Persistent organic pollutants

Aberrant DNA methylation underlying potential health effects

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CHAPTER 1

General introduction

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Background

Fish consumption is an important source of proteins, minerals and especially omega-3 fatty acids such as EPA and DHA. Dietary guidelines, including the advice of weekly consumption of fatty fish,[1] are designed for an adequate intake of nutrients and to protect against diet-related diseases. Nevertheless, fish also contains environmental contaminants, which might counteract the positive effects of fish or even cause adverse health effects.

In the Netherlands, the main rivers, including the Rhine-Meuse delta, are highly contaminated due to a high degree of urbanization and industrialization along these rivers. Lipophilic contaminants do not dissolve in water and therefore accumulate in the sediments of these rivers and estuaries. Consequently, predators living closely to these sediments are more exposed to lipophilic contaminants than other fish. Eel is a bottom dwelling carnivorous animal and enters the Netherlands as glass eel after a long journey from the Sargasso Sea. Once settled down, eel stays at the same location for a long time. They have a long life span, and do not spawn at their settled location, but only produce offspring after their long return journey to the Sargasso Sea. During reproduction, a substantial amount of the lipophilic contaminants are passed on to the offspring. The absence of spawning, together with the other characteristics and high lipid content, make eel particularly prone to bioaccumulation of lipophilic contaminants resulting in high levels.[2-4] Although many contaminant levels are declining in the environment, due to the long half-lives it is estimated that it will take until 2200 until certain contaminant levels in the river Rhine will disappear under the detection levels.[2] This indicates that these contaminants will pose a problem for the environment and fish quality for still a long time. Smoking eel is a popular tradition in the Netherlands, but eel consumption might be an important pathway of exposure to contaminants, such as persistent organic pollutants (POPs). Authorities were therefore concerned about the implications for human health due to consumption of contaminated eel, as risk assessors showed in 2007 that safe levels could be exceeded.[5] A ban on eel fishing in certain high-polluted areas was implemented in 2011 after the broadcasting of a documentary about this topic. However, there is a continuous debate about the necessity of this ban, the actual

accumulation of these contaminants in eel consumers and the potential adverse health effects.

Categories of persistent organic pollutants (POPs) and health effects

POPs are mainly men-made chemicals that have in common that they resist degradation through environmental processes, remaining intact for long periods of time. Due to their persistence and semi-volatile properties, facilitating long-range transport, these compounds are ubiquitously present in the environment and also found in remote places where there is no human activity nor chemical sources. Most POPs are lipophilic, enabling bioaccumulation in fatty tissues. Over 90% of human exposure to POPs comes from food consumption,[6] with high contributions from fish caught in polluted areas (Fig. 1).[7,8] Due to biomagnification especially marine top predators have very high levels of POPs. Therefore, the highest exposed populations are found around the artic, where traditional foods include marine mammals, such as seal and whale blubber.[9-11] In the Netherlands, the waste incinerations used to be a main distributing source of POP-exposure (mainly dioxins, explained in next paragraph). This caused elevated levels in cow's milk and subsequently considerably high dioxin levels in the Dutch population.[12] Nowadays, dairy products are still the main exposure sources to POPs, although fish has the highest pollution level based on product basis.[8,13] The highest POP levels are measured in shrimp, eel and herring, and therefore especially frequent fish consumers may accumulate POPs above safe levels.[5,7,8] The following paragraphs will describe different POP categories and adverse health effects related to these POPs.

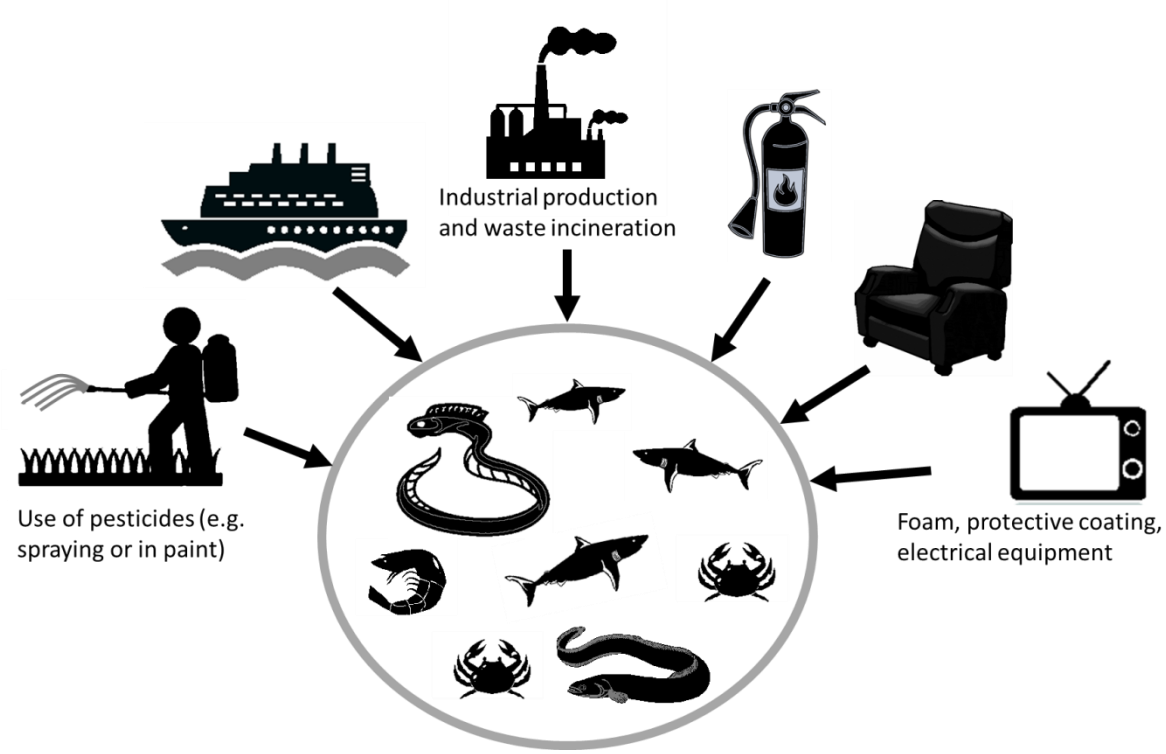


Figure 1. Schematic overview of sources of persistent organic pollutants (POPs). Some POPs, such as TBT and PFOS, are used in pesticides which might be sprayed but can also be used in paint, e.g. for boats. A major source of POPs used to be industrial production, but POPs can also originate as an unwanted by-product in waste incineration or fossil fuel combustion. PFOS was used in fire-fighting foams, but also in protective coatings in furniture and carpet. Flame retardants are also used in furniture and in electrical equipment. The arrows indicate the POPs entering the environment, where they settle down in sediments and subsequently accumulate in eel and other organisms.

Dioxins and PCBs

Dioxins refer to a group of 75 polychlorinated dibenzo-p-dioxins (PCDD) congeners and 135 polychlorinated dibenzofurans (PCDF) congeners, of which 17 are of toxicological concern. Furthermore, there are 209 different polychlorinated biphenyls (PCB) congeners, from which 12 have properties similar to dioxins (DL-PCBs). The toxicological active dioxins and dioxin-like compounds share a common mechanism involving binding to a cytoplasmic receptor protein called the aryl hydrocarbon (AH) receptor, although with different potencies.[13] Therefore toxic equivalency factors (TEFs) have been introduced that indicate the relative potency compared to 2,3,7,8-tetrachlorodibenzodioxin (TCDD) which is the most potent dioxin.[14]

Dioxins and DL-PCBs have received considerable attention from the European Commission, which are regulated as one class because the congeners are present in the environment and food as mixtures. Toxic equivalents (TEQ) were introduced to sum up the toxicity, using the TEF values, of all these congeners. The maximum level for the sum of PCDD/Fs and DL-PCBs in eel was set at 10 pg TEQ/g wet weight (ww) in 2012, while other seafood items have to comply to 6.5 pg TEQ/g ww.[15] The majority of the eel from the Rhine-Meuse delta does not comply with the maximum levels,[4,16] and risk assessors concluded that consumption of one portion eel per month would lead to accumulation of the compounds in humans above estimated safe levels.[5] The safe level is derived from animal reproduction and developmental studies and set to protect the developing unborn child.

Dioxins have been referred to as 'the most toxic man-made compounds' and, therefore, have generated much concern regarding their potential health risks. TCDD and certain PCBs are classified as a 'known human carcinogen',[17] and these compounds are also related to endocrine disruption, neurological disorders, and immune impairment.[18] PCBs are slowly metabolized by phase I enzymes intro hydroxylated (OH-) PCBs and by phase II enzymes into e.g. methylsulfony-PCBs (MeSO2-PCBs). These metabolites are not lipophilic, however, some OH-PCBs are retained in blood[19] and can bind to thyroid transport proteins explaining effects on thyroid hormones.[20]

PFAS

Perfluoroalkyl substances (PFAS) are fluorinated organic compounds that have the unique property that they contain both lipid- and water-repellent characteristics. PFASs, especially perfluorooctanoic acid (PFOA) and perfluoroctane sulphonate (PFOS), were therefore widely used in industrial and consumer applications, including stain-resistant coatings for fabrics and carpets, oil-resistant coatings for paper products approved for food contact, fire-fighting foams, and insecticides.[21] Unlike other POPs, PFASs bind to blood proteins and are mainly stored in the liver, kidneys, and biliary system.[22] Adverse health effects include developmental toxicity, thyroid disturbance and altered lipid metabolism.[23]

Brominated flame retardants

Flame retardants include a diverse group of compounds used to prevent or slow down the development of fires. Two major classes of brominated flame retardants include polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecanes (HBCDs). The lower brominated congeners (between 1 and 5 bromine-substitutions per molecule) have a higher bioavailability than the higher brominated congeners.[24] PBDEs are generally used as mixtures containing multiple PBDEs. HBCD is composed of three stereoisomers: α-, β-, and γ-HBCD, which have high capacity of bioaccumulation. The main target for these compounds is the liver and thyroid hormone homeostasis, but also reproductive and nervous system effects are important. For PBDEs also the OH-metabolites are important, as they have a high similarity with thyroid hormones.[24]

Organotin

Organotins, such as tributyltin (TBT) and triphenyltin (TPT), have been widely used as heat stabilizers, wood preservatives and pesticides. TBT got considerably more attention when the phenomenon of imposex was reported, meaning that male sex characteristics were superimposed on normal female gastropods. Organotins can exert endocrine disruption due to inhibition of aromatase, but have also immunological, neurotoxic and obesity-related effects.[25,26]

Mercury

The main source of the widespread mercury is coal combustion, where mercury is present in trace amounts. Mercury is not bioavailable, whereas methylmercury (MeHg) can be adsorbed by the gastrointestinal tract and cross the blood-brain barrier. The bulk of MeHg within natural systems originates from methylation of mercury by bacteria within aquatic sediments.[27] MeHg bioaccumulates mainly in muscle tissue due to protein binding, rather than fatty tissue. MeHg exposure is related to nervous system damage in adults and impaired neurological development in infants, to an impaired immune system, and endocrine disruption.[28]

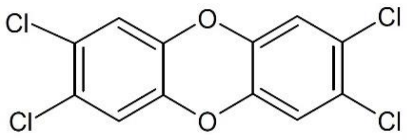
Considerations of test compounds for thesis

Eight different POPs present in the human seafood chain, and especially eel, were chosen to represent the different POP groups and were included in the experiments described in this thesis. Within the different groups the compounds were chosen based on their relative abundance in polluted fish species (Table 1 and Fig. 2). These include the dioxin TCDD and the dioxin-like PCB126;[29] the non-dioxin-like PCB153;[2] PFOS;[3] BDE-47;[30] HBCD;[31] TBT;[32] and the organometallic MeHg.[33] Most of these POPs are nowadays strictly regulated within Europe and other countries, however, restriction and regulation is in general less strict in less developed countries. Furthermore, due to the persistence of these POPs, they are only slowly degraded and these POPs will remain in the environment for still a long time. Most of these POPs also have long half-lives in humans, indicating there are slowly excreted from the human body (Table 1).

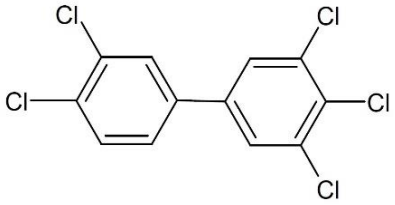
Table 1. POPs representing the most abundant contaminants in eel within different POP groups.

Compound	Use	Half-life in humans	Stockholm Convention[a]	Restriction / elimination Europe
TCDD	By-product	8 years[34]	Annex C	Emission requirements (1987)[35,36]
PCBs	Industrial	10-15 years[37,38]	Annex A / C	Restricted (1985), disposal close system (2010)[39]
PFOS	Industrial	4-10 years[40]	Annex B	Forbidden (2008)[41]
BDE-47	Flame retardant (electronic devices / upholstery)	2-5 years[42]	Annex A	Forbidden (2004)[43]
HBCD	Flame retardant (polystyrene foams / textile)	0.2 years[42]	Annex A (with certain exemptions)	Forbidden (2015), authorized use allowed[44]
TBT	Biocide	?	-	Banned in anti-fouling paints (2008)[45] Forbidden for new, but not existing products (2010)[46]
MeHg	Natural + by-product	0.3 years[47]	-	Emission requirements, restriction mercury (2008)[48]

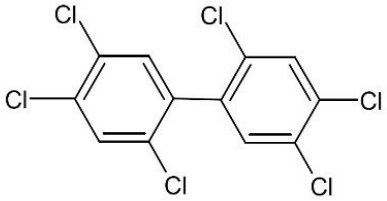
[a] Annex A: Elimination of the production and use; Annex B: Restricted production and use; Annex C: Reduction of unintentional release.



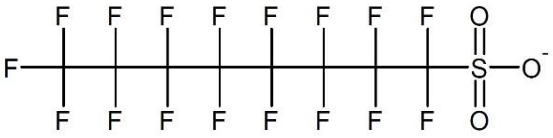
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)



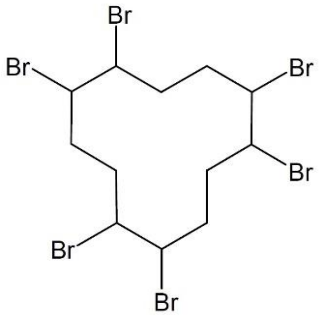
3,3',4,4',5-pentachlorobiphenyl (PCB126)



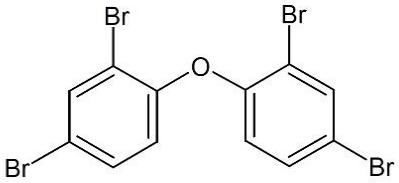
2,2',4,4',5,5'-hexachlorobiphenyl (PCB153)



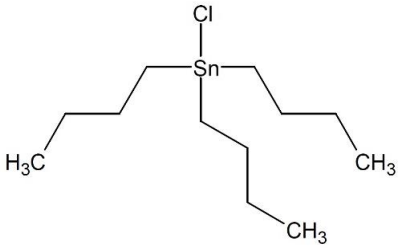
Heptadecafluorooctanesulfonic acid (PFOS)



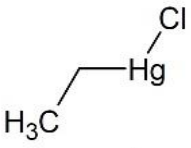
Hexabromocyclododecane (HBCD)



2,2',4,4'-tetrabromodiphenyl ether (BDE-47)



Tributyl chlorotin (TBT)



Methylmercury(II) chloride (MeHg)

Figure 2: Molecular structures of the tested POPs.[49] Eight different POPs present in the human seafood chain were chosen based on their relative abundance in polluted eel and different mechanisms of action.

Mechanism of action behind adverse health effects

The adverse health effects of POPs, described in the previous sections, can be attributed to different mechanisms of action. Although some of these mechanisms are clear, like dioxins binding to the AH receptor,[13] hydroxylated metabolites binding to thyroid transport proteins,[20] and PFASs resembling fatty acids,[50] various long-term or delayed adverse health effects cannot be explained by these mechanisms of action. Exposure to POPs may for example increase the risk of certain cancers, however, POPs are not genotoxic and the exact mechanism is not elucidated.[17] Also, exposure of POPs in adults has been associated with an increased risk of the metabolic syndrome.[51] Furthermore, prenatal exposure can cause immunological disturbances later in life indicating long-lasting effects at the stem cell level.[52]

Epigenetic phenomena, such as DNA methylation, have been proposed as a possible molecular mechanism underlying adverse health effects.[53] Already in 1989, the role of DNA methylation in dioxin response was suggested.[54] Nevertheless, this possible mechanism of action in relation to adverse health effects is still not elucidated. In this thesis, we therefore hypothesized that epigenetic mechanisms causing persistent changes in biological functions might explain described long-lasting or delayed adverse health effects of POPs.

Epigenetics

Epigenetics is an emerging field of science that studies heritable or stable, long-term changes caused by the activation and deactivation of genes without any change in the underlying DNA. The word epigenetics is of Greek origin and literally means over or above (epi) the genome.

Epigenetics is important in determining phenotype and packaging of the DNA. There are three major epigenetic modifications that work together: DNA methylation, histone modifications and non-coding RNAs (ncRNAs).[55] DNA methylation is the most stable epigenetic modification and therefore of high interest as a possible mechanism of action for long-lasting adverse health effects, while both histone modifications and ncRNAs are more flexible marks. Histone modifications play an important role in chromatin state, subsequently facilitating or blocking gene expression.[56] ncRNAs are involved in

transcriptional and post-transcriptional silencing, e.g. by affecting mRNA stability or targeting mRNA for degradation.[57]

DNA methylation

DNA methylation refers to the addition of a methyl group to cytosine in a CpG dinucleotide. DNA methylation marks are transmitted to daughter cells during somatic cell division, and possibly also from one generation to the next.[58] DNA methylation has long been considered to be a stable, persistent and heritable mark that can arise by adding methyl groups, but cannot be removed. More recently the removal of the methyl group by active demethylation enzymes was shown.[59] CpG dinucleotides tend to cluster in regions called CpG islands (CGIs), but there are also CpGs outside these CpG-rich areas in low-CpG-dense areas (referred to as open sea).

Methylation of DNA can change the functional states of regulatory regions and has a complicated relationship with gene expression. Methylation is involved in many forms of stable epigenetic repression, such as imprinting, X chromosome inactivation and silencing of repetitive DNA.[60] Global DNA methylation is essential for stabilization of the genome, and loss of global methylation (hypomethylation) has been related to diseases such as cancer, psychiatric disorders and atherosclerosis.[61]

DNA methylation close to the promoter region or transcription start site (TSS) is expected to decrease gene expression, and severe hypermethylation of these regions can fully inhibit the gene function.[60,62] When the promoter region is not methylated, gene expression is regulated by transcription factors. It is important to note that some genes have CGIs at their TSSs, while other genes are CpG-poor at the TSS. CpG density might influence fluctuations in methylation levels and subsequently affect gene regulation in different ways.[60] The quantitative relationship between promoter methylation and gene expression is thus more complicated than once assumed and the details have not been fully worked out.

DNA methylation is in general more abundant in the gene body compared to the TSS, indicating that transcription initiation but not elongation seems to be sensitive to silencing through DNA hypermethylation. Methylation in the gene body is currently considered to affect gene expression by stimulating transcription elongation or by

influencing alternative splicing, and can both result in an increase or decrease of gene expression.[63]

The role of DNA methylation in intergenic regions (IGR) is poorly understood,[60] but it may be relevant for cell-type discriminatory patterns.[64] Furthermore, about half of the enhancers are present in IGRs,[65] and DNA methylation in the enhancer region can exert different effects because enhancers can regulate multiple genes. Enhancers might activate a promoter from a distance, but therefore both methylation states are relevant. Differential methylation in enhancers therefore does not have a straightforward relationship with gene expression, however, the variable DNA methylation state in enhancers suggest an important role of DNA methylation in altering expression of multiple genes.[66,67] Furthermore, for many genes the enhancers are not yet identified, and enhancers can be thousands of kilobases away from their target genes, resulting in a highly complex relationship between DNA methylation and gene expression.[68]

When exposure to POPs affect DNA methylation, this might cause a persistent change in biological functions due to affected gene regulation. To date, there are multiple observational studies relating global methylation levels to POP exposure, but the results are not consistent (Table 2). Global methylation is difficult to interpret because an overall loss of DNA methylation might occur together with gene-specific increases in DNA methylation. POP-induced changes in gene-specific DNA methylation is measured for only a couple of genes in human studies, but experimental animal studies (Table 3) and *in vitro* experiments (Table 4) suggest that POPs might affect gene-specific DNA methylation, although the consequence of this aberrant DNA methylation it is not yet elucidated.

Table 2. Human observational studies measuring global or gene-specific DNA methylation associated with levels of persistent organic pollutants (POPs).

Compound	Tissue	DNA methylation	Method	n	Group (origin)
Mixture of POPs[69]	Whole blood	↓ Global	*Alu* elements[a]	70	Adults (Greenlandic Inuit)
Mixture of POPs[70]	Whole blood	↓ Global	*Alu* elements[a]	86	Adults (South-Korea)
Total TEQ levels[71]	Blood	↑ Global	LUMA[b]	524	Adults (Sweden)
OC pesticides[72]	Leukocytes	↓ Global	LUMA[b]	403	Adult women (Japan)
OC pesticides PCBs[73]	Whole blood	↓ *MGMT* U-shaped *MGMT*	Methylation-specific PCR	368	Adults (South-Korea)
PCB153[74]	Sperm	↔ Global	Repetitive elements	209	Adult men (Greenland, Poland, Ukraine)
PCBs[75]	Placenta	↔ Global ↔ *IGF2/H19*	LUMA[b] + Pyrosequencing	109	Fetoplacental (USA)
PFOS[76]	Leukocytes	↑ Global	*LINE-1*[a] elements	685	Adults (USA)
PFOA[77]	Umbilical cord serum	↓ Global	ELISA[c]	113	Newborns (USA)
PFASs[78]	Sperm	↔ Global	Repetitive elements	209	Adult men (Greenland, Poland, Ukraine)
BDE-47[79]	Cord blood leukocytes	↓ *TNFα* promoter	PCR and sequencing	46	Newborns (African-American)
PBDE[80]	Blood clot	↔ Global	*Alu* and *LINE-1* elements[a]	358	Children (Mexican-American)
PBDEs[75]	Placenta	↑ Global ↔IGF2/H19	LUMA[b] + Pyrosequencing	108	Fetoplacental (USA)
Mercury[81]	Buccal mucosa	↓ *SEPP1* promoter	Pyrosequencing	131	Adults (USA)
MeHg[82]	Cord blood clot	↓*TCEANC2*	Methylation array (CHARM)	141	Newborns (USA)

↑ increase, ↓ decrease, ↔ no change

[a] *Alu* and *LINE-1* elements are repetitive elements that can be used as a surrogate marker for global DNA methylation.

[b] LUminometric Methylation Assay (LUMA) measures global DNA methylation levels.

[c] Enzyme-Linked Immuno Sorbent Assay (ELISA) measures global DNA methylation levels.

Table 3. Experimental animal studies assessing the relationship between gene-specific DNA methylation and persistent organic pollutants (POPs).

Compound	DNA methylation	Method	Health effects	Species (organ)
TCDD[83,84]	50 DMRs	MeDIP[a] Tiling Array	Sexual maturation	Rats (sperm)
TCDD[85]	↓ *Ddx54* ↑ *Ill7rd, Bankl, Zfpl28, Ralgds*	Arbitrarily Primed PCR	Suppressed immune response	Mice (splenocytes)
TCDD[86]	↔ *Snrpn, Peg3, Igf2r* (sperm) ↑ *Igf2r* (muscle) ↑ *Igf2r* + *Peg3* (liver)	Pyrosequencing	Decreased sperm count	Mice (sperm, liver, tibialis anterior muscle)
TCDD[87]	↔ *αGSU, LHβ*	Restriction enzymes, PCR	Attenuated steroidogenesis	Rats (fetal pituitary)
TCDD[88]	↓ *lrata, thumpd1* ↑ *tjp1, usp5, krt5, spryd4, adra2a, col2a1* (liver) ↓*cdhrl*↑ *taar20p* (embryo)	CpG island tiling array	Larvae died post hatching	Zebrafish (liver or embryo)
TCDD[89]	↓*Cpne4* ↑ *Kcnkl3, Abcg1, Ubash3b, Ly86, Dscaml1, Fbxl16*	MeDIP[a] Array	-	Rat (liver)
TCDD[90]	↓ *Igf2* (body) ↑ *Igf2* (enhancer blocking region)	Bisulfite sequencing	Decreased liver weight, elevated AST	Rats (liver)
TCDD[91]	↓ *c-fos* ↑ *ahrr* ↔ *ahr2*	Bisulfite PCR	-	Zebrafish (embryos)
TCDD[92]	↑ *H19/Igf2*	Bisulfite sequencing	↓ fetal weight	Mice embryos
TCDD[93]	↑ *Brca1*	Methylated-specific PCR	Disrupted mammary gland morphology	Rat (mammary tissue)
PCBs[94]	↓↑ *Cdkn2a-p16INK4a*	Pyrosequencing	Hepatic inflammation	Rat (liver)
PCBs[95]	↔ *AR*	Bisulfite PCR	-	Rats (liver)
BDE-47[96]	↓ *Mt-co2, LIRn, Bdnf* ↑ *Nr3c1* ↔ Majority of genes	Pyrosequencing	-	Rats (frontal lobe brain)
TBT[97]	↓ *Fabp4* ↔ *Pparg2*	Restriction enzymes, PCR	Increased lipid accumulation	Mice (mADSCs)
TBT[98]	↑ *vtg1, cyp19a2* ↔ *vasa*	Pyrosequencing	Embryo-toxicity	Zebrafish (embryos)
PFOS[99]	↑ *GSTP* promoter ↔ *p16*	Sequencing	Decreased body weight	Rats (liver)
MeHg[94]	↓↑ *Cdkn2a-p16INK4a*	Pyrosequencing	Hepatic inflammation	Rats (liver)
MeHg[100]	↑ *BDNF* promoter	PCR, primer extension	Impaired behavior	Mice (hippocampus)
MeHg[88]	↓ 32 genes ↑ no genes (liver) ↓20 genes ↑ 5 genes (embryo F1)	CpG island tiling array	-	Zebrafish (liver or embryo)

↑ increase, ↓ decrease, ↔ no change

[a] Methylated DNA immunoprecipitation (MeDIP) is used to enrich methylated DNA sequences.

Table 4. *In vitro* experiments investigating the relationship between gene-specific DNA methylation and persistent organic pollutants (POPs).

Compound	DNA methylation	Method	Health related effects	Cell type (origin)
TCDD[101]	↑ *p16INK4a* promoter	Methylation-specific PCR	Diminished senescence	HEK (human keratinocytes)
TCDD[102]	↓ *Foxp3* ↑ *Il-17*	Methylation-specific PCR	Increased regulatory T cells	Primary mice T lymphocytes
TCDD[103]	↑ *BRCA1*	Methylation-specific PCR	-	MCF-7 (human breast cancer cells)
BDE-47[104]	↓ *Pparg2* ↔ *Lep*	MS-HRM[a]	↑ adipocyte differentiation	3T3-L1 (mouse preadipocytes)
HBCD[105]	↔ 10 genes	Methylation-specific PCR	-	Primary human hepatocytes and HepG2 cells

↑ increase, ↓ decrease, ↔ no change

[a] Methylation-Sensitive High Resolution Melting (MS-HRM) analysis is based on a net temperature shift with different methylation levels.

Thesis outline

Frequent consumption of eel might cause accumulation of POPs to levels that are not regarded safe and could potentially affect DNA methylation. The aim of this thesis is to elucidate whether seafood-related POPs can induce aberrant DNA methylation in high-exposed eel consumers, potentially causing adverse health effects. Understanding the mechanism of action of POPs can facilitate risk assessment and give more insight in health effects associated with chronic exposure.

The main objectives of this thesis were to:

1) Study whether frequent consumption of eel from polluted areas would lead to POP levels above regarded safe levels
2) Investigate mechanisms of action of POPs focusing on gene expression and DNA methylation (using *in vitro* studies)
3) Determine aberrant DNA methylation between eel consumers from high-polluted areas compared to consumers from low-polluted areas

This chapter, **chapter 1**, presents background information, a short summary about the different POPs tested, information about DNA methylation and its function, the objectives of the studies, and the outline of the thesis.

Chapter 2 describes the effects of the POPs, including two mixtures, on steroidogenesis and focusses on functional effects (hormone levels) of altered gene regulation. DNA methylation was not measured due to the short exposure time, which was expected not to be sufficient for long-term epigenetic marks.

Therefore, in **chapter 3**, a differentiation experiment was performed enabling long-term exposure to POPs. Human mesenchymal stem cells (hMSCs) were differentiated into mature adipocytes and the association between functional endpoints (differentiation proficiency), gene expression, and DNA methylation was assessed. Because it was still unclear how DNA methylation would be affected by the differentiation process, DNA methylation profiles were first established in unexposed cells before and after differentiation (**chapter 3A**). We subsequently measured DNA methylation in

differentiated adipocytes that were continuously (11 days) exposed to different POPs (**chapter 3B**).

We performed a human study to investigate the accumulation of POPs in frequent eel consumers of low- and high-polluted areas (**chapter 4**). After establishing which POP levels were elevated in the men consuming eel from high-polluted areas, we investigated differences in DNA methylation profiles and measured biomarkers related to potential adverse health outcomes (**chapter 5**).

At the end, the data obtained in this thesis is discussed together with data available in the literature (**chapter 6**). Suggestions for future research are provided as well as implications of our research for eel consumers and policy makers.

Finally, **chapter 7** summarizes the content of this thesis in English and in Dutch.

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CHAPTER 2

Steroid hormone related effects of marine persistent organic pollutants in human H295R adrenocortical carcinoma cells

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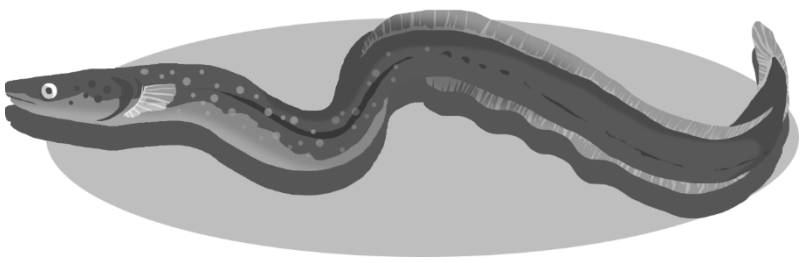

Abstract

Persistent organic pollutants (POPs) such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), polychlorobiphenyl (PCB) 126 and 153, perfluorooctanesulfonic acid (PFOS), hexabromocyclododecane (HBCD), 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47), tributyltin (TBT), and methylmercury (MeHg) can be accumulated in seafood and then form a main source for human exposure. Some POPs have been associated with changes in steroid hormone levels in both humans and animals. This study describes the *in vitro* effects of these POPs and mixtures thereof in H295R adrenocortical carcinoma cells. Relative responses for 13 steroid hormones and 7 genes involved in the steroidogenic pathway, and *CYP1A1*, were analyzed. PFOS induced the most pronounced effects on steroid hormone levels by significantly affecting 9 out of 13 hormone levels measured, with the largest increases found for 17β-estradiol, corticosterone, and cortisol. Furthermore, TCDD, both PCBs, and TBT significantly altered steroidogenesis. Increased steroid hormone levels were accompanied by related increased gene expression levels. The differently expressed genes were *MC2R*, *CYP11B1, CYP11B2*, and *CYP19A1* and changes in gene expression levels were more sensitive than changes in hormone levels. The POP mixtures tested showed mostly additive effects, especially for DHEA and 17β-estradiol levels. This study shows that some seafood POPs are capable of altering steroidogenesis in H295R cells at concentrations that mixtures might reach in human blood, suggesting that adverse health effects cannot be excluded.

Introduction

Environmental persistent organic pollutants (POPs), either historical or currently in use, accumulate in fatty tissues causing seafood to be a main source for human exposure.[1-3] This is of major concern, because many of these marine POPs have been related to negative health effects such as reduced cognitive development, immune toxicity, neurological disorders, cancer, and endocrine disruption.[4,5] In addition to the well-known mechanism for dioxin-like compounds via the aryl hydrocarbon receptor (AhR),[6,7] other mechanisms of toxic action have been suggested to cause adverse health effects, including disruption of thyroid[8] and steroid hormone systems.[9] Such mechanisms can be inappropriate activation or antagonism of the nuclear steroid receptors, modulating nuclear receptor coactivators, or interference with key enzymes involved in steroid hormone synthesis and metabolism.[10] Several POPs have been shown to affect hormone levels in humans and animals.[11-13] To date, most studies focused on the effects of single POPs on levels of only a few different steroid hormones,[14-16] or extracted mixtures of only partially identified POPs were tested.[17,18] Investigating single compounds is important to elucidate the mechanisms of action as different compounds may counteract each other's mechanisms. Humans, on the other hand, are exposed to mixtures so it is important to elucidate combined actions as well.

In this study we investigated the effects of single POPs and mixtures thereof on steroidogenesis. Eight different POPs present in the human seafood chain were chosen based on their relative abundance in polluted fish species. These include the dioxin-like compounds TCDD and PCB 126;[19] the non-dioxin-like PCB 153;[20] the perfluorinated compound heptadecafluorooctanesulfonic acid (PFOS);[21] the brominated flame retardants hexabromocyclododecane (HBCD)[22] and 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47);[23] the biocide tributyl chlorotin (TBT),[24] and the organometallic MeHg.[25] The composition of mixtures to which people are exposed depends on the origin and type of polluted seafood that is consumed. Therefore, there is not one mixture that is most relevant to test, but still it is important to test whether compounds in a mixture can induce interactive effects. The *in vitro* model used was the H295R human adrenocortical carcinoma cell line, chosen because it expresses all hormones found in the adult adrenal

cortex and the gonads and all key enzymes involved in steroidogenesis.[26] The functionality of this bioassay is consistent with most results observed *in vivo*.[27] The endpoints we studied with the H295R model are corticosteroid synthesis and the production of sex steroid hormones. The assay has been fully validated to evaluate effects on testosterone and estradiol production.[28] However, more elaborate approaches provide a screen for endocrine disruption and can identify potential targets of the test compound.[29] In this study we measured steroidogenesis disruption as hormone production of 13 steroid hormones plus expression levels of 7 key steroidogenic genes for 8 individual POPs and mixtures thereof.

Materials and methods

Chemicals

TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin, CAS 1746-01-6) was purchased from AccuStandard (New Haven, USA) and PCB 153 (2,2′,4,4′,5,5′-hexachlorobiphenyl, CAS 35065-27-1), PFOS (heptadecafluorooctanesulfonic acid potassium salt, CAS 2795-39-3), TBT (tributyl chlorotin, CAS 1461-22-9), and MeHg (methylmercury(II) chloride, CAS 115-09-3) were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). PCB 126 (3,3',4,4',5-pentachlorobiphenyl, CAS 57465-28-8) was purchased from Promochem (Wesel, Germany). HBCD (hexabromocyclododecane, technical mixture) and BDE-47 (2,2′,4,4′-tetrabromodiphenyl ether, CAS 5436-43-1) were kindly provided by Professor Åke Bergman (Stockholm University, Sweden) within the framework of the EU FIRE project.[30] Forskolin (CAS 66575-29-9) and prochloraz (CAS 67747-09-5) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions for all chemicals were prepared in dimethylsulfoxide (DMSO) (Acros Organics, Belgium). Pregnenolone, 17α-OH-pregnenolone, progesterone, 17α-OH-progesterone, dehydroepiandrosterone (DHEA), androstenedione, testosterone, 11-deoxycorticosterone, corticosterone, 11-deoxycortisol and cortisol were obtained from Steraloids (Newport, RI, USA). The deuterium labelled internal steroid standards were from CDN isotopes (Point-Claire, Canada). Derivatisation reagent consisted of 1 mg 4-dimethyl-aminopyridine, 5 mg 2-methyl-6-nitrobenzoic anhydride and 3 mg picolinic acid in 1 mL tetrahydrofuran after

which 10 µL of triethylamine was added. All were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture and exposure

H295R human adrenocortical carcinoma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured according to the standardized protocol approved by the OECD.[28] Briefly, cells were routinely grown in 75cm^2 culture flasks containing DMEM/F12 (without phenol-red, Sigma, Zwijndrecht, The Netherlands) supplemented with 1.2g/l $NaHCO_3$, 1% Insulin Transferrin Selenium (ITS+ premix), and 2.5% NuSerum (BD Biosciences, Bedford, MA) at 37°C and 5% CO_2 atmosphere. Cells were subcultured when 80% confluency was reached. For subcultivation, cells were washed twice with PBS, detached using trypsin-EDTA (0.25/0.05%, v/v) (Difco, NJ), and seeded in a 1:3 ratio. After thawing frozen stocks (passage 5), cells were cultured for at least four additional passages prior to testing and cells were not used after passage 13. For experiments, 3 ml of 3×10^5 cells/ml was seeded in 6-well plates (Greiner bio-one, Frickenhausen, Germany). After 24h, medium was replaced by 2 mL exposure medium containing a single POP or a mixture thereof dissolved in DMSO. The final concentration of DMSO in the medium was 0.25%. Three independent experiments were performed and in each experiment three concentrations were tested for both the single POPs and POP mixtures. Concentrations were chosen in two steps, a first selection of concentrations was based on literature study, followed by cytotoxicity testing to ensure experiments were performed at non-toxic concentrations. Two mixtures of different combinations of these compounds were tested, one with only four compounds which all affected steroidogenesis (mixture A) and one with all eight compounds (mixture B). Mixtures A1 and B1 consist of the middle concentration of the concentration range chosen for the individual POP tests based on the absence of cytotoxicity. So mixture A1 consisted of TCDD (30 nM), PCB 126 (3 µM), PCB 153 (3 µM) and PFOS (100 µM) and mixture B1 consisted of these 4 POPs and HBCD (300 nM), BDE-47 (300 nM), TBT (10 nM), and MeHg (30 nM). Mixture A1 or B1 were 3 times diluted to obtain mixture A3 or B3 and 10 times diluted to obtain mixture A10 or B10. Forskolin, a known adenylate cyclase inducer, and prochloraz, a known inhibitor of multiple CYPs involved in steroidogenesis such as CYP17, were run as positive and

negative controls. After 48h of exposure, as suggested by the OECD, the medium was collected and stored at –80°C until hormone analysis. The cells were immediately lysed in Trizol (Invitrogen, Breda, The Netherlands), transferred to a fresh vial, and stored at –80°C until RNA extraction.

Cell viability assays

For cytotoxicity testing, cells were seeded in a 96-well plate using 100 µL of 3×10^5 cells/ml. After 24h, medium was replaced by 200 µL medium containing the individual POPs or mixtures thereof in triplicate. The highest concentrations tested were 100 nM for TCDD, 10 µM for both PCBs, 600 µM for PFOS, 1 µM for HBCD, 1 µM for BDE-47, 100 nM for TBT, 100 nM for MeHg, and the highest test concentration of both mixtures. Cytotoxic effects from three independent experiments were compared to the solvent control (SC) and Triton X-100 (0.1%) was included as a positive control. After 48h, cytotoxic effects of the test compounds were evaluated with two cytotoxicity assays that measure different endpoints. The assays applied were the WST-1 cytotoxicity assay (Roche Diagnostics, Mannheim, Germany) based on the activity of mitochondrial dehydrogenase enzymes and the ATPlite assay (PerkinElmer, Groningen, The Netherlands) based on the principle that ATP is present in all metabolically active cells. For the WST-1 assay, 20 µl of WST-1 reagent (WST-1 kit content) was added, and after 1h absorbance was measured at 450 nm using a Synergy HT multi-detection microplate reader (BioTek Instruments Inc.). For the ATPlite assay, 100 µl medium was removed from every well, and 50 µl mammalian cell lysis buffer (ATPlite kit content) was added. After 5-min incubation on an orbital plate shaker, 50 µl of substrate solution (ATPlite kit content) was added, and the plate was shaken again for 5 min. After 10-min incubation in the dark, luminescence was measured at 590 nm using the same microplate reader. To ensure that further experiments would not be performed at cytotoxic concentrations, the highest concentrations included did not deviate more than 20% from the SC in either the WST-1 or the ATPlite assay.

LC-MS/MS hormone analysis

Hormone levels of pregnenolone, 17α-OH-pregnenolone, progesterone, 17α-OH-progesterone, DHEA, androstenedione, testosterone, 11-deoxycorticosterone,

corticosterone, 11-deoxycortisol, and cortisol were measured in the cell culture medium following a method previously described.[31] Calibration standards were prepared by spiking 900 µl of supplemented DMEM/F12 medium with a mix of steroid standards, resulting in concentrations of 0; 10; 25; 50; 100; 250; 500; 1000; 2500; 5000; 10,000; 25,000; 50,000 and 100,000 pg/mL. Standards as well as samples (also 900 µL) were spiked with 22.5 µL ^{13}C labelled internal standard mix (10 ng/mL) and filled up to 1 mL with MilliQ water. Calibration standards and samples were subjected to solid-phase extraction (SPE) using OASIS HLB cartridges (Waters, 60 mg) in 96-wells format previously conditioned with methanol and Milli-Q water. After washing with Milli-Q water, methanol/water/acetic acid (55:43:2, v/v/v %), methanol/water/25% ammonia (30:62:8, v/v/v %), and acetonitrile/water (35:65, v/v %) the free steroids were eluted with acetone. The eluate was evaporated to dryness at 45°C under nitrogen, and picolinoyl derivatisation was achieved by incubating the dried sample extracts with 35 µL of derivatisation reagent (see chemicals section) for 45 min at room temperature. The reaction was terminated by adding a 5% ammonia solution and samples were directly analyzed on the LC-MS/MS as described by Blokland *et al*,[32] with a change in the gradient of the LC mobile phase. The gradient was: 0-0.2 min 5% B (90/10 v/v-% acetonitrile/0.1% formic acid), 0.2-0.5 min, linear increase to 20% B, 0.5-3.5 min, linear increase to 80% B, 3.5-3.6 min, increase to 95% B with a hold of 0.45 min, after which the gradient returned in 0.05 min to 5% B with a final hold of 0.9 min. For androstenedione and 11-deoxycortisol analysis samples were diluted with 200 µL tetrahydrofuran/ammonia (35:50, v/v %) before measurement. Steroid hormone concentrations were calculated using a linear calibration curve and corrected for loss during the sample extraction based on the isotope labelled internal standard. Accuracy was calculated by dividing the mean of the determined concentration by the actual concentration times 100% and should be between 70 – 130%. The precision of the method was calculated as the coefficient of variation (CV) and should not exceed 30%. The range was set at the concentrations of the calibration curve where the method could operate with acceptable accuracy, precision, within the linear range of the curve (r^2 > 0.99), and with a signal-to-noise (S/N) ratio greater than 10. The limit of quantification (LOQ) was set at the lowest value of the range and samples with an S/N ratio lower than 10 were assigned a value equivalent to the LOQ in order to calculate fold changes.

Hormone measurement EIAs

17β-Estradiol and aldosterone levels in medium were analyzed using enzyme immunoassays (EIAs) according to the manufacturer's recommendations. Cross-reactivity of the 17β-estradiol kit (Oxford Biochemical Research) reported by the manufacturer was 1.0% for testosterone and lower for other hormones. Cross-reactivity for the aldosterone measurement (Cayman Chemical) was reported to be 1.1% for corticosterone and lower for other hormones. Prior to hormone analysis, the medium samples were thawed and kept on ice. The kits were modified by replacing the standard curve with standards prepared in cell culture medium. Data were linearized using a logit transformation followed by a linear regression fit. Accuracy and precision (see LC-MS/MS hormone analysis) were calculated for both assays.

RNA isolation and cDNA synthesis

Total RNA was isolated according to the manufacturer's (Invitrogen) instructions using a chloroform / isopropyl alcohol extraction. RNA was dissolved in Milli-Q water and the RNA concentration was measured using a NanoDrop ND-1000 UV–vis spectrophotometer (Isogen, Maarssen, The Netherlands). Samples with a 260/280 value around 2.0 and with a 260/230 ratio > 2.0 were regarded to be of good quality resulting in good PCR results. First-strand cDNA was synthesized from 1 μg of total RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Germany) following the supplier's protocol.

Real-Time quantitative PCR

Primer sequences for *CYP1A1* and 7 key enzymes involved in steroidogenesis were retrieved from literature or from the Harvard PrimerBank (http://pga.mgh.harvard.edu /primerbank) (Table S1). Primers were tested for specificity by BLAST analysis using NCBI PrimerBLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast). Real-time quantitative PCR (qPCR) was performed using SensiMix SYBR (GC Biotech, Alphen A/D Rijn, The Netherlands) and a CFX384 Real-Time PCR detection system (Bio-Rad laboratories BV, Veenendaal, The Netherlands). 2 μL cDNA was used in a total volume of 8 μL, with a primer concentration of 600 nM. The following thermal cycling

conditions were used: 10 min at 95°C, followed by 40 cycles of 95°C for 10s and 60°C for 15s. Melting curve analysis was performed to assure no non-specific signals were detected. Relative expression was calculated using a three-fold dilution series of pooled cDNA to correct for the efficiency of the PCR reaction. The reactions were performed in duplicate and all samples were normalized to *HPRT* (hypoxanthine phosphoribosyltransferase) expression. Relative changes in gene expression levels were expressed as fold change compared to the SC.

