Drake et al. BMC Medicine (2015) 13:18
DOI 10.1186/s12916-014-0251-x

BMC Medicine

RESEARCH ARTICLE Open Access

In utero exposure to cigarette chemicals induces sex-specific disruption of one-carbon metabolism and DNA methylation in the human fetal liver

Amanda J Drake[1*], Peter J O'Shaughnessy[2], Siladitya Bhattacharya[3], Ana Monteiro[2], David Kerrigan[1], Sven Goetz[4], Andrea Raab[4], Stewart M Rhind[5^], Kevin D Sinclair[6], Andrew A Meharg[7], Jörg Feldmann[4] and Paul A Fowler[8*]

Abstract

Background: Maternal smoking is one of the most important modifiable risk factors for low birthweight, which is strongly associated with increased cardiometabolic disease risk in adulthood. Maternal smoking reduces the levels of the methyl donor vitamin B12 and is associated with altered DNA methylation at birth. Altered DNA methylation may be an important mechanism underlying increased disease susceptibility; however, the extent to which this can be induced in the developing fetus is unknown.

Methods: In this retrospective study, we measured concentrations of cobalt, vitamin B12, and mRNA transcripts encoding key enzymes in the 1-carbon cycle in 55 fetal human livers obtained from 11 to 21 weeks of gestation elective terminations and matched for gestation and maternal smoking. DNA methylation was measured at critical regions known to be susceptible to the *in utero* environment. Homocysteine concentrations were analyzed in plasma from 60 fetuses.

Results: In addition to identifying baseline sex differences, we found that maternal smoking was associated with sex-specific alterations of fetal liver vitamin B12, plasma homocysteine and expression of enzymes in the 1-carbon cycle in fetal liver. In the majority of the measured parameters which showed a sex difference, maternal smoking reduced the magnitude of that difference. Maternal smoking also altered DNA methylation at the imprinted gene *IGF2* and the glucocorticoid receptor (GR/*NR3C1*).

Conclusions: Our unique data strengthen studies linking *in utero* exposures to altered DNA methylation by showing, for the first time, that such changes are present in fetal life and in a key metabolic target tissue, human fetal liver. Furthermore, these data propose a novel mechanism by which such changes are induced, namely through alterations in methyl donor availability and changes in 1-carbon metabolism.

Keywords: DNA methylation, Liver, Maternal smoking, Vitamin B12

* Correspondence: mandy.drake@ed.ac.uk; p.a.fowler@abdn.ac.uk
^Deceased
[1]Endocrinology Unit, University/BHF Centre for Cardiovascular Science, University of Edinburgh, Queen's Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ, UK
[8]Division of Applied Medicine, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, UK
Full list of author information is available at the end of the article

Background

Exposure to an adverse environment *in utero* leading to a reduction in birthweight is associated with a marked increase in later susceptibility to cardiometabolic and neuroendocrine disorders [1]. Smoking in pregnancy reduces birthweight, body length, and head circumference at term [2] and is associated with increased cardiovascular disease risk in the offspring [3,4]. Thus, in addition to being one of the most important modifiable risk factors for low birthweight, maternal smoking is potentially a critical risk factor for a substantial burden of non-communicable disease in adulthood. Despite public health advice, the prevalence of smoking during pregnancy remains high: in developed countries up to 25% of pregnant women smoke and fewer than 4% stop smoking while pregnant [5].

There is growing interest in the concept that alterations in the epigenome, particularly changes in DNA methylation, may be an important mechanism linking the early life environment with later disease risk. Smoking affects DNA methylation in adults [6] and recent studies in children and adults exposed to cigarette smoke *in utero* have reported alterations in global and site-specific DNA methylation [7-10]. One potential pathway by which exposure to adverse environmental conditions, including cigarette smoke *in utero*, might impact on DNA methylation is through altered availability of methyl donors provided by the 1-carbon pathway [11]. In animal models, the availability of methyl donors and 1-carbon substrates and co-factors, such as vitamin B12, during pregnancy is associated with differences in DNA methylation in the offspring [12,13], and this may also be relevant to humans [14,15]. Maternal vitamin B12, a key co-factor in the 1-carbon cycle, is the major determinant of neonatal vitamin B12 concentrations [16] and maternal smoking reduces the concentrations of vitamin B12 in pregnant women [17] and their infants [18].

Based on initial observations of depleted concentrations of cobalt (the central metal ion in vitamin B12) in second-trimester human fetal livers, reported for the first time in this article, we hypothesized that exposure to maternal cigarette smoking would also reduce fetal vitamin B12 levels. This, in turn, may be associated with changes in 1-carbon metabolism and with DNA methylation in the fetal liver – a key metabolic target tissue. Furthermore, since exposure to maternal smoking during pregnancy has sex-specific effects on fetal growth [19,20], the expression of steroidogenic and metabolic genes in fetal liver [21,22] and on the later risk of being overweight/obese [23], we postulated that any effects would be sex-specific. We sought to test these hypotheses directly through study of the effects of prenatal smoke exposure on the fetal liver.

Methods

Sample collection

The collection of fetal material was approved by the NHS Grampian Research Ethics Committees (REC 04/S0802/21). Women seeking elective, medical, terminations of pregnancy were recruited with full written, informed, consent by nurses working independently at Aberdeen Pregnancy Counselling Service. There was no change in patient treatment or care associated with recruitment to the study and women were able to withdraw from the study at any point. Fetal normality was determined at ultrasound scan 2 to 9 days prior to the termination of pregnancy. Women bearing abnormal fetuses were not included in the study. Only fetuses from normally-progressing pregnancies, from women over 16 years of age with a good understanding of English, and between 11 to 21 weeks of gestation, were collected. Fetuses were transported to the laboratory within 30 minutes of delivery, weighed, crown-rump length recorded, and sexed. Blood samples were collected by cardiac puncture *ex vivo* and plasma was initially stored at −20°C and then transferred to −85°C after assay. Livers were snap-frozen in liquid nitrogen and stored at −85°C.

Study design

For local ethical and logistic reasons it is not possible to prospectively collect specific fetuses for each study; therefore, studies were performed retrospectively by rigorous selection of already collected fetal tissues, samples, and data. To ensure accurate classification of fetuses, exposure to cigarette smoke was determined by measuring fetal plasma cotinine [24]. From a population of 228 fetuses available, two subpopulations were formed based on the availability of: (A) liver tissue (55 fetuses [21,22]) and (B) plasma measurements (60 fetuses). Of the 60 fetuses in (B), 18 fetuses overlapped with the 55 livers in (A). Within each subpopulation, the studied fetuses were matched for gestational age, fetal sex, and maternal smoking/fetal plasma cotinine (Table 1). The aim of this matching was to ensure that each of the four main analytical groupings (male, non-smoke-exposed; male, smoke-exposed; female, non-smoke-exposed; female, smoke-exposed) contained similar numbers of fetuses representing similar gestational ages, enabling two-way ANOVA to be used (see Statistical analysis section below). We were unable to collect data on maternal diet under the study ethics but the women were matched for age and body mass index across the four main analytical groupings (Table 1).

