Early life exposure to cigarette smoke is associated with lung function changes and primes gene expression and DNA methylation changes at *Cyp1a1* upon exposure later in life

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Abstract

Prenatal and early life exposure to cigarette smoke (CS) have repeatedly been shown to induce stable, long-term changes in DNA methylation (DNAm) in offspring. It has been hypothesized that these changes might be functionally related to the known outcomes of prenatal and early life CS exposure, which include impaired lung development, altered lung function and increased risk of asthma and wheeze. However, to date, few studies have examined DNAm changes induced by prenatal CS in tissues of the lung, and even fewer have attempted to examine the specific influences of prenatal versus early postnatal exposures. Here, we have established a mouse model of CS exposure which isolates the effects of prenatal and postnatal CS exposures in early life. We have used this model to measure the effects of prenatal and/or postnatal exposures on lung function and immune cell infiltration as well as DNAm and expression of *Cyp1a1*, a candidate gene previously observed to demonstrate DNAm differences upon CS exposure in humans. Our study revealed that exposure to CS prenatally and in the early postnatal period cause distinct and long-lasting differences in offspring lung function, gene expression and lung *Cyp1a1* DNAm, which are reestablished upon re-exposure to CS in

adulthood. This study creates a testable mouse model which can be used to investigate the effects of prenatal and early postnatal CS exposures, and will contribute to the design of intervention strategies to mediate these detrimental effects.

Introduction

The developmental origins of health and disease (DOHaD) hypothesis postulates that environmental insults in the intrauterine and early postnatal environments program the developing fetus through changes in cellular function and structure, and that these changes can last through adulthood[1,2]. Numerous studies have shown that factors such as maternal nutrition, infections, stress and environmental exposure to toxins such as cigarette smoke (CS) can in fact produce long-lasting health consequences in offspring[3,4]. CS is a common environmental toxin in humans with about 9.5% of women of reproductive age (18-34 years) reported as active smokers in Canada in 2021[5]. Cigarette smoke is made up of thousands of components which have detrimental effects, some of which can also cross the placenta and affect offspring development[6–10].

Prenatal CS exposure alters offspring growth and development, lung function, and is associated with airway hyperresponsiveness, asthma and wheeze in children[11–13]. In some cases, these outcomes persist into adulthood, suggesting that prenatal CS 'leaves a mark' on the offspring, a process that has been called *biological embedding*[14–16]. Epigenetic modifications have been identified as potential mechanisms by which biological embedding takes place[16,17]. The three main components of the epigenome – DNA methylation (DNAm), histone modifications, and non-coding RNAs[18] - are dynamic and reflect past environmental exposures, meaning that environmental insults in early life can elicit changes in epigenetic marks which cells would propagate over time, and thus could translate to disease phenotypes in later life.

47 CS exposure *in utero* has been associated with differential DNAm across thousands of genes in
48 exposed children, suggesting that DNAm changes might directly or indirectly link early life CS
49 exposure with later life outcomes[19–21 21–25]. While most epigenome-wide association studies (EWAS)
50 investigating association between maternal smoking and DNAm have been conducted in newborn
51 umbilical cord blood[19–25], others have also reported differential DNAm in whole blood[26], placenta[27],
52 nasal epithelia[28], buccal cells and peripheral blood granulocytes of adults[29,30]. The largest EWAS study
53 to date which investigated effects of prenatal smoke exposure on umbilical cord blood DNAm was a
54 meta-analysis across 13 cohorts (n = 6685) in the Pregnancy and Childhood Epigenetics Consortium
55 (PACE)[20]. Results from the PACE study reported differential methylation at over 6000 CpGs, with
56 some of the top sites being Aryl Hydrocarbon Receptor Repressor (*AHRR*), Cytochrome P450 1A1
57 (*CYP1A1*), Myosin 1G (*MYO1G*) and Growth Factor Independent 1 Transcriptional Repressor (*GFI1*).
58 These genes have also been associated with maternal smoking in other similar EWAS studies[19,20,23,31].
59 They are important in detoxification, apoptosis and cell proliferation, and have been linked to the
60 development of orofacial clefts, oncogenesis and asthma[19,20,31]. Existing studies including PACE could
61 not investigate the effects of prenatal smoking after birth, nor could it measure DNAm in tissues that
62 might be more proximal to CS-mediated disease, such as the lungs.

63 While there have been human studies outlining the long-term effects of prenatal CS exposure on
64 offspring, one challenge of these studies is their inability to effectively account for the effects of the
65 postnatal environment. One study which measured the long-term effects of prenatal CS on offspring
66 lung function found no significant association between second-hand smoke exposure while pregnant
67 and long-term offspring lung function after controlling for postnatal smoke exposure[32]. It is difficult in
68 human studies to completely rule out the effect of postnatal smoke exposure since prenatal and
69 postnatal exposures are typically very similar[33,34]. Therefore, there is a need to investigate the
70 differences between DNAm signatures and health outcomes observed due to prenatal versus postnatal

71 CS exposure, especially since these exposures have been shown to produce mixed/confounding
72 effects[35–38]. Animal models provide a solution to these problems by allowing the study of the effects of
73 prenatal versus postnatal CS exposure, and enable research into longitudinal, cross-tissue and sex-
74 specific effects of early life CS exposure.

75 Here, we have created a mouse model to study the effects of prenatal, postnatal, and combined early
76 life CS on offspring DNAm and lung phenotype. Taken together, our mouse model successfully
77 recapitulates some of the phenotypes identified in humans exposed to prenatal CS but provided further
78 information on the dynamics underlying early life CS exposure. We show that early life CS exposure
79 alters offspring DNAm at *Cyp1a1*, lung function and gene expression in patterns specific to their early
80 life exposure periods. Critically, we show that the early life period is critical for creating lasting
81 memories, and that exposure to CS in adulthood recapitulates patterns set by early life CS exposure.

82

83 **Materials and Methods**

84 **Animals**

85 This animal experiment was approved by the Animal Research Ethics and Compliance Committee of
86 the University of Manitoba. Balb/C mice (Charles River Laboratories, Massachusetts, United States)
87 were supplied with standard laboratory chow and clean water *ad libitum* and housed, four mice of a
88 single sex per cage unless otherwise noted, in individually ventilated cages in a 12-hour light/dark
89 cycle throughout the duration of the experiment.

90 **Development of murine model of early life CS exposure**

91 Standard 1R6F research cigarettes (University of Kentucky, Lexington, KY) were used for this
92 experiment. Cigarette smoke was delivered via the use of the SCIREQ InExpose smoking robot

93 (SCIREQ, Montreal, QC, Canada). Female mice were left to acclimate to their environment for 1
94 week, and then divided into 2 groups: 16 control and 16 CS-exposed mice. Beginning at 8 weeks,
95 control mice were exposed to room air in a foreign cage, and CS mice were exposed to whole body CS
96 twice daily. Total estimated particulate matter per the certificate of analysis from the cigarette
97 manufacturer was 46.8 mg/cigarette, which we administered with a flow rate of 2L/min and one puff
98 per minute, with approximately 6 puffs per cigarette. CS mice were exposed for a total period of 9
99 weeks, beginning with a pre-pregnancy period of 3 weeks, after which they were mated (2 females to 1
100 male) with male mice. Once a vaginal plug was achieved, we separated female mice into individual
101 cages for the rest of the duration of the experiment. We continued all smoke and room air exposures on
102 CS and control mice respectively, throughout mating, pregnancy, birth, and stopped at weaning (3
103 weeks post-birth). At birth, we culled offspring to 6 offspring per litter, and cross-fostered half of CS
104 offspring with half of control offspring, matching offspring by birth date to reduce potential of
105 rejection. Pups were marked with a toe tattoo to identify birth group. Using our cross-fostering
106 strategy, we generated 4 distinct offspring groups: control with no CS exposure ("Con"), offspring
107 exposed to prenatal CS only ("Pre"), offspring exposed to postnatal CS only ("Post"), and offspring
108 that received both prenatal and postnatal CS exposures ("Full") Throughout the experiment, offspring
109 were never directly exposed to CS, but were indirectly exposed *in utero*, or via breastmilk or fur from
110 the dams. We sacrificed and collected tissues from all offspring not cross-fostered at birth. Dams and
111 offspring were weighed weekly. Three weeks after birth, offspring were weaned, CS exposures
112 stopped, and dams underwent lung function testing followed by tissue collection. Pups were housed by
113 sex, 4-6 to a cage. While original plans were to assess phenotype at 8 weeks, of age, due to SARS-
114 CoV2-related lab lockdowns animals were maintained until 16-20 weeks of age, at which point lung
115 function and tissue collection were conducted.

116 **CS re-exposure experiment**

117 Female offspring which were not euthanized at 16-20 weeks were left undisturbed until 60 weeks of
118 age. Half of these female adult offspring were re-exposed for 2 hours to full body CS for 5 consecutive
119 days per week, for 21 days in total. The other half were not re-exposed to CS, and served as the adult
120 control group. At the end of CS exposure, lung function, lavage and tissue collection were also
121 performed.

122 **Transthoracic echocardiography**

123 As described previously[39], serial echocardiography was performed using a Vevo 2100 ultrasound
124 (Fujifilm-Visual Sonics, ON Canada) equipped with a 30 MHz transducer to assess cardiac
125 morphology and function in 12-14 week-old mice. Imaging by a blinded and experienced sonographer
126 was performed under mild anesthesia (induced with 3% isoflurane and 1.0L/min oxygen and
127 maintained at 1 – 1.5% isoflurane and 1.0 L/min oxygen) during echocardiography. Pulse Wave
128 Doppler Mode was used to measure the velocity time integral of blood flow passing through the right
129 ventricular outflow tract (RVOT). Vevo software was used to determine pulmonary acceleration times
130 (PAT), pulmonary ejection times (PET), and ventricle thickness from the RVOT for each subject.
131 Measurements of PAT and PET were taken from three different doppler waves for each subject and
132 ventricle thickness was measured from three image stills. The average of these three measurements
133 was then recorded.

134 **Lung function measurement**

135 As previously described[40,41], mice were anesthetized with sodium pentobarbital and lung function
136 measured using the SCREQ Flexivent small animal ventilator (SCIREQ Inc., Montreal, QC, Canada).
137 Total airway resistance (Rrs), Newtonian resistance (Rn), tissue resistance (G) and elastance (H) were
138 measured at baseline in response to nebulized saline, and changes in these parameters were also
139 measured in response to increasing concentrations of nebulized methacholine (3 to 50 mg/ml).

140 **Bronchoalveolar lavage**

141 Following lung function measurement, mouse lungs were washed twice through a tracheal cannula
142 with 1 ml of phosphate buffer saline (PBS) each wash. The bronchoalveolar lavage fluid (BALF) was
143 then centrifuged at 4°C at 1200 rpm for 10 minutes to obtain cell-free supernatants which were stored
144 at -20°C for later cytokine analysis. Cell pellets were resuspended in 1 ml of PBS and total cell counts
145 were performed using a hemocytometer. Differential cell counts were performed by placing 100ul of
146 the resuspended pellets on glass slides using cytospin columns, and staining cells using a modified
147 Wright-Giemsa stain (Hema 3 Stat Pack). Cell differentials were counted using a Carl Zeiss Axio
148 Observer ZI microscope.

149 **Blood and tissue collection and preparation**

150 Whole blood was collected from dams at pup weaning and from offspring at 16 and 63 weeks. Blood
151 was collected by severing the abdominal aorta, into EDTA-coated tubes, centrifuged at 4000 rpm for
152 15 minutes to obtain plasma. Blood cell pellets and plasma were snap-frozen in dry ice and stored at -
153 80°C for future measurements. Left, inferior, postcaval and middle and superior lung lobes were
154 collected separately and snap-frozen in dry ice for DNAm and gene expression analyses.

155 **DNA/RNA isolation from lungs and blood**

156 DNA and RNA were extracted from mouse tissues using the Invitrogen DNA and RNA isolation kits
157 respectively. Left lungs were homogenized using the Qiagen Tissue Lyser II, followed by
158 simultaneous DNA and RNA isolation using same Invitrogen DNA and RNA isolation kits. DNA and
159 RNA were quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, USA).

160 **Maternal cotinine measurement**

161 To test the concentration of nicotine in the dams, dam cotinine levels were measured in plasma 48
162 hours after smoking cessation at weaning of offspring, and again 13 weeks later in a subset of dams.
163 Plasma cotinine was measured using a commercially available ELISA kit (CalBiotech, Spring Valley,
164 CA), according to the manufacturer's instructions, and the concentration of cotinine in each sample
165 was measured at 450 nm.

166 **Selection of mouse candidate genes to measure DNAm**

167 To obtain candidate genes at which to measure DNAm, we selected 2 of the top differentially
168 methylated CpGs from the meta-analysis conducted in human cohorts[20]. The 2 chosen human CpGs
169 with some of the largest effect sizes were selected from genome build GRCh37/hg19: *AHRR* at
170 position chr5:373378 (cg05575921) and *CYP1A1* at position chr15:75019251 (cg22549041). We then
171 blasted the resulting human sequence against the *Mus musculus* genome assembly on NCBI. Using R
172 studio version 3.6, we performed a muscle[42] alignment between human and resulting mouse sequences
173 to detect mouse CpGs which exactly aligned with or were closest in position to the human CpGs. The
174 2 mouse CpGs were selected from genome build GRCm38/mm10 based on results of the alignment:
175 position chr13:74260517 for *Ahrr* and position chr9:57696335 for *Cyp1a1.* Importantly, the *Ahrr* gene
176 is not conserved between human and mice, and while we selected the closest mouse CpG, it may not
177 be comparable to the human position. To select a CpG position for the mouse control gene, we
178 selected a human CpG position which was not differentially methylated in newborn cord blood (human
179 genome GRCh38/hg38, *PRKAA1* cg13345558, position chr5:40796738), as reported in the PACE
180 study. We then identified a corresponding mouse CpG (genome build GRCm39/mm39 position
181 chr15:5174566) as described above.

182 **Measurement of the effects of cross-fostering**

183 Since we cross-fostered offspring at birth to create our model for early life CS exposure, we needed to
184 rule out any potential effects of the cross-fostering process on offspring. 4 female and 2 male Balb/C
185 mice were purchased at 6 weeks of age, separate from the mice used for the major experiment. They
186 were left to acclimate for 2 weeks, fed and housed as described above. At 8 weeks of age, mice were
187 placed together (2 females to 1 male) and mated for 3 days. At birth, we culled offspring to 6 offspring
188 per litter, and cross-fostered half of resulting offspring, matching offspring by birth date to reduce
189 potential of rejection. Pups were marked with a toe tattoo to identify birth group. Cross-fostering led to
190 the generation of 2 groups of offspring: non cross-fostered and cross-fostered groups. We performed
191 lung lavage, sacrificed and collected tissues from all offspring at 8 weeks of age.

192 **Measurement of candidate gene DNAm**

193 500 ng of DNA isolated from the left lungs was treated with sodium bisulfite (Zymo Research) to
194 generate bisulfite-converted DNA (bcDNA), following the manufacturer's protocol. Following
195 conversion, bcDNA was then amplified by PCR using the following conditions: 95°C for 15 min,
196 followed by 45 cycles of 95°C for 30 secs, 58°C for 30 secs, 72°C for 30 secs, and then followed by
197 72°C for 5 min. Mouse *Ahrr* and *Cyp1a1* primers were generated using the PyroMark Assay Design
198 software version 2.0 (Qiagen). Primer sequences can be found in Table S1. DNAm at candidate genes
199 was measured using the Qiagen Q48 pyrosequencer. Prior to DNAm measurement in samples, CpG
200 assays were validated in duplicates using mixtures of completely methylated and completely
201 unmethylated control DNA (0%, 25%, 50%, 75%, and 100% methylated). After validation, sample
202 DNAm was then measured using the optimized assay conditions.

203 **Gene expression measurement**

204 200ng of lung mRNA was treated with ezDNase (Thermo Fisher Scientific, Inc.) and then converted to
205 cDNA using Maxima cDNA synthesis Kit (Thermo Fisher Scientific, Inc.) according to the

206 manufacturer's protocol. Quantitative real-time RT-PCR (qPCR) was performed on the QuantStudio 3
207 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA), using the SYBR-Green Master
208 Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) to determine the relative expression levels
209 of *Ahrr* and *Cyp1a1* in lung tissues. *β-actin* and *Eif2a* were used as reference and normalization
210 controls. Samples were run in duplicates under the following cycling conditions: Holding stage, 1
211 cycle of 50°C for 2 min and 95°C for 2 min; cycling stage, 40 cycles of 95°C for 15 sec, 57°C for 15
212 sec and 72°C for 1 min; and melt curve stage, 95°C for 15 sec, 60°C for 1 min and 95°C for 15. The
213 primer sequences used can be found in Table S2. *Ahrr* and *Cyp1a1* mRNA levels were quantified
214 using the $\Delta\Delta^{Cq}$ method[43,44] and normalized against the mean *β-actin* and *Eif2a* levels in the same
215 sample.

216 **Statistical analyses**

217 Statistical analysis was done using R (version 3.6.1). Percent DNAm at each CpG was averaged across
218 duplicates and between-group differences were analyzed using a one-way ANOVA. Lung function and
219 differential cell count data were analyzed using two-way ANOVA, and multiple comparison was
220 performed between groups at each methacholine dose. P values < 0.05 considered significant.
221 Adjustment for litter size effects was conducted on significant values using simple linear regression.
222 Figures were also produced in R, using the *ggplot2* package.

223

224 **RESULTS**

225 **Development of a protocol to study the effects of early life CS exposures**

226 We created a mouse model to study the effects of early life CS exposure on offspring, and specifically,
227 to isolate prenatal and postnatal CS exposures. Exposure of Balb/C dams to whole-body CS was
228 carried out for 9 weeks, beginning 3 weeks before mating and pregnancy, and lasting till 3 weeks after

229 birth (Figure 1A). To isolate the effects of prenatal and postnatal-only smoke exposures, we cross-
230 fostered half of the offspring born to control and smoke-exposed dams at birth, with toe tattoos to
231 identify cross fostered offspring (Figure 1A). All cross-fostering was carried out no more than 24
232 hours after birth of matched control and CS-exposed litters. Only one litter, including both cross
233 fostered and not cross fostered offspring was lost. All smoke-exposed dams tolerated smoke exposures
234 well, and our exposure paradigm did not result in loss of any dams. Since mating resulted in variable
235 plug/conception dates, we ensured that all analyses reported here were carried out on offspring born no
236 more than 48 hours apart. COVID-19 related lab lockdowns occurred shortly after weaning of the
237 majority of litters, resulting in changes to planned timelines.

