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The effect of (prenatal) smoke exposure on lung progenitor cell behavior and Cyp2a5 gene regulation

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**THE EFFECT OF (PRENATAL) SMOKE EXPOSURE
ON LUNG PROGENITOR CELL BEHAVIOR AND
CYP2A5 GENE REGULATION**

Khosbayar Lkhagvadorj

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university of
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The effect of (prenatal) smoke exposure on lung progenitor cell behavior and *Cyp2a5* gene regulation

PhD thesis

to obtain the degree of PhD at the
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and in accordance with
the decision by the College of Deans.

This thesis will be defended in public on
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*Dedicated to my beloved
parents & family*

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CHAPTER 1

General introduction and scope of the thesis

Maternal smoking during pregnancy

1

Maternal smoking through pregnancy is one of the most widespread prenatal insults in the world [1]. Although epidemiological studies have shown that the prevalence of maternal smoking appears to be slightly decreasing overall in the last decades [2], current studies have shown that the prevalence of maternal smoking during pregnancy in western countries remains at more than 10% in the last few years [3–5]. Besides, in low-income countries, the prevalence of maternal smoking during pregnancy is estimated even higher [2].

Maternal smoking during pregnancy has been found to have a number of potentially negative effects on fetus, such as low birth weight, preterm birth, birth defects and an increased risk of developing pulmonary diseases [6–8]. Interestingly, maternal smoking during pregnancy has additionally been shown to be a risk factor for cigarette smoke addiction in later in life, potentially creating a vicious circle. Kandel *et al.* showed in the Child Health and Development and The New York State Follow-Up cohorts that offspring who had been *in utero* exposed to cigarette smoke had an increased risk for nicotine dependence in later in life [9,10] along with a higher chance for developing chronic obstructive pulmonary disease (COPD) [11,12].

One of the proposed underlying mechanisms for prenatal smoke exposure (PSE) induced risk for COPD and aberrant smoking behavior could be epigenetic modifications, which have been shown for a large number of genes in peripheral blood, placenta and buccal cells in humans [13,14] and in mouse models [15,16]. Although epidemiology and *in vivo* experiments highlighted adverse outcomes of PSE in relation to the smoke-related diseases, the pathogenesis of diseases and related signaling pathways have not yet been fully described.

Lung development and early life exposure

Lung development starts from a single sheet of endodermal cells in the embryonic foregut [17], which can be divided into five stages, namely the embryonic -, pseudoglandular -, canalicular -, saccular - and the alveolar stage, which are briefly described in Table 1. In the mouse, primary lung buds are identified around E9.5. During the embryonic stage, the primitive lung bud branches to form the two lung buds. In the pseudoglandular stage, the two lung buds begin to grow and differentiate into airway trees with thousands of terminal branches [18]. This is followed by the canalicular stage, for which the

main pattern of this stage is the formation of terminal branches of the airway trees and early development of the lung parenchyma, as the gas exchange areas. The saccular and alveolar stages run through the formation of primitive terminal airspaces and the production of surfactant by alveolar epithelial type 2 cells (AECII). This stage prepares the fetus for birth by increasing lung parenchyma growth, thinning of connective tissue between the airspaces and further maturation of the surfactant system. Finally, the full maturation of the alveoli occurs during the alveolarization stage in the postnatal period.

Table 1. *The five structural phases of lung formation[19].*

Phase	Events	Mouse
Embryonic	Formation of the lung buds and major bronchi, division of tracheal-esophageal tube	E9-E11.5
Pseudoglandular	Proliferation of bronchial branches, acinar tubules and buds; vasculogenesis and innervation	E11.5-E16.5
Canalicular	Organization of the pulmonary vascular bed, pulmonary acinus, and increasing innervation, beginning of alveolar epithelium development	E16.5-E17.5
Saccular	Dilatation of peripheral airspaces, differentiation of the respiratory epithelium and increasing vascularity of the saccules, surfactant synthesis	E17.5-PN5
Alveolar	Increase of number and size of the alveoli, maturation of the pulmonary vascular system	PN5-PN28
<i>E = embryonic; PN = postnatal</i>		

In addition, lung development is required for proximodistal patterning, which includes a variety of lung epithelial cell types [20]. The lung epithelial cell plays a variety of physiological roles, including repair, and regeneration of the lung epithelial cells. Of particular interest is this latter function governed by lung epithelial stem/progenitor cells, i.e., basal cell, pulmonary neuroendocrine cell, and AECII (reviewed in [21,22]). These cells are capable of self-renewal and differentiate to mature lung epithelial cell types in order to repair epithelial injury and maintain plasticity [23,24].

There is increasing evidence that exposure of children to smoke *in utero* has substantial adverse effects on respiratory health. Many studies have documented that PSE is associated with a decreased lung function, airway obstruction, and airway hyperresponsiveness in children [11,12,25–27]. One of the underlying mechanism for the PSE-induced effects is aberrant lung development, as

shown by numerous studies in experimental animal models [28–32]. In these models, PSE was shown in mice to increase the airway muscle layer [8] and enlarge the airspace size, leading to aberrant alveolarization [31]. A similar effect was seen in rhesus monkeys, in which *in utero* exposure to nicotine resulted in lung hypoplasia and a reduction of the gas-exchange surface in the lung [32]. Several pathways have been proposed to play a role in impaired lung development [33–35], including the epidermal growth factor receptor (EGFR) signaling pathway[36], the Wnt signaling pathway [37] as well as the bone morphogenetic protein (BMP) signaling pathway[38]. In this study we focused on the role of the EGFR signaling pathway as we found PSE-induced differential expression of EGFR ligands EGF and AREG in our mouse model.

The role of amphiregulin (AREG) and (EGF)-induced EGFR signaling in lung development and epithelial cell biology.

The EGFR signaling pathway has been identified as a central regulator of lung epithelial cell growth and differentiation during lung development[39]. Moreover, EGFR and its ligands are expressed during both the prenatal and postnatal periods of lung development and give rise to the different bronchiolar and alveolar epithelial cells, as well as the vascular smooth muscle[40]. Experiments in EGFR-deficient mice showed an immature development of the lung, which included deficient alveolarization, loss of surfactant expression, and alveolar collapse, which is comparable to the pathology of neonatal respiratory distress syndrome[36].

EGFR signaling is mostly induced by two competitive ligands, i.e., epidermal growth factor (EGF) and amphiregulin (AREG)[41]. EGF has a higher affinity for the EGFR than AREG[42]. Those ligands were originally described as epithelial cell-derived factors, and recent data showed that AREG can additionally be expressed by multiple populations of activated immune cells in a variety of inflammatory conditions[43]. Moreover, both ligands have distinct biological functions. EGF is involved in epithelial cell differentiation and maturation[39,44,45], and both EGF and EGFR are higher expressed in immature lungs compared to adult lungs, as shown in the mouse[36]. In contrast, AREG has been described to promote cell proliferation and hyperplasia[44,46]. Several studies have highlighted that AREG is secreted by alveolar macrophages[47], mast cells[48], and T helper (Th2) 2 cells[49] and can behave both as a paracrine and autocrine factor[50]. Recent data have

suggested an important role for AREG in (smoking-related) chronic lung diseases[51–53], showing that AREG treatment of cultured human airway basal cells promoted basal cell and mucous cell hyperplasia, altered ciliated cell differentiation and impaired barrier integrity [50].

Maternal smoking during pregnancy and prenatal (re) programming

Developmental Origins of Health and Disease (DOHaD) hypothesis.

Prenatal programming occurs during embryonic and fetal development and it has a crucial and profound impact on the risk of disease development later in life, which depends on the *in utero* environmental conditions. This concept was originally proposed as the “Barker Hypothesis”. David Barker was the first to describe that poor maternal nutrition during pregnancy in early life imposed a higher risk of developing ischemic heart diseases later in life[54]. Until now, epidemiological and experimental animal studies have shown that maternal stressors during pregnancy such as poor nutrition, drug addiction, hypoxia, alcohol, and maternal smoking, contribute to the development of chronic diseases in offspring[55,56]. The Barker hypothesis is currently recognized as the Developmental Origins of Health and Disease (DOHaD) hypothesis. Increasing evidence suggests that epigenetic mechanisms mediate prenatal programming[57].

Epigenetics

Epigenetics describes the heritable changes in gene expression or cellular phenotype caused by mechanisms that do not alter the underlying DNA sequence. Epigenetics includes histone modifications, DNA methylation and RNA-based mechanisms (lncRNAs / miRs)[58], as shown in figure 1. Altered epigenetic gene regulation is one of the processes that has been implicated to underlie the DOHaD hypothesis by changing gene expression levels leading to the development of disease. DNA methylation is the most extensively studied epigenetic mechanism in the control of gene expression by altering the methylation. DNA methylation is a process in which a methyl group ($-CH_3$) is added to cytosine residues in cytosine-phosphate-guanine (CpG) dinucleotides which is catalyzed by DNA methyltransferase (DNMT) family members. In contrast, the ten-eleven translocation (TET) methylcytosine dioxygenase family of enzymes are considered to play a major role in maintaining of active DNA demethylation patterns (reviewed in[59]). DNA demethylation is a process that removes the methyl group from cytosines in DNA. These DNMT and TET

enzymes are thought to perform an opposite role in DNA methylation patterns. In mammals, approximately 70% of CpG dinucleotides are methylated[60], which occurs in high density CpGs (CpG islands) at the gene transcription start site. The levels of DNA methylation play a role in gene silencing and activation, which are tightly controlled by a tissue and development specific manner[61].

Figure 1. Epigenetic mechanisms in context of the DOHaD theory.

Developmentally induced epigenetic modifications of DNA are generally stable during mitotic cell divisions and could continue throughout a lifetime. Therefore, early life triggers during embryonic development, such as *in utero* exposure to cigarette smoke, initiating altered DNA methylation levels in certain genes, can pose for chronic diseases. A longitudinal study in young adults of both sexes showed that PSE-induced differentially methylated CpGs in peripheral blood observed around the age of 30 persisted into middle age (around age 48) [62,63]. Although these results suggest that some of the PSE-associated methylation changes are irreversible and unaffected by age, the mechanisms underlying the adverse effects of PSE are yet not fully described. It has been proposed that PSE may cause stable alterations within the gene expression profile affecting further protein expression throughout the life-course[64].

CYP2A5 regulation and nicotine metabolism.

Cigarette smoke is a toxic and carcinogenic mixture of more than 5000 individual components[65], of which almost all components can enter the fetal circulation after maternal cigarette smoke exposure[66]. Among these, nicotine

is the most likely cause of deficits in growth and neural development, which have long-term effects on brain function, cognition, and behavior[67]. One of the main actions of nicotine, as described by pharmaco-kinetics studies, is the binding to nicotine receptors, which stimulates dopamine release in the central nervous system, the area of the brain responsible for rewarding properties[68], which could lead to nicotine dependence (reviewed in[69,70]). Nicotine dependence is generally associated with a higher nicotine metabolism[71–73], as persons with a faster rate of nicotine conversion (i.e. a faster elimination of nicotine from the body), experience more severe nicotine withdrawal symptoms and a greater subjective reward from the cigarette smoked following deprivation (reviewed in[72]). In contrast, persons with a slower rate of nicotine conversion display higher smoking cessation rates due to mild nicotine withdrawal symptoms[71]. These effects would explain why persons with a higher nicotine metabolism have a more persistent smoking behavior when compared to those with a lower nicotine metabolism. Smoking behavior and nicotine dependence have been studied extensively in relation to smoke-related diseases[25,74].

In humans, ~90% of nicotine is converted by the Cytochrome P450 2A6 (CYP2A6) enzyme in the liver[75]. So far, the human has three functional genes (*CYP2A6*, *CYP2A7*, and *CYP2A13*), and the mouse has four (*Cyp2a4*, *Cyp2a5*, *Cyp2a12*, and *Cyp2a22*), which have been identified in the different organs[76,77]. In the mouse, the CYP2A5 is considered the orthologue gene of the human CYP2A6[78]. The regulation of the mouse CYP2A5 and the human CYP2A6 is complex as it metabolizes several therapeutic drugs and toxic substances, such as nicotine, nitrosamines, and aflatoxins. In the mouse, the majority of nicotine components (80% - 90%) is metabolized by the CYP2A5 enzyme in the liver[79]. The reaction of nicotine conversion takes place in two phases. Nicotine is first metabolized to nicotine- $\Delta 1'$ (5')-iminium ion, which subsequently is converted to cotinine by aldehyde oxidase[80]. Cotinine is further metabolized to trans-3'-hydroxycotinine in a process that is entirely mediated by the CYP2A5 enzyme[81], as described in Figure 2.

Figure 2. The major pathway of nicotine conversion.

The tissue distribution of CYP2A5 expression and enzyme activity is rather limited and most abundant in the liver. Extrahepatic CYP2A5 expression is described in olfactory mucous, kidney and lung tissue[82,83].

Scope of the thesis

This thesis covers two main topics, which are summarized in figure 3. In the first part we investigated the effect of (prenatal) smoke exposure on airway and alveolar epithelial cell differentiation. In the second part, we investigated the effect of pre- and/or postnatal smoke exposure on *Cyp2a5* DNA methylation in relation to the *in vitro* nicotine conversion throughout the life-course.

In part 1, **chapter 2**, the aim was to investigate the adverse effects of PSE on lung epithelial cell development in neonatal offspring and explore the possible role of AREG-induced EGFR signaling using AREG-treated mouse lung organoids. Subsequently, in **chapter 3**, a start was made to investigate the role of AREG-induced EGFR signaling on AECII behavior in COPD, using AREG-treated human lung organoids.

In part 2 of the thesis, we investigated the prenatal programming effects of PSE on *Cyp2a5* DNA methylation in relation to the hepatic nicotine metabolism across three developmental stages. In **chapter 4**, the effect of PSE on promoter methylation profiles of *Cyp2a5* was investigated in fetal, neonatal and adult offspring and linked to its gene expression and the functional readout of *in vitro* nicotine metabolism in the liver. We found that PSE induced persistent *Cyp2a5* promoter methylation, which was accompanied by a higher conversion rate of nicotine-to-cotinine in the liver of neonatal and adult male offspring. In **chapter 5**, we added the effect of postnatal smoke exposure to our study to investigate whether postnatal smoke exposure further impaired the PSE-induced effects on nicotine metabolism, as well as *Cyp2a5* DNA methylation along with gene expression in the liver. In **chapter 6**, our question related to the PSE-induced effects on *Cyp2a5* methylation and its gene expression was extended to the lung to investigate whether the prenatal programming effect across three developmental stages was organ-specific. Here, we describe for the lung a sex-dependent response to PSE regarding *Cyp2a5* mRNA expression, while DNA methylation profiles were not greatly altered across three developmental stages. In **chapter 7**, the results are summarized, discussed and put in perspective for future experiments.

Figure 3. Thesis overview

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CHAPTER 2

Prenatal smoke exposure dysregulates lung epithelial cell differentiation in mouse offspring – Role for AREG-induced EGFR signaling

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Abstract

Prenatal smoke exposure is a risk factor for impaired lung development in children. Recent studies have indicated that amphiregulin (AREG), which is a ligand of the epidermal growth factor receptor (EGFR), has a regulatory role in airway epithelial cell differentiation. In this study, we investigated the effect of prenatal smoke exposure on lung epithelial cell differentiation and linked this with AREG-EGFR signaling in 1-day old mouse offspring. Bronchial and alveolar epithelial cell differentiation were assessed by immunohistochemistry. *Areg*, epidermal growth factor (*Egf*) as well as mRNA expression of specific markers for bronchial and alveolar epithelial cells were assessed by RT-qPCR. Results in neonatal lungs were validated in an AREG-treated 3D mouse lung organoid model. We found that prenatal smoke exposure reduced the number of ciliated cells and the expression of the cilia-related transcription factor *Foxj1*, whereas it resulted in higher expression of mucus-related transcription factors *Spdef* and *Foxm1* in lung. Moreover, prenatally smoke exposed offspring had higher numbers of alveolar epithelial type II cells (AECII) and lower expression of the AECI-related *Pdpn* and *Gramd2* markers. This was accompanied by higher expression of *Areg* and lower expression of *Egf* in prenatal smoke exposed offspring. In bronchial organoids, AREG treatment resulted in fewer ciliated cells and more basal cells when compared non-treated bronchiolar organoids. In alveolar organoids, AREG treatment led to more AECII cells than non-treated AECII cells. Taken together, the observed impaired bronchial and alveolar cell development in prenatally smoke-exposed neonatal offspring may be induced by increased AREG-EGFR signaling.

Keywords: Prenatal smoke exposure; Amphiregulin; Epithelial cell differentiation; Lung organoids.

Introduction

Epidemiological studies have shown that prenatal smoke exposure is associated with asthma in children and adolescents, as well as with chronic obstructive pulmonary disease (COPD) later in life [1–5]. One of the underlying mechanisms could be an aberrant development of the lung, as shown by a number of studies in experimental animal models [6–10]. In these models, prenatal smoke exposure was shown to increase the airway muscle layer [11] and enlarge the airspace size, suggesting an alveolarization defect [9]. A similar effect was seen in rhesus monkeys, in which *in utero* exposure to nicotine resulted in lung hypoplasia and a reduction of the gas-exchange surface in the lung [10].

Epidermal growth factor receptor (EGFR) signaling regulates lung morphogenesis to give rise to the branching of airways and alveolarization [12]. In addition, it also has been suggested to be a crucial regulator of goblet cell hyperplasia [13] and mucous hypersecretion [14] in cigarette smoke-exposed airway epithelium. EGFR signaling is mostly induced by two competitive ligands, i.e. amphiregulin (AREG) and epidermal growth factor (EGF) [15,16]. Each ligand has distinct biological functions, i.e. AREG is known to promote cell proliferation, apoptosis, and hyperplasia [17,18], whereas EGF has a higher affinity to EGFR than AREG and is involved in epithelial cell differentiation [19,20]. AREG is a polypeptide growth factor, which belongs to the EGF family. AREG is expressed by activated immune cells, such as alveolar macrophages [18,21], mast cells [22], and T helper 2 (Th2) cells [23], and it is regulator as paracrine and autocrine factors in chronic lung inflammation and wound repair. Studies have highlighted AREG-induced EGFR activation through various environmental effects, such as smoking [24], injuries, and infections [21,25,26]. Recently, Zuo *et al.* showed that AREG-induced EGFR activation drives airway epithelial cell differentiation towards basal -and mucous cell hyperplasia in an *in vitro* model of the air-liquid interface [24].

To date, no studies have addressed the role of the AREG-EGFR signaling pathway in prenatally smoke-induced aberrant lung development. With this project, we aimed to investigate the effect of prenatal smoke exposure on lung epithelial cell differentiation (bronchiolar and alveolar) and its link with AREG-induced EGFR signaling in 1-day old mouse offspring.

Material and methods

Animals

Female and male C57Bl/6 mice, age 8-10 weeks, were obtained from Harlan (Horst, The Netherlands). Mice had access to standard food and water ad libitum. The animal study was approved by the Institutional Animal Care and Use Committee of the University of Groningen (Permit Number: 6589a) and was performed under strict governmental and international guidelines on animal experimentation.

Cigarette smoke exposure

Mainstream cigarette smoke was generated using a TE-10 smoke exposure system of Teague Enterprises Smoke Exposure System (Woodland, California, USA). Female mice were exposed to fresh air (n=3) or cigarette smoke (n=4) in two sessions of 50 minutes with a 3h interval between both exposures per day in which smoke of 10 cigarettes was generated per session. Mice were exposed from 7 days before mating until the day of sacrifice. The adaption protocol included exposure to 3 cigarettes per session the first day, 5 cigarettes the second day, 7 cigarettes the third day and 10 cigarettes the fourth day and thereafter. Smoking 10 cigarettes in one session generated total particulate matter counts of 211 ± 30 mg/m³ and a CO level of 205 ± 35 parts per million where CO levels of 241 parts per million were reached at the max. Kentucky 2R4F research-reference filtered cigarettes (The Tobacco Research Institute, University of Kentucky, Lexington, Kentucky, USA) were used. For experimental purposes, female mice were treated with 1.25 IU pregnant mare's serum gonadotropin and 1.25 IU human chorionic gonadotrophin to induce simultaneous cycling. To induce pregnancy, 1 female was housed with 1 male. Mating was confirmed by vaginal plug detection. Smoke exposure remained constant during the total pregnancy. Mothers and offspring were not exposed to cigarette smoke after offspring was born. Offspring (n=16 from non-smoking mothers, n=24 from smoking mothers) was sacrificed one day after birth. The left lung was used for qRT-PCR analyses. The right lung was fixed in formalin and embedded in paraffin for immunohistochemically and immunofluorescence analyses.

qRT-PCR analysis in lung tissue

Total RNA was isolated from lung tissue using an RNA isolation trizol kit (Invitrogen, USA). cDNA was reverse transcribed using a Superscript-

II Reverse Transcriptase kit (Invitrogen, USA). To measure the expression of *Gapdh* (Mm99999915_g1), *Foxj1* (Mm01267279_m1), *Scgb1a1* (Cc10, Club cell, Mm00442046_m1), *Foxm1* (Mm00514924_m1), *Spdef* (Mm00600221_m1), *Ppdn* (Mm00447558_m1), *Aqp5* (Mm00437578_m1), *Gramd2* (Mm01174485_m1), *Nkx2.1* (Mm00447558_m1), *Egf* (Mm00438696_m1), *Areg* (Mm 01354339_m1), *Egfr* (Mm01187858_m1), on demand Gene Expression Assays were used (T, USA). PCR reactions were performed in triplicate in a volume of 10 μ L consisting of 2 μ L of MilliQ water, 5 μ L PCR master mix (Eurogentec, Seraing, Belgium), 0.5 μ L assay mix (life technologies, USA), and 2.5 μ L cDNA. Runs were performed by a LightCycler® 480 Real-Time PCR System (Roche, Basel, Switzerland). Data were analyzed with LightCycler® 480 SW 1.5 software (Roche, Basel, Switzerland) and the Fitpoints method. RNA data were normalized to *Gapdh* mRNA expression using $2^{-\Delta C_p}$ (C_p means crossing points). Undetectable C_p values of the genes of interest (>40) were interpreted as the maximum C_p value.

Lung organoids culture

Mouse lung organoids were grown as described previously [27,28]. For the lung organoids, EpCAM⁺ cells were isolated from lungs of pathogen free male, (n=3) and female (n=2) mice that were between 8 and 12 weeks old. Briefly, ~10,000 EpCAM⁺ mouse lung cells were seeded with an equal number of fibroblasts per insert in 100 μ L growth factor-reduced Matrigel, diluted 1:1 with DMEM/F12 containing 5% FBS into transwell inserts for 24-well. In the treated lung organoids groups, AREG (10ng/ml; R&D System, Minneapolis, USA) was freshly added to the lung organoids every 2 days. All mouse lung organoid cultures were maintained at 37°C in a humidified incubator with 5% CO₂. After 14 days, lung organoids were fixed in acetone that was diluted 1:1 (v/v) with methanol for 15 min at -20°C. For bronchial epithelial cell analyses, organoids were prepared for the histology of cross-sectional membranes, as published previously [29]. The lung organoids were stabilized in 2% agarose, dehydrated, and paraffin-embedded. Organoids were stained for basal cells (KRT5), club cells (CC10), ciliated cells (tubulin), AECI (PDPN) and AECII (pro-SPC) All used antibodies are listed in Table 1.

Table 1. *List of primary and secondary antibodies used in the present study*

Antigen	Host and binder recognition	Dilution	Company
Primary antibody			
Tubulin	Mouse, anti-acetylated a-tubulin	1:10000 IHC, IF	Sigma-Aldrich, T7451
CC10	Rabbit, anti-club cell (10 kD)	1:6000 IHC	Millipore, 07-623
CC10	Goat, anti-club cell	1:200 IF	Santa-Gruz, sc-365992
ProSP-C	Rabbit, anti-SP-C	1:200 IHC	Millipore, AB3786
Pdpn	Hamster, anti-Podoplanin	1:5000 IHC, IF	Thermo Scientific, 14-5381-82
KRT5	Chicken, anti- Keratin 5	1:200 IHC, IF	Biolegend, 905901
Nkx2.1	Mouse, anti- Nkx2.1	1:200 IHC, IF	Thermo Scientific, MA5-13961
Amphiregulin	Rabbit, anti-amphiregulin	1:100 IHC, IF	Boster, A01787
Amphiregulin	Rabbit, anti-amphiregulin	1:50 IF	Biorbyt, orb4539
Ki67	Rat anti-Ki67	1:200 IF	Biolegend, 652402
Secondary antibody			
Rabbit	Anti-goat HRP	1:100 IHC	Dako, P0449
Goat	Anti-rabbit HRP	1:100 IHC	Dako, P0448
Goat	Anti-hamster HRP	1:200 IHC	Southern Biotech, 6061-05
Rabbit	Anti-Goat BIOT	1:100 IHC	Southern Biotech, 6160-08
Donkey	Anti-Rat BIOT	1:100 IF, IHC	Southern Biotech, 6430-08
	Streptavidin-HRP	1:300 IHC	Dako, P0397
Rabbit	Anti-rat HRP	1:100 IHC	Dako, P0450
Donkey	Anti-goat Alexa Fluor 488	1:100 IF	Thermo Scientific, A11055
Donkey	Anti-rabbit Alexa Fluor 488	1:100 IF	Thermo Scientific, A21206
	Streptavidin, Alexa Fluor 555	1:300 IF	Thermo Scientific, S21381

IF, immunofluorescence; IHC, immunohistochemistry.

Immunohistochemistry (IHC) and Immunofluorescence (IF) staining

Lung tissue. Sections (3 μm) of formalin-fixed and paraffin-embedded lung tissue were stained for ciliated cell, club cells, AECI and AECII using standard IHC method. Briefly, slides were deparaffinized and put in citrate (pH 6.0) and Tris-HCl (pH 7.4) buffer in a microwave oven for 15min. After cooling, slides were incubated in 0.3% H_2O_2 in PBS for 30 min. To visualize ciliated cells (Tubulin), club cells (CC10), AECII (pro-SPC and NKX2.1), AECI (PDPN), proliferation marker (Ki67) with AECII cells and AREG, they were incubated with the specific primary antibodies, as listed in Table 1. As a second step, binding of primary antibodies was detected by using enzyme horseradish peroxidase (HRP) and the biotinylated conjugated secondary antibodies for 30 min. For the color reaction, the secondary antibodies were detected by A 0.05% diaminobenzidine (DAB, Sigma-Aldrich, The Netherlands) and

3-amino-9-ethylcarbazole (AEC, Sigma Aldrich) solutions. For all quantification's analysis of lung and organoid slides, one slide per mouse was used and slides were coded and examiners were blinded to groups in all quantifications. Numbers of tubulin and CC10 positive cells in all airways were counted manually, and total the number of CC10 and tubulin positive cells were corrected for per mm airway. These analyses were assessed by using Aperio-ImageScope viewing software (Aperio, 11.2.0.780, Vista, USA) and TissueFaxs imaging system (Tissue Gnostics, Austria). Numbers of pro-SPC, NKX2.1, AREG, PDPN, and Pro-SPC/Ki67 positive cells were analyzed by using morphometric analysis in Aperio-ImageScope viewing and ImageJ software, and corrected for the total area of the lung tissue per section.

Lung organoids

Paraffin-embedded organoids were stained double or triple stained with primary antibodies against KRT5 (basal cell), CC10 (club cell) and tubulin (Ciliated cell). The expression of these markers was visualized by staining with fluorescent secondary antibodies and the mounting medium containing DAPI (Abcam, Cambridge, UK). Immunofluorescence (IF) slides were scanned using a TissueFaxs imaging system (Tissue Gnostics, Austria), and images were acquired using a Leica SP8 (Leica) confocal and a Leica 4000b microscopes at 40x magnification. For alveolar organoids, organoid cultures were washed with PBS and blocked in PBS with 5% (w/v) BSA. Primary antibodies against pro-SPC (AECII) and PDPN (AECI) were diluted in PBS buffer with 0.1% BSA and 0.1% Triton, and the organoids cultures were incubated with primary antibodies

at 4°C overnight. The next day, the organoids were washed with PBS for 30 min, and AECI and AECII were detected using fluorescent secondary antibodies and the mounting medium containing DAPI. The alveolar organoids' images were acquired using the Leica SP8 confocal microscope at 40x magnification. Processed channels were merged or split using ImageJ software. Positive cells were counted manually and positive cell numbers were corrected for the total number cells of bronchial and alveolar epithelial cells in mouse lung organoids.

