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General Discussion
Main Aims

It is hypothesized that persistent epigenetic changes induced by (early) environmental conditions may partly underlie the association between development and disease. Animal studies showed that prenatal nutrition may induce a persistent change in DNA methylation which in turn affects the expression level of genes known to be implicated in the disease mechanism. The main aim of this thesis was to investigate if the prenatal environment is also associated with DNA methylation changes in humans at genes and pathways implicated in disease.

To this end we first characterized the normal variation, patterns and stability of DNA methylation at sixteen loci in blood and buccal swaps (Chapter 2), as to learn if DNA methylation patterns in whole blood are suitable for study in human cohorts. To test for an influence of the environment on DNA methylation we first performed a proof-of-principle study by measuring the imprinted insulin like growth factor 2 differentially methylated region (IGF2 DMR) in the Dutch Hunger Winter Families Study. We measured DNA methylation in individuals exposed early or late in gestation to famine and a same-sex sibling as control (Chapter 3). We extended this measurement to all sixteen characterized loci to learn which period of development is the most sensitive and to investigate if famine exposure may be associated in a sex-specific and timing dependent fashion with DNA methylation, similar to the phenotypic associations (Chapter 4). Intra-uterine growth restriction (IUGR) is hypothesized to be a sign of prenatal malnutrition. Therefore we measured DNA methylation at four loci associated with famine in young adults born following IUGR from the Dutch national cohort of growth restricted and preterm born children (the POPS study) to test if our findings in the Dutch Famine relate to this pregnancy outcome (Chapter 5).

Besides relating our findings to more contemporary complications during pregnancy, we also wished to contrast the influence of early environment (nurture) and genetic variation (nature) on DNA methylation. We therefore extended our analyses to regulatory regions within and flanking the IGF2 locus in The Dutch Hunger Winter Families Study (Chapter 6). Finally,
we characterized the regions at which DNA methylation is sensitive to prenatal famine by extending our measurements to a genome-scale, which subsequently also allowed us to comprehensively test DNA methylation along whole pathways of related genes and investigate the relationship between DNA methylation and phenotypes affected by famine exposure (Chapter 7).

The first steps: candidate loci

We started with characterizing the variation, patterns and stability of DNA methylation in whole blood (Chapter 2). We selected 16 loci involved in metabolism, development and growth with some loci situated around retrotransposons or within imprinted regions. These latter features were put forward as especially liable following experiments on prenatal nutrition in animals. An additional important selection criterion was that CpG dinucleotides either overlapped important DNA methylation sensitive transcription factor binding sites or were shown in literature to correlate with gene expression in vitro or in vivo. DNA methylation was a quantitative trait and we observed correlation both within and between loci, highly analogues to linkage disequilibrium for genetic variation. Interestingly, DNA methylation in blood was correlated with that in buccal for some loci and more importantly, was stable over time and for most loci not dependent on blood cell heterogeneity. DNA methylation is suitable for study in human epidemiological studies.

*IGF2 DMR methylation is associated with early gestational famine exposure*

*IGF2* regulates the amount of nutrients transferred over the placenta to the developing embryo, making it an attractive candidate for epigenetic modifications in relation to nutritional disturbances. Moreover, DNA methylation at the *IGF2* DMR in blood was found to mark DNA methylation patterns in buccal cells (Chapter 2) while others found that it marked that in colon, where it is also linked to *IGF2* expression. We hoped that this covariance stemmed from the phenomenon that differences induced early in
development may be transmitted to other tissues\textsuperscript{10}. Since early development is epigenetically the most dynamic period\textsuperscript{11}, we hypothesized that famine exposure during early development is associated with DNA methylation at \textit{IGF2} DMR.

We were the first to report for humans a DNA methylation difference associated with a prenatal environmental exposure (Chapter 3). A decrease in methylation at \textit{IGF2} DMR was found in individuals exposed from conception up to 10 weeks into development as compared to their prenatally unexposed same-sex siblings (**Figure 1A**). To further test our hypothesis we also tested sibling pairs of which one sibling was exposed during the last trimester of pregnancy. In correspondence with our hypothesis that early development is the most sensitive period we found no effect on DNA methylation as a result of famine exposure during the last trimester (**Figure 1B**). Interestingly, not all sibling pairs showed differential DNA methylation following early gestational famine exposure of one of the siblings (**Figure 1A**). This is analogues to results from experiments in the \textit{agouti} and \textit{axin fused} mice models where not all mice exposed to prenatal folic acid supplementation showed a change in DNA methylation associated with prenatal environmental exposure (**Figure 1C and 1D**). These findings in mice, and ours in humans, indicate either a stochastic or individual-specific component to the epigenetic response to an environmental challenge.