Hepatic metal content analysis and cobalt speciation

Livers were homogenized using a TissueLyser (Qiagen Ltd., Crawley, UK) and were freeze-dried and stored at −80°C [21]. For total element determination, 100 mg of liver was

Table 1 Morphological data for the mothers and fetuses (mean ± SEM) at census date and then for those allocated to the liver and plasma cysteine and homocysteine analyses populations

Population	Characteristic	Male fetuses		Female fetuses	
		Control	Smoke-exposed	Control	Smoke-exposed
Whole population at census date	*N*	57	66	49	56
Maternal indices	Age (years)	24 ± 1	25 ± 1	24 ± 1	24 ± 1
	Body mass index (BMI) (kg/m^2)	25 ± 1	25 ± 1	24 ± 1	25 ± 1
	Cigarettes/day	0	11 ± 1	0	12 ± 1
Fetal indices	Age (weeks)	14.5 ± 0.3	15.1 ± 0.3	14.9 ± 0.3	14.9 ± 0.3
	Weight (g)	73 ± 10[a]	95 ± 9[b]	87 ± 11	92 ± 12
	Crown-rump length (CRL, mm)	94 ± 4[a]	106 ± 4[b]	101 ± 4	102 ± 4
	Ponderal index (weight g/[CRL cm^3])	0.067 ± 0.002	0.067 ± 0.002	0.069 ± 0.003	0.067 ± 0.002
	Plasma cotinine (ng/mL)	3 ± 1[a]	40 ± 2[b]	4 ± 1[a]	43 ± 2[b]
Sub-population: methylation and metal	*N*	14	16	14	11
Maternal indices	Age (years)	21 ± 1	26 ± 2	25 ± 1	26 ± 2
	BMI (kg/m^2)	25 ± 2	26 ± 1	24 ± 1	25 ± 2
	Cigarettes/day	0	16 ± 2	0	16 ± 2
Fetal indices	Age (weeks)	14.0 ± 0.6	14.3 ± 0.4	15.3 ± 0.7	15.8 ± 0.9
	Weight (g)	62 ± 18	74 ± 14	100 ± 20	151 ± 34
	CRL (mm)	93 ± 8	97 ± 6	103 ± 8	121 ± 9
	Ponderal index (weight g/[CRL cm^3])	0.063 ± 0.004	0.070 ± 0.003	0.080 ± 0.007	0.071 ± 0.003
	Plasma cotinine (ng/mL)	3 ± 1[a]	44 ± 3[b]	3 ± 1[a]	48 ± 3[b]
Sub-population: plasma cysteine and homocysteine	*N*	14	17	13	16
Maternal indices	Age (years)	25 ± 2	24 ± 1	24 ± 2	24 ± 1
	BMI (kg/m^2)	26 ± 2	25 ± 2	24 ± 1	24 ± 1
	Cigarettes/day	0	13 ± 1	0	11 ± 1
Fetal indices	Age (weeks)	15.2 ± 0.7	14.9 ± 0.6	15.6 ± 0.7	15.4 ± 0.6
	Weight (g)	102 ± 24	89 ± 18	105 ± 21	101 ± 19
	CRL (mm)	108 ± 8	105 ± 7	109 ± 8	121 ± 9
	Ponderal index (weight g/[CRL cm^3])	0.074 ± 0.005	0.064 ± 0.002	0.065 ± 0.003	0.065 ± 0.003
	Plasma cotinine (ng/mL)	5 ± 1[a]	44 ± 3[b]	4 ± 3[a]	41 ± 3[b]

[a,b]Values in the same row that do not share a superscript letter are significantly different (P <0.05) due to maternal cigarette smoking. Absence of superscript letters indicates no significant differences.

mixed with 1 mL concentrated nitric acid and pre-digested overnight. Hydrogen peroxide (2 mL) was added and the samples were digested using a MARS5 microwave oven. The digest was diluted to 10 mL with water (18 MΩ cm, MilliQ). Elemental standards for calibration were prepared by appropriate dilutions of multi-element standard Merck XXI (Merck, UK) and single element solutions of Mo (CPI, single element solutions) with 5% nitric acid. The samples and standards were measured using an Agilent 7500c inductively coupled plasma mass spectrometry (ICP-MS) with on-line addition of Rh-solution (20 μg/L) as an internal standard. The ICP-MS was used with H_2 as reaction gas to remove molecular interferences of ArCl on As (m/z 75) and ArAr on Se (m/z 78). Certified standard reference materials (RM8415, TORT-2, DORM-3, BCR-185R, NIST 1,577b) were used to check reproducibility and accuracy, with both shown to be better than 5%. The hepatic essential element content was expressed as ng/g dry matter (dm). Cobalamin in its vitamin B12 form was extracted from homogenized liver (30 to 100 mg) using 2.5 mg papain and 10 μL 1% NaCN dissolved in 600 μL acetate buffer (pH 4.0). The mixture was heated to 57°C for 3 h and subsequently boiled in a water-bath for 10 min and centrifuged. Different cobalt species were separated using an Agilent Eclipse XDB-C8 (150 × 4.6 mm) column with an eluent containing 0.1% (v/v) formic acid in a gradient of 10% to 70% MeOH in 20 min. The column was held at 30°C in a column oven, the injection volume was 100 μL, and the flow rate was 1 mL/min. Cobalt detection was performed

through an Element 2 ICP-MS (Thermo, Bremen). The internal standard (Rh in 1% nitric acid) was added post-column via a T-piece before the nebulizer. The column effluent was split after the column with three quarters going into the UV/electrospray ionization (ESI)-MS and one quarter into the ICP-MS using a short length of peek tubing. ESI-MS (Orbitrap Discovery, Thermo) was used for some samples in positive ES-FT-mode (resolution 30,000) from 100 to 2,000 m/z using MS/MS in FT-mode (resolution 7,500) to aid identification of the eluting vitamin B12. Quantification was performed after calibration with a cobalt standard solution and corrected for the different elution conditions for the gradient [25]. The limits of detection for every cobalamin species was 0.9 μg Co/kg dm or 1.5 nmol/kg (calculated from 3 × SD) while reproducibility was better than ±15%.

Plasma cysteine and homocysteine determination

Fetal plasma homocysteine and cysteine were analyzed using an Agilent 1100 HPLC system (Agilent Technologies, Stockport, UK) according to previously published in-house protocols [26] based on the original method of Pfeiffer et al. [27]. Peak integration was performed using Agilent ChemStation software (Agilent Technologies).

Real-time quantitative PCR

Total RNA was extracted from frozen fetal liver samples (10 to 20 mg) using TRIzol (Life Technologies, Paisley, UK). Reverse transcription, primer design, and real-time PCR were as previously described [28-30] and the primers used are shown in Additional file 1: Table S1. Data were normalized against a validated combination of housekeeping genes (B2M, PMM1, TBP) as described previously [21].

Analysis of DNA methylation

DNA was extracted from fetal liver using a Qiagen AllPrep DNA/RNA/Protein mini kit (Qiagen) following tissue homogenization using a Qiagen TissueLyser (Qiagen) and following the manufacturer's instructions [31]. Briefly, 1 μg of DNA was subjected to bisulfite conversion using the Epitect Bisulfite Kit (Qiagen). Pyrosequencing was performed to analyze DNA methylation across a number of regions (Figure 1) as previously described for regions known to be important in controlling the expression of insulin-like growth factor 2 (IGF2): the IGF2 differentially methylated regions (DMRs) and the H19 imprinting control region (ICR) [32] and for exons 1(C) and 1(F) of the *NR3C1* glucocorticoid receptor (GR) promoter [31]. All primers were purchased from Eurogentec (Southampton, UK). Pyrosequencing was carried out using SQA reagents (Qiagen) on the PSQ™ HS-96A. Data were analyzed using Pyro Q-CpG Software (Qiagen). Background non-conversion levels were determined by inclusion of non-cytosine-guanine dinucleotide (CpG) cytosine controls in all assays and were between 1 and 3%.

