View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Nottingham ePrints

Daniel, Zoe and Akyol, Asli and McMullen, Sarah and Langley-Evans, Simon C, (2013) Exposure of neonatal rats to maternal cafeteria feeding during suckling alters hepatic gene expression and DNA methylation in the insulin signalling pathway. Genes and Nutrition, 9 (1). 365/1-365/10. ISSN 1555-8932

Access from the University of Nottingham repository:
http://eprints.nottingham.ac.uk/28478/3/DNAMethylation.pdf

Copyright and reuse:

The Nottingham ePrints service makes this work by researchers of the University of Nottingham available open access under the following conditions.

- Copyright and all moral rights to the version of the paper presented here belong to the individual author(s) and/or other copyright owners.
- To the extent reasonable and practicable the material made available in Nottingham ePrints has been checked for eligibility before being made available.
- Copies of full items can be used for personal research or study, educational, or not-for-profit purposes without prior permission or charge provided that the authors, title and full bibliographic details are credited, a hyperlink and/or URL is given for the original metadata page and the content is not changed in any way.
- Quotations or similar reproductions must be sufficiently acknowledged.

Please see our full end user licence at:
http://eprints.nottingham.ac.uk/end_user_agreement.pdf

A note on versions:

The version presented here may differ from the published version or from the version of record. If you wish to cite this item you are advised to consult the publisher's version. Please see the repository url above for details on accessing the published version and note that access may require a subscription.

For more information, please contact eprints@nottingham.ac.uk

EXPOSURE OF NEONATAL RATS TO MATERNAL CAFETERIA FEEDING DURING SUCKLING ALTERS HEPATIC GENE EXPRESSION AND DNA METHYLATION IN THE INSULIN SIGNALLING PATHWAY.

Zoe C Daniel, Asli Akyol[2], Sarah McMullen, Simon C Langley-Evans[1]

School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, LE12 5RD United Kingdom

[1]Correspondence to:

Professor Simon Langley-Evans, Simon.Langley-Evans@Nottingham.ac.uk, +44 (0)115 9516139

[2]Current affiliation, Department of Nutrition and Dietetics, Hacettepe University, Turkey.

RUNNING TITLE: Early cafeteria feeding programmes hepatic insulin signalling.

KEYWORDS: Programming, epigenetics, insulin signalling, cafeteria diet

6820 words, 3 Tables, 3 Figures

Pre-submission copy

Abstract

Nutrition in early life is a determinant of lifelong physiological and metabolic function. Diseases that are associated with ageing may, therefore, have their antecedents in maternal nutrition during pregnancy and lactation. Rat mothers were fed either a standard laboratory chow diet (C) or a cafeteria diet (O) based upon a varied panel of highly palatable human foods, during lactation. Their offspring were then weaned onto chow or cafeteria diet giving four groups of animals (CC, CO, OC, OO n=9-10). Livers were harvested 10 weeks post-weaning for assessment of gene and protein expression, and DNA methylation. Cafeteria feeding post-weaning impaired glucose tolerance and was associated with sex-specific altered mRNA expression of peroxisome proliferator activated receptor gamma (*PPARg*) and components of the insulin-signalling pathway (*Irs2, Akt1* and *IrB*). Exposure to the cafeteria diet during the suckling period modified the later response to the dietary challenge. Post-weaning cafeteria feeding only down-regulated *IrB* when associated with cafeteria feeding during suckling (group OO, interaction of diet in weaning and lactation P=0.041). Responses to cafeteria diet during both phases of the experiment varied between males and females. Global DNA methylation was altered in the liver following cafeteria feeding in the post-weaning period, in males but not females. Methylation of the *IrB* promoter was increased in group OC, but not OO (P=0.036). The findings of this study add to a growing evidence base that suggests tissue function across the lifespan a product of cumulative modifications to the epigenome and transcriptome, which may be both tissue and sex-specific.

250 words

Pre-submission copy

Introduction

The concept of the developmental origins of health and disease, founded on the observation that low birthweight and other sequelae of maternal undernutrition are associated with cardiovascular disease and type-2 diabetes, is strongly supported by animal experiments (McMullen and Langley-Evans, 2010). Variation in the quality or quantity of nutrient provision during pregnancy has a strong programming effect upon long-term health and well-being in the offspring of rodents, pigs, sheep and non-human primates (McMullen and Langley-Evans, 2010).

Much of the work to date that has investigated the impact of maternal nutrition as a programming factor that determines physiological and metabolic function, has focused upon maternal undernutrition. Food restriction (Vickers *et al.*, 2001), protein restriction (Langley and Jackson, 1994) and iron deficiency (Gambling *et al.*, 2003) in rodent pregnancy all elicit similar phenotypes in the resulting offspring, including cardiovascular dysfunction (Yates *et al.*, 2008, Torrens *et al.*, 2009) impaired glucose homeostasis (Fernandez-Twinn *et al.*, 2005) and renal impairments (Langley-Evans *et al.*, 1999). A smaller body of work has shown that maternal over-feeding, generally with high fat diets, has similar programming effects in rats and mice (Samuelsson *et al.*, 2008, Shankar *et al.*, 2008). Work in our laboratory has demonstrated that feeding a cafeteria diet (a varying menu of highly palatable human foods) can impact upon metabolic function, with changes in glucose homeostasis as determined by intraperitoneal glucose tolerance test (Akyol *et al.*, 2012). This work established that fetal life is not the only period of sensitivity to the maternal diet. Feeding a

Pre-submission copy

cafeteria diet during lactation in the rat also altered responses to a glucose load and had powerful effects upon feeding and other behaviours (Wright *et al*., 2011a, 2011b).

The most favoured explanation for associations between early dietary exposures and later physiological and metabolic function is that early diet impacts upon epigenetic marks, and hence gene expression (Szyf, 2009, Lillycrop and Burdge, 2011). There is good evidence from animal models of undernutrition during fetal life that maternal diet can alter the epigenome, particularly DNA methylation, and this may establish changes in gene expression that permanently modify tissue structure, or reset the responses to dietary and age-related challenges that occur later in life (Sinclair *et al*., 2007, Lillycrop *et al*., 2007, Bogdarina *et al*., 2010). Exposure to high fat diets has been shown to alter DNA methylation and histone marks in rodents, non-human primates and primates, with the brain being particularly sensitive to dietary influences. (Seki *et al*., 2012, Carlin *et al*., 2013, Langie *et al*., 2013, Jacobsen *et al.,* 2012) The theory that epigenetic marks are solely vulnerable to nutritional programming in fetal life is now discounted as the epigenome responds to dietary cues at all life stages.

Whilst there is strong evidence of altered DNA methylation patterns across the whole genome in response to variation in maternal nutrition during pregnancy, it is not clear how wide the "window" for epigenetic programming in response to nutrition may be. Certainly there is some evidence that a degree of plasticity remains into the pubertal growth phase in rats, as epigenetic marks on the peroxisome proliferator activated receptor alpha promoter respond to the folate

Pre-submission copy

content of the diet during this period (Burdge *et al.*, 2009). With a long period of sensitivity to diet, it is of interest to determine whether the state of the epigenome following one set of dietary stimuli, for example in fetal life, can influence the response of the epigenome to a stimulus at a later stage.

