Examinations looking for late neuro-developmental effects in arctic children exposed to Poly Chlorinated Biphenyls (PCB's) and Mercury in early life show inconsistent results.

The epigenetic approach as new kid on the block.

Minor Arctic Studies 2016.
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ABSTRACT

PolyChlorinatedBiphenyls (PCB's) and methylmercury are very easily transmitted from mother to child during pregnancy and with mother's milk. Both pollutants are lipophilic and considered very harmful for the developing central nervous system during gestation and the first 6 months after birth. With examinations shortly after birth showing neurodevelopmental deficits, several longer lasting cohort studies were undertaken. Only one was performed in the arctic region of Nunavik, North Western Canada. Besides PCB's, mercury was measured in mothers' cord blood, at the age of 5 to 7 and at 11 years. All concentrations were compared to a large number of neurodevelopmental and neurophysiological examinations and tests performed at these later ages. Multivariate analyses occasionally showed a significant correlation between the level of PCB 153, being the most abundant, or mercury and some of the tests investigating a certain aspect of the neurodevelopment of the children. However, these results were not consistent at the different ages and for the particular test. It is concluded that a dose response relation in this relatively low grade intoxication is lacking and that the classical neurodevelopmental testing systems are not consistent enough to draw firm conclusions, despite persisting concerns about the detrimental effects of these pollutants. Recently performed epigenetic investigations found significant relations between abnormalities in the methylation of DNA in the placenta and outcome of the pregnancy. In addition, altered placental DNA methylation was associated with lethargy, levels of attention, quality of movement and arousal in the newborn. It is speculated that an epigenetic approach can be an alternative for intensive neurodevelopmental and neurophysiological investigations in mother-child cohorts. Hopefully this new research field can provide better insights in the complex relation between low-grade intoxications with PCB's or methylmercury and neurodevelopment of the children later in their life.

INTRODUCTION

Persistent Organic Pollutants (POP's) are identified since the start of industrialisation. After many toxic incidents we have learned that certain substances may be very harmful for mammals. Besides POP's, heavy metals as Mercury, Lead and Arsenic are proven to be toxic as well. All these substances are transported over long distances, spread in the marine food web and have shown to accumulate in tissues. Arctic mammals appeared to be of particular risk as it has been shown that they can contain very high concentrations of POP's and heavy metals which may persist in their bodies for a very long time [1].

In the 1980's Poly Chlorinated Biphenyls (PCB's) became of special interest because negative effects on neuromotor development were shown in very young babies of highly industrialized countries. Significant effects were seen despite only moderately elevated levels of PCB's in these babies' blood. These studies were confirmed in the mid 1990's [2]. Great concerns arose what might be the late effects on human health in general and neuromotor development in particular, because of the severe consequences for the rest of the lives of exposed individuals.

Heavy metals are known to be toxic in general and to the central nervous system in particular. Acute intoxications with high levels cause acute and severe neurological symptoms as muscle weakness, ataxia and visual plus hearing impairments. But even moderately elevated levels were shown to have impact on human health and development.

While toxicological knowledge and new insights in the neurodevelopment of babies and young children improved over the years, it became very clear that the developing brain of the fetus and newborn are vulnerable and very susceptible for PCB's and Mercury in particular. On the other hand, toxicological research in humans could prove only a distinct relation between a toxic substance and certain symptoms in acute intoxications with a high dose of a certain single substance. In chronic low-grade intoxications effects on the level of individuals

are difficult to demonstrate and very large, long lasting cohort studies are needed to show the final effects on populations.
In this paper the focus will be on the existing knowledge of late neurodevelopmental effects of PCB's and Mercury in children exposed to these substances early in life. For the readability a separate description of the impact of PCB's and mercury has been made.
Accumulating insights about the relation of environment and human health and how the environmental influences can lead to changes in the human genome is of recent date. The quick expanding availability of new genetic techniques and recent results of their application in toxicology look promising for a better understanding of the complex relation of pollutants and human health, and for neurodevelopment of children in particular.

POLY CHLORINATED BIPHENYLS (PCB's)

PCB's are environmental contaminants persisting for a long time due to their resistance to biological and chemical degradation. It is a class of chemical compounds in which a biphenyl molecule has a number of chloride atoms attached to it. This number can be highly variable from 1 to 10 and so a large number of different compounds are possible. More then 200 different compounds called congeners have been identified and categorized in different ways according tot their degree of chlorination and the positions of the chloride atoms in the molecule [3].

