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The first 1000 days and beyond

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CHAPTER 10

GENERAL DISCUSSION

In the 1990s, professor David Barker introduced the foetal programming or developmental origins of health and disease (DOHAD) hypothesis, which states that adverse intrauterine exposures and reduced foetal growth can be related to long-term increased risk of chronic diseases later in life[1]. Barker showed that a low birth weight was associated with hypertension, coronary heart disease and insulin resistance[1-3], which are all related to overweight. In this thesis we focused on effects of foetal programming on infant growth and the development of overweight later in life. However, we did not only study foetal programming, we also tested which underlying biological mechanisms might be responsible and we confirmed that DNA methylation partly mediates the effects of foetal programming. Additionally, we determined how lifestyle factors during infancy and childhood influence the development of childhood overweight against a programmed background assumed to make the child more susceptible or resilient to external obesogenic influences.

Early life exposures, such as intrauterine exposures or exposures during early postnatal life, can affect the risk of obesity later in life. Examples of intrauterine exposures are maternal smoking during pregnancy, gestational weight gain and gestational diabetes[4]. Examples of early postnatal exposures are accelerated infant growth and bottle-feeding instead of breastfeeding, as discussed in **Chapter 6**. Recent research on foetal programming has shifted towards investigating the lasting effects of these influences and their underlying biological mechanisms such as differential DNA methylation[5].

EPIGENETIC EPIDEMIOLOGY

Epigenetic epidemiology is currently one of the "hottest" fields in epidemiology, mostly because it has huge potential to find biological explanations for previously observed associations between (intrauterine) exposures and diseases[6-8]. Epigenetics (and in particular DNA methylation) may constitute not only a good biomarker of exposure but also a good predictor of diseases. As such, several studies showed that DNA methylation was associated with overweight[5,9-11]. Combining traditional observational epidemiology with epigenetics may therefore provide new insights in biological mechanisms of exposure-outcome associations. This combination is now possible because of the development of high-throughput arrays. Initially, investigation and measurement of DNA methylation was limited to basic science, but these arrays allowed for its inclusion in large-scale epidemiological studies, resulting in the field of epigenetic epidemiology. Sebert et al. already hypothesized that DNA methylation could be the "missing link"

between early programming and metabolic health[12]. This theory has recently been supported by results from epigenome-wide association studies (EWAS) in humans[13-16]. Part of the initial evidence came from the famous Dutch hunger winter studies, showing that prenatal famine exposure could be related to differential DNA methylation in the offspring, which then could be linked to birth weight and LDL serum cholesterol levels[16]. One example of the combination of traditional epidemiology and epigenetics in this thesis is the association between maternal smoking during pregnancy and birth weight in **Chapter 6,** which we confirmed in our EWAS on maternal smoking in **Chapter 2**. In the observational study (**Chapter 6**) we found that children of smoking mothers had a lower birth weight and that this difference in size remained until at least two years of age. We then showed that differences in DNA methylation are part of the biological mechanism underlying this difference in birth weight caused by maternal smoking during pregnancy (**Chapter 2**). This example shows that epigenetic epidemiology has the potential to improve biological insight into the many associations between exposures and diseases that have been observed and described in the past decades. Therefore, in the context of this thesis, the addition of epigenetics to epidemiology offers great promise to biologically explain the developmental origins of childhood growth and weight status[5].

DNA METHYLATION AS BIOMARKER OF EXPOSURE

Rapid developments are occurring in the field of epigenetic epidemiology including the development of high-throughput measurements and corresponding software packages to analyse the results[17,18]. Furthermore, cohorts with EWAS data increasingly collaborate in international consortia in order to perform meta-analyses that increase the power and generalizability of our findings. These developments will increase the possibilities to find more differentially methylated genes. The effects in epigenetics have substantial clinical relevance. For example, we showed in **Chapter 2** that DNA methylation could explain 20% of the 200 grams lower birth weight in offspring of smoking mothers compared to offspring of non-smoking mothers. This is a much larger effect size than those found in traditional genetic epidemiology, in which a maximum of only 10% of the variance in BMI could so far be explained by many genetic polymorphisms[11]. Shah and colleagues recently showed that methylation profiles and genetic profiles predicted adult BMI in an additive manner[11]. Ideally, a combination of epigenetic epidemiology and the more traditional genetic and exposure-disease epidemiology could add to the reduction of the worldwide obesity epidemic, because the more we know about the predictors of obesity and underlying mechanisms, the better we can target our prevention programmes[11]. This

might especially be interesting for a complex disease like obesity, where many factors seem to play a role[19]. In conclusion, DNA methylation may offer a viable alternative as a "biomarker" of intrauterine growth restriction, may be directly related to the underlying regulatory processes that are affected and may constitute a mechanism through which effects of environmental exposures are carried forward in life.

