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Impact of epidemiologically identified mixtures of endocrine disrupting chemicals on metabolic programming

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Abstract

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We are ubiquitously exposed to a plethora of endocrine-disrupting chemicals (EDCs), i.e. substances that alter the function(s) of the endocrine system. While ample evidence show individual EDC's influence on developmental processes resulting in adverse health outcomes, less is known about the effects of human-relevant EDC mixtures exposure. Additionally, there is a lack of appropriate methodology to assess the hazard and risk of complex mixtures.

This doctoral project aimed to examine the effects of two EDC mixtures and to compare individual components to its mixture, on the developing metabolic system. And to investigate additivity approach for predicting effects of complex mixtures.

Studied EDC mixtures (G and G1) were previously identified using Swedish Environmental Longitudinal Mother and Child Asthma and Allergy (SELMA) study data, based on their association with lower birth weight. In this thesis, these mixtures, and mixture G1's components, were tested for effects on adipogenesis and underlying epigenetic and transcriptional changes in human mesenchymal stem cells (hMSCs) and on metabolic rate in zebrafish larvae.

In hMSCs, both mixtures induced adipogenesis at concentrations corresponding to SELMA cohort measured levels. Mixture G induced early transcriptional changes of over 1000 genes in a dose-dependent manner. These genes significantly overlapped with glucocorticoid-regulated genes and were involved in early osteogenesis. Mixture G1 induced significant DNA methylation changes at 713 positions and in six genomic regions in genes whose expression or methylation was previously associated with obesity or MSC differentiation. In zebrafish larvae, mixture G1 increased oxygen consumption rate. Compared to mixture G1, none of its individual components showed equally large effects on adipogenesis or metabolic rate. However, mixture G1 effect on both endpoints could be adequately predicted by the additivity model using experimental data from its constituents.

In conclusion, this doctoral project showed that mixtures corresponding to human real-life exposures, in terms of proportions and concentrations, can induce molecular, cellular, and whole-organism changes relevant to developmental metabolic programming, which could underlie adverse outcomes later in life. The results emphasise that mixtures matter and should be accounted for in regulatory risk assessments, and provide support for additivity models as a pragmatic approach to mixture risk assessment.

Keywords: Endocrine disrupting chemicals, Mixtures, Metabolism, Developmental Toxicology, Epigenetics

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*To my parents, Natasha and Vadim,
and to my brother, Rodion.*

List of Papers

This thesis is based on the following paper/manuscripts, which are referred to in the text by their Roman numerals.

- I. **Lizunkova, P***, Caporale, N*, Engdahl, E., Borbély, G., Cheroni, C., Germain, PL., Lindh, C., Gennings, C., Bornehag, CG., Testa, G., Rüegg, J. (2023) A Mixture of Endocrine Disrupting Chemicals Associated with Lower Birth Weight Induces Adipogenesis and Transcriptional Changes in Human Mesenchymal Stem Cells. *Manuscript*.
* *co-first authors*
- II. **Lizunkova, P.**, Engdahl, E., Borbély, G., Gennings, C., Lindh, C., Bornehag, C. G., Rüegg, J. (2022) A Mixture of Endocrine Disrupting Chemicals Associated with Lower Birth Weight in Children Induces Adipogenesis and DNA Methylation Changes in Human Mesenchymal Stem Cells. *International journal of molecular sciences*, 23(4):2320.
- III. Wennemo, T*, **Lizunkova, P***, Lindh, C., Gennings, C., Bornehag, CG., Sturve, J., Engdahl, E., Rüegg, J. (2023) A human-relevant complex mixture affects metabolic endpoints in human mesenchymal stem cells and zebrafish (*Danio rerio*) differently than its single compounds. *Manuscript*.
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- IV. **Lizunkova, P***, Mistry, S*, Wennemo, T., Lindh, C., Bornehag, CG., Sturve, J., Engdahl, E., Rüegg, J., Gennings, C. (2023) Additivity provides a good approximation for the effect of a complex, human-relevant chemical mixture on metabolic outcomes. *Manuscript*.
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Introduction

Rising prevalence of metabolic diseases and adiposity

The increasing prevalence of metabolic diseases including those of metabolic syndrome (MetS), is a growing worldwide concern in public health ^{1,2}. MetS is a complex of interconnected cardiometabolic disorders associated with excess adiposity. Increased adiposity is excessive accumulation of body fat, and can commonly be classed as overweight or obesity, depending on the measurements of body mass index and waist circumference ³. The prevalence of overweight and obese individuals, both in childhood and adult stages, between the years 1975 and 2016 has increased in nearly every country in the world and is currently at epidemic levels globally ⁴⁻⁶. Adverse health effects of excessive body fat are particularly linked to the accumulation of visceral and ectopic fat as well as adipocyte hypertrophy (increase in adipocyte cell size) and the inflammatory phenotype of white adipose tissue ⁷⁻¹¹.

The global increase in prevalence of excessive adiposity is often attributed to lifestyle habits, such as diet and low physical activity, and to a lesser degree to genetic predisposition. However, in recent years' numerous epidemiological studies have found associations between adverse prenatal environmental conditions and increased susceptibility of the fetus to develop obesity and metabolic syndrome disorders later in life ¹²⁻²⁶. Many of these studies were influenced by the hypothesis put forth by epidemiologist David Barker and colleagues, commonly known as the 'Developmental Origins of Adult Health and Disease' (DOHaD) ²⁷⁻²⁹. DOHaD is the theory describing the link between intrauterine conditions and susceptibility to adult onset of non-communicable diseases ³⁰. In particular, DOHaD postulates that environmental influences such as maternal nutrition, during critical developmental periods like embryonic development, fetal growth, and early infancy, can influence vulnerability to diseases later in life.

Barker and Osmond (1986) conducted a pioneering study that led to the development of the DOHaD. They have shown a positive association between infant mortality rates and ischemic heart disease, concluding that poor nutrition during early life enhances vulnerability to the effects of an affluent diet ²⁷. Furthermore, several epidemiological studies have consistently linked low birth weight (LBW), which refers to infants weighing less than 2500 g at birth,

with cardiovascular disease morbidity and mortality later in life ^{31–39}. These findings supported the notion that birth weight serves as an initial indicator of fetal growth quality within the intrauterine environment as well as an indicator of the fetus's morbidity and mortality ^{40–42}. Numerous epidemiological studies have observed a similar association between LBW and an increased risk for several adult-onset diseases, such as adiposity, diabetes, cardiovascular diseases as well as changed postnatal weight gain ^{43–50}. Additionally, maternal undernutrition was shown to not only lead to fetal growth restriction but also changed responsiveness of various fetal tissues to the placental and fetal hormones ^{51–53}. These changes contributed to abnormal organ development and were linked to a heightened risk of developing cardiovascular disease and obesity later in life ^{28,54}. In summary, adverse prenatal environmental conditions, along with lifestyle factors, have been proposed as key contributors to the development of adiposity and adult onset of metabolic disorders.

Endocrine disrupting chemicals

Humans and wildlife are continuously exposed to chemicals that were shown to, or suspected of, interfering with the endocrine system, known as Endocrine Disrupting Chemicals (EDCs). The EDC is defined as “an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny or (sub) populations” ⁵⁵. Exposure to EDCs occurs via omnipresent sources in everyday life, such as consumer products like clothing, electronics, personal care products, as well as building materials, foods, and food-contact materials ^{56,57}. Numerous studies worldwide have shown the presence of various EDCs in human blood, urine, as well as in breast milk, amniotic fluid, and cord blood which indicates exposures during sensitive windows of developmental programming in prenatal and early postnatal periods ^{58–62}. Since hormones play a crucial role in guiding growth and development, disruptions in the balance of hormonal regulation can result in long-lasting health adversities ^{63 57,64–69}. Indeed, exposure to EDCs has been associated with, among others, interference with sexual, neurological, and metabolic development ^{60,60,70–74}.

The EDCs studied in this project are from eight different chemical classes as listed in Table 1 in the Methods section. A brief overview of some of these EDCs is provided below.

Phthalates like diethylhexyl phthalate (DEHP) and diethyl phthalate (DEP) are man-made chemical esters of phthalic acid. While predominantly utilized as plasticizers, phthalates can also be detected in various other products including adhesives, paints, rubber goods, toys, flooring, food packaging materials, medical devices, and sports equipment ⁷⁵. Due to their extensive use and

the tendency to leach from products, phthalates are frequently detected in water, air, food, and dust, leading to human exposure from multiple sources ⁷⁵. Ample human, as well as animal studies, have shown adverse reproductive and developmental effects as a result of exposure to various phthalates ⁷⁵. Since the 1990s the use of several phthalates was restricted from certain products sold in the European Union (EU) owing to their recognized endocrine-disrupting properties ⁷⁶. Due to the diverse and heavy use of phthalates in consumer and industrial products, the demand for non-phthalate plasticizers emerged. Non-phthalate plasticizers, such as diisononyl cyclohexane-1,2-dicarboxylate (DINCH), belong to a chemical class that serves as an alternative to phthalate plasticizers ⁷⁷. Similarly to phthalates, non-phthalate plasticizers are found in a wide variety of products, therefore human exposure to them also occurs via many different sources. The non-phthalate plasticisers were produced to be a safer alternative to phthalate plasticisers but the knowledge about their toxicological properties remains relatively limited. Hence, it is still a question of whether these chemicals are safe replacements or regrettable substitutions ⁷⁷. For example, DINCH was initially introduced as a safer substitution to phthalates like DEHP, however, recent studies have shown that DINCH is not a biologically inert molecule and exerted more adverse metabolic effects than DEHP on lipid homeostasis, adipogenesis, and the inflammatory state of the adult mammary gland in the gestational exposed Sprague Dawley rats ⁷⁷⁻⁸².

Per- and polyfluoroalkyl substances (PFASs) are highly fluorinated compounds characterized by extreme stability ⁸³. These chemicals are extensively employed for their hydrophobic and oil-repellent characteristics in various consumer products such as single-use food containers, cooking utensils, outdoor clothing, building materials, paints, and furniture ⁸³. PFASs possess a stable chemical structure resulting in the accumulation of these chemicals in the environment as well as bioaccumulation throughout the food chain since the start of their production ⁸⁴. Due to their extensive use and non-biodegradability PFASs are frequently detected in water, air, dust, seafood, and on contaminated surfaces, leading to human exposure from multiple sources ⁸³. Exposure to PFASs is associated with an array of adverse health effects as demonstrated by several longitudinal studies conducted on the people living near to the West Virginia DuPont Washington Works fluorotelomer plant ⁸⁵⁻⁸⁸. These studies showed strong associations between exposure to perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) and high cholesterol, thyroid dysfunction, pregnancy-induced hypertension, lower birth weight, and testicular cancer ^{50,64,89-94}.

Pesticides are chemical substances used to eradicate various unwanted organisms such as insects, weeds, and fungi for agricultural and public health purposes ⁹⁵. Organo-chlorine pesticides (OCPs) such as hexachlorobenzene

(HCB) are chlorinated compounds that were used as a safer alternative to arsenic-based pesticides ⁹⁶. OCPs belong to the class of persistent organic pollutants (POPs) and hence, are highly resistant to degradation resulting in high persistence in the environment and bioaccumulation in various organisms, including humans ⁹⁷. While many OCPs have been banned from use in the EU, these chemicals are still detected in soil, air, groundwater, wildlife, and certain foods like fish ⁹⁸. Numerous studies showed associations between OCP exposure and adverse health effects such as thyroid dysfunction, type 2 diabetes, neurotoxicity, reproductive disorders, autoimmune diseases, and a series of cancers such as of the breast, prostate, and stomach ^{97,99(p),100–106}.