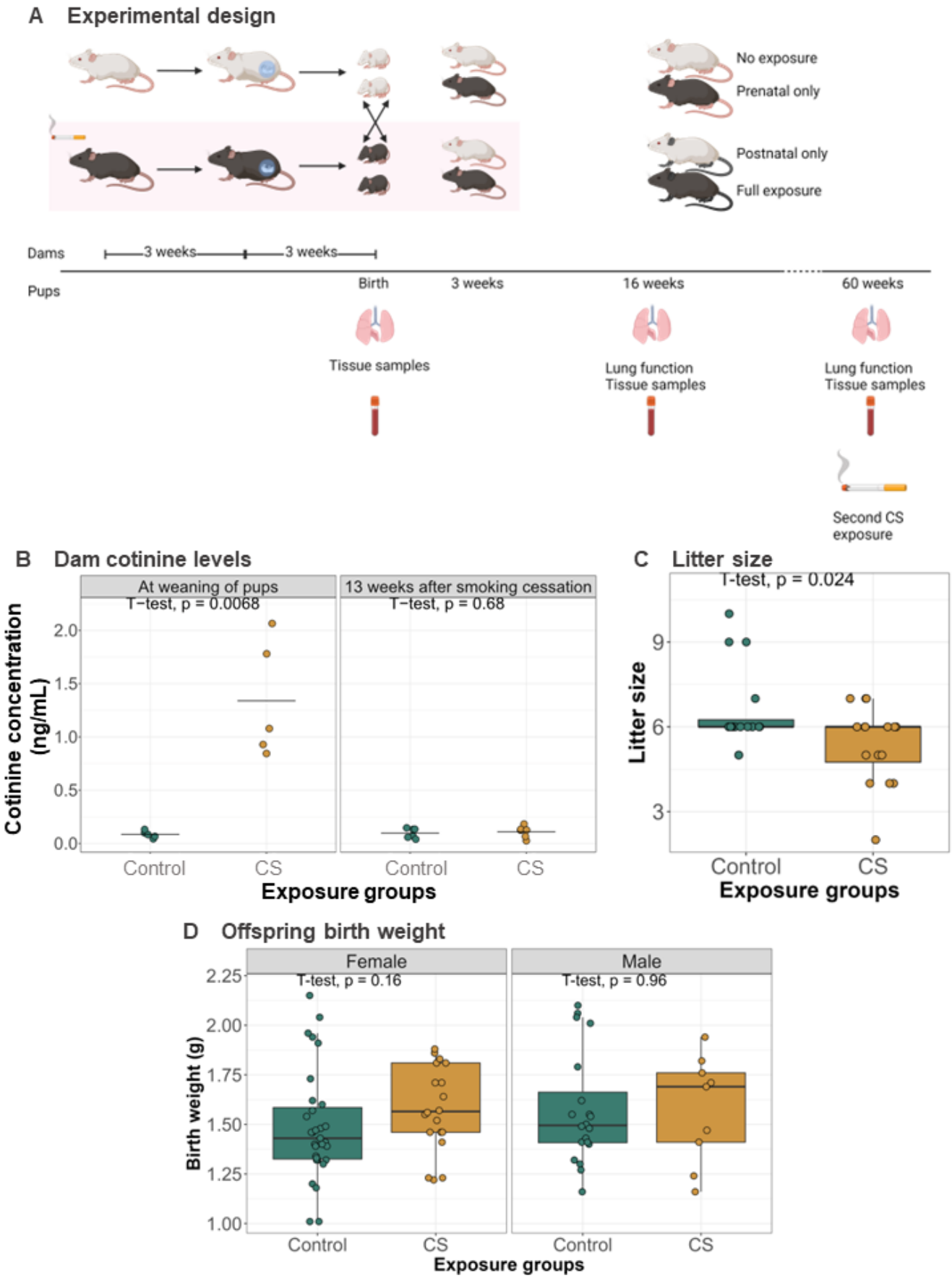
238 To verify absorption of CS components, we measured cotinine levels in plasma collected from 10
239 dams 48 hours after smoking cessation (3 weeks after birth of pups). The remaining 12 dams' cotinine
240 levels were measured 13 weeks after smoking cessation (Figure 1B). Dams which were exposed to CS
241 had 15 times more elevated plasma cotinine levels (Mean = 1.34, SD = 0.55) 48 hours after smoking
242 cessation compared to control dams (Mean = 0.09, SD = 0.03) (Figure 1B, $p = 0.0068$). The relatively
243 low levels of cotinine observed overall in the smoke-exposed dams can be attributed to the collection
244 of plasma from dams 48 hours after smoking, with the reported half-life of cotinine being about 24
245 hours[45,46]. Cotinine levels measured 13 weeks after CS exposure showed no significant difference
246 between control and smoking dams (Figure 1B).

247 Maternal smoking can reduce litter size[47,48] and pup birth weight in mice, which might have indirect
248 effects on pup development[48,49]. To ensure that our DNAm and phenotypic analyses are not
249 confounded by such effects, we tested the effects of our smoking model on offspring birth weight and
250 litter size. We found a slightly larger litter size in control dams (Mean = 6.56, SD = 1.46) than smoke-
251 exposed ones (Mean = 5.38, SD = 1.36) (Figure 1C, T-test $p = 0.024$). Birthweight in male and female
252 animals was slightly but not significantly larger in CS than control pups, perhaps due to the smaller

253 litter size (Figure 1D). We performed sensitivity tests in downstream analyses to identify the potential

254 effects of litter size.

255


A Experimental design
No exposure
Prenatal only
Postnatal only
Full exposure
Dams
3 weeks
3 weeks
Pups
Birth
3 weeks
16 weeks
60 weeks
Tissue samples
Lung function
Tissue samples
Lung function
Tissue samples
Second CS exposure
B Dam cotinine levels
At weaning of pups
T−test, p = 0.0068
13 weeks after smoking cessation
T−test, p = 0.68
Cotinine concentration (ng/mL)
2.0
1.5
1.0
0.5
0.0
Control
CS
Control
CS
Exposure groups
C Litter size
T-test, p = 0.024
Litter size
9
6
3
Control
CS
Exposure groups
D Offspring birth weight
Female
T-test, p = 0.16
Male
T-test, p = 0.96
Birth weight (g)
2.25
2.00
1.75
1.50
1.25
1.00
Control
CS
Control
CS
Exposure groups

256

257 Figure 1: Development of a mouse model to study the effects of early life CS exposure. (A)
258 Experimental design: Adult female mice were exposed to CS for 9 weeks, starting 3 weeks prior to
259 mating and ending 3 weeks after birth. Half of control offspring were cross-fostered at birth with half
260 of the CS-exposed offspring to generate 4 groups of offspring: control, prenatal CS-exposed, postnatal
261 CS-exposed and combined prenatal and postnatal CS-exposed groups. Lungs, blood and other tissues
262 were collected from dams and offspring at birth and at 16 weeks of age, after lung function
263 measurement. At age 60 weeks, half of the remaining female offspring were re-exposed to CS for 3
264 weeks, followed by lung function and tissue collection. (B) Dam cotinine levels measured in plasma
265 48 hours after smoking cessation and 13 weeks after. (C) Litter size of control and smoke-exposed
266 dams. Student t-test was used to measure differences in litter size between control and smoke-exposed
267 dams. (D) Weight of control and CS-exposed male and female offspring at birth. Differences in birth
268 weight were analyzed using student t-tests within sexes.

269

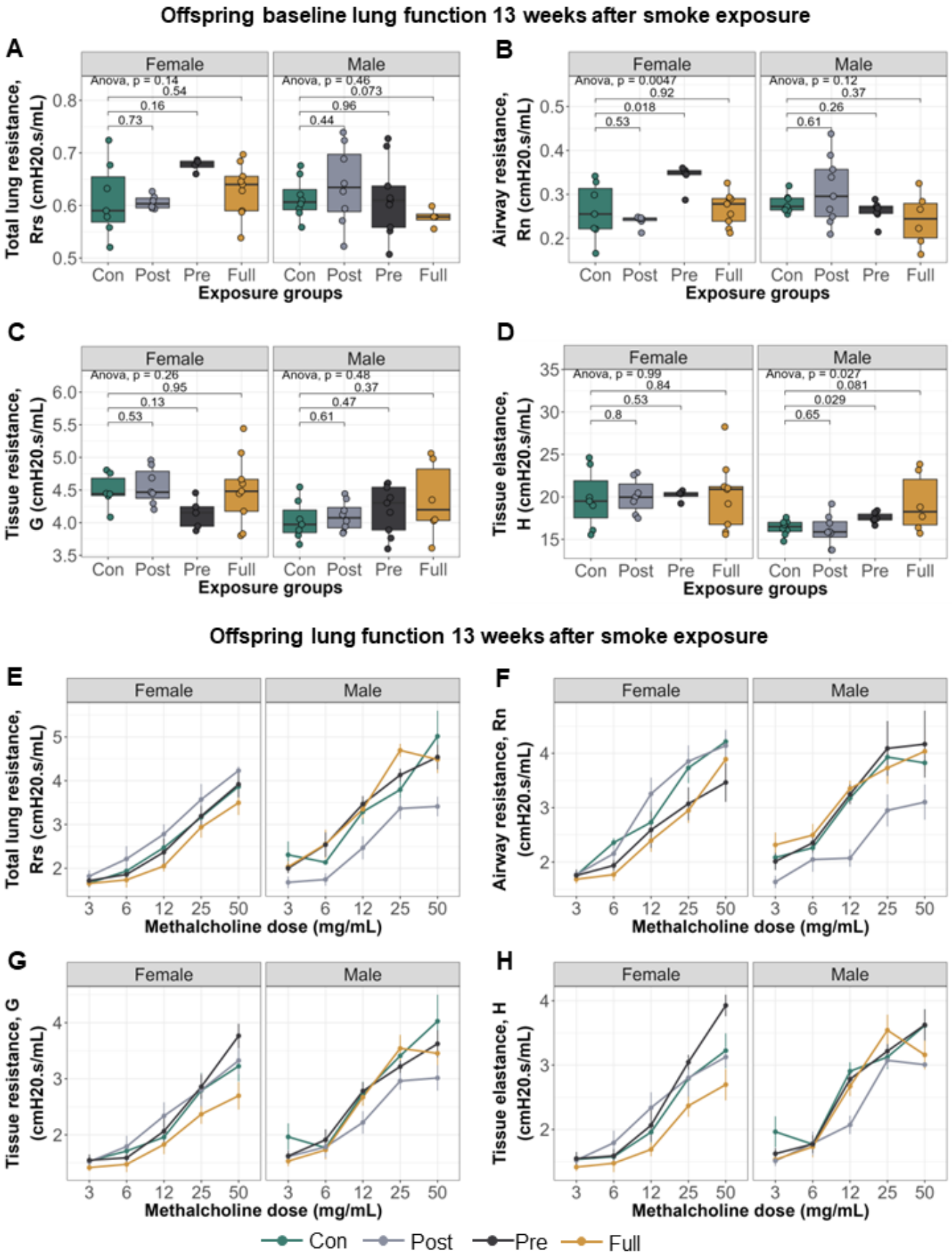
270 **Offspring lung phenotype at 16 weeks of age, 13 weeks after cessation of smoke exposure**

271 At baseline, only male offspring with prenatal CS exposure and female offspring with prenatal CS
272 exposure had increased elastance (Figure 2D, $p = 0.029$) and airway resistance (Figure 2B, $p = 0.018$),
273 respectively, compared to control offspring. When methacholine was introduced however, male
274 offspring in the postnatal CS group showed significant decrease in responsiveness for total lung
275 resistance, airway resistance and tissue elastance (Figures 2E, 2F and 2H), while fully exposed male
276 offspring showed increased methacholine sensitivity for total lung resistance (Figure 2E). Similarly,
277 full CS exposure induced decreased methacholine sensitivity for airway resistance in female offspring
278 (Figures 2F and 2H).

279 When examining immune cell infiltration in the lung, female offspring exposed to postnatal only CS
280 had significantly elevated eosinophils (Mean = 1691, SD = 1647) compared to control females (Mean
281 = 475, SD = 800) (Figure 3B, $p = 0.0046$), while male offspring with full CS exposure had
282 significantly elevated lymphocytes (Mean = 3479, SD = 3064) compared to control males (Mean =
283 601, SD = 659) (Figure 3D, $p = 0.029$).

284 Together, our data suggested a mild phenotype induced by 9-week early life smoke exposure in
285 indirectly exposed offspring, as observed by persistent decline in lung function and increased
286 infiltration of immune cells into the lungs. Similar analyses in dams are in the Supplementary Results.

287


Offspring baseline lung function 13 weeks after smoke exposure
A
Female
Male
Anova, p = 0.14
0.54
0.16
0.73
Anova, p = 0.46
0.073
0.96
0.44
Total lung resistance, Rrs (cmH20.s/mL)
Con Post Pre Full
Exposure groups
B
Anova, p = 0.0047
0.92
0.018
0.53
Anova, p = 0.12
0.37
0.26
0.61
Airway resistance, Rn (cmH20.s/mL)
C
Anova, p = 0.26
0.95
0.13
0.53
Anova, p = 0.48
0.37
0.47
0.61
Tissue resistance, G (cmH20.s/mL)
D
Anova, p = 0.99
0.84
0.53
0.8
Anova, p = 0.027
0.081
0.029
0.65
Tissue elastance, H (cmH20.s/mL)
Offspring lung function 13 weeks after smoke exposure
E
Total lung resistance, Rrs (cmH20.s/mL)
3 6 12 25 50
Methalcholine dose (mg/mL)
F
Airway resistance, Rn (cmH20.s/mL)
G
Tissue resistance, G (cmH20.s/mL)
H
Tissue elastance, H (cmH20.s/mL)
Con Post Pre Full

288

Figure 2: Early life exposure to CS alters sensitivity to methacholine up to 13 weeks after cessation of smoke exposure. Lung function parameters (A) Total lung resistance at baseline. (B) Airway resistance at baseline. Female offspring exposed to prenatal CS had significantly higher Rn (Mean = 0.34, SD = 0.03) compared to female controls (Mean = 0.26, SD = 0.06). (C) Tissue resistance at baseline. (D) Alveolar elastance at baseline. Male offspring exposed to prenatal CS had significantly higher H (Mean = 17.50, SD = 0.63) compared to male controls (Mean = 16.40, SD = 0.91). (E) Total lung resistance with methacholine. (F) Airway resistance with methacholine. (G) Tissue resistance with methacholine. (H) Alveolar elastance with methacholine. Lung function was assessed by taking 90th percentile values in response to injection of saline into the lungs (baseline) and in response to increasing doses of methacholine. Lung function data was analyzed in a sex-disaggregated manner using a two-way ANOVA, followed by multiple comparisons between groups at each methacholine dose where significant.

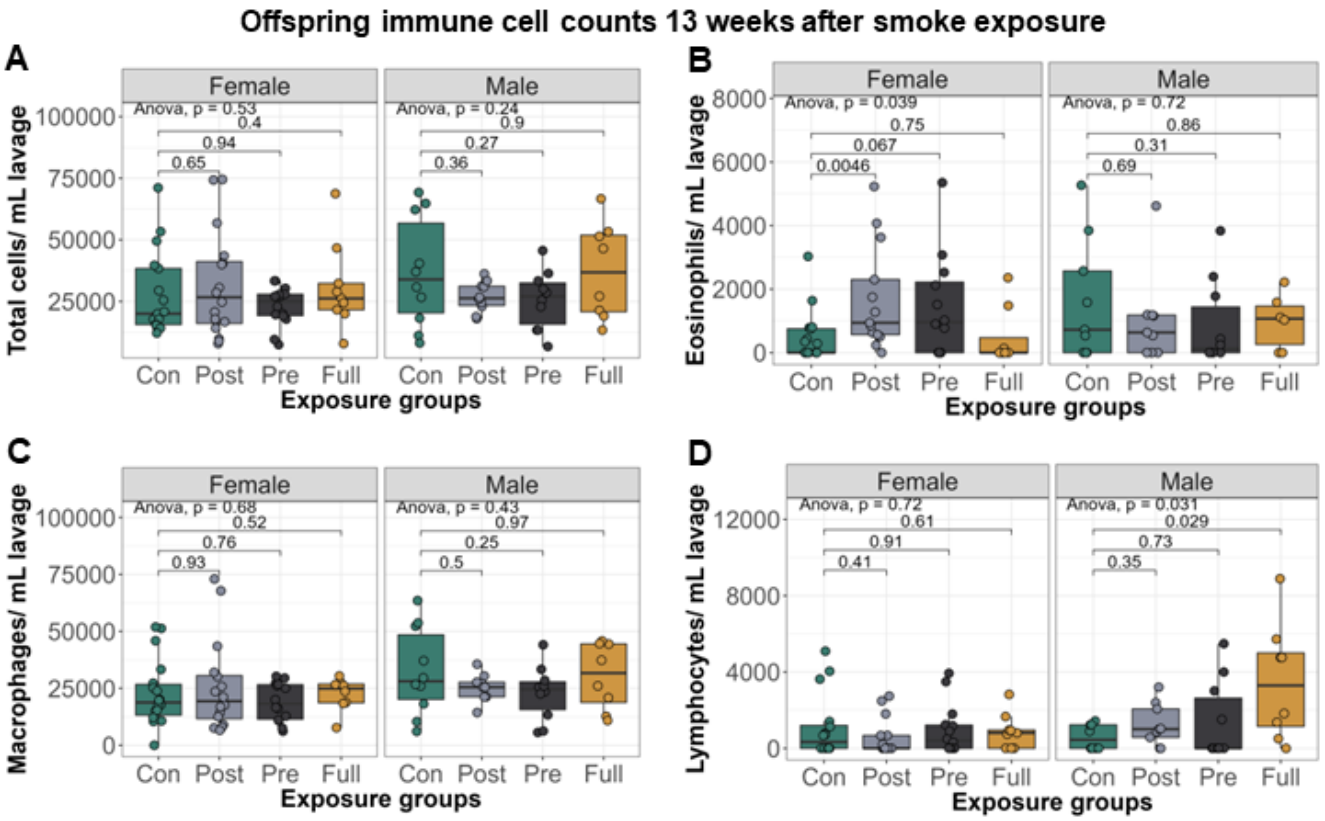


Figure 3: Early life exposure to CS increases immune cell infiltration into offspring lungs, up to 13 weeks after smoke exposure. (A) Total immune cells per mL lavage. (B) Eosinophils per mL lavage. (C) Macrophages per mL lavage. (D) Lymphocytes per mL lavage. Differential cell counts were normalized to lavage volume. Pairwise comparisons were conducted using t-tests.

Impact of CS on offspring cardiac morphometry and function

To determine whether CS exposure affected cardiac morphometry or function we used echocardiography to image the heart and examine left ventricular and right ventricular structural and functional parameters. In males, there were no effects of smoke exposure on left ventricle (LV) mass or other LV structural parameters (Table S3). In females, prenatal CS exposure reduced the intraventricular septum, the LV internal diameter and LV volume (Table S4). On the other hand, postnatal CS exposure increased LV posterior wall thickness in females. Overall, these structural

316 changes did not impact LV mass in females. Regarding LV functional parameters, in the males,
317 prenatal CS exposure significantly increased LV ejection fraction and cardiac output, though postnatal
318 CS and the combination of pre- and postnatal CS did not affect these parameters (Table S3). However,
319 in females, CS exposure did not affect LV function in any of the groups (Table S4). Next, right
320 ventricle (RV) morphometry and function was examined. In both males and females, RV wall
321 thickness was increased in prenatal CS exposed offspring and by postnatal CS as well as the
322 combination of prenatal and postnatal CS (Tables S3, S4). CS did not alter RV function and
323 hemodynamics in males (Table S3). Female RV function and hemodynamic parameters were more
324 sensitive to the effects of CS. Prenatal CS exposure increased the pulmonary vessel peak velocity and
325 peak pressure (Table S4). In addition, prenatal CS exposure followed by postnatal CS also increased
326 pulmonary valve peak velocity and peak pressure in females. Pulmonary acceleration time was
327 significantly reduced by postnatal CS and the combination of prenatal and postnatal CS in females.