Statistical methods

For statistical evaluation of the different groups, a two-tailed Mann-Whitney U-test was used (GraphPad Prism 7.02 Software, San Diego, CA and SPSS Statistics 23, IBM, The Netherlands). Differences between the prenatal smoke-exposed offspring and controls were assessed separately in males and females with a two-sided Mann-Whitney U-test. A value of $p < 0.05$ was considered significant. Relative gene expression ($2^{-\Delta Ct}$ method) and standard error of the mean (SEM) were calculated in Microsoft® Office Excel 2016.

Results

To investigate how prenatal smoke exposure affects on lung development, we began by exploring the bronchial epithelial cell development, which was visualized by IHC analyses of tubulin-expressing (ciliated) cells and CC10-expressing (club) cells, as shown in Figures 1A and C. Prenatally smoke-exposed neonates had lower numbers of ciliated cells when compared to the controls ($P < 0.001$; Figure 1B), which was observed in both male and female offspring ($P < 0.0001$; Figure 1b). This was accompanied by lower expression of *Foxj1*, especially in prenatally smoke-exposed male offspring ($P = 0.03$; Figure 1E, male: $P = 0.04$; Figure 1e). In contrast, no effect of prenatal smoke exposure was observed on the number of club cells (Figure 1D and 1d) or expression of *Scgb1a1* mRNA levels (club cell marker) in both groups (data not shown). Prenatally smoke-exposed neonates did have higher mRNA expression of other key players in secretory cell formation such as *Foxm1* ($p = 0.014$, Figure 1F, male: $P = 0.01$, female: $P = 0.04$; Figure 1f) and *Spdef* ($P < 0.001$; Figure 1G, male: $P < 0.001$; Figure 1g).

Figure 1. Ciliated cell (A) and club cell (C) were stained by tubulin and CC10 markers in paraffin-embedded slide with DAB (brown) substrate. The number of tubulin+ (B, and b) and CC10+ (D and d) cells which were determined in lung tissue of prenatally exposed to cigarette smoke or not, original magnification 40x. E and e present gene expression of *Foxj1*, a marker for ciliated cells the differentiation markers. Genes that drive secretory cell development, gene expression of the transcription factor *Foxm1* (F and f), and *Spdef* (G and g) were analyzed by real-time qPCR in RNA, isolated from lung tissue. A lowercase letter indicates a sex-dependent (male, female) difference in the data. Data represent means of expression in offspring of air-exposed mothers (open symbols) or smoke-exposed mothers (closed symbols). Circle (○) symbol(s)=male, triangle (Δ) symbol(s)=female. Data are shown as individual values. If not stated otherwise, the difference between groups was not significant. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (Mann-Whitney U-test).

For alveolar epithelial cell development, we asked whether AECII (Figures 2A and C) and AECI (Figure 3A) differentiation was affected by prenatal smoke exposure in this mouse model. Prenatally smoke-exposed offspring had a higher number of pro-SPC (AECII) and NKX2.1 (AECII)-positive cells in both sexes compared to their control groups ($p = 0.001$; Figure 2B, both sexes: $P < 0.05$; Figure 2b and $p = 0.0005$; Figure 2D, male: $P = 0.05$, female: $P = 0.006$; Figure 2d). To investigate whether the higher AECII number was due to more

proliferating AECII cells, we double stained AECII (pro-SPC) cells with the proliferation marker Ki67, as shown in Figure 2E. In prenatally smoke-exposed neonates, the number of double positive (Ki67/SPC) cells was higher than in the control group ($P = 0.045$; Figure 1F and f). In contrast, the number of PDPN positive (AECI) cells was not affected by prenatal smoke exposure (Figure 3B and 3b) but lower mRNA levels of the *Pdpn* and *Gramd2* were observed in prenatally smoke-exposed offspring when compared to the control group ($P < 0.0001$; Figure 3C, male: $P = 0.04$, female: $P = 0.006$; Figure 3c and $P < 0.0001$, Figure 3E, both sexes: $P < 0.001$; Figure 3e). *Aqp5* mRNA levels were not affected by prenatal smoke exposure (Figure 3D and 3d).

Figure 2. AECII cells were stained by pro-SPC (A) and Nkx2.1 (C) markers in paraffin-embedded slide with DAB (brown) substrate, original magnification 40x. The number of SPC⁺ (B and b) and Nkx2.1⁺ (D and d) cells significantly increased in neonatal offspring from smoke-exposed mothers and compared to control neonates. E presents proliferation marker (Ki67, green) with AECII (pro-SPC, red). Ki67/SPC positive cells were increased in prenatally smoke-exposed neonates when compared to control neonates, as shown in Figure F and f. A lowercase letter indicates a sex-dependent (male, female) difference in the data. Data represent means of expression in offspring of air-exposed mothers (open symbols) or smoke-exposed mothers (closed symbols). Circle (○) symbol(s)=male, triangle (Δ) symbol(s)=female. Data are shown

as individual values. If not stated otherwise, the difference between groups was not significant. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (Mann-Whitney U-test).

We next investigated the role of AREG in the prenatal smoke-induced aberrant cell differentiation. First, we identified AREG-expressing cells, which could be mainly observed in the lung parenchyma, as shown in Figure 4A. A higher number of AREG-expressing cells was found in prenatally smoke-exposed offspring when compared to the control group ($P = 0.04$, Figure 4B), which was most pronounced in females ($P = 0.012$, Figure 4b).

Figure 3. The number of PDPN⁺ cells (B and b) was quantified in a paraffin-embedded slide with DAB (brown, A) substrate, original magnification 40x. The *Pdpn* (C and c) *Aqp5* (D and d) and *Grand2* (E and e) mRNA levels were analyzed by real-time qPCR in isolated RNA from lung tissue. A lowercase letter indicates a sex-dependent (male, female) difference in the data. Data represent means of expression in offspring of air-exposed mothers (open symbols) or smoke-exposed mothers (closed symbols). Circle (○) symbol(s)=male, triangle (Δ) symbol(s)=female. Data are shown as individual values. If not stated otherwise, the difference between groups was not significant. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ (Mann-Whitney U-test).

Furthermore, higher *Areg* mRNA levels were found in prenatally smoke-exposed offspring when compared to the control group in both sexes ($P = 0.0014$, Figure 4C; both sexes: $P < 0.05$, Figure 4c). In contrast, lower *Egf* mRNA levels were detected in prenatally smoke-exposed offspring ($P = 0.0018$, Figure 4D, female: $P = 0.02$; Figure 4d) when compared to the control group. *Egfr* mRNA levels were not affected by prenatal smoke

Figure 4. A presents IHC staining of AREG-expressing cells (red). The number of AREG-expressing cells (B and b) was quantified in lung tissues of neonatal offspring from smoke-exposed mothers and air-exposed mothers, original magnification 40x. The red arrow shows AREG-expressing cells in lung tissue. Gene expression levels of *Areg* (C and c), *Egf* (D and d) and *Egfr* (E and e) were analyzed by real-time qPCR in RNA, isolated from lung tissue. A lowercase letter indicates a sex-dependent (male, female) difference in the data. Data represent means of expression in offspring of air-exposed mothers (open symbols) or smoke-exposed mothers (closed symbols). Circle ($\circ$) symbol(s)=male, triangle (Δ) symbol(s)=female. Data are shown as individual values. If not stated otherwise, the difference between groups was not significant. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ (Mann-Whitney U-test).

exposure (Figure 4E and 4e). Based on these results, we next asked whether indeed AREG was able to affect lung epithelial cell differentiation.

To answer this question, we used a 3D mouse lung organoid model and compared epithelial cell differentiation in AREG-treated and non-treated lung organoids. Epithelial cell differentiation in these mouse lung organoids was assessed 14 days after treatment as shown in Figure 5A. In bronchiolar organoids, ciliated, club and basal cells were visualized in cross-section slides using triple IF staining for tubulin, CC10 and KRT5, as shown in Figure 5B. After counting whole organoids, we found lower numbers of tubulin-positive cells ($P < 0.001$, Figure 5D) and higher numbers of KRT5 positive cells ($P = 0.02$, Figure 5C) in AREG-treated lung organoids when compared to non-treated lung organoids. AREG treatment did not affect the number of CC10-positive cells in bronchiolar organoids (Figure 5E). Furthermore, we assessed alveolar epithelial cell differentiation for which pro-SPC (AECII) and PDPN (AECI)-positive cells (Figure 5F) were counted in AREG-treated and non-

treated groups. More pro-SPC positive cells were detected in AREG-treated alveolar organoids when compared to non-treated alveolar organoids ($P = 0.03$, Figure 5G), while no effect was seen for the number of PDPN-positive cells (Figure 5H).

Figure 5. 3D mouse organoids formation and lung epithelial cell differentiation in AREG-treated and non-treated groups. Representative light and confocal microscopes pictures show mouse lung organoids formation at 14 days (A, AREG-treated organoids). Original magnification 20x or 40x. The representative picture shows triple IF staining (B) in bronchiolar organoids and double IF staining (F) in alveolar organoids. The number of Tubulin⁺ (C, green cells), CC10⁺ (D, yellow cells) and KRT5⁺ (E, red cells) were in bronchiolar mouse organoids. Quantification analysis of pro-SPC⁺ (G) and PDPN⁺ (H) was performed in alveolar mouse organoids. Data are shown as individual values. If not stated otherwise, the difference between groups was not significant. * $P \leq 0.05$, * $P \leq 0.001$ (Mann-Whitney U-test).

Discussion

In the present study, we evaluated the adverse effect of maternal smoking during pregnancy on lung epithelial cell development in neonatal offspring, and investigated the possible role of AREG on epithelial development in this process. With respect to *bronchial* epithelial cells, prenatal smoke exposure resulted in fewer ciliated cells and downregulation of *Foxj1*, which was accompanied by higher expression of secretory transcription factors *Spdef* and *Foxm1*. Regarding *alveolar* development, a higher number of AECII was found, whereas lower *Pdpn* and *Gramd2* mRNA levels were observed in prenatally smoke-exposed neonates. These epithelial cell alterations may be mediated through AREG-induced EGFR signaling, as in prenatally smoke-exposed offspring higher numbers of AREG-expressing cells were found and higher *Areg* mRNA levels were accompanied by a lower expression of *Egf*. A possible role of AREG in epithelial cell differentiation was confirmed in mouse lung organoids, showing that AREG suppressed the number of ciliated and increased the number of AECII.

Ciliated cells are necessary for proper mucociliary clearance of particles and pathogens [30], and cilia dysfunction or a decrease of ciliated cell numbers may account for repeated chest infections and tissue destruction in the lung [31]. Indeed, epidemiological studies have shown that children born from a mother that smoked during pregnancy have a higher risk to suffer from asthma and airway infections [32,33]. Our data is also supported by a previous *in vitro* study in which primary human bronchial epithelial cells were exposed to cigarette smoke extract during differentiation at the air-liquid interface [34]. In that study, cigarette smoke extract exposure was shown to reduce the number of ciliated cells, while it increased the number of club cells and goblet cells. In our study, lower numbers of ciliated cells were accompanied by lower expression of *Foxj1*. In mice, club cells are thought to be progenitors of ciliated cells and goblet cells [35,36] which are the two major cell types in the proximal airways of the mouse. Therefore, more club cells in prenatally smoke-exposed offspring were expected. However, the lower numbers of ciliated cells were not accompanied by the presence of more CC10-positive club cells in our mice, which may be due to a large variation in club cell numbers between the different mice. However, prenatal smoke exposure did increase the expression of *Foxm1* and *Spdef*. *Foxm1*, a transcription factor of the Forkhead box family, plays an important role during embryonic development of alveolar surfactant

homeostasis and monocyte/macrophage recruitment [37]. Moreover, FOXM1 is known to promote SPDEF activity, a master transcription factor that regulates goblet cell differentiation in the airway epithelium [38]. Therefore, higher expression of *Foxm1* could have contributed to the higher expression of *Spdef* in offspring from smoke-exposed mothers that we found.

We next assessed the distribution of alveolar epithelial cells in our model. In the current study, prenatal smoke exposure increased the number of AECII, as well as the number of proliferating AECII cells. This result was accompanied by lower *Pdpn* and *Gramd2* mRNA expression in 1-day-old mouse offspring. Similarly, studies have shown that AECII proliferate to restore the alveolar epithelium in response to cigarette smoke-induced lung injuries, whereas AECI are more susceptible to die from the same exposure [39,40]. In our study, however, the number of AECI was not different in prenatal smoke-exposed mice compared to controls. A similar effect was seen in prenatal nicotine-exposed rat offspring [41]. In that study, intrauterine exposure to nicotine increased AECII proliferation and altered the metabolic activity of surfactant synthesis in AECII. Furthermore, in rhesus monkeys, intrauterine nicotine exposure resulted in a higher number of AECII and a higher mean linear intercept of airspaces, which was accompanied by a reduced alveolar complexity in the lung [10]. These models all show a preference for AECII self-renewal after injury induced by a intrauterine exposure. In the case this preference persists in the alveolarization stage, it could impair differentiation from AECII to AECI after injury and limit the regenerative capacity of the lung later in life.

Our results are also in line with human epidemiological studies, showing that prenatal smoke exposure is associated with deficits of the functional residual capacity of the lung and tidal flow volumes [42,43], which could be a high risk for an insufficient gas exchange in prenatally smoke-exposed infants.

Although prior reports have described that AREG promotes lung fibrosis [17], and mucous cell metaplasia in the airway [44], the role of AREG in lung development is not understood. To our knowledge, this is the first study showing that prenatal smoke exposure increased the number of AREG-expressing cells in the lung. This result is consistent with human studies in the lung, showing that urban fine particulate matter [45] and cigarette smoke [24] can stimulate AREG mRNA expression. In addition, AREG was shown to be increased in sputum samples from children with asthma [46]. The implications of more AREG-expressing cells in our model could be that

AREG, as a paracrine factor, promoted *Spdef* expression, a required regulator for club cell and goblet cell differentiation [44], which explains the reduced ciliated cell number in prenatal smoke exposed offspring. This is consistent with a study by Zuo *et al.* where they assessed the role of EGFR signaling in human airway basal cell differentiation upon AREG and EGF treatment in an air-liquid interface cell model and found that AREG-induced EGFR signaling promoted mucous cell hyperplasia and suppressed ciliated cell differentiation [24]. Another study showed that EGF-induced EGFR activation altered the morphology of airway basal cells with the loss of airway intercellular contacts [20]. Interestingly, in our model, prenatally smoke-exposed offspring had higher *Areg* mRNA levels which was accompanied by lower *Egf* mRNA levels. These results could implicate that the relative lack of EGF supports the binding of AREG to EGFR, for which the receptor normally has a lower affinity compared to EGF [16].

In this mouse model, for the vast majority of parameters we found no sex-differences, while prenatal smoke exposure induced more AREG-expressing cells in female neonates when compared to controls. This is of interest as a prior study reported that the treatment of progesterone was a critical host factor mediating production of AREG by epithelial cells and pulmonary tissue repair following infection with sublethal influenza A virus infection (9). These data suggest that sex hormones might have a role in AREG promoted proliferation of lung epithelial cells.

AREG has been described in lung epithelial and mesenchymal cells from embryonic mice [19], where it stimulates epithelial and mesenchymal cell proliferation, particularly at the tips of growing lung buds. This suggests that the location of AREG expressing cells and its target cells are an important factor in determining regulation of AREG. In the current study, our findings further elaborate on the unrecognized role of a cross-talk between AREG expressing cells and AECII responder cells regarding differentiation and proliferation, which might have a negative effect on the regenerative capacity of the lung later in life.

To validate our data obtained in the prenatally smoke-exposed model, and to further investigate the direct effects of AREG on epithelial cell differentiation, we used a 3D mouse lung organoid model. In this model, isolated AECII cells were cultured with fibroblasts for 14 days until lung organoids were formed [28]. From Day 1 on, they were treated with AREG protein or PBS, every other

day for 14 days. We found more basal cells and lower ciliated cell numbers in AREG-treated lung organoids, suggesting that AREG selectively supports the proliferation of basal cells, which may be at the expense of ciliated cell differentiation. This result supports both our results in prenatal smoke exposed offspring as it supports the study from Zuo *et al*, in the 2D air liquid interface cultured AREG-treated airway epithelium [24]. To elaborate on the role of AREG in AECII differentiation in our organoid model, it is of interest to note that AREG is upregulated in lung fibrosis and linked with lung fibroblast proliferation [17]. Because AREG can promote fibroblast proliferation, it is possible that AREG induces fibroblast activation in our AREG-treated lung organoids, which might have a negative impact on epithelial cell differentiation during lung organoid formation. Indeed, the efficiency of lung organoid-forming was shown to be reduced by TGF- β pretreatment of fibroblasts in the same model [47]. However, further studies are needed to determine the effect of AREG on fibroblast activation and lung epithelial cell differentiation in lung organoids.

Next to our results in the alveolar organoids, a higher number of AECII cells was observed in AREG-treated alveolar lung organoids, whereas the number of AECI was not altered. This observation regarding AECII stem cell proliferation fits with the earlier described stimulating effect of AREG on basal cell numbers, which are important for airway wall regeneration. Therefore, more studies are needed to further investigate the impact of AREG/EGF-induced EGFR signaling on basal-and alveolar epithelial cell proliferation, remodeling and regeneration in the lung. It should be noted that for AREG-treated and non-treated 3D lung organoid cultures, EpCAM⁺ cells were isolated from adult mice, which may be a confounding factor in this study to understand the role of AREG in.

To conclude, we provide evidence that prenatal smoke exposure impairs lung development in the offspring in which increased AREG/EGFR signaling may have a role. During *bronchiolar* development, prenatal smoke exposure resulted in fewer ciliated cells and lower expression of cilia-related transcription factor of *Foxj1*, whereas it promoted *Foxm1* and *Spdef* expression, as key players in secretory cell formation. During *alveolar* development, prenatal smoke exposure resulted in more AECII which was accompanied by lower expression of AECI-related markers *Pdpn* and *Gramd2*, which may cause a delay in alveolar development. As the AREG/EGFR signaling pathway

relates to impaired epithelial cell differentiation, AREG regulation during lung development may provide a novel intervention for aberrant lung development in children from smoking mothers.

Abbreviations: Alveolar epithelial cell (AEC), chronic obstructive pulmonary disease (COPD), amphiregulin (AREG),

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Disclosure

The authors declare they have no competing financial interests.

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CHAPTER 3

Alveolar epithelial type II cell behavior in COPD

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Abstract

Chronic obstructive pulmonary disease (COPD) is a progressive, life-threatening disease characterized by several phenotypes, including the destruction of alveolar tissue in the lung parenchyma resulting in emphysema. Emphysema is likely partly due to a reduced repair capacity of resident lung stem cells in the alveolar niche i.e., the alveolar epithelial type II cell (AECII) and the mesenchymal stromal cell. Previous studies have indicated that amphiregulin (AREG) and epidermal growth factor (EGF), which are ligands of the epidermal growth factor receptor (EGFR) that is present on, amongst others, AECII, have a regulatory role in epithelial cell differentiation and proliferation. In this study, we evaluated the hypothesis that impaired alveolar regeneration in patients with COPD could be explained by aberrant AREG-EGFR signaling in AECIIs.

First, we examined human lung tissue from Stage IV COPD patients and non-COPD control subjects for protein expression of AREG, EGF, EGFR and pro-SPC (AECII marker) by immunohistochemical (IHC) staining. We found that COPD patients had a lower number of pro-SPC positive cells per surface of lung parenchyma and EGFR-expressing AECII per total cell number of lung parenchyma when compared to non-COPD controls. No differences were found in the number of AREG-expressing cells per surface of lung parenchyma and EGF-expressing cells per total cell number of lung parenchyma.

To further investigate whether the function of AECII was impaired in COPD, AECII isolated from patients with COPD and non-COPD controls were cultured as organoids and treated with or without AREG. We found that AECII-derived organoids from COPD patients were significantly smaller in size than organoids from controls. In non-COPD controls, AREG treatment appeared to result in smaller organoids compared to untreated organoids. Immunofluorescent staining of the organoids revealed that the AECII proportion within the total cell number was not different between COPD or AREG-treated groups.

To conclude, our findings indicate that patients with severe, stage IV, COPD have fewer EGFR-expressing AECII in the lung tissue, which could be associated with a reduced ability to form organoids as we observed in COPD patients. More insight regarding the growth and formation of human AECII-derived organoids in COPD is necessary before we can conclude that EGFR signaling could be a new intervention for alveolar regeneration in patients with emphysema.

Keywords: Alveolar epithelial type II cell; Amphiregulin; Epidermal growth factor receptor; COPD; Human lung organoids.

Introduction

Chronic obstructive pulmonary disease (COPD) is a smoking-related progressive disease and a leading cause of death worldwide[1]. COPD is characterized by tissue remodeling [2], inflammation [3], and emphysema due to destruction of alveolar walls [4]. The emphysematous destruction of lung tissue is a major characteristic in the pathogenesis of severe COPD [5–8] and is shown by a reduced presence of epithelial and endothelial cells in the alveolar wall which is associated with impaired alveolar regeneration[9,10]. The alveolar epithelium consists of two major cell types: cuboidal surfactant-producing AECII and elongated alveolar type I cells (AECI) that facilitate gas exchange. AECII serve as endogenous lung alveolar epithelial stem cells and can self-renew or differentiate into AECI post injury and repair [11]. In COPD patients, the AECII stem cell function is thought to be affected, contributing to impaired lung tissue repair [6,12].

Epidermal growth factor receptor (EGFR) signaling regulates lung morphogenesis giving rise to the branching of airways and to alveolarization [13]. Moreover, EGFR signaling is involved in stem cell proliferation as well as maintenance of alveolar epithelial regeneration[14]. EGFR signaling is mostly activated by the two cognate ligands amphiregulin (AREG) and epidermal growth factor (EGF) [15,16]. Each ligand has distinct biological functions, i.e. EGF-induced signaling enhances AECII differentiation and maturation [17], whereas AREG is known to promote epithelial cell proliferation and hyperplasia [18,19]. Both EGF and EGFR were found to be higher expressed in immature lungs when compared with adult lungs, consistent with lung growth and differentiation [17]. Several studies have highlighted AREG-induced EGFR activation after various environmental effects, such as smoking [19] and other injuries [20].

We previously reported that the number of AREG-expressing cells was higher in prenatally smoke-exposed neonatal mouse lungs [21]. This higher number of AREG expressing cells was accompanied by more AECII cells in the alveolar compartment, as well as more Keratin 5⁺ basal cells and less ciliated cells in the airways, suggesting that AREG promotes epithelial cell proliferation instead of differentiation. In an effort to define the possible role of AREG in aberrant epithelial cell development in our prenatally smoke-exposed neonates, AECII-derived mouse organoids were treated with AREG, which resulted in more AECII and basal cells than non-treated organoids. However,

in these experiments, AECII were derived from healthy (adult) mice that are not expected to be functionally impaired. Furthermore, the responsiveness of mouse-derived AECII to AREG treatment in mouse organoids could be very different to the responsiveness of human AECII to AREG. Therefore, in the current study, we aimed to investigate the possible role of AREG-induced EGFR signaling in impaired alveolar regeneration in patients with COPD compared to non-COPD subjects.

Material and methods

Human subject and Ethics

All human lung tissue specimens used for histology were derived from 11 non-COPD control and 14 COPD patients undergoing tumor resection surgery and lung transplantation, respectively, at University Medical Center Groningen (UMCG). The study protocol was consistent with the Research Code of the UMCG (<https://www.umcg.nl/EN/Research/Researchers/General/ResearchCode>) and national ethics. All COPD patients were Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage IV, defined as a FEV1%pred < 30% and FEV1/FVC ratio < 70%.

Immunohistochemistry (IHC) in the human lung tissue.

Lung tissue

Sections (3 μ m) of formalin-fixed and paraffin-embedded human lung tissue were stained for AREG, EGF, EGFR and pro-SCP using the IHC method as described previously[21]. To visualize AREG-expressing cells, AECII (pro-SPC), and EGFR-and-EGF-expressing cells in the lungs, lung sections were incubated with the specific primary antibodies, as listed in Table 2. As a second step, binding of primary antibodies was detected by using the enzyme horseradish peroxidase (HRP) and the biotinylated conjugated secondary antibodies for 30 min. For the color reaction, the secondary antibodies were detected by 3-amino-9-ethylcarbazole (AEC, Sigma Aldrich) and by A 0.05% diaminobenzidine (DAB, Sigma-Aldrich, The Netherlands) solutions. Stained slides were all scanned using a Nanazoomer 2.0-HT slide (Hamamatsu Photonics) scanner. Numbers of AECII, AREG-expressing, EGF-and EGFR-expressing positive cells in the lung parenchyma were detected by the QuPath v0.2.3 software (open-source software, <https://qupath.github.io/>). Firstly, both the number of negatively stained cells and the surface of the lung

parenchyma were automatically analyzed in lung parenchyma using QuPath software. Subsequently, numbers of pro-SPC-, AREG-, and EGF positive cells were automatically counted in the lung parenchyma using QuPath software and corrected for both the total cell number and the total surface of lung parenchyma per section. For EGFR-expressing AECII, five random fields were analyzed per lung, and the QuPath software automatically counted negatively stained cells in selected areas. The EGFR-expressing AECII were manually counted based on AECII location and shape in the lung parenchyma and corrected for the total cell number in the lung parenchyma.

Table 1. *Patient characteristics*

	Donor	group	Age	Gender	Smoking status	Pack-years	ICS history	FEV1 %	FEV1/FVC%
Immunohistochemistry analysis	1	control	50	male	ex-smoker	NA	no	102	86.6
	2	control	59	female	ex-smoker	40	no	85	72.9
	3	control	43	female	ex-smoker	15	NA	121	75
	4	control	62	male	ex-smoker	15	NA	99	79
	5	control	44	female	ex-smoker	25	NA	105	75
	6	control	62	female	ex-smoker	NA	NA	119	69
	7	control	57	female	ex-smoker	10	yes	NA	NA
	8	control	55	male	ex-smoker	15	NA	91	76
	9	control	50	female	ex-smoker	NA	NA	127	79
	10	COPD IV	49	female	ex-smoker	30	no	12.2	23.6
	11	COPD IV	47	female	ex-smoker	28	yes	25.3	42.1
	12	COPD IV	52	male	ex-smoker	40	yes	15.4	20.5
	13	COPD IV	50	female	ex-smoker	7.5	no	NA	NA
	14	COPD IV	48	female	ex-smoker	6	yes	25.7	25.8
	15	COPD IV	55	female	ex-smoker	22	no	20.4	24
	16	COPD IV	55	male	ex-smoker	NA	no	17.4	33.9
	17	COPD IV	53	female	ex-smoker	8.25	no	14.2	45.5
	18	COPD IV	55	female	ex-smoker	54	yes	29.9	43.3
	19	COPD IV	48	male	ex-smoker	25	yes	12	23
	20	COPD IV	54	male	ex-smoker	20	yes	14	68
	21	COPD IV	51	female	ex-smoker	42	no	28.3	28.7
Lung organoids	1	control	73	male	current-smoker	32	no	108	78
	2	control	52	female	current-smoker	NA	no	84	73
	3	COPD IV	66	male	ex-smoker	15	NA	12	21
	4	COPD IV	61	female	ex-smoker	54	NA	13	32

FEV1 is forced expiratory volume in 1 second, FVC is forced vital capacity, ICS is inhaled corticosteroid.

Table 2. *List of primary and secondary antibodies used in the present study*

Antigen	Host and binder recognition	Dilution	Company
Primary antibody			
Pro-SPC	Rabbit, anti-SP-C	1:400 IHC	Millipore, AB3786
EGFR	Rabbit, anti-EGFR	1:100 IHC	Abcam, 14-5381-82
EGF	Rabbit, anti- EGF	1:500 IHC	Novusbio, NBP1-19806
Amphiregulin	Mouse, anti- AREG	1:50 IHC	Santa Cruz, sc-74501
Secondary antibody			
Donkey	Anti-mouse AP	1:100 IHC	Dako, P0449
Goat	Anti-rabbit HRP	1:100 IHC	Dako, P0448
Rabbit	Anti-Goat BIOT	1:100 IHC	Southern Biotech, 6160-08
	Streptavidin-HRP	1:300 IHC	Dako, P0397
Donkey	Anti-rabbit Alexa Fluor 5	1:100 IF	Thermo Scientific, A11055
Donkey	Anti-rabbit Alexa Fluor 488	1:100 IF	Thermo Scientific, A21206
	Streptavidin, Alexa Fluor 555	1:300 IF	Thermo Scientific, S21381

IF, immunofluorescence; IHC, immunohistochemistry.

