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DNA methylation analysis using bisulphite-based amplicon sequencing of individuals exposed to maternal tobacco use during pregnancy, and offspring conduct problems in childhood and adolescence†

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ABSTRACT

Maternal tobacco smoking during pregnancy is a large driver of health inequalities and a higher prevalence of conduct problem (CP) has been observed in exposed offspring. Further, maternal tobacco use during pregnancy can also alter offspring DNA methylation. However, currently, limited molecular evidence has been found to support this observation. Thus we aim to examine the association between maternal tobacco use in pregnancy and offspring CP, to determine whether offspring CP is mediated by tobacco exposure-induced DNA methylation differences. Understanding the etiology of the association between maternal tobacco use and offspring CP will be crucial in the early identification and treatment of CP in children and adolescents. Here, a sub group of $N = 96$ individuals was sourced from the Christchurch Health and Development Study, a longitudinal birth cohort studied for over 40 years in New Zealand. Whole blood samples underwent bisulphite-based amplicon sequencing at 10 loci known to play a role in neurodevelopment, or which had associations with CP phenotypes. We identified significant ($P < 0.05$) differential DNA methylation at specific CpG sites in *CYP1A1*, *ASH2L* and *MEF2C* in individuals with CP who were exposed to tobacco *in utero*. We conclude that environmentally-induced DNA methylation differences could play a role in the observed link between maternal tobacco use during pregnancy and childhood/adolescent CP. However, larger sample sizes are needed to produce an adequate amount of power to investigate this interaction further.

Keywords: conduct disorder, developmental biology, DNA, DNA methylation, environmental epigenetics, epigenetics, pregnancy, tobacco exposure.

Introduction

Mothers who smoked tobacco during pregnancy have a higher prevalence of offspring developing a conduct problem (CP) phenotype compared to mothers who did not smoke (Wakschlag *et al.* 1997). This association has been proven in several different cohort studies and the observations have remained following adjustment for various other confounding factors, for example, socio economic status, maternal age, substance abuse, parental anti-social personality, and maladaptive parenting (Wakschlag *et al.* 1997; Joelsson *et al.* 2016). However, there is limited molecular evidence to suggest a link between *in utero* tobacco exposure and offspring conduct disorder, thus a direct link between *in utero* tobacco exposure and CP remains elusive. Previously we conducted a pilot study assessing differential DNA methylation in a small cohort of individuals who were exposed to tobacco *in utero*, with sub-groups of individuals defined as having high

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CP scores (Noble *et al.* 2021). We found nominally significant DNA methylation changes in several genes associated with neurodevelopment (Noble *et al.* 2021). Due to the limitations of using a small sample size combined with an array containing a large number of loci, results were underpowered, therefore observations were unable to reach genome wide significance. However, the biological relevance of these nominally significant CpG loci to the CP phenotype, combined with further research that has suggested an epigenetic link between *in utero* tobacco exposure and ADHD (Sengupta *et al.* 2017), implies that the link between DNA methylation and CP development in tobacco-exposed offspring should be investigated more fully.

Here, we further pursue this hypothesis, by exploring differential methylation in genes that have known roles during *in utero* neurodevelopment and CP phenotypes, to understand whether DNA methylation may help explain the relationship between *in utero* tobacco exposure and development of CP in offspring. We applied a targeted approach *via* bisulphite-based amplicon sequencing (BSAS) of regions of genes involved in neurodevelopment. Amplicon sequencing has the ability to interrogate a region of the genome, therefore specifically targeting consecutive CpG sites in a row. We then assessed differential methylation in the DNA of participants from the Christchurch Health and Development Study (CHDS) whose mothers consumed tobacco during pregnancy, with high and low CP scores, and compared this to controls who were not exposed. This approach allowed us to specifically ask whether DNA methylation at genes involved in neurodevelopment and CP phenotypes are specifically differentially methylated in the DNA of offspring with CP, who were exposed to tobacco *in utero*. A significant interaction here would provide further support of a role for DNA methylation in the link between *in utero* exposure and CP development, something thathas so far proved elusive.

Methods

Ethics declarations

All aspects of the study were approved by the Southern Health and Disability Ethics Committee, under application number CTB/04/11/234/AM10 'Collection of DNA in the Christchurch Health and Development Study'.