328

329 **DNAm and gene expression at birth and 16 weeks of age in candidate genes**

330 Results from human studies have shown that maternal smoking causes decreased and increased DNAm
331 of *AHRR* and *CYP1A1* respectively, in newborn umbilical cord blood[19–21,23,26], and that these
332 differential methylation patterns persist in adult blood at midlife[24]. The effects of smoking on the
333 human lungs is less well documented, as only one study to our knowledge has reported increased
334 *AHRR* and *CYP1A1* DNAm in nasal epithelia of adult smokers[28]. Therefore, as DNAm is species and
335 tissue-specific, and studies investigating effects of prenatal CS exposure on the lungs is lacking, we
336 measured *Ahrr* and *Cyp1a1* DNAm in the lungs and blood of offspring at birth immediately after CS
337 exposure and at 16 weeks of age (13 weeks after cessation of smoke exposure). Since DNAm is also
338 associated with regulation of gene expression, we also measured *Ahrr* and *Cyp1a1* expression in dam
339 and offspring lungs at both timepoints.

340 At birth, *Ahrr* and *Cyp1a1* DNAm in the lung and blood were not significantly different between
341 control and smoke-exposed offspring (Figure 4A and 4C). However, both male and female offspring
342 born to CS-exposed dams showed lung *Cyp1a1* expression levels which were over 20 times higher that
343 controls (Figure 4B).

344 We next measured *Ahrr* and *Cyp1a1* methylation levels in lungs and blood of offspring at 16 weeks of
345 age to determine longitudinal effects of early life smoke exposure on offspring (Figure 4E – 4H).
346 There were no significant differences in *Ahrr* DNAm between control and CS-exposed groups in
347 offspring blood 13 weeks after cessation of smoke exposure, and found (Supplementary figure 4B). In
348 the lung in male offspring only, *Ahrr* DNAm in prenatally exposed offspring was significantly higher
349 than offspring exposed only postnatally (Figure 4G, $p = 0.03221$), but none of the CS-exposed
350 offspring showed significantly altered *Ahrr* DNAm compared to control offspring. In female offspring
351 at 16 weeks, those exposed to prenatal or postnatal CS had higher *Cyp1a1* DNAm in the lung
352 compared to controls (Figure 4E), while offspring with full CS exposure showed significantly
353 decreased *Cyp1a1* methylation compared to either postnatal (Figure 4E, $p = 0.02$) or prenatal groups
354 (Figure 4E, $p = 0.0049$). These differences remained significant even after adjusting for possible
355 effects of litter size on DNAm using linear regression. The decrease in *Cyp1a1* DNAm in the lung in
356 fully exposed female offspring, but not in cross-fostered offspring, is similar to the direction of
357 *Cyp1a1* DNAm in dams (Supplementary figure 5).

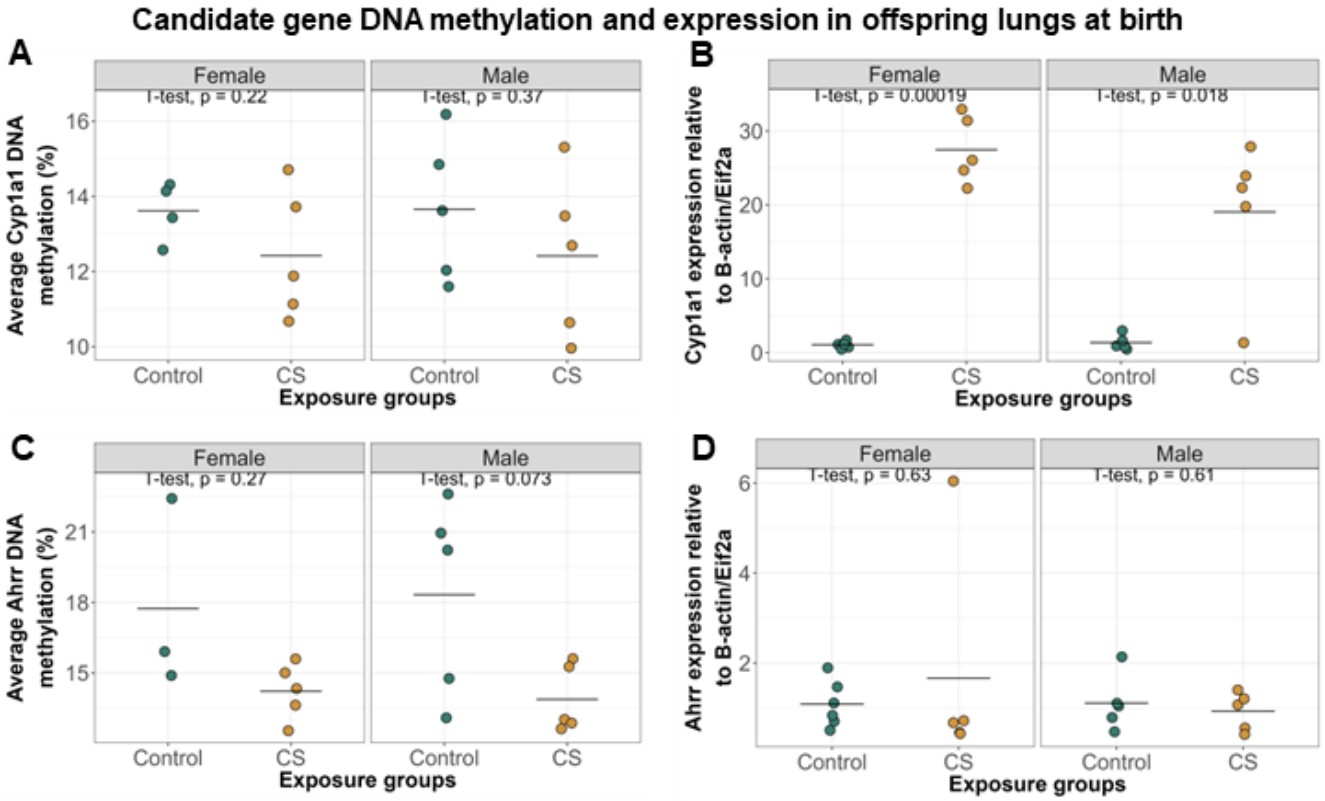
358 We observed the same trend when we measured offspring lung *Cyp1a1* expression: increased
359 expression levels of *Cyp1a1* in the postnatal only and prenatal only CS groups but decreased
360 expression in the lungs of offspring exposed to both prenatal and postnatal CS, compared to controls,
361 though these differences were not statistically significant (Figure 4F). To show that the differences in
362 candidate gene DNAm observed in offspring was not because of changes in global DNAm, we
363 measured DNAm at a control gene, *Prkaa1*, which has not been associated with smoking. As expected,

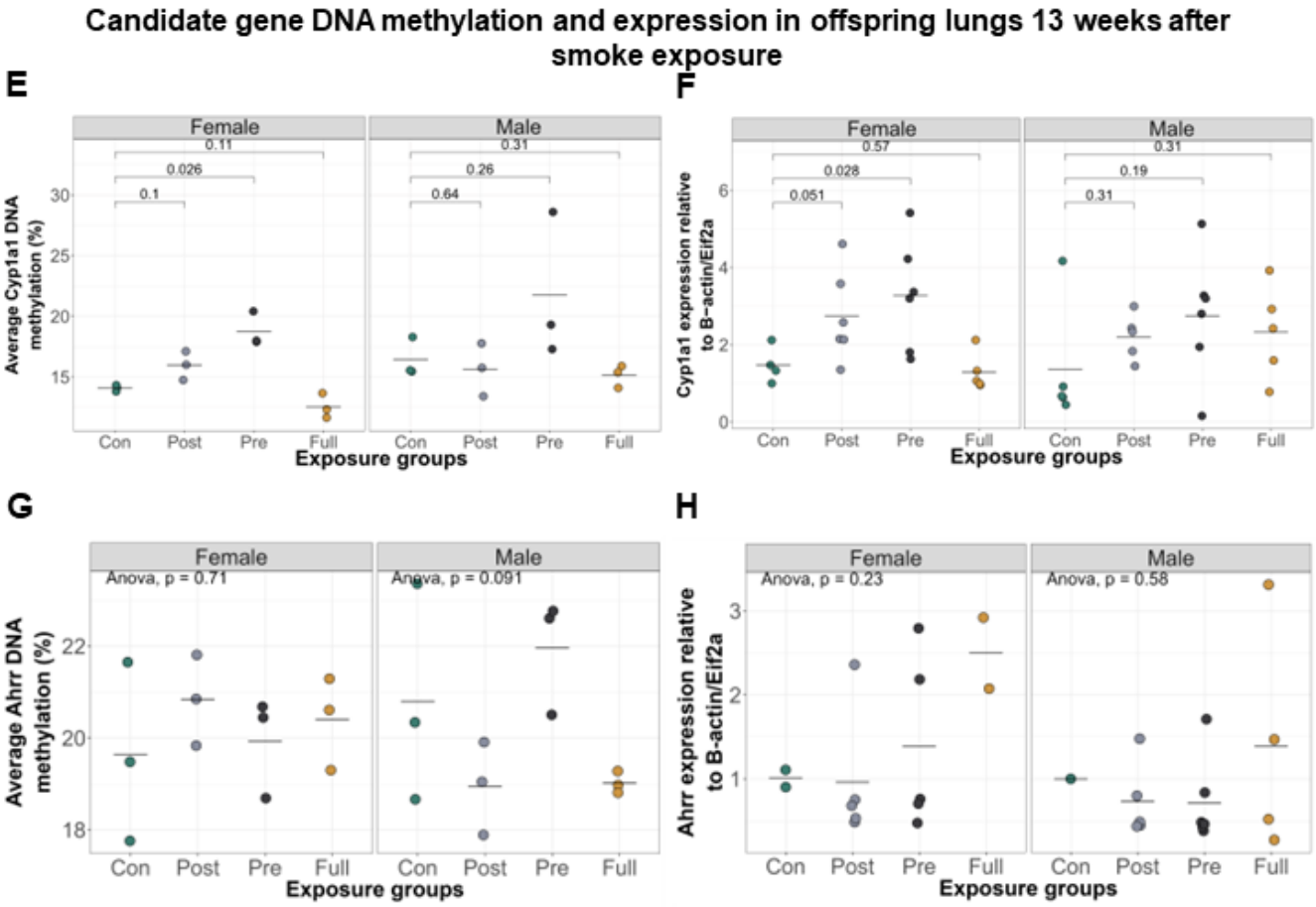
364 *Prkaa1* methylation in lungs of offspring exposed to both prenatal and postnatal CS was not
365 significantly different from *Prkaa1* methylation in control offspring (Supplementary Figure 6).

366 To rule out the potential effects of cross-fostering on DNAm, we cross fostered litters without any
367 exposures and measured *Cyp1a1* DNAm in lungs. We found no differences in *Cyp1a1* DNAm
368 between cross-fostered and non-cross fostered offspring (Supplementary figure 7).

369 This data suggested that early-life exposure to CS creates long lasting lung DNAm and gene
370 expression changes at one of our two candidate genes.

371


Candidate gene DNA methylation and expression in offspring lungs at birth
A
Female
Male
T-test, p = 0.22
T-test, p = 0.37
Average Cyp1a1 DNA methylation (%)
Control
CS
Exposure groups
B
T-test, p = 0.00019
T-test, p = 0.018
Cyp1a1 expression relative to B-actin/Eif2a
C
T-test, p = 0.27
T-test, p = 0.073
Average Ahrr DNA methylation (%)
D
T-test, p = 0.63
T-test, p = 0.61
Ahrr expression relative to B-actin/Eif2a


Candidate gene DNA methylation and expression in offspring lungs 13 weeks after smoke exposure
E
Female
Male
0.11
0.026
0.1
0.31
0.26
0.64
Average Cyp1a1 DNA methylation (%)
Con
Post
Pre
Full
Exposure groups
F
0.57
0.028
0.051
0.31
0.19
0.31
Cyp1a1 expression relative to B-actin/Eif2a
G
Anova, p = 0.71
Anova, p = 0.091
Average Ahrr DNA methylation (%)
H
Anova, p = 0.23
Anova, p = 0.58
Ahrr expression relative to B-actin/Eif2a

372

373 Figure 4: Early life exposure to CS significantly alters lung *Cyp1a1* expression at birth and *Cyp1a1*
374 DNAm 13 weeks after smoke cessation. (A) *Cyp1a1* DNAm in offspring lungs at birth. (B) *Cyp1a1*
375 expression in offspring lungs at birth was significantly increased in CS-exposed males (Mean = 19.00,
376 SD = 10.3) and females (Mean = 27.50, SD = 4.54) offspring, compared to control males (Mean =
377 1.36, SD = 0.93) and control females (Mean = 1.47, SD = 0.43). (C) *Ahrr* DNAm in offspring lungs at
378 birth. (D) *Ahrr* expression in offspring lungs at birth. (E) *Cyp1a1* DNAm in offspring lungs at 16
379 weeks of age. Prenatal and postnatal CS exposure caused an increase in female lung *Cyp1a1* DNAm,
380 while fully exposed offspring showed a decrease. Only prenatally exposed female offspring had
381 significantly elevated *Cyp1a1* DNAm (Mean = 18.80, SD = 1.42) compared to control females (Mean
382 = 14.10, SD = 0.26). (F) *Cyp1a1* expression in offspring lungs at 16 weeks of age. Prenatally exposed
383 female offspring had significantly elevated lung *Cyp1a1* expression levels (Mean = 3.28, SD = 1.44)
384 compared to control females (Mean = 1.48, SD = 0.47). (G) *Ahrr* DNAm in offspring lungs at 16
385 weeks of age. (H) *Ahrr* expression in offspring lungs at 16 weeks of age. One-way ANOVA was used
386 to compare DNAm values between groups ($p<0.05$ was significant), followed by two-group
387 comparisons where significant.

388

389 **Acute re-exposure to CS at 60 weeks alters lung function and re-establishes DNAm patterns**

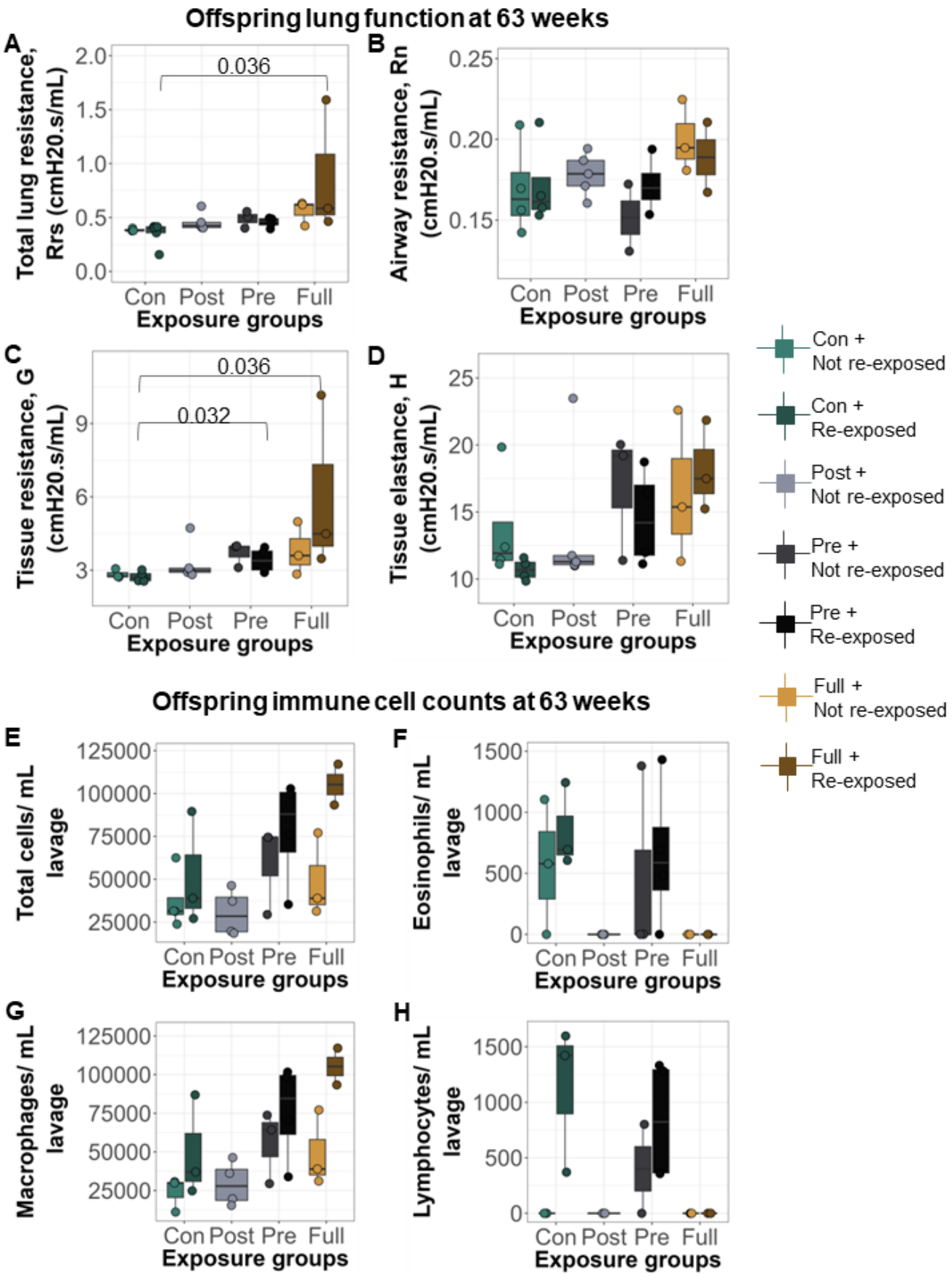
390 There has been some evidence in humans that maternal smoking in pregnancy induces persistent
391 DNAm changes until midlife, regardless of smoking habits later in life[24]. We thus sought to test what
392 effects re-exposure to CS in adulthood would have on the lung phenotypic and DNAm patterns set by
393 early life CS exposure. To achieve this, at 60 weeks of age, we re-exposed half of the offspring
394 remaining to CS for 3 weeks, followed by lung function, differential immune cell analyses and DNAm
395 measurement. At the time of re-exposure, we had a limited number of male offspring remaining, and
396 so the following experiments were conducted solely on female offspring. We also had a low number of

397 animals in the exclusively postnatal CS group, so they contribute to the baseline data only at 60 weeks
398 of age.