AECII isolation and human lung organoids

Human peripheral lung tissues were obtained from 2 non-COPD and 2 COPD stage IV patients undergoing tumor resection and lung transplant surgeries, as previously described above. Lung tissues were dissociated and homogenized with a multi-tissue dissociation kit 1 (Miltenyi #130-110-201) using a GentleMACS Octo dissociator at 37 °C (Miltenyi #130-096-427). To specifically isolate the population of AECII, the cell suspension was depleted of CD45 and CD31 cells (fibroblasts and macrophages/leukocytes) using a magnetic-activated cell sorter (MACS, Miltenyi Biotec) and CD45 and CD31 microbeads (130-045-801 and 130-091-935 Miltenyi Biotec) according to manufacturer instructions. Subsequently, CD326 (EpCAM) beads (130-061-101, Miltenyi Biotec) were used to enrich EpCAM positive AECII as previously described[22,23]. The isolated cells were confirmed to be 90% pure as assessed by a positive staining of pro-SPC (Millipore, AB3786) on cytopspins of the isolated cells.

Human lung organoids were grown as described previously [24,25]. Briefly, ~5000 EpCAM⁺ human lung cells were seeded with an equal number of MRC5 fibroblasts per insert in 100µl growth factor-reduced Matrigel, diluted 1:1 with DMEM/F12 containing 5% FBS were added to for a 24-well cell culture insert. In the treated lung organoid groups, AREG (10ng/ml; #262-AR, R&D System, Minneapolis, USA) was freshly added to the lung organoids every 2-3 days. All human lung organoid cultures were maintained at 37°C in a humidified

incubator with 5% CO₂. After 14 days, lung organoids were fixed in acetone that was diluted 1:1 (v/v) with methanol for 15 min at -20°C. The human lung organoids were stained with pro-SPC for detection of AECII.

Quantification and immunofluorescence staining of lung organoids

The organoid diameter and the total number of organoids were counted manually per well after 14 days using a light microscope at 10 x magnification. Consequently, alveolar organoid cultures were washed with PBS and blocked in PBS with 5% (w/v) BSA. Primary antibody against pro-SPC (AECII) was diluted in PBS buffer with 0.1% BSA and 0.1% Triton, and the organoids cultures were incubated with primary antibodies at 4°C overnight. The next day, the organoids were washed with PBS for 30 min, and incubated with fluorescent secondary antibodies and the mounting medium containing DAPI (Abcam, Cambridge, UK). The alveolar organoids' images were acquired using the Leica SP8 confocal microscope at 40x or 63x magnification. Processed channels were merged or split using ImageJ software. Positive cells were counted manually, and positive cell numbers were corrected for the alveolar organoids' total cell number.

Statistical methods

For statistical evaluation of the different groups, a two-tailed Mann-Whitney U-test was used (GraphPad Prism 8.1 Software, San Diego). A value of $p < 0.05$ was considered significant.

Results

Patients' characteristics.

The characteristics of the lung donors are shown in table 1. We obtained lung tissues from 21 patients for IHC analysis and from 4 patients for lung organoids culture. The characteristics of COPD patients were similar compared with their matched control groups in terms of age and sex.

A lower number of EGFR-expressing AECII was detected in lung tissues of patients with COPD.

To assess a role for EGFR signaling in the impaired AECII behavior in COPD, AECII and EGFR-expressing AECII cells were visualized by IHC staining of pro-SPC (AECII) and EGFR, as shown in Figures 1A and C. We found a trend towards lower number of AECII in lung tissue from patients with

COPD when compared to lung tissue from control patients ($p = 0.05$; Figure 1B). Within the AECII population, significantly fewer EGFR-expressing cells were detected in lung tissue from patients with COPD when compared to non-COPD control lungs ($p = 0.038$; Figure 1D). Furthermore, the presence of EGF and AREG protein expression was investigated in two groups (Figure 2A and C). We observed that AREG and EGF were mostly expressed by alveolar macrophages and to a lesser extent also in AECII in the lung tissues from COPD and non-COPD patients. No difference was detected in the numbers of EGF-expressing and AREG-expressing cells when comparing lung tissue from patients with COPD to non-COPD control lungs (Figure 2B and D).

Figure 1. A. Representative immunohistochemical staining of AECII (blue cells expressing pro-SPC, A) and EGFR-expressing AECII (AECII is blue, A and EGFR is red, C) on paraffin-embedded lung slides from non-COPD ($n=10$) and COPD patients ($n=12$). The number of AECII positive cells (B) and EGFR-positive cells (D) were determined comparing COPD and non-COPD, original magnification 40x. The red arrowheads show examples of AECII positive cells and EGFR-expressing cells in the lungs. Data represent the number of AECII positive cells and EGFR-expressing cells in non-COPD (open symbols) or COPD (closed symbols). The whiskers plots show the median with minimum and maximum values. If not stated otherwise, the difference between groups was not significant. $p \leq 0.05$ was considered significant (Mann-Whitney U-test).

Figure 2. Representative immunohistochemical stainings of EGF (red, A) and AREG-expressing AECII (AECII is blue and AREG is red, C) on paraffin-embedded lung slides from non-COPD (EGF: $n=7$, AREG; $n=10$) and COPD patients (EGF: $n=9$, AREG; $n=11$). The proportion of EGF positive cells (B) and AREG positive cells (D) were determined comparing COPD and non-COPD, original magnification 40x. The red arrowheads show examples of EGF positive cells and AREG-expressing in the lungs. Data represent the number of positive cells in non-COPD (open symbols) or COPD (closed symbols). The whiskers plots show the median with minimum and maximum values. If not stated otherwise, the difference between groups was not significant (Mann-Whitney U-test).

Patients with COPD ($n=2$) have smaller lung organoids.

We next investigated AECII functionality and compared their response to AREG treatment in a human AECII-derived lung organoid model from lung tissue of patients with COPD and non-COPD ($n=2$ per group). The size of lung organoids and the presence of AECII (pro-SPC positive cells) and AECI (PDPN positive cells) in lung organoids was assessed after 14 days of culture. Figure 3A, B, C and D shows the presence of AECII in the non-treated and AREG treated COPD and control organoids, whereas PDPN-positive AECI were not found. We were limited in the number of donors for these experiments, but when combining all organoids for the 2 donors. We demonstrated that AECII-derived organoids from patients with COPD were significantly smaller in size compared to lung organoids from non-COPD controls ($p < 0.001$; Figure 3E). In addition, we demonstrated that AREG treatment resulted in significantly smaller organoids in the controls ($p = 0.005$; Figure 3F) but not in the COPD patients (Figure 3G).

Figure 3. 3D human lung organoids formation (A-D) and alveolar organoids (a-d) in AREG-treated and non-treated groups derived from non-COPD ($n=2$) and COPD ($n=2$) patients. Representative light and confocal microscopes pictures show human lung organoids formation at 14 days (A, a and B, b: AREG-treated organoids; C, c and D, d: non-treated organoids). The representative picture shows the pro-SPC IF staining (a-d) in alveolar organoids. The size of lung organoids measured in AECII-derived lung organoids from non-COPD and COPD patients (E). The size of AREG-treated and non-treated organoids were determined in AECII-derived lung organoids from non-COPD (F) and COPD patients (G). Quantitative analysis of pro-SPC positive cells in alveolar organoids derived from non-COPD (H) and COPD (I). The size of alveolar organoids appears to differ between donor 3 and donor 4 with COPD patients (J). Data represent the number of positive cells in non-COPD (open symbols) or COPD (closed symbols). The whiskers plots show the median with minimum and maximum values. If not stated otherwise, the difference between groups was not significant (Mann-Whitney U-test).

The proportion of AECII (pro-SPC positive cells as shown in red) did not differ between AREG-treated and non-treated AECII-derived organoids (Figure 3H and I). In addition, for COPD, the organoid size and the AECII proportion in the organoids appeared to be donor-dependent (Figure 3I and J), whereas this was not the case for non-COPD (data not shown).

Discussion

In the present study, we made an attempt to evaluate EGFR signaling related to AECII behavior in COPD. In lung tissue, we found fewer AECII in patients with COPD compared to non-COPD controls, which was accompanied with a lower proportion of EGFR-expressing cells within the AECII population. With

respect to the EGFR ligands AREG and EGF, the numbers of EGF-expressing cells and AREG-expressing cells were not different between both groups. AREG and EGF were mostly expressed by other cells than AECII, e.g., alveolar macrophages. In lung organoids, the size of AECII-derived organoids from the COPD patients seemed to be smaller and less responsive to AREG treatment.

Regarding the lower number of AECII that we found in tissue from patients with COPD, this was to be expected. All COPD patients suffered from severe emphysema and their lung function was very poor with an FEV1% pred < 30%. From the literature we know that in response to (smoking-related) injury of AECI, AECII are stimulated to multiply and differentiate into AECI [26]. However, long-term repeated injury is known to impair this cell replacement system and the ability to repair [27].

From the AECII that were present in lung tissue of patients with COPD, we found that fewer of them expressed EGFR. The EGFR signaling is important for AECII proliferation, but we do not know whether AECII are still able to proliferate in the absence of EGFR expression. Other signaling pathways like the WNT/ β -catenin, VEGFR and IGF/Akt1 signaling pathways are also involved in lung tissue repair [10,12,28,29]. A previous study by the group of Königshoff showed a lower mRNA expression of WNT receptor FZD4 of whole lung homogenates from COPD compared to non-COPD [10]. In mouse AECII, they subsequently found that reduced FZD4 expression, along with reduced WNT/ β -catenin activity in these cells, inhibited AECII to AECI cell differentiation and organoid formation, which suggests that lower FZD4 expression in COPD contributes to impaired alveolar repair capacity. WNT/ β -catenin and EGFR signaling could function in parallel to activate AECII in response to alveolar injury. An additional investigation of the expression of WNT-related receptors in our COPD and control groups could be of interest.

As mentioned earlier, little is known about the capacity of AECII for self-renewal and repair in emphysematous destruction in the context of EGFR activation. The absence of EGFR signaling in EGFR-knockout mice was shown to result in immature development of the lung, including deficient pulmonary alveolarization, loss of surfactant production and alveolar collapse [30]. In addition, Zuo and colleagues assessed the role of EGFR signaling in human airway basal cell differentiation upon AREG and EGF treatment in an air-liquid interface cell model. The authors found that AREG-induced EGFR signaling promoted basal cell- and club cell hyperplasia and suppressed ciliated cell

differentiation [19]. In AECII, EGFR activation may be important for AECII proliferation to repair damaged alveoli in lung tissue of patients with COPD. Indeed, Tushar and Krasnow demonstrated that murine AECII treated with EGF resulted in more AECII proliferation, whereas an EGFR blocking antibody inhibited AECII proliferation [14]. We did, however, show that patients with COPD had lower numbers of AECII in lung tissue. A study by Imai *et al.* showed more cell proliferation (PCNA staining) in emphysematous lungs [31], but this was not correlated with surface area, suggesting that a disbalance in the kinetics of both cell loss (apoptosis) and cell replacement (proliferation) may be a critical feature of emphysema pathogenesis.

The EGFR signaling pathway is mostly activated by two cognate ligands, AREG and EGF[15,16]. EGF has a higher affinity for EGFR than AREG [15]. We did not investigate in detail the source of AREG and EGF in this study, but we did observe that AREG was mostly expressed by immune cells, mainly alveolar macrophages. Indeed, prior studies showed that AREG is expressed by activated immune cells, such as alveolar macrophages [32,33], mast cells [34], T helper 2 (Th2) cells [35], Foxp3 expressing T regulatory T cells[36] and those AREG-expressing cells have a regulatory role in chronic lung inflammation and wound repair. As a result, it is worth investigating the source of AREG, and in particular the role of AREG-expressing macrophages, as alveolar macrophages are known to have a role in wound healing and tissue regeneration [33,37].

In a pilot study, we further investigated the direct effects of AREG on AECII differentiation in AECII-derived organoids from patients with COPD and non-COPD (n=2 per group). In this model, isolated AECII cells were cultured with MRC5 fibroblasts for 14 days until lung organoids were formed [25]. In non-COPD controls, we found that AREG treatment resulted in smaller AECII-derived organoids whereas the proportion of AECII was not affected. This was in contrast to what we found in mouse lung organoids where AREG stimulated AECII proliferation [21]. In COPD, this effect of AREG on organoid size was not seen, maybe as their ability to grow was already impaired. A more detailed investigation into the presence and exact composition of subtypes of AECII and AEI is necessary to get a better understanding what determines the growth and size of an organoid.

As we did not observe AEI (PDPN positive cells) in any of the organoids, AREG may selectively have prevented the differentiation from AECII to AEI. The absence of AEI, however, still needs to be confirmed by another AEI

marker (e.g Aquaporin 5). Interestingly, regarding the size of lung organoids and the proportion of AECII in lung organoids, a donor-dependent effect was observed in the COPD pilot group. The organoids obtained from the COPD patient with 54 pack years were smaller and had a lower AECII proportion than the organoids obtained from the COPD patient with only 15 pack years. It is possible that a reduced ability to form lung organoids may be associated with a longer duration of smoking and higher number of cigarettes smoked. Obviously, we need more donors to confirm this observation.

To conclude, patients with COPD had a lower number of EGFR-expressing AECII in lung tissue compared non-COPD controls, as indicated by our IHC experiments on lung tissue. This lower number of EGFR- expressing AECII in COPD could explain a possible impaired ability to form organoids. Clearly, more experiments are needed to establish the link between EGFR signaling and AECII proliferation, as well as their connection with impaired alveolar regeneration in COPD.

Abbreviations: Alveolar epithelial cell (AEC), chronic obstructive pulmonary disease (COPD), amphiregulin (AREG), epidermal growth factor (EGF), epidermal growth factor receptor (EGFR).

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Disclosure

The authors declare no competing financial interests.

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CHAPTER 4

Prenatal smoke exposure induces persistent *Cyp2a5* methylation and increases nicotine metabolism in the liver of neonatal and adult male offspring

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Abstract

Prenatal smoke exposure (PSE) is a risk factor for nicotine dependence. One susceptibility gene for nicotine dependence is Cytochrome P450 (CYP) 2A6, an enzyme responsible for the conversion of nicotine to cotinine and nicotine clearance in the liver. Higher activity of the CYP2A6 enzyme is associated with nicotine dependence, but no research has addressed the PSE effects on the *CYP2A6* gene or its mouse homologue *Cyp2a5*. We hypothesized that PSE affects *Cyp2a5* promoter methylation, *Cyp2a5* mRNA levels, and nicotine metabolism in offspring. We used a smoke-exposed pregnant mouse model. RNA, DNA, and microsomal protein were isolated from liver tissue of fetal, neonatal and adult offspring. Enzyme activity, *Cyp2a5* mRNA levels and *Cyp2a5* methylation status of six CpG sites within the promoter region were analyzed via HPLC, RT-PCR and bisulfite pyrosequencing. Our data show that PSE induced higher cotinine levels in livers of male neonatal and adult offspring compared to controls. PSE-induced cotinine levels in neonates correlated with *Cyp2a5* mRNA expression and promoter methylation at CpG-7 and CpG+45. PSE increased methylation in almost all CpG sites in fetal offspring, and this effect persisted at CpG-74 in male neonatal and adult offspring. Our results indicate that male offspring of mothers which were exposed to cigarette smoke during pregnancy have a higher hepatic nicotine metabolism, which could be regulated by DNA methylation. Given the detected persistence into adulthood, extrapolation to the human situation suggests that sons born from smoking mothers could be more susceptible to nicotine dependence later in life.

Keywords: Prenatal smoke exposure; epigenetics; cotinine; CYP2A5; nicotine dependence; sex difference; mouse model

Introduction

Nicotine is the most prominent and the primary psychoactive component in cigarette smoke, which is primarily metabolized by the enzyme Cytochrome P450 (CYP) 2A6[1–3]. The encoding gene *CYP2A6* has been identified as a susceptibility gene for nicotine dependence[4], and variation in *CYP2A6* predicts heavier cigarette consumption[5,6] and delayed smoking cessation[7], which are both related to nicotine dependence[4,8]. Up until today, a number of pharmacogenetics studies in humans have shown that nicotine dependence relies on alterations in nicotine metabolism[9], which in turn has been associated with higher CYP2A6 enzyme activity[10,11].

Cyp2a5, the mouse homologue of *CYP2A6*, plays a prominent role in the clearance of nicotine in the liver, as CYP2A5 protein converts 80% of nicotine to cotinine [12,13]. This conversion requires the production of nicotine- Δ^1 '(5')-iminium ion first, which is then subsequently converted to cotinine by aldehyde oxidase[12]. Similar to *CYP2A6*, *Cyp2a5* is primarily expressed in the liver[14].

Until now, many studies have described the adverse effects of smoking during pregnancy on fetal development[15], metabolic disease[16,17], smoking behavior[18,19] and the risk to develop the smoking-related diseases such as chronic obstructive pulmonary disease (COPD)[20]. These observations are supported by experimental mouse studies from our own group[15,21,22] and others[23–25].

Despite the above-mentioned plethora of studies, relatively little is known about the underlying mechanisms that contribute to the impact of prenatal smoke exposure (PSE) on smoking behavior and nicotine dependence. However, following the prenatal programming paradigm regarding prenatal epigenetic changes such as alterations in DNA methylation patterns and their role in onset of diseases, it is suggested that changes in DNA methylation could be one of the contributing mechanisms in dependency establishment. Thus far, a number of birth cohort studies indeed reported aberrantly methylated CpG sites in peripheral blood[26], placenta[27] and buccal cells[28] from children of smoking mothers. Furthermore, in the mouse studies from our group, PSE was shown to induce differential gene regulation and DNA methylation profiles of a variety of genes[29,30], but this effect was not shown by other studies[31].

However, to our knowledge, nothing has been reported on the effect of PSE on nicotine metabolism and possible nicotine dependence. Therefore, we investigated the effect of PSE on hepatic *Cyp2a5* gene expression, promoter

methylation and enzyme activity in male and female offspring from smoke exposed pregnant mice. To establish persistence of the PSE effect, this was investigated across three developmental stages, namely fetal (E17.5), neonatal (D3) and adult (D140).

Results

Higher *Cyp2a5* mRNA expression and promoter methylation in male PSE offspring.

PSE increased *Cyp2a5* mRNA levels in the liver of neonates ($p = 0.029$, Figure 1C), which was most prominent in male offspring ($p = 0.015$, Figure 1D). PSE had no effect on *Cyp2a5* mRNA steady state in fetal (Figure 1A and 1B) and adult livers of both sexes (Figure 1E and F). Interestingly, when comparing control male and female adult groups, females had a higher *Cyp2a5* mRNA content than male adult offspring ($p < 0.0001$, Figure 1F).

Figure 1. *Cyp2a5* expression in liver of PSE and control in E17.5, D3 mice and adult offspring.

Cyp2a5 mRNA expression in whole liver of fetal offspring (E17.5, A and B), neonatal offspring (D3, C and D) and adult offspring (E and F) in PSE (closed symbols) and control groups (open symbols). Data are shown as individual values. If not stated otherwise, the comparison of shown groups was not significant. (Mann-Whitney U-test). * $p \leq 0.05$, *** $p \leq 0.001$ (Mann-Whitney U-test). Circle (○) symbol(s)=male, square (□) symbol(s)=female.

To investigate whether the observed PSE-induced mRNA levels were associated with *Cyp2a5* promoter methylation, the percentage of methylation of six CpG sites (CpG-614, CpG-589, CpG-542, CpG-74, CpG-7 and CpG+45) was measured using bisulfite pyrosequencing.

Figure 2. Sex-dependent *Cyp2a5* promoter methylation across three time points (E17.5, D3 mice and adult offspring). DNA was isolated from whole liver of fetuses (E17.5, A), neonatal offspring (D3, B) and adult offspring (C) in PSE (closed symbols) and control groups (open symbols). DNA was subjected to bisulfite sequencing-based methylation analysis of the *Cyp2a5* promoter region and the percentage of DNA methylation was assessed. Data of the 6 targeted CpG-sites are presented per sex and exposure as individual values with median as a horizontal line. CpG-site annotations are relative to the ATG start codon. If not stated otherwise, the comparison of shown groups was not significant. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (Mann-Whitney U-test). Circle (○) symbol(s)=male, square (□) symbol(s)=female.

In *fetal* livers, PSE-induced *Cyp2a5* hypermethylation, both in male and female offspring at CpG-614 (male: $p = 0.0009$, fold-change=1.06, female: $p = 0.001$, fold-change=1.05; Figure 2A), CpG-589 (male: $p = 0.0005$, fold-change=1.06, female: $p = 0.003$, fold-change=1.08; Figure 2A), and CpG-542 (male: $p = 0.001$, fold-change=1.1, female: $p = 0.004$, fold-change=1.1; Figure 2A). PSE-induced hypermethylation was found in male fetuses at CpG-74 ($p = 0.01$, fold-change=1.1, Figure 2A), but not in females. In contrast, in females, PSE induced hypermethylation at CpG-7 ($p = 0.04$, fold-change=1.12, Figure 2A) and CpG+45 ($p = 0.05$, fold-change=1.17, Figure 2A). In *neonatal* livers, PSE-induced *Cyp2a5* hypermethylation was detected in both male and female mice at CpG-74 (male: $p = 0.01$, fold-change=1.1, female: $p = 0.03$, fold-change=1.07; Figure 2B). In male neonates, PSE-induced hypermethylation was found at CpG-614 ($p = 0.05$, fold-change=1.03, Figure 2B) and CpG-542 ($p = 0.04$, fold-change=1.05, Figure 2B). In females, PSE-induced hypermethylation was found at CpG+45 ($p = 0.04$, fold-change=1.17, Figure 2B). All other analyzed CpG sites of the *Cyp2a5* promoter were not found to be influenced by PSE (Figure 2B). In *adult* livers, higher methylation of CpG-74 was detected in PSE male offspring ($p = 0.03$, fold-change=1.21, Figure 2C), while no PSE effect was observed in all other analyzed CpG sites, both in male and female offspring (Figure 2C). In addition, we investigated whether these differentially methylated CpG sites were mediated by DNA methyltransferase (DNMT) enzymes. *Dnmt1* expression was lower in PSE male fetal offspring, compared to the control group (Supplementary Figure 4A and B), while no differences were detected in the neonates and adult offspring (Supplementary Figure 4C-F). Expression of *Dnmt3a* mRNA and *Dnmt3b* mRNA was not affected by PSE (Supplementary Figure 5A-F and Figure 6A-F).

Table 1. Sequences of primers used in bisulfite-based methylation analysis

Gene	Targeted CpG-sites	Sequence 5'-3'
<i>Cyp2a5</i>	CpG-614 to -542	For: TTTGTGTTTGTGTTTGTAGTGTGGGATTA
		Rev: CCCCATCCACAACCACTTCTT
		Seq: GTTGGGATTATAGGTTTATATTA
		Sequence to analyze: TTATATTYGATTTTGGGAGTTTTTAATGAAGAGGATTTGAATTTAAGGATGYGAGAA GTGGAGATTT TAGGGTTATYGG
	CpG-74 to +45	For: AGTGGATAGTTTGGAGGTGAAAT
		Rev: ACAACTTTCCTAAAAACTTTCTCTACTTC
		Seq1: GGAGGTGAAATAGTTGTATAATTAA
		Seq2: TAGTTATTATTGTTTGTATTATTAT
		Sequence to analyze: S1: GATTAAAGTTYGTTTTTTGTGTTTGGATGTATAAAAGTAAGTTAATT
		S2: TTATYGTATTAGTTGATTTAGGATTTTTTTGGTGGTTGAGTGGTTTTTTAGYGTITTTGGTTTT

Table 2. Correlations between *Cyp2a5* mRNA expression and promoter methylation in fetal (E17.5), neonatal (D3) and adult offspring liver.

correlation of / with	<i>Cyp2a5</i> (2-dCT)	E17.5									
			all	all male	all female	all control	all PSE	male control	male PSE	female control	female PSE
<i>Cyp2a5</i> promoter methylation [%]	CpG-614	r	-0.02	-0.23	0.17	0.17	-0.29	-0.23	-0.45	0.65	-0.13
		p-value	ns	ns	ns	ns	ns	ns	ns	0.02*	ns
	CpG-589	r	0.23	0.04	0.38	0.46	0.19	0.32	-0.1	0.57	0.47
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-542	r	0.11	-0.38	0.41	0.26	0.1	-0.56	-0.38	0.70	0.23
		p-value	ns	ns	ns	ns	ns	ns	ns	0.01*	ns
	CpG-74	r	0.21	0.19	0.25	0.35	0.05	-0.05	0.13	0.48	0.06
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-7	r	0.02	-0.04	0.01	0.19	-0.12	0.07	-0.15	0.40	-0.14
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG+45	r	0.25	0.1	0.38	0.24	0.20	-0.25	0.16	0.44	0.29
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
correlation of / with	<i>Cyp2a5</i> (2-dCT)	D3									
			all	all male	all female	all control	all PSE	male control	male PSE	female control	female PSE
<i>Cyp2a5</i> promoter methylation [%]	CpG-614	r	0.29	0.48	0.16	0.26	0.05	0.08	0.13	0.07	0.04
		p-value	ns	0.02*	ns	ns	ns	ns	ns	ns	ns
	CpG-589	r	0.28	0.38	0.27	0.21	0.15	0.12	0.31	0.04	0.26
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-542	r	0.24	0.33	0.23	0.48	0.12	0.52	0.33	0.32	0.08
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-74	r	0.35	0.17	0.02	0.37	0.24	-0.19	0.50	-0.39	-0.02
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-7	r	0.12	0.17	0.00	0.03	0.37	0.23	0.51	-0.61	0.47
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG+45	r	0.27	0.4	-0.04	0.29	0.23	0.09	0.77	0.11	-0.27
		p-value	ns	ns	ns	ns	ns	ns	0.009**	ns	ns
correlation of / with	<i>Cyp2a5</i> (2-dCT)	Adult									
			all	all male	all female	all control	all PSE	male control	male PSE	female control	female PSE
<i>Cyp2a5</i> promoter methylation [%]	CpG-614	r	0.22	0.12	0.21	0.02	0.83	0.18	0.2	0.15	-0.81
		p-value	ns	ns	ns	ns	0.003**	ns	ns	ns	ns
	CpG-589	r	0.24	0.17	0.33	0.38	-0.11	0.24	-0.12	0.29	0.31
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-542	r	-0.18	0.1	0.01	-0.36	0.22	0.22	-0.60	0.1	-0.31
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-74	r	-0.41	0.43	-0.11	-0.17	-0.82	0.62	0.15	0.41	-0.6
		p-value	ns	ns	ns	ns	0.004**	ns	ns	ns	ns
	CpG-7	r	-0.42	0.03	-0.47	-0.32	-0.85	0.11	-0.66	-0.64	0.61
		p-value	ns	ns	ns	ns	0.004**	ns	ns	ns	ns
	CpG+45	r	-0.44	0.12	0.11	-0.57	-0.57	0.1	-0.1	-0.28	0.4
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns

* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. P-values were adjusted by the Bonferroni method.

Linking *Cyp2a5* mRNA levels with methylation across three time points, in PSE male neonates, *Cyp2a5* mRNA content was positively correlated with methylation at CpG+45 site ($r = 0.77$, $p = 0.009$), as shown in Table 2 and Supplementary Figure 1C. This correlation was not seen when PSE and control male neonates were taken together (Table 2, Supplementary Figure 1A), or within male neonate controls only (Table 2, Supplementary Figure 1B). For CpG-614, methylation in all male neonatal livers correlated positively with *Cyp2a5* mRNA expression ($r = 0.48$, $p = 0.027$, Table 2), while no correlations were found in PSE female neonates. In PSE adult offspring, methylation levels of CpG-614 were positively correlated with *Cyp2a5* expression ($r = 0.83$, $p = 0.003$, Table 2), while no correlations were observed in combined and control adult offspring. Furthermore, negative correlations were observed for CpG-74 ($r = -0.81$, $p = 0.004$, Table 2) and CpG-7 ($r = -0.84$, $p = 0.004$, Table 2) in PSE adult offspring, while no additional correlations were detected in both adult offspring groups. In fetuses, positive correlations were found at CpG-614 ($r = 0.65$, $p = 0.029$, Table 2) and CpG-542 in control female fetal livers ($r = 0.7$, $p = 0.016$, Table 2) while no correlation was found in PSE fetal livers.

