Perinatal risk factors for childhood overweight development

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ADIM	Adipocyte Differentiation Initiation Medium
adj.MR	adjusted Mean Ratio
adj.OR	adjusted Odds ratio
adj.RR	adjusted Risk Ratio
ADMM	Adipocyte Maintenance Medium
AT	Adipose Tissue
BAT	Brown Adipose Tissue
BM	Basal Medium
BMI	Body Mass Index
BPA	Bisphenol A
BuP	Butylparaben
C	Carnitine
C/EBP	CCAAT/Enhancer-Binding-Protein
CI	Confidence Interval
Con	Control
DES	Diethylstilbestrol
EDC	Endocrine Disrupting Chemicals
EOSS	Edminton Obesity Staging System
EtOH	Ethanol
EtP	Ethylparaben
FACS	Fluorescence-activated Cell Sorting
GEE	Generalized Estimating Equation
IBMX	3-Isobutyl-1-methylxanthine
IFNγ	Interferon γ
IgE	Immunglobulin E
IL	Interleukin
IRS2	Insulin receptor substrate 2
LINA	Lifestyle and Environmental Factors and their Influence on Newborns Allergy Risk
LPL	Lipoprotein Lipase

LPS	Lipopolysaccharide
MeP	Methylparaben
MEST	Mesoderm Specific Transcript
MSC	Mesenchymal Stem Cell
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyoltetrazoliumbromid
PHBA	p-Hydroxybenzoic Acid
PHA	Phytohemagglutinin
PCR	Polymerase Chain Reaction
PPAR	Peroxisome Proliferator-activated Receptor
PrP	Propylparaben
PSQ	Perceived Stress Questionnaire
RT	Room Temperature
RXR	Retinoic x Receptor
s.e.m.	Standard Error of Means
SCCS	European Committee on Consumer Safety
SD	Standard Deviation
SES	Socio-economic Status
SREBP	Sterol Regulatory Element-binding Protein
T2DM	Type 2 Diabetes Mellitus
TBT	Tributyltin
TDI	Tolerable Daily Intake
TG	Triglyceride
TNF	Tumour Necrosis Factor
UGT	UDP-glucuronosyltransferase
UPL	Universal Probe Library
WAT	White Adipose Tissue
WHO	World Health Organisation

1. INTRODUCTION

1.1. History of the obesity epidemic

Obesity rates have been growing during the last decades all over the world and have almost doubled since 1980 [1, 2]. Once considered to be a problem of the western industrial countries, obesity is also on the raise in middle- and low-income countries with most people living in countries, where overweight and obesity kill more people than underweight. Obesity is a so called non-communicable disease, stating that it is a non-infectious but chronic disease. In 2015 about 68 % of all deaths worldwide were caused by non-communicable diseases, with about 4-5 million deaths directly assigned to overweight or obesity [1, 3].

In 2014 about 39 % (more than 1.9 billion) of the world's adult population was overweight and about 13 % of them (over 600 million) were obese [4]. In the European Union overweight affects more than 50 % and obesity more than 20 % of all adults with 60 % overweight and 23 % obese adults in Germany in 2011 [5].

Particular attention should be paid to obesity prevalence of children. In the past 30 years there was a tremendous increase in childhood obesity prevalence in the USA and other countries. A high percentage of these obese children will stay obese in adulthood, setting them on high risk for serious comorbidities and shorten their life expectancy [6, 7]. In 2014 about 12 % of all German children age 2 to 10 years already gained overweight and 5% of children were obese [8].

Overweight and obesity are huge risk factors for and accompanied by serious comorbidities, like type 2 diabetes mellitus, high blood pressure, cardiovascular diseases, fatty liver disease, dementia and cancer [9, 10]. In that context, many epidemiological studies have investigated causes of the raising obesity prevalence around the world. In summary the scientific community agrees that risk factors for obesity development are multifactorial including genetic, environmental and social influences, like less physical activity, excessive calorie consumption, stress, infections and exposures to endocrine disrupting environmental pollutants (Figure 1) seem to be major contributors in the disease pathology as reviewed in [11, 12].

