Summary of Results

Introduction

Early environmental conditions and growth parameters at birth are associated with adult human disease risk in epidemiological studies. Generally, a low birth weight is taken in such studies as a proxy for an adverse environment during development, a view that is hotly debated and has lead to much discussion on the nature of these associations. The study of historic famines and in particular the Dutch Famine has shown that exposure during specific developmental time-frames is associated with a higher risk on metabolic disease and a poorer mental health later in life. Most of these associations are independent of birth weight. Indeed, also other defined prenatal exposures, like smoking and maternal obesity, are associated with variation in traits which have been related to famine exposure during early development.
It is hypothesized that persistent epigenetic differences induced by environmental challenges partly underlie such epidemiological observations. Animal studies have shown the basic principle; prenatal nutrition was found to modulate histone and DNA methylation marks at genes functionally implicated with disease. In this thesis, we addressed the contribution of epigenetic mechanisms to the link between environmental challenges in early life and adult health in humans by studying DNA methylation in individuals prenatally exposed to the Dutch Famine and an unexposed same-sex sibling as control. We also address if individuals with in uterine growth restriction show similar DNA methylation patterns as prenatal famine exposure.

Chapter 2: variation, patterns and stability

First we described variation of DNA methylation in whole blood, which is arguably the most widely available source for epigenetic research in well-characterized biobanks that are currently available. We studied a selection of 16 candidate loci that are epigenetically regulated and have roles in metabolism and development. We discovered that DNA methylation i) is variable in the population ii) is relatively stable over time and iii) is frequently not confounded by the cellular heterogeneity of whole blood samples.

Between CpG dinucleotides a high correlation was found, making it likely that a limited number of CpG dinucleotides can be measured to gauge the methylation status of a region (cf. genetic linkage disequilibrium). We also found a high covariance for a subset of loci between blood and buccal cells, which stem from a different embryonic lineage, indicating that there are genomic regions for which DNA methylation in blood may be used as a proxy for DNA methylation in other tissues.

Chapter 3: A persistent DNA methylation difference

No epigenetic differences associated with (prenatal) environmental conditions were reported on in humans; therefore we wished to set a proof-of-principle. The Dutch Famine, a severe 6 month famine at the end of WWII, provides a defined and severe prenatal environmental exposure. Therefore we measured DNA methylation of the *insulin like growth factor* 2 (*IGF2*), a crucial regulator of fetal growth, in whole blood of 60 individuals exposed early and 62 individuals exposed late in gestation to the Dutch Famine. For each individual we also measured a prenatally unexposed same-sex sibling as control. We hypothesized that early gestation would be the most sensitive period. Work in animals show that this period is the most dynamic period for establishing epigenetic marks and DNA methylation differences induced early in development may be passed on soma-wide.
Early gestational famine exposure was associated with a decrease in *IGF2* DNA methylation six decades post exposure. This was the first evidence that the epigenome may be persistently altered during early development by the environment, providing a candidate mechanism linking development and later disease in humans. Late gestational famine exposure was not associated with *IGF2* DNA methylation, which was in line with our hypothesis.

Chapter 4: Epigenetic differences associated with Famine are common and time- and sex-specific

Next we explored if the *IGF2* DMR association was unique, or that DNA methylation is frequently altered by famine exposure and includes associations with non-imprinted regions. Moreover, associations should mirror the epidemiological literature and should also include sex-specific and timing independent associations if epigenetic change is to be the mechanism underlying the link between development and disease. We therefore extended our investigation in exposure discordant sibling pairs to the additional 15 loci characterized in chapter 2.

DNA methylation was associated with prenatal famine exposure at additional imprinted (*INSIGF*, *GNASAS* and *MEG3*) and non-imprinted regions (*LEP*, *ABCA1* and *IL10*). Overall, the DNA methylation differences were modest on a molecular scale (<4%), but sizeable relatively to the variation in the population and included increases in DNA methylation. Most associations were restricted to early gestational famine exposure and sex-specificity of the associations was common. One association, with DNA methylation at the *leptin* promoter, was independent of the gestational timing of the famine exposure. These results give strength to our *IGF2* DMR study and position epigenetics as a candidate mechanism to explain the association between early development and later disease in humans.

Chapter 5: Generalizability, no association with prenatal growth restriction

A low birth weight, or in uterine growth restriction (IUGR) has been linked to an increased risk of adult metabolic and cardiovascular disease. IUGR is often taken as a proxy for prenatal malnutrition or seen as a sign of a suboptimal prenatal environment. Therefore we tested the relevance of famine-associated DNA methylation differences in contemporary cases of IUGR. We resorted to the developmental extreme of preterm birth (<32 weeks), because most DNA methylation differences were found after early

developmental famine exposure. We measured DNA methylation at *IGF2* DMR, *GNASAS*, *LEP* and *IL10* in preterm IUGR and non-growth restricted individuals. No differences were found. Our results add to a growing literature showing that IUGR may not necessarily find its basis in prenatal malnutrition. IUGR may arise as a result of many different causes and may therefore represent a heterogenic etiology.

Chapter 6: nature & nurture both influence DNA methylation

DNA methylation is also influenced by genetic variation. Therefore we wised to investigate the influence of genetic variation (Nature) on DNA methylation and contrast its influence with that of the environment (Nurture). First, we extended our *IGF2* DMR finding to five regulatory regions across the imprinted *IGF2*/*H19* locus. DNA methylation at most regulatory sites was associated with prenatal famine exposure and their methylation was correlated, indicating that epigenetic fine-tuning may involve larger regions. Next, we measured the genetic variation around *IGF2* and *H19*. DNA methylation at some regulatory sites was also associated with single nucleotides polymorphisms and these associations were similar in effect size to the famine associations. The associations of DNA methylation with prenatal famine and genetic variation were independent and additive. We were the first to show that Nature's and Nurture's influence on DNA methylation may co-exist. Findings from epigenetic association studies may thus have an environmental and genetic component and we should therefore not rush to exclude an influence of environmental factors on DNA methylation levels if a SNP is found to influence DNA methylation at a particular locus.

Chapter 7: epigenome-wide characterization

We aimed to learn at which genomic annotations DNA methylation differences associated with early famine exposure occur the most and if associations do not only extend across larger regions, but also across pathways. DNA

methylation of 1.2 million CpG dinucleotides was assessed by next generation sequencing in 24 individuals exposed to the Dutch Famine in early gestation and 24 unexposed same-sex sibling controls of which one sibling was conceived during the famine. Famine associated DNA methylation changes clustered at regions with a regulatory potential. Increases in DNA methylation were commonly observed and most differentially methylated regions were in gene-bodies. Even within the time-window of the first trimester we found evidence of an effect of the timing of the exposure on DNA methylation. DNA methylation at loci mapping to *CDH23*, *SMAD7*, *INSR*, *CPT1A*, *RFTN1* and *KLF13* were associated to famine in individuals conceived before April 1945, two months before the famine's end. The differences were smaller than 5%, but the associations extended to the pathways these genes belong to, which were pathways related to growth and lipid metabolism. Moreover, we found tentative associations between methylation at *INSR* with birth weight and methylation at *CPT1A* with LDL cholesterol levels. The latter association was almost identical in the prenatally exposed and their unexposed same-sex siblings. If replicated, *CPT1A* methylation may thus prove a LDL quantitative trait locus contributing to the higher LDL levels in the exposed. Modest DNA methylation differences thus extend across biologically relevant pathways and may be linked to phenotypes of interest in relation to early famine exposure.