Our next question addresses the potential persistence of DNA methylation levels across three developmental stages, fetal, neonatal and adult. As shown in Figure 3, all of CpG sites showed a variable pattern at the three different stages (p -values < 0.0001 , Figure 3). For CpG-614, CpG-589 and CpG-542 sites, basal levels of DNA methylation increased from fetal to adulthood (p -values < 0.0001 , Figure 3A-C) in both sexes, while it was not affected by PSE (Figure 3A-C). In control male and female offspring, methylation patterns of CpG-74 and CpG-7 increased from fetal to neonatal stage, but reversed to hypomethylated in adulthood (p -values < 0.0001 , Figure 3D and E). Interestingly, PSE effects were detected for CpG-74 across three developmental stages in male offspring, (p -values < 0.05 , Figure 3D), whereas at CpG-74, the PSE effect on methylation disappeared in female fetus and adults (Figure 3D). For CpG+45, hypermethylation was observed at the neonatal stage when compared to neonates across fetal to neonatal stage ($p < 0.01$, Figure 3F), which was only observed in male offspring of both the control and PSE groups. (Figure 3F). In female controls, methylation at CpG+45 remained stable across three time points, while PSE increased methylation at both the fetal and neonatal stages (p -values < 0.05 , Figure 3F).

Figure 3. Time points comparisons of sex-dependent *Cyp2a5* promoter methylation status in PSE and control groups. The ANOVA test was used to do the comparison analysis among the fetal stage (E17.5), neonatal period (D3 means three days after birth) and adulthood (D140 means 140 days later after birth). P -values < 0.0001 were detected by ANOVA test in all of CpG sites over three time points. Mann Whitney U-test was used to test the comparisons between two time points in control (open symbols) and PSE (closed symbols) groups for 6 promoter methylation CpG sites. Data are represented as mean $\pm$ SEM; CpG-site annotations are relative to the ATG start codon. $*p \leq 0.05$, $***p \leq 0.001$ (Mann-Whitney U-test). Circle (○) symbol(s)=male, square (□) symbol(s)=female.

Prenatal smoke exposure increased the nicotine metabolism in neonatal and adult male offspring.

Our final question aimed towards the understanding of whether altered *Cyp2a5* mRNA levels, as observed in PSE neonatal offspring, was accompanied by an aberrant nicotine metabolism. This research question is based on the understanding that the enzyme CYP2A5 is responsible for conversion of nicotine after smoke exposure. Conversion of the *in vitro* nicotine metabolism to cotinine was measured by HPLC-L and MS, as shown in Figures 4C and D.

Interestingly, PSE-induced cotinine formation to higher levels in livers of male neonates when compared to controls ($p = 0.058$; Figure 4F). In female neonates, this PSE effect was not seen. This PSE-induced cotinine formation was even more pronounced in PSE male adult offspring ($p = 0.03$; Figure 4G) when compared to the male adult controls. In contrast, cotinine formation was decreased in PSE female offspring, when compared to female adult controls ($p = 0.03$; Figure 4G).

Figure 4. Optimization and the cotinine formation levels of *in vitro* nicotine metabolism in E17.5, D3 mice and adult offspring. Cotinine formation of nicotine metabolism of fetuses (E17.5), three-day-old offspring (D3) and adult offspring in PSE and control groups. The nicotine metabolism was measured

by HPLC-L as described in the material and methods section. The assay was optimized with respect to incubation time (A) and cytosolic protein concentration (B). Arrows at the X-axis indicate conditions used for actual experiments. C presents the HPLC chromatogram separation of a blank sample containing the standards nicotine and cotinine and the cotinine was produced in liver microsomes of PSE male neonatal offspring. D presents the cotinine analysis by LCMS. Cotinine, corresponding with m/z 177 was detected both in standard cotinine and in the microsome sample. The levels of cotinine formation were measured in fetal (E), three-day-old offspring (F) and adult offspring (G) of both groups. Circle ($\circ$) symbol(s)=male, square ($\square$) symbol(s)=female. Data are shown as individual values. If not stated otherwise, the difference between groups was not significant. * $p \leq 0.05$, ** $p \leq 0.01$ (Mann-Whitney U-test). Open symbol(s)=control group, closed symbol(s)=PSE.

A comparison of basal levels of cotinine formation in male and female controls showed that female neonates and adults had a higher nicotine metabolism than their male counter parts (neonates: $p = 0.007$; adults: $p < 0.0001$; Figure 4F and G). In fetuses, the nicotine metabolism was not affected by PSE (Figure 4E), nor were there differences in control levels of cotinine formation in males compared to females.

In fetal livers, the level of cotinine formation correlated positively to *Cyp2a5* expression when data of all fetuses were plotted together ($r = 0.45$, $p = 0.003$; Supplementary Figure 2A, Table 3). This correlation was mainly driven by the control female fetuses ($r = 0.78$, $p = 0.01$; Supplementary Figure 2C, Table 3), as no correlation was detected in PSE groups. Furthermore, a lower correlation was also seen in control when male and female fetuses were combined ($r = 0.57$, $p = 0.01$; Supplementary Figure 2B, Table 3). In male neonates, PSE-induced cotinine formation correlated positively to *Cyp2a5* expression ($r = 0.81$, $p = 0.01$; Supplementary Figure 2E, Table 3), whereas no significant correlation was observed when data from all male neonates were combined ($r = 0.53$, $p = \text{ns}$; Supplementary Figure 2D, Table 3). In control adult offspring, the level of the cotinine formation positively correlated with *Cyp2a5* expression ($r = 0.67$, $p = 0.002$; Figure 2G, Table 3), while no correlation was detected when data from PSE and control mice were combined, as shown in Supplementary Figure 2F.

Levels of DNA methylation versus levels of cotinine formation shows that, in all cases, strong correlations were found in both male PSE neonatal and adult mice. In PSE *neonatal* males, levels of cotinine formation correlated strongly with CpG+45 and CpG-7 methylation ($r = 0.86$, $p = 0.008$ and $r = 0.83$, $p \leq 0.01$; Table 3), whereas it strongly negatively correlated with CpG-614 methylation in PSE female neonates ($r = -0.79$, $p = 0.01$; Table 3). In contrast, methylation of CpG-7 ($r = 0.70$, $p \leq 0.01$; Table 3) and CpG+45 ($r = 0.77$, p

≤ 0.001 ; Table 3) correlated with cotinine levels in all male neonatal groups, while CpG-614 ($r = -0.63$, $p = 0.009$; Table 3) and CpG-589 ($r = -0.63$, $p = 0.009$, Table 3) negatively correlated with cotinine levels in all female neonates. In *adult* offspring, a majority of correlations of cotinine formation levels with DNA methylation levels were detected in PSE adult offspring. Promoter methylation at CpG-614 was negatively correlated with cotinine levels in PSE adult offspring ($r = -0.75$, $p = 0.01$, Table 3). In contrast, for CpG-74, promoter methylation correlated positively with cotinine levels ($r = -0.75$, $p = 0.01$, Table 3), while no other correlations were observed in PSE adult offspring (Table 3).

4

Comparison of gene expression, methylation profiles and cotinine formation of nicotine metabolism.

Table 3. Correlations between cotinine levels, *Cyp2a5* mRNA expression and promoter methylation in fetal (E17.5), neonatal (D3) and adult offspring liver.

correlation of / with	Cotinine formation (nmol/min/mg)	E17.5								
		all	all male	all female	all control	all PSE	male control	male PSE	female control	female PSE
Cyp2a5 promoter methylation [%]	Cyp2a5 (2-dCT)	r	0.45	0.47	0.41	0.57	0.33	0.41	0.46	0.78
		p-value	0.00**	0.03*	ns	0.01**	ns	ns	ns	0.01**
	CpG-614	r	-0.15	-0.21	-0.14	0.30	-0.07	-0.41	0.19	0.44
		p-value	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-589	r	-0.59	0.01	-0.10	0.30	-0.05	0.07	0.28	0.65
		p-value	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-542	r	0.15	0.17	0.03	0.36	-0.09	0.55	-0.18	0.87
		p-value	ns	ns	ns	ns	ns	ns	ns	0.00***
	CpG-74	r	0.21	0.31	0.10	0.12	0.26	0.36	0.30	-0.03
		p-value	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-7	r	0.14	0.26	0.01	0.54	-0.19	0.42	0.08	0.75
		p-value	ns	ns	ns	0.02*	ns	ns	ns	ns
	CpG+45	r	0.31	0.34	0.28	0.4	0.37	0.19	0.57	0.57
		p-value	ns	ns	ns	ns	ns	ns	ns	ns
correlation of / with	Cotinine formation (nmol/min/mg)	D3								
		all	all male	all female	all control	all PSE	male control	male PSE	female control	female PSE
Cyp2a5 promoter methylation [%]	Cyp2a5 (2-dCT)	r	0.16	0.53	-0.12	0.01	0.21	0.21	0.81	0.14
		p-value	ns	ns	ns	ns	ns	ns	0.01**	ns
	CpG-614	r	-0.10	0.46	-0.63	0.14	-0.30	0.59	0.21	-0.43
		p-value	ns	ns	0.00***	ns	ns	ns	ns	0.01**
	CpG-589	r	-0.14	0.48	-0.63	0.01	-0.28	0.69	0.20	-0.61
		p-value	ns	ns	0.00***	ns	ns	ns	ns	ns
	CpG-542	r	-0.26	-0.38	-0.42	-0.18	-0.26	0.20	-0.28	-0.54
		p-value	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-74	r	-0.29	-0.01	-0.29	-0.57	-0.12	-0.03	-0.28	-0.36
		p-value	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-7	r	0.47	0.70	0.30	0.38	0.29	0.46	0.83	0.32
		p-value	ns	0.00***	ns	ns	ns	ns	0.00***	ns
	CpG+45	r	0.19	0.77	-0.52	0.12	0.21	0.80	0.86	-0.64
		p-value	ns	0.00***	ns	ns	ns	ns	0.00***	ns

correlation of / with	Cotinine formation (nmol/min/mg)	Adult									
		all	all male	all female	all control	all PSE	male control	male PSE	female control	female PSE	
<i>Cyp2a5</i> promoter methylation [%]	<i>Cyp2a5</i> (2-dCT)	r	0.23	0.26	0.21	0.67	-0.67	0.21	-0.37	0.47	-0.41
		p-value	ns	ns	ns	0.00**	ns	ns	ns	ns	ns
	C p G -	r	-0.24	-0.42	-0.12	0.02	-0.75	-0.50	0.30	0.42	-0.89
	614	p-value	ns	ns	ns	ns	0.01**	ns	ns	ns	ns
	C p G -	r	0.04	0.05	-0.09	0.24	-0.35	-0.15	-0.78	0.07	-0.53
	589	p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	C p G -	r	-0.37	-0.37	-0.21	-0.33	-0.51	-0.16	0.00	0.28	-0.90
	542	p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-74	r	-0.19	-0.18	0.64	-0.28	0.73	-0.17	-0.52	0.48	0.90
		p-value	ns	ns	0.01**	ns	0.01**	ns	ns	ns	ns
	CpG-7	r	0.017	0.21	0.01	-0.17	0.55	0.19	-0.41	-0.22	0.89
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG+45	r	-0.03	0.23	0.21	-0.15	0.31	0.65	-0.20	0.03	0.80
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns

* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. *P*-values were adjusted by the Bonferroni method.

Comparisons of the *Cyp2a5* mRNA expression, promoter methylation levels and the nicotine metabolism across three time points in control offspring shows that mRNA levels of *Cyp2a5* and the level of cotinine formation were much higher in adult offspring, when compared to fetal and neonatal offspring, in both sexes (p -values < 0.0001 , Supplementary Figure 3A and B). In addition, basal levels of cotinine formation were higher in female neonates ($p = 0.0002$; Supplementary Figure 3B), when compared to female fetuses. This was accompanied with higher methylation levels of all measured CpG sites in both sexes, except for CpG+45 (p -values ≤ 0.01 ; Supplementary Figure 3C and D). In adult offspring, hypermethylation was detected at three CpG sites (p -values < 0.0001 ; CpG-614, CpG-589 and CpG-542), compared to fetuses and neonates (Supplementary Figure 3C). Inversely, promoter methylation was lower at two CpG sites (p -values ≤ 0.0001 ; CpG-74 and CpG-7) in adult offspring, for both sexes, compared to fetuses and neonates, as shown in Supplementary Figure 3C and D.

Discussion

In this study, we found that PSE increased the nicotine metabolism in male neonatal and adult but not in fetal offspring. This observation correlated with higher *Cyp2a5* mRNA levels along with elevated promoter methylation in neonates but not in adults. In addition, while the PSE-induced nicotine metabolism increased from the fetal developmental stage to adulthood, partial

to almost complete waning of the PSE-induced *Cyp2a5* promoter methylation was observed over time, which was sex dependent. Finally, female neonates and adult mice had a higher hepatic nicotine metabolism than their male counterparts, and PSE had a downregulatory effect on nicotine metabolism in female adults.

Our observation that PSE increased the nicotine metabolism in neonatal and adult offspring could be relevant with respect to human smoking behavior, as a higher nicotine metabolism is generally associated with heavier smoking, higher dependency to nicotine, and more difficulty to quit cigarette smoking[32,33]. Indeed, Kandel *et al.* reported aberrant smoking behavior in children born to smoking mothers, in which PSE daughters had a higher risk to start smoking, and the smoking behavior was more persistent than in sons in adolescence age[19]. Whether the sex-difference in smoking behavior of PSE adolescence is based on epigenetically regulation of genes is worth to further investigate in human subjects.

In our mouse study, the PSE effect on nicotine metabolism was also sex-dependent since the effect was only seen in male offspring. This is of interest as in a study by Klein *et al.* in preadolescent mice, prenatal nicotine exposure induced the preference for drinking nicotine-containing water, an effect that was only seen in males, while female mice appeared to be unresponsive to treatment[25]. In addition, in a study where pregnant rats were treated with nicotine, only male hyperactive offspring exhibited a significant increase in the cortical receptor densities whereas the hyperactive females did not. Evidence suggests that only male offspring of nicotine-exposed dams are also susceptible to neurochemical effects of intrauterine nicotine exposure[34].

In the current study, for the CpG sites that were affected by PSE, methylation levels were increased, which was seen at three time points in both sexes. Promoter methylation is usually associated with repressed gene expression, but recent findings[17,22] suggest that this association could also occur inversely, which is in line with our previous studies[29,30] and studies by others[35]. Interestingly, in three development stages, PSE caused a persistent increase of methylation at CpG-74 when compared to control groups. This confirms human studies [26,36] showing that PSE-induced differentially methylated CpG sites in *MYO1G* and *CNTNAP2* were persistent at birth, during childhood and during adolescence in the replication cohort. Indeed, the causal link between DNA (de) methylation and gene expression and even further protein activation is complex,

given that DNA methylation is only one of the known epigenetic modifications. Other modes include histone modifications, chromatin remodeling and RNA-based mechanisms (lncRNAs / miRs). All of these modes seem to be interlinked, but their chronological order and the exact mechanism(s) that may connect these modes still need to be described to their full extent.

In this study, the PSE effect was the most striking in male neonates. PSE induced *Cyp2a5* mRNA expression was correlated with both CpG+45 methylation as well as with elevated cotinine levels. As this effect was not seen in females, it indicates that this CpG site may play a regulatory role in sex-dependent regulation of *Cyp2a5* expression. Studies have reported sex-dependent methylation patterns of CpG sites in the livers of male and female mice[37,38]. It is known that sex hormones can play a major role in regulating sex-dependent DNA methylation, as described in the mouse model by Takasugi *et al*[39]. In that study, male mice, treated with female-like growth hormone showed differential methylation of CpG sites of *Cyp17a1*, *Cxcl11* and *Nmt* in the liver when compared to non-treated male mice, and this observation was also described in other organs[40]. In addition, Koudsi *et al.* demonstrated DNA methylation changes in regulating *CYP2A6* expression in a pilot study of human livers. In that study, no prominent role for DNA methylation changes was observed with respect to the regulation of *CYP2A6* gene expression in human livers[41]. Based on this finding, a lack of association between DNA methylation and *CYP2A6* expression suggests that other gene areas, such as the proximal promotor CpG sites (1000-100 bp) in relation to the gene's transcription start site might be relevant in regulating *CYP2A6* expression, which needs to be investigated further. Moreover, in murine hepatocytes, two major regions of *Cyp2a5* regulation were observed in the promoter, which are the distal (AHR and USF-1 within 2700-1500bp) and the proximal region (HNF, NF-1 and C/EBP within 1000-10bp), as described by Arpiainen *et al.* [42]. Therefore, Arpiainen and colleagues compared the distal promoter region with the proximal promoter region in the transcriptional regulation of *Cyp2a5* expression, showing HNF, NF-1 and C/EBP transcription factors within the proximal region are the key activators of the *Cyp2a5* expression, when compared to the distal region.

Next to this, we assessed mRNA expression of the DNA methyltransferases across three developmental stages. There are two different DNA methylation processes: maintenance (DNMT1) and de novo (DNMT3a and DNMT3b).

Interestingly, lower *Dnmt1* levels were observed in male fetuses only, supporting a study in which it has been shown that *Dnmt1* enzyme can be reduced by current cigarette smoke exposure, which downregulated target genes in *in vitro* and *ex vivo* studies [43,44]. This result could be considered as the “current smoke” exposure effect, which was present in the fetuses. As neonatal and adult offspring have not been exposed after they were born, this current smoke effect would not apply to these groups.

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In our study, we observed that nicotine metabolism increased over time, as adult mice had a higher cotinine formation than neonates and fetuses. In addition, the PSE effect on nicotine metabolism was also observed in neonatal and adult offspring but not in fetal offspring. This result suggests that offspring in the fetal stage had a very low nicotine metabolism, which was not induced by PSE. Indeed, in a human study by Dempsey *et al.*, it was reported that the half-life of nicotine in serum is three to four times longer in newborns than in adults[45], indicating that fetal and newborn liver might have lesser nicotine clearance than adult[46]. In addition, during pregnancy, the metabolic clearance of nicotine is relatively high[47], resulting in fetal nicotine clearance being primarily controlled by the mother’s hepatic blood flow.

With respect to *Cyp2a5* methylation, this is the first study to report on methylation of *Cyp2a5* over time and after PSE. We found a decline in the number of differentially methylated CpG sites over time after PSE, suggesting that PSE-induced *Cyp2a5* methylation is reversible over time. One explanation could be a diverse cellular composition in all three developmental stages, which is a limitation of this study and which was also as reported in previous studies[30,48]. Indeed, in the human study, a similar result was described by Richmond and colleagues when comparing PSE newborns to adolescent offspring[26]. They found that differentially methylated CpG sites in PSE newborns have been identified in *AHRR*, *MYO1G*, *GFII*, *CNTNAP2*, *KLF13*, *CYP1A1* and *ATP9A* genes, which are known as smoke related genes. In that study, CpG sites in less than half of the smoking related genes showed reversibility of the methylation pattern in PSE adolescents when compared to PSE newborns.

With respect to the functionality of the *Cyp2a5* gene in fetal mice, no PSE effect was seen on nicotine metabolism, although methylation was higher in four out of six CpG sites, both in male and female PSE groups. The potential of PSE to induce fetal programming has been addressed in multiple studies, but

a differential effect in males and females has only occasionally been evaluated by our research group[29,30] and others[49,50]. The present study shows that PSE-induced methylation was observed mostly in fetuses. This result could be due to the ‘current’ smoke effect, as fetuses were exposed to smoke until they were terminated. From the literature, it is known that current smoking has a profound effect on DNA methylation which for a number of genes disappears after smoking cessation [e.g., ref.[51]]. Similar effects have been described by us for *Igf1* and *Igf1r* methylation in the same pups[30]. This ‘current’ smoke exposure effect is not present in the neonates and adults which have not been exposed to smoke after they were born. However, neonates may have been exposed to nicotine via the mothers’ milk as nicotine could have been ingested, when mothers cleaned their fur. The waning of the PSE effect over time could therefore also be explained by the ‘current’ nicotine exposure at the fetal and neonatal stages and the absence of ‘current’ nicotine exposure in adults.

We found that female neonates and female adults had a higher nicotine metabolism, which supports previous studies in humans[52,53], and which suggests that the nicotine and cotinine clearance is higher in women than in men. Indeed, other CYP enzymes may have a role in nicotine metabolism [13], and may explain the differences seen between males and females. For instance, Z. Liu *et al.* tested the ability of CYP2B6 to metabolize nicotine in the CYP2B6-humanized mouse model [54] showing that the nicotine-induced cotinine formation was increased in males when compared to females. In contrast, a clinical study showed that livers from female donors had a higher level of CYP2B6 mRNA, protein and enzyme activity compared to male donors [55]. Moreover, CYP2B6 expression is known to be stimulated by estrogen hormones [56]. However, as the CYP2B6 is a considerably lower activity enzyme in nicotine metabolism when compared to the CYP2A6 enzyme [13], we do not consider a major role for this enzyme in our model. Unfortunately, the molecular mechanism that underlies different CYP2B6 regulation in the mouse is not clear. In addition, in humans, the inherent gender difference in smoking initiation and nicotine addiction was well documented in the epidemiological studies [57,58], showing that women have greater vulnerability for heritability of smoking initiation/persistence compared to males (women 66% vs. man 49%) [59].

The nicotine metabolism is also higher in pregnant women than in non-pregnant women[47,60], suggesting a dose-dependent effect of the sex hormones

estrogen and progesterone on CYP2A6 regulation. Indeed, Higashi *et al.* showed that *CYP2A6* mRNA was induced by estradiol in human hepatocytes after specific binding of the estrogen receptor to the putative estrogen response element (ERE) on the *CYP2A6* gene[61]. The sex hormone may also have a direct effect on the nicotine metabolism in rodents. In a rat experiment, ovariectomized animals showed a lower behavior motivation effect of nicotine intake than controls [62]. Moreover, in another study, higher plasma nicotine levels were measured in intact female rats compared to male rats, and the sex difference was attenuated in ovariectomized animals[63]. Although we expected otherwise, a lower nicotine metabolism was observed in PSE female adult offspring, compared to controls. This might have resulted from PSE-induced ovarian steroidogenesis[64,65]. Indeed, studies in rats have demonstrated that *in utero* exposure to cigarette smoke can result in decreased estrogen hormone[66] in female offspring, explaining a different effect of PSE in females.

There were limitations to the study. Throughout three development stages, the cellular compositions within different liver zones may be a confounding factor. Therefore, it would be interesting to assess DNA methylation patterns and nicotine metabolism in different cell compounds within a specific liver zone in future studies. In addition, fetal and neonatal offspring only provided us with limited material to do all analyses within one mouse. For the *in vitro* nicotine metabolism experiment, firstly, we optimized the required ratio of enzymes on higher metabolic active specimens. Selected optimized ratios were used in the actual *in vitro* nicotine metabolism experiment.

Conclusion: In this study, we have shown for the first time that male neonatal and adult offspring from mothers that smoked during pregnancy have a higher nicotine metabolism. In neonates this was accompanied by a higher *Cyp2a5* gene expression, which was correlated with higher DNA methylation. As studies have shown a positive relation between higher nicotine metabolism and increased nicotine dependency, this could imply that in human subjects, male offspring from smoking mothers have a higher chance of developing tobacco addiction when they start smoking.

Materials and Methods

Animals & smoke exposure

C57BL/6J mice (48 females and 48 males) were obtained from Harlan (Horst, The Netherlands) at 6 weeks of age and housed under standard conditions, with food and water provided *ad libitum* and at a 12-hour light/dark cycle. The experimental setup was approved by the local committee on animal experimentation (DEC6589 B & C; University of Groningen, Groningen, The Netherlands) and governmental and international guidelines on animal experimentation.

Mainstream cigarette smoke was generated by a Teague¹⁰ smoking machine (Tobacco and Health Research Institute of the University of Kentucky, Lexington, KY, USA). Over a period of 7 days, 20 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. On adjustment day 5, after the end of the smoking session, all female mice were injected with pregnant mare's serum gonadotropin (PMSG, 1.25 i.u.) to stimulate ovulation, and on day 7 with human chorionic gonadotropin (hCG, 1.25 i.u.) to induce ovulation. Subsequently, female mice were housed overnight at a 1:1 mating ratio with males. Mating was confirmed by the presence of a vaginal plug the following morning.

Throughout gestation, female mice were housed in groups and exposed to air or whole-body smoking sessions twice a day, 7 days per week. After delivery, dams and their offspring were no longer exposed to cigarette smoke. Dams were separately housed, with their offspring.

A total of 48 fetuses (24 males and 24 females) from 5 smoke-exposed dams and 4 control dams were collected at embryonic stage 17.5 (E17.5) and dams were euthanized under anesthesia. A total of 42 pups at postnatal day 3 (D3) were randomly selected from prenatally smoke exposed (11 male, 8 female) and control (11 male, 12 female) groups for collection of the liver. Another 34 pups were randomly selected from 20 smoke-exposed (6 male, 6 female) and 19 control (10 male, 12 female) mice that were exposed to air in the Teague¹⁰ from 8 weeks of age for the following 12 weeks (adulthood) until euthanized for collection of the liver. The livers were immediately frozen in liquid nitrogen and stored at -80 °C until further use.

Genetic determination of mouse sex

DNA from the tail of E17.5, D3 and adult mice was obtained by SDS denaturation, high salt extraction, and precipitation. DNA was amplified as described by Lambert *et al.*[67]., using two primer sets which are specific for the genes *Sry* (forward: 5'-TGGTCCC GTGGTGAGAGGC-'3, reverse: 5'-TATGTGATGGCATGTGGGTTCC-'3) and *IL3* (forward: 5'-GGGACTCCAAGCTTCAATCA-'3, reverse: 5'-TGGAGGAGGAAGAAAA GCAA-'3).

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Isolation of DNA and RNA

DNA and RNA were isolated using the All Prep DNA/RNA Mini Kit (Qiagen, Cat No. 80204), according to the manufacturer's protocol.

mRNA expression and Pyrosequencing-based bisulfite PCR analysis

Gene expression analysis in whole liver mRNA isolates was done via qPCR using qPCR MasterMix Plus (Eurogentec, Seraing, Belgium) with commercially available primers for *Cyp2a4/5* (Mm00487248_m1, TaqMan® Gene Expression Assay, Applied Biosystems, Foster City, CA, USA). Primers and probes for *Dnmt1*, *Dnmt3a* and *Dnmt3b* were obtained from Invitrogen (Breda, The Netherlands), which are described in details elsewhere [68]. Detection of amplification reactions was performed using the LightCycler® 480 System (Roche Diagnostics GmbH, Mannheim, Germany) with cycling conditions as follows: for 2 min at 50°C, for 10 min at 90°C, for 15s at 40 cycles of 95°C and for 1 min at 60°C. Reactions were performed in triplicate for each sample with *Gapdh* (Mm99999915_m1, TaqMan® Gene Expression Assay, Applied Biosystems, Foster City, CA, USA) as a reference gene.

DNA in liver tissue of fetal, neonatal and adult mice was isolated and prepared for bisulfite-based methylation analysis. For the assessment of promoter methylation levels of *Cyp2a5*, bisulfite sequencing primers were designed using PyroMark assay design software (version 2.0, Qiagen). Selection of CpG-sites was based on manual identification of CpG-dinucleotides, using ENSEMBL genome web browser (Ensembl 83: Dec 2015) and transcript location for the identification of gene promoter regions. Extracted genomic DNA was converted with sodium bisulfite (EZ DNA methylation Direct™, Zymo Research, Irvine, CA), following the manufacturer's instructions. In short, the bisulfite conversion was carried out in the dark at 98°C for 10 minutes and 64°C for 3.5 hours,

followed by desulphonation of the converted DNA. Gene amplification was done using HotStarTaq® MasterMix (Kit Qiagen, Venlo, The Netherlands). CpG-sites were identified manually in the 600bp promoter region of the mouse *Cyp2a5* gene (ENSMUSG00000005547), and assessment of DNA methylation levels was performed on the PyroMarkQ24 (Qiagen) instrument. Relative levels of methylation at each CpG-site were analyzed with PyroMark Q24 2.0.6 software. The amplification and sequencing primers used in this study are listed in Supplementary Table 1.

Microsomal membrane preparation

Microsomal membranes were obtained from homogenized livers of embryonic, neonatal and adult mice for nicotine functional assay, as previously described[1][69]. Briefly, the homogenized liver tissue was centrifuged at 9000g for 20 minutes at 4°C in phosphate buffer with EDTA. The supernatant fractions were collected and centrifuged at 100,000g for 90 minutes at 4°C. The resultant pellets (microsomes) were collected and resuspended in phosphate buffer without EDTA. The collected supernatants (cytosolic fractions) were pooled for further use as source of aldehyde oxidase reaction in nicotine functional analysis. The content of microsomal protein was determined in each sample with a RC DC protein assay (BIO-rad kit RC DC protein Assay).