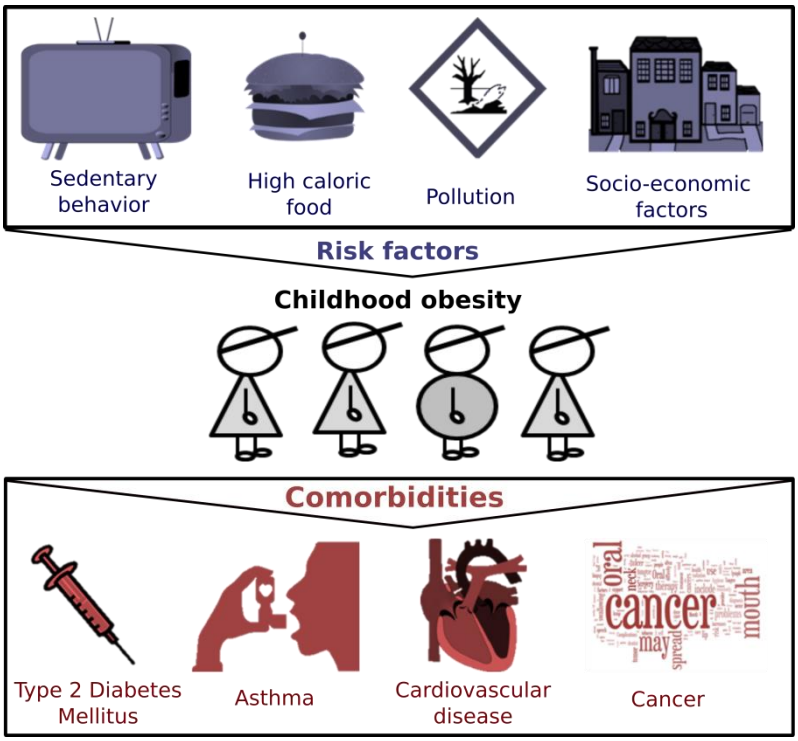


Figure 1: General risk factors and comorbidities of childhood obesity.

1.2. Obesity definition

Obesity is a chronic condition in which excess body fat tissue has accumulated to the extent that it may have a negative effect on health. Overweight and obesity are generally defined via the body mass index (BMI). The BMI is calculated by dividing a person's body weight in kilograms by the square of his body height in meter (kg/m^2) [13].

An adult person with a BMI of 18.5-25 kg/m^2 is considered to be normal weight, with a BMI of 25-30 kg/m^2 to be overweight and with a BMI of > 30 kg/m^2 to be obese [14]. Although the BMI has been criticized widely because of missing information about body fat/ muscle percentage and general health parameters, it is still commonly used in epidemiological studies [15].

For the categorization of children, their age and gender need to be taken into account, because BMI distribution varies widely during maturation [16]. Relevant thresholds are derived from large reference populations by the World Health Organization (WHO) [17] or the German Health Interview and Examination Survey for Children and Adolescents (KIGGS) [18] that give an average BMI for boys and girls at a certain age, as well as a distribution below and above the average. These reference BMIs are usually defined as z-scores. Considering the WHO reference a BMI z-score of -1 to 1 equals the average BMI of a boy or girl at that age, a BMI z-score of >1 equals >+1 SD of the average and a corresponding BMI

of >25 kg/cm^2 (overweight), a BMI z-score of >2 equals >+2 SD of the average and a BMI of >30 kg/cm^2 (obese).

In addition to this very stringent classification of overweight and obesity depending on the BMI, a new classification system has been proposed by AM Sharma in 2009 [19]. The Edmonton Obesity Staging System (EOSS) aims to classify overweight and obesity into four stages with no, moderate, significant or severe comorbidities, functional limitations and psychological symptoms of obesity. The EOSS shall help clinicians to make rational decisions about obesity management of an individual person, because the BMI does not reliably reflect the obesity stage in an individual. Nevertheless, BMI and BMI z-scores are still a sufficient tool to record overweight and obesity rates in population based epidemiological studies [20, 21].

1.3. Adipose tissue biology

Adipose tissue (AT) is a heterogeneous tissue with mesoderm origin and important metabolic, endocrine but also inflammatory function. It consists of one third adipocytes and two third varies cells, comprising pre-adipocytes, endothelial cells, multipotent stem cells, fibroblasts and cells of the immune system [22]. AT can be distinguished in white adipose tissue (WAT) for energy storage and mobilization of triglycerides and brown adipose tissue (BAT) for energy expenditure and fat burning [23]. WAT adipocytes contain one single, large lipid droplet that fulfils about 90 % of the cytoplasm, and the nucleus, which can be found squeezed at the periphery of the cell. WAT can be further distinguished due to its localization within the body mainly in visceral and subcutaneous WAT. Both have distinct functions, with increased visceral WAT being associated with increased risk for insulin resistance and cardiovascular diseases [24]. BAT adipocytes are smaller than WAT adipocytes and contain several lipid droplets, a roundish nucleus and large mitochondria for heat production.
In lean human adults AT accounts for 8-18 % of body weight in males and 14-28 % of body weight in females with predominantly WAT and a few BAT spots mainly around the heart, aorta and at the base of the neck [25]. However, human BAT undergoes age related morphological and functional changes towards WAT, with the highest percentage of BAT at birth [26].