Statistical analysis

Statistical significance of reduced cell viability was calculated using an unpaired Student's *t*-test. Differences between treatments and SCs for hormone levels and gene expression analysis were calculated as fold change compared to the SC and evaluated using a one way ANOVA followed by a two-sided Dunnett's test. Differences were considered significant at a p-value < 0.05 and highly significant at a p-value < 0.01. Statistical analyses were performed with IBM SPSS Statistics version 20.

Results

Cell viability

Upon exposure of H295R cells no decrease in cell viability was observed with either the WST-1 or the ATPlite assay for any of the concentrations tested for TCDD, PCB 126, PCB 153, HBCD, BDE-47, TBT, MeHg, and both mixtures. PFOS showed decreased viability for concentrations ≥ 300 µM (data not shown). The highest non-cytotoxic concentration of PFOS in our experimental setup (200 µM) was used for further experiments.

Quality controls

The basal hormone productions in H295R cells exposed for 48h to the SC can be found in Table 1. Production was highest for 11-deoxycortisol (200 ng/mL), followed by androstenedione (43 ng/mL), and lowest for 17β-estradiol (143 pg/mL) and progesterone (600 pg/mL). The LOQ, reported as the lower range, was low enough for all hormones to significantly determine down-regulation compared to the SC (Table 2A and Fig. 1).

Table 1. Hormone production in H295R cells (absolute and as percentage of the total hormone production) and the range, precision and accuracy of the measurements from three independent experiments.

Hormone	Range standard curve (pg/mL)[a]	Maximum RSD[b] (%)	Mean accuracy (%)	Hormone levels (pg/mL)[c]	Relative hormone levels (%)[d]
Pregnenolone	2,500 – 25,000	12	100 – 125	5,229 ± 274	1.7 ± 0.2
17α-OH-Pregnenolone	2,500 – 25,000	13	89 – 105	9,640 ± 472	3.1 ± 0.3
Progesterone	50 – 10,000	29	81 – 111	600 ± 52	0.2 ± 0.0
17α-OH-Progesterone	25 – 25,000	26	87 – 130	4,360 ± 221	1.4 ± 0.1
DHEA	1,000 – 25,000	31	94 – 127	3,538 ± 154	1.2 ± 0.1
Androstenedione[e]	500 – 100,000	8	78 – 105	42,897 ± 4073	14 ± 2.3
Testosterone	100 – 10,000	9	94 – 118	1,689 ± 305	0.6 ± 0.2
17β-Estradiol[f]	20 – 2,000	16	94 – 112	143 ± 8	0.04 ± 0.0
11-Deoxycorticosterone	50 – 10,000	11	93 – 125	9,383 ± 163	3.1 ± 0.1
Corticosterone	500 – 25,000	24	93 – 117	3,616 ± 515	1.2 ± 0.3
Aldosterone[f]	7.8 – 1,000	17	97 – 108	606 ± 104	0.2 ± 0.1
11-Deoxycortisol[e]	2,500 – 100,000	11	89 – 108	200,451 ± 13,120	66 ± 7.4
Cortisol	250 – 25,000	30	92 – 107	24,402 ± 5,704	8.0 ± 3.2

[a] Range was determined as those concentrations of the calibration curve where the method could operate with acceptable precision, accuracy and within the linear range of the curve ($r^2 > 0.99$), and with a signal-to-noise (S/N) ratio greater than 10.
[b] Relative standard deviation (RSD) is calculated as a measurement of precision.
[c] Basal hormone levels measured in culture medium after 48h exposure to the solvent control (0.25% DMSO) (mean ± SE).
[d] Relative hormone levels (%) compared to the sum of all measured basal hormones levels.
[e] Androstenedione and 11-deoxycortisol levels were measured after dilution of the samples.
[f] 17β-Estradiol and aldosterone levels were measured using an EIA kit.

The hormone measurements for 17β-estradiol and testosterone were in compliance with the requirements of the OECD guidelines regarding LOQ, fold change, precision and accuracy.[28] Forskolin significantly induced the levels of all hormones, except progesterone, as well as the gene expression levels of all measured genes involved in steroidogenesis (Table 2A and 2B). Prochloraz significantly reduced all hormone levels, except for pregnenolone and 11-deoxycorticosterone, while none of the gene expression levels were decreased (Table 2A and 2B).

Table 2A. Hormone production in H295R cells exposed for 48h to 10 µM forskolin or 1 µM prochloraz from three independent experiments.

	Forskolin		Prochloraz	
Hormone	Fold change[a]	SE	Fold change[a,b]	SE
Pregnenolone	2.1*	0.2	1.2	0.1
17α-OH-Pregnenolone	6.4**	1.0	< -5.1*	1.2
Progesterone	1.3	0.1	10.0**	0.6
17α-OH-Progesterone	3.8**	0.8	-2.0**	0.4
DHEA	28.8**	9.6	< -5.8*	2.3
Androstenedione	6.5**	1.1	-31.9**	5.5
Testosterone	3.6*	0.8	< -17.9*	6.1
17β-Estradiol	11.0**	1.6	-2.6**	0.4
11-Deoxycorticosterone	2.6**	0.1	-1.1	0.1
Corticosterone	6.6**	1.9	< -7.1**	1.9
Aldosterone	3.9**	0.7	-3.5**	0.3
11-Deoxycortisol	3.1**	0.6	-16.0**	4.6
Cortisol	4.8**	0.7	< -57.1*	17.7

[a] Fold changes are calculated compared to the DMSO solvent control.
[b] Fold induction indicating smaller than (<) are calculated using the limit of quantification.
* p value < 0.05 ** p value < 0.01

Table 2B. Gene expression changes in H295R cells exposed for 48h to 10 µM forskolin or 1 µM prochloraz from three independent experiments.

	Forskolin		Prochloraz	
Gene	Fold change[a]	SE	Fold change[a]	SE
CYP1A1	1.5	0.3	6.7**	1.5
MC2R	29.5**	11.7	2.2	1.1
StAR	6.4**	0.6	-1.1	0.1
CYP11A1	3.1**	0.1	1.0	0.1
HSD3B2	28.4**	5.0	1.2	0.2
CYP11B1	131.2**	40.3	1.1	0.3
CYP11B2	29.1**	7.3	2.0	0.4
CYP19A1	18.4**	3.9	1.8*	0.2

[a] Fold changes are calculated compared to the DMSO solvent control.
* p value < 0.05 ** p value < 0.01

Effects of POPs on Steroid Synthesis

Three POPs (HBCD, BDE-47 and MeHg) did not significantly influence any of the hormone levels (Fig. 1 and Table S2). Interestingly, PFOS had the most pronounced effects of all POPs tested and altered almost all hormones of the steroid biosynthesis pathway. The hormones at the end of the pathway, aldosterone, cortisol, testosterone and 17β-estradiol, as well as corticosterone and androstenedione, were up-regulated after exposure to PFOS. Hormones in the beginning of the pathway, 17α-OH-pregnenolone, pregnenolone and 11-deoxycorticosterone were significantly down-

regulated. TCDD and PCB 126 significantly up-regulated progesterone (1.4-fold) and 17β-estradiol (1.8-fold), respectively. PCB 153 and TBT did not up-regulate any hormone levels, but decreased several hormone levels. PCB 153 had the most pronounced effect on 17α-OH-progesterone (1.8-fold down-regulation) and testosterone (1.5-fold down-regulation). TBT down-regulated pregnenolone (-1.7-fold), progesterone and 17α-OH-progesterone (both -1.5-fold). Mixtures A and B had comparable effects and each raised or lowered levels of several of the hormones produced (Fig. S1). 17α-OH-Pregnenolone and DHEA were significantly down-regulated after exposure to mixtures of POPs, and progesterone, 17β-estradiol and corticosterone levels were significantly increased. In addition, mixture B decreased pregnenolone levels and raised aldosterone levels.

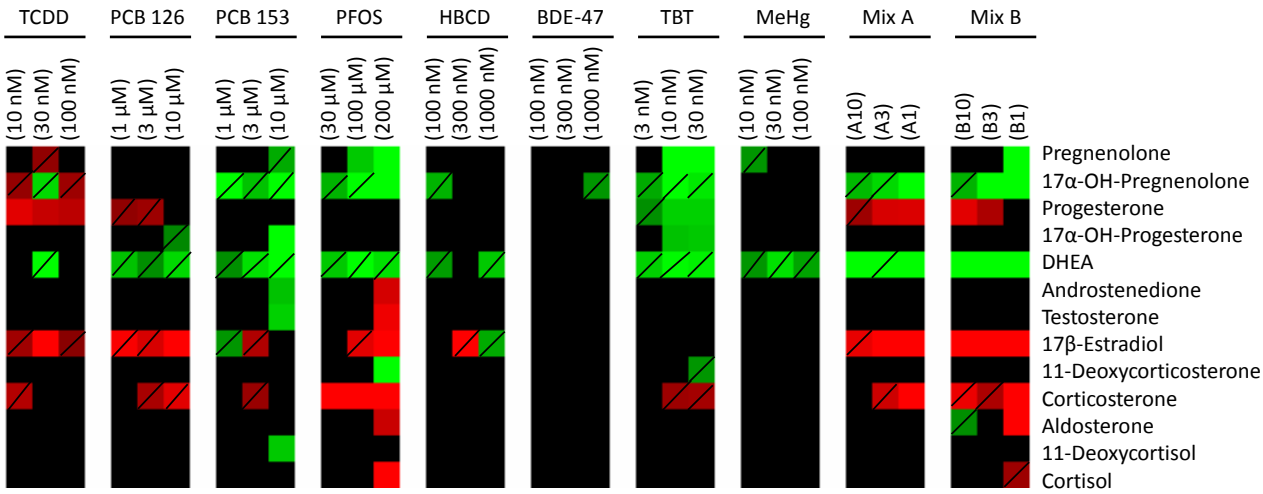

Figure 1. Heat map showing changes in individual hormone levels after exposure of H295R cells for 48h to eight POPs and two mixtures thereof as determined by LC-MS/MS or EIA. Color scale ranges from increased (red) to decreased (green) steroid levels. The brightest color intensity indicates ≥ 2 fold change; black spots represent a fold change between -1.3 and 1.3. Colored boxes with a strikethrough represent non-significant changes in hormone levels, red or green boxes without strikethrough indicate statistically significant changes ($p < 0.05$). Values represent three independent experiments.

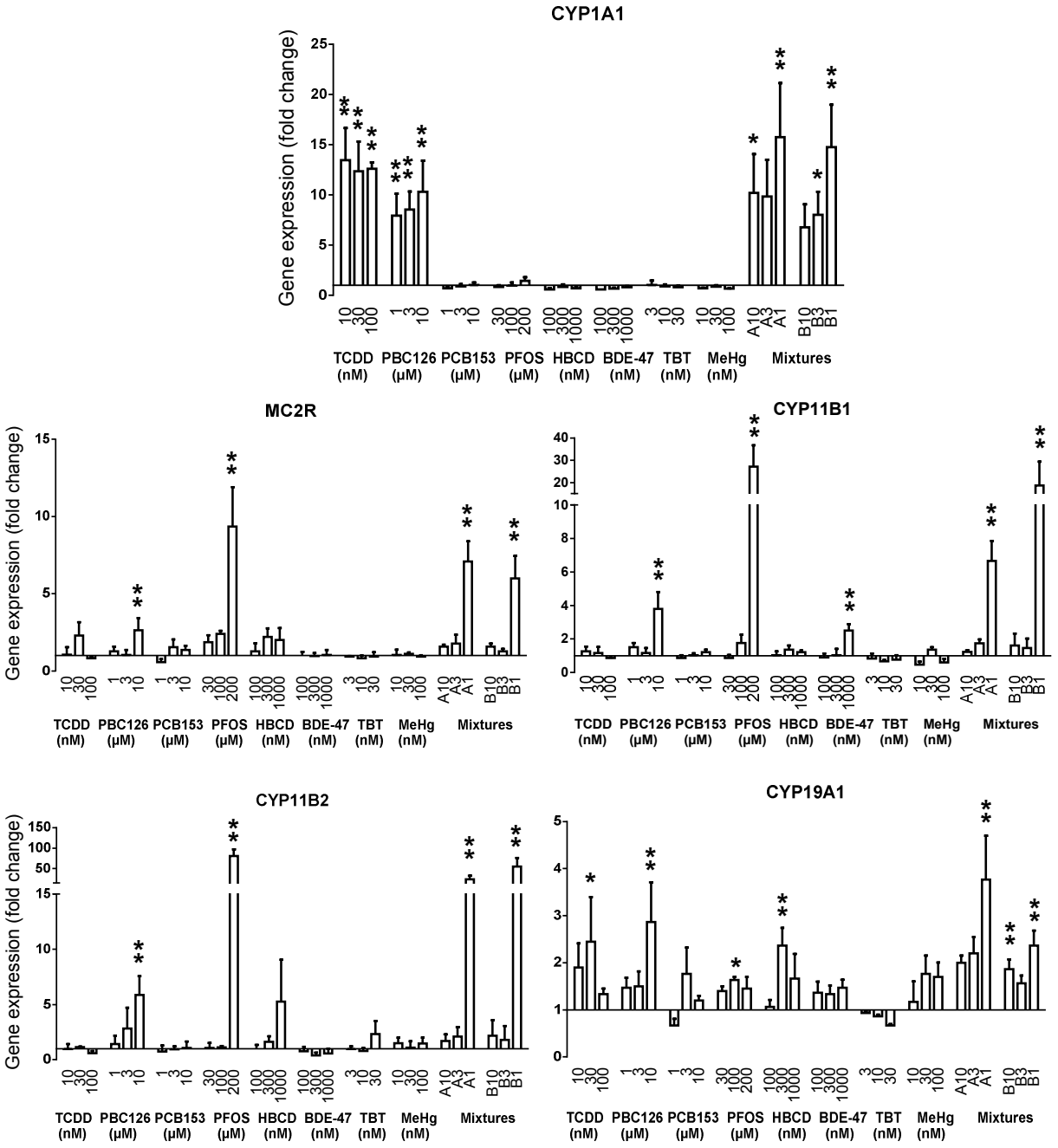

Figure 2. Gene expression changes in H295R cells exposed for 48h to eight POPs and mixtures A and B. Gene expression levels were normalized for *HPRT* expression. Values represent fold change (mean + SE) compared to the solvent control (0.25% DMSO) from three independent experiments. * p-value < 0.05 ** p-value < 0.01

Effects of POPs on Steroid Related Gene Expression Levels

Fig. 2 gives an overview of the changes in gene expression levels found. *CYP1A1*, an indicator for exposure to AhR agonists such as dioxin-like compounds, was up-regulated after exposure to TCDD, PCB 126 and both mixture A and B. Expression levels of the steroid related genes *MC2R* (3-9 fold change), *CYP11B1* (3-27 fold change), and *CYP11B2* (6-81 fold change) were up-regulated by PCB 126 (10 μM), PFOS (200 μM) and both mixtures (highest concentration). Furthermore, PCB 126 and mixture A and B up-regulated *CYP19A1* resulting in a 2.9-, 3.9-, and 2.3-fold induction, respectively. No significant changes in expression levels were found for *StAR*, *CYP11A1*, and *HSD3B2* after exposure to any of the tested POPs (Fig. S2).

Discussion

The effects of persistent organic pollutants (POPs) and two mixtures thereof on steroidogenesis were studied using the enhanced H295R screening assay. For this purpose, 13 steroid hormone levels and 7 steroid-related genes were measured.

In absolute quantities the H295R cells produced higher levels of adrenal steroids than of sex steroids, which is in accordance with the basal hormone levels reported by Xing *et al.*[33] Forskolin and prochloraz also gave comparable changes in hormone levels and gene expression levels as reported in previous studies,[27,34] with the exception that prochloraz did not decrease gene expression levels after 48h exposure. Earlier research reported small decreases in expression levels of steroidogenic related genes, but not for all genes a dose-response relationship was observed.[35] In our study prochloraz, which is a known AhR agonist, did induce *CYP1A1* as was reported before.[36] The main endocrine modulating mode of action of prochloraz, however, is not by inducing *CYP1A1* but by decreasing *CYP17* and *CYP21* activity.[35] All three test concentrations of both dioxin-like compounds (TCDD and PCB126) induced *CYP1A1* expression approximately ten-fold, suggesting that the lowest concentrations already induced maximal AhR activation. Since TCDD and PCB 126 induced very different steroidogenic profiles, this suggests that

the hormone alteration mechanisms in H295R cells are not, at least not solely, AhR related. This observation is in agreement with previous research.[37]

The compounds HBCD, BDE-47, and MeHg did not significantly alter any hormone level, which is in accordance with the finding that they hardly changed expression of genes involved in steroidogenesis. Previous research showed that metabolites of BDE-47 can affect steroidogenesis in H295R cells.[38,39] It is known that H295R cells possess little CYP1B activity,[40] which is apparently not enough to produce metabolites of BDE-47 for measurable effects on steroidogenesis. The limited production of OH-metabolites *in vitro* has been shown before.[41] In our experiments MeHg (up to 100 nM) had no effect, while Knazicka *et al*[42] reported lowered testosterone production in H295R cells exposed to ≥ 1 μM mercury chloride. It cannot be excluded that the MeHg concentrations in our study (up to 100 nM) were below effect levels.

TCDD had little effect in the H295R cells, while TCDD exposure in humans is negatively correlated with testosterone levels.[43,44] *In vivo* TCDD has been shown to decrease hormone production in mouse antral follicles[45] and the number and size of Leydig cells in rats, which is likely to affect testosterone production.[46] TCDD might affect steroidogenesis by interfering with the hypothalamic-pituitary-adrenal (HPA) axis, but these effects cannot be investigated by the current H295R cell model.

PCB 126 exposure increased CYP19A1 expression which corresponds with previous research.[47] This also is in agreement with the increased 17β-estradiol levels we found, comparable to another study, exposing H295R cells for 48h to 4 μM PCB126.[15] In addition, Kraugerud and colleagues reported increased aldosterone and small increases in cortisol and progesterone levels. In a different study, exposing H295R cells for 10 days, the cortisol and aldosterone levels increased time-dependently.[48] Therefore, the 48h exposure in the current study could have been too short. In our study PCB 153 not only decreased testosterone levels, which is in accordance to earlier research in H295R cells,[15] but also androstenedione, 17α-OH-progesterone, and 11-deoxycortisol levels.

PFOS induced the most pronounced effects of the tested POPs on steroid hormone levels and gene expression. Earlier studies reported similar effects of PFOS on 17β-estradiol, at lower concentrations (up to 300 nM),[49] and testosterone, at higher

concentrations (up to 600 μM).[16] At lower concentrations than we tested (300 nM), PFOS was shown to decrease testosterone levels in H295R cells.[49] Kraugerud et al. reported no change in cortisol levels after exposure to PFOS,[16] which we did find, but this could also be due to their less sensitive detection method using a radioimmunoassay with cross-reactivity, which is not a problem in LC-MS/MS measurements as we applied.[50] Levels of PFOS in human serum of occupationally exposed workers were as high as 114 μg/mL (= 212 μM), but for the general population median values are not higher than 53 ng/mL (around 100 nM).[51] Indeed, PFOS is negatively associated with serum testosterone levels in humans, but no effects on other reproductive hormones have been shown.[52] Already our lowest test concentration (30 μM) affected steroid hormone production, and this is the first study to show that PFOS has the ability to alter most of the steroid hormone levels and gene expression levels in H295R cells.

The effects of TBT on hormone production and gene expression have, to the best of our knowledge, not been shown before in H295R cells. TBT lowered pregnenolone, 17α-OH-progesterone, and progesterone levels significantly, but in bovine adrenal cells increased 17α-OH-progesterone levels have been seen.[53] The fact that *CYP19A1* gene expression levels were not significantly altered in our study was to be expected, as our exposure concentrations were lower (up to 30 nM) than reported for inhibition of aromatase activity by TBT.[54] The TBT levels reported for human blood, as high as 85 ng/mL (261 nM) with a geometric mean of 6.6 ng/mL (20 nM),[55] are higher than the lowest observed effect concentration of 10 nM in the H295R cells. As *in vivo* studies also indicate that TBT targets multiple steroidogenic tissues, including testosterone levels and steroidogenic enzymes in the testes,[56] endocrine disruption in humans by TBT cannot be excluded.

DHEA levels were significantly decreased after exposure to either of both mixtures, but were not significantly changed after exposure to any of the single POPs. As no cytotoxicity was observed, this suggests an additive effect if this effect can be replicated. Also for 17β-estradiol the mixture effect of the compounds was additive. Mixture B consisted of mixture A plus HBCD, BDE-47, and MeHg that individually did not alter hormone levels, and TBT that individually decreased pregnenolone, 17α-OH-progesterone, and progesterone. The latter were also decreased by exposure to mixture

B, suggesting that TBT is responsible for the difference in response between the mixtures A and B. Aldosterone levels, which were only induced with the highest PFOS concentration tested (200 μM), were significantly increased after exposure to mixture B (PFOS concentration of 100 μM) and not mixture A. Although synergistic effects in mixture B cannot be excluded, the effects might also be explained by addition of effects of the individual compounds just below the level of quantification.

An overview of how POPs modulated the steroidogenic pathway is presented in Figs. 3A and S3. Increased expression of steroidogenesis related genes did not always correspond to increased hormone levels, but only when the gene expression changes were relatively high (>10 fold for *CYP11B1* and *CYP11B2*), indicating that gene expression changes might be more sensitive to POP exposure than changes in hormone levels. Many steroidogenic related genes are still up-regulated after 48h exposure, except *HSD3B2* which was shown to return to basal levels after 48h.[57] Increased hormone levels were always accompanied by increased gene expression levels, suggesting that increased hormone levels are, at least partly, caused by increased gene expression levels which is biologically plausible. On the other hand, decreased hormone levels did not coincide with decreased gene expression levels. This is not surprising, as a decrease in hormone levels is not necessarily due to decreased hormone production, but could also be the consequence of increased conversion into subsequent steroid hormones further down the pathway. This was also shown by the enzyme conversion flows in Fig. 3B. PFOS clearly up-regulates the enzymes toward the end of the pathway (e.g. 11β-hydroxylase and 18-hydroxylase) without lowering the total hormone levels as shown by the stable cholesterol side-chain enzyme. This suggests that PFOS lowers hormone levels by increasing the conversion towards subsequent hormones.

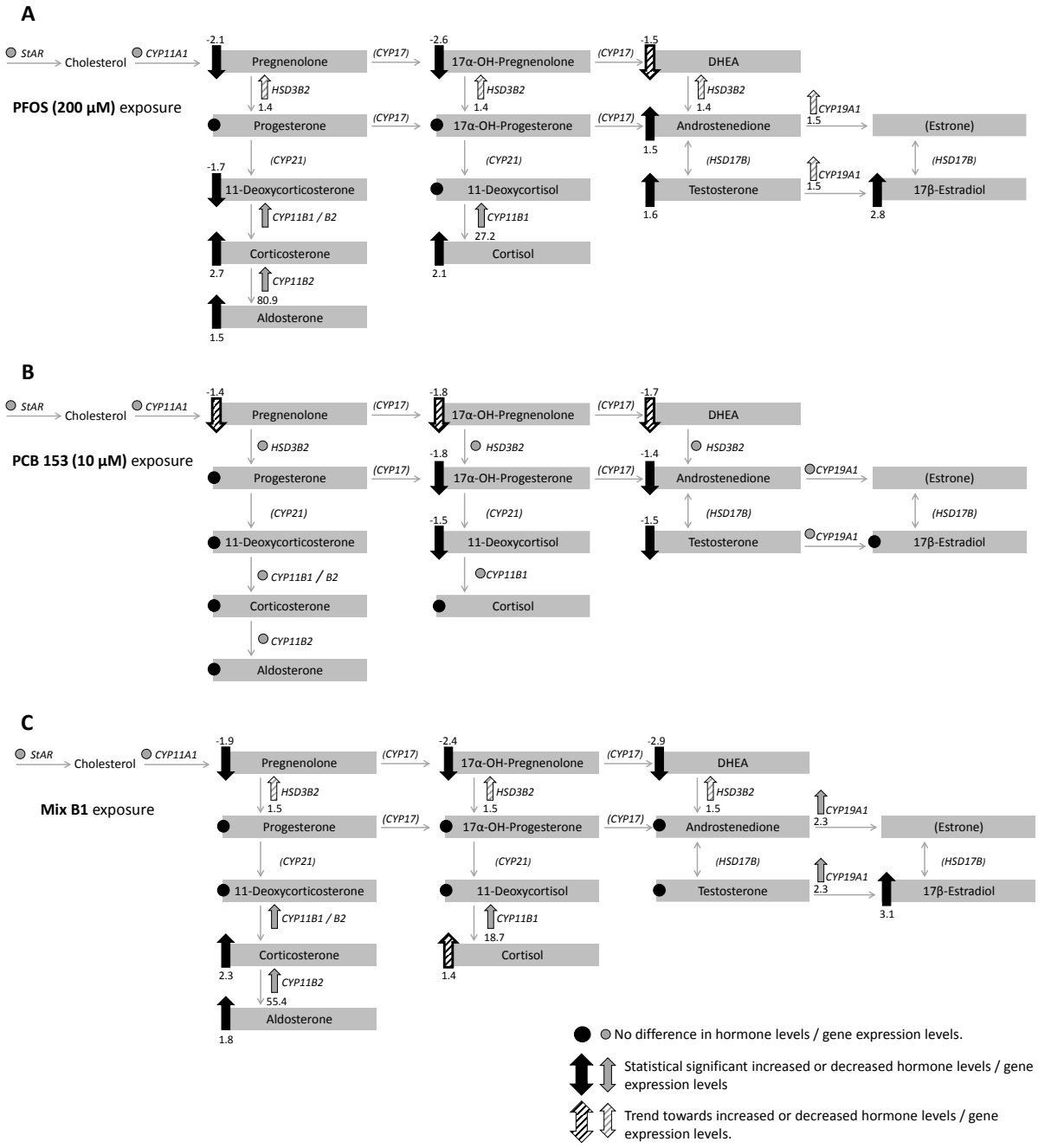

Figure 3A. Steroidogenic pathway in H295R cells exposed for 48h to POPs. Hormones and genes between brackets were not measured. Arrows indicate increased or decreased hormone or gene expression levels after exposure to A) PFOS; B) PCB 153; and C) Mix B1 from three independent experiments.

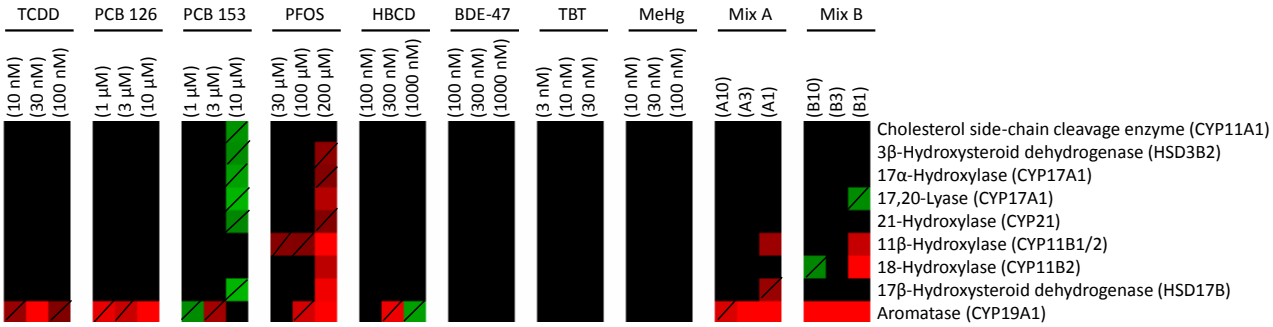

Figure 3B. Heat map showing changes in groups of hormone levels. For each enzyme involved in steroidogenesis, the corresponding hormone levels are grouped. For example, the enzyme encoded by *CYP11A1* (cholesterol side-chain cleavage enzyme) is relevant for all steroid hormones, so the sum of all measured hormones is used in this heat map. The HSD17B enzyme (17β-hydroxysteroid dehydrogenase) is only relevant for 17β-estradiol and testosterone production, so only the sum of these two hormones was calculated. The fold change was then calculated compared to the solvent control. Color scale ranges from increased (red) to decreased (green) enzyme conversion. The brightest color intensity indicates ≥ 2 fold change; black spots represent a fold change between -1.3 and 1.3. Colored boxes with a strikethrough represent non-significant changes in hormone levels, red or green boxes without strikethrough indicate statistically significant changes ($p < 0.05$). Values represent groups of hormones measured in three independent experiments. Absolute numbers are provided in Table S2.

Most *in vitro* studies, including the H295R assay, lack certain *in vivo* features such as the formation of POP metabolites and feedback mechanisms at the level of the HPA axis. For example, in our study melanocortin 2 receptor (*MC2R*) gene expression levels were induced by PCB 126 and PFOS. This receptor is activated by adrenocorticotropic hormone (ACTH)[58] which is not produced by H295R cells. Therefore up-regulation of *MC2R* in this study is not expected to result in a functional effect. Up-regulation of *MC2R in vivo*, however, could result in over-stimulation of the adrenocortical cells in the presence of ACTH, leading to an unbalanced steroid hormone synthesis. These, and other indirect effects among different tissues, make development of an *in vitro* battery of tests to study endocrine disruption with relevance for the *in vivo* situation very challenging, but not impossible as has been reasoned for studying thyroid hormone disruption.[41] In addition to multiple effect mechanisms, also multiple xenobiotic compounds, including their bioactive OH-metabolites, are present together in real life and, as shown in this study, may lead to additive effects. For example, average PCB serum

levels in humans are around 210 ng/mL (approximately 0.6 μM),[59] which is only about 10 times lower than the lowest observed effect concentration of 10 μM in our experiments for the individual compounds PCB 126 and PCB 153. Therefore, it cannot be excluded that human steroidogenesis can be affected by PCBs and related (OH-) POPs, present as mixtures at effect levels.[3]

In conclusion, this study shows that ubiquitous seafood POPs, such as TCDD, PCB 126, PCB 153, PFOS, TBT and mixtures thereof, are capable of altering steroidogenesis in H295R cells at non-cytotoxic and relevant concentrations. The overall effects of the mixtures in this study suggest additivity, not synergy. As POPs have been reported to affect steroidogenesis via several mechanisms in different tissues, adverse effects in humans cannot be excluded.

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Supporting Information

Primer sequences and their product length are provided in Table S1. Hormone levels measured after exposure to the POP mixtures are presented in Fig. S1. Gene expression data of genes not affected by any of the POPs are displayed in Fig. S2. Table S2 contains information of all tested POPs and their corresponding hormone levels, which is the basis of both heat maps (Figs. 1 and 3B). Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tiv.2015.03.002.

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CHAPTER 3A

Comprehensive DNA methylation and gene expression profiling in differentiating human adipocytes

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Abstract

Insight into the processes controlling adipogenesis is important in the battle against the obesity epidemic and its related disorders. The transcriptional regulatory cascade involved in adipocyte differentiation has been extensively studied, however the mechanisms driving the transcription activation are still poorly understood. In this study we explored the involvement of DNA methylation in transcriptional regulation during adipocyte differentiation of primary human mesenchymal stem cells (hMSCs). Genome-wide changes in DNA methylation were measured using the Illumina 450K BeadChip. In addition, expression of 84 adipogenic genes was determined, of which 43 genes showed significant expression changes during the differentiation process. Among these 43 differentially expressed genes, differentially methylated regions (DMRs) were detected in only 3 genes. By comparing genome-wide DNA methylation profiles in undifferentiated and differentiated adipocytes 793 significant DMRs were detected. Pathway analysis revealed the adipogenesis pathway as the most statistically significant, although only a small number of genes were differentially methylated. Genome-wide DNA methylation changes for single probes were most often located in intergenic regions, and underrepresented close to the transcription start site. In conclusion, DNA methylation remained relatively stable during adipocyte differentiation, implying that changes in DNA methylation are not the underlying mechanism regulating gene expression during adipocyte differentiation.

Introduction

Obesity, a condition characterized by excessive accumulation of adipose tissue, is a critical issue, causing a wide range of adverse health effects. Adipose tissue accumulation results from an increased size as well as number of adipocytes. Adipocyte numbers increase when stem cells are triggered to differentiate into the adipogenic pathway.[1] Adipocytes mainly arise from mesenchymal stem cells (MSCs) through a well-orchestrated process of transcriptional regulation. *C/EBPA* and *PPARG* are key regulators that are responsible for the activation of a number of adipocyte-specific genes.[2] The exact mechanisms underlying transcriptional activation are only partially elucidated. In embryonic stem cells, DNA methylation is associated with differentiation[3] and *de novo* methylation rapidly silences genes related to pluripotency.[4] Less is known about the mechanisms responsible for adult stem cell differentiation. DNA methylation is known to be related to gene expression, but its causality in this relationship is yet not fully understood. In general, it is thought that methylation in the immediate vicinity of the transcription start site (TSS) blocks initiation, but this is dependent on which transcription factors are binding.[5,6] Methylation in the gene body is currently considered to influence gene expression by stimulating transcription elongation and alternative splicing.[7] The role of DNA methylation in intergenic regions (IGR) is only poorly understood, but methylation in repetitive regions is considered important for genomic stability.[5]

DNA methylation changes during adipocyte differentiation have been reported, indicating this might be one of the underlying mechanisms of transcriptional regulation.[8] CpG dinucleotides located in the *LEP*,[9-11] the *GLUT4*,[12] and the *PPARG2* promoter[10,13] become hypomethylated during differentiation. The fact that obesity is associated with DNA methylation changes[14,15] implies that DNA methylation changes can be relevant for human health. To our knowledge, only one genome-wide study was performed addressing DNA methylation in relation to gene expression during the differentiation into adipocytes.[16] Takada *et al.* found methylation changes when adipose derived stem cells were induced to become pluripotent stem cells, but only few changes were detected upon differentiation. In our study we tested cells from a different origin,

namely pluripotent MSCs which can differentiate into adipocytes, chondrocytes, and osteoblasts. When MSC differentiate into adipocytes they first go through the so called 'commitment phase' where the cells become preadipocytes. Subsequently the preadipocytes enter the actual differentiation phase where they mature into functional adipocytes. Fully understanding the normal development of MSCs into mature adipocytes is crucial to study the suggested role of environmental and lifestyle factors (e.g. diet, exercise, pollutants) in triggering of and interfering with adipocyte differentiation, possibly resulting in obesity and adverse health effects.

In this paper we report for the first time genome-wide DNA methylation profiles in primary bone marrow-derived human MSCs (hMSCs) preceding through both the commitment and differentiation phase of adipocyte differentiation. Compared to previous research we focused not mainly on DNA methylation close to the promoter region or in single CpG sites, but emphasized the identification of differentially methylated regions (DMRs), without predefining these regions. DMRs are stretches of DNA with different methylation patterns compared to the control samples, and can provide insight into important biological processes. Furthermore, we determined the relationship between DNA methylation and gene expression of 84 relevant adipogenic genes.

Materials and methods

Cell culture and differentiation

Poietics™ human mesenchymal stem cells (hMSC; Lot No 2F3478) were obtained from Lonza Walkersville Inc. at passage 2. These cells were cultured in MSCGM (Lonza, Walkersville, USA) growth medium, which contained fetal bovine serum (FBS) and L-glutamine, in a humidified incubator with 5% CO2 at 37°C. Cells were subcultured once per week and medium was refreshed once a week. For the differentiation experiments, only cells from passage 9 were used, because DNA methylation profiles were shown to depend of the number of passages.[17] Differentiation experiments were repeated four times to make sure independent results were obtained. Cells were seeded with a density

of 3.800 cells/cm^2 and grown for four days until fully confluent. Differentiation reagents were purchased from Sigma-Aldrich, Zwijndrecht, the Netherlands, unless otherwise specified. Exposure to 5-aza-2′-deoxycytidine (5-aza-dC, 1 µM), used as a positive control for DNA hypomethylation and dissolved in DMSO, was started within 24h after seeding and was continuously present during differentiation (Fig. 1A). Differentiation was induced (at day 0) with an adipogenic induction medium for hMSCs consisting of DMEM supplemented with L-glutamine (1%), FCS (10%), dexamethasone (1 µM), human insulin (5 µg/mL; Insuman Rapid (100 IE/mL), pharmacy), 3-isobuty-l-methyl-xanthine (IBMX, 0.5 mM), and rosiglitazone (1 µM; Sanbio B.V, Uden, the Netherlands). Medium was refreshed twice a week, until day 10 when differentiation was ended.

Quantification of lipid accumulation

At day 10 of adipocyte differentiation, cells cultured in 12-wells plates were fixated with 4% formalin in PBS for 10 minutes. Cells were washed with PBS and Oil-Red-O dye (3 mg/mL, 60% isopropanol, 40% distilled water) was added for 10 minutes. After staining, cells were washed with water multiple times to remove unbound dye and photographed. Subsequently, plates were air-dried. To each well, 350 µL of isopropanol was added to extract the Oil-Red-O. After dissolving, two aliquots of 100 µL were transferred to a 96-well plate and the optical absorbance was measured at 520 nm. Oil-Red-O staining of undifferentiated cells grown in parallel culture was used as a negative control and was subtracted from the measurements. Differences in lipid accumulation between 5-aza-dC treatment and the solvent control were calculated using a student's t-test from four independent experiments.

RNA isolation and cDNA synthesis

Total RNA was isolated from 60 mm dishes at day 0 (just before differentiation was induced), at day 2 and at day 10 of differentiation using Trizol (Invitrogen, Merelbeke, Belgium) according to the manufacturer's instructions (Fig. 1A). RNA was dissolved in Milli-Q water and the RNA concentration was measured using a NanoDrop ND-1000 UV–vis spectrophotometer (Isogen, Maarssen, the Netherlands). First-strand cDNA was synthesized from 1 µg of total RNA using the RT2 First Strand Kit (Qiagen, Venlo, the Netherlands) following the supplier's protocol.

RT² profiler™ PCR assay

The adipogenesis RT2 profiler PCR Array (PAHS-049ZE-4, Qiagen, Venlo, the Netherlands) was used according to the manufacturer's instructions to profile the expression of 84 key genes involved in the differentiation and maintenance of mature adipocytes plus five housekeeping genes. Expression of genes that needed more than 35 cycles to reach the threshold was regarded as below the detection limit. Gene expression data were normalized against the geometric mean of the references genes *B2M* and *HPRT1*, because the other 3 reference genes included did not remain stable over time. Statistically significant differences between days of differentiation were calculated using a student's t-test on the log-transformed values of the expression normalized for the reference gene. Relative changes in gene expression were expressed as fold changes on a $\log_2$ scale compared to day 0. A $\log_2$ fold change of 1 represents a two-fold increase in gene expression, whereas a $\log_2$ fold change of -1 represents a two-fold reduction in expression. When no expression was measured at either day 0, day 2 or day 10 of differentiation, fold changes were calculated with a value of 35 cycles.

Real-Time Quantitative PCR

Primers (Table S1) were tested for specificity by BLAST analysis using NCBI PrimerBLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast). Real-time quantitative PCR (qPCR) was performed using SensiMix SYBR (GC Biotech, Alphen A/D Rijn, The Netherlands) and a CFX384 Real-Time PCR detection system (Bio-Rad laboratories BV, Veenendaal, The Netherlands). 2 µL cDNA was used in a total volume of 8 µL, with a primer concentration of 600 nM. The following thermal cycling conditions were used: 10 min at 95°C, followed by 40 cycles of 95°C for 10s and 60°C for 15s. Melting curve analysis was performed to assure no non-specific signals were detected. Relative expression was calculated using a three-fold dilution series of pooled cDNA to correct for the efficiency of the PCR reaction. The reactions were performed in duplicate and further data analysis was performed similar to the PCR array.

DNA isolation

Genomic DNA was isolated from cells in 60 mm culture dishes just before differentiation started (day 0) and at day 10 of differentiation (Fig. 1A) using the DNeasy blood and

tissue kit (Qiagen, Venlo, the Netherlands). Culture dishes were rinsed twice with PBS, followed by adding 500 µL of mastermix, containing PBS, proteinase K (>60mAU/mL), and RNAse (200 units/mL), and 500 µL lysis buffer (buffer AL). The following steps of the DNA isolation were performed according to the manufacturer's protocol. DNA was eluted in 25 µL AE buffer and the concentration was determined using a NanoDrop ND-1000 UV–vis spectrophotometer.

Genome-wide DNA methylation for single probes

DNA methylation was analyzed using the Illumina Infinium Human Methylation 450K BeadChip (Infinium Inc., San Diego, CA, USA), which addresses 485,512 methylation sites. DNA samples (500 ng) were prepared in 45 µL and the bisulfite conversion (EZ DNA Methylation Kit, Zymo Research, Freiburg, Germany) and the BeadChips were processed by the Human Genotyping Facility of the Erasmus University Medical Centre (Rotterdam, the Netherlands). DNA from the positive control, 5-aza-dC, was pooled from two independent experiments, resulting in two samples measured. From the other samples, the four independent experiments were measured separately. To minimize batch effects, samples were randomly allocated to two chips. Intensity iDAT files were processed using R-based open software (R 3.1.2), including 450K Chip Analysis Methylation Pipeline (ChAMP) (version 1.1.2) and Limma (Linear Models for Microarray Data) (version 3.22.1). Autosomes and the X and Y chromosomes were included in the analysis. ChAMP[18] was used for pre-processing and the normalization of the raw data with SWAN.[19] Probes with a detection *p*-value of > 0.01 in >1% of the samples were filtered out, as well as the probes with a beadcount < 3 in 5% of the samples. Finally, 483,914 probes were included in the analysis and annotated based on the ilmn12.hg19 annotation.[20] The multidimensional scaling (MDS) plot, based on the 1000 most variable probes, showed that one sample clustered separately and was therefore excluded from further analysis. The Limma package was used to identify differentially methylated CpG sites. DNA methylation at day 10 of differentiation was compared to DNA methylation profiles of day 0. The statistical analyses were performed unpaired and adjusted for multiple testing with the Benjamini-Hochberg (BH) method.[21] A false discovery rate (FDR) value of 0.05 was used as the threshold to define statistical

significance. The methylation (β) values were constrained to lie between 0 (completely unmethylated, equal to 0%) and 1 (completely methylated, equal to 100%).

Differentially methylated regions (DMRs)

In addition to the approach of identifying methylation differences in single probes, we used the DMRcate package from Bioconductor to detect DMRs.[22] This method does not use predefined regions, but identifies the most differentially methylated regions across the genome based on tunable kernel smoothing of the differential methylation signal. The measured probes are identified independent of the direction of the methylation change and distance from each other. DMRs are defined as regions of at least 5 probes with a distance of consecutive probes less than 1000 nucleotides. To gain more insight into the biological relevance regarding the genome-wide approach, we further selected DMRs with an absolute difference in methylation of ≥ 5% in at least 1 probe. The statistical tests for the DMRs also were adjusted for multiple testing with the BH method by applying a FDR of 0.05.

