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and the Environment
Ministry of Health, Welfare and Sport

Contribution to risk of chronic disease by epigenetic programming

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Colophon

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Rapport in het kort

Bijdrage aan risico op chronische ziekten door epigenetische programmering.

Epigenetische programmering is een fysiek mechanisme dat ervoor zorgt dat lichaamsfuncties goed zijn aangepast aan invloeden uit de omgeving. Het kan worden voorgesteld als schakelaars die de activiteit van celfuncties bepalen. Als het schakelbord van de cel van een kind tijdens de zwangerschap niet goed wordt ingesteld, kan dit later in het leven negatieve gevolgen hebben. De cel functioneert dan niet goed, oftewel gaat minder adequaat op de omgeving reageren. Zulke celverstoringen kunnen op verschillende manieren ontstaan, bijvoorbeeld door ongebalanceerde voeding, chemische verontreinigingen in voedsel, en tabaksrook. Epigenetische programmering kan zo het risico vergroten dat iemand een chronische ziekte ontwikkelt, zoals overgewicht, diabetes, hart- en vaatziekten, sommige allergieën en kanker.

Uit literatuuronderzoek van het RIVM blijkt dat de verstoring van de epigenetische programmering een reëel fenomeen is. Hoe groot de bijdrage uit de omgeving op verstoringen is, is nog niet duidelijk. Wel kan dit gegeven de toename van de genoemde aandoeningen in de laatste jaren helpen verklaren. Er zijn technieken beschikbaar die gebruikt kunnen worden om de bijdrage van omgevingsfactoren aan verstoring van het epigenetisch programma te meten en in de literatuur worden hiervoor meerdere testmethoden beschreven. Het RIVM stelt voor om deze testmethoden nader uit te werken. Met goede testmethoden kunnen in de toekomst risico's van blootstellingen tijdens zwangerschappen beter worden ingeschat en gerichte beleidsadviezen worden opgesteld.

Het belang van epigenetische programmering bleek voor het eerst bij kinderen van zwangere vrouwen die in de hongerwinter van de Tweede Wereldoorlog ondervoed waren. Deze kinderen bleken later in hun leven vaker te lijden aan chronische ziekten als diabetes dan leeftijdsgenoten die de ondervoeding niet hoefden te doorstaan. Een ander voorbeeld is een tijdens de zwangerschap doorgemaakte blootstelling aan een vocht- en vuilafstotend middel, waarna vaccinaties minder effectief bleken te zijn op het moment dat de kinderen de schoolleeftijd hebben bereikt.

Trefwoorden: prenatale programmering, epigenetica, voedselcontaminanten, toxicologie, testmethoden

Abstract

Contribution to risk of chronic disease by epigenetic programming

Epigenetic programming is a physical mechanism that enables adaptation of body functions to environmental conditions. It can be considered as switches that determine the activity of cell functions. Inproper adjustment of the cellular switch board early in life can have negative consequences later in life. The cell will not function properly, it will not respond adequately on external stimuli. There are various ways that can lead to such disruption of cell functions, including imbalanced diet, chemical contaminants in food, and tobacco smoke. Through this mechanism, epigenetic programming can contribute to the risk of chronic disease, such as overweight, diabetes, heart failure, some allergies, and cancer.

A literature review by RIVM shows that disruption of epigenetic programming is undisputed. However, it is not yet possible to estimate the actual contribution of environmental conditions on such disruption. On the other hand, this paradigm could contribute to the explanation of rising trends of chronic disease in recent years. Techniques are available to measure the contribution of environmental factors to disruption of epigenetic programming, and in literature, several methods have been proposed to fill that gap. RIVM proposes further development of such methods. Validated test methods could be used to improve estimation of the risk of environmental exposures during pregnancy, and thus contribute to regulatory advice.

The importance of epigenetic programming was first suggested in children of mothers who experienced undernutrition during pregnancy at the time of the Dutch Hunger Winter. As middle aged adults, these children had higher rates of chronic diseases such as diabetes as compared to people of the same age who did not suffer the undernutrition. Another example is prenatal exposure to a repellent, which is associated with reduced vaccination efficiency at school age.

Key words: prenatal programming, epigenetics, food contaminants, toxicology, test methods

Contents

Summary

There is growing evidence that early life ‘environmental’ factors can contribute to future health and disease of the adult human being. Such environmental factors include prenatal maternal diet, food contaminants and maternal stress. They act through modulation of the epigenetic make-up of the organism, which determines cell and body functions. The epigenome can thus be considered as a filtering layer, determining the expression of the genome, or as a set of switches through which genes are turned on and off. This epigenetic make-up is largely defined during early life stages, and therefore early life factors can contribute to prenatal programming of an increased sensitivity to develop chronic disease.
The evidence for the existence of epigenetic programming comes from epidemiological observations, which started with the association of fetal undernutrition during the Dutch hunger winter and increased prevalence of various chronic diseases in adult life. The evidence is further built by observations from animal and in vitro experiments, showing effects of toxicant exposure on the epigenetic profile.
While this should be reason for concern, which indeed has been expressed by regulatory authorities, mainly at scientific conferences, prenatal programming is at present not considered as an end point for regulation. An important reason is that epigenetic endpoints are not covered by current toxicological test screens. This in turn is due to uncertainties in understanding of the impact of epigenetic disruption on cellular and systemic functions, and thus of the contribution of epigenetic disruption to development of chronic disease. Since this gap in toxicological testing has been acknowledged, conditions for valid and informative testing have been defined, leading to proposal of applicable testing models and strategies.
In this report, we explore the rationale and possibilities for detection of risk through epigenetic programming by early contaminant exposure, and propose further research to explore the practical possibilities to describe such risk in qualitative and quantitative terms.

1 Introduction

There is growing evidence that environmental conditions (including lifestyle) can contribute to an increased risk to develop non-communicable (chronic) diseases. These environmental conditions include (maternal) diet, dietary contaminants, stress, etc., and they do not only act during adult life (direct association) but also early in (fetal) life (indirect association). The consequences of this 'environmentally' induced or facilitated sensitivity may indeed be serious, in view of the rising trends of incidences of various chronic diseases – worldwide and in the Netherlands. Some important examples are overweight/obesity, diabetes, cardiovascular diseases, some allergies, and cancer. In the Netherlands, overweight showed an increase of 50% during the past 30 years, and in 2010 50% (males) and 40% (females) of adults over 30 years were overweight. Particularly alarming is the rising trend of overweight in young people, of which around 13% showed overweight in 2010.
Diabetes, both type 1 and 2, also shows an increasing trend, particularly over the past 15-20 years. Presently, around 1 million people in the Netherlands are diagnosed with diabetes, and this number is expected to increase up to 1.3 million people in 2025. Also, the age at which diabetes is diagnosed is decreasing, and obstetricians observe a higher prevalence of gestational diabetes.
Asthma is an example of an allergic disease which showed a high increase, particularly between 1984-1997 (levelling off afterwards).
The incidence of all cancers is slowly increasing in young children (particularly leukemia and brain cancer), and the risk in young adult men shows a strong increase, particularly due to more testicular cancer, leukemia, lymphoma, and melanoma. In adults, there is a strong increase in breast cancer.
Autism spectrum disorders (ASD) show a high cumulative incidence, with approximately 1% of all children affected in USA and the Netherlands. ASD has long been considered as a genetically determined disease, but new data, e.g. from twin studies, suggest at least a contribution of prenatal factors (Van der Ven et al. 2012, Beaudet et al, 2013).
Many risk factors for these diseases are known, such as a sedentary lifestyle and a high calorie diet associated with overweight, or a high age of first pregnancy and abstinence of breast feeding associated with breast cancer. However, the contribution of environmental conditions is, until now, insufficiently established. Particularly the action of environmental conditions at early life stages may be of specific interest because both epidemiological and experimental data suggest that modifications during developmental life stages may underlie disease at later ages.
Because of such alerts on this so-called prenatal programming, which were expressed in literature and at scientific conferences, NVWA requested to give an outline of the issue, and further substantiate the impact of prenatal programming, in view of possible regulatory actions (commission 2012-9.4.26).