The present study utilized tissue from the study previously reported by Akyol and colleagues (2012), as a preliminary investigation to establish the possible mechanistic basis of programming by maternal over-feeding in the lactation period. Specifically we investigated the hypothesis that the observed metabolic phenotype is linked to cafeteria diet-induced changes in the insulin signalling pathway that are not just responses to current diet. The study also aimed to establish whether the epigenome is sensitive to maternal over-feeding during lactation or post-weaning.

Pre-submission copy

Materials and Methods

Experimental protocol and sample preparation

This paper describes a study which utilized a subset of tissue harvested from rats aged 3 months that had been subject to either maternal cafeteria diet feeding or a standard commercial chow diet during suckling. The full experiment which generated the tissue, has been previously published (Akyol *et al.*, 2012). The protocol is described briefly below.

The animal experiments were performed under licence from the Home Office in accordance with the 1986 Animals (Scientific Procedures) Act. Virgin female Wistar rats (aged 3 weeks) were fed standard laboratory chow diet (Harlan UK)

from weaning until mating at age 10 weeks. On giving birth, all of the resulting litters were culled to a maximum of 8 pups (4 males and 4 females where possible, randomly selected) and were randomly allocated to be fed either a control chow diet alone (n=11, chow control; C), or a control chow diet alongside an obesogenic random selection of highly energetic and palatable human foods (n=12, cafeteria diet; O), as previously described by Akyol et al., (2009, 2012). All litters were weaned at 3 weeks of age and then half of the offspring from each litter were weaned onto the standard chow diet and the remaining weanlings were allocated to receive chow and cafeteria diet. There were four groups of animals considered in this study; control in both lactation and post-weaning (CC); control in lactation, cafeteria post-weaning (CO); cafeteria during lactation, control post-weaning (OC) and cafeteria diet in both lactation and post-weaning (OO).

At the age of 13 weeks, all offspring were subject to an intraperitoneal glucose tolerance test, following an 18 hour fast, and were then culled (Akyol *et al.,* 2012). Liver was rapidly dissected and snap-frozen in liquid nitrogen before storage at -80 ℃. RNA was prepared from frozen tissue using Qiagen AllPrep kits according to the manufacturers instructions. DNA was extracted using Qiagen DNeasy kits. Protein extractions were performed by homogenising ground sample with 5 volumes of extraction buffer (150mM NaCl, 50mM HEPES, 2.5mM EDTA, 10% glycerol, 1% Triton, 1mM Na_3VO_4, 10mM NaF). After addition of 2 volumes SDS mix (glycerol, 1M Tris/HCl, 10% SDS, 0.154g/ml dithiothreitol 2:1.25:4:1 v:v:v:v), the samples were centrifuged at 13000 rpm for 10 mins at 4 ℃. Protein content of supernatant was determined using the method of Lowry

Pre-submission copy

et al., (1951). Protein, DNA and RNA were all prepared from the same samples, with only one liver per litter selected for this study.

Quantitative real-time PCR

The transcription of a number of target genes was determined by quantitative real-time PCR. The targets included the insulin receptor (*IrB*) and the glucose transporter *Glut2* (Kahn, 1997) as markers of the insulin-signalling pathway (insulin receptor substrate 2;*Irs2* and the alpha serine/threonine-protein kinase;*Akt1* had been previously measured in the same tissues, Akyol *et al.*, 2012). The expression of peroxisome proliferator activated receptor alpha (*PPARa*) was determined as this transcription factor regulates hepatic glucose metabolism (Viana Abranches *et al.*, 2011). Peroxisome proliferator activated receptor gamma (*PPARg*) expression was also measured as the effects of *PPARg* on adipocytes increase insulin sensitivity and the production of cytokines that regulate glucose homeostasis (Floyd and Stephens, 2012). Expression of acetyl CoA carboxylase 1 (*Acc1*) and carnitine-palmitoyl transferase 1a (*Cpt1a*) was determined as these are downstream targets of *PPARa* (Menendez *et al.*, 2012).

RNA was reverse transcribed using Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, UK). Real-time PCR primers were designed for *PPARa*, *PPARg*, *Glut2*, *IrB*, *ACC1* and *Cpt1a* using Primer Express software (version 1.5; Applied Biosystems) from the RNA sequence and were purchased from Sigma (UK). The primer sequences were as follows: Rat *PPARa* Fwd: CCACGAAGCCTACCTGAAGAACT Rvr: ACAAAAGGCGGATTGTTGCT; Rat *PPARg* gamma Fwd: GAATACCAAAGTGCGATCAAAGTAGA Rvr:

Pre-submission copy

GGCCATGAGGGAGTTTGAAG; Rat *Glut2* Fwd: TCAGCCAGCCTGTGTATGCA Rvr: TCCACAAGCAGCACAGAGACA; Rat *IrB* Fwd: GGATTATTGTCTCAAAGGGCTGAA Rvr: CGTCATACTCACTCTGATTGTGCTT; Rat *ACC1* Fwd: CCATTGGTATTGGGGCTTAC Rvr: CCGACCAAGGACTTTGTTG; Rat *Cpt1a* Fwd: CATTACAAGGACATGGGCAAGTT Rvr:TGTCCTCCCTTCTCGGAAGA. Primers were ordered from MWG Biotech, (Germany). Primer sequences for the housekeeping gene (cyclophilin) are published elsewhere (Austin and Langley-Evans, 2011). Real Time PCR was performed using a Lightcycler 480 PCR machine (Roche, UK) and SYBRgreen, as previously described (Swali *et al*., 2012). Expression of the genes of interest were normalised to the housekeeping gene, expression of which was unaltered in response to the dietary treatments.

A Cayman Chemicals DNA Methylation EIA kit was used to estimate crude global DNA methylation in the samples. This assay measures 5-methyl-2'-deoxy cytidine in DNA. Methylation-specific real-time PCR was performed for *PPARa*, *PPARg* and *IrB* using the SABiosciences EpiTect Methyl II PCR Primer assay, following the manufacturers instructions.

Western blotting

Protein expression was determined using Western blot, as described previously (Ryan *et al*., 2012). Primary antibodies were PPARa (Cayman Chemicals), PPARg (Santa Cruz), Glut2 (Millipore) and Insulin receptor beta (Cell Signalling). Expression was normalized against beta actin (Sigma) as a housekeeper. For Glut2, IrB, PPARa and PPARg the secondary antibody was anti-rabbit and for

Pre-submission copy

beta-actin it was anti-mouse (both from Amersham). Signal was quantified using ECL Select (Amersham) developed on Hyperfilm (Amersham).

Statistical analysis

All data are presented as mean ± SEM. Data were analysed by three way analysis of variance (SPSS 20) to examine the influence of offspring sex, maternal diet during lactation and offspring diet from weaning until 3 months of age. P≤0.05 was accepted as statistically significant.

