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Epigenetic regulation of *POMC*; implications for nutritional programming, obesity and metabolic disease

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ABSTRACT

Proopiomelanocortin (*POMC*) is a key mediator of satiety. Epigenetic marks such as DNA methylation may modulate *POMC* expression and provide a biological link between early life exposures and later phenotype. Animal studies suggest epigenetic marks at *POMC* are influenced by maternal energy excess and restriction, prenatal stress and Triclosan exposure. Postnatal factors including energy excess, folate, vitamin A, conjugated linoleic acid and leptin may also affect *POMC* methylation. Recent human studies suggest *POMC* DNA methylation is influenced by maternal nutrition in early pregnancy and associated with childhood and adult obesity. Studies in children propose a link between *POMC* DNA methylation and elevated lipids and insulin, independent of body habitus. This review brings together evidence from animal and human studies and suggests that *POMC* is sensitive to nutritional programming and is associated with a wide range of weight-related and metabolic outcomes.

1. Background

Proopiomelanocortin (*POMC*), a pro-hormone, gives rise to numerous active peptides and hormones with a wide range of physiological actions (Toda et al., 2017). In the brain, *POMC* is expressed in the arcuate nucleus (ARC) of the hypothalamus, the pituitary gland and the brain stem. Tissue-specific post-translational proteolysis of *POMC* gives rise to the active hormones ACTH (adrenocorticotropic hormone), α-, β– and γ- MSH (melanocyte stimulating hormone) and β – endorphin (see Fig. 1). Different populations of *POMC* neurons produce different amounts of the active hormones dependent on the expression levels of prohormone convertases e.g. corticotrophs of the anterior pituitary produce predominantly ACTH, whereas melanotrophs of the hypothalamus produce predominantly α- and β-MSH (Toda et al., 2017; Cone, 2005).

POMC is a key component of the melanocortin system (Mountjoy, 2015); a complex network of systemic signals and neural pathways that regulate food intake and energy balance (see Fig. 2). *POMC* neurons in the ARC of the hypothalamus integrate peripheral signals such as leptin (Balthasar et al., 2004), glucose (Parton et al., 2007) and insulin (Qiu et al., 2014), and regulate energy balance by inducing satiety and increasing energy expenditure (Toda et al., 2017). Satiety is mediated via the actions of α- and β-MSH on melanocortin 4 receptors (MC4R) in the paraventricular nucleus (PVN) of the hypothalamus (Schioth et al.,

Abbreviations: 5hmC, 5-hydroxymethylcytosine; 5mC, 5-methylcytosine; ACTH, adrenocorticotropic hormone; *AgRP*, agouti-related peptide; AN, anorexia nervosa; ARC, arcuate nucleus of the hypothalamus; A^{vy}, agouti viable yellow; $Axin^{Fu}$, axin-fused; BEC, buccal epithelial cells; BMI, body mass index; CAF, Cafeteria diet; C-C, control diet – control diet (pre and postnatally); CLA, conjugated linoleic acid; CpG, cytosine-phosphate-guanine; CREB1, cAMP responsive element binding protein 1; DIO, diet induced obesity; DNMT1, DNA methyltransferase 1; DR, diet resistant; E2F, E2 Factor; ERRα, estrogen-related receptor alpha; FS-FS, high fat - high sucrose diet (pre and postnatally); H3K9, Histone3 Lysine9; HA, high vitamin A; HC, high carbohydrate; HDAC, histone deacetylase; HDL, high density lipoprotein; HFD, high fat diet; HFol, high folate diet; HOMA-IR, homeostatic model assessment of insulin resistance; HV, high vitamin; IAP, intracisternal A particle; JAK, Janus Kinase; LA, linoleic acid; MBD1, methyl binding domain protein 1; MC4R, melanocortin 4 receptors; Mecp2, methyl CpG binding protein; ME, metastable epiallele; MM, mother's milk; MSH, melanocyte stimulating hormone; nPE, neural *POMC* enhancer; NF-KB, nuclear factor K-B; *NPY*, neuropeptide-Y; PAR, predictive adaptive response; PBC, peripheral blood cell; PNS, Prenatal stress; *POMC*, proopiomelanocortin; PUFA, polyunsaturated fatty acids; PVN, paraventricular nucleus of the hypothalamus; RELA, v-rel reticuloendotheliosis viral oncogene homologue A (avian); RV, recommended intake of vitamins; SAH, S-adenosyl homocysteine; SAM, S-adenosyl methionine; SETDB1, SET domain binding 1; SFA, saturated fatty acid; Sp1, Specificity Protein 1; SRY, sex determining region Y; STAT3, signal transducer and activator of transcription 3; VMR, variably methylated region; Y^{NPAR}, non-paring region of the Y chromosome

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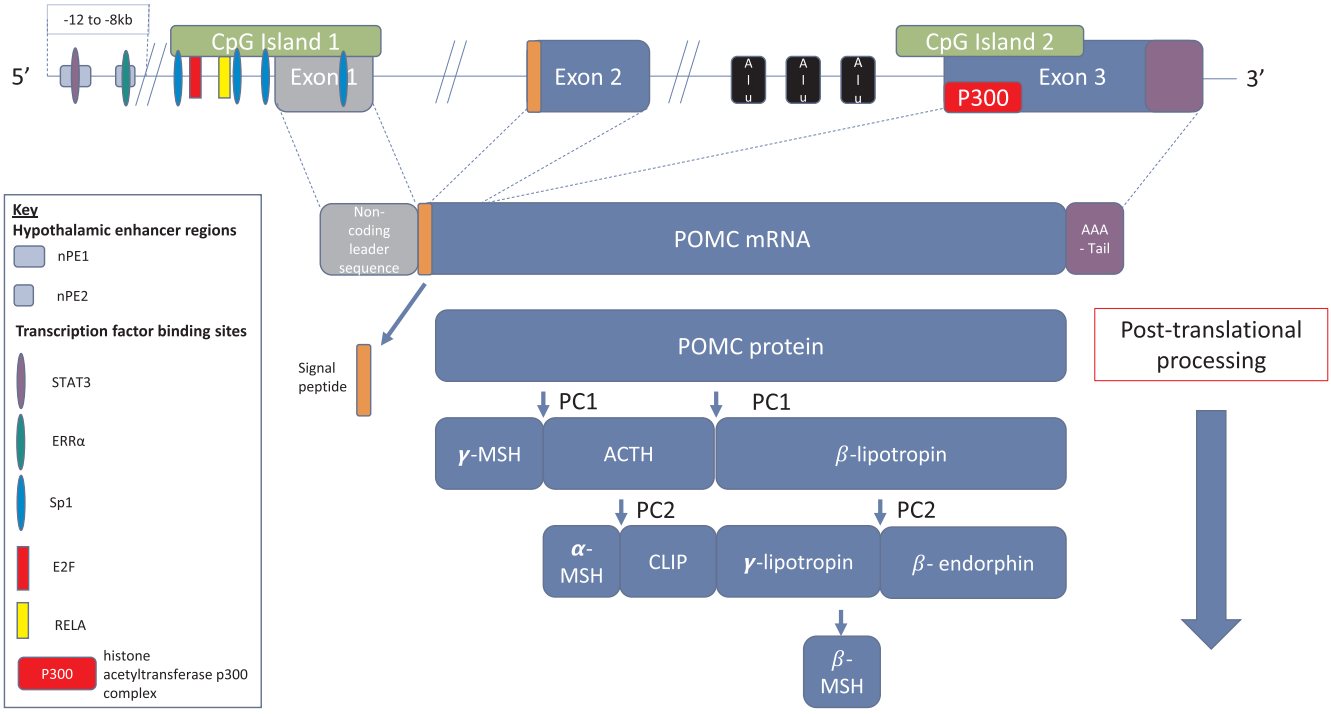

Fig. 1. Human *POMC* Gene; transcription, translation and post-translational processes. The human *POMC* gene consists of 3 exons and 2 large introns and is located between chromosome 2:25,383,722 to 25,391,722 (hg19, reverse strand). There are two CpG islands related to the *POMC* gene; the first in the promoter region and second over the boundary of intron2/exon3. Exon 1 (87 bp) contains a non-coding sequence and produces a short leader sequence that binds the ribosome at the start of translation. Exon 2 (152 bp) gives rise to a small signal peptide and forms the N-terminal end of the *POMC* peptide. Exon 3 (835 bp) produces the majority of the *POMC* peptide as well the signal for the addition of the poly-A tail. The figure provides schematic representation of the key transcriptional enhancers and binding sites related to leptin signalling and hypothalamic expression of neuropeptides (discussed in this article). Key: CpG; cytosine-guanine dinucleotide, Alu; Alu element, P300; P300 complex binding domain, *POMC*; Proopiomelanocortin, PC1; Prohormone convertase 1, PC2; Prohormone convertase 2, -MSH; -melanocyte stimulating hormone, ACTH; Adrenocorticotropic hormone, CLIP; corticotropin-like intermediate peptide, AAA-tail; poly-A tail, nPE; neuro *POMC* enhancer, STAT3, Signal transducer and activator of transcription 3, ERRα; estrogen-related receptor alpha, Sp1; Specificity Protein 1, E2F; E2 Factor, RELA; v-rel reticuloendotheliosis viral oncogene homologue A (avian).

2002; Biebermann et al., 2006). An opposing group of orexigenic neurons (Agouti-related peptide (*AgRP*) and neuropeptide-Y (*NPY*)) receive systemic inputs from ghrelin (released from enteroendocrine cells) and have the opposite action by increasing appetite and decreasing energy expenditure by antagonising MC4R and by direct inhibition of satiety neurons in the PVN (Toda et al., 2017; Andermann and Lowell, 2017). Perturbations of the melanocortin system can lead to disorders of energy balance such as obesity. For example, individuals with bi-allelic loss of function mutations in *POMC* demonstrate early hyperphagia, severe obesity (due to α-/β-MSH deficiency) and central adrenal insufficiency (due to ACTH deficiency) (Krude et al., 1998). It has been demonstrated that heterozygote variant carriers have an increased risk of developing obesity but with no adrenal insufficiency (Farooqi et al., 2006; Krude et al., 2003) suggesting a gene dosage effect on energy balance.

Epigenetic processes, including DNA methylation, histone modification, chromatin remodelling and RNA-based mechanisms can affect gene expression (Gibney and Nolan, 2010). DNA methylation is widely studied in animals and humans and occurs primarily at cytosine-guanine (CG) dinucleotides (CpG methylation). DNA methylation is usually associated with condensed heterochromatin and subsequent gene silencing or reduced gene expression (Jones, 2012). DNA methylation is mitotically heritable (Riggs and Porter, 1996) and is influenced by genetic and environmental factors (Hannon et al., 2018; Jaenisch and Bird, 2003; James et al., 2018).

In humans, the *POMC* gene is found on chromosome 2p23 and consists of three exons. Exon 3 codes for the majority of translated mRNA (see Fig. 1) and functionally-relevant peptides i.e. ACTH, α-/β-MSH, β – endorphin (Takahashi et al., 1983). The gene contains two CpG islands (areas of the genome with high CpG density), the first in the 5′-promoter region and the second at the boundary of intron2/exon3 (Kuehnen and Krude, 2012).

POMC expression is controlled by different and independent transcriptional enhancers in the pituitary and hypothalamus (de Souza et al., 2005) (see Fig. 1). Of particular relevance to *POMC*'s role in energy balance is regulation of gene expression in the hypothalamus. In the hypothalamus, two proximal enhancers; neural *POMC* enhancer 1 (nPE1) and 2 (nPE2) regulate *POMC* expression. nPE1 contains a STAT3 (Signal transducer and activator of transcription – 3) binding site, important for leptin signalling. Leptin's transcriptional activation of *POMC* is dependent on the Janus kinase (JAK)-STAT pathway with STAT 3 and 5 especially relevant for mediating satiety (Ladyman and Grattan, 2013). STAT activation of *POMC* transcription is also dependent on Specificity Protein 1 (Sp1) binding to a region in the *POMC* promoter (Yang et al., 2009). nPE2 is a shorter genomic region and contains a binding site for the estrogen-related receptor alpha (ERRα) (de Souza et al., 2005; Drouin, 2016). These enhancer and promoter binding regions are functionally conserved in rodents and humans (de Souza et al., 2005; Hill, 2010; Bumaschny et al., 2007). In both humans and mice, additional transcriptional enhancer activity has also been identified at the boundary of intron2/exon3. This region contains a histone acetyltransferase p300 complex binding site that is associated with gene activation (Kuehnen et al., 2012; Lacaze-Masmonteil et al., 1987). The majority of animal studies (see Tables 1 and 2) concentrate on the promoter region, but more recently, human studies have examined other regulatory regions.