399 Offspring that got full CS exposure in early life, followed by re-exposure to CS at 60 weeks, had
400 significantly increased tissue resistance (Figure 5C) and total lung resistance (Figure 5A) levels at
401 baseline, while re-exposed prenatal CS offspring had increased tissue resistance compared to offspring
402 who had no early life CS exposure (controls), but were exposed to CS at 60 weeks (Figure 5). Immune
403 cell infiltration specifically by macrophages was increased only in animals with full early life CS
404 exposure who were also re-exposed later in life, but this increase was not statistically significant (p =
405 0.1223) (Figure 5E - 5H).

406 DNAm and gene expression at *Cyp1a1* showed a similar pattern, they were altered only in offspring
407 with full CS exposure in early life who were also re-exposed to CS (Figure 6A, p = 0.036). This
408 decrease in DNAm was not significant after adjusting for possible effects of litter size on DNAm using
409 linear regression, but it is unlikely that litter size continues to influence DNAm at 60 weeks of age.
410 Interestingly, the *Cyp1a1* DNAm patterns established upon re-exposure to CS in adulthood were
411 similar to those observed at 16 weeks.

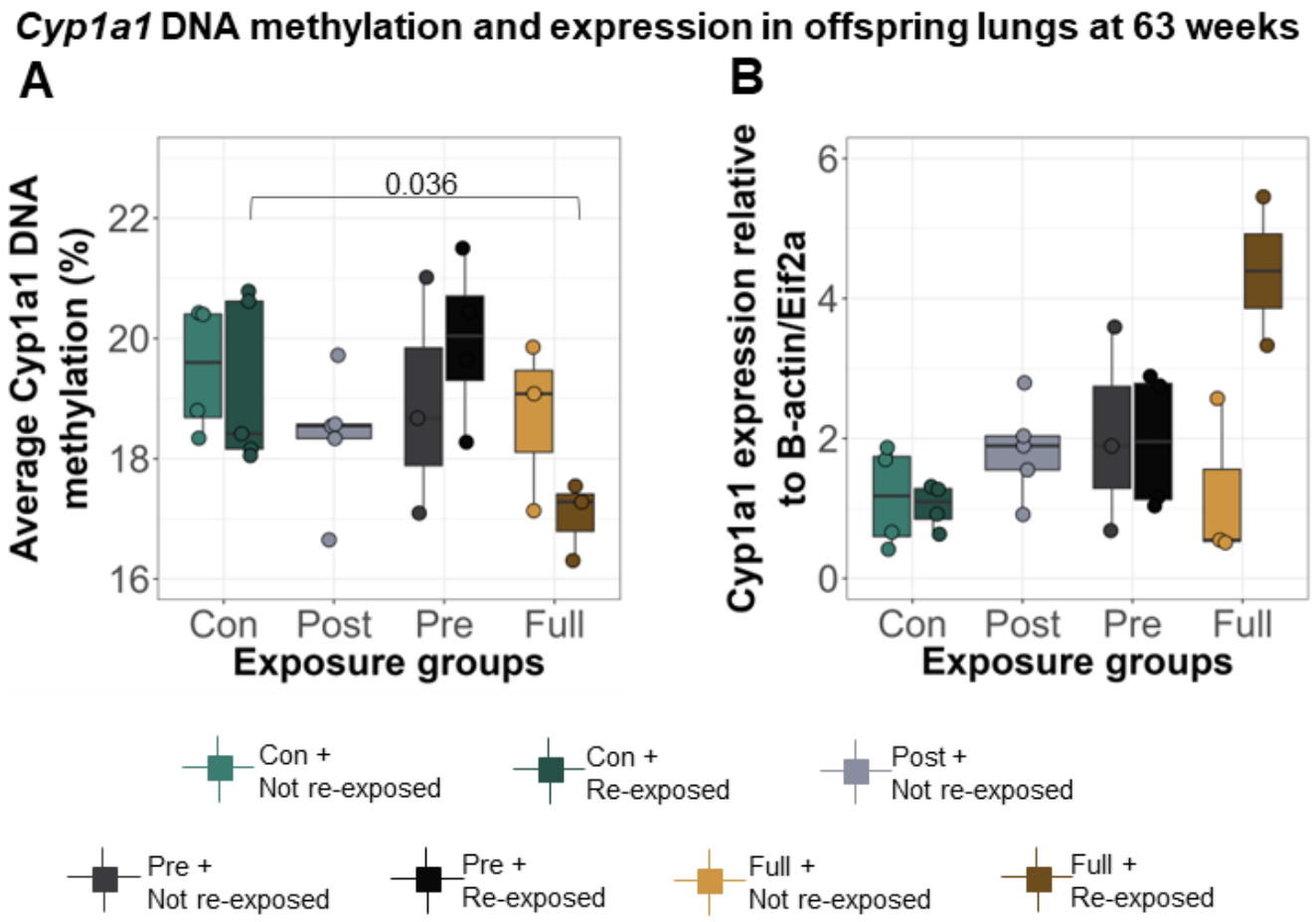
412 Taken together, this data demonstrates that early life exposure to CS induces transient changes in lung
413 phenotype and *Cyp1a1* DNAm, which become re-established upon personal smoking in adulthood.


Offspring lung function at 63 weeks
A
Total lung resistance, Rrs (cmH20.s/mL)
0.036
Con Post Pre Full
Exposure groups
B
Airway resistance, Rn (cmH20.s/mL)
Con Post Pre Full
Exposure groups
C
Tissue resistance, G (cmH20.s/mL)
0.036
0.032
Con Post Pre Full
Exposure groups
D
Tissue elastance, H (cmH20.s/mL)
Con Post Pre Full
Exposure groups
Con + Not re-exposed
Con + Re-exposed
Post + Not re-exposed
Pre + Not re-exposed
Pre + Re-exposed
Full + Not re-exposed
Full + Re-exposed
Offspring immune cell counts at 63 weeks
E
Total cells/ mL lavage
Con Post Pre Full
Exposure groups
F
Eosinophils/ mL lavage
Con Post Pre Full
Exposure groups
G
Macrophages/ mL lavage
Con Post Pre Full
Exposure groups
H
Lymphocytes/ mL lavage
Con Post Pre Full
Exposure groups

414

415 Figure 5: Acute re-exposure to CS at 60 weeks induced significant alterations in baseline lung function
416 compared to early life control offspring which were exposed to CS in adulthood. Lung function
417 parameters at baseline (A) total lung resistance. Offspring with full early life CS exposure and re-
418 exposed to CS at 63 weeks had higher Rrs (Mean = 0.88, SD = 0.62) compared to early life control
419 offspring exposed to CS at 63 weeks only (Mean = 0.35, SD = 0.11). (B) airway resistance. (C) tissue
420 resistance. Offspring with full early life CS exposure and re-exposed to CS at 63 weeks had higher G
421 (Mean = 6.04, SD = 3.60) compared to early life control offspring exposed to CS at 63 weeks only
422 (Mean = 2.74, SD = 0.20). Offspring exposed to prenatal CS early in life and re-exposed to CS at 63
423 weeks had higher G (Mean = 3.40, SD = 0.51) compared to early life control offspring exposed to CS
424 at 63 weeks only. (D) alveolar elastance. (E) Total immune cells per mL lavage. (F) Eosinophils per
425 mL lavage. (G) Macrophages per mL lavage. (H) Lymphocytes per mL lavage. For lung function, 90th
426 percentile values were calculated in response to saline administered into the lungs. Differential cell
427 counts were normalized to lavage volume.

428


Cyp1a1 DNA methylation and expression in offspring lungs at 63 weeks
A
B
Average Cyp1a1 DNA methylation (%)
0.036
22
20
18
16
Con
Post
Pre
Full
Exposure groups
Cyp1a1 expression relative to B-actin/Eif2a
6
4
2
0
Con
Post
Pre
Full
Exposure groups
Con + Not re-exposed
Con + Re-exposed
Post + Not re-exposed
Pre + Not re-exposed
Pre + Re-exposed
Full + Not re-exposed
Full + Re-exposed

429

430 Figure 6: Re-exposure of offspring to CS at 60 weeks induced changes in lung DNAm which were
431 similar to those observed at 16 weeks. (A) *Cyp1a1* DNAm in offspring lungs at 63 weeks. Offspring
432 with full early life CS exposure and re-exposed to CS at 63 weeks had higher lung *Cyp1a1* DNAm
433 (Mean = 17.00, SD = 0.65) compared to early life control offspring exposed to CS at 63 weeks only
434 (Mean = 19.20, SD = 1.37). (B) *Cyp1a1* expression in offspring lungs at 63 weeks. Differences in
435 DNAm and expression were calculated using a one-way ANOVA, followed by two-group
436 comparisons.

437

438 **DISCUSSION**

439 The global burden of smoking still remains significant, despite numerous campaigns against its
440 use, and smoking in pregnancy predisposes offspring to harmful health outcomes[55–57]. CS components
441 have been known to pass through the placenta to the developing fetus, but it is not yet clear what
442 specific mechanisms link CS exposure to offspring health[27,58–60]. Past studies have shown that prenatal
443 exposure to CS induces differential DNAm in newborn umbilical cord blood, placenta, buccal and
444 nasal epithelia, and adult peripheral blood granulocytes[20,23,28,29,59]. However, previous studies in
445 humans have not been able to isolate the influence of postnatal exposure to smoke, as prenatal and
446 postnatal exposures tend to be highly correlated in humans. In addition, while the effects of maternal
447 smoking on peripheral tissues are well documented, less is known about the effects on the lungs,
448 despite significant epidemiological evidence linking maternal smoking to long term lung health,
449 including asthma and COPD[61–65]. Since we know that each tissue has a unique epigenetic signature[66–
450 74], it is essential to measure the effects of early life smoke exposure in the lungs. Therefore, we created
451 a mouse model to study the independent effects of prenatal and postnatal CS exposure that would also
452 allow us to measure lung phenotype and DNAm specifically in the lung. We used cross-fostering to
453 isolate prenatal and postnatal exposures rather than altering exposure to CS in the dams to avoid the

454 effects of nicotine withdrawal on the dams and pups[75–78]. Here, we showed that exposure to CS at
455 different phases early in life alters offspring lung function, DNAm and gene expression profiles, and
456 that these early life exposures prime the lungs such that a secondary smoke exposure in adulthood re-
457 establishes some of these outcomes. Tables S5 and S6 depict summaries of our findings in offspring
458 and dams respectively.

459 Cigarette smoking has been shown to cause airway hyperresponsiveness and altered lung
460 function in offspring[12,63,79–81]. In our model, we noted sex-specific differences in specific
461 manifestations of lung function in offspring even after cessation of smoke exposure for 13 weeks,
462 similar to those that have previously been reported in other human and mouse studies[82–85]. Many of
463 these studies have identified female lungs as being less sensitive to the effects of environmental toxins
464 such as from CS. This is hypothesized to be because important protective factors in lung development,
465 such as surfactant production, begin earlier in females than males[86–88]. In our model however, the sex-
466 specific differences we observed in lung function and immune cell infiltration were more spread out,
467 with female offspring exhibiting differences in certain parameters but not males, and vice versa. The
468 observed differences in our results compared to previous studies may be as a result of differences in
469 the study designs- for instance, the fact that we completely isolated CS exposures by cross fostering, as
470 opposed to these other studies.

471 In our study, lung function defects in female offspring persisted until 16 weeks of age, which is
472 supported by previous studies that have reported persistent effects of CS, both in human and animal
473 studies[63,85,94]. In one such study, maternal smoking led to a decrease in offspring lung function which
474 lasted till 21 years of age[65]. Similarly, another longitudinal study found persistently decreased forced
475 expiratory flows in 8800 children aged 8-12 years of age who were exposed to CS *in utero*[94]. We
476 found that the lung function alterations observed in female offspring did not last till 60 weeks, but
477 reappeared upon re-exposure to CS at 60 weeks of age. While we do not know when these differences

478 disappeared, these results suggest that early-life CS exposure alone may not be enough to induce life-
479 long lung function alterations that last until 60 weeks, but may prime the lungs so that once offspring
480 are re-exposed to CS in adulthood, lung function is altered. This idea of long term priming has been
481 well documented in the past[84,95,96], but few studies have extended exposures as long as those shown
482 here. In a similar light, one study showed that early-life secondhand smoke exposure did not alter lung
483 function until the administration of a postnatal allergen (*A. fumigatus* extract)[96]. Another study found
484 that *in utero* exposure to second-hand smoke exacerbated allergic response and lung function deficits
485 in offspring when exposed to ovalbumin at 23 weeks of age[82].

486 In addition to lung phenotype, it is important to investigate the effects of CS on DNAm, as this
487 could give insights into the underlying mechanisms of disease development. Cytochrome P450 (Cyp)
488 enzymes are hemoproteins which are involved in metabolism of drugs and other xenobiotics primarily
489 in the liver[97]. Its major substrates are polyaromatic hydrocarbons (PAHs) and nitrosamines (major
490 carcinogenic components of CS[98]), and activation by these ligands usually occurs via an aryl
491 hydrocarbon receptor (Ahr)-dependent pathway[99,100]. There are many subclasses of the Cyp enzymes,
492 but CYP1A1 is extensively studied as it is expressed at basal levels in extra-hepatic tissues[101] and is
493 highly inducible, leading to the speculation that it may be primarily responsible for xenobiotic
494 metabolism in extra-hepatic tissues[102]. When activated by Ahr, CYP1A1 directly hydroxylates or
495 oxidizes its bound ligand, leading to its detoxification[103–105]. CS is a potent inducer of the Ahr-Cyp
496 pathway both in humans and murine models[106–108], so it follows that measuring the effects of CS on
497 DNAm in these genes would be informative on CS-induced phenotypes.

498 The dynamics of *Cyp1a1* DNAm and expression over the life course in CS-exposed lungs
499 revealed unexpected patterns. First, we noted that maternal smoking did not alter offspring *Ahrr* or
500 *Cyp1a1* DNAm in the lung at birth at our chosen CpGs but causes a 20-fold increase in expression of
501 just *Cyp1a1*. At 16 weeks, offspring exposed to only prenatal CS or only postnatal CS showed a slight

502 increase in *Cyp1a1* DNAm (and expression), while those exposed to both prenatal and postnatal CS
503 showed decreased *Cyp1a1* DNAm and expression in their left lungs compared to control offspring. We
504 tested the effects of cross-fostering independently and saw no difference in *Cyp1a1* DNAm, which
505 implies these differences are not an effect of cross-fostering itself. These opposing results could
506 possibly be explained by the *developmental mismatch hypothesis*, which surmises that traits that
507 develop in an organism in one environment may be disadvantageous in a different environment[109–111].
508 In other words, the mismatch between prenatal and postnatal environments in the cross-fostered
509 offspring would confer different characteristics from offspring with matched prenatal and postnatal
510 environments. The fact that the *Cyp1a1* DNAm in the prenatal CS only group and postnatal CS only
511 group changes in the same direction emphasizes the need for future animal models to isolate and study
512 them separately, as it is evident that they produce different effects and can be difficult to differentiate
513 in more long-term studies. The increase in *Cyp1a1* DNAm observed in the prenatal CS only group is in
514 fact in line with many human studies which have measured prenatal CS-induced DNAm changes in
515 newborn umbilical cord blood[20–23,25]. We cannot make comparisons to other mouse studies however,
516 as none so far has investigated effects of CS on *Cyp1a1* DNAm.
517 The fact that these differences in offspring *Cyp1a1* DNAm were observed 13 weeks after
518 smoke exposure cessation shows that similar to the lung function results, the effects of early life CS on
519 offspring *Cyp1a1* DNAm are long-lasting. Another mouse study similarly showed that maternal
520 smoking induced changes in *Cyp2a5* DNAm methylation in mouse liver which persisted till 140 days
521 after smoking[112]. The evidence of persistent effects of CS in humans is more abundant, as human
522 studies have shown that maternal smoking causes changes in the offspring epigenome which can last
523 till mid-life[21,23,24]. It is worth noting that our observed differences in *Cyp1a1* DNAm were not
524 persistent till 16 weeks in male offspring, suggesting that the effects of maternal smoking on offspring
525 DNAm could be sex-specific[66,113,114] .

526 Finally, we observed that while early life CS-induced *Cyp1a1* DNAm changes do not persist
527 till 60 weeks, CS-exposed animals develop impaired lung function with age. In addition, acute
528 exposure to CS in adulthood re-established DNAm patterns observed at 16 weeks and was associated
529 with an immune cell infiltration phenotype that was dependent on both early life and later life CS
530 exposure. The disappearance of the *Cyp1a1* DNAm patterns at 60 weeks and re-establishment by acute
531 smoke exposure proves that DNAm cannot be the epigenetic mechanism responsible for this response.
532 The next most likely candidate for a mechanism linking early life exposure with later life response is
533 histone modifications. One study has in fact shown that early life smoke exposure in rats causes
534 chromatin remodeling, marked by increased histone acetylation and altered transcription factor binding
535 in the lungs[115]. The DNAm and lung phenotype changes observed at this timepoint, in particular the
536 immune cell infiltration phenotype, are further evidence of priming. Some studies have suggested that
537 disappearance of detrimental phenotypes over time is an adaptation mechanism in response to
538 adversity[116,117], which allows affected individuals to function as close to optimal as possible, even
539 though they may show some altered phenotypic patterns. However, the underlying detrimental
540 signatures are embedded until a secondary stimulant is introduced[116,117].
541 Taken together, our results represent a novel model for the DOHaD hypothesis, which surmises
542 that early life adversities or environment can shape the offspring phenotype such that it will produce
543 long term consequences on an individual. While some hypotheses such as the thrifty phenotype[118] and
544 adaptive response[119] have been used to provide reasons why there are long-lasting health conditions
545 following early-life adversity, the underlying mechanisms are largely unknown[120]. Epigenetic
546 mechanisms like DNAm are suspected to play major roles in this phenomenon and there are many
547 studies which have investigated the effects of early life environment on offspring DNAm. For
548 example, early life smoke exposure and air pollution exposure in general has been shown to cause
549 conditions like asthma and cancer in adulthood, and many studies have suggested DNAm as the

underlying mechanism[20,31,121,122]. However, we have shown that in our model DNAm was not responsible for long-term CS-associated phenotype, and future work will investigate other epigenetic marks.

One limitation of this study is that we measured DNAm in whole mouse lungs. While this gives us a whole picture, it does not tell what specific lung structural cell type is responsible for the observed changes in DNAm. This is an important question, as the lungs are made up of numerous cell types, and DNAm is cell type-specific[123–125]. It is unlikely that immune cell infiltration is the cause of the observed DNAm changes, as lungs were lavaged to remove alveolar immune cells, and the relative number of immune cells residing in lung tissue is very small. In addition, in a similar pilot study we conducted prior to this experiment, we found that *Cyp1a1* DNAm in the blood of adult female control mice is higher than *Cyp1a1* DNAm in lungs, and as a result, infiltration of immune cells from the blood into lungs is unlikely to be the cause of the decrease in lung *Cyp1a1* DNAm in the present study (Supplementary figure 8). A second limitation is that as our model involves administration of heavy doses of CS and so we cannot extrapolate these results to analyze the effects of light or moderate smoking.