In this report, the plausibility of the newly recognized risk of prenatal programming of adult disease will be explored, particularly focusing on early life exposures. The extent of the problem will be evaluated, and the final aim is to address the need to characterize the contribution of prenatal environmental (dietary) factors to an increased sensitivity to develop disease later in life.

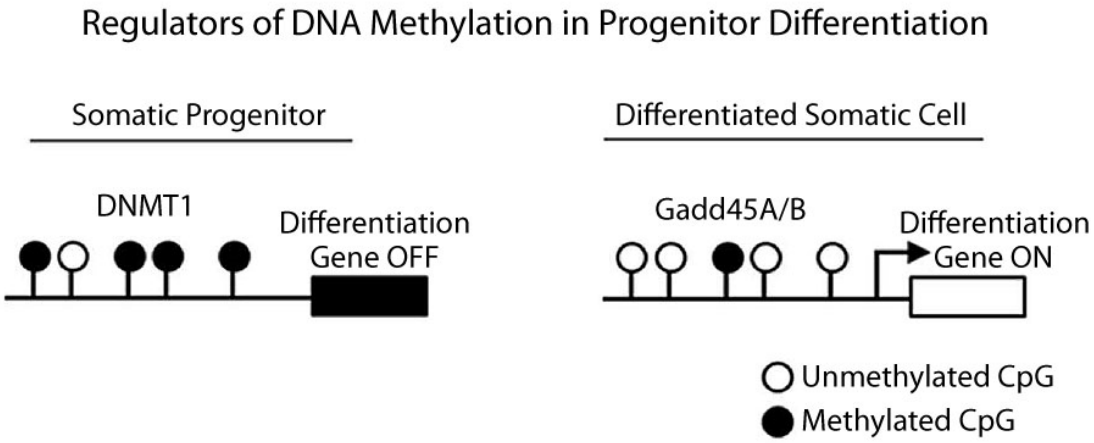

Figure 1: Model for the role of regulators of DNA methylation, as an example in somatic progenitor differentiation. In the undifferentiated progenitor state (left), DNA methylation at differentiation gene regulatory elements suppresses their expression (switch is OFF). This repression is enforced by the DNA methyltransferase DNMT1. In the differentiated state (right), DNA is demethylated (action of Gadd45A/B), the promoter de-repressed and subsequently, differentiation gene transcription is induced (switch is ON). Epigenetic switches of gene expression act similarly across the genome to regulate various cell functions. After: Khavari, 2010.

2 The concept of prenatal programming

Functioning of a biological organism is determined by 1) its genetic make-up and 2) canalization of the genetic potential through epigenetic mechanisms. The latter result in changes in gene expression or cellular phenotype by mechanisms other than changes in the underlying DNA sequence. Examples of epigenetic changes are DNA methylation and histone modification, both of which serve to regulate gene expression without altering the underlying DNA sequence. Epigenetic mechanisms can be thought of as switches that turn genes on and off (Figure 1). The entirety of switches then forms a virtual layer around the genome that defines its actual functionality. This layer is partly *static* (switches in a fixed state) and inheritable over subsequent generations of cells or even between parents and offspring, and partly *plastic* (switches conditionally on or off), allowing for adaptations to actual needs. Thus, static epigenome defines the differentiated phenotype of specialized cells, and thus the phenotype of the organism, whereas plastic epigenetic modifications contribute to instantaneous cellular responses to actual environmental events (e.g. DNA methylation changes in muscle after exercise (Barrès et al., 2012)). Conclusive evidence supporting epigenetics shows that these mechanisms can enable the effects of parents' experiences to be passed down to subsequent generations.
DNA methylation and histone modifications at least in part interact to regulate the accessibility and expression of genes. A third regulating layer is formed by micro-RNAs (miRNAs), which are small non-coding RNAs, mainly interfering with RNA stability and translation.

The static epigenetic make-up is developed early in life, when differentiating and developing cells, tissues and organs settle for an optimal adaptation of the organism to its future life in its particular environment. In this way, the genomic potential of the organism is fine-tuned, and this phenomenon is known as early life programming.
Early life programming should be considered a highly regulated process, intrinsically allowing for adaptations to environmental influences, which should be understood as either the internal environment of the fetus or the external

Table 1: Examples of adverse early life programming in humans.

cohort	**early life stressor**	**→ late effect**	**reference**
Dutch hunger winter	maternal/fetal malnutrition	cardiovascular disease, glucose intolerance, blood coagulation, high stress responsiveness, obesity, breast cancer, obstructive airway disease at middle age	Roseboom, 2006
Holocaust survivors	infancy stress	breast cancer, colon cancer at middle age	Keinan-Boker, 2009
Ecuador floriculture	maternal pesticide exposure	learning disabilities at school age	Harari, 2010
Faroese fish eaters	maternal PFOS exposure	decreased vaccination response at 5-7 y	Grandjean, 2012

(maternal) environment. As an example, the ambient temperature contributes to sex determination in cold-blooded animals through modulation of DNA methylation of estrogen regulating genes (Navarro-Martín, 2011). Responsiveness of the epigenetic processes in early life to environmental conditions implies that abnormal environmental conditions may produce misregulation of the organism's adaptive program and thus to adverse health effects. Such adverse health effects may be apparent immediately, or only after a latency, when the organism accumulates other adverse hits on top of its hampered program. This concept is known as prenatal programming of health and disease, or developmental origins of health and disease (DOHaD).

The concept of DOHaD emerged only in the most recent years. One of the earliest observations in the field was in the Dutch Hunger winter cohort, where prenatal suffering of undernutrition appeared to be associated with a higher prevalence of a wide scope of chronic diseases in adult life (Table 1). In another cohort from World War II, Jewish children that survived the Holocaust, it was observed that the stress in early life imposed by life in the concentration camps was associated with a higher prevalence of cancer at middle age, and moreover, that the age window in which the stress was experienced determined development of specific types of cancer. Two more recent epidemiological observations are the higher incidence of learning deficits in children born from floriculture workers in Ecuador who were exposed to pesticides during pregnancy, and the decreased vaccination response in children on the Faroese Islands that were prenatally exposed to PFOS.
The relative novelty of the subject and the growing interest in DOHaD can be derived from the publication boost in recent years (Figure 2; search terms explained in Appendix Table A.1). It stands without doubt that early life programming is a valid and realistic phenomenon, and the concept is underpinned through mechanistic information.