EDC mixtures

Although evidence mounts for the harmful effects of individual EDCs, the reality that human exposure entails a complex mixture of chemicals has initially been overlooked ¹⁰⁷. However, in recent years, there has been a notable shift in focus toward understanding the potential impacts of EDC mixtures as well ¹⁰⁸. EDC mixtures can exhibit diverse chemical interactions, including additive, synergistic, or antagonistic effects ¹⁰⁹. These complex chemical interactions, when the chemical in question is present with several other chemicals that may contribute to the same health adversity, may therefore be underestimated. Numerous studies have shown adverse combined effects of EDC mixtures even when individual chemicals within the mixture were below levels of concern ^{110–112}. For example, the combined effect of five synthetic steroidal pharmaceuticals significantly inhibited egg production in *Pimephales promelas* even when each steroidal pharmaceutical in the mixture was present at concentrations that, individually, did not yield statistically significant effects—a demonstration of producing a combined impact from otherwise inconsequential concentrations ¹¹⁰. Similarly, eleven xenoestrogens induced a change of 17ss-estradiol action within a yeast estrogen system assay, even though the concentration of each xenoestrogen was below its no-observed-effect concentration ¹¹³. Furthermore, a growing number of studies show that even mixtures comprising chemicals from heterogeneous classes with distinct modes of action can produce more potent combined effects that surpass the individual impacts of each component within the mixture ^{114,115}.

In the study conducted by Caporale et al. (2022), an innovative approach was employed, integrating both epidemiological and experimental data to formulate a mixture-centered risk assessment strategy for heterogeneous mixtures ¹¹⁶. This investigation systematically established a connection between early pregnancy EDC mixture exposure and language delay in children. The process involved two sequential steps: firstly, identifying an EDC

mixture linked to language delay through epidemiological findings; secondly, subjecting this mixture to experimental testing at concentrations and mixing ratios relevant to human exposure. The EDC mixture consisted of chemicals from different classes and at different concentrations, reflecting real-life exposure. The experimental assessments revealed significant disruptions within hormone-regulated networks in human brain organoids, *Xenopus laevis*, and *Danio rerio* models, as well as behavioral changes. Upon reevaluating the epidemiological data in light of the experimental outcomes, up to 54 % of children within the cohort exhibited exposures at or exceeding levels of concern. Thus, even when individual concentrations of chemicals are below the regulatory threshold, their simultaneous exposure effects may produce long-lasting health adversity^{57,109,117,118}.

Before a chemical is introduced into the market, its safety for both humans and the environment needs to be evaluated. Despite the extensive array of regulatory toxicological examinations conducted to assess the safety of individual chemicals, there are concerns regarding combined unintentional mixture effects, as such exposures are not normally considered^{119–121}. Hence, exposure to unintentional chemical mixtures, including of EDCs, is a major unmet problem requiring immediate regulatory action. One of the significant regulatory obstacles as formulated by European Food Safety Authority (Efsa) (2013) is limited toxicological data from studies focused on real-life human-relevant chemical mixtures exploring whole mixture and component-based approaches for toxicity assessment^{119,121}.

One challenge concerning chemical mixtures is to identify the truly harmful ones among the infinite number of possible mixtures to which humans are exposed. The mixtures studied in this doctoral thesis were identified within the project EDC MixRisk (<http://edcmixrisk.ki.se/>) and prepared by the EDC-MixRisk partner at the Division of Occupational and Environmental Medicine at Lund University. The mixtures were established using data from The Swedish Environmental Longitudinal Mother and Child Asthma and Allergy (SELMA) study where associations between prenatal exposure and lower birth weight were found^{122,123}. Mixture G was composed of 9 individual compounds while mixture G1 of 14. The primary components of the mixture G1 were PFOA, HCB, MBP (metabolite of DEP) and MBzP (metabolite of BBzP), together receiving 57 % of the weight in the mixture. The primary components of the mixture G were MEP and MBP, together constituting 50 % of the weight in the mixture. The full contents of mixture G1 and mixture G are shown in Table 1. The concentrations of all included chemicals were designed to reflect the geometric mean of the serum concentration measured in the SELMA mothers.

EDCs as obesogens

In 2002, Baillie-Hamilton proposed a connection between the rise in obesity prevalence and the rise in new industrial chemicals, suggesting that these chemicals, obesogens, may disrupt weight-control mechanisms¹²⁴. Grun and Blumberg (2006) have developed this theory further into the environmental obesogen hypothesis, describing that early exposure to synthetic chemicals could predispose individuals to increased fat mass and body weight¹²⁵. During fetal development, the programming of fat deposits occurs. Adipose tissue, an essential endocrine organ, first appears in human fetuses around gestational week 14. It plays a crucial role in the body's homeostasis and metabolism. The adipose tissue continues to increase throughout gestation in a linear relationship to fetal growth rate. As described above, numerous *in vivo* and *in vitro* studies have demonstrated that certain EDCs can act as obesogens and induce adipogenesis and weight gain, illustrating their role in obesity development.

In the last decades, evidence has increased that EDCs can disrupt metabolic functions. Several epidemiological studies have shown that prenatal exposure to EDCs like per- and polyfluoroalkyl substances (PFAS) and polychlorinated biphenyls (PCBs) is associated with LBW of the offspring, as well as with a higher risk of adiposity in childhood^{126–131}. Similarly, prenatal exposure to diethyl phthalate (DEP), di-n-butyl phthalate (DBP), butyl benzyl phthalate, di(2-ethylhexyl) phthalate (DEHP), dichlorodiphenyldichloroethylene (DDE) and 1,1,1-trichloro-2,2-bis(p-chlorophenyl)-ethane (p,p'-DDT) has been associated with increase in BMI at 12 year old^{132,133}. These epidemiological findings are corroborated by experimental results indicating that several EDCs can indeed alter the metabolic programming *in utero* (Angle, et al., 2013; Mackay, et al., 2013). Perinatal exposure of C57BL/6J female mice to p,p'-DDT and DDE led to persistent thermogenic impairment in brown adipose tissue¹³⁴. Perinatal exposure to DEHP increased body weight, liver weight, and fat pads weight in C57BL/6J mice¹³⁵. Similarly, DEHP-exposed female C3H/N mice had a significant increase in body weight, food intake, and visceral adipose tissue¹³⁶. EDCs from several different classes were shown to upregulate mesenchymal stem cell (MSC) and preadipocyte differentiation into adipocytes. p,p'-DDT induced a concentration-dependent increase in the nuclear levels of both PPARGamma and C/EBPalpha protein levels that led to an increase in 3T3-L1 adipocyte differentiation¹³⁷. PCBs 153 and 180 and a mixture of PCB induced an increase in lipid content in mature 3T3-L1 adipocytes¹³⁸. Benzyl butyl phthalate (BBP) in a dose-dependent manner induced epigenetic stress and upregulated lipid accumulation and adipogenesis in MSCs¹³⁹.

Mechanism of action of EDCs

EDCs belong to heterogeneous classes of synthetic and natural chemicals that do not share the same chemical structure. EDC mechanisms of action remain an active area of research with currently no complete understanding. EDCs have the potential to affect every possible cellular hormonal pathway, including the interference with the hormone receptors¹⁴⁰. Hormones exert their effects by binding to a specific receptor or receptors¹⁴¹. For example, steroid hormones exert their effects by interacting with specific intracellular receptors, namely nuclear receptors (NRs)¹⁴¹. Upon entering the target cell, typically through passive diffusion due to their lipid-soluble nature, steroid hormones bind to their corresponding receptor proteins within the cytoplasm or the nucleus. In the nucleus, the hormone-receptor complex acts as a transcription factor, modulating the expression of target genes by binding to the hormone response elements. The NRs include the estrogen receptors, glucocorticoid receptors, thyroid hormone receptors, and peroxisome proliferator-activated receptors among others¹⁴¹. Certain EDCs can directly bind to these receptors, acting either as agonists to enhance hormone effects or as antagonists to inhibit them^{56,65,73,142,143}. Kojima and colleagues (2004) conducted a large-scale study examining the agonistic and antagonistic activity of 200 pesticides on human estrogen receptor alpha (hERalpha), human estrogen receptor β (hERbeta) and human androgen receptor (hAR). Of the tested pesticides, 47 displayed hERalpha and 33 hERbeta mediated estrogenic activity. While none of the pesticides showed a direct hAR-mediated androgenic activity, 66 of them inhibited the transcriptional activity induced by 5 α -dihydrotestosterone¹⁴⁴. Direct action on NRs has been linked to the disruption of metabolic endpoints. For example, numerous experimental studies have shown the binding ability of EDCs to peroxisome proliferator-activated receptor gamma (PPAR γ), a key regulator of adipogenesis, and hence, the implication of these chemicals in the promotion of obese phenotype^{93,145–150}. Furthermore, Kassotis and colleagues (2019) have reported inhibition of thyroid receptor beta (TR β) by a mixture of flame retardants that increased triglyceride accumulation and pre-adipocyte proliferation¹⁵¹.

EDCs can also influence the concentration of endogenous free active hormone concentrations for example by competing for binding to transport proteins in the blood, disrupting the biosynthesis or degradation of circulating hormone-binding transport proteins in the blood, hence, impacting both the overall concentration of hormones and/or their free active fraction within the body. For example, thyroid hormones (THs) control transcription of many target genes and hold significant importance in cellular proliferation and differentiation, growth and developmental processes, maintenance of homeostasis, and metabolism^{87,152–156}. Triiodothyronine (T3), an active form of TH, and thyroxine (T4), a less active form of TH, are synthesized in the thyroid gland and

are the major forms of human THs. The thyroid gland releases THs mostly in the form of T4 153,156. The primary route for T3 synthesis involves the 5'-deiodination of the outer ring of T4 by deiodinase enzymes. Studies have shown EDC's ability to disrupt T4 conversion to T3, resulting in an adverse reduction of availability of the active form of THs 151,157–159. For example, treatment of female Wistar rats with butylparaben led to a dose-dependent decrease in D1 activity in the kidney 160. Furthermore, EDCs were shown to affect TH signaling via other mechanisms, such as affecting THs synthesis and circulating levels, obstructing enzymatic activity of thyroid peroxidase, and iodine transport 87,157,159,161–163.

The availability of a hormone is dependent on several factors such as hormone biosynthesis, hormone transport to target tissues, levels of hormone-binding proteins, and hormone breakdown. All these processes were shown to be disrupted by EDCs 55,65,73,140,141.

Epigenetics

The term epigenetics was first coined by the developmental biologist Conrad H. Waddington which he defined as “the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being” 164,165. In 1957, Waddington presented the concept of the epigenetic landscape, where he illustrated how a cell, depicted as a ball, navigates a landscape of valleys and ridges whose shape represents the whole complex of developmental processes of the cell (Figure 1) 166. The term epigenetics refers to mechanisms, epigenetic marks, that regulate gene transcription, and thus cell differentiation processes as well as tissue and organ development.

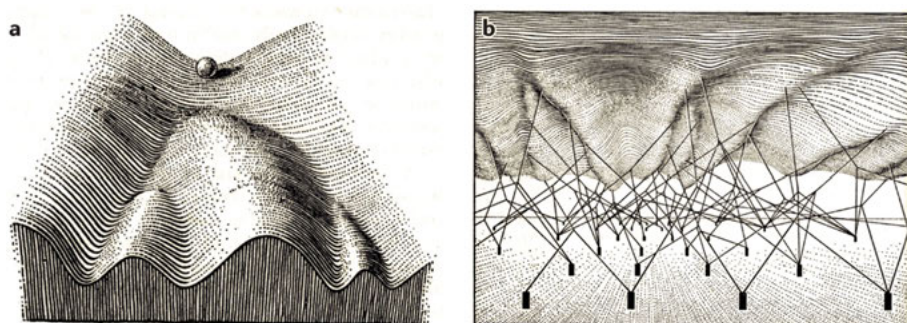


Figure 1: The epigenetic landscape proposed by Conrad Waddington. A. The cell developmental process is represented as a series of decisions that are represented as different paths and “forks” that the cell moves through, represented as a ball. B. the activity of genes, represented as pegs beneath the hills and valleys, shape the landscape's contours and dynamics. Modified from ¹⁶⁷.