Results

Differential expression of adipogenic markers in differentiated hMSCs

To explore gene expression in relation to DNA methylation during adipocyte differentiation, adipogenesis was induced in hMSCs (see Fig. 1A for exposure regime). Oil-red-O staining of the differentiating cells revealed weak lipid accumulation at day 2 while at day 10 clear lipid accumulation was observed in the majority of the cells (Fig. 1B). A panel of 84 adipogenic genes was analyzed with the RT2 profiler PCR Array, but expression levels of 10 genes were below the detection limit (Ct value > 35) and therefore excluded. Of the remaining 74 genes, 43 showed significant ($p < 0.05$) differential expression at either day 2 and/or day 10 of differentiation compared to day 0. Gene expression changes were most pronounced at day 10 compared to day 2 of differentiation. The subset of 43 genes included 26 up-regulated (Fig. 1C / Table S3) and 17 down-regulated genes (Fig. 1D / Table S3) at day 10 of differentiation. The up-regulated genes included key regulators of adipogenesis (*CEBPA* and *PPARG*),

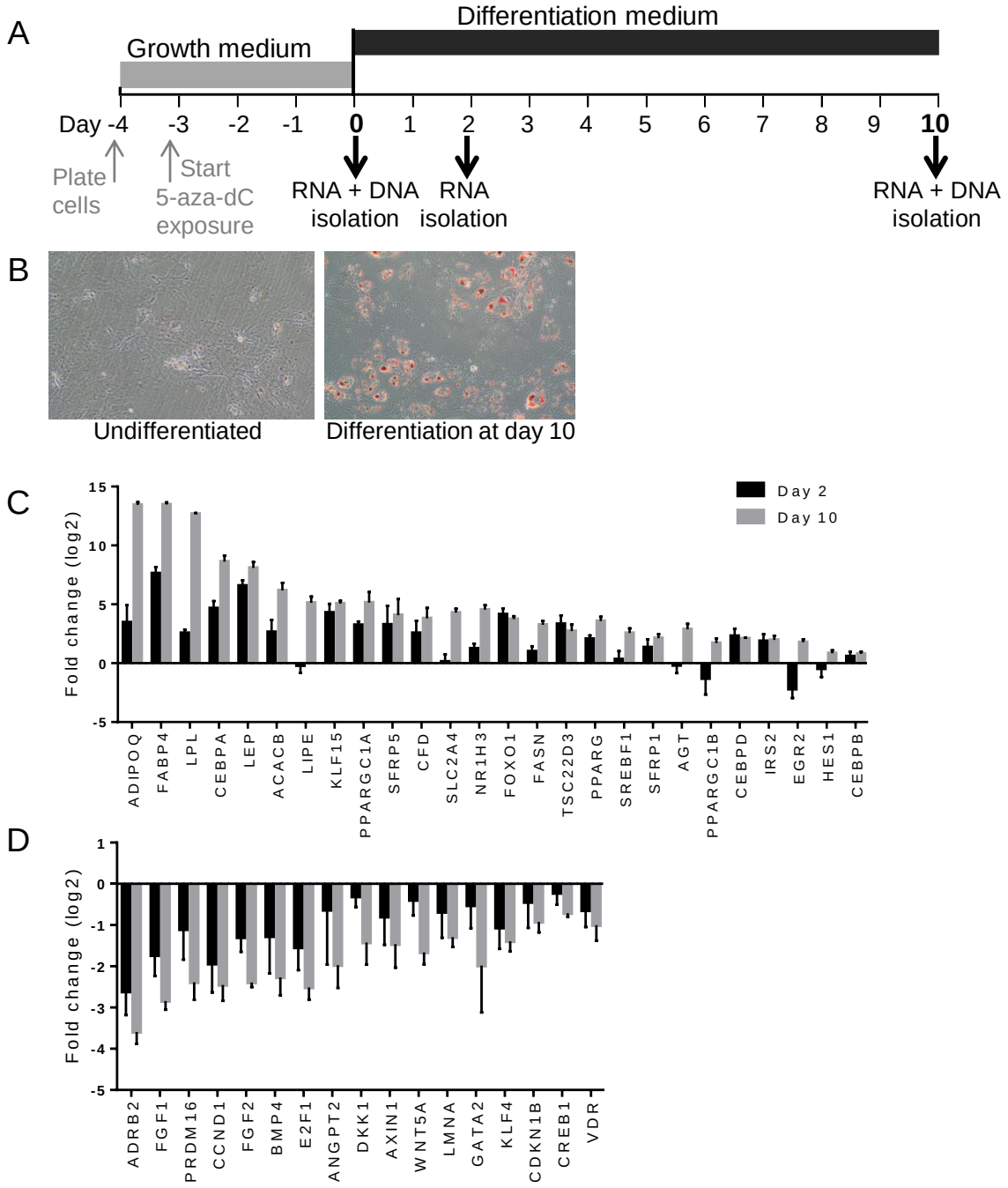

Fig. 1. Adipogenic markers in human mesenchymal stem cells (hMSCs) at day 2 and day 10 of adipocyte differentiation. **A)** Schematic representation of exposure regime. **B)** Representative pictures of Oil-red-O stained lipid droplets in undifferentiated and differentiated hMSCs cells at day 10. **C)** Adipogenic gene expression at day 2 and day 10 of differentiation. Only genes that were significantly up-regulated at day 10 of differentiation ($p < 0.05$) are shown. Expression of 84 genes related to adipogenesis was measured and expression levels were normalized for HPRT1 and B2M expression. Values represent the $\log_2$ fold change (mean + SD) compared to day 0 from three independent experiments. **D)** Relative expression changes for genes that were significantly down-regulated at day 10 of differentiation ($p < 0.05$), together with the fold changes at day 2. Fold changes of all measured genes with their level of significance can be found in Table S3.

adipokines (*ADIPOQ*, *CFD*, and *LEP*), various PPARG target genes (e.g. *FASN*, *IRS2*, *LIPE*) and multiple pro-adipogenic genes relevant for the late stage of adipocyte differentiation (e.g. *ACACB*, *FABP4*, *SLC2A4*). Fluctuating results were observed for pro-adipogenic genes that are important for the commitment or early stage of adipogenesis. *EGR2*, an immediate early gene responsible for up-regulation of *CEBPB*, was down-regulated at day 2 and up-regulated at day 10. The early stage anti-adipogenic genes *CDKN1B* and *GATA2* were down-regulated upon differentiation as was the late stage anti-adipogenic gene *ADRB2*. Our data revealed that expression of most *WNT*s, known to block induction of key adipogenic transcription factors, was below the detection limit and only *WNT5A* expression levels were measurably reduced during the differentiation process.

In short, hMSCs successfully differentiated into mature adipocytes as shown by the lipid accumulation and the expression profiles of the adipogenic genes.

No clear association between gene expression and DNA methylation of known adipocyte-related genes

Genome-wide DNA methylation was examined by applying Illumina 450K BeadChip analysis, comparing hMSCs at day 0 to differentiated adipocytes at day 10. Day 10 was chosen based on the pronounced gene expression changes and because DNA is expected to be a long-term epigenetic mark. None of the 483,914 single probes were statistically significant different (adjusted *p*-value < 0.05) in any of the above described 84 adipogenic genes after adjusting for the false discovery rate (FDR). We therefore focused on DNA methylation of the top 1000 probes with the lowest non-adjusted *p*-value, and located probes annotated to one or more of the 84 adipogenic genes (Table S2). This revealed 7 probes that can be allocated to 5 different genes. The probe with the lowest non-adjusted *p*-value (p = 2.4E-05) was detected in the 5'untranslated region (UTR) of *LEP*.

Although our results did not reveal significantly differentially methylated probes with an adjusted *p*-value < 0.05, we identified 8 DMRs (adjusted *p*-value < 0.05) with multiple consecutive probes in 6 of the 84 earlier mentioned adipogenic genes (Table 1). The 6 adipogenic genes containing DMRs included 3 down-regulated genes, no up-regulated

genes, 2 genes without significant changed expression, and 1 gene with expression below the detection limit (Fig. 2). As explained earlier, the relationship between DNA methylation and gene expression depends on the specific location within or outside the genes. We found that most regions that were differentially methylated during adipocyte differentiation were located in the gene body. *AXIN1* and *PRDM16* contained DMRs with increased methylation in the gene body (Table 1) and these genes showed a concordant decrease in gene expression (Fig. 1D), while *CCND1* showed a decrease in gene body methylation (Fig. 3) together with a decrease in gene expression (Fig. 1D). Furthermore, we detected three DMRs (*FOXC2, GATA3,*and *IRS1*) in close proximity to the TSS or in the first exon of the corresponding genes (Fig. 3). Gene expression for *FOXC2* and *IRS1* was not significantly changed and below the detection limit for *GATA3*.

Overall, a minority of the adipogenic genes were differentially methylated during adipocyte differentiation. DNA methylation changes close to the TSS or in the gene body could not be associated with differential gene expression.

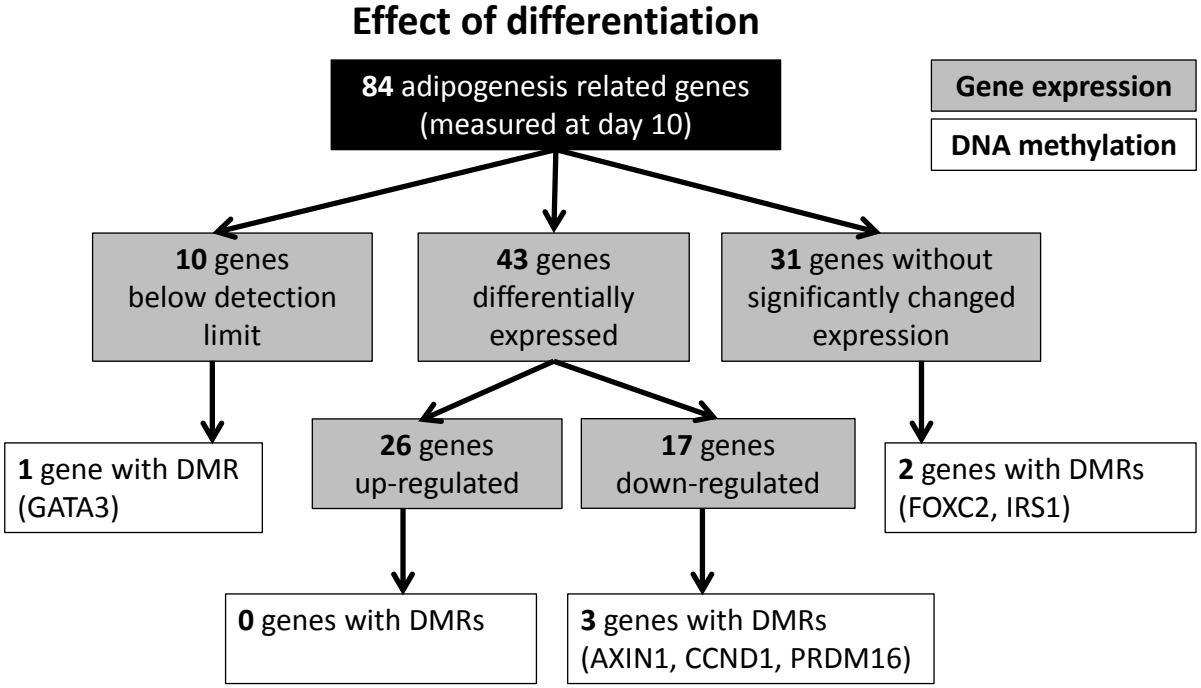

Fig. 2. Results of changes in gene expression (grey boxes) and differentially methylated regions (DMRs) (white boxes) of 84 adipogenesis related genes at day 10 of hMSC differentiation compared to undifferentiated cells at day 0.

Table 1. Differentially methylated regions (DMRs) found with the DMRcate package in the 84 genes related to adipogenesis comparing undifferentiated hMSCs at day 0 to differentiated adipocytes at day 10.

Gene	Chromosome: position[1]	Relation-ship to gene	No. probes in DMR	No. probes on array	*p*-value[2]	DNA methy-lation change[3] (%)	Gene expression
AXIN1	chr16:367990-368761	Body	6	62	1.2E-03	4.1	↓ d10
CCND1	chr11:69460638-69461451	Body	6	69	1.4E-02	-5.3	↓ d2/10
FOXC2	chr16:86598792-86599976	TSS1500	9	16	1.3E-02	-8.7	ns[4]
GATA3 / FLJ45983	chr10:8095755-8096650	TSS1500 TSS200	29	29	2.2E-02	6.7	na[5]
IRS1	chr2:227661213-227662083	1stExon	5	34	1.7E-02	-11.2	ns[4]
PRDM16	chr1:3276413-3276786	Body	6	636	3.3E-02	5.5	↓ d10
PRDM16	chr1:3330724-3331278	Body	5	636	2.5E-06	4.6	↓ d10
PRDM16	chr1:3347146-3347994	Body	5	636	4.1E-03	4.7	↓ d10

TSS200 /1500 = 200 / 1500 base pairs from the transcription start site.
[1] Annotation according to Illmn12.hg19 identifiers.
[2] *p*-value is the mean BH-adjusted *p*-value of the probes in the DMR
[3] maximal change in DNA methylation (beta %) for the probes within the DMR
[4] ns: no significant change in gene expression was found
[5] na: gene expression levels were below the limit of detection

Numerous genome-wide DNA methylation changes were found, spread out over different pathways

The genome-wide DNA methylation analysis of the undifferentiated (d0) and differentiated (d10) hMSCs did not reveal significantly differentially methylated single probes based on the adjusted *p*-value. However, we identified 793 statistically significant DMRs. The number of probes per DMR for the top 10 most significant DMRs reached up to 13 consecutive probes, and the strongest magnitude change in DNA methylation was 11.6% (Table 2 / Table S4).

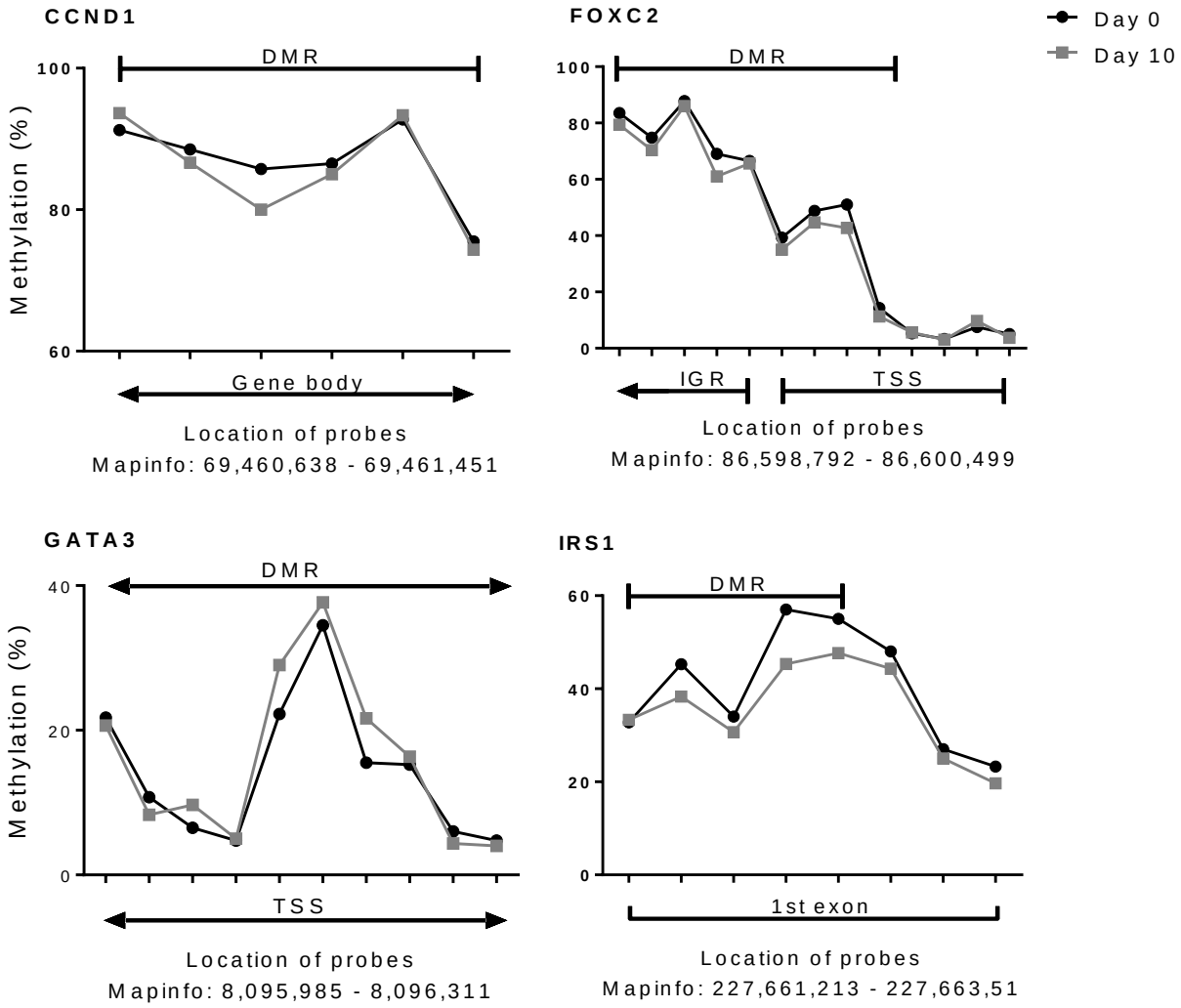

Fig. 3. Examples of differentially methylated regions (DMRs) in adipogenic related genes after differentiation into mature adipocytes at day 10 (grey line) compared to non-differentiated hMSCs at day 0 (black line). Each point indicates a single probe and the line represents the average of at least three independent experiments. The DMRs were identified using the DMRcate package and the location of the probes is based on genome h19/GRCh37.

Table 2. Differentially methylated regions (DMRs) found with the DMRcate package in all measured genes comparing undifferentiated hMSCs at day 0 to differentiated adipocytes at day 10. The top 10 is presented of DMRs with a minimum of 5% methylation difference for one probe. A table with all 258 DMRs can be found in Table S4.

Gene	Chromosome: position[1]	Relation-ship to gene	No. probes in DMR	No. probes on array	*p*-value[2]	DNA methylation change (%)
CAND2	chr3:12856409-12856862	Body	6	33	9.4E-07	7.6
TBX15	chr1:119521928-119522855	5'UTR	6	61	1.5E-06	-9.3
-	chr4:188953092-188953969	-	8	13	1.2E-05	11.2
HS3ST3B1	chr17:14202916-14203711	TSS1500	5	54	2.7E-05	7.4
LOC388965	chr2:84517321-84517950	TSS1500 TSS200 Body	9	10	5.1E-05	-8.2
CASZ1	chr1:10707454-10708091	3'UTR Body	6	176	7.2E-05	6.4
OCA2	chr15:28344273-28344730	5'UTR 1stExon TSS200 TSS1500	13	95	1.5E-04	5.3
LOC100130522	chr18:77905119-77905947	TSS1500 TSS200 Body	11	20	2.4E-04	-11.6
TMEM87B	chr2:112811068-112812167	TSS1500	7	22	2.5E-04	6.8
CRTAC1	chr10:99695981-99696260	Body	5	46	2.5E-04	5.4

3'/5'UTR = 3'/5' untranslated region. TSS200 /1500 = 200 / 1500 base pairs from the transcription start site

[1]Annotation according to Illmn12.hg19 identifiers.

[2]*p*-value is the mean BH-adjusted *p*-value of the probes in the DMR

Interestingly, a DMR in the top 10 of most significant DMRs was located in *TBX15*, a mesodermal developmental gene, which can influence adipocyte differentiation and lipid accumulation[23] and is associated with obesity.[24] Another DMR was located in *CRTAC1*, which can be used as a marker to distinguish between MSCs and osteoblasts.[25] Besides these genes within the top 10, there were also certain DMRs which consisted of 15 consecutive probes or more (Table S4). One of these DMRs was locating in *SLC1A3,* previously found to be up-regulated upon differentiation in hMSCs.[26] Remarkably, the DMR related to *HOXA5* consisted of 32 consecutive probes. *HOXA5* is a developmental gene and its expression in hMSCs was shown before,[27] as well as the differential

expression in adipocytes depending on lipid accumulation.[28] Furthermore, a DMR was found for *DYRK1B* which is expected to be involved in adipocyte differentiation in mouse cells.[29] For more in depth analysis, we performed Ingenuity Pathway Analysis (IPA, Qiagen) which revealed the adipogenesis pathway as the most significant (*p*-value 1.26E-03) based on the DMRs. However, only 6 (*ATG5, EBF1, FGFR4, FOXC2, HDAC4, TNF*) out of 127 known genes in this pathway were differentially methylated (Fig. 4). To determine whether the DMRs annotated to these genes showed differential expression we performed additional qPCR analysis (Table 3). We detected differential expression for *TBX15, CRTAC1, EBF1,* and *DYRK1B*, however altered gene expression was both related to increased and decreased methylation levels.

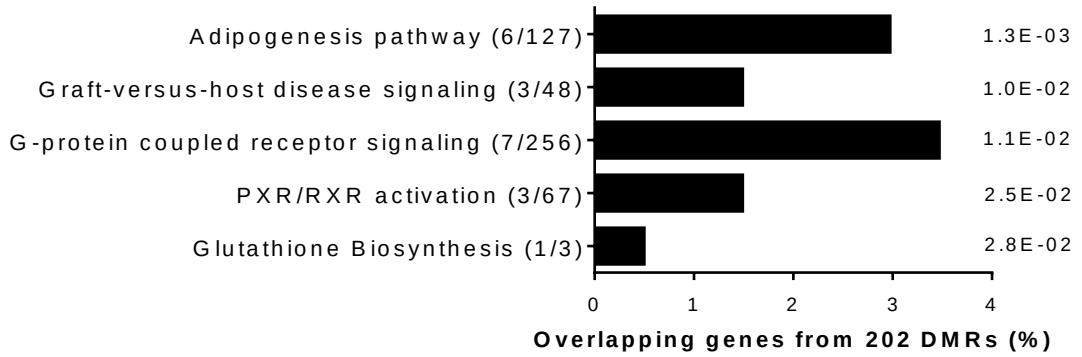

Fig. 4. The 5 most significant pathways detected in the differentially methylated regions (DMRs; number of genes detected / number of genes present in pathway) upon differentiation following Ingenuity Pathway Analysis (IPA). Non-differentiated hMSCs (d0) were compared to differentiated adipocytes (d10). In total, 258 DMRs with a minimum of 5% methylation difference was included which resulted in 202 unique genes. The pathways are presented as the percentage of overlapping genes between the DMRs and the pathway as well as the statistical significance.

Table 3. Differentially methylated regions (DMRs) found with the DMRcate package with a genome-wide analysis comparing undifferentiated hMSCs at day 0 to differentiated adipocytes at day 10. Genes were chosen for additional transcriptional analysis based on their possible relevance for hMSCs and adipocytes and had to apply to either one of the three following criteria: 1) present in the top 10 of most significant DMRs (*TBX15* and *CRTAC1*); 2) detected as relevant for the adipogenesis pathway based on the Ingenuity Pathway Analysis (*ATG5, EBF1,* and *HDAC4*); 3) genes associated with a DMR of minimal 15 probes (*SLC1A3, HOXA5, DYK1B*).

Gene	Chromosome: position[1]	Relation-ship to gene	No. probes in DMR	No. probes on array	*p*-value[2]	DNA methy-lation change (%)	Gene expression
TBX15	chr1:119521928-119522855	5'UTR	6	61	1.5E-06	-9.3	↑ d2/d10
CRTAC1	chr10:99695981-99696260	Body	5	46	2.5E-04	5.4	↑ d10
ATG5	chr6:106773518-106773949	5'UTR 1stExon TSS200 TSS1500	8	22	4.1E-02	-5.0%	ns[3]
EBF1	chr5:158526263-158526693	Body 1stExon 5'UTR	5	57	1.5E-02	10.7%	↑ d2/d10
HDAC4	chr2:240029626-240029909	Body	5	425	2.3E-02	5.8%	ns[3]
SLC1A3	chr5:36606102-36607417	TSS1500 TSS200 1stExon 5'UTR	19	28	2.0E-02	5.6%	ns[3]
HOXA5	chr7:27183794-27184737	TSS1500	32	51	1.7E-02	-9.4%	ns[3]
DYRK1B	chr19:40324226-40325405	5'UTR 1stExon TSS200 3'UTR TSS1500 Body	15	16	1.9E-03	6.2%	↓ d10

3'/5'UTR = 3'/5' untranslated region. TSS200 /1500 = 200 / 1500 base pairs from the transcription start site.

[1] Annotation according to Illmn12.hg19 identifiers.

[2] *p*-value is the mean BH-adjusted *p*-value of the probes in the DMR

[3] ns: no significant change in gene expression was found

5-aza-dC treatment induced strong hypomethylation in differentiating hMSCs

As a positive control for changes in DNA methylation, adipocyte differentiation in hMSCs was induced in the presence of 5-aza-dC, a well-known DNMT1 inhibitor. Indeed, the Illumina 450K BeadChip analysis revealed significant (FDR adjusted *p*-value < 0.05) differential methylation of 26,982 probes and 13,769 statistically significant

DMRs upon 5-aza-dC-treatment (Table 4). The differentially methylated probes were not randomly distributed over the probes present on the BeadChip (Fig. 5A). 5-aza-dC-treatment predominantly affected methylation in the gene body, while differentiation predominantly affected methylation in the IGR. Methylation around the TSS was underrepresented for both adipocyte differentiation and 5-aza-dC-treatment. Of the 1000 most differentially methylated probes upon differentiation at day 10, 664 showed an increase in methylation, while 5-aza-dC treatment induced hypomethylation of nearly all (99.7%) significantly changed probes.

Table 4. Number of differentially methylated probes and differentially methylated regions (DMRs) found, measured with Illumina 450K BeadChip, during adipocyte differentiation (comparison of day 0 with day 10) and with 5-aza-dC treatment (comparison solvent control and 5-aza-dC treatment at day 10).

	Adipocyte differentiation	**5-aza-dC treatment**
Significant single probes	0	26,982
Significant DMRs	793	13,796
Significant DMRs + β difference ≥ (-) 5%	258	11,868

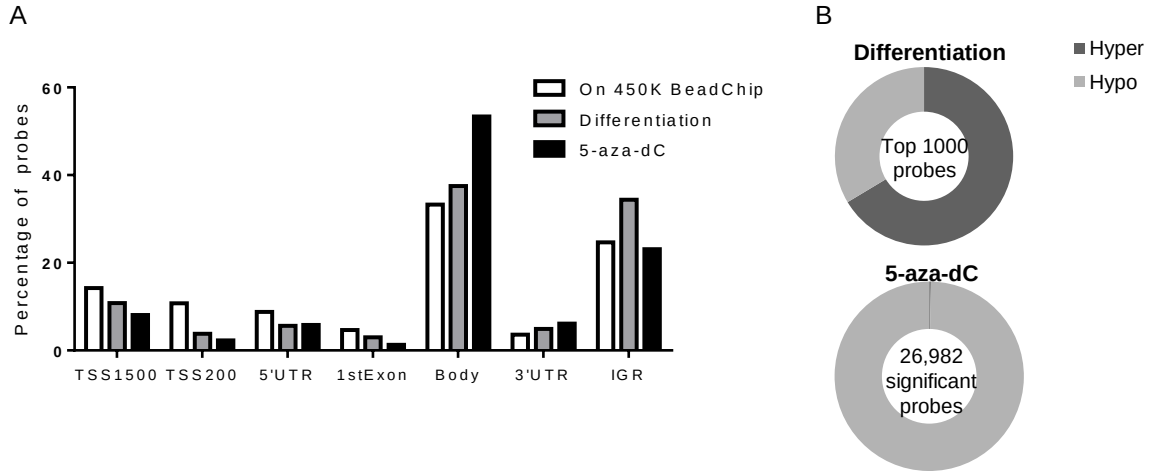

Fig. 5. DNA methylation features. **A)** Features of the 1000 probes with the highest statistical significance after adipocyte differentiation (grey bars), and of the statistically significant probes after treatment with 5-aza-dC (black bars) compared to all considered probes on the Illumina 450K BeadChip (white bars). TSS = transcription start site. UTR = untranslated region. IGR = intergenic region. **B)** Number of hypo- and hypermethylated probes following differentiation (top) or 5-aza-dC treatment (bottom).

After establishing that 5-aza-dC successfully induced DNA hypomethylation, we investigated the effect of this DNMT inhibitor on the adipocyte differentiation and the expression levels of the adipogenic markers. Strong inhibition of adipocyte differentiation was observed after 5-aza-dC-treatment, visible as a 31% reduced lipid storage compared to the solvent control (Fig. 6A), and the decreased expression levels of the key adipogenic regulators *CEBPA* and *ADIPOQ*. Gene expression significantly changed for 17 of the 84 genes, while 11 genes were below the limit of detection (Fig. 6B). Of these 17 genes, 9 were related to a DMR. All DMRs showed a decrease in methylation, while gene expression levels were both increased as well as decreased (Fig. 6B). Fig. 7 shows that, out of the 84 adipogenic genes, 43 genes contained a DMR with a minimum beta difference of 5% in at least one of the probes, but DNA methylation changes were not related to gene expression levels. DNA hypomethylation occurred in the proximity of the TSS for *AGT, SFRP1, SFRP5, SIRT2,* and *TSC22D3*, and except for *SFRP5,* this coincided with a decrease in gene expression (Table S5). In summary, 5-aza-dC caused strong hypomethylation in most adipogenic genes, but no concomitant change in gene expression was found.

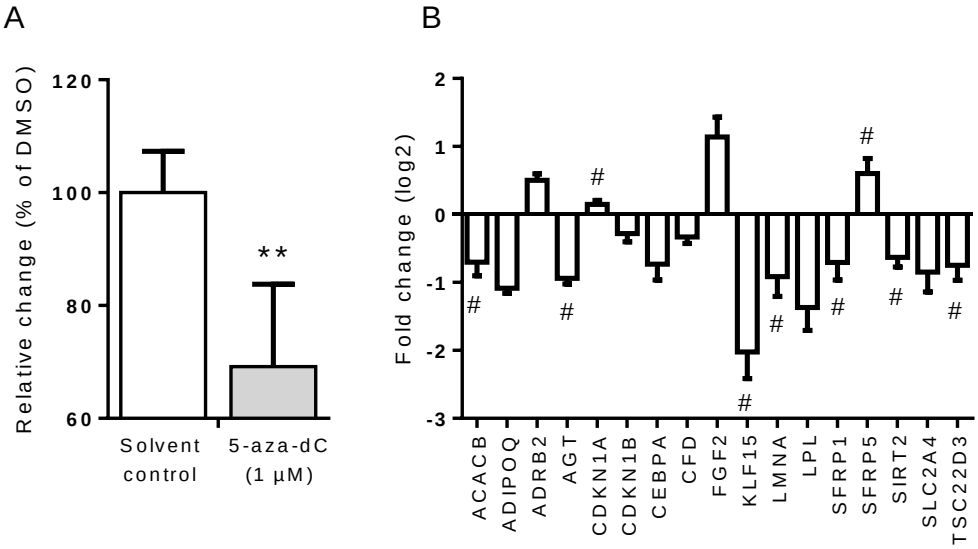

Fig. 6. Adipogenic markers in human mesenchymal stem cells (hMSCs) after continuous exposure to 5-aza-dC (1 µM). **A)** Lipid accumulation at day 10 of adipocyte differentiation, quantified by Oil-Red-O staining (mean + SD from four independent experiments). ** $p < 0.001$. **B)** Gene expression changes (all $p < 0.05$) at day 10 of differentiation, normalized for HPRT1 and B2M expression. Values represent the $\log_2$

fold change (mean + SD from three independent experiments) compared to the solvent control at day 10. # indicates that this gene also has a significant differentially methylated region (DMR). All DMRs found in any of the 84 adipogenic genes are presented in Table S5.

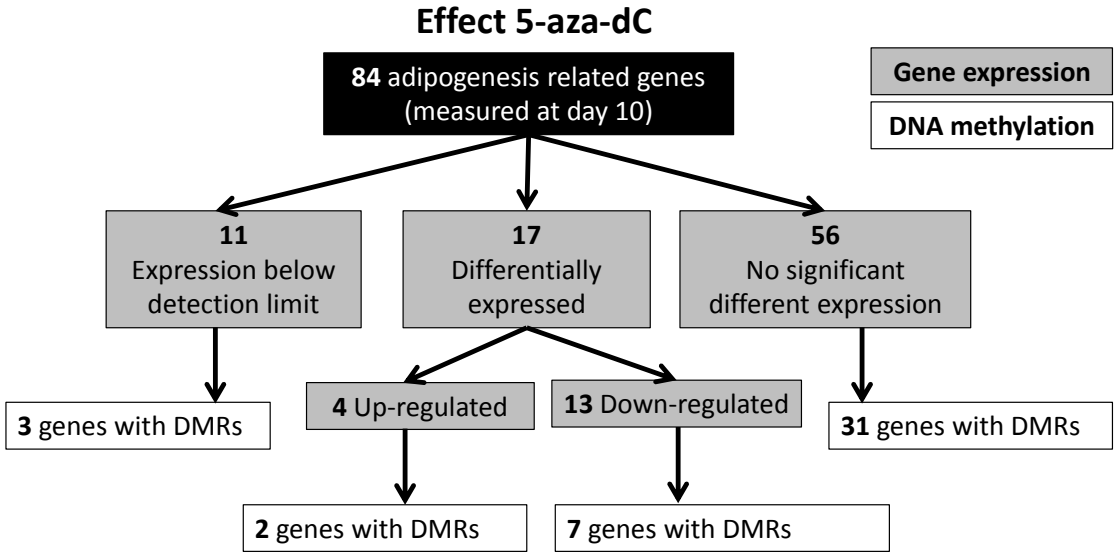

Fig. 7. Results of changes in gene expression (grey boxes) and differentially methylated regions (DMRs) with a minimum beta difference of 5% (white boxes) of 84 adipogenesis related genes found after exposure to 5-aza-dC (1 µM) at day 10 of differentiation compared to non-treated differentiated adipocytes at day 10. All DMRs found in any of the 84 adipogenic genes are presented in Table S5.

Discussion

Primary human mesenchymal stem cells (hMSCs) were used to assess genome-wide changes in DNA methylation upon differentiation into mature adipocytes, and to explore the relationship between DNA methylation and gene expression of adipogenic genes.

The examined adipogenic markers changed as expected during the differentiation process, shown by lipid droplet formation and the gene expression profiles.[2] Out of the total panel of 84 adipogenic genes, 43 genes revealed differential expression during adipocyte differentiation, of which 3 genes had a significant DMR. Furthermore, we detected DMRs in 2 genes that were not differentially expressed. In general, we predominantly identified an increase of DNA methylation, i.e. hypermethylation, during

differentiation. Other studies, mostly using mouse cells like 3T3-L1 cells, predominantly detected hypomethylation in specific adipogenic genes.[9-13] The 3T3-L1 cells, however, are already committed to the adipocyte lineage which possibly results in a different initial methylation pattern compared to multipotent cells.[30]

Our results show that DNA methylation and gene expression changed independently from each other during adipocyte differentiation. This is in accordance with the study of Takada *et al*. (2014), who also did not find a clear relationship between the two phenomena in adipose-derived stem cells.[16] Furthermore, also during myogenic differentiation, no association between gene expression and DNA methylation was found.[31] DMRs in the promoter region are proposed to correlate with gene expression changes.[5] Since the majority of the observed DNA methylation alterations in this study were not located in the proximity of the TSS, it is not surprising that DNA methylation changes could not be associated with gene expression alterations. However, also in the few DMRs detected in close proximity to the TSS, the association with gene expression was lacking. Perhaps the DMRs were too small to influence expression, as other probes measured around the TSS were not differentially methylated, as for example showed for *FOXC2* (Fig. 3). We therefore also investigated gene expression for some DMRs than contained a minimum of 15 consecutive probes, however, this did not improve the association between gene expression and DNA methylation. Even for our positive control, 5-aza-dC-treatment, which caused a strong effect on DNA methylation, no clear association with gene expression was observed. It has to be noted that 5-aza-dC does not only inhibit DNA methylation as it can also exert other effects, such as inhibition of adipocyte differentiation. This could be a reason for the lack of association between DNA methylation and gene expression after 5-aza-dC exposure.

The majority of the identified DNA methylation changes in our study were found in IGRs. DNA methylation in the IGR is important for genomic stability, but earlier research also found cell-type discriminatory patterns in IGRs outside CpG islands.[32] This might indicate that methylation in the IGR is important for the differentiation process, or alternatively, is the consequence of the differentiation. More research is necessary to further elucidate the relationship between differential DNA methylation in IGRs and

gene expression levels, and to determine the functional consequences of DNA methylation in IGRs.

The precise role of DNA methylation within the gene body is far from being understood, but it has been suggested to be involved in transcript elongation, alternative splicing and enhancer activation.[7] We found DNA methylation changes in the gene body for genes that were not differentially expressed and for genes with increased or decreased gene expression levels, suggesting there might be a rather complex interaction.

No statistically significant changes in methylation were found in single probes with the adjusted *p*-values for multiple testing. This might be explained by small effects and limited number of samples, in combination with the variation in heterogeneous cell populations after adipocyte differentiation.[33] Differentiation of hMSCs results in a heterogeneous cell population including, apart from fully differentiated adipocytes, also less differentiated cell types with concomitant DNA methylation profiles. Furthermore, identification of DMRs is often more sensitive to reveal small changes in DNA methylation, because it takes consecutive probes into account. This also makes DMRs more biologically relevant than differentially methylated single probes. By exploring genome-wide DNA methylation we found 793 DMRs during differentiation, and 258 of these DMRs displayed at least a DNA methylation change of 5%. DMRs were not strikingly (6 DMRs detected in 127 adipogenic genes), although significantly, overrepresented in adipocyte-related genes. *EBF1* gene expression was increased together with DNA methylation, but *ATG5* and *HDAC4* expression was not affected. This suggests that specific DNA methylation changes might be important for the differentiation process, independent from transcriptional regulation. Relevant genes in de top 10 of detected DMRs included *TBX15* and *CRTAC*. The methylation status of these genes might be important to determine cell fate,[23,25] and was associated with increased gene expression.

The limited DNA methylation changes found are in accordance with previous studies.[8,16] Takada *et al*., however, reported DMRs in *ADIPOQ*, which we could not detect in our experiment because their particular probes are not present on the 450K BeadChip. Although we studied DNA methylation extensively by measuring a large number of

probes, this only covered about 1% of the CpG sites present in the genome. It is therefore possible that DNA methylation changed at one or more of the unmeasured probes. Furthermore, the 450K BeadChip cannot distinguish DNA methylation from hydroxymethylation. Hydroxymethylation might be an intermediate stage of active demethylation and does not necessarily have the same function as DNA methylation. However, hydroxymethylation only represents a small fraction of total methylation in mammalian tissue[34] and in cultured cells.[35]

It has been suggested that DNA methylation during adipogenesis is most important in the commitment stage, and less in the differentiation phase.[16] However, our experiments with the hMSCs included both stages, and stable long-term DNA methylation in the commitment stage would still be present at the end of differentiation. If DNA methylation is not as stable as thought,[4] DNA methylation changes in the commitment phase might be missed if measured at day 10. For a very limited subset of probes in the *BRD2* gene it was shown that DNA methylation decreased in the first few days of adipogenesis, and increased in mature adipocytes, indicating DNA methylation can be a dynamic process.[36]

Our data suggest that DNA methylation might not be the most important factor regulating adipocyte differentiation, in contrast to differentiation of embryonic stem cells.[3] Similarly, in hMSCs, differentiating into chondrocytes, DNA methylation in the promoter region did not correlate with changes in gene expression, but the chromatin structure had changed.[37] It has also been shown that during adipocyte differentiation the chromatin landscape is modulated[38] and that gene expression changes correlated with chromatin marks.[39,40] *PPARG*, which is an important regulator in adipogenesis, was also found to be related to chromatin structure and histone marks.[41] We previously showed that also during aging changes in genome-wide DNA methylation and gene expression occur independently.[42] Taken altogether, these findings suggest that changes in DNA methylation are often insufficient to regulate gene expression. The complex interaction between DNA methylation, histone modifications and noncoding RNA should be studied to fully comprehend epigenetic effects on gene expression.

In conclusion, genome-wide DNA methylation analysis showed that DNA methylation was relatively stable during adipocyte differentiation, although some significant changes were observed. The pronounced differences in gene expression levels of 84 adipocyte-related genes could not be explained by DNA methylation, suggesting that other mechanisms play a more important role.

Acknowledgements

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Supplementary data

Primer sequences and their product length are provided in Table S1. Single probes identified for any of the 84 adipogenic related genes from the top 1000 are given in Table S2. Gene symbols of the 84 adipogenic genes with their description, followed by the average fold change and the *p*-value, can be found in Table S3. The 258 genome-wide detected DMRs, with a minimum beta difference of 5%, are described in Table S4. DMRs with a minimum beta difference of 5%, found in the 84 adipogenic genes after 5-aza-dC treatment, and the association with gene expression, are provided in Table S5. Supplementary information can be found, in the online version, at http://dx.doi.org/10.1002/jcb.25568. The data sets supporting the DNA methylation results of this article are available in the Gene Expression Omnibus (GEO) with accession number GSE74609.

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CHAPTER 3B

Persistent organic pollutants alter DNA methylation during human adipocyte differentiation

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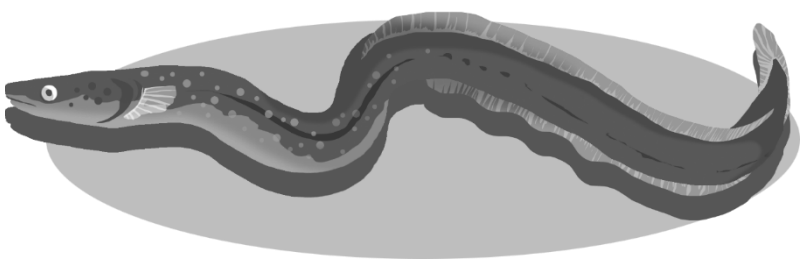

Abstract

Ubiquitous persistent organic pollutants (POPs) can accumulate in humans where they might influence differentiation of adipocytes. The aim of this study was to investigate whether DNA methylation is one of the underlying mechanisms by which POPs affect adipocyte differentiation, and to what extent DNA methylation can be related to gene transcription. Adipocyte differentiation was induced in two human cell models with continuous exposure to different POPs throughout differentiation. From the seven tested POPs, perfluorooctanesulfonic acid (PFOS) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) decreased lipid accumulation, while tributyltin (TBT) increased lipid accumulation. In human mesenchymal stem cells (hMSCs), TCDD and TBT induced opposite gene expression profiles, whereas after PFOS exposure gene expression remained relatively stable. Genome-wide DNA methylation analysis showed that all three POPs affected DNA methylation patterns in adipogenic and other genes, but without concomitant gene expression changes. Differential methylation was predominantly detected in intergenic regions, where the biological relevance of alterations in DNA methylation is unclear. This study demonstrates that POPs, at environmentally relevant levels, are able to induce differential DNA methylation in human differentiating adipocytes.

Introduction

Persistent organic pollutants (POPs) can cause a variety of adverse health effects in humans, often because of their endocrine disrupting properties.[1] The common route for exposure to POPs in the general population is consumption of contaminated food, especially fish and seafood.[2,3] Most POPs are highly lipophilic, except for perfluorinated compounds, and accumulate in adipose tissue. Adipose tissue is not merely an energy depot, but has essential endocrine functions.[4] Many POPs display obesogenic effects leading to alterations in body weight[5] and induction of obesity-associated conditions.[6] Previous research has shown that mature adipocytes are marginally responsive to POPs, while precursor cells are strongly affected by POP exposure.[7] This suggests that POPs exert their negative health effects mainly in sensitive early stages, which is supported by the observation that numerous POPs are capable of affecting adipocyte differentiation.[8-10]

Early life development is commonly acknowledged to be a critical window of susceptibility to adverse health effects,[5,11] and early POP exposure in adipose and other tissues has been shown to cause effects in a later life-stage or even induce transgenerational effects.[12,13] Emerging evidence indicates that aberrant epigenetic regulation of critical genes during the developmental phase might lead to adiposity later in life.[14] A well-studied epigenetic modification is DNA methylation. DNA methylation refers to the addition of a methyl group to cytosine in a CpG dinucleotide and has a complex relationship with gene expression. DNA methylation close to the transcription start site (TSS) is expected to inhibit gene expression, although this is dependent on the transcription factors that are binding.[15,16] Methylation in the gene body is currently considered to affect gene expression by stimulating transcription elongation or alternative splicing.[17] The role of DNA methylation in intergenic regions (IGR) is poorly understood, but methylation in repetitive regions is important for genomic stability.[15] Results of previous studies indicate that the observed effects of POPs on adipocyte differentiation may be explained by alterations in DNA methylation. TBT has been shown to decrease DNA methylation in the promoter/enhancer region of *Fabp4*, increase the expression of this gene, and enhance adipocyte differentiation in mouse

stromal cells.[18] Furthermore, TBT exposure resulted in decreased global methylation in 3T3-L1 cells.[10] BDE47, on the other hand, did not significantly alter global DNA methylation,[10] but decreased methylation levels in the *Pparg* promoter in 3T3-L1 cells.[19] Altogether, these findings suggest that DNA methylation might be an underlying mechanism resulting in POP-induced adverse health effects. However, the genome-wide effects of POPs on DNA methylation in differentiating adipocytes have not been studied in detail before.

The aim of this study was to investigate the effects of POPs in differentiating human adipocytes on genome-wide DNA methylation as well as gene transcription of relevant adipogenic genes. We first screened seven ubiquitous POPs with different modes of action (namely TCDD, PFOS, TBT, HBCD, PCB153, BDE47, and MeHg) for their effects on adipocyte differentiation using two human cell models. Simpson-Golabi-Behmel syndrome (SGBS) cells are human preadipocytes that can differentiate into mature adipocytes. Human mesenchymal stem cells (hMSCs), derived from bone marrow, are primary cells which can differentiate into multiple cell types including adipocytes and osteoblasts. The hMSCs first have to commit and become preadipocytes, before they can differentiate into mature adipocytes. We first identified which of the seven candidate POPs affected adipocyte differentiation in the two cell types. Subsequently, we measured the effects of selected POPS on the expression profiles of 84 relevant adipogenic genes as well as genome-wide DNA methylation.

Materials and methods

Chemicals

TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin, CAS 1746-01-6) was purchased from AccuStandard (New Haven, USA). PCB153 (2,2′,4,4′,5,5′-hexachlorobiphenyl, CAS 35065-27-1), PFOS (heptadecafluorooctanesulfonic acid potassium salt, CAS 2795-39-3), TBT (tributyl chlorotin, CAS 1461-22-9), MeHg (methylmercury(II) chloride, CAS 115-09-3) and differentiation reagents were obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands) unless otherwise mentioned. HBCD (hexabromocyclododecane, technical

mixture) and BDE47 (2,2′,4,4′-tetrabromodiphenyl ether, CAS 5436-43-1) were kindly provided by Professor Åke Bergman (Stockholm University, Sweden). Stock solutions for all POPs and differentiation reagents were prepared in dimethyl sulfoxide (DMSO; Acros Organics, Geel, Belgium).