However, the available information is fragmented, mainly addresses cases or is focused on limited aspects of DOHaD. There is no systematic appraisal of the importance of environmental influences on early life programming, be it dietary,

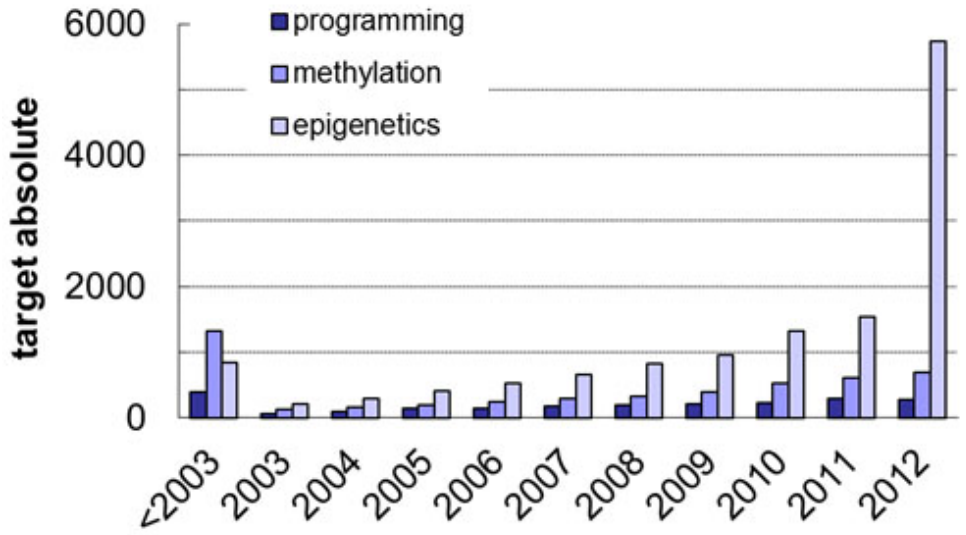

Figure 2: Number of publications in Pubmed.

chemical exposures, stress etc. There is also no systematic evaluation of the importance or relevance of the contribution of early programming to development of disease, and thus not of the risk associated with epigenetic modulation. Consequently, *no adequate assessment has been performed of the importance or relevance to include prenatal programming as an endpoint in toxicological testing strategies.*

3 Early life programming in literature

Programming is the (phenotypic) outcome of epigenetic events. The epigenome in turn is defined by a number of molecular mechanisms that regulate gene expression, which in the context of this report are limited to mechanisms that regulate the access of transcription factors to promoter regions of the genes, and particularly methylation of DNA, which is by far the most investigated mechanism. The most meaningful distinction in Pubmed searches is between a) programming related terms, which explicitly consider the relation between early life causes and effects that appear with a latency, and b) methylation and epigenetics related terms, which mostly consider mechanistic events. With that in mind, searches can be directed that distinguish the role of epigenetics/programming in health or in disease, related to 1) types of diseases, 2) causes of disrupted programming, and 3) research models (see Appendix 1: Pubmed analysis).
The conclusions are that:

- Early life programming is most frequently considered in the context of disease rather than of health.
- Major health subjects related to programming are immune, musculo-skeletal, neuromotor and learning functions.
- Major disease subjects related to programming are in the domain of metabolic disease (including obesity, diabetes, hypertension and vascular disease), cancer, neuropsychiatric dysfunctionality, asthma, allergy and osteoporosis.
- Dietary/nutritional factors and endocrine disruptors are mostly considered as determining factors for early life programming, followed by early life exposure to pharmaceuticals, maternal stress, smoking, alcohol and metabolic disorders.
- Most information comes from human studies (epidemiology), followed by experimental *in vivo* and *in vitro* models.
- There is no systematic appraisal of the importance or relevance of the phenomenon of early life programming for future life health and disease, and models to describe this importance or relevance are at the hypothetical stage (see below).
- Early life programming is not implemented in toxicological testing, nor is there systematic evaluation of the necessity for toxicological testing in this area (see chapter 7).

It is also obvious that there are important knowledge gaps (see e.g. Priestley et al, 2012), which need to be bridged in order to assess the importance of DOHaD in terms of causes, risks of exposures, and public health. A first and general knowledge gap is the incomplete understanding of the epigenetic processes, particularly the incomplete understanding of the consequences of modulation of epigenetic systems (both adaptive and adverse, see chapter 4), relating to normal variability.

4 Impact of epigenetic modifications

The static epigenetic make-up of a cell defines the boundaries of its functionality (a liver cell will only have hepatic functions and never act as a muscle cell). This static make-up is laid down early in the differentiation process (Figure 3: open black arrow) and is heritable in a clonal way, that is, daughter cells of the liver cell will have the same static epigenetic make-up. However, a second epigenetic layer is more dynamic and enables the cell to respond to environmental signals in an adaptive way (black waved line). Thus, a muscle cell receives its muscle program during early embryogenesis, but physical exercise later in life produces epigenetic fine-tuning enabling the cell to contract more powerful. These differences explain why the time window of disruption of programming is crucial for the severity of consecutive effects.

In chapter 2, it was introduced that the epigenetic program can be disrupted by environmental factors such as nutritional deficits or chemical contaminants, with possible adverse consequences. Now it can be understood that depending on the

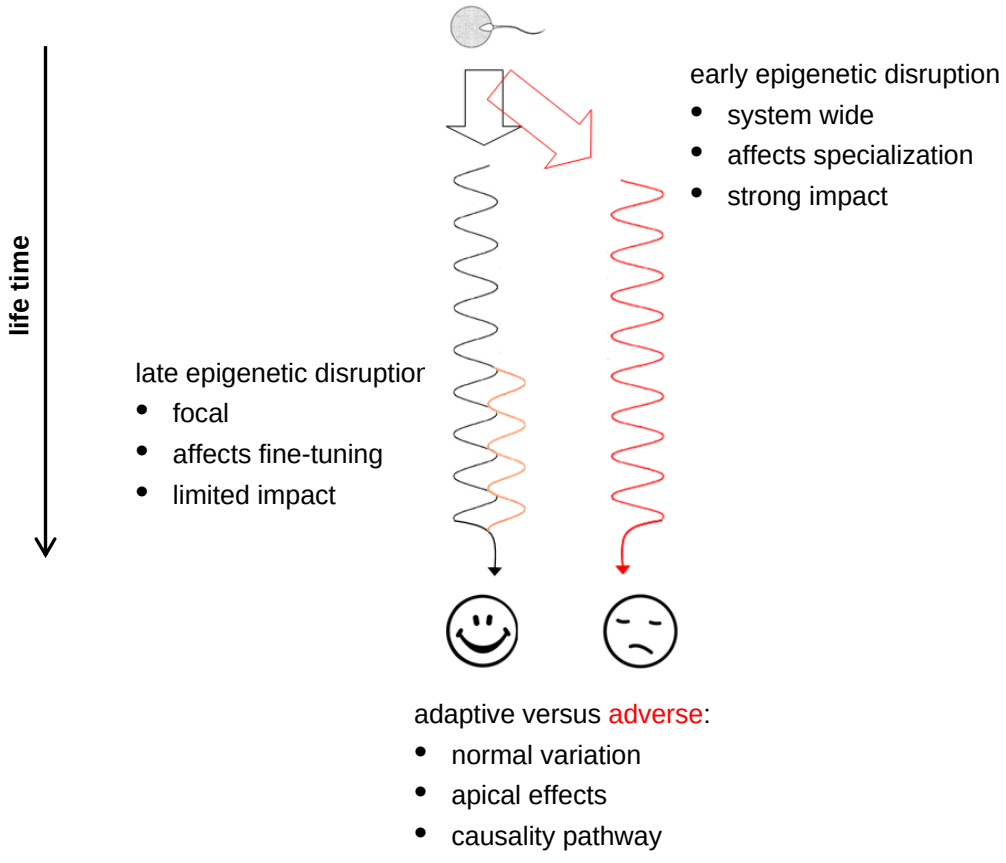

Figure 3: Life stage dependent impact of epigenetic programming.

window of exposure, the same factor can either disrupt the static epigenetic profile when acting at an early life stage, or the dynamic epigenetic profile when acting later in life. Late epigenetic disruption (orange variation of the black wavy line) is more likely to act on a specific set of receptive cells (focal) instead of the whole organism and only affect the fine-tuning of those cells. The impact on the function of those cells and on the body as a whole will therefore be limited. On the other hand, early epigenetic disruption is more likely to affect the static epigenetic program and hit early, undifferentiated cells, or cells that are at the basis of a clonal expansion (open red arrow). Early epigenetic hits may therefore have more systemic effects and affect specialized functions of cells. Further dynamic epigenetic regulation will then be beyond the normal variation (red wavy line). The impact of epigenetic disruption early in life will therefore be more profound than disruption later in life, and indeed more likely lead to adverse health effects. This implies that exposure early in life to certain food components, food contaminants, or chemical compounds that are otherwise ingested, might have more profound adverse health effects than we have assumed until now.