Sample

A sub-group of individuals from the CHDS were selected for this study (Table 1). This longitudinal study originally included 97% of all the children (N = 1265) born in the Christchurch, New Zealand, urban region during a period in mid-1977 and has been studied at 24 time points from birth to age 40 (N = 987 at age 30). All participants were aged between 28 and 30 when blood samples and DNA was extracted.

For the subsets studied in this report, CHDS participants were chosen based on their *in utero* tobacco exposure status, their adult smoking status, and their CP scores. Group 1 consisted of individuals who were exposed *in utero* to tobacco smoke, and never smokers at the time blood samples were taken (N = 32). Group 2 consisted of individuals who were exposed *in utero* to tobacco smoke and were themselves regular smokers at the time the blood was taken (N = 32). Group 3 consisted of individuals who were not exposed to tobacco *in utero*, and never smokers at the time blood was taken (N = 32). *In utero* tobacco exposure was defined as 10+ cigarettes per day throughout pregnancy. Within each group of 32, 16 individuals were selected with a 'high' score on a measure of childhood CP at age 7–9 years and 16 with a 'low' score. Severity of childhood CP was assessed using an instrument that combined selected items from the Rutter and Conners child behaviour checklists (Conners 1969, 1970; Rutter *et al.* 1970; Fergusson *et al.* 1991)

Table 1. CHDS subsets selected for analysis.

	Group 1 Exposed *in utero* and never smokers n = 32	Group 2 Exposed *in utero* and a regular smoker n = 32	Group 3 Not *exposed in utero* and never smokers n = 32
Sex			
Male	69%	72%	60%
Female	31%	28%	40%
Tobacco smoking status at the time of blood collection			
Never	100%	0%	100%
Regular	0%	100%	0%
Conduct problem score (CPS)			
Low CPS (<46)	n = 16 (42–46)	n = 16 (42–46)	n = 16 (41–43)
High CPS (>53)	n = 16 (53–75)	n = 16 (60–85)	n = 16 (53–68)

The range of conduct problem scores in each category is indicated in brackets. A score of 53 or more is the top quartile for CP, a score of 60 or more the top decile for CP.

as completed by parents and teachers at annual intervals from 7–9 years. Parental and teacher reports were summed and averaged over the 3 years (Fergusson *et al.* 2005) to derive a robust scale measure of the extent to which the child exhibited conduct disordered/oppositional behaviours [mean (s.d.) = 50.1(7.9); range 41–97]. The behaviours sampled by the measures include many behaviours encompassed by the diagnostic classification, including violence toward peers and authority figures, fire setting, damage to the property of others, an unwillingness to follow rules or commands, and related behaviours. High conduct problem scores reflect the reporting of a larger number of these problems by parents and teachers. For the purposes of this report a 'high' score was defined as falling into the top quartile of the score distribution (scores > 53) and a 'low' score was defined as scores < 46.

Bisulphite-based amplicon sequencing

Bisulphite-based amplicon sequencing (BSAS) was carried out as described (Noble *et al.* 2021). Briefly, DNA was extracted from whole blood samples using the Kingfisher Flex System (Thermo Scientific, Waltham, MA, USA). DNA was quantified *via* nanodrop (Thermo Scientific). 500 ng of DNA underwent bisulphite treatment using the EZ DNA Methylation-Gold kit (Zymo Research, Irvine, CA, USA) as per the manufacturer's instructions. DNA samples were then diluted to a final concentration of 100 ng/μL.

Amplicons for sequencing (Table 2 and Supplementary Table S1) were picked based upon several criteria: (i) previously published differential DNA methylation in response to *in utero* tobacco smoking; (ii) known associations with *in utero* brain development, and; (iii) known associations with CP phenotypes. Primers were then designed to flank the CpG sites of interest, ~350 base pairs (bp) in total, or to amplify the promoter region of the gene if a specific CpG site was not known. Multiple pairs of primers were designed to amplify larger regions.

Bisulphite-converted DNA was amplified *via* PCR, using KAPA Taq HotStart DNA Polymerase (Sigma-Aldrich, St Louis, MO, USA) under the following conditions: 95°C for 10 min, 95°C for 30 s, 59°C for 20 s, 72°C for 7 min, and held at 4°C using the Mastercycler Nexus (Eppendorf, Macquarie Park, Australia). This was then cycled a total of 40 times. PCR products were purified with the Zymo DNA Clean & Concentrator Kit™ (Zymo Research, Irvine, CA, USA).