In vitro assay for nicotine metabolism

The metabolism of nicotine follows two steps, the first of which is the conversion of nicotine to nicotine- $\Delta^{1' (5')}$ -iminium ion by *Cyp2a5* in the liver. Thereafter, the nicotine- $\Delta^{1' (5')}$ -iminium ion is converted to cotinine by aldehyde oxidase, which is present in cytosolic fraction. The liver microsomal protein was incubated with the pooled cytosolic fraction and nicotine containing solution. To ensure that the nicotine conversion was linear with time, and aldehyde oxidase availability was not rate-limiting, 45 minutes with incubation time (optimization: 15, 30, 45 and 60 min) and 1 mg/ml with concentration of cytosolic protein (optimization: 0.25, 0.5, 1, 2, 3, 4, 6, 8 and 10mg/ml)[69] were used, as shown in Figure 4A and B. Briefly, a normalized 0.5mg/ml liver microsomal protein and 80µl cytosolic fraction with a protein content of 1.0 mg/ml were added to a freshly prepared nicotine containing solution 80 µl, which is a mix of 4.4µl S(-)-nicotine ($\geq 99\%$, N3876, Sigma-Aldrich, St.Louis, MO), 1.5ml demineralized water and 28.5ml of 100mM sodium phosphate buffer pH

7.4. The mixture was supplemented with phosphate buffer (pH 7.4) to a total volume of 220 μ l. After preheating this mixture at 37°C for 5 minutes, 180 μ l of freshly prepared NADPH in phosphate buffer containing 12.3mM magnesium chloride (Sigma-Aldrich, St. Louis, MO), 55.8mM potassium chloride, 1.17mM D-Glucose-6-phosphate ($\geq$ 98%, G7250, Sigma-Aldrich, St. Louis, MO) and 5.2 μ l glucose-6-phosphate dehydrogenase (Roche, Germany; Grade II from yeast) were added to the mixture for supplying the energy to recycle the CYP's active site after oxidation. This mixture (400 μ l in total) was incubated at 37°C for 45 minutes in an incubator for the actual biotransformation.

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Analysis for HPLC and LCMS

For the analysis of the biotransformation products of nicotine metabolism, the samples were prepared as previously described by E.S. Messina *et al.*[1]. and H. Raunio *et al.*[2]. Briefly, the samples were alkalized by adding 20 μ l of 10M sodium hydroxide and mixing for 2-3 seconds on a vortex, to stop the reaction after the biotransformation assay, and to deprotonate the analytes. From this, the uncharged analytes were extracted in a two-step extraction. Each extraction step consisted of adding 1ml of dichloromethane, mixing thoroughly for 60 seconds on a vortex, spinning for 3 minutes at 10,000g to separate the layers, after which the organic (lower) phase was collected. 20 μ l of 2.5M hydrochloric acid was added to the combined dichloromethane fractions, as suggested by A.M. Massadeh *et al.*[70]. Before evaporating the solvent under a nitrogen atmosphere overnight at room temperature until dryness. Dried samples were reconstituted in 100 μ l of HPLC buffer, which is a mix of 15% acetonitrile and 85% of 20mM phosphate buffer, pH 3.0, containing 1,0 g/L heptane sulfonate sodium salt acid. The sample was analyzed by reverse phase HPLC with UV detection (245nm), as shown in Figure 4C. The separation was performed on HPLC-L, a Hitachi system consisting of a L2130 pump (set at isocratic elution of 0,6ml/min) and a L2300 column oven set at 25 °C. To confirm the identity of the peak in the chromatogram a fragment of the cotinine fraction from HPLC system was sent to Interfaculty Mass Spectrometry Center (IMSC) at the University of Groningen. The sample was dried again under a nitrogen atmosphere and reconstituted in eluent (water and acetonitrile both containing formic acid) for the liquid chromatography separation. The sample was injected into LCMS system, which was measured in positive mode from m/z 100-1000 and parallel reaction monitoring was included in the analysis for targeted analysis of the compounds with m/z 177. We identified that cotinine

of the nicotine metabolism experiment displayed the same retention time as the cotinine standard. In the LCMS analysis, both the cotinine standard and the cotinine of the metabolism experiment were detected at an m/z of 177.10 (Figure 4D).

Calculations and statistical methods

For statistical evaluation of the different groups, two-tailed Mann-Whitney U-test and Spearman's correlation test were used (GraphPad Prism 7.02 Software, San Diego, CA and SPSS Statistics 23, IBM, The Netherlands). Comparisons of the methylation data among three different time points were conducted using one-way Analysis of Variance (ANOVA). P values were adjusted by the Bonferroni method. $P \leq 0.05$ was considered significant. Relative gene expression ($2^{-\Delta C_t}$ method) as well as mean percent methylation and standard error of the mean (SEM) were calculated in Microsoft® Office Excel 2003.

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Supplementary figures.

Figure 1. Correlations of *Cyp2a5* expression with *Cyp2a5* CpG#45 in PSE male neonates.

The correlation between *Cyp2a5* mRNA expression and CpG#45 methylation is shown for all groups (A), control group (B) and PSE group of male neonates(C). Data were analyzed by Spearman's correlation test. P-values were adjusted by the Bonferroni method. Open symbols(s)=control group, closed symbols(s)= PSE.

Figure 2. Correlations analysis of *Cyp2a5* expression and the levels of cotinine in fetuses, neonates and adults. Cotinine formation from nicotine in liver microsomal protein and *Cyp2a5* mRNA levels were measured in whole liver from fetal offspring (E17.5), neonatal offspring (D3) and adult offspring in PSE and control group. The cotinine formation of E17.5 (**A**) correlated with *Cyp2a5* expression when all mice (control and PSE) were plotted together. **B**, all (female and male) controls and **C**, control female groups presented a correlation with *Cyp2a5* mRNA expression and cotinine formation in fetuses (E17.5). In contrast, cotinine formation correlated with *Cyp2a5* mRNA levels in both (control and PSE) male neonates and in PSE male neonates, as shown in **D** and **E**. **F** presents the correlation of cotinine formation with *Cyp2a5* mRNA expression in all adult offspring (control and PSE) and **G** presents the correlation for control adult offspring (male and female). Circle ($\circ$) symbol(s)=male, square ($\square$) symbol(s)=female. Data were analyzed by Spearman's correlation test. Open symbol(s)=control group, closed symbol(s)=PSE. P-values were adjusted by the Bonferroni method.

Figure 3. Comparison of *Cyp2a5* gene expressions and cotinine formation between control fetuses, neonates and adults. **A:** presents comparison of *Cyp2a5* mRNA levels for three time points in control group. **B:** presents the comparison between cotinine levels at three time points in control groups. The sex-dependent comparison of *Cyp2a5* promoter regions between E17.5, D3 and adult mice in male (**C**) and female (**D**) control groups. Data of the 6 targeted CpG-sites are presented per sex and exposure as individual values with median as a horizontal line. Circle (○) symbol(s)=male, square (□) symbol(s)=female. If not stated otherwise, the comparison of shown groups was not significant. * $p \leq 0.05$, *** $p \leq 0.001$, (Mann-Whitney U-test). Open symbol(s)=control group.

Figure 4. Comparison of *Dnmt1* mRNA expressions between control fetuses, neonates and adults. *Dnmt1* expression in whole liver of fetal offspring (E17.5, **A** and **B**), three-days-old offspring (D3, **C** and **D**) and adult offspring (**E** and **F**) in PSE (closed symbols) and control groups (open symbols). Data are shown as individual values. If not stated otherwise, the comparison of shown groups was not significant. (Mann-Whitney U-test). Circle (○) symbol(s)=male, square (□) symbol(s)=female.

Figure 5. Comparison of *Dnmt3a* mRNA expressions between control fetuses, neonates and adults. *Dnmt3a* expression in whole liver of fetal offspring (E17.5, **A** and **B**), three-days-old offspring (D3, **C** and **D**) and adult offspring (**E** and **F**) in PSE (closed symbols) and control groups (open symbols). Data are shown as individual values. If not stated otherwise, the comparison of shown groups was not significant. (Mann-Whitney U-test). Circle (○) symbol(s)=male, square (□) symbol(s)=female.

Figure 6. Comparison of *Dnmt3b* mRNA expressions between control fetuses, neonates and adults. *Dnmt3b* mRNA expression in whole liver of fetal offspring (E17.5, **A** and **B**), neonatal offspring (D3, **C** and **D**) and adult offspring (**E** and **F**) in PSE (closed symbols) and control groups (open symbols). Data are shown as individual values. If not stated otherwise, the comparison of shown groups was not significant. (Mann-Whitney U-test). Circle (○) symbol(s)=male, square (□) symbol(s)=female.

CHAPTER 5

Postnatal smoke exposure further increases the hepatic nicotine metabolism in prenatal smoke exposed male offspring and is linked with aberrant *Cyp2a5* methylation

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Abstract

Prenatal smoke exposure (PreSE) is a risk factor for nicotine dependence, which is further enhanced by postnatal smoke exposure (PostSE). One susceptibility gene is Cytochrome P450 (CYP) 2A6, an enzyme responsible for the conversion of nicotine to cotinine in the liver. Higher CYP2A6 activity is associated with nicotine dependence and could be regulated through DNA methylation. In this study we investigated whether PostSE further impaired PreSE-induced effects on nicotine metabolism, *Cyp2a5* mRNA expression and DNA methylation. Using a mouse model where prenatally smoke-exposed adult offspring was exposed to cigarette smoke for 3 months, enzyme activity, *Cyp2a5* mRNA levels, and promoter methylation were measured in the liver tissue. We found that in male offspring, PostSE increased PreSE-induced cotinine levels and *Cyp2a5* mRNA expression. In addition, both PostSE and PreSE changed *Cyp2a5* DNA methylation in male groups. PreSE however decreased cotinine levels whereas it had no effect on *Cyp2a5* mRNA expression or methylation. These adverse outcomes of PreSE and PostSE were most prominent in females. When translated to the human situation, the combined effect of prenatal and adolescent smoke exposure could lead to an accelerated risk for higher nicotine dependence later in life.

1. Introduction

Nicotine dependence is a complex disease, which is driven by an interplay of different causes and factors. Underlying mechanisms include genetic risk factors [1], as well as environmental exposures such as maternal smoking during pregnancy [2,3]. Human studies indicated that prenatal smoke exposure (PreSE) is associated with a higher probability of nicotine addiction [3], and a greater daily consumption of nicotine [4], as nicotine is the most prominent toxicant and psychoactive component in cigarette smoke. Cytochrome P450 (CYP) 2A6 is an enzyme responsible for the clearance of nicotine in the liver [5] by conversion of nicotine into cotinine [6], and *CYP2A6* mRNA expression has been shown to be increased after cigarette smoke exposure [7]. Higher CYP2A6 activity is associated with high smoking intensity [8,9], which is linked with alterations in the nicotine metabolism [10,11].

Among the members of the CYP2A family, mouse CYP2A5 is the orthologue to human CYP2A6 and is generally believed to be the major nicotine-to-cotinine metabolizer in the mouse, accounting for ~80% hepatic turnover [12]. Inhibition of CYP2A5 was shown to reduce the nicotine elimination rate along with prolonged nicotine-induced pharmacological effects [13], as nicotine circulated longer in the test body [14].

Thus far, aberrant DNA methylation patterns have been associated with deregulated gene levels, as shown in prenatally smoke-exposed children in birth cohort studies [15–17]. Prenatal programming is now thought to be one of the missing links to set the stage for metabolic disease development, albeit that the underlying mechanisms are not yet fully described. Previously, our studies have demonstrated that PreSE increased cotinine conversion rate, which was accompanied by aberrant methylation profiles and deregulated *Cyp2a5* mRNA expression in livers from fetal, neonatal and adult offspring [18]. In this study, we asked the question whether postnatal smoking (PostSE) would further enhance the effects of PreSE, as it has been shown that children of mothers who smoke during pregnancy will most likely be exposed to cigarette smoke while growing up [3,19] and have a higher chance to start smoking during adolescence [20].

2. Results

PreSE males had more cotinine formation compared to their controls ($p = 0.03$, Figure 1A), whereas in PreSE female offspring, cotinine levels were lower ($p =$

0.04, Figure 1B). Interestingly, in males, PostSE further enhanced the PreSE-induced cotinine levels, (positive interaction, ($p = 0.002$, Figure 1A), while no interactive effects were observed between PreSE and PostSE female offspring (Figure 1B). *Cyp2a5* mRNA levels in PostSE groups ($p = 0.012$, Figure 1C) were additionally increased, while no PostSE effect on mRNA was seen in female offspring (Figure 1D). Basal levels of cotinine formation and mRNA expression was higher in females than in males ($p < 0.01$, Figure 3A and $p < 0.001$, Figure 3B). A positive correlation was found between the cotinine conversion in all control groups and *Cyp2a5* mRNA levels ($r = 0.67$, $p = 0.001$; Table 1), while this correlation was not seen in both pre- and postnatal smoke exposed groups.

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Figure 1. Cotinine formation rate of in vitro nicotine metabolism in the liver from (prenatal and postnatal) smoke exposed and control groups. Cotinine formation of hepatic nicotine metabolism and *Cyp2a5* mRNA expression were measured in liver from control, postnatal smoke exposed (PostSE), prenatally smoke exposed (PreSE), and the combined with prenatally smoke exposed and postnatal smoke exposed (PreSE/PostSE) mice. The cotinine formation rates (A, male and B, female) and mRNA expression (C, male and D, female) were measured by high performance liquid chromatography (HPLC) and qRT-PCR analysis as

described in the Section 4. Circle (○) symbol(s) = male, square (□) symbol(s) = female. The linear regression analysis was performed among all groups. A positive interaction indicates a significant interaction between the effect of smoking during pregnancy and postnatal smoke exposure. Data are shown as individual values. If not stated otherwise, the comparison of shown groups was not significant. * $p \leq 0.05$, (Mann–Whitney *U*-test). Open symbol(s) = control group, closed symbol(s) = (prenatally) smoke exposed group

To investigate whether higher mRNA expression in PostSE male offspring groups was supported by epigenetic changes, *Cyp2a5* promoter methylation levels of six CpG sites (CpG-614, CpG-589, CpG-589, CpG-542, CpG-74, CpG-7, and CpG + 45) were assessed, as described in the Section 4. PreSE male offspring had higher methylation levels at CpG-589 ($p = 0.02$, Figure 2B) and CpG-74 ($p = 0.01$, Figure 2D) when compared to control male offspring, while no differences were observed in PreSE female offspring (Figure 2G–L). PostSE male offspring had higher methylation levels at CpG-589 ($p = 0.01$, Figure 2B), CpG-74 ($p = 0.01$, Figure 2D) and CpG-7 ($p = 0.04$, Figure 2E), whereas in female offspring, PostSE groups had higher methylation at CpG-542 ($p = 0.01$, Figure 2I) and CpG-7 ($p = 0.02$, Figure 2K). In contrast, PostSE males had lower methylated CpG-542 ($p = 0.01$, Figure 2C).

Figure 2. Sex-dependent *Cyp2a5* promoter methylation in the liver from (prenatally) smoke exposed- and control groups. DNA was isolated from whole liver of male (A–F) and female (G–L) and from smoke exposed (prenatal and/or postnatal: closed symbols) and control groups (open symbols). DNA was subjected to bisulfite sequencing-based methylation analysis of the *Cyp2a5* promoter region and the percentage of DNA methylation was assessed. Data of the 6 targeted CpG-sites are presented per sex and exposure as individual values with median as a horizontal line. CpG-site annotations are relative to the ATG start codon. If not stated otherwise, the comparison of shown groups was not significant. A linear regression analysis was performed among all groups. Circle (○) symbol(s) = male, square (□) symbol(s) = female. Open symbol(s) = control group, closed symbol(s) = (prenatally) smoke exposed group.

Figure 3. Comparisons of *Cyp2a5* mRNA expression and promoter methylation status between control male and female in the liver. (A,B) figures represent comparisons of cotinine levels and *Cyp2a5* mRNA expression between male and female in control liver. (C) figure represents sex-dependent comparisons of *Cyp2a5* promoter regions between male and female in control liver. Data of the 6 targeted CpG-sites are presented per sex and exposure as individual values with median as a horizontal line. Circle (○) symbol(s) = male, square (□) symbol(s) = female. If not stated otherwise, the comparison of shown groups was not significant. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (Mann–Whitney *U*-test). Open symbol(s) = control group.

Basal levels of methylation of CpG-542, CpG-74 and CpG + 45 sites were higher in female offspring than in male offspring (all, p -values < 0.05 , Figure 3C). Based on these differentially methylated CpG sites in promoter region of *Cyp2a5*, we expanded our evaluation of mRNA expression and assessed whether DNA methyltransferase (DNMT) expression levels are affected by pre- and postnatal smoke exposures (Figure 4). It appeared that *Dnmt3a* mRNA levels were lower in the PostSE offspring, independent of their sexes (male: $p = 0.01$, Figure 4C, and female: $p = 0.03$, Figure 4D), while no differences were detected in PreSE offspring. Moreover, *Dnmt1* and *Dnmt3b* mRNA levels were not affected by PreSE and PostSE in both male and female offspring, as shown in Figure 4A,B,E,F.

Figure 4. *Dnmt1*, *Dnmt3a* and *Dnmt3b* mRNA expressions were measured in whole liver from control, PreSE, PostSE and PreSE/PostSE offspring. *Dnmt1* (A, male and B, female), *Dnmt3a* (C, male and D, female) and *Dnmt3b* (E, male and F, female) mRNA were quantified by qRT-PCR analysis as described in Section 4. A linear regression analysis was performed among all groups. Circle (○) symbol(s) = male, square (□) symbol(s) = female. If not stated otherwise, the comparison of shown groups was not significant.

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In all PreSE groups, *Cyp2a5* mRNA levels correlated with CpG-614 methylation ($r = 0.83$, $p = 0.003$; Table 1), and negatively correlated with methylation at CpG-74 and CpG-7 ($r = -0.82$, $p = 0.004$ and $r = -0.85$, $p = 0.004$; Table 1). In PostSE males, *Cyp2a5* mRNA levels correlated with CpG + 45 methylation ($r = 0.89$, $p = 0.00$; Table 1), whereas in PostSE females, *Cyp2a5* mRNA levels correlated with CpG-614 and CpG-589 methylation ($r = 0.76$, $p = 0.00$; $r = 0.93$, $p = 0.003$, Table 1). *Cyp2a5* mRNA levels from the PreSE/PostSE male group correlated with CpG-589 methylation ($r = 0.9$, $p = 0.02$; Table 1). CpG-589 methylation levels correlated also with *Cyp2a5* mRNA levels in the PreSE females ($r = 0.93$, $p = 0.00$; Table 1).

Table 1. Correlations between cotinine levels, *Cyp2a5* mRNA expression and promoter methylation in liver

Correlation of/with		All (Male and Female)						Male			Female						
		All	All	All	All	All PreSE + PostSE	All	Control	PreSE	PostSE	PreSE + PostSE	Control	PreSE	PostSE	PreSE + PostSE		
<i>Cyp2a5</i> (2-ΔCT)																	
Cotinine formation (nmol/min/mg)	r	0.10	0.67	0.02	0.83	0.39	-0.31	0.06	0.38	0.20	-0.25	-0.06	0.44	0.12	0.82	0.76	-0.63
	p value	ns	0.00	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
CpG-614	r	0.20	0.02	0.83	0.39	0.39	-0.31	0.06	0.38	0.20	-0.25	-0.06	0.44	0.12	0.82	0.76	0.53
	p value	ns	ns	ns	0.00	ns	ns	ns	ns	ns	ns	ns	0.00	ns	ns	0.00	ns
CpG-589	r	0.27	0.38	-0.10	0.23	0.48	0.31	0.03	-0.11	0.08	0.9	0.55	0.29	0.32	0.93	0.22	0.22
	p value	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.02	0.00	ns	ns	0.00	ns
CpG-542	r	-0.01	-0.36	0.22	0.12	0.12	0.11	-0.21	0.20	-0.60	-0.31	-0.29	0.04	0.04	-0.31	-0.20	0.68
	p value	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
CpG-74	r	-0.36	-0.17	-0.82	-0.26	-0.50	-0.50	0.17	0.72	0.16	-0.16	-0.09	0.15	0.08	-0.60	0.26	0.34
	p value	0.00	ns	0.00	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
CpG-7	r	-0.20	-0.33	-0.85	-0.03	-0.32	-0.32	0.19	0.35	-0.67	-0.03	0.03	-0.35	-0.67	-0.63	-0.39	-0.46
	p value	ns	ns	0.00	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
CpG + 45	r	-0.09	-0.01	-0.57	0.04	-0.55	-0.55	0.52	0.25	-0.10	0.89	-0.26	0.11	0.12	0.40	0.10	-0.60
	p value	ns	ns	ns	ns	ns	ns	0.00	ns	ns	0.00	ns	ns	ns	ns	ns	ns

p-values were adjusted by the Holm-Bonferroni method, ns= not significant.

3. Discussion

In our study, we found that PreSE increased cotinine formation levels, which was further enhanced by PostSE in PreSE offspring. This phenomenon was only observed in males. Higher nicotine metabolism was accompanied by higher *Cyp2a5* mRNA levels in PostSE male groups. In females, PostSE had no effect on the nicotine metabolism, and the PreSE group had lower cotinine formation than the other groups. Finally, *Cyp2a5* mRNA levels and differential DNA methylation patterns were more pronounced in PostSE male offspring than in PreSE offspring.

In this study, the increased nicotine metabolism in PreSE male offspring was further enhanced by personal smoke exposure of these mice. Extrapolating our observation to the human context, PreSE combined with PostSE could imply a serious risk for adverse smoking behavior, as a higher nicotine metabolism was associated with heavier smoking habits, and smokers who metabolize nicotine more rapidly may have difficulties quitting cigarette smoking than slower nicotine metabolizers [1,21,22]. Regarding the impact of PreSE alone on smoking behavior, maternal smoking during pregnancy selectively increased the probability that adolescent offspring would smoke and would persist in smoking. Concerning sex differences, an effect of PreSE on smoking behavior was stronger in adolescent daughters than in sons [3]. Other human studies support sex differences in nicotine dependence, showing that nonpharmacological effects, such as sex hormones and environmental factors have a stronger impact on drug and nicotine dependence in females than in males [23,24]. Considering that environmental factors are much more influential in humans as compared to laboratory animals, animal models are very useful to elucidate the biological relevance in nicotine dependence. Several experimental studies have assessed the impact of prenatal nicotine exposure on nicotine dependence in rat offspring [4,25]. Klein et al. described a preference of nicotine-containing water in prenatal nicotine exposed adolescent offspring, compared to non-exposed offspring. This effect was seen in males but not in female offspring [4]. In addition, prenatal nicotine-exposed hyperactive male offspring exhibited a significant increase in nicotine cholinergic receptors, while the effect was not seen in prenatal nicotine-exposed female offspring [25]. In accordance with our results, a potential combined effect of both pre- and postnatal smoke exposure on the nicotine metabolism is worth further exploration and may reveal important mechanisms of nicotine addiction in (early) adulthood.

In this study, we detected more PostSE-induced changes in *Cyp2a5* promoter methylation changes than PreSE-induced changes. In a human study, a similar result was described by Richmond and colleagues when comparing prenatally smoke-exposed newborns and adults [15]. In PreSE newborns, differentially methylated CpG sites were identified in *AHRR*, *MYO1G*, *GFII*, *CNTNAP2*, *KLF13*, *CYP1A1* and *ATP9A* genes, which are known as smoke related genes. In that study, CpG sites in less than half of the smoking related genes have shown reversibility of methylation in PreSE offspring when compared to PreSE newborns. These findings could be an explanation for the current study showing that a majority of PreSE-induced CpG sites reverted to normal methylation “methylation recovery” after years of not being exposed to cigarette smoke [15,26,27], while differentially methylated CpG sites were more pronounced after PostSE. In addition, a current cigarette smoke exposure is known to be a powerful modifier for DNA methylation [28]. The effect of current smoke exposure has been associated with differential DNA in peripheral blood cells in multiple cross-sectional studies [29,30]. It should be noted in our study that PreSE-induced differentially methylated CpG sites of *Cyp2a5* have not been impaired by PostSE in both sexes.

In addition, we investigated the expression of DNMT enzymes in this mouse model. In mammals, DNA methylation is primarily catalyzed by three active DNMTs, which are maintenance methyltransferase (DNMT1) and *de novo* methyltransferase (DNMT3a and DNMT3b). Here, the expression of *Dnmt3a* mRNA was reduced by PostSE, while no significant changes were detected in PreSE offspring. The downregulatory effect of PostSE on expression of *Dnmt3a* is consistent with two previous in vitro studies, showing that exposure to cigarette smoke extract significantly reduced mRNA levels of *Dnmt3a* and *Dnmt3b* enzymes in embryonic orofacial cells [31] and mouse embryonic fibroblast cells [32]. The reduction in *Dnmt3a* and *Dnmt3b* mRNA levels were associated with decreased global and gene-specific DNA methylation patterns.

It is well known that smoke exposure effects on DNA methylation and gene expression are sex-dependent, as described by our research group [18,33,34] and others [28–30,35,36]. Similarly, we found that the distinct effects of PreSE and/ or PostSE on *Cyp2a5* methylation, mRNA levels and the nicotine conversion rate were different in male and female offspring. One interesting observation was that PreSE female offspring had a lower cotinine formation rate compared to controls. As a study in rats showed that early life smoke exposure

caused impaired ovarian steroidogenesis [37], our result could be explained by a lower level of ovarian hormones in PreSE female offspring, leading to lower CYP2A5 activity. Another interesting observation was that female control offspring had a higher hepatic cotinine conversion and *Cyp2a5* mRNA expression as compared to males. This supports previous studies in humans [38–40] where female livers had a higher *CYP2A6* gene expression and enzyme activity than males [40]. This result is likely due to the effect of sex hormones on metabolic activity towards nicotine metabolism [40]. In addition to this, a study in rats demonstrated that ovariectomized female rats have a lower behavior motivation effect of nicotine intake than intact female rats [41,42].

Some limitations should be mentioned in the present study. Firstly, hepatic CYP2A5 protein levels were not measured to support a change in *Cyp2a5* mRNA levels. Secondly, RNA and DNA were isolated from whole liver tissue and a variation in the cellular composition of the liver in the different groups could be a confounding factor for the DNA methylation analysis.

For the analyzed CpG sites, only the proximal CpG sites were investigated in *Cyp2a5* gene. However, although both the distal and proximal regions were described to regulate *Cyp2a5* in murine hepatocytes [43], the key activators of the *Cyp2a5* expression are within the proximal regions when compared to the distal region [44]. A final limitation is that blood and urine samples were not collected immediately after the final smoke exposure. They could have been an additional source for nicotine-to-cotinine conversion analyses as shown by in vivo mouse studies [12,14].

Materials and Methods

4.1. Animals & Smoke Exposure

A total of 48 female and 48 male C57BL/6J mice were obtained from Harlan (Horst, The Netherlands) at 6 weeks of age, housed under standard conditions with food and water provided ad libitum and with a 12-h light/dark cycle. The experimental setup was approved by the local committee on animal experimentation (DEC6589 B & 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, USA). Over 7 days, randomly selected primiparous female mice were adjusted to cigarette smoke by increasing the number of

smoked cigarettes step-wise (3R4 cigarettes; 2.45 mg nicotine/cigarette) from 2 to 5 every smoking session. At day 5 after the end of the second smoking session, all female mice were injected with pregnant mare's serum gonadotropin (PMSG, 1.25 IU) to stimulate ovulation and at day 7 with human chorionic gonadotropin (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 at the following morning.

Throughout gestation, female mice were housed in groups and exposed to two air or whole-body smoking sessions per day, 7 days per week. After delivery, dams were no longer exposed to cigarette smoke and housed individually for the following 8 weeks. Offspring ($n = 46$ from non-smoking mothers, $n = 25$ from smoking mothers) were whole-body exposed to air (16 males and 18 females) or smoke (19 males and 18 females) for 12 weeks, 5 days a week. A total of 71 adult offspring exhibited from 22 control (10 males and 12 females), 12 PreSE (6 males and 6 females), 24 PostSE (12 males and 12 females) and 13 Pre- and PostSE (7 males and 6 females), as shown in Figure 5. Offspring were euthanized at 20 weeks for collection of liver tissues. The material was immediately frozen in liquid nitrogen and stored at -80°C until further use.