1.3.1. Adipocyte differentiation

Adipocytes generally derive from multipotent mesenchymal stem cells (MSC) that can undergo a multistep process to either produce cells from the adipocyte, chondrocyte or osteocyte linage [27, 28] (Figure 2). Early signals for lineage commitment are not very well studied, but bone morphogenetic factors (BMP) and Wnt molecules seem to have important functions [29]. There is also evidence that at least some adipocytes around the salivary gland

and ears are derived from neural crest cells with ectoderm origin [30]. However, once committed to the adipose lineage cells develop into pre-adipocytes that are not lipid filled but peroxisome proliferator-activated receptor γ (PPARγ) positive and with very restricted differentiation potential [31]. Once they are appropriately stimulated, they undergo several rounds of mitosis (mitotic clonal expansion) and differentiate into mature adipocytes [32]. In humans this final differentiation starts in late embryogenesis but mostly perinatal and in early childhood. In contrast, mouse and rat pre-adipocytes differentiate exclusively after birth [27].

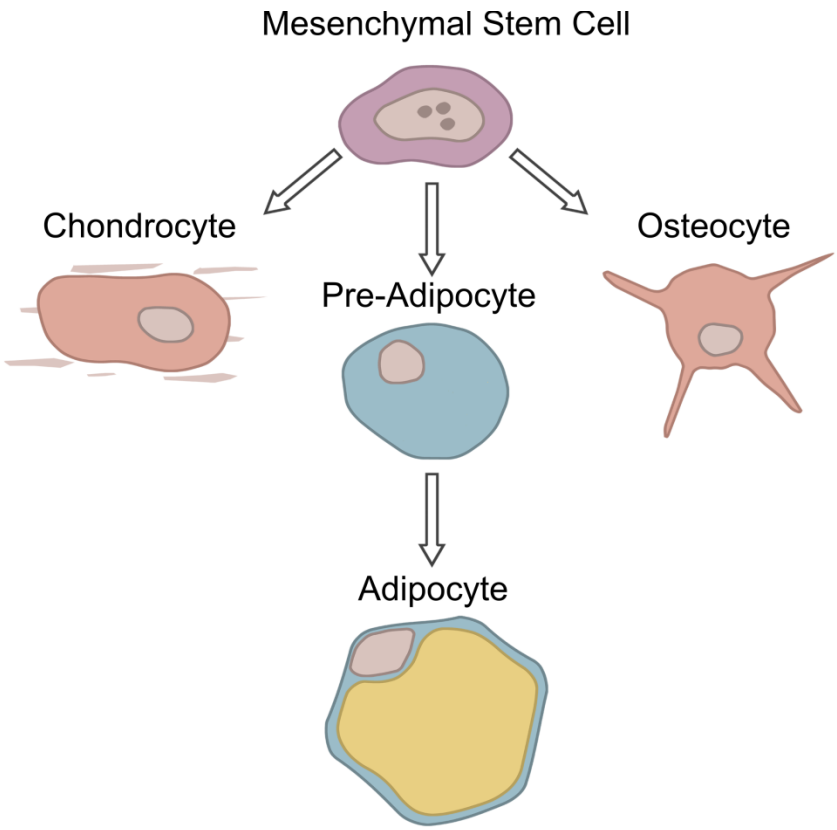


Figure 2: Mesenchymal stem cell differentiation potential.