Statistical analysis

Statistical analyses of data were performed using JMP 9.0.2 software (Thomas Learning, London, UK) [21,22]. Normality of data distribution was tested with the Shapiro-Wilk test and non-normally distributed data were log-transformed and re-checked for normality prior to analysis by ANOVA and Tukey-Kramer honestly significant difference and T-tests. Where data were not normalized, or the variances remained unequal, non-parametric tests were performed (Wilcoxon Test). Care was taken to ensure that no comparison involved groups with any difference between smokers and non-smokers in terms of stage of gestation, avoiding bias. Because

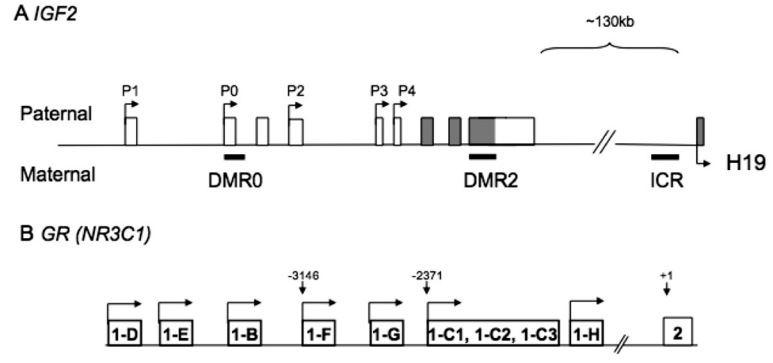

Figure 1 Schematic diagrams showing of regions in which methylation was assessed including IGF2 and exons 1(F) and 1(C) of the *GR* promoter. (A) The *IGF2* gene comprises multiple transcripts originating from promoters (P0-P4) which splice into common protein-coding exons (shaded in grey). The H19 ICR is situated distal to IGF2. Regions of differential methylation are shown underlined. **(B)** The *GR* gene also comprises multiple alternate untranslated first exons as indicated (B-H) [33]. Percentage methylation was assessed at CpGs within exons 1F, 1-C1 and 1-C3.

most of the parameters investigated showed developmental changes in expression across the second trimester, two-way ANOVA was used to test the combined effects of gestational age (weeks) and maternal smoking (yes/no and confirmed by fetal plasma cotinine assay) on morphological and biochemical data and on transcript levels.

Results

1-carbon metabolism

Effects of fetal sex

Vitamin B12 is an essential component of the 1-carbon cycle; it is required for the transfer of the C1 unit from methyltetrahydrofolate to methionine by methionine synthase (MTR; EC 2.1.1.13), and is a rate-limiting co-factor for methionine synthase reductase (MTRR; EC 1.16.1.8). Levels of vitamin B12 and cobalt (the central metal ion in vitamin B12) were significantly lower in liver from non-exposed male fetuses than in non-exposed female fetuses (Figure 2A, B). Vitamin B12 acts as a co-factor for the conversion of homocysteine to methionine and homocysteine is converted to cysteine through the actions of cystathionine-β-synthase (EC 4.2.1.22) and cystathionine gamma-lyase (CTH; EC 4.4.1.1). We therefore also measured fetal plasma homocysteine and cysteine. Plasma homocysteine and cysteine levels were higher in non-exposed males compared to females (Figure 2D, E).

Effects of smoke exposure

In females, both vitamin B12 and cobalt concentrations correlated significantly and inversely with fetal cotinine ($r = -0.593$, $P = 0.002$ and $r = -0.510$, $P = 0.009$, respectively) and levels of both were significantly reduced by maternal cigarette smoking to resemble the levels seen in males (Figure 2A, B and Additional file 2: Table S2). In contrast, maternal smoking was associated with a significant increase in the vitamin B12/cobalt ratio across the second trimester in males (Additional file 3: Figure S1). This reflects a decrease in the proportion of cobalt that is not bound as cobalamin and demonstrates that maternal smoking reduces non-cobalamin cobalt uptake. We also measured fetal plasma homocysteine and cysteine (to which homocysteine is converted through the actions of cystathionine-β-synthase and CTH). The sex differences in plasma homocysteine and cysteine were abolished by exposure to maternal smoking (Figure 2D, E).

Expression of 1-carbon cycle genes and DNA methyltransferases

Alterations in the expression of key genes in the 1-carbon cycle (Additional file 4: Figure S2) might alter the availability of methyl groups and impact on DNA methylation. We therefore measured the expression of mRNA transcripts encoding enzymes in the 1-carbon cycle in fetal liver (Figure 3A–I).

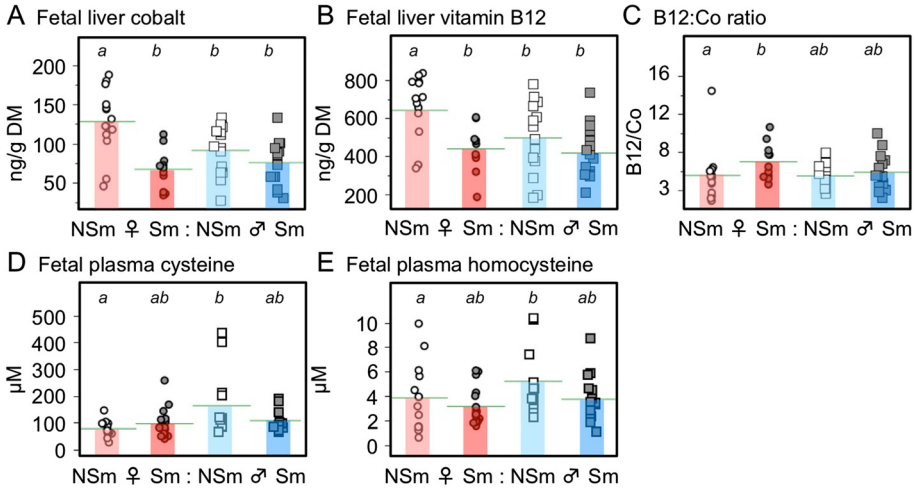

Figure 2 Concentrations of cobalt (Co) and vitamin B12 (B12) in the human fetal liver. (A) and **(B)** show the effects of sex and maternal smoking on Co and B12, respectively. The ratio between B12 and Co **(C)** was lower in females and increased to male levels by smoke-exposure in females. Circulating fetal cysteine **(D)** and homocysteine **(E)** concentrations were significantly higher in male fetuses and this was lost when the mothers smoked. Data from individual fetuses (**A–D**: 11–16 fetuses per group; **E–F**: 13–17 fetuses per group) are shown, and the horizontal bar represents the mean of the group. Significant differences between groups are indicated by letters in the boxes above each graph. Within each element or compound, groups that do not share a letter are significantly ($P < 0.05$) different. NSm, represents control fetuses from non-smoking mothers; Sm, represents smoke-exposed fetuses from mothers who smoked during pregnancy. Fetal sex is denoted by the appropriate symbol.