Since the publication of this finding others have linked \textit{IGF2} methylation to birth weight\textsuperscript{12,13} and brain morphology\textsuperscript{14}, which is of interest since early gestational exposure to famine does not lead to a lower birth weight (Chapter 3) while it is associated with brain morphology\textsuperscript{15}, schizophrenia\textsuperscript{15} and a poorer mental health\textsuperscript{16}.

\textit{Most associations are limited to early gestation}

Next, we wished to see if famine generally affects DNA methylation of genes including those in non-imprinted genomic regions. Moreover, if the associations between famine exposure and DNA methylation are to be relevant they should mirror the phenotypic associations with sex-specific and
Figure 1. The stochastic nature of famine associated DNA methylation differences

In panels (A) and (B) the within pair differences is plotted along the estimate of conception of the prenatally or birth of the exposed sibling of the pair. **A.** Early exposure leads to a shift in the average within pair difference below the zero line. Lots of variation remains and several pairs do not show a decrease in DNA methylation in the exposed pairs. **B.** No overall difference in within pair methylation is seen after late gestational famine exposure. **C.** The pattern observed after early famine exposure (A) is similar to the influence of folic acid supplementation on the *agouti* locus. **D.** Again also for the axin fused locus a similar pattern is found to *IGF2* (A) and *agouti* (C). Not all mice in a litter show an increase in DNA methylation after folic acid supplementation during pregnancy at the agouti and axin fused loci, lots of variation remains, just as seen in the famine pairs.
timing independent associations. We extended our *IGF2* DMR study to the additional 15 candidate loci characterized in Chapter 2. In total, seven out of the sixteen candidate loci showed an association with prenatal famine exposure (Chapter 4). Most associations were found with early gestational famine exposure, but we identified several sex specific associations and one timing independent association between famine exposure and DNA methylation, also a first in human studies. Moreover, imprinted and non-imprinted regions were affected. All seven genes (*IGF2*, *GNASAS*, *INSIGF*, *LEP*, *IL10* and *ABCA1*) are involved in development and implicated in disease. Thus, our findings mirror animal studies\(^{17,18}\) and the patterns observed in the epidemiological literature for famine exposure\(^{19}\). Our observations give credence to the hypothesis that the associations between development and disease may be explained by epigenetic mechanisms\(^1\).

**IUGR and prenatal malnutrition**

We asked ourselves the question if our findings may be extrapolated to other more contemporary exposures and pregnancy complications (Chapter 5). Others have successfully found associations of DNA methylation with folic acid supplementation at the *IGF2* DMR\(^{13}\) and with gestational diabetes at the *LEP*\(^{20}\) gene. In the epidemiological literature IUGR and a low birth weight are regarded as a proxy for a poor prenatal nutrition\(^{21}\) and associated to adult health. However, early famine exposure does not lead to a lower birth weight\(^{22}\) while the epidemiological findings in famine cohorts often entail associations that are either independent on the gestational timing or restricted to early gestational famine exposure\(^{23}\). Therefore we tested DNA methylation at loci associated with prenatal famine exposure in the context of IUGR, to examine whether the etiology of IUGR and prenatal famine exposure may have a similar component.
DNA methylation at famine loci is not associated with IUGR

Since our results indicated that early gestation is the most sensitive period we resorted to the developmental extreme of early growth restriction. From the POPS cohort we compared DNA methylation at 19 years of age of growth restricted and non-growth restricted individuals that were born before week 32 of gestation. We did not find differences between the two groups for DNA methylation at \textit{IGF2}, \textit{GNASAS}, \textit{LEP} and \textit{INSIGF} at which DNA methylation was associated with prenatal famine exposure during early or both early and late gestation.

Although we took special care to avoid individuals treated with glucocorticoids, which changes the transport of folic acid and choline over the placenta and may influence DNA methylation, pregnancies were affected by intoxications with drugs or alcohol, which may have confounded our analyses. Furthermore, the study was limited in the number of subjects studied and since the individuals were unrelated we had less power than in the famine study to detect DNA methylation differences. However, the contrast in birth weight and body size was considerable and others have also studied the \textit{IGF2} locus in the context of prenatal growth restriction and found no differences. An explanation for our negative findings may also lie in the heterogeneity of causes underlying IUGR in the POPS cohort, which included intoxications, hypertension, placental insufficiencies and (acute) infections.

Different etiologies of IUGR?