Epigenetic marks do not entail DNA sequence alterations, can be heritable and/or reversible, and can be affected by environmental factors thus serving as a dynamic bridge connecting gene expression and environmental cues¹⁶⁷. One of the most stable and hence studied epigenetic processes that is involved in gene expression regulation is DNA methylation¹⁶⁸. DNA methylation refers to the covalent addition of a methyl group to the 5-cytosine residue in the context of a cytosine-guanine dinucleotide (CpG) by DNA methyltransferases (DNMTs), which can result in conformational changes to the chromatin¹⁶⁹. Depending on the genomic location of the DNA methylation, it can result in either gene transcription activation or suppression, for example, by affecting the accessibility of transcription factor binding sites¹⁶⁸.

During fetal development, epigenetic processes establish and maintain the differentiation of cells into specialized types that constitute the various organs and tissues of the body. These processes are tightly regulated and ensure that cells acquire the appropriate identity and function. Notably, the fetal epigenome undergoes dynamic changes as it responds to signals from the maternal environment, such as nutrients, hormones, and even exposure to chemicals or stressors^{170,171}. The intricate interplay between environmental factors and epigenetics during fetal development has profound implications for health and disease. Environmental cues can trigger modifications in the epigenetic marks, leading to alterations in gene expression patterns that persist beyond birth and into adulthood^{68,172–174}. There is an accumulating number of studies showing that altered epigenetic programming can be persistent and contribute to an individual's susceptibility to disease in adult life^{169,175–177}.

Developmental effects of exposure to diethylstilbestrol (DES) and bisphenol-A (BPA) have been extensively studied and serve as good examples of the persistent effects of adverse *in utero* exposure mediated by epigenetic changes. DES, a synthetic estrogen, was prescribed between the 1940s and 1970s to prevent miscarriage. DES was later associated with vaginal clear cell adenocarcinoma and genital tract malformations in women that were exposed *in utero*^{178–180}. Notably, DES impacts the Homeobox (HOX) gene family that is involved in embryonic development^{180,181}. In particular, DES disrupts HOXA10 expression through induction of hypermethylation in the promoter of the *HOXA10* gene and this aberrant methylation persists from fetal development into adulthood^{180,182–184}.

While exposure to DES was intentional, exposure to BPA is unintentional as it is a highly prevalent chemical used in commonly utilized items like baby bottles and food containers^{185,186}. BPA's effects were observed in developmentally exposed rodent models resulting in reproductive tract maldevelop-

ment and linked to advanced puberty, altered mammary development, and reproductive function changes^{187–190}. In humans, gestational exposure to BPA was associated with recurrent miscarriages, endometrial hyperplasia, obesity, diabetes, and heart disease^{191–193}. In subsequent studies *in utero* BPA exposure was found to permanently impact HOXA10 expression via induction of hypomethylation of the promoter of *HOXA10* gene^{181,194,195}. Importantly, adult exposure to BPA did not lead to altered DNA methylation of the *HOXA10* gene, indicating that *in utero* exposure is a critical developmental window for epigenetic sensitivity to BPA effects¹⁹⁵.

An EDC can disrupt a hormone action via inducing epigenetic changes^{140,142,196}. For instance, the insecticide methoxychlor increased the expression of DNA Methyltransferase 3 Beta, as well as induced hypermethylation of the ERbeta promoter regions in the ovaries of developmentally exposed Fischer (CDF) inbred rats¹⁹⁷. Moreover, gestational exposure of Sprague Dawley dams to DEHP led to decreased DNA methylation in the promoter of the mineralocorticoid receptor, which was associated with decreased mineralocorticoid receptor mRNA levels in offspring testes. Given the crucial role of the mineralocorticoid receptor in Leydig cell testosterone synthesis, this inhibition could contribute to suppressed testosterone production in adulthood¹⁹⁸. Continuous exposure to mixtures of EDCs, particularly during stages of heightened sensitivity and critical life phases, has the potential for enduring modifications. The lasting alterations caused by EDCs could be transmitted to the next generation through epigenetic mechanisms^{157,197}. The complete understanding of molecular mechanisms of EDC-driven health adversity remains unclear. Understanding the underlying mechanisms is crucial for developing targeted strategies to mitigate the potential health risks associated with EDC exposure.

Experimental models to address metabolic programming effects

Human MSCs have emerged as a valuable experimental model for investigating the effects on metabolic programming¹⁹⁹. MSCs are multipotent stem cells that are found in a number of human tissues. These cells can be experimentally differentiated along mesodermal lineage, which include cells of tissues like adipose, bone, and skeletal muscle²⁰⁰. MSCs serve as a primary origin for adipocyte generation, playing a central role in the adipogenesis process in adult animals²⁰¹. In humans, the development of adipose tissue starts in mid-gestation, and the shift in MSC differentiation during that period could have a profound impact on fetal tissue development^{202–204}. Hence, this model has

been chosen as a relevant model for testing developmental effects on adipose tissue in humans.

Zebrafish (*Danio rerio*) is a commonly used experimental model for studying metabolic programming effects²⁰⁵. Notably, over 70% of the human genome has orthologs in zebrafish, and many physiological processes are shared between fish and mammals, underscoring the model's physiological relevance²⁰⁶. Zebrafish embryos are small, develop rapidly, and transparent making them suitable for an array of toxicological studies including chemical-induced toxicity assessments²⁰⁷. Additionally, zebrafish possess functional conservation in adipose biology making it a suitable model for studying metabolic disorders^{208,209 208–210}.

Aims of the Thesis

This doctoral project aimed at examining and comparing the effects of human-relevant EDC mixtures, established from pregnancy cohort data, and individual EDCs in human-relevant concentrations and mixing ratios on the developing metabolic system, linking molecular (transcriptional and epigenetic) and physiological readouts in a cell model and in zebrafish. Additionally, the aim was to address if additivity models could be used for more accurate EDC mixture hazard and risk assessment. The aims were separated into four studies with the following objectives:

Study I: Identify early transcriptional changes induced by mixture G in MSCs and link them to differentiation to adipocytes

Study II: Identify epigenetic changes induced by mixture G1 in MSCs and link them to differentiation to adipocytes

Study III: Identify metabolic effects induced by mixture G1 as well as its single components in MSCs and zebrafish

Study IV: Assess whether additivity of single component effects would accurately predict mixture G1 effect on the metabolic endpoints

Methods

Endocrine disrupting chemicals

To identify which chemical mixtures are relevant for human real-life exposure and fetal growth, two EDC mixtures were identified within the project EDC MixRisk (<http://edcmixrisk.ki.se/>) and established using data from the SELMA study. The full procedures are described in ^{122,123,211} and **Paper II**. Briefly, for both mixtures, in the urine and serum of week 10 pregnant SELMA women the chemicals of concern were measured and those associated with lower birth weight in their offspring were identified based on weighted quantile sum (WQS) regression, a method for ascertaining empirical weights for a weighted sum of quantile concentrations linked to health outcomes ²¹². As described by Bornehag et al. (2019), urine compounds were converted to serum concentrations using estimated daily intake, while serum-measured chemicals were employed as-is ¹²³. Finally, the mixture proportions of these chemicals were determined using SELMA mothers' serum geometric mean levels, thus 1 X SELMA concentration reflects their mean exposure at week 10 of pregnancy.

Firstly, 20 analytes that included ten phthalate metabolites, two phenols, and eight PFASs were measured in the samples collected from the pregnant SELMA women. Using the WQS regression a subset of measured analytes associated with lower birth weight was identified ¹²³. This subset of analytes was called mixture G and consisted of nine chemicals (Table 1).

Secondly, the EDC MixRisk extended their chemical analyses and reanalysed the urine and serum samples from the biobank of the same SELMA women. The new analysis included 41 analytes (compared to previous 20). Several classes of chemicals were additionally measured such as polycyclic aromatic hydrocarbons (PAHS), organochlorine pesticides, and PCBs. The same analysis as for mixture G was conducted, and a subset of analytes negatively associated with birth weight was identified, called mixture G1. Mixture G1 consisted of 14 chemicals (Table 1).

A 1 molar (1 M) solution of both mixtures in DMSO (Sigma-Aldrich, Saint Louis, MO, USA) was prepared for experimental use by the EDC-MixRisk partner at the Division of Occupational and Environmental Medicine at Lund

University. There was 1 batch of mixture G that was used in **Paper I**. There were 3 batches of mixture G1: batch 1 was used in **Paper I**, batch 2 in **Paper II**, and batch 3 in **Paper II** validation experiments with nine genes and in **Paper III**.

Table 1. Individual chemicals and their concentrations within mixture G and mixture G1. The concentrations (Conc.) are in relation to 1X SELMA exposure.

Chemical Class	Analytes measured	Analytes name	Conc. Mix G1 (nM)	Conc. Mix G (nM)
Phthalates	MEP	Monoethyl phthalate	29,7	27,6
	MBP	Monobutyl phthalate	26,4	23,1
	MBzP	Monobenzyl phthalate	5,3	10,7
	MEHP	Mono-(2-ethylhexyl) phthalate	19,0	14,6
	MINP	Di-isononyl phthalate	-	21,1
Plasticizer	MINCH	2-4-Methyl-7-oxyooctyl-oxycarbonyl-cyclohexane carboxylic acid	0,5	-
	DPP	Diphenylphosphate	0,5	-
Antibacterial	Triclosan		0,3	3,1
PAH	2OHPH	2-Hydroxyphenanthrene	1,3	-
Pesticide	3-PBA	3-Phenoxybenzoic acid	0,1	-
PFAS	PFOA	Perfluorooctanoic acid	3,6	3,4
	PFOS	Perfluorooctane sulfonate	9,7	10,5
	PFHxS	Perfluorohexanesulphonic acid	3,0	3,3
Organo-chlorine pesticide	HCB	Hexachlorobenzene	0,1	-
	p,p' DDE	Dichlorodiphenyltrichloroethane	0,5	-

Mesenchymal stem cells

Cell culture

Human mesenchymal stem cells (MSCs) were used as a cell model for adipogenesis. For **Paper I** and **Paper II**, bone marrow-derived human MSCs (bmMSCs) from two donors were generously provided by Dr. Katarina Leblanc from Karolinska Institutet, Stockholm, Sweden. bmMSCs were cultured in growth media comprising DMEM supplemented with 10% Fetal Bovine Serum (FBS), 1% penicillin-streptomycin, and 2% l-glutamine at 37 °C in an incubator with 5% (v/v) CO₂. In **Paper I**, human iPSC-MSCs were also used. These were obtained and cultured from neural crest stem cells derived from iPSCs according to the protocol described by ²¹³ and previously used in ²¹⁴.

The human iPSC-MSCs were cultured in DMEM/F12 (Gibco®) medium with 10% Fetal Bovine Serum (FBS), 1% penicillin-streptomycin, and 2% l-glutamine at 37 °C in an incubator with 5% (v/v) CO₂. These bmMSCs and iPSC-MSCs were used in experiments in cell passages 5 - 8.

For **Paper II** validation of the nine genes, for **Paper III**, and **Paper IV** bmMSCs were purchased from PromoCell in passage 2. These bmMSCs were cultured in MSC growth medium 2 (PromoCell) with 10% Supplement Mix (PromoCell) according to manufacturer's instructions. These bmMSCs were used in experiments in cell passage 5.

For **Papers I and II**, bmMSCs and iPSC-MSCs were seeded in 96-well plates with black-walled μ CLEAR bottoms (Greiner Bio One, Kremsmünster, Austria) or 6-well plates. Treatment media (TM) consisted of DMEM (Gibco, Waltham, MA, USA) supplemented with 10% charcoal stripped FBS (DCC, Gibco, Waltham, MA, USA), 1% penicillin-streptomycin (Gibco, Waltham, MA, USA), and 2% l-glutamine (Gibco, Waltham, MA, USA).

For **Paper I**, exposure to mixture G was carried out using exposure media, consisting of TM supplemented with mixture G in DMSO at concentrations of 10 nM (0.1X), 100 nM (1X), 1 μ M (10X), 10 μ M (100X), and 100 μ M (1000X). The exposure continued for 14 - 21 days for lipid droplet accumulation assay and for 48 hour for transcriptional assay.

For **Paper II**, exposure to mixture G1 was carried out using exposure media, consisting of TM supplemented with mixture G1 in DMSO at concentrations of 100 nM (1X), 1 μ M (10X), 10 μ M (100X), and 100 μ M (1000X). The exposure continued for 21 days.