Results

The original experiment which generated the tissues for this study evaluated the impact of cafeteria diet prior to and during pregnancy, in addition to considering the effects of the diet during lactation upon glucose homeostasis in the offspring. As the current study used a subset of groups from the original study, the glucose tolerance data from those groups was reanalysed to focus on the interaction between maternal diet during lactation and post-weaning diet (Figure 1). After adjustment for sex, there was a significant interaction of the diet in lactation and post-weaning (P=0.039). Compared to group CO, glucose tolerance was significantly impaired by cafeteria feeding in both periods (OO). Glucose tolerance was not significantly different to group CC in any of the groups, although group CO cleared an i.p. glucose load moderately faster.

Our previously published work suggested that exposure to cafeteria diet at key stages of life altered expression of two components of the insulin signalling pathway (*Irs2* and *Akt1*, Akyol *et al.*, 2012). Confining the analysis to just the four

Pre-submission copy

groups of rats in this study confirmed the sensitivity of these genes to cafeteria diet as *Irs2* was strongly up-regulated by post-weaning cafeteria diet in females only (2.64-fold, *P*<0.001, Table 1). *Akt1* expression was up-regulated by both cafeteria diet in lactation and post-weaning, but again only in females (interaction sex x post-weaning diet x lactation diet P<0.05, Table 1).). The effects of cafeteria diet were not observed when the diet was fed in both the lactation and post-weaning periods, hence expression was increased in groups OC (6.8-fold) and CO (2.9-fold), but not OO. *Irs2* expression was correlated with the area under the glucose tolerance curve (r=0.450, *P*=0.006) Expression of two other components of the insulin signalling pathway indicated that *Glut2* expression was not influenced by cafeteria feeding (Figs. 2D & 3D), but *IrB* expression was influenced by sex, lactation diet and post-weaning diet (interaction P=0.041, Table 1, Figs 2C & 3C). In females it was only the animals suckled by cafeteria fed mothers that were sensitive to post-weaning cafeteria diet, but these OO females had reduced (20% lower) expression of *IrB* compared to CC.

The mRNA expression of *PPARa* differed markedly between male and female animals (lower in females) but was not significantly influenced by diet post weaning. Exposure to cafeteria diet in lactation reduced *PPARa* expression (P=0.038), as shown by the significant effect in males of group OO (Table 1, Figs 2A & 3A). Similarly *PPARg* was expressed at a lower level in females than males (25% lower, Table 1, Figs 2B & 3B) and specifically in males of group CO expression was increased (1.37-fold) by feeding the cafeteria diet post-weaning (sex x post-weaning diet interaction P=0.05). Given the significant effect of the

Pre-submission copy

lactation diet upon *PPARa* expression the mRNA expression of the downstream targets *Acc1* and *Cpt1a* was evaluated. Whilst *Acc1* expression was unaffected by sex or diet at either stage of life (Table 1, Figs 2E & 3E), *Cpt1a* expression (Table 1, Figs 2F & 3F) was influenced by the interaction of sex and the post-weaning diet. In males of group OC expression was significantly lower (55%) than in all other groups, whilst in females of the same group expression tended to be higher (significant compared to OO). Surprisingly there was no correlation between expression of *PPARa* and either of the downstream targets, which may reflect the differential effects of the dietary exposures upon protein expression.

As shown in Table 2, global DNA methylation was significantly influenced by the post-weaning diet. In males but not females (interaction of sex x post-weaning diet *P*=0018), Post-weaning cafeteria diet significantly reduced global methylation. Methylation specific PCR found no significant effects of cafeteria diet upon *PPARa* , where little methylation was detected in any of the groups (data not shown). There was some evidence that exposure to the cafeteria diet during lactation, followed by weaning onto chow, increased methylation of *PPARg* (group OC), but this was not statistically significant (P=0.069, Table 2). In contrast there was a clear sensitivity of the *IrB* promoter to effects of diet upon methylation. As with *PPARg*, the OC group exhibited greater methylation than in all other groups (interaction of lactation and post-weaning diet P=0.036).

Table 3 shows the expression of PPARa, PPARg, Glut2 and IrB at the protein level. There were no significant effects of sex, maternal diet during lactation or offspring diet from weaning upon these proteins.

Pre-submission copy

Discussion

The cafeteria feeding protocol for rodents was originally developed by Rothwell and Stock (1979), as a means of generating obesity for the study of diet-induced thermogenesis. Cafeteria feeding promotes obesity by overcoming the ability of rodents to regulate energy intake, as it provides a diet with high palatability and novelty. Our study replicated the original protocol and cafeteria feeding involved the addition of a constantly varied panel of high sugar/high fat foods to standard laboratory chow diet. Although the protocol has critics because, by definition, the self-selection from a panel of varied foods makes it difficult to standardise actual food consumption between animals, it provides a good approximation of the drivers of weight gain and associated metabolic consequences in humans. A variety of feeding protocols using differing nutrient composition have been used to induce obesity in rodent models, including purified high fat diets (HFD), chow diets with supplemented fat, and cafeteria style self-selection diets. The literature (Buettner *et al*., 2007) demonstrates consistent evidence that a metabolic phenotype similar to that observed in the human can be induced, despite rodents not exhibiting progression to an atherosclerotic state. In addition to being high fat, the cafeteria diet is high in sodium, sugars, saturated fat and cholesterol, and low in fibre and micronutrients. It is therefore considered to be more closely aligned with dietary patterns observed in the humans than conventional purified high fat diets (Crozier *et al*., 2006,Kant 2004). Importantly, this model of diet-induced obesity does not rely on very high intakes of a particular type or source of fat. Therefore, whilst cafeteria feeding is not appropriate for studies focusing on the role of specific nutrients, it

Pre-submission copy

is considered the most effective tool for modelling the effects of 'non-prudent' dietary patterns and diet-induced obesity in humans (Sampey *et al*., 2011).

The study examined two key hypotheses and the data generated supports the assertion that maternal cafeteria feeding during lactation programmes metabolic function through long-term alteration of expression of the insulin-signalling and other metabolic pathways. We have also confirmed that the epigenome may be sensitive to this form of dietary challenge during the suckling phase in rodents. This corresponds with work demonstrating that high fat diets can impact upon histone acetylation and DNA methylation in rodents and macaques (Aagaard-Tillery *et al*., 2008, Vucetic *et al*., 2010, 2011).

From a metabolic perspective the animals most affected by the cafeteria diet were in the OO group. The slower clearance of a glucose load in this group is likely to be explained by their lower *IrB* expression. Clearly changes in expression of the insulin-signalling pathway (*IrB, Irs2, Akt1*) are contributing to the metabolic response to the diet and a role for *Glut2* has been excluded. As previously reported (Akyol *et al*., 2012), the differences in metabolic response to glucose are more likely to be due to this programming of the insulin-signalling pathway or glucose metabolism as there were no gross effects of the cafeteria diet upon other aspects of metabolic function. Whilst the post-weaning cafeteria diet promoted fat deposition, there was no effect of the diet in lactation on body fatness. Abdominal fat deposition in OC rats was no different to CC, and in CO was no different to OO (Akyol *et al.,* 2012). The data critically demonstrates that the transcriptional and metabolic effects seen in OO animals are not just a

Pre-submission copy

response to the diet at the time of measurement, as the nature of the response also depended upon the dietary exposure during lactation.