Following PCR, DNA was cleaned up with Agencourt® AMPure® XP beads (Beckman Coulter, Brea, CA, USA) and washed with 80% ethanol and allowed to air-dry. DNA was then eluted with 52.5 μL of 10 mM Tris pH 8.5 before being placed back into the magnetic stand. Once the supernatant had cleared, 50 μL was aliquoted for the

Table 2. Genes selected to investigate the link between *in utero* tobacco exposure and CP.

Gene	Function	Significance
AHRR (Joubert *et al.* 2012; de Vocht *et al.* 2015; Richmond *et al.* 2015; Rotroff *et al.* 2016; van Otterdijk *et al.* 2017)	Mediates toxicity of dioxin (found in cigarette smoke)	Hypomethylated in tobacco smokers and their offspring
ASH2L (Li *et al.* 2019)	Histone lysine methyltransferase	Associated with schizophrenia
BDNF (Jiao *et al.* 2016; Skogstrand *et al.* 2019)	Nerve growth factor	Promotes neuronal survival. Implicated in neurodegenerative disease
CNTNAP2 (Richmond *et al.* 2015; Joubert *et al.* 2016; Rzehak *et al.* 2016)	Neurexin family – functions in vertebrate nervous system	Implicated in schizophrenia, autism, ADHD, intellectual disability. Hypomethylated in offspring of maternal smoking
CYP1A1 (Suter *et al.* 2010; Joubert, *et al.* 2012; de Vocht *et al.* 2015; Richmond *et al.* 2015; Rotroff *et al.* 2016; van Otterdijk *et al.* 2017)	Monooxygenase – expression is induced by hydrocarbons found in cigarette smoke	Hypomethylated in offspring of maternal smoking
DUSP6 (Demontis *et al.* 2019)	Protein phosphatase, cellular proliferation and differentiation	Regulates neurotransmitter homeostasis
GFI1 (Joubert,*et al* 2012; Rotroff *et al.* 2016; van Otterdijk *et al.* 2017)	Zinc finger protein – transcriptional repressor	Part of a complex that controls histone modifications and gene silencing. Hypermethylated in offspring of maternal smoking
GRIN2B (Riva *et al.* 2015)	Glutamate receptor – expressed early in the brain and is required for normal brain development	Mutations associated with autism, ADHD, schizophrenia
MEF2C (Demontis *et al.* 2019)	MEF2C is associated with hippocampal-dependent learning and memory	MEF2C is crucial for normal neuronal development. Associated with ADHD
PRDM8 (Joubert *et al.* 2016)	Histone methyltransferase – controls expression of genes involved in neural development and neuronal differentiation	Hypomethylated in offspring of maternal smoking

experiment. DNA samples were quantified using the Quant-iT™ PicoGreen™ dsDNA Assay kit (Thermo Fisher) using the FLUROstar® Omega (BMG Labtech, Mornington, Australia). Samples were processed using the Illumina MiSeq™ 500 cycle Kit V2 and sequenced on the Illumina MiSeq™ system by Massey Genome Service (Palmerston North, New Zealand). Illumina MiSeq™ sequences were trimmed using SolexaQA++ software (Cox *et al.* 2010) and aligned to FASTA bisulphite converted reference sequences using the package Bowtie2 (ver. 2.3.4.3) Each individual read was then aligned to all reference sequences using the methylation-specific package Bismark (Krueger and Andrews 2011).

Statistics

Differential DNA methylation was assessed using the package edgeR (Chen *et al.* 2017). Coverage level was set to greater or equal to '8' across unmethylated and methylated counts, as recommended by (Chen *et al.* 2017). Two models were used – the first was a bivariate model, to assess differences between the *in utero* exposed to tobacco compared to the non-exposed control group (model 1).

$$Y \sim U + \mathrm{AS} + e$$

The second was a multiple regression to assess the interaction term *in utero* maternal smoke exposure and offspring conduct problem score (high or low, model 2).

$$Y \sim U + C + \mathrm{AS} + U{:}C + e$$

where Y is defined as the methylation M ratio, U is the exposed/unexposed *in utero* to maternal smoking, C is conduct problem score with high conduct problem score < 53 and low conduct problem core < 46, AS is adult smoker/non-smoker and e is the unexplained variation or error term.