Cell culture, differentiation and exposure

Human SGBS preadipocytes were kindly provided by Professor Wabitsch (University of Ulm, Germany) and cultured and differentiated as described previously.[20] Poietics™ human mesenchymal stem cells (hMSC; Lot No 2F3478) were obtained from Lonza Walkersville Inc. at passage 2 and cultured in MSCGM growth medium (Lonza, Walkersville, USA). Adipogenic induction medium for hMSCs consisted of DMEM supplemented with L-glutamine (1%), FCS (10%), dexamethasone (1 µM), human insulin (5 µg/mL; Insuman Rapid (100 IE/mL), Sanofi-Aventis, Frankfurt am Main, Germany), 3-isobuty-l-methyl-xanthine (IBMX, 0.5 mM), and rosiglitazone (1 µM; Sanbio B.V, Uden, the Netherlands). Osteogenic induction medium consisted of DMEM supplemented with L-glutamine (1%), FCS (10%), dexamethasone (100 nM), ascorbic acid (50 µg/mL), vitamin D (10 nM; Merck Chemicals, Amsterdam, the Netherlands), and β-glycerophosphate (12.5 mM). Cells were seeded with a density of 3.800 cells/cm^2 and grown until confluency. Both confluent SGBS cells and hMSCs were pre-treated for 24h with the POPs after which differentiation was initiated (day 0). Exposure medium was refreshed twice a week, until day 10 when the experiment was ended. During differentiation cells were continuously exposed to POPs or the solvent control (0.1% DMSO). All endpoints were assessed in mature adipocytes at day 10 of differentiation because the most pronounced differences can be expected towards the end of differentiation and after prolonged POP exposure. Gene expression was also measured at day 2 because these changes are expected to be highly dynamic. A schematic overview of the experimental setup for hMSCs can be found in Fig. 1, because SGBS cells were only used for the Oil-Red-O staining to validate pro- and anti- adipogenic effects of POPs with another human cell strain.

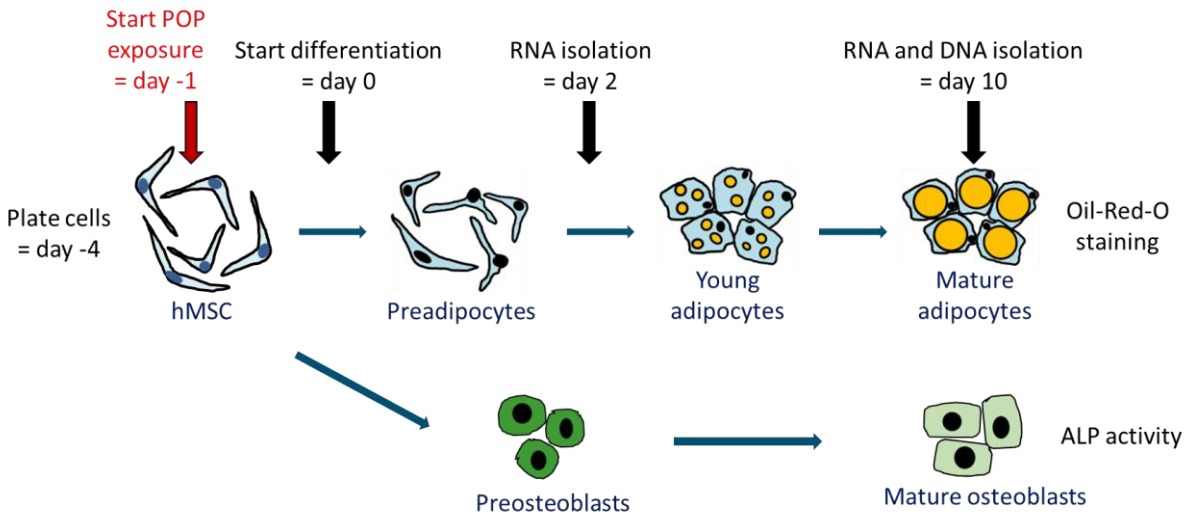

Fig. 1. Experimental set-up for hMSCs. Cells were plated (day -4) and grown to confluence. When cells were confluent (day -1) POP exposure started and was continued until the experiment ended. Differentiation into adipocytes or osteoblasts was initiated at day 0. During the adipocyte differentiation, RNA was isolated at day 2 and day 10, DNA was isolated at day 10, and Oil-red-O staining was performed at day 10. The alkaline phosphatase (ALP) activity was measured at day 10 of osteoblast differentiation.

Cell viability assays

For cytotoxicity testing, undifferentiated cells in 96-wells plates were exposed to the individual POPs for 48h before 3-(4,5-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the cells and incubated for another hour. Formazan crystals were then solubilized with DMSO and the optical density was measured at 562 nm and corrected for background absorption at 620 nm. Cytotoxic effects of at least three independent experiments were compared to the solvent control and TritonX-100 (0.1%) was included as a positive control. To ensure that further experiments would not be performed at cytotoxic concentrations, the viability of the highest exposure concentrations included did not deviate more than 20% from the viability of the solvent control.

Lipid accumulation

At day 10 of adipocyte differentiation, cells in 12-wells plates were stained with Oil-Red-O (3 mg/mL, 60% isopropanol, 40% distilled water) and photographed. Isopropanol was

added to extract the Oil-Red-O and the optical absorbance was measured at 520 nm. Oil-Red-O staining of undifferentiated cells grown in parallel was subtracted from the measurements. The amount of differentiation of POP-exposed cells was calculated as the percentage of the solvent control from at least three independent differentiation experiments. Statistical significance of the differences between the treatments and the solvent control were determined with a one-way ANOVA followed by Dunnett's post test.

Alkaline phosphatase assay

Alkaline phosphatase (ALP) activity was measured in 96-wells plates at day 10 of osteoblast differentiation from at least three independent experiments. Cells were fixated in paraformaldehyde (4%) before p-nitrophenyl phosphate was added (30 min, 37°C). The reaction was stopped by sodium hydroxide (0.5 M) and absorbance was measured at 405 nm in a microplate reader. ALP activity from undifferentiated cells was subtracted from the absorbance measurement. The differentiation of treated cells was calculated as percentage of the solvent control, and statistical significance was determined with a one-way ANOVA followed by Dunnett's post test.

RNA isolation and cDNA synthesis

Total RNA was isolated from 60 mm dishes at day 2 and at day 10 of hMSC differentiation using Trizol (Invitrogen, Merelbeke, Belgium) according to the manufacturer's instructions. RNA was dissolved in RNase-free water and the concentration was measured using a NanoDrop ND-1000 UV–vis spectrophotometer (Isogen, Maarssen, the Netherlands). First-strand cDNA was synthesized from 1 µg of total RNA using the RT2 First Strand Kit (Qiagen, Venlo, the Netherlands) following the supplier's protocol.

RT2 profiler™ PCR array

The expression of 84 key adipogenic genes was measured in three independent experiments with the adipogenesis RT2 profiler PCR Array (PAHS-049ZE-4, Qiagen, Venlo, the Netherlands) according to the manufacturer's instructions. Genes that required more than 35 cycles to reach the threshold were regarded as below the

detection limit. Gene expression was normalized against the geometric mean of the reference genes *B2M* and *HPRT1* at day 2 and against *CFD* for day 10, because the other reference genes did not remain stable over time. Relative changes in gene expression are expressed as fold changes compared to the solvent control of that respective day. When no expression was detected for one of the treatments, fold changes were calculated with a value of 35 cycles. Differentially expressed genes were identified using a student's t-test on the log2-transformed values.

Genome-wide DNA methylation for differentially methylated single positions (DMPs)

Genomic DNA was isolated from four independent experiments at day 10 of hMSC differentiation using the DNeasy blood and tissue kit according to the manufacturer's protocol (Qiagen, Venlo, the Netherlands). Only cells from passage 9 were used, because DNA methylation profiles depend on the passage number.[21] DNA concentration and quality was determined using a NanoDrop ND-1000 UV–vis spectrophotometer. DNA methylation was analyzed using the Illumina Infinium HumanMethylation450 BeadChip (Infinium Inc., San Diego, USA), which addresses 485,512 cytosine positions, and is cross-validated against conventional quantitative pyrosequencing.[22] Bisulfite conversion, performed with 500 ng DNA using the EZ DNA Methylation Kit (Zymo Research, Freiburg, Germany), and processing of the BeadChip was done by the Human Genotyping Facility of the Erasmus University Medical Centre (Rotterdam, the Netherlands). Intensity iDAT files were processed using R-based open software (R 3.1.2). The 450K Chip Analysis Methylation Pipeline (ChAMP) (version 1.1.2) was used for pre-processing[23] and the normalization of the raw data was performed with SWAN.[24] After filtering positions that did not meet the selection criteria (detection p-value > 0.01 in >1% of the samples and bead count < 3 in 5% of the samples), 483,914 positions were included in the analysis. The positions were annotated based on the ilmn12.hg19 annotation.[25] The multidimensional scaling (MDS) plot based on the 1000 most variable positions showed that one sample in the solvent controls clustered separately and this sample was therefore excluded from further analyses. The identification of differentially methylated positions (DMPs) after POP exposure was performed using Linear Models for Microarray Data (limma, version 3.22.1). The statistical analyses were performed unpaired and adjusted for multiple testing with the Benjamini-Hochberg (BH)

method.[26] A false discovery rate (FDR) value of 0.05 was used as the threshold to define statistical significance. Furthermore, we considered potential DMPs with a non-adjusted *p*-value below 1E-04. The methylation (β) values were constrained to lie between 0% (completely unmethylated) and 100% (completely methylated). The data sets supporting the results of this article are available in the Gene Expression Omnibus (GEO) with accession number GSE75133.

Differentially methylated regions (DMRs)

In addition to the identification of methylation differences in single positions, we used the DMRcate package to identify differentially methylated regions (DMRs).[27] This method does not use predefined regions, but identifies DMRs across the genome based on tunable kernel smoothing of the differential methylation signal, independent of the direction of the methylation change and distance from each other. We defined DMRs as regions of at least five positions with a distance between consecutive positions less than 1000 nucleotides. Statistical tests were adjusted for multiple testing using a FDR of 0.05 with the BH method. Pathway analyses were performed for all genes annotated to the DMRs with a FDR < 0.05 using Ingenuity Pathway Analysis (IPA). All canonical pathways detected with a *p*-value < 0.001 (Fisher Exact Test) are described.

Results and discussion

Adipocyte differentiation was affected by TCDD, PFOS, and TBT

To investigate the adipogenesis-interfering effects of seven different POPs, adipocyte differentiation was induced in two human cell models in the presence of non-toxic POP concentrations (Fig. A1). Exposure to TCDD (≥ 0.1 nM) showed a concentration-dependent decrease in lipid accumulation in both hMSCs and SGBS cells (Fig. 2). This result is in accordance with previous studies showing an inhibitory effect of TCDD on adipocyte differentiation in human as well as mouse cells.[8,9] Also PFOS (≥ 10 µM) reduced lipid accumulation in both cell models. Previous studies in 3T3-L1 cells found no effect of PFOS on differentiation[10] or reported smaller lipid droplets.[28] TBT, on the contrary, induced adipocyte differentiation in both cell lines, but stronger in hMSCs (≥

3 nM). This effect was expected, because a well-described mechanism of action for TBT is activation of PPAR-gamma, which is a key regulator of adipogenesis.[29] HBCD, PCB153, BDE47, and MeHg did not affect lipid accumulation in both hMSCs and SGBS cells (Fig. 2). Bastos Sales *et al.* also did not find effects for HBCD,[10] but they and others observed PCB153 and BDE47 exposure to modestly induce adipogenesis in 3T3-L1 cells.[30,31] It has previously been shown that inorganic mercury (HgCl2) (≥ 5 µM) negatively affects adipocyte differentiation.[32] Organic MeHg, however, is more relevant regarding human exposure from seafood. MeHg is more cytotoxic than $HgCl_2$,[33] so we could not test whether MeHg at higher concentrations than 100 nM would influence adipocyte differentiation. In conclusion, we found that three out of the seven tested POPs affected lipid accumulation in both human cell models.

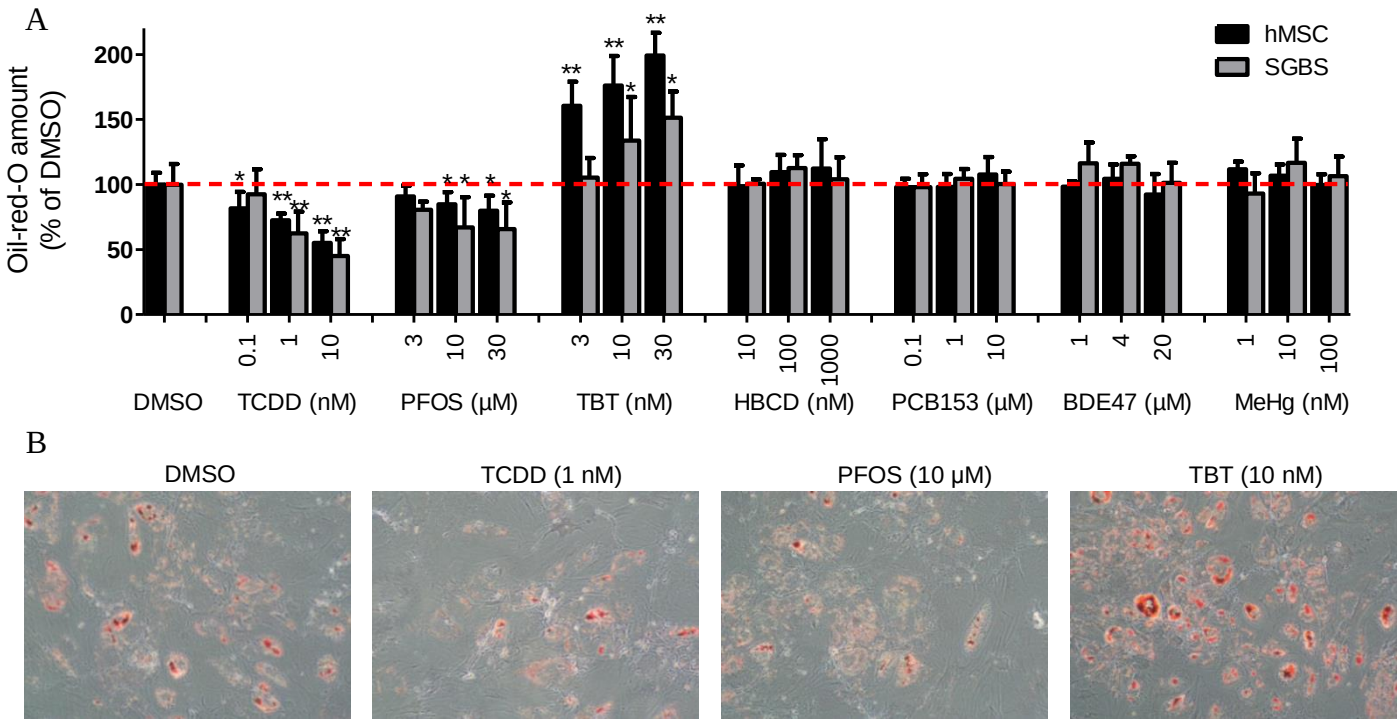

Fig. 2. (A) Effect of POP-treatment on adipocyte differentiation in hMSCs and SGBS cells. Lipid accumulation was measured with an Oil-Red-O staining, which was quantified from at least three independent experiments (mean + SD). Statistical significance was determined with a one-way ANOVA followed by Dunnett's post test compared to the solvent control. * $p < 0.05$ ** $p < 0.001$ **(B)** Representative pictures of stained hMSCs at day 10 of differentiation (original magnitude 100x).

Osteoblast differentiation was affected by TCDD, TBT, and HBCD

To investigate whether the POPs specifically affected adipocyte differentiation, or interfered with differentiation in general, osteoblast differentiation was induced in hMSCs in the presence of the same seven POPs. Since SGBS cells are preadipocytes that cannot be differentiated into osteoblasts they were not included in this experiment. ALP activity, a marker of osteoblast differentiation, was decreased due to TCDD (≥ 1 nM) exposure (Fig. 3), which is in accordance with previous studies.[34] This result suggests that TCDD interferes with differentiation in general, possibly through the aryl hydrocarbon receptor.[35-37] No effect of PFOS exposure on ALP activity was observed, suggesting that this compound specifically affects adipogenesis. TBT (≥ 3 nM) decreased osteoblast differentiation as expected, given it is a very strong PPAR-gamma inducer.[18,38] PPAR-gamma is an important promoter of adipocyte differentiation and, concomitantly, inhibits alternative differentiation pathways.[39] Although HBCD (1 µM) did not affect adipocyte differentiation, it clearly induced ALP activity, which is, according to our knowledge, not reported before with regard to osteoblast differentiation. The increase in osteoblast differentiation might be one of the underlying mechanisms by which HBCD increases bone mineral density.[40] PCB153, BDE47 and MeHg did not affect differentiation towards osteoblasts in hMSCs.

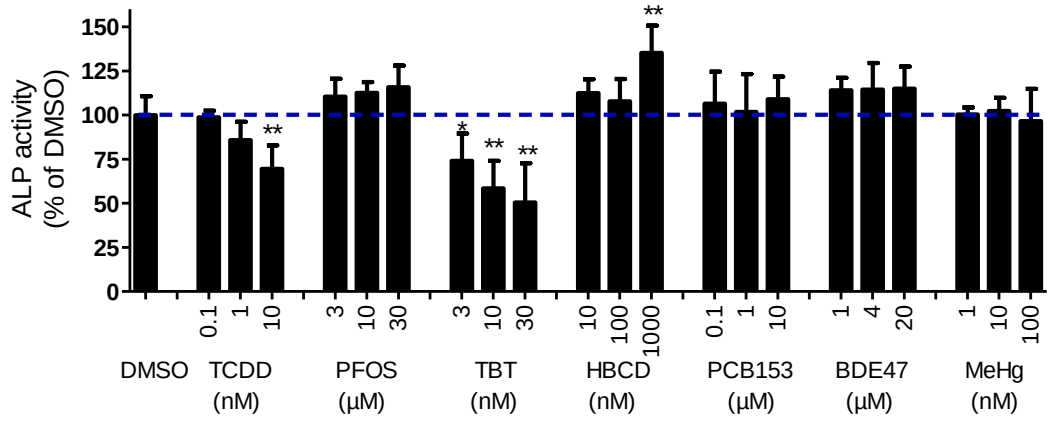

Fig. 3. Effect of POP-treatment on osteoblast differentiation in hMSCs as determined with alkaline phosphatase (ALP) activity from at least three independent experiments (mean + SD). Statistical significance was determined with a one-way ANOVA followed by Dunnett's post test compared to the solvent control. * $p < 0.05$ ** $p < 0.001$

TCDD and TBT displayed opposite effects on gene expression regulation, while PFOS had minor effects

To gain insight in the molecular mechanisms responsible for the effects of the three POPs on adipocyte differentiation, differentiating hMSCs were continuously exposed to TCDD (1 nM), PFOS (10 µM), and TBT (10 nM), because at these concentrations the POPs had a pronounced effect on lipid accumulation (Fig. 2). hMSCs were chosen over SGBS cells because these are primary cells and therefore a representative biological model for human health. Gene expression of 84 adipogenic genes was analyzed at day 2 and day 10 of adipocyte differentiation. Fig. 4 shows that at day 10 of adipocyte differentiation, expression of pro-adipogenesis genes was in general down-regulated after TCDD exposure, relatively stable after PFOS exposure, and up-regulated after TBT exposure. The anti-adipogenesis genes showed the opposite effect and were up-regulated by TCDD at both time points and mostly inhibited due to TBT exposure at day 2. Furthermore, TBT caused a striking up-regulation of pro-adipogenesis genes at day 2 of differentiation, indicating that this POP exerts its effects mainly during early adipocyte differentiation.

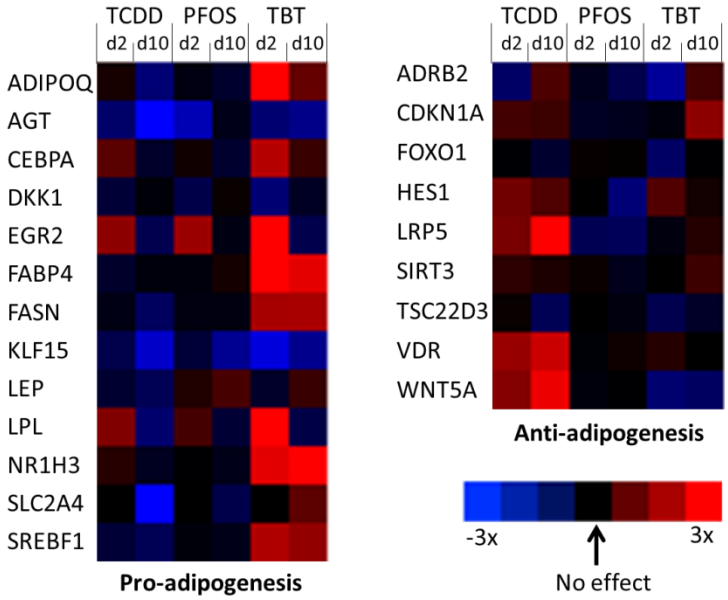

Fig. 4. Gene expression of 84 adipogenic genes was measured at day 2 and 10 of adipocyte differentiation in hMSCs after continuous exposure to either TCDD (1 nM), PFOS (10 µM) or TBT (10 nM). Only genes displaying differentially expression (p-value < 0.05, student's t-test) after exposure to one of the POPs are presented in this heat map. Values represent the fold change compared to the corresponding solvent control from three independent experiments. Color scale ranges from decreased (blue) to increased (red) expression.

Differential expression induced by PFOS-treatment was limited to *HESI* and *LEP* at day 10 (Appendix B). Lipid accumulation was less impaired after PFOS exposure than after TCDD exposure. It can be speculated that PFOS does not inhibit the differentiation process, but that the decreased lipid content was attributable to a decreased lipid droplet size.[28] This could be explained by the fatty acid resemblance of PFOS which disturbs lipid metabolism.[41] In conclusion, the obtained gene expression profiles suggest that all three compounds may affect adipocytes by different mechanisms.

DNA methylation in adipogenic genes was affected by all three POPs

Genome-wide DNA methylation was measured at day 10 of differentiation, to investigate stable DNA methylation differences due to POP exposure. We first searched for differentially methylated regions (DMRs) in the panel of 84 adipogenic genes. DMRs in response to TCDD, PFOS, and TBT-treatment were observed in 4, 2 and 3 adipogenic genes respectively (Fig. 5).

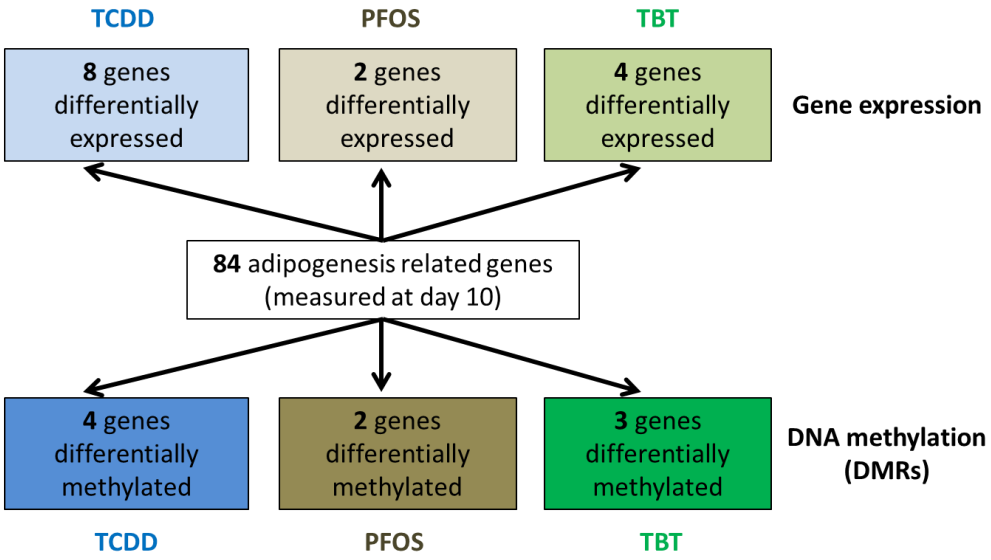

Fig. 5. Schematic overview of the 84 adipogenic genes at day 10 of hMSC differentiation after continuous exposure to TCDD (1 nM), PFOS (10 µM) or TBT (10 nM). The number of genes which were differentially expressed or contain a differentially methylated region (DMR) is displayed.

Of these genes only the pro-adipogenic gene *DKK1*, which was differentially methylated at day 10 after PFOS exposure, displayed differential expression at day 2 but not at day

10. The largest DMR, indicating a higher chance of being biological relevant, was detected in *DLK1* after TCDD exposure (Table 1). *DLK1* is a negative regulator of adipogenesis and its expression is maintained in undifferentiated hMSCs and decreases immediately upon differentiation.[42] The detected DMR in *DLK1,* with in general decreased methylation levels, consisted of 21 consecutive positions close to the TSS, which supposedly results in enhanced gene expression.[15] Theoretically, increased *DLK1* expression would imply inhibition of adipocyte differentiation, which was indeed the result of TCDD exposure. However, in our study gene expression levels for *DLK1* were below the limit of detection, and therefore a possible enhancement in gene expression could not be evaluated.

After TBT exposure DMRs were detected in three brown adipocyte differentiation markers, of which the *SRC* gene contains a DMR close to the TSS (Table 1). However, none of the genes displayed a change in gene expression upon TBT exposure. This finding might be explained by the observation that TBT affected gene expression mainly in the early stage of adipocyte differentiation (i.e. day 2 of differentiation). If DNA methylation would be the mechanism of action, this would indicate a very dynamic methylation process, since most genes were not differentially expressed anymore at day 10 of differentiation. Therefore, other mechanisms of action are more likely to be responsible for the observed changes in gene expression, such as binding of TBT to PPAR-gamma. A previous study, however, reported the relationship between *Fabp4* expression and DNA methylation in adipose derived stem cells of TBT-treated mice.[18] We also found increased expression of *FABP4,* but no effect on DNA methylation.

The differential methylation is expected to be an effect of POP exposure, and not the result of inhibiting or enhancing adipocyte differentiation, because differential methylation in these genes was not found in unexposed cells differentiating towards adipocytes.[43,44] Overall, the tested POPs affected DNA methylation in multiple adipogenic genes but without an associated change in gene expression. Likewise, for the differentially expressed genes no change in DNA methylation was detected. This points to the complex relationship between DNA methylation and gene expression, which is also not straightforward for methylation in the promoter region.

Table 1. Differentially methylated regions (DMRs) found with the DMRcate package in the 84 adipogenic genes comparing POP exposure to the solvent control at day 10 of adipocyte differentiation in hMSCs.

Gene	Relationship to gene[1,2]	Chromosome: position[3]	No. positions in DMR	No. positions on BeadChip	*p*-value[4]	Max change (%)[5]
TCDD (1 nM)						
DLK1	TSS1500,1stExon 5'UTR	chr14:101191797-101193432	21	41	2.1E-03	-8.9%
EGR2	Body,5'UTR 1stExon,TSS200 TSS1500	chr10:64575442-64576514	12	33	1.7E-02	-9.7%
PRDM16	Body	chr1:3303301-3304131	6	636	2.1E-02	-7.7%
SFRP5	5'UTR,1stExon TSS200,TSS1500	chr10:99531645-99532174	10	23	2.7E-02	5.9%
PFOS (10 µM)						
AXIN1	Body	chr16:351867-352684	6	62	2.5E-02	-3.1%
DKK1	TSS1500,TSS200 5'UTR,1stExon	chr10:54073150-54074079	10	19	1.7E-02	-6.1%
TBT (10 nM)						
INSR	Body	chr19:7267351-7268027	5	42	1.3E-03	-3.7%
PRDM16	Body	chr1:3104999-3105326	5	636	3.6E-02	4.8%
SRC	TSS1500,TSS200 1stExon,5'UTR	chr20:35972536-35973318	7	35	1.4E-02	-5.3%

[1] TSS200 /1500 = 200 / 1500 base pairs from the transcription start site.
[2] UTR= untranslated region
[3] Annotation according to Ilmn12.hg19 identifiers.
[4] *p*-value is the mean BH-adjusted *p*-value of all positions in the DMR
[5] Maximal change in DNA methylation (beta %) for the positions within the DMR

Pathway analysis revealed DMRs in many different gene functions

After analyzing the DMRs found in the adipogenic genes, we extended our DMR analysis with a genome-wide approach. We identified 346, 440, and 303 DMRs for TCDD, PFOS and TBT respectively (Appendix C). Pathway analyses, performed on the genes annotated to these DMRs, did not reveal the adipogenesis pathway for any of the three POPs. For TCDD the significant pathways were 'p53 signaling' (*p*-value = 8.4E-04), 'STAT3 pathway' (*p*-value = 9.5E-04) , and 'interferon signaling' (*p*-value = 2.3E-03). For

PFOS the most significant pathways were 'molecular mechanisms of cancer' (*p*-value = 1.4E-04), 'G1/S checkpoint regulation' (*p*-value = 2.7E-04), 'GADD45 signaling' (*p*-value = 4.7E-04), and 'IGF-1 signaling' (*p*-value = 6.9E-04). In the early stages of adipogenesis, insulin functions mainly through IGF1, but the ratio of receptors for IGF1 and insulin shifts as differentiation proceeds.[45] This could possibly be affected in PFOS-exposed adipocytes resulting in altered insulin sensitivity. For TBT the most significant pathways included 'neuroprotective role of THOP1 in Alzeimer's disease' (*p*-value = 2.4E-03), 'caeolar-mediated Endocytosis Signaling' (*p*-value = 3.2E-03), and 'mTOR signaling' (*p*-value = 5.1E-03). Overall, these data suggest that genes with a DMR are randomly distributed in the genome and DNA methylation is not overrepresented in adipogenic genes.

Exposure to TCDD and PFOS, but not TBT, induced hypomethylation

For further in-depth analyses we searched for single differentially methylated positions (DMPs). We assessed DMPs based on the non-adjusted *p*-value (< 1E-04), because after adjusting for multiple comparisons all FDRs were above 0.05. For TCDD, we detected 27 potential DMPs, including a position in *DLK1* and eight positions in intergenic regions. PFOS exposure resulted in 45 potential DMPs, including a position in *DKK1* and 15 positions in intergenic regions. After TBT exposure, 16 potential DMPs were found, for which nine positions are in intergenic regions (Appendix D).

We determined hypo- and hypermethylation (i.e. decreased and increased levels in methylation, respectively) compared to the solvent control based on the top 1000 DMPs with the lowest non-adjusted *p*-values. Exposure to both TCDD and PFOS caused hypomethylation of the majority of the positions (Fig. 6A). In contrast, TBT-treatment resulted in both hypo- and hypermethylation. Hypomethylation of the promoter region might result in a transcriptionally permissive state by making DNA accessible for transcription factors. However, without any transcription factors actually binding these regions, DNA hypomethylation in itself does not necessarily change the expression of these genes.[46] Furthermore, hypomethylation also occurred in the gene body, and the lack of association with gene expression shows once more the complex relationship.[17]

Hypomethylation in IGRs, on the other hand, might lead to genomic instability, increasing the chance of cancer development.[15]

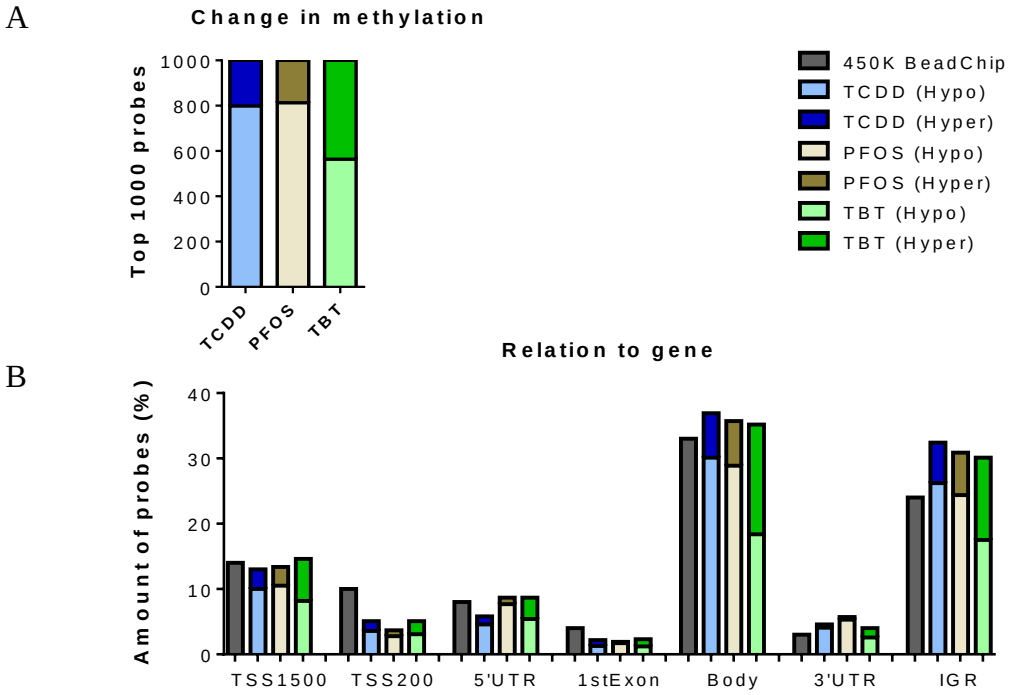

Fig. 6. Genome-wide DNA methylation analysis in differentiated hMSCs. **(A)** The number of hypo- and hypermethylated positions, based on the top 1000 most statistically significant positions, after continuous exposure to TCDD (1 nM), PFOS (10 µM) or TBT (10 nM) at day 10 of adipocyte differentiation. **(B)** Features of the 1000 positions with the highest statistical significance after continuous POP exposure at differentiation day 10 compared to all considered positions on the Illumina 450K BeadChip (indicated with '450K BeadChip').

Subsequently, we explored the location of the top 1000 DMPs in relation to the genes (Fig. 6B). In general, all three POPs affected relatively few DMPs close to the TSS and in the 1st exon, which is in agreement with the lack of concomitant gene expression changes. After POP exposure, there were more DMPs detected in the gene bodies and in the IGRs relative to the considered positions on the BeadChip. The function of DNA methylation in the IGR is currently unclear, but besides the importance for genomic stability, it is relevant for cell-type discriminatory patterns.[47] Furthermore, about half of the enhancers are present in IGRs[48], and differential methylation in enhancers might affect expression of multiple genes.[49,50] More research is needed to fully understand the

implication of differential methylation in IGRs, and subsequently the consequences of POPs interfering with DNA methylation.

Relevance of tested exposure concentrations

It is complicated to compare *in vitro* exposure levels to real-life environmental exposures, because *in vitro* studies lack the formation of POP metabolites and feed-back mechanisms from e.g. thyroid and steroid hormones.[51] Additionally, POP levels in humans are usually assessed per gram lipid, in both serum and adipose samples, and not in molar concentrations as for *in vitro* experiments. For determining the real-life relevance of the tested POP concentrations, we compared molar concentrations with measured blood levels in humans. However, adipose tissue levels of POPs can be a thousand fold higher per gram tissue than corresponding concentrations in serum.[1] TCDD levels are reported up to concentrations of 280 pM in human blood, with a median of 20 pM based on a 0.5% lipid content.[52] Our analysis revealed effects on adipocyte differentiation at concentrations of 100 pM and higher. This concentration is less than an order of magnitude from human median levels, while higher exposed individuals exceed this effect level. Median exposure levels for PFOS are around 100 nM, and only occupationally exposed workers have levels above the tested effect concentration of 10 µM.[53] For TBT, human median levels are around 17 nM in blood,[54] which is above the concentration of 3 nM that affected both adipocyte and osteoblast differentiation. This indicates that TCDD and TBT by itself might exert adverse health effects in the general environmentally exposed human population. In this study we explored the effects of single POPs, while in the real-life situation humans are exposure to a combination of POPs, and therefore additive effects cannot be excluded.[55] In a previous study, it was shown that TBT and TCDD have an agonistic inhibitory effect in osteoblast differentiation,[34] which might also be the case for other POPs and adipocyte differentiation. Therefore, even lower body burdens than the concentrations indicated may cause biological effects in adipose tissue due to both additivity and specific accumulation of POPs in adipose tissue. This is in agreement with studies demonstrating that POPs, at environmental levels, could contribute to obesity and metabolic disorders.[56]

Conclusions

Our data suggest that TCDD, PFOS, and TBT at relevant exposure levels can interfere with adipocyte differentiation, gene expression, and DNA methylation. HBCD, PCB153, BDE47, and MeHg did not alter adipocyte differentiation in both hMSCs and SGBS cells. Although no clear association between DNA methylation and gene expression was found, our results support the hypothesis that POPs can interfere with DNA methylation.

Acknowledgements

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Supporting information

Appendices can be found online after acceptation of this paper. Fig. A.1 shows the results of cytotoxicity testing in hMSCs and SGBS cells. Appendix B contains the gene description of the 84 adipogenic genes and the expression fold change together with the *p*-values of the POP-exposed cells. The DMRs found after genome-wide DNA methylation analyses for all three POPs are presented in Appendix C. The DMPs (*p*-value < 1E-04) detected for TCDD, PFOS, and TBT can be found in Appendix D.

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CHAPTER 4

Accumulation of persistent organic pollutants in consumers of eel from polluted rivers or relatively clean eel

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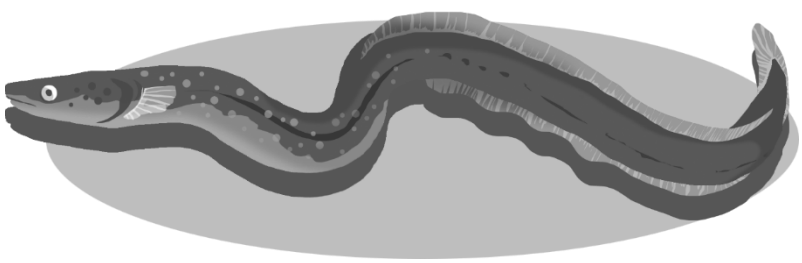

Abstract

Background Globally, many rivers are seriously contaminated with persistent organic pollutants (POPs) known to accumulate in aquatic food chains. In the Netherlands, toxicological risks of human exposure to dioxins and dioxin-like compounds led to a ban on commercial eel fishing in the Rhine-Meuse delta. The necessity of this ban as well as expected elevated POP levels resulting from contaminated eel consumption is, however, debated.

Objectives The aim of this study is to investigate differences in serum POP levels in consumers of eel from high-polluted areas to consumers of eel from low-polluted areas or aquaculture.

Methods In total 80 Dutch men were included, aged 40 to 70 years and with a habitual eel consumption of at least one portion (150 g) per month. Dioxins and dioxin-like compounds were measured in serum of all participants with the DR CALUX bioassay, validated with GC-MS. For a subgroup of 38 participants extensive POP measurements were performed, resulting in 30 POPs for which at least 60% of the participants had levels above the detection limit.

Results Levels of dioxins and dioxin-like compounds, polychlorinated biphenyls (PCBs) with their hydroxylated (OH) metabolites, and perfluoroalkyl substances (PFASs) were, up to ten times, higher in consumers of eel from pullulated areas compared to controls.

Conclusions This study is the first to reveal that (past) consumption of eel from polluted rivers resulted in high body burdens of dioxins, PCBs, and PFASs. The high levels of dioxins and dioxin-like compounds as well as the OH-PCBs are of health concern.

Introduction

Persistent organic pollutants (POPs) are a group of mostly lipophilic compounds that are resistant to degradation and accumulate in the environment and the food chain. POPs include dioxins, polychlorinated biphenyls (PCBs), organochlorine (OC) pesticides, brominated flame retardants, and perfluoroalkyl substances (PFASs). These chemicals are known for their potency to cause various adverse health effects, including endocrine disorders, cancer, and neurodevelopmental problems.[1] Many POPs are regulated by the Stockholm Convention, and POP levels in the environment have been decreasing over the past decades.[2] However, their occurrence in the environment is still a major concern. A high degree of urbanization and industrialization along European rivers have caused the sediments of the main Dutch rivers and estuaries to be highly polluted. Over 90% of human exposure to POPs comes from food consumption,[3] with high contributions from fish caught in polluted areas.[4,5] Eel has eco-physiological features such as bottom dwelling, being a long-living predator, and having a high lipid content, which attributes to high accumulation of contaminants.[6-8]

Dioxins (polychlorinated dibenzo-p-dioxins/dibenzofurans (PCDD/Fs)) and dioxin-like (DL-) PCBs in food have received considerable attention from the European Commission. PCDD/Fs are highly toxic, although with different toxic potencies, as expressed in a range of toxic equivalency factors (TEFs).[9] DL-PCBs, including non-ortho (NO) and mono-ortho (MO) PCBs, have properties similar to PCDD/Fs and therefore also assigned TEF values. The NO-PCBs have a higher dioxin-like potency than the MO-PCBs.[9] To reduce levels in food and hence human exposure, maximum levels (MLs) for PCDD/Fs and DL-PCBs in various food items have been established using toxic equivalents (TEQs) to sum up the different TEF values. The ML for the sum of PCDD/Fs and DL-PCBs in eel was set at 10 pg TEQ/g wet weight (ww) in 2012, while other seafood items have to comply to 6.5 pg TEQ/g ww.[10] Several studies showed that the majority of eel from the Rhine-Meuse delta did not comply with the ML.[8,11] Following this discovery, a risk assessment was performed, including the potential impact on the body burden. Long-term consumption of one portion eel (150 gram) per month from these high-polluted areas (average level of 29 pg TEQ/g eel) was estimated to result in a body

burden of 7.6 ng TEQ/kg body weight (bw), compared to the Dutch background body burden of 3.0 ng TEQ/kg bw.[12] Moderate eel consumers were therefore expected to reach POP levels above the safe body burden of 4 ng TEQ/kg bw (extrapolated from rat studies), implying that adverse health effects could not be excluded.[13,14] After a documentary was broadcasted raising awareness, the Dutch government decided on a complete ban on eel fishing in the seriously polluted fishing areas from 2011 onwards. Despite this ban, it is expected that people who consumed this eel in the past will still have elevated levels of PCDD/Fs, PCBs and other POPs in their body due to the long half-lives of these pollutants (approximately 10-15 years for PCBs).[15] As the necessity of the prohibited eel fishing is still debated, we decided to study the actual body burdens in eel consumers to verify the expectations expressed in the former risk assessment. The aim of this study was to compare blood POP levels in men who consumed eel from high-polluted areas (now closed for eel fishing) with men consuming eel from aquaculture and relatively low-polluted areas (areas open for eel fisheries).

Methods

Study population

Eligible participants were all Dutch men aged from 40 to 70, with a habitual eel consumption of at least one portion (150 gram) a month since many years (at least until the implemented ban on eel fishing). Age range was based on Hoogenboom *et al*, who showed that body burden increases due to eel consumption, but remains relatively stable from 40 to 70 years of age.[12] Men were invited to participate through professional and recreational fishermen associations, because these men are more likely to know the origin of the eel, and through advertisements in local newspapers and webpages. A total of 80 men were included after checking whether they met the inclusion criteria. This study was approved by the Medical Ethical Committee of Wageningen University, and written informed consent was obtained from all participants before inclusion in the study.

Participants filled out a questionnaire at home about their fish consumption habits, including the origin of the eel (either aquaculture or the area where the eel was caught). Height and weight were measured using standard methods in light clothes. Blood samples were obtained by venipuncture after overnight fasting, and serum for the persistent organic pollutant (POP) analyses was separated by centrifugation within 6 hours after collection. Samples were stored at -80^{0}C until further analyses. Total cholesterol and triglycerides levels were measured in plasma in a clinical chemistry laboratory (Hospital Gelderse Vallei, Ede, the Netherlands).

DR CALUX bioassay

Total levels of dioxins and dioxin-like compounds were measured for all 80 subjects using the DR CALUX bioassay at RIKILT Wageningen UR, which is validated for food and feed, as described previously.[16] In short, aliquots of 5 mL serum were extracted twice with hexane and purified on a column containing 10 g acid silica (33% H_2SO_4) with 1 g dried Na_2SO_4 on top. The extracts were dried in a SpeedVac with 10 µL DMSO as a keeper, which was later mixed with cell culture medium. Control samples of butter fat with different levels of a mix of PCDD/Fs and dl-PCBs (0.5; 17; 39; and 88 pg TEQ/g lipid), and with a similar absolute amount of fat, were included and extracted in the same way. p-GudLuc transfected H4IIE-cells were obtained from Wageningen University[17] and are similar to those sold by Biodetection Systems (BDS, Amsterdam, The Netherlands). Cells were cultured in a 96-wells plate and exposed to the extract (1% DMSO) in quadruplicate for 24h. The cells were lysed and the luciferase content was measured in a Luminoskan (Labsystems). Total levels of dioxin and dioxin-like compounds were estimated from a calibration curve of the reference butter fat samples included in each clean-up series. These estimated levels were expressed in bioanalytical equivalents (BEQs) to acknowledge the fact that they were determined with a bioassay and not with gas chromatography high resolution mass spectrometry (GC-HRMS). In the bioassay also other compounds that pass the selective clean-up may contribute to the response. BEQ levels were adjusted for lipid content, calculated based on triglycerides and total cholesterol levels as described by Philips *et al*[18] and recommended by AMAP[19], which correlates well to gravimetric determination.[20,21]

Validation bioassay with GC-HRMS and congener patterns

Eight pooled samples as well as two individual samples with the highest estimated BEQ levels (> 100 pg BEQ/g lipid) were measured with GC-HRMS at RIKILT Wageningen UR, which is ISO/IEC 17025 accredited for the analysis of PCDD/F and PCB containing extracts (L014). GC-HRMS was first of all performed to validate the bioassay results, and secondly to compare congener patterns. Pooled serum included samples from all low- and high-exposed men (n=34 and n=21, respectively) and the subgroup for which the extensive POP measurements were performed (n=14 for low- and n=13 for high-exposed men). Furthermore, samples were pooled based on individual BEQ levels to get sub-groups for the low- and high-exposed group with the same average BEQ levels (20 and 45 pg BEQ/g lipid). Levels of PCDD/Fs and dl-PCBs were determined in 3 to 12 mL serum. Prior to extraction, the samples were spiked with ^{13}C- isotope labelled internal standard for each congener. Extraction of lipids was performed as previously described.[22] After evaporation of the solvents, the residues (lipids) were dissolved in hexane and the crude extract was purified using an automated clean-up (PowerPrep system, Fluid Management Systems, Waltham, USA). Extracts were purified on an acid silica column, a neutral silica column, a basic alumina column and an activated carbon/Celite column. For the elution from the columns, custom made solvents and mixtures were used, respectively being hexane, hexane/dichloromethane (1:1, v/v), ethyl acetate/toluene (1:1, v/v) and toluene. The volume of the final extract was reduced using an automated evaporation system with fixed endpoint of 0.5 ml. The recovery standards, ^{13}C-labelled 1,2,3,4-TCDD and 2,3,4,6,7,8-HxCDF, were added and the volume of the extract was again reduced to 0.5 ml. PCDD/F and PCB analyses were performed by GC-HRMS using an Agilent (Wilmington, USA) 6890 Series gas chromatograph and an AutoSpec Ultima high resolution mass spectrometer (Waters, Milford, USA) operated at a resolution of 10,000 (10% peak valley). The GC column was a DB5 MS (60 m, 0.25 mm i.d., 0.25 µm; J&W, Folson, USA). The mass spectrometer was operated in electron impact ionization mode, using selected-ion monitoring. Further GC-HRMS details can be found elsewhere.[23] A large volume injector (LVI) capable to inject 100 µl was used to inject the sample on the GC. Data were converted to TEQ levels using the TEFs of 2005[9] and expressed per gram lipid using the calculated lipid content.

