DISSERTATION

Analysis of POMC DNA methylation variability and the impact on hypothalamic body weight regulation

Analyse der POMC DNA Methylierungsvariabilität und die Bedeutung für die hypothalamische Körpergewichtsregulation

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List of abbreviations

AgRP	Agouti related peptide
AHCY	Adenosylhomocysteinase
BDNF	Brain-derived neurotrophic factor
BMI	Body mass index
C1	Carbon-1
cDNA	Complementary DNA
CpG	Cytosine-Guanine dinucleotide
DMEM	Dulbecco’s modified Eagle’s medium
DNA	Desoxyribonucleic acid
EWAS	Epigenome-wide association study
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GWAS	Genome-wide association study
hESC	Human embryonic stem cell
HNR	Heinz Nixdorf Recall
HPLC	High performance liquid chromatography
IAP	Intra-cisternal A particle
ICM	Inner cell mass
KLF17	Kruppel like factor 17
LEPR	Leptin receptor
LIF	Leukemia inhibitory factor
MAT2A	Methionine adenosyltransferase 2A
MC4R	Melanocortin 4 receptor
MSH	Melanocyte stimulating hormone
NKX2.1	NK2 homeobox 1
OR	Odds ratio
PBAT	Post-bisulfite adapter tagging
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PCSK1	Proprotein convertase subtilisin/kexin type 1
POMC	Proopiomelanocortin
PRS	Polygenic risk scores

RNA	Ribonucleic acid
RRBS	Reduced representation bisulfite sequencing
RT-PCR	Reverse transcription-PCR
scRNAseq	Single cell RNA sequencing
SHMT1	Serine hydroxymethyltransferase 1
SNP	Single nucleotide polymorphism
TBP	TATA-binding protein
TET1	Ten-eleven translocation methylcytosine dioxygenase 1
TFCP2L1	Transcription factor CP2 like 1
VMR	Variable methylated region

Abstract

Obesity is a disease with a rapidly increasing prevalence worldwide, accompanied by comorbidities like cardiovascular diseases or diabetes mellitus type 2. This represents an enormous burden for health care systems. However, the underlying mechanisms that might explain the individual risk for the development of obesity remain mainly unclear. proopimelanocortin (*POMC*) plays a central role in hypothalamic satiety regulation through the leptin-melanocortin pathway. Mutations within the *POMC* gene are leading to severe early-onset obesity and can be successfully treated with a melanocortin 4 receptor (MC4R) agonist. In addition to rare genetic mutations, epigenetic variations of the *POMC* gene correlate with body weight. The DNA methylation at the variable methylated region (VMR) at the beginning of exon 3 of the *POMC* gene was significantly increased in obese children and adults compared to normal weight controls, which was confirmed in post-mortem hypothalamic neurons.

In the present PhD-project, in order to characterize this epigenetic variant more specifically, we provide evidence that the *POMC* methylation variability seems to occur independently of any genetic variant by conducting a genome-wide-association study. This data argues for a stochastic setting of the *POMC* DNA methylation signature during the early in utero stadium, which is supported by our evidence of high methylation concordance between monozygotic twins, sharing this period on a cellular level, as opposed to dizygotic twins. Moreover, the difference in methylation between obese and normal-weight individuals has been reproduced, and a sex-specific effect was uncovered.

To disentangle the relationship between increased methylation and neuronal function and model the in utero establishment of methylation patterns, we established a stem cell based model. Assessing the transition of naïve human embryonic stem cells (hESC) blending into primed hESCs and then further differentiation into hypothalamic-like neurons, we could mimic the dynamics observed in human embryonic tissue using publicly available whole methylome data sets. Furthermore, we analyzed the impact of nutritional factors during early DNA methylation patterning. Carbon-1 (C1)-metabolite concentrations were varied during the formative transition phase prior to differentiation. The developed stem cell model is used to study the susceptible early embryonic phase, modeled between the naïve cell stage and the formative transition. Moreover, we evaluated the *POMC* expression of differentiated neurons according to their *POMC* DNA methylation

and observed a significant negative correlation between DNA methylation and *POMC* expression.
Taken together, this data supports the hypothesis that increased *POMC* methylation is an epigenetic risk factor for developing obesity, which might be related to impaired function of the leptin-melanocortin pathway.

Zusammenfassung

Die Prävalenz von Adipositas nimmt weltweit stark zu und stellt durch die damit einhergehenden Begleiterkrankungen wie Herz-Kreislauf-Erkrankungen oder Diabetes mellitus Typ 2 eine enorme Belastung für die Gesundheitssysteme dar. Die zugrundeliegenden Mechanismen, die das individuelle Risiko für die Entwicklung von Adipositas erklären könnten, sind jedoch nach wie vor weitgehend unklar. Proopimelanocortin (*POMC*) spielt über den Leptin-Melanocortin-Weg eine zentrale Rolle in der hypothalamischen Sättigungsregulation. Mutationen im *POMC* Gen führen zu schwerer, früh einsetzender Adipositas und können erfolgreich mit einem Melanocortin 4 Rezeptor (MC4R) -Agonisten behandelt werden. Zusätzlich zu genetischen Mutationen korrelieren auch epigenetische Variationen des *POMC* Gens mit dem Körpergewicht. Die DNA-Methylierung an der variabel methylierten Region (VMR) am Anfang von Exon 3 des *POMC* Gens war bei Adipösen im Vergleich zu normalgewichtigen Kontrollen signifikant erhöht, was in postmortem hypothalamischen Neuronen bestätigt wurde.

Um diese epigenetische Variante genauer zu charakterisieren, haben wir im vorliegenden Dissertationsprojekt durch eine genomweite Assoziationsstudie nachgewiesen, dass die *POMC* Methylierungsvariabilität unabhängig von einer genetischen Variante aufzutreten scheint. Diese Daten sprechen für eine stochastische Einstellung der *POMC* DNA Methylierungssignatur während des frühen in utero Stadiums, was durch unseren Nachweis einer hohen Methylierungskonkordanz zwischen eineiigen Zwillingen unterstützt wird. Darüber hinaus konnte der Unterschied in der Methylierung zwischen adipösen und normalgewichtigen Personen reproduziert und ein geschlechtsspezifischer Effekt aufgedeckt werden.

Um den Zusammenhang zwischen erhöhter Methylierung und neuronaler Funktion zu entschlüsseln und die in utero Etablierung von Methylierungsmustern zu modellieren, haben wir ein stammzellbasiertes Modell entwickelt. Indem wir den Übergang von naiven

humanen embryonalen Stammzellen (hESC) in geprimte hESCs und dann die weitere Differenzierung in hypothalamusähnliche Neuronen untersuchten, konnten wir die im menschlichen Embryonalgewebe beobachtete Dynamik mit Hilfe von öffentlich verfügbaren Methylom-Datensätzen nachahmen. Darüber hinaus analysierten wir den Einfluss von Ernährungsfaktoren während der frühen Etablierung der DNA Methylierung. Die Konzentrationen von Kohlenstoff 1 (C1)-Metaboliten wurden während der formativen Übergangsphase vor der Differenzierung variiert. Darüber hinaus haben wir die *POMC*-Expression der differenzierten Neuronen anhand ihrer *POMC* DNA Methylierung bewertet und eine signifikante negative Korrelation zwischen DNA Methylierung und *POMC*-Expression festgestellt.

Zusammengenommen unterstützen diese Daten die Hypothese, dass eine erhöhte *POMC* Methylierung ein epigenetischer Risikofaktor für die Entwicklung von Adipositas ist, der mit einer gestörten Funktion des Leptin-Melanocortin-Signalwegs zusammenhängen könnte.

1 Introduction

1.1 Introduction to the topic

One of the major health challenges of our time is the exponential global increase in the proportion of people who are overweight or obese. According to the World Health Organization, the prevalence of obesity has almost tripled worldwide since 1975 (1). In addition to the associated physical comorbidities, such as diabetes mellitus type 2 and cardiovascular diseases, individuals affected by obesity face a substantial psychological burden due to extensive stigmatization, primarily observed in the global North. Weight stigma itself imposes notable detrimental health consequences, including increased risk of depression and further weight gain (2). This stigmatization predominantly stems from the presumption that excessive body weight is indicative of a lack of willpower and self-control. This presumption is challenged by the strong evidence that weight – also in today´s obesogenic environment - is mostly biologically regulated. However, the highlighted role of biological weight regulation should be framed within the current endemic obesity context. The increase in body weight observed over the past five decades is unlikely attributed to genetic changes within this relatively brief timeframe. Instead changing environmental conditions, including abundant availability of food, social-economic factors and reduced levels of physical activity, are the determining factors. However, the extent to which people gain weight due to changing environmental conditions can be dependent on their genetic background. Epidemiological indications in this regard are provided by the altered weight distribution of the US population in 1985 compared to 2014 (3). Not only was the mean value of the weight distribution increased, but the scatter parameter of the distribution has also changed, showing the increased variability of the phenotype in the population (3). Accordingly, the changed environmental conditions have different degrees of influence on the weight gain of the individual (3). To enhance our comprehension of the pathophysiology of obesity, it is crucial to explore the biological factors that contribute to this divergent response to environmental changes. This exploration should encompass not only genetic variants but also epigenetic factors, as they may play a significant role in shaping the underlying mechanisms and the individual risk high body weight.