Due to *POMC*'s involvement in multiple physiological processes, epigenetic changes at *POMC* are associated with a diverse range of

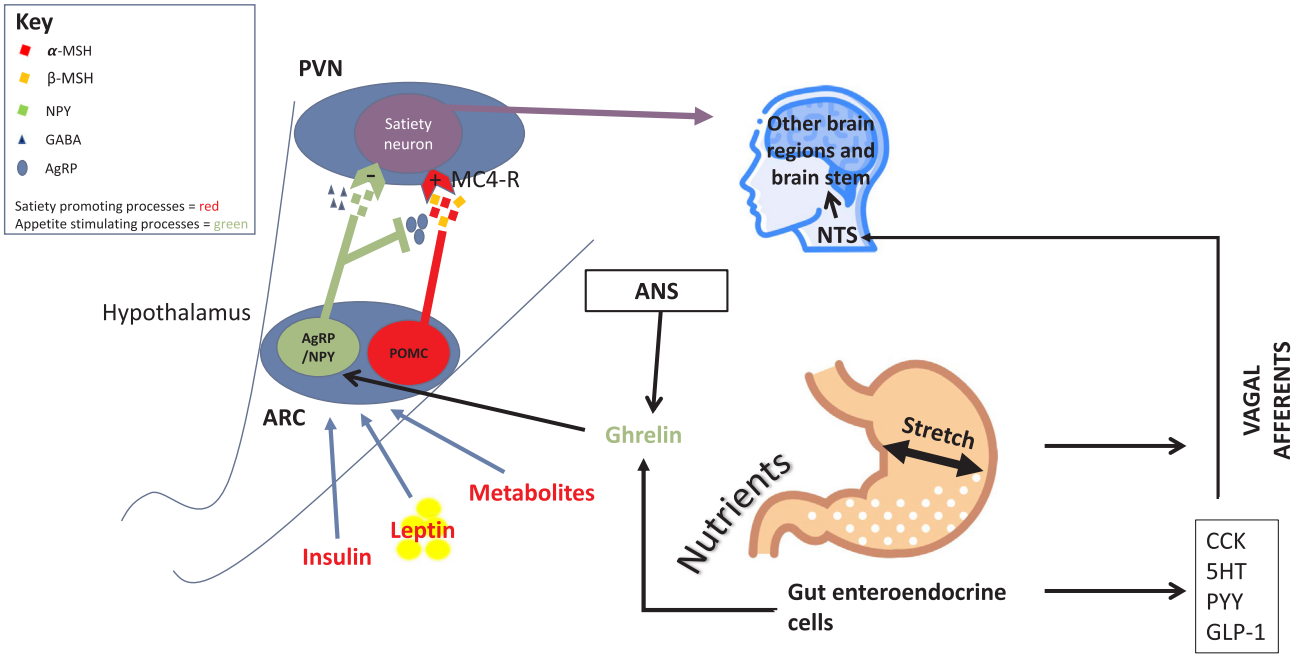

Fig. 2. Schematic diagram of the melanocortin system and the control of appetite and satiety. At the level of the hypothalamus, appetite and satiety regulating neurons (POMC, AgRP/NPY) of the arcuate nucleus (ARC) send projections to the paraventricular nucleus (PVN), The anorectic POMC expressing neurons are responsive to systemic signals including leptin, insulin and metabolites (such as glucose), ***α*** and β-MSH (derived from POMC) mediates the satiety signal via the action on MC4R. AgRP/NPY expressing neurons respond to ghrelin (which is predominantly under the control of the autonomic nervous system (ANS)), a hormone released by enteroendocrine cells that acts to increase appetite. AgRP antagonises the action of ***α***-MSH at MC4R, whilst the neurotransmitters NPY and GABA convey an orexigenic signal via PVN neurons. Meal termination (satiation) is brought about via activation of vagal afferents from stomach stretch receptors and nutrient-induced release of enteroendocrine factors (CCK, 5HT, PYY, GLP-1). The vagal afferents send projections to the NTS (nucleus tractus solitarii) to bring about meal termination. Abbreviations: POMC; Proopiomelanocortin, AgRP; Agouti-related peptide, NPY; neuropeptide-Y, ***α***-MSH; alpha-melanocyte stimulating hormone, β-MSH; beta-melanocyte stimulating hormone, MC4R; melanocortin 4 receptors, GABA; gamma-aminobutyric acid, NTS; nucleus tractus solitarii, CCK; cholecystokinin, 5HT; 5-hydroxytryptamine, PYY; Peptide YY, GLP-1; glucagon-like peptide 1, ARC; arcuate nucleus of the hypothalamus, PVN; paraventricular nucleus of the hypothalamus, ANS; autonomic nervous system.

phenotypes. In this review, we present evidence linking epigenetic regulation of *POMC* to nutritional programming, obesity, energy balance and metabolic outcomes.

2. Methods

A MEDLINE database search was conducted using the following search terms; (((epigen*) OR methylation)) AND (((*POMC*) OR Proopiomelanocortin) OR Pro-opiomelanocortin)) on 12th November 2018. This search produced 181 articles. The purpose of this systematic narrative review is to summarise the evidence, in both animals and humans, of epigenetic regulation of *POMC* relevant to nutritional programming, energy balance, obesity and metabolic outcomes. Therefore, only articles that referred to obesity, body weight or BMI (body mass index) or energy balance, adiposity, lipid or glucose metabolism were subsequently included. Articles concerning intergenerational or transgenerational processes were also included as these are relevant to nutritional programming. Articles with no specific reference to *POMC* epigenetic processes (such as DNA methylation or histone modifications) were excluded. Reference sections of included studies were also reviewed, and additional relevant papers were included if not found during the initial MEDLINE search.

3. Animal studies

3.1. Prenatal exposures, epigenetic alterations in the POMC gene and offspring phenotype

Animal studies have allowed researchers to manipulate the fetal environment and examine subsequent postnatal phenotypes under controlled conditions. Fetal adaptation to the *in utero* environment may present a survival advantage by the organism being better prepared for the anticipated *ex utero* environment (Gluckman and Hanson, 2004). For example, a consequence of fetal adaptation to prenatal energy restriction might be for offspring to be better adapted to postnatal nutritional scarcity by, for instance, maintaining a smaller body size and having greater energy efficiency and increased appetite. This could result in later obesity if there is a mismatch between *in utero* and *ex utero* environments and forms the basis of the predictive adaptive response (PAR) hypothesis (Gluckman et al., 2005). Alternatively, environmentally induced epigenetic changes may simply be a consequence of the exposure on normal epigenetic development (Guerrero-Bosagna, 2017). Either way, a better understanding of how epigenetic mechanisms can mediate links between prenatal exposures and postnatal phenotype will have important implications for human health. A number of animal studies have examined how altering the *in utero* nutritional and/or prenatal environment influences *POMC* epigenetic marks and the subsequent phenotype in the offspring (see Table 1).

3.1.1. Maternal energy excess

Maternal overnutrition, induced by feeding pregnant rats high energy diets, has been shown to lead to persistent changes in offspring phenotype with accompanying epigenetic changes (Gali Ramamoorthy et al., 2018; Marco et al., 2014; Zheng et al., 2015).

Rat pups exposed to high fat diet (HFD) *in utero* have higher *POMC* DNA methylation and a shift towards an obese phenotype as adults. This was demonstrated in a study by Marco et al. (2014) who showed that, compared to controls, the offspring of mothers fed HFD in pregnancy had a higher birth weight, ate more postnatally and had higher body weight up to 110 days of age. Offspring from the HFD group

Table 1
Animal models of prenatal exposures, epigenetic changes in *POMC* gene and associated phenotype.

Animal model	DNA Tissue source	Animal number*, †	Prenatal Exposure	*POMC* epigenetic changes in offspring	Offspring phenotype	Reference
Rat	ARC	Offspring: n = 7–11 per group (HFD-C & C-C)	Maternal Overnutrition	• ↑*POMC* promoter methylation persisted into adulthood	• Maternal HFD led to ↑birth weight, ↑calorific intake, ↑ adult body weight, vulnerability to HFD	Marco et al. (2014)
Rat	Hypothalamus	Offspring: n = 10 per group (C-C & FS-FS)	Maternal Overnutrition	• FS-FS diet led to ↓*POMC* methylation	• FS-FS led to ↑body weight into adulthood, impaired glucose handling and insulin resistance	Zheng et al. (2015)
Rat	ARC	Offspring: n = 5–13 per group (C-C & HFD-C)	Maternal Overnutrition	• ↑*POMC* promoter methylation, ↑H3K9me2, ↑MBD1 & ↑SETDB1 • Lean controls had ↑5hme *POMC* promoter levels	• Maternal HFD led to ↑birth weight, ↑calorific intake, ↑ adult body weight	Marco et al. (2016)
Rat	ARC	Offspring: n = 6 per group (Maternal HFD & C)	Maternal Overnutrition	• ↓DNMT1, ↓HDAC, ↓*POMC* expression in adulthood	• Maternal HFD led to ↑ adiposity, ↑ body weight	Desai et al. (2016)
Rat	ARC and PVN	Offspring: n = 6–22 per group (LFD & HFD)	Maternal Overnutrition	• ↑*POMC* promoter methylation persisted into adulthood	• Maternal HFD led to ↑ adiposity, ↑ body weight, vulnerability to HFD	Gali Ramamoorthy et al. (2018)
Sheep	Hypothalamus	Mothers: n = 6–11 per group (CR & C)	Maternal Undernutrition	• ↓*POMC* promoter methylation, ↑histone acetylation of H3K9	• Not known	Stevens et al. (2010)
Rat	Hypothalamus	Offspring: n = 40 per group (C-C, CR-C and CR-CR)	Maternal Undernutrition	• No changes in hypothalamic methylation in *POMC*	• ↓Birth weight and ↑ body weight by day 16 of life	Coupé et al. (2010)
Sheep	ARC	Offspring: n = 7–9 per group (C & underfed)	Maternal Undernutrition	• ↓*POMC* promoter methylation, ↑histone acetylation of H3K9, ↓DNMT1	• Not known	Begum et al. (2012)
Rat	Hypothalamus	Offspring: n = 10–14 per group (RV, HV, HA)	High Vitamin	• HA postnatal diet led to offspring ↓*POMC* promoter methylation	• Maternal HV diet led to ↑ body weight post weaning, ↑ calorific intake – attenuated with HV or HA postnatal diet	Sánchez-Hernández et al. (2014)
Rat	Hypothalamus	Offspring: n = 8–14 per group (RV-RV, HV-RV, HV-HFol, HV-HV)	High Folate	• HFol-HFol diet led to ↓*POMC* promoter methylation, ↓*POMC* expression in adulthood	• Maternal HFol diet led to ↑calorific intake, ↑adult body weight, impaired glucose handling – attenuated with HFol postnatal diet	Cho et al. (2013b)
Rat	Hypothalamus	Offspring: n = 6–8 per group (C & PNS)	Prenatal stress	• Interaction of methylation with PNS and postnatal HFD at CpG site in *POMC* promoter • HFD led to ↑*POMC* promoter methylation in non-stressed rats	• PNS led to ↑ adiposity, ↑insulin resistance	Paternain et al. (2012)
Rat	ARC and PVN	Offspring: n = 4–8 per group (BWMC, SEDC, RWC, ABA)	Prenatal stress	• No difference in *POMC* methylation between groups	• PNS rats showed ↓ food intake and ↑ weight loss when exposed to ABA	Boersma et al. (2016)
Rat	ARC and PVN	Offspring: n = 10 per group (Triclosan 4 or 8 mg/kg/day & C)	Drug exposure (Triclosan)	• ↑*POMC* promoter methylation, ↓*POMC* expression	• Triclosan exposure associated with ↑Calorific intake, ↑ adult body weight, metabolic syndrome in adulthood	Hua et al. (2019)

Key: ARC; arcuate nucleus of the hypothalamus, PVN; paraventricular nucleus of the hypothalamus, HFD; High Fat Diet, C; Control diet, FS; high fat, high sucrose diet, LFD; low fat diet, CR; calorie restricted diet, RV; recommended vitamin diet, HA; High Vitamin A diet, HV; High Vitamin diet, HFol High folate, BWMC; Body Weight Matched Controls, SEDC; Sedentary Controls, RWC; Running Wheel Controls, ABA; Activity Based Anorexia, PNS; Pre Natal Stress, MBD1; methyl binding protein 1, SETDB1; SET domain binding 1, 5hme; 5-hydroxymethylcytosine, DNMT1; DNA methyltransferase 1, HDAC; histone deacetylase.