5 Distribution of epigenetic modifications

A further importance of the window of induction is that theoretically, epigenetic modifications induced early in life are distributed over, and should be detectable in a wide variety of cell types (Figure 4).
Consequently, when monitoring epigenetic changes, effects in a random, accessible cell type should be a proxy for effects in non-accessible cell types. There is, however, no systematic evidence to support this assumption, although it implicitly underlies the analysis of peripheral white blood cells, buccal cells or serum DNA in epidemiological research.

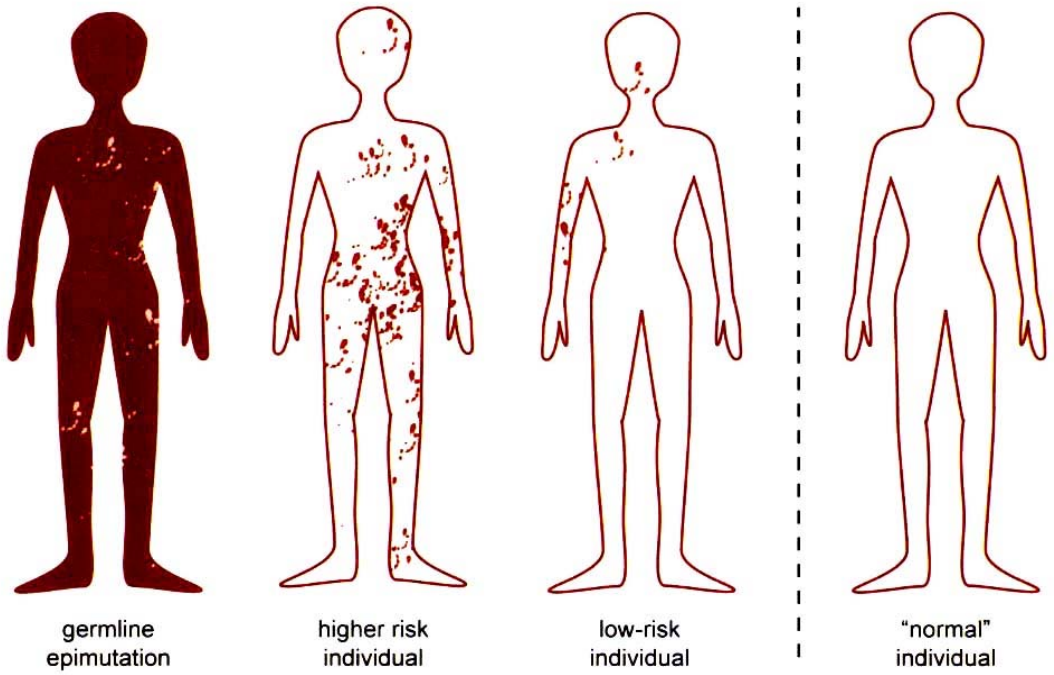

Figure 4: The 'somatic burden' hypothesis predicts that the proportion of cells affected by an epimutation determines disease risk. Every gene in every individual may lie on a spectrum of epigenetic mosaicism created by stochastic errors and environmental exposures. Some individuals carry a germline epimutation that affects all of their cells, or most cell if some cells have reverted to normal – their risk of disease is high. Most individuals carry some level of mosaicism for epigmutation, whereby only a proportion of cells is affected. Epimutation-free individuals may not be normal at all, but rather extremely rare: we may all be walking mosaics for epimutation at any number of genes. After Martin et al, 2011.

6 The process of epigenetic disruption

In principle, epigenetic disruption can result from directed, targeted processes or from more generalized loss of control of epigenetic profile definition (Figure 5). In the scenario that epigenetic disruption is considered as a targeted process, the sequence of events involves activation of specific molecular pathways, leading to regulation of specific enzymes that modify specific epigenetic marks and subsequent downstream regulation of expression of specific genes. These events involve specialized cells in which the targeted molecular pathways are active. In the end, the dysregulated gene expression leads to specific homeostatic alterations with development of a specific outcome (chronic disease). Several lines of research explore this route of epigenetic disruption. As such, it underlies for instance the hypothesis that exposure to endocrine disrupting chemicals early in life can lead to obesity later in life (EU-FP7 project OBELIX). Vice versa, it also relates to the observed epigenetic regulation of expression of the estrogen receptor in health (e.g. sex dependent development of the brain) and disease (e.g. breast cancer). Epigenetic regulation of the estrogen receptor may provide an illustrative case for reviewing directed, targeted epigenetic disruption.

In the alternative scenario, epigenetic disruption as a more generalized, stochastic process, molecular pathways may be less clearly defined, targets more global (not a specific genetic locus or a specific process, not in a specific cell type), and the outcome in terms of health and disease more random. This route funnels down to global effects, such as genome wide modulation of methylation, which is implicated in a multitude of disease states and is also associated with certain exposures (e.g. tobacco smoke, alcohol). Genome wide methylation may provide an illustrative case for reviewing stochastic, non-targeted epigenetic disruption.

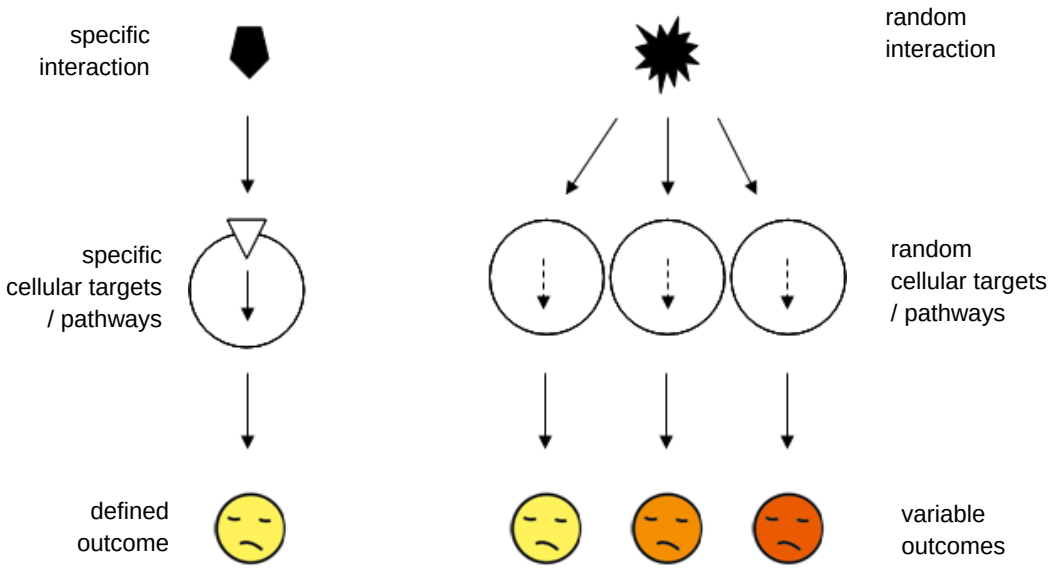

Figure 5: Targeted versus random epigenetic disruption.