1.2 Current state of research

1.2.1 Genetics of obesity

To understand these biological differences, genetic variability is the first consideration. Twin studies play a central role in this regard, as the expected heritability of a certain phenotype can be estimated by comparing mono - and dizygotic twins. In terms of body mass index (BMI), a comprehensive meta-analysis describes that the heritability amounts to approximately 0.47 to 0.9, whereby both, population-related aspects, and factors of the different study designs, could be identified for the large variability of the estimators between the individual studies. An increased genetic influence on BMI during childhood and adolescence was also identified (4). Another study includes the skewness of the BMI distribution in its model of a twin study and presents a two-class solution under the skew-t mixture distribution. They find a very high heritability in the normal weight range and a lower heritability in overweight and obese individuals (5). These results are also reproduced in a large Finnish twin cohort, emphasizing that heritability can vary in different BMI ranges (6). Overall, a high genetic influence on the interindividual variability of BMI can be assumed. In 1997, the identification of congenital leptin deficiency as the initial monogenic form of obesity in humans, along with subsequent discoveries of other candidate genes, provided valuable insights into the physiological mechanisms governing satiety regulation in the hypothalamus (7-9). In summary, the leptin-melanocortin pathway is a central mechanism through which leptin signals satiety and energy balance. It involves the interplay between anorexigenic and orexigenic neurons in the hypothalamus, with the activation of POMC neurons and the MC4R receptor leading to appetite suppression and increased energy expenditure.

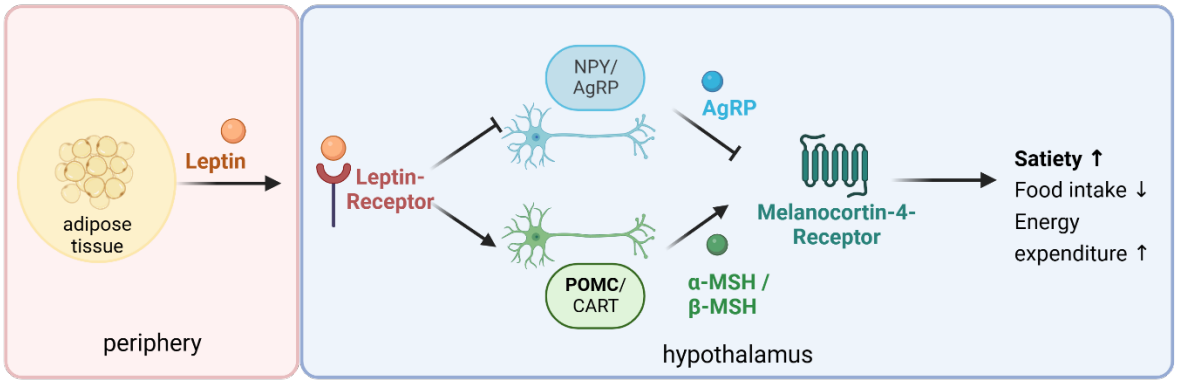

Figure 1: Leptin melanocortin pathway. Leptin is secreted by adipose tissue and, by binding to its receptors in the arcuate nucelus of the hypothalamus, activates anorexigenic neurons that produce POMC. POMC is cleaved into several peptides, including α- and β- melanocyte stimulating hormone (MSH). α- and β- MSH binds to MC4R on target neurons. MC4R activation stimulates pathways that suppress appetite and increase energy expenditure. At the same time, orexigenic neurons that produce agouti related peptide (AgRP) are inhibited. AgRP is an inverse agonist to the MC4R receptor and promotes hunger. The activation of POMC neurons and subsequent MC4R activation leads to reduced appetite and increased energy expenditure, while inhibition of AgRP-containing neurons contributes to the suppression of hunger signals. When leptin levels are high (indicating sufficient energy stores), the leptin-melanocortin pathway is activated, resulting in reduced appetite and an increase in metabolism. Conversely, low leptin levels lead to increased activity of orexigenic neurons and a subsequent increase in hunger. Graphical representation of the signaling pathway was generated by myself using information from Yeo et al., 2021 and Cowley et al., 2001 (10, 11).

However, it is important to note that monogenic forms of obesity account for only a minority of individuals affected by obesity (12). This raises the question of the genetic background of so-called common or polygenic obesity. To investigate this, extensive genome-wide association studies have been carried out, particularly in the last 15 years. In 2015, Locke et al. identified a total of 97 loci that correlate significantly with BMI in a meta-analysis involving 340,000 individuals. However, these single nucleotide polymorphisms (SNPs) explain only about 2.7% of the BMI variability in the population. It should be noted that common variants often have a minimal effect and are therefore difficult to detect, whereas rare variants with somewhat stronger effects are difficult to study due to their rare occurrence. However, extrapolations from this work show that about 21% of the variability can be explained by other common variants (13). The overall picture is that even the lower estimates of BMI heritability from twin studies can only be partially explained by genetic variation. This conceptual problem is called “missing heritability”. Explanations range from the need for more comprehensive genome-wide association studies (GWAS)

to other mechanisms, such as epigenetic changes, which may contribute to heritability in twin studies but are not reflected in genetic changes.

1.2.2 Epigenetic paradigms, limitations and metastable epialleles

A definition of the term epigenetics was formed at the Cold Spring Harbor Laboratory conference in 2008 as followed: “An epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence” (14). Though there are still many different uses of the term epigenetics, there are mainly two distinct schools, the molecular epigenetics and the system epigenetics. While molecular epigenetics focuses on biomolecular marks on the chromatin (15), system epigenetics concentrates on the regulation and maintenance of a specific cell state, as described in the early 20th century by Conrad Waddingtons epigenetic landscape (16). In a recent Perspective Huang emphasizes that recent advances suggest that the two separate epigenetics schools may represent various aspects of the same principle of gene control (17). Huang underlines the role of stability in epigenetic control. This stability can consist of cis-acting gene regulation at one locus through molecular bistable memory switches, as well as trans-acting gene regulatory networks. His conceptual framework highlights the importance of in-depth characterization of epigenetic loci of interest and their stability, in contrast to arbitrary associations of single methylated DNA bases with phenotypes.
These conceptual thoughts help to identify limitations and pitfalls in epigenetic studies. Studies which address the correlation of a certain phenotype with the methylation of Cytosine-Guanine dinucleotide (CpG) positions all over the genome are also called epigenome-wide association studies (EWAS). One example in the context of obesity is a study from Dick et al., where genome-wide methylation was analyzed in relation to BMI (18). These studies imply that considerable limitations exist when thinking about epigenetic variants as risk factors for obesity. Not only are some epigenetic variants identified so far based on genetic variants, but it is also important to distinguish between cause and consequence of DNA methylation variability. Particularly relevant for functional characterization is also cell type specificity, which complicates study designs immensely and is therefore not sufficiently reflected. Another consideration is that most epigenome-wide association studies, including Dick et al., assess DNA methylation by the Illumina Infinium HumanMethylation450 array. These arrays cover 485,577 CpGs which are distributed over the whole genome, however, this is only a fraction of all CpGs and some regions are systematically underrepresented, HM450 for example, lacks coverage of distal regulatory

elements (19). This means that it is very difficult to evaluate the real biological consequences from these EWAS.

Opposite this, a very profound concept is that of metastable epialleles. Metastable epialleles are distinct loci that display high interindividual variability of DNA methylation levels in a population while being stable in an intraindividual longitudinal and tissue-specific context. These loci are additionally linked to the proximity of transposable elements, which are mobile DNA sequences usually silenced through DNA methylation (20, 21). While being independent of genetic influences, there are examples of environmental influences in utero like maternal methyl metabolism (C1-metabolism) having effects on the offspring's DNA methylation levels at metastable epialleles (22). The Agouti (A^{vy}) mouse model represents a decisive milestone as a proof of concept and a remarkable example of a metastable epiallele (figure 1). The spontaneous insertion of a retrotransposon (Intracisternal A Particle, IAP) results in distinct DNA methylation levels, which affect the gene expression of the agouti gene (23). Genetically identical mice that differ solely in their DNA methylation at the agouti gene show a different phenotype in terms of body weight and coat color (24, 25). The agouti gene plays a central role in hypothalamic satiety regulation via its homology with AgRP, functionally linking the DNA methylation of the murine A^{vy} gene with the mice's body weight (26, 27).

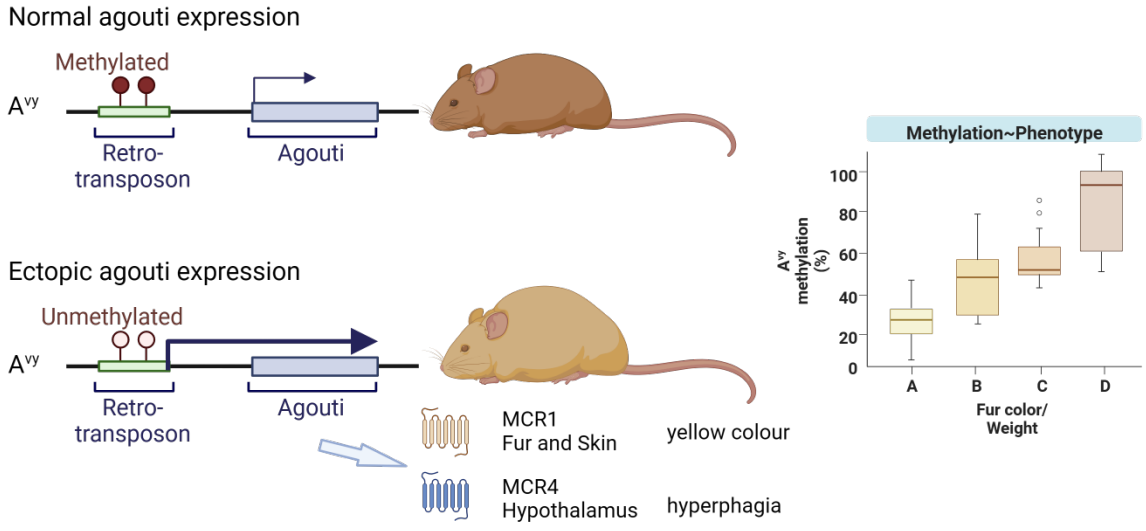

Figure 2: Agouti (A^{vy}) mouse model. Spontaneous insertion of a retrotransposon upstream of the agouti gene leads to differences in phenotype in genetically identical mice. The alternative promotor of the retrotransposon is constitutively active if not repressed through methylation, leading to differences in expression depending on methylation levels. Figure modified according to Jirtle et al., 2007 (28).