Recently it was shown in rats that IUGR induced by placental insufficiency or malnutrition is associated with different effects on \textit{igf2} and \textit{lep} expression. Also in the phenotypic consequences nuance differences can be found. A large meta-analysis of rat studies on hypertension using either caloric or protein restriction to induce IUGR found that protein restriction affected diastolic blood pressure, while caloric restriction affected both diastolic and systolic blood pressure. Such studies indicate that IUGR caused by different exposures may result in a different etiology of the IUGR.
Also in humans, the epidemiological observations relating birth weight and IUGR with adult disease\textsuperscript{21,28} may be the result of a mixture of developmental complications, as they were done in developed economies where malnutrition is unlikely to be a major underlying cause of IUGR. Indeed, twin studies have shown for developed economies that associations between birth weight and disease are driven by a considerable genetic component\textsuperscript{29-32}. Some have argued that twin pregnancies are not suitable to study in this context\textsuperscript{33}, since both siblings may be considered growth restricted and have a higher pre- and postnatal mortality rate. However, recent studies have shown that adult all-cause mortality and cardiovascular and diabetes specific mortality is not different in twins, nor is the incidence of disease\textsuperscript{37,38}. Future successful studies may need to focus on IUGR as a result of one clear factor. Indeed, focused studies in animals\textsuperscript{39} and humans\textsuperscript{40} on IUGR as a result of placental insufficiency have successfully discovered epigenetic changes. The epigenetic consequences of the diverse exposures and complications leading to IUGR may then be compared to discover common mechanisms leading to disease. Studying the unique consequences on both the epigenetic and phenotypic level will be likewise insightful.

**Characterizing loci sensitive to prenatal famine exposure**

Therefore we set out to characterize the effect of early famine exposure on DNA methylation in more detail. We focused our measurements on the 60 sibling pairs of which one sibling was exposed to famine during early gestation. We expanded our measurements to most of the regulatory regions in and around \textit{IGF2} (Chapter 6) and to a genome-scale (Chapter 7).

\textit{Nature and Nurture may both influence DNA methylation}

It is known that DNA sequence variation may influence DNA methylation levels\textsuperscript{41} and we described before the onset of this thesis that DNA methylation at \textit{IGF2} DMR is heritable\textsuperscript{34}, while \textit{IGF2} DMR methylation is also sensitive to
the early prenatal environment (Chapter 3). Therefore we measured both DNA methylation and genetic variation to contrast the influence of genetic variation (Nature) with that of prenatal famine exposure (Nurture) (Chapter 6). DNA methylation at most regulatory regions in IGF2/H19 was associated both with early famine exposure and single nucleotide polymorphisms (SNPs). We showed that the associations were independent of each other although they may occur at the same CpG dinucleotides. The effect sizes of the associations with prenatal famine exposure and genetic variation were highly similar. Findings from epigenetic association studies may therefore relate to genetic or environmental influences and to both.

**Characterizing regions sensitive to early famine exposure**

In Chapter 4 we found associations between prenatal famine exposure and DNA methylation at imprinted and non-imprinted regions and we wondered if there are certain genomic regions more sensitive to prenatal famine exposure. To this end we expanded our measurements to 1.2M CpG sites in 24 of the 60 sibling pairs of which one sibling was exposed during early gestation (Chapter 7). Genomic regions with a regulatory potential were especially sensitive. Promoters devoid of CpG islands, exons, enhancers (especially those active around implantation) and regions with an open chromatin status, as identified by DNase1 and FAIRE-seq, were associated with prenatal famine exposure. Regions with an open chromatin structure are often regulatory and the associations are therefore more likely to be functionally relevant.

Imprinted regions, retrotransposon insertion sites and regions of extreme epigenetic variability have been suggested to be especially sensitive to environmental conditions during development. We found no evidence that associations with early famine exposure are restricted to these genomic features (Chapter 4 and 7). Recently, systematic investigations of imprinted regions following prenatal malnutrition in mice also found no evidence for imprinted regions as particularly sensitive.
**Modest effects of exposure**

The more thorough investigations in-depth (Chapter 6) and in scope (Chapter 7) concurred with the candidate gene findings that within pair DNA methylation differences are small on a molecular scale. Although some larger DNA methylation differences were identified in our genome-wide dataset (Chapter 7), the majority was smaller than 5%. Others did also find some larger DNA methylation differences (>20%) among 27K CpG dinucleotides as a result of prenatal micronutrient supplementation and smoking\(^{39-41}\), but these studies did not validate these findings by an independent method and performed their analyses on sub divisions of already small datasets. All-in-all the evidence points towards small effects of prenatal environmental exposures on DNA methylation and it is sensible to discuss how such small DNA methylation differences may be biologically relevant.