RESILIENCE VERSUS SUSCEPTIBILITY

Foetal programming is thought to translate into a child's predisposition that is either characterised by resilience against or susceptibility to the obesogenic environment and/or unhealthy lifestyle during all subsequent stages of life[20]. It can be hypothesised that the difference between susceptibility and resilience originates in the biological memory of differential DNA methylation related to (unhealthy) intrauterine exposures[5,6,13]. Thus, a resilient child can be considered biologically programmed to resist adverse environmental influences and is protected against developing overweight in response to the obesogenic environment and an unhealthy lifestyle. On the contrary, susceptibility is characterised by an increased vulnerability to these adverse obesogenic exposures, increasing the risk of obesity. Examples of obesogenic behaviours could be low levels of physical activity, short sleep duration or unhealthy dietary habits[21-23]. The concept of resilience was illustrated in this thesis by the fact that not all infants and toddlers with unhealthy dietary habits showed an increased growth velocity in the first 2 years of life (**Chapter 6**) or a higher risk of overweight at six years of age (**Chapter 8**). We conclude that a combination of a susceptible predisposition with unhealthy dietary habits and low physical activity will likely be most detrimental for a child's growth and weight status.

Another important concept in the DOHAD research field is catch-up growth, which often occurs after intrauterine growth restriction and increases the risk of obesity and cardiometabolic problems later in life[2,24,25]. In **Chapter 6** we found that growth in the first two years of life was indeed dependent on intrauterine exposures and foetal development. Newborns with a low birth weight showed an increased growth velocity in the first six months, while high birth weight newborns showed a decreased growth velocity in that period. We also observed a clear difference in birth weight and consecutive growth between children of smoking versus non-smoking mothers, as previously observed[26]. In contrast, maternal BMI determined the starting point with a higher maternal BMI related to a higher birth weight, but did not affect the growth velocity after birth. Thus, the higher weight in newborns from overweight mothers remained high

during the first two years of life. Interestingly, BMI of the father started to play a role from six months onwards, potentially indicating the increasing importance of the family lifestyle from this time point. However, it could also imply a genetic component[27] that is "overruled" in the first six months as this early period is dominated by the influence of the intrauterine development and the type of milk feeding[26]. Independent of the starting point, accelerated deviation from an individual's growth curve is unhealthy as the obesity epidemic is not only a question of the obese becoming fatter, but also of lean individuals becoming less lean[28]. Given the clear distinction in growth curves and its dependence on the starting point determined by intrauterine development we believe it is important for future studies to stratify growth curves on quartiles of exposure or birth weight, instead of modelling one overall growth curve for the entire group. This will allow the illustration of both positive and negative growth in different strata.

CAUSAL EFFECT VERSUS REVERSE CAUSATION

In spite of the abovementioned potential of DNA methylation to explain the biological mechanism underlying increased chronic disease risk, the causal pathway in epigenetic epidemiology is often not clear. For example, in adults it is difficult to distinguish whether obesity causes differential DNA methylation, or whether differential DNA methylation causes an increase in BMI. However, by studying DNA methylation in cord blood and its association with diseases later in life, the chronological order makes it plausible that cord blood DNA methylation differences cause childhood overweight rather than the other way around. Within the context of the foetal programming hypothesis, the same logic applies to adverse exposures such as maternal smoking during pregnancy causing differences in cord blood DNA methylation, which in turn lead to increased disease risks during childhood and adulthood, e.g. via a lower birth weight. In a more general sense, epigenetic epidemiology provides a very promising model to biologically explain the effect of a wide range of adverse intrauterine exposure on risk of metabolic diseases in the context of the foetal programming hypothesis. However, as in all observational studies, confounding (by factors such as diet and activity patterns) should be kept in mind as an issue that can act at multiple stages of the life course.