This model was fitted with both ANOVA parameters and with contrasts between *in utero* exposure groups (exposed–non-exposed) within CP score levels. Top tables were constructed using the topTags function in edgeR, Log fold change, average log counts per million, and in some cases F statistic and were calculated and nominal significance was given for $P < 0.05$, these were then corrected using FDR. Scatter plots with the inclusion of confidence intervals were constructed from log transformed normalised methylated and unmethylated counts. Differential methylation was also assessed for adult tobacco smoking status, this was determined by using a linear model with just AS and e (Table S3).

Results

Here we assessed DNA methylation within 10 separate genes (Table 2). DNA sequence data for 15 amplicons from these 10 genes (Table S1) was generated, comprising a total of 280 CpG sites. These CpG sites included a combination of sites previously identified as differentially methylated, as well as amplification of all CpGs within the promoter region of genes associated with *in utero* neurodevelopment and CP phenotypes (Table 2). Differential methylation across these CpG sites was calculated to address whether any were specifically differentially methylated in individuals with CP, in response to *in utero* tobacco exposure.

Quantification of DNA methylation at previously reported CpG sites in response to *in utero* exposure to tobacco

Initially, we attempted to validate in our cohort (age ~28–30 years) five CpG sites that have been previously reported to be differentially methylated in the DNA of cord blood from newborns, and whole blood from children and adolescents (ages newborn to 17) in response to *in utero* tobacco exposure (Table 1). Data were partitioned into those individuals exposed *in utero*, and those who were not (model 1), to assess whether or not BSAS could detect previously reported CpG sites (Table 3).

Aryl-hydrocarbon receptor repressor (*AHRR*) (cg05575921) displayed a 3.1% decrease in DNA methylation between exposed and non-exposed individuals, at a nominal P value of 0.02. This site has previously been identified as hypomethylated in adult tobacco smokers, as well as in postnatal cord blood samples between *in utero* tobacco-exposed and non-exposed individuals. Differential methylation found between adult smokers compared to non-smoking controls in this

Table 3. Previously reported CpG sites showing differential DNA methylation in response to *in utero* tobacco exposure, and their average methylation values in individuals from this cohort.

Gene	Illumina ID	Exposed *in utero* β value	Non-exposed *in utero* β value	β difference	*P* value
AHRR	cg05575921	72.287	75.448	−3.161	0.022
CNTNAP2	cg2594950	3.845	3.860	−0.014	0.991
CYP1A1	cg05549655	26.894	21.699	5.195	0.425
GFI1	cg09935388	75.151	75.330	−0.582	0.055
GFI1	cg09662411	95.837	97.400	−1.583	0.274

study are found in Table S3. The probe cg05549655 in the gene Cytochrome P450, family 1, subfamily A (*CYP1A1*) displayed a 5.19% increase in DNA methylation in the *in utero* exposed group, however, this site did not reach nominal statistical significance in our cohort. Cg09935388 and cg09662411 in the gene, Growth Factor Independent 1 (*GFI1*) were unable to be replicated as differentially methylated between the exposed and the non-exposed groups (no significant change in β values). Both CpG sites show hypomethylation, supporting previous observations of differential methylation within this gene. Contactin-associated protein-like 2 (*CNTNAP2*) (cg2594950) was similarly unable to be validated in our cohort using the method BSAS. Results of model 1 contain all CpG sites analysed in the 10 gene regions using BSAS are found in Table S2.

Differentially methylated CpGs under the interaction of *in utero* tobacco exposure and CP

Differential methylation dependent on both *in utero* exposure and CP score was found at 10 loci in six genes at nominal significance level, none were significant after correcting for false discovery rate (Table 4).

Of these CpG sites, five of the 10 CpG sites were found in the following genes: *CYP1A1*, *GFI1*, ASH2 like histone lysine methyltransferase complex subunit (*ASH2L*) *and* Glutamate Inotropic Receptor NMDA Type Subunit 2B (*GRIN2b*). Differential methylation was observed between *in utero* exposed and non-exposed associated with high conduct scores. No nominal significance from the interaction was observed in association with low conduct scores. The top three CpG sites with nominal significance under the interaction are displayed in Fig. 1. Here, differential methylation is found in response to high CP score and no differences are seen between the exposed and non-exposed low CP groups (Fig. 1).