In conclusion, this study has created an effective model for DOHaD to investigate the effects of early life CS exposure, providing an essential foundation for future animal studies. Using this model, we have shown that the effects of early life smoke exposure are embedded into the offspring, producing phenotypes which are re-established by smoking in adulthood. Future studies are needed to investigate these effects across the mouse epigenome and in particular, investigate the effects of early life CS on chromatin accessibility and specific histone marks or variants, to identify the specific mechanism responsible for the long-term persistence of response due to early life CS exposure.

574 **Supplementary Results**

575 **Dam lung phenotype immediately after 9 weeks of smoking and 13 weeks after smoking**
576 **cessation**

577 Several studies have shown that personal smoking alters lung function parameters and induces airway
578 hyperresponsiveness[50–52]. Therefore, we assessed the effects of CS exposure on the lungs by measuring
579 lung function and immune cell infiltration.

580 We first measured the effects of personal smoking on dam lung function and immune cell infiltration,
581 to serve as a baseline for these measurements in their offspring. We found that direct CS exposure did
582 not alter dam lung function immediately after smoking (Supplementary figure 1), but over time
583 induced decreased methacholine responsiveness in dam lungs, as observed by significant alterations in
584 tissue resistance and total lung resistance values 13 weeks after smoking cessation (Supplementary
585 figure 2). Personal smoking for 9 weeks also caused a 4-fold and 8-fold increase in eosinophils and
586 lymphocytes respectively immediately after smoking, compared to controls (Supplementary figure 3).
587 The immune cell infiltration differences in dams however, did not persist 13 weeks after smoking
588 cessation (Supplementary figure 3).

589 Together, this data showed a mild phenotype induced by 9-week smoke exposure in dams who were
590 directly exposed to CS, as observed by increased infiltration of immune cells into dam lungs
591 immediately after smoking and persistent decline in lung function.

592

593 **Candidate gene DNAm and expression in dams 13 weeks after smoking cessation**

594 To determine whether DNAm changes reflect early life CS exposure in the mouse lung, we identified
595 candidate genes to assess via pyrosequencing. We selected two CpGs with the biggest effect sizes from

596 a large meta-analysis of prenatal CS exposure conducted in human cohorts; *AHRR* at human position
597 373378 (mouse *Ahrr* position 74260517) which is not well conserved between humans and mice, and
598 *CYP1A1* at human position 75019251 (mouse *Cyp1a1* position 57696335). In addition, we chose
599 position 40796738 (corresponding to the mouse gene *Prkaa1* position 5174566) as a CpG which is not
600 known to demonstrate DNAm changes in response to CS in human studies as a negative control[20,53,54].

601 We measured *Ahrr* DNAm in dam blood, since the corresponding human locus (cg05575921) is
602 known to be significantly altered in blood of humans exposed to CS[19–21,23]. However, we found no
603 significant differences in *Ahrr* DNAm in dam blood 13 weeks after smoking cessation (Supplementary
604 figure 4A).

605 On the other hand, chronic exposure to CS for 9 weeks led to a significant decrease in *Cyp1a1* DNAm
606 in the left lungs of exposed dams (Mean = 15.00, SD = 1.07), compared to controls (Mean = 17.60, SD
607 = 0.58) which lasted up to 13 weeks after smoking cessation (Supplementary figure 5, $p = 0.033$).
608 Similarly, direct smoke exposure caused a slight but not significant decrease in dam lung *Ahrr* DNAm
609 (Supplementary figure 5). *Ahrr* and *Cyp1a1* expression in the lungs of CS-exposed dams was also
610 slightly but not significantly elevated (Supplementary figure 5).

611 These results thus show that personal smoking for 9 weeks causes alterations in DNAm which persist
612 for 13 weeks after smoking cessation.

613

614

615

616

617

618 Table S1: Mouse pyrosequencing primers

Gene	**Primer sequence**
Ahrr	Forward: AGTTGTTGTGGTTTTTGATAAAAAGAG
	Reverse: /5BiosG/AAACACAATATACAAAAATCCAACTCAT
	Sequencing: AGGGATTTATTGGGG
Cyp1a1	Forward: TTTTGTTTGAGAGTAAAGGTTTGAATAG
	Reverse: /5BiosG/ACACCTACAAAACAACCAACTAA
	Sequencing: GTTTATATTTTTAGTGGTAGGAT
Prkaa1	Forward: AGGAAGTTTGGAGTTGATAAGAA
	Reverse: /5BiosG/CTCCCCTAAAAATAACACAACAAC
	Sequencing: ATTATATAGTAAGTTAATTGAGTTT

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629 Table S2: Mouse qPCR primers

Gene	**Primer sequence**
Ahrr	Forward: GGACAAACAGATAGGAGCCACT
	Reverse: GCTGTTCTGTGCTCCAGTTCA
Cyp1a1	Forward: CAAGCGAAAGTGCATCGGAG
	Reverse: GAGGACCAGAAGACCGCATC
β -actin	Forward: AGAAGAGCTATGAGCTGCCTG
	Reverse: TAGAGGTCTTTACGGATGTCAACG
Eif2a	Forward: TCCCAAAAGCAGTTTGCCTTG
	Reverse: TTGTAGGTTAGGCGTCCCAG

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640

641 Table S3: Male offspring echocardiography parameters

	Control	Prenatal Smoke	Postnatal Smoke	Prenatal and Postnatal Smoke	Prenatal Smoke	Postnatal Smoke	Interaction
Body Weight (g)	26.0 ± 0.13	25.9 ± 0.13	25.7 ± 0.06	25.7 ± 0.12	0.8872	0.4964	0.8655
LV mass (mg)	89.1 ± 0.9	89.2 ± 1.4	85.6 ± 0.8	82.6 ± 1.3	0.6957	0.1840	0.6716
LVPWd (mm)	0.75 ± 0.01	0.76 ± 0.01	0.73 ± 0.01	0.67 ± 0.01	0.4383	0.1099	0.3685
LVAWd (mm)	0.74 ± 0.01	0.75 ± 0.01	0.79 ± 0.01	0.73 ± 0.01	0.4717	0.5564	0.2913
IVSd (mm)	0.66 ± 0.01	0.69 ± 0.01	0.71 ± 0.01	0.68 ± 0.01	0.9911	0.5504	0.2704
LVIDd (mm)	4.10 ± 0.02	4.06 ± 0.02	4.02 ± 0.01	4.00 ± 0.01	0.5881	0.2374	0.9119
LV Vol (ul)	73.5 ± 0.5	69.8 ± 0.8	68.8 ± 0.6	66.6 ± 0.5	0.2022	0.0914	0.7522
EF (%)	51.8 ± 0.6	56.2 ± 0.7	53.5 ± 0.5	54.9 ± 0.4	0.0352	0.3285	0.8389
FS (%)	26.3 ± 0.4	29.2 ± 0.5	28.2 ± 0.3	29.2 ± 0.3	0.1095	0.4307	0.4225
CO (ml/min)	14.9 ± 0.3	17.0 ± 0.2	15.8 ± 0.1	16.3 ± 0.2	0.0347	0.8675	0.2108

SV (ul)	38.5 ± 0.6	40.5 ± 0.5	37.9 ± 0.4	39.5 ± 0.4	0.1241	0.6471	0.4787
IVCT (ms)	8.1 ± 0.3	9.9 ± 0.2	12.1 ± 0.3	11.3 ± 0.3	0.6817	0.0198	0.2509
IVRT (ms)	15.2 ± 0.3	13.7 ± 0.3	15.3 ± 0.2	14.2 ± 0.2	0.1118	0.7505	0.8026
E/A	2.47 ± 0.07	2.05 ± 0.05	1.88 ± 0.03	2.16 ± 0.05	0.6882	0.1546	0.0419
E'/A'	1.15 ± 0.03	1.12 ± 0.03	1.03 ± 0.02	1.00 ± 0.02	0.5589	0.1929	0.8663
E/E'	47.2 ± 0.6	42.8 ± 0.9	41.1 ± 0.5	40.3 ± 0.8	0.2331	0.0520	0.3970
MPI	0.44 ± 0.01	0.43 ± 0.01	0.51 ± 0.01	0.46 ± 0.01	0.3947	0.0944	0.4189
PAT (ms)	15.8 ± 0.2	15.7 ± 0.2	16.8 ± 0.2	15.7 ± 0.1	0.3675	0.4982	0.4769
PET (ms)	62.2 ± 0.5	60.3 ± 0.4	61.2 ± 0.3	59.6 ± 0.5	0.2517	0.5760	0.9120
PAT/PET	0.26 ± 0.01	0.26 ± 0.01	0.27 ± 0.01	0.26 ± 0.01	0.8646	0.2981	0.5046
PV Peak Velocity (mmHg)	608.0 ± 6.1	669.9 ± 7.7	637.3 ± 5.1	639.0 ± 3.0	0.1371	0.9716	0.1595

PV Peak Pressure (mmHg)	1.49 ± 0.03	1.70 ± 0.04	1.65 ± 0.03	1.64 ± 0.02	0.3104	0.6548	0.2682
RVWT (mm)	0.50 ± 0.01	0.52 ± 0.01	0.53 ± 0.00	0.57 ± 0.01	0.0005	< 0.0001	0.2881
RVOT (mm)	1.24 ± 0.01	1.19 ± 0.01	1.15 ± 0.01	1.20 ± 0.01	0.9959	0.2501	0.1421
MPAP (mmHg)	80.2 ± 0.13	80.3 ± 0.12	79.6 ± 0.11	80.3 ± 0.08	0.3678	0.4982	0.4769
HR (bpm)	390 ± 4.1	405 ± 3.8	413 ± 2.6	416 ± 3.6	0.7402	0.9087	0.2510

Abbreviations: HR-heart rate; LV- left ventricle; LVID- LV internal diameter; LVAW- LV anterior wall; LVPW- LV posterior wall; IVS- interventricular septum; EF- ejection fraction; FS- fractional shortening; CO- cardiac output; SV- stroke volume; IVRT- isovolumic relaxation time; E/A; ratio of mitral valve peak velocities measured by pulsed wave doppler during the early (E) and atrial (A) peaks; E'/A'- ratio of mitral valve peak velocities measured by tissue doppler during the early (E') and atrial (A') peaks; E/E'- ratio of early mitral inflow velocity (E) and mitral annular early diastolic velocity (E') measured by tissue doppler; MPI- myocardial performance index; LV Vol- end diastolic volume; bpm- beats per minute; PAT- pulmonary acceleration time; PET- pulmonary ejection time; RVWT- right ventricle wall thickness; RVOT- right ventricular outflow tract; MPAP- Mean pulmonary arterial pressure

654 Table S4: Female offspring echocardiography parameters

	Control	Prenatal Smoke	Postnatal Smoke	Prenatal and Postnatal Smoke	Prenatal Smoke	Postnatal Smoke	Interaction
Body Weight (g)	21.4 ± 0.04	21.3 ± 0.07	21.1 ± 0.06	20.5 ± 0.07	0.2274	0.0658	0.4613
LV mass (mg)	68.1 ± 0.3	67.5 ± 0.5	69.0 ± 0.5	68.0 ± 0.6	0.7459	0.6094	0.9065
LVPWd (mm)	0.61 ± 0.002	0.63 ± 0.003	0.66 ± 0.005	0.69 ± 0.005	0.1866	0.0010	0.9604
LVAWd (mm)	0.65 ± 0.003	0.61 ± 0.003	0.65 ± 0.003	0.67 ± 0.005	0.5532	0.0733	0.1731
IVSd (mm)	0.65 ± 0.003	0.62 ± 0.003	0.67 ± 0.003	0.65 ± 0.004	0.05	0.1592	0.6054
LVIDd (mm)	3.7 ± 0.01	3.6 ± 0.01	3.7 ± 0.01	3.6 ± 0.01	0.0072	0.5422	0.3386
LV Vol (ul)	57.8 ± 0.15	55.9 ± 0.28	58.9 ± 0.34	53.9 ± 0.38	0.0112	0.7292	0.2413
EF (%)	57.1 ± 0.2	57.0 ± 0.4	55.3 ± 0.3	59.2 ± 0.3	0.1831	0.8515	0.1593
FS (%)	29.0 ± 0.1	28.7 ± 0.2	28.4 ± 0.2	30.9 ± 0.2	0.2157	0.3742	0.1142

CO (ml/min)	13.4 ± 0.07	12.5 ± 0.09	13.2 ± 0.09	13.2 ± 0.11	0.3023	0.5938	0.2920
SV (ul)	34.1 ± 0.13	32.9 ± 0.25	33.3 ± 0.24	33.2 ± 0.24	0.5342	0.8299	0.6036
IVCT (ms)	10.6 ± 0.14	10.3 ± 0.16	11.4 ± 0.15	9.4 ± 0.18	0.1162	0.9182	0.2574
IVRT (ms)	13.9 ± 0.09	14.8 ± 0.14	13.4 ± 0.10	14.0 ± 0.12	0.1287	0.1935	0.7663
E/A	2.44 ± 0.02	2.37 ± 0.03	2.38 ± 0.03	2.07 ± 0.03	0.2039	0.2141	0.2858
E'/A'	1.04 ± 0.01	1.08 ± 0.01	1.05 ± 0.01	1.11 ± 0.01	0.3278	0.6450	0.8636
E/E'	41.0 ± 0.31	39.9 ± 0.40	41.2 ± 0.33	39.5 ± 0.40	0.4263	0.9619	0.8685
MPI	0.44 ± 0.003	0.44 ± 0.004	0.45 ± 0.004	0.44 ± 0.004	0.6185	0.9261	0.9677
PAT (ms)	16.9 ± 0.10	15.9 ± 0.08	15.5 ± 0.07	15.0 ± 0.08	0.0888	0.0105	0.4307
PET (ms)	63.0 ± 0.14	64.2 ± 0.17	62.1 ± 0.22	62.0 ± 0.17	0.5380	0.0826	0.4368
PAT/PET	0.27 ± 0.002	0.25 ± 0.001	0.25 ± 0.001	0.24 ± 0.001	0.0903	0.0894	0.3316

PV Peak Vel (mm/s)	580.0 ± 2.3	615.1 ± 3.9	605.6 ± 2.7	635.6 ± 3.4	0.0294	0.1213	0.8618
PV Peak Pressure (mmHg)	1.36 ± 0.01	1.58 ± 0.02	1.53 ± 0.01	1.69 ± 0.02	0.0080	0.0494	0.7303
RVWT (mm)	0.46 ± 0.000	0.49 ± 0.002	0.51 ± 0.003	0.54 ± 0.002	0.0004	< 0.0001	0.2874
RVOT (mm)	1.08 ± 0.004	1.13 ± 0.004	1.10 ± 0.004	1.14 ± 0.005	0.0609	0.4914	0.5624
MPAP (mmHg)	79.5 ± 0.06	79.7 ± 0.07	80.1 ± 0.05	80.5 ± 0.06	0.4103	0.0265	0.7468
HR (bpm)	393 ± 1.1	389 ± 1.5	398 ± 1.6	397 ± 1.3	0.7349	0.3194	0.8034

Abbreviations: HR-heart rate; LV- left ventricle; LVID- LV internal diameter; LVAW- LV anterior wall; LVPW- LV posterior wall; IVS- interventricular septum; EF- ejection fraction; FS- fractional shortening; CO- cardiac output; SV- stroke volume; IVRT- isovolumic relaxation time; E/A; ratio of mitral valve peak velocities measured by pulsed wave doppler during the early (E) and atrial (A) peaks; E'/A'- ratio of mitral valve peak velocities measured by tissue doppler during the early (E') and atrial (A') peaks; E/E'- ratio of early mitral inflow velocity (E) and mitral annular early diastolic velocity (E') measured by tissue doppler; MPI- myocardial performance index; LV Vol- end diastolic volume; bpm- beats per minute; PAT- pulmonary acceleration time; PET- pulmonary ejection time; RVWT- right ventricle wall thickness; RVOT- right ventricular outflow tract; MPAP- Mean pulmonary arterial pressure

Table S5: Summary of results from offspring

		Birth		**16 weeks**						**60 weeks**	
		Male	Female	Male			Female			Female	
		CS	CS	Post	Pre	Full	Post	Pre	Full	Pre	Full
Lung function at baseline	Rrs										↑
	Rn						↑				
	G									↑	↑
	H			↑							
Lung function with methacholine	Rrs			↓		↑					
	Rn			↓				↓	↓		
	G										
	H			↓							
Lung immune cell infiltration	Macrophages										
	Eosinophils						↑				
	Lymphocytes					↑					
	Total cells								*		
Lung DNAm	*Cyp1a1* DNAm						↑	↑* *	↓		↓
Lung gene expression	*Cyp1a1* expression	↑	↑				↑	↑			

At birth and 16 weeks, arrow directions are relative to control groups. At 60 weeks, arrows are relative to early-life control offspring exposed to CS in adulthood only.

* = borderline significance.

671 Table S6: Summary of results from dams

		Immediately after 9 weeks of smoke exposure	**13 weeks after smoking cessation**
		CS-exposed	CS-exposed
Lung function with methacholine	Rrs		⬇
	Rn		
	G		⬇
	H		
Lung immune cell infiltration	Macrophages		
	Eosinophils	⬆	
	Lymphocytes	⬆	
	Total cells		
Lung DNAm	*Cyp1a1* DNAm		⬇
Lung gene expression	*Cyp1a1* expression		

672 Arrow directions are relative to control groups. Lung function data reported here are results from the

673 methacholine responsiveness test, not baseline.