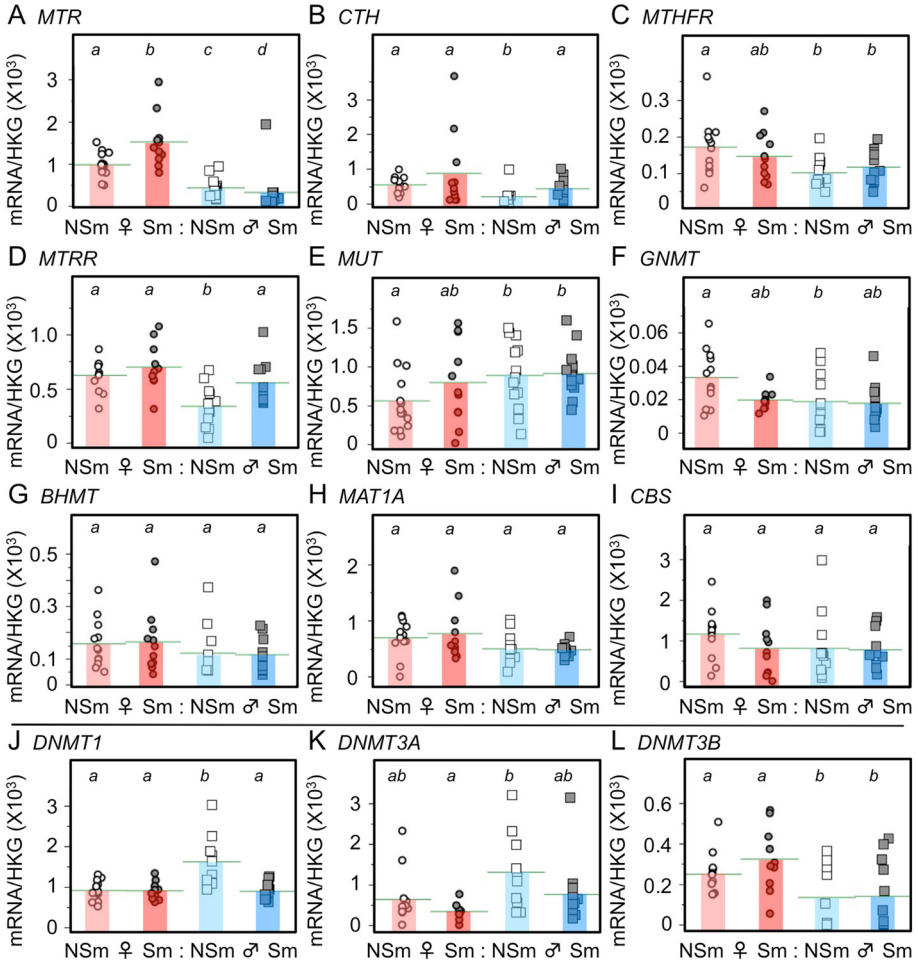

Figure 3 Expression of selected transcripts encoding members of the 1-Carbon cycle (A-I) and DNA methylation enzymes, (J) DNMT1, (K) DNMT3A, (L) DNMT3B, in the human fetal liver and effects of sex and maternal smoking. Real-time PCR was used to measure specific transcript levels as described in the text. Data from individual fetuses (11–16 fetuses per group) are shown, and the horizontal bar represents the mean of the group. Significant differences between groups are indicated by letters in the boxes above each graph. Within each transcript, groups that do not share a letter are significantly (P <0.05) different. NSm, represents control fetuses from non-smoking mothers; Sm, represents smoke-exposed fetuses from mothers who smoked during pregnancy. Fetal sex is denoted by the appropriate symbol.

Effects of fetal sex

Sex differences in transcript levels were apparent, with lower expression of *MTR*, *MTRR*, methylene-THF-reductase (*MTHFR*), glycine N-methyltransferase (*GNMT*), and *CTH* in non-exposed males compared to females. In addition, expression of the DNA methyltransferase *DNMT1* was higher and the expression of *DNMT3B* was lower in males compared to females (Figure 3J–L).

Effects of smoke exposure

Exposure to maternal smoking was associated with sex-specific changes in mRNA transcript levels: in males, smoke exposure resulted in an increase in the expression of *CTH* and *MTRR* to female levels but also resulted in a decrease in the expression of *MTR*. In females, smoke exposure resulted in an increase in the expression of *MTR* and a decrease in the expression of *GNMT* to male levels. The expression of *DNMT1* in males was reduced to female levels by exposure to maternal smoking but there were no effects of smoking on the expression of *DNMT3A or DNMT3B* (Figure 3J–L).

Expression of IGF2 and NR3C1 (GR)

Effects of fetal sex

The expression of the *IGF2* transcript was higher in males compared to females (Figure 4A) whereas expression of the glucocorticoid receptor (*GR/NR3C1*) was lower in males (P = 0.022, Figure 4B) compared to females.

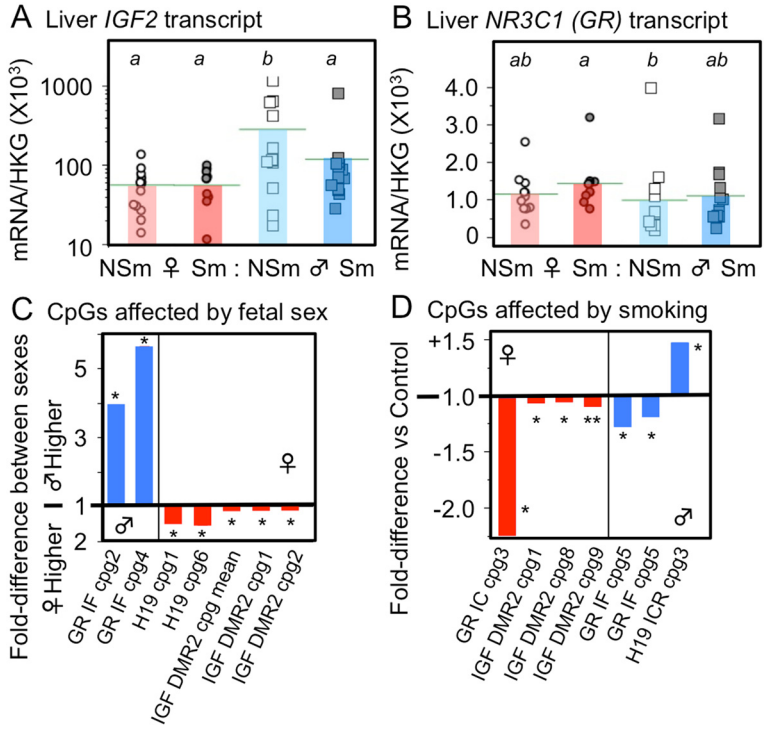

Figure 4 Effects of fetal sex and maternal smoking on expression of transcripts encoding *IGF2* (A) and *GR* (B), and on DNA methylation (C and D) in the human fetal liver. Real-time PCR was used to measure specific transcript levels and pyrosequencing was used to analyze DNA methylation as described in the text. Data from individual fetuses (11–16 fetuses per group) are shown, and the horizontal bar represents the mean of the group. In A and B, significant differences between groups are indicated by letters in the boxes above each graph. Within each transcript, groups that do not share a letter are significantly (P <0.05) different. NSm, represents control fetuses from non-smoking mothers; Sm, represents smoke-exposed fetuses from mothers who smoked during pregnancy. Fetal sex is denoted by the appropriate symbol.

Effects of smoke exposure

The expression of *IGF2* transcript was decreased by smoke exposure in males but there was no effect in females (Figure 4A). There was no effect of smoking on the expression of *GR* (Figure 4B).

DNA methylation of IGF2 and NR3C1 (GR)

Pyrosequencing was used to quantify DNA methylation at the DMRs known to be important in the control of *IGF2* expression: DMR0, DMR2, and the *H19* ICR, which lies upstream of the *H19* gene. There was a significant (P <0.05) increase in DNA methylation at *IGF2* DMR0 with gestational age (Additional file 5: Figure S3A). Levels of DNA methylation at exons 1C and 1F of the *GR/NR3C1* promoter were very low as previously published [31], consistent with the status of the *GR* promoter as a dense CpG island. Methylation at exon 1C increased with gestational age (Additional file 5: Figure S3B).