7 Towards testing for epigenetic toxicity hazard

Clearly, it is currently worldwide being recognized that epigenetic programming by environmental factors might have a deleterious effect on human health, and that such effects should be considered in the regulatory perspective. Therefore, many models and techniques are now being explored for their potential to screen for epigenetic toxicity (see Appendix 1: Pubmed analysis), and their applicability was subject of workshops by ILSI-HESI and the US National Academy of Sciences. As summarized by Priestley et al. (2012), a number of issues relating to epigenetic testing were identified by these workshops. These included:

- incomplete understanding of the epigenetic processes with key data and knowledge gaps;
- differences between cells, tissues, organs, individuals, strains and species, regarding DNA methylation, histone modifications, and miRNA (essential to normal biological processes in differentiation and development);
- little understanding of the consequences of modulation of epigenetic systems (both adaptive and adverse), relating to the normal variability; and hence uncertainty of distinguishing false and true positives/negatives;
- a lack of definition of adversity in terms of dose response (relevant effect dose) and reversibility;
- a lack of definition of endpoints or mechanisms to be targeted in screens of epigenetic processes;
- a lack of translation: what is the relevance of testing models for predicting risk in humans? and for long-term effects to public health? what follow-up testing strategies would be employed to address these uncertainties?
- insufficient validation of epigenetic tests for inclusion into the regulatory process, specifically, no single test is ideal for all epigenetic effects.

Similar considerations led Rasoulpour et al. (2011) to define conditions for a viable epigenetic screening assay, including:

- It has to be medium- or high-throughput.
- It should have potential to capture, and be able to distinguish, all components of the epigenome (i.e. DNA methylation, histone modifications, and miRNAs). Clearly, these factors can work complementary or in opposition toward regulation of gene expression; therefore, understanding one piece of the epigenetic triangle is limited at best.
- It should be quantitative in order to differentiate between adaptive and adverse changes, in other words, distinguish between normal variation of the epigenetic profile and variations beyond normal.
- It should enable data interpretation in the context of dose response and no-observed-epigenetic-effect levels/thresholds.
- It should correlate with disease states (relate modulations of the epigenetic profile to apical effects) and be relevant to humans.

With this in mind, the authors proposed some major models to be assayed for their screening potential for epigenetic modifications and programming of health and disease (Table 2), in view of implementation in a comprehensive testing strategy.

The same uncertainties were expressed at a recent workshop of the European Centre for Ecotoxicology and Toxicology of Chemicals (Ecetoc). In his summary

of that workshop, one of the key scientists Prof. Boobis stated: “There are differing views on how often interference with epigenetic mechanisms underlies chemical toxicity. Similarly, it is not clear how often chemically-induced changes in epigenetic processes translate into an adverse effect… Understanding epigenetic mechanisms may help improve risk assessment and reduce uncertainty in areas such as interspecies extrapolation, life stage sensitivity, human relevance and inter-individual variability. In the absence of such knowledge uncertainty may be greater but can still be addressed, by using more conservative assumptions, as at present… there needs to be an investigative strategy to confirm the relevance of any epigenetic changes observed in the toxicity of the compound. As knowledge increases, such *epigenetic changes will eventually become more predictive.*” (ECETOC Workshop Report 23, 2012; cited with permission).

Table 2: Overview of selected approaches for epigenetic toxicity hazard identification screening (adapted from: Epigenetic screening in product safety assessment: are we there yet? Rasoulpour RJ et al., 2011).

Test models	**Throughput**	**Strengths**	**Weaknesses**
Regular mouse models	Medium (quantitative); low (qualitative)	• assesses critical period of epigenetic programming (i.e. development); • assessment of development, apical end points, transgenerational.	• significant animal use for a screening tool; • qualitative end point subject to observer bias and drift; • quantitative bisulfite sequencing (low throughput) needed for confirmation.
Axin1^{Fu}, *A^{vy}* IAP mouse models	Medium (quantitative); low (qualitative)	• assesses critical period of epigenetic programming (i.e. development); • easily read phenotype.	
Zebrafish (worms, honey-bees, …)	High	• amenable to diverse array of molecular techniques; • assessment of development, apical end points, transgenerational.	• human relevance of non-mammalian models?
Embryonic stem cells [1]	Medium	• in vitro; multiple rodent and human lines available; • assessment of epigenetic regulated differentiation.	• heterogeneity of stem cell cultures; • reliance on feeder layers.
In silico modelling	Very high	• no animal use; • very high throughput.	• no adequate models currently exist; • comprehensive tools are decades away from reality.

[1] Note: we are currently exploring the feasibility of umbilical cord derived mesenchymal stem cells as a human based in vitro test model. A major advantage of this model is that for exploration purposes, a large supply of umbilical cords is available, providing the opportunity to analyse epigenetic consequences of maternal conditions (smoking, diabetes, diet….) in child derived material.

8 Implications for regulatory policy

In regulatory policy, prenatal programming is currently not considered as a mechanism that can contribute to adverse health effects. As a consequence, no mandatory tests are performed for (new) chemical compounds and pharmaceuticals. This is due to the fact that prenatal programming is a relatively newly recognized element that may be relevant for public health, and, therefore, there are no regulatory test paradigms yet to screen for prenatal programming of adverse effects later in life. However, there is a growing suspicion that prenatal programming might be a relevant factor, contributing to the rising trends of many chronic diseases, and as a consequence, should be integrated as an endpoint in mandatory toxicity testing. Key element is that exposure at a sensitive and vulnerable life stage (i.e. in the unborn child) to dietary factors, food contaminants or lifestyle factors, can cause a perturbation of the normal epigenetic programming, leading to increased sensitivity to develop disease later in life. Many of these causative factors are potentially subject to regulation.

In the past few years, the Society of Toxicology (SOT) organized three conferences on the subject under the title Prenatal Programming and Toxicity (PPTOX). At the most recent PPTOX-3 meeting (Paris, May 2012), the French food agency ANSES announced that it defined fifty key priority compounds in relation to programming, i.e. where investigation is imperative in relation to non-communicable disease, and in sensitive, vulnerable populations. Generally, the opinions expressed at this meeting were progressive regarding the need for action. The consensus paper drafted by the organizers regarding the current scientific insight in this field and the implications for future research and public health, concluded that the developmental paradigm has reached the stage where the data, while not complete, are sufficiently robust and replicable across species, including humans, to require a policy and public health response. The current pandemic of non-communicable diseases and the increased prevalence of important dysfunctions demand an open interrogation of why current interventions appear insufficient. We now know that disease risk can be induced very early in the life course and that it is modifiable by nutrients and environmental chemical exposures (along with drugs, infections, and other types of stresses).

A new approach towards disease prevention is needed, with a new emphasis on early development. A rational methodology is to improve nutrition and reduce environmental chemical exposures pre-pregnancy, during pregnancy and during the first few years of life. This change is likely to have a very large impact on reducing disease incidence and the cost of health care, while at the same time increasing the quality of life at a global level (Barouki et al, 2012).