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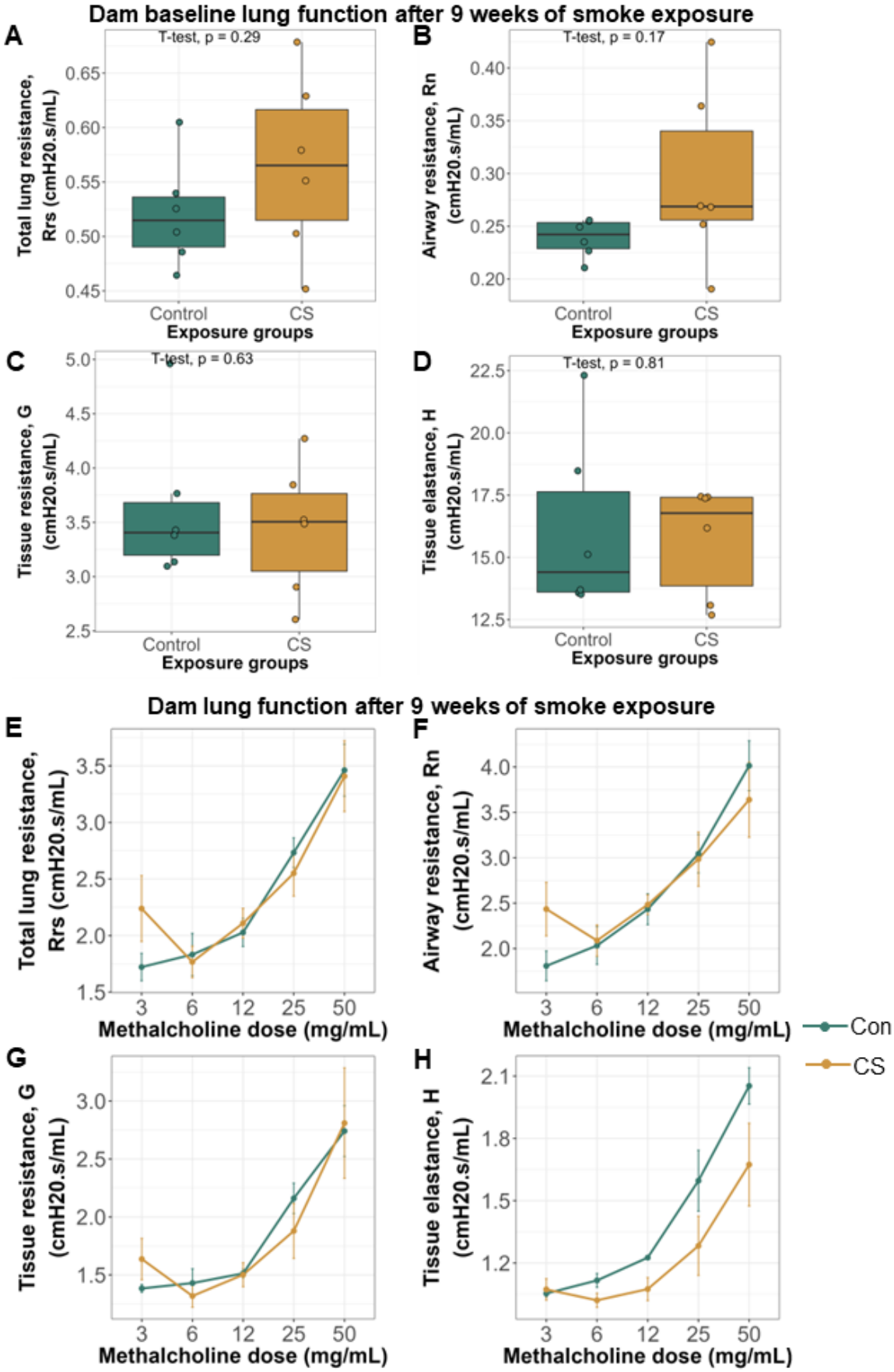
Dam baseline lung function after 9 weeks of smoke exposure
A
T-test, p = 0.29
Total lung resistance, Rrs (cmH20.s/mL)
0.65
0.60
0.55
0.50
0.45
Control
CS
Exposure groups
B
T-test, p = 0.17
Airway resistance, Rn (cmH20.s/mL)
0.40
0.35
0.30
0.25
0.20
Control
CS
Exposure groups
C
T-test, p = 0.63
Tissue resistance, G (cmH20.s/mL)
5.0
4.5
4.0
3.5
3.0
2.5
Control
CS
Exposure groups
D
T-test, p = 0.81
Tissue elastance, H (cmH20.s/mL)
22.5
20.0
17.5
15.0
12.5
Control
CS
Exposure groups
Dam lung function after 9 weeks of smoke exposure
E
Total lung resistance, Rrs (cmH20.s/mL)
3.5
3.0
2.5
2.0
1.5
3
6
12
25
50
Methalcholine dose (mg/mL)
F
Airway resistance, Rn (cmH20.s/mL)
4.0
3.5
3.0
2.5
2.0
3
6
12
25
50
Methalcholine dose (mg/mL)
Con
CS
G
Tissue resistance, G (cmH20.s/mL)
3.0
2.5
2.0
1.5
3
6
12
25
50
Methalcholine dose (mg/mL)
H
Tissue elastance, H (cmH20.s/mL)
2.1
1.8
1.5
1.2
3
6
12
25
50
Methalcholine dose (mg/mL)

678

679 Supplementary figure 1: Personal smoking for 9 weeks does not significantly alter lung function in
680 dams immediately after smoking. Lung function parameters (A) Total lung resistance at baseline. (B)
681 Airway resistance at baseline. (C) Tissue resistance at baseline. (D) Alveolar elastance at baseline. (E)
682 Total lung resistance with methacholine. (F) Airway resistance with methacholine. (G) Tissue
683 resistance with methacholine. (H) Alveolar elastance with methacholine. Lung function values were
684 measured by calculating the 90th percentile in response to inhalation of saline into the lungs.

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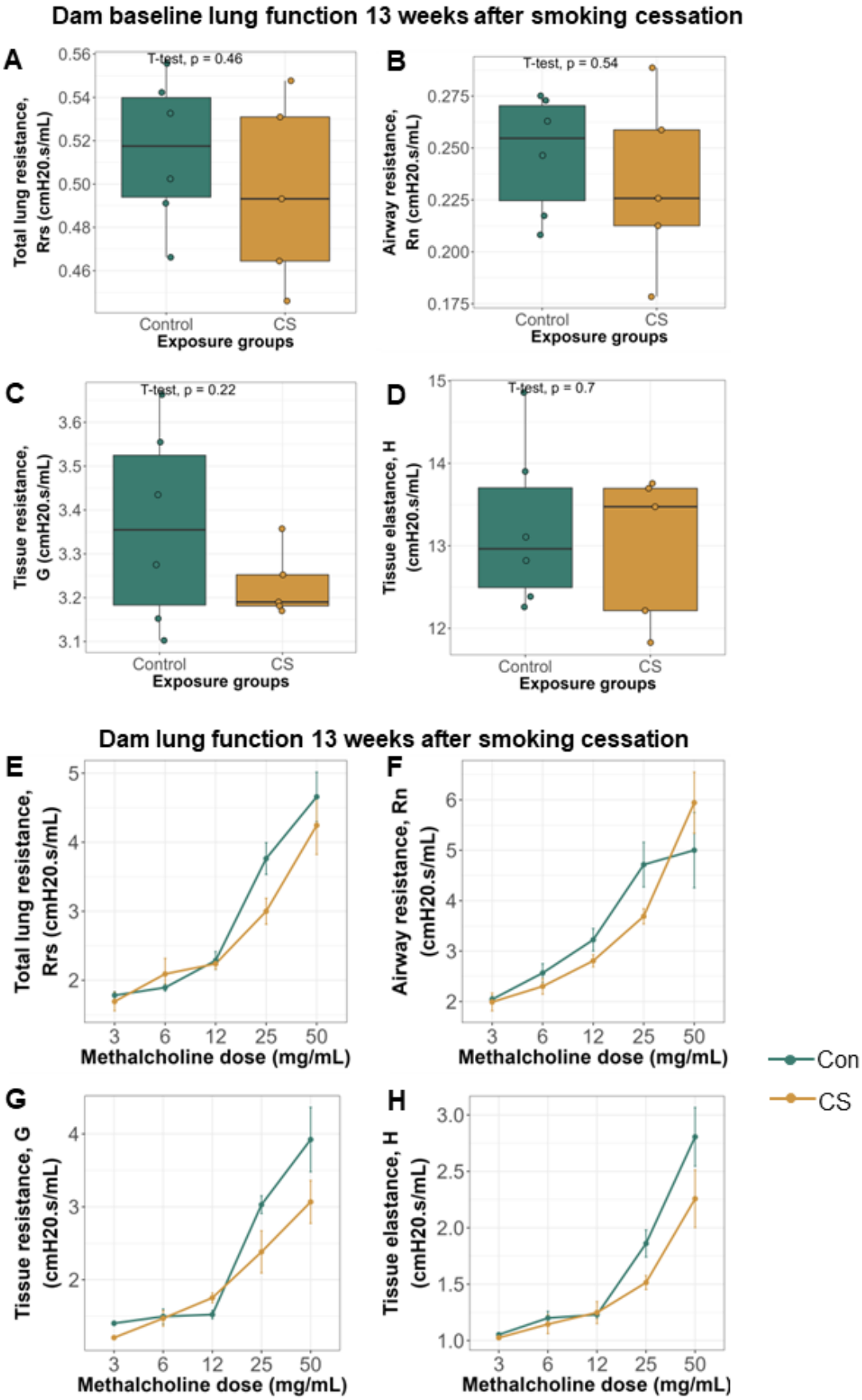
Dam baseline lung function 13 weeks after smoking cessation
A
T-test, p = 0.46
Total lung resistance, Rrs (cmH20.s/mL)
Control
CS
Exposure groups
B
T-test, p = 0.54
Airway resistance, Rn (cmH20.s/mL)
Control
CS
Exposure groups
C
T-test, p = 0.22
Tissue resistance, G (cmH20.s/mL)
Control
CS
Exposure groups
D
T-test, p = 0.7
Tissue elastance, H (cmH20.s/mL)
Control
CS
Exposure groups
Dam lung function 13 weeks after smoking cessation
E
Total lung resistance, Rrs (cmH20.s/mL)
Methalcholine dose (mg/mL)
F
Airway resistance, Rn (cmH20.s/mL)
Methalcholine dose (mg/mL)
Con
CS
G
Tissue resistance, G (cmH20.s/mL)
Methalcholine dose (mg/mL)
H
Tissue elastance, H (cmH20.s/mL)
Methalcholine dose (mg/mL)

687

688 Supplementary figure 2: Personal smoking affects dam lung function up to 13 weeks after smoking
689 cessation. Lung function parameters. (A) Total lung resistance at baseline. (B) Airway resistance at
690 baseline. (C) Tissue resistance at baseline. (D) Alveolar elastance at baseline. (E) Total lung resistance
691 with methacholine. (F) Airway resistance with methacholine. (G) Tissue resistance with methacholine.
692 (H) Alveolar elastance with methacholine. Lung function values were measured by calculating the 90th
693 percentile in response to inhalation of saline into the lungs.

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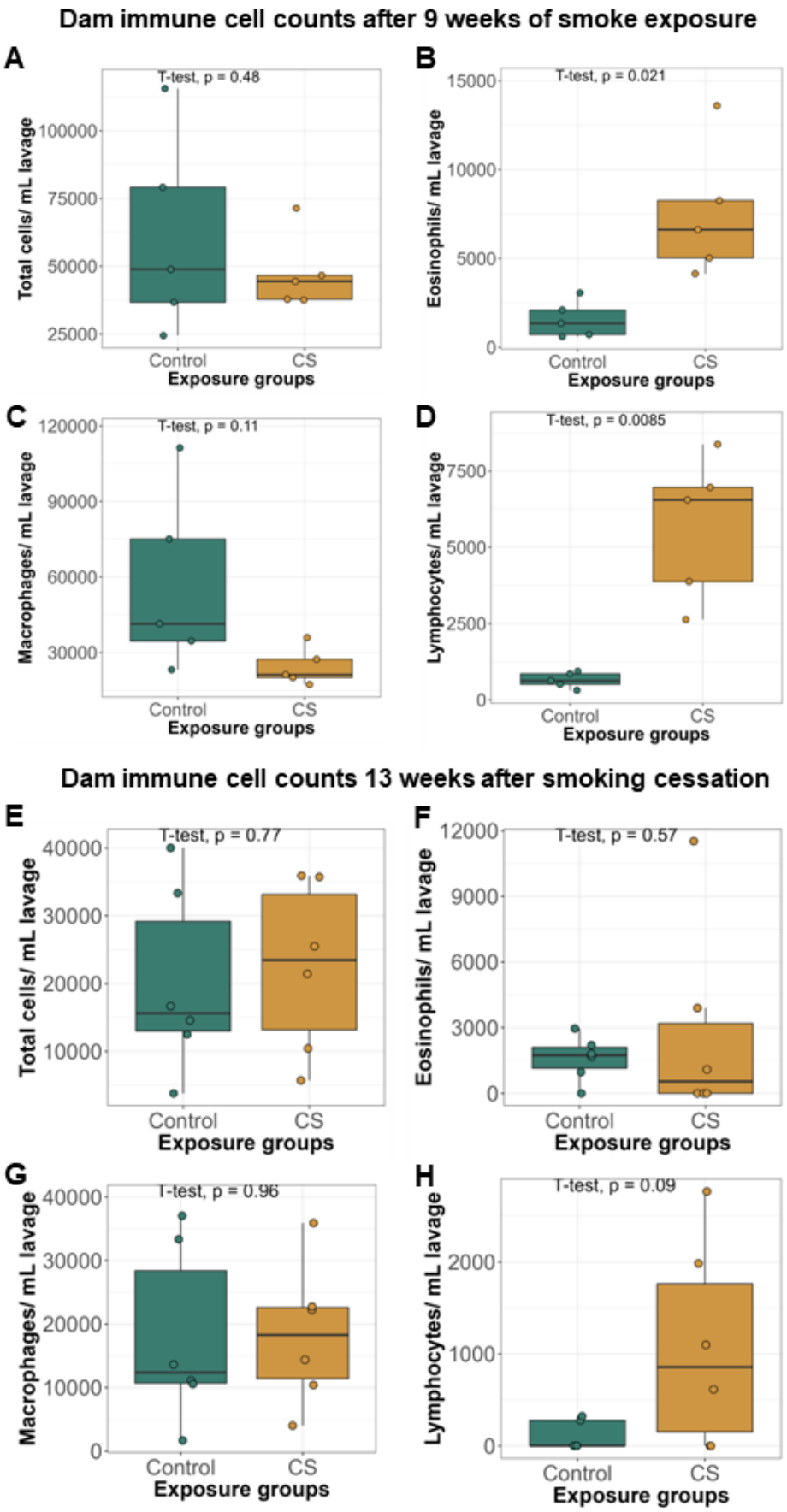
Dam immune cell counts after 9 weeks of smoke exposure
A
T-test, p = 0.48
Total cells/ mL lavage
100000
75000
50000
25000
Control
CS
Exposure groups
B
T-test, p = 0.021
Eosinophils/ mL lavage
15000
10000
5000
0
Control
CS
Exposure groups
C
T-test, p = 0.11
Macrophages/ mL lavage
120000
90000
60000
30000
Control
CS
Exposure groups
D
T-test, p = 0.0085
Lymphocytes/ mL lavage
7500
5000
2500
0
Control
CS
Exposure groups
Dam immune cell counts 13 weeks after smoking cessation
E
T-test, p = 0.77
Total cells/ mL lavage
40000
30000
20000
10000
Control
CS
Exposure groups
F
T-test, p = 0.57
Eosinophils/ mL lavage
12000
9000
6000
3000
0
Control
CS
Exposure groups
G
T-test, p = 0.96
Macrophages/ mL lavage
40000
30000
20000
10000
0
Control
CS
Exposure groups
H
T-test, p = 0.09
Lymphocytes/ mL lavage
2000
1000
0
Control
CS
Exposure groups

698

699 Supplementary figure 3: Personal smoking causes immediate but transient changes to immune cell
700 infiltration into dam lungs. Direct exposure to CS increases immune cell infiltration into the lungs
701 immediately after smoking, but these effects disappear 13 weeks after smoking. (A) Total immune
702 cells per mL lavage immediately after smoking. (B) Eosinophils per mL lavage in the CS-exposed
703 dams (Mean = 7527.94, SD = 3735.92) was 4 times more elevated than control dams (Mean =
704 1568.64, SD = 1028.79) immediately after smoking. (C) Macrophages per mL lavage immediately
705 after smoking. (D) Lymphocytes per mL lavage in the CS-exposed dams (Mean = 5679.51, SD =
706 2357.40) was over 8 times elevated compared to control dams (Mean = 648.84, SD = 255.68)
707 immediately after smoking. (E) Total immune cells per mL lavage 13 weeks after smoking cessation.
708 (F) Eosinophils per mL lavage 13 weeks after smoking cessation. (G) Macrophages per mL lavage 13
709 weeks after smoking cessation. (H) Lymphocytes per mL lavage 13 weeks after smoking cessation.
710 Differential cell counts were normalized to lavage volume. Two-group comparisons were conducted
711 using a student t-test.

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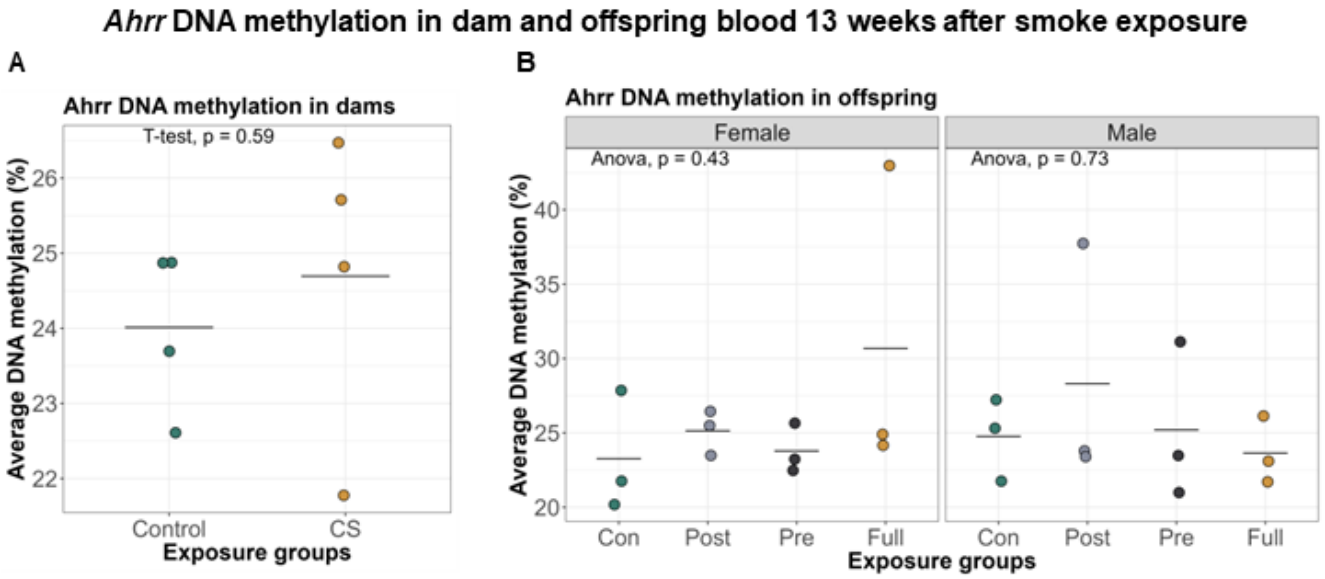
Ahrr DNA methylation in dam and offspring blood 13 weeks after smoke exposure
A
Ahrr DNA methylation in dams
T-test, p = 0.59
Average DNA methylation (%)
26
25
24
23
22
Control
CS
Exposure groups
B
Ahrr DNA methylation in offspring
Female
Male
Anova, p = 0.43
Anova, p = 0.73
Average DNA methylation (%)
40
35
30
25
20
Con
Post
Pre
Full
Con
Post
Pre
Full
Exposure groups

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718 Supplementary figure 4: Smoking did not alter *Ahrr* DNAm in dam or offspring blood 13 weeks after
719 smoke cessation. (A) *Ahrr* DNAm in dam blood 13 weeks after smoking cessation. (B) *Ahrr* DNAm in
720 offspring blood 13 weeks after cessation of smoke exposure.