† Diets separated by a hyphen refer to diet pre and post natally.

* Range of numbers in each experimental group reflects the different numbers used for different aspects of the experimental design.

Table 2
Animal models of postnatal exposures, epigenetic changes in *POMC* gene and associated phenotype.

Animal model	DNA Tissue source	Animal number*	Postnatal Exposure	*POMC* epigenetic changes	Phenotype	Reference
Rat	Hypothalamus	n = 9 per group (SL & C)	Energy excess	• SL ↑ *POMC* promoter methylation compared to controls • Methylation impeded the anorectic effects of leptin and insulin resulting in ↓ *POMC* expression	• SL rats demonstrated ↑ body weight, glucose, leptin and insulin by week of age	Plagemann et al. (2009)
Rat	ARC	n = 16–18 per group (HFD & C)	Energy excess	• HFD ↑ methylation at a Sp1 binding site which impeded Sp1 binding	• HFD fed rats from the neonatal period into adulthood demonstrated ↑ weight, ↑ insulin and ↑ leptin	Marco et al. (2013)
Mouse	ARC	n = 20 per group (LFD & HFD)	Energy excess	• HFD ↑ methylation at/near to RELA and Sp1 binding sites • HFD-induced chronic inflammation inhibits activation of *POMC* transcription	• Acute inflammation suppresses food intake	Shi et al. (2013)
Rat	Hypothalamus	n = 8 per group (HC & C)	Energy excess	• By day 16, HC rats ↓ *POMC* gene H3K9 gene acetylation and no difference in *POMC* promoter methylation compared to controls	• HC diet group had ↑ body weight, ↑ insulin and ↑ leptin by day 100.	Mahmood et al. (2013)
Rat	Hypothalamus	C group (n = 6) HFD (n = 50)	Energy excess	• DR rats had an overall ↓ *POMC* methylation compared to DIO rats	• DR rats consumed less than DIO rats, had similar weight to controls	Cifani et al. (2015)
Rat	ARC and PVN	n = 16 per group (CAF & C)	Energy excess	• CAF associated with ↓ *POMC* promoter methylation and ↑ expression of *POMC*	• CAF fed rats ↑body weight and ↑energy intake compared to controls	Lazzarino et al. (2017)
Rat	Hypothalamus	n = 9–12 per group (C, CR, SL, AL)	Energy restriction	• No differences in *POMC* methylation between the groups	• Post weaning CR group had comparable food intake and ↓ weight gain compared to controls when fed AL	Liu et al. (2013)
Mouse	Hypothalamus	n = 5 per group (CR, AL, CR-AL)	Energy restriction	• No differences in *POMC* methylation between the groups	• No comment	Unnikrishnan et al. (2017)
Mouse	Adipose tissue and Hypothalamus	n = 15 per group (n3 PUFA deficient, Soy Oil, Soy/Fish Oil, Fish Oil, C)	Fatty acid	• n-3 PUFA normalised *POMC* expression in obese mice • No differences in *POMC* methylation between the groups	• n-3 PUFA fed mice had ↓ body weight compared n-6 PUFA fed mice	Fan et al. (2011)
Mouse	Hypothalamus	n = 16 per group (LA & CLA)	Fatty acid	• CLA diet led to ↑*POMC* promoter methylation and ↓ *POMC* expression compared to LA fed group	• CLA fed mice ↑adult body weight and impaired glucose homeostasis compared to controls	Zhang et al. (2014)
Rat	Hypothalamus	n = 6–8 per group (Control: HFD & NF, Leptin: HFD & NF)	Leptin	• Leptin treated rats demonstrated ↑ *POMC* methylation when fed a normal diet and ↓ *POMC* methylation when fed HFD	• Leptin treatment led to ↓ body weight and ↓ energy intake in adulthood compared to controls	Palou et al. (2011)

Key: ARC; arcuate nucleus of the hypothalamus, PVN; paraventricular nucleus of the hypothalamus, SL; small litters, C; Control diet HFD; High Fat Diet, LFD; Low Fat Diet, HC; high carbohydrate, DIO; diet induced obesity, DR; diet resistant, CAF; Cafeteria Diet, CR; Calorie restricted diet, AL; ad libitum, PUFA; polyunsaturated fatty acids, LA; linoleic acid, CLA; Conjugated linoleic acid, NF; normal feeds, RELA; v-rel reticuloendotheliosis viral oncogene homologue A, Sp1; specificity protein 1, STAT3; Signal transducer and activator of transcription 3.

* Range of numbers in each experimental group reflects the different numbers used for different aspects of the experimental design.

demonstrated ARC *POMC* hypermethylation across the promoter region (with significantly higher methylation in 5 CpGs) and this hypermethylation was maintained into adulthood suggesting that *POMC* methylation is a stable epigenetic mark. When these pups were subsequently exposed to an HFD challenge, they consumed more and gained more weight than offspring of controls. Despite differences in *POMC* methylation, there were no differences in *POMC* expression. However, the HFD group had higher levels of leptin though this did not lead to higher *POMC* expression as might be expected. Only female offspring were examined for the hormonal, POMC expression and methylation analyses. This study provides evidence of an association between *POMC* hypermethylation (influenced by prenatal HFD) and increased body weight in offspring.

A further study by Marco et al. (2016), replicated the earlier finding that offspring of HFD fed rats had higher body weight into adulthood and had higher ARC *POMC* promoter methylation (with significantly higher methylation in 3 CpGs) compared to controls, but went further by postulating a process of dual epigenetic silencing with higher levels of H3K9me2 (an epigenetic mark associated with transcriptional repression) at the *POMC* promoter in the ARC. These epigenetic changes were associated with lower *POMC* expression and higher body weight. Interestingly, lean control offspring had higher levels of 5-hydroxymethylcytosine (5hmC), an epigenetic mark predominantly found in the central nervous system that is associated with increased gene transcription (Shi et al., 2017; Mellen et al., 2012). Body weight was positively correlated with *POMC* promoter 5-methylcytosine (5mC) and negatively correlated with *POMC* promoter 5hmC levels. The study also demonstrated higher levels of methyl binding domain protein 1 (MBD1) binding in the *POMC* promoter and SET domain binding 1 (SETDB1, a histone methyltransferase) in HFD offspring and showed that this complex of MBD1 and SETDB1 promoted histone methylation. There were lower levels of the MBD1-SETDB1 complex in lean controls, suggesting that higher levels of 5hmC inhibited binding at this region leading to greater expression of *POMC* and a stronger satiety signal. This study gives evidence of an association between levels of 5mC and 5hmC in the *POMC* promoter and offspring's body weight. It further supports a relationship between prenatal HFD exposure and increased levels of 5mC in the *POMC* promoter.

In those exposed to a high energy diet *in utero, a* high energy diet postnatally may have a modulatory effect on *POMC* methylation. Evidence for this came from Zheng et al. (2015) who produced two groups of rats; FS-FS (high fat, high sucrose diet fed to mothers and offspring from weaning) and C-C (control diet fed to mothers and offspring from weaning). The FS-FS group demonstrated higher body weight in adulthood, though with higher expression of *POMC* and lower average methylation of the *POMC* promoter compared to controls (mean methylation ± SD = FS-FS 37.5 ± 1.7% vs C-C 46.3 ± 3.5%, $p = 0.03$). Despite examining the same genomic region, this is different to the observation reported by Marco et al. (2014, 2016), where increased *POMC* methylation was reported in offspring exposed to HFD *in utero*. In the studies by Marco et al. (2014, 2016), pups were weaned onto a normal calorie diet (not high energy) and therefore the disparate postnatal diets may explain the difference in *POMC* methylation reported following high energy exposure *in utero*. Another potential explanation is that Zheng et al. (2015) studied male rats from weaning, in contrast to the use of female rats (for hormonal and POMC expression/methylation analysis) examined in Marco et al. (2014).

Further evidence of differential effects of pre- and postnatal dietary exposures on offspring *POMC* methylation was highlighted in a recent study by Gali Ramamoorthy et al. (2018) who reported hypermethylation in the *POMC* promoter and enhancer regions in 3-week-old male pups from HFD fed mothers. This methylation pattern in the promoter (but not the enhancer) was conserved into adulthood and suggests a potential critical period of prenatal programming. In this study, post weaning HFD also led to hypermethylation in the *POMC* promoter of male pups from the control group, though there were no additive effects of post-weaning HFD on *POMC* methylation in pups exposed to HFD prenatally. This study gives further evidence that maternal HFD is associated with increased methylation at regulatory regions of *POMC* and that increased *POMC* methylation is associated with increased body weight and food intake into adulthood.

Other epigenetic pathways have been shown to be influenced by maternal HFD. Desai et al. (2016), showed lower expression of hypothalamic DNA methyltransferase 1 (DNMT1) and histone deacetylase (HDAC, a class of enzyme that removes acetyl groups from histones and is associated with gene suppression) in offspring exposed to HFD *in utero*. In this study, as expected, male offspring of HFD mothers, when milk fed by the same HFD mothers, developed markedly increased weight and adiposity compared to controls. On day 1 of life, those offspring from HFD fed mothers showed lower expression of hypothalamic DNMT1 and increased *AgRP* expression compared to controls. However, *POMC* expression was the same in both groups. By 6 months, hypothalamic levels of HDAC were significantly reduced in the offspring of HFD mothers. The higher expression of *AgRP* was maintained but now also with reduced *POMC* expression compared to controls. This study provides additional evidence that prenatal HFD influences offspring's epigenetic processes at *POMC*. The changes were not associated with altered *POMC* expression until adulthood, though there were marked differences in body weight between the two groups.

As well as effects on offspring bodyweight and adiposity, maternal high energy diet has been shown to lead to impaired glucose homeostasis and greater insulin resistance in progeny. This has been evidenced by Gali Ramamoorthy et al. (2018) who reported greater insulin resistance from 8 weeks of age that persisted into adulthood in rats exposed to HFD *in utero*. Furthermore, Zheng et al., demonstrated that *POMC* methylation was negatively associated with glucose response to a glucose load in a study in rats (Zheng et al., 2015).

In summary, there is a strong evidence of an association between prenatal HFD and offspring hypermethylation in regulatory regions of *POMC* that persist until adulthood (Gali Ramamoorthy et al., 2018; Marco et al., 2014, 2016). A potential modulatory effect of postnatal FS diet on *POMC* methylation has been reported (Zheng et al., 2015), however Gali Ramamoorthy et al. (2018) demonstrated no additional influence from a postnatal high energy diet on the hypermethylation already seen in those with prenatal HFD exposure.

3.1.2. Maternal energy restriction

Maternal undernutrition was first demonstrated to be associated with offspring *POMC* hypomethylation and increased histone acetylation of H3K9 (associated with increased gene transcription), by Stevens et al. using a sheep model with analysis of fetal hypothalamic tissue (Stevens et al., 2010). However, these observed epigenetic changes were not associated with any change in *POMC* expression compared to controls (normally fed ewes). Importantly, their study found that alterations to the periconceptional nutritional environment, even when applied to a narrow window, can alter *POMC* gene methylation in later pregnancy. The study demonstrated that undernutrition exposure from as little as 2 days and up to 30 days before conception, was associated with significant *POMC* hypomethylation (64% lower than controls) in fetuses by day 133–135 of gestation. Pregnancies were terminated between day 131–135 of gestation, so an assessment of the postnatal phenotype was not made. Though 10-month old sheep from undernourished mothers (using the same protocol), had impaired glucose handling and increased body weight, it is not known if this was associated with alterations in *POMC* methylation or expression (Todd et al., 2009).