Effects of fetal sex

Sex differences in DNA methylation were apparent at the H19 ICR and IGF2 DMR2, where methylation was lower in males than females. Conversely, at the *GR* promoter there was higher DNA methylation within exon 1F (with a similar trend for exon IC, $P = 0.056$) in males, consistent with the lower transcript levels of this gene (Figure 4C).

Effects of smoke exposure

In males, smoke exposure significantly increased DNA methylation at one CpG within the *H19* ICR (Figure 4D), whereas in females, smoke exposure was associated with decreased methylation at several CpGs within *IGF2* DMR2. Additionally, at the *GR* promoter, there were small changes in DNA methylation associated with maternal smoking at specific CpGs within exon 1F in males and at one CpG in exon 1C in females (Figure 4D).

Discussion

Vitamin B12 is an essential component of the 1-carbon cycle and data from both human and animal studies strongly support the concept that maternal and/or fetal vitamin B12 availability has important influences on health outcomes, including fetal growth, neurodevelopment, and longer term cardiometabolic disease risk [34-36]. Herein, we show, for the first time, that maternal smoking, which is known to reduce the concentrations of vitamin B12 in pregnant women and their infants [17,18], is associated

with widespread effects on 1-carbon metabolism, including alterations in levels of enzyme transcripts and cofactors. Importantly, we also show that these effects are present during early development and in a key target tissue, the fetal liver.

The precise role of *in utero* methyl donor availability and/or effects on the 1-carbon pathway in the programming of later disease risk remains unknown; however, one important mechanism may be through changes in DNA methylation [11]. In animal models, dietary availability of methyl donors during pregnancy has a profound influence on both phenotype and DNA methylation in offspring [12,37]. In humans, maternal vitamin B12 concentrations correlate inversely with global DNA methylation in umbilical cord blood [15], and an inverse correlation between cord plasma homocysteine concentrations and genome-wide DNA methylation at repetitive sequences has been reported [38]. Additionally, genome-wide and candidate gene studies have identified alterations in DNA methylation in association with maternal smoking in accessible tissues at birth, including cord blood, buccal cells, and placenta [8-10]. Our findings that maternal smoking is associated with effects on DNA methylation strengthen and extend previous studies by showing that such changes are detectable in the fetal liver, a major target organ for developmental programming effects [39,40], and that they are present during early development.

The imprinted gene *IGF2* has a major role in mediating fetal growth [41] and altered DNA methylation at the *IGF2* DMRs is known to be associated with human syndromic growth disorders, including Silver Russell and Beckwith Wiedemann syndromes [42]. Recent data suggest that more subtle alterations in DNA methylation at *IGF2* are associated with patterns of fetal growth within the normal range [43-46] and some studies report that infants born small for gestational age have reduced DNA methylation at *IGF2* in cord blood [43]. Thus, reduced *IGF2* methylation in fetal liver during early-mid gestation could be one mechanism for the reduction in birthweight seen with prenatal smoke exposure. DNA methylation at *IGF2* appears to be particularly sensitive to the prenatal environment [9,47,48] and our data support the concept that methylation at *IGF2* may be a useful marker of *in utero* exposures [9]. DNA methylation at *GR* may also be influenced by diverse environmental cues in early life and animal and human studies suggest that downstream effects on the expression of *GR* may play a role in mediating the associations between the early life environment and subsequent cardiometabolic and neuropsychiatric disorders [31,39,49,50]. The methylation changes we report at both *IGF2* and *GR* in association with smoke exposure are relatively small; however, they resemble those described in previous studies, including studies of prenatal smoke exposure [9,47,51]. Additionally, the observed alterations in DNA methylation were region- and CpG site-specific, consistent with reports in both animal models and human studies [31,52]. In agreement with previously published studies in humans, DNA methylation levels did not necessarily predict changes in transcript levels, notably at *IGF2*, suggesting that there are alternative and/or additional mechanisms driving changes in transcript expression [53]. Rather, such changes in DNA methylation may result in genes being 'poised' for activation in response to future events [53,54].

Sex differences were evident in DNA methylation, which was higher at *IGF2* and lower at *GR* in females compared with males and the most marked changes in DNA methylation in association with maternal smoking occurred at these loci. Sexually dimorphic changes in DNA methylation have been reported as a consequence of early life nutritional challenges and exposure to maternal smoking [9,51,55,56]. The proposed mechanisms to explain these observations include differences in the timing and speed of development, the influence of sex steroids, especially androgens, and/or sex chromosome complement [22,57]. Our results suggest that sex differences in the concentrations of vitamin B12 and homocysteine and in the expression of genes important in 1-carbon metabolism and DNA methylation could also be important in determining the sex-specific effects of exposure to an adverse environment in early life. Consistent with our previous studies [21,22], the smoke-induced changes in cobalt, vitamin B12, and homocysteine, and in the expression of enzymes involved in both the 1-carbon cycle and DNA methylation, resulted in profiles which resembled those seen in the opposite sex (summarized in Figure 5). These clearly suggest that smoke exposure results in masculinization of the female liver and feminization of the male liver (Figure 5).

Temporal changes in gene expression and DNA methylation indicate that there may be specific windows of susceptibility during development and, importantly, that this is likely to differ between the sexes. Allelic methylation patterns at the *IGF2* DMRs arise early in embryogenesis and change progressively during development [58] and here we show that this is also the case for *GR* between 12 and 20 weeks of gestation. Thus, since a worryingly high proportion of women continue to smoke throughout pregnancy, the observed effects on DNA methylation may change or become amplified with ongoing exposure. Additionally, the temporal changes in DNA methylation profiles across gestation could be one explanation for the differences between studies with respect to the association of DNA methylation at *IGF2* and fetal growth [9,59]. Whilst the data shown here represent a single, albeit unique, 10-week snap-shot of the effects of maternal smoking, they are consistent with previous