Discussion

In utero tobacco exposure is known to alter DNA methylation at the genome-wide level in offspring (Joubert *et al.* 2012; Richmond *et al.* 2015; Joubert *et al.* 2016). The later-life implications of these tobacco-induced DNA methylation changes are unclear, however, an association between *in utero* tobacco exposure and CP has previously been observed (Sengupta *et al.* 2017). Given the complex etiology of CP phenotypes (Acosta *et al.* 2004; Beaver *et al.* 2007; Salvatore and Dick 2018) and the vast array of socioeconomic variables associated with tobacco use (Lantz *et al.* 1998), proving a causal link between maternal smoking and offspring CP is inherently challenging. Previously we quantified tobacco-induced DNA methylation changes that associate with CP phenotypes in offspring exposed to tobacco *in utero* (*via* maternal smoking) using the Illumina EPIC array, with results indicating that methylation was altered at the gene Fast Kinase Domain 1 (*FASTKD1*), which may have roles in neurodevelopment and CP phenotypes. However, due to a combination of a comparatively small sample size relative to the number of loci on the array, only nominal significance was observed. Thus, since the array data suggested a role for DNA methylation in the link between *in utero* tobacco exposure and CP, here we identified a panel of genes with known roles in neurodevelopment and CP phenotypes, and sought to determine whether DNA methylation is specifically altered at phenotypically relevant loci. Our previous research indicated that BSAS is an accurate tool through which to target amplicon-specific differential methylation, so here

Table 4. CpG sites where differential methylation between conduct problem scores differs with *in utero* exposure at $P < 0.05$.

Gene	CpG location	Interaction(1)		Low CPS(2)		High CPS(3)	
		Log FC	*P* value	Log FC	*P* value	Log FC	*P* value
CYP1A1	Chr15, 75019290	−2.013	0.010	0.344	0.493	−1.669	0.005
GFI1	Chr1, 92947705	−0.957	0.011	0.002	0.992	−0.955	0.001
ASH2L	Chr8, 37962878	1.257	0.024	−0.447	0.253	0.811	0.042
MEF2C	Chr5, 88179596	−1.679	0.040	0.678	0.174	−1.000	0.122
DUSP6	Chr12, 89746588	−1.444	0.041	0.864	0.107	−0.580	0.204
ASH2L	Chr8, 37962657	−0.199	0.042	0.052	0.455	−0.147	0.033
CYP1A1	Chr15, 75019127	−1.221	0.045	0.403	0.319	−0.819	0.072
ASH2L	Chr8, 37962901	1.250	0.046	−0.561	0.205	0.688	0.121
GRIN2b	Chr12, 14133359	2.711	0.048	0.121	0.903	2.832	0.004
MEF2C	Chr5, 88179541	−1.336	0.050	0.615	0.139	−0.720	0.190

Log fold change (FC) and *P* values (unadjusted) from log ratio tests for the effect on normalised methylation ratios of: (1) interaction between *in utero* exposure and conduct problem score, (2) *in utero* exposed–non-exposed contrast within low CPS and (3) within high CPS participants. Loci with nominally significant ($P < 0.05$) interaction shown, all FDR *P* values > 0.05.