1.2.3 *POMC* DNA methylation as metastable epiallele

Hypothalamic satiety control relies heavily on the leptin-melanocortin signaling pathway, in which *POMC* plays a central role (10). *POMC* codes for various melanocortins (α-/β-MSH), which act as satiety neurotransmitters in the hypothalamus through activation of MC4R. Bi-allelic mutations of the *POMC* gene lead to severe early-onset obesity through impaired hypothalamic satiety regulation (9). Since 2021, treatment with the MC4R agonist setmelanotide in patients with bi-allelic mutations in *POMC*, Leptin receptor (*LEPR*) and Proprotein Convertase Subtilisin/Kexin Type 1 (*PCSK1*) has been approved by the European Medicines Agency and the United States Food and Drug Administration, resulting in immense weight loss due to a regained feeling of satiety and a reduction in food intake (29). Previous work by our research group was able to show that increased DNA methylation at the Intron2-Exon3 boundary of the *POMC* gene is associated with an increased individual BMI (30). Obese children and adults show higher *POMC* methylation than normal-weight individuals. This finding is reproducible in human postmortem hypothalamus samples (31) and adipose tissue (32). The *POMC* VMR classifies as a metastable epiallele since longitudinal stability was shown and the intraindividual levels of *POMC* VMR methylation significantly correlate in tissues from different germ lines (31). The *POMC* metastable epiallele is associated with the presence of ALU elements, which are, like IAPs, transposable elements (33). Individual *POMC* DNA methylation levels are most likely to be established in the early embryonic phase, in utero. Generally, maternal methylation is typically low in the oocyte, while paternal methylation in the sperm is erased post-fertilization. Subsequently, the embryo's methylation patterns are re-established after gastrulation (34, 35) (figure 3). This emphasizes the significance of the timeframe in examining epigenetic variations. A correlation was found between the level of *POMC* methylation and the concentrations of C1 metabolites in the mother's blood at the time of conception (31). There is a similarity between this and the A^{vy} mouse model described above. In these mice, too, it could be shown that modifications of the maternal diet (regarding metabolites of the C-1 metabolism such as folate) lead to a change in DNA methylation in the agouti gene of the offspring (36). In contrast to the mouse experiments, where female mice receive modified diet compositions before and during pregnancy, the association of maternal C1 metabolism at the time of conception with *POMC* methylation is based on a cohort study in The Gambia. The population's diet in The Gambia varies seasonally; hence, nutrition varies according to the time of year (dry and rainy seasons),

depending on the available products from the agricultural crop. These seasonal differences in diet lead to changes in trace element and metabolite composition in the blood and influence the birth weight and body weight of the population. In collaboration with Prof. Andrew Prentice, Kühnen et al. studied the DNA methylation of children whose mothers had different C1 metabolite compositions in their serum at the time of conception. It was observed that the methylation intensity of the *POMC* locus in this population correlates with certain metabolites of the C1 metabolism like betaine or the S-adenosylmethionine / S-adenosylhomocysteine ratio (31).

1.2.4 Comprehensive assessment of the literature on *POMC* methylation

Thus, there is evidence that in children and adults, hypermethylation of the *POMC* gene at the intron2-exon3 transition is associated with obesity, with a high probability that it is a human metastable epiallele and is influenced by the periconceptional nutritional status of the mother. In other studies examining other regions, like the *POMC* promoter of the gene, the association between body weight and *POMC* DNA methylation has not been observed (37, 38). Crujeiras et al. describe an association between hypermethylation in the promoter region of the *POMC* gene and weight gain after a weight loss intervention (39). McFadden et al. show that a high-fat diet in male rats increases DNA methylation from the *POMC* promoter and decreases gene expression in the hypothalamus (40). Nevertheless, they found that CRISPR-dCas9-TET1-mediated demethylation from the *POMC* promoter was not sufficient to prevent abnormal weight gain following a high-fat diet. Furthermore, CRISPR-dCas9-DNMT3a-mediated methylation from the *POMC* promoter did not alter weight gain after a normal or high-fat diet. Overall, these results thus suggest that high-fat diet-induced changes in *POMC* promoter DNA methylation in rats are a consequence of, but do not directly contribute to, the development of obesity. In a randomised controlled trial, the influence of diets based on saturated vs. unsaturated fatty acids on methylation in adipose tissue was investigated. Only the diet with unsaturated fatty acids led to an average change in *POMC* methylation (41). Interestingly, Sharma et al. were able to show a strong correlation between *POMC* DNA methylation in the region of chr2:25,384,001–25,385,000 (hg19) and the expression of *POMC* in adipose tissue of a human cohort (32). This region corresponds exactly to the intron2-exon3 transition of the *POMC* gene, which is the region of the metastable epiallele.

In summary, different associations between *POMC* DNA methylation and obesity were presented. These results seem to be mainly dependent on the region of the gene and the

species studied. At the *POMC* promoter region, contradictory results were observed, whereas the associations of *POMC* DNA methylation at the intron-2-exon3 transition appear reproducible in humans. This phenomenon seems to be related to the specific metastable epiallele characteristics at the intron2-exon3 transition, which depend on ALU elements upstream of exon3, which are not present in all species (30). This highlights the relevance of reproducible effects in appropriate cohorts and thoughtful characterization. Descriptions of associations of genetic polymorphisms with DNA methylation in other loci suggest a possible mechanism for how genetic polymorphisms lead to a particular phenotype secondarily via epigenetics. My PhD project focuses on a primarily epigenetic variation of the *POMC* gene at the intron2-exon3 transition. This raises the need to exclude genetic influence to complete the descriptive characterization of the epigenetic variant as a metastable epiallele and the need to establish a suitable human model, as the methylation patterns of the *POMC* gene differ in different species, to explore possible functional mechanisms of how *POMC* DNA methylation is related to the development of obesity.

1.3 Aim

1.3.1 Objectives

This leads to two overarching questions that will be addressed in this thesis:
1. what factors influence the emergence of different levels of *POMC* VMR DNA methylation and
2. does the level of DNA methylation at the *POMC* VMR have functional effects?
This is done against the background of two hypotheses: First, that *POMC* methylation is not determined by individual genetic background, arises stochastically in utero and can be influenced by C1 metabolites; second, that *POMC* methylation affects gene expression, for example, through changes in chromatin organization or transcription factor binding, and thus influences the production of the saturation messenger MSH.

1.3.2 Methodological approach

Different methodological approaches were employed to address these aims.
Firstly, the association of genetic background with the level of DNA methylation could be analyzed in large human cohorts, with the possibility of genome-wide association studies and twin cohorts. In the twin cohorts, we compared the intra-twin correlation of monozygotic vs. dizygotic twin pairs. This gave us information on whether identical twins are more

similar than dizygotic twins, which would mean that *POMC* methylation may contribute to missing heritability if no genetic explanation for this feature is identified. To assess possible genetic explanations, the correlation between individual SNPs and the methylation levels of different CpG positions was characterized in the Heinz Nixdorf Recall (HNR) cohort, in which we analyzed *POMC* methylation through pyrosequencing of more than 1000 samples. This cohort was used to assess additional considerations such as sex-based differences in *POMC* methylation and the connection to BMI.

Secondly, one major aim was to investigate how increased methylation functionally affects the *POMC* gene expression and which mechanisms underlie the establishment of the methylation pattern. To mimic the physiological level of *POMC* DNA methylation during stem cell differentiation, we first reverted the conventionally cultured hESCs to the stage of pre-implantation blastocysts. These cells are the referred to as naïve hESCs in contrast to conventional primed hESCs and are characterized by various features, for example low DNA methylation. In the following stepwise differentiation to *POMC* expressing neurons, the *POMC* DNA methylation was expected to re-establish. This previously undescribed method aimed to mimic the dynamics of *POMC* methylation in utero. Following the establishment of the in vitro model, the functional implications of methylation levels on RNA expression of POMC-neurons were investigated.

2 Material and methods

The content of this material and methods section has been previously described in the associated publication (42). Here I will recapitulate the descriptions of the experiments and analyses that I conducted or majorly contributed to and that led to the results described in this thesis. These were also reported in the attached publication (42).

2.1 Human cohorts

Cohort of the HNR Study: A substantial population-based research cohort called the Heinz Nixdorf Recall Project was started in 2000 and has already been extensively documented (43). 4814 men and women, drawn at random from three German cities (Essen, Mühlheim an der Ruhr, and Bochum), make up the cohort. Participants ranged in age from 45 to 75 at baseline. Pyrosequencing was used to check 1383 samples from the HNR cohort for DNA methylation at the *POMC* VMR (CpG-2 to +7). (Table S1). 1083 pyrosequenced samples had genome wide genotyping data available, which were used in the *POMC* methylation whole genome SNP association study.
Twin Cohorts: From HealthTwist©, blood samples were obtained from 32 pairs of monozygotic twins and 38 pairs of dizygotic twins (MZ: 22 females and 42 males; mean BMI: 24.64 3.45 kg/m^2; DZ: 48 females and 28 males; mean BMI: 25.1 4.46 kg/m^2). The PAX8 methylation of 37 MZ samples and 34 DZ samples was examined.

2.2 Stem cell based model

Since conventional hESCs display an artificially high methylation pattern, the cells were reset to a naïve state similar to the pre-implantation epiblast stage. Naïve embryonic stem cells undergo a distinct intermediate "formative" cell state upon exit from pluripotency, which resembles the in vivo time course of epiblast implantation prior to gastrulation. The cells are then able to respond to inductive signals, allowing subsequent differentiation into *POMC*-expressing neurons.