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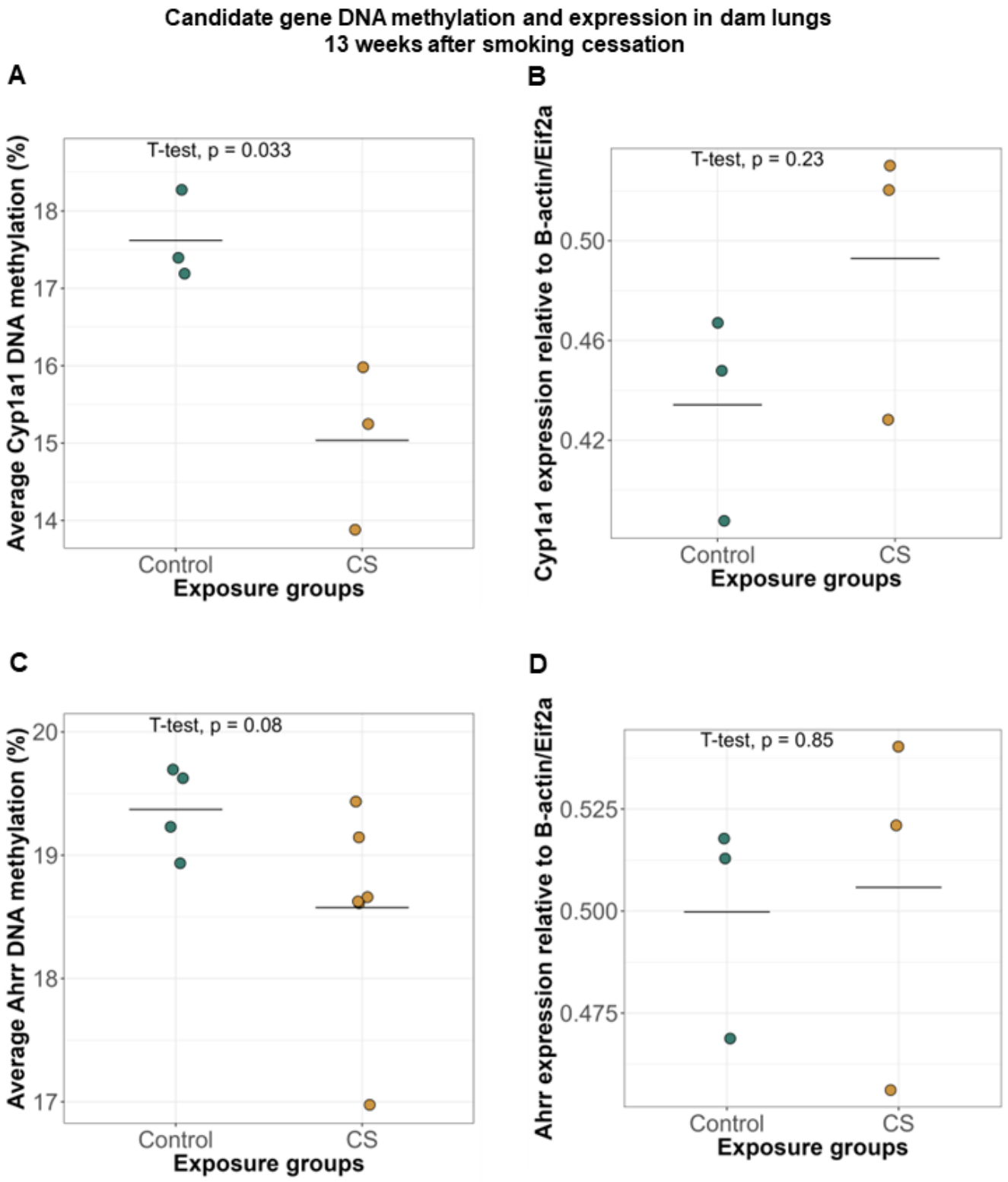
Candidate gene DNA methylation and expression in dam lungs
13 weeks after smoking cessation
A
T-test, p = 0.033
Average Cyp1a1 DNA methylation (%)
18
17
16
15
14
Control
CS
Exposure groups
B
T-test, p = 0.23
Cyp1a1 expression relative to B-actin/Eif2a
0.50
0.46
0.42
Control
CS
Exposure groups
C
T-test, p = 0.08
Average Ahrr DNA methylation (%)
20
19
18
17
Control
CS
Exposure groups
D
T-test, p = 0.85
Ahrr expression relative to B-actin/Eif2a
0.525
0.500
0.475
Control
CS
Exposure groups

738

739 Supplementary figure 5: CS exposure caused reduced *Cyp1a1* methylation in dam lungs 13 weeks after
740 smoking cessation. (A) *Cyp1a1* DNAm in dam lungs 13 weeks after smoking cessation. (B) *Cyp1a1*
741 expression in dam lungs 13 weeks after smoking cessation. (C) *Ahrr* DNAm in dam lungs 13 weeks
742 after smoking cessation. (D) *Ahrr* expression in dam lungs 13 weeks after smoking cessation.

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759 ***Prkaa1 DNA methylation in offspring lungs after 13 weeks of smoking cessation***

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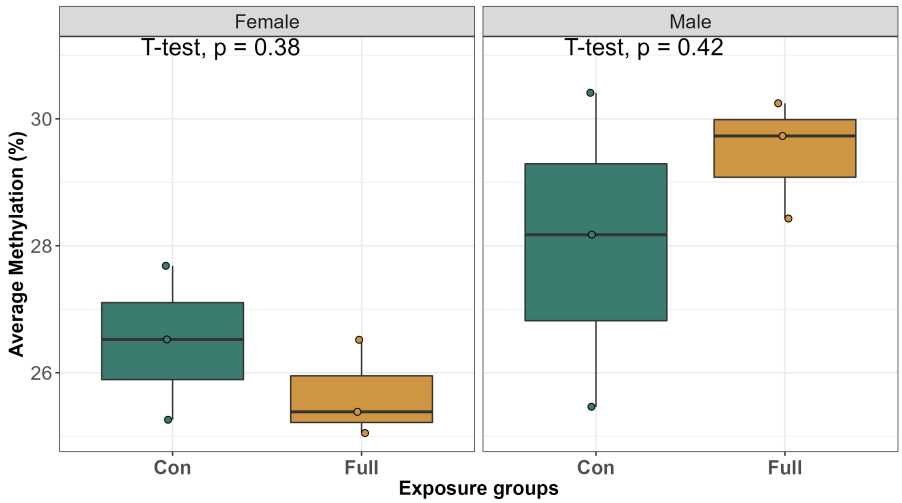

761 762 763 764 765

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767 Supplementary figure 6: Early life exposure to CS did not significantly alter offspring lung DNAm at

768 the control gene, *Prkaa1*.

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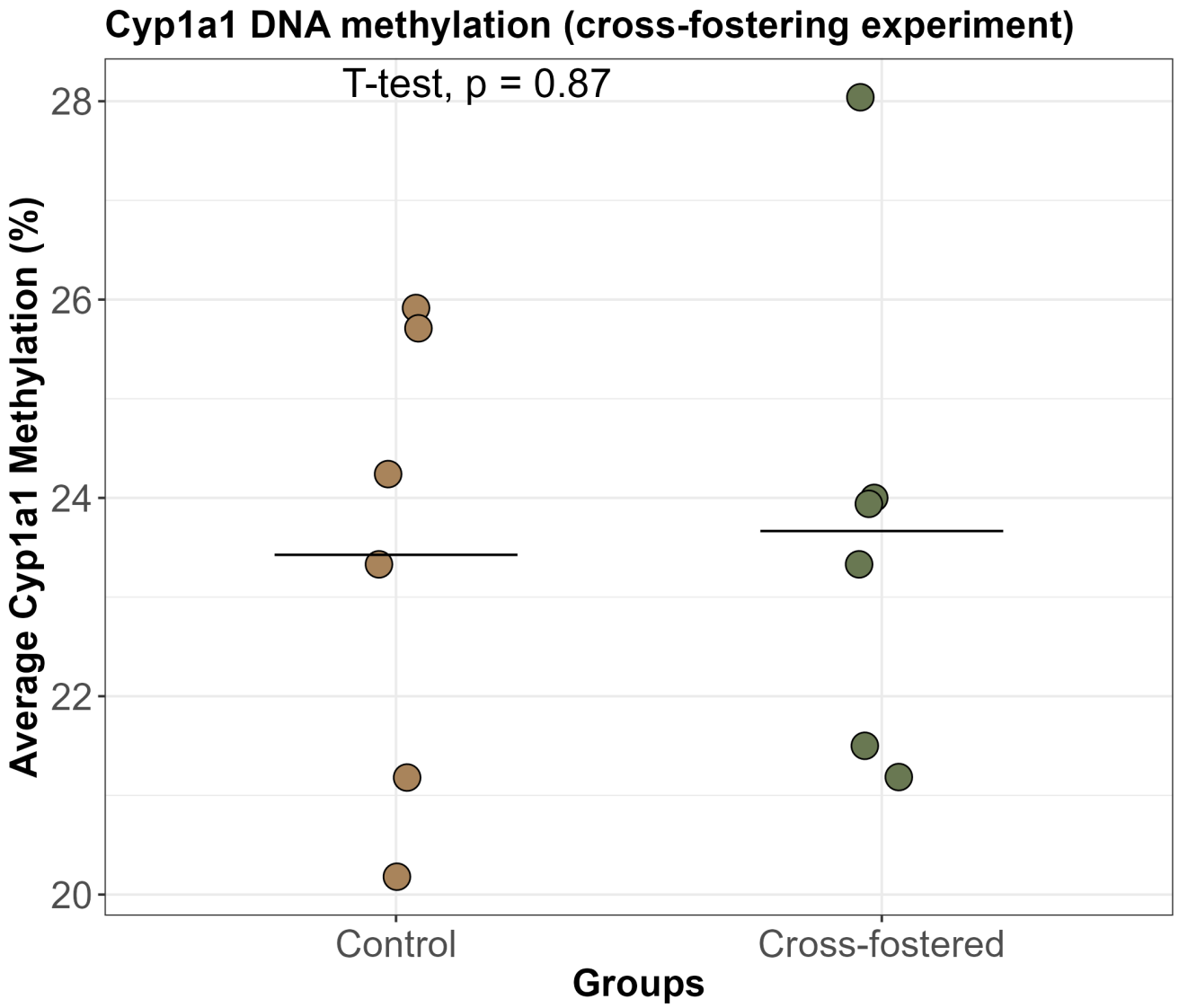
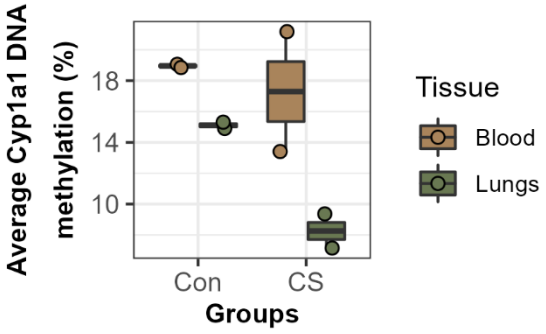


Supplementary figure 7: Cross-fostering did not significantly affect *Cyp1a1* DNAm in mouse lungs. To rule out potential effects of cross-fostering on offspring DNAm, we measured *Cyp1a1* DNAm in lungs of cross-fostered and non-cross fostered control Balb/C mice at 8 weeks of age. Differences in DNAm were measured using a student t-test.



Supplementary figure 8: Pilot study - DNAm in mouse blood is higher than in the lungs. In a previous pilot study like the present experiment, we exposed adult female BAlb/c mice to CS twice daily for 3 weeks, immediately followed by lung function and lavage as described above. We measured DNAm in dam blood and lungs and data was analyzed as described above. Similar to dams in the present study, we also found that exposure of adult mice to CS for 3 weeks caused a decrease in lung *Cyp1a1* DNAm.

REFERENCES

1. Roseboom, T. J. *et al.* Effects of Prenatal Exposure to the Dutch Famine on Adult Disease in Later Life: An Overview. *Twin Research and Human Genetics* **4**, 293–298 (2001).
2. Nielsen, C. H., Larsen, A. & Nielsen, A. L. DNA methylation alterations in response to prenatal exposure of maternal cigarette smoking: A persistent epigenetic impact on health from maternal lifestyle? *Arch Toxicol* **90**, 231–245 (2016).
3. Gluckman, P. D., Hanson, M. A., Cooper, C. & Thornburg, K. L. Effect of in utero and early-life conditions on adult health and disease. *The New England journal of medicine* **359**, 61–73 (2008).
4. Mandy, M. & Nyirenda, M. Developmental Origins of Health and Disease: the relevance to developing nations. *International Health* **10**, 66–70 (2018).
5. Government of Canada, S. C. Smokers, by age group. https://www150.statcan.gc.ca/t1/tbl1/en/tv.action?pid=1310009610 (2018).
6. Harris, J. E. Cigarette Smoke Components and Disease: Cigarette Smoke I s More Than a Triad of Tar, Nicotine, and Carbon Monoxide. 17.
7. Swan, G. E. & Lessov-Schlaggar, C. N. The Effects of Tobacco Smoke and Nicotine on Cognition and the Brain. *Neuropsychol Rev* **17**, 259–273 (2007).
8. Cheraghi, M. & Salvi, S. Environmental tobacco smoke (ETS) and respiratory health in children. *Eur J Pediatr* **168**, 897–905 (2009).
9. Ekblad, M., Korkeila, J. & Lehtonen, L. Smoking during pregnancy affects foetal brain development. *Acta Paediatrica* **104**, 12–18 (2015).
10. Rogers, J. M. Tobacco and pregnancy. *Reproductive Toxicology* **28**, 152–160 (2009).
11. CDCTobaccoFree. 2014 SGR: The Health Consequences of Smoking—50 Years of Progress. *Centers for Disease Control and Prevention* https://www.cdc.gov/tobacco/data_statistics/sgr/50th-anniversary/index.htm (2021).
12. Singh, S. P. *et al.* Prenatal Cigarette Smoke Decreases Lung cAMP and Increases Airway Hyperresponsiveness. *Am J Respir Crit Care Med* **168**, 342–347 (2003).
13. Lawder, R., Whyte, B., Wood, R., Fischbacher, C. & Tappin, D. M. Impact of maternal smoking on early childhood health: a retrospective cohort linked dataset analysis of 697 003 children born in Scotland 1997–2009. *BMJ Open* **9**, (2019).
14. Hertzman, C. & Wiens, M. Child development and long-term outcomes: a population health perspective and summary of successful interventions. *Soc Sci Med* **43**, 1083–1095 (1996).
15. Aristizabal, M. J. *et al.* Biological embedding of experience: A primer on epigenetics. *PNAS* (2019) doi:10.1073/pnas.1820838116.
16. Demetriou, C. A. *et al.* Biological embedding of early-life exposures and disease risk in humans: a role for DNA methylation. *Eur. J. Clin. Invest.* **45**, 303–332 (2015).
17. Cunliffe, V. T. Experience-sensitive epigenetic mechanisms, developmental plasticity, and the biological embedding of chronic disease risk. *WIREs Systems Biology and Medicine* **7**, 53–71 (2015).
18. Kim, J. K., Samaranayake, M. & Pradhan, S. Epigenetic mechanisms in mammals. *Cell. Mol. Life Sci.* **66**, 596 (2008).
19. Joubert, B. R. *et al.* 450K epigenome-wide scan identifies differential DNA methylation in newborns related to maternal smoking during pregnancy. *Environmental health perspectives* **120**, 1425–1431 (2012).
20. Joubert, B. R. *et al.* DNA Methylation in Newborns and Maternal Smoking in Pregnancy: Genome-wide Consortium Meta-analysis. *American journal of human genetics* **98**, 680–696 (2016).

21. Richmond, R. C. *et al.* Prenatal exposure to maternal smoking and offspring DNA methylation across the lifecourse: findings from the Avon Longitudinal Study of Parents and Children (ALSPAC). *Hum Mol Genet* **24**, 2201–2217 (2015).
22. Wiklund, P. *et al.* DNA methylation links prenatal smoking exposure to later life health outcomes in offspring. *Clinical Epigenetics* **11**, 97 (2019).
23. Lee, K. W. K. *et al.* Prenatal Exposure to Maternal Cigarette Smoking and DNA Methylation: Epigenome-Wide Association in a Discovery Sample of Adolescents and Replication in an Independent Cohort at Birth through 17 Years of Age. *Environ Health Perspect* **123**, 193–199 (2015).
24. Tehranifar, P. *et al.* Maternal cigarette smoking during pregnancy and offspring DNA methylation in midlife. *Epigenetics : official journal of the DNA Methylation Society* **13**, 129–134 (2018).
25. Richmond, R. C., Suderman, M., Langdon, R., Relton, C. L. & Davey Smith, G. DNA methylation as a marker for prenatal smoke exposure in adults. *Int J Epidemiol* **47**, 1120–1130 (2018).
26. Rauschert, S. *et al.* Maternal Smoking During Pregnancy Induces Persistent Epigenetic Changes Into Adolescence, Independent of Postnatal Smoke Exposure and Is Associated With Cardiometabolic Risk. *Frontiers in Genetics* **10**, (2019).
27. Suter, M. *et al.* IN UTERO TOBACCO EXPOSURE EPIGENETICALLY MODIFIES PLACENTAL CYP1A1 EXPRESSION. *Metabolism* **59**, 1481–1490 (2010).
28. Reddy, K. D. *et al.* Current Smoking Alters Gene Expression and DNA Methylation in the Nasal Epithelium of Patients with Asthma. *Am J Respir Cell Mol Biol* **65**, 366–377 (2021).
29. Breton, C. V. *et al.* Prenatal tobacco smoke exposure affects global and gene-specific DNA methylation. *Am J Respir Crit Care Med* **180**, 462–467 (2009).
30. Flom, J. D. *et al.* Prenatal smoke exposure and genomic DNA methylation in a multiethnic birth cohort. *Cancer Epidemiol Biomarkers Prev* **20**, 2518–2523 (2011).
31. Neophytou, A. M. *et al.* In utero tobacco smoke exposure, DNA methylation, and asthma in Latino children. *Environmental Epidemiology* **3**, e048 (2019).
32. Sherrill, D. L. *et al.* Longitudinal effects of passive smoking on pulmonary function in New Zealand children. *Am Rev Respir Dis* **145**, 1136–1141 (1992).
33. Knopik, V. S. *et al.* Contributions of parental alcoholism, prenatal substance exposure, and genetic transmission to child ADHD risk: a female twin study. *Psychol. Med.* **35**, 625–635 (2005).
34. Agrawal, A. *et al.* Correlates of cigarette smoking during pregnancy and its genetic and environmental overlap with nicotine dependence. *Nicotine Tob Res* **10**, 567–578 (2008).
35. Vardavas, C. I. *et al.* The independent role of prenatal and postnatal exposure to active and passive smoking on the development of early wheeze in children. *The European respiratory journal* **48**, 115–124 (2016).
36. Joad, J. P., Ji, C. M., Kott, K. S., Bric, J. M. & Pinkerton, K. E. In Utero and Postnatal Effects of Sidestream Cigarette Smoke Exposure on Lung Function, Hyperresponsiveness, and Neuroendocrine Cells in Rats. *Toxicology and Applied Pharmacology* **132**, 63–71 (1995).
37. Burke, H. *et al.* Prenatal and passive smoke exposure and incidence of asthma and wheeze: systematic review and meta-analysis. *Pediatrics* **129**, 735–744 (2012).
38. Eskenazi, B. & Castorina, R. Association of prenatal maternal or postnatal child environmental tobacco smoke exposure and neurodevelopmental and behavioral problems in children. *Environmental Health Perspectives* **107**, 991–1000 (1999).
39. Tomczyk, M. M. *et al.* Mitochondrial Sirtuin-3 (SIRT3) Prevents Doxorubicin-Induced Dilated Cardiomyopathy by Modulating Protein Acetylation and Oxidative Stress. *Circulation: Heart Failure* **15**, e008547 (2022).
40. Ma, Y. *et al.* Sustained suppression of IL-13 by a vaccine attenuates airway inflammation and remodeling in mice. *Am J Respir Cell Mol Biol* **48**, 540–549 (2013).