**Modest effects: epigenetic fine-tuning?**

It was suggested that small epigenetic changes across genes and pathways (epigenetic ‘fine-tuning’) may accumulatively act to shift gene-expression\(^{42}\). The methylation effects associated with early prenatal famine exposure across the regulatory regions of \(IGF2/H19\) were correlated (Chapter 6) and in our genome-wide measurement we found associations that extended towards entire pathways (Chapter 7). Recently a study across 27K CpG dinucleotides on prenatal tobacco smoke exposure made similar observations\(^{43}\). DNA methylation differences associated with prenatal tobacco exposure were modest (<10%) and were enriched across pathways that were found to be differentially expressed. This study, together with Chapters 6 and 7, gives strong empirical support for the hypothesis that modest epigenetic effects of an exposure may act accumulatively in genomic regions and pathways.
Modest effects: phenotypic associations

Such accumulative effects of exposure may explain why a limited variation and <10% differences in DNA methylation at regions studied in Chapters 3 to 6 have been related to type 1 and 2 diabetes (INSIGF)\textsuperscript{44,45}, HDL cholesterol, hypercholesterolemia and prior cardiovascular disease (ABCA1)\textsuperscript{46}, birth weight (IGF2)\textsuperscript{12,13}, serum levels (IGF2, LEP)\textsuperscript{12,20} and the risk of myocardial infarction (GNASAS, INSIGF)\textsuperscript{55} in other populations. Indeed, in Chapter 7 we found associations for DNA methylation at CPT1A and INSR with early gestational famine exposure that extended to multiple lipid and cholesterol (for CPT1A) and growth related (for INSR) pathways. CPT1A methylation was also tentatively associated with LDL cholesterol and INSR methylation with birth weight. These associations may be argued to stem from the multiple effects in DNA methylation along the relevant pathways, a hypothesis we were underpowered to test in the genome-wide data of Chapter 7 because we only had genome-wide data for 48 individuals and the within pair differences were only present in the 18 pairs with a sibling conceived before April '45 during the Famine, reducing the studies' power even further. For CPT1A the association with LDL was almost identical in the 60 individuals exposed during early gestation and the 60 unexposed same-sex siblings. Therefore the CPT1A P-DMR may be a quantitative trait locus (QTL) for LDL and the higher DNA methylation levels following early gestational famine exposure at CPT1A may therefore have contributed to the higher LDL cholesterol levels in the exposed\textsuperscript{47,48}. For the P-DMR at INSR we only had birth weight data for the exposed. It is promising that the direction of the association between INSR methylation and birth weight within the exposed is compatible with the interpretation that the increased methylation at INSR following early famine exposure may have contributed to the possibly higher birth weight in these individuals (Chapter 3). However, this latter inference is highly speculative.

The two associations were small on a molecular scale (CPT1A-LDL: 2.4%/1 mmol*l\textsuperscript{-1}; INSR-birth weight: 0.4%/100 gram), but sizeable when expressed
relative to the normal variation in the population (0.3 SD/1 SD; 0.5 SD/1 SD) and explained up to 7.7% and 11.1% of the phenotypic variation. Such effect sizes are comparable to those reported for DNA methylation in cord blood at one CpG site in \textit{RXRA} associating with prenatal maternal carbohydrate intake and childhood fat mass\textsuperscript{40} and \textit{IGF2} DMR methylation with prenatal folic acid supplementation and birth weight\textsuperscript{13}.

\textbf{Modest effects make epigenetic association studies challenging}

The modest size of the DNA methylation differences and coefficients of the associations reported by us and others show a real future challenge. The techniques to measure DNA methylation are based on bisulfite conversion, which degrades DNA and can easily fail to deliver a complete conversion, which subsequently influences the quantification of DNA methylation levels. Getting the technical aspects wrong for just a subset of measurement can easily lead to false positive and false negative findings. Detailed descriptions of the technical aspects of the study design and execution should be included in publications as to gauge the possible robustness of the reported findings.

\textbf{Methodological issues: The Dutch Hunger Winter Families Study}

The Dutch Hunger Winter Families study\textsuperscript{4} includes same-sex sibling pairs of which one sibling was exposed during either early or late gestation and presents a powerful epidemiological design and a clear extreme environmental exposure to investigate the influence of early and late gestational malnutrition on DNA methylation. This design matches for early familial conditions, sex and partially for genetic variation. However, when interpreting findings several aspects should be considered.
**Post-natal exposure?**

Our current analyses included prenatally unexposed same-sex siblings as controls. Roughly half of them were born before the famine, and thus most likely exposed as a child. Recently it was shown that self-reported childhood exposure to the Dutch Famine was associated with an increased risk for obesity and diabetes\(^{50,51}\). Chinese famine studies have also reported that famine exposure during early childhood is associated with the metabolic syndrome\(^{61,62}\) and BMI\(^{52}\), although no effect on postnatal exposure was found on T2D risk\(^{53}\). Results are sometimes not replicated between Chinese famine studies\(^{65,66}\). Nonetheless, it is prudent in light of these studies to take post-natal exposure into consideration in analyses.