Figure 5. Study plan. The exposed offspring are divided into four group, which are air exposed offspring (control, $n = 22$), postnatal smoke exposed offspring (PostSE, $n = 24$), prenatally smoke exposed offspring (PreSE, $n = 12$), and the combined with prenatally smoke exposed and postnatal smoke exposed offspring (PreSE/PostSE, $n = 13$). Arrows indicate periods of exposure to air exposure (open box) and cigarette smoke exposure (closed box).

4.2. mRNA Expression and Pyrosequencing-Based Bisulfite PCR Analysis

DNA and RNA were isolated using the All Prep DNA/RNA Mini Kit (Qiagen, Cat No. 80204), according to the manufacturer's protocol. Gene expression analysis in whole liver mRNA isolates was done via qPCR using qPCR MasterMix Plus (Eurogentec, Seraing, Belgium) with commercially available primers for *Cyp2a4/5* (Mm00487248_m1, TaqMan® Gene Expression Assay, Applied Biosystems, Foster City, CA, USA), *Dnmt1*, *Dnmt3a* and *Dnmt3b* (Invitrogen, The Netherlands), and normalized to the housekeeping gene *Gapdh* (Mm99999915_m1, TaqMan® Gene Expression Assay, Applied Biosystems, Foster City, CA, USA). Detection of amplification reactions was performed using the LightCycler® 480 System (Roche Diagnostics GmbH, Mannheim, Germany), as previously described [18].

4.3. Isolation and Preparation of DNA of Liver Tissues for Bisulfite-Based Methylation Analysis

For the assessment of promoter methylation levels of *Cyp2a5*, bisulfite sequencing primers were designed using PyroMark assay design software (version 2.0, Qiagen). The selection of CpG-sites was based on manual identification of CpG-dinucleotides, using the ENSEMBL genome web browser (Ensembl 83: December 2015) and transcript location for the identification of gene promoter regions. Extracted genomic DNA from liver was converted with sodium bisulfite (EZ DNA methylation Direct™, Zymo Research, Irvine, CA), following the manufacturer's instructions. In short, the bisulfite conversion was carried out in the dark at 98 °C for 10 min and 64 °C for 3.5 h, followed by desulphonation of the converted DNA. Gene amplification was done using HotStarTaq® MasterMix (Kit Qiagen, Venlo, The Netherlands). CpG-sites were identified manually in the 600bp promoter region of the mouse *Cyp2a5* gene (ENSMUSG00000005547), and assessment of DNA methylation levels was performed on the PyroMarkQ24 (Qiagen) instrument. Relative levels of methylation at each CpG-site were analyzed with PyroMark Q24 2.0.6 software. The used amplification and sequencing primers used in this study are listed in Table 2.

Table 2. *Sequences of primers used in bisulfite-based methylation analysis*

Gene Targeted CpG-Sites	Sequence 5'-3'
<i>Cyp2a5</i> CpG-614 to -542	For: TTTGTGTTTGTGTTTGAGTGTGGGATTA Rev: CCCCATCCACAACCATCTT Seq: GTTGGGATTATAGGTTTATATTA Sequence to analyze: TTATATTYGATTTTGGGAGTTTTTAATGAAGAGGATTTGAATTTAAGGATGYGAGAA GTGGAGATT TAGGGTTATYGG
<i>Cyp2a5</i> CpG-74 to +45	For: AGTGGATAGTTTGGAGGTGAAAT Rev: ACAACTTTCCTAAAACTTCTCTACTTC Seq1: GGAGGTGAAATAGTTGTATAATTA Seq2: TAGTTATTATTGTTTGTATTATTAT Sequence to analyze: S1: GATTAAAGTTYGTTTTTTTGTGTTTTGGATGTATAAAAGTAAGTTAATT S2: TATYGTTATTATGTTGATTTTAGGATTTTTTTGGTGGTTGTAGTGGTTTTTTTAGYGTGTTGGTTTT

5

4.4. Microsome Preparation and In Vitro Assay for Nicotine Metabolism

Microsomes were obtained from homogenized livers of 20-week-old mice to measure nicotine metabolism, as previously described [5,18]. Briefly, the homogenized liver tissue was centrifuged at 9000× *g* for 20 min at 4 °C in phosphate buffer with EDTA. The supernatant fractions were collected and centrifuged at 100,000× *g* for 90 min at 4 °C. The resulting pellets (microsomes) were collected and re-suspended in phosphate buffer without EDTA. The collected supernatants (cytosolic fractions) were pooled for further use as a source of aldehyde oxidase activity for nicotine metabolism. The content of microsomal protein was determined in each sample with a reducing agent and detergent compatible (RC DC) protein assay (RC DC protein Assay, Bio-Rad, The Netherlands). In this study, we used the in vitro oxidation assay for nicotine-to-cotinine conversion, as adapted from previous mouse studies [14,45]. As cotinine is the major primary metabolite of nicotine metabolism in mice and humans [46], this approach has the clear advantage that the relative contribution of CYP2A5 (CYP2A6 in human) to cotinine formation can be estimated. The metabolism of nicotine follows two steps, the first of which is the conversion of nicotine to nicotine- $\Delta 1'(5')$ -iminium ion by CYP2A5 in the liver.

Thereafter, the nicotine- $\Delta 1'(5')$ -iminium ion was converted to cotinine by aldehyde oxidase, which is present in the cytosolic fraction. The liver microsomal protein was incubated with the pooled cytosolic fraction and nicotine containing solution. To ensure that the nicotine conversion was linear

with time, and aldehyde oxidase availability was not rate-limiting, a 45-min incubation time was selected from the tested experimental durations (15, 30, 45 and 60min), and 1mg/mL was selected as the optimal final concentration of cytosolic protein from a tested range between 0.25, 0.5, 1, 2, 3, 4, 6, and 8 mg/mL. These optimizations of nicotine-to-cotinine conversion were originally examined in the liver from PreSE embryonic and neonatal mice [18]. For the assay, a normalized 0.5 mg/mL liver microsomal protein and 80 μ L cytosolic fraction with a protein content of 1.0 mg/mL were added to 80 μ L of a freshly prepared nicotine containing solution, which is a mix of 4.4 μ L S(-)-nicotine ($\geq 99\%$, N3876, Sigma-Aldrich, St.Louis, MO, USA), 1.5 mL demineralized water and 28.5 mL of 100 mM sodium phosphate buffer pH 7.4. The mixture was supplemented with phosphate buffer (pH 7.4) to a total volume of 220 μ L. After preheating this mixture at 37 °C for 5 min, 180 μ L of freshly prepared NADPH in phosphate buffer containing 12.3 mM magnesium chloride (Sigma-Aldrich, St.Louis, MO, USA), 55.8 mM potassium chloride, 1.17 mM D-Glucose-6-phosphate ($\geq 98\%$, G7250, Sigma-Aldrich, St.Louis, MO, USA) and 5.2 μ L glucose-6-phosphate dehydrogenase (Roche, Germany; Grade II from yeast) were added to the mixture for supplying the energy to recycle the CYP's active site after oxidation. This mixture (400 μ L in total) was incubated at 37 °C for 45 min in an incubator for the actual biotransformation.

4.5. Nicotine Metabolite Analysis by HPLC and LCMS

For the analysis of the biotransformation products of nicotine metabolism, the samples were prepared as previously described by Messina et al. [6] and H. Raunio et al. [14]. The samples from the biotransformation experiment were alkalinized by adding 20 μ L of 10 M sodium hydroxide and mixing for 2–3 s on a vortex, to stop the reaction after the biotransformation assay, and to deprotonate the analytes. From this, the uncharged analytes were extracted in a two-step extraction. Each extraction step consisted of adding 1ml of dichloromethane, mixing thoroughly for 60 s on a vortex, spinning for 3 min at 10,000 g to separate the layers, after which the organic (lower) phase was collected. An amount of 20 μ L of 2.5 M hydrochloric acid was added to the combined dichloromethane fractions, as suggested by Massadeh et al. [47], before evaporating the solvent under a nitrogen atmosphere overnight at room temperature until dryness. Dried samples were reconstituted in 100 μ L of HPLC buffer, which is a mix of 15% acetonitrile and 85% of 20 mM phosphate buffer, pH 3.0, containing 1.0 g/L heptane sulfonate sodium salt acid. The sample was

analyzed by reverse phase HPLC with UV detection (245 nm), as shown in Figure 6A.

Figure 6. Cotinine formation rate of in vitro nicotine metabolism. The nicotine metabolism was measured by HPLC-L as described in the Section 4. (A) presents the HPLC chromatogram separation of the cotinine, which was produced in liver microsomes of a PreSE male mouse (B) presents the cotinine analysis by LCMS. Cotinine, corresponding with m/z 177 was detected both in a blank sample containing standard cotinine and in the microsome sample.

The separation was performed by HPLC-L, a Hitachi system consisting of an L2130 pump (set at isocratic elution of 0,6 mL/min) and an L2300 column oven set at 25 °C. To confirm the identity of the peak in the chromatogram a fragment of the cotinine fraction from HPLC system was sent to Interfaculty Mass Spectrometry Center (IMSC) at the University of Groningen. The sample was dried again under a nitrogen atmosphere and reconstituted in eluent (water and acetonitrile both containing formic acid) for the liquid chromatography separation. The sample was injected into the LCMS system, which was measured in positive mode from m/z 100–1000 and parallel reaction monitoring was included in the analysis for targeted analysis of the compounds with m/z 177. We identified that cotinine of the nicotine metabolism experiment displayed the same retention time as the cotinine standard. In the LCMS analysis, both the cotinine standard and the cotinine of the metabolism experiment were detected at an m/z of 177.10 (Figure 6B).

4.6. Statistical Methods

For statistical evaluation of the different groups, we chose two-tailed Mann-Whitney U-test for comparisons of two groups. For four groups, we performed a multiple linear regression analysis, the latter to distinguish between a positive interaction and a negative interaction. Spearman's correlation test was used for correlation analysis. p values were adjusted by the Holm–Bonferroni method, and a value of $p \leq 0.05$ was considered significant (GraphPad Prism 7.0 Software, San Diego, CA and SPSS Statistics 23, IBM, The Netherlands). Relative gene expression ($2^{-\Delta C_t}$ method) as well as mean percent methylation and standard error of the mean (SEM) were calculated in Microsoft® Office Excel 2016.

5. Conclusions

This study presents that male offspring from mothers who were exposed to smoke during pregnancy have a higher nicotine metabolism which is further increased by 3 months of smoke exposure at adulthood. The higher nicotine metabolism is accompanied by higher *Cyp2a5* mRNA expression, which could be regulated with DNA methylation. Our study emphasizes that adverse outcomes of prenatal smoke exposure and postnatal smoke exposure are sex-dependent. When considering the impact of our observations on human health, the combined effect of prenatal and postnatal smoke exposure could lead to an accelerated risk for higher nicotine dependence later in life.

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CHAPTER 6

Prenatal smoke exposure sex-dependently affects mRNA levels and promoter methylation of *Cyp2a5* in the lung across the life-course.

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Abstract

Prenatal smoke exposure (PSE) is a potential risk factor for chronic obstructive pulmonary disease (COPD). In humans, the enzyme CYP2A6 is involved in the detoxification of cigarette smoke by the conversion of nicotine into cotinine. However, a higher CYP2A6 expression and nicotine metabolism in smokers is also a risk for development of COPD and lung cancer as it is linked to heavier cigarette consumption and a higher level of nicotine dependence. CYP2A5 enzyme, the mouse orthologue of CYP2A6, is also increased after exposure to cigarette smoke, both in the liver and the respiratory organ. Recently, we showed in our mouse model that PSE induced hepatic *Cyp2a5* expression and nicotine conversion in the liver. In this study, we aimed to follow up on our *Cyp2a5* studies in the liver and investigated the PSE effect on lung *Cyp2a5* mRNA levels and promoter methylation in fetal, neonatal and adult offspring. C57BL/6 mice were exposed to fresh air or smoke from 3 weeks before conception until delivery. DNA and RNA were isolated from lung tissue from 48 fetuses (E17.5), 42 neonates (D3) and 34 adult mice (D140). We found that PSE affected *Cyp2a5* mRNA levels in males only. In PSE male neonatal offspring, *Cyp2a5* mRNA levels were lower, compared to controls. In contrast, PSE adult male offspring had higher *Cyp2a5* mRNA levels, which was strongly inversely correlated with methylation of CpG-614 and CpG-7. PSE caused hypomethylation at CpG-74 in female fetuses, while no effect was found on the other analyzed CpG sites across the three different developmental stages. Baseline *Cyp2a5* promoter methylation was higher in adult mice for the majority of methylation sites when compared to fetal and neonatal offspring.

Our results in neonates where lower PSE-induced *Cyp2a5* levels were found could imply an increased risk for lung injury as the lung is less protected against the toxic effects of nicotine exposure. In adults, higher PSE-induced *Cyp2a5* mRNA levels could be a result of PSE-induced *Cyp2a5* hypomethylation, whereas in general, methylation was not much affected by PSE, sex and developmental stage.

Keywords: “epigenetics” “*Cyp2a5*” “lung” “fetal programming” “prenatal smoke exposure”

Introduction

Cigarette smoking is the main risk factor for chronic obstructive pulmonary disease (COPD), as nicotine is both the most addictive component and the most prominent substance in cigarette smoke[1]. Cytochrome P450 (CYP) 2A6 is an enzyme that is responsible for the clearance of nicotine in the human liver[2]. Although the function of CYP2A6 has been studied extensively in the liver, little is known about its presence and regulation in lung tissue. Mouse CYP2A5, the orthologue of *CYP2A6*, is primarily expressed in the liver, but extrahepatic expression was shown in the nasal olfactory mucosa[3][4], as well as in lung epithelium[5]. CYP2A5 is known to be a catalyst for tobacco specific-components, such as 3-methylindole[6], which is a selective toxicant for lung epithelial cells in mice[7].

Epidemiological studies have shown a clear adverse association between maternal smoking during pregnancy, an impaired lung function in the offspring[8,9] and a higher risk to develop COPD later in life[10–12]. This is supported by experiments in prenatally smoke-exposed mouse offspring by us[13,14] and others[15,16]. Underlying mechanisms that explain that children from mothers that smoked during pregnancy have an increased risk to develop COPD later in life are not entirely clear, but studies have suggested that prenatal programming may play a role. It is well known that prenatal smoke exposure (PSE) results in differential DNA methylation[14,17], which was shown to be associated with deregulated gene levels in a variety of genes[18][19][20]. According to our prior study, PSE induced aberrant *Cyp2a5* methylation which was accompanied by a higher nicotine metabolism in the liver in the offspring[21], as shown in chapter 4. As PSE impairs lung function in humans across the life course[12], and as CYP2A6 was shown to be expressed in lung tissue[22], our investigations into *Cyp2a5* were extended to the lung. The aim of our study was to investigate the PSE effect on lung *Cyp2a5* mRNA levels and *Cyp2a5* promoter methylation in fetal, neonatal and adult mouse offspring.

Materials and Methods

Animals & smoke exposure

C57BL/6J mice (48 females and 48 males) were obtained from Harlan (Horst, The Netherlands) at 6 weeks of age and housed under standard conditions, with food and water provided ad libitum and at a 12-hour light/dark cycle.

The experimental setup was approved by the local committee on animal experimentation (DEC6589 B & C; University of Groningen, Groningen, The Netherlands) and governmental and international guidelines on animal experimentation.

Mainstream cigarette smoke was generated by a Teague10 smoking machine (Tobacco and Health Research Institute of the University of Kentucky, Lexington, KY, USA). Over a period of 7 days, 20 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. On adjustment day 5, after the end of the smoking session, all female mice were injected with pregnant mare's serum gonadotropin (PMSG, 1.25 i.u.) to stimulate ovulation, and on day 7 with human chorionic gonadotropin (hCG, 1.25 i.u.) to induce ovulation. Subsequently, female mice were housed overnight at a 1:1 mating ratio with males. Mating was confirmed by the presence of a vaginal plug the following morning.

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Throughout gestation, female mice were housed in groups and exposed to air or whole-body smoking sessions twice a day, 7 days per week. After delivery, dams and their offspring were no longer exposed to cigarette smoke. Dams were separately housed, with their offspring.

A total of 48 fetuses (24 males and 24 females) from 5 smoke-exposed dams and 4 control dams were collected at embryonic stage 17.5 (E17.5) and dams were euthanized under anesthesia. A total of 42 pups at postnatal day 3 (D3) were randomly selected from prenatally smoke exposed (11 male, 8 female) and control (11 male, 12 female) groups for collection of the lung. Another 34 pups were randomly selected from 20 prenatally smoke-exposed (6 male, 6 female) and 19 control (10 male, 12 female) mice that were exposed to air in the Teague10 from 8 weeks of age for the following 12 weeks (adulthood) until euthanized for collection of the lung. The lungs were immediately frozen in liquid nitrogen and stored at -80 °C until further use.

Genetic determination of mouse sex

DNA from the tail of E17.5, D3 and adult mice was obtained by SDS denaturation, high salt extraction, and precipitation. DNA was amplified as described by Lambert et al[23]. using two primer sets, which are specific for the genes Sry (forward: 5'-TGGTCCC

GTGGTGAGAGGC-’3, reverse: 5’-TATGTGATGGCATGTGGGTTCC-’3) and IL3 (forward: 5’-GGGACTCCAAGCTTCAATCA-’3, reverse: 5’-TGGAGGAGGAAGAAAA GCAA-’3).

mRNA expression and Pyrosequencing-based bisulfite PCR analysis.

DNA and RNA were isolated using the All Prep DNA/RNA Mini Kit (Qiagen, Cat No. 80204), according to the manufacturer’s protocol. Gene expression analysis in whole lung mRNA isolates was done via qPCR using qPCR MasterMix Plus (Eurogentec, Seraing, Belgium) with commercially available primers for *Cyp2a4/5* (Mm00487248_m1, TaqMan® Gene Expression Assay, Applied Biosystems, Foster City, CA, USA). Detection of amplification reactions was performed using the LightCycler® 480 System (Roche Diagnostics GmbH, Mannheim, Germany) with cycling conditions as follows: for 2 min at 50°C, for 10 min at 90°C, for 15s at 40 cycles of 95°C and for 1 min at 60°C. Reactions were performed in triplicate for each sample with *Gapdh* (Mm99999915_m1, TaqMan® Gene Expression Assay, Applied Biosystems, Foster City, CA, USA) as a reference gene. DNA in lung tissue of fetal, neonatal and adult mice was isolated and prepared for bisulfite-based methylation analysis. For the assessment of promoter methylation levels of *Cyp2a5*, bisulfite sequencing primers were designed using PyroMark assay design software (version 2.0, Qiagen). The selection of CpG-sites was based on manual identification of CpG-dinucleotides, using ENSEMBL genome web browser (Ensembl 83: Dec 2015) and transcript location for the identification of gene promoter regions. Extracted genomic DNA was converted with sodium bisulfite (EZ DNA methylation Direct™, Zymo Research, Irvine, CA), following the manufacturer’s instructions. In short, the bisulfite conversion was carried out in the dark at 98°C for 10 minutes and 64°C for 3.5 hours, followed by desulphonation of the converted DNA. Gene amplification was done using HotStarTaq® MasterMix (Kit Qiagen, Venlo, The Netherlands). CpG-sites were identified manually in the 600bp promoter region of the mouse *Cyp2a5* gene (ENSMUSG00000005547), and assessment of DNA methylation levels was performed on the PyroMarkQ24 (Qiagen) instrument. Relative levels of methylation at each CpG-site were analyzed with PyroMark Q24 2.0.6 software. The amplification and sequencing primers used in this study are listed in **Table 1**.

Calculations and statistical methods

For statistical evaluation of the different groups, two-tailed Mann-Whitney U-test and Spearman's correlation test were used (GraphPad Prism 8.02 Software, San Diego, CA and SPSS Statistics 23, IBM, The Netherlands). Comparisons of the *Cyp2a5* methylation and *Cyp2a5* mRNA levels data among three different time points were conducted using one-way Analysis of Variance (ANOVA). P values were adjusted by the Bonferroni method. $P \leq 0.05$ was considered significant. Relative gene expression ($2^{-\Delta Ct}$ method) as well as mean percent methylation and standard error of the mean (SEM) were calculated in Microsoft® Office Excel 2016.

Results

PSE decreased *Cyp2a5* mRNA levels in male neonatal mice ($p=0.0014$; **Figure 1B**) when compared to control male neonates, whereas no difference was found in female neonates (**Figure 1E**). This effect was not detected in fetal lungs of both sexes (**Figures 1A and D**). In contrast, *Cyp2a5* mRNA levels were higher in PSE male adult lungs ($p=0.032$, **Figure 1C**), while no PSE effect was detected in female adult lungs (**Figure 1F**).

Figure 1. *Cyp2a5* expression in the lung of PSE and control in E17.5, D3 mice and adult offspring. *Cyp2a5* mRNA expression in whole lung of fetal offspring (E17.5, **A**: male and **D**: female), neonatal offspring (D3, **B**: male and **E**: female) and adult offspring (**C**: male and **F**: female) in PSE (closed symbols) and control groups (open symbols). Data are shown as individual values. If not stated otherwise, the comparison of shown groups was not significant. (Mann-Whitney U-test). * $p \leq 0.05$, ** $p \leq 0.01$ (Mann-Whitney U-test). Circle (○) symbol(s)=male, square (□) symbol(s)=female.

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For *Cyp2a5* DNA methylation in fetal offspring, PSE caused hypomethylation only at *Cyp2a5* CpG-74 in female fetuses ($p=0.002$, **Figure 2**), but not in male fetuses. In addition, lower methylation levels were detected in female control adults at CpG-+45 when compared to male control adults ($p=0.025$, **Figure 2C**). In neonatal and adult offspring, all analyzed CpG-sites were not affected by PSE in their methylation profiles (**Figures 2B and C**). No significant difference was detected in all analyzed CpG sites when comparing male and female offspring.

Figure 2. Sex-dependent *Cyp2a5* promoter methylation across three time points (E17.5, D3 mice and adult offspring). DNA was isolated from whole lung of fetuses (E17.5, **A**), neonatal offspring (D3, **B**) and adult offspring (**C**) in PSE (closed symbols) and control groups (open symbols). DNA was subjected to bisulfite sequencing-based methylation analysis of the *Cyp2a5* promoter region and the percentage of DNA methylation was assessed. Data of the 6 targeted CpG sites are presented per sex and exposure as individual values with median as a horizontal line. CpG-site annotations are relative to the ATG start codon. If not stated otherwise, the comparison of shown groups was not significant. * $p \leq 0.05$, ** $p \leq 0.01$ (Mann-Whitney U-test). Circle ($\circ$) symbol(s)=male, square ($\square$) symbol(s)=female

Although no PSE-induced effects on methylation were shown to persist across three development stages, for baseline DNA methylation levels, five out of six CpG sites were differentially methylated across three developmental stages, in both sexes (all p -values ≤ 0.001 , for CpG-614, -542, -74, -7, and CpG+45; **Figure 3A-B** and **E-K**). *Cyp2a5* hypermethylation was found at CpG-614, CpG-7 and hypomethylation was found at CpG-542, CpG-74 in adult offspring, when compared to the fetal and neonatal offspring (all p -values ≤ 0.001 , **Figure 3**). Moreover, no differential methylation levels were found at CpG-589 in both female and male offspring and CpG+45 in female offspring across three time points (**Figure 3C, D** and **L**). In addition, comparing embryonic to the neonatal stage, PSE-induced hypermethylation was observed at CpG-74 in female offspring, while this effect was not detected in the control female offspring ($p=0.03$, **Figure 3H**). In contrast, for CpG-7, hypermethylation was observed in the embryonic to the neonatal stage in the control female offspring, whereas this effect was not detected after PSE ($p=0.037$, **Figure 3J**). Regarding baseline gene expression levels, *Cyp2a5* mRNA levels were differentially expressed across three developmental stages (male: $p = 0.0016$ and female: $p = 0.002$). In male offspring, *Cyp2a5* mRNA levels were higher in fetal offspring compared to neonates ($p=0.001$, **Figure 4A**), while in female offspring, *Cyp2a5* mRNA levels were higher adulthood compared to neonates ($p<0.001$, **Figure 4B**).

Figure 3. Time points comparisons of sex-dependent *Cyp2a5* promoter methylation status in PSE and control groups (A-L). The ANOVA test was used to do the comparison analysis among the E17.5, D3 and adult groups. P-values were detected by ANOVA test in all of CpG sites over three time points. Mann Whitney U-test was used to test the comparisons between two time points in control (open symbols) and PSE (closed symbols) groups for 6 promoter methylation CpG sites. Data are represented as mean $\pm$ SEM * $p \leq 0.05$, (Mann-Whitney U-test). Circle ($\circ$) symbol(s)=male, square ($\square$) symbol(s)=female.

Comparing *Cyp2a5* mRNA levels in control male and female offspring, no significant difference was detected in the fetal offspring. However, male neonates had lower *Cyp2a5* mRNA levels than female neonates ($p=0.001$, **Figure 4C**), whereas male adult offspring had higher *Cyp2a5* mRNA levels than female offspring ($p=0.001$, **Figure 4C**).

Promoter methylation was correlated with mRNA levels across three different time points, as shown in **Table 2**. Strong negative correlations were found between mRNA levels and methylation at CpG-614 and CpG-7 in PSE male adults (p values; $p<0.001$ $r=0.9$ and $r=0.93$; **Table 2**), but not in female adults. No significant correlation was seen for the other CpG sites (**Table 2**).

Figure 4. The sex-dependent comparison of *Cyp2a5* mRNA levels between fetuses, neonates and adults (**A**: male and **B**: female). The ANOVA test was used to do the comparison analysis among the E17.5, D3 and adult groups. Comparison of *Cyp2a5* mRNA levels between male and female offspring (**C** and **D**). Mann-Whitney U-test was used to test the comparisons between two time points in control (open symbols) and PSE (closed symbols) groups. Data are represented as mean $\pm$ SEM; ** $p \leq 0.01$, *** $p \leq 0.001$ (Mann-Whitney U-test). Circle ($\circ$) symbol(s)=male, square ($\square$) symbol(s)=female.

Table 1. Sequences of primers used in bisulfite-based methylation analysis

Gene Targeted CpG-sites	Sequence 5'-3'
Cyp2a5 CpG-614 to -542	For: TTTGTGTTTGTGTTTGTAGTGTTGGGATTA Rev: CCCCATCCACAACCATTCCTT Seq: GTTGGGATTATAGGTTTATATTA
	Sequence to analyze: TTATATTYGATTTTGGGAGTTTTTAATGAAGAGGATTTTGAATTAAGGATGYGAGAA GTGGAGATTT TAGGGTTATYGG
CpG-74 to +45	For: AGTGGATAGTTTGGAGGTGAAAT Rev: ACAACTTTCCTAAAACTTCTCTACTTC Seq1: GGAGGTGAAATAGTTGTATAATTAA Seq2: TAGTTATTATTGTTTGTATTATTAT
	Sequence to analyze: S1: GATTAAAGTTYGTTTTTTGTTTTTGGATGTATAAAAGTAAGTTAATT S2: TTATYGTATTATGTTGATTTTAGGATTTTTTTGGTGGTTGTAGTGGTTTTTTTATAGYGTTTTGGTTTTT

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Table 2. Correlations between Cyp2a5 mRNA expression and promoter methylation in fetal (E17.5), neonatal (D3) and adult offspring lung.