Extensive POP analyses

Further analyses for individual POPs were performed for 38 participants. Samples were selected based on age and BEQ values. Participants were divided in three age groups (40-49, 50-59, 60-70) to ensure age differences between groups would not affect POP results as body burden is related to age.[24] Based on bioassay results, participants were divided in having a low body burden (median around 13 pg BEQ/g lipid), medium body burden (median 2x higher than low body burden), and high body burden (median 4x higher than low body burden, detailed information in Table S4). After this stratified sampling, we used a simple random sampling for each stratum to choose the 38 participants.

The individual POP analyses were performed at the Laboratory of Environmental Toxicology at the Norwegian University of Life Sciences (NMBU), which is ISO/IEC 17025 accredited for the analyses of PCBs, organochlorine (OC) pesticides, and brominated flame retardants (BFRs) (TEST 137).[25] The extraction method for most POPs, excluding perfluoroalkyl substances (PFASs), was performed with 4 mL of serum and is based on a liquid/liquid lipid extraction with acetone and cyclohexane (3:2), sodium chloride (NaCl) and 10 mL 1 M sulphuric acid (H_2SO_4) which is described by Polder *et al.*[26] An additional extraction with potassium hydroxide (1 M) was performed to extract the hydroxylated metabolites.[27] The analyses were performed with GC, followed by Electron-Capture Detection (ECD, for analyzing PCBs and OC pesticides) and by low resolution MS (LRMS) quadrupole detector for OH-PCBs, BFRs and for confirmation of certain PCBs and OC pesticides. The limit of detection (LOD) was set at three times the noise levels and recoveries ranged from 77 to 116%. Lipid determination was done gravimetrically and these results correlated well with the calculated lipid content based on triglycerides and total cholesterol levels (spearman r = 0.79; calculated fat percentage = 0.75*gravimetrically determined fat percentage + 0.15). To make sure the results within this study are comparable, the calculated lipid content was used for normalization in all experiments.

The extraction for PFASs analyses was performed with 1 mL of serum using methanol as is described by Bytingsvik *et al.*[28] The cleaning of fat and other matrix constituents was

performed with a package material called Envi-Carb. The following internal standards were added to all samples: $^{13}C_4$ PFOS, $^{18}O_2$ PFHxS, $^{13}C_8$ FOSA, $^{13}C_2$ PFDoDA, $^{13}C_4$ PFUdA, $^{13}C_4$ PFDA, $^{13}C_5$ PFNA, $^{13}C_4$ PFOA, $^{13}C_4$ PFHxA, and $^{13}C_4$ PFHpA (Wellington Laboratories, Guelph, Canada). $^{13}C_2$ PFDoA was also used for PFTrDA and PFTeDA, while $^{18}O_2$ PFHxS was also used for PFBS. Control AMAP Ring Test samples were included, following the same procedures, and these samples complied with the quality criteria.[19] Quantification was performed with LC-MS with ESI ion source (6460 Triple Quadrupole LC-MS, Agilent Technologies, Santa Clara, USA) using a Supelco Discovery C18 column (150 mm x 2.1 mm, 5 µm, Sigma-Aldrich, Oslo, Norway). Recoveries ranged from 93 to 119% and results were normalized for serum wet weight (ww).

Data analysis

Only compounds quantified in more than 60% of the samples are reported and used in statistical analyses. To ensure that values below the LOD did not affect the overall results, they were replaced by random imputed values with a log-normal distribution between zero and the LOD, before the contaminant data were normalized to lipid content of individual samples.[29,30] All POP levels were log10-transformed for statistical and graphical purposes. After log-transformation not all POPs were normally distributed as verified with the Kolmogorov-Smirnov test. Therefore, non-parametric tests were used. The Mann-Whitney *U* test was performed to compare the low-exposed to the high-exposed group and the Spearman rank correlation was used to test the statistical significance for the correlation between the BEQ levels and age. Differences were assumed to be statistically significant when the *p*-value is below 0.05. To convert the well-accepted safe body burden of 4 ng TEQ/kg bw to levels in serum (15.1 pg TEQ/g lipid) for comparison reasons, a 26.5% body fat content was used.[31,32]

Results

Study characteristics

In total 80 eligible men responded to our research invitation and eventually participated in this study. Of these men, 34 consumed eel exclusively from areas open for eel fishery

or aquaculture (low-exposed group) and 21 consumed eel exclusively from high-polluted areas where the ban on eel fishing is now implemented (high-exposed group). Other participants consumed eel from both areas or were not aware of the origin of the eel. The majority of the men consumed eel once or twice per month, and 20% consumed eel at least five times per month. Most men also consumed other fish. Age and BMI were comparable between the low- and high-exposed groups (Table S1).

Dioxins and dioxin-like compounds

Total levels of dioxins and dioxin-like compounds are expressed in bioanalytical equivalent (BEQ) when measured with the bioassay, and in toxic equivalent (TEQ) when analyzed with GC-HRMS. These values correlated well (Spearman r = 0.95; BEQ = 0.95*TEQ + 4.0), based on ten samples analyzed with both techniques. The BEQ values followed a normal distribution (Fig. 1A) and were in general increased in the high-exposed men (Fig. 1B), with levels up to 145 pg BEQ/g lipid (164 TEQ/g lipid based on GC-HRMS). Based on the pooled samples from the low- and high-exposed men, the high-exposed men had on average 2.5 times higher TEQ levels. In the low-exposed group, especially the dioxins contributed to the TEQ level (Fig. 1C). For the high-exposed group the majority of the TEQ came from DL-PCBs, more specifically the NO-PCBs (Table S2). The MO-PCBs, however, showed a higher relative increase, namely 2.3-times compared to 1.5-times for the NO-PCBs comparing percentages of high- to low-exposed. Also other PCBs were present at relatively higher levels as shown by the ratio of PCB153 and TEQ (Fig. S1).

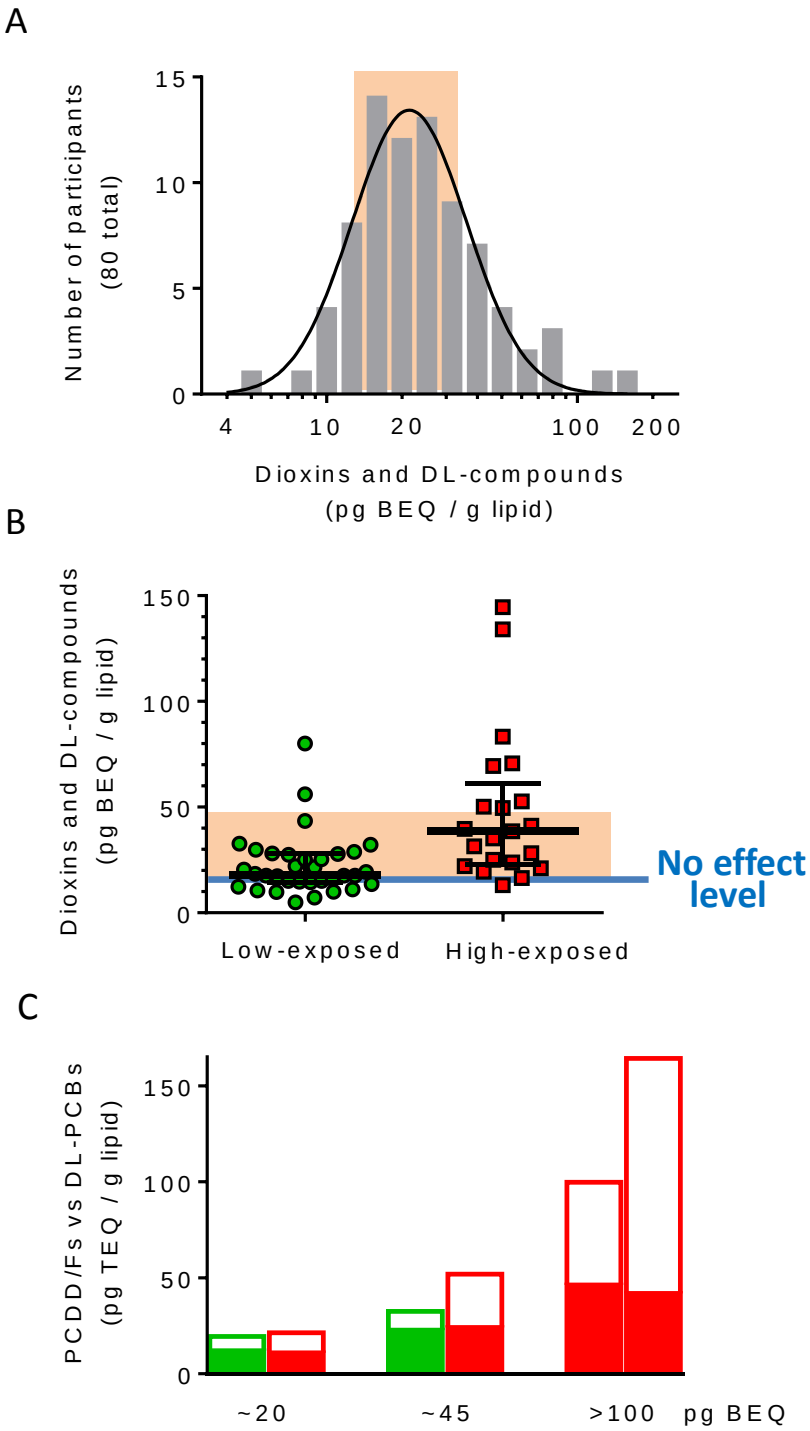

Figure 1. (**A**) Frequency distribution of dioxins and dioxin-like (DL) compounds determined for all 80 participants. The light orange box presents European background levels reported in the 21st century.[33] (**B**) Levels of dioxins and other dioxin-like (DL) compounds for the 55 participants that ate eel exclusively from either low- (green circles) or high-polluted areas (red squares). The black lines indicate the median with the interquartile ranges. The blue line indicates the extrapolated safe blood level.[13] (**C**) The contribution of PCDD/Fs (filled bar) and DL-PCBs (clear bar) to TEQ levels in pooled blood from men with 20 and 45 pg BEQ/g lipid, and two individual samples (>100 pg BEQ/g lipid, presented separately) from men that consumed eel from low- (green) and high-polluted areas (red). Details can be found in Table S3.

Individual persistent organic pollutants (POPs)

Of the 48 POPs measured in the subgroup of 38 men (14 low-exposed, 13 high-exposed, and 11 with unknown origin of the majority of the consumed eel), 30 POPs had levels above the LOD in at least 60% of the samples (Table S3). Levels of the OC pesticides: β-HCH, mirex, oxychlordane, and trans-nonachlor, did not differ between the two groups (Fig. S2). Levels of HCB and DDT, but not DDE, were significantly elevated in the high-exposed men (Fig. 2). Furthermore, all PCBs (except for PCB 52) were significantly higher in the high-exposed men, with fold differences ranging up to 10.6 (Fig. 2). Concomitant increases of the internal OH-PCB metabolites were observed, amounting to an eight-times increase for the sum of the four measured OH-PCBs (Fig. 3).

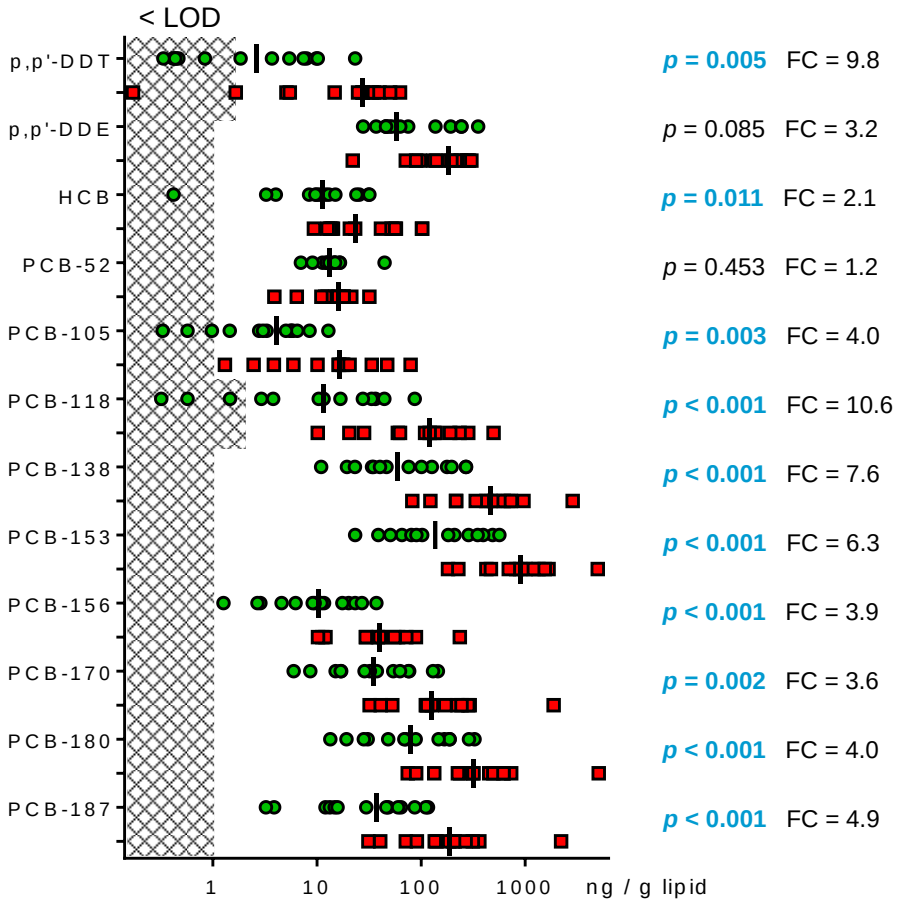

Figure 2. DDT, DDE, HCB, and PCB levels in men who ate eel from low- (green circles) and high-polluted areas (red squares). The *p*-value in blue indicates a significant difference (Mann-Whitney *U* test). The fold change (FC) is calculated by dividing the median (indicated black line) of the high- by that of the low-exposed group. Samples in the grey quadrant are below the limit of detection (<LOD).

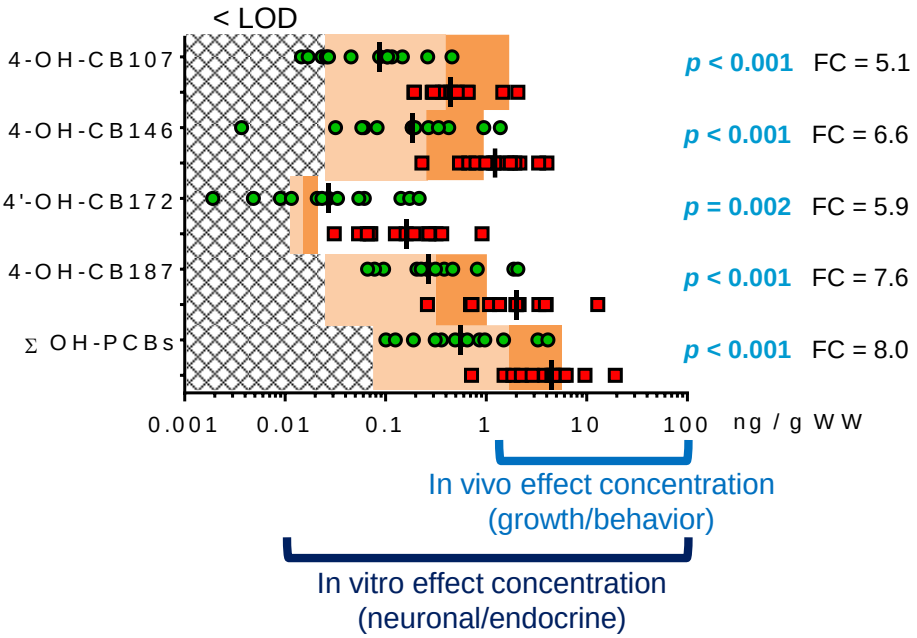

Figure 3. OH-PCB levels in male consumers of eel from low- (green circles) and high-polluted areas (red squares), expressed as ng/g ww because OH-PCBs do not accumulate in lipids. Blue *p*-values indicate a significant difference (Mann-Whitney *U* test). The fold change (FC) is calculated by dividing the median (indicated black line) of the high- by that of the low-exposed group. ∑ OH-PCBs is the sum of the 4 measured OH-PCBs. Samples in the grey quadrant are below the limit of detection (<LOD). The light orange area represents world-wide background levels of OH-PCBs in the 21st century, while the dark orange represents levels in high-exposed populations (average median high-exposed levels to maximum median levels reported in the literature).[34] The *in vivo* as well as the *in vitro* effect concentrations are also based on a literature review.[34]

Two PBDEs, BDE-47 and BDE-153, were detectable in at least 60% of the men but levels were not significantly different between the groups (Fig. S3). Furthermore, two perfluorinated alkylsulfates and six perfluorinated alkylacids were detectable (Fig. S4). The median levels of most PFASs were significantly elevated, up to 3.5 times in the high-exposed men. Detailed information for all 30 POPs can be found in Table S4.

Discussion

Our research is the first to reveal that men who consumed eel from high-polluted areas in the Netherlands have, up to ten times, higher body burdens of dioxins, (OH-)PCBs and PFASs compared to men consuming eel from relatively clean areas or aquaculture. Although risk assessment and management mainly focus on dioxins and PCBs, we show that also body burden of hydroxylated PCBs might be of health concern.

The dioxins and dioxin-like levels observed in the low-exposed men are comparable to background levels observed in other European studies (orange block, Fig 1A).[33] The high-exposed men had on average 2.5 times higher TEQ levels, which is in agreement with the predictions made in a previous risk assessment on eel consumption.[12] Total dioxins and dioxin-like levels, however, were lower than those reported in fishermen consuming fish from the high-polluted Baltic Sea.[35] The Baltic Sea herring contains similar to higher TEQ levels than eel, and is consumed more frequently, explaining the even higher levels in consumers.[36]

The median BEQ levels of both groups in our study (18 and 39 pg BEQ/g lipid, for the low- and high-exposed respectively), are above the estimated safe level of 15 pg TEQ/g lipid. Although fish consumption is considered healthy because of its nutritional value, frequent consumption of fish with levels above the ML is expected to result in TEQ body burdens that exceed the safe level.[12,37] The safe level is derived from reproduction studies and aims to protect the developing unborn child.[13] The actual safe level for adults may be higher but this was not specifically addressed in the risk assessment.

Men in this study consumed eel for many years, and given the long half-life, the body burden could include POPs from even higher contaminated eel consumed in the past and from other food. Interestingly, in the low-exposed group the BEQ levels are positively correlated with age, but not in the high-exposed group (Fig S5). This could be explained by recent intake of high-polluted eel, while the low-exposed group might have benefited more from the generally decreased POP levels in food products.

To confirm that eel is the actual source of the elevated POP levels, we compared the POP profiles in men and eel. In general, TEQ exposure from aquatic species mainly

originates from DL-PCBs, while in terrestrial sources (such as meat and dairy) PCDD/Fs and DL-PCBs have an equal contribution.[38] Indeed, for the average Dutch consumer the main sources contributing to PCDDD/Fs are dairy products, while fish contributes to most of the DL-PCBs.[37] In our study the major contribution to the TEQ levels in the high-exposed men came from DL-PCBs, indicating fish as a likely source, while in low-exposed men the PCDD/Fs contributed most to the TEQ. Additional indication for eel as the source of the higher TEQ levels is the relatively high contribution of MO-PCBs within the DL-PCBs. In farmed eel and other fish, NO-PCBs predominate, but wild eel has relatively high levels of MO-PCBs.[11] The PCB profiles in the high-exposed men followed the eel profiles, while the PCDD/F profiles were different between men and eel (Fig. 4). This, together with the increasing PCB153 to TEQ ratio in the high-exposed men (Fig. S1), indicates that eel is the most likely source of the elevated TEQ and PCB levels.

The most pronounced differences between the low- and high-exposed men were observed for the PCBs and OH-PCBs, followed by the PFASs. Levels of PFAS in the low-exposed group are comparable to levels measured in the USA,[39] while the high-exposed men had PFOS levels well above general population levels.[40] It is, however, not yet known above which levels adverse effects could occur.[41] Brominated flame retardant levels did not significantly differ between the groups, which is in accordance with earlier reports that regular eel consumptions does not cause health risks from PBDEs and HBCD.[14] Besides food, significant exposure to PBDEs occurs from house dust due to e.g. electronics or carpets releasing flame retardants,[42] while PCB exposure is almost fully limited to the food chain.[3] The highest PCB-exposed populations are found around the artic, where traditional foods include marine mammals, such as seal and whale blubber.[43-45] Interestingly, levels of PCBs and their OH-metabolites were higher in Dutch men consuming eel from high-polluted areas than in men consuming marine mammals, and were thus among the highest levels measured in the 21st century (orange blocks, Fig. 3).[34] This could be related to the higher PCB biotransformation capacity in mammals compared to fish,[46] resulting in higher PCB levels in fish, which will be metabolized by the consumers who subsequently retain the OH-metabolites in their body.

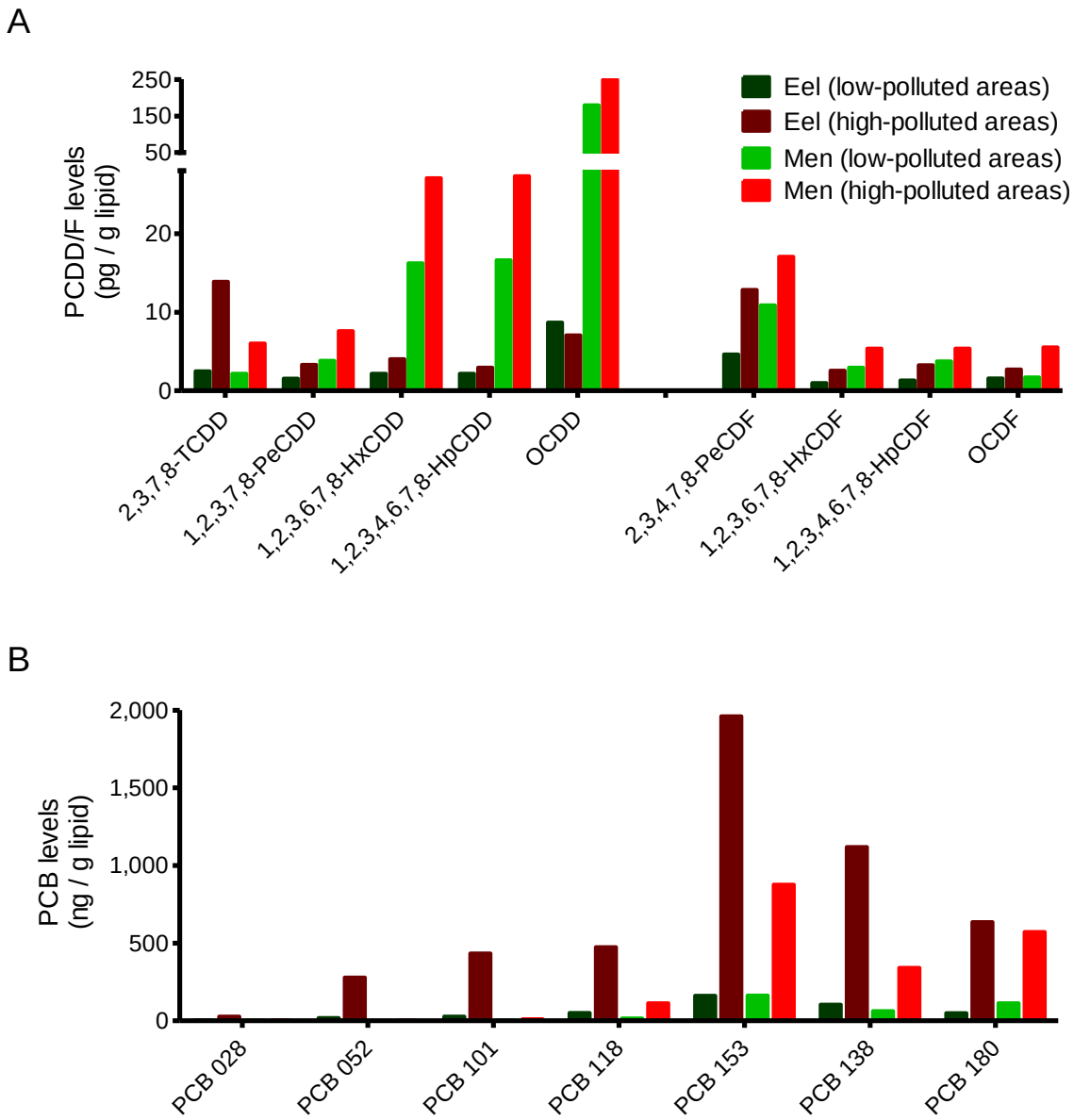

Figure 4. Profiles of (**A**) PCDD/Fs that occurred in levels higher than 5 pg/g lipid in high-exposed men and (**B**) the seven indicator PCBs in both eel and men. Eel data are derived from 360 (pooled) samples, collected between 2001 and 2012. The average levels in eel are calculated for both low- (dark green bars) and high-polluted areas (dark red bars) (unpublished data from monitoring program). Human data are determined in pooled samples for low- (n=34, bright green bars) and high-polluted men (n=21, bright red bars) (this study).

OH-PCBs have been associated with several adverse health effects in animal studies, especially related to development, growth and behavior.[34] OH-PCBs can mimic thyroid hormones and have a high affinity for thyroid hormone transport proteins.[47] In all men the sum of the OH-metabolites are expected to give adverse health effects based on *in vitro* studies, and based on animal studies adverse health effects might be expected in the majority of the men (Fig 3). Furthermore, the (OH-)PCB levels in the high-exposed eel consumers were slightly higher than those reported for Inuit from Canada, where negative effects on thyroid hormone parameters were identified, especially a reduction of triiodothyronine.[48] As already explained, there is a special concern for the most vulnerable humans, especially the unborn child exposed through its mother. Unborn children of women with a comparable eel consumption as the men in this study are likely not only exposed to dioxins but also to OH-PCBs above safe levels.

Our results show that consumption of eel from high-polluted areas is not safe, and therefore we support the ban (in the Netherlands and France) on catching eel in high-polluted areas. Several other countries (e.g. Belgium and Germany), however, still do not have a ban on catching eel from high-polluted areas, only a recommendation not to consume this eel. Indications exist that recreational fishermen are still catching high-polluted eel for consumption, and we recommend more strict regulation to prevent consumption of these eels. Most eel sold in the Netherlands nowadays comes from aquaculture and to a limited extent from low-polluted areas. Our results reveal that consumption of this eel indeed does not lead to a high increase of background POP levels, as was predicted before.[12]

Conclusions

From a public health perspective, weekly fish consumption as advised by the WHO[49] can be encouraged for all fish with levels well below the legal limits. However, to protect human health, appropriate measures should be taken to prevent consumption of fish from high-polluted areas world-wide.

Acknowledgements

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Supplemental material

Table S1. Characteristics of study population. Values are given as median (min-max) or in number of participants per exposure category (percentage).

		Total	Low-exposed*	High-exposed*
Number of participants		80	34	21
Age (years)	Med (min-max)	59 (40-70)	58 (41-70)	62 (40-70)
Body Mass Index (kg/m^2)	Med (min-max)	29.2 (21.4-45.2)	29.3 (21.8-41.3)	29.5 (23.0-35.3)
Fishermen	Professional	26 (33)	10 (29)	13 (62)
n (%)	Sport	27 (34)	9 (26)	8 (38)
	No	27 (34)	15 (44)	0 (0)
Frequency eel	1-2x per month	51 (64)	23 (68)	10 (48)
consumption	3-4x per month	13 (16)	2 (6)	7 (33)
n (%)	>5x per month	16 (20)	9 (26)	4 (19)
Frequency consumption	<1x per week	44 (55)	17 (50)	14 (67)
other fatty fish	1-2x per week	34 (43)	16 (47)	6 (29)
n (%)	3-4x per week	2 (3)	1 (3)	1 (5)
Frequency consumption	<1x per week	33 (41)	12 (35)	9 (43)
of semi-fatty fish	1-2x per week	44 (55)	20 (59)	12 (57)
n (%)	3-4x per week	3 (4)	2 (6)	0 (0)
Frequency consumption	<1x per week	44 (55)	21 (62)	11 (52)
of lean fish	1-2x per week	34 (43)	13 (38)	10 (48)
n (%)	3-4x per week	2 (3)	0 (0)	1 (5)

* The low-exposed group ate eel exclusively from aquaculture and/or from the areas regarded safe for eel consumption. The high-exposed group ate eel exclusively from areas now banned for eel fishing. The other participants consumed eel from both areas or were not aware of the origin of the eel.

Table S2. Levels of PCDD/Fs, non-ortho (NO) and mono-ortho (MO) PCBs as measured by GC-HRMS. Absolute values are given in pg TEQ/g lipid and relative contribution to the total TEQ is given between brackets. Numbers above the table indicate the approximate total BEQ level based on the bioassay. For the low and middle group (20 and 45 pg BEQ/g lipid) pooled samples were measured with the same BEQ levels for both the low- (green columns) and high-exposed group (red columns). The two individuals with BEQ levels above 100 pg BEQ/g lipid were measured separately.

	~20 pg BEQ/g lipid		*~45 pg BEQ/g lipid*		*>100 pg BEQ/g lipid*	
	Low-exposed (n=8)	*High-exposed* (n=7)	*Low-exposed* (n=3)	*High-exposed* (n=5)	*High-exposed* (n=1)	*High-exposed* (n=1)
PCDD/F	12.0 (61%)	10.9 (51%)	22.7 (70%)	24.1 (46%)	46.3 (46%)	41.9 (26%)
NO-PCB	6.1 (31%)	8.2 (38%)	7.8 (24%)	20.6 (40%)	39.7 (40%)	85.0 (52%)
MO-PCB	1.5 (7%)	2.3 (11%)	2.0 (6%)	7.2 (14%)	13.8 (14%)	37.3 (23%)

Table S3. List of all 48 persistent organic pollutants (POPs) measured in serum samples, together with the limit of detection (LOD), the percentage of samples with levels above the LOD and the maximum levels measured.

	LOD (ng/g ww)	**Samples above LOD** (%)	**Max levels** (ng/g ww)	**Max levels** (ng/g lipid)
OC pesticides				
p,p'-DDE	0.01	100	2.4	353
p,p'-DDT	0.01	68	0.53	62.6
HCB	0.01	97	0.86	102
β-HCH	0.02	100	0.29	36.9
Mirex	0.01	84	0.23	29.9
Oxychlordane	0.01	82	0.32	46.1
Trans-nonachlor	0.01	100	0.39	49.2
PCBs				
PCB-28	0.02	39	0.12	14.8
PCB-52	0.01	100	0.29	44.5
PCB-101	0.02	55	0.65	82.2
PCB-105	0.01	82	0.63	79.3
PCB-118	0.02	74	3.9	497
PCB-138 (/ 163)	0.01	100	22.8	2870
PCB-153	0.02	100	39.4	4960
PCB-156	0.01	100	1.9	235
PCB-170	0.02	100	14.9	1880
PCB-180	0.01	100	40.3	5080
PCB-187	0.01	100	17.6	2210
OH-compounds				
3-OH-CB118	0.02	46	0.38	/
3'-OH-CB138	0.04	57	1.7	/
3'-OH-CB180	0.01	57	0.57	/
4-OH-CB107	0.02	92	2.1	/
4'-OH-CB130	0.03	0	-	/
4-OH-CB146	0.02	95	4.0	/
4'-OH-CB159	0.00	51	0.03	/
4'-OH-CB172	0.01	84	0.91	/
4-OH-CB187	0.02	100	12.8	/
6-OH-BDE47	0.02	46	0.13	/
Brominated compounds				
BDE-28	0.01	0	-	-
BDE-47	0.01	66	0.07	8.3
BDE-99	0.01	5	0.02	2.7
BDE-100	0.01	21	0.04	6.9
BDE-153	0.01	63	0.14	17.7
BDE-154	0.01	0	-	-
HBCD	0.19	13	0.64	80.7

PFASs				
PFOS	0.13	100	259	/
PFHxS	0.20	100	16.6	/
PFBS	1.00	0	-	/
FOSA	0.12	0	-	/
PFTrDA	0.04	86	0.56	/
PFDoDA	0.04	78	1.5	/
PFUdA	0.20	84	5.2	/
PFDA	0.05	100	12.8	/
PFNA	0.06	100	9.4	/
PFOA	0.20	100	17.8	/
PFHxA	0.10	0	-	/
PFHpA	0.06	35	0.4	/
PFTeDA	0.04	46	0.3	/

Table S4. Results of extensive POP measurements in all participants as well as in the men consuming eel from relatively low- and high-polluted areas.

		All measured participants			*Low-exposed*			*High-exposed*		
	N (all / low / high)	25p	**Median**	75p	25p	**Median**	75p	25p	**Median**	75p
DR CALUX (pg BEQ / g lipid)	80 / 34 / 21	16.3	**22.3**	32.3	14.5	**18.0**	27.7	23.8	**38.5**	52.7
DR CALUX (pg BEQ / g lipid)	38 / 14 / 13	17.4	**27.3**	42.5	14.4	**18.5**	30.8	31.4	**49.6**	70.7
OC (ng / g lipid)										
p,p'- DDE	38 / 14 / 13	51.4	**111.7**	227.8	46.0	**57.7**	178.2	97.2	**182.1**	241.7
p,p'-DDT	38 / 14 / 13	0.9	**5.2**	21.3	0.4	**2.8**	7.8	5.5	**27.2**	35.2
HCB	38 / 14 / 13	10.1	**13.8**	25.3	8.7	**11.3**	21.6	13.8	**23.3**	56.4
β-HCH	38 / 14 / 13	6.3	**9.6**	16.7	6.3	**11.2**	17.6	6.9	**13.7**	19.8
Mirex	38 / 14 / 13	3.4	**5.7**	9.2	3.4	**7.2**	11.1	2.8	**8.8**	23.0
Oxychlordane	38 / 14 / 13	1.8	**4.0**	8.6	1.5	**4.9**	7.5	11.8	**13.2**	18.5
Trans-nonachlor	38 / 14 / 13	7.4	**11.8**	18.3	7.1	**11.3**	21.4	3.7	**5.7**	6.2
PCBs (ng / g lipid)										
PCB-52	38 / 14 / 13	11.3	**12.6**	16.2	12.4	**13.3**	14.9	12.0	**16.0**	17.9
PCB-105	38 / 14 / 13	2.5	**5.6**	12.3	1.8	**4.2**	5.8	5.9	**16.4**	20.5
PCB-118	38 / 14 / 13	3.2	**25.8**	80.8	3.1	**11.4**	32.0	59.8	**121.0**	190.2
PCB-138	38 / 14 / 13	42	**152**	318	34	**61**	165	217	**465**	689
PCB-153	38 / 14 / 13	93	**317**	662	69	**142**	333	468	**896**	1340
PCB-156	38 / 14 / 13	7	**15**	35	5	**10**	19	29	**40**	60
PCB-170	38 / 14 / 13	28	**58**	123	17	**35**	72	111	**126**	245
PCB-180	38 / 14 / 13	63	**157**	306	35	**80**	183	225	**319**	617
PCB-187	38 / 14 / 13	16	**62**	131	14	**38**	63	91	**186**	267
OH-PCBs (ng / g ww)										
4-OH-CB107	37 / 13 / 13	0.0	**0.1**	0.4	0.0	**0.1**	0.1	0.3	**0.4**	0.6
4-OH-CB146	37 / 13 / 13	0.1	**0.3**	1.1	0.1	**0.2**	0.3	0.8	**1.2**	1.9
4'-OH-CB172	37 / 13 / 13	0.0	**0.1**	0.1	0.0	**0.0**	0.1	0.1	**0.2**	0.3
4-OH-CB187	37 / 13 / 13	0.2	**0.5**	1.9	0.2	**0.3**	0.5	1.1	**2.0**	3.3
BDEs (ng / g lipid)										
BDE-47	38 / 14 / 13	0.6	**1.6**	2.6	0.3	**1.0**	1.9	1.6	**2.0**	2.7
BDE-153	38 / 14 / 13	1.2	**3.1**	5.3	0.9	**2.4**	4.6	2.6	**4.5**	10.8
PFASs (ng / g ww)										
PFOS	37 / 14 / 13	10.6	**38.8**	82.3	10.6	**33.6**	55.0	71.8	**82.3**	104.5
PFHxS	37 / 14 / 13	2.5	**3.6**	6.5	2.6	**3.3**	3.9	4.0	**6.2**	9.5
PFTrDA	37 / 14 / 13	0.1	**0.1**	0.2	0.0	**0.1**	0.1	0.1	**0.3**	0.4
PFDoDA	37 / 14 / 13	0.1	**0.2**	0.4	0.0	**0.2**	0.2	0.3	**0.5**	0.9
PFUdA	37 / 14 / 13	0.3	**0.6**	1.4	0.3	**0.7**	1.2	0.6	**1.4**	2.5
PFDA	37 / 14 / 13	0.6	**1.7**	4.8	0.6	**1.9**	3.4	1.9	**4.8**	6.7
PFNA	37 / 14 / 13	1.0	**1.5**	2.5	0.9	**1.4**	3.6	1.7	**2.2**	3.4
PFOA	37 / 14 / 13	2.9	**4.2**	5.9	2.7	**3.6**	5.0	4.8	**5.5**	7.0

All measured participants: all men who consumed eel at least once per month, independent of the origin on the eel:
Low-exposed: men who only consumed eel from aquaculture or wild eel from areas from which consumption is regarded to be safe.
High-exposed: men who only consumed wild eel from areas that are now closed for consumption due to high POP levels.

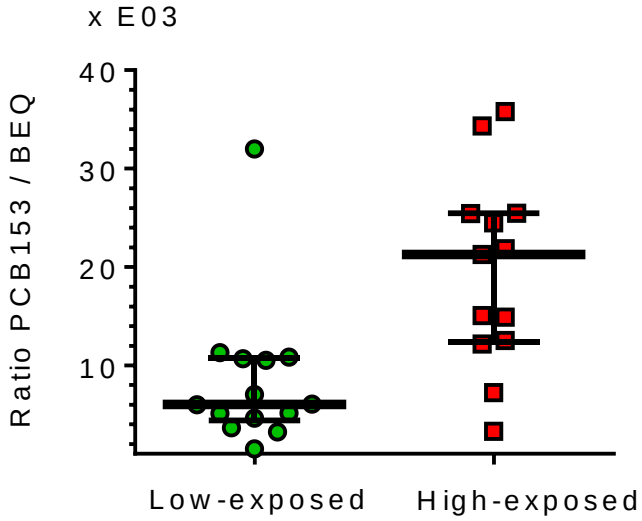

Figure S1. The ratio of PCB153, used as indicator PCB, and BEQ levels determined with the bioassay from 27 participants (low- and high-exposed men only) for which the extended POP measurements were performed comparing the low- (green circles) to the high-exposed men (red squares). The black lines indicate the median with the interquartile range.

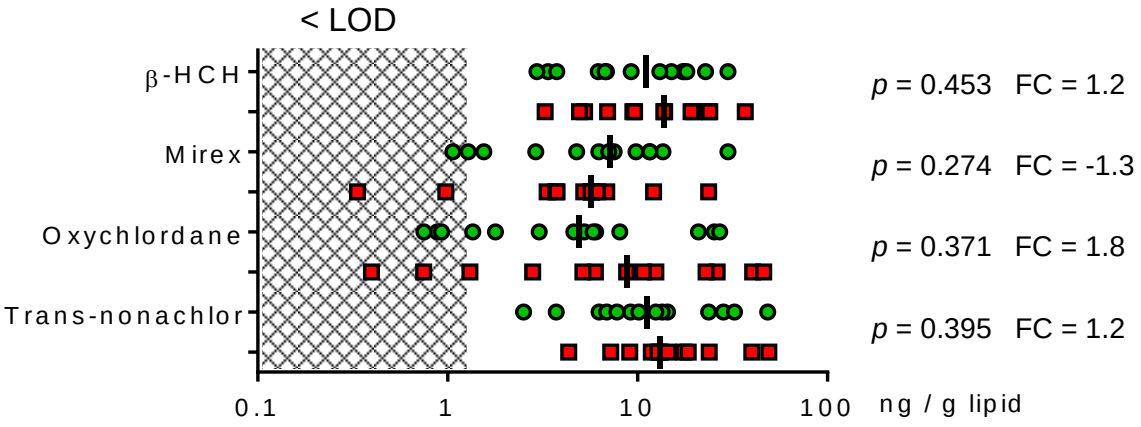

Figure S2. Levels of OC pesticides in low- (green circles) and high-exposed men (red squares). The *p*-value is calculated with a Mann-Whitney *U* test and the fold change (FC) is calculated by dividing the median (indicated line) of the high- by that of the low-exposed group. Samples in the grey quadrant are below the limit of detection (<LOD).

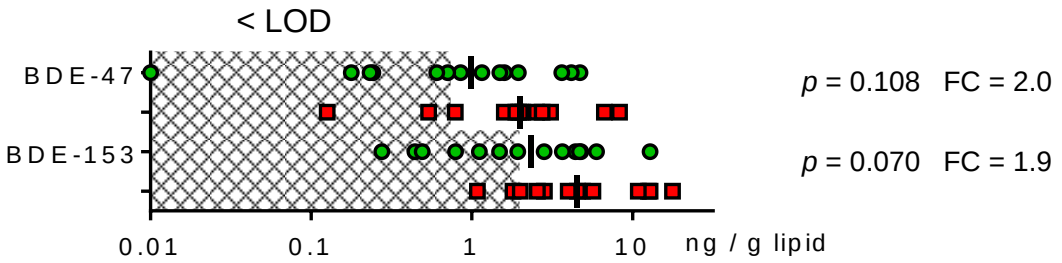

Figure S3. Levels of polybrominated diphenyl ethers (PBDEs) in low- (green circles) and high-exposed men (red squares). The *p*-value is calculated with a Mann-Whitney *U* test and the fold change (FC) is calculated by dividing the median (indicated line) of the high- by that of the low-exposed group. Samples in the grey quadrant are below the limit of detection (<LOD).

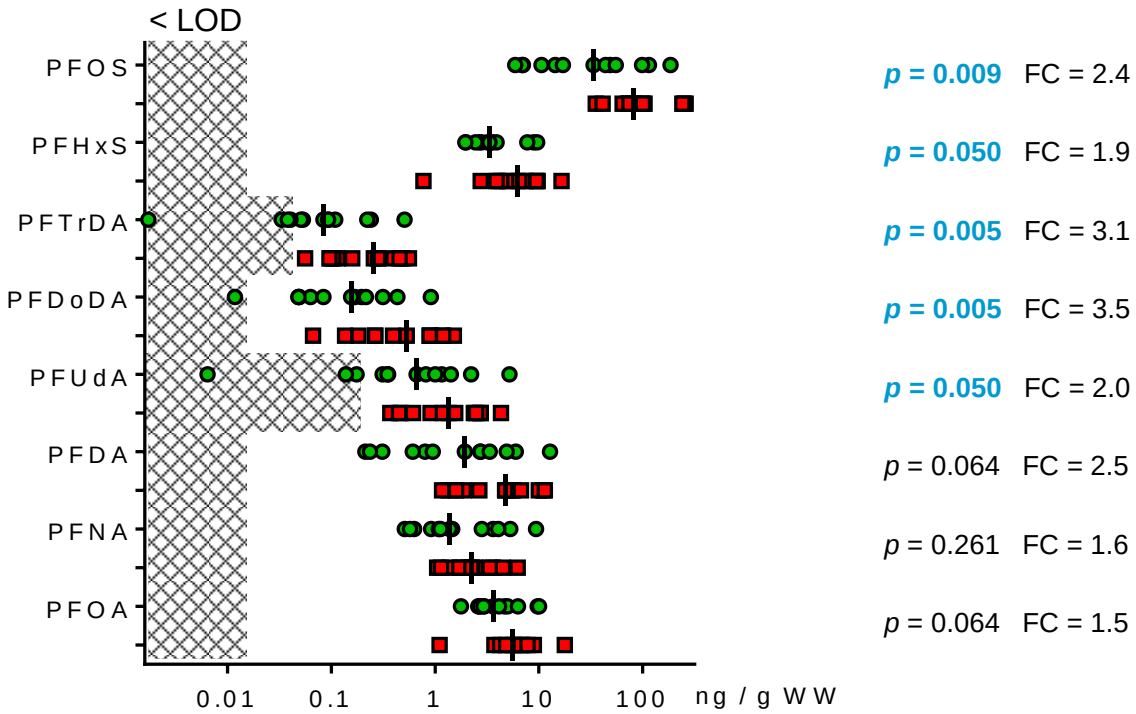

Figure S4. Levels of perfluoroalkyl substances (PFASs) in low- (green circles) and high-exposed men (red squares). The *p*-value in blue indicates a significant difference calculated with a Mann-Whitney *U* test. The fold change (FC) is calculated by dividing the median (indicated line) of the high- by that of the low-exposed group. Samples in the grey quadrant are below the limit of detection (<LOD).