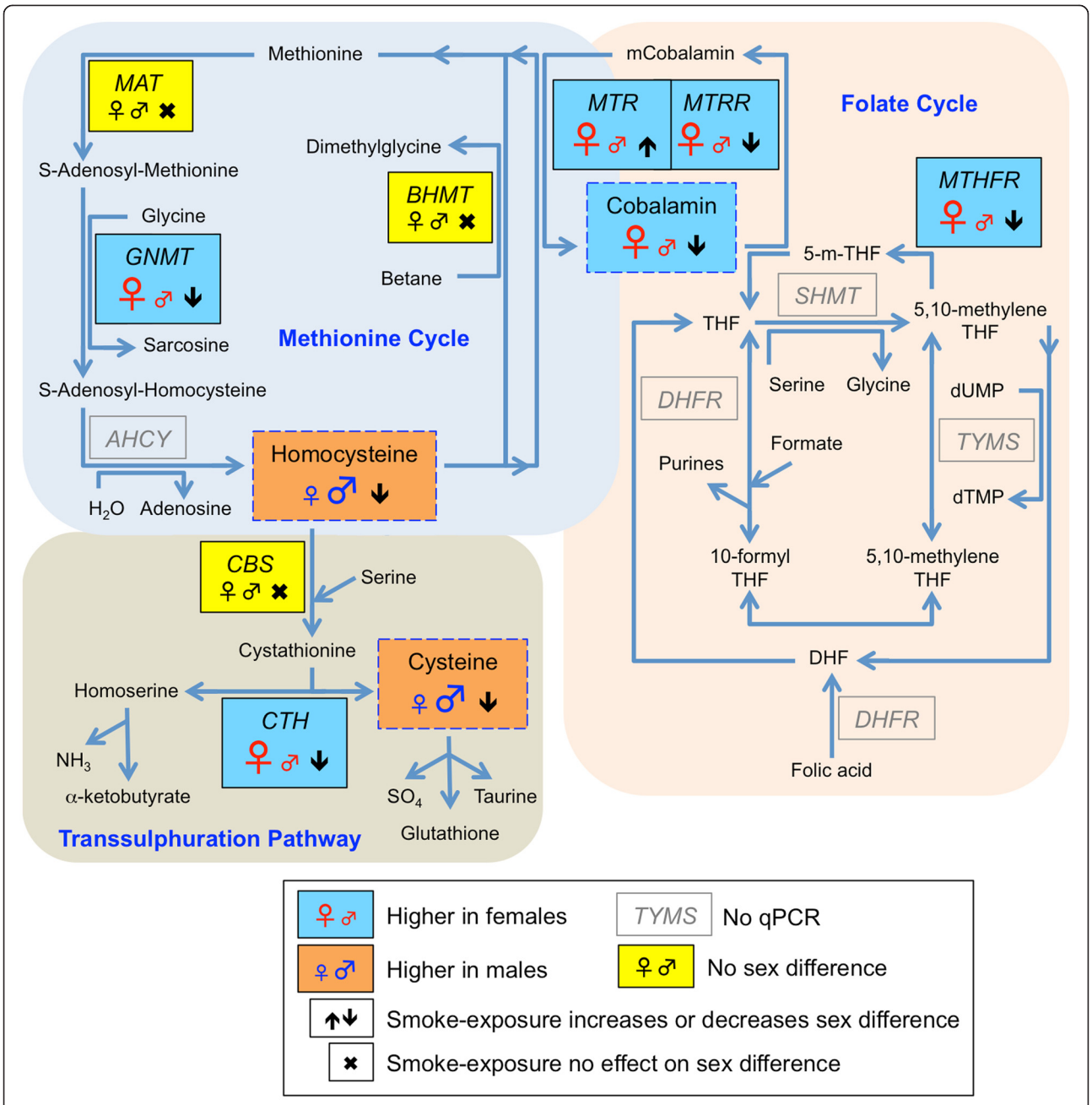

Figure 5 Summary of effects of fetal sex and maternal smoking on the human fetal second trimester liver 1-carbon metabolism pathway. Redrawn and modified from [10]. In 7 out of 8 genes or compounds showing sex differences, maternal smoking reduced the magnitude of the difference. Together with sex differences and sex-specific effects of maternal smoking on DNA methylation this demonstrates the sensitivity of the fetal liver to maternal smoking.

studies showing that maternal smoking is associated with altered DNA methylation in blood, buccal cells, and placenta in exposed offspring at birth [8]. Importantly, these changes may be persistent and/or evolve postnatally since DNA methylation patterns in peripheral blood and buccal cells in young children and in peripheral blood in adolescence and young adulthood also associate with *in utero* exposure to maternal smoking [7,10,60,61]. Furthermore, some studies report that methylation differences only become apparent some considerable time after *in utero* environmental exposures [51].

Conclusions

Our novel data greatly extend previous studies linking *in utero* exposures with altered DNA methylation by showing, for the first time, that such changes are present

comparatively early in fetal life and, importantly, that these occur in a key metabolic target tissue, the liver. These changes are likely to have a direct effect on fetal development and, if persistent, may have long-term effects on health. Additionally these data propose an important new mechanism by which these changes might be induced, through alterations in methyl donor availability and changes in the 1-carbon cycle.

Additional files

Additional file 1: Primers used for qPCR.

Additional file 2: Fetal human hepatic essential element content.

Additional file 3: Maternal smoking is associated with a significant increase in the ration between hepatic Co and vitamin B12 in males fetuses only across the second trimester. Males are shown by squares, females by circles, controls are open, smoke-exposed are shaded. "NSm" represents control fetuses from non-smoking mothers; "Sm" represents smoke-exposed fetuses from mothers who smoked during pregnancy. Fetal sex is denoted by the appropriate symbol.

Additional file 4: Summary of the 1-carbon metabolism cycle. Simplified and modified diagram based on Steegers-Theunissen RPM, Twight J, Pestinger V and Sinclair KD. The periconceptional period, reproduction and long-term health of offspring: the importance of one-carbon metabolism. *Human Reproduction Update* 19,640-655 (2013).

Additional file 5: Fetal liver methylation levels of specific or mean CpGs for *IGF2* (A) and *GR* (B) icrease significantly across the second trimester. Males are shown by squares, females by circles, controls are open, smoke-exposed are shaded.

Abbreviations

CpG: Cytosine-guanine dinucleotide; CTH: Cystathionine gamma-lyase; DMR: Differentially methylated region; DNMT: DNA methyltransferase; ESI: Electrospray ionization; GNMT: Glycine N-methyltransferase; GR: Glucocorticoid receptor (*NR3C1*); ICP-MS: Inductively coupled plasma mass spectrometry; ICR: Imprinting control region; IGF2: Insulin-like growth factor 2; MTHFR: Methylene-THF-reductase; MTR: methionine synthase; MTRR: Methionine synthase reductase.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AJD participated in study design and coordination, assisted with the methylation analysis, assisted in data interpretation, and helped to draft the manuscript. PJOS participated in study design, transcript analysis, assisted in data interpretation, and helped to draft the manuscript. PAF conceived of the study and participated in its design and coordination, performed the statistical analysis, data interpretation, and helped to draft the manuscript. SB participated in study design and helped to draft the manuscript. AM carried out the qPCR. DK performed methylation analysis. SG carried out the essential elements analysis. AR carried out the essential elements analysis and developed B12 methods. SMR participated in study design and data interpretation and helped to draft the manuscript. KDS carried out the cysteine and homocysteine analyses, assisted in data interpretation, and helped to draft the manuscript. AM participated in study design, essential element data interpretation, and helped to draft the manuscript. JF participated in study design, developed B12 methods, and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank Ms Margaret Fraser, Ms Samantha Flannigan, and Dr Wing Yee Kwong for their expert assistance. The staff at Grampian NHS Pregnancy Counselling Service were essential for collecting fetuses. We thank Professor Geoffrey Hammond and Dr Marc Simard, University of British Colombia for helpful comments on the manuscript.

Supported by grants as follows: Scottish Senior Clinical Fellowship (AJD); Chief Scientist Office (Scottish Executive, CZG/1/109 to PAF, & CZG/4/742 (PAF & PJOS); NHS Grampian Endowments 08/02 (PAF, SB & PJOS); the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement no 212885 (PAF & SMR); the Medical Research Council grants MR/L010011/1 (PAF & PJOS) and MR/K018310/1 (AJD). None of the funding bodies played any role in the design, collection, analysis, and interpretation of data, in the writing of the manuscript, nor in the decision to submit the manuscript for publication.

Author details

[1]Endocrinology Unit, University/BHF Centre for Cardiovascular Science, University of Edinburgh, Queen's Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ, UK. [2]Institute of Biodiversity, Animal Health & Comparative Medicine, College of Medical, Veterinary & Life Sciences, University of Glasgow, Glasgow G61 1QH, UK. [3]Division of Applied Health Sciences, Aberdeen Maternity Hospital, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, UK. [4]TESLA (Trace Element Speciation Laboratory) and Marine Biodiscovery Laboratory, University of Aberdeen, Aberdeen, Scotland AB24 3UE, UK. [5]The James Hutton Institute, Craigiebuckler, Aberdeen AB15 8QH, UK. [6]School of Biosciences, Sutton Bonington Campus, University of Nottingham, Loughborough LE12 5RD, UK. [7]Institute for Global Food Security, Queen's University Belfast, David Keir Building, Malone, Road, Belfast BT9 5BN, UK. [8]Division of Applied Medicine, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, UK.