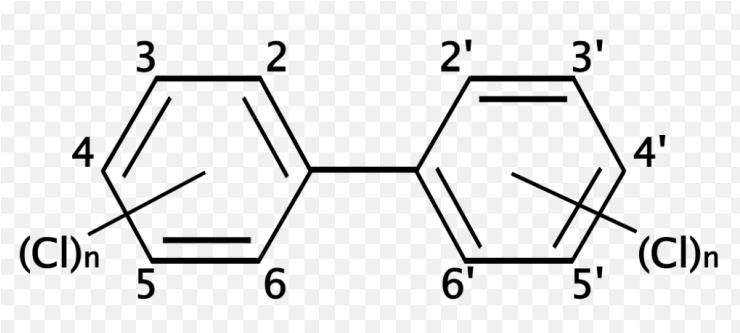

Fig 1. Basic molecule structure of PCB's

In their review Faroon et al. [4] explain the structure of PCB's and give a nice overview of concentrations and the neurobehavioral affects in animals, adults and mother child cohorts in different highly industrialized countries.
Animal studies, including primates, have shown that PCB's affect behaviour but also higher cognitive functions such as memory, learning and attention. Even the very low dose of 0.01 and 0.005 mg/kg per day of PCB 153 respectively PCB 128 during a 90 day test of this single congeners, gave decreased levels of one of the most important neurotransmitters dopamine. All areas of the brain were equally affected without preference.
From the Michigan Mother-Child study, the Oswego Newborn and development project, the NCBMFP, the Dutch Mother-Child study and a German study, plus related information from the Taiwan accidental poisoning, a number of abnormal observations were made. Shortly after birth hypoactive reflexes, more motor immaturity, less stable states and a greater amount of startles were recorded. At the age of 4 years prenatal exposures to PCB's could be linked to poorer performance on both the Verbal and the Memory scales of the Mc Carthy scales. At the age of 11 years children scored lower at full scale and verbal IQ. These results plus poorer reading word comprehension were linked to prenatal PCB exposure.
Adult fish-eaters from the Michigan area aged 50-90 years old however showed no visual-motor or fine motor abnormalities but it is likely that they ingested PCB's later in life.
Many studies showed equal levels of PCB's in maternal and cord blood. So mothers easily transmit these substances tot their offspring during the entire period of gestation.

With mother's milk the baby also ingests PCB's and the amount depends on the total amount stored in the mother's body, mainly the fat tissues, together with the actual intake the mother gets from her diet. At the age of six month Ayotte et al. [4] found that 72% of the babies plasma level of PBC 153 could be predicted from a multivariate model including maternal PCB-153 plasma concentration, duration of breast feeding and the sum of 2 skin-fold thickness measurements in the baby; the last being an index of the infants body fat mass. When the skin fold measurements not were included into the model only 36% of the babies plasma PCB level could be predicted.
Reasons why fetus and young children are more susceptible to pollution with both hydro- and lipophilic substances are the nearly absence and only gradually increasing function of the so-called blood-brain barrier. The brain is protected against all kinds of medications, toxic substances and invaders transported in an individual's blood due to specific functions in the inner wall (endothelium) of the vessels in the brain. But only at the age of approximately 4 months, the blood brain barrier is expected to function at the adult level [7]. Second, in the first months after birth massive myelinisation of the central nervous system and peripheral nerves occur. The growth of the head circumference with 1 cm per month during the first half year of live is the most important exponent of this process. As myelin contains a very high percentage of lipids, it is obvious why lipophilic pollutants can accumulate here.
Mother's milk also has proven to contain different but often high concentrations of PCB's especially lipophilic, than in their blood.
Therefore the gestational and lactation period with mother's milk are considered the most risky periods concerning transmission from mother to child of all kinds of unwished substances.