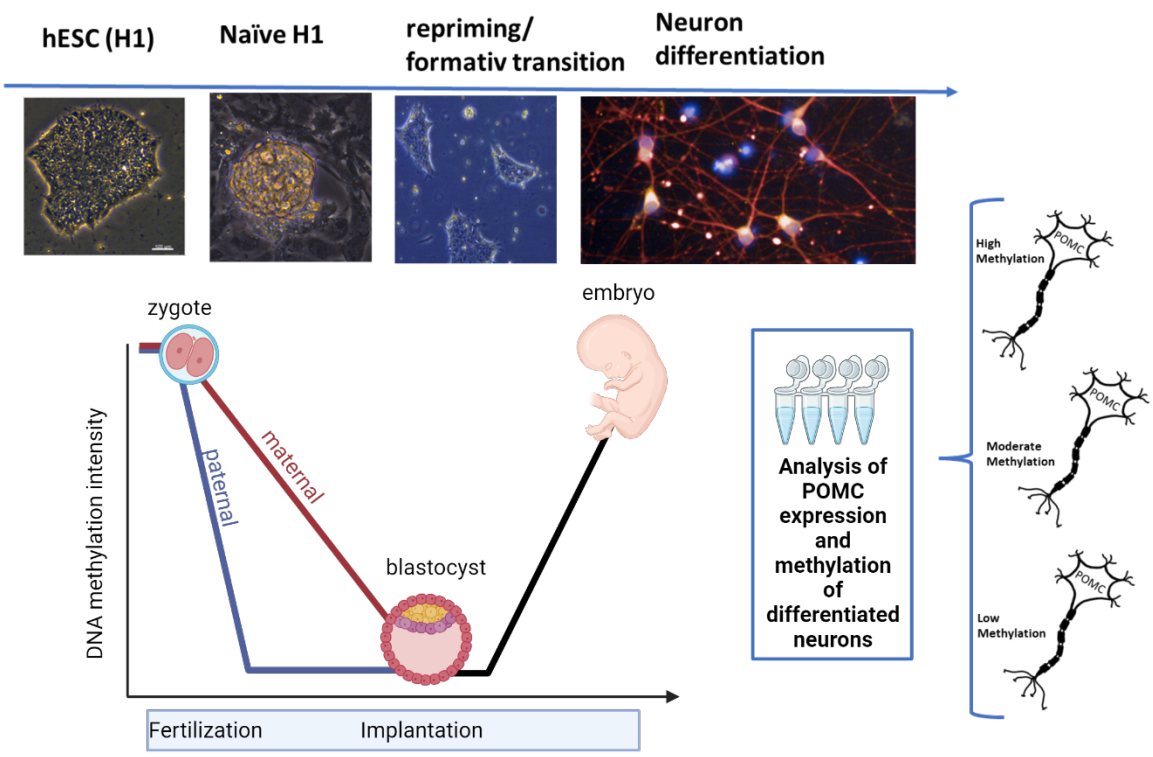

Figure 3: Schematic display of the experimental design for the stem cell based model. Neuronal differentiation after resetting hESCs in a naïve state was evaluated as a potential model to mimic physiological early embryonic methylation dynamics. In the next step, differentially methylated neurons can be analyzed in terms of their DNA methylation and RNA expression. Figure modified after Zeng et al., 2019 and Lechner et al., 2023 (35, 42).

2.2.1 Transfer of human embryonic stem cells into naïve state

H1 (WA01) hESCs were grown in E8-medium on Geltrex-coated 6-Well plates. The medium was chemically split every 3–4 days with 0.5 mM EDTA and changed every day with one double-feed each week.

Using NaiveCult from StemCell Technologies and following the manufacturer's instructions, which are based on published techniques, hESCs (WA01) were reset to a naïve state of pluripotency (44). Simply put, hESCs were grown in a hypoxic environment (5% O2, 5% CO2) on a surface of inactive mouse embryonic feeder cells (CF-1). The protocol consists of subsequent changes in medium composition. During the complete protocol, the cells are cultured in N2B27 medium supplemented with Leukemia Inhibitory Factor (LIF) and MEK-inhibitor (PD0325901). During the first days, the cells are treated with Valproic acid (Stem Cell Technologies) for temporary histone deacetylase inhibition. The medium is then extended with a Protein Kinase C inhibitor (Gö6983) and a Glycogen Synthase Kinase 3-inhibitor (CHIR99021) in a low concentration, with the subsequent

addition of a Wnt/beta-catenin inhibitor (IWP2) to stabilize the newly formed naïve colonies. As GSK3-inhibition enhances WNT/beta-catenin signaling while IWP2 inhibits it, the sensitivity of this regulatory balance between pluripotent self-renewal and priming and differentiation is pronounced. In this medium, uniform dome-shaped cells were expanded following a successful transfer into the naïve stage. To increase cell survival during the enzyme passaging process, a Rho-Kinase inhibitor (Y-27632) was added to the Trypsin Like Enzyme (TrypLE, Gibco) solution for 24 hours. The naïve cells were expanded and cryopreserved as a cell bank to provide the same starting point for each subsequent experiment.

2.2.2 Formative transition of naïve-like stem cells

Naïve H1 (WA01) cells were dissociated with TrypLE and 1.6 x 10^4/cm^2 were seeded on Geltrex™-coated plates in t2iIGö Medium supplemented with 10µM Y-27632. After 24 hours, the medium was changed to t2iIGö without additional Y-27632. After 48 hours, the culture medium was changed to E8 medium. For further experiments with modification of C1-metabolite concentrations, different E8 media compositions (SFX-E8) were created as described below. Formative transition with E8 was previously described by Guo et al. In parallel, we performed formative transition with N2B27+ WNT-inhibition based on Rostovskaya et al., which is a further development of their group's own protocol describing a better yield (45). Since both protocols worked well and we aimed for modification of the medium for our following experiments, we focused on formative transition with E8 instead of N2B27+ WNT-inhibition. The formative transition was conducted for a total of 10–30 days.

2.2.3 Modification of carbon-1 metabolite concentrations during the formative transition phase

After hESC H1 cells achieved a naïve state, the concentrations of C1 metabolites in cell culture media were varied during the formative transition phase. Established stem cell medium formulations (like E8) have extremely high and saturated C1 metabolite concentrations. In order to create E8 cell culture media with lower C1 metabolite contents, we employed a Dulbecco’s modified Eagle’s medium (DMEM) formulation that had been depleted of C1 metabolites. We adopted a mixture (1:1) of ordinary HAMs-F12 media and DMEM deprived of C1 metabolites (SFX-E8 depleted) because the standard E8 medium is based on DMEM-F12 and hESCs are not viable in a DMEM-only E8 medium lacking

HAMs-F12. In comparison to the components of the commonly used E8 medium, the C1-depleted E8 medium had lower quantities of folate (1.4 M), methionine (20 M), and choline chloride (50 M). C1-depleted E8 (SFX-E8-depleted) was used as the basal medium during the formative transition. To analyze the effects of C1 metabolites, each C1 metabolite (folate, choline, methionine, and betaine) was added individually to SFX-E8-depleted medium. As a corresponding control group, basal medium fully supplemented with C1 metabolites was prepared (based on a depleted formulation supplemented with SFX-E8), like conventional E8 cell culture medium. Changes in morphology, viability and cell growth during the protocol were controlled by performing parallel formative transitions in conventional E8. The exact media compositions are listed in detail in the material and methods section of Lechner et al. (42).

2.2.4 Differentiation into *POMC*-expressing neurons

According to methods that have been published, hESCs were differentiated into neurons that resemble a 2D in vitro culture similar to human hypothalamic cells (46, 47). In E8 media, hESC H1 cells were planted on Geltrex-coated culture dishes and then grown until confluent. Subsequently, E8 medium containing FGF was switched to knock-out serum replacement medium (Gibco), and 10 M SB 431542 and 2.5 M LDN 193189 were added and acted as dual SMAD inhibitors, which facilitate the induction of neuroectodermal growth. Meanwhile, ventralization was induced for eight days by concurrently adding 100 ng/ml SHH (R&D) and 2 M purmorphamine (Stemolecule). Starting on day nine, Notch-inhibition (DAPT; Tocris) enabled progenitor cells to further differentiate into NK2 Homeobox 1 (NKX2.1)-expressing cells. On day 13, $1x10^5$/cm2 cells were replated in N2B27 media with DAPT, a small molecule inhibitor, for the next few days, followed by subsequent Brain-derived Neurotrophic Factor (BDNF, R&D) addition on poly-L-ornithine/laminine coated plates for further maturation. On day 26, neurons expressing *POMC* were collected or preserved for staining.

2.3 Biomolecular analysis

2.3.1 DNA methylation analysis

The EZ DNA Methylation Kit (ZYMO Research) was used to bisulfite-convert 200–500ng of genomic DNA, which was then eluted in 30 l of high performance liquid chromatography (HPLC) water. The DNA was isolated from cell pellets (for in vitro experiments) or blood

samples (for human cohorts). According to the manufacturer's instructions, pyrosequencing with PyromarkQ24 was performed after a polymerase chain reaction (PCR) using 2–6 µl of converted DNA. The analysis of the samples was done in a random and balanced order to lessen the impact of any potential batch effects. Software from QIAGEN Pyromark was used to examine the results. Oligo sequences are listed in the supplementary material section of Lechner et al. (42).

2.3.2 Immunofluorescence

Cultured cells were preserved with 4% PFA for 15 minutes at room temperature in 96-well or 24-well plates for undifferentiated cells or differentiated neurons, respectively, and then washed with phosphate-buffered saline (PBS). The primary antibody was incubated overnight after a 30 minutes blocking period at 4°C in a blocking buffer solution containing 10% donkey serum in PBST (PBS+ 0.2% Triton X-100). Following three PBST washes, cells were incubated for 1 hour at room temperature with secondary antibodies at a 1:250 dilution in PBS + 0.1% Triton. After the cells had been washed three times with PBS, they were treated for five minutes at room temperature with (DAPI in hESCs)/Hoechst 1:10000 in PBS. Leica's DMIL Fluor-Microscope and Opera Phenix High-Content Screening System (Perkin Elmer©) were used for imaging.