Adipocyte differentiation is regulated by an orchestra of transcription factors and signalling molecules. One of the earliest events in adipogenesis is the induction of a cascade of several CCAAT/Enhancer-Binding-Protein (CEBP) family members [33]. Early induction of CEBPβ and CEBPδ induces the expression of kruppel-like factor 5 (KLF5), CEBPα and PPARγ [34]. PPARγ, the so called “master regulator” of adipogenesis, is both necessary and sufficient for adipocyte differentiation and no other regulator has been found to promote adipogenesis in absence of PPARγ [35, 36]. In addition, most pro-adipogenic factors seem to enhance PPARγ expression or activity. PPARγ exists in two splice variants, PPARγ2 is exclusively found in adipocytes, whereas PPARγ1 is also expressed in other cell types, but their distinct roles are not understood so far. PPARγ and CEBPα enter a positive feedback loop to maintain an adipose phenotype [37]. CEBPα accumulates and is phosphorylated by cyclin

D3, leading to the inhibition of proliferation and entering the final differentiation phase [38]. PPARγ forms an obligate heterodimer with retinoic x receptor α (RXRα) to bind at PPAR responds elements (PPRE) in target genes. Almost all genes involved in lipid and glucose metabolism have PPREs and approximately 63 % of PPARγ target sites are also bound by CEBPα [39]. In addition many other transcription factors are known to be involved in adipogenesis, like kruppel-like factors (KLF), sterol regulatory element-binding protein (SREBP) or early B-cell factors (EBF) [22].

Adipogenesis can be induced by different signalling molecules and their appropriate receptor and down-stream pathways *in vitro* [40]. Dexamethasone is traditionally used to activate the glucocorticoid receptor pathway which directly activates CEBPβ; 3-isobutyl-1-methylxanthine (IBMX) is a phosphodiesterase inhibitor [41] which raises intracellular cAMP levels and activates the cyclic cAMP response element-binding protein (CREB) and KLF4 signalling [42], both being pro-adipogenic and CEBPβ-activating. Insulin is added as an important regulator of adipogenesis. Insulin acts via the insulin receptor substrate (IRS), which is bound to the insulin receptor and also activates CREB via phosphatidylinositol-3-kinases (PI3K) and protein kinase B (PKB) [43]. Additionally a PPARγ agonist can be added to improve adipocyte differentiation efficiency. Troglitazone or rosiglitazone are common PPARγ agonists used in cell culture models [44].

1.3.2. Adipose tissue function and adipokines

The main functions of mature adipose tissue are triglyceride storage or release under excess caloric or fasting conditions respectively, thermoregulation and mechanic organ protection but also the regulation of many metabolic pathways through various adipokines [45]. Adipokines are secretory proteins that are produced by cells of the adipose tissue, some of them exclusively by adipocytes [46]. Since the discovery of the first adipokines leptin in 1994 [47] and adiponectin in 1995 [48], more than 600 potential adipokines have been discovered [49]. Adipokines are involved in the regulation of appetite and satiety, energy expenditure, fat distribution, insulin signalling, inflammation, blood pressure and hemostasis [50, 51]. Furthermore, they regulate adipogenesis, cell migration in the adipose tissue, adipocyte metabolism and target many other pathways in other organs like the liver, brain or muscle [50].

Some of the best studied adipokines that will be considered here are leptin and adiponectin, but there are hundreds more that cannot be described in detail in this context. Leptin was identified as the secretory protein, which the extremely obese *ob/ob* mouse model lacks to secrete [47]. A mutation in the leptin gene, which is also found in isolated cases of super obese humans [52], leads to a leptin deficiency and consequently excessive weight gain. Leptin is a 16 kDa protein with 167 amino acids and is exclusively produced by adipocytes in

direct proportion to body fat mass. It has an important inverse effect on appetite and food intake regulation and a direct positive effect on satiety, energy expenditure and foetal growth. Therefore high circulating leptin level reduce appetite emergence in the hypothalamus [53].

Adiponectin is also almost exclusively produced by adipocytes and has insulin-sensitizing, anti-inflammatory and anti-apoptotic properties [48]. In addition, it increases energy expenditure, enhances insulin production and correlates negatively with body weight, metabolic syndrome, blood pressure and serum lipids. Adiponectin is a 30 kDa protein with 244 amino acids and acts via two G protein-coupled receptors AdipoR1 and AdipoR2, which can be found in adipose tissue, skeletal muscle but also in brain, heart, spleen, kidney, liver etc. [54].

1.3.3. Adipose tissue in obesity

Obesity is characterized by two types of WAT development: hyperplasia and hypertrophy (see Figure 3) [45]. Hyperplasia is the increase in adipocyte number by pre-adipocyte or stem cell differentiation. Hypertrophy is an increase in adipocyte volume by increased triglyceride (TG) storage. It was believed, that the adipocyte number stays constant in adulthood and weight changes are mostly due to changes in adipocyte volume. But recent research indicates that both hyperplasia and hypertrophy contribute to an increasing AT in a dynamic and highly regulated process [55], even though deep information about the mechanisms behind are still missing.