With some delay compared to the industrialized areas, POP's including PCB's and heavy metals appeared in the arctic, leading to a number of research programs funded by different countries in the artic area. The difficult methodology of toxicological research in humans together with funding and the different views of governments and experts about the best methods to investigate and monitor this problem, have lead to a wide variety of programs and approaches of this threat to the artic people.
In a number of subsequent reports, the last in 2015, the Arctic Monitoring and Assessment Programme (AMAP) summarizes all these human health related programs and gives an overview of the present knowledge on human health in the arctic [6].
Although recently some decrease in most PCB levels in blood of people living in the arctic have been reported, the AMAP Technical report from 2014 [5] points to an only slight decrease of all monitored PCB's in arctic air in combination of persisting high "production" of these substances in the industrialized world, with the highest impact on artic pollution coming from northern Europe and America. In figure 2 the graph for PCB 153 concentrations measured at different artic monitoring locations is shown.
The most likely explanation for the reported decrease in their blood levels of PCB's is that women in childbearing age have changed their dietary intake with less traditional foods after a gourvernmental advise. The contamination in all other live in the artic will continue and eventually can show up in the human food web.
In the Nunavik Child Development Study (NSDS) a prospective mother-child cohort is investigated. Inclusion of pregnant women was from 1994 until 2001. At birth, 6, 12 month of age and also at the age of 5 and 11 years blood was taken respectively from mothers, the umbilical cord and from the children at these moments. From this cohort a number of reports of the investigation of the neurodevelopment of the children are published. This is the only cohort known in the arctic, where this follow-up has been performed so stringent.

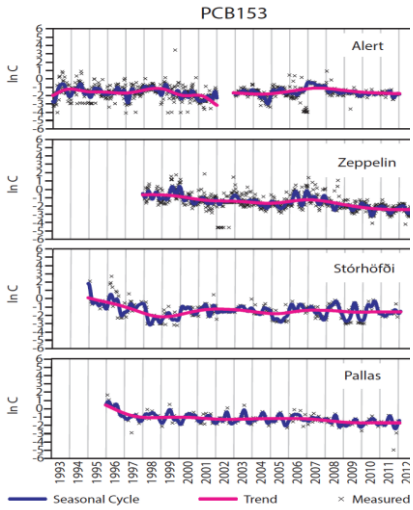

Figure 2. PCB 153 concentration in the air measured over the years at different arctic locations.

At 5 years of age, Verner et al. [8] showed that PCB 153 had increased from a mean concentration of 122 ng/g lipids (range 22-490) in cord blood to 155 ng/g lipids (range 10-1467) at the age of 5. Video recordings of children undergoing fine motor testing assessed inattention and activity. Authors included 98 children in this report and could not find significant relations between PCB 153 and inattention and activity at these ages.
In the paper of Saint-Amour at al. [9] a part of the NSCD cohort was investigated between 5 and 6 years using visual evoked potentials (VEP's) in comparison with a number of blood tests. In the model smoking, binge drinking and use of marijuana of the mother during pregnancy was included. In this group cord blood level PCB 153 was in mean 98.02 ug/kg lipids (range 85.76-112.04) and only slightly decreased to 83.17 ug/kg lipids (range 63.85-108.32) at the age of 5. The multivariate analysis showed small but significant changes in the latency of the N75 and P100 components pointing to alterations of signal transduction in the visual system of the children. However, a relation of these alterations with the PCB 153 level was not obvious.
Plusquellec et al. [10] and Despres et al. [11] also investigated participants of the same cohort at the age of 5 years. They showed a positive correlation between prenatal exposure to PCB 153 and increased states of unhappiness and anxiety during the testing sessions [10]. However no abnormalities of gross and fine motor functions plus neurodevelopment were significantly correlated with the levels of PCB's at the moment of testing[11].
At the age of 11 years a bigger number of tests were performed in members of the NSDS and reported in 5 papers [12-16]. After statistical adjustments VEP's showed no significant association with PBC exposure [12].
However, visual recognition memory testing showed a small but significant impairment and a relation with prenatal PCB exposure [13].
Boucher at al. [14] reported a significant relation between current PCB 153 concentrations, slower reaction time and electrophysiologic signals during the performance of specific attention and fine motor tasks. In the Stanfort-Binet Coping subtest, the Santa Ana Form Board and the Finger Tapping Test as exponents for fine motor testing [15], significant relations with current PCB concentrations were shown. Information processing tested in an auditory oddball protocol [16] appeared to have no relation with antenatal and current concentrations of PCB 's.