We have stratified our analyses for \(IGF2\) and the other candidate genes, finding no effect of post-natal exposure on DNA methylation or on the associations reported (data not shown). These analyses are, however, limited due to the small number of individuals with postnatal famine exposure in these datasets. Moreover, the age range of these individuals at the time of the famine was large (0-20y), while the postnatal associations are mostly found in a younger age range. Indeed, post-natal growth has been shown to have a marked effect on the associations between birth weight and later health\(^{54}\). The effect of early childhood famine exposure remains an interesting issue to explore in the larger Dutch Hunger Winter Families study.

**An appetite for DNA methylation?**

Since the onset of this thesis it was found that adult DNA methylation levels can be influenced by diet\(^{55}\) and longitudinal variations in BMI\(^{56}\). Nutrient restriction and famine exposure during early gestation may lead to a greater appetite for more energy dense foods in animals and possibly humans\(^{57}\). The association between early famine exposure and nutrition preferences awaits further investigation, as the AMC Dutch Famine cohort did\(^{71}\) and the Dutch Hunger Winter Families study did not find evidence for a higher intake of energy dense foods following early famine exposure\(^{72}\). In Chapter 7 we
controlled the famine associations for the amount of calories, fat, protein and carbohydrates in the current diet as to gain insight in this issue. The associations were not influenced by this possible confounder even though the percentage of fat and carbohydrates was associated with DNA methylation at five out of six regions chosen for validation (data not shown).

**Time of origin: direct or accumulating over the life-course**

Animal experiments have shown that besides arising immediately post-exposure, differences may accumulate following the exposure across the lifetime. Other studies in humans on the loci identified by us in Chapter 3 and 4 suggest that the differences may have arisen at or closely after exposure, rather than accumulated during life. Gestational diabetes was associated with LEP promoter methylation and maternal folic acid intake and prenatal smoking with DNA methylation at various DMRs around IGF2/H19 in newborns and young children. Furthermore, analyses of sibling pairs with a smaller age difference resulted in finding larger famine associated DNA methylation differences (Chapter 3 & data not shown), indicating that the differences do not become more pronounced with increasing age. However, measuring DNA from new samples from the same sibling pairs will be necessary to rule out the possibility that the DNA methylation differences may slowly accumulate across the life-course.

**Time of origin: early gestation or tissue specificity?**

Our results suggest that periconceptional period is the period at which most differential methylation following famine exposure may be found (Chapter 4). This period may even be confined to the period after conception (Chapter 7). Animal studies show that the blastocyst period is a very sensitive period and that DNA methylation changes induced here may be passed to tissues not yet formed during this developmental stage. Because of this mitotic inheritance during development caution needs to be taken when designating the early developmental period as the most sensitive in humans. It is possible
that famine exposure late in gestation has a larger effect on DNA methylation, but perhaps only in specific tissues. To further support our hypothesis that early development is the most sensitive period of development in humans we would need to measure DNA methylation from different tissues in both the early and late gestational exposure groups.

**Time of origin: pin-pointing**

No influence on DNA methylation was found in individuals conceived during April and May 1945, the last two months of the famine (Chapter 7). However, we are not able to pin-point the exact developmental time-point at which famine exposure is associated with DNA methylation. The official rations were steadily increasing during May and we do not know when the amount of calories was sufficient. Furthermore, the used estimate of conception, the last menstrual period, has an uncertainty of 2 weeks. The DNA methylation data also does not allow us to pin-point a certain developmental event. The differentially methylated regions in Chapter 7 were enriched for genes changing expression during the first week of human development and for those during organogenesis (>5 weeks). Moreover, enhancers active around implantation (week 1-2) were also associated with famine exposure as a group. Experiments on developing blastocysts will be required to gain more precise insight in this matter.