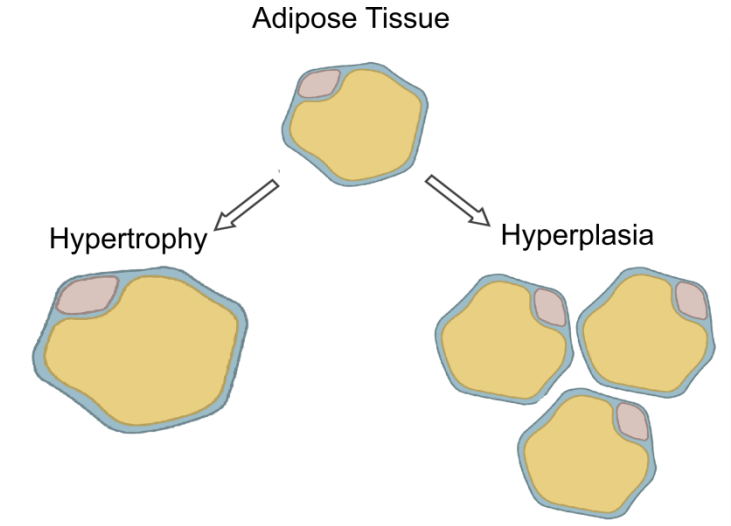


Figure 3. Adipose tissue hypertrophy and hyperplasia in obesity.

Furthermore obesity is associated with adipose tissue dysfunctions, like a loss of insulin sensitivity, elevated inflammatory markers and adipokine secretion that do contribute to the development of a metabolic syndrome, including cardiovascular diseases, type 2 diabetes mellitus and other diseases [45].

In addition, obesity is characterized by a chronic inflammatory state of the adipose tissue with impact on the systemic inflammation status [56-58]. There is a shift in the adipose tissue immune cell population from a mainly Th2 phenotype (anti-inflammatory) in lean subjects to a predominantly Th1 phenotype (inflammatory) in obese subjects. Lean adipose tissue is soaked by immune cells that inhibit inflammation and promote a Th2 response by secreting IL4, IL5 and IL13 [59, 60]. In obese adipose tissue immune cells, predominantly neutrophils, macrophages and natural killer cells, accumulate and induce an inflammatory Th1 response by secreting interferon γ (IFNγ) and tumour necrosis factor α (TNFα), with unfavourable effects for insulin sensitivity and metabolism in general [45].

1.4. Risk factors for overweight development in children

Obesity is an endocrine disease involving dysregulation of food intake, nutrient metabolism and storage, energy expenditure, hunger but also gut microbiota and immunology. Perturbation of this complex metabolic system especially during critical windows in development can lead to permanent changes and an increased likelihood to develop overweight [11, 61, 62]. One critical and tremendously important window for disruptions is the pre- and postnatal period with immune system and organ development, as well as the establishment of the whole endocrine system [63-65]. Disruptions within that critical window can for example be implemented by the foetal environment (placenta function and nutrient supply), endocrine disrupting compounds (EDCs) or through psychological mechanisms like adverse behaviour pattern development [11].