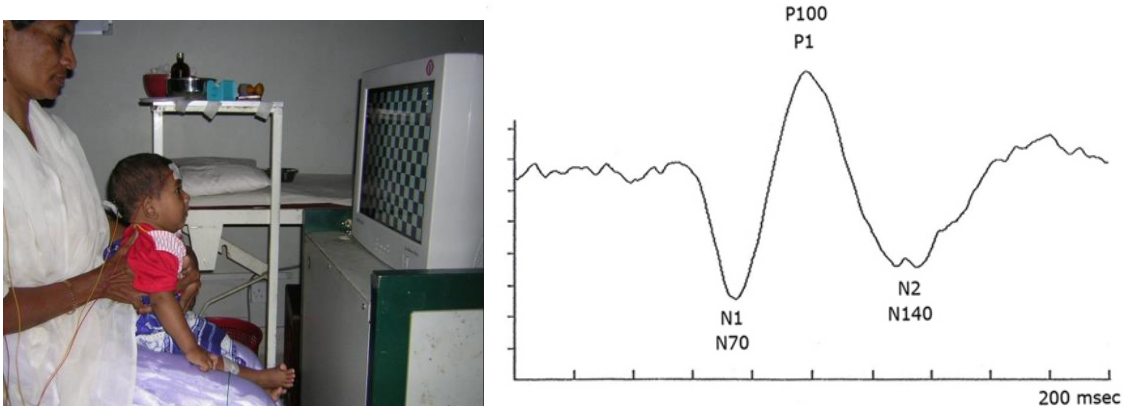

Figure 3. Visual evoked potential tested in a small child. In the right graph a typical result of a recording.

The diets of childbearing women in the Faroer Islands are comparable to artic people and these populations are monitored in the AMAP program, with interesting scientific results. In the different Faroese mother-child cohorts a number of pollutants were investigated and it was concluded that noticed neurobehavioral deficits at the age of 7 years were more likely caused by mercury than PCB's, as after correction for the mercury influence in the model, no significant relations with PCB's could be detected [17].
In summary, in all the studies performed in this mother-child cohort, multivariate analysis incidentally showed significant relations between PBC concentrations in cord blood or blood taken at the age when later neurodevelopmental examination was performed, however the correlation coefficients were low. Moreover, these significant correlations were not consistent in the different studies of the Nunavik cohort.
Although any, even the slightest, positive correlation shown between neurodevelopmental impairment and pollutants, in this case PCB's, should be of great concern, the question still is how much individuals are effected and how much the found associations affect their later daily live. Ofcourse the number of participants, difficult methodology and a number of logistic problems may have a negative impact on the solidity of the data. As Cogliano [18] states in his critical paper; "Still, important data gaps remain most notably for nursing infants and for inhaled PCBs" and he appeals to more research needed to be done for a better collective understanding of mechanisms and interactions.

Ref no.	n	age	Cord PCB-153 Mean and (range) ng/g lipids	Child PCB-153 Mean and (range) ng/g lipids	Cord MeHg Mean and (range) ug/l	Child MeHg Mean and (range) ug/l
8	97	5	122 (22-490)	153 (10-1487)	23 (2-104)	9 (0-38)
9	78	5-6	98.0 (85.7-112.0)	83.2(63.9-108.3)	18.2(14.5-22.0)	6.4(4.9-8.33)
10	110	5	120 (21.6-407.4)	153(7.5-779.8)	22.2 (1.8-104.0)	9.6(0.2-38.2)
11	109	4-6	123.6(21.6-652.6)	160.5(7.5-1467.2)	22.2(1.8-104.0)	9.6(0.2-38.2)
12	148	10.9	127.7(21.6-653.6)	79.8(4.1-809.5)	21.0(2-89)	5(0-34)
13	92	11	114.8(12.0-550.9)	na	22.5(2.4-97.3)	na
14	196	11.3	117.7(9.7-653.6)	72.0(3.5-431.4)	21.2(1.0-99.3)	4.6(0.1-34.1)
15	255	11.3	124.3(9.7-653-6)	73.6(3.5-809.5)	21.4(1.0-99.3)	4.8(0.1-34.1)

Table 1. PCB-153 and methylmercury (MeHg) data from the Nunavik cohort. na=not available.