For **Paper II** validation, **Paper III**, and **Paper IV**, bmMSCs were seeded in CellView black-walled 96 well plates (Greiner Bio One, Kremsmünster, Austria). Exposure medium with mixture G1 consisted of TM supplemented with mixture G1 in DMSO at concentrations of 100 nM (1X), 1 μ M (10X), 10 μ M (100X), and 100 μ M (1000X); or mixture G1 individual chemicals in DMSO at concentrations 1X, 10X, 100X, and 1000X (1 X concentration of each chemical is indicated in Table 1). The exposure continued for 21 days. For all experiments, the exposure media was changed every second or third day.

Lipid droplet accumulation

For **Papers I, II, III**, and **IV** the lipid droplet accumulation in MSCs and in iPSC-MSCs was quantified by fluorescent microscopy, as described previously [114] as well as in corresponding papers. Briefly, cells in the 96 well plates were stained with 10 μ g/mL BODIPY 493/503 (Gibco®, Waltham,

MA, USA) and 2 $\mu\text{g/mL}$ of Hoechst 33,342 (Gibco®, Waltham, MA, USA). The images were taken in FITC and DAPI channels at 10X magnification using the Image Xpress Micro High-Content Analysis System. For **Papers I and II**, images were analyzed using the MetaXpress High-Content Image Acquisition and Analysis software (Molecular Devices, Sunnyvale, CA, USA), and for **Papers III and IV** using the MetaXpress Image Analysis Software (version MX6.6.3) (Molecular Devices, Sunnyvale, CA, USA).

Lipid droplets were quantified by measuring the integrated granule intensity, and this value was normalized to nuclei count. For each treatment condition, the lipid accumulation per cell is presented as a ratio compared to the lipid accumulation per cell of the DMSO control on the same plate. **Papers I, II, and III** the significance was determined using Kruskal-Wallis rank sum test followed by Dunns post hoc test, with adjustments made using the Benjamini-Hochberg method, when compared to the DMSO control ($p < 0.05$). Additionally, for **Paper III** Spearman's rank correlation test was used to determine if there was a monotonic dose response.

RNA extraction and RNAseq analysis

For **Paper I** the total RNA was isolated with the RNeasy Micro kit (Qiagen) according to the manufacturer's instructions. RNA sequencing (RNAseq) was performed with the Illumina HiSeq 2000 platform. RNA-seq quantification was performed directly from the reads using Salmon 6.1, applying the hg38 Refseq annotation. Only genes with at least 20 reads in at least 2 samples were included, and small (<200nt) genes, ribosomal RNA genes, and fusion genes were excluded. Differential expression analysis was performed on the estimated counts after TMM normalization with edgeR 216. The fold change patterns for the core cluster were plotted to show the dose-response patterns. Gene Ontology (GO) over-representation analyses were performed with topGO R package version 2.52.0 on the differentially expressed genes (DEGs).

DNA extraction and DNA methylation analysis

For **Paper II** the bmMSCs that were grown in 6-well plates underwent DNA extraction using the AllPrep DNA/RNA micro kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. In summary, cultured cells were lysed and the resultant lysate was preserved at -80°C . Upon thawing, 250 μL of RLT buffer was added, and the lysate was then transferred to a spin column, and DNA isolation steps were followed as per manufacturer's instructions. The elution of DNA was done using an EB buffer.

Genome-Wide DNA methylation analysis

Genome-wide DNA methylation analysis was used in **Paper II** to investigate epigenetic effects of mixture G1 in bmMSCs. The analysis on the Illumina EPIC Array was executed by the Bioinformatics and Expression Analysis (BEA) core facility at Karolinska Institutet, Stockholm, Sweden. Briefly, the DNA was normalized and preprocessed by BEA using the Chip Analysis Methylation Pipeline (ChAMP), which includes quality control checks, batch corrections for both the slide and the array variations. The allocation of Differentially Methylated Positions (DMPs) was established relative to the reference genome annotation for Illumina's EPIC methylation array (Homo sapiens genome assembly GRCh37, hg19). The extent of methylation at individual CpG sites was represented as β value and ranges from 0 to 1, with 0 signifying no methylation and 1 representing 100 % methylation of all analysed strands at the specific position. The Delta Beta value, denoted as $\Delta\beta$, shows the difference between the average β values of the treatment (mixture G1) and the control samples. In essence, a negative $\Delta\beta$ value indicates hypomethylation, less methylation in the treatment sample compared to the control, while a positive $\Delta\beta$ value indicates hypermethylation, more methylation in the treatment sample compared to the control.

To identify statistical differences, multiple testing correction was done with the false discovery rate (FDR), enabling the identification of DMPs based on variations in DNA methylation averages between the treatment groups (mixture G1 1X, 10X, and 1000X) and the control (DMSO) group ($\Delta\beta \geq 0.1$ and $\text{FDR} < 0.05$). A principal component analysis (PCA) was conducted to assess sample clustering in R, version 4.0.5. For the distribution of DMPs and the creation of volcano plots, the base genome annotation for Illumina's EPIC methylation array (Homo sapiens genome assembly GRCh37, hg19) and the ggplot2 package were used respectively.

Regional methylation changes, Differentially Methylated Regions (DMRs), were identified using the ChAMP package²¹⁵. In brief, default settings were applied with criteria for DMR being seven or more successive DMPs within a 300-base pair genomic span with Family Wise Error Rate < 0.2 . Genes stemming from the DMR analysis, with an adjusted p-value threshold of 0.05, underwent further testing for the enrichment of gene ontology categories using the GO.db annotation package, according to default settings. Both DMR and GO analyses were conducted on pooled data from both donors, and to mitigate any sex bias, sex chromosomes were excluded.

Pyrosequencing

For **Paper II** validation experiments, pyrosequencing analysis was conducted using the PyroMark Q24 ID system (Qiagen, Hilden, Germany), as per the manufacturer's instructions. Briefly, the polymerase chain reaction (PCR) was performed using PyroMark PCR Kit (200) (Qiagen Cat No. 978703, Hilden, Germany) and a T100 Thermal Cycler (Bio-Rad, Hercules, California). The PCR products (10 μ l) were mixed with binding buffer (Qiagen, Hilden, Germany), Streptavidin Sepharose beads (Cytiva), and Milli-Q water. A sequencing primer solution (25 μ l), diluted to 0.3 μ M in Annealing buffer (Qiagen, Hilden, Germany), was loaded on a pyrosequencing 24-well plate. The PCR product was then added to the pyrosequencing plate and loaded into the PyroMark Q24 ID machine. The substrate, enzyme, and nucleotides from the PyroMark Gold Q24 Reagents (Qiagen, Hilden, Germany) were added into the reagent cartridge according to the plate-specific layout determined by the PyroMark Q24 software (version 5.0). The PCR and pyrosequencing conditions were optimized for all CpG assays and are shown in Table 2.

Table 2: Primer sequences and annealing temperature for PCR for pyrosequencing assays. Where F stands for forward primer, R stands for biotinylated reverse primer, and S for sequencing primer.

Primer	Sequence	Annealing T (°C)
F ACSM3	5'-TGTAGGAAATTTGGAAAATTATAGGAAGTA-3'	58°C
R ACSM3	5'-ACCCATACTACTTTTAAATCCAAACTT-3'	58°C
S ACSM3	5'-GGTTTAGAGGGGTTTT-3'	-
F RPL28	5'-TGGGAGATTTTTGAGGGTAGATAG-3'	58°C
R RPL28	5'-ATTATTTTCCCCTCACTCTCAT-3'	58°C
S RPL28	5'-TTAGGTAGTAGAGATTAGTT-3'	-
F BCLAF1	5'-AGGGAATTTAGTGGATTAAGAAAGT-3'	58°C
R BCLAF1	5'-ACTACATTAATAATCCAACCTCCTAATAT-3'	58°C
S BCLAF1	5'-ATTGTTTTTAATTTTGATTGTGT-3'	-
F MAF	5'-GAGGGTGTGTATTTAATTAATTTGTATT-3'	60°C
R MAF	5'-TCCCCTCCCTAAACAAATCC-3'	60°C
S MAF	5'-GAGAAAATTAGGGGGT-3'	-
F MYOF	5'-GAGAAGGTTTTTTGGTTTAAATATGTGA-3'	60°C
R MYOF	5'-CCATCCAAATACCCTCAACTCTTAA-3'	60°C
S MYOF	5'-GTTTTTTTTGGTTTTAGAATT-3'	-
F PGM1	5'-AGTGAAAAAGGAATTTTATTGGTTTATGT-3'	58°C
R PGM1	5'-AACTTAAACTCTCCTCAATTCTAATACT_3'	58°C
S PGM1	5'-GTTATTGAAAAGTTTAAGAGAAT-3'	-
F HCFC1	5'-AGTTTGTGTAGGAGGAGTTG-3'	60°C

R HCFC1	5'-TAACCTCAAACCCTACCCTACCTA_3'	60°C
S HCFC1	5'-AGGTATAGTTTTATTAGTTTTGT-3'	80°C
F PTPRJ	5'-GTGTTAATGATGGTGGTGATGGTAT-3'	56°C
R PTPRJ	5'-ACCACAACATAAAACCCTATCTCT-3'	56°C
S PTPRJ	5'-GAAAATAAGTAGATGAGTAGG-3'	-
F HOXA5	5'-TGTTATTTAAGAGAGGTAGGGATATTGT-3'	60°C
R HOXA5	5'-TTTCCCCCTTTTACAACCCCTCTTATT-3'	60°C
S HOXA5	5'-GAGAGGTAGGGATATTGTTT-3'	-

Zebrafish

Zebrafish husbandry

For **Papers III** and **IV**, the adult wild-type zebrafish of the AB strain were housed within the SciLifeLab zebrafish facilities at Uppsala University (<http://www.scilifelab.se/facilities/zebrafish/>) and were maintained following their guidelines. Zebrafish embryos were cultured in a light-free incubator set at 29.5 °C. Embryos were kept in the incubator at 29.5 °C in zebrafish embryo medium (ZEM), composed of 0.994 mM magnesium (II) sulfate heptahydrate, 0.151 mM monopotassium phosphate, 0.042 mM disodium phosphate, 0.986 mM calcium chloride dihydrate, 0.503 mM potassium chloride, and 15 mM sodium chloride. At 24 hours post fertilization (hpf), half of the ZEM medium was exchanged and the dead embryos were carefully removed.

Zebrafish larvae were subjected to exposure to mixture G1 as well as its individual compounds upon hatching at 48 hpf. The concentrations utilized for mixture G1 were set at 0.01X (1 nM), 0.1X (10 nM), 1X (100 nM), 10X (1 µM), and 100X (10 µM) the geometric mean of the serum levels determined in the SELMA mothers. Similarly, the mixture G1 individual chemicals were used in DMSO at concentrations 1X, 10X, 100X, and 1000X (1 X concentration of each chemical is indicated in Table 1). The zebrafish larvae underwent exposure to these compounds for a duration of 48 hours, from 48 hpf until 96 hpf.

Respirometry assay

The oxygen consumption rate was assessed using the Microplate system (Loligo®). Briefly, all measurements and calibrations were conducted at 28.5 °C in the absence of light. Calibration of the Loligo® microplate system involved purging ZEM with air for 30 minutes for the 100% oxygenated sample, while ZEM contained 0.159 M sodium sulfite for the 0% oxygenated sample. The plate was thoroughly rinsed with deionised water between calibrations as well as respirometry measurements.

For each well, 40 μ l of ZEM and one zebrafish larva were introduced, with the well volume subsequently raised to 80 μ l using ZEM. Exposed zebrafish larvae were added to the plate in a randomized manner. Each concentration had 5 to 7 biological replicates, encompassing 9 to 16 technical replicates each. Microseal 'B' PCR Plate Sealing Film adhesive optical (bio-rad #MSB1001) was used to seal the plate. Measurements persisted until all wells exhibited oxygenation levels below 70% or reached this threshold. Following the measurements, tricaine was employed for euthanizing the zebrafish larvae.