The available evidence for the contribution of environmental factors to prenatal programming for health and disease appears to be weighed differently among scientists and by regulatory authorities across the world. Complex, multi-factorial issues may be reduced to simple cause-and-effect cases in some opinions, whereas the lack of full and consistent reproducibility, or the lack of strong conventional toxicological effects are a reason to withhold certain substances from any connotation as programming factors in other opinions. In that respect, the tendency in the US and Canada to attribute the obesity epidemic to exposure to so-called obesogens early in life is an example of

possible oversimplification of a complex problem. On the other hand, the highly critical attitude in the EU towards possible programming effects of a substance with weak endocrine activity such as bisphenol-A may be an example of high prudence for labelling. Both sides may be a reflection of the non-linear nature of epigenetic programming, i.e. exposure to a programming factor may lead to stochastic multi-site epigenetic modifications, or, multiple switches may be turned in random combinations, leading to a complex phenotypic translation. Presently available testing procedures are not tuned to reveal such effects in a consistent and unambiguous way. This underlines the necessity for new strategies to delineate the particular, and potentially important risk of prenatal programming.

9 Potential study strategy and study cases

The problem of prenatal programming is probably rather complex, not a matter of addressing a single food contaminant or a single dietary factor. Theme at the PPTOX-3 meeting was that dietary factors and chemical contaminants interact in the developing child and should be considered in concert. Still, through analysis of sub-questions, more insight can be gained into the matter whether prenatal programming should be considered in regulatory policy, i.e. be included in hazard screening strategies.
Based on our review of the current literature and publicly available information, it can be concluded that while there is wide consensus on the paradigm that insults early in life can program humans for development of chronic disease later in life, some uncertainties remain to be resolved before epigenetic testing can be implemented in regulatory risk assessment. One important uncertainty is *to what extent* external ('environmental') factors early in life contribute to risk of disease in future life. To address that issue, and consequently the need to include epigenetic programming in hazard screening strategies, we propose the following sub-questions:

- What new or adapted existing test models can be used to screen for disruption of normal programming by chemicals or food components?
- Can we, using these test models, provide experimental proof-of-principle that prenatal exposure contributes to increased risk of chronic disease in later life via epigenetic programming?
- Can we, using these models, estimate to what extent external ('environmental') factors early in life contribute to risk of disease in future life through epigenetic mechanisms?

To this aim, we propose to elaborate on one or two selected cases for which the following background information is available:

- established or suspected association between causative factor and (adverse) health effect in humans (epidemiology) and/or animals (experimental toxicology);
- established or suspected link between causative factor and epigenetic modifications (i.e. DNA methylation or histone modifications)

The analysis of literature given in Appendix 1 directs to important environmental factors that can contribute to early life programming (causes; Appendix 1, Figure A.4A, B). These include diet/nutrition, endocrine disruptors (as food contaminants), pharmaceuticals, maternal stress, smoking and alcohol intake, maternal metabolic disorders, and gut conditions (microbiome). The most clear combination of such causative factors with health or disease outcomes (given in Appendix 1, Figure A.3A,B), i.e. disorders related to the metabolic syndrome (obesity, diabetes, other metabolic, hypertension, vascular diseases), cancer, neurodegenerative and psychiatric conditions, asthma and allergy, and osteoporosis, should lead to the most informative cases to explore in a follow-up project.

Practically, several strategies can be applied to build a proof of principle:

1. Start with causative factors with known relevance to prenatal programming and apply these in the defined test models, to quantify their epigenetic modulating effects. Then validate in epidemiology.
   Examples of such known causative factors are: prenatal exposure to tobacco smoke and to folic acid (FA). Cigarette smoke contains several epimutagens

(compounds that can stably affect the epigenetic profile), such as reactive aldehydes and other cigarette smoke derived oxidants. Prenatal exposure to cigarette smoke has adverse health effects with a possible epigenetic contribution, such as low birth weight and respiratory disease. FA is a micronutrient, and maternal FA supplementation before and during pregnancy is recommended to protect against neural tube defects. However, epidemiological studies have shown associations between high maternal folate blood levels and immunological disease (atopic dermatitis, eczema, asthma, respiratory infections) and insulin resistance in children. Experimental support for prenatal programming of both cigarette smoke and FA comes from animal and in vitro studies (Burdge et al. 2012, Ly et al. 2012, McKay et al. 2011). FA or other dietary methyl donors can be considered as a background variable in studies with other programming factors.

2. Start with adverse health outcome for which a contribution of prenatal environmental factors is suspected. Analyse suspected contributing prenatal factors, and test these in the selected models, and parallel in epidemiology. Prenatal programming of adult obesity, diabetes or related metabolic diseases, either by specific maternal diets or dietary contaminants, may represent a case in this approach. Literature data and studies at RIVM have revealed that programming of body weight changes and metabolic disease can be modelled in experimental rodent models. However, the relative contribution of epigenetic mechanisms to the development of disease in human studies remains uncertain until now.

3. Start with a set of priority contaminants in food. Test these in selected models and compare their relative potential to modify the epigenetic profile. Identify and validate the most active substance and verify in epidemiology. As stated above, the French agency ANSES announced at the PPTOX-3 2012 conference that it defined fifty key priority compounds in relation to programming, i.e. where investigation in relation to non-communicable disease, and in sensitive, vulnerable populations is imperative. The EFSA website (http://www.efsa.europa.eu/en/contam/contamtopics.htm) also lists a number of priority food contaminants, and similar lists are available in literature (e.g. Kantiani 2010). From these lists, a proposed test set of contaminants should include **acrylamide**, **furan, 3-monochloropropane-1,2 diol esters** (3-MCPD), brominated flame retardants (particularly **polybrominated diphenyl ethers**), **dioxins**, **furans** (other than furan), **polychlorinated biphenyls** (PCBs), **metals** (arsenic, cadmium, lead, mercury, tin), **perfluorinated compounds**, and **antibacterials** and other **veterinary drug residues.** The epidemiological verification in this approach can be achieved in birth cohorts where fetal contaminant exposure is analysed. Such cohorts are available in the EU-OBELIX project or from collaborating parties, e.g. in the Farese cohort.

Further exploration of such cases may particularly address the higher sensitivity of the early (fetal) life stages compared to adults for epigenetic programming effects.

Interestingly, the effects of known prenatal exposures on the epigenetic profile in the child can be directly studied in umbilical cords, independently of the selected approach.

10 Conclusions

There is growing evidence that early life environmental factors can contribute to future health and disease of the adult human individual. Such environmental factors include prenatal maternal diet, food contaminants and maternal stress. In this way, early life factors can contribute to prenatal programming of an increased sensitivity to develop non-communicable (chronic) diseases, and this may be of ultimate societal relevance in view of the present rise and pandemic of such diseases, particularly obesity and related metabolic disease, cancer, and autism.

However, at present, prenatal programming is not considered as an end point for regulation, partly because it is not covered by current toxicological test screens. This in turn is due to uncertainties in understanding of the impact of epigenetic disruption on cellular and systemic functions, of the distribution of epigenetic disruptive effects within the body, and of mechanism of epigenetic disruption. Epigenetic programming may not follow established toxicological paradigms, and requires consideration of complex, indirect mechanisms. However, building on what is known, existing screening and test models could be employed in dedicated cases to at least partially reveal and quantify epigenetic disruption by prenatal exposure factors. There are multiple strategies to achieve this goal, all building on specific conditions including:

- availability of background data;
- prospect of a human-based approach:
  - informative data available in collaborating epidemiological cohorts;
  - biomaterial from informative individuals available for epigenetic analysis;
  - *in vivo* exposed human mesenchymal stem cells accessible for *in vitro* challenge experiments;
- prospect of an experimental approach, building on models recommended in literature.