**The nature of the exposure**

The Dutch Famine offers a quasi-experimental setting to study the effects of well-defined extreme nutritional changes during gestation. Although malnutrition is arguably the largest component to the exposure, the other possible contributors should not be excluded, like the lack of heating during winter and stress. Dutch women normally gave birth at home, while the prenatally exposed individuals studied were born in hospital, indicating that their home situation may have been deemed unsuitable for child birth at the time of the famine.
Moreover, the frequent increases in DNA methylation observed (Chapter 4, 6 and 7) also hint to the fact that the effect on DNA methylation may not be a simple result of nutritional shortages in methyl-donors and essential co-factors. This may hint that maternal characteristics, like available fat reserves and size, may be important. Indeed, it may be hypothesized that increases in certain energy carriers freed from the fat reserves as a result of starvation, like free fatty acids and 3-hydroxybutyrate\textsuperscript{65}, may be related to some of the observed DNA methylation differences between the prenatally exposed and unexposed siblings. In vitro experiments in embryonic stem cell during differentiation\textsuperscript{66}, embryoid bodies\textsuperscript{67} and on bovine blastocyst development\textsuperscript{68}, may be performed to test the effect of shortages and surpluses of certain nutritional compounds and metabolites on DNA methylation.

\textit{5-hydroxymethylation}

During the earliest embryonic stages massive active demethylation of the paternal genome leads to various oxidation products of 5-methylcytosine (mC), the main of which is 5-hydroxy methylcytosine (5hmC), but also higher oxidation products in the form of 5-formyl- and 5-carboxycytosine have been discovered. 5hmC is a stable base and is abundant in embryonic stem cell and is rapidly depleted as the genome is remethylated during blastocyst development and implantation\textsuperscript{69}. It is believed that 5hmC is an intermediate for complete demethylation and rapid remethylation\textsuperscript{70} and present at low levels in non-neural adult cells\textsuperscript{71}. With bisulfite treatment you cannot distinguish between 5hmC and mC. It may therefore be possible that some of the effects reported may be related to differences in 5hmC levels rather than mC, but considering the low levels of 5hmC in adult blood it should have a limited bearing on our results.

Rather than a possible confounder, the influence of nutritional compounds on the mechanism of 5hmC formation and depletion during development may be interesting to study. Recently it was shown that 5hmC levels in embryonic stem cells are influenced by vitamin C levels \textit{in vitro}\textsuperscript{72} as one of the main enzymes implicated in 5hmC formation, \textit{tet1}, is driven to a higher efficiency.
by higher vitamin C levels\textsuperscript{73}. The effect of nutrient shortages and surpluses on \textit{tet1} and its functional partners are prime targets for detailed functional studies on the possible molecular mechanism behind the observed DNA methylation patterns induced by the prenatal environment.

**Bringing epigenetic studies in humans to the next level**

Epigenetic epidemiology will surely benefit from larger study sizes and the scrutiny of replication, as was recently shown with the first epigenome-wide association study (EWAS) with multiple replications on the effect of smoking\textsuperscript{84,85}. But population size alone will not be enough to discover robust associations, since our and the above mentioned studies are well powered considering that the effect sizes discovered were around 0.5 standard deviations.

\textit{Epigenetic epidemiology is like epidemiology}

The epigenome, including DNA methylation, is highly dynamic. Recently it was shown that DNA methylation at genes involved in immunity can fluctuate within as little as four days\textsuperscript{74}. This means that any analysis within and between cohorts can be easily confounded by a myriad of factors, like the time between DNA sampling and phenotyping and dietary differences between populations and ethnic groups. This touches on a dilemma of epidemiology in general, whether it is more efficient to perform studies on several smaller but well-characterized cohorts or on larger cohorts in which important covariates may be missing, a brute force strategy successfully employed in genetic epidemiology. For epigenetic studies the first approach may prove the most suitable one.
Considerations on the ideal cohort study for EWAS

For epigenetic epidemiology it would help to know a lot about the current and past environmental conditions of individuals within a cohort study, as environmental conditions are among the most likely confounders. Secondly the availability of other –omics datasets, in particular for genome-wide expression, is essential. Such datasets may help in assessing the functionality of epigenetic marks associated in EWAS, as it is currently unclear at which genomic regions epigenetic variation has biological consequences.

The ideal cohort would also include some longitudinal and tissue sampling. The first can help with building a case for the causality of epigenetic marks within a disease process, as epigenetic variation may merely reflect disease progression. Tissue sampling is required to ascertain if an association relates to variation in a tissue relevant to the phenotypic association. Sampling should ideally also entail the collection of viable cells for cell culture, since such samples may help to elucidate the molecular mechanism underlying an association. Fibroblasts can be isolated from skin biopsies and could be used to test for an influence of epigenetic marks on the binding of transcription factors and the activity of a genomic region. More mechanistic insight may be gained from mesenchymal stem cells isolated from cryo-preserved muscle biopsies. These mesenchymal stem cells can be differentiated into muscle, fat, cardiomyocytes and even ectodermal tissues, and offer room for extensive experimentation. Such experiments are of interest, as both animal studies and human studies have yet to uncover the mechanism underlying the epigenetic changes described.