Using a similar protocol to the present study, Bayol *et al.*, (2010) found sex-specific effects of cafeteria diet upon insulin-signalling and glucose metabolism in rats. However, in the Bayol study insulin receptor expression was *increased* by cafeteria feeding at any stage of life in males and *Irs2* was down-regulated by cafeteria diet in females. Effects of the diet on expression of PPARg and glucose transporters in adipose tissue were also reported (Bayol *et al.*, 2008), with evidence that the dietary exposure of rats during fetal life and suckling could modify the later response to cafeteria feeding. The differences between the current study and the work by Bayol and colleagues, which are also reflected in the metabolic phenotypes that were observed (Akyol *et al.*, 2012, Bayol *et al.*, 2010), are most likely to be explained by variation in the nature of the cafeteria feeding regimens and different timing of exposure.

Earlier work in which the cafeteria diet was fed specifically during lactation in rats has shown this to be a period where maternal overfeeding can have a longer term programming effect upon the developing offspring. Cafeteria diet exposure during suckling alters anxiety-related behaviours in adult rats and has a profound impact upon the behavioural satiety sequence in adult females (Wright *et al.*, 2011a, 2011b). These effects suggest a programming effect upon specific pathways in the hippocampus and hypothalamus. In the present study we found that exposure to cafeteria feeding during lactation had a lasting modulatory impact upon glucose homeostasis and a programming effect upon the expression

Pre-submission copy

of *IrB* and *PPARa* in the liver. These effects, which favoured impairment of glucose tolerance in rats weaned onto cafeteria diet, persisted for 10 weeks beyond the period of exposure to the diet. The mechanism through which the suckling diet exerts these effects is open to debate. The signal from mother to pups that diet is altered could be behavioural, or may be mediated by changes to milk composition (Weaver *et al*., 2004, Hernandez *et al*., 2012). There is also likely to be a short period immediately prior to weaning where the pups are also able to directly consume the cafeteria foods and this may contribute to the longer-term programming effect. The present study has shown that, at least in liver, there are lasting effects of the exposure upon transcription of genes in the insulin-signalling and other metabolic pathways.

The reanalysis of mRNA expression data in this study showed that *Irs2* was up-regulated in response to post-weaning cafeteria feeding and we would suggest that this is likely to be a compensatory response to maintain glucose homeostasis. *Akt1* expression also responded to cafeteria feeding post-weaning, again an adaptation that would be expected to maintain homeostasis in the face of increasing adiposity and dietary challenge. There was clear variation in response dependent upon the lactation exposure. Differential responses to the post-weaning dietary challenge between males and females were noted with respect to the nature of adaptive responses to over-feeding. Males up-regulated *PPARg* rather than the *Irs2*/*Akt* pathway. This would provide a means of alternative disposal in glucose adipose tissue rather than liver. It is not clear why the regulatory responses to cafeteria diet should be sex-dependent. Studies of *Irs2* knockout mice, however, show differences between males and females in

Pre-submission copy

terms of metabolic and behavioural phenotypes (Masaki *et al.*, 2004). There are also reports of sex-differences in Akt1 responses to drug treatments (Chen *et al.*, 2013) and activation in the heart (Foryst-Ludwig *et al.*, 2011). Similarly the responses of PPARg deficient mice to agonist treatment and the expression of PPARg in adipose tissue have been reported to vary between males and females (Duan *et al.*, 2010, Kadowaki *et al.*, 2007). Typically males develop insulin resistance at a younger age than females, a phenomenon previously noted in the programming literature (Fernandez-Twinn *et al.*, 2005)

The assessment of global DNA methylation did not note any effects of cafeteria diet during lactation. This may be unremarkable as simple assays of 5-methyl-2'-deoxy cytidine are considerably less sensitive than sequencing based methods that were beyond the scope of this investigation. However, post-weaning diet did influence global methylation, consistent with reports of Vucetic *et al.*, 2010). It is of interest that this effect was seen only in males. *IrB* was the only specific gene target for which an effect of the diet upon DNA methylation was noted. However, whilst methylation was increased, there was no associated decrease in mRNA expression. In fact quite the opposite was apparent. Discordance of DNA methylation and mRNA expression has been widely reported (Carone *et al.*, 2010, Zhou *et al.*, 2011). The discordance suggests that the interplay between DNA methylation and gene expression is complex. Differences in methylation may only, for example, bring about expression changes during a metabolic challenge or in response to a specific stimulus. Furthermore, the effect of methylation on expression of a specific gene will also depend upon the histone marks associated with that promoter. Liver is not a homogenous tissue and

Pre-submission copy

contains many distinct cell types. It is important to note that methylation and expression may differ between different cell types and this may contribute to the discordance in the data. Programmed changes in the numbers and distribution of hepatocytes and non-parenchymal cells might contribute to some of the variation in gene expression observed between the CC/CO and OC/OO groups. Tissue remodelling is an established feature of programming associated with variation in maternal nutrition during rodent development (McMullen and Langley-Evans, 2010, McArdle *et al.*, 2011).

A general lack of major effects of the diet during lactation upon methylation of DNA might also result from the window of epigenetic sensitivity being much wider that originally suspected. If the diet fed during weaning can also change epigenetic marks, and possibly in a different manner to earlier phases, then effects of diet in lactation could be masked or erased. Investigation of this would require a different experimental design with earlier timepoints to consider the epigenome during periods of transition from one diet to another. The study of Jacobsen *et al.*, (2012) showed that when healthy young men were fed a short-term high-fat diet there were changes in genome-wide methylation in skeletal muscle, which reversed very slowly and were still present 8 weeks after the withdrawal of the diet. This is consistent with the view that dietary effects on the epigenome at one point in life can determine the response to nutritional cues, and the impact of the diet upon the genome at another. With ageing, the nature of the epigenome will reflect an accumulation of methylation patterns that are generated by dietary and environmental exposures across the lifecourse.

Pre-submission copy

It is clear from the data presented in this paper that there was marked discordance between the mRNA and protein measurements, with none of the gene expression changes verified at the protein level. This is not entirely unsurprising as a number of studies have shown that at most only 40% of the protein levels in mammalian cells are related to mRNA expression even in homogenous tissues and cultures. (Vogel *et al.,* 2010). Protein concentration is the product of transcription, mRNA stability, translation, post-translational modification and protein degradation. Transcription factors, such as PPARa and PPARg, are among a large group of proteins where both mRNA and protein have high rates of turnover (Schwanhauser *et al.,*2011). This applies also to cyclins which are apparent targets for nutritional programming in rats (Swali *et al*., 2011) The discordance of mRNA and protein data in this study could limit the capacity of the study to establish the underlying basis of physiological and metabolic phenotype, but does not lessen the significance of the observed effect of a highly variable, human-type, cafeteria diet upon transcription and the epigenome.

Feeding a cafeteria diet based upon highly palatable human foods to rats during lactation modulates the response of their offspring to the same dietary pattern post-weaning. Programmed changes in hepatic gene expression underpin these differential responses and may be, in part, driven by differences in DNA methylation. The study adds to a growing evidence base that suggests tissue function across the lifespan is determined by cumulative modifications to the epigenome and hence transcriptome, which may be both tissue and sex-specific.