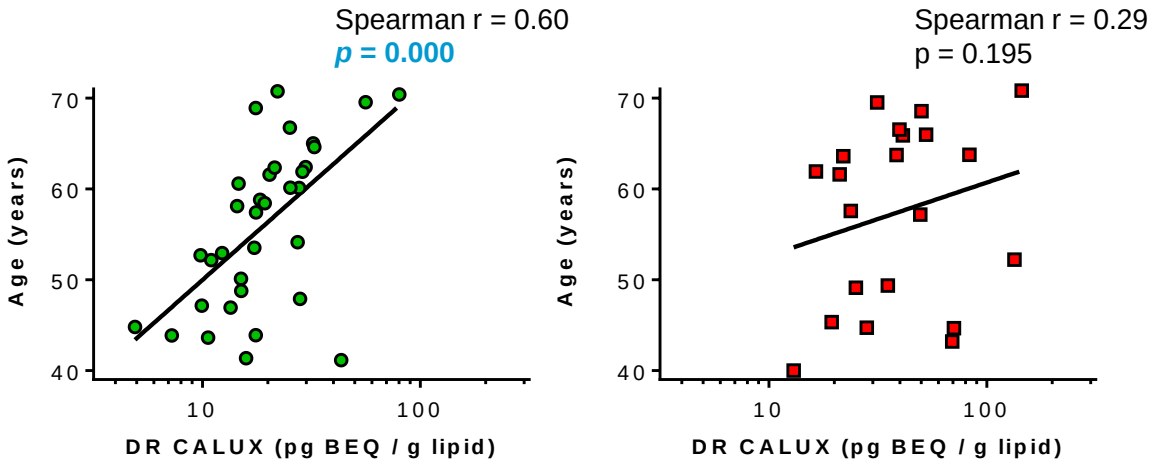

Figure S5. Relationship between age and BEQ levels for both the low- (green dots) and high-exposed (red squares) groups. The *p*-value in blue indicates a significant difference calculated with Spearman's nonparametric correlation.

CHAPTER 5

Epigenome-wide association between DNA methylation in leukocytes and serum levels of persistent organic pollutants in Dutch men

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Manuscript in preparation

Abstract

Background Consumption of polluted fish may lead to high levels of persistent organic pollutants (POPs) in humans, where they can cause adverse health effects. The underlying mechanisms are yet not fully elucidated, although altered DNA methylation has been suggested as a possible contributor to a variety of adverse health effects.

Objectives The aim of this study was to evaluate the relationship between serum POP levels and genome-wide DNA methylation as well as clinical parameters.

Methods In total, 80 Dutch men were included who regularly consumed eel from either high-polluted areas, and subsequently had elevated POP levels, or from low-polluted areas. Serum POP levels were determined and clinical parameters related to e.g. hormone levels and liver enzymes were included as biomarkers for adverse health effects. The Infinium 450K BeadChip was used to measure genome-wide DNA methylation in a subset of 34 men.

Results Most clinical parameters were not associated with serum POP levels. Multiple genes, however, were differentially methylated in men with higher POP levels. Several of these genes are involved in carcinogenesis (e.g. *BRCA1, MAGEE2*, *HOXA5*), the immune system (e.g. *RNF39*, *HLA-DQB1*), retinol homeostasis (*DHRS4L2*), or in metabolism (*CYP1A1*).

Conclusions This is the first human study to investigate genome-wide DNA methylation profiles associated with serum POP levels. We conclude that elevated POP levels, due to consumption of high-polluted food products, affect DNA methylation profiles in adult men, which can potentially be involved in adverse health effects.

Introduction

Persistent organic pollutants (POPs) are widely present in the environment and humans are mainly exposed to POPs through the consumption of food, especially fish and seafood.[1] In Dutch men, serum POP levels were shown to be increased in consumers of high-polluted eel, coming from rivers with a high degree of urbanization and industrialization.[2] Especially polychlorobiphenyls (PCBs) (median increases 4-10 times), PFOS (median increase 2.4 times), and the total amount of dioxins and dioxin-like compounds (median increase 2.1 times) were found to be elevated in these men compared to men consuming eel from relatively low-polluted areas or aquaculture. In 2011, a ban on eel fishing in the high-polluted areas was implemented after risk assessors concluded that the consumption of one portion eel per month would lead to unsafe levels of especially dioxins and dioxin-like compounds.[3] The safe level is derived from animal reproduction studies,[4] and there is a lack of information regarding exact safe levels in humans. We therefore studied whether the elevated POP levels in adult men, related to eel consumption, are associated with clinical parameters as an indication for adverse health effects.

In adults, POPs can cause many adverse health effects of which the majority are related to endocrine disruption, neurological disorders, immune function and cancer.[5] More specifically, hydroxylated (OH-) PCBs might affect thyroid and retinol regulation,[6] while dioxins and PFOS might affect testosterone levels,[7,8] liver enzymes,[9,10] hematocrit (Ht) and hemoglobin (Hg) levels.[11,12] Furthermore, POP mixtures were shown to interfere with glucose and HDL cholesterol levels, and possibly attribute to diabetes mellitus type II.[13] Although some mechanisms behind adverse health related to POP-exposure are clear, like dioxins binding to the aryl hydrocarbon receptor,[14] OH-PCBs binding to thyroid transport proteins,[6] and PFOS resembling fatty acids,[15] not all adverse health effects can be explained by these mechanisms of action. Epigenetic phenomena, such as DNA methylation, have been proposed as a possible molecular mechanism underlying adverse health effects.[16] DNA methylation is a heritable modification, which refers to the addition of a methyl group to cytosine in a CpG dinucleotide. Observational studies have indeed shown that POPs can affect global DNA methylation, and both hypo- (decrease)

and hyper- (increase) methylation have been found.[17-20] Furthermore, animal studies showed that dioxin,[21] PCBs,[22] and PFOS[23] might cause adverse health effects due to gene-specific methylation changes. Also *in vitro* experiments showed that POPs can affect gene-specific DNA methylation.[24,25]

Therefore, we hypothesized that the higher POP levels in men who consumed eel from high-polluted areas might affect DNA methylation and possibly explain adverse health effects. In this study, we measured clinical parameters (e.g. hormone levels and liver enzymes) as well as DNA methylation in blood collected from male eel consumers and associated these outcomes with serum POP levels. This is the first study to investigate genome-wide DNA methylation in humans environmentally exposed to POPs.

Material and methods

Study population

The population consisted of 80 male Dutch participants aged 40 to 70 years, with a habitual eel consumption of at least one portion a month since many years.[2] Men were invited to participate through professional and recreational fishermen associations, because these men are more likely to know the origin of the eel, and through advertisements in local newspapers and webpages. Participants filled out a questionnaire at home about fish consumption habits (including the origin of the eel indicating whether it came from an area where nowadays a ban on catching eel is implemented or not), smoking habits, alcohol consumption and medication use. Blood samples were obtained by venipuncture after overnight fasting. Furthermore, height and weight were measured using standard methods in light clothes. Waist circumference was measured midway between the iliac crest and the costal margin at the end of gentle expiration and hip circumference was measured over the great trochanters. This study was approved by the Medical Ethical Committee of Wageningen University, and written informed consent was obtained from all participants before inclusion in the study.

Serum POP levels

Serum samples were separated by centrifugation within 6 hours after blood collection and full details regarding POP measurements are described previously.[2] In short, the total dioxin-like toxic potency of compounds in blood was measured for all 80 participants with the DR CALUX bioassay at RIKILT Institute for Food Safety[26] and expressed in bioanalytical equivalent (BEQ) per gram lipid. Extensive analyses for individual POPs were performed in a subset of 38 participants with an age distribution that represents the whole study population. Stratification for age is important because POP levels are related to age[27] as is DNA methylation.[28,29] First, all participants were categorized in three age groups (40-49, 50-59, 60-70 years), and secondly by the measured BEQ levels with equal numbers of participants for low, medium or high levels (Table S1). The 38 participants were randomly selected from each of the categories.

The extensive POP measurements included 48 individual compounds and were performed at the Laboratory of Environmental Toxicology at the Norwegian University of Life Sciences (NMBU), according to the requirements of the ISO/IEC 17025 accreditation (TEST 137). Of the 48 POPs, there were 30 compounds for which levels in at least 60% of the participants were above the detection limit, and therefore used in the statistical analyses (Table S1).The seven indicator PCBs, recommended by the European Union Community Bureau of Reference based on their relatively high concentrations in technical mixtures and their wide chlorination range, were measured with GC-MS after liquid-liquid extraction and expressed per gram lipid.[30] PFOS was measured with LC-MS after methanol extraction and expressed per gram wet weight (ww).[31]

Clinical parameters

Liver parameters (AST, ALT, GGT), HDL cholesterol, and thyroid hormones (free T4 and TSH levels) were determined in venous blood collected in lithium heparin vacutainers. Blood collected in a sodium fluoride / potassium oxalate vacutainer was analyzed for glucose, while testosterone and retinol were quantified in serum. Whole blood collected in an EDTA vacutainer was used to measure hemoglobin (Hb), hematocrit (Ht), and white blood cell counts (WBC). All samples were transferred to the clinical chemistry

lab at the Hospital Gelderse Vallei (Ede, the Netherlands) within 3 hours after blood sampling.

Associations between total BEQ levels as well as all individual POPs and the clinical parameters were investigated using multivariable linear regression analyses. The association between log-transformed POP levels and log-transformed parameters was assessed while adjusting for age and waist-to-hip ratio, because these covariates had statistical significant effects on some of the clinical parameters measured. The standardized β coefficients are reported for the linear regression analyses and can be used to interpret the influence of the POP levels on the clinical parameters. Participants with known or expected diabetes mellitus (6 out of 80) were excluded from the analyses regarding the relationship between glucose and POP levels.

DNA isolation

Two participants were excluded for DNA methylation analysis since their WBC showed deviating ratios (percentage of either monocytes, lymphocytes, or granulocytes outside two times the standard deviation of the population). Therefore, a total of 36 participants were included, for which extensive POP measurement were performed (Fig. S1). Blood was collected in EDTA vacutainers and stored at 4^{0}C until processing. The buffy coat was separated by centrifugation within 6h after collection and stored at -80^{0}C. Genomic DNA was isolated from the buffy coat using the DNeasy blood and tissue kit according to the manufacturer's protocol (Qiagen, Venlo, the Netherlands). DNA concentration and quality was determined using a NanoDrop ND-1000 UV–vis spectrophotometer.

DNA methylation measurements

DNA methylation was analyzed using the Illumina Infinium HumanMethylation450 BeadChip (Infinium Inc., San Diego, USA), which addresses 485,512 cytosine positions. For the 36 participants, a total of 500 ng DNA was sent to the Human Genotyping Facility of the Erasmus University Medical Centre (Rotterdam, the Netherlands) who performed the bisulfite conversion (EZ DNA Methylation Kit, Zymo Research, Freiburg, Germany) and processed the BeadChip. Multidimensional scaling revealed two potential

samples mix-ups which were excluded from the analysis, resulting in a total of 34 remaining samples (Fig S1).

Data analysis was conducted as described previously.[32] In short, the minfi package (v1.12.0)[33] was used to process the intensity iDAT files in R-based open software (R 3.1.2). Autosomes and the X and Y chromosomes were included in the analysis, but positions containing a SNP (based on the dbSNP version 137,[34] at the CpG interrogation and/or the single nucleotide extension with a frequency of at least 1% were excluded. Finally, there were 467,974 positions included in the analyses and annotated based on the ilmn12.hg19 annotation.[35] Differentially methylated positions were identified as a function of log-transformed BEQ levels, sum of seven indicator PCBs, and PFOS levels, using the dmpFinder function for continuous phenotypes with a linear regression analyses adjusted for age and leukocyte counts (percentage of monocytes, lymphocytes, and granulocytes). Statistical analyses were adjusted for multiple testing with the Benjamini-Hochberg (BH) method.[36] A false discovery rate (FDR) value of 0.05 was used as the threshold to define statistical significance. Furthermore, we considered positions with a non-adjusted *p*-value below 1E-04. M-values (log2 intensity ratios) were used for statistical testing,[37] while β-values were used for visualization purposes. These β-values range from 0 % (completely unmethylated) to 100 % (completely methylated). All the β-values as well as single iDAT files and other relevant information are available through the Gene Expression Omnibus (GEO) repository with accession number GSE79329.

Differentially methylated regions

Identification of differentially methylated regions (DMRs) was performed with the DMRcate package (v1.2.0) from Bioconductor.[38] This method identifies DMRs across the genome based on tunable kernel smoothing. For this study, DMRs were defined as regions of at least 5 positions with a distance of consecutive positions less than 200 nucleotides. Statistical analyses were adjusted for multiple testing with the BH method and a FDR value of 0.05 was used as the threshold to define statistically significant DMRs.

Results

The 80 men included in this study showed strong variation in their serum levels of dioxins and dioxin-like compounds (range from 4.9 to 145 pg BEQ / g lipid). Demographic characteristics and measured POP levels can be found in Table 1. Men with 'low' or 'high' levels of dioxins and dioxin-like compounds (based on a median-split) did not differ regarding their demographic characteristics.

Table 1. Characteristics of the men studied. Values are given as median (25-75 percentile) or in number (plus percentage) of participants.

		All participants	**Participants with DNA methylation analyses**
Number of participants (n)		80	34
Age (years)		58.6 (49.0 – 63.9)	57.3 (49.2 – 65.5)
Body Mass Index (kg/m^2)		29.2 (26.5 – 31.0)	29.4 (26.7 – 32.3)
Waist-to-hip ratio		0.98 (0.93 – 1.02)	0.98 (0.94 – 1.00)
Smoking habits	Current	11 (14)	5 (15)
	Former	48 (60)	21 (62)
	Never	21 (26)	8 (24)
Alcohol consumption	Yes	69 (86)	32 (94)
	No	11 (14)	2 (5)
Medication	Yes	38 (48)	17 (50)
(at least once a month)	No	42 (53)	17 (50)
Serum levels of persistent organic pollutants (POPs)			
Dioxins and dioxin-like compounds	Low*	16.2 (12.8 – 18.6)	17.1 (12.2 – 19.3)
(pg BEQ / g lipid)	High*	32.4 (27.4 – 49.7)	49.6 (35.2 – 70.7)
Sum of 7 indicator PCBs	Low*		246 (115 – 371)
(ng / g lipid)	High*		1635 (1095 – 2484)
PFOS levels	Low*		14.3 (6.9 – 33.6)
(ng / g wet weight)	High*		96.9 (71.8 – 114)

* For graphical, but not statistical, purposes participants were divided with a median-split in two groups, where low indicates the lower half of the group and high the upper half of the group. BEQ = bioanalytical equivalent

We first analyzed the BEQ levels for all 80 men and the selected panel of clinical parameters previously shown to be affected by POP exposure (ALT, AST, GGT, glucose, HDL cholesterol, Hb, Ht, testosterone, TSH, free T4, and retinol) and detected no statistically significant associations (Table S2). For the 38 participants for whom extensive POP analyses were performed (see flow diagram Fig. S1), we analyzed an extensive panel of 30 POPs in relation to the same clinical parameters. Again, for most POPs no statistically significant associations were detected. Statistically significant associations were only found for two organochlorine pesticides, namely HCB levels which was inversely associated with HDL cholesterol and trans-nonachlor which was inversely associated to both Ht and Hb levels (Table S2). The biological effects, however, were moderate with standardized β coefficients ranging from -0.30 to -0.50.

We continued with genome-wide DNA methylation analyses to determine whether BEQ levels, PCBs, and PFOS were associated with differentially methylated positions or regions. No statistically significant positions were detected after correction for multiple testing (FDR < 0.05). However, we found 22, 17, and 29 positions with a *p*-value below 1E-04 that were associated with BEQ levels, PCBs, and PFOS, respectively (Fig. S2-4).

We further analyzed the DNA methylation differences based on the top 1000 positions with the lowest *p*-value in more detail. Higher levels of all three POP groups were associated with hypermethylation of most of the positions (Fig. 1A). Approximately 42% of the top 1000 positions associated to either BEQ levels, PCBs, or PFOS, were located in CpG islands (CGIs), compared to 31% of the positions on the 450K BeadChip (Fig. 1B). With higher levels of POPs, slightly fewer positions were detected in the open sea and shelf as compared to all available positions on the 450K BeadChip.

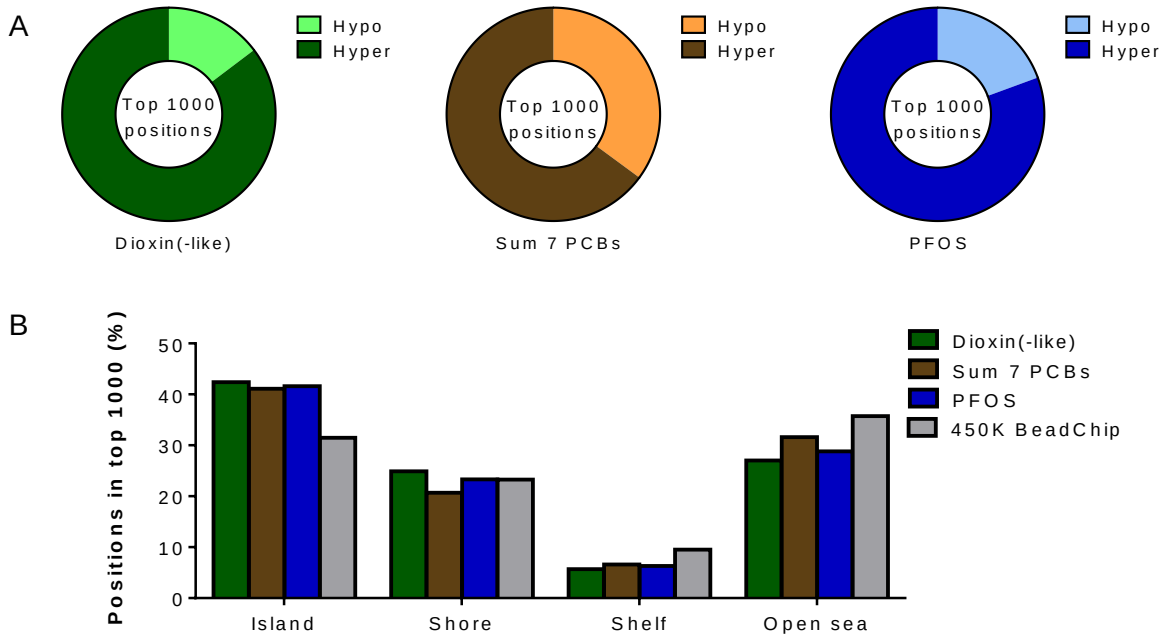

Figure 1. General features of genome-wide DNA methylation associations with higher POP levels. **(A)** The fraction of hypo- (inverse association with POP levels) and hyper methylated (positively associated with POP levels) positions, based on the top 1000 most statistically significant positions, determined with a continuous analyses for serum levels of dioxins and dioxin-like compounds, the sum of seven indicator PCBs, and PFOS. **(B)** Percentages of positions expressed in relationship to CpG density, for the top 1000 positions with the highest statistical significance, for the three POP groups (colored bars) as well as all considered positions on the Illumina 450K BeadChip (grey bars). 'Islands' indicate the CpG islands (CGI), 'Shore' the CGI shores (0-2 kb from CGI), 'Shelf' the CGI shelves (2-4 kb from CGI) and 'Open sea' the single positions outside CGIs.

We continued our analysis by searching for regions, rather than single positions, that are differentially methylated upon higher POP levels. In total, we detected 13, 8, and, 37 DMRs for BEQ levels, PCBs, and PFOS, respectively (Table S3-5). Interestingly, three regions were detected for all three POP groups, namely *DHRS4L2*, *ZNF85*, and *BRCA1*/*NBR2* (Fig. 2). *DHRS4L2* encodes for a retinol oxidoreductase protein and *ZNF85* encodes for a zinc finger protein. *BRCA1* is a tumor suppressor gene, while *NBR2* (the neighbor of *BRCA1*) does not encode a protein.

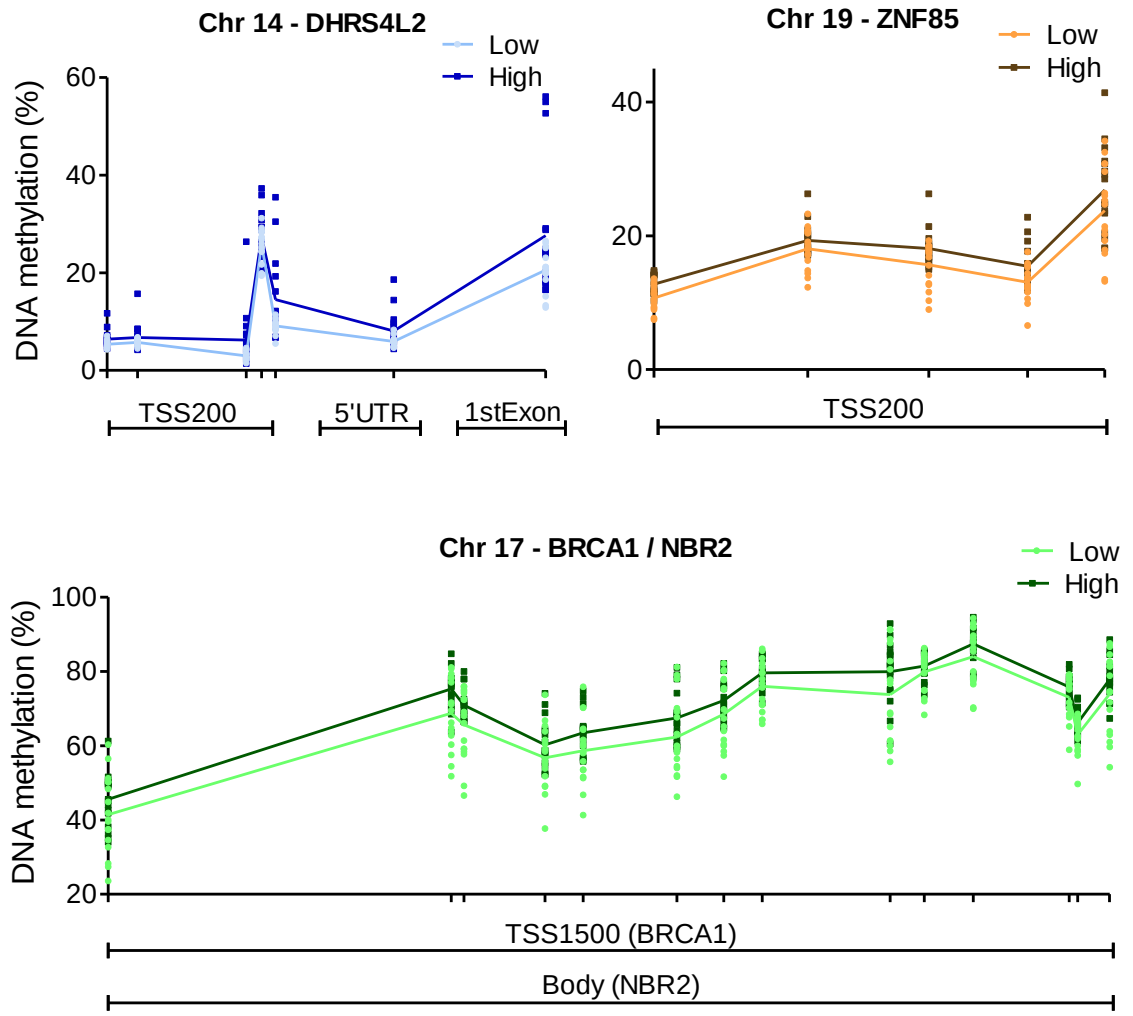

Figure 2. Representative differentially methylated regions detected in leukocytes in relation to PFOS (DHRS4L2), the sum of seven indicator PCBs (ZNF85), and to dioxins and dioxin-like compounds (BRCA1 / NBR2), although similar DMRs were detected in relation to the other POPs (Table S2-4). Each dot represents methylation rate at the indicated position of individual samples and the lines show the average methylation levels. The light color represents the low-exposed group, while the dark color represents the high-exposed group based on the serum levels (median-split). 5'UTR, five prime untranslated region; Chr, chromosome; TSS200, 200 base pairs from the transcription start site; TSS1500, 1500 base pairs from the transcription start site

Most of the detected DMRs are annotated to a known gene, however, these genes are involved in different biological functions. Nevertheless, there were multiple cancer-related genes containing a DMR apart from *BRCA1*, including *MAGEE2, NWD1, ZNF300, HOXA5, NET1, RHOBTB1, EDNRB,* and *SFRP2* (summary in Fig. 3). Furthermore, DMRs were found in genes with an immune-related function (such as *RNF39, HLA-DQB1, (PUS7L)/IRAK, MPZL2, TFCP2, LY6G5C, HCG9, and ZNF416*). Also, a DMR was found in

CYP1A1, which plays an important role in the metabolism of many chemicals (Fig. 4). In addition, some genes characteristic for germ-line cells were differentially methylated like *HFM1* and *ESX1*, as well as genes associated with neurodevelopment, such as *PRRT1* and *SLC6A11*.

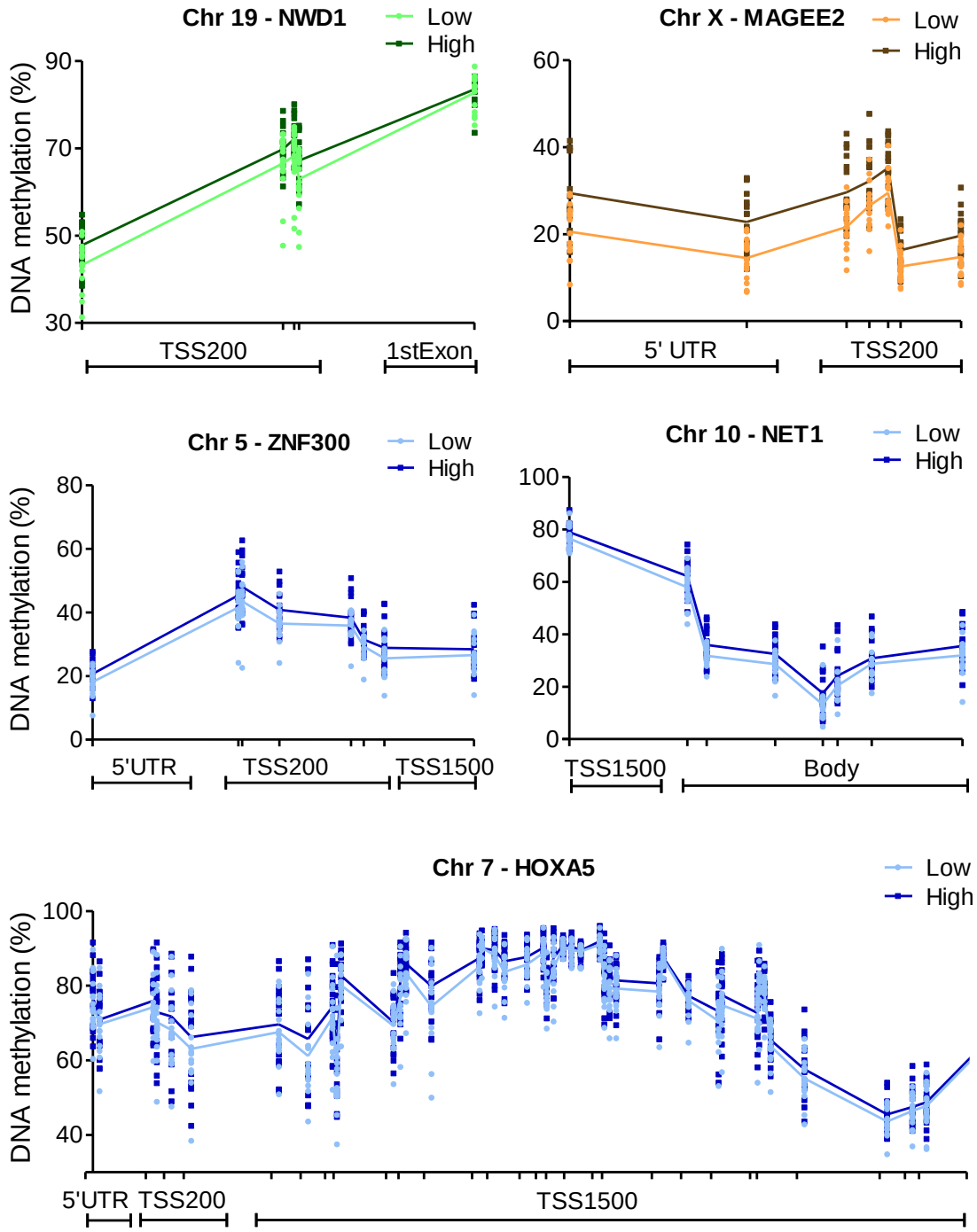

Figure 3. Differentially methylated regions (DMRs) detected in genes which have a relationship with cancer. DMRs were found in relation to dioxins and dioxin-like compounds (green lines), sum of PCBs (beige lines), and PFOS (blue lines). Each dot

represents methylation rate at the indicated position of individual samples and the lines show the average methylation levels. The light color represents the low-exposed group, while the dark color represents the high-exposed group (median-split). 5'UTR, five prime untranslated region; Chr, chromosome; TSS200, 200 base pairs from the transcription start site; TSS1500, 1500 base pairs from the transcription start site

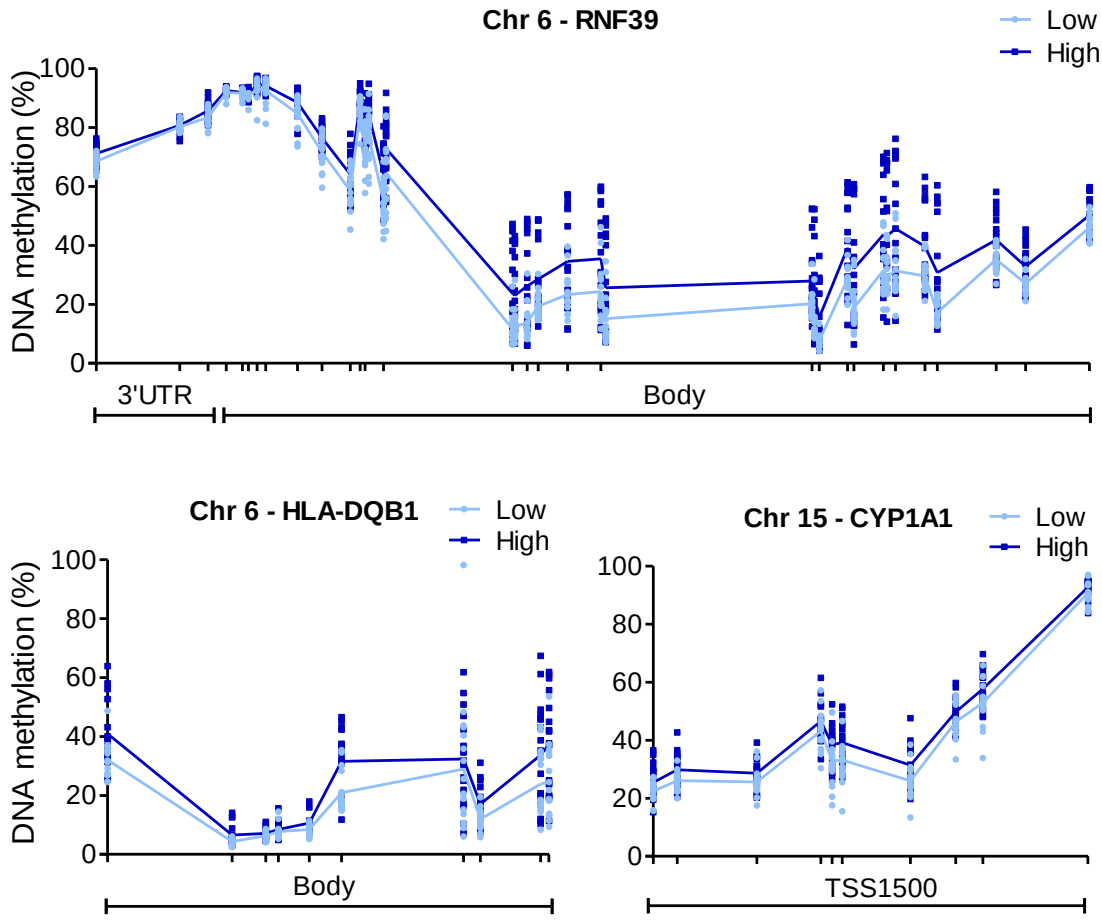

Figure 4. Differentially methylated regions (DMRs) detected in genes related to the immune system (RNF39 and HLA-DQB1) and metabolism (CYP1A1) associated with PFOS levels. Each dot represents methylation rate at the indicated position of individual samples and the lines show the average methylation levels. The light color represents the low-exposed group, while the dark color represents the high-exposed group (median-split).
3'UTR, three prime untranslated region; Chr, chromosome; TSS1500, 1500 base pairs from the transcription start site

Discussion

The present study showed that higher levels of dioxins and dioxin-like compounds, sum of seven indicator PCBs, and PFOS were associated with differentially methylated regions in the DNA. These pollutants, however, did not affect the selected clinical parameters such as hormone levels and liver enzymes. Altered DNA methylation was mostly detected in genes that could be related to other potential adverse health effects, such as cancer and immune responses.

Although thyroid-related health effects are mostly observed in early life stage, we expected adverse health effects with these, sometimes very high, POP levels based on animal studies[39] and a human study with slightly lower (OH-) PCB levels.[40] Furthermore, both PFOS and dioxin were shown before to negatively affect testosterone levels,[7,8] but we could not reproduce this effect. With regard to the effects of POPs on liver enzymes,[9,10] Ht, and Hb levels,[11,12] the findings in literature are inconsistent. In our study, we only found moderate associations between HCB and HDL cholesterol, and trans-nonachlor and both Hb and Ht levels. More pronounced adverse health effects could be expected during sensitive stages such as early development. These parameters are under tight homeostatic regulation, and the men possibly were able to compensate. Furthermore, the small sample size of our study could have attributed to the lack of power to detect an association between clinical parameters and POP levels. We conclude that in this study POP levels, including very high PCB levels, did not cause measurable effects in adult men on the clinical parameters studied.

We found three DMRs with increased methylation levels associated with higher levels of all three POP groups. One of these DMRs was located in the promoter of *DHRS4L2*, relevant for retinol homeostasis, and it has been previously shown that this gene is transcriptionally controlled by DNA methylation.[41] In our study, retinol levels were not affected. In otters, plasma retinol levels were also not correlated to dioxins or PCBs, but hepatic retinol levels were dramatically decreased with increasing pollutant levels.[42] Although *DHRS4L2* is only one of many genes involved in retinol homeostasis, and we measured DNA methylation in leukocytes instead of a more relevant tissue such as the

liver, hypermethylation of this region associated with elevated POP levels, could theoretically be related to a disturbed retinol balance.

Also *ZNF85* was hypermethylated in men with higher POP levels. Zinc finger (ZNF) proteins have many key functions and they are important as transcriptional regulators.[43] *ZNF85* is especially expressed in testicular tissues, with the highest expression levels described in germ-cell tumors and fetal tissue.[44] This gene might have a developmental function, and due to its interaction with many nuclear receptors, differential methylation could potentially lead to a diverse range of adverse health effects.

The third encoding gene detected for all three POP groups was *BRCA1*. Dioxin-exposed rat offspring shows hypermethylation of this gene and reduced gene expression.[45] Also in human breast cancer cells, hypermethylation of *BRCA1* was detected after dioxin exposure.[24] However, while we detected the hypermethylation in the CGI, Papoutsis et al. reported enhanced methylation in the shore. *BRCA1* expression is frequently silenced in breast carcinomas, while somatic mutations of this gene are not reported. Promoter hypermethylation of *BRCA1* is associated with tumor progression,[46] and it was shown that *BRCA1* hypermethylation in leukocytes is associated with an increased breast cancer risk.[47] Another gene that was differentially methylated was *HOXA5,* which is best known for its developmental role during embryogenesis, but its protein has been shown to be able to increase the expression of the tumor suppressor p53. Methylation of *HOXA5* results in loss of expression of both HOXA5 and p53 and is also related to human breast cancer.[48] Although men are not very likely to develop breast cancer, similar results can be expected in women, and our results suggest that DNA methylation changes might be an underlying mechanism in breast cancer development due to POP exposure.[49]

In addition, we also detected DMRs in other genes possibly related to cancer development. *NWD1*, with the highest expression in male reproductive organs, is related to androgen receptor levels and might be involved in prostate cancer.[50] Melanoma antigen gene (MAGE) belongs to the larger family of cancer-germline antigens that are frequently overexpressed in cancer. *ZNF300* hypermethylation is related to downregulation of this gene during toxicant-induced malignant formation,[51] and hypermethylation of *NET1* might affect DNA repair. The association between POPs and

DNA methylation in cancer-related genes might explain part of the carcinogenic mechanisms these compounds have without being genotoxic.[52] Interestingly, also a DMR in *CYP1A1* was detected. A relationship between the role of PFOS and *CYP1A1* in breast cancer has been proposed, possibly explained by the important role of *CYP1A1* in the metabolism of many pollutants.[53] DNA methylation of *CYP1A1* has been described to influence expression levels, which subsequently could both affect the half-lives of pollutants and alter the amount of carcinogenic metabolites produced.[54]

Furthermore, dioxins and PCBs are known to suppress immune function.[55] A relatively large DMR with a distinct profile in the high-exposed men was detected for *RNF39*, a transcription factor in the major histocompatibility complex. For both *RNF39*[56] and *HLA-DQB1*[57] methylation status might be related to poor immune responses.

The cross-sectional nature of our study makes it impossible to determine whether the DNA methylation profiles are a direct result of the elevated POP levels. Furthermore, most differentially methylated genes are not primarily relevant in leukocytes but in other tissues. Additionally, we must consider that the different POPs investigated are correlated with each other, and therefore the interpretation of the results with regard to which POP exerts which effect should be done with caution. Linking DNA methylation and clinical parameters to POP levels is difficult due to the latency between exposure and the manifestation of health consequences. POP levels are very persistent in the body, making current exposure levels a good estimate of past exposures, but significant changes in life style habits interfering with POP levels cannot be excluded. A strength of our study, on the other hand, is the large variation in POP levels in our population, enabling us to investigate DNA methylation in low- to high-exposed men.

Conclusions

Persistent organic pollutants in environmentally exposed men are associated with DNA methylation patterns, but not clinical parameters. Genes containing differentially methylated regions were involved in different biological functions, and health conditions among which cancer and immune functioning. These findings may imply

that DNA methylation might indeed be an underlying mechanism of POP-induced health effects and this will be a good starting point for future research.

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Supplemental material

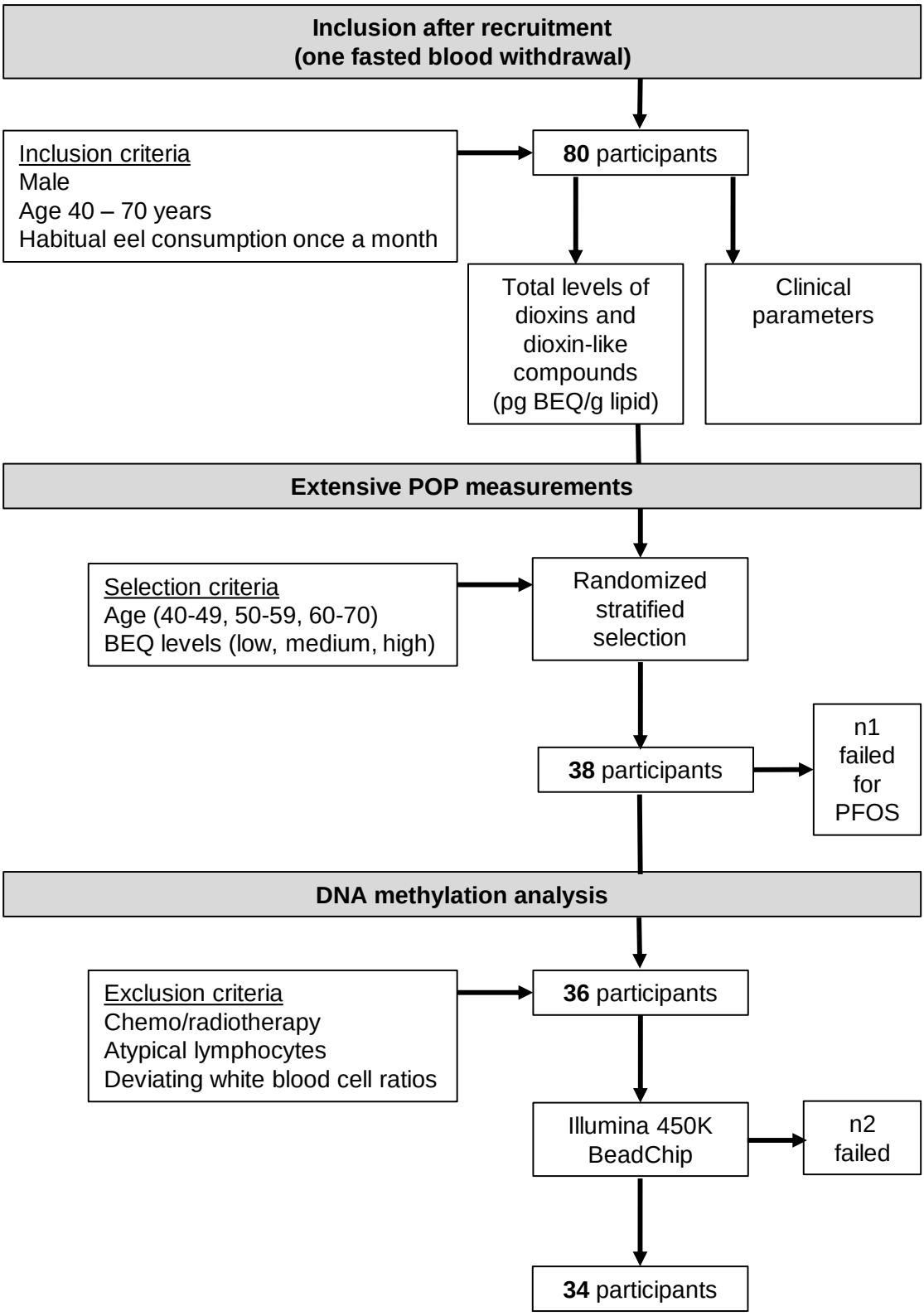


Figure S1. Flow diagram for the selection of the participants.

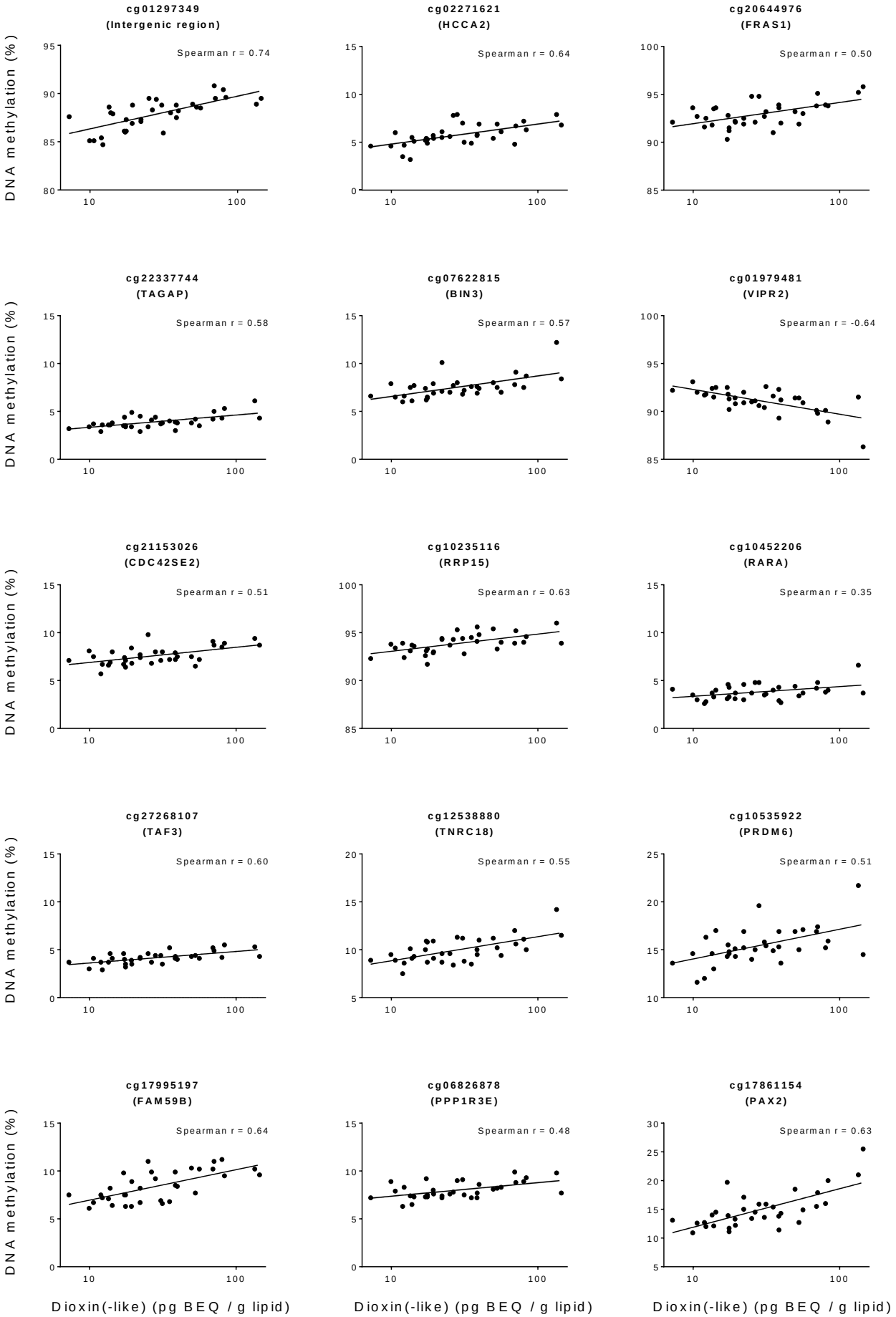
Table S1. Results of the extensive POP measurements. Participants were split in three groups, based on the total BEQ body burden (pg BEQ/g lipid), for stratified selection. Based on this selection 38 participants (out of the total of 80) were chosen for extensive POP measurements.