2.3.4 Quantitative reverse transcription-PCR (RT-PCR)

Cell pellets were harvested according to one experimental unit (as described below) and directly frozen with dry ice. The RNAeasy Micro or Mini Kit from Qiagen was used to extract the ribonucleic acid (RNA). For complementary DNA (cDNA) synthesis, ReverseTranscriptionSystem (Promega) was employed with 100–400 ng RNA. SYBR-Green and QuantStudio (both from Thermofisher) were used for the quantitative PCR. 5 ng of cDNA was used in a 10µl batch and quantitative RT-PCR was performed for all three genes at a temperature of 66°C. There is a list of oligo sequences for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), TATA-binding protein (TBP) and *POMC* in the supplemental material section of Lechner et al. (42). Technical triplicates were carried out, and the delta-Ct technique was used to normalize *POMC* expression to GAPDH and TBP. For the analysis, Graphpad Prism (Version 9) was utilized. The differentiated neuron samples evaluated were derived from the experimental setup in which C1 metabolite concentrations were altered.

2.3.5 RNA array

For each condition using depleted E8 medium and added C1 metabolites (separately betaine, folate, methionine, and choline), RNA samples originating from three independent experiments, were analyzed during the formative phase (after day 10-14 and after day 20-30 just before the start of differentiation), resulting in n = 3 for each formative state condition per time point. Using Illumina's HT-12 v4 BeadChip technology, direct hybridization assays were used to assess the expression profiles of RNA samples. Utilizing GenomeStudio and the R packages lumi and limma, bead-array data was processed, analyzed and visualized.

2.3.6 Single cell RNA sequencing

Single cell sequencing was used to analyze five samples, comprising differentiated, primed, and naïve hESC. Naïve H1 cells were grown on MEFs in NaiveCult expansion medium, with one sample taken prior to the change to the formative state. Parallel cultivation of conventionally primed hESC H1 was commenced, and samples for the primed and formative batches of cells were acquired during seeding for differentiation into *POMC*-expressing neurons. Using TrypLE to separate the cells on differentiation day 26, a single cell suspension was obtained by carefully pipetting the cells up and down. Cells were counted manually on a microscope with a Neubauer Chamber since the single cell suspension of the harvested differentiated neurons also contained structures looking like contracted demolished neurites, which could be mistaken for cells and therefore needed careful distinguishing. Following the manufacturer's instructions, single cell processing and gene expression library construction were carried out (Chromium Next GEM Single Cell 3´ by 10X Genomics™). Illumina HiSeq was used to sequence the libraries. Subsequent bioinformatic processing was performed by Benedikt Obermayer, as described in Lechner et al. (42).

2.4 Statistical analysis

The statistical analyses are described in detail in the associated publication (42). Here I will repeat the descriptions of the analyses that I carried out myself and that led to the results described in this thesis, which were also first reported in the attached publication (42).

2.4.1 General description

The data is displayed as mean +/- SD in all graphs, unless otherwise stated. Each graph shows the number of experimental units per group as "n." Statistical tests were run on experimental units. Cells from a single culture dish were used as experimental units in cell culture research. For example, I divided differentiated neurons that were harvested from a single 6-well plate, into two cell pellets. These pellets were then used for analysis of both DNA and RNA. Each formative and differentiation experiment was run through its entirety at least three times, beginning with the naïve condition. Repetition of a sub-step, such as repeated differentiation from the same stock of formative cells, was not considered to be a separate experiment. Standard statistical tests were used, with the prerequisites for the respective tests checked and results corrected for multiple testing as indicated in each figure legend. Only the analysis of the RNA array data requires a more detailed explanation of how the principles of good scientific practice were complied with. I analyzed the expression of C1-metabolism related genes by filtering the RNA array data for a list of genes of interest containing all genes shown in supplemental table 3 of the associated paper. Since all raw values of these genes were visible to me, I considered all genes of the primary list for multiple testing adjustment to avoid any bias from my side towards further gene selection for group wise comparisons. The statistical testing shown in figure 5 was accordingly not only adjusted for multiple testing within each shown one-way ANOVA but for all genes of this list with a false discovery rate of 0.05. To check presumptions for ANOVA I performed Shapiro-Wilk normality test for each C1- metabolite group. For the genes Adenosylhomocysteinase (AHCY) and Serine hydroxymethyltransferase 1 (SHMT1), at least one group failed normal distribution. In this case I performed Kruskal-Wallis with subsequent uncorrected Dunn´s test. Otherwise, I performed a One-Way ANOVA for each gene with Fisher´s uncorrected LSD test as post-hoc test. The reference group was the control (adjusted) group. From all tests, I created a stack of all 55 p values, which was then analyzed for a false discovery rate of 5% with two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli to correct for cumulated alpha error. This led to 19 discoveries which are available in the data repository of the associated paper (42).

2.4.2 Analysis of human *in vivo* DNA methylation from embryonic tissue

To access in vivo data for inner cell mass (ICM) and post-implantation embryos, I downloaded methylation data from Guo et al. (48) from GEO (accession number GSE49828). This included reduced representation bisulfite sequencing (RRBS) and whole genome-wide bisulfite sequencing (WGBS) data. In addition, I retrieved Post-bisulfite adapter tagging (PBAT) data from Zhu et al. (49) from GEO (accession number GSE81233) for samples labeled as ICM and 7 weeks, 9 weeks, and 11 weeks (heart). I filtered the data with Git Bash for Chr2: 25383722 – 25391559 (hg19) to obtain only information about the *POMC* VMR. Afterwards I analyzed and visualized the data in R version 4.2.2. I defined five as the minimum coverage for each CpG and samples assigned the samples to three different developmental stages: ICM, post-implantation and differentiated samples (embryonic heart and liver). This has already been described in the associated paper (42).

3 Results

3.1 Genetic influence on DNA methylation

The DNA methylation of the *POMC* locus is mainly characterized by its interindividual variability with simultaneous intraindividual stability. While metastable epialleles are not genetically determined, more and more associations between individual SNPs and the methylation levels of different CpG positions have been reported in recent years (50). To obtain clarification regarding the genetic influence on the methylation of the *POMC* locus, 1083 samples from the HNR study, for which genome-wide SNP array data were available, were pyrosequenced. This showed an association of methylation with SNPs from the EFR3B-*POMC* region (rs13428823, rs17046887, rs934778) for the two CpG positions located on the intron. This genetic influence was not detectable for the exon CpG positions. The Manhattan plots for this result are displayed in figure 1B and supplemental figure 2 of the associated paper (42). In addition to genome-wide SNP association studies, twin cohorts provide a promising resource to investigate genetic influences on DNA methylation. We examined 32 monozygotic and 38 dizygotic twin pairs with respect to their *POMC* DNA methylation and found a strong correlation of monozygotic twins ($r = 0.78$; $p < 0.0001$) in contrast to no significant correlation of the dizygotic twins ($r = 0.31$, $p = 0.0582$) with respect to the mean value of the CpG positions of the *POMC* intron2-exon3 region (CpG+1 to CpG+7) located in exon 3. This is shown in figures 2E-F of Lechner et al.(42). Pearson correlation was used as a statistical test. Normality was tested with a Shapiro-Wilk test (MZ twin pairs: $W = 0.97$, $p = 0.62$; DZ twin pairs: $W = 0.98$, $p = 0.67$). At the two CpG positions located in intron 2, we also observed a very strong correlation in monozygotic twins ($r = 0.71$, $p < 0.0001$) and a weaker correlation of dizygotic twins ($r = 0.34$, $p = 0.0389$). These results are displayed in supplemental figure S3A-B (42). As a control, we analyzed the known metastable epiallele locus PAX8 (51) in the same twin samples and saw a strong correlation in monozygotic twins ($r = 0.91$, $p < 0.0001$) and a weaker correlation in dizygotic twins ($r = 0.36$, $p = 0.035$), shown in supplemental figure S3C-D (42).

3.2 Reproduction of the association between body weight phenotype and DNA methylation

In addition to the GWAS, the analysis of the HNR cohort, a representative cohort of adults aged 45 to 75 years, also provided the opportunity to verify the previous results regarding the association of DNA methylation with body weight. This showed a sex-specific effect, with women showing higher overall *POMC* DNA methylation than men (Tukey post-hoc test, mean difference (95% CI) = 5.357 (4.16-6.56), $p < 0.0001$). Furthermore, if males and females are analyzed separately in the HNR cohort, the association between BMI and *POMC* methylation can only be reproduced in women. Significantly higher methylation levels are observed in women with a BMI above 35 kg/m^2 compared to women with a BMI below 35 kg/m^2 (Tukey post-hoc test, mean difference (95% CI) = 2.934 (0.33-5.54), $p = 0.023$). Without predefined specific cut-off values for DNA methylation, we dichotomized mean *POMC* methylation (CpG +1 to CpG+7) using the median of the individual sex subgroups and divided them into low vs. high methylation (female median = 29.7%). The subsequent Odds Ratio (OR) calculation shows a significantly increased chance of obesity in women with high methylation (vs. low methylation) with OR = 1.46 (risk for BMI > 30 kg/m^2) and OR = 1.44 (risk for BMI > 35 kg/m^2). These results are shown in figure 2A-D of the associated paper (42).

3.3 Establishment of stem cell based modeling of *POMC* methylation

To obtain a human model to study the functional characteristics of *POMC* DNA methylation, we first induced naïve human embryonic stem cells and then differentiated them into *POMC*-expressing neurons. While conventional hESC lines are cultivated in a primed stage, it is useful for studies of DNA methylation to set the cells back one step in their development to achieve low DNA methylation as a starting point for subsequent differentiations. The following sections first state the results of the characterization of the different cell stages and the applicability of the model, and then present the actual findings concerning the causes and consequences of variable *POMC* DNA methylation.