Begum et al. (2012), replicated the findings of *POMC* hypomethylation and increased H3K9 acetylation but also reported lower DNMT activity in the hypothalamus of both male and female offspring of energy restricted ewes (restricted between day −60 to +30 relative to conception). Similar to Stevens et al., the fetuses were euthanised between day 131–135 of gestation so there was no assessment of postnatal

phenotype, however this study gives further evidence of an effect of periconceptional undernutrition on offspring's epigenetic marks at the *POMC* gene.

However, Coupé et al. (2010) reported no changes in DNA methylation in hypothalamic feeding-related genes including in the *POMC* promoter or enhancer regions, in the postnatal male rat hypothalamus in intrauterine growth restricted rats. Though the exact timing of calorific restriction was not clear, this would not support a modulatory effect of undernutrition on *POMC* methylation in rats.

Sex specific alterations in *POMC* expression are reported in offspring of energy restricted sheep, though the mechanism remains unclear. Begum et al. (2013) found that adult sheep exposed to undernutrition in early pregnancy demonstrated lower *POMC* expression in males (with increased fat mass in adulthood) but no difference in females (Begum et al., 2013). However, there was no assessment of *POMC* methylation as a mediator of these changes in gene expression. Óvilo et al. (2014) found female offspring (but not males) of late gestation nutritionally-restricted Iberian sows had a lower hypothalamic expression of *POMC*. There was increased body weight and fat but again there was no measurement of *POMC* methylation. Sexual dimorphism in hypothalamic circuits has been described and extensively reviewed (Chowen et al., 2019). Deletion of ERRα from *POMC* neurons has no effect on male mice but leads to increased body weight, hyperphagia and increased lean mass in females (Xu et al., 2011). One possibility is that epigenetic changes involving the ERR are responsible for the sexual dimorphic response to maternal energy restriction. Alternatively, circulating estrogen could modulate the central actions of insulin or glucose on *POMC* neurons postnatally (Ramamoorthy et al., 2015).

In summary, there is strong evidence from the studies of Stevens et al. (2010) and Begum et al. (2012) to suggest that periconceptional undernutrition has a modulatory effect on late gestational *POMC* epigenetic marks including hypomethylation, increased H3K9 acetylation and lower DNMT activity. There is limited evidence of an association between these changes and a subsequent postnatal phenotype. Evidence of phenotypic changes were highlighted by Begum et al. (2013), with higher fat mass and lower *POMC* expression in offspring of energy restricted ewes but it is not clear if this was mediated by epigenetic changes at *POMC* (Begum et al., 2013).

3.1.3. Mismatch in pre and postnatal vitamin and folate diet

A set of interlocking pathways, collectively known as one-carbon metabolism, provide methyl groups for methylation reactions including the methylation of cytosine bases and histone tails that in turn influence gene expression. These methylation reactions are controlled by methyltransferases that act on methyl groups produced by the conversion of S-adenosyl methionine (SAM) to S-adenosyl homocysteine (SAH). One-carbon metabolism is dependent on a number of nutrients and vitamins including folate, B12, B6, B2 and choline (Friso et al., 2017 Apr) (see Fig. 3). It has been postulated that alterations in folate or other one-carbon metabolite levels pre or postnatally could mediate epigenetic changes, putatively by altering the availability of methyl groups. For example in humans, maternal concentrations of folate in early pregnancy have been associated with offspring's DNA methylation and birth weight, illustrating the potential importance of prenatal exposure to one-carbon related nutrients and vitamins on DNA methylation and phenotype (Hoyo et al., 2014).

A mismatch between a high vitamin diet (HV) *in utero* and recommended vitamin diet (RV) in postnatal life has been shown to be associated with obesity in rats (Cho et al., 2013a; Sanchez-Hernandez et al., 2015, 2014). Maintaining a similarly high vitamin intake in the postnatal diet can attenuate the development of the obese phenotype and can normalise *POMC* expression to that of controls. This was elegantly shown in a rat model by Cho et al. (2013a) who showed that male offspring of rats fed HV, a 10-fold higher recommended intake of multivitamins ('AIN-93G' containing 13 vitamins (Reeves, 1997) including Vitamin A, D, Riboflavin, Choline and Folic acid) in pregnancy, who were then weaned to RV, demonstrated higher body weight, food consumption and higher glucose response to a glucose load than those born to mothers fed the RV intake in pregnancy. Conversely, HV or HFol (high folate) postnatally prevented these associated phenotypic changes. Interestingly, postnatal HFol diet did not normalise *POMC* expression as the HV diet did. There was no difference in global hypothalamic DNA methylation between any of the dietary groups, though *POMC* methylation was not measured specifically.

Furthermore, it has been shown that HFol diet postnatally, alters *POMC* methylation in rats. This was evidenced by Cho et al. (2013b), who gave male offspring of mothers fed HFol diet, either RV or HFol and compared them to controls fed RV pre and postnatally. HFol-RV rats demonstrated significantly higher food intake and body weight compared to controls and a 20% lower glucose response to an insulin load at 7 weeks of age (no differences at week 12 or 16 of life). HFol-HFol rats showed lower food intake and body weight compared to controls and demonstrated a significantly lower glucose response to a glucose load compared to HFol-RV and controls at week 10 (though no differences at week 14 or 18 of life). HFol in gestation reduced hypothalamic *POMC* expression compared to RV. The HFol – HFol groups had lower hypothalamic *POMC* methylation compared to the RV-RV and HFol-RV groups, providing evidence for ongoing postnatal plasticity in *POMC* DNA methylation mediated by increased dietary folate (even though global DNA methylation was not altered). It should be noted that although statistically significant, the differences in *POMC* methylation between the groups were small (HFol-HFol had 4% lower mean methylation compared to the other groups). This study supports an association between *POMC* methylation and a glucose response to a glucose load (glucose to *POMC* methylation coefficient of 0.7, $p = 0.03$).

There is evidence that vitamin A can mediate epigenetic changes as it can increase glycine N-methyltransferase enzyme expression, which can lead to loss of methyl groups (Rowling et al., 2002). In male rats exposed to a HV diet *in utero*, a postnatal diet high in vitamin A (HA, 10-fold higher vitamin A than recommended) can lower methylation at *POMC*, as demonstrated by Sánchez-Hernández et al. (2014). This study found that postnatal diets of either HV or HA, led to reduced post weaning weight gain and reduced food intake compared to male rats fed RV diets. HA postnatal diet was associated with significantly lower hypothalamic *POMC* promoter methylation compared to RV and HV, though the difference in mean methylation between the groups was small (3%). HV postnatal diet was associated with higher levels of *POMC* expression compared to RV but there was no difference in *POMC* expression between HV and HA diet. Though higher *POMC* gene expression was seen in the HV group compared to the control group, there was no difference in *POMC* promoter methylation, suggesting other epigenetic mechanisms (e.g. histone modifications) could account for the difference in expression. This study does support a modest effect of a postnatal HA diet in lowering mean *POMC* methylation and that a mismatch in vitamin diets between pre and postnatal period is associated with weight gain and increased food intake into adulthood.

In summary, there is strong evidence that a mismatch of HV diet prenatally and RV diet postnatally is associated with increased body weight and higher energy intake into adulthood (Cho et al., 2013a, 2013b; Sanchez-Hernandez et al., 2015, 2014). There is evidence that high concentrations of vitamin A (Sánchez-Hernández et al., 2014) and folate (Cho et al., 2013b) postnatally have a modest effect to lower *POMC* methylation in those exposed to HV diet prenatally.

3.1.4. Prenatal stress

Prenatal stress (PNS) is associated with chronic disease, such as obesity, in adulthood (Tamashiro and Moran, 2010). Epigenetic changes could be a conduit between PNS exposures and later phenotype. There is indeed evidence of epigenetic changes at *POMC* associated with PNS that predispose an individual to obesity when the animal is exposed to an obesogenic environment, such as HFD. Paternain

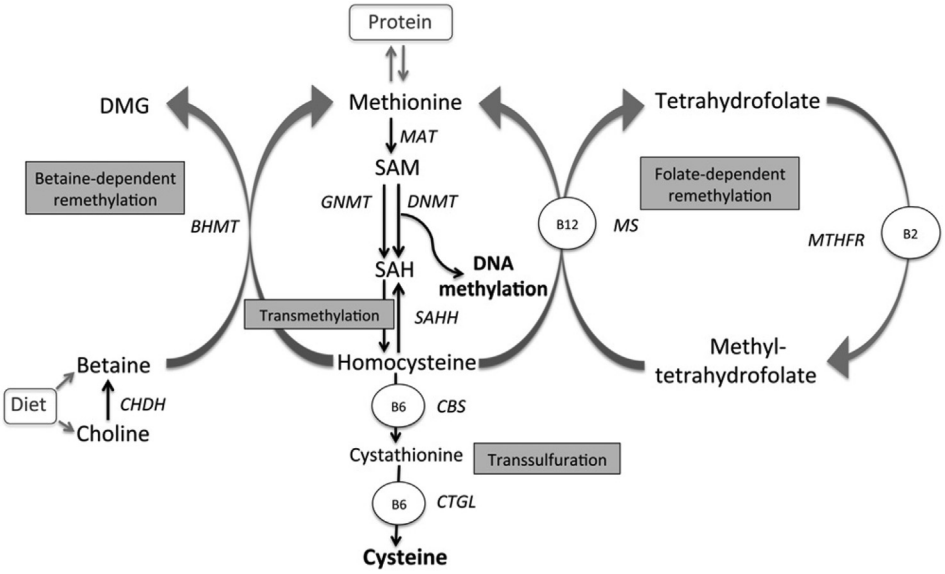

Fig. 3. An overview of one-carbon metabolism. Key: BHMT, betaine-homocysteine methyltransferase; B2, riboflavin; B6, vitamin B-6; B12, vitamin B-12; CBS, cystathionine-β-synthase; CHDH, choline dehydrogenase; CTGL, cystathionine-γ-lyase; DMG, dimethylglycine; DNMT, DNA methyltransferases; GNMT, glycine-N-methyltransferase; MAT, methionine adenosyltrasferase; MTHFR, methylenetetrahydrofolate reductase; MS, methionine synthase; SAH, S-adenosylhomocysteine; SAHH, S-adenosylhomocysteine hydrolase; SAM, S-adenosylmethionine. Reproduced with permission from Paula Dominguez-Salas, Sophie E Moore, Darren Cole, Kerry-Ann da Costa, Sharon E Cox, Roger A Dyer, Anthony JC Fulford, Sheila M Innis, Robert A Waterland, Steven H Zeisel, Andrew M Prentice, Branwen J Hennig, DNA methylation potential: dietary intake and blood concentrations of one-carbon metabolites and cofactors in rural African women, The American Journal of Clinical Nutrition, Volume 97, Issue 6, June 2013, Pages 1217–1227, https://doi.org/10.3945/ajcn.112.048462.

et al showed that female PNS rats fed an HFD as adults demonstrated higher adiposity and greater insulin resistance when compared to non-PNS controls (Paternain et al., 2012). HFD in adulthood led to increased hypothalamic *POMC* expression in both PNS rats and controls, but with an interaction between PNS, HFD and methylation at a single CpG site in the *POMC* promoter after adjustment for multiple testing. This study thus provides limited evidence that *POMC* methylation changes are influenced by PNS leading to increased susceptibility to HFD and the subsequent postnatal obese phenotype.

PNS has been shown to lead to greater weight loss in offspring when subjected to energy restriction. Boersma et al demonstrated that compared to controls, PNS male and female rats showed lower food intake and greater weight loss when subsequently exposed to activity-based anorexia (ABA); experimental conditions where food is limited to 1.5 h per day and a running wheel in placed in their cage postnatally (Boersma et al., 2016). ABA induced a reduction in *POMC* expression and an increased expression of *NPY* in all groups. There was no difference in *POMC* promoter methylation between groups. This study does not support the hypothesis that *POMC* methylation changes are associated with PNS. PNS does appear to influence postnatal energy balance under conditions of ABA, though this does not appear to be mediated by methylation changes at the *POMC* gene.