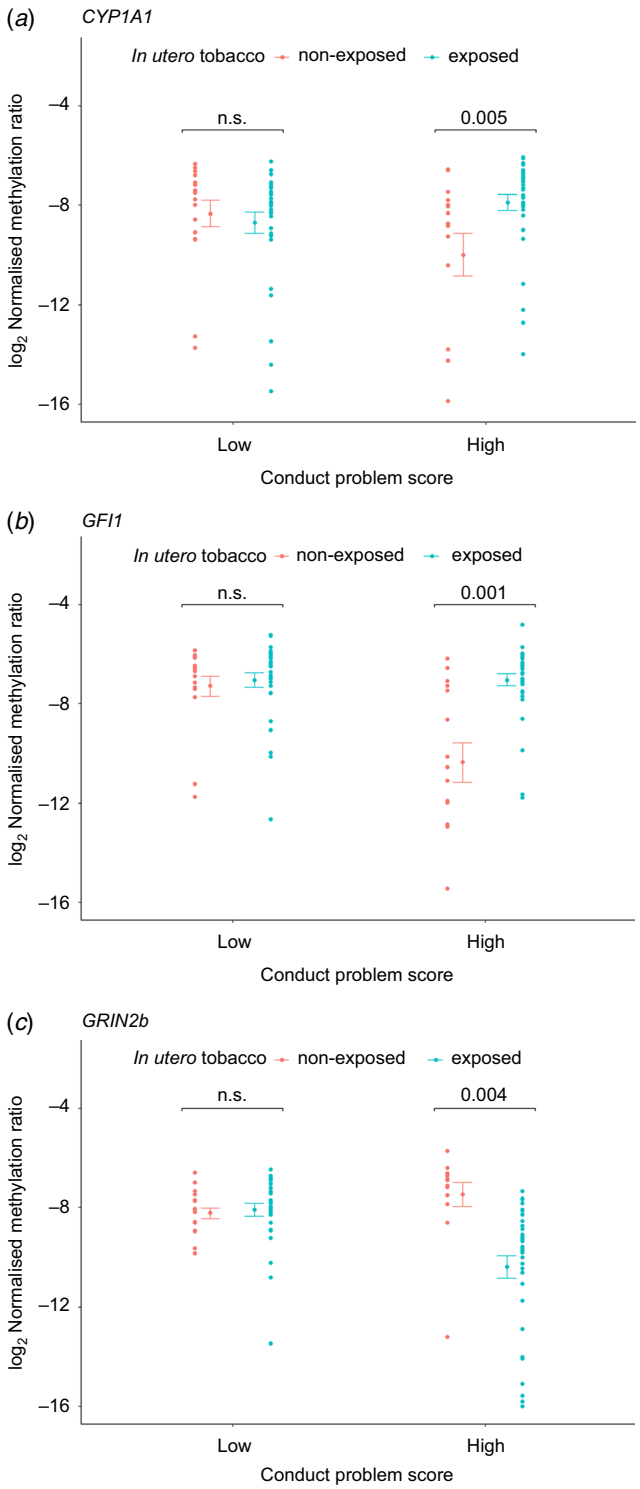

Fig. 1. Differential methylation with *in utero* tobacco exposure for individuals with high conduct problem score that is not observed in individuals with low conduct problem score. (*a*) *CYP1A1* (Chr15, 75019290), (*b*) *GFI1* (Chr1, 92947705) and (*c*), *GRIN2b* (Chr12, 14133359).

we used targeted BSAS to quantify differential methylation that is specific to the interaction between high CP score and *in utero* tobacco exposure.

Validation of previously identified differentially methylated CpG's from *in utero* tobacco exposure

First, we asked whether differentially methylated CpGs that have been previously associated with *in utero* tobacco exposure were supported by this cohort. Here, we present validation of differential methylation of a CpG site within the gene *AHRR* (cg05575921). *AHRR* is a well-defined tobacco smoking gene, which is consistently represented in tobacco methylation data. *AHRR* has previously been found to be differentially methylated in response to *in utero* tobacco exposure (Joubert *et al.* 2012; de Vocht *et al.* 2015; Richmond *et al.* 2015). In both of our analyses we included adult smoking as a covariate in our models. It is, however, very difficult to interpret the exact cause of differential methylation as we do also find several sites in *AHRR* to be differentially methylated when we assess adult tobacco smoking compared to controls (Table S3).

Four other CpG sites investigated here due to previous association with *in utero* tobacco exposure were not differentially methylated in our data. However, the direction of methylation change was supported at all five sites investigated (Rotroff *et al.* 2016; Tehranifar *et al.* 2018; Rauschert *et al.* 2019). We suggest that further investigation in a larger cohort may lead to nominal significance at the sites in *CYP1A1*, *CNTNAP2* and *GFI1*.

Identification of *in utero* exposure-related differentially methylated CpG sites that are specific to individuals with high CP scores

Epidemiological data suggest that there is an increased association between *in utero* tobacco exposure and behavioural disorder in children and adolescents (Mick *et al.* 2002; Carter *et al.* 2008). Thus, here, we investigated DNA methylation changes induced by *in utero* tobacco exposure as a potential molecular mechanism of dysfunction that could link the phenotypic trait of CP to maternal tobacco use during pregnancy. We therefore analysed DNA methylation patterns within our gene panel in response to *in utero* tobacco exposure and its interaction with CP status. A total of 10 CpG sites in seven genes were found to display nominal significance in DNA methylation in response to *in utero* tobacco exposure and CP in this cohort (Table 4).