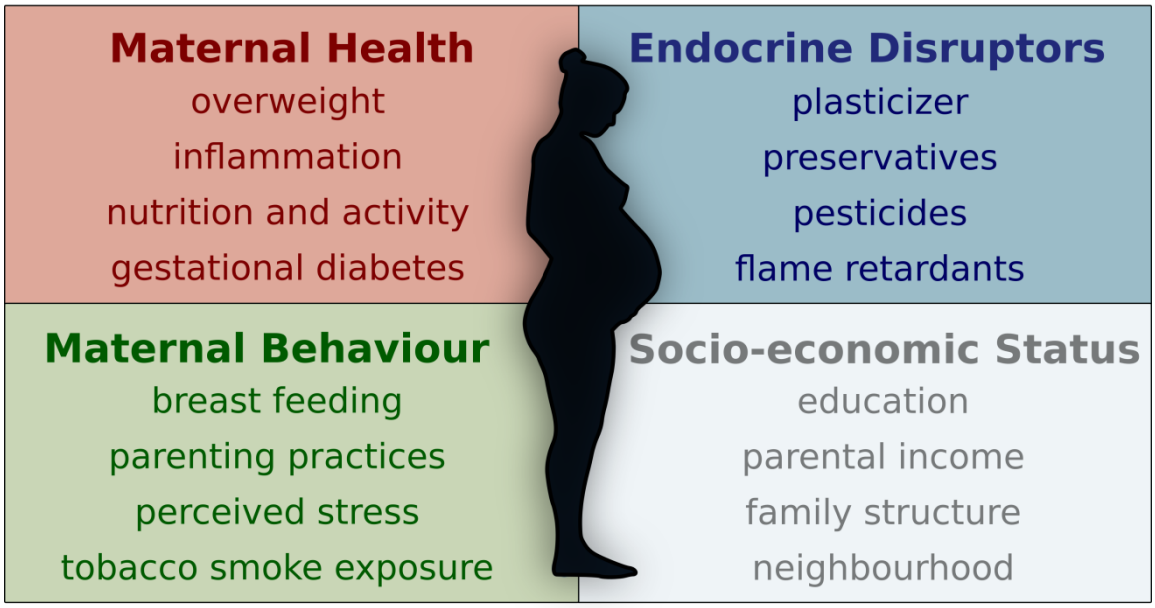


Figure 4: Selection of potential pre- and perinatal risk factors of childhood obesity.

However, many of these factors are already well studied and not all of them can be investigated within only one study. Therefore this thesis focuses on perinatal risk factors for overweight development like maternal inflammation during pregnancy, prenatal exposure to endocrine disruptors and perinatal maternal stress that have not been extensively studied so far, thereby considering known risk factors like aspects of the socio-economic status and maternal smoking during pregnancy (Figure 4). The mentioned risk factors are described in more detail below but can of course not account for all risk factors in their entirety.

1.4.1. Prenatal risk factors for overweight development

During the prenatal period the developing foetus needs to be protected against external hazards since this time is very sensitive for adverse environmental influences [64-66]. In mammals, this protection barrier is given by the placenta. The placenta connects the foetus to the maternal uterine wall for nutrient supply, thermo-regulation, gas-exchange and waste elimination via the maternal blood supply. A great number of studies have investigated the association of foetal malnutrition and obesity rates. Subjects that were born in times of prenatal famine or experienced prenatal starvation due to clinical reasons had a higher risk for overweight development later on [67]. Furthermore, subjects who are born to obese mothers are set on higher risk for overweight development [68]. Both observations suggest that metabolic priming takes place already *in utero* and the foetus adjusts to its available nutrient supply very early in its development [61, 62].

Next to the nutrient supply, the maternal health and immune status is important for foetal priming [12]. In particular maternal inflammatory states during pregnancy have been associated with the onset of civilization diseases like asthma or allergies [69-71]. But the maternal immune status during pregnancy has also been discussed as a contributing factor for the high prevalence of childhood obesity [72-74]. However, data on the involvement of the innate (IL-6, IL8, IL10 or TNFα) or adaptive (IFNγ, IL4, IL5 or IL13) immune status is inconsistent or even missing out some important associations in general: For example, Dahlgren et al. found that increased maternal IL6 was associated with overweight development in rat offspring [75], whereas Danielsen et al. observed no association between maternal inflammatory markers like TNFα, IL1β and IL6 and overweight development in 20 year-old children [76]. Interestingly, whether the maternal Th2 cytokines IL4, IL5 and IL13 during pregnancy may play a role in children's obesity development has not been studied so far. The hypothesis that cytokines of the adaptive immune system (IL4, IL5 and IL13) may act in the context of obesity, was derived from results of mechanistic studies. As described above, obesity is characterized by a chronic inflammatory state of the adipose tissue with impact also on the systemic inflammation status. In adipose tissue of lean subjects IL4, IL5 and IL13 have been described to function anti-inflammatorily. In detail, IL4 was able to

stabilize local immune response, maintain insulin sensitivity or limit further adipogenesis [77, 78]. Also IL4 and IL13 production has been shown to limit diet-induced inflammation and insulin resistance in mice [79].

In addition to the endogenous maternal factors also external environmental substances are able to either pass the placenta-blood barrier, like ingredients in tobacco smoke or plastics [80-82], or change placenta function and nutrient supply [83, 84], when maternal exposure during pregnancy occurs. Some of these substances may thereby act as endocrine disrupting chemicals and possess obesogenic properties.