The computation of the oxygen consumption rate was conducted using MicroResp™ version 1 (Loligo systems). Only data from wells displaying linear regressions with an R^2 value exceeding 0.95 were considered. The average metabolic rate, denoted as MO2 (pmol/min), of the DMSO group served as the basis for calculating the fold change per biological replicate. A Kruskal-Wallis test was used, followed by Dunn's test with p-values adjusted using the Benjamini-Hochberg method. To test for a monotonic dose-response, a Spearman's rank correlation test was used.

Statistical modeling

For **Paper IV** computation modeling analysis, we used a Gompertz function for single chemicals and for the mixture to estimate the additivity model. The Gompertz function ranges between 0 and 1 and takes a sigmoid shape. To facilitate comparisons, the results were adjusted relative to the control group's averages. When assessing individual EDCs, the Gompertz function was applied with specific constraints, ensuring that the model accurately estimated their effects. For mixtures of these EDCs, a more intricate version of the Gompertz function was used. A test of additivity was performed to determine whether the mixture's effects aligned with what would be expected based on the individual EDCs responses.

Results

Paper I and II: EDC mixtures induced lipid droplet accumulation in MSCs

To explore the impact of EDC mixtures on the adipogenic potential of MSCs, the bmMSCs, obtained from two donors were exposed to five different concentrations of mixture G or G1 for a duration of up to 21 days. Adipogenesis was assessed by quantifying lipid droplet accumulation through high-content imaging.

Both mixtures significantly increased lipid droplet accumulation in bmMSCs in dose dependent manner starting from human-relevant concentrations of 1X SELMA (**Paper I** and **Paper II**).

Paper I: Early transcriptional changes induced by mixture G in MSCs

To investigate if exposure to mixture G can change gene expression in MSCs that could underlie the earlier observed adipogenic effects, RNA-Seq was conducted on bmMSCs and iPSCs-derived MSCs. Exposure to five concentrations of mixture G (0.1X, 1X, 10X, 100X, 1000X) for 48 hours in two distinct MSC models led to significant transcriptional changes. A set of 1604 DEGs was identified across both cell types that exhibit the same dose-response pattern to mixture G exposure. The core DEGs showed slightly more down-regulated ($n = 807$) than upregulated ($n = 797$) genes, exhibiting a consistent pattern across concentrations from 1X to 1000X of mixture G. These findings demonstrate that mixture G altered the expression of 1604 genes across diverse MSC lines originating from different sources, reflecting concentrations relevant to those found in SELMA women.

GO over-representation analyses on the set of 1604 DEGs showed that the upregulated genes were enriched in 130 GO terms, while downregulated genes were enriched in 104 GO terms. The top eight enriched GO terms for upregulated DEGs were primarily associated with cell division. Conversely, the downregulated DEGs were significantly enriched in GO terms related to cell

adhesion and modulation of the extracellular matrix. When compared to genes induced during adipogenesis and osteogenesis, mixture G-upregulated genes overlapped with downregulated genes in both pathways.

The overlap of mixture G-induced DEGs with genes regulated by relevant hormonal pathways was investigated. Mixture G DEGs significantly overlapped with corticoid- and estrogen-regulated genes, with implications for adipogenesis and osteogenesis. Notably, glucocorticoid disruption's effects on these pathways are less explored in the context of EDCs. Some of the mixture G-induced DEGs enriched with corticoid-regulated genes were shown to be involved in osteogenesis. In conclusion, mixture G was shown to affect gene expression in MSCs, hinting at the early transcriptional role of mixture G suppressing osteogenesis rather than inducing adipogenesis.

Paper II: Epigenetic changes linked to adipocyte differentiation by mixture G1 in MSCs

To investigate if exposure to mixture G1 can induce epigenetic changes in bmMSCs that could underlie the earlier observed adipogenic effects, Illumina EPIC analysis was conducted on bmMSCs. Exposure to mixture G1 induced significant alterations in DNA methylation patterns in bmMSCs, at all three tested concentrations of mixture G1: 1X, 10X, and 1000X. Principal component analysis revealed possible non-monotonic effects on DNA methylation, where similar pattern was observed at the lowest and highest concentrations. The largest number of DMPs were detected in cells exposed to 1X mixture G1, followed by 1000X, and 10X concentrations. Most (%) DMPs were hypomethylated. Only eight DMPs were shared across the three concentrations. While between 1X and 1000X mixture G, there were 150 shared DMPs, primarily associated with gene regions important for osteogenesis, adipogenesis, thermogenesis, fatty acid metabolism, obesity, and glucose metabolism. The distribution of these DMPs showed enrichment in intergenic regions and open sea areas, while depletion was observed in CpG island and exon regions. This comprehensive analysis underscores the complex and concentration-dependent epigenetic effects of mixture G1 on bmMSCs, revealing potential mechanistic links to metabolic programming.

Six hypomethylated DMRs were found and were associated with genes like *HOXA11AS/HOXA11*, *PM20D1*, *PANCR*, *HOXA5*, *RP11-134D3.2*, and *RPL28* upon mixture G1 1X exposure. Notably, *HOXA11AS*, *HOXA5*, and *PM20D1* are known to influence adipocyte differentiation and metabolism regulation, while *RPL28*'s expression has shown an inverse correlation with

BMI. No DMRs emerged from mixture G1 10X exposure, while mixture G1 1000X induced hypomethylated DMRs in *HOXA11AS/HOXA11* and *PANCR*.

GO enrichment analysis of genes in the mixture G1 1X-induced DMRs revealed significant enrichment in three major GO categories: biological process (BP), cellular component (CC), and molecular function (MF). Specifically, mixture G1 1X altered methylation of genes related to metabolic processes involving nucleic acids, gene expression regulation, protein targeting to the endoplasmic reticulum, endocrine system development, and cellular macromolecule metabolic processes.

From the above genome-wide DNA methylation analysis, nine individual genes were chosen for validation of DNA methylation changes using pyrosequencing targeted analysis. In order to achieve that, bmMSCs from two new donors were employed in a new set of triplicate experiments using a slightly different cell culture protocol, as earlier described in the methods section. These were the genes that in the Illumina EPIC analysis had the highest DNA methylation difference in comparison to the control as well as some of the DMR genes. The selected genes for methylation analysis were *MAF*, *MYOF*, *ACSM3*, *HCFC1*, *HOXA5*, *PGM1*, *RPL28*, *BCLAF1*, and *PTPRJ*. In the pyrosequencing analysis there were no observed statistically significant changes in DNA methylation compared to the control group of any of the assessed CpGs sites after exposure to mixture G1 (Supplementary Figure 1). Notably, certain CpG sites within distinct genes exhibited statistically significant variations between donors.

Paper III: Mixture G1 and its components induced metabolic effects in MSCs and zebrafish

As shown in **Paper III**, exposure to mixture G1 induced a significant dose-dependent increase in lipid accumulation, particularly at 1000x SELMA concentrations, with a 2.9-fold increase. Hence, next the effects on lipid droplet accumulation of each of the chemicals composing mixture G1 in their respective concentrations were investigated. Among the individual compounds, only MEHP showed a significant and monotonic increase in lipid accumulation at 1000X SELMA concentration. Other single chemicals did not significantly affect lipid droplet accumulation in bmMSCs. This suggests that the combined effect of mixture G1 is greater than any effect produced by its individual component. MEHP may be a driver in the observed mixture G1 effect on lipid droplet accumulation in bmMSCs.

To investigate the effect of developmental exposure to mixture G1 on the whole organism metabolic rate, respirometry experiments were conducted on zebrafish larvae. Exposure to mixture G1 resulted in a significant dose-dependent increase in zebrafish larvae's oxygen consumption. Statistically significant increases were observed at 10X (1.1-fold) and 100X (1.23-fold) concentrations (**Paper III**). Notably, individual compounds MBzP and PFOS also significantly affected oxygen consumption. PFOS 100X developmental exposure led to a similar increase as mixture G1, while MBzP induced a decrease in oxygen consumption at 0.1X concentration. Other compounds did not elicit significant changes in oxygen consumption. Similarly, to the adipogenesis assay results, the combined effect of mixture G1 was greater than any effect produced by its individual component. However, the individual chemicals that induced significant effects were different from the lipid droplet accumulation assay, highlighting the complex metabolic effects of mixture G1 and its components on different metabolic endpoints.

Paper IV: Additivity model predicted mixture G1 effects on metabolic endpoints in MSCs and zebrafish

Next, we examined the applicability of the additivity model in predicting the outcomes of mixture G1 using the experimental data of its individual chemicals in both the adipogenesis and respirometry assays. This mixture reflects "real-world" human exposure comprising compounds from various classes that likely exhibit distinct modes of action (MoA).

The additivity model adequately predicted dose-response combined effect of a complex and human-relevant mixture G1 using the experimental data from individual compounds for both MSCs adipogenesis and zebrafish respirometry. Interestingly, the estimated dose-response curve appeared to be lower than the prediction based on additivity for both assays, implying an antagonistic influence. The test for additivity was statistically rejected for adipogenesis assay, indicating a significant departure from the additivity model. Nonetheless, the estimated dose-response curve still adequately predicted the mixture effect. The test for additivity was statistically accepted for respirometry assay.

Discussion

We are daily exposed to a large number of endocrine-disrupting chemicals that might not pose a risk individually, as they occur below their regulatory thresholds ²¹⁷. However, accumulating evidence shows that the cocktails of chemicals in our environment might have significant effects on the health of humans and wildlife ^{109,110,116,120,217}. Additionally, accumulating evidence shows that there are sensitive windows of exposure rendering individuals more vulnerable to environmental stressors where hormonal signaling plays a central role, implicating a particular concern for developmental exposure to EDCs ^{68,74,132,176,218,219}. We are currently facing the challenge of: 1. limited knowledge about the developmental exposure to mixtures of EDCs and links to adverse health outcomes ^{64,220} 2. lack of appropriate methodology to accurately assess the hazard and risk of existing chemicals in present mixtures and new chemicals before they reach the market, considering that they will be added to already present chemical mixtures ^{221,222}.

This doctoral project aimed to address these limitations by firstly, experimentally examining and comparing the effects of an epidemiologically identified EDC mixture and its individual components in human-relevant concentrations on the developing metabolic system; and secondly, by addressing if additivity models could be used for more accurate assessment of real-life chemical mixtures.

Effects of mixtures G and G1 on hMSCs adipogenesis

Both mixtures of EDCs were associated with lower birth weight in Swedish children of the SELMA cohort, implying that they could interfere with growth and metabolism during gestational development ¹²². Furthermore, Mentor et al (2020) have demonstrated that developmental exposure to mixture G induced adipogenesis in zebrafish ²²³. LBW infants tend to have a higher accumulation of visceral adipose tissue, which is associated with an increased risk of metabolic disorders such as obesity later in life ^{224,225}. This heightened risk is potentially attributed to adipose tissue maldevelopment, involving alterations in body composition and adipocyte number/size, resembling the pattern

observed in visceral obesity.^{33,36,40,44,45,49,54,219,226} Therefore, we aimed to determine the effects of both EDC mixtures on adipogenesis, the process of fat cell differentiation and accumulation, a crucial determinant of adipose tissue development, which in turn affects metabolic health. We found that both EDC mixtures, mixture G and mixture G1, significantly induced lipid droplet accumulation in human bone marrow derived MSCs (**Paper I** and **II**). To the best of our knowledge, this is the first study that showed that two EDC mixtures reflecting real-life human exposure as well as associated with lower birth weight, induced adipogenesis in human bmMSCs.

Previous studies have shown that almost all EDCs that were part of mixture G and G1, induced adipogenesis or lipid droplet accumulation when tested individually or in small combinations with other EDCs at concentrations such as 1 μ M of DDE, 1 μ M of MEHP, 100 μ M of MBzP, 50 μ M of MINCH, 40 μ M of PFOA, 80 μ M of PFHxS, and 200 μ M of PFOS^{139,145,146,227,227,228,228–231,231–236}. Studies using triclosan, however, showed contradicting results where anti-adipogenic effects were reported in *in vitro* studies using 0.156 to 2.5 μ M in hMSCs as well as 50 μ M in 3T3-L1 adipocytes while 0.35 mM triclosan exposure in mice in combination with high-fat diet resulted in larger abdominal white adipose tissue. The majority of previous studies focusing on individual EDCs present in the mixtures have detected significant effects at μ M concentrations. In contrast, we have shown that epidemiologically identified heterogeneous mixtures induced significant effects already at 100 nM, where the concentrations of the individual components were significantly lower. This suggests that the combined mixture possesses a greater potency compared to the individual chemicals, as indicated by the results.