3.3.1 *POMC*-expressing differentiated neurons originating from naïve hESCs resemble their in vivo counterparts

The cells of different stages in the proposed model were assessed according to published characteristics to make sure that the cell model is generally applicable. We show that

naïve cells display dome-shaped colonies in light microscopy, expression of naïve pluripotency markers such as Transcription Factor CP2 Like 1 (TFCP2L1) and KLF17 (Kruppel Like Factor 17) both immunohistochemically and in single cell RNA sequencing (scRNAseq) and low methylation patterns at the *POMC* gene. Immunofluorescence is shown in supplemental figure S6 and scRNAseq results are displayed in supplemental figure S7 of the associated paper (42). In scRNA sequencing analysis, pluripotent primed hESCs strongly overlap with pluripotent cells after formative transition, and the profiles of differentiated POMC neurons are congruent, regardless of whether they originate from primed or from formative transition cells. These results are shown in figures 3G-I of the associated publication (42).

Whole-genome methylation datasets generated from human embryos showed very low *POMC* methylation (CpG positions -2 to +7 levels) for ICM and a distinct methylation pattern for differentiated embryonic cells. *POMC* methylation of naïve hESCs displays values comparable to the ICM, and differentiated *POMC* expressing neurons show a pattern similar to the observed pattern of their in vivo counterpart. This result is presented in figures 3J-K of the associated publication (42). In addition, the differentiated neurons show DNA methylation in a similar range to that observed in human samples. In contrast, neurons differentiated directly from primed hESCs showed artificially high mean *POMC* VMR DNA methylation levels of over 75%, which is not encountered in human tissues such as blood cells or the post-mortem hypothalamus (31). Accordingly, the model is shown to be suitable to reproduce the methylation dynamics at the *POMC* locus.

3.3.2 Functional characterization of differentiated *POMC*-expressing neurons

As described in Lechner et al., the cells adopted a neuronal morphology after day 13 of neuronal differentiation, and by day 26 the cell cultures contained a high proportion of *POMC*-expressing neurons in scRNA sequencing (42). Immunofluorescence analysis demonstrated POMC processing leading to α-MSH production in active neurons, and liquid chromatography tandem mass spectrometry confirmed the presence of desacetylated α-MSH in the media of POMC+ neuronal culture. These results are shown in figure 3B-D and supplemental figure S5 and scRNAseq results are shown in figure 3 and supplemental figure S8 of Lechner et al. (42). No relevant differences concerning these RNA and protein expressions were seen between cells that were reset to a naïve stage before differentiation and cells that were differentiated directly from primed stage.

Differentiated neurons from the primed stage showed mean *POMC* methylation levels (CpG +1 to +7) of over 75%, whereas reset cells established physiological levels of around 50% mean *POMC* methylation. This is displayed in figure 3F of the associated paper (42).
Within *POMC* expressing neurons with naïve hESCs as origin, a significant negative correlation between the level of *POMC* DNA methylation and *POMC* RNA expression is evident (Pearson correlation coefficient: $r = -0.39$, $p = 0.013$). This is shown in figure 3E of the associated paper. Normality was tested with a Shapiro-Wilk test ($W = 0.97$, $p = 0.14$). This was evaluated by quantitative RT-PCR normalized to GAPDH and TBP as housekeeping genes.

3.3.3 Challenging with C1-metabolites lead to *POMC* methylation differences and altered expression profiles of enzymes involved in C1-metabolism

Previous studies in The Gambia were able to link maternal C1 metabolism at conception with infant DNA methylation. This raises the question of whether DNA methylation in the in vitro stem cell model can be influenced by modifying the C1 metabolites in the culture medium. Since the common cell culture media are oversaturated with C1 metabolites, we prepared a homemade E8 medium, which has the lowest possible concentrations of methionine, folate, betaine and choline. In addition, different media were prepared by adding single metabolites in high concentrations to present a clear metabolite challenge. The cells were cultured with the different media during the formative transition, and then they were all differentiated into *POMC*-expressing neurons under the same conditions.
Only minor differences in the transformative stage between the different conditions occurred, with *POMC* mean methylation levels between 10 and 25%. During differentiation all conditions significantly gained methylation (nested t test with different time points as columns [1] and C1 metabolite groups as subcolumns [2], $p[1] < 0.0001$, $p[2] < 0.0001$). Differentiated neurons in the adjusted, fully supplemented, group showed significantly lower methylation compared to each of the other conditions (One-way ANOVA with supplemental control E8 (SFX-E8 suppl.) as the reference group. Post hoc testing and adjusting were performed with Dunnett's multiple comparison testing.) The biggest differences were observed between the supplemented control and the depleted + folate group (mean diff = -14.49; adjusted $p < 0.0001$) and the completely depleted group (mean diff = -11.68; adjusted $p < 0.0001$). The data is shown in detail in figure 4C of Lechner et al. (42). These differences in differentiated cell state were not observed in

the control loci, which were characterized by hypo- or hypermethylation. This is shown in detail in supplemental figure S10 of Lechner et al. (42). The global RNA expression of the different groups shows only minor differences, although it is noticeable that the methionine-supplemented group forms a cluster with the control group. Principal component analysis and hierarchical cluster analysis are displayed in supplemental figure S11 of Lechner et al. (42). Interestingly, expression differences between the methionine-supplemented groups and the methionine-deficient groups are evident in the expression of enzymes of the C1 metabolism. A heatmap of the RNA expression of key C1 metabolism enzymes is shown in figure 5B of the associated paper (42). Methionine adenosyltransferase 2A (MAT2A), for example, is significantly higher expressed in depleted cells compared to the adjusted control group (one-way ANOVA, SFX-E8 suppl. as the reference group and post-hoc 5 % FDR adjustment, adjusted $p = 0.0027$), whereas Ten-eleven translocation methylcytosine dioxygenase 1 (TET1) seems to be regulated in the opposite way (adjusted $p = 0.0043$). These results are displayed in figures 5C-E of the associated paper (42).

4 Discussion

4.1 Interpretation of results and embedding into the current state of research

The present study evaluates the origin and consequences of variable degrees of *POMC* DNA methylation. The results have already been discussed in the attached publication, and some of the categorizations and interpretations presented in the following have already been elaborated there (42).

4.1.1 Epigenetic variation as an obesity risk factor

One aim of the cohort-based analysis was to evaluate the association of *POMC* VMR with obesity. The previously reported association between increased *POMC* DNA methylation and higher weight was replicated, demonstrating a remarkable sex-specific effect. There is a significantly higher methylation at the *POMC* VMR in females than in males, and the association of *POMC* methylation with BMI is also more pronounced in women.

Until now, there has been no molecular explanation for the sex-specific effects, even though this has been seen in many epigenetic studies. The high overlap of the statistical position-scatter parameters of normal and overweight women (see figure 2C of Lechner et al. [42]) clarifies that there is no high prognostic significance of DNA methylation regarding weight development. Therefore, the effect of varying levels of *POMC* methylation can be classified as small and reminds of the characteristics of genetic risk variants. The genetic risk variant with the highest effect is the FTO SNP with an odds ratio to be obese of 1.6 per allele (52). This is comparable to the odds ratio of high POMC methylation that we observed in women in the HNR cohort (OR = 1.46 risk for BMI > 30 kg/m^2; OR = 1.44 risk for BMI > 35 kg/m^2) (42).

Research into the biological risk factors for obesity is both important in explaining the causes of obesity and may have implications for treatment options.

Although the individual effect of each SNP is small, it is worth taking a genome-wide view of all genetic risk factors together to assess the relevance of the concept of "risk factors". The GWAS mentioned in the introduction provided a foundation for other groups to develop polygenic risk scores (PRS) (13, 53). Using this score, subjects can be ranked according to their genetic susceptibility to weight and obesity. In human cohorts such as the UK Biobank, this results in a weight difference of 13 kg between the top and bottom deciles(53). Polygenic risk scores have also been used in other studies to demonstrate the

interaction between environmental factors and genetic risk in the development of obesity. Dashti et al. showed that the difference in BMI between individuals with a high versus a low obesogenic lifestyle significantly differed between patients with a high PRS (3.18 kg/m2) compared to patients in the lowest decile of PRS (1.55 kg/m2) (54). In another study, the same group showed that high PRS was prospectively associated with greater increases in BMI. Evidence for the potential for behavioral interventions to ameliorate PRS-related differences in weight gain was provided with a 12-month randomized controlled behavioral intervention, where significant differences between high and low genetic risk groups could only be observed in the control group (55). This highlights that individuals are not equally predisposed to gaining weight due to lifestyle choices, and interventions could reduce the impact of biological differences in susceptibility to obesity. Epigenetic risk factors have not yet been incorporated into these polygenic risk scores. The pitfalls of EWAS are discussed in detail in the limitations section. Amongst others, there is the necessity for a conceptual background, like metastable epialleles, for accurate characterization of epigenetic variation when drawing conclusions about the risk for a particular disease.

Taken together, these considerations suggest that for the development of useful treatment options, besides legislative measures improving lifestyle conditions and thus attenuating the influence of biological risk, it may be promising to assess the individual's risk that certain pathways are impaired by genetic and epigenetic components. Based on the hypothesis that increased *POMC* methylation levels would lead to impaired function of the leptin melanocortin pathway, we evaluated within our investigator-initiated study (NCT02507492) if five individuals with *POMC* hypermethylation, who had never been able to stabilize body weight for a longer period and in whom a monogenic cause for obesity has been excluded, would respond to treatment with the MC4R agonist Setmelanotide. This treatment led to a reduction in hunger scores and a reduction in body weight of 4.7 ± 2.2% compared to pre-study weight over a mean treatment duration of 38.4 ± 26 weeks (42). The data and study protocol are provided in the associated paper. This aspect is not included in the results presented above, since my involvement in the clinical study was marginal. Even though treated participants lost weight, increased *POMC* VMR methylation should rather be considered a risk factor for obesity, since the results display much lower weight loss than in patients with monogenic obesity treated with MC4R agonists (29). However, much larger, randomized, placebo-controlled studies are needed to

evaluate whether MC4R agonist treatment might be beneficial for individuals with increased *POMC* methylation.