Received: 30 July 2014 Accepted: 9 December 2014
Published online: 29 January 2015

References

1. Barker DJ, Osmond C. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. Lancet. 1986;1:1077–81.
2. Pringle PJ, Geary MPP, Rodeck CH, Kingdom JCP, Kayamba-Kay's S, Hindmarsh PC. The influence of cigarette smoking on antenatal growth, birth size, and the insulin-like growth factor axis. J Clin Endocrinol Metabol. 2005;90:2556–62.
3. Brion M-JA, Leary SD, Lawlor DA, Smith GD, Ness AR. Modifiable maternal exposures and offspring blood pressure: A review of epidemiological studies of maternal age, diet, and smoking. Pediatr Res. 2008;63:593–8.
4. Mamun AA, O'Callaghan MJ, Williams GM, Najman JM. Maternal smoking during pregnancy predicts adult offspring cardiovascular risk factors – evidence from a community-based large birth cohort study. PLoS One. 2012;7:e41106.
5. Tappin D, MacAskill S, Bauld L, Eadie D, Shipton D, Galbraith L. Smoking prevalence and smoking cessation services for pregnant women in Scotland. Subst Abuse Treat Prev Pol. 2010;5:1.
6. Wan ES, Qiu W, Baccarelli A, Carey VJ, Bacherman H, Rennard SI, et al. Cigarette smoking behaviors and time since quitting are associated with differential DNA methylation across the human genome. Hum Mol Genet. 2012;21:3073–82.
7. Flom JD, Ferris JS, Liao Y, Tehranifar P, Richards CB, Cho YH, et al. Prenatal smoke exposure and genomic DNA methylation in a multiethnic birth cohort. Canc Epidemiol Biomarkers Prev. 2011;20:2518–23.
8. Joubert BR, Håberg SE, Nilsen RM, Wang X, Vollset SE, Murphy SK, et al. 450 K Epigenome-wide scan identifies differential DNA methylation in newborns related to maternal smoking during pregnancy. Environ Health Perspect. 2012;120:1425–31.
9. Murphy SK, Adigun A, Huang Z, Overcash F, Wang F, Jirtle RL, et al. Gender-specific methylation differences in relation to prenatal exposure to cigarette smoke. Gene. 2012;494:36–43.
10. Novakovic B, Ryan J, Pereira N, Boughton B, Craig JM, Saffery R. Postnatal stability, tissue, and time specific effects of *AHRR* methylation change in response to maternal smoking in pregnancy. Epigenetics. 2014;9:377–86.
11. Steegers-Theunissen RPM, Twigt J, Pestinger V, Sinclair KD. The periconceptional period, reproduction and long-term health of offspring: the importance of one-carbon metabolism. Hum Reprod Update. 2013;19:640–55.
12. Sinclair KD, Allegrucci C, Singh R, Gardner DS, Sebastian S, Bispham J, et al. DNA methylation, insulin resistance, and blood pressure in offspring