In summary, the study by Paternain et al. (2012), provides only limited evidence of an interaction between PNS and *POMC* methylation changes. However, both studies do provide evidence that PNS rats have greater adiposity when fed HFD as adults though conversely are more susceptible to weight loss under periods of nutritional scarcity. Whether these changes are mediated by *POMC* epigenetic alterations (other than DNA methylation) warrants further study.

3.1.5. Maternal drug exposure

Triclosan is a broad-spectrum antibiotic agent found in household and personal hygiene products (US Food and Drug Administration, 2018). Exposure to the drug prenatally is thought to influence birth weight in humans (Etzel et al., 2017), though the influence on epigenetic changes and later phenotype is little explored. Hua et al., showed a link between prenatal triclosan exposure and later obese phenotype in both male and female rats. Rats exposed to Triclosan in early/mid gestation (gestational day 6–14) had lower birth weight, but by day 30 of life showed increased mean *POMC* methylation (significantly higher in 6 CpGs across the *POMC* promoter), reduced *POMC* expression and subsequent early hyperphagic obesity and metabolic syndrome (Hua et al., 2019). This study gives support to the idea of an environmental agent acting prenatally to influence *POMC* methylation which is associated with the development of obesity and metabolic syndrome later in life.

3.2. Postnatal exposures, epigenetic alterations in the POMC gene and phenotype

There is also evidence that postnatal factors can influence *POMC* epigenetic marks and influence weight-related phenotype (see Table 2). One possibility is that developmental plasticity continues into the postnatal period with an epigenetic adaptive response continuing beyond the 'classical' window of fetal programming. If this is the case, then alterations to *POMC* epigenetic marks in the postnatal period would allow the organism to more accurately *predict, adapt* and *respond* to future nutritional requirements.

Alternatively, postnatal changes may represent more widespread alterations to the epigenome brought about by environmental factors and may not target *POMC* specifically. Yet another explanation is that epigenetic changes are due to reverse causation where postnatal phenotype influences the epigenome.

3.2.1. Energy excess

Overnutrition during infancy, in animals and humans, is associated with the later development of obesity (Ojha et al., 2013). There is evidence that *POMC* hypermethylation, induced by postnatal energy excess, may suppress the satiety response by impeding the action of peripheral signals such as leptin and insulin. This was demonstrated in a rodent model (Plagemann et al., 2009) whereby postnatal energy excess was induced by artificially creating 'small litters' (SL) of 3 pups per mother (as opposed to 10 pups per mother). Rats placed into SL from day 2 of life, demonstrated higher body weight, glucose, leptin and insulin by day 7 of life (Plagemann et al., 2009). Higher *POMC* promoter methylation, including in regions associated with nuclear factor K-B (NF-KB) and Sp1 binding on the *POMC* gene, was observed compared to controls (even after Bonferroni correction for multiple testing). Sp1 binding is crucial to mediate the effect of circulating leptin via STAT3-Sp1-complex formation. There was an inverse relationship between *POMC* methylation at the Sp1 binding site and *POMC* expression per unit of leptin and insulin, suggesting that methylation at this site impedes the anorectic effects of leptin and insulin resulting in lower *POMC* expression. This study supports a link between energy

excess in the postnatal period and epigenetic changes at transcriptional regulatory sites in the *POMC* promoter.

Marco et al. (2013), used a rat over-feeding model and further demonstrated the interaction of *POMC* methylation, Sp1 binding and *POMC* expression. Male rats fed an HFD from the neonatal period into adulthood demonstrated increased weight, insulin and leptin with hypermethylation at 6 CpGs in the *POMC* promoter including at a Sp1 binding site. However, there was not the expected increase in hypothalamic *POMC* expression despite significantly higher levels of leptin, Sp1 and insulin (factors associated with upregulation of *POMC* expression (Balthasar et al., 2004; Qiu et al., 2014; Yang et al., 2009). ChIP analysis demonstrated reduced Sp1 binding in the HFD group, despite higher levels of circulating Sp1, suggesting hypermethylation impeded the formation of the Sp1 complex. This study supports a link between energy excess in the postnatal period and methylation changes at CpGs near a key transcriptional regulatory site (Sp1) in the *POMC* promoter.

NF-KB is released in response to both acute and chronic inflammation. Though acute inflammation is associated with reduced food intake (Grossberg et al., 2010), obesity is associated with chronic low grade inflammation (Ellulu et al., 2017). RELA (v-rel reticuloendotheliosis viral oncogene homologue A (avian)) is a subunit of NF-KB. Shi et al. (2013), demonstrated that during acute inflammation, male mice showed reduced appetite and increased *POMC* expression. Using a STAT3 (Signal transducer and activator of transcription 3) knock out model they demonstrated that this upregulation of *POMC* was independent of the STAT3 pathway (mediated by leptin) but instead RELA upregulated *POMC* transcription by directly binding to the *POMC* promoter. In the same study, they showed that HFD male mice (modelling the chronic inflammation seen in obesity) had hypermethylation of the *POMC* promoter which subsequently impeded RELA-mediated *POMC* transcriptional activation. This study supports a link between energy excess and increased methylation in the *POMC* promoter but also proposes an alternative means of *POMC* transcriptional regulation by direct binding of RELA to the promoter region.

Mahmood et al. (2013) demonstrated a potential postnatal programming effect of high carbohydrate (HC) feeds on melanocortin system neuropeptides and adult body weight, though mediated predominantly through epigenetic changes in the *NPY* (neuropeptide-Y) gene. Female rats were fed with either HC or mother's milk (MM), and then weaned onto standard feed from day 24. By day 16, HC rats had higher *NPY* H3K9 acetylation, lower *NPY* gene methylation, lower *POMC* gene H3K9 gene acetylation but no difference in *POMC* promoter methylation. By day 100, only *NPY* methylation differences persisted (with increased expression of *NPY*) with significantly higher body weight in those fed HC diet compared to those fed MM in the postnatal period. This study suggests that a high energy diet can induce epigenetic changes at the *POMC* gene but these changes do not persist beyond the immediate postnatal period. Importantly, sustained epigenetic changes were only seen in the *NPY* gene, and not *POMC*.

It has been suggested that epigenetic changes could 'predispose' an individual to overconsumption in an obesogenic environment (Stöger, 2008 Dec). Cifani et al., demonstrated there are epigenetic differences in *POMC* and *NPY*, in those rats who are obesity-resistant or obesity-prone on exposure to an obesogenic environment (Cifani et al., 2015). Male rats were fed a high energy feed for 5 weeks and classified as to whether they developed obesity or did not (diet induced obesity: DIO or diet resistant: DR). The DR rats consumed less, had similar weight to controls but also had decreased hypothalamic expression of *NPY* by 5 weeks (with hypermethylation at one CpG in *NPY*) and higher expression of *POMC* at 21 weeks (the mean *POMC* methylation was significantly lower though the difference was very small (~1%), however there were still significant differences at 4 CpG sites even after Bonferroni correction) compared to DIO rats. This study suggests that different neuropeptide gene methylation and expression could account for the divergent eating behaviour and weight gain, though overall differences in *POMC* methylation were small.

There is further evidence that high energy diets induce epigenetic changes that produce increased expression of orexigenic neuropeptides (*AgRP/NPY*), overriding any increased expression of the anorectic *POMC*, and leading overall to overconsumption and increased body weight. Lazzarino et al. (2017) fed female rats a CAF diet (Cafeteria diet, modelled on Western diet of high palatability and highly energy dense foods) from weaning and demonstrated an increased expression of *POMC* and reduced *AgRP* expression at the level of the ARC but increased expression of *AgRP/NPY* in the PVN. This was accompanied by lower methylation in the *POMC* promoter of the ARC and lower methylation in the *NPY* promoter in the PVN. CAF fed rats had higher body weight from week 10 and consistently consumed a higher energy intake compared to controls. Of note, the methylation of CpGs associated with Sp1 binding (thought to mediate anorectic effects of leptin) were not measured in this study. This study suggests that in response to CAF diet epigenetic and gene expression changes effect both *AgRP/NPY* and *POMC,* though the higher energy intake observed was mediated by higher *AgRP/NPY* expression in the PVN and not counteracted by increased *POMC* expression in the ARC.

In summary, the studies by Plagemann et al. (2009), Marco et al. (2013) and Zheng et al. (2015) provide the strongest evidence for nutritionally-driven postnatal changes to *POMC* epigenetic marks. However, it is not clear if the diet itself or the increased body weight leads to the epigenetic differences observed. Other studies suggest that *AgRP/NPY* genes are more susceptible to postnatal overnutrition than *POMC* and may be more implicated with the postnatal phenotype (Mahmood et al., 2013; Lazzarino et al., 2017).

3.2.2. Energy restriction

There are data from humans that suggest periods of extreme calorific restriction may lead to remission of type 2 diabetes mellitus (Lean et al., 2018) and a sustained reduction in BMI (Willi et al., 2004) long after the end of dietary intervention. Animal models suggest this 'metabolic memory' may be mediated via epigenetic changes in the appetite regulating neuropeptides. Liu et al. (2013), explored if periods of calorific restriction in the post weaning period could mitigate the development of obesity in adult life associated with SL during suckling. Male rats given moderate calorific restriction (24% reduction in daily calorific intake for 49 days) in the post weaning period, showed comparable food intake and slower weight gain compared to controls when subsequently fed ad libitum, such that they did not reach the weight of controls by day 140 of life. Furthermore, they had similar levels of insulin, leptin and expression of *NPY* and *POMC* to controls. In both control and calorific restriction groups there was increased methylation in the *NPY* promoter at a CpG important for E2F (E2 Factor) binding, compared to those from SL with no restriction. E2F is a transcription factor known to regulate *NPY* expression. There were no differences in *POMC* promoter methylation between the groups. This study demonstrates that a period of calorific restriction may attenuate the hyperphagia and obese phenotype associated with SL, though suggests that this does not appear to be mediated by alterations in *POMC* gene methylation.

However, Unnikrishnan et al., reported 65% lower hypothalamic *POMC* expression following short term dietary restriction in male mice (Unnikrishnan et al., 2017). These alterations in *POMC* gene expression persisted even after 2 months of ad libitum feeding. There was no associated difference in DNA methylation at the *POMC* promoter, though DNA methylation was analysed from whole hypothalamic tissue and not specifically the ARC where *POMC* is expressed in the hypothalamus.

These two studies suggest that postnatal energy restriction does not lead to changes in *POMC* methylation but predominantly effects methylation marks at *NPY* (similar to studies of postnatal energy excess (Mahmood et al., 2013; Lazzarino et al., 2017). Reported alterations in *POMC* gene expression could be related to other epigenetic processes not measured in these studies.

3.2.3. Fatty acids

Diets high in n–6 polyunsaturated fatty acids (PUFA) found in foods such as sunflower oil, and low in n–3 PUFA (found in fish oil for example) have been implicated in a number of chronic diseases including obesity (Ailhaud et al., 2006). N-3 PUFA has been shown to reduce leptin expression (Ukropec et al., 2003), which is elevated in obesity due to the increased adipose tissue mass. Leptin regulates the anorectic actions of *POMC* via the long form leptin receptor on *POMC* neurons, and it has been postulated that elevated leptin in obesity represents a form of leptin resistance (Myers et al., 2012). Fan et al., demonstrated that the addition of n-3 PUFA to the diet of obese male mice normalised *POMC* expression and leptin to comparable levels seen in controls (Fan et al., 2011). N-3 PUFA fed mice had lower weight compared to those fed n-6 PUFA. The changes in gene expression were not related to a difference in methylation in either *POMC* or the leptin promoter from adipose tissue.