		Low body burden (BEQ)			*Medium body burden (BEQ)*			*High body burden (BEQ)*		
	N	25p	**Median**	75p	25p	**Median**	75p	25p	**Median**	75p
DR CALUX (pg BEQ/g lipid)	38	11.6	**13.6**	17.1	22.1	**25.8**	30.8	40.6	**54.4**	77.7
Organochlorine pesticides (ng / g lipid)										
p,p'-DDE	38	43.6	**54.5**	83.6	66.5	**111.7**	156.2	137.8	**240.2**	277.1
p,p'-DDT	38	0.5	**2.0**	4.1	0.5	**6.5**	12.7	2.6	**22.7**	34.8
HCB	38	7.0	**9.8**	11.9	12.9	**13.8**	21.3	14.7	**30.7**	55.3
β-HCH	38	6.0	**9.2**	11.9	6.4	**9.0**	14.2	7.3	**13.3**	22.0
Mirex	38	3.2	**4.9**	7.1	3.5	**5.9**	10.3	4.1	**5.9**	10.8
Oxychlordane	38	1.6	**2.0**	3.7	2.2	**4.0**	12.4	3.7	**6.2**	12.0
Trans-nonachlor	38	4.8	**9.0**	11.9	7.7	**11.0**	21.2	12.1	**14.4**	18.3
PCBs (ng / g lipid)										
PCB-52	38	8.7	**11.4**	13.0	11.9	**12.5**	17.0	11.9	**15.4**	17.5
PCB-105	38	1.4	**2.5**	3.7	3.7	**5.3**	7.0	6.3	**16.4**	19.9
PCB-118	38	1.4	**2.4**	15.6	11.1	**24.4**	48.2	26.4	**117.4**	177.1
PCB-138	38	20	**30**	65	78	**161**	217	197	**456**	673
PCB-153	38	44	**66**	139	170	**317**	441	418	**851**	1309
PCB-156	38	3	**5**	10	11	**19**	30	14	**35**	57
PCB-170	38	13	**23**	39	32	**58**	104	77	**129**	210
PCB-180	38	28	**43**	80	76	**157**	229	194	**322**	484
PCB-187	38	5	**15**	44	28	**56**	93	64	**149**	209
Sum 7 indicator PCBs	38	108	**171**	301	352	**686**	982	858	**1771**	2686
OH-PCBs (ng / g ww)										
4-OH-CB107	37	0.0	**0.0**	0.1	0.1	**0.1**	0.3	0.3	**0.5**	0.6
4-OH-CB146	37	0.0	**0.1**	0.2	0.2	**0.4**	0.7	0.5	**1.2**	1.9
4'-OH-CB172	37	0.0	**0.0**	0.0	0.0	**0.1**	0.1	0.1	**0.1**	0.2
4-OH-CB187	37	0.1	**0.2**	0.3	0.3	**0.7**	0.9	0.6	**1.9**	2.1
BDEs (ng / g lipid)										
BDE-47	38	0.5	**0.9**	1.5	0.2	**1.5**	2.0	1.9	**2.7**	3.6
BDE-153	38	1.0	**1.5**	3.0	1.2	**2.4**	4.7	3.7	**5.8**	10.2
PFASs (ng / g ww)										
PFOS	37	5.8	**7.0**	21.1	38.1	**55.0**	89.6	35.4	**74.8**	164.8
PFHxS	37	2.7	**4.3**	7.1	2.7	**3.5**	5.2	2.0	**3.6**	7.0
PFTrDA	37	0.0	**0.0**	0.1	0.1	**0.1**	0.2	0.1	**0.1**	0.3
PFDoDA	37	0.0	**0.0**	0.1	0.2	**0.2**	0.5	0.2	**0.3**	0.9
PFUdA	37	0.1	**0.2**	0.3	0.6	**0.9**	1.8	0.5	**1.0**	2.2
PFDA	37	0.3	**0.4**	1.0	1.6	**2.8**	5.1	1.6	**3.4**	8.9
PFNA	37	0.6	**0.8**	1.1	1.6	**2.1**	2.6	1.5	**2.3**	4.3
PFOA	37	2.6	**3.3**	4.1	4.6	**5.5**	7.5	4.1	**4.9**	6.9

Table S2. Summary of the association (standardized β coefficients of linear regression) between several (groups of) persistent organic pollutants (POPs) and biomarkers related to health. All β estimates are corrected for age and waist-to-hip ratio. * *p*-value < 0.05

Biomarker	Dioxin(-like)[a]	Sum 7 PCBs[b]	PFOS[c]	HCB[b]	Trans-nonachlor[b]
ALT	0.009	0.039	0.011	-0.071	-0.073
AST	-0.029	0.129	0.190	0.061	0.164
GGT	0.135	0.094	0.129	0.033	-0.051
Glucose	-0.035	-0.333	-0.104	-0.068	-0.059
HDL cholesterol	-0.121	-0.128	0.005	-0.304 *	0.199
Hb	-0.180	-0.257	-0.112	-0.225	-0.501 *
Ht	-0.197	-0.269	-0.095	-0.273	-0.457 *
Testosterone	-0.173	-0.116	-0.209	-0.192	-0.014
TSH	0.145	-0.100	0.015	0.226	-0.351
Free T4	-0.128	-0.070	-0.116	-0.002	0.100
Retinol	0.174	0.224	0.205	-0.055	0.154

[a] Association based on n = 80.
[b] Association based on n = 38.
[c] Association based on n = 37.


cg01297349
(Intergenic region)
Spearman r = 0.74
cg02271621
(HCCA2)
Spearman r = 0.64
cg20644976
(FRAS1)
Spearman r = 0.50
cg22337744
(TAGAP)
Spearman r = 0.58
cg07622815
(BIN3)
Spearman r = 0.57
cg01979481
(VIPR2)
Spearman r = -0.64
cg21153026
(CDC42SE2)
Spearman r = 0.51
cg10235116
(RRP15)
Spearman r = 0.63
cg10452206
(RARA)
Spearman r = 0.35
cg27268107
(TAF3)
Spearman r = 0.60
cg12538880
(TNRC18)
Spearman r = 0.55
cg10535922
(PRDM6)
Spearman r = 0.51
cg17995197
(FAM59B)
Spearman r = 0.64
cg06826878
(PPP1R3E)
Spearman r = 0.48
cg17861154
(PAX2)
Spearman r = 0.63
DNA methylation (%)
Dioxin(-like) (pg BEQ / g lipid)

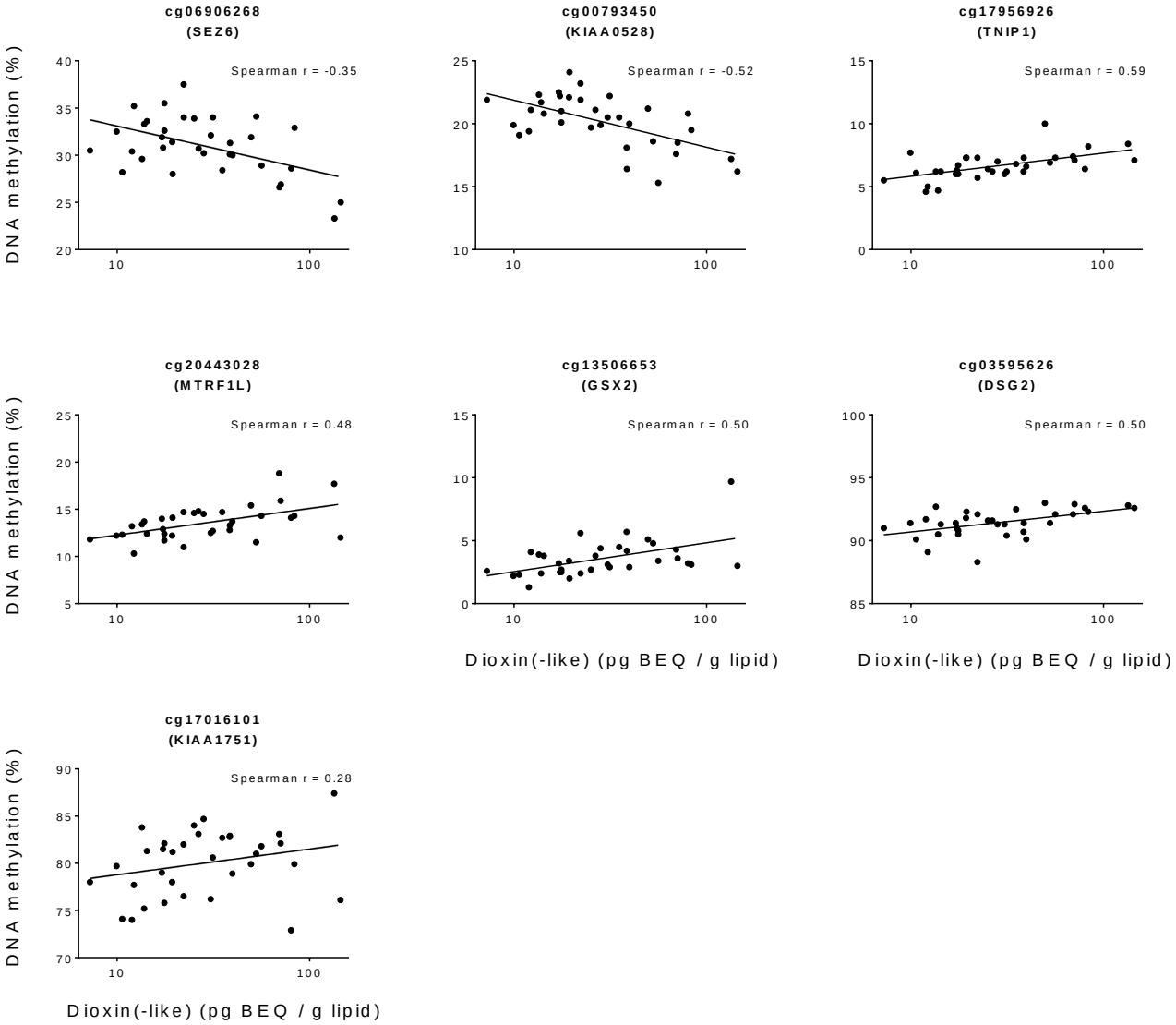
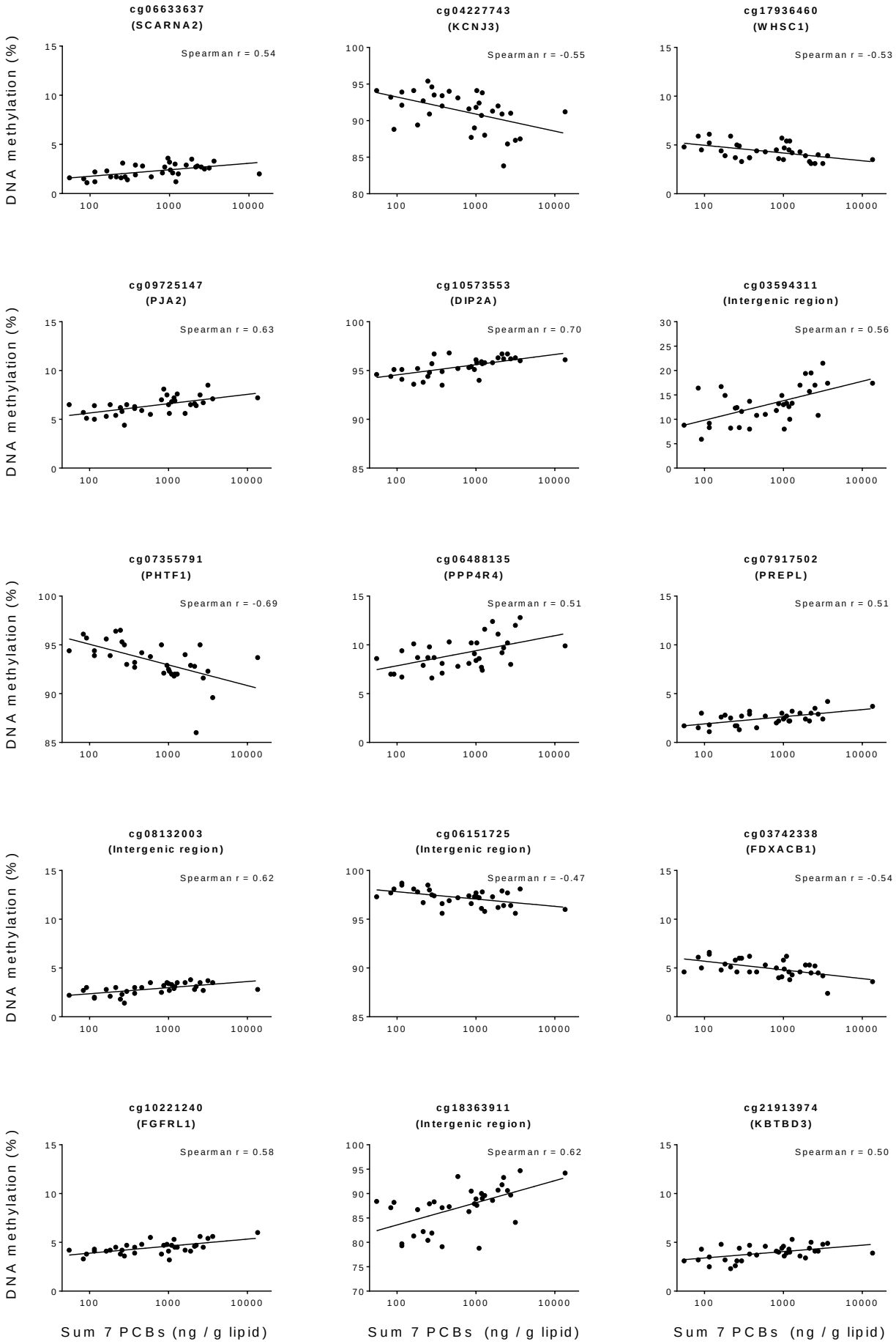


Figure S2. Differentially methylated positions (*p*-value < 1E-04) with increasing levels of dioxins and dioxin-like compounds. BEQ = bioanalytical equivalent (sum of dioxins and dioxin-like compounds)


cg06633637
(SCARNA2)
Spearman r = 0.54
cg04227743
(KCNJ3)
Spearman r = -0.55
cg17936460
(WHSC1)
Spearman r = -0.53
cg09725147
(PJA2)
Spearman r = 0.63
cg10573553
(DIP2A)
Spearman r = 0.70
cg03594311
(Intergenic region)
Spearman r = 0.56
cg07355791
(PHTF1)
Spearman r = -0.69
cg06488135
(PPP4R4)
Spearman r = 0.51
cg07917502
(PREPL)
Spearman r = 0.51
cg08132003
(Intergenic region)
Spearman r = 0.62
cg06151725
(Intergenic region)
Spearman r = -0.47
cg03742338
(FDXACB1)
Spearman r = -0.54
cg10221240
(FGFRL1)
Spearman r = 0.58
cg18363911
(Intergenic region)
Spearman r = 0.62
cg21913974
(KBTBD3)
Spearman r = 0.50
DNA methylation (%)
Sum 7 PCBs (ng / g lipid)

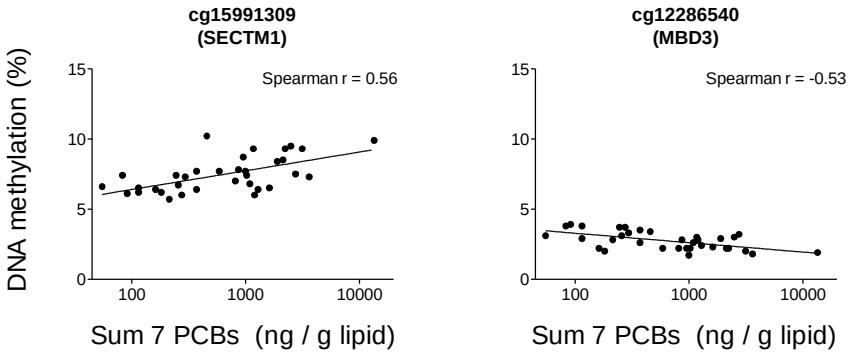
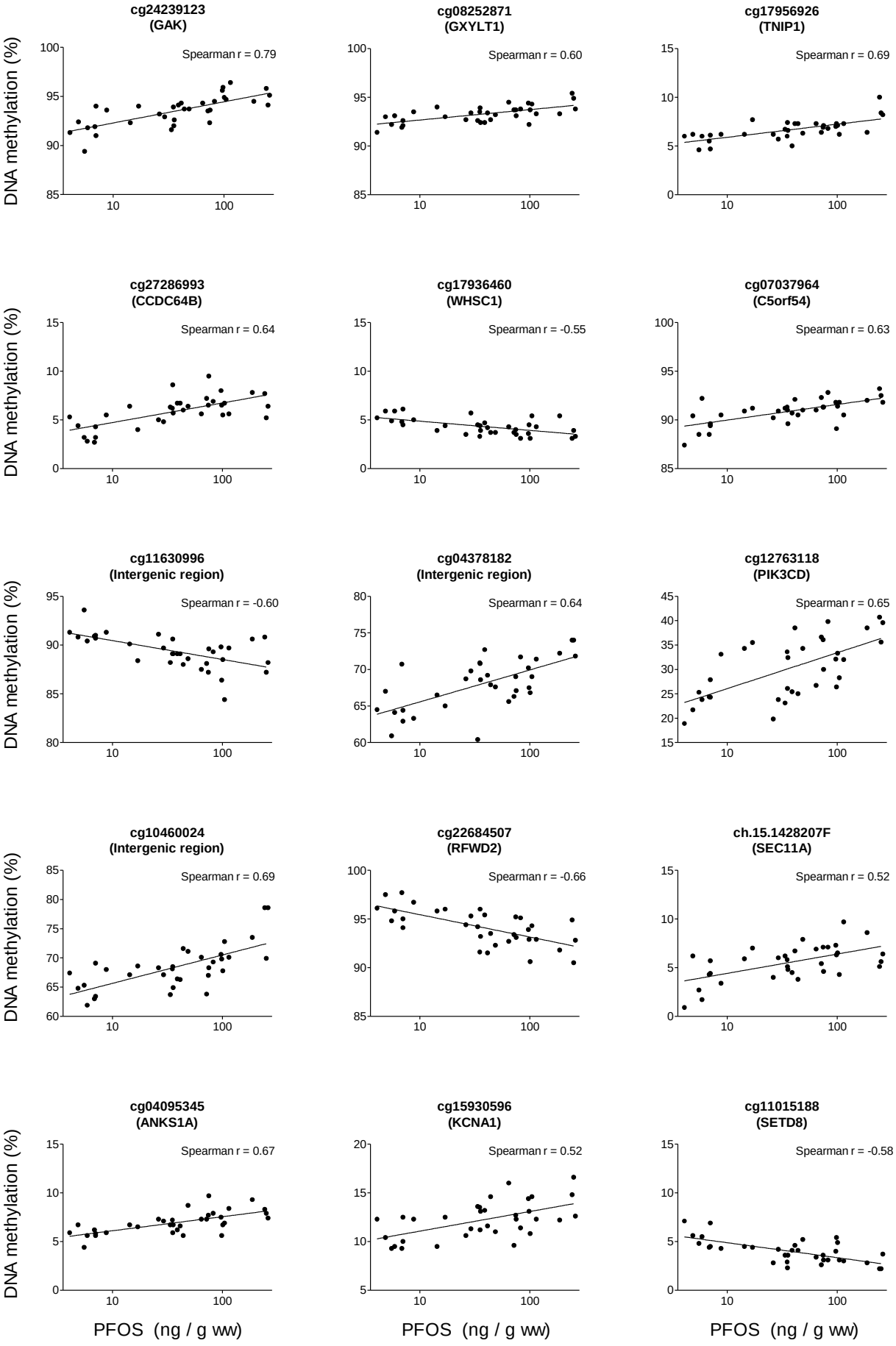


Figure S3. Differentially methylated positions (*p*-value < 1E-04) with increasing levels of the seven indicator PCBs.


cg24239123
(GAK)
Spearman r = 0.79
cg08252871
(GXYLT1)
Spearman r = 0.60
cg17956926
(TNIP1)
Spearman r = 0.69
cg27286993
(CCDC64B)
Spearman r = 0.64
cg17936460
(WHSC1)
Spearman r = -0.55
cg07037964
(C5orf54)
Spearman r = 0.63
cg11630996
(Intergenic region)
Spearman r = -0.60
cg04378182
(Intergenic region)
Spearman r = 0.64
cg12763118
(PIK3CD)
Spearman r = 0.65
cg10460024
(Intergenic region)
Spearman r = 0.69
cg22684507
(RFWD2)
Spearman r = -0.66
ch.15.1428207F
(SEC11A)
Spearman r = 0.52
cg04095345
(ANKS1A)
Spearman r = 0.67
cg15930596
(KCNA1)
Spearman r = 0.52
cg11015188
(SETD8)
Spearman r = -0.58
DNA methylation (%)
PFOS (ng / g ww)

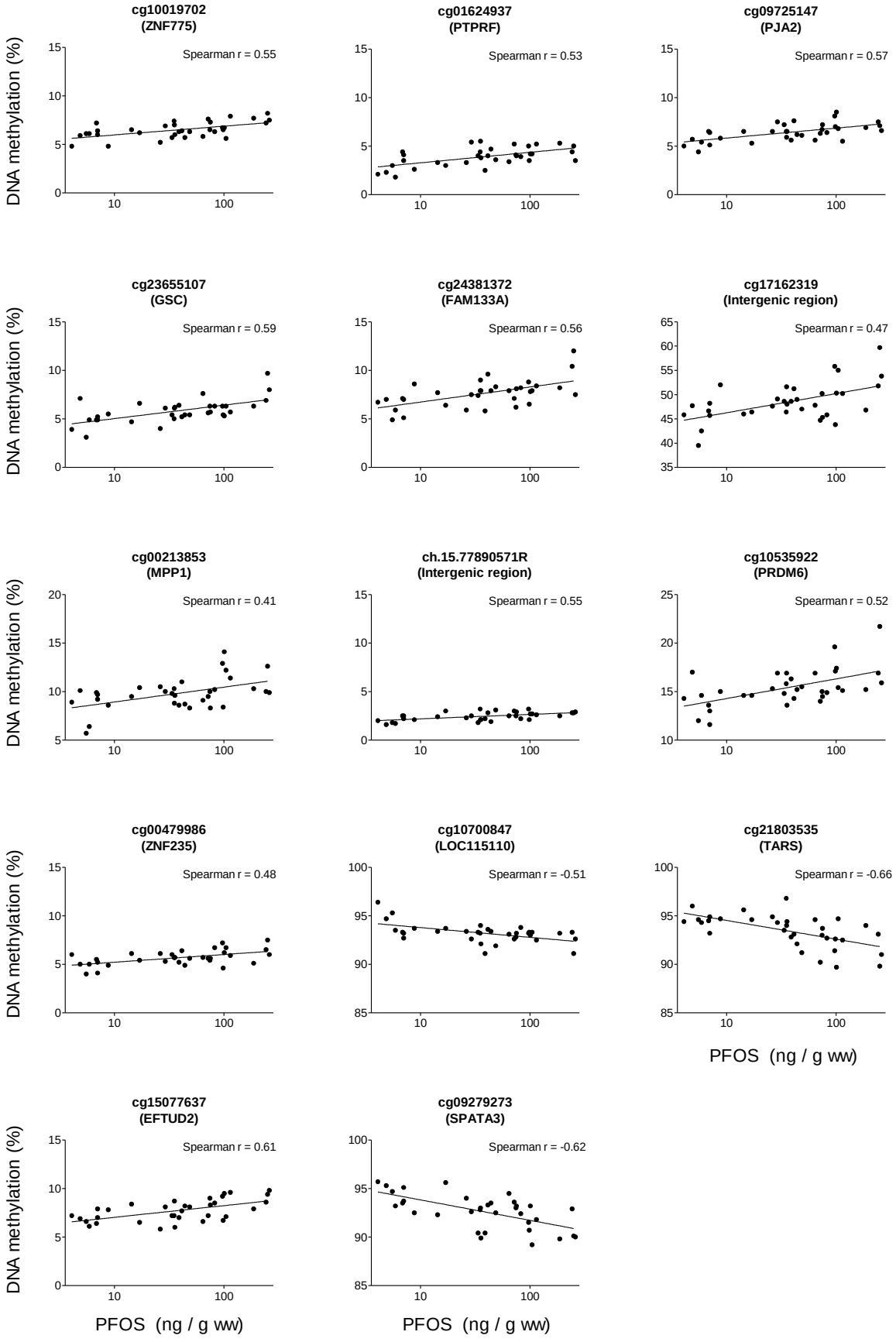


Figure S4. Differentially methylated positions (*p*-value < 1E-04) with increasing levels of PFOS. ww = wet weight

Table S3. Differentially methylated regions (DMRs) in leukocytes in relation to the sum of dioxins and dioxin-like compounds as measured with the bioassay.

Gene	Relation to gene	Chr	Start[a]	End[a]	Nr. probes	Mean *p*-value	Change[b]
LOC284798	TSS1500 TSS200 Body	20	25,129,400	25,129,562	7	7.2E-04	4.5%
NIPAL4	TSS200	5	156,886,970	156,887,005	5	8.2E-04	5.7%
BRCA1 NBR2	TSS1500 Body	17	41,277,974	41,278,444	14	4.6E-03	14.3%
ZNF85	TSS200 5'UTR 1stExon	19	21,106,002	21,106,120	7	4.7E-03	6.9%
MAGEE2	5'UTR 1stExon TSS200	X	75,005,028	75,005,131	6	5.5E-03	9.4%
PUS7L IRAK4	1stExon 5'UTR TSS1500 TSS200	12	44,152,509	44,152,715	7	6.1E-03	5.2%
-	-	5	71,146,767	71,146,876	6	7.1E-03	-8.0%
NWD1	TSS200 1stExon 5'UTR	19	16,830,613	16,830,859	5	7.7E-03	9.3%
GJB6	5'UTR TSS200	13	20,805,380	20,805,487	6	9.9E-03	7.9%
-	-	6	29,818,086	29,818,300	6	1.1E-02	12.2%
PLOD2	TSS1500	3	145,879,568	145,879,710	5	1.7E-02	10.1%
DHRS4L2	TSS200 5'UTR 1stExon Body	14	24,457,993	24,458,299	6	1.9E-02	13.7%
CYorf15A	TSS1500 TSS200	Y	21,728,952	21,729,069	5	2.3E-02	4.9%

3'UTR, three prime untranslated region; 5'UTR, five prime untranslated region; Chr, chromosome; TSS200, 200 basepairs from the transcription start site; TSS1500, 1500 basepairs from the transcription start site

[a] Position of start and end of the DMR according to Hg19 annotation

[b] The maximum DNA methylation change within the DMR

Table S4. Differentially methylated regions (DMRs) in leukocytes in relation to the sum of seven indicator PCBs.

Gene	Relation to gene	Chr	Start[a]	End[a]	Nr. probes	Mean *p*-value	Change[b]
MAGEE2	5'UTR 1stExon TSS200	17	41,277,974	41,278,563	7	5.6E-04	6.9%
BRCA1 NBR2	TSS1500 Body	X	75,004,943	75,005,131	16	7.2E-03	10.3%
DHRS4L2	TSS200 5'UTR 1stExon	14	24,457,993	24,458,208	5	9.0E-03	8.3%
ESX1	5'UTR 1stExon TSS200	X	35,937,617	35,937,949	6	1.5E-02	3.1%
CXorf22	TSS1500 TSS200 5'UTR 1stExon	4	1,004,609	1,004,703	9	1.7E-02	4.2%
FGFRL1	TSS1500	8	1,765,217	1,765,387	5	1.8E-02	0.7%
MIR596	TSS200	X	103,499,545	103,499,650	7	1.9E-02	1.9%
ZNF85	TSS200	19	21,106,002	21,106,043	5	4.2E-02	2.8%

3'UTR, three prime untranslated region; 5'UTR, five prime untranslated region; Chr, chromosome; TSS200, 200 basepairs from the transcription start site; TSS1500, 1500 basepairs from the transcription start site

[a] Position of start and end of the DMR according to Hg19 annotation

[b] The maximum DNA methylation change within the DMR

Table S5. Differentially methylated regions (DMRs) in leukocytes in relation to PFOS.

Gene	Relation to gene	Chr	Start[a]	End[a]	Nr. probes	Mean *p*-value	Change[b]
SERPINB6	TSS200	6	2,972,097	2,972,195	6	3.6E-05	3.8%
RNF39	3'UTR Body	6	30,038,791	30,039,600	36	1.3E-04	14.5%
ZNF300	5'UTR 1stExon TSS200 TSS1500	5	150,284,302	150,284,600	8	4.0E-04	5.9%
MPZL2	1stExon 5'UTR TSS200	11	118,134,959	118,135,203	5	2.0E-03	3.5%
MIR596	TSS1500 TSS200 Body	8	1,765,066	1,765,477	11	2.1E-03	4.2%
-	-	6	106,441,441	106,441,618	5	2.3E-03	5.8%
HAS3	5'UTR TSS200 1stExon	16	69,141,250	69,141,478	7	2.3E-03	3.7%
HLA-DQB1	Body	6	32,632,848	32,633,163	10	3.2E-03	12.1%
HCG9	Body	6	29,943,209	29,943,480	9	4.3E-03	4.8%
BRCA1 NBR2	TSS1500 Body	17	41,277,847	41,278,622	20	4.9E-03	10.8%
ZNF85	TSS200 5'UTR 1stExon	19	21,106,002	21,106,120	7	5.3E-03	4.4%
HOXA5	5'UTR 1stExon TSS200 TSS1500	7	27,183,262	27,184,821	44	5.7E-03	9.3%
NET1	TSS1500 Body TSS200 5'UTR 1stExon	10	5,488,225	5,488,628	8	7.2E-03	5.1%
CLDN9	TSS200 1stExon 5'UTR	16	3,062,349	3,062,795	7	7.6E-03	8.8%
C10orf26	TSS200 Body 5'UTR 1stExon	10	104,535,792	104,536,052	8	7.9E-03	4.5%
SUOX	TSS1500 TSS200 5'UTR 1stExon	12	56,390,798	56,391,077	10	8.9E-03	4.6%
TFCP2	TSS200 TSS1500	12	51,566,731	51,566,990	8	9.0E-03	-7.0%
LY6G5C	TSS1500	6	31,648,544	31,648,767	11	9.0E-03	1.9%
HCG9	Body	6	29,944,917	29,945,155	12	9.7E-03	3.2%
-	-	6	29,818,154	29,818,300	5	1.1E-02	6.7%
DHRS4L2	TSS200 5'UTR 1stExon	14	24,457,893	24,458,208	7	1.2E-02	6.8%

C5orf35	TSS1500 TSS200	5	56,204,834	56,204,925	5	1.3E-02	4.7%
RHOBTB1	Body TSS200 TSS1500	10	62,704,143	62,704,289	6	1.3E-02	1.6%
CYP1A1	TSS1500	15	75,019,070	75,019,376	10	1.3E-02	6.0%
C10orf53	TSS1500 TSS200	10	50,887,472	50,887,578	5	1.4E-02	4.1%
EDNRB	5'UTR TSS200 TSS1500	13	78,493,100	78,493,365	16	1.5E-02	2.5%
HORMAD2	TSS1500 TSS200	22	30,476,206	30,476,345	7	1.6E-02	5.1%
ZNF880	TSS200 5'UTR 1stExon	19	52,873,019	52,873,173	6	1.7E-02	1.6%
POT1	TSS200	7	124,570,047	124,570,181	6	2.1E-02	1.1%
SFRP2	TSS1500	4	154,711,563	154,711,675	5	2.5E-02	4.2%
MIR7-3 C19orf30	TSS1500 Body	19	4,769,592	4,769,690	6	2.6E-02	3.3%
TRPC6	5'UTR 1stExon TSS200	11	101,454,626	101,454,733	5	2.8E-02	4.3%
ZNF416	1stExon 5'UTR TSS200	19	58,090,229	58,090,357	5	3.2E-02	0.6%
PRRT1	3'UTR Body	6	32,116,918	32,117,049	6	3.4E-02	4.1%
C22orf45 UPB1	TSS200 TSS1500	22	24,890,794	24,890,833	6	3.8E-02	4.1%
TSTD1	5'UTR 1stExon TSS200	1	161,008,705	161,008,826	5	4.0E-02	-2.0%
SLC6A11	TSS1500 TSS200	3	10,857,688	10,857,719	5	4.0E-02	3.9%

3'UTR, three prime untranslated region; 5'UTR, five prime untranslated region; Chr, chromosome; TSS200, 200 base pairs from the transcription start site; TSS1500, 1500 base pairs from the transcription start site

[a] Position of start and end of the DMR according to Hg19 annotation

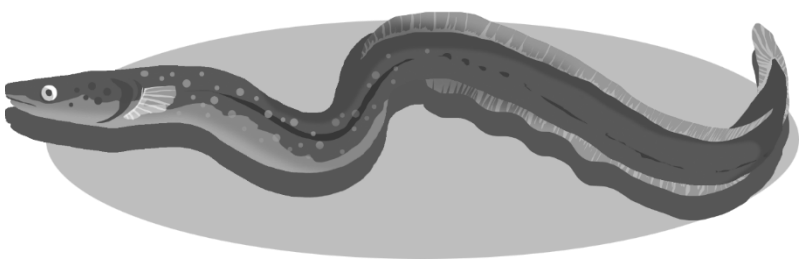
[b] The maximum DNA methylation change within the DMR

CHAPTER 6

General discussion

Conclusion

Future perspectives



General discussion

This thesis studied the hypothesis that DNA methylation might be an underlying mode of action by which persistent organic pollutants (POPs) exert some of their delayed or long-lasting adverse health effects. The thesis consists of 2 parts: 1) *in vitro* studies with eight abundant seafood POPs to identify gene expression and DNA methylation changes related to functional endpoints and 2) a human observational study in men to investigate whether consumption of eel from polluted areas lead to higher POP levels than considered safe and whether these POP levels were associated with aberrant DNA methylation.

A short overview of the results from this thesis is presented in Table 1. The *in vitro* studies (part 1) were performed using H295R cells for steroidogenesis-related effects and with SGBS cells and hMSCs for determining effects on adipocyte differentiation. We detected only minor effects for TCDD and the dioxin-like PCB126 on steroidogenesis, but TCDD was a strong inhibitor of adipocyte differentiation. The non-dioxin-like PCB153 decreased steroid hormone levels, but did not affect adipocyte differentiation. PFOS had the strongest impact on steroidogenesis, and affected 9 out of 13 measured hormone levels. PFOS also inhibited lipid accumulation in differentiating adipocytes, although not as strong as TCDD. Both flame retardants as well as MeHg did not affect steroidogenesis or adipocyte differentiation, but HBCD was the only compound to induce osteoblast differentiation. TBT, on the other hand, had minor effects on steroidogenesis and strong effects on adipocyte and osteoblast differentiation. In chapter 2, two POP mixtures were investigated in addition to the single compounds tested, and revealed the occurrence of additive effects, expected to be relevant to humans. Besides the functional endpoints, chapter 2 showed that all increased hormone levels were accompanied by increased gene expression levels, while decreased hormone levels did not coincide with decreased gene expression. Gene expression profiles in chapter 3B supported the effects on functional endpoints for TCDD and TBT. Pro-adipogenesis genes were down-regulated after TCDD exposure and up-regulated after TBT exposure, and anti-adipogenesis genes were up-regulated after TCDD exposure. Although differentiation in embryonic stem cells is frequently attributed to DNA

methylation changes,[1] the differentiation of hMSCs to adipocytes was only associated with minor changes in DNA methylation (Chapter 3A). Even though DNA methylation was not severely altered during adipocyte differentiation, POP exposure was associated with DNA methylation changes (Chapter 3B). These changes in DNA methylation could, however, not be linked to gene expression changes.

The human study (part 2) showed that consumers of eel from high-polluted areas had 2.5 times higher levels of dioxin and dioxin-like compounds than consumers of eel from low-polluted areas or aquaculture, confirming predictions of a risk assessment performed in 2007 (Chapter 4).[2] PCB levels were up to ten times higher in eel consumers from high-polluted areas and were among the highest measured world-wide, and also PFAS levels were increased. DNA methylation in leukocytes was therefore investigated in relation to these three POP groups (dioxin and dioxin-like compounds, sum of seven indicator PCBs, and PFOS levels) in chapter 5. Serum POP levels were associated with DNA methylation of genes related to cancer, the immune system, early development and neurological disorders. The aberrant DNA methylation in leukocytes with elevated blood dioxin levels were different from the DNA methylation effects in TCDD exposed adipocytes. For PFOS, differentially methylated regions (DMRs) were detected in a gene within the major histocompatibility complex class I region (*RNF39*) and one gene with an unknown function (*C10orf26*) in both adipocytes and leukocytes (chapters 3B and 5). In our human study, hormone levels and liver enzymes were not associated with any of the POP levels.

Table 1. Summary of the results from the different chapters.

Compound	***Chapter 2***[a] Steroidogenesis	***Chapter 3***[a] Adipocyte differentiation	***Chapter 4***[c] Fold elevated in poll. eel consumers	***Chapter 5*** Aberrant DNA methylation in eel consumers
TCDD	↑ Progesterone	↓ Adipocyte diff ↓ Osteoblast diff Expr: 8 genes DMRs: 4 adipogenic genes	↑2.8x	13 DMRs[e] ↔ Clinical parameters
PCB126	↑ Estradiol	-	↑3.4x	-
PCB153	↓OH-Progesterone, androstenedione, testosterone, deoxycortisol	↔	↑6.3x	8 DMRs[f] ↔ Clinical parameters
PFOS	↓ (OH-)pregnenolone, deoxycorticosterone ↑ androstenedione, testosterone, estradiol, corticosterone, (deoxy-) cortisol	↓ Adipocyte diff ↔ Osteoblast diff Expr: 2 genes DMRs: 2 adipogenic genes	↑2.4x	37 DMRs ↔ Clinical parameters
BDE-47	↔	↔	↔	- DMRs ↔ Clinical parameters
HBCD	↔	↔ Adipocyte diff ↑ Osteoblast diff	-[d]	-
TBT	↓ Pregnenolone, (OH-) progesterone	↑ Adipocyte diff ↓ Osteoblast diff Expr: 4 genes DMRs: 3 adipogenic genes	-	-
MeHg	↔	↔	-	-

↔ No significant effect; – Not measured; Expr = Significantly differential gene expression in adipogenic gene; DMR = differentially methylated region; diff = differentiation

[a] Measured in human H295R Adrenocortical Carcinoma Cells
[b] Measured in SGBS (Simpson-Golabi-Behmel syndrome) cells and hMSCs (human mesenchymal stem cells)
[c] Men consuming eel from high-polluted areas were compared to men consuming eel from low-polluted areas or aquaculture.
[d] In the majority of the samples HBCD levels were below the detection limit
[e] Based on sum of dioxins and dioxin-like compounds
[f] Based on the sum of seven indicator PCBs

Accumulation of POPs in frequent eel consumers and potential health effects

The serum levels of dioxins and dioxin-like compounds in men who consumed eel from high-polluted areas were 2.5 times higher than in men consuming eel from low-polluted areas or aquaculture, which confirmed the predictions made in the risk assessment that was relevant for the implementation of the ban in 2011.[2] The consequence of the accumulation of these compounds is that levels in these men are above the levels regarded as safe, derived from animal reproduction and developmental studies. It can be speculated that the actual safe level for adults may be higher, however, endometriosis was found in adult rhesus monkeys at levels just above the level considered safe.[3] Safe levels are not specifically quantified for adults, and it is difficult to predict at which levels adult men are susceptible to adverse health effects.

Also PFAS levels were higher in the high-exposed men and well above background levels in the general population,[4] however it is not known whether these levels could cause adverse health effects.[5] Based on the *in vitro* experiments we performed, adverse health effects would be expected at much higher levels than we detected in these men. The most pronounced *in vitro* effects regarding steroidogenesis were detected after PFOS exposure (200 µM ≈ 100 µg/mL) such as up-regulation of testosterone. The maximum levels of PFOS detected in the human study were much lower (260 ng/mL), and no significant association with testosterone was detected. The experiments with the H295R cells show a possible mechanism of action by which PFOS may exert adverse health effects, e.g. by stimulating hormone production by inducing CYP enzymes.

Two POPs that were studied in the *in vitro* experiments could not yet be measured in the human study within the available time. TBT levels still are of interest, because potential adverse effects were detected after exposing cells to concentrations that could be reached in humans. For MeHg no effects were detected during the *in vitro* testing. Although MeHg is the most abundant metal in seafood, levels in eel were decreased during the last decade and are well-below the established consumption levels.[6]

The most striking results in the human study were the, up to ten times, higher PCB levels (and subsequently the hydroxylated (OH)-PCBs) in the eel consumers from the high-polluted areas. We showed that it is highly likely that eel is the source of the increased POP levels due to the profiles of the PCBs in combination with TEQ levels. Interestingly, levels of PCBs and their OH-metabolites were higher in Dutch men from our study consuming eel from high-polluted areas than in men consuming marine mammals, and were among the highest levels measured world-wide.[7] OH-PCBs have been associated with several adverse health effects in animal studies, especially during early development.[7] POP levels in women are comparable[8] or only slightly different[9] than POP levels in men, and it is therefore a reasonable assumption that women with a similar frequency of eel consumption have POP levels within the same range as these men. Approximately 50% of the OH-PCBs in maternal plasma are transferred across the placenta to the fetus.[10] These levels are quite high and fall within the range were adverse health effects are to be expected based on animal studies.[7] Thyroid disturbance in animals is expected to be mainly due to the binding of the OH-PCBs to transthyretin (TTR), facilitating active transport throughout the whole body, and subsequently exerting a broad range of adverse health effects.[11] OH-BDEs were not significantly elevated due to consumption of eel from high-polluted areas in our study, while they may have a higher potency than OH-PCBs for thyroxine-binding globulin (TBG) binding, the main thyroid hormone transporter in humans.[12] Effects of hydroxylated metabolites on the thyroid hormone regulation have been observed in infants[13] as well as in adults.[14,15]

We did not observe a marked correlation between POP levels and thyroid hormone levels in the adult men in our human study. The sample size of the human study was based on expected variation and differences in serum POP levels between eel consumers from low- and high-polluted areas. Based on the number of participants included, the minimal detectable effect regarding the clinical parameters can be calculated. Including 80 participants, a correlation coefficient of 0.31 can be detected for the association between clinical parameters and POP levels, and for the sub-group of 38 participants for which extensive POP measurements were performed, a correlation coefficient of 0.44 can be detected. A correlation coefficient below 0.3 (=r^2 of 0.09, indicating that 9% of

the variance in the relationship can be accounted for by these variables) may be considered to be not biologically relevant, while a correlation coefficient between 0.3 and 0.5 (=r^2 of 0.25) indicates a moderate effect. We can therefore conclude that the high POP levels do not have strong effects on thyroid hormone levels, however, due to the sample size, small and moderate effects cannot be excluded. The biological endpoints, however, are under homeostatic control and moderate effects might still be relevant due to the broad range of adverse health effects that could be related to a disturbed homeostasis. However, due to feed-back mechanisms regulating homeostasis no health effects have to occur in exposed men. Also for in utero exposed children, affected thyroid hormones were described to be transient,[16] in contrast with neurodevelopmental problems,[17] indicating adaptation is possible.

In addition to the thyroid hormone related biomarkers, other clinical parameters were measured. In previous epidemiological studies, PFOS and dioxins were shown to negatively affect testosterone levels,[18,19] liver enzymes,[20,21] Ht, and Hb levels.[22,23] Previous studies, however, were inconsistent, and effects were relatively small. We conclude that in this study POP levels, including high PCB levels, did not cause strong effects in adult men on the clinical parameters studied.