Epigenetic fine-tuning has consequences for the data analyses

If epigenetics acts mainly by small changes across large regions and along entire pathways more focus should be placed on how to analyze data. The current statistical models for grouped analyses are complicated and still limited in their ability to capture all the correlations in DNA methylation data. We grouped DNA methylation on new genome-wide annotations coming out
of various genome wide annotation efforts, including the ENCODE project\textsuperscript{36}, but still relied on a simplification of the data to perform pathway level tests (Chapter 7). More focus will need to be placed on statistical methodologies for grouped and pathway based analyses to tackle epigenome datasets.

**Evolutionary considerations**

The differentially methylated regions we discovered map to genes and their pathways with key roles in growth and energy allocation, including *INSR*, *LEP*, *IGF2* and *INS*, arguing for an evolutionary component to our findings.

*Thrifty genes: selection?*

Some of these particular genes were hypothesized to be involved in metabolic disease in the thrifty genotype hypothesis. This hypothesis states that our ancestors evolved under a frugal diet and that our metabolism is thus genetically ill adapted to deal with our modern energy rich diet, causing obesity and metabolic disease\textsuperscript{31,90,91}. However, no links between the increase in metabolic disease and genetic variation has been found in populations undergoing a shift from traditional to affluent “Western” diets\textsuperscript{78}. Recently this hypothesis was overhauled\textsuperscript{79}, stating that under adverse conditions during early gestation embryos with a thrifty genotype are selected. The author refers to our Dutch Hunger Winter Families study as the ideal setting to test this hypothesis. Indeed we have already taken some steps in addressing the issue of genetic selection. In Chapter 6 we searched for differences in genotype frequency in the *IGF2/H19* region in exposed and non-exposed individuals, finding no differences. We have also measured SNPs taken from GWAS on body composition and various glucose and lipid traits and as of yet found no differences between prenatally exposed and unexposed individuals (*data not shown*). To comprehensively address this issue SNP genotyping arrays could be run to search for evidence of genetic selection\textsuperscript{79}, using algorithms developed for genetic anthropology.
There is little doubt that selection may have occurred as the number of new pregnancies dropped 50% during the famine\textsuperscript{80}. However, selection on another genomic level should also be considered in light of the increasing number of studies finding epigenetic differences following early gestational exposure. Under nutrition reduces cell numbers in pre- and post implantation rat embryos\textsuperscript{81}. Upon famine exposure cells may be selected with a favorable epigenetic signature for growth. We found that differential methylation was enriched along growth related pathways (Chapter 7). Moreover we found associations between early gestational famine exposure and key growth and insulin signaling genes \textit{IGF2} (Chapter 3), \textit{INS} (Chapter 4) and \textit{INSR} (Chapter 7). Again, experiments in animal blastocyst models should give insight in this matter by applying more or less nutrients and measuring markers for apoptosis and the final DNA methylation signatures of the cultured blastocyst.

**Thrifty phenotypes: phenotypic plasticity**

The thrifty genotype theory has been largely replaced by the thrifty phenotype hypothesis\textsuperscript{82} that led to the so-called developmental origins hypothesis of adult disease (DOHaD), which introduces modern ideas on phenotypic plasticity into Medicine\textsuperscript{28}. The most modern version of the DOHaD hypothesis states that developmental time-frames exist during which the fetus makes persistent adaptations to the perceived environment (predictive adaptive plasticity), disease follows when the adult environment does not match that during development\textsuperscript{96}. This hypothesis is well founded in observations in shorter lived animal models and also supported by some observations in humans. Individuals exposed during early development to famine in China, but adhere to a ‘frugal’ diet as adults are less likely to develop disease than those adhering to an affluent Western diet\textsuperscript{83,84}. The same principle is seen in the Gambia following seasonal food shortages\textsuperscript{85}. Moreover, individuals from under privileged backgrounds show a less severe phenotype upon starvation in childhood than those from a more wealthy background\textsuperscript{86}. 
Epigenetic fine-tuning of metabolic networks is seen as a candidate mechanism for adaptive phenotypic plasticity, as metabolic and growth related gene networks are subject to intense selection, which may have resulted in genetic canalization and an extreme robustness for genetic mutations\textsuperscript{42}. Indeed, leptin was put forward as a prime candidate for epigenetic fine-tuning and we found DNA methylation at the leptin promoter associated with prenatal famine exposure (Chapter 4). Moreover, small changes across regulatory and gene networks were hypothesized to be the mechanism by which this fine-tuning occurs, which matches with our observations (Chapter 6 and 7).