determined by maternal periconceptional B vitamin and methionine status. Proc Natl Acad Sci Unit States Am. 2007;104:19351–6.
13. Waterland RA, Dolinoy DC, Lin JR, Smith CA, Shi X, Tahiliani KG. Maternal methyl supplements increase offspring DNA methylation at Axin fused. Genesis. 2006;44:401–6.
14. Boeke CE, Baccarelli A, Kleinman KP, Burris HH, Litonjua AA, Rifas-Shiman SL, et al. Gestational intake of methyl donors and global LINE-1 DNA methylation in maternal and cord blood: prospective results from a folate-replete population. Epigenetics. 2012;7:253–60.
15. McKay JA, Groom A, Potter C, Coneyworth LJ, Ford D, Mathers JC, et al. Genetic and non-genetic influences during pregnancy on infant global and site specific DNA methylation: role for folate gene variants and vitamin B12. PLoS One. 2012;7:e33290.
16. Monsen A-LB, Ueland PM, Vollset SE, Guttormsen AB, Markestad T, Solheim E, et al. Determinants of cobalamin status in newborns. Pediatrics. 2001;108:624–30.
17. van Wersch JWJ, Janssens Y, Zandvoort JA. Folic acid, vitamin B12, and homocysteine in smoking and non-smoking pregnant women. Eur J Obstet Gynecol Reprod Biol. 2002;103:18–21.
18. Bjørke Monsen AL, Vollset SE, Refsum H, Markestad T, Ueland PM. Hematological parameters and cobalamin status in infants born to smoking mothers. Neonatology. 2004;85:249–55.
19. Voigt M, Hermanussen M, Wittwer-Backofen U, Fusch C, Hesse V. Sex-specific differences in birth weight due to maternal smoking during pregnancy. Eur J Pediatr. 2006;165:757–61.
20. Zarén B, Lindmark G, Bakketeig L. Maternal smoking affects fetal growth more in the male fetus. Paediatr Perinat Epidemiol. 2000;14:118–26.
21. O'Shaughnessy PJ, Monteiro A, Bhattacharya S, Fowler PA. Maternal smoking and fetal sex significantly affect metabolic enzyme expression in the human fetal liver. J Clin Endocrinol Metabol. 2011;96:2851–60.
22. O'Shaughnessy PJ, Monteiro A, Bhattacharya S, Fraser MJ, Fowler PA. Steroidogenic enzyme expression in the human fetal liver and potential role in the endocrinology of pregnancy. Mol Hum Reprod. 2013;19:177–87.
23. Danielzik S, Czerwinski-Mast M, Langnase K, Dilba B, Muller MJ. Parental overweight, socioeconomic status and high birth weight are the major determinants of overweight and obesity in 5-7/y-old children: baseline data of the Kiel Obesity Prevention Study (KOPS). Int J Obes Relat Metab Disord. 2004;28:1494–502.
24. Fowler PA, Cassie S, Rhind SM, Brewer MJ, Collinson JM, Lea RG, et al. Maternal smoking during pregnancy specifically reduces human fetal desert hedgehog gene expression during testis development. J Clin Endocrinol Metabol. 2008;93:619–26.
25. Bluemlein K, Raab A, Meharg A, Charnock J, Feldmann J. Can we trust mass spectrometry for determination of arsenic peptides in plants: comparison of LC–ICP–MS and LC–ES-MS/ICP–MS with XANES/EXAFS in analysis of Thunbergia alata. Anal Bioanal Chem. 2008;390:1739–51.
26. Kanakkaparambil R, Singh R, Li D, Webb R, Sinclair KD. B-vitamin and homocysteine status determines ovarian response to gonadotropin treatment in sheep. Biol Reprod. 2009;80:743–52.
27. Pfeiffer CM, Huff DL, Gunter EW. Rapid and accurate HPLC assay for plasma total homocysteine and cysteine in a clinical laboratory setting. Clin Chem. 1999;45:290–2.
28. Baker P, O'Shaughnessy P. Role of gonadotrophins in regulating numbers of Leydig and Sertoli cells during fetal and postnatal development in mice. Reproduction. 2001;122:227–34.
29. O'Shaughnessy PJ, Willerton L, Baker PJ. Changes in Leydig cell gene expression during development in the mouse. Biol Reprod. 2002;66:966–75.
30. O'Shaughnessy PJ, Baker PJ, Monteiro A, Cassie S, Bhattacharya S, Fowler PA. Developmental changes in human fetal testicular cell numbers and messenger ribonucleic acid levels during the second trimester. J Clin Endocrinol Metabol. 2007;92:4792–801.
31. Drake AJ, McPherson RC, Godfrey KM, Cooper C, Lillycrop KA, Hanson MA, et al. An unbalanced maternal diet in pregnancy associates with offspring epigenetic changes in genes controlling glucocorticoid action and foetal growth. Clin Endocrinol (Oxf). 2012;77:808–15.
32. Dupont JM, Tost J, Jammes H, Gut IG. De novo quantitative bisulphite sequencing using the pyrosequencing technology. Anal Biochem. 2004;333:119–27.
33. Turner JD, Muller CP. Structure of the glucocorticoid receptor (NR3C1) gene 5′ untranslated region: identification, and tissue distribution of multiple new human exon 1. J Mol Endocrinol. 2005;35:283–92.
34. Muthayya S, Kurpad AV, Duggan CP, Bosch RJ, Dwarkanath P, Mhaskar A, et al. Low maternal vitamin B12 status is associated with intrauterine growth retardation in urban South Indians. Eur J Clin Nutr. 2006;60:791–801.
35. Bhate V, Deshpande S, Bhat D, Joshi N, Ladkat R, Watve S, et al. Vitamin B12 status of pregnant Indian women and cognitive function in their 9-year-old children. Food Nutr Bull. 2008;29:249–54.
36. Yajnik CS, Deshpande SS, Jackson AA, Refsum H, Rao S, Fisher DJ, et al. Vitamin B12 and folate concentrations during pregnancy and insulin resistance in the offspring: the Pune Maternal Nutrition Study. Diabetologia. 2008;51:29–38.
37. Waterland RA, Jirtle RL. Transposable elements: targets for early nutritional effects on epigenetic gene regulation. Mol Cell Biol. 2003;23:5293–300.
38. Fryer AA, Nafee TM, Ismail KMK, Carroll WD, Emes RD, Farrell WE. LINE-1 DNA methylation is inversely correlated with cord plasma homocysteine in man: a preliminary study. Epigenetics. 2009;4:394–8.
39. Lillycrop KA, Phillips ES, Jackson AA, Hanson MA, Burdge GC. Dietary protein restriction of pregnant rats induces and folic acid supplementation prevents epigenetic modification of hepatic gene expression in the offspring. J Nutr. 2005;135:1382–6.
40. Drake AJ, Raubenheimer PJ, Kerrigan D, McInnes KJ, Seckl JR, Walker BR. Prenatal dexamethasone programs expression of genes in liver and adipose tissue and increased hepatic lipid accumulation but not obesity on a high-fat diet. Endocrinology. 2010;151:1581–7.
41. DeChiara TM, Robertson EJ, Efstratiadis A. Parental imprinting of the mouse insulin-like growth factor II gene. Cell. 1991;64:849–59.
42. Murrell A, Ito Y, Verde G, Huddleston J, Woodfine K, Silengo MC, et al. Distinct methylation changes at the IGF2-H19 locus in congenital growth disorders and cancer. PLoS One. 2008;3:e1849.
43. Bouwland-Both MI, van Mil NH, Stolk L, Eilers PHC, Verbiest MMPJ, Heijmans BT, et al. DNA methylation of IGF2DMR and H19 is associated with fetal and infant growth: The Generation R Study. PLoS One. 2013;8:e81731.
44. Guo L, Choufani S, Ferreira J, Smith A, Chitayat D, Shuman C, et al. Altered gene expression and methylation of the human chromosome 11 imprinted region in small for gestational age (SGA) placentae. Dev Biol. 2008;320:79–91.
45. Bourque DK, Avila L, Peñaherrera M, von Dadelszen P, Robinson WP. Decreased placental methylation at the H19/IGF2 imprinting control region is associated with normotensive intrauterine growth restriction but not preeclampsia. Placenta. 2010;31:197–202.
46. Koukoura O, Sifakis S, Soufla G, Zaravinos A, Apostolidou S, Jones A, et al. Loss of imprinting and aberrant methylation of IGF2 in placentas from pregnancies complicated with fetal growth restriction. Int J Mol Med. 2011;28:481–7.
47. Heijmans BT, Tobi EW, Stein AD, Putter H, Blauw GJ, Susser ES, et al. Persistent epigenetic differences associated with prenatal exposure to famine in humans. Proc Natl Acad Sci U S A. 2008;105:17046–9.
48. Soubry A, Murphy S, Huang Z, Murtha A, Schildkraut J, Jirtle R, et al. The effects of depression and use of antidepressive medicines during pregnancy on the methylation status of the IGF2 imprinted control regions in the offspring. Clinical Epigenetics. 2011;3:2.
49. Oberlander TF, Weinberg J, Papsdorf M, Grunau R, Misri S, Devlin AM. Prenatal exposure to maternal depression, neonatal methylation of human glucocorticoid receptor gene (NR3C1) and infant cortisol stress responses. Epigenetics. 2008;3:1–9.
50. McGowan PO, Sasaki A, D'Alessio AC, Dymov S, Labonte B, Szyf M, et al. Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. Nat Neurosci. 2009;12:342–8.
51. Khulan B, Cooper WN, Skinner BM, Bauer J, Owens S, Prentice AM, et al. Periconceptional maternal micronutrient supplementation is associated with widespread gender related changes in the epigenome: a study of a unique resource in the Gambia. Hum Mol Genet. 2012;21:2086–101.
52. Weaver ICG, Cervoni N, Champagne F, D'Alessio AC, Sharma S, Seckl JR, et al. Epigenetic programming by maternal behavior. Nat Neurosci. 2004;7:847–54.
53. Lam LL, Emberly E, Fraser HB, Neumann SM, Chen E, Miller GE, et al. Factors underlying variable DNA methylation in a human community cohort. Proc Natl Acad Sci. 2012;109:17253–60.
54. Thomassin H, Flavin M, Espinas ML, Grange T. Glucocorticoid-induced DNA demethylation and gene memory during development. EMBO J. 2001;20:1974–83.

55. Tobi EW, Lumey LH, Talens RP, Kremer D, Putter H, Stein AD, et al. DNA methylation differences after exposure to prenatal famine are common and timing- and sex-specific. Hum Mol Genet. 2009;18:4046–53.
56. Heijmans BT, Kremer D, Tobi EW, Boomsma DI, Slagboom PE. Heritable rather than age-related environmental and stochastic factors dominate variation in DNA methylation of the human IGF2/H19 locus. Hum Mol Genet. 2007;16:547–54.
57. Aiken CE, Ozanne SE. Sex differences in developmental programming models. Reproduction. 2013;145:R1–R13.
58. Feil R, Walter J, Allen N, Reik W. Developmental control of allelic methylation in the imprinted mouse Igf2 and H19 genes. Development. 1994;120:2933–43.
59. Tobi EW, Heijmans BT, Kremer D, Putter H, de Waal HAD-v, Finken MJJ, et al. DNA methylation of IGF2, GNASAS, INSIGF and LEP and being born small for gestational age. Epigenetics. 2011;6:171–6.
60. Breton CV, Byun H-M, Wenten M, Pan F, Yang A, Gilliland FD. Prenatal tobacco smoke exposure affects global and gene-specific DNA methylation. Am J Respir Crit Care Med. 2009;180:462–7.
61. Terry MB, Ferris JS, Pilsner R, Flom JD, Tehranifar P, Santella RM, et al. Genomic DNA methylation among women in a multiethnic New York City birth cohort. Cancer Epidemiol Biomarkers Prev. 2008;17:2306–10.