We also observed difficulties in distinguishing causality versus reverse causation in **Chapter 8** on the predictive value of dietary habits for childhood growth. With our combined food score based on dietary habits at 11 or 24 months, prediction of overweight status at two or six years of age was difficult. We observed that unhealthier

dietary habits were associated with a lower weight status at two years of age. From an energy-balance point of view this was unexpected, but it might be explained by reverse causation. It is likely that dietary habits are constantly changing based on current weight status. Thus, if a child has a high weight, parents might feel the need to restrict energy intake to diminish growth. Or if a child has a low weight, parents might feel the need to increase the child's energy intake to ensure a "healthy" growth. This resulted in a higher risk of accelerated growth between two and six years of age for children with (constant) unhealthier dietary habits compared to children with (constant) healthy dietary habits. It is debatable, however, whether extra growth in low weight children is healthy, because although these children remain in the normal BMI range, their body composition could negatively change.

HOLISTIC APPROACH OF DIETARY HABITS AS PREDICTORS OF OVERWEIGHT

Traditional nutrition research has mainly focused on isolated intake of single micro- and macronutrients. However, in the past decades nutrition research has shifted from this single nutrient approach towards a more holistic approach[29]. This paradigm shift is important as nutrients interact with each other in food products and whole diets[30]. The single nutrient approach is distant from the public's understanding and better suitable for mechanistic research. Additionally, exact quantification of nutrient intake is difficult in toddlers, because of large day-to-day variation[31]. However, the holistic approach examines "markers" instead of detailed measurements, it is closer to actual daily food intake and thus very suitable for translational research.

In **Chapter 8** we investigated whether dietary behavior of toddlers predicted growth between two and six years of age, known to be an important determinant of overweight later in life[32]. We showed that an easy-to-use tool that measured the key elements of toddlers' diet indeed predicted differences in growth. The added value of this study lies in its simplicity of data collection on dietary habits. Thus an easy-to-measure overall idea of dietary habits, as in our food score, is preferred as a tool for prediction of childhood growth and future research may reveal additional dietary markers. Importantly, the ability to predict the risk at a very early age (in toddlers) may facilitate development of early prevention programs for these children.

LIMITATIONS OF EPIGENETIC EPIDEMIOLOGY

Epigenome-wide association studies in humans are often conducted using peripheral blood or cord blood in newborns, which both consist of a mixture of cell types. This measurement of DNA methylation in blood samples can be seen as a limitation because heterogeneity in cell type composition typically is associated with methylation differences, and both cell type composition as well as methylation could be differentially affected by exposures like maternal smoking. However, blood is the best available source of biomaterial in humans and by adjusting the analyses for estimated cell type proportions we could partly obviate problems with cell type heterogeneity [33]. Furthermore, DNA methylation is one of the processes in a chain of omics processes[34]. A relatively small change in DNA methylation may cause a larger effect on gene expression. This partly depends on the genomic region where the methylation differences appear, e.g. small changes in the promoter region of a gene could have large effects in gene expression[35]. The results of our EWASs should therefore be followed up by studying them in a more detailed manner, e.g. by comparing methylation effects in different tissues (e.g., using mouse models), by studying the regions around the top CpGs in more detail using pyrosequencing or by examining the link between DNA methylation and gene expression.

IMPLICATIONS FOR FUTURE RESEARCH AND PUBLIC HEALTH

Throughout this thesis the importance of research on the foetal programming hypothesis is discussed. In this context, the first 1000 days of life, starting from the first day of pregnancy until two years after birth, have gained attention in the past years[36]. For example, in 2013 the World Health Organization published a guide for nutritional interventions in this critical time window during which all organs are formed and most of the programming is presumed to take place[37,38]. Consequently, adverse exposures during these first 1000 days could have long-lasting effect throughout life, partly via the "biological memory" of DNA methylation[5]. This "1000 days" phenomenon should be given even more attention in future research and may need to be extended to even earlier stages before conception. For the development of obesity the growth between two and six years of age is another critical time window. An important focus should be on distinguishing the most important age windows in this vulnerable period, during which susceptibility to adverse exposures is most critical. This approach would ideally result in "windows of opportunity" for better prediction of overweight later in life and for future

prevention. A theoretical model of these windows of opportunity is illustrated in **Figure 1**. Based on this thesis, the main focus of public health and prevention research should shift towards the periconceptional, intrauterine and early postnatal (infant) periods as windows of opportunity. We now know that part of healthy ageing already starts during pregnancy.