4.1.2 Functional implications from stem cell based model

To evaluate if the observed increased risk of developing obesity is due to impairment of leptin-melanocortin signaling, we used the above-mentioned stem cell based model to mimic physiological methylation levels. We found a negative correlation between *POMC* methylation and expression in differentiated hypothalamic-like neurons. This supports the hypothesis that increased *POMC* methylation impairs hypothalamic satiety regulation.
In order to make statements about the functional implications, it is important to take a closer look at the different protocols. On the one hand, differentiated neurons were derived from naïve cells via the formative stage and thus developed a DNA methylation level comparable to physiological levels. On the other hand, neurons were also differentiated directly from conventional primed hESCs as a control. While the neurons hardly differ in function, morphology and global RNA expression, the neurons from primed hESCs retain their artificially high *POMC* DNA methylation. Since both populations produce alpha-MSH and do not differ in their *POMC* RNA expression, it can be concluded that *POMC* DNA methylation at this region is not one of the crucial on-off regulators of *POMC* expression. However, within *POMC* expressing neurons with naïve hESCs as origin, a significant negative correlation between the level of *POMC* DNA methylation and *POMC* RNA expression is shown.
This result fits well with the results that Sharma et al. see in adipose tissue mentioned in the introduction. They demonstrated a strong correlation between *POMC* DNA methylation in the region of the *POMC* VMR and *POMC* expression in the adipose tissue of a human cohort (32). The actual tissue of interest in this context is the hypothalamus, which is difficult to obtain samples from in living individuals. Kuhnen et al. therefore analyzed post-mortem hypothalamic neurons and showed a negative correlation of BMI with *POMC* VMR methylation in α-MSH positive cells, while no analysis of RNA expression profiles was presented (31).

4.1.3 Genetic independence though high concordance of *POMC* VMR in monozygotic twins

The conducted GWAS disclosed single SNPs at CpG positions -2 and -1 associated with methylation levels. However, this genetic effect vanishes with the onset of the exon. As

initially proposed for metastable epialles (56), this is, to our knowledge, the first described human metastable epiallel that occurs independently of genetic variation. Gunasekara et al. proposed to define metastable epialleles in humans regardless of whether they are genetically or stochastically mediated, given the difficulty of excluding genetic influences in human populations (57). While this approach also has advantages, it is still noteworthy that loci seem to exist in humans that fulfill all criteria for metastable epialleles.

Analysis of twin studies with correlation patterns, e.g., monozygotic twins, may, if genetic causes were excluded, as in the *POMC* VMR, point towards other early in utero effects. The twin cohort analyzed by us provides evidence that monozygotic twins display very similar intensities of *POMC* VMR methylation, though dizygotic twins do not correlate with their twin partner regarding *POMC* VMR methylation. This phenomenon has been described previously for other loci and is termed “epigenetic supersimilarity”. Van Baak et al. identified genomic regions where the epigenetic similarity of monozygotic twins exceeded what could be explained by their genetic identity (58). This "epigenetic supersimilarity" appears to be due to the locus-establishment of epigenetic patterns before embryo cleavage during twinning. These regions are further distinguished by increased responsiveness to the periconceptional environment and are primarily located in sub-telomeric regions of the genome. Furthermore, van Baak et al. observed in a prospective cohort that blood DNA methylation at these regions had a 4-fold higher enrichment of associations with developing various types of cancer compared to control clusters of CpGs (58). This link from the epigenetic signature established early in utero to the phenotype puts the results from previous heritability studies from twin studies in a new perspective. This provides an approach to explaining the “missing heritability” concept presented in the introduction and highlights the importance of the early embryonic period for these loci.

4.1.4 Early in utero establishment of *POMC* VMR DNA methylation and hESC model

In order to investigate the early embryonic period in the humane stem cell model, we established a protocol mimicking early embryonic methylation dynamics as an advancement of previous studies. We show that human embryonic stem cells with reset to naïve stage before neuronal differentiation represent a suitable model to mimic physiological methylation levels. *POMC* DNA methylation at the different stages from naïve to differentiated cells is shown to be comparable to publicly available embryonic WGBS in vivo data. Up to our knowledge, – this is the first time that this strategy has been used to characterize disease related methylation variants.

To investigate this early embryonic phase in connection with the establishment of DNA methylation, Kessler et al. followed the methylation dynamics at metastable epialleles during different developmental stages of human in vitro fertilization embryos (59). Interestingly, the same methylation dynamics were seen for a known subset of CpG regions sensitive to the periconceptional environment. In short: metastable epialleles present significantly lower methylation during gastrulation and develop a high proportion of intermediate methylation status compared to control loci, which are mostly either very highly or very poorly methylated. To investigate the emergence of intermediate methylation closer, Kessler et al. have developed an index with which they can measure the homogeneity of methylation on individual DNA reads (59). They conclude that MEs show higher molecule-specific methylation, suggesting that the intermediate methylation states are most likely emerging from intratissue variegation effects. Taken together, Kessler et al. conclude that these results suggest that the phase of gastrulation plays a critical role in the establishment of the intermediate methylation status of metastable epialleles and that this intermediate methylation status is driven by intratissue variegation effects. Furthermore, they state that their analysis suggests that "interindividual variation in MEs is influenced by at least three factors: stochastic or probabilistic processes, periconceptional environmental exposures, and genomic context."

Our results from the in vitro model align with the results of Kessler et al., showing very low methylation levels during naïve cell state with the subsequent emergence of intermediate methylation at *POMC* VMR. Our control locus chosen from in vitro data developed high methylation levels at the end of differentiation, as expected. This substantiates the evidence of Kessler et al. and van Baak et al. that the early in utero period seems to be crucial for the establishment of *POMC* VMR methylation and that this period seems most promising as a time frame where interindividual variability is emerging.

Taken together, epigenetic supersimilarity without genetic dependence and methylation dynamics in vivo and in vitro suggest that interindividual variability of the *POMC* VMR occurs during early embryonic development in utero.

4.1.5 Carbon-1 metabolite challenging in vitro

In a second step, we manipulated the system and modulated key C1 metabolite concentrations during the formative transition state, which corresponds to the embryonic period in humans where methylation patterning is taking place. Interestingly, methylation levels

were remarkably stable despite severe C1 metabolite changes. Contrary to our expectations, the lowest methylation levels were even found in neurons originating from fully supplemented formative stages. RNA expression analysis provided evidence for intracellular compensational gene expression changes of key enzymes like MAT2A, TET1 and DNA Methyltransferase 3 Beta. This means that this data is pointing towards an unexpectedly robust system and compensational response mechanisms after such a strong “environmental” modification to keep DNA methylation levels stable. These results support recent observations made by Anne Ferguson-Smith and her team, where severe stimuli (also C1 metabolite concentration modifications) were not associated with remarkable changes in DNA methylation in mice (60).

To place these in vitro results in the current state of research, it is worth looking at specific human cohort studies. In the previous work of our group, in cooperation with Andrew Prentice and the MRC Unit in The Gambia, the relationship between C1 metabolites and *POMC* VMR DNA methylation was investigated (31). The cohort studied in The Gambia is notable for its excellent characterization and a recurrent change in the dietary habits of the population depending on the season. A dry season can be distinguished from a rainy season, with a lower nutrient supply during the rainy season (61). The levels of the different C1 metabolites in the blood of the mothers also vary according to the seasons and can be examined at the time of conception. This showed a positive correlation of *POMC* VMR methylation with betaine, SAM/SAH ratio, and conception presented in the rainy season (31). An interesting connection to the results in the in vitro model is that the rainy season represents the "starvation" phase, and cells exposed to metabolite deficiency also showed higher DNA methylation at the end of *POMC* differentiation than cells exposed to adjusted metabolite concentrations. Another cohort study in Bangladesh also showed a correlation of *POMC* DNA methylation with the seasons of conception (62). Conception during the monsoon, the season with the highest food security, was associated with reduced *POMC* methylation. In addition, household wealth was associated with reduced *POMC* methylation. Taken together, the results of the in vitro and cohort experiments suggest that undersupply could lead to increased *POMC* DNA methylation levels. The exact mechanisms of this association remain unclear and represent an exciting research question for the future.

4.2 Limitations

4.2.1 General remarks about epigenetic studies

As mentioned in the introduction, some conceptual limitations need to be considered in epigenetic studies. This includes the fact that epigenetic variations can be tissue-specific and longitudinally instable. Furthermore, the emergence of methylation variability can have many origins, for example, methylation at certain loci can be dependent on an individual's genetic background or the consequence of a certain phenotype or environmental exposure.

To determine the relevance of epigenetic variants as the origin of disease and for development of treatment strategies, the causality of DNA methylation needs to be considered. Some studies address how genetic variants affect DNA methylation, leading to differential expression. For example, Nikpay et al. describe rare variants within 2p23.3 that may impact obesity by making site cg01884057 more susceptible to methylation, which the authors suggest subsequently decreases expression of *POMC*, *ADCY3*, and *DNAJC27* (63). Other studies focus on the effect of adiposity on DNA methylation, trying to link associated diseases to obesity. Wahl et al. demonstrated that BMI is associated with widespread changes in DNA methylation while stating that most alterations in DNA methylation may be the consequence of adiposity rather than the cause (64).

Therefore, we addressed these limitations as well as possible. Tissue specificity was considered in that the neurons produced in the in vitro model should represent the target tissue in humans as closely as possible. The in vitro model also allows longitudinal tracking of methylation dynamics. For the longitudinal stability of *POMC* VMR methylation in human blood, Kuehnen et al. showed that methylation values from newborn screening cards highly correlate with intraindividual methylation values later in life (31). The dependence on genetic background was addressed in a genome-wide SNP association study, which is especially relevant since we also interpreted the results of twin studies. Whether DNA methylation is more of a consequence or cause of obesity is addressed by the assessment in the in vitro model. Also, the high longitudinal stability hints at the fact that increased *POMC* methylation is not a consequence of obesity.