We therefore propose to employ combined epidemiological and experimental methods to explore dedicated cases in depth, with the aim to better estimate the relative risk for development of chronic disease later in life, associated with exposure to prenatal factors. This should answer the question how prenatal programming can be implemented as an endpoint in regulatory testing.

11 References

- Barrès R, Yan J, Egan B, Treebak JT, Rasmussen M, Fritz T, Caidahl K, Krook A, O'Gorman DJ, Zierath JR. Acute exercise remodels promoter methylation in human skeletal muscle. Cell Metab. 2012;15:405-411.
- Barouki R, Gluckman PD, Grandjean P, Hanson M, Heindel JJ. Developmental origins of non-communicable disease: Implications for research and public health. Environ Health. 2012;11:42.
- Beaudet AL. A Neuronal Carnitine Deficiency Hypothesis for Autism. Lecture at Keystone Symposium Nutrition, Epigenetics and Human Disease, Feb 2013, Santa Fe, NM USA.
- Burdge GC, Lillycrop KA. Folic acid supplementation in pregnancy: are there devils in the detail? Br J Nutr. 2012;108:1924-1930.
- Carmichael, N (Ed.). Epigenetics and Chemical Safety, 5-6 December 2011, Rome. European Centre For Ecotoxicology And Toxicology Of Chemicals (ECETOC), Workshop Report No. 23, Brussels, 2012, ISSN-2078-7200-23 (print), ISSN-2078-7219-23 (online).
- Grandjean P, Andersen EW, Budtz-Jørgensen E, Nielsen F, Mølbak K, Weihe P, Heilmann C. Serum vaccine antibody concentrations in children exposed to perfluorinated compounds. JAMA. 2012;307:391-397.
- Harari R, Julvez J, Murata K, Barr D, Bellinger DC, Debes F, Grandjean P. Neurobehavioral deficits and increased blood pressure in school-age children prenatally exposed to pesticides. Environ Health Perspect. 2010;118:890-896.
- Keinan-Boker L, Vin-Raviv N, Liphshitz I, Linn S, Barchana M. Cancer incidence in Israeli Jewish survivors of World War II. J Natl Cancer Inst. 2009;101:1489-1500.
- Kantiani L, Llorca M, Sanchís J, Farré M, Barceló D. Emerging food contaminants: a review. Anal Bioanal Chem. 2010;398:2413-2427.
- Khavari DA, Sen GL, Rinn JL. DNA methylation and epigenetic control of cellular differentiation. Cell Cycle. 2010;9:3880-3883.
- Ly A, Hoyt L, Crowell J, Kim YI. Folate and DNA methylation. Antioxid Redox Signal. 2012;17:302-326.
- Martin DI, Cropley JE, Suter CM. Epigenetics in disease: leader or follower? Epigenetics. 2011;6:843-848.
- McKay JA, Mathers JC. Diet induced epigenetic changes and their implications for health. Acta Physiol (Oxf). 2011;202:103-118.
- Navarro-Martín L, Viñas J, Ribas L, Díaz N, Gutiérrez A, Di Croce L, Piferrer F. DNA methylation of the gonadal aromatase (cyp19a) promoter is involved in temperature-dependent sex ratio shifts in the European sea bass. PLoS Genet. 2011;7:e1002447.
- Priestley CC, Anderton M, Doherty AT, Duffy P, Mellor HR, Powell H, Roberts R. Epigenetics – relevance to drug safety science. Toxicol. Res., 2012;1: 23-31.
- Rasoulpour RJ, LeBaron MJ, Ellis-Hutchings RG, Klapacz J, Gollapudi BB. Epigenetic screening in product safety assessment: are we there yet? Toxicol Mech Methods. 2011;21:298-311.
- Roseboom T, de Rooij S, Painter R. The Dutch famine and its long-term consequences for adult health. Early Hum Dev. 2006;82:485-491.
- Van der Ven E, Termorshuizen F, Laan W, Breetvelt EJ, van Os J, Selten JP. An incidence study of diagnosed autism-spectrum disorders among immigrants to the Netherlands. Acta Psychiatr Scand. 2012. [Epub ahead of print].

12 Appendix 1: Pubmed analysis

When targeting Pubmed searches at papers focusing on either programming, methylation, or epigenetics, it appears that there is some overlap, particularly between epigenetics on one hand and programming and methylation on the other hand, but these items appear to be considered largely separately (high ratio of unique hits, see Table A.1). The most meaningful separation, however, is between programming related terms, which explicitly consider the relation between early life causes and effects that appear with a latency, and on the other hand methylation and epigenetics related terms, which mostly consider mechanistic events.

Table A.1: Key search term characteristics.

Target term	**Search**	**Total hits**	**% unique hits**	**% overlap with other two terms**
programming	(programming AND environment AND (offspring OR DNA methylation OR epigene* OR prenatal OR postnatal OR maternal)) OR (programming AND early life) OR (programming[title] AND (perinatal[ti] OR prenatal[ti] OR epigen*[ti] OR develop*[ti] OR fetal[ti] OR offspring[ti])) OR (developmental origi*[Title])	2,054	81.0	1.2
methylation	DNA methylation[ti]	4,586	57.0	6.0
epigenetics	epigen*	31,198	92.5	35.4

Search was updated until end of 2012.

As implicated above, early life programming is involved in determination of appropriate functioning of the organism in its environment, and inappropriate programming leads to malfunctioning. Thus, programming can be approached from either the health or disease perspective. However, it appears that most publications in the area focus on disease rather than on health, which is true for both the programming search and the total programming/methylation/epigenetics search (Figure A.1A,B).

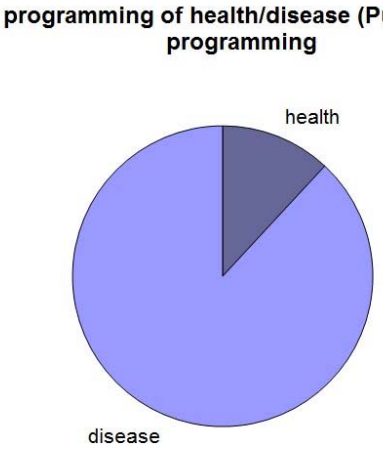

Figure A.1A

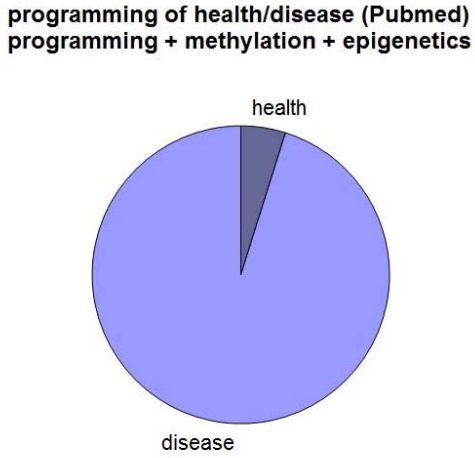

Figure A.1B

Health functions (small segment in Figure A.1) that occur most in literature are immune functioning, musculo-skeletal functioning, neuromotor and learning issues, puberty development, and overall fitness, in that order, and this distribution is similar in the programming search and the total programming/methylation/epigenetics search (Figure A.2A, B).