1.4.2. Endocrine disrupting chemicals and obesity

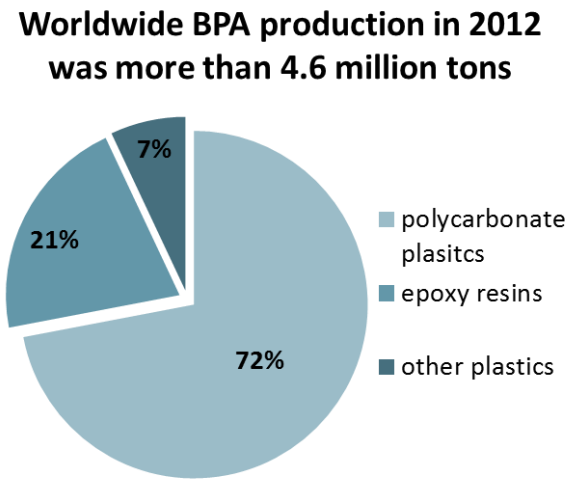
Many intervention studies have shown that once being obese 90 % of patients are unable to lose weight permanently [85, 86]. Even through a restriction in calorie intake and increased energy expenditure are still the most frequent recommendations for weight loss, there is evidence that factors like environmental pollutants are heavily underestimated in obesity treatment as well as prevention [87]. Endocrine disrupting chemicals (EDCs) are mostly synthetic chemicals that are able to interact with the human endocrine system and possess hormone like activity. EDCs can be found in plastics, furniture and clothing but also in food, cosmetic products and drugs. They are added to products as plasticizer, for antimicrobial and antifungal function, as flame retardants etc. and enter the body via food and water intake, skin absorption or inhalation [88, 89].

In 2015, a multidisciplinary group of scientists met in Parma, Italy to discuss concerns about the relationship of EDCs and metabolic diseases including obesity. They stated that it is of very high need to understand the impact of environmental chemicals on disease development [88]. Exposure during the critical prenatal period may be particularly harmful to the developing foetus and alter metabolic priming permanently. They further deeply encouraged that more attention should be paid on identifying relevant underlying mechanisms of EDC function to offer better prevention strategies.

1.4.2.1. Bisphenol A

The EDC bisphenol A (BPA) is a chemical used in the manufacture of polycarbonate plastics and epoxy resins. It was originally designed in the late 19th century and considered for use as synthetic oestrogen replacement in humans in the 1930s. Later on, BPA became one of the most important components in the manufacture of polycarbonate plastics with an overall annual production of about 4.6 million tons per year in 2012 [90]. It is released from consumer products and human exposure has been reported ubiquitously in industrialized countries [91, 92]. BPA has been detected in human blood, urine, adipose tissue, as well as in placental tissue, amniotic fluids and breastmilk [91], suggesting that exposure already

starts during the sensitive perinatal time window. Large cohort studies in the USA were able to detect BPA or its metabolites in 92.6 % of all participants with highest concentrations seen in children (see Figure 5) [92].



Estimated daily intake of BPA (µg/kg body weight/day)

Infant (0-6 months) formula-fed	1-11
Infant (0-6 months) breast-fed	0.2-1
Child (1.5-6 years)	0.043-14.7
Adult	0.008-1.5

Figure 5: Worldwide production and estimated daily intake of bisphenol A.

BPA is categorized as an endocrine disruptor and potential obesogen because of its ability to affect hormones and receptors involved in the onset of obesity, including oestrogen, glucocorticoid and PPARγ signalling [65, 66]. Furthermore, BPA might be able to affect reproduction, metabolism, immunology and neurobiology as well as cancer development. Therefore the tolerable daily intake (TDI) for BPA has been reduced for several times over the last years to 4 µg/kg of body weight/day. In 2015 the European Food Agency (EFSA) declared that "…BPA poses no health risk to consumers of any age group (including unborn children, infants and adolescents) at current exposure levels…" [93]. But this evaluation is mostly build on toxicological investigations, not considering endocrine disrupting properties. However, in 2016 BPA was proposed for re-classification as "Repotoxic 1B" i.e. "presumed human reproductive toxicant" in 2018 by the Risk Assessment Committee (RCA) of the European Chemicals Agency (ECHA) [94] and several states already banned BPA from consumer products, especially for those in childcare use.