MSCs play a pivotal role in adipose tissue development starting from the gestational period^{203,204,237–239}. Adipocytes as well as the adipose tissue are responsible for an array of significant functions such as managing body weight by releasing numerous proteins like leptin, resistin, adiponectin, and apelin, synthesis of estrogen, storage of steroid hormone, and secretion of adipokines in immunological responses^{240–243}. Our results show that human-relevant mixtures of EDCs can induce adipogenesis in MSCs, which could potentially contribute to obesity. Increased adipogenesis is also a characteristic of an obese phenotype, and obesity is further a risk factor associated with low birth weight^{224,240,242}. In summary, an increase in adipogenesis could lead to excessive adipose tissue, that in turn may result in modified body composition and heightened visceral fat accumulation, thus mirroring the mechanism observed in individuals with metabolic syndrome, suggesting a potential connection to the susceptibility of adult-onset diseases in infants born with low birth weight^{9,10,16,72,224,240,244,245}. Hence, the finding of increased lipid droplet accumulation

upon EDC mixtures exposure could have broader implications for understanding the mechanisms underlying metabolic programming and the potential role of EDCs in contributing to metabolic disorders.

Early effects of exposure to mixture G on gene expression in MSCs

Adipogenesis is a highly regulated process controlled by transcriptional factors and epigenetic processes ²⁴⁶. To understand potential mechanisms by which EDCs induce lipid droplet accumulation, we investigated early transcriptional changes upon mixture G exposure in two MSCs models, hMSCs from bone marrow as well as two iPSCs derived MSCs cell lines (**Paper I**). Two different cell modes were used in order to enhance the robustness of our findings and reduce the potential biases associated with using a specific cell model. Bone marrow derived MSCs are adult derived stem cells and are commonly employed MSCs model, iPSCs derived MSCs were generated by reprogramming adult somatic cells back to a pluripotent state followed by induced differentiation into MSCs ^{247,248}. We showed that the exposure to mixture G induced significant transcriptional modifications in genes shared between both MSC lines within 48 hours of exposure. These genes followed the same concentration dependent patterns in a dose-dependent manner. While we did not see induction of adipogenesis related pathways, some of the downregulated genes enriched osteogenesis related pathways. The upregulated genes were related to cell proliferation. Finally, we showed that DEGs significantly overlapped with corticoid- and estrogen-regulated genes.

Most of the individual EDCs that are part of mixture G have previously been shown to increase adipogenesis via transcriptional regulation of PPAR γ signaling, a master regulator of adipogenesis. However, our results indicate that the mechanism of early effects of mixture G may be different to the ones reported earlier for single EDCs.

Mixture G increased expression of genes linked to cell proliferation. A study conducted by Marquez et al (2017) showed that cell cycle related gene expression was downregulated at 48 h post adipogenic induction ²³⁷. However, upon treatment of MSCs with basic fibroblast growth factor, an exogenous growth factor known to enhance expression of cell cycle genes, a promotion of adipogenesis during the first 48 hours was observed, in contrast to a significant inhibition of adipogenesis during adipogenic commitment phase, days 3–6. Therefore, this study could help speculate that the mixture G effect leads to an increased pool of cells that could further be committed to adipogenesis. The observed differences in gene expression between the 48-hour exposure and

the cellular changes seen at 21-day exposure underscore the importance of considering both short-term and long-term effects of EDCs. Given the intricate nature of EDC actions, it is clear that more research is needed to elucidate the mechanisms underlying these effects. This includes exploring the specific EDC components within the mixture, their interactions with cellular pathways, and potential epigenetic changes that may be involved.

The significant overlap of the mixture G upregulated genes with corticoid- and estrogen-regulated genes indicates that this EDC mixture may be mimicking or interfering with those hormone signaling pathways. Although estrogen signaling is widely recognized as a prime target for EDCs, less is known about the impact of EDCs on glucocorticoid signaling. The adrenal glands produce steroid hormones known as glucocorticoids (GCs). They play essential roles in behavior, immunity, and metabolism by binding to corticoid receptors in various target cells throughout the body, including the adipocytes²⁴⁹. Furthermore, the effects of GCs on adipose tissue are multifaceted and depend on a range of physiological or pathophysiological conditions, as well as the specific fat depots involved, either enhancing or diminishing lipid storage within the specific adipose tissues²⁵⁰. Additionally, there is also a connection between excess GCs and the development of metabolic disease such as seen in Cushing's syndrome that is currently not well understood²⁵¹. Furthermore, it is well documented that excess GC influences MSCs' cell fate decisions favoring adipogenic differentiation^{252–254}. Two *in vivo* studies investigated the exact role of GR in adipose tissue development and have reported that while GR and GC can promote adipocyte differentiation, they are not required for the development of brown and white adipose tissues^{255,256}. However, there is limited knowledge about the initial events triggered by GCs in MSCs differentiation²⁵⁰. When it comes to the concentrations of GCs and their effect of MSCs differentiation fate, physiological GC concentrations were found to stimulate osteoblast proliferation and osteogenic differentiation while, excessive or pharmacological concentrations were found to induce osteoblast and osteocyte apoptosis, as well as reduced proliferation, and inhibition of osteoprogenitor cell differentiation²⁵⁷. Hence, our finding of overlap between the genes affected by mixture G and those regulated by GCs could indicate that prolonged exposure to the EDC mixture could lead to significant changes in MSCs fate, favoring adipocyte differentiation, as seen in our previous study of induced lipid droplet accumulation after 21 days of exposure to mixture G1.

Effects of mixture G1 exposure on DNA methylation in MSCs

To further understand the potential mechanisms by which mixture G1 increases lipid droplet accumulation, we investigated DNA methylation changes induced by mixture G1 after 21 days of exposure (**Paper II**). We showed that mixture G1 exposure at concentrations relevant to those found in SELMA mothers induced DNA methylation changes, as measured by Illumina EPIC bead array, in genes important for metabolic functions.

Mixture G1 exhibited differential effects at distinct concentrations, implying a potential non-monotonic response. However, this effect was not observed in lipid droplet accumulation assay. Hence, we cannot conclusively determine non-monotonic effects on DNA methylation in hMSCs. Nonetheless, in the context of EDCs, a non-monotonic dose-response relationship presents a significant challenge in toxicological and risk assessments. Non-monotonic dose response can result due to several molecular mechanisms such as opposing effects induced by multiple receptors differing by their affinity or dose-dependent metabolism modulation²⁵⁸.

This type of response implies that the biological effects of EDCs do not consistently increase or decrease with increasing dose, and instead, they exhibit complex patterns of response. Traditional toxicological assessments often assume a linear dose-response relationship, which means that higher doses are expected to have more substantial effects. When dealing with non-monotonic responses, relying solely on linear models may underestimate the potential risks associated with EDC exposure. Therefore, these complex responses need to be carefully considered in risk assessments. Additionally, we showed that mixture G1 1X SELMA induced an equally potent effect as mixture G1 1000 X SELMA. Considering that EDCs are often encountered at low doses in real-world environmental exposures, non-monotonic responses highlight the importance of studying these chemicals across a broad range of concentrations. Furthermore, in order to better understand potential health risks and EDCs effects, environmentally relevant concentrations must be considered.

We have shown that six hypomethylated regions (DMRs) were associated with genes linked to adipogenesis, metabolic functions, birth weight, and obesity. One DMR significantly overlapped with the *PM20D1* gene promoter, a regulator of mitochondrial respiration and thermogenesis in adipose tissues. Additionally, DMRs overlapping with *HOXA11*, *HOXA5*, *RPL28*, and *PANCR* genes suggest implications for MSC differentiation and adipose tissue function. Gene ontology analysis revealed enrichment of differentially methylated genes in molecular and cellular metabolism pathways, with endocrine system development and metabolic regulation on organismal levels. Notably,

pathways associated with protein localization to the endoplasmic reticulum (ER) were identified, potentially linking mixture G1 to ER stress, adipogenesis, and obesity. Despite limitations in the absence of quantification of intracellular mixture G1 concentration, this study provides significant insights into the metabolic effects and epigenetic alterations induced by mixture G1, showcasing the complex interactions between EDCs and metabolic programming.

There is well documented increasing incidence of obesity and its potential association with exposure to EDCs known as "obesogens." Epigenetic changes, such as DNA methylation, could be the intermediaries linking EDC exposure to obesity development in susceptible individuals. We have shown that exposure to a human-relevant EDC mixture associated with lower birth weight and exposure during early-life development, induced epigenetic modifications in human MSCs. It is known that DNA methylation can be persistent in mitosis, resulting in life long heritable effects. Therefore, these epigenetic alterations, induced by EDCs exposure, could underlie the disruption of the adipose tissue development.

Illumina EPIC Bead Array is a good method for *de novo* analysis however, it offers low resolution for single CpG methylation changes. Therefore, we next wanted to investigate if the DNA methylation changes seen with Illumina EPIC Bead Array could also be detected using a pyrosequencing method, a targeted DNA methylation analysis technique that offers high resolution on single CpG sites. However, in the validation analysis on the nine genes using pyrosequencing, none of the genes showed a similar significant dose-dependent DNA methylation changes that were previously seen with Illumina EPIC Bead Array method. This could be a result of using different donors of MSCs in the validation experiments, as well as that the experimental conditions were slightly different in the validation experiments. This highlights the potential variability between the four donors and the importance of considering inter-individual differences in studies with human derived cell lines. These findings emphasize the need for further research into the EDC effects on epigenetic mechanisms and their context-specificity. Additionally, understanding the factors that influence epigenetic responses will improve the reliability and applicability of epigenetic endpoints in chemical testing.

Effects of mixture G1 exposure on respirometry in zebrafish

To further elucidate the metabolic effects of mixture G1, we extended our investigation to a whole organism model, employing zebrafish larvae as a sensitive indicator of metabolic alterations. Oxygen consumption is tightly linked

to metabolic activities, reflecting the rate at which an organism utilizes energy at a resting metabolic state. By evaluating how mixture G1 impacts oxygen consumption in zebrafish, we gained a broader understanding of its potential to disrupt metabolism at a whole organism level. We showed that in zebrafish larvae mixture G1 significantly increased oxygen consumption (**Paper III**). An observed increase in oxygen consumption of zebrafish larvae following developmental exposure to mixture G1 could indicate a range of significant metabolic changes. The increase might stem from enhanced energy demand for growth and development, potentially indicating altered metabolic homeostasis. For example, an increase in oxygen consumption could be a result of heightened oxidative stress²⁵⁹. Oxidative stress has been associated with various metabolic conditions, including obesity, cardiovascular diseases, and type 2 diabetes^{260,261}.

Phthalates, among other EDCs, have been identified as contributors to oxidative stress, potentially influencing the endocrine system²⁶². Oxidative stress results from excessive accumulation of free radicals, including reactive oxygen species (ROS) and nitrogen species (RNS), which can originate from both internal sources like cellular metabolism and external factors like radiation and chemicals²⁶¹. Several previous studies have shown that many EDCs of mixture G1 induced oxidative stress in cell or animal models^{263–267}. ROS together with RNS plays an important role in the development of the placenta²⁶⁸. Throughout the gestational period as a result of the continuously increasing metabolic activity of the placenta and the fetus, there is a paired continuous increase in the amounts of production of the ROS/RNS as well as antioxidants that remove them²⁶⁹. The inadequate clearance and hence buildup of ROS/RNS is associated with several pregnancy complications such as miscarriage and fetal growth restriction^{270–272}. Furthermore, a study conducted on the maternal first trimester exposure burden to a mixture of EDCs found significant association with maternal as well as neonatal ROS/RNS²⁷³. Thus, the findings suggest that developmental exposure to mixture G1 can elevated oxygen consumption at human-relevant concentrations, and could potentially be implicated in oxidative stress and related metabolic disorders.