Clinical health-related parameters assessed in this thesis may be influenced by many factors. Blood may not reflect effects in tissues of interest as POP-related health effects can be exerted in different organs. Three of the tested POPs affected *in vitro* adipocyte differentiation, but this was not an endpoint in the human study. We did not study other functional endpoints upon differentiation such as adipokines. For PCB77 it was shown in 3T3-L1 cells that differentiation was induced as well as various proinflammatory cytokines, which might explain metabolic disorders in humans.[24] These cytokines can be measured in human blood, although small effects are likely obscured by the high variability. Other human studies have associated POPs to obesity and the metabolic syndrome,[25-27] but glucose levels in our study were not affected. However, a glucose tolerance test or a full metabolomics approach would provide more insight. Furthermore, clinical parameters to predict e.g. future cancer do not exist. Chronic exposure to dioxins has been shown to increase several types of cancer,[28] and the elevated levels in our consumers of eel from high-polluted areas might cause an increase

in cancer incidence.[29] Therefore, adverse health effects due to the elevated POP levels cannot be excluded based on our results.

Aberrant DNA methylation

The human study as well as the *in vitro* differentiation experiment showed an association between POP levels and aberrant DNA methylation. To investigate DNA methylation we used the Infinium 450K BeadChip, which is a commonly applied method for genome-wide DNA methylation analysis, because it is informative, not very expensive, and there are well-developed statistical pipelines ensuring standardized analysis. This method correlates well with whole-genome bisulfite sequencing[30] and is cross-validated against conventional quantitative pyrosequencing.[31] A limitation of the 450K BeadChip is the low (1-2%) coverage of the total CpG sites, although 99% of the known genes are included. The CpGs present on the BeadChip are biased towards regions with high CpG content, where inter-individual and inter-tissue variation is expected to be low compared to CpG-poor areas, which are underrepresented in the 450K BeadChip.[32] Relatively many DNA methylation changes were detected in intergenic regions upon adipocyte differentiation. Intergenic regions (IGRs) are relevant with regard to cell-type discriminatory patterns[33] and about half of the enhancers are present in IGRs.[34] Enhancers can show differential methylation near genes with tissue-specific expression[35] and underrepresentation of the enhancer areas might have attributed to the fact that we only detected minor DNA methylation changes during adipocyte differentiation *in vitro*. However, a previous study which used a more elaborate DNA methylation analysis also did not detect pronounced effects on DNA methylation in differentiating adipocytes and no concomitant gene expression changes.[36] Furthermore, DNA methylation might be more dynamic than expected.[37] To test this, future research should include more time points during adipocyte differentiation.

DNA methylation analysis in our adipocyte study was performed in a heterogeneous cell population including, besides the differentiated adipocytes, also undifferentiated hMSCs, and possibly spontaneous differentiated osteoblasts. Isolation of differentiated

adipocytes by e.g. fluorescence activated cell sorting (FACS) would provide a homogenous cell population, ensuring aberrant DNA methylation is not the consequence of cell-specific DNA methylation patterns. Cell sorting would most likely not affect DNA methylation, however, this procedure may induce stress and affect gene expression.[38-40] Genes that were differentially methylated after POP exposure, were, however, not differentially methylated during differentiation and therefore the different cell composition has likely not affected the results. In the human study, DNA methylation was measured in leukocytes. We measured the leukocyte composition in each participant to statistically adjust for different cell compositions.

In vitro DNA methylation versus methylation in the human study

Most genes that were found to be differentially methylated upon POP-exposure during *in vitro* adipocyte differentiation, were not differentially methylated in the human study. This is not necessarily surprising as DNA methylation is tissue-specific (even though both cell types originate from the mesoderm), and DNA methylation changes can occur in one tissue but not in another.[41,42] There were two genes for which DMRs were associated with PFOS levels in both studies. The function of one gene is yet unknown, the other gene, *RNF39*, had a DMR containing 36 positions and was hypermethylated in the human study. The DMR within *RNF39 in vitro* contained 5 probes and was hypomethylated upon PFOS exposure. Methylation status of this gene has been related to immune responses,[43] which is a relevant outcome for leukocytes, and will be explained in more detail below.

In vitro POP-exposure mainly resulted in hypomethylation, while higher POP-levels in our human study were mainly related to hypermethylation. This may be explained by the differences in the location of the aberrant DNA methylation. *In vitro* exposure had the most significant effects in CpGs located in the open sea and were either related to the gene body or detected within IGRs, while most significant effects in the human study were detected in CGIs and were annotated close to the TSS or 5'UTR. DNA methylation in CGIs is in general less abundant compared to methylation in the open sea. Overall, hypermethylation is more likely to occur in CGIs, where hypomethylation mainly occurs in the gene body and open sea.[44]

The majority of the men in our study were either professional or recreational fishermen who started fishing at a young age. It is possible that they already consumed the highly contaminated eel since their childhood and possibly their parents too. Therefore prenatal exposure in these men might be expected, and we cannot exclude that aberrant DNA methylation already was established during sensitive early stages and continued during their adult life.

Implications of altered DNA methylation for health

Most of the detected differentially methylated regions (DMRs) were not related to the clinical parameters measured. We detected a DMR in the promoter of *DHRS4L2*, relevant for retinol homeostasis, and it has been previously shown that this gene is transcriptionally controlled by DNA methylation.[45] We did not find differences in retinol levels between the low- and high-exposed men. Interestingly, all men had generally high retinol levels, probably due to the frequent eel consumption. Also liver enzymes were not altered due to the higher POP levels, but *CYP1A1* was differentially methylated. CYP1A1 belongs to the phase I xenobiotic metabolizing enzymes which play important roles in detoxification as well as biosynthesis of endogenous steroid hormones.[46] CYP1A1 is especially important for metabolizing the aromatic lipophilic compounds, such as dioxins and dioxin-like compounds. These compounds have a strong affinity for the AH receptor and are therefore potent inducers of CYP1A1 enzymes. Some of the PCBs, however, may become more harmful after being hydroxylated during this phase I metabolism (that also includes other CYP enzymes), until they are detoxified by phase II enzymes. Increased DNA methylation in CYP1A1 might alter expression[47] and subsequently affect the half-lives of some pollutants and alter the amount of metabolites produced.

The most striking differences in DNA methylation between the low- and high-exposed men were observed in genes related to cancer, the immune system, and neurological and developmental regulation. These are adverse health effects that can be expected upon POP-exposure,[48] but we did not measure clinical parameters related to these health outcomes. We observed promoter hypermethylation of *BRCA1* in men with high POP levels, which is associated with tumor progression.[49] It was shown that *BRCA1*

hypermethylation in leukocytes is associated with breast cancer related molecular changes.[50] Also *HOXA5* was hypermethylated, which is related to loss of expression of the tumor suppressor gene p53 and to breast cancer.[51] Although men do not often develop breast cancer, similar results can be expected in women. Our results suggest POPs could be involved in breast cancer development due to aberrant DNA methylation.[52] Aberrant methylation in these and other genes related to cancer development could be one of the mechanisms by which POPs cause cancer since they are not genotoxic[28] and could partly explain the increased cancer risk as calculated by the EPA.[29] Since DNA methylation was measured in leukocytes, and not the relevant tissue for many cancers, this finding only has biological meaning when other tissues show the same aberrant DNA methylation. Leukocytes are the relevant tissue for the immune response and differential methylation in leukocytes has been related to antibody response.[53] Dioxins and PCBs have also been related to immune suppression,[54-56] and we detected multiple genes with aberrant DNA methylation related to the immune system. Both *RNF39*[43], with an especially distinct profile between the low- and high-exposed men, and *HLA-DQB1*[57] were detected amongst other genes, and the methylation status of these genes might be involved in immune responses. Whether these changes in DNA methylation cause health effects in these men could be tested by measuring the immune response, such as antibody levels, after a vaccination.

Association between DNA methylation, other epigenetic marks, and gene expression

The trending field of epigenetic research has exploded in the last years, with to date 15,567 publications filed in the Medline database with the keyword 'epigenetics', of which 11,228 are new publications since the start of this PhD thesis project. The amount of research that is currently performed regarding epigenetics brings an enormous advancement in understanding the role of epigenetics and its biological meaning. The main focus in this thesis was DNA methylation, however, other epigenetic marks include histone modifications and non-coding RNAs (ncRNAs) and might also affect gene regulation.[58,59]

The relationship between DNA methylation and gene expression is highly complex, depending on many factors such as the CpG content and the region within the genome.

Promoter methylation in the CGI and gene expression is extensively-studied, where increased methylation decreases gene expression, although decreased methylation does not induce expression.[60] This is the case for the approximately 70% of the promoter regions that contain CGIs, while high methylation in CpG-poor promoters does not inhibit expression. Gene body methylation, on the other hand, might still regulate expression in these genes. It has recently been suggested that gene body methylation might be more predictive towards gene regulation than promoter methylation.[61] In general, Lou *et al.* found that DNA methylation was well-correlated to gene expression when methylation levels are high (indicating very low expression levels) or when gene expression levels are extremely high (indicating very low methylation levels). However, the majority of the genes have both low methylation and low expression. Lacking clear signatures from DNA methylation alone, genes with medium expression are difficult to classify.

The relationship between DNA methylation and gene expression is also dependent on histone modifications and ncRNAs. There is a complex interplay between DNA methylation and histone modifications. Genome-wide DNA methylation profiles suggest that DNA methylation is well-correlated with histone methylation patterns, and that the establishment of new DNA methylation is, in part, regulated by histone methylation.[58,62] Some histone methylation marks may protect from active demethylation, while other marks prevent the binding of DNA methyltransferases, thereby blocking DNA methylation.[58]

Certain histone modifications are reasonable predictors of gene expression by themselves, and can better predict which genes have medium expression levels than DNA methylation can predict this. However, the combination of both DNA methylation and histone modifications increases the accuracy of predicting gene expression.[61] This suggests that understanding aberrant DNA methylation in genes is relevant to predict gene expression and possibly health related effects, however, elucidating the combination of DNA methylation and histone modifications could lead to better predictive models. Strong indications have been published that histone modifications might be affected by POP levels, even without affecting DNA methylation,[63-65] supporting the notion that follow-up research should include both epigenetic marks.

Also non-coding (nc) RNAs can regulate chromatin structure and can mediate the recruitment of histone and DNA methyltransferases.[66] The exact mechanisms by which ncRNAs interact with other epigenetic marks is, however, not fully understood. ncRNAs provide a fast way of altering gene expression[67], and the implications for long-term adverse health effects are not yet known. At this point, the best method to predict gene expression is probably by applying a combined analysis of histone modification and DNA methylation, without including ncRNAs. Methylation, on the other hand, can also influence the expression of ncRNAs and this might be one of the possible ways in which differential methylation might exert health effects.[68] This shows once more the complex interplay between the different epigenetic marks which are all associated with each other. Expression of multiple ncRNAs were shown to be induced in Chinese chemical workers exposed to elevated levels of PFAS,[69] and ncRNAs were also affected by different POPs in animal studies.[70-73] POPs are therefore expected to be not only associated with DNA methylation, but also with other epigenetic marks.

With this in mind, the question remains whether DNA methylation is merely a biomarker of exposure, an adaptation to this exposure (non-adverse, or even potentially beneficial), or an actual adverse health effect (either direct or indirect). Direct effect of POPs on DNA methylation could include affecting DNMT activity, which is suggested to be under hormonal control,[74] or altering SAM availability. Some of these POPs can bind to steroid receptors and subsequently also affect histone enzymes (e.g. Jaridlb) which in their turn can affect histone marks.[75] Indirectly these histone modifications could affect DNA methylation. In summary, POPs may alter the epigenetic state by regulating the activity of various transcription factors, subsequently changing expression or activity of DNA or histone methyltransferases.

Overall conclusion

The aim of this thesis was to elucidate whether seafood-related POPs can induce aberrant DNA methylation in high-exposed eel consumers, potentially causing adverse health effects. With the use of *in vitro* models it was shown that TCDD and TBT affected both steroidogenesis and adipocyte differentiation at environmentally relevant

concentrations. Although PFOS exerted even more pronounced effects, the *in vitro* concentrations needed to induce these effects were approximately 400 times higher than found in blood. This thesis does, however, show possible modes of action of PFOS that were not shown before. For these three POPs we also detected a few DNA methylation changes in differentiating adipocytes, but this could not be related to differential gene expression in the adipocytes.

In the human study we revealed that consumption of eel from the polluted areas indeed caused high internal levels of certain POPs: especially dioxins, PCB and PFAS levels were elevated. We detected differential methylation in a number of genes, with strong differences and large DNA regions in multiple genes related to cancer and the immune system. We measured general clinical parameters that were not specifically related to these endpoints, including e.g. hormone levels and liver enzymes. These parameters were not affected. In short, POP levels are seriously elevated in men consuming polluted eel and POPs are associated with aberrant DNA methylation. Further clarifications are needed to determine the possible role of DNA methylation in health implications.

Implications for public health

Implications for consumers

The frequent consumption of eel from the high-polluted areas, where currently a ban is implemented, causes high accumulation of certain POPs. Although we did not find effects on the clinical parameters measured, adverse health effects cannot be excluded based on the current literature as safe levels were exceeded. The safe level is set to protect unborn and developing children. Therefore, especially girls and women of childbearing age should not consume high-polluted eel. The safe level for adults is not exactly known, for example for tumor promoting effects. Exceedance of the safe level does not mean that adverse health effects attributed to the higher POP levels will be encountered. It does, however, mean that an increased risk of developing certain health effects cannot be excluded. We showed that higher POP levels were associated with

aberrant DNA methylation patterns. The biological meaning of DNA methylation at this point is unknown, however, it could be related to cancer and immune disorders.

There is no action to take for people who consumed this eel in the past. POP levels are excreted slowly from the body (half-life of 4 to 15 years), and besides limiting new exposures, the process of elimination cannot be accelerated. The consumption of food products, including eel, from regulated markers (e.g. supermarket, and not from own catch) will generally have low POP levels due to strict regulations and controls. When consuming products low in POPs, this gives the body a chance to excrete more POPs than are newly accumulated, eventually lowering the POP levels in the body.

Implications for policy makers

The internal POP levels measured in frequent eel consumers were clearly elevated for dioxins and dioxin-like compounds, (OH-)PCBs, and PFASs. Up to now, the focus of risk assessors was mainly on dioxins and dioxin-like compounds. Our study was the first to actual measure POP levels in frequent consumers of high-polluted eel, and we could validate the predictions made in an earlier performed risk assessment.[2] This confirms that the ban on eel fishing was implemented on correct assumptions. As long as the levels in eel are above the European legal limit, a ban on consumption of this eel is advisable.

We established that men consuming eel just below the European levels, mainly from Lake IJssel, had a body burden of dioxins and dioxin-like compounds just above the safe level. The European Commission (EC) established maximum levels of 6.5 pg TEQ/g ww for fish, whereas wild eel has to comply to 10 pg TEQ/g ww.[76] Meat and dairy products have to comply to lower limits than set for fish, because of more frequent consumption in the general population. For most Dutch consumers the main contribution to dioxins and dioxin-like compounds comes from dairy products,[77] while for Belgium consumers fish is the main source.[78] The levels set by the EC protect the majority of the Dutch population. Frequent fish and/or eel consumers, however, could still accumulate POP levels above the accepted safe level, when the fish or eel consumed have levels just below the EC limit. The low-exposed men from our study had levels just above the safe level, with the consumption of at least one portion eel per month. It is not ideal to

communicate a maximum fish consumption to prevent people from exceeding the safe level, since weekly consumption of fatty fish has also many beneficial health effects, and this might give the impression that products on the market are not actual safe. Ideally, legal levels of pollutants in eel should be lowered to levels found in other fish (6.5 pg TEQ/g ww) to protect frequent consumers from accumulating high levels of POPs.

The EC legal limit for the sum of the six indicator PCBs (excluding PCB118) is set at 75 ng/g ww in wild eel. Adverse health effects of PCBs can especially be expected from their OH-metabolites. This is relevant because the main pollutants present in eel are PCBs and not dioxins. OH-PCBs are formed in the consumers, not in the eel, and therefore the legal limit should indeed be set for PCB levels. Policy makers and scientists should take the formation and toxicity of OH-PCBs into account for future risk assessments, to ensure that set levels indeed guarantee consumer safety.

In the Netherlands, a ban on eel fishing in the high-polluted areas is implemented and eel from these areas do not reach the market since 2011. Indications exist that recreational fishermen are still catching highly-polluted eel for consumption, and we recommend additional measures to prevent consumption of these eels. Belgium and Germany do not have such a ban, while POP levels in eel are similar, but it is highly advisable to make anglers aware of the possible risks associated with frequent consumption of polluted eel.[79,80] The information obtained in this study is also applicable for other high-polluted areas, such as the Baltic Sea and the Great Lakes, where fish is equally or even higher polluted.

The policy measures to decrease POP levels in the environment seem to be effective, as multiple POP levels in the environment, in food, and in humans are decreasing.[81] It will, however, still take years before POP levels in the Dutch river sediments are low enough to ensure safe fish again. Sanitation of rivers is a possibility to manually remove POP levels, although this is not cheap and might temporarily disturb ecosystems.

Future perspectives

The main findings of this study are the accumulation of POPs due to eel consumption and the association between POP levels and aberrant DNA methylation. In the *in vitro* studies, DNA methylation and gene expression could not be related, but gene expression was not measured in the human study. As most DNA methylation differences were detected in CGIs, of which many are close to the TSS, gene expression analysis in the eel consumers could provide additional insight into a possible association between DNA methylation, gene expression and possibly health effects. Now that we revealed that POPs are associated with gene-specific DNA methylation, it would be highly informative to measure histone modifications as well as other DNA marks. The bisulfite conversion used in this thesis does not distinguish between methylation and hydroxymethylation, while the two have different functionalities.[82] Additional measurements of histone modification and DNA hydroxy-methylation could first of all be done for the genes that were differentially methylated, before a more expensive and elaborate genome-wide analysis is performed. There is a need for a model that can predict gene expression based on DNA methylation profiles and histone modifications. The NIH Roadmap Epigenomics Program is a good example of an effort to provide an epigenome map, by combining human reference epigenomes from many different tissues.[35] With the help of a comprehensive overview, epigenetic marks might become predictive for gene regulation.

The next step is elucidating the potential health effects related to these epigenetic marks. First, the knowledge to translate epigenetic marks measured in blood to other relevant tissues should be obtained. The epigenome map, including over 100 different human tissues and cell-types, might be a good step forward to interpret DNA methylation in blood with regard to health effects related to other tissues. Subsequently, an association between epigenetic marks and health effects can be established by measuring health outcomes related to these marks. In our human study this could be done by measuring the immune response in these men. Current biomarkers related to cancer are not conclusive, and our sample size is far too small to perform a follow-up study to determine how many participants eventually develop cancer. An option would

be to use existing cohort studies with established biobanks and follow people over time, measuring a whole-range of possible adverse health outcomes. As POPs remain very stable, POP levels could be measured in stored blood from these biobanks. However, to measure a broad range of POPs, a relatively large amount of blood is needed. It is therefore important to use very sensitive techniques and to establish the most relevant POPs to use the available material sparingly.

The use of a prospective cohort study can also help to eliminate the question whether DNA methylation is a cause or consequence of the adverse health effects, however, this may still be difficult when DNA methylation is an adaptive protective mechanism and the disease still occurs. To determine a causal relationship, *in vitro* experiments or animal studies can be performed. When gene-specific DNA methylation can be artificially altered, the consequences can become clear.

Our human study focussed on men between 40 and 70 years old, which is a relatively homogenous group and not representative for the whole population. Results might be different in women due to differences in hormonal responses. Research with pregnant women and later their children might provide more information about the safe level in humans for the unborn and developing child. This is, however, also more challenging because lactation is a known factor to lower POP levels in these women, and therefore the number of pregnancies influences the outcome.[83] Detailed epigenomics in combination with metabolomics to determine a broad range of potential adverse health effects could be used to test whether sex-specific effects can be expected and to translate the data from this study to other populations.

Before epigenetic marks can be included in standard risk assessments, more information about the biological relevance is needed. At this point epigenetic marks raise more questions, than they give clear answers. It should become clear that there is a causal, and preferably a dose-response, relationship between epigenetic marks and a certain health outcome, or the epigenetic marks should lead to increased susceptibility of disease in certain populations. Epigenetic marks in risk assessment are most important for compounds who do not show immediate toxicity, but can exert health effects after

long-term exposure, and should therefore be able to predict adverse health effects before they arise.

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CHAPTER 7

Summary (English)

Nederlandse samenvatting

List of abbreviations

Summary

Wild caught fish, especially marine fish, can contain high levels of persistent organic pollutants (POPs). In the Netherlands, especially eel from the main rivers have high POP levels. This led to a ban in 2011 on eel fishing due to health concerns. Many of the marine POPs have been related to adverse health effects such as endocrine disruption, neurodevelopmental problems, immune suppression and cancer. Although some mechanisms of action of POPs are clear, like dioxins binding to the aryl hydrocarbon receptor and OH-PCBs binding to thyroid transport proteins, not all adverse health effects can be explained by these mechanisms of action. Epigenetic phenomena, such as DNA methylation, have been proposed as a possible molecular mechanism underlying adverse health effects. DNA methylation is a heritable modification, which refers to the addition of a methyl group to cytosine in a CpG dinucleotide. Observational studies have indeed shown that POPs can affect global DNA methylation, although results are inconsistent. Some animal studies as well as *in vitro* experiments suggest that POPs can affect gene-specific DNA methylation, however, the biological significance and relevance for humans is not clear. Therefore, this thesis aimed to 1) study the accumulation of POPs in men consuming eel from high-polluted areas 2) elucidate whether seafood-related POPs can induce aberrant DNA methylation and 3) to determine whether DNA methylation is related to functional endpoints and gene expression *in vitro*.

For this purpose eight POPs that are abundantly present in seafood were chosen, namely 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), polychlorobiphenyl (PCB) 126 and 153, perfluorooctanesulfonic acid (PFOS), hexabromocyclododecane (HBCD), 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47), tributyltin (TBT), and methylmercury (MeHg). **Chapter 2** describes the *in vitro* effects of these POPs and mixtures thereof in H295R adrenocortical carcinoma cells. Relative responses for 13 steroid hormones and 7 genes involved in the steroidogenic pathway, and *CYP1A1*, were analysed. PFOS induced the most pronounced effects on steroid hormone levels by significantly affecting 9 out of 13 hormone levels measured, with the largest increases found for 17β-estradiol, corticosterone, and cortisol. Furthermore, TCDD, both PCBs, and TBT significantly

altered steroidogenesis. Increased steroid hormone levels were accompanied by related increased gene expression levels. The differently expressed genes were *MC2R, CYP11B1, CYP11B2*, and *CYP19A1* and changes in gene expression levels were more sensitive than changes in hormone levels. The POP mixtures tested showed mostly additive effects, especially for DHEA and 17β-estradiol levels. This study shows that some seafood POPs are capable of altering steroidogenesis in H295R cells at concentrations that mixtures might reach in human blood, suggesting that adverse health effects cannot be excluded. DNA methylation was not measured in this study due to the short exposure time, which was expected not to be sufficient for long-term epigenetic marks. Therefore, in **chapters 3A** and **3B** a differentiation experiment was performed enabling long-term exposure to POPs. Human mesenchymal stem cells (hMSCs) were differentiated into mature adipocytes over a time-course of 10 days. The transcriptional regulatory cascade involved in adipocyte differentiation has been extensively studied, however the mechanisms driving the transcription are poorly understood. In chapter 3A we therefore first explored the involvement of DNA methylation in transcriptional regulation during adipocyte differentiation. Genome-wide changes in DNA methylation were measured as well as the expression of adipogenic genes. The majority of these genes showed significant expression changes during the differentiation process. There were, however, only a couple of these differentially expressed genes that were differentially methylated. Genome-wide DNA methylation changes were most often located in intergenic regions, and underrepresented close to the transcription start site. This suggested that changes in DNA methylation are not the underlying mechanism regulating gene expression during adipocyte differentiation. Nevertheless, we explored DNA methylation differences after continuous exposure to POPs to investigate whether this could be an underlying mechanism by which POPs affect adipocyte differentiation. TCDD and PFOS decreased lipid accumulation, while TBT increased lipid accumulation. TCDD and TBT induced opposite gene expression profiles, whereas after PFOS exposure gene expression remained relatively stable. Genome-wide DNA methylation analysis showed that all three POPs affected DNA methylation patterns in adipogenic and other genes, but without concomitant gene expression changes. Differential methylation was again predominantly detected in intergenic regions, where the biological relevance of alterations in DNA methylation is unclear. This study demonstrated that POPs, at

environmentally relevant levels, are able to induce differential DNA methylation in differentiating adipocytes. However, the biological relevance of this aberrant DNA methylation remains unclear.

The *in vitro* results showed a proof of principle that POPs could be capable of altering DNA methylation. To this date, no human studies were performed investigating the relationship between POP levels and genome-wide DNA methylation. In order to investigate this, we first measured POP levels in eel consumers from the high-polluted areas (areas with a ban on eel fishing) and compared these levels to men consuming eel from low-polluted areas or aquaculture (**chapter 4**). We aimed to investigate the accumulation of these POPs and determine whether the predictions made in an earlier risk assessment were valid. This was indeed the case as levels of dioxins and dioxin-like compounds were on average 2.5 times higher in men consuming eel from high-polluted areas. Furthermore, PCBs with their hydroxylated metabolites, and perfluoroalkyl substances (PFASs) were, up to ten times, higher in these consumers. Especially the high levels of dioxins and dioxin-like compounds as well as the OH-PCBs are expected to be of health concern. We continued this research in **chapter 5** by associating all the measured POPs to clinical parameters related to e.g. thyroid hormones and liver enzymes, but found no relationship. Subsequently, we investigated the association between dioxins and dioxin-like compounds, the sum of seven indicator PCBs, and PFOS with genome-wide DNA methylation. We detected a number of differentially methylated regions (DMRs) related to genes involved in carcinogenesis (e.g. *BRCA1, MAGEE2*, *HOXA5*), the immune system (e.g. *RNF39*, *HLA-DQB1*), in retinol homeostasis (*DHRS4L2*), or in metabolism (*CYP1A1*). In contrast to the *in vitro* data, most significant effects were detected in CpG islands and were annotated close to the promoter region. This suggests that the differential methylation might be related to differential expression and possibly induce adverse health effects. The hypermethylation of some of these gene related to cancer could be an explanation of the carcinogenic effects that are observed with POP exposure.

Based on the results of this thesis we can conclude that the consumption of eel from high-polluted areas lead to accumulation of POPs above safe levels and that POP levels are associated with gene-specific DNA methylation *in vitro* as well as in environmentally

exposed men. More research, however, is needed to fully elucidate the biological implications of this aberrant DNA methylation. A first step can be to measure histone modifications, as these two epigenetic marks together are likely better in predicting gene expression. The second step can be to investigate the potential health effects related to these epigenetic marks and to determine whether there is a causal relationship. Although at this point there is a lack of knowledge with regard to health effects caused by DNA methylation, the consumption of eel from these high-polluted areas is ill-advised, because adverse health effects cannot be excluded based on our results and can even be expected based on literature.

Nederlandse samenvatting

Wild gevangen vis, voornamelijk zeevis, kan hoge gehaltes bevatten van persistente organische verontreinigingen (afgekort tot POPs, gebaseerd op het Engelse 'Persistent Organic Pollutants'). In Nederland heeft voornamelijk paling uit de grote rivieren hoge POP gehaltes. Dit heeft in 2011 geleid tot een vangstverbod op paling in bepaalde gebieden, omdat POPs schadelijk voor de gezondheid kunnen zijn. Verschillende van deze POPs zijn gerelateerd aan negatieve gezondheidseffecten zoals hormoon-verstoring, neurologische ontwikkelingsproblemen, onderdrukking van het immuun-systeem en kanker. Ook al zijn sommige werkingsmechanismen van deze POPs bekend, zoals dioxines die binden aan de aryl hydrocarbon receptor en OH-PCBs die binden aan schildklier transporteiwitten, niet alle nadelige gezondheidseffecten kunnen hiermee verklaard worden. Epigenetische processen, zoals DNA methylatie, zijn mogelijke moleculaire mechanismen die ten grondslag liggen aan de negatieve gezondheidseffecten. DNA methylatie is een erfelijke verandering, waarbij een methylgroup bindt aan cytosine in een CpG dinucleotide. Observationeel onderzoek heeft inderdaad aangetoond dat POPs effect hebben op globale DNA methylatie, ook al zijn de resultaten niet consistent. Experimentele dierstudies en *in vitro* (letterlijk *in glas*, heeft betrekking op gekweekte lichaamscellen) onderzoek heeft aanwijzingen opgeleverd dat POPs gen-specifieke DNA methylatie kunnen beïnvloeden, maar de biologische betekenis en de relevantie voor mensen is nog niet duidelijk. Deze dissertatie heeft daarom als doel om 1) de opstapeling van POPs in mannen te bestuderen die paling hebben geconsumeerd uit sterk verontreinigde gebieden 2) uitzoeken of deze POPs voor veranderingen in DNA methylatie kunnen zorgen en 3) bepalen of DNA methylatie gerelateerd is aan functionele gezondheidsmetingen en gen expressie *in vitro*.

Om dit doel te bereiken hebben we acht POPs gekozen die relatief veel voorkomen in vis, namelijk 2,3,7,8-tetrachloor-dibenzo-p-dioxine (TCDD), polychloorbifenyl (PCB) 126 en 153, perfluoroctaansulfonzuur (PFOS), hexabromocyclododecaan (HBCD), 2,2′,4,4′-tetrabromodifenyl ether (BDE-47), tributyltin (TBT), en methylkwik (MeHg). **Hoofdstuk 2** beschrijft *in vitro* effecten van deze POPs en 2 mixen hiervan in H295R

bijnierschorscellen. De relatieve effecten op 13 steroïdhormonen en 7 steroïdhormoon-gevoelige genen en *CYP1A1* zijn geanalyseerd. PFOS induceerde de meest opvallende effecten op de steroïdhormonen en veranderde de niveaus van 9 van de 13 gemeten hormonen, met de grootste veranderingen op oestradiol, corticosteron en cortisol. TCDD, beide PCBs en TBT hadden ook significante effecten op de steroïdhormonen. Verhoogde hormoon niveaus gingen samen met verhoogde genexpressie. De door de POPs beïnvloede genen waren *MC2R*, *CYP11B1*, *CYP11B2* en *CYP19A1*. Veranderingen in genexpressie waren gevoeliger dan veranderingen in hormoon niveaus. De geteste POP mixen lieten additieve effecten zien, voornamelijk voor DHEA en oestradiol niveaus. Deze studie laat zien dat sommige POPs, bij gehaltes die in humaan bloed bereikt kunnen worden, in staat zijn om niveaus van steroïdhormonen in H295R cellen te veranderen, en negatieve gezondheidseffecten kunnen daarom niet worden uitgesloten. DNA methylatie was niet gemeten in deze studie omdat het hier een kortdurende blootstelling betreft, en de verwachting was dat de blootstelling te kort was om chronische epigenetische veranderingen te meten. In **hoofdstuk 3A** en **3B** is daarom een differentiatie experiment uitgevoerd die een langdurige blootstelling aan POPs mogelijk maakte. Humane mesenchymale stamcellen (hMSCs) zijn gedurende 10 dagen gedifferentieerd in volwassen vetcellen. De genexpressie betrokken bij deze vetceldifferentiatie is al uitgebreid onderzocht, maar het mechanisme achter deze genexpressie is nog niet bekend. In hoofdstuk 3A is daarom als eerste onderzocht of DNA methylatie betrokken is bij het reguleren van genexpressie tijdens deze vetceldifferentiatie. Genoom-brede veranderingen in DNA methylatie zijn gemeten tegelijkertijd met de expressie van genen gerelateerd aan vetcellen. Het merendeel van deze genen lieten significante genexpressie veranderingen zien tijdens de differentiatie. Slechts enkele van deze genen waren echter anders gemethyleerd. Genoom-brede DNA methylatie veranderingen werden voornamelijk gevonden in regio's tussen genen, en werden niet vaak gevonden dicht bij de promotor regio van genen. Deze resultaten laten zien dat veranderingen in DNA methylatie waarschijnlijk niet het onderliggende mechanisme zijn om genexpressie te sturen tijdens vetceldifferentiatie. Desalniettemin hebben we DNA methylatie veranderingen onderzocht na continue blootstellingen aan verschillende POPs om te onderzoeken of DNA methylatie een relevant mechanisme kan zijn waarmee POPs vetceldifferentiatie beïnvloeden. TCDD en PFOS verminderden

vetopslag in vetcellen, terwijl TBT zorgde voor een toename in de vetopslag. TCDD en TBT hadden ook een tegenovergesteld effect op genexpressie, terwijl er na de PFOS blootstelling weinig verschillen te zien waren. Genoom-brede DNA methylatie analyses laten zien dat alle drie deze POPs DNA methylatie beïnvloeden in zowel vetcel gerelateerde genen als ook in andere genen. Deze DNA methylatie verschillen kwamen niet overeen met veranderingen in genexpressie. Ook in deze studie zijn de verschillen in DNA methylatie voornamelijk gevonden in regio's tussen genen, en op deze locatie in het genoom is de biologische relevantie van een veranderd DNA methylatie patroon nog niet duidelijk. Dit onderzoek laat zien dat POPs, bij relevante concentraties, in staat zijn om veranderingen aan te brengen in DNA methylatie tijdens vetceldifferentiatie. De biologische relevantie van dit afwijkende DNA methylatie patroon blijft onduidelijk.

De *in vitro* resultaten geven aanwijzingen voor de hypothese dat POPs in staat zijn om veranderingen in DNA methylatie aan te brengen. Er zijn echter nog geen humane onderzoeken uitgevoerd om te onderzoeken wat de relatie is tussen POP gehaltes in mensen en genoom-brede DNA methylatie. Om dit te kunnen onderzoeken, hebben we eerst POP gehaltes gemeten in palingconsumenten uit verontreinigde gebieden (namelijk gebieden waar een vangstverbod geldt). Deze POP gehaltes hebben we vergeleken met POP gehaltes gemeten in mannen die paling consumeren uit relatief schonen gebieden of kweekpaling (**hoofdstuk 4**). Het doel was om de opstapeling van de POPs in het lichaam te onderzoeken en te bepalen of de schattingen die in eerdere risico-evaluaties gemaakt zijn kloppen. Dit bleek inderdaad te kloppen aangezien de gehaltes van dioxines en dioxine-achtige stoffen gemiddeld 2,5 maal hoger waren in mannen die paling uit verontreinigde gebieden geconsumeerd hadden vergeleken met mannen die paling hebben geconsumeerd uit relatief schone gebieden. Verder waren PCBs, met hun gehydroxyleerde (OH) metabolieten, en de geperfluoreerde stoffen (zoals PFOS) tot wel tien maal hoger in de consumenten uit de verontreinigde gebieden. Voornamelijk de hoge gehaltes van de dioxines en dioxine-achtige stoffen als ook de OH-PCBs zijn mogelijk schadelijk voor de gezondheid. Dit onderzoek hebben we voortgezet in **hoofdstuk 5** door het effect van alle gemeten POPs te bekijken op klinische bloedwaarden zoals schildklierhormonen en leverenzymen, maar we vonden hier geen effect op. Vervolgens hebben we gekeken of dioxines en dioxine-achtige

stoffen, het totaal van zeven indicator PCBs, en PFOS een effect hadden op genoom-brede DNA methylatie. We vonden verschillende regio's in het DNA die anders gemethyleerd waren en deze genen waren voornamelijk gerelateerd aan kanker (*BRCA1, MAGEE2*, *HOXA5*), het immuunsysteem (*RNF39*, *HLA-DQB1*), retinol homeostase (*DHRS4L2*), of bij de metabolisme van deze stoffen (*CYP1A1*). In tegenstelling tot de *in vitro* resultaten vonden we de meeste significante effecten in CpG eilanden en waren de meeste effecten dicht in de buurt van de promotor regio. Deze veranderde DNA methylatie patronen kunnen dan ook gerelateerd zijn aan genexpressie en mogelijk kunnen ze ten grondslag liggen aan negatieve gezondheidseffecten. De hypermethylatie van sommige van deze genen gerelateerd aan kanker kunnen een verklaring zijn voor de verhoogde kans op kanker die geobserveerd is met hogere gehaltes van deze POPs in het lichaam.

Gebaseerd op de resultaten beschreven in deze dissertatie kunnen we concluderen dat de consumptie van paling uit de verontreinigde gebieden leidt tot hogere gehaltes van POPs die boven het veilige niveau uitkomen en dat deze POP gehaltes gerelateerd zijn aan gen-specifieke DNA methylatie *in vitro* en in de mannelijke palingconsumenten. Meer onderzoek is nodig om de volledige biologische consequenties van deze veranderde DNA methylatie te ontrafelen. Een eerste stap hiervoor kan zijn om histon modificaties te meten, omdat die samen met de DNA methylatie waarschijnlijk een betere voorspelling zijn voor genexpressie. Een tweede stap zou kunnen zijn om de potentiele gezondheidseffecten gerelateerd aan deze epigenetische mechanismen te bestuderen om vast te stellen of er een oorzakelijk verband is. Ook al is er op dit moment nog een gebrek aan kennis betreffende de gezondheidseffecten veroorzaakt door een veranderd DNA methylatie profiel, de consumptie van paling uit de verontreinigde gebieden is sterk af te raden. Negatieve gezondheidseffecten kunnen namelijk niet worden uitgesloten gebaseerd op onze resultaten en kunnen zelfs worden verwacht op basis van de literatuur.

List of abbreviations

3'UTR: 3' untranslated region
5-aza-dC: 5-aza-2′-deoxycytidine
5'UTR: 5'untranslated region
AH: aryl hydrocarbon
ALP: alkaline phosphatase
ALT: Alanine aminotransferase
AST: Aspartate aminotransferase
BDE47: 2,2′,4,4′-tetrabromodiphenyl ether
BEQ: bioanalytical equivalent
BH method: Benjamini-Hochberg method
CGI: CpG Island
ChAMP: Chip Analysis Methylation Pipeline
CV: coefficient of variation
DHA: docosahexaenoic acid
DHEA: dehydroepiandrosterone
DL: dioxin-like
DMP: differentially methylated position
DMR: differentially methylated region
DMSO: dimethyl sulfoxide
DNA: deoxyribonucleic acid
DNMTI: DNA methyltransferase 1
DR CALUX: dioxin responsive chemical-activated luciferase gene expression
EC: European Commission
EIA: enzyme immunoassay
ELISA: Enzyme-Linked Immuno Sorbent Assay
EPA: eicosapentaenoic acid
FDR: false discovery rate
GC-MS: gas chromatography mass spectrometry
GEO: Gene Expression Omnibus
GGT: gamma-glutamyl transferase
HBCD: hexabromocyclododecane
HDL: high density lipoprotein
Hg: hemoglobin
HgCl2: inorganic mercury
hMSCs: human mesenchymal stem cells
HPA: hypothalamic-pituitary-adrenal
Ht: hematocrit
IBMX: 3-isobuty-l-methyl-xanthine
IGR: intergenic region
IPA: Ingenuity Pathway Analysis
LC-MS: liquid chromatography mass spectrometry
Limma: Linear Models for Microarray Data
LINE-1: long interspersed nuclear element 1
LUMA: LUminometric Methylation Assay
LOD: limit of detection
LOQ: limit of quantification
MC2R: melanocortin 2 receptor
MeHg: methylmercury
MTT: 3-(4,5-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MDS: multidimensional scaling
MeDIP: methylated DNA immune-precipitation
ML: maximum levels
MO: mono-ortho
MS-HRM: Methylation-Sensitive High Resolution Melting
NO: non-ortho
OC: organochlorine
OECD: organization for economic co-operation and development
OH: hydroxylated
PBDE: polybrominated diphenyl ether
PCB: polychlorobiphenyl
PCB153: 2,2′,4,4′,5,5′-hexachlorobiphenyl
PCDD: polychlorinated dibenzo-p-dioxin
PCDF: polychlorinated dibenzofurans
PCR: polymerase chain reaction
PFAS: perfluoroalkyl substance
PFOS: perfluorooctanesulfonic acid
POPs: persistent organic pollutants
RNA: ribonucleic acid
mRNA: messenger RNA
ncRNA: non-coding RNA
SC: solvent control
SGBS, Simpson-Golabi-Behmel syndrome
S/N: signal-to-noise ratio
SPE: solid-phase extraction
TBT: tributyltin

TBG: thyroxine-binding globulin
TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TEF: toxic equivalency factor
TEQ: toxic equivalents
TSH: thyroid stimulating hormone
TSS: transcription start site
TTR: transthyretin
UTR: untranslated region
WBC: white blood cell count

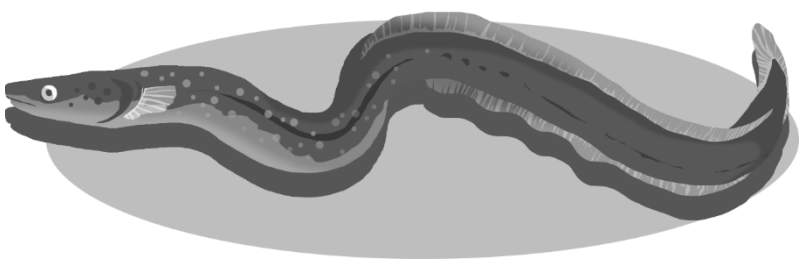
Appendices

Acknowledgements

About the author

List of publications

Overview of completed training activities



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About the author

Myrthe van den Dungen was born on 28 October 1987 in Venlo. After completing her secondary education at College Den Hulster in Venlo, she started in 2005 with her study Biomedical Sciences at the Radboud University in Nijmegen. In 2008 Myrthe started her master Biomedical Sciences with a major in Toxicology. She performed her internships at the Department of Clinical Pharmacy to investigate drug-drug interactions and later at the National Institute for Public Health and the Environment (RIVM) where she worked on alternative methods for animal testing. After her graduation in 2011 she started in 2012 with her PhD research at Wageningen University at the Division of Toxicology (later Sub-department of Environmental Technology and the Marine Animal Ecology Group) and the Division of Human Nutrition. During her PhD, Myrthe followed a postgraduate program and registered as toxicologist-in-training, to become European Registered Toxicologist (ERT) after graduation. Currently, Myrthe is working as a Toxicologist within the Global Product Regulatory and Quality Management department of DSM Food Specialties.

List of publications

Van den Dungen MW, Murk AJ, Kok DE, Steegenga WT. *Comprehensive DNA methylation and gene expression profiling in differentiating human adipocytes.* J Cell Biochem. 2016.

Van den Dungen MW, Rijk JCW, Kampman E, Steegenga WT, Murk AJ. *Steroid hormone related effects of marine persistent organic pollutants in human H295R adrenocortical carcinoma cells.* Toxicol In Vitro. 2015; 29(4):769-78.

Zwart EP, Schaap MM, van den Dungen MW, Braakhuis HM, White PA, van Steeg H, van Benthem J, Luijten M. *Proliferating primary hepatocytes from the pUR288 lacZ plasmid mouse are valuable tools for genotoxicity assessment in vitro.* Environ Mol Mutagen. 2012; 53(5):1-8

Brüggemann RJ, van Luin M, Colbers EP, van den Dungen MW, Pharo C, Schouwenberg BJ, Burger DM. *Effect of posaconazole on the pharmacokinetics of fosamprenavir and vice versa in healthy volunteers.* J Antimicrob Chemother. 2010; 65(10):2188-94.

Van den Dungen MW, Murk AJ, Kok DE, Steegenga WT. *Persistent organic pollutants alter DNA methylation during human adipocyte differentiation.* Submitted.

Van den Dungen MW, Kok DE, Polder A, Hoogenboom LAP, van Leeuwen SPJ, Steegenga WT, Kampman E, Murk AJ. *Accumulation of persistent organic pollutants in consumers of eel from polluted rivers or relatively clean eel*. Submitted.

Van den Dungen MW, Murk AJ, Kampman E, Steegenga WT, Kok DE. *Epigenome-wide association between DNA methylation in leukocytes and serum levels of persistent organic pollutants in Dutch men.* Submitted.

Overview of completed training activities

Discipline specific courses

Epidemiology	Postgraduate Education in Toxicology (PET)	2012
Risk assessment	PET	2012
Environmental Toxicology	Wageningen University	2012
Toxicogenomics	PET	2013
Epigenesis & Epigenetics	VLAG	2014

Meetings and conferences

Dutch society of Toxicology (NVT) annual meeting (2012)
Dutch society of Toxicology (NVT) annual meeting (2013), poster presentation
Dutch society of Toxicology (NVT) annual meeting (2015), poster and speed presentation
Society of Toxicology (SOT), San Diego, USA (2015), oral presentation

General courses

VLAG PhD week	VLAG	2012
Philosophy and Ethics of Food Science and Technology	VLAG	2014
Afstudeervak organiseren en begeleiden	EduWeb	2013
Life Sciences with Industry Workshop	STW	2014
Information Literacy including Endnote Introduction	WGS	2012
Techniques for writing and presenting a scientific paper	WGS	2013
Reviewing a Scientific Paper	WGS	2014

Optional activities

Preparing PhD research proposal	Wageningen University	2012
Organizing international PhD excursion	Wageningen University	2013
Methodology Nutrition Research	Wageningen University	2012
Attending Scientific Presentations	Wageningen University	2012-2016
Discussion Group Epigenetics	Wageningen University	2012-2015

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