In the 10 CpG sites we identified under the interaction, *CYP1A1* showed greater magnitude differential methylation in high CP scores (exposed *in utero* vs non-exposed with high CPS), with reduced, reversed or no evidence of differential methylation at the same sites with low CP score. This indicates that within the observed nominal methylation changes the interaction was being driven in the high CP score group. One gene *ASH2L*, contained three nominally significantly differentially methylated CpG sites,

and *CYP1A1* and Myocyte enhancer factor 2C (*MEF2C*) both had two.

CYP1A1 is a well-established marker for *in utero* tobacco smoke exposure (Lee *et al.* 2015; Richmond *et al.* 2015, 2018; Tehranifar *et al.* 2018). Neither of the two sites we observed in this study have probes at these locations on the Illumina array system, thus emphasising a benefit of amplicon sequencing compared to an array-based method. Variant differences in *CYP1A1* have previously been associated with child behavioural problems at age 2, from prenatal maternal environmental tobacco smoke (Hsieh *et al.* 2010). This highlights the need for this gene to be further investigated for its role in the development of conduct problems following *in utero* tobacco exposure.

Three CpG sites from the gene *ASH2L* showed in consistent levels of differential methylation in response to *in utero* tobacco exposure and CP, with two displaying hyper- and one hypomethylation. *ASH2L* has been found to interact with *MEF2C* to mediate changes in histone 3 lysine 4 trimethylation (H3K4me3) (Jung *et al.* 2016). Here, we detected nominal significance at two CpG sites within *MEF2C* (chr5, 88179596 and 88179541). Both of these sites were associated with a greater level of hypomethylation in participants who were exposed to tobacco *in utero* with high CP scores in this cohort, although not at the FDR significance level. *MEF2C* plays a role in neural crest formation during development, where tissue-specific inactivation of the gene results in embryonic lethality (Verzi *et al.* 2007). Further, *MEF2* interacts with oxytocin, which is affiliated with prosocial behaviours (Kosfeld *et al.* 2005; Zak *et al.* 2007). Alterations to oxytocin have been shown to change the morphology of neurons *via MEF2A* (Meyer *et al.* 2018, 2020). Functional roles of the gene in relation to early neuronal development still remain unclear, however it is thought to play a crucial role (Harrington *et al.* 2016). Recent research in animal models suggests that nicotine-dependent induction of the *ASH2L* and *MEF2C* complex during development induces alterations that could lead to fundamental changes in the brain.

There are several limitations in this study thatshould be addressed. Firstly, the lack of significance after adjustment for multiple testing impacted our ability to draw firm conclusions around the association between *in utero* tobacco exposure and CP. However, these data indicated that this association is worth pursuing in a larger cohort. An additional subgroup of individuals who were not exposed to tobacco *in utero*, but who are smokers in adulthood, would add strength to the study, but were not available to us at the time the study was undertaken. Assessing a brain-related phenotype using whole blood to sample can be a problem due to cellular heterogeneity, however as with most retrospective human studies of DNA methylation, this was unable to be acquired in this instance.

Our study design is limited by the ~21-year age discrepancy between when CP diagnosis (age 7–9) occurred and when whole blood sampling was undertaken (age 28–30), furthermore, as in most retrospective studies on exposure-induced DNA methylation changes in humans, a more biologically relevant tissue was not available or feasible. Both of these factors may have impacted our findings, and ideally this study would have been better suited to be undertaken at CP diagnosis (age 7–9). However, investigating the association between exposure, methylation and CP in adults is still intriguing as this indicates the stability of some developmentally induced methylation changes (e.g. *AHRR*), which prior to this study had been demonstrated

Thus, while we cannot assert causality in this study, our targeted approach shows that *in utero* tobacco exposure may be altering methylation at CpG sites associated with neural phenotypes thatpersist into adulthood, and are associated with increased risk of CP.

Conclusion

Here we have presented preliminary data to suggest that the association between maternal tobacco use during pregnancy and the development of CP in children and adolescents may in part be mediated by altered DNA methylation, induced by *in utero* tobacco exposure during development, at genes that have roles in *in utero* brain development and CP phenotypes. We acknowledge the limitations of this study described above, however, the data presented here are suggestive of a role for DNA methylation in the link between *in utero* tobacco exposure and offspring CP. Our findings should stimulate further study using larger sample sizes.

Supplementary material

Supplementary material is available online.

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