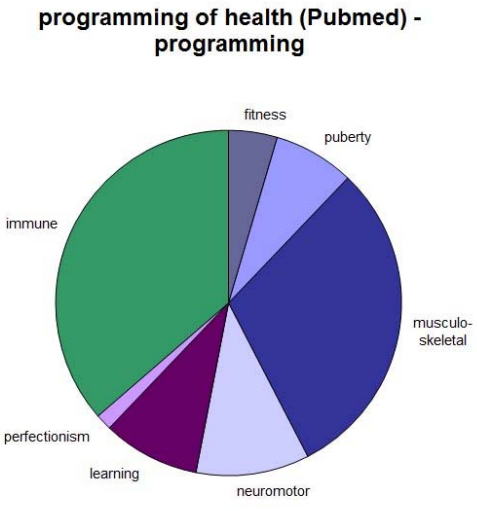

Figure A.2A

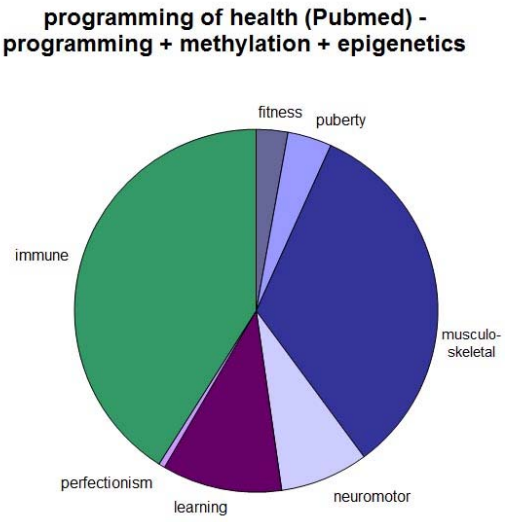

Figure A.2B

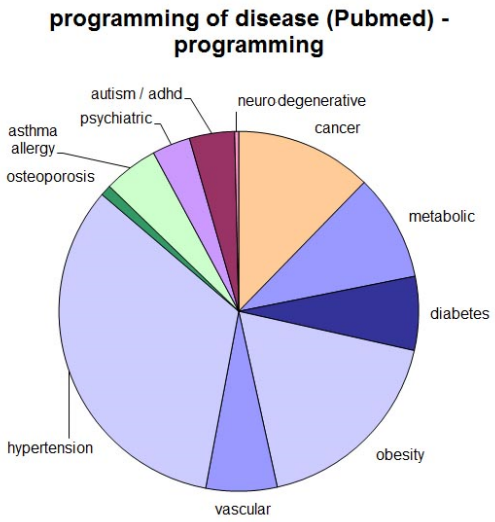

Figure A.3A

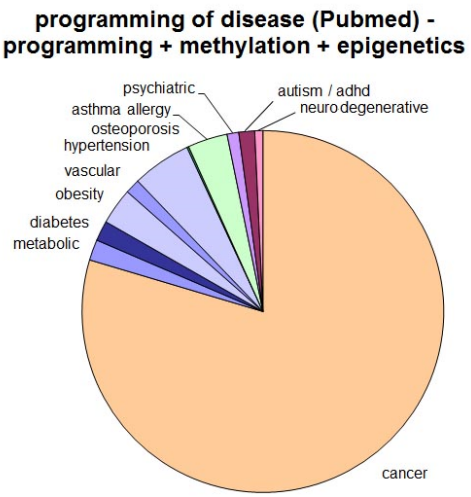

Figure A.3B

Regarding diseases (large portion in Figure A.1), disorders related to the metabolic syndrome (obesity, diabetes, other metabolic, hypertension, vascular diseases) are by far most frequently published in relation to programming (74%), with cancer, neurodegenerative and psychiatric conditions, asthma and allergy, and osteoporosis, making up the remainder (Figure A.3A). However, in the total programming/methylation/epigenetics search, cancer is the subject of 80% of the publications, with the other subjects making up the remaining 20% (Figure A.3B).

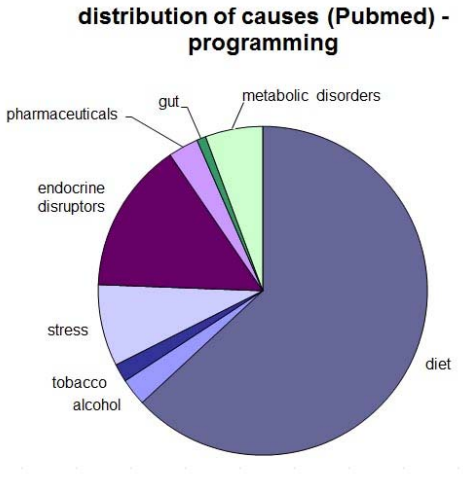

Figure A.4A

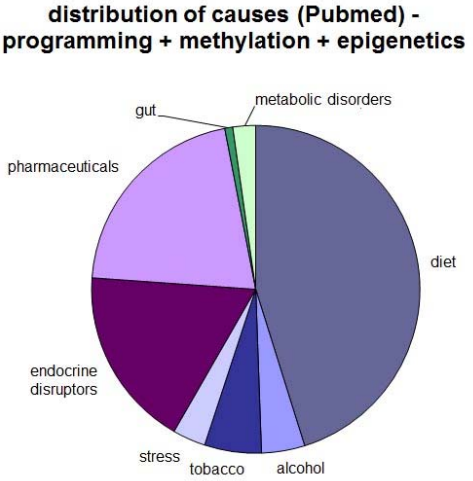

Figure A.4B

When environmental factors that contribute to early life programming (causes) are searched, it appears that diet/nutrition are mostly considered (Figure A.4A, B), with endocrine disruptors as the second most occurring subject (together with pharmaceuticals for the total programming/methylation/epigenetics search, Figure A.4B). Factors receiving less attention are maternal stress, smoking and alcohol intake, maternal metabolic disorders, and gut conditions (microbiome).

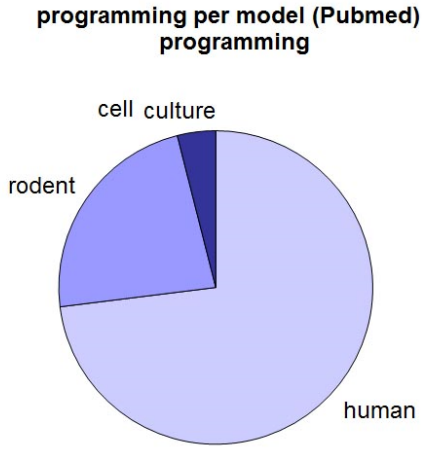

Figure A.5A

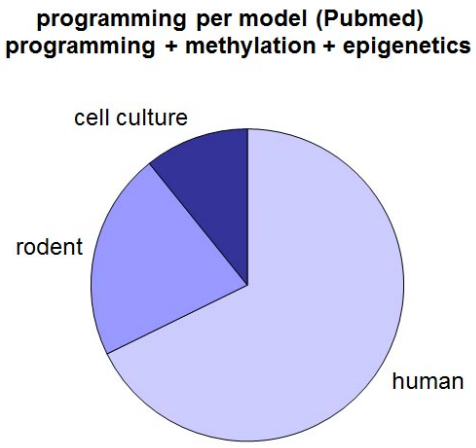

Figure A.5B

Finally, when relating the key search targets to models in which they have been investigated, it appears that most programming studies concern human subjects, followed by rodent models, then *in vitro* models(Figure 5A,B), with similar ratios for programming per se (73%, 23% and 4%, respectively) and the total programming/methylation/epigenetics search (63%, 23%, and 14%, respectively).

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