Effects of mixture G1 vs individual chemical effects on metabolic endpoints

The heterogeneous composition of mixture G1 suggests that these chemicals could act through various mechanisms as well as have complex interactions. Hence, we investigated and compared the metabolic effects of mixture G1's individual components to the effects of mixture G1 using both the adipogenesis as well as the respirometry assays (**Paper III**). Furthermore, we assessed

the applicability of the additivity model in predicting the effects of mixture G1 on metabolic endpoints in cellular and whole-organism assays (**Paper IV**).

We showed that the human-relevant mixture G1 affected metabolic endpoints both in human MSCs as well as developmentally exposed zebrafish larvae, and that the mixture effect was more potent than that of any of the individual EDCs by themselves in corresponding concentrations. Among the individual compounds, MEHP showed a similar increase in adipogenesis assay to mixture G1 effect. In zebrafish larvae, PFOS showed a similar increase in oxygen consumption to mixture G1 effect, while MBzP reduced oxygen consumption, and MEHP exhibited no effect on oxygen consumption. This suggests that the effects on the different measured metabolic endpoints were most likely induced via different modes of action, explaining why the individual EDCs compared to the mixture effect differently in the two assays.

Previous studies have shown that MEHP can promote adipogenesis and activate PPAR γ ^{147,231}. Additionally, previous studies have shown that EDCs can affect both the oxidative stress and thyroid hormone signaling^{274,159,275}. A reduction in thyroid hormones can lead to decreased glucose, fat, and protein metabolism, resulting in a lower metabolic rate and reduced oxygen consumption²⁷⁶. MBzP, for which we showed decreased oxygen consumption, has been associated with a lower ratio of T4 to T3 and decreased T4 levels in epidemiological studies^{277,278}. Moreover, the parent compound of MBzP, butylbenzyl phthalate, exhibited a T3 antagonistic effect in a *Xenopus laevis* reporter cell line at 20 μ M¹⁶¹. These findings suggest that the ability of MBzP to reduce oxygen consumption might involve a decrease in thyroid hormone signaling as a potential mechanism.

We assessed the applicability of the additivity model in predicting the effects of mixture G1 on metabolic endpoints in cellular and whole-organism assays (**Paper IV**). We found that, in general, the additivity model provided a reasonable approximation of the combined effect of the mixture. However, the test of additivity was rejected for the adipogenesis assay due to the less potent effect of the mixture compared to the predicted additive effect. The estimated dose-response curves for mixture G1 were consistently lower than those predicted under additivity for both the adipogenesis and respirometry assays, suggesting antagonistic interactions among the mixture components. We identified specific individual compounds within the mixture that could potentially drive the observed effects, including DDE, DPP, HCB, MBP, MEHP, MEP, MINCH, and PBA for the adipogenesis assay, and DDE, DPP, Triclosan, and PFOS for the respirometry assay. Notably, one compound in each assay, PFHxS for adipogenesis and MBzP for respirometry, showed an opposing effect to mixture G1, indicating a potential role in generating an antagonistic effect.

The choice of a statistical method when comparing mixture effects to individual chemical effects significantly impacts the results, underlining the importance of this decision. The Gompertz model is employed to fit a sigmoidal curve, enabling the calculation of the significance of the dose-response curve for each individual chemical. Consequently, this approach treats the entire dose-response curve as a unified entity, focusing on the overall trend. In contrast, Kruskal-Wallis test evaluates the significance across all individual concentrations of a chemical, dissecting the data into separate concentration points. These differing strategies lead to distinct outcomes in **Paper III** and **Paper IV** due to their contrasting approaches to analyzing the dose-response relationship.

To ensure relevant and comprehensive assessment, unlike many previous EDC mixture studies that often employed binary combinations or chemicals from the same class, our approach utilized individual chemicals in proportions reflecting real-life human exposure, as determined by associations with lower birth weight in the SELMA study. Importantly, we avoided data extrapolation by comparing effects only within the experimentally tested concentrations.

The application of mixture risk assessment is a critical challenge for regulatory bodies. The dose addition model in quantitative predictive approaches has been gaining traction to facilitate this process. While the additivity model has often been associated for simpler mixtures composed of chemicals with known same MoA, we demonstrated its effectiveness for complex mixtures involving chemicals with different MoA. Our findings align with previous studies that showed the dose addition model's aptitude for predicting the cumulative effects of mixtures with different MoAs. This approach provides a conservative estimate of potential risks, supported by the consistent observation that mixtures frequently exhibit greater impacts than single compounds. Therefore, relying on the assumption of additive effects in heterogeneous EDC mixtures can enhance the reliability of mixture risk assessment methods, contributing to a more comprehensive understanding of their potential risks.

Concluding remarks and future perspectives

We are daily exposed to a large number of endocrine disrupting chemicals that might not pose a risk individually, when they occur below their regulatory thresholds²¹⁷. However, accumulating evidence shows that mixtures of chemicals in our environment might have significant effects on health and development in humans and wildlife^{109,110,116,120,217}. Additionally, accumulating evidence highlights a particular concern for developmental exposure to EDCs, where disruptions to hormonal signaling can lead to permanent effects^{68,74,132,176,218,219}. We are currently facing the challenge of: 1. limited knowledge about the developmental exposure to mixtures of EDCs and links to adverse health outcomes^{64,220}. 2. lack of appropriate methodology to accurately assess the hazard and risk of existing chemicals in present mixtures and new chemicals before they reach the market, considering that they will be added to already present chemical mixtures^{221,222}.

We have shown that an epidemiologically identified mixtures of EDCs associated with lower birth weight induced lipid droplet accumulation, transcriptional as well as epigenetic changes in hMSCs and increased oxygen consumption in developmentally exposed zebrafish larvae. Given that excessive adipogenesis during developmental stages could increase the risk of obesity and related metabolic disorders later in life, such findings could indicate that the EDC mixtures have the capacity to influence long-term metabolic health through its impact on early cellular differentiation processes. This finding could have broader implications for understanding the mechanisms underlying metabolic programming and the potential role of EDCs in contributing to metabolic disorders.

This thesis also showed that the EDC mixture had a stronger impact on adipogenesis and oxygen consumption than any of its individual components, and the additivity model adequately predicted the mixture's effects. This work has significant implications for understanding the broader health consequences of EDC exposure and highlights the importance of considering mixtures rather than individual chemicals in risk assessments. It underscores the need for continued research in this field to understand the mechanisms underlying EDC-induced health effects and to implement better risk assessment strategies for

reducing their impact on human health. Finally, this thesis shows that combining observational data in epidemiological investigations with experimental studies in cell and animal models may be an important approach for coming closer to causal relationships between environmental complex exposures and human health risks.

There is a pressing need to comprehensively investigate the consequences of developmental exposure to human-relevant EDC mixtures, including exposures during different trimester periods, postnatal, as well as evaluating other sensitive windows such as exposure during puberty and menopause. More research should focus on establishing links between such exposures and adverse health outcomes. Additionally, the effects of mixtures of EDCs on oxidative stress should be assessed in relation to gestational exposure, lower birth weight, and adipose tissue development in metabolic disease.

The current regulations do not adequately address the hazards posed by EDC mixtures. Developing more precise and comprehensive methodologies for assessing the risks of existing and new chemicals, especially in the context of co-occurring mixtures, is crucial. This includes considering the cumulative effects of multiple chemicals already present in the environment.

Svensk sammanfattning

Människor exponeras kontinuerligt för många typer av kemikalier i vardagen från olika källor, t.ex. via mat, vatten, luft och genom hudkontakt. Vissa av dessa kemikalier kan påverka kroppens hormonsystem och dessa kemikalier kallas för hormonstörandekemikalier (endocrine disrupting chemicals; EDC). Med tanke på den viktiga roll som hormoner spelar i mänsklig utveckling är exponering för dessa kemikalier under de tider då celler och organ utvecklas särskilt oroande. Många studier har visat att enskilda kemikalier kan påverka normala utvecklingsprocesser, vilket kan leda till sjukdomar. Det finns dock mindre information tillgänglig om vilka effekter blandningar av EDC har på utvecklingen av celler och organ, trots att människan kontinuerligt exponeras för blandningar av kemikalier. Detta bidrar delvis till det faktum att statliga myndigheter inte har lämpliga metoder och därför inte korrekt bedömer riskerna med exponering för dessa kemiska blandningar.

Därför syftar denna avhandling för det första till att undersöka effekterna av två EDC-blandningar på metabol utveckling via experiment på stamceller och fiskyngel. För det andra jämfördes effekterna av en av EDC-blandningarna med effekterna av dess individuella kemikalier. För det tredje, inom ramen för det större projektet RACH-Mix som detta doktorandprojekt ingick i, undersökte vi om additivitetsmodellen kunde förutsäga EDC blandning effekter baserat på de individuella kemikaliedata. Additivitetsmodellen är en väletablerad metod känd för sin tillförlitlighet för att förutsäga de kombinerade effekterna av blandningar där kemikalier har liknande egenskaper. Vi syftade till att undersöka om denna metod skulle kunna utöka sin prediktiva noggrannhet till komplexa blandningar sammansatta av kemikalier med olika egenskaper som är mer anpassade till verkliga mänskliga exponeringar.

De EDC-blandningar som studerats i detta doktorandprojekt identifierades inom projektet EDC-MixRisk (<http://edcmixrisk.ki.se/>), där man med hjälp av data från den svenska SELMA studien har kopplat blandningar av kemikalier i blodet hos gravida kvinnor med lägre födelsevikt hos barnen, en tidig markör för prenatal metabol programmering. I den här avhandlingen testades dessa blandningar, sk. Blandning G och G1, samt blandning G1:s alla individuella kemikalier experimentellt i mänskliga mesenkymala stamceller (hMSCs) och i zebrafisk-yngel. hMSCs kan bli fett-, ben-, muskel- eller broskceller, och

återspeglar viktiga processer i organutveckling hos fostret under graviditeten. Utveckling av stamceller till fettceller studerades, samt hur kemikalierna påverkar genuttryck och epigenetiska mönster. I zebrafiskyngel studerades syreupptag, ett mått på kroppens energiomsättning i vila.

I stamcellerna ökade blandning G fettcellsutveckling och förändrade uttryck av över 1000 gener på ett dosberoende sätt, redan vid de mänskligt relevanta koncentrationerna. Överraskande nog observerade vi inte ökat uttryck av gener relaterade till fettcellers utveckling. Några av generna som minskade i uttryck av blandning G är dock gener som visats vara involverade i den tidiga utvecklingen av mesenkymala stamceller mot benceller. Dessutom förändrades uttryck av gener som regleras av kortikoider, en grupp hormoner där några är kända för att vara involverade i bencellsutveckling. Därför kan den observerade utvecklingen av stamceller mot fettceller bero på en minskad benägenhet att utvecklas till benceller. Blandning G1 i koncentration som återspeglar mänsklig exponering i verkligheten inducerade epigenetiska förändringar vid 713 positioner i genomet, och i sex olika regioner i gener vars uttryck eller metylering tidigare har associerats med fetma och metabola sjukdomar. Dessutom, på samma sätt som blandning G, ökade blandning G1 fettcellsutvecklingen i hMSCs. Effekten av Blandning G1 var större än någon av dess individuella kemikalier. I zebrafiskyngel ökad syreförbrukningshastigheten med blandning G1, men två av de enskilda kemikalierna resulterade i en minskad syreförbrukningshastighet, vilket var i motsats till effekten av blandning G1. Slutligen visade denna avhandling att blandningens G1-effekt på fettcellsutveckling och syreförbrukning kunde förutsägas på ett adekvat sätt av additivitetsmodellen med hjälp av experimentella data från dess individuella kemikalier, särskilt vid lägre koncentrationer. Vid högre koncentrationer av blandningen förutspådde additivitetsmodellen mer effekt än vad som uppmättes.