MERCURY

Mercury neurotoxicity is known for a long time; the first report on neurodevelopmental neurotoxicity in children was in Sweden in 1952 and shortly there after in Minamata, Japan. The fetus and young children appeared to be very vulnerable to mercury and antenatal intoxication was shown. Severe neuromotor developmental delay and mental retardation were part of the clinical picture and the effects persisted livelong [19]. The chemical and biological behaviour of many different mercury compounds are described in more detail by Counter, Buchanan [20] and Oostdam [21].

Methylmercury as the most prevalent molecule is of greatest interest. This compound is nearly instantly accumulated in the brain of rats after intravenous injection. Concentrations in maternal and cord blood from the babies they gave birth too, are of equal concentrations and an extremely high correlation coefficient of 1.0 was found. So, there is no doubt about the very easy transportation into and potential detrimental effects in the fetus and younger children at least for higher concentrations of methylmercury.

As methylmercury is lipophilic, accumulation in fatty tissues like the brain especially before birth and shortly there after is explained in the previous paragraph.

Women from tradionally fish-eating societies have elevated methylmercury levels in their blood and the Faroese mother-child cohort is one of the three known cohort studies, but the only one close to the arctic [19].

The mechanism of moderate level human toxicity is not clear at present and it has been assumed that prenatal methylmercury may disrupt calcium homeostasis or alter the homeostasis of glutamate. Reactive oxygen species may be generated by methylmercury with oxidative stress as a consequence [22].

There is a striking difference in available literature of mercury versus PCB induced developmental adverse effects in children, although the negative effects particular of methylmercury look much more convincing.

Both the Nunavik and the Faroese birth cohorts were started to investigate the impact of PCB's. In the studies mercury was measured as well. So the results for the impact of mercury are secondary endpoints, because the studies were not primarily designed with this goal in mind.

In the Nunavik cohort at 5-6 years of age the methylmercury level at the time of testing was significantly associated with shorter latencies of the N75 and the P100 component of VEP's [9]. It was calculated that one unit increase in the methylmercury blood level caused a decrease in latency of 3-4 msec. Gross motor development and neurological examination were not influenced by PCB or mercury levels at birth and the age between 4 and 6 years old [11].

A study in 43 children at the age of 7-12 years old was performed in Qaanaaq, Greenland [23]. Clinical neurological examination was normal. Neuropsychological tests showed possible exposure related effects, only in a few tests reaching statistical significance.

Conflicting results of the VEP examinations at 11 years of age [12] compared to tests performed at the age of 4-6 years [9] were reported and only significant deficits in the N75 amplitude and not latency at the highest level of contrast in the test was found. In their paper from 2016, Boucher at al. [15] reported that a higher current mercury level was independently associated with a poorer performance of the Finger Tapping Test.

In the 2014 paper [13] only loosely the data of the Wechsler Intelligence Scale were mentioned with a full IQ of 88 with a range from 72-108. This means that the mean IQ is nearly 1 standard deviation lower than expected. Whether this is a population based observation remains unclear as no correlation with mercury was discussed.

In the Faroese cohort examined at the age of 7 years Grandjean at al. [24] found comparable levels for mercury in cord blood and at the age of testing as were measured in the Nunavik cohort. However, their cohort had data from 894 babies at birth and 903 at the age of 7. In 9 children a neurological diagnosis (mainly epilepsy) was reported and these children were

excluded from the analysis. General neurological examination and both gross and fine motor functions were in the normal range and showed no relation with mercury as with visual acuity. A number of subtle mercury related neuropsychological dysfunctions in the domains of language, attention, memory and to a lesser extent in visio spatial and fine motor functions were detected. No full-scale IQ tests were performed. The authors concluded that in the presence of mercury concentrations that are considered as safe, PCB neurotoxicity is difficult to detect. In a later paper of these authors, they express their point of view that methylmercury neurotoxicity is independent of PCB exposure [25].
PCB's have half-lives are between 4 and 6 years and concentrations in humans show a slightly declining tendency but the concern about mercury is at least as big as in the past which is stated in the AMAP 2015 report [6]. Although the reported half-life of methylmercury with 40-50 days is much shorter than of PCB's, the mercury molecule itself as an element remains intact and can easily form new compounds that are neurotoxic again.
From this point of view the AMAP recommendations [6] deserve it to be realized.
Despite all this exsisting literature it still remains difficult to conclude that the levels of the contaminants are fully responsible for the detected neurodevelopmental deficits recognized in the children at the age of 7 until 11 years. Therefore, new ways should be searched for to find better explanations of these deficits in relation to the toxicity of PCB's and mercury.