Conjugated linoleic acid (CLA) is a fatty acid associated with the development of metabolic disease (Tsuboyama-Kasaoka et al., 2000) and has been implicated in postnatal programming (Poulos et al., 2001) in murine models. There is evidence from Zhang et al. (2014) to suggest that postnatal nutritional exposure to CLA causes *POMC* promoter hypermethylation, preventing Sp1 complex formation which in turn leads to reduced *POMC* expression and an increase in food intake. Lactating mice were fed a diet rich in either CLA or LA (linoleic acid) which altered milk composition such that CLA fed mice had increased milk glucose and insulin, lower milk lactose and triglycerides and altered fatty acid composition compared to LA fed mice. CLA fed pups had significantly lower *POMC* expression compared to LA fed pups with hypermethylation of two promoter CpGs. This region of the *POMC* promoter is at the location of a Sp1 binding site and ChIP assays demonstrated reduced Sp1-*POMC* promoter complex formation in CLA fed pups. Interestingly, CLA fed pups had low SAH (formed after a methyl group is removed from SAM in methylation reactions, see Fig. 3) and it was suggested this may explain the observed hypermethylation in this group. In the same study, CLA fed mice initially had lower body weight and restricted growth, but increased food intake. However, they were heavier as adults with impaired glucose homeostasis compared to controls. This study does support a modulatory effect of dietary CLAs on the *POMC* methylation and gene expression with an association with obesity in later life.

There is limited evidence that postnatal dietary fatty acids influence *POMC* gene methylation. However, Zhang et al. (2014) provide the strongest link with dietary CLAs associated with alterations at key regulatory regions of the *POMC* promoter.

3.2.4. Leptin

Due to leptin's known association with upregulation of *POMC*, Palou et al investigated if leptin treatment could influence postnatal programming of hypothalamic hormones including *POMC* (Palou et al., 2011). Leptin treatment in the suckling period led to lower body weight and food consumption in adulthood compared to controls. After the suckling period, male rats were fed either normal diet or HFD. At a CpG position close to the Sp1 binding site, leptin-treated rats demonstrated significantly higher methylation when fed a normal diet and lower methylation when fed HFD (compared to non-leptin treated animals). However, there were no significant correlations between *POMC* methylation and *POMC* expression. The authors note these observations should be treated with caution until confirmed by other independent studies and significance was only reached when comparing individual groups (as overall there was a non-significant interaction using two-way ANOVA tests).

3.3. Other epigenetic regulatory factors and weight-related phenotypes

3.3.1. Methyl CpG binding protein (Mecp2)

Mecp2 was thought to be a repressor of *POMC* activity acting by recruitment of a repressor complex (Wu et al., 2014; Gangisetty et al., 2014) to methylated DNA. However, a study by Wang et al found that Mecp2 can act as a transcriptional activator in the hypothalamus (Wang et al., 2014). Male mice with a specific Mecp2 knock out (KO) in *POMC* neurons had higher body weight, energy intake, leptin and body fat percentage. The Mecp2 KO mice had higher *POMC* promoter methylation and lower *POMC* expression. Co-transfection of wild type Mecp2 and cAMP responsive element binding protein 1 (CREB1) led to increased *POMC* promoter activity, significantly more than when transfected with Mecp2 or CREB1 alone. This suggests that the activating properties of Mecp2 are dependent on an interaction with CREB1 to increase *POMC* expression. In contrast to previous studies (Wu et al., 2014; Gangisetty et al., 2014), this study specifically altered Mecp2 expression in *POMC* neurons and therefore better demonstrates the effect in this neuronal group.

3.4. Summary of evidence from animal studies

From the evidence in animal models, it is clear there is a complex relationship between pre- and postnatal exposures that influence epigenetic processes associated with the *POMC* gene.

Epigenetic processes and marks are sensitive to prenatal nutritional and environmental exposures and have a consequence on later adult phenotype. There is evidence from these animal studies that supports the PAR hypothesis that mismatched *in utero* and *ex utero* environments are associated with obesity and poor metabolic health and can be to some degree explained by epigenetic changes in *POMC*. For example, there is considerable evidence that prenatal exposures to energy excess (Marco et al., 2014, 2016; Gali Ramamoorthy et al., 2018) and periconceptional and early pregnancy energy restriction (Stevens et al., 2010; Begum et al., 2012) have different effects on *POMC* epigenetic marks and are associated with postnatal phenotype. A mismatch in pre and postnatal vitamin levels have been shown to be associated with higher body weight and increased food intake (Cho et al., 2013a, 2013b; Sanchez-Hernandez et al., 2015, 2014), and there is modest effects of vitamin A (Sánchez-Hernández et al., 2014) and folate (Cho et al., 2013b) to lower *POMC* methylation postnatally. Though limited to one study (Hua et al., 2019), the modulatory effect of Triclosan on *POMC* methylation appears significant and is strongly associated with obesity in adulthood.

The evidence of postnatal exposures influencing *POMC* epigenetic marks is more limited. Evidence from Plagemann et al. (2009) and Marco et al. (2013) provide the strongest link between postnatal energy excess and changes to DNA methylation at *POMC*, however other studies report that *AgRP/NPY* genes appear more sensitive than *POMC* to postnatal energy imbalance (Mahmood et al., 2013; Lazzarino et al., 2017; Liu et al., 2013; Unnikrishnan et al., 2017). The study by Zhang et al. (2014) suggests that dietary CLAs are the strongest specific nutritional factor to mediate methylation changes at *POMC* gene postnatally.

It is important to consider the potential for reverse causation when interpreting results from epigenetic studies. An approach to minimising the potential for reverse causation effects is to measure epigenetic marks (e.g. DNA methylation) prior to manifestation of the disease or phenotype. One advantage of animal studies is the ability to access functionally relevant tissue (such as ARC samples when examining *POMC*), however this may prevent assessment of epigenetic marks prior to the development of a particular phenotype as the animal is euthanised. To circumvent this, researchers often use subgroups of animals who are euthanised at different time points. Marco et al. (2014), euthanised a proportion of rodents on weaning and maintained a group into adulthood allowing comparisons of methylation before the adult phenotype developed. In this study, the same *POMC* promoter hypermethylation was seen at weaning and in adulthood suggesting that the methylation predated the development of the adult phenotype.

In all of the animal studies, samples harvested were either from the

whole hypothalamus, ARC or PVN. One issue with analysing whole hypothalamus is that cell heterogeneity (i.e. the presence of cells that are not exclusively ARC or PVN cells) may dampen any positive signal and could risk type 2 errors, or conversely increase the risk of type 1 errors in the case of confounding due to changing cell populations between treatment groups.

There are a number of studies which report no association between DNA methylation and *POMC* expression, suggesting that other epigenetic processes could drive differential expression. However, studies that accounted for leptin or insulin levels when interpreting *POMC* gene expression more often found significant differences (Plagemann et al., 2009). Mechanistic studies are required to fully understand how alterations in *POMC* epigenetic pathways influence gene expression or phenotype, for example by assessing the effect of gene 'knock out' or 'knock down' models that target specific epigenetic processes. An exemplar of this approach is demonstrated by Wang et al. (2014) where the mechanism by which Mecp2 and CREB1 influence *POMC* methylation, gene expression and phenotype was elucidated using an MECP2 knock out in mouse POMC neurons.

It is important to note that whilst many studies did account for multiple comparisons (e.g., Gali Ramamoorthy et al., 2018; Marco et al., 2014, 2016, 2013; Begum et al., 2012; Paternain et al., 2012; Boersma et al., 2016; Plagemann et al., 2009; Cifani et al., 2015; Liu et al., 2013; Unnikrishnan et al., 2017; Palou et al., 2011) some studies did not (Desai et al., 2016; Hua et al., 2019; Shi et al., 2013; Mahmood et al., 2013; Lazzarino et al., 2017; Fan et al., 2011; Zhang et al., 2014). The need for multiple testing corrections can be context dependent since methylation levels within genomic regions such as CpG islands may be correlated, suggesting that a correction isn't justified.

4. Human studies

The establishment and maintenance of cell-specific gene expression profiles is a key function of epigenetic processes, and epigenetic marks including DNA methylation are therefore generally tissue-specific. Thus a significant challenge in human epigenetic studies is selecting relevant accessible tissue to examine. Peripheral blood cell (PBC), buccal epithelial cells (BEC) or hair follicles are often used as they are easily accessed and minimally invasive. However, the degree of tissue discordance in DNA methylation can be a problematic and limit how much can be inferred from an accessible tissue (such as PBC) about the tissue of interest (i.e. the hypothalamus for *POMC*'s regulation of energy balance). In this respect, the use of human cadaveric samples can be useful to understand the correlation in methylation between the accessible tissue and the tissue of interest (Kühnen et al., 2016a). An assessment of two or more samples originating from different germ layers e.g. PBC (mesoderm) and BEC (ectoderm) can be used to identify systemic methylation patterns.

4.1. Nutritional influences on the POMC gene – Evidence in humans

As with animal studies (see Tables 1 and 2), there is evidence in humans that *POMC* gene methylation is associated with nutrition in early pregnancy and diet into adulthood (see Table 3).

4.1.1. Influence of periconceptional diet on the POMC gene

A body of work in mouse and humans has focussed on nutrition-associated changes in DNA methylation at metastable epialleles (MEs). MEs are epigenetic loci with characteristic methylation patterns that are established early in embryonic development (Rakyan et al., 2002) that are often associated with neighbouring transposable elements (Waterland and Jirtle, 2003).

The most robust evidence for MEs comes from the Agouti viable yellow (A^{vy}) (Waterland and Jirtle, 2003) and Axin-fused ($Axin^{Fu}$) mouse models (Waterland et al., 2006). For example, isogenic A^{vy} mice show variable agouti expression dependent on DNA methylation at a cryptic promoter within an IAP (intracisternal A particle (IAP)) upstream of the *agouti* gene. A maternal diet rich in methyl donor nutrients folate, B12, choline and betaine gives rise to increased methylation at the IAP in A^{vy} offspring (Waterland and Jirtle, 2003). The degree of methylation is associated with phenotype such that hypomethylation leads to increased ectopic agouti expression, yellow fur and an obese phenotype. In contrast, hypermethylation at the same region is associated with reduced expression and a lean mouse with brown or mottled fur.

A number of human studies have demonstrated associations between periconceptional diet and DNA methylation at putative MEs (Finer et al., 2016; Silver et al., 2015; Dominguez-Salas et al., 2014), and interestingly there are multiple lines of evidence, including sensitivity to maternal nutrition, that *POMC* is a putative human metastable epiallele.

Firstly, as with the *agouti* mouse, there is evidence that the methylation state of the *POMC* gene in humans is sensitive to maternal diet in early pregnancy. Mother-child paired blood samples from a Gambian cohort demonstrated an association between early pregnancy one-carbon metabolite concentrations in maternal plasma and offspring PBC *POMC* methylation (Kühnen et al., 2016a). Specifically, there was a significant negative correlation for SAH and positive correlations with betaine and the ratio of SAM to SAH at a region spanning the intron2/exon3 boundary of the *POMC* gene. Offspring DNA methylation was also associated with Gambian season of conception, with lower DNA methylation at the *POMC* VMR (variably methylated region) in children conceived in the dry season compared to those conceived in the rainy season (Kühnen et al., 2016a). In contrast to animal models of maternal overnutrition (Gali Ramamoorthy et al., 2018; Marco et al., 2014; Zheng et al., 2015), neither maternal body weight at conception nor weight change in pregnancy correlated with offspring *POMC* methylation (Kühnen et al., 2016a).

Secondly, *POMC* methylation appears to be set very early in embryonic development. Evidence for this comes from post mortem samples that demonstrate *POMC* DNA methylation is highly correlated across tissues originating from different germ cell layers i.e. brain – ectoderm, and kidney or PBC – mesoderm (Kühnen et al., 2016a). This suggests the methylation state was set prior to the separation of the germ layers at gastrulation; the canonical feature of an ME.

Thirdly, methylation at the *POMC* VMR is thought to be largely independent of genotype, at least in *cis*, with similar methylation patterns across genetically diverse cohorts (Kühnen et al., 2016a). It is interesting to note that MEs were originally identified in isogenic mice and defined as being independent of genotype. While current evidence suggests this may be true at the *POMC* VMR, it has recently been proposed that MEs may be sensitive both to environment and local genotype (Kessler et al., 2018).

Fourthly, *POMC* methylation is associated with the presence of neighbouring transposable elements. A considerable proportion of the human genome consists of transposable elements such as retrotransposons. The most common retrotransposons in the human genome are Alu elements (Price et al., 2004). Kühnen et al., observed three Alu elements in intron 2 (Kuehnen and Krude, 2012) of the *POMC* gene (see Fig. 1). These Alu elements are only found in humans and higher primates (e.g. chimpanzees) and are not found in more distant primates or mice. Those species with Alu elements, demonstrated the same hypermethylation pattern of intron 2, however those without Alu elements showed hypomethylation (Kuehnen et al., 2012) suggesting that the presence of Alu elements drives hypermethylation in this region. This demonstrates a similar pattern seen in MEs where alteration in gene expression is driven by methylation at retrotransposons, similar to that seen with the IAP retrotransposon in the A^{vy} mouse (Morgan et al., 1999).

