signaling pathways. A novel therapeutic approach for asthma. *Am J Respir Cell Mol Biol* 50: 667–677, 2014. doi:10.1165/rcmb.2013-0397TR.
27. **Lee KW, Pausova Z.** Cigarette smoking and DNA methylation. *Front Genet* 4: 132, 2013. doi:10.3389/fgene.2013.00132.
28. **Lee KW, Richmond R, Hu P, French L, Shin J, Bourdon C, Reischl E, Waldenberger M, Zeilinger S, Gaunt T, McArdle W, Ring S, Woodward G, Bouchard L, Gaudet D, Smith GD, Relton C, Paus T, Pausova Z.** Prenatal exposure to maternal cigarette smoking and DNA methylation: epigenome-wide association in a discovery sample of adolescents and replication in an independent cohort at birth through 17 years of age. *Environ Health Perspect* 123: 193–199, 2015. doi:10.1289/ehp.1408614.
29. **Martinez FO, Gordon S.** The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep* 6: 13, 2014. doi:10.12703/P6-13.
30. **Maselli DJ, Bhatt SP, Anzueto A, Bowler RP, DeMeo DL, Diaz AA, Dransfield MT, Fawzy A, Foreman MG, Hanania NA, Hersh CP, Kim V, Kinney GL, Putcha N, Wan ES, Wells JM, Westney GE, Young KA, Silverman EK, Han MK, Make BJ.** Clinical epidemiology of COPD: insights from 10 years of the COPD gene study. *Chest* 156: 228–238, 2019. doi:10.1016/j.chest.2019.04.135.
31. **McDonald JH.** Multiple comparisons. In: *Handbook of Biological Statistics* (3rd ed.). Baltimore, MD: Sparky House, 2014, p. 254–260.
32. **Meyer KF, Krauss-Etschmann S, Kooistra W, Reinders-Luinge M, Timens W, Kobzik L, Plösch T, Hylkema MN.** Prenatal exposure to tobacco smoke sex dependently influences methylation and mRNA levels of the *Igf* axis in lungs of mouse offspring. *Am J Physiol Lung Cell Mol Physiol* 312: L542–L555, 2017. doi:10.1152/ajplung.00271.2016.
33. **Meyer KF, Verkaik-Schakel RN, Timens W, Kobzik L, Plösch T, Hylkema MN.** The fetal programming effect of prenatal smoking on Igf1r and Igf1 methylation is organ- and sex-specific. *Epigenetics* 12: 1076–1091, 2017. doi:10.1080/15592294.2017.1403691.
34. **Nielsen CH, Larsen A, Nielsen AL.** DNA methylation alterations in response to prenatal exposure of maternal cigarette smoking: A persistent epigenetic impact on health from maternal lifestyle? *Arch Toxicol* 90: 231–245, 2016. doi:10.1007/s00204-014-1426-0.
35. **Noël A, Xiao R, Perveen Z, Zaman H, Le Donne V, Penn A.** Sex-specific lung functional changes in adult mice exposed only to second-hand smoke in utero. *Respir Res* 18: 104, 2017. doi:10.1186/s12931-017-0591-0.
36. **Novakovic B, Ryan J, Pereira N, Boughton B, Craig JM, Saffery R.** Postnatal stability, tissue, and time specific effects of AHRR methylation change in response to maternal smoking in pregnancy. *Epigenetics* 9: 377–386, 2014. doi:10.4161/epi.27248.
37. **Oken E, Levitan EB, Gillman MW.** Maternal smoking during pregnancy and child overweight: systematic review and meta-analysis. *Int J Obes* 32: 201–210, 2008. doi:10.1038/sj.ijo.0803760.
38. **Papi A, Bellettato CM, Braccioni F, Romagnoli M, Casolari P, Caramori G, Fabbri LM, Johnston SL.** Infections and airway inflammation in chronic obstructive pulmonary disease severe exacerbations. *Am J Respir Crit Care Med* 173: 1114–1121, 2006. doi:10.1164/rccm.200506-859OC.
39. **Richmond RC, Simpkin AJ, Woodward G, Gaunt TR, Lyttleton O, McArdle WL, Ring SM, Smith AD, Timpson NJ, Tilling K, Davey Smith G, Relton CL.** Prenatal exposure to maternal smoking and offspring DNA methylation across the lifecourse: findings from the Avon Longitudinal Study of Parents and Children (ALSPAC). *Hum Mol Genet* 24: 2201–2217, 2015. doi:10.1093/hmg/ddu739.
40. **Richmond RC, Suderman M, Langdon R, Relton CL, Davey Smith G.** DNA methylation as a marker for prenatal smoke exposure in adults. *Int J Epidemiol* 47: 1120–1130, 2018. doi:10.1093/ije/dyy091.
41. **Rzehak P, Saffery R, Reischl E, Covic M, Wahl S, Grote V, Xhonneux A, Langhendries JP, Ferre N, Closa-Monasterolo R, Verduci E, Riva E, Socha P, Gruszfeld D, Koletzko B; European Childhood Obesity Trial Study group.** Maternal smoking during pregnancy and DNA-methylation in children at age 5.5 years: epigenome-wide-analysis in the European Childhood Obesity Project (CHOP)-study. *PLoS One* 11: e0155554, 2016. doi:10.1371/journal.pone.0155554.
42. **Suter MA, Aagaard K.** What changes in DNA methylation take place in individuals exposed to maternal smoking in utero? *Epigenomics* 4: 115–118, 2012. doi:10.2217/epi.12.7.
43. **Weber M, Hellmann I, Stadler MB, Ramos L, Pääbo S, Rebhan M, Schübeler D.** Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. *Nat Genet* 39: 457–466, 2007. doi:10.1038/ng1990.
44. **Xiao FH, Wang HT, Kong QP.** Dynamic DNA methylation during aging: A "prophet" of age-related outcomes. *Front Genet* 10: 107, 2019. doi:10.3389/fgene.2019.00107.
45. **Ziller MJ, Gu H, Müller F, Donaghey J, Tsai LTY, Kohlbacher O, De Jager PL, Rosen ED, Bennett DA, Bernstein BE, Gnirke A, Meissner A.** Charting a dynamic DNA methylation landscape of the human genome. *Nature* 500: 477–481, 2013. doi:10.1038/nature12433.