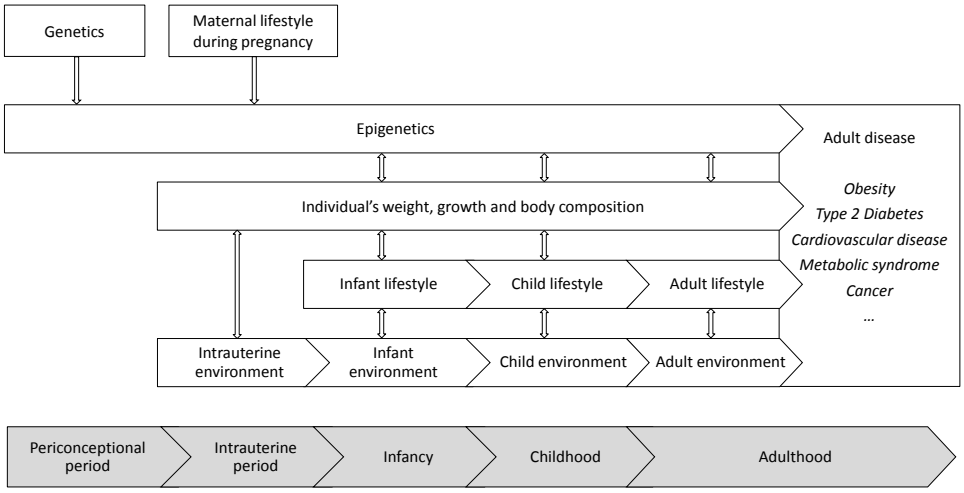

FIGURE 1. Windows of opportunity for obesity prevention.

Apart from investigating the most optimal windows of opportunity, great potential lies in unravelling the biological mechanism driving foetal programming such as DNA methylation. Based on the studies reported in the current thesis we now know that DNA methylation is affected by intrauterine exposures (e.g. maternal smoking), associated with health outcomes (e.g. birth weight), and that it may mediate the effects of exposures on health outcomes. However, it is not yet known whether the differential methylation pattern associated with adverse exposures remains stable over time. That is, are those CpG sites in cord blood associated with e.g. maternal smoking also differentially methylated during childhood, adolescence and adulthood? And can differential methylation at birth predict obesity later in life? Such information may prove to be valuable for improved prediction (complementary to information on intrauterine exposures, lifestyle and genetics). Therefore, future studies should aim to study the stability of differential DNA methylation throughout childhood and into adulthood. Finally, future studies should investigate whether knowledge on stability of DNA methylation will improve predictive performance of epigenetic risk scores. This information from repeated DNA methylation measurements will ideally provide insight into the most

promising window of opportunity for prevention efforts countering adverse effects of foetal programming and DNA methylation. Such repeated measurements would also allow for a better theoretical model to distinguish causality and reverse causation. Such a study design could possibly also give us an answer to the question how long-lasting the effects of foetal programming are. One of the most important questions to study in the future would be which lifestyle interventions are effective in preventing obesity in children that have been foetally programmed to be susceptible to the obesogenic environment.

CONCLUSION

In the current thesis we have shown that maternal lifestyle during pregnancy can alter DNA methylation of the offspring, which in turn could program the child to be resilient or vulnerable to the obesogenic environment later in life. A combination of adverse foetal programming and unhealthy infant and family lifestyle would be detrimental to childhood overweight. Therefore, a holistic approach of measuring (maternal and infant) dietary habits, combined with other pre- and early postnatal lifestyle factors in combination with genetic markers and epigenetic biomarkers of the programming effect of exposures can optimize the prediction of overweight, with the first 1000 days offering the most promising window of opportunity for prevention.

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