Importantly, assessment of *POMC* methylation in new-born and adolescent blood samples demonstrated that the methylation pattern appears stable, suggesting that associations with postnatal phenotypes

Table 3
Summary table of human studies of *POMC* gene methylation in relation to energy balance and metabolic disease outcomes.

Study type and participants	DNA Tissue Source	Outcomes and summary of findings	Illustration of region of interest in *POMC* gene[†]	Reference
Case control Anorexia Nervosa; recovered (n = 30) & acutely underweight (n = 31) vs. normal weight adult women (n = 30)	PBC	• Mean promotor *POMC* methylation was not different across nutritional states nor across disease groups • ↓*POMC* mRNA expression in those with malnutrition and hypoleptinaemia		Ehrlich et al. (2010)
Case control Anorexia Nervosa; recovered (n = 21) & acutely underweight (n = 40) vs. normal weight adult women n = 54	PBC	• Mean promotor *POMC* methylation was not different across nutritional states nor across disease groups but negatively associated with smoking		Ehrlich et al. (2012)
Two Case control studies Obese vs. normal weight children (n = 71 vs. n = 36 & n = 100 vs. n = 54)	PBC	• Higher *POMC* methylation in obese vs. normal weight • *POMC* methylation stable in childhood • *POMC* methylation set early in development • *POMC* hypermethylation associated with reduced *POMC* expression		Kuehnen et al. (2012)
Longitudinal cohort study Children from birth to mid-childhood (n = 90)	PBC	• Higher triglycerides and insulin in the high *POMC* methylation group compared to mid and low *POMC* methylation groups • No difference in BMI or adiposity between groups • *POMC* methylation highly correlated from birth to mid childhood		Yoo et al. (2014)
Case control study Adults following a weight loss intervention; 'regainers (n = 7) vs non-regainers (n = 11)'	PBC	• Higher methylation in regainers vs. non regainers • % weight regain correlated with % *POMC* methylation		Crujeiras et al. (2013)
Case control study Adults obese (n = 103) vs. normal weight (n = 125) **Cross sectional study of cadaveric ARC samples and BMI (n = 41)** **Brain and Kidney samples to assess *POMC* methylation across different germ layers (n = 16)** **Longitudinal cohort** Methylation assessed from birth to adolescence (n = 52) **Mother-Child pairs from Gambia** n = 144 mother- child pairs	PBC ARC Brain & Kidney PBC PBC	• Positive correlation between BMI and *POMC* methylation in both PBC and ARC samples • Non-tissue specificity of *POMC* methylation • *POMC* methylation stable through childhood • *POMC* associated with one-carbon metabolites around conception		Kühnen et al. (2016a)
Cross sectional Obese children (n = 82)	PBC	• No correlation between *POMC* methylation and BMI		Ács et al. (2017)
Randomised controlled trial Healthy normal weight adults, SFA (n = 17) vs PUFA (n = 14) diets	Adipose tissue	• PUFA but not SFA diet altered mean methylation at *POMC*	Illumina Human Methylation 450 K array (26 Individual CpGs related to POMC gene, 6 significant CpGs[†])	Perfilyev et al. (2017)
Case control study Overweight/obese (n = 41) vs. normal weight (n = 79) children	PBC	• Lower methylation in overweight/obese groups compared to normal weight • Association between hypermethylation in CpG site 2 & lower HDL-cholesterol levels		Kwon et al. (2018)

Key: ARC; arcuate nucleus of the hypothalamus, PBC; peripheral blood cells, *POMC*; Proopiomelanocortin, BMI; Body Mass Index, ARC; arcuate nucleus, CpG; cytosine-guanine dinucleotide, PUFA; polyunsaturated fatty acids SFA; saturated fatty acid, HDL; high density lipoprotein.

[†] Where reported, the genomic coordinates refer to hg19 genome build; Ehrlich et al. (2010, 2012): 68 CpGs between chr2:25,392,258 – 25,391,492, Kuehnen et al. (2012): 10 CpGs between chr2:25,384,508–25,384,832, Yoo et al. (2014): 4 CpGs between chr2:25383999–25384108, (Crujeiras et al., 2013): 2 CpGs demonstrated significance between chr2:25,391,046–25,391,545 (total of 52 CpGs in this genomic region), Kühnen et al. (2016a): 9 CpGs between chr2:25,384,508–25,384,832, Ács et al. (2017): CpG Island in exon 1, (Perfilyev et al., 2017) CpGs at chr2:25,391,670, chr2:25,384,809, chr2:25,389,989, chr2:25,384,293, chr2:25,384,762, chr2:25,391,505 (Kwon et al., 2018) CpGs between chr2: 25383999–25384108.

are not driven by reverse causation effects (Kühnen et al., 2016a). This is further supported by Yoo et al. (2014), who assessed *POMC* methylation in a longitudinal birth cohort and showed that methylation was highly correlated from birth to childhood (r = 0.80, p = 0.0001).

4.1.2. POMC methylation and diet in adulthood

SFA (saturated fatty acid) diets are associated with increased visceral and hepatic steatosis and PUFA (polyunsaturated fatty acids) diets are associated with increased lean mass (Rosqvist et al., 2014). There is evidence that these diets may influence DNA methylation. The LIPOGAIN study (Perfilyev et al., 2017), a double-blind randomised controlled trial, gave healthy normal weight adults 7 weeks of a daily muffin either high in SFA (n = 17) or PUFA (n = 14). Adipose tissue samples were taken at baseline and at the end of intervention and used for methylation analysis. There was increased global methylation in adipose tissue following both diets. However, *POMC* was one of a number of genes where mean methylation in adipose tissue increased only in response to PUFA and not SFA treatment.

4.2. POMC methylation and weight-related phenotypes

The majority of evidence related to *POMC* epigenetics and weight regulation has come from animal models. However, more recently, studies have explored the role of epigenetic regulation of *POMC* and weight-related phenotypes in humans (see Table 3).

4.2.1. Obesity

Kuehnen et al. (2012) were first to examine the relationship between *POMC* DNA methylation and obesity in humans. In a case control study comparing 71 obese and 36 normal weight children, they reported a significant difference in average PBC *POMC* DNA methylation at a VMR overlapping the boundary of intron2/exon3 (average methylation 25% normal weight vs. 40% obese, $p < 0.001$). This finding was replicated in a second case control study in children with comparable results. An association between *POMC* hypermethylation at the VMR and BMI was demonstrated in both PBC and cadaveric MSH neurons in adults (Kühnen et al., 2016a). The effect size was largest in MSH neurons, where a 10% increase in methylation was associated with a 2.8 kg/m^2 increase in BMI (Kühnen et al., 2016a). Kühnen et al., also demonstrated that hypermethylation at the VMR decreased histone acetyltransferase P300 binding at the VMR (see Fig. 1), leading to reduced expression of *POMC* from PBC. P300 is an enzyme that promotes transcription through histone acetylation (Kuehnen et al., 2012). There was no association between BMI and DNA methylation in the CpG island in the *POMC* promoter region (Kuehnen et al., 2012).

The association of BMI and *POMC* methylation appears dependent on the region of the gene studied. For example, a recent study by Ács et al. (2017) examining 82 obese children aged 3–18 years old did not show any correlation between PBC *POMC* methylation and BMI. This study examined DNA methylation at exon 1 of the *POMC* gene rather than the intron2/exon3 region studied by Kühnen et al. (2016a). Another possible explanation for the lack of correlation between *POMC* methylation and BMI is that this study examined obese children with BMI > 95th percentile, and there may not be a strong correlation with methylation and BMI at these extremes of weight compared to comparisons made with normal weight individuals or the general population. Furthermore, Yoo et al. (2014) reported no significant association between BMI or percentage body fat with *POMC* methylation in exon 3. A possible explanation is that the genomic region studied is downstream of the region associated with P300 binding (Kuehnen et al., 2012, 2016). In this study, PBC *POMC* methylation (at 4 CpG sites in exon 3) measured in cord blood was subdivided into categories of either high (> 90th centile), mid or low (< 10th centile) methylation. Birth weights (p = 0.01) and ponderal indices (p = 0.01) in the high *POMC* methylation group were significantly lower than in the mid-methylation group, but there were no differences in BMI z-score or percentage body fat between the groups by mid-childhood. It should be noted that there were only 10 children in each of the high and low groups and no clear justification was given for splitting the participants into methylation categories, rather than using methylation as a continuous variable. In a recent case control study of 79 controls and 41 overweight/obese children aged 7–9 years, Kwon et al. (2018) compared PBC *POMC* methylation (4 CpG sites in exon 3 and a similar region to Yoo et al. (2014) and reported significantly lower methylation in the overweight/obese groups compared to normal weight individuals. This genomic region is downstream from intron2/exon3 border and again would not have included the region associated with P300 binding. Though statistically significant, mean differences in methylation were small, for example the methylation difference at *POMC* CpG site 2 was 50.3% (normal weight) vs 49.1% (overweight/obese), $p < 0.001$. These differences highlight the need to study defined CpGs and it should be noted that there is limited coverage of the regions described in the studies above on methylation arrays such as the Illumina EPIC Array.

As mentioned above, there is evidence that *POMC* methylation is stable through infancy into adolescence and that hypermethylation at *POMC* may predate the onset of obesity (Kuehnen et al., 2012; Yoo et al., 2014). Kühnen et al., examined PBC DNA from individuals aged 5 or 13 years who later became obese and 8 of 21 individuals had the hypermethylation variant many years before the onset of obesity, implying that *POMC* hypermethylation is not merely a consequence of increased body mass (Kuehnen et al., 2012).

In summary, the strongest evidence of an association between *POMC* methylation and BMI comes from the case control studies of Kuehnen et al. (2012) and Kühnen et al. (2016a). Other studies examine different regions of the gene where there is limited evidence for an association with body weight (Yoo et al., 2014; Kwon et al., 2018).

4.2.2. Metabolic outcomes

There are reported associations between *POMC* methylation (in exon 3) and metabolic outcomes that appear independent of BMI or adiposity. Yoo et al. (2014), reported significantly higher fasting triglycerides (TG) (though not total cholesterol) in children aged 7–9 years in the high and middle level *POMC* methylation groups compared to the low methylation group; high vs low (TG = 113.89 mg/dl vs 57.97 mg/dl, p = 0.03) and middle vs low (TG = 67.29 mg/dl vs 57.97 mg/dl, p = 0.01). This is despite there being no difference in BMI or percentage body fat between the groups. Kwon et al. (2018), reported significant association between methylation at a CpG site in exon 3 and lower HDL (high density lipoprotein) cholesterol levels ($\beta = -0.23$, p = 0.048) after adjusting for age, gender and BMI. Interestingly, polymorphisms in melanocortin signalling pathways have been linked to altered lipid metabolism, independent of body habitus suggesting a possible distinct causal mechanism (Perez-Martinez et al., 2011).

There is evidence of an association between elevated insulin and increased *POMC* methylation. Yoo et al. (2014), demonstrated higher fasting insulin levels in the high and middle *POMC* methylation groups compared to low methylation group; high vs low (insulin 10.13 μIU/ml vs 7.1 μIU/ml vs p = 0.05) and mid vs low (insulin = 7.64 μIU/ml vs. 7.1 μIU/ml, p = 0.02). A non-significant trend to greater insulin resistance (homeostatic model assessment of insulin resistance (HOMA-IR)) between high (p = 0.09) and mid (p = 0.06) groups compared to low methylation group was also observed, and no significant difference in blood glucose was reported between the different methylation groups.

4.2.3. POMC methylation as a predictor of successful weight loss intervention

Crujeiras et al. (2013) explored the utility of *POMC* methylation as a potential biomarker for success in weight loss interventions. Eighteen men enrolled in a dietary intervention programme who successfully lost more than 5% of their body weight were reviewed at 32 weeks post

intervention. Participants were divided into two groups: regainers (regained more than 10% body weight) and non-regainers (regained less than 10% body weight). Higher *POMC* promoter methylation was seen in regainers vs. non-regainers ($p = 0.02$) with a percentage body weight regain to methylation correlation coefficient of 0.6. Interestingly, there was an opposite trend for *NPY* gene methylation.