		E17.5								
Correlation of/with	Cyp2a5 (2-dCT)	All	All control	All PSE	All Male	Male control	Male PSE	All Female	Female control	Female PSE
Cyp2a5 promoter methylation (%)	CpG-614 r	0.3	0.46	0.22	0.36	0.79	-0.15	0.26	0.15	0.27
	p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-589 r	0.3	0.34	0.34	0.2	0.52	0.01	0.32	0.18	0.45
	p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-542 r	-0.16	-0.34	-0.25	-0.29	-0.57	-0.31	-0.09	-0.32	-0.27
	p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-74 r	-0.1	-0.33	0.05	-0.49	-0.61	-0.44	0.28	-0.26	0.22
	p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-7 r	0.17	-0.17	0.46	0.45	0.5	0.41	0.02	-0.38	0.56
	p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG+45 r	-0.02	-0.34	0.27	-0.05	-0.55	0.38	0.04	-0.21	0.31
	p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns

			D3								
Correlation of/with	<i>Cyp2a5</i> (2-dCT)		All	All control	All PSE	All Male	Male control	Male PSE	All Female	Female control	Female PSE
<i>Cyp2a5</i> promotor methylation (%)	CpG-614	r	-0.02	0.09	-0.09	0.12	0.12	-0.05	-0.06	-0.19	-0.12
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-589	r	0.01	-0.13	0.01	0.16	0.18	0.03	-0.1	-0.3	-0.35
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-542	r	-0.05	-0.17	0	-0.04	0.15	-0.35	0.05	-0.33	0.09
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-74	r	-0.02	-0.04	-0.1	0.13	0.17	-0.23	-0.2	-0.21	-0.2
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-7	r	0.25	0.23	0.19	0.48	0.65	0.46	-0.02	-0.21	0.05
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG+45	r	-0.14	-0.29	-0.14	0	0.1	-0.11	-0.26	-0.57	-0.02
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
			Adult								
Correlation of/with	<i>Cyp2a5</i> (2-dCT)		All	All control	All PSE	All Male	Male control	Male PSE	All Female	Female control	Female PSE
<i>Cyp2a5</i> promotor methylation (%)	CpG-614	r	-0.17	0.17	-0.14	-0.15	0.32	-0.99	-0.13	0.32	-0.27
		p-value	ns	ns	ns	ns	ns	0.001	ns	ns	ns
	CpG-589	r	0.09	-0.31	-0.25	0.14	-0.31	0.83	0.09	-0.31	0.49
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-542	r	0.2	-0.02	,302	0.27	0.2	0.62	0.17	0.2	,551
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-74	r	-0.13	-0.01	-0.23	-0.34	-0.38	-0.52	0.1	-0.38	-0.2
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-7	r	-0.32	-0.52	-0.24	-0.42	-0.1	-0.93	-0.24	-0.1	-0.2
		p-value	ns	ns	ns	ns	ns	0.008	ns	ns	ns
	CpG+45	r	-0.1	-0.4	0.21	-0.2	-0.4	0.1	-0.02	-0.4	0.09
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns

P-values were adjusted by the Holm-Bonferroni method.

Discussion

This study aimed to evaluate the prenatal programming effect of maternal smoking during pregnancy on *Cyp2a5* methylation and its gene expression in the lung of murine fetal, neonatal and adult offspring. Only minor PSE-induced programming effects across three developmental stages were found, while lower *Cyp2a5* mRNA levels in neonatal offspring and higher *Cyp2a5* mRNA levels in PSE male adult offspring were observed. In addition, basal *Cyp2a5* methylation profiles were sex-dependently differentially methylated across these three stages, which was not seen in PSE offspring.

In the present study, PSE affected methylation of only one CpG site and only in female fetal offspring. In these mice, lower CpG-74 could be attributed to a ‘current’ smoke effect, as fetuses were (in utero) exposed to smoke until they were terminated. In our related and recently published study on *Cyp2a5* promoter methylation in the liver tissue in the same pups[24], similar effects have been described for CpG-614, -589, -542, -74, -7 and +45 sites, showing again that the majority of differentially methylated *Cyp2a5* CpG sites were observed in PSE fetal offspring. However, the number of PSE-induced *Cyp2a5* methylation changes in the lung are rather limited, which suggests that the lung is less susceptible to PSE-induced *Cyp2a5* methylation. This could indicate that the fetal lung is less exposed to nicotine, or that the lung has a less important function in the nicotine metabolism than the liver. In addition, two promoter regions in *Cyp2a5* gene have been identified to activate the transcription of the gene[25]. The proximal promoter region is suggested to play a role in murine hepatocytes, whereas the distal promoter region may have a regulatory role of *Cyp2a5* expression for the lung tissue. As a result, the regulation of the distal promoter DNA methylation of *Cyp2a5* remains to be investigated.

Above all, we found more baseline *Cyp2a5* CpG sites to be differentially methylated in adults than in fetal and neonatal offspring. In the liver tissue, we found a similar distinct *Cyp2a5* methylation profile between adulthood and the neonatal stage[24]. As might be expected, a diverse cellular composition of the lung could be a confounding factor for distinct *Cyp2a5* methylation levels across three developmental stages.

In the current study, there was no persistent effect for PSE-induced *Cyp2a5* mRNA levels across three development stages, while lower *Cyp2a5* mRNA levels were detected in neonatal male offspring. In PSE neonatal offspring, lower *Cyp2a5* levels were found, which could imply an increased risk for lung disease development when offspring would be further exposed to cigarette smoke. From mouse studies by Turk and Zhou, we know that CYP2A5 in the lung is necessary to metabolize toxicants[7,26,27]. Therefore, lower levels of CYP2A5 could be a risk in PSE neonatal offspring, as they could be less protected against toxic effects. However, also higher levels of *Cyp2a5*, as we found in PSE adult offspring, could pose a risk for lung diseases. Indeed, Xin and colleagues demonstrated in a mouse model[6] that a higher CYP2A5 enzyme activity plays an important role in the 3-methylindole’s metabolism, for which its breakdown products appeared to be toxic and carcinogenic in

the lung epithelium. Taking together, these findings might provide a basis for predicting the consequences of CYP2A5 enzyme activity in the PSE-induced lung epithelium throughout neonatal and adulthood stages.

Unfortunately, we were not able to analyze the CYP2A5 enzyme activity in the lungs from our mice, and from the literature nothing is known about the toxicity effects related to PSE-induced CYP2A5 activity in the lung.

Regarding the sex-dependent PSE-induced effects in our study, in a mouse model where the CYP-linked metabolism was studied in the lung after exposure to a low dose of the pesticide diflubenzuron, responsiveness and induction of CYP isoforms, amongst CYP2A5, was less in male mice. In contrast, female mice had a better tolerance to low dose pesticides[28]. In the present study, increased *Cyp2a5* mRNA levels were detected in female adult offspring when compared to male adult offspring. Although enzyme activity was not measured in the lung tissue of our mice, van Iersel *et al.* showed that the conversion of coumarin to 7-hydroxycoumarin was higher in female mice than in males[29], demonstrating a sex-dependent effect for CYP2A5 enzyme activity as the CYP2A5 enzyme is a selective metabolizer for coumarin.

In conclusion, our data show that PSE affects *Cyp2a5* mRNA levels in male neonatal and adult offspring. In neonates, lower PSE-induced *Cyp2a5* levels could imply an increased risk for lung diseases as the lung could be less protected against the toxic effects of smoke exposure. In adults, PSE-induced *Cyp2a5* hypomethylation could underlie higher PSE-induced *Cyp2a5* mRNA levels, whereas in general, DNA methylation profiles were not extensively affected by PSE throughout three developmental stages. This sex-specific difference has received little attention so far. This may be particularly relevant for predicting the consequences of detoxification processes in prenatally smoke-exposed children, which can facilitate further studies.

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CHAPTER 7

Summary, General discussion and Future perspectives

Summary

Maternal smoking during pregnancy has been associated with impaired lung development in the offspring as well as specific aspects of smoking behavior later in life. In the first part of the thesis (**chapters 2-3**), the role of the epidermal growth factor receptor (EGFR) signaling was investigated in lung epithelial cell development and regeneration. This was addressed in prenatally smoke-exposed neonatal mouse offspring as well as in patients with chronic obstructive pulmonary disease (COPD). In the second part of the thesis (**chapters 4-6**), the effects of prenatal smoke exposure (PSE) on *Cyp2a5* promoter methylation were investigated in the lung and liver, as well as the nicotine metabolism at fetal, neonatal and adult developmental stages. In a combination of assessments, we examined a causal link between altered DNA methylation and the functional readout of *in vitro* nicotine metabolism. Furthermore, in adult offspring exposed to cigarette smoke for 12 weeks, the effect of current cigarette smoke exposure on PSE-induced *Cyp2a5* promoter methylation was investigated in the lung and liver and linked with *in vitro* nicotine conversion, which is a measure for nicotine metabolism.

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In **chapter 2**, we assessed the effect of PSE on lung epithelial cell development and linked this with amphiregulin (AREG) expression and EGFR signaling activation. We provide evidence that prenatal smoke exposure resulted in more AREG expression, fewer ciliated cells and lower expression of the cilia-related transcription factor *Foxj1*. Regarding alveolar development, prenatal smoke exposure induced more AECII, which was accompanied by lower expression of the AECI-related markers *Pdpr* and *Gramd2*. These results obtained from PSE neonatal lungs were confirmed in an AECII-derived healthy adult mouse lung organoid model, showing that AREG treatment of organoids resulted in fewer ciliated cells and more basal cells than non-treated bronchiolar organoids. In alveolar organoids, AREG treatment led to more AECII cells than non-treated AECII cells.

In **chapter 3**, we investigated AECII behavior in COPD patients in relation to AREG expression and EGFR signaling. In lung tissue, we found a lower number of AECII in COPD than in non-COPD lung. Within the AECII population, a lower proportion of cells expressed EGFR in COPD. No differences were found in the numbers of AREG-expressing and EGF-expressing cells when comparing COPD to non-COPD. Subsequently, in a small pilot study, the direct effect of AREG on AECII cell proliferation was examined

in human AECII-derived organoids. Alveolar organoids from COPD patients seemed to be smaller in size than organoids from non-COPD lungs. AREG treatment likely led to a smaller size of alveolar organoids in non-COPD when compared to non-treated organoids. In contrast, no difference was found in the proportion of AECII nor the total number of cells in the alveolar organoids.

In **Chapter 4**, we focused on the PSE-induced *Cyp2a5* methylation states in relation to *Cyp2a5* mRNA levels and the nicotine metabolism in the liver across the life course of mice. We found that PSE increased the nicotine metabolism in male neonatal and adult offspring. These results correlated with higher *Cyp2a5* mRNA levels along with elevated PSE-induced promoter methylation. We also found that PSE-induced promoter methylation of a specific CpG site in the liver was stable across three developmental stages. Moreover, our studies showed that the observed effects were sex-specific, stressing the increased susceptibility of male offspring. **Chapter 5** reveals, as an extension on the outcome of **chapter 4**, that postnatal smoke exposure further impaired the PSE-induced effects on nicotine metabolism, as well as *Cyp2a5* DNA methylation along with gene expression in the liver. The PSE-induced *Cyp2a5* methylation states were not much affected by postnatal smoke exposure.

Finally, in **chapter 6**, we investigated the *Cyp2a5* methylation states and *Cyp2a5* mRNA levels in the lung, across three developmental stages. PSE-induced *Cyp2a5* mRNA levels were lower in neonatal mouse offspring, whereas it was higher expressed in PSE adult offspring. This effect was seen in males only. PSE induced hypomethylation at CpG-74 in female fetuses, while no effect was found on the other analyzed CpG sites across the three different stages.

General discussion

The effect of (prenatal) smoke exposure on lung epithelial cell differentiation and tissue regeneration.

PSE has been associated with many adverse outcomes such as incidences of pneumonia[1], bronchitis[2] but also impaired lung function[3], suggesting a role for PSE-induced aberrant lung development in offspring, which potentially could last for life. In **chapter 2**, we provide evidence that PSE neonatal offspring had a lower number of the ciliated cell and less expression of cilia-related transcription factor *Foxj*. The ciliated cells in the airways play a role in the proper mucociliary clearance of inhaled particles and pathogens. Therefore, these results might explain that children from mothers that smoked during pregnancy have a higher hospitalization rate due to respiratory infections, specifically during the first year of life[1,4]. With respect to alveolar epithelial cell development, PSE increased the number and proportion of proliferating AECII along with lower mRNA expression of the AECI-related markers *Pdpm* and *Grmd2*. This suggests that PSE induced AECII proliferation rather than AECII-to-AECI differentiation, which could result in a limited regenerative capacity of the lung later in life. In addition, we investigated epithelial cell distribution in lung tissues from PSE adult offspring at 12 weeks of age. PSE-induced effects seen in neonates regarding the number of ciliated cells, the number of AECII, and cilia-related *Foxj1* mRNA levels were not observed in adult offspring (data not shown), suggesting that features of aberrant lung development do not have to be permanent.

EGFR and its ligands, EGF and AREG, have been identified as central regulators of epithelial cell proliferation[5], as well as differentiation during lung development and tissue regeneration[6]. Upon binding of its ligand, EGFR activates PI3K/AKT, MAPK/ERK, and JAK/STAT3 downstream signaling cascades to regulate different biological processes (reviewed by Wee *et al.*[7]). Indeed, blocking EGFR in a mouse-derived AECII culture prevented AECII proliferation after stimulation with EGFR ligands TGF α , HB-EGF, and NRG1[8]. Of interest, AECII differentiation into AECI was not affected by EGFR inhibition. These results suggest that EGFR signaling is a selective regulator of AECII proliferation, which we could confirm in our mouse lung organoids derived from pathogen-free mice (**chapter 2**).

Although AREG expression was barely detectable in healthy lung epithelium[9], recent data show that AREG can be expressed by multiple

immune cells, such as alveolar macrophages[10,11], mast cells[12], T helper 2 (Th2)[13] and Foxp3 expressing T regulatory cells[14] in a variety of inflammatory conditions, upon exposure to smoking[9], injuries and infections[15,16] in the lung. Also in COPD, AREG is expressed by a variety of immune cells in the lung, as shown in figure 1. These data were obtained from the single-cell RNA sequencing database from the COPD Cell Atlas (open-source database, <https://p2med.shinyapps.io/copd-cell-atlas/>).

Figure 1. Boxplot figure shows AREG expression in single-cell RNA sequencing database from lung immune cells of patients with COPD and non-COPD. Data represent means of AREG expression in lung tissues of patients with COPD and non-COPD.

Figure 2. A). Representative pictures of a double immunofluorescence staining of IRF5 (M1 macrophage) and AREG and B). YM1 (M2 macrophage) and AREG in control and prenatally smoke-exposed lung. Data represent means of expression in offspring of air-exposed mothers (open symbols) or smoke-exposed mothers (closed symbols). Data are shown as individual values. If not stated otherwise, the difference between groups was not significant. * $p < 0.05$ (Mann-Whitney U-test).

In the mouse, we identified AREG in M1 and M2 alveolar macrophages, as shown in figures 2A and B. We found a higher number of AREG-expressing M2 macrophages in lung from prenatally smoke-exposed offspring compared to the control group ($p=0.017$; Figure 2b), while the number of AREG-expressing M1 macrophages was not affected by PSE (Figure 2a).

This result is in line with a prior study reporting that AREG is expressed by classically activated macrophages, which suppressed epithelial cell apoptosis in lipopolysaccharide-induced mouse lung injury[17]. It could be that macrophage-derived AREG contributed to the PSE-induced aberrant lung epithelial cell development in our model, but we cannot rule out other activated immune cells as a source of AREG production in the lung.

In addition, other signaling pathways are known to affect epithelial cell differentiation, i.e., platelet-derived growth factor (PDGF)[18], Wnt signaling[19], fibroblast growth factor (FGF)[20], vascular endothelial growth

factor (VEGF)[21] and the insulin growth factor receptor (IGFR) signaling pathway[22] and it is of interest to include these pathways in future studies into the PSE-associated dysregulation of epithelial cell differentiation during lung development. Although PSE could be linked to differential mRNA expression and DNA methylation of genes related to the Wnt[23,24] and the IGFR[25–27] signaling pathways, a direct connection to epithelial cell differentiation was not made in these studies.

Alveolar epithelial cell type II cell behavior in COPD patients.

AECII behavior and AREG responsiveness were additionally investigated in patients with COPD (**chapter 3**). The AECII serve as (one of) the endogenous lung stem cells, as AECII are able to self-renew and /or differentiate into AECI in order to restore the alveolar epithelial barrier after injury[28]. To our knowledge, a role for EGFR signaling in AECII self-renewal or differentiation in general, or in COPD, has not been investigated. EGFR signaling is, however, described to be upregulated in lung adenocarcinoma[29,30], the major form of lung cancer, and plays a significant role in promoting cell proliferation and opposing apoptosis[29]. In addition, knockout of EGFR in the A549 adenocarcinoma cell line, that is considered to be originated from AECII (reviewed in[31]), resulted in PI3K/Akt and ERK/MAPK downstream cascades and reduced AECII cell proliferation[30]. These data suggest that EGFR signaling selectively stimulates cell proliferation of AECII[32]. In COPD, we showed that fewer EGFR-expressing AECII were present in lung tissue when compared to non-COPD controls. As the number of AECII were also lower in these patients, it is tempting to speculate that EGFR signaling is important in AECII proliferation in response to alveolar injury in COPD.

As we were not able to show the presence of AECI cells in human organoids, it could be that 14 days of culture was not enough to support AECII-to-AECI differentiation. Other groups have established AECII-derived long-term organoid cultures from non-COPD patients[28,33,34], suggesting that human AECII are capable of growing from 21 to 89 days and maintain their stem cell behavior in 3D organoids culture. However, we do not know whether AECII-derived lung organoids from COPD could grow over an extended period.

Another explanation could be that we used the not the best antibody or AECI marker. Zacharias et al. reported that inhibition of the Wnt signaling increases the size of human alveolar organoids, which is determined by a decreased number of

AECII and an increased number of Aqp5⁺ AECI in human alveolar organoids[33]. For future studies the Aqp5 marker should be tested for AECI visualization.

With respect to the functionality of the bronchiolar basal progenitor (KRT5) cell, we did not investigate this in our studies described in this thesis. However, Ghosh *et al.* investigated basal cell behavior in patients with COPD [35] using basal cell clones in a 3D organoid culture. They show that basal cells isolated from COPD patients were less likely to make a clone and have smaller clones than the basal cell clones from non-COPD patients.

Taken together, impaired functionality of lung progenitor cells may occur in COPD patients, suggesting that progenitor exhaustion is involved in COPD pathogenesis. Until now, functionality studies of AECII performed in COPD progenitor cells are still relatively rare, and AECII behavior in COPD remains to be investigated.

Maternal smoking and prenatal programming.

Prenatal (re)programming

7

Epidemiological[36,37]and experimental[38,39] studies have related the risk for development of asthma and COPD (partly) to prenatal (re) programming as a result of prenatal smoke exposure. PSE offspring were shown to have epigenetic modifications of genes involved in lung development and tissue regeneration[25,26,40], immunologic abnormalities[41–43] and detoxifying xenobiotic substances[41,44]. As a link to the risk for COPD, PSE is also associated with several behavioral alterations in the offspring during the lifespan, including alterations in cognitive processes, such as learning, and memory, and impulsivity, as shown both in humans[45,46] and in animal models[47–49]. Such alterations could explain the higher nicotine dependence in offspring from smoking mothers later in life[36,50].

In this thesis (**Chapter 4 and 5**), we were able to link PSE-induced *Cyp2a5* methylation with hepatic *Cyp2a5* mRNA levels and nicotine conversion in mouse offspring across three developmental stages. One limitation of our study was that the experimental setup prevented us from investigating the actual smoking behavior or nicotine dependence in our mice. However, several pharmacogenetics studies in humans and animal models have shown that smoking behavior and/or nicotine intake are directly affected by alterations in the nicotine metabolism[51–54]. Therefore, our studies showing that PSE is

associated with *Cyp2a5* methylation, *Cyp2a5* mRNA expression, and nicotine conversion are relevant and may help explain that children from smoking mothers are more likely to become addicted to nicotine once they start smoking.

Despite our findings, to prove a causal link between DNA (de)methylation and gene silencing or activation is difficult, given that DNA methylation is one out of many epigenetic modifications. Other modes include histone modifications and RNA-based mechanisms (lncRNAs / miRNAs). Indeed, several post-transcription inhibitors are well known for *Cyp2a5/CYP2A6* mRNA expression, such as protein heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), which plays an essential role in *Cyp2a5* mRNA stabilization. Glisovic *et al.* demonstrated that when transcription was inhibited, hnRNP A1 was re-localized from the nucleus to the cytoplasm, and the CYP2A5 mRNA was reduced by binding to hnRNPA1 in cultured mouse primary hepatocytes[55]. It is possible that environmental smoke exposure, including PSE, induces hnRNP A1, which could regulate *Cyp2a5/CYP2A6*mRNA levels.

DNA methylation: stability and organ-specificity

In **chapter 4**, we showed that PSE induced differential methylation of six CpG sites in the promoter region of *Cyp2a5* in the liver, of which differential methylation at CpG-74 persisted across three developmental stages. This PSE had no effect on *Cyp2a5* methylation in the lung (**chapter 6**), where only differential methylation was found in one CpG site when comparing male and female controls. Regarding site-specific CpG methylation that can persist over time, similar results are described in humans, in which global and site-specific aberrant DNA methylation was found in prenatally smoke-exposed children[56,57], and the persistence of CpG-site specific differential DNA methylation was found at birth, seven years, and 17 years of age[58]. Moreover, in a large longitudinal cohort with genome-wide methylation data, Richmond et al. found PSE-induced differentially methylated at 15 CpG sites in 11 gene regions, i.e., *MYO1G*, *FRMD4A*, *CYP1A1*, *CNTNAP2*, *AHRR* from peripheral blood samples, which persisted from around the age of 30 into at least age of 48, both men and women[41]. Together with our current data, these results provide evidence that PSE can establish persistent changes in the DNA methylation pattern, which can be used as a biomarker to predict who will develop a chronic disease, as shown in **chapter 4**. Although PSE-induced DNA methylation changes could be related to later adverse outcomes in animal models showing behavioral and cognitive deficits[48], increased nicotine

metabolism[44], and increased brain hypoxic-ischemic injury[59] (reviewed in[60]), these direct links in humans are very limited[61]. Therefore, further studies in humans are needed to investigate the biological relevancies for persistent DNA methylation changes in the corresponding tissues.

With respect to organ-specificity, *Cyp2a5* mRNA levels in controls were significantly higher in the liver than the lung, in both male and female offspring ($p < 0.001$, Figure 3A). As shown in figures 3B and C, organ-specific differential methylation was found at five out of six CpG sites, regardless of their exposure ($p < 0.001$, figure 3B and C). Moreover, for three developmental stages, PSE had a higher impact on *Cyp2a5* mRNA levels in the liver than in the lung. These results support our previously reported data on *Igf1* DNA methylation that was performed in the same mouse model[62]. This means that DNA methylation responsiveness to *in utero* smoke exposure is organ-specific, and given that each organ has a unique epigenetic signature, this likely reflects different organ functions[62,63].

Figure 3. Comparison between liver and lung tissues regarding *Cyp2a5* mRNA levels (A) and *Cyp2a5* promoter methylation (B and C) in control- and prenatally smoke-exposed adult offspring.

For *Cyp2a5* mRNA levels, data are shown as individual values. Data of the six targeted CpG-sites are presented per organ and group as mean $\pm$ SEM. If not stated otherwise, the comparison of shown groups was not significant. *** $p \leq 0.001$ (Mann-Whitney U-test).

The additional effect of postnatal smoke exposure in PSE mice.

Following **chapter 5**, we investigated the effect of postnatal smoke exposure on the hepatic *in vitro* nicotine metabolism, as well as *Cyp2a5* methylation and *Cyp2a5* mRNA, comparing male and female adult offspring. We found that postnatal smoke exposure increased the conversion rate of nicotine-to-cotinine in the PSE liver from male adult offspring. In contrast, postnatal smoke exposure did not affect PSE-induced *Cyp2a5* methylation nor the PSE-induced *Cyp2a5* mRNA levels. Interestingly, postnatal smoke exposure had a greater effect on hepatic *Cyp2a5* methylation than PSE-induced *Cyp2a5* methylation. This phenomenon was also seen in the lung tissue from adult offspring after postnatal smoke exposure, as shown in figure 4.

Figure 4. Postnatal smoke exposure (PostSE) effect on *Cyp2a5* mRNA expression (A and B) and promoter methylation (C-H) in the lung from adult offspring that was exposed to cigarette smoke between 8 and 22 weeks of age.

Data of the six targeted CpG-sites are presented per sex and exposure as individual values with the median as a horizontal line. Circle (○) symbol(s)=male, square (□) symbol(s)=female. Mann-Whitney U-test was used to test the comparisons between control (open symbols) and PostSE (closed symbols) groups for mRNA levels and promoter methylation CpG sites. If not stated otherwise, the comparison of shown groups was not significant. * $p \leq 0.05$, ** $p \leq 0.01$ (Mann-Whitney U-test).

In the lung, postnatal smoke exposure increased *Cyp2a5* mRNA levels from male adult offspring ($p = 0.009$, Figure 4A). This effect was not observed in postnatally smoke-exposed female adult offspring (Figure 4B). For *Cyp2a5*

promoter methylation, two out of six CpG sites were affected by postnatal smoke exposure in both sexes (Figures 4G, and H). Interestingly, postnatal smoke exposure decreased DNA methylation levels at CpG-7 ($p = 0.03$, Figure 4G) and CpG+45 ($p = 0.03$, Figure 4H) in male offspring, whereas postnatal smoke exposure in females increased methylation levels at CpG-7 ($p = 0.01$, Figure 4G) and CpG+45 ($p = 0.02$, Figure 4H). In addition, PSE-induced *Cyp2a5* CpG sites were not impaired by postnatal smoke exposure in lung from adult offspring, both sexes (data not shown). We detected a less effect in PSE-induced *Cyp2a5* methylation levels in the lung across three developmental stages compared to postnatal smoke-exposed *Cyp2a5* methylation levels in the lung from adult offspring (data not shown).

Our results revealed that postnatal (current) smoke exposure in adult offspring had a more pronounced effect on DNA methylation than PSE, which was organ-dependent. This pattern is consistent with human studies where current smoking could be directly associated with differential DNA methylation in peripheral blood cells, as shown in multiple cross-sectional studies[64,65]. This means that DNA methylation levels seem to reflect active smoking, which was shown to be related to the number of cigarettes smoked[66], as well as the time and number of pack years[64]. Interestingly, after smoking cessation, the majority of the differentially methylated CpG sites observed in an analysis of current vs. non-smokers returned to the level of non-smokers within five years of smoking cessation from blood-derived samples[67].

Sex-specific differences

The effect of pre-and/or postnatal smoke exposure on the *in vitro* hepatic nicotine metabolism, as described in **chapters 4 and 5**, was clearly sex-dependent. In male neonatal and adult offspring, PSE increased the conversion rate of nicotine-to-cotinine, whereas in females this effect was not seen (neonates) or even decreased (adults). Control females (neonates and adults) also had a higher *in vitro* hepatic nicotine metabolism than their male counterparts. This is of interest as it supports studies showing that female sex hormones significantly increased affect CYP2A6[68] and CYP2A5 activity. In addition, sex differences in nicotine dependence were demonstrated in multiple human studies[reviewed in 69,70] where nonpharmacological effects, such as sex hormones and environmental factors, have a stronger impact on nicotine dependence in females than males[67,70]. In female mice, sexually dimorphic *Cyp2a5* expression was reported in the liver as well as higher CYP2A5

activity than males[71]. This phenomenon was confirmed in our studies where female adult offspring had higher *Cyp2a5*mRNA levels in both liver and lung. Overall, these results suggest that a higher nicotine metabolism results in a shorter circulation of nicotine in female mice, and an increased risk for nicotine addiction. A sex-difference in nicotine and tobacco addiction has been documented in epidemiological studies showing that although females smoke fewer cigarettes per day when they start smoking compared to males, they become nicotine-dependent much faster[72].

Finally, our studies showed a further increase in hepatic nicotine metabolism by postnatal smoke exposure in the male adult offspring (**chapter 5**). The relevance of these findings could be important when translating to the human situation, as a higher nicotine metabolism is associated with heavier smokers, and smokers who metabolize nicotine more rapidly may have difficulty in quitting cigarettes than slower nicotine metabolizers[73–75]. Human CYP2A6 gene variability has additionally been associated with tobacco consumption and reported in multiple racial/ethnic populations[73,76]. Therefore, it would be interesting to focus on determining the functional genetic variation at the CYP2A6 in smokers linked with *CYP2A6* DNA methylation pattern. Despite this, Sellers and Tyndale have demonstrated that inhibition of the CYP2A6 and CYP2A5 enzymes would slow nicotine conversion, prolong the duration that nicotine remains in the brain, and lead to a reduction in the number of cigarettes smoked[53]. Few compounds, such as methoxsalen and selegiline, have proven to inhibit nicotine metabolism[77,78], and therefore, these compounds can be used in the smoking related-diseases, facilitating smoking cessation.