4.2.2 Internal validity

Aspects increasing internal validity were considered according to the QUEST criteria (65). However, given methodological restrictions, it was not possible to randomize all samples

during experiments or analysis, especially for the analysis of the HNR cohort and the experimental design of the hESCs model.

The samples used for genome-wide SNP analysis were planned for additional analysis of *POMC* methylation in relation to individuals' BMI. Because we were blinded to statistical analysis, it was only after pyrosequencing the 1083 samples that we realized there was a selection bias in which individuals were genotyped from the HNR cohort, such that severely obese individuals (BMI < 35) were underrepresented. Therefore, 300 additional individuals from the HNR cohort were added to the study subset. For cost and material reasons, not all samples, including the new 300 samples, could be re-randomized and analyzed again. Nevertheless, to avoid batch effects, all pyrosequencing performed later was controlled and matched with the previously analyzed samples. In the cell culture experiments with different C1 metabolite concentrations, it was not possible to blind the different culture conditions because, firstly, the different growth characteristics were obvious upon daily visual inspection, and secondly, the effort for blinding the different reagents was disproportionately high. To ensure reliable analysis, samples were randomised and blinded after collection.

A weakness of the in vitro model is that the experiments were only carried out on one cell line. The H1 (WA01) cell line was chosen because it is one of the best characterized lines. It is known that distinct iPSC and hESC lines behave differently, and it is therefore important to validate the results in follow-up studies in other cell lines. The data still seems reliable, as the results compare well with in vivo embryonic data. However, as this is a male cell line and the sex-specific effect of *POMC* methylation is becoming more and more apparent, it is a very interesting aspect to integrate female cell lines in follow-up studies.

Since the conditions with differently challenged media did not grow with the same doubling rate, it was not possible to assure that all cells had the same time frame of formative transition. It is not yet clear if it is of significance for how long cells are cultured in formative transition with respect to methylation patterns. Rostovskaya et al. indicate that a capacitation of 10 days should be sufficient to ensure that the cells are reactive to inductive cues (45). But they also propose a different protocol for formative transition using N2B27 medium with WNT-inhibition, which we did not use for the challenging because it was not possible to adjust the metabolite concentrations this way.

4.2.3 External validation

One major limitation of this study is that the functional characterization is not performed in vivo, for example, in a mouse model. Therefore, consequences can only be considered at the cell culture level and cannot be assessed in the context of the metabolic interplay. The pattern of high to low methylation at the intron 2 exon 3 border at the *POMC* VMR and the resulting emergence of DNA methylation variability are unmistakably linked to their proximity to retrotransposons (ALU elements in intron 2) (30). These ALU elements are not present in the mouse genome, which is why the methylation pattern is different in mice and they are not a suitable model. Although this lack of an in vivo model represents a limitation, the primary strength of the study is that we have directly established a human model, which provides higher translational potential through its biomedical closeness. In addition to the scientific superiority of the model, another advantage of the hESC model is that animal testing is replaced, thus complying with the 3Rs principle.

4.3 Relevance and novelty

This hESC based system, for the first time, may provide the opportunity to functionally characterize identified epigenetic variants and evaluate the relevance of environmental factors for epigenetic changes. This model, which encompasses the naïve and formative cell states, is particularly interesting because it offers the possibility to study the very early in utero emergence of *POMC* DNA methylation and to assess the function of differentially methylated *POMC* neurons. Furthermore, this can be an essential new tool to improve evidence and investigate how and if environmental cues affect epigenetic marks during early human embryonic development elsewhere in the genome. Due to its novelty and applicability to study further molecular mechanisms underlying epigenetic homeostasis, the presented hESC protocol is most relevant to a broad spectrum of researchers interested in stem cell energetics, epigenetics and C1 metabolism.

The translational approach of our study, with the combination of basic research techniques with well-characterized clinical and genetic data from large cohorts, increases the clinical relevance of the presented findings and may be fascinating as a concept for clinically active scientists in the fields of obesity, metabolism and personalized medicine.

4.4 Outlook

To understand how differences in *POMC* DNA methylation lead to alterations in RNA expression, it is necessary to assess gene regulation on a chromatin level, including histone marks and transcription factor binding. Especially interesting is the recently published evidence hinting at a second mechanism by which DNA methylation influences transcription. Before, the major mechanism was thought to work as indirect repression through the sequence-independent recruitment of histone deacetylases to methylated DNA. Opposing this, the group of Prof. Schübeler could show that direct repression through direct obstruction of transcription factor binding by cytosine methylation within their motif seems to be even more crucial in gene and repeat repression (66). In this context, it will be very useful to use the hESC model to evaluate the consequence of DNA methylation on the binding of certain methylation sensitive transcription factors like BANP or CREB1 (67).

To address whether there are more loci displaying the same characteristics as the *POMC* metastable epiallele and if they are also associated with phenotypical variance, a next step will be to characterize the introduced stem cell based model on a genome wide level and integrate the data bioinformatically with twin data. The *POMC* locus, in its depth of characterization, displays a proof of concept for other loci, which could lead to a paradigm shift in what we see as contributors to human phenotypic variance and disease etiology.

5 Conclusions

In conclusion, we show that *POMC* DNA methylation is established early in utero and strengthen the hypothesis that increased *POMC* methylation represents an epigenetic risk factor for developing obesity, which may be associated with compromised functionality of the leptin melanocortin pathway.

Firstly, we investigated which factors influence the emergence of different levels of *POMC* VMR DNA methylation. The epigenetic supersimilarity of monozygotic twins in the absence of genetic dependence and the parallels between the in vitro and in vivo methylation dynamics suggest that the inter-individual variability in the *POMC* VMR occurs during early embryonic development in utero. Our initial hypothesis that C1 metabolites influence *POMC* VMR methylation levels is only partially supported, and these results highlight that epigenetic variation at *POMC* is more stable than expected, and different regulatory mechanisms are revealed.

Secondly, we aimed to investigate whether the level of DNA methylation at the *POMC* VMR functionally affects hypothalamic satiety regulation. The shown correlation between increased *POMC* DNA methylation and obesity in females, accompanied by the inverse association between *POMC* DNA methylation and RNA expression, indicates a functional relevance. Nevertheless, because of the marginal impact observed in all utilized approaches, it appears that the functional effect is relatively small, suggesting that elevated *POMC* methylation should be considered more of a risk factor. This aligns with the lower treatment response of patients with increased *POMC* methylation treated with MC4R agonists compared to those with bi-allelic genetic *POMC* mutations. However, it is suggested that stratified multimodal therapy concepts, which involve medication of pathways at risk of impairment, could be advantageous for patients.

In the future, the established stem cell model and detailed characterization of the *POMC* VMR can potentially serve as proof of concept to reveal other human metastable epialleles that are pathologically relevant. Additionally, it can help resolve pending epigenetic mechanistic questions such as sex specificity, environmental influences, and the molecular mechanisms that links DNA methylation at metastable epialleles to gene expression.

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Statutory Declaration

“I, Lara Katharina Lechner, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic: „Analysis of POMC DNA methylation variability and the impact on hypothalamic body weight regulation“ / „Analyse der POMC DNA Methylierungsvariabilität und die Bedeutung für die hypothalamische Körpergewichtsregulation “, independently and without the support of third parties, and that I used no other sources and aids than those stated.
All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; http://www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.”

Date Signature

Declaration of your own contribution to the publications

Lara Katharina Lechner contributed the following to the below listed publications:

Publication: Lechner L, Opitz R, Silver MJ, Krabusch PM, Prentice AM, Field MS, Stachelscheid H, Leitão E, Schröder C, Fernandez Vallone V, Horsthemke B, Jöckel KH, Schmidt B, Nöthen MM, Hoffmann P, Herms S, Kleyn PW, Megges M, Blume-Peytavi U, Weiss K, Mai K, Blankenstein O, Obermayer B, Wiegand S, Kühnen P. Early-set POMC methylation variability is accompanied by increased risk for obesity and is addressable by MC4R agonist treatment. Sci Transl Med. 2023

Contribution in detail: Planning and execution of cell culture experiments (hands-on support for POMC differentiation by Rita Oeltjen), biomolecular analyses of cohorts and cell culture samples: Pyrosequencing (support by Rita Oeltjen), Immunofluorescence staining (support by Rita Oeltjen and Oilvia Hertel), Microscopy, qPCRs, Sample and library preparation for scRNAseq (support by Valeria Fernandez Vallone), Statistical analysis, Visualization and preparation of figures, Editing of the manuscript, Reviewing process.
From my performance of the experiments/sample analysis and my statistical analysis, the results for Figures 2A-F, 3E, 4B-C, 5B-E, and Supplemental Figures S3, S4, S10, S11, S12, S13, S14 were obtained. I conducted the experiments and visualization of Figures 3A-C, 3F, and Supplemental Figures S5, S6. I performed the bioinformatic visualization of publicly available in vivo data in comparison with the data from my own experiments that gave rise to Figure 3J-K. Pyrosequencing of the samples for Figure 1, and Supplemental Figures S1C, S2 or stem cell differentiations and library preparation of the samples for Figure 3G-I, and Supplemental Figures S7, S8 was performed by me; bioinformatic analysis and preparation of these figures were performed by others as described in the publication.

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Publication list

Lechner L, Opitz R, Silver MJ, Krabusch PM, Prentice AM, Field MS, Stachelscheid H, Leitão E, Schröder C, Fernandez Vallone V, Horsthemke B, Jöckel KH, Schmidt B, Nöthen MM, Hoffmann P, Herms S, Kleyn PW, Megges M, Blume-Peytavi U, Weiss K, Mai K, Blankenstein O, Obermayer B, Wiegand S, Kühnen P. Early-set POMC methylation variability is accompanied by increased risk for obesity and is addressable by MC4R agonist treatment. **Sci Transl Med**. 2023 Jul 19;15(705):eadg1659. doi: 10.1126/scitranslmed.adg1659. Epub 2023 Jul 19. PMID: 37467315.
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