EPIGENETICS, PCB'S, MERCURY AND NEURODEVELOPMENT

Epigenetics is the study of heritable changes in gene activity without alterations in the genetic code. Through epigenetic marks environmental factors like diet, stress and prenatal nutrition can make an imprint on genes passed from one generation to the next.
Epigenetic changes can be attributed or accumulated within cells of different tissues, all having specific patterns of epigenetic modification. There are several epigenetic marks. Most important up to now are chemically by methylation and via proteins, histones, which play a role in the folding and by this means of the susceptibility for replication of DNA. Only the fixed amino acid combination of cytosine and guanine linked to one-another in the DNA by phosphorus (CpG) can be methylated. Areas with a high concentration of the formation of CpGs are called CpG islands [26]. Because these islands are close to the locations of promoter genes, there is a strong correlation between CpG and transcription initiation.
Hypermethylation of an CpG island leads to the inability for a stimulus to reach the promoter gene and in this way blocks the transcription of the gene.

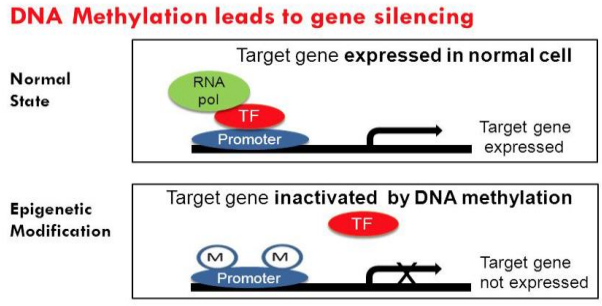

Figure 4. Hypermethylation of an CpG island depresses gene expression.

Shortly after fertilisation a nearly total de-methylation of DNA takes place in the blastocystic stage of the developing embryo, followed by quick reprogramming and maternal versus parental genetic imprinting. Because this de-methylation is not absolute as imprinted epigenetic marks remain intact and 1 percent of genes are not demethylated, alterations in the epigenome can start already from this point. By this process influences from the environment, sometimes from much earlier date, can lead to phenotypical changes. This process is also

linked to evolutionary changes that can be expressed much quicker than changes in DNA sequence [27].

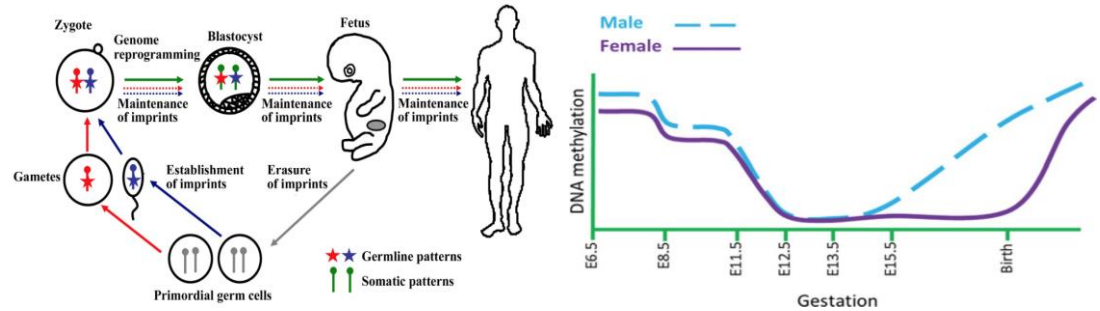

Figure 5; The process of de- and re-methylation of DNA in gametes, embryo and fetus.

In figure 6 is shown how an insult, for instance intoxication with mercury, which takes place in a female, pregnant of a female fetus, can efect three generations at the same time. And before we can speak of an inherited epigenetic change, this change therefore still must be observed in the 4th generation.