Pre-submission copy

Acknowledgements

This work was funded by a small project grant from Diabetes UK.

Compliance with Ethics Guidelines

Zoe Daniel confirms that she has no conflict of interest. Asli Akyol confirms that she has no conflict of interest. Sarah McMullen confirms that she has no conflict of interest. Simon Langley-Evans declares that he has no conflict of interest. All institutional and national guidelines for the care and use of laboratory animals were followed.

References

Author AA, Author BB (1999) Title of paper. Jnl abbrev style 101:99-100.

Akyol A, Langley-Evans SC, McMullen S (2009) Obesity induced by cafeteria feeding and pregnancy outcome in the rat. Br J Nutr 102:1601-1610.

Akyol A, McMullen S, Langley-Evans SC (2012) Glucose intolerance associated with early-life exposure to maternal cafeteria feeding is dependent upon post-weaning diet. Br J Nutr 107:964-978.

Austin RM, Langley-Evans SC (2011) Cultured neonatal rat cardiomyocytes display differences in glucose uptake and sensitivity to dexamethasone related to maternal diet. J DOHAD 2:190-194.

Bayol SA, Simbi BH, Bertrand JA, Stickland NC (2008) Offspring from mothers fed a 'junk food' diet in pregnancy and lactation exhibit exacerbated adiposity that is more pronounced in females. J Physiol 586:3219-3230.

Pre-submission copy

Bayol SA, Simbi BH, Fowkes RC, Stickland NC (2010) A maternal "junk food" diet in pregnancy and lactation promotes nonalcoholic Fatty liver disease in rat offspring. Endocrinology 151:1451-1461.

Buettner R, Scholmerich J, Bollheimer LC (2007) High-fat diets: modeling the metabolic disorders of human obesity in rodents. Obesity15:798-808.

Bogdarina I, Haase A, Langley-Evans S, Clark AJ (2010) Glucocorticoid effects on the programming of AT1b angiotensin receptor gene methylation and expression in the rat. PLoS One. 2010 Feb 16;5(2):e9237.

Burdge GC, Lillycrop KA, Phillips ES, Slater-Jefferies JL, Jackson AA, Hanson MA (2009). Folic acid supplementation during the juvenile-pubertal period in rats modifies the phenotype and epigenotype induced by prenatal nutrition. J Nutr 139:1054-1060.

Carlin J, George R, Reyes TM (2013). Methyl donor supplementation blocks the adverse effects of maternal high fat diet on offspring physiology. PLoS One 8:e63549.

Carone BR, Fauquier L, Habib N, Shea JM, Hart CE, et al. (2010) Paternally induced transgenerational environmental reprogramming of metabolic gene expression in mammals. Cell 143:1084-1096

Chen YW, Kao HY, Min MY, Lai WS (2013). A Sex- and Region-Specific Role of Akt1 in the Modulation of Methamphetamine-Induced Hyperlocomotion and Striatal Neuronal Activity: Implications in Schizophrenia and Methamphetamine-Induced Psychosis. Schizophr Bull In Press.

Crozier SR, Robinson SM, Borland SE, Inskip HM; SWS Study Group (2006). Dietary patterns in the Southampton Women's Survey. Eur J Clin Nutr 60:1391-1399.

Duan SZ, Usher MG, Foley EL 4th, Milstone DS, Brosius FC 3rd, Mortensen RM (2010). Sex dimorphic actions of rosiglitazone in generalised peroxisome proliferator-activated receptor-gamma (PPAR-gamma)-deficient mice. Diabetologia 53:1493-1505.

Fernandez-Twinn DS, Wayman A, Ekizoglou S, Martin MS, Hales CN, Ozanne SE (2005).

Pre-submission copy

Maternal protein restriction leads to hyperinsulinemia and reduced insulin-signaling protein expression in 21-mo-old female rat offspring. Am J Physiol Regul Integr Comp Physiol 288:R368-R373.

Floyd ZE, Stephens JM (2012). Controlling a master switch of adipocyte development and insulin sensitivity: covalent modifications of PPARγ. Biochim Biophys Acta 1822:1090-10955.

Foryst-Ludwig A, Kreissl MC, Sprang C, Thalke B, Böhm C, Benz V, Gürgen D, Dragun D, Schubert C, Mai K, Stawowy P, Spranger J, Regitz-Zagrosek V, Unger T, Kintscher U (2011). Sex differences in physiological cardiac hypertrophy are associated with exercise-mediated changes in energy substrate availability. Am J Physiol Heart Circ Physiol 301:H115-H122.

Gambling L, Dunford S, Wallace DI, Zuur G, Solanky N, Srai SK, McArdle HJ (2003). Iron deficiency during pregnancy affects postnatal blood pressure in the rat. J Physiol 552:603-610.

Hernandez LL, Grayson BE, Yadav E, Seeley RJ, Horseman ND (2012). High fat diet alters lactation outcomes: possible involvement of inflammatory and serotonergic pathways. PLoS One 7:e32598.

Jacobsen SC, Brøns C, Bork-Jensen J, Ribel-Madsen R, Yang B, Lara E, Hall E, Calvanese V, Nilsson E, Jørgensen SW, Mandrup S, Ling C, Fernandez AF, Fraga MF, Poulsen P, Vaag A (2012). Effects of short-term high-fat overfeeding on genome-wide DNA methylation in the skeletal muscle of healthy young men. Diabetologia 55:3341-3349.

Kadowaki K, Fukino K, Negishi E, Ueno K (2007). Sex differences in PPARgamma expressions in rat adipose tissues. Biol Pharm Bull 30:818-820.

Kahn A (1997) Transcriptional regulation by glucose in the liver. Biochimie 79: 113-118.

Kant AK (2004) Dietary patterns and health outcomes. J Am Diet Assoc 104:615-635.

Langie SA, Achterfeldt S, Gorniak JP, Halley-Hogg KJ, Oxley D, van Schooten FJ, Godschalk RW, McKay JA, Mathers JC (2013) Maternal folate depletion and high-fat feeding from

Pre-submission copy

weaning affects DNA methylation and DNA repair in brain of adult offspring. FASEB J27:3323-3334.

Langley SC, Jackson AA (1994) Increased systolic blood pressure in adult rats induced by fetal exposure to maternal low protein diets. Clin Sci 86:217-222

Langley-Evans SC, Welham SJ, Jackson AA (1999) Fetal exposure to a maternal low protein diet impairs nephrogenesis and promotes hypertension in the rat. Life Sci 64:965-974.

Lillycrop KA, Burdge GC (2011) Epigenetic changes in early life and future risk of obesity. Int J Obes 35:72-83.

Lillycrop KA, Slater-Jefferies JL, Hanson MA, Godfrey KM, Jackson AA, Burdge GC (2007). Induction of altered epigenetic regulation of the hepatic glucocorticoid receptor in the offspring of rats fed a protein-restricted diet during pregnancy suggests that reduced DNA methyltransferase-1 expression is involved in impaired DNA methylation and changes in histone modifications. Br J Nutr 97:1064-1073.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265-275.