41. Ryu, M. H. *et al.* Chronic exposure to perfluorinated compounds: Impact on airway hyperresponsiveness and inflammation. *Am J Physiol Lung Cell Mol Physiol* **307**, L765-774 (2014).
42. MUSCLE: multiple sequence alignment with improved accuracy and speed | IEEE Conference Publication | IEEE Xplore. https://ieeexplore.ieee.org/abstract/document/1332560.
43. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402–408 (2001).
44. Wang, R. *et al.* Long non-coding RNA HOX transcript antisense RNA promotes expression of 14-3-3σ in non-small cell lung cancer. *Experimental and Therapeutic Medicine* **14**, 4503–4508 (2017).
45. Leong, J. W. *et al.* The Elimination Half-Life of Urinary Cotinine in Children of Tobacco-Smoking Mothers. *Pulmonary Pharmacology & Therapeutics* **11**, 287–290 (1998).
46. Benowitz, N. L. Cotinine as a Biomarker of Environmental Tobacco Smoke Exposure. *Epidemiologic Reviews* **18**, 188–204 (1996).
47. Camlin, N. J. *et al.* Maternal Smoke Exposure Impairs the Long-Term Fertility of Female Offspring in a Murine Model1. *Biology of Reproduction* **94**, 39, 1–12 (2016).
48. Mays, C. E. Effects of Sidestream Smoke on Pregnant Mice and their Offspring. *Proceedings of the Indiana Academy of Science* **95**, 529–536 (1985).
49. Esposito, E. R., Horn, K. H., Greene, R. M. & Pisano, M. M. An animal model of cigarette smoke-induced in utero growth retardation. *Toxicology* **246**, 193–202 (2008).
50. Melgert, B. N. *et al.* Short-Term Smoke Exposure Attenuates Ovalbumin-Induced Airway Inflammation in Allergic Mice. *Am J Respir Cell Mol Biol* **30**, 880–885 (2004).
51. Blacquière, M. J. *et al.* Maternal smoking during pregnancy induces airway remodelling in mice offspring. *European Respiratory Journal* **33**, 1133–1140 (2009).
52. Lei, Y., Cao, Y.-X., Xu, C.-B. & Zhang, Y. The Raf-1 inhibitor GW5074 and dexamethasone suppress sidestream smoke-induced airway hyperresponsiveness in mice. *Respir Res* **9**, 71 (2008).
53. Dere, E., Forgacs, A. L., Zacharewski, T. R. & Burgoon, L. D. Genome-Wide Computational Analysis of Dioxin Response Element Location and Distribution in the Human, Mouse, and Rat Genomes. *Chem. Res. Toxicol.* **24**, 494–504 (2011).
54. Sun, Y. V., Boverhof, D. R., Burgoon, L. D., Fielden, M. R. & Zacharewski, T. R. Comparative analysis of dioxin response elements in human, mouse and rat genomic sequences. *Nucleic Acids Research* **32**, 4512–4523 (2004).
55. Metzger, M. J., Halperin, A. C., Manhart, L. E. & Hawes, S. E. Association of Maternal Smoking during Pregnancy with Infant Hospitalization and Mortality Due to Infectious Diseases. *Pediatr Infect Dis J* **32**, e1–e7 (2013).
56. Agrawal, A. *et al.* The Effects of Maternal Smoking During Pregnancy on Offspring Outcomes. *Prev Med* **50**, 13 (2010).
57. Janbazacyabar, H. *et al.* The Effects of Maternal Smoking on Pregnancy and Offspring: Possible Role for EGF? *Frontiers in Cell and Developmental Biology* **9**, (2021).
58. Suter, M. A. & Aagaard, K. M. The impact of tobacco chemicals and nicotine on placental development. *Prenat Diagn* **40**, 1193–1200 (2020).
59. Suter, M. *et al.* Maternal tobacco use modestly alters correlated epigenome-wide placental DNA methylation and gene expression. *Epigenetics* **6**, 1284–1294 (2011).
60. Luck, W., Nau, H., Hansen, R. & Steldinger, R. Extent of nicotine and cotinine transfer to the human fetus, placenta and amniotic fluid of smoking mothers. *Dev Pharmacol Ther* **8**, 384–395 (1985).
61. Beyer, D., Mitfessel, H. & Gillissen, A. Maternal smoking promotes chronic obstructive lung disease in the offspring as adults. *Eur J Med Res* **14**, 27–31 (2009).

62. Toppila-Salmi, S. *et al.* Maternal smoking during pregnancy affects adult onset of asthma in offspring: a follow up from birth to age 46 years. *European Respiratory Journal* **55**, (2020).
63. Gilliland, F. D., Li, Y.-F. & Peters, J. M. Effects of Maternal Smoking during Pregnancy and Environmental Tobacco Smoke on Asthma and Wheezing in Children. *Am J Respir Crit Care Med* **163**, 429–436 (2001).
64. Neuman, Å. *et al.* Maternal smoking in pregnancy and asthma in preschool children: a pooled analysis of eight birth cohorts. *Am J Respir Crit Care Med* **186**, 1037–1043 (2012).
65. Hayatbakhsh, M. R. *et al.* Maternal smoking during and after pregnancy and lung function in early adulthood: a prospective study. *Thorax* **64**, 810–814 (2009).
66. Meyer, K. F. *et al.* The fetal programming effect of prenatal smoking on Igf1r and Igf1 methylation is organ- and sex-specific. *Epigenetics* **12**, 1076–1091 (2017).
67. Lin, P.-I., Shu, H. & Mersha, T. B. Comparing DNA methylation profiles across different tissues associated with the diagnosis of pediatric asthma. *Sci Rep* **10**, 151 (2020).
68. Song, F. *et al.* Tissue specific differentially methylated regions (TDMR): Changes in DNA methylation during development. *Genomics* **93**, 130–139 (2009).
69. Novakovic, B. *et al.* Postnatal stability, tissue, and time specific effects of AHRR methylation change in response to maternal smoking in pregnancy. *Epigenetics : official journal of the DNA Methylation Society* **9**, 377–386 (2014).
70. Armstrong, D. A., Lesseur, C., Conradt, E., Lester, B. M. & Marsit, C. J. Global and gene-specific DNA methylation across multiple tissues in early infancy: implications for children's health research. *FASEB J* **28**, 2088–2097 (2014).
71. Zhou, J. *et al.* Tissue-specific DNA methylation is conserved across human, mouse, and rat, and driven by primary sequence conservation. *BMC Genomics* **18**, 724 (2017).
72. Wan, J. *et al.* Characterization of tissue-specific differential DNA methylation suggests distinct modes of positive and negative gene expression regulation. *BMC Genomics* **16**, 49 (2015).
73. Herzog, E. M. *et al.* The tissue-specific aspect of genome-wide DNA methylation in newborn and placental tissues: implications for epigenetic epidemiologic studies. *Journal of Developmental Origins of Health and Disease* **12**, 113–123 (2021).
74. Herzog, E. *et al.* Tissue-specific DNA methylation profiles in newborns. *Clinical epigenetics* **5**, (2013).
75. Chellian, R. *et al.* Rodent models for nicotine withdrawal. *J Psychopharmacol* **35**, 1169–1187 (2021).
76. McLaughlin, I., Dani, J. A. & De Biasi, M. Nicotine Withdrawal. in *The Neuropharmacology of Nicotine Dependence* (eds. Balfour, D. J. K. & Munafò, M. R.) 99–123 (Springer International Publishing, 2015). doi:10.1007/978-3-319-13482-6_4.
77. Paolini, M. & De Biasi, M. Mechanistic insights into nicotine withdrawal. *Biochemical Pharmacology* **82**, 996–1007 (2011).
78. Jacobsen, L. K. *et al.* Effects of smoking and smoking abstinence on cognition in adolescent tobacco smokers. *Biological Psychiatry* **57**, 56–66 (2005).
79. Tager, I. B., Weiss, S. T., Muñoz, A., Rosner, B. & Speizer, F. E. Longitudinal Study of the Effects of Maternal Smoking on Pulmonary Function in Children. *New England Journal of Medicine* **309**, 699–703 (1983).
80. McEvoy, C. T. & Spindel, E. R. Pulmonary Effects of Maternal Smoking on the Fetus and Child: Effects on Lung Development, Respiratory Morbidities, and Life Long Lung Health. *Paediatric Respiratory Reviews* **21**, 27–33 (2017).
81. Goksör, E., Åmark, M., Alm, B., Gustafsson, P. M. & Wennergren, G. The impact of pre- and post-natal smoke exposure on future asthma and bronchial hyper-responsiveness. *Acta Paediatrica* **96**, 1030–1035 (2007).

82. Xiao, R., Noël, A., Perveen, Z. & Penn, A. L. In utero exposure to second-hand smoke activates pro-asthmatic and oncogenic miRNAs in adult asthmatic mice. *Environ Mol Mutagen* **57**, 190–199 (2016).
83. Leon Hsu, H.-H. *et al.* Prenatal Particulate Air Pollution and Asthma Onset in Urban Children. Identifying Sensitive Windows and Sex Differences. *Am J Respir Crit Care Med* **192**, 1052–1059 (2015).
84. Xiao, R. *et al.* In utero exposure to second-hand smoke aggravates the response to ovalbumin in adult mice. *Am J Respir Cell Mol Biol* **49**, 1102–1109 (2013).
85. Noël, A. *et al.* Sex-specific lung functional changes in adult mice exposed only to second-hand smoke in utero. *Respir Res* **18**, 104 (2017).
86. Nielsen, H. C. Androgen receptors influence the production of pulmonary surfactant in the testicular feminization mouse fetus. *J Clin Invest* **76**, 177–181 (1985).
87. Torday, J. S. & Nielsen, H. C. The Sex Difference in Fetal Lung Surfactant Production. *Experimental Lung Research* **12**, 1–19 (1987).
88. Carey, M. A. *et al.* The impact of sex and sex hormones on lung physiology and disease: lessons from animal studies. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **293**, L272–L278 (2007).
89. Thun, M. J. *et al.* Lung Cancer Death Rates in Lifelong Nonsmokers. *JNCI: Journal of the National Cancer Institute* **98**, 691–699 (2006).
90. Carey, M. A. *et al.* It's all about sex: gender, lung development and lung disease. *Trends in Endocrinology & Metabolism* **18**, 308–313 (2007).
91. Mandhane, P. J., Greene, J. M., Cowan, J. O., Taylor, D. R. & Sears, M. R. Sex Differences in Factors Associated with Childhood- and Adolescent-Onset Wheeze. *Am J Respir Crit Care Med* **172**, 45–54 (2005).
92. Almqvist, C., Worm, M., Leynaert, B. & working group of GA2LEN WP 2.5 Gender. Impact of gender on asthma in childhood and adolescence: a GA2LEN review. *Allergy* **63**, 47–57 (2008).
93. Sennhauser, F. H. & Kühni, C. E. Prevalence of respiratory symptoms in Swiss children: Is bronchial asthma really more prevalent in boys? *Pediatric Pulmonology* **19**, 161–166 (1995).
94. Cunningham, J., Dockery, D. W. & Speizer, F. E. Maternal Smoking during Pregnancy as a Predictor of Lung Function in Children. *American Journal of Epidemiology* **139**, 1139–1152 (1994).
95. Drummond, D. *et al.* Combined Effects of in Utero and Adolescent Tobacco Smoke Exposure on Lung Function in C57Bl/6J Mice. *Environ Health Perspect* **125**, 392–399 (2017).
96. Singh, S. P. *et al.* Maternal Exposure to Secondhand Cigarette Smoke Primes the Lung for Induction of Phosphodiesterase-4D5 Isozyme and Exacerbated Th2 Responses: Rolipram Attenuates the Airway Hyperreactivity and Muscarinic Receptor Expression but Not Lung Inflammation and Atopy. *The Journal of Immunology* **183**, 2115–2121 (2009).
97. Maideen, N. M. P. Tobacco smoking and its drug interactions with comedications involving CYP and UGT enzymes and nicotine. *World Journal of Pharmacology* **8**, 14–25 (2019).
98. Anttila, S. *et al.* Methylation of Cytochrome P4501A1 Promoter in the Lung Is Associated with Tobacco Smoking. *Cancer Research* **63**, 8623–8628 (2003).
99. Hankinson, O. The aryl hydrocarbon receptor complex. *Annu Rev Pharmacol Toxicol* **35**, 307–340 (1995).
100. Kim, J. Expression of cytochromes P450 1A1 and 1B1 in human lung from smokers, non-smokers, and ex-smokers. *Toxicology and Applied Pharmacology* **199**, 210–219 (2004).
101. Rendic, S. & Di Carlo, F. J. Human cytochrome P450 enzymes: a status report summarizing their reactions, substrates, inducers, and inhibitors. *Drug Metab Rev* **29**, 413–580 (1997).

1040 102. Okey, A. B. Enzyme induction in the cytochrome P-450 system. *Pharmacology &*
1041 *Therapeutics* **45**, 241–298 (1990).
1042 103. Ohashi, H. *et al.* The aryl hydrocarbon receptor–cytochrome P450 1A1 pathway controls lipid
1043 accumulation and enhances the permissiveness for hepatitis C virus assembly. *J Biol Chem* **293**,
1044 19559–19571 (2018).
1045 104. Rendic, S. & Guengerich, F. P. Contributions of Human Enzymes in Carcinogen Metabolism.
1046 *ACS Publications* https://pubs.acs.org/doi/pdf/10.1021/tx300132k (2012) doi:10.1021/tx300132k.
1047 105. Nebert, D. W. *et al.* Role of the aromatic hydrocarbon receptor and [Ah] gene battery in the
1048 oxidative stress response, cell cycle control, and apoptosis. *Biochemical Pharmacology* **59**, 65–85
1049 (2000).
1050 106. Uno, S., Sakurai, K., Nebert, D. W. & Makishima, M. Protective role of cytochrome P450 1A1
1051 (CYP1A1) against benzo[a]pyrene-induced toxicity in mouse aorta. *Toxicology* **316**, 34–42 (2014).
1052 107. Uno, S. *et al.* Oral Benzo[a]pyrene in Cyp1 Knockout Mouse Lines: CYP1A1 Important in
1053 Detoxication, CYP1B1 Metabolism Required for Immune Damage Independent of Total-Body
1054 Burden and Clearance Rate. *Mol Pharmacol* **69**, 1103–1114 (2006).
1055 108. Neuhold, L. A., Shirayoshi, Y., Ozato, K., Jones, J. E. & Nebert, D. W. Regulation of mouse
1056 CYP1A1 gene expression by dioxin: requirement of two cis-acting elements during induction.
1057 *Molecular and Cellular Biology* **9**, 2378–2386 (1989).
1058 109. Gluckman, P. D., Hanson, M. A. & Low, F. M. Evolutionary and developmental mismatches
1059 are consequences of adaptive developmental plasticity in humans and have implications for later
1060 disease risk. *Philosophical Transactions of the Royal Society B: Biological Sciences* **374**,
1061 20180109 (2019).
1062 110. Frankenhuis, W. E., Nettle, D. & McNamara, J. M. Echoes of Early Life: Recent Insights From
1063 Mathematical Modeling. *Child Development* **89**, 1504–1518 (2018).
1064 111. Godfrey, K. M., Lillycrop, K. A., Burdge, G. C., Gluckman, P. D. & Hanson, M. A. Epigenetic
1065 Mechanisms and the Mismatch Concept of the Developmental Origins of Health and Disease.
1066 *Pediatr Res* **61**, 5–10 (2007).
1067 112. Lkhagvadorj, K. *et al.* Prenatal smoke exposure induces persistent Cyp2a5 methylation and
1068 increases nicotine metabolism in the liver of neonatal and adult male offspring. *Epigenetics* **15**,
1069 1370–1385 (2020).
1070 113. Zeng, Z. *et al.* Prenatal smoke effect on mouse offspring Igf1 promoter methylation from fetal
1071 stage to adulthood is organ and sex specific. *American Journal of Physiology-Lung Cellular and*
1072 *Molecular Physiology* **318**, L549–L561 (2020).
1073 114. Dehmel, S. *et al.* Intrauterine smoke exposure deregulates lung function, pulmonary
1074 transcriptomes, and in particular insulin-like growth factor (IGF)-1 in a sex-specific manner. *Sci*
1075 *Rep* **8**, 7547 (2018).
1076 115. Marwick, J. A. *et al.* Cigarette Smoke Alters Chromatin Remodeling and Induces
1077 Proinflammatory Genes in Rat Lungs. *Am J Respir Cell Mol Biol* **31**, 633–642 (2004).
1078 116. Meldrum, D. R. *et al.* Adaptive and maladaptive mechanisms of cellular priming. *Ann Surg*
1079 **226**, 587–598 (1997).
1080 117. Kentner, A. C., Cryan, J. F. & Brummelte, S. Resilience Priming: Translational models for
1081 understanding resiliency and adaptation to early-life adversity. *Dev Psychobiol* **61**, 350–375
1082 (2019).
1083 118. Hales, C. N. & Barker, D. J. P. The thrifty phenotype hypothesis: Type 2 diabetes. *British*
1084 *Medical Bulletin* **60**, 5–20 (2001).
1085 119. Gluckman, P. D. & Hanson, M. A. Living with the Past: Evolution, Development, and Patterns
1086 of Disease. *Science* **305**, 1733–1736 (2004).

1087 120. Hsu, C.-N. & Tain, Y.-L. Animal Models for DOHaD Research: Focus on Hypertension of
1088 Developmental Origins. *Biomedicines* **9**, 623 (2021).
1089 121. Lavigne, É. *et al.* Maternal exposure to ambient air pollution and risk of early childhood
1090 cancers: A population-based study in Ontario, Canada. *Environ Int* **100**, 139–147 (2017).
1091 122. Erickson, A. C. & Sbihi, H. Biological embedding, the air we breathe, and carcinogenesis. *The*
1092 *Lancet Planetary Health* **2**, e149–e150 (2018).
1093 123. Bloushtain-Qimron, N. *et al.* Cell type-specific DNA methylation patterns in the human breast.
1094 *Proceedings of the National Academy of Sciences* **105**, 14076–14081 (2008).
1095 124. Gervin, K. *et al.* Cell type specific DNA methylation in cord blood: a 450K-reference data set
1096 and cell count-based validation of estimated cell type composition. *Epigenetics : official journal of*
1097 *the DNA Methylation Society* 0 (2016).
1098 125. Novak, P. *et al.* Cell-Type Specific DNA Methylation Patterns Define Human Breast Cellular
1099 Identity. *PLOS ONE* **7**, e52299 (2012).
1100