4.2.4. POMC methylation and anorexia nervosa

Individuals with anorexia nervosa (AN) reportedly have low levels of folate (Castro et al., 2004) and elevated homocysteine (Frieling et al., 2005); important components of one-carbon metabolism pathways (see Fig. 3). Furthermore, lower global DNA methylation has been reported in AN compared to normal weight controls (Frieling et al., 2007). This combined with *POMC*'s influence on appetite regulation makes the gene a key candidate to explore epigenetic influence on the development of AN. Ehrlich et al. (2010), explored the relationship of PBC *POMC* promoter DNA methylation and expression of *POMC* mRNA in both acutely admitted and weight recovered women with AN and normal weight female controls. Mean *POMC* promoter methylation was neither different across nutritional states nor across disease groups. *POMC* mRNA expression was decreased in those with undernutrition and hypoleptinaemia. The study demonstrated that expression of *POMC* is linked to nutritional state rather than a distinct feature of AN.

Ehrlich et al. (2012) later confirmed observations from the earlier study that there was no effect in women of undernutrition or a diagnosis of AN on PBC *POMC* promoter DNA methylation. However, they did observe significant associations between cigarette smoking and PBC *POMC* DNA methylation. Overall PBC *POMC* promoter methylation was negatively associated with average number of cigarettes smoked per day ($\rho = -0.287$, $p = 0.002$). Nicotine is known to induce hypophagia and this is thought to be mediated through *POMC* neuronal activation (Mineur et al., 2011; Huang et al., 2011). Smokers have a lower body weight compared to non-smokers (Chiolero et al., 2008) and weight gain following smoking cessation has been observed (Filozof et al., 2004). This is a cross sectional study and as such the direction of causality between smoking and *POMC* methylation is not known.

In summary, these studies do not suggest an association with *POMC* methylation and AN diagnosis.

5. Summary of evidence from human studies

There is evidence in both children and adults that hypermethylation at the *POMC* VMR of intron2/exon3 is associated with obesity (Kuehnen et al., 2012, Kühnen et al., 2016a). There are a number of lines of evidence to suggest that this region of the *POMC* gene is a putative human ME and influenced by periconceptional nutritional status. An association between *POMC* gene methylation and body weight was not replicated in other studies that examined different regions of the gene (Yoo et al., 2014; Ács et al., 2017; Kwon et al., 2018).

Results from two studies in children show an association between *POMC* methylation in exon 3 and altered levels of lipids (Yoo et al., 2014; Kwon et al., 2018). There is also preliminary evidence in children that *POMC* methylation is associated with elevated fasting insulin (Yoo et al., 2014). Crujeiras et al. (2013), report an association between hypermethylation in the *POMC* promoter and weight regain after a weight management intervention which demonstrates how *POMC* methylation measurement could inform clinical risk stratification and help guide tailored interventions.

Cell specific methylation patterns in heterogenous samples are a potential confounder in epigenetic studies and are generally accounted for in epigenome-wide association studies by adjusting for cell composition estimates derived from the DNA methylation data itself (Teschendorff and Zheng, 2017). The region of intron2/exon3 described by Kuhnen et al is a putative ME, and as such demonstrated cross-tissue concordance in DNA methylation patterns in tissues derived from all 3 germ layers, notably between PBC and the hypothalamus (Kühnen et al., 2016a). Therefore, in this instance correcting for cell composition should not be necessary as the methylation pattern is thought to be systemic. The only human study to correct for cell composition was Perfilyev et al. (2017) who used a reference-free method to correct for potential differences in adipose tissue cell type composition. Furthermore, a comparison of POMC methylation in the promoter or exon 1 between the hypothalamus and PBC has not been made before.

Periconception and gestation represent key windows where there is the potential for the prenatal environment to influence epigenetic reprogramming. However environmental and nutritional factors may influence DNA methylation throughout adult life (Martin and Fry, 2018). For example, methylation at a number of *POMC* CpGs was associated with PUFA diet in a study in adults as described above (Perfilyev et al., 2017). Thus while there is evidence that the *POMC* ME region may be stable from periconception to late adolescence (Kühnen et al., 2016a; Yoo et al., 2014), sensitivity to specific postnatal factors, and stability beyond this period warrants further study.

Further study is also needed to establish causative links between *POMC* methylation, altered gene expression and subsequent phenotype. Though Kuehnen et al. (2012), did see lower expression of *POMC* in PBC from hypermethylated individuals many studies do not examine *POMC* expression (Yoo et al., 2014; Crujeiras et al., 2013; Perfilyev et al., 2017; Ács et al., 2017; Kwon et al., 2018) or find no association between methylation and expression (Ehrlich et al., 2010). Exploration of how *POMC* methylation could influence energy consumption or the satiety response has also not been studied before in humans.

5.1. Transgenerational inheritance of epigenetic marks at the POMC gene

Maternal exposures before and during pregnancy including environmental (e.g. toxins or stress) or nutritional factors can induce epigenetic changes in the offspring. In this case transmission of epigenetic changes is referred to as inter-generational epigenetic inheritance (Heard and Martienssen, 2014). In exposed mothers (F0), developing offspring (F1) and their germ cells (F2) are also exposed. Transmission of epigenetic changes resulting from maternal exposure in F0 that persists into the F3 generation or beyond (i.e. no direct exposure) is termed transgenerational inheritance. In contrast, where epigenetic changes result from an exposure in F0 males, effects in F1 are inter-generational whereas those apparent in F2 and beyond will be trans-generational (Heard and Martienssen, 2014). Evidence for trans-generational epigenetic inheritance in humans is currently lacking and may be extremely rare due to epigenetic reprogramming at conception and during germ cell development (Horsthemke, 2018).

Evidence for human inter-generational inheritance through the maternal line has been described above. There is provisional evidence from animal and human studies to suggest *POMC* epigenetic marks may be transmitted across generations and mediated via the paternal line. Firstly, evidence from animal models on the effect of fetal alcohol exposure on *POMC* epigenetic marks suggests the potential for transgenerational epigenetic inheritance via the male germline. Secondly, evidence from family trios in humans demonstrates a significant correlation between offspring PBC *POMC* methylation and paternal, but not maternal *POMC* methylation.

5.1.1. Fetal alcohol syndrome (FAS) and epigenetic inheritance

Recent studies have implicated a role for appetite regulating neuropeptides (including *POMC*) in alcohol dependence and craving (Hillemacher et al., 2010; Fortuna, 2010). *POMC* promoter methylation has been shown to be associated with craving in those with alcohol dependency (Muschler et al., 2014, 2010). In human studies, paternal alcohol dependency has been associated with alterations in the hypothalamic-pituitary axis including changes to ACTH secretion (a derivative of *POMC*) in offspring (Hernandez-Avila et al., 2002; Zimmermann et al., 2004). FAS is seen more frequently in the F2 generation of an alcohol abusing mother (i.e. her grandchildren)

compared to F2 controls (Kvigne et al., 2008). Govorko et al. (2012) explored the possibility of intergenerational or transgenerational effects from alcohol exposure in a rat model by establishing two germlines: (1) breeding male fetal alcohol exposed rats and their male offspring with unexposed females and (2) breeding female fetal alcohol exposed rats and their female offspring with unexposed males. Hypermethylation of the *POMC* promoter and reduced *POMC* expression were seen in both female and male offspring in the F1 generation (of alcohol consuming mothers), but this pattern continued in male progeny in F2 and F3 from the male germline only. Thus a transgenerational effect was only seen via the male exposed germline. *POMC* promoter methylation was higher in sperm of male rats (F1-F3) from the male exposed germline suggesting a possible mechanism of epigenetic inheritance via methylation differences in the sperm. It is postulated that maternal fetal alcohol exposure to the developing progeny leads to transgenerational epigenetic transmission through the male germline thereafter (Mead and Sarkar, 2014).

Interestingly, maternal supplementation with choline (a one-carbon metabolite) altered the epigenome of the offspring such that *POMC* methylation and expression, and DNMT1 and Mecp2 levels were no different to non-alcohol exposed rats (Bekdash et al., 2013). It is known that alcohol interferes with one-carbon metabolism (Fowler et al., 2012) and therefore it seems intuitive to consider supplementation to normalise the levels. Additionally, giving the alcohol consuming pregnant rat either DNMT1 inhibitor or HDAC inhibitor reversed the methylation and expression changes in the offspring caused by prenatal alcohol exposure, suggesting a potential for future interventions (Govorko et al., 2012). This demonstrates an interesting point of principle, albeit in rats, that changing the metabolome during gestation can alter the offspring epigenome in relation to *POMC* and mitigate the effects of alcohol. Were similar effects to be evident in humans, this could lead to a major new approach in preventative medicine with targeted maternal nutritional interventions to favourably influence the offspring's epigenome and break the intergenerational risk of diseases like obesity, as suggested by observed links between maternal concentrations of one-carbon metabolites, *POMC* methylation and obesity in humans (Kühnen et al., 2016a).

5.1.2. Human family trios

Kühnen et al. (2016a) examined 47 mother-father-offspring trios and demonstrated significant correlation with offspring PBC *POMC* methylation and the father's PBC *POMC* methylation but not the mother's, suggesting a potential intergenerational influence from the father. However, sperm methylation at this region was significantly lower than PBC, suggesting that the apparent paternal inheritance of epigenetic marks seen in the offspring was not mediated through sperm methylation. One potential mechanism is through modifications to sperm RNAs (Chen et al., 2016).

5.1.3. Y chromosome-linked patriline inheritance

Studies suggest a possible link between *POMC* expression, methylation and areas of the Y chromosome. The non-paring region of the Y chromosome (Y^{NPAR}) is exclusively transmitted between fathers and sons and includes functional genes such as SRY (sex determining region). Previous studies have suggested a possible interaction between SRY-androgen receptor binding and *POMC* methylation (Muschler et al., 2014). A study in mice has demonstrated a significant Y^{NPAR} influence on brain β – endorphin (a derivative of *POMC*, see Fig. 1) levels (Botbol et al., 2011), suggesting a possible interaction with genetic polymorphisms in Y^{NPAR} on β – endorphin expression. Alternatively, it has been postulated that epigenetic changes on the Y^{NPAR} chromosome (for example caused by alcohol exposure) may influence *POMC* expression and/or methylation in the offspring and be a potential mechanism for epigenetic inheritance via the male line (Sarkar, 2016).

6. Conclusions

POMC is a key mediator of satiety and perturbations in the melanocortin system have been associated with dysregulation of energy balance. In animal models *POMC* gene methylation has been shown to be influenced by the prenatal and postnatal environment and associated with subsequent weight and appetite related phenotype in adulthood. In humans periconceptional nutrition has been associated with offspring methylation at *POMC*. Human studies have often demonstrated contradictory associations between *POMC* methylation and BMI and these appear to be dependent on the region of the gene studied. Therefore care should be exercised when selecting genomic regions for study. More prospective studies are needed to examine the influence of *POMC* DNA methylation on energy balance. Early studies suggest that *POMC* is an interesting candidate for exploring inter and transgenerational epigenetic inheritance in humans and future research should elucidate potential mechanisms for this.

There are potential clinical applications for using *POMC* epigenetic testing as a biomarker for early identification of obesity risk and as a predictor of response to obesity interventions. There are also potential pharmacological options with Setmelanotide, a MC4R agonist, demonstrating success in treating those with *POMC* deficiency (Kühnen et al., 2016b), although it is yet to be established if this could prove an option for those with *POMC* hypermethylation. Looking to the future, a better understanding of nutritional factors influencing the epigenetic regulation of *POMC* could pave the way for maternal and paternal nutritional interventions that would provide a more favourable epigenotype, so reducing the risk of obesity in the next generation.

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Declaration of Competing Interest

None.

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The images of the stomach and brain in Fig. 2 were made by Smashicons available from www.flaticon.com. The protein image in the graphical abstract was adapted from an icon from www.icons8.com. The rodent and human icons in the graphical abstract were made by koksikoks available from www.iconfinder.com.

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