Masaki T, Chiba S, Noguchi H, Yasuda T, Tobe K, Suzuki R, Kadowaki T, Yoshimatsu H (2004) Obesity in insulin receptor substrate-2-deficient mice: disrupted control of arcuate nucleus neuropeptides. Obes Res 12: 878-885.

McArdle HJ, Gambling L, Lang C, Swali A, Langley-Evans SC, McMullen S (2011) A common cause for a common phenotype- the gatekeeper hypothesis in fetal programming. Medical Hypotheses 78: 88-94.

McMullen S, Langley-Evans SC (2010) The developmental origins of adult disease. Medical Principles and Practice. 19, 87-98.

Menendez-Gutierrez MP, Roszer T, Ricote M (2012). Biology and therapeutic applications of peroxisome proliferator- activated receptors. Curr Top Med Chem 12; 548-584.

Pre-submission copy

Rothwell NJ, Stock MJ (1979) Regulation of energy balance in two models of reversible obesity in the rat. J Comp Physiol Psychol 93:1024-1034.

Ryan KJP, Elmes MJ, Gardner DS, Langley-Evans SC (2012) The effects of prenatal protein restriction on ß-adrenergic signalling of the adult rat heart during ischaemia. J Nutr Metab 12:397389.

Sampey BP, Vanhoose AM, Winfield HM, Freemerman AJ, Muehlbauer MJ, Fueger PT, Newgard CB, Makowski L (2011). Cafeteria diet is a robust model of human metabolic syndrome with liver and adipose inflammation: comparison to high-fat diet. Obesity 19:1109-1117.

Samuelsson AM, Matthews PA, Argenton M, Christie MR, McConnell JM, Jansen EH, Piersma AH, Ozanne SE, Twinn DF, Remacle C, Rowlerson A, Poston L, Taylor PD (2008). Diet-induced obesity in female mice leads to offspring hyperphagia, adiposity, hypertension, and insulin resistance: a novel murine model of developmental programming. Hypertension 51: 383-392.

Schwanhäusser B, Busse D, Li N, Dittmar G, Schuchhardt J, Wolf J, Chen W, Selbach M (2011) Global quantification of mammalian gene expression control. Nature 473:337-342.

Seki Y, Williams L, Vuguin PM, Charron MJ (2012) Minireview: Epigenetic programming of diabetes and obesity: animal models. Endocrinology 153:1031-1038.

Shankar K, Harrell A, Liu X, Gilchrist JM, Ronis MJ, Badger TM (2008) Maternal obesity at conception programs obesity in the offspring. Am J Physiol Regul Integr Comp Physiol 294:R528-R538.

Sinclair KD, Allegrucci C, Singh R, Gardner DS, Sebastian S, Bispham J, Thurston A, Huntley JF, Rees WD, Maloney CA, Lea RG, Craigon J, McEvoy TG, Young LE (2007) DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status. Proc Natl Acad Sci U S A 104:19351-19356.

Pre-submission copy

Swali A, McMullen S, Hayes H, Gambling L, McArdle H, Langley-Evans (2011) Cell cycle regulation and cytoskeletal remodelling are critical processes in the nutritional programming of embryonic development. PlosOne 6: e23189

Swali A, McMullen S, Hayes H, Gambling L, McArdle HJ and Langley-Evans SC (2012) Processes Underlying the Nutritional Programming of Embryonic Development by Iron Deficiency in the Rat. PLOS One 7: e48133.

Szyf M (2009). The early life environment and the epigenome. Biochim Biophys Acta 1790:878-885

Torrens C, Hanson MA, Gluckman PD, Vickers MH (2009) Maternal undernutrition leads to endothelial dysfunction in adult male rat offspring independent of postnatal diet. Br J Nutr 101:27-33.

Viana Abranches M, Esteves de Oliveira FC, Bressan J (2011) Peroxisome proliferator-activated receptor: effects on nutritional homeostasis, obesity and diabetes mellitus. Nutr Hosp 26:271-279.

Vickers MH, Breier BH, McCarthy D, Gluckman PD (2003) Sedentary behavior during postnatal life is determined by the prenatal environment and exacerbated by postnatal hypercaloric nutrition. Am J Physiol Regul Integr Comp Physiol 285:R271-R273.

Vogel C, Abreu Rde S, Ko D, Le SY, Shapiro BA, Burns SC, Sandhu D, Boutz DR, Marcotte EM, Penalva LO (2010) Sequence signatures and mRNA concentration can explain two-thirds of protein abundance variation in a human cell line. Mol Syst Biol 6:400.

Weaver IC, Cervoni N, Champagne FA, D'Alessio AC, Sharma S, Seckl JR, Dymov S, Szyf M, Meaney MJ (2004) Epigenetic programming by maternal behavior. Nat Neurosci 7:847-854.

Wright T, Langley-Evans SC, Voigt JP (2011a) The impact of maternal cafeteria diet on anxiety-related behaviour and exploration in the offspring. Physiology and Behaviour 103:164-172.

Pre-submission copy

Wright T, Fone K, Langley-Evans SC, Voigt JP (2011b) Exposure to maternal consumption of cafeteria diet during the lactation period programmes feeding behaviour in the rat. Int J Develop Neurosci 29:785-793.

Yates Z, Tarling EJ, Langley-Evans SC, Salter AM (2008) Maternal undernutrition programmes atherosclerosis in the Apo E*3 Leiden mouse. Br J Nutr 101:1185-1194.

Zhou Z, Yuan Q, Mash DC, Goldman D (2011) Substance-specific and shared transcription and epigenetic changes in the human hippocampus chronically exposed to cocaine and alcohol. Proc Natl Acad Sci U S A 108: 6626-6631.

Pre-submission copy

Figure legends

Figure 1. Area under the curve from glucose tolerance test.

Data are shown as mean±SEM for 9-10 observations per group. Data is shown for males and females combined (no significant effect of sex) and adjusted for sex. * indicates significantly different to OC (P<0.05). Glucose tolerance area under the curve was influence by an interaction of lactation and post-weaning diet (P=0.039). Reanalysis of data from Akyol *et al*., 2012.

Figure 2. mRNA expression of insulin signalling pathway-related genes in male rat liver at 3 months of age.

Data are shown as mean±SEM for 4-5 observations per group. * indicates significantly different to CC (P<0.05), § significantly different to CO (P<0.05), ! significantly different to OC (P<0.05).

A. *PPARa* expression was different in males and females (P=0.019) and was influenced by exposure to cafeteria diet during lactation (P=0.038).

B. *PPARg* expression was different in males and females (P=0.002) and was influenced by the interaction of sex and cafeteria diet post-weaning (P=0.050).