Regarding CYP2A5 enzyme activity in the lung organ, however, we were not able to isolate CYP2A5 enzymes due to the small size of lung tissues were available from this mouse model (**chapter 6**). All CYP enzymes, including CYP2A5, play a role in early cellular defense against lung toxicity. Studies in animal models have been shown that CYP enzymes are preferentially localized in bronchial epithelial cells, AECII, and macrophages[79,80], where some lung toxicants are inactivated and metabolized by lung CYP enzymes. Therefore, it is interesting to study whether PSE could impair CYP enzyme activity in the lung from offspring, as the susceptibility of the lung to environmental exposures depends on the metabolic balance between toxication and detoxication pathways[80–82].

Future Perspectives

In part one of this thesis, we set out a first set of experiments in our PSE mouse model and in lung specimens of patients with COPD to investigate the role of the AREG-EGFR signaling in lung development and regeneration. Because of time limitations, we conducted only a small pilot study to investigate AECII functionality in COPD, which left us with more questions than answers. For instance, why were organoids from patients with COPD smaller, and why seemed they respond differently to AREG than mouse organoids. In addition, it may be worthwhile to further investigate AECII responsiveness to other EGFR ligands, such as EGF, TGF- α . Also, other pathways, such as WNT/ β -catenin[83] and IGFR signaling[84], could be of interest to focus on when studying lung progenitor cell function in COPD.

In the second part of the thesis, we described the (re)programming effects of (prenatal) smoke exposure on *Cyp2a5* regulation the nicotine metabolism. Also, here, our studies left us with more questions to be answered. As we clearly demonstrated that smoke effects are organ and sex-dependent, it would be interesting to assess DNA methylation patterns in different cell types[85] or within a specific region of the organ in future studies. In addition, as we investigated only the proximal region of *Cyp2a5* DNA methylation, as we have shown in **chapters 4-6**, the distal region of *Cyp2a5* would be interestingly to investigate for *Cyp2a5* methylation as well, in relation to *Cyp2a5* expression. Besides, DNA methylation is the most extensively studied epigenetic mark in gene expression. Given that other epigenetic modification mechanisms are involved in regulating transcriptional activity, such as histone modifications or microRNA, it would be interesting to add these marks in future studies. Altered histone modifications and deregulated microRNA expression patterns may all play a role in prenatal (re)programming, and were shown to be affected by (prenatal) smoke exposure[86,87].

Another interesting observation in our mouse studies was that CYP2A5 seems to be regulated in a sex-dependent manner[88]. To our knowledge, a sex-specific hepatic *CYP2A6* DNA methylation and mRNA expression pattern has not been reported in the human subjects, and it would also be interesting to focus on sex differences when performing human *in vitro* studies. Finally, it can be interesting to clarify whether PSE or smoking impairs CYP enzyme activity in lung tissue.

In conclusion, we have provided evidence in mouse and human (*in vitro*) models that (prenatal) smoke exposure is one of the triggers for abnormal lung development, tissue regeneration and nicotine dependence, for which aberrant methylation of specific genes could be an underlying factor that requires further investigation. If our results in the mouse were translated to the human situation, smoking during pregnancy would pose a threat to the unborn child, as it not only affects the development of the fetal lung, but may also increase the risk of nicotine addiction when the offspring starts smoking.

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Nederlandse
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CHAPTER 8

**Dutch/Mongolian
summary**

Acknowledgements

About the author

Nederlandse samenvatting

Helaas rookt 10% van de zwangere vrouwen ondanks sterke aanwijzingen dat het roken van de moeder tijdens de zwangerschap de groei en de ontwikkeling van het ongeboren kind, de foetus, beïnvloedt. De acute negatieve effecten van roken tijdens de zwangerschap variëren van het krijgen van een miskraam tot een slechtere ontwikkeling en werking van de organen van het ongeboren kind, zoals de longen en de lever. Kinderen van rokende moeders hebben een slechtere longfunctie en hebben daarmee een hoger risico op de ontwikkeling van infecties in de long, benauwdheid en astma. Daarnaast heeft het nageslacht van rokende moeders een verhoogde kans op een nicotine verslaving wanneer ze zelf gaan roken. Hiermee is roken tijdens de zwangerschap een risicofactor voor de ontwikkeling van de chronisch rook-gerelateerde ziekte COPD later in het leven. Dit suggereert dat de kiem van het ontstaan van COPD op de volwassen leeftijd al gelegd wordt tijdens de ontwikkeling in de baarmoeder. De mechanismen hierachter zijn nog niet volledig begrepen, maar bevolkingsonderzoek heeft laten zien dat roken tijdens de zwangerschap effect heeft op het DNA van het pasgeboren kind. Het blijkt dat regio's in het DNA die genen harder of juist zachter laten werken, de zogeheten promotorregio's, verstoord zijn in kinderen van rokende moeders. Epigenetische mechanismen zoals veranderingen in DNA methylering zouden hieraan ten grondslag kunnen liggen. Dit kan als gevolg hebben dat er meer of minder van een bepaald eiwit gemaakt wordt in kinderen van rokende moeders, een effect dat ook onderliggend kan zijn aan de verstoorde orgaan ontwikkeling van het ongeboren kind.

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Doel van het onderzoek

Het doel van het onderzoek beschreven in dit proefschrift was tweeledig. In het eerste deel werden de effecten van (prenatale) rookblootstelling op de ontwikkeling en het herstellend vermogen van de luchtwegen en longblaasje in kaart gebracht in een muismodel en in personen met COPD.

In het tweede deel werd het effect van pre- en / of postnatale rookblootstelling onderzocht op de activiteit, expressie en methylering van het gen Cyp2a5 dat belangrijk is voor het onschadelijk maken van giftige stoffen zoals die voorkomen in sigaretten rook.

Roken tijdens de zwangerschap en long ontwikkeling

Bevolkingsonderzoek laat zien dat kinderen van moeders die roken tijdens de zwangerschap een verhoogd risico hebben op het krijgen van luchtweg infecties, benauwdheid en astma. Daarnaast is gebleken dat de longfunctie van jonge kinderen van rokende moeders slechter is in vergelijking met kinderen van moeders die niet rookten tijdens de zwangerschap en dat deze achterstand aanwezig blijft gedurende de rest van het leven. Verschillende proefdier modellen hebben aangetoond dat blootstelling aan sigarettenrook tijdens de zwangerschap effect heeft op de expressie van genen die belangrijk zijn voor een juiste ontwikkeling van de long. Dit zijn grotendeels dezelfde genen die belangrijk zijn bij herstel van longweefsel na een chronische schadelijke blootstelling zoals roken, later in het leven. Vroege blootstelling, dus vóór de geboorte, heeft hiermee potentiële gevolgen op lange termijn die van invloed kunnen zijn voor de ontwikkeling van een longziekte op de volwassen leeftijd. Wanneer personen met een rokende moeder zelf ook gaan roken dan wordt het risico op COPD verder verhoogd.

Hoofdstuk 2 beschrijft een studie in muizen waarbij het effect van blootstelling aan sigarettenrook tijdens de zwangerschap op de ontwikkeling van het luchtwegepitheel, de laag cellen die de bekleding vormt van de luchtwegen, werd onderzocht in nakomelingen. Deze studie liet een verstoorde ontwikkeling van epitheelcellen zien in pasgeboren nakomelingen (pups) van moeders die blootgesteld waren aan rook tijdens de zwangerschap. Deze pups hadden een lager aantal trilhaardragende epitheelcellen in de luchtwegen dat gepaard ging met een lagere expressie van het gen *Foxj1* dat belangrijk is voor de aanmaak van trilharen. Trilhaardragende cellen helpen de long schoon te houden door slijm, stof, microben en andere schadelijke stoffen naar de mond te transporteren. Bovendien hadden rook-blootgestelde pups een hogere expressie van de genen *Spdef* en *Foxm1*. Dit zijn genen die betrokken zijn bij de aanleg en ontwikkeling van de slijm producerende cel, ook wel slijmbekercel genoemd. Hoewel er nog geen slijmbekercellen aanwezig waren in de longen van de pasgeboren muizen kan deze bevinding wel de verhoogde gevoeligheid voor het ontstaan op latere leeftijd van teveel van deze slijmbekercellen verklaren.

Naast een verlaagd aantal trilhaardragende cellen in de luchtwegen hadden pups van rook blootgestelde muizen ook meer alveolaire type 2 cellen. De longblaasjes (alveoli) zijn opgebouwd uit 2 soorten epitheelcellen:

alveolaire epitheelcel type 1 (AECI), nodig voor de uitwisseling van zuurstof en koolstofdioxide en alveolaire epitheelcel type 2 (AECII), nodig voor de productie van surfactant. Surfactant verlaagt de oppervlaktespanning van de AECI cellen en bevordert ontplooiing van de longblaasjes bij inademen zodat daarvoor minder kracht nodig is. AECII hebben daarnaast een stamcel functie in de long en kunnen zich, in het geval van schade, vermeerderen en/of veranderen in AECI. De hogere aantallen AECII in rook blootgestelde pups kan er op duiden dat deze cellen minder in staat zijn om AECI te vormen die nodig zijn voor de vorming van een longblaasje na schade. Rook blootgestelde pups hadden ook een hogere concentratie van de groeifactor amfireguline (AREG). Dit is interessant aangezien *in vitro* studies met humane luchtwegepitheel cellen hebben aangetoond dat AREG de basale stamcel in de luchtweg stimuleert tot vorming van slijm producerende cellen ten koste van trilhaardragende cellen. Dit konden we reproduceren in een muizen long organoid model (“mini long” model) waarbij longstamcellen die in aanwezigheid van AREG werden gekweekt meer slijm producerende cellen maakten en minder trilhaar dragend epitheel. Ook bleek in dit organoid model dat AREG de groei van AECII stimuleerde ten koste van AECI.

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Roken en activiteit van stamcellen in COPD

In **hoofdstuk 3** werd de studie naar het effect van AREG op AECII activiteit voortgezet in longweefsel en geïsoleerde AECII van patiënten met de rook-gerelateerde chronische longziekte COPD. Allereerst vonden we in personen met COPD een lager aantal AECII hadden in longweefsel in vergelijking met controle personen zonder COPD. Daarnaast was in de COPD groep het aantal AECII die de AREG receptor EGFR tot expressie brachten lager dan bij de controles. Dit kan betekenen dat AECII stamcellen in COPD slechter kunnen reageren op AREG terwijl AREG dat nodig is voor vermeerdering van de AECII. De COPD patiënten beschreven in dit hoofdstuk hadden emfyseem dat ontstaat door (rook-gerelateerde) beschadiging van de longblaasjes waardoor ademen moeilijker wordt. Het aantal cellen in long weefsel dat AREG produceerde was gelijk in COPD en controles.

Van controles en 2 personen met COPD werden ook AECII vers geïsoleerd en in kweek gebracht, met of zonder AREG, en na 14 dagen de grootte van de verschillende long organoïden in kaart gebracht. Long organoïden van COPD patiënten waren kleiner dan die van controles. AREG behandeling resulteerde

in kleinere organoids in controles maar niet in COPD en de proportie AECII in organoids was niet verschillend. Deze pilot studie moet uitgebreid moeten worden voordat conclusies getrokken kunnen worden met betrekking tot een mogelijk verminderde activiteit van AECII in COPD als verklaring voor verminderde regeneratie van beschadigde longblaasjes en het ontstaan van emfyseem.

Roken tijdens de zwangerschap, Cyp2a5 en nicotine verslaving

Het enzym cytochroom p450 2A6 (CYP2A6) speelt een belangrijke rol bij het ontgiften van sigaretten rook aangezien het nicotine omzet in cotinine in de lever. De snelheid waarmee nicotine omzetting plaatsvindt is geassocieerd met de gevoeligheid voor nicotine verslaving, het aantal sigaretten dat gerookt wordt per dag en het gemak waarmee gestopt kan worden met roken. Personen met een hoge activiteit van het CYP2A6 enzym blijken sneller nicotine af te breken in de lever en roken meer sigaretten per dag om het nicotine gehalte in het bloed constant te houden. Ook hebben deze personen meer moeite met het stoppen met roken en zijn zij gevoeliger voor het krijgen van COPD en longkanker. Uit onderzoek is gebleken dat nakomelingen van moeders die roken tijdens de zwangerschap op jongere leeftijd gaan roken en meer moeite hebben met het stoppen met roken. Aangezien onduidelijk is of dit rookgedrag in het nageslacht gerelateerd is aan een hogere omzetting van nicotine en of methylering van *CYP2A6* in de lever onderliggend kan zijn aan een hoge CYP2A6 expressie werd dit verder onderzocht met behulp van ons rookmodel in de muis. Dit onderzoek werd verricht in lever en longen van foetale (1 dag voor geboorte), neonatale (3 dagen oud) en volwassen (6 maanden oud) nakomelingen van rook-blootgesteld moeders en controles.

In **hoofdstuk 4** hebben we het effect van roken tijdens de zwangerschap onderzocht op gen expressie van *Cyp2a5* (synoniem van CYP2A6 in de muis), DNA methylering en de afbraak van nicotine door de lever. We vonden dat de effecten van rookblootstelling tijdens de zwangerschap op *Cyp2a5* methylering het grootst is in de foetale lever. Daarnaast hadden neonatale pups van rook-blootgestelde moeders een verhoogde *Cyp2a5* gen expressie en dit gold alleen voor het mannelijke geslacht. Meest interessant was dat de afbraaksnelheid van nicotine hoger was in neonatale en volwassen nakomelingen van rook-blootgestelde moeders in vergelijking met controles en dat dit effect alleen te zien was in het mannelijk nageslacht. Vertaald naar de mens zou dit

kunnen betekenen dat mannelijke nakomelingen van rokende moeders gevoeliger zijn voor een nicotine verslaving wanneer ze zelf ook gaan roken. Vrouwelijke nakomelingen van rokende moeders hadden een lagere nicotine omzettingssnelheid en dat kan betekenen dat zij juist slechter in staat zijn tot het ontgiften van schadelijke stoffen in sigaretten rook. Een hierdoor verhoogde aanwezigheid van giftige stoffen zou een schadelijk effect kunnen hebben op de ontwikkeling van de andere organen en mogelijk zelfs een risico kunnen zijn voor ontwikkeling van ziekte later in het leven.

In **hoofdstuk 5** werden de volwassen nakomelingen gedurende 3 maanden ook zelf blootgesteld aan sigaretten rook en zagen we dat dit het effect op de nicotine metabolisme in mannelijk nageslacht van de rokende moeder alleen maar versterkte. Ten slotte onderzochten we in **hoofdstuk 6** de *Cyp2a5* methylering en expressie in de long en vonden we dat adulte nakomelingen van rook-blootgestelde moeders een hogere *Cyp2a5* gen expressie hadden terwijl dat in neonatale pups verlaagd was. Een effect van roken tijdens de zwangerschap werd wederom voornamelijk gezien in het mannelijk nageslacht maar het effect op DNA methylering was minimaal in de long in vergelijking met de lever.

In **hoofdstuk 7** worden de resultaten bediscussieerd in een algemene discussie.

Товч хураангуй

Уушгины архаг бөглөрөлтөт өвчин (УАБӨ) нь дэлхийн хүн амын өвчлөл, нас баралтын голлох шалтгаан бөгөөд 2020 он гэхэд нас баралтын шалтгааны гуравдугаар байранд орно гэж ДЭМБ-ын тайланд тэмдэглэгдсэн байна. УАБӨ үүсгэх үндсэн шалтгаан тамхидалт боловч өвчлөлийн 10 – 20% хувийг тамхины бус шалтгаан эзэлдэг гэж *Global Initiative for Chronic Obstructive Lung Disease (GOLD)*-н удирдамжинд тэмдэглэсэн байдаг. Тамхины бус шалтгаанд: генетик, хүйс, мэргэжлийн шалтгаан, ургийн хөгжлийн үед шууд тамхидалт болон дан тамхидалтанд өртөх, уушгины дутуу хөгжил зэрэг олон хүчин зүйл нөлөөлдөг гэж үздэг.

Жирэмсэн эхийн тамхидалт буюу ургийн хөгжлийн үед эхийн тамхидалт болон дан тамхидалтанд удаан хугацаанд өртөх нь УАБӨ, астма болон цаашлаад тамхинд донтох өвчнийг үүсгэх шалтгаан болдог гэж үздэг ч эмгэг жамыг судалсан суурь судалгаанууд хангалтгүй байна. Эдгээр эмгэг жамуудын нэг нь эпигенетикийн зохицуулгийн алдагдалтай холбоотой үүсдэг гэж үздэг. Эпигенетик (*epigenetic*) гэдэг эпи (*epi*) - дээр/ нэмэлт/ латин үгнээс гаралтай бөгөөд генетикийн нэмэлт зохицуулга гэж нэрлэж болох юм. Өөрөөр хэлбэл дезоксирибонуклейны хүчил (ДНХ)-ийн нуклеотидийн дараалалд өөрчлөлт үүсгэхгүйгээр генийн экспрессийг зохицуулах механизм юм. Одоогоор ДНХ-н метилжилт (*DNA methylation*) нь хамгийн их судлагдаж байгаа эпигенетикийн зохицуулгуудын нэг бөгөөд судлаачид энэхүү метилжилтын түвшин болон тогтворжилт нь ургийн хөгжлийн үед тогтдог гэж үздэг. Ургийн хөгжлийн үед тамхидалт болон дан тамхидалтанд өртсөн хүүхдэд ДНХ-ийн метилжилтийн өөрчлөлтүүд нь хүний захын цусанд, ихэс, амны хөндийн эсүүдэд тогтмол ажиглагддаг бөгөөд энэхүү өөрчлөлт нь хүүхдэд цаашид архаг өвчинүүдийг үүсгэх үндсэн шалтгаануудыг нэг байж болох юм. ДНХ-ийн метилжилтийн түвшин өөрчлөгдсөнөөр генийн экспресс дарангуйлагдах эсвэл сэдээгдэх нөхцлүүдээр улмаар уургийн нийлэгжилтын зохицуулга алдагддаг гэж тайлбарладаг.

Энэхүү судалгааны ажлын эхний хэсэгт уушгины үүдэл эсүүдийн хөгжлийг ургийн хөгжлийн үед шууд тамхидалтанд өртсөн хулганы загвар дээр (**2-р бүлэг**), болон УАБӨ-тэй өвчтэйнээс ялгаж авсан уушгины үүдэл эсийн үйл ажиллагааг (**3-р бүлэг**) амфирегулин (*AREG*)-ээр өдөөгдсөн эпидермисийн өсөлтийн рецептор (*EGFR*) – ийн дохио дамжуулалтын идэвхижлтэй холбож судалсан. Эдгээрээс гарсан үр дүнг хүний болон

хулганы уушгины органоидын (*in vitro*) загварууд дээр баталгаажуулсан. Судалгааны ажлын хоёрдугаар хэсэгт (**4 - 6 бүлэг**) ургийн хөгжлийн үед эхийн тамхидалт нь ургийн *Cyp2a5* генийн промотор хэсэгт байрлах метилжилтийн түвшинд үзүүлэх нөлөөллийг хулганы хөгжлийн 3 шатанд буюу ураг, нярайн болон насанд хүрсэн хулганд элэгний никотины солилцооны өөрчлөлтэй холбон судалсан.

Ургийн хөгжлийн үед шууд тамхидалтанд өртөхөд ургийн гуурсан хоолойн сормууст эсүүдийн тоо цөөрч сормууст эсийн генийн экспрессүүд багассан нь ажиглагдсан. Харин цулцангийн хөгжлийн үед тамхидалтанд өртөхөд II-р хэлбэрийн цулцангийн эсийн тоо ихсэж, I-р хэлбэрийн цулцангийн эсийн генийн экспрессүүд буурсан нь ажиглагдсан. Эдгээр үр дүнг хулганы уушгины үүдэл эсээс өсгөвөрлөсөн уушгины органоид загварт амфирегулин уургийн нөлөөг судлахад гуурсан хоолойн органоидод сормууст эсийн тоо цөөрч, суурийн эсүүдийн тоо ихэссэн бол уушгины цулцангийн органоидуудад II-р хэлбэрийн цулцангийн эсийн тоо ихэссэн үр дүн ажиглагдсан (**2-р бүлэг**). Манай судалгааны 3-р бүлэгт УАБӨ-ны хүнд хэлбэрийн IV үе шатанд оношлогдсон өвчтөнгүүдийн уушгинд II-р хэлбэрийн цулцангийн эсийн үйл ажиллагааг эпидермисийн өсөлтийн рецепторийн дохио дамжуулалттай холбон судалсан. Судалгаанд УАБӨ-ны хүнд хэлбэрийн IV үе шатанд оношлогдсон өвчтөнгүүдийн уушгинд II-р хэлбэрийн цулцангийн эсийн тоо хяналтын бүлэгтэй харьцуулахад цөөрсөн байсан ба эдгээрийн эсүүдэд эпидермисийн өсөлтийн рецептор агуулсан II-р хэлбэрийн цулцангийн эсийн тоо багассан нь бас ажиглагдсан. Харин УАБӨ-ны IV үе шатанд оношлогдсон өвчтөнгүүдийн уушгинд амфирегулины уургийн болон эпидермисийн өсөлтийн фактор (*EGF*)-ын уургийн хэмжээ хяналтын бүлэгтэй харьцуулахад өөрчлөлт ажиглагдаагүй. УАБӨ-ны IV үе шатанд оношлогдсон өвчтөнгүүдийн уушгины үүдэл эсээс өсгөвөрлөсөн уушгины цулцангийн органоидын хэмжээ нь хяналтын бүлэгтэй харьцуулахад буурсан нь ажиглагдсан.

1 болон **2 бүлгүүдийг** дүгнэхэд уушгины хөгжлийн үед шууд тамхидалтанд өртөхөд болон УАБӨ-ны IV үе шатанд оношлогдсон өвчтөнгүүдээс ялгаж авсан уушгины II-р хэлбэрийн цулцангийн эсийн үйл ажиллагааны чадвар нь алдагдсан байна. Эдгээр уушгины үүдэл эсийн үйл ажиллагааны алдагдалд амфирегулинээр өдөөгдсөн эпидермисийн

өсөлтийн рецепторийн дохио дамжуулалтын идэвхжил чухал үүрэгтэй байгааг нь уушгины органомйдын туршилтаар батлагдаж байна.

4-р бүлэгт бид ургийн хөгжлийн үед дэхь тамхидалтанд өртөхөд *Cyp2a5* генийн промотор хэсгийн метилжилтын өөрчлөлтийг, *Cyp2a5* генийн экспрессийн түвшин болон элэгний никотины метаболизмийн өөрчлөлттэй холбож хулганы хөгжлийн 3 шатанд судалсан. Элэгний *CYP2A6/ Cyp2a5* генийн экспресс болон уургийн нийлэгжилт ихсэлт нь элэгний никотины үйл ажиллагааг эрчимжүүлэх замаар тамхи буюу никотинд донтох эсрдэлийг нэмэгдүүлдэг гэж үздэг. Энэхүү судалгаанд ургийн хөгжлийн үед шууд тамхидалтанд өртөх нь нярай болон насанд хүрсэн хулганд никотины солилцоог нэмэгдүүлсэн үзүүлэлт гарсан. Ургийн хөгжлийн үед тамхидалтанд өртөх нь *Cyp2a5* генийн промотор хэсгийн метилжилтийн түвшинг ихэсгэсэн үзүүлэлт гарсан бөгөөд энэ өөрчлөлт нь *Cyp2a5* генийн экспрессийн түвшинтэй хамааралтай байгаа нь энэхүү судалгаанд ажиглагдсан (**4-р бүлэг**). Эдгээр *Cyp2a5* генийн промотор хэсгийн метилжилтийн болон элэгний никотины солилцооны өөрчлөлт нь зөвхөн эр хүйсийн хулганд ажиглагдсан. Энэ хулганы загварыг насанд хүрсэн үед нь дахин тамхины утаанд дахин өртүүлэхэд элэгний никотины метаболизмыг улам нэмэгдүүлж байгаа нь ажиглагдсан (**5-р бүлэг**). Эцэст нь 6-р бүлэгт бид энэ хулганы загвар дээр уушгинд дахь *Cyp2a5* генийн промотор CpG-74 хэсэгт метилжилт нь багассан үзүүлэлт ураг үед илэрсэн бол *Cyp2a5* генийн экспресс өөрчлөлтийг зөвхөн насанд хүрсэн хулганд ажиглагдсан. Насанд хүрсэн хулганы уушгинд *Cyp2a5* генийн промотор метилжилтын түвшинд өөрчлөлт илрээгүй.

Эдгээр үр дүнг (**4-6 бүлэг**) дүгнэхэд ургийн хөгжлийн шууд тамхидалтанд удаан хугацаанд өртөх нь *Cyp2a5* генийн промотор хэсэгт дэхь метилжилтийн түвшин өөрчдөгдсөн ба энэ үр дүн нь элэгний никотины бодисын солилцоог нэмэгдүүлдүүлэхтэй хамааралтай байх нь энэхүү хулганы загварт ажиглагдаж байна.

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About the author

Khosbayar Lkhagvadorj was born on July 25, 1986 in Ulaanbaatar city, Mongolia. He graduated as Medical Doctor from the Mongolian National University of Medical Sciences (MNUMS), Mongolia. Subsequently, he received a master's degree (MSc) and specialized as a pulmonologist at the same university. He has been a lecturer in the Department of Pulmonology and Allergology at MNUSM since 2010. Besides, he has been a pulmonologist in the Third State Central Hospital of Mongolia since 2012.

In 2016, he acquired the scholarship from the Mongolian State Training Fund for his PhD projects and started his PhD study in the Department of Pathology and Medical Biology, University Medical Center Groningen (UMCG), the Netherlands. His research project study focused on the effects of (early life) smoke exposure in mouse models and lung progenitor cell behavior in COPD and was supervised by Dr. M.N. Hylkema and Prof. T. Plosch. The thesis is entitled “The effect of (prenatal) smoke exposure on lung progenitor cell behavior and *Cyp2a5* gene regulation”.

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Peer-reviewed publications

- Lkhagvadorj K, Meyer KF, Verweij LP, Kooistra W, Reinders-Luinge M, Dijkhuizen HW, de Graaf IAM, Plösch T, Hylkema MN. **“Prenatal smoke exposure induces persistent *Cyp2a5* methylation and increases nicotine metabolism in the liver of neonatal and adult male offspring”** *Epigenetics*. 2020 Dec;15(12):1370-1385.
- Lkhagvadorj K, Zeng Z, Song J, Reinders-Luinge M, Kooistra W, Song S, Krauss-Etschmann S, Melgert BN, Cao J, Hylkema MN. **“Prenatal smoke exposure dysregulates lung epithelial cell differentiation in mouse offspring: role for AREG-induced EGFR signaling”** *Am J Physiol Lung Cell Mol Physiol*. 2020 Oct 1;319(4)
- Lkhagvadorj K, Zeng Z, Meyer KF, Verweij LP, Kooistra W, Reinders-Luinge M, Dijkhuizen HW, de Graaf IAM, Plösch T, Hylkema MN. **“Postnatal Smoke Exposure Further Increases the Hepatic Nicotine**

Metabolism in Prenatally Smoke Exposed Male Offspring and Is Linked with Aberrant Cyp2a5 Methylation” *Int J Mol Sci.* 2020 Dec 26;22(1)

- 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.* 2020 Mar 1;318(3)

Abstracts, grants and presentations at conferences.

- Lkhagvadorj, K., Zeng, Z., Song, J., Reinders-Luingers, M., Kooistra, W., Krauss-Etschmann, S., Cao, J. & Hylkema, M., **Prenatal Smoke Exposure Dysregulates Lung Epithelial Cell Differentiation - Role for the AREG/EGFR Axis**, 2018, *American Journal of Respiratory and Critical Care Medicine.* 197, 2 p.
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- Netherlands Respiratory conference, Longdagen 2018 Cytochrome P450 2A5 methylation and gene expression profile is organ-specific and affected by maternal smoking during pregnancy. (poster presentation)
- Netherlands Respiratory conference, Longdagen 2018 Prenatal smoke exposure dysregulates lung epithelial cell differentiation – Role for AREG-EGFR axis (oral presentation)

- European Respiratory Society International Congress 2018 conference. Paris, France, Cytochrome *P450 2A5* methylation and gene expression profile is organ-specific and affected by maternal smoking during pregnancy (poster presentation).
- Bronchitis X symposium - Prevention and resolution of lung disease, Groningen 2019, the Netherlands. Pre- and post-natal smoke exposure increases nicotine metabolism in male mice. (Poster presentation)
- Netherlands Respiratory Society, Dutch Lung conference 2021, Alveolar epithelial type II cell behavior in chronic obstructive pulmonary disease. (poster presentation)