Sammanfattningsvis visade detta projekt att en blandning som återspeglar mänskliga verkliga exponeringar, både vad gäller proportioner och koncentrationer, kan ge molekylära och fysiologiska förändringar. Dessa förändringar kan vara relevanta under utvecklingsperioder som kan leda till ökad risk att utveckla metabola sjukdomar senare i livet. Dessutom visar resultaten att även om kemikalier som testats var för sig inte gav signifikanta negativa effekter så gav dessa kemikalier i kombination statistiskt signifikanta effekter på fettcellsutveckling och syrekonsumtion. Slutligen visade denna avhandling tillämpbarheten av additivitetsmodellen för att förutsäga effekter av en blandning av kemikalier med olika egenskaper. Dessa resultat styrker de ökande bevisen för att EDC, särskilt i blandningar, påverkar det utvecklande metabola systemet, och understryker vikten av att beakta effekterna av kemikalier i kombination.

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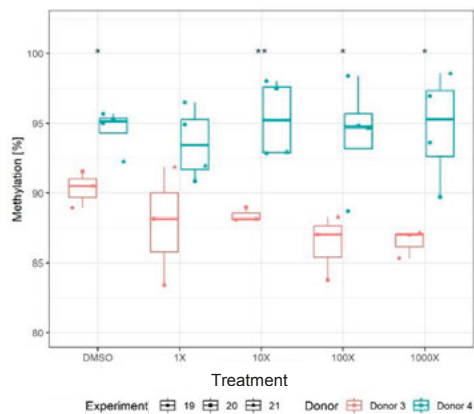
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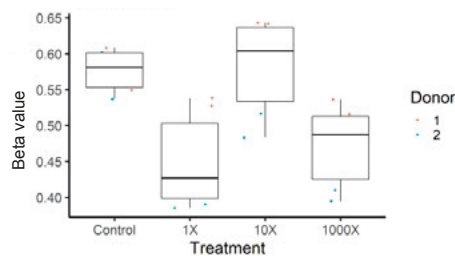
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Supplementary Information

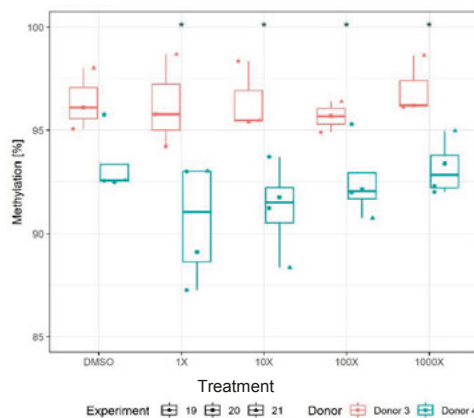
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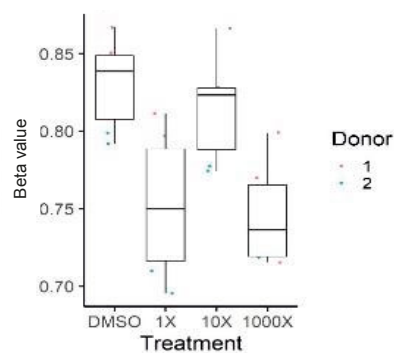
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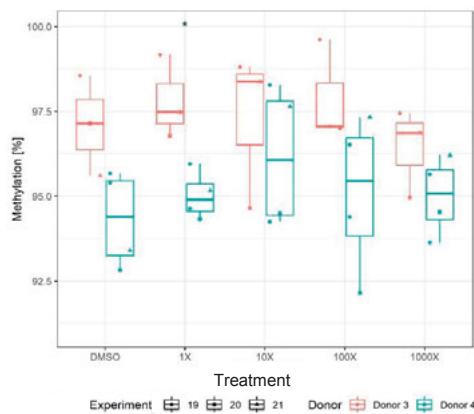
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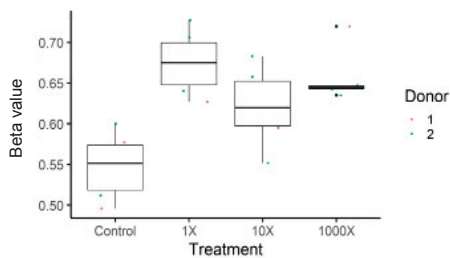
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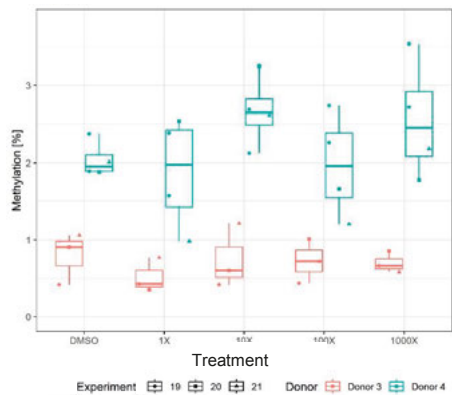
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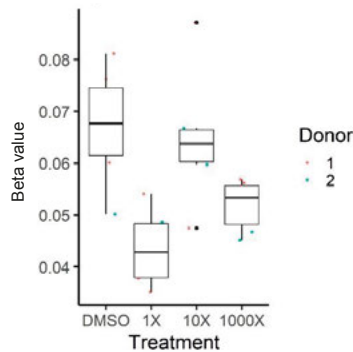
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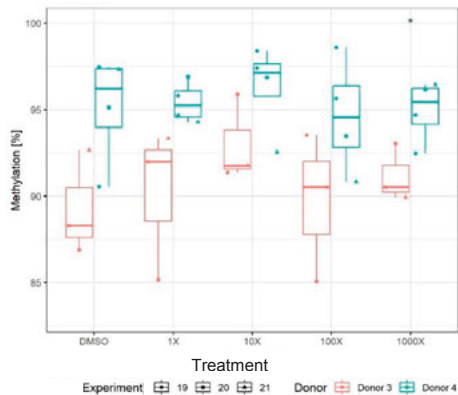
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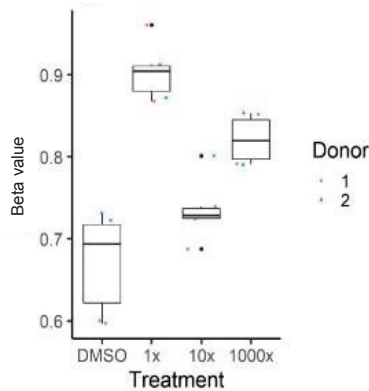
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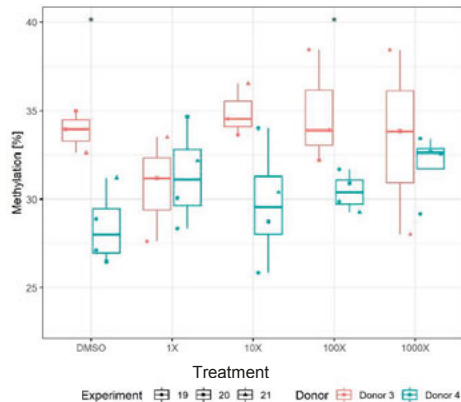
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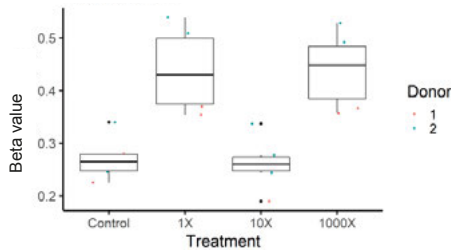
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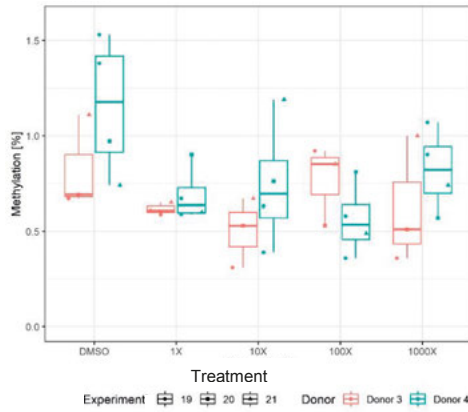
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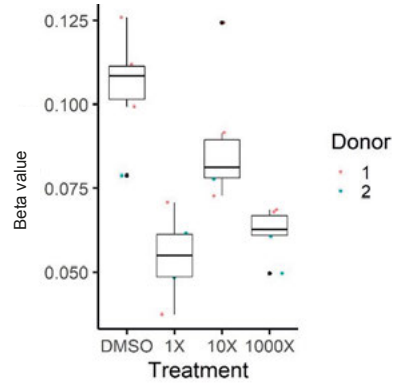
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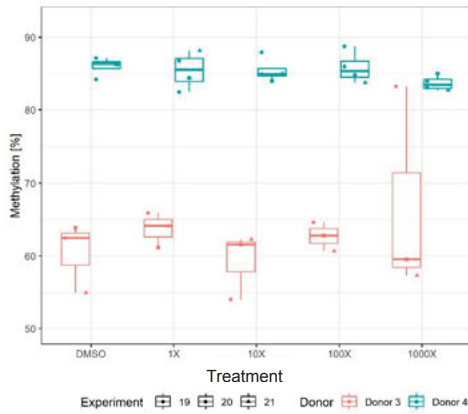
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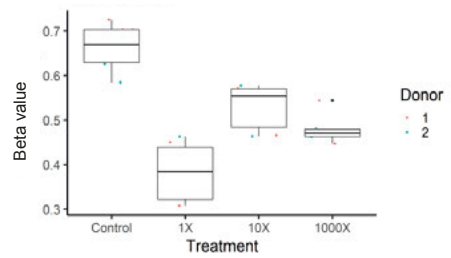
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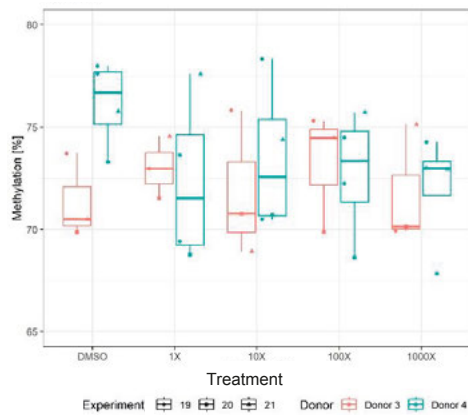
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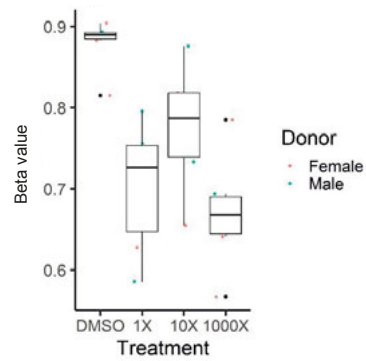
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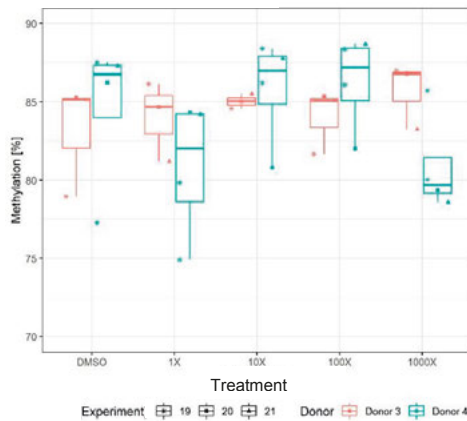
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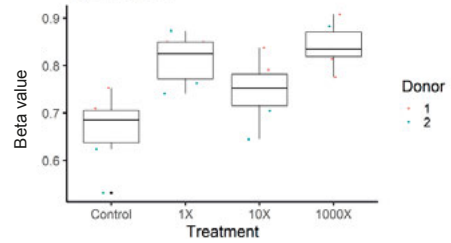
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Pyrosequencing: MYOF



Illumina ID cg03362798 (MYOF)



Figures S1: The comparison between the CpG cites analysed with pyrosequencing and those identified in Illumina EPIC array. The Left panel contains the DNA methylation levels measured by pyrosequencing; the right panel contains corresponding DNA methylation levels identified by Illumina EPIC array. The donors of bmMSCs are indicated as "Donor". Treatment contains the control, DMSO, and mixture G1 at various concentrations.

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