C. *Glut2* expression was not influenced by sex or dietary exposures.

D. *IrB* expression was influenced by the interaction of sex, diet in lactation and diet post-weaning (P=0.041).

E. *Acc1* expression was not influenced by sex or dietary exposures.

F. *Cpt1a* expression was influenced by sex (P=0.026) and the interaction of sex and post-weaning diet (P=0.023).

Pre-submission copy

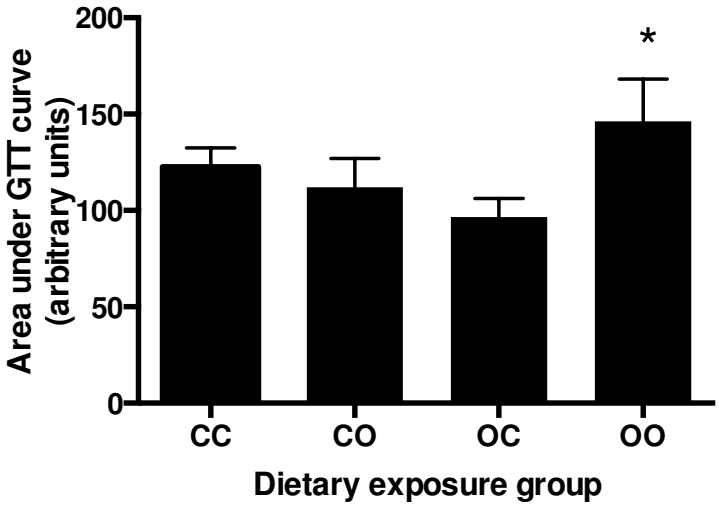
Figure 3. mRNA expression of insulin signalling pathway-related genes in female rat liver at 3 months of age.

Data are shown as mean±SEM for 4-5 observations per group. * indicates significantly different to CC (P<0.05), § significantly different to CO (P<0.05), ! significantly different to OC (P<0.05).

For full legend see Figure 2.

Pre-submission copy

Figure 1



Pre-submission copy

Figure 2

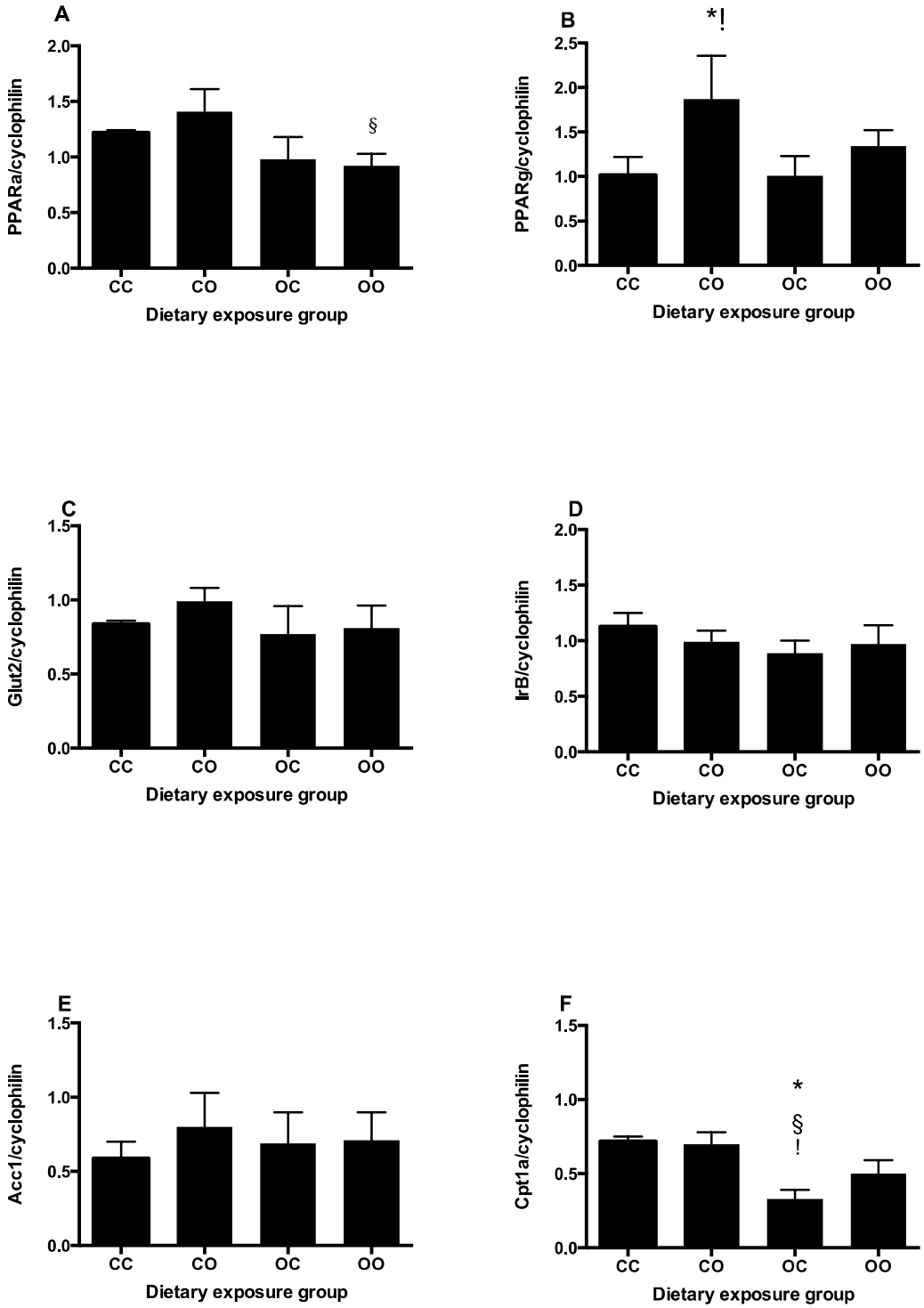

A
PPARa/cyclophilin
2.0
1.5
1.0
0.5
0.0
§
CC
CO
OC
OO
Dietary exposure group
B
PPARg/cyclophilin
2.5
2.0
1.5
1.0
0.5
0.0
*!
CC
CO
OC
OO
Dietary exposure group
C
Glut2/cyclophilin
1.5
1.0
0.5
0.0
CC
CO
OC
OO
Dietary exposure group
D
IrB/cyclophilin
2.0
1.5
1.0
0.5
0.0
CC
CO
OC
OO
Dietary exposure group
E
Acc1/cyclophilin
1.5
1.0
0.5
0.0
CC
CO
OC
OO
Dietary exposure group
F
Cpt1a/cyclophilin
1.5
1.0
0.5
0.0
*
§
!
CC
CO
OC
OO
Dietary exposure group