**Phenotypic inertia**

However, predictive adaptive phenotypic plasticity makes little evolutionary sense in a long lived species as *homo sapiens* and it could only have arisen when famine is a very common phenomenon\textsuperscript{87}. This is a debated issue, as famine most likely only became frequent after the advent of agriculture\textsuperscript{88}, a period too short for predictive adaptive plasticity to have evolved in man\textsuperscript{78} considering our long lifespan and reproductive cycle\textsuperscript{89}. Furthermore, predictive adaptive plasticity would logically entail that the epigenetic code is reset every generation, while evidence is accumulating that epigenetic adaptations to environmental circumstances may sometimes be passed to the next generation\textsuperscript{47,103,104}, even when the environmental circumstance that induced the change is no longer present. Also on the epidemiological level the evidence for transgenerational inheritance is building, although still inherently weak\textsuperscript{90}. Kuzawa *et al.* shows that there may have been a selective advantage for long-living primates to be able to adapt to gradual differences in climate over generations\textsuperscript{89,91}. The envisioned mechanism would allow one generation to adapt to the environment, but never over-act. Key in this hypothesis of ‘phenotypic inertia’ is the data collected on the buffering capacity of the womb\textsuperscript{92} and the transgenerational information contained in the maternal body composition of mammals (maternal constraint), which would allow for
a slow transgenerational adaptation to robust environmental differences\textsuperscript{93}. Epigenetic change in response to environmental circumstances is hypothesized to be one of the mechanisms by which the adaptation and buffering may occur\textsuperscript{91}. Measurements in sperm of prenatally exposed men and in the children of all prenatally exposed may help to gain insight in this phenomenon in \textit{Homo sapiens}.

\textit{Don’t forget about mum}

For individuals conceived during the famine, like those studied extensively in this thesis, the mothers may be where the selection has acted, resulting in a selected offspring group. They may have had more fat reserves at the time of the famine, and the BMI of the mother at the time of pregnancy has an influence on the child’s later adiposity\textsuperscript{94}. These mothers may also have had access to better nutrition. Furthermore, the mothers of the individuals conceived during the famine may be more fertile, although investigations on this issue have been largely inconclusive\textsuperscript{23}.

For the studies in this thesis it is good to emphasize that we compared the individuals conceived during the famine with a same-sex sibling, so that genetic and familial factors are at least partially similar. Furthermore, there are a numbers of animal studies that show that epigenetic differences may arise in the fetus as a result of a nutritional challenge. Both may argue for an interpretation that the associations presented have arisen, at least in part, as a result of the famine exposure, and not solely as a result of maternal characteristics.

\textbf{Conclusion and Future prospects}

We successfully identified regions at which the DNA methylation levels are associated with prenatal famine exposure six decades post exposure. We discovered that these associations may be timing dependent and sex specific, mimicking the reported epidemiological findings. The regions identified map to biologically relevant genes and pathways for the phenotypes associated with
prenatal famine and we discovered tentative associations with phenotypes also associated with prenatal famine exposure. The Dutch Hunger Winter Families study offers one of the best epidemiological designs to serve as a discovery cohort for DNA methylation variation linked to both developmental adversity and adult disease. We aim to scale our DNA methylation measurements to the entire cohort and test associations in other cohorts. These cohort scale analyses have now become possible by the 450K Illumina DNA methylation micro-array\textsuperscript{95}, which allows the cost-effective measurement of 450K CpG dinucleotides in large sample sizes. Associations should be robust and consistent and taken further. For we need to relate associations in the Dutch Hunger Winter Families study to other prenatal exposures, measure longitudinal samples to infer causality, investigate relevant tissues and set up models to uncover the molecular mechanisms behind the associations if we are to understand the biology behind the associations and move on to designing possible interventions.

In the introduction of this thesis we referred to the work by Waddington (Chapter 1, Box 1), who tried to conceptually merge embryology, evolution and genetics and who coined the term ‘epigenetics’ to refer to this interdisciplinary endeavor. The Dutch Hunger Winter Study offers the unique opportunity to study epigenetics in its most modern sense, which is very much in the spirit of Waddington. We should try to link embryology, genetics, cell biology and evolution and through this interdisciplinary approach, as a true ‘epigenetic’ endeavor, enhance our insight in the mechanisms underlying disease and the continuing evolution of \textit{Homo sapiens} in its modern environment.
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