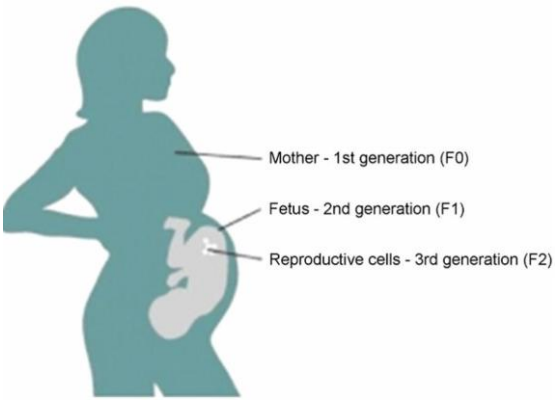

Figure 6. The epigentic inheritance route.

In recent years aberrations of the DNA methylation marks have been shown to be the underlying mechanism of different, phenotypically known, neurological diseases or syndromes [28]. Besides these, Mendelian inherited genetic disorders in the DNA replication process have been reported for disorders in the histone machinery and also the genetic imprinting process. It is concluded that there are many interconnections between at least a number of (epi)genetic layers.
Parallel to this, the field of neural and behavioural epigenetics shows a very quick accumulation of knowledge expressed in many papers. Links between epigenetic changes and behavioural abnormalities for instance attention deficit hyperactivity disorder (ADHD) at one side and psychiatric illnesses as autism spectrum illnesses on the other, have been made [29]. Very recently it was published that ambient particulate matter can induce hypo- and hypermethylation and abnormal mRNA expression in the promoter region of autism candidate genes [30].
Epigenetic insights have been generated during gestation and linked to placental function and pregnancy outcome [31]. In this review the different steps from demethylation in the embryonic phase and present knowledge of the epigenetic impact on placenta function and development of a healthy fetus is described. Most of the abnormalities and diseases often seen during pregnancy and the epigenetic role are also presented.
Effects of pollutants on the feto-placental unit with the epigenetic mechanism as underlying course are presented in the paper of Kappils et al [32]. A significantly positive association

between global DNA methylation and the total level of polybrominated diphenyl ethers was found. For the expression between H19, a maternally expressed non-coding RNA that is located downstream of the Insulin like Growth Factor 2 gene, and total PCB levels a significant correlation was also shown.
Also very recently Paquette et al. [33, 34] again reported that variable DNA methylation in the human placenta is associated with neurobehavioral in the newborn. Altered placental DNA methylation was associated with lethargy, attention, quality of movement and arousal in the newborn.
In the umbilical cord of infants exposed to mercury and arsenic, a doubling in maternal toenail mercury resulted in hypermethylation of 85% of loci located in the north shore regions of the top 100 CpG associated islands [22]. The authors state that even mercury and arsenic at low levels can have impact on the epigenome. However, the potential relation with newborn neurobehavioral has not been investigated in this paper. Although it looks reasonable to connect the epigenetic changes to alterations in the neonatal neurobehavioral, this is speculative at the moment. Moreover, what these early-recognized abnormalities mean for later life remains difficult to predict.

CONCLUSION AND FUTURE DIRECTION

After correlations between levels of total PCB's in cord blood and neurodevelopmental deficits at the age of 4 months were detected, great concerns about the eventual long-term effects of these pollutants in antenatally exposed children arose. Several mother-child cohort studies with a follow-up period until 11 years of age were started and neuromotor functions, behaviour and global development in children were investigated. In these studies besides PCB's, levels of methylmercury was measured as well.
Classic neurodevelopment investigating techniques have shown inconsistent results regarding the neurodevelopmental outcome at the age of 11 years in arctic children, who were antenatally exposed to PCB's and methylmercury.
Lack of dose-response relations and the inconsistent outcome in neurodevelopmental tests in children exposed to these pollutants during pregnancy and the first months after birth, ask for more research. Persistent concern about the toxicity and eventual life-long effects underline this.
The recently developed techniques and knowledge in the (neuro)epigenetic field look very promising in this perspective and preliminary data show relations between hypermethylated areas in DNA of the placenta and abnormalities in attention, lethargy, quality of movement and arousal in the baby shortly after birth. However, we need to remain critical how to interpret these findings [35].
Expansion of our knowledge how dangerous different kinds of POP's, mercury and other heavy metals are, even at a low dose, is urgently needed [18]. Previously, long-lasting cohort studies starting before conception and continued until adulthood were the only way to answer these questions properly. Let us hope that well-designed epigenetic studies can provide us the answers, in a considerably shorter time.

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