Figure 3

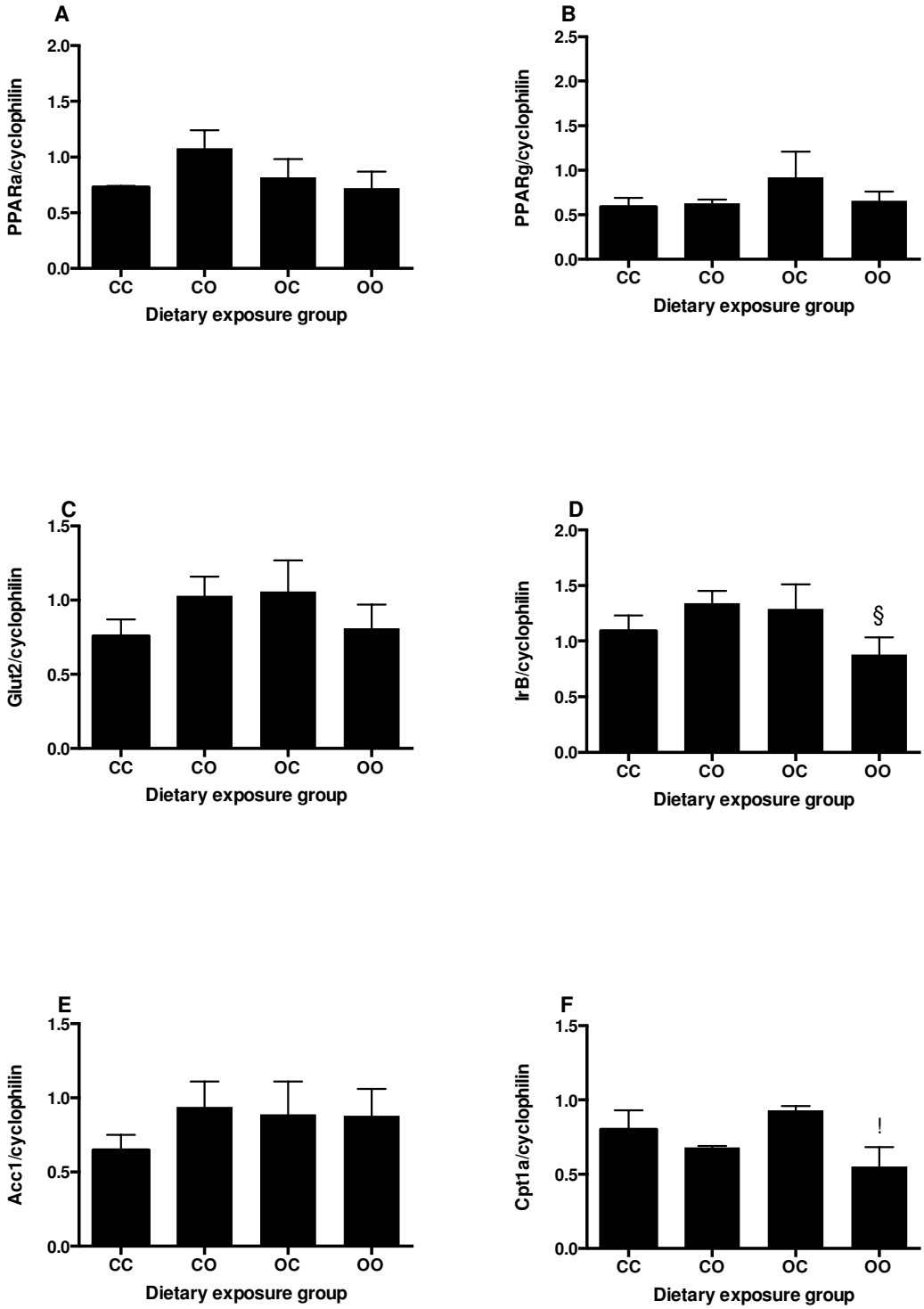

A
PPARa/cyclophilin
2.0
1.5
1.0
0.5
0.0
CC
CO
OC
OO
Dietary exposure group
B
PPARg/cyclophilin
2.5
2.0
1.5
1.0
0.5
0.0
CC
CO
OC
OO
Dietary exposure group
C
Glut2/cyclophilin
1.5
1.0
0.5
0.0
CC
CO
OC
OO
Dietary exposure group
D
IrB/cyclophilin
2.0
1.5
1.0
0.5
0.0
§
CC
CO
OC
OO
Dietary exposure group
E
Acc1/cyclophilin
1.5
1.0
0.5
0.0
CC
CO
OC
OO
Dietary exposure group
F
Cpt1a/cyclophilin
1.5
1.0
0.5
0.0
!
CC
CO
OC
OO
Dietary exposure group

Table 1. Impact of cafeteria diet during lactation and post-weaning on mRNA expression

Gene	*P* effect of sex	Lactation Fold Change (OC and OO vs CC and CO)	*P* effect of cafeteria diet during lactation	Post-weaning Fold Change (CO and OO vs CC and OC)	*P* effect of cafeteria diet post-weaning
Irs2	0.002	-1.29	NS	1.63§	0.043
Akt1	NS	2.45*€	0.008	-1.51*€	NS
IrB	NS	-1.04+	NS	-1.12+	NS
Glut2	NS	1.06	NS	1.07	NS
PPARa	0.019	-1.29	0.038	1.10	NS
Cpt1a	0.026	1.30	0.059	1.06§	NS
Acc1	NS	1.05	NS	1.17	NS
PPARg	0.002	-1.04	NS	1.27§	NS

Data are shown as Fold Change (- denotes down-regulation). *P* values for lactation and post-weaning effects are univariate analyses. Interaction terms are not shown in this table, see legend Figure 2, but are indicated by § (sex x post-weaning, $P<0.05$), € (lactation x post-weaning, $P<0.05$) and * (sex x lactation x post-weaning, $P<0.05$).

Pre-submission copy

Table 1 DNA Methylation in liver of 3 month old rats

	Dietary exposure groups							
	CC		CO		OC		OO	
	Male	Female	Male	Female	Male	Female	Male	Female
Global methylation (pg MDC/µg DNA)	190±11	174±12	154±4	176±4	166±12	160±6	120±11	212±5
Methylation PPARg (%)	0.47±0.17	0.42±0.20	0.61±0.38	0.48±0.23	9.24±6.63	9.19±6.62	0.27±0.04	0.21±0.04
Methylation IrB (%)	0.60±0.14	0.54±0.12	1.43±0.68	0.91±0.68	13.1±7.72	13.1±7.68	0.46±0.05	0.49±0.02

Data are shown as mean±SEM for 5-6 observations per group. Global methylation was influenced by the interaction of sex and post-weaning diet (P=0.018). Methylation of PPARg was not influenced by sex or diet at any stage. IrB methylation was significantly influenced by the interaction of diet in lactation and weaning (P=0.036), with univariate influences of lactation (P=0.058) and post-weaning (P=0.054) diets approaching significance. MDC- 5-methyl-2'-deoxy cytidine .

Pre-submission copy

Table 2. Protein expression in liver of 3 month old rats

	Dietary exposure groups							
	CC		CO		OC		OO	
	Male	Female	Male	Female	Male	Female	Male	Female
IrB	0.95±0.09	1.16±0.09	0.85±0.08	1.07±0.15	0.92±0.11	1.24±0.10	1.05±0.19	0.99±0.13
PPARa	1.32±0.17	1.26±0.13	1.21±0.24	1.30±0.6	0.92±0.09	1.08±0.04	1.36±0.28	1.20±0.06
PPARg	1.16±0.22	1.05±0.24	1.01±0.23	1.01±0.24	1.09±0.23	1.37±0.20	1.21±0.33	1.16±0.26
Glut2	1.07±0.03	1.08±0.04	1.06±0.03	1.07±0.04	1.05±0.03	1.08±0.04	1.10±0.03	1.20±0.05

Data are shown as mean±SEM for 4-5 observations per group. All protein expression was normalised to expression of beta-actin.

Expression of all proteins, including the housekeeper beta-actin was not influenced by sex or dietary exposures.

Pre-submission copy