BPA is one of the most studied EDCs although results are very inconsistent and controversially discussed. Also only a few studies exist on human prenatal exposure. Some epidemiological studies report an increased risk for obesity development in early childhood after high prenatal BPA exposure [95-97], while other studies associate high prenatal BPA concentration with a lower BMI in pre-puberty girls [98-100]. Also animal studies are not consistent, reporting either increased body weight in new-born and adult mice [101] or suggesting gender specific effects on weight development [102]. *In vitro* studies found either

a promoting [103, 104] or no effect on adipogenesis for BPA exposed pre-adipocytes or MSC [105].

1.4.2.2. Parabens

Another group of EDCs are parabens, a group of alkyl esters of p-hydroxybenzoic acid (PHBA) that are extensively used as preservatives with antimicrobial and antifungal properties in cosmetic products, toiletries, food (E214-219) and pharmaceuticals. A survey in 1995 found parabens in 99 % of all leave-on cosmetics and in 77 % of rinse-off products [106]. The most abundant parabens are methylparaben and propylparaben, followed by butylparaben. Parabens enter the human body mainly through ingestion or skin absorption and can commonly be detected in urine, blood and breast milk [107, 108]. Parabens have endocrine disrupting properties and there is evidence that parabens might have adverse effects on breast cancer [109], allergy development [110] and obesity [111]. We can divide parabens accordingly to their alkyl chain in short chain (methylparaben MeP and ethylparaben EtP) and long chain parabens (propylparaben PrP and butylparaben BuP) (see Figure 6) which are suspected to act on different pathways thereby being involved in the onset of different diseases. So far parabens have been reported to activate the glucocorticoid receptor, oestrogen pathways and PPARγ [111-113], but the literature is sparse on endocrine disrupting properties of parabens.

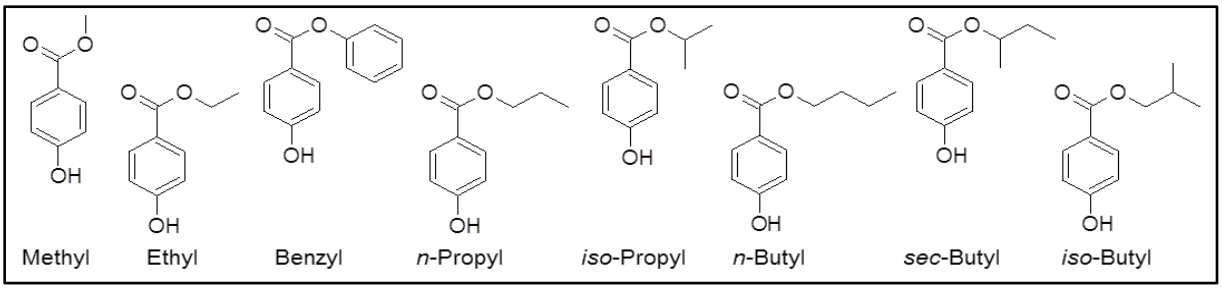


Figure 6: Chemical structure of parabens.

The European committee on consumer safety (SCCS) set the group TDI for MeP and EtP to 0-10 mg/kg body weight/day in 2004 [114]. There was uncertainty about a safe dose for PrP and BuP and no TDI was defined. In cosmetics the maximum authorized concentrations for MeP and EtP are 0.4 % for one ester and 0.8 % when used in combination. The use of PrP and BuP as preservatives in cosmetic products was considered as safe for humans as long as their sum does not exceed 0.19 %. However there are concerns about the endocrine modifying effects of PrP and BuP, which do appear to increase with chain length [114]. A recent re-evaluation of parabens by the SCCS in 2013 did not lead to a change in the maximum authorized concentrations [115] but concern was raised for the exposure to children under 6 months of age. The human risk was not yet evaluated for isopropyl-, isobutyl-, phenyl-, benzylparaben and pentylparaben.

Although there is uncertainty about the safety and endocrine disrupting properties especially about the long chain parabens PrP and BuP, studies that address these issues are sparse. A literature review revealed only 10 publications listed in *pubmed* for the association of parabens and obesity from 2008–2016. In 2013, Hu et al. reported an increased differentiation of 3T3-L1 mouse fibroblasts in adipocytes under BuP exposure [111]. Furthermore he proposed a glucocorticoid mediated pathway of BuP, which is in accordance with a study from Klopcic et al. conducted in 2015 [113]. They found that PrP and BuP showed glucocorticoid effects at 1 µM but only BuP showed effects at 10 nM as well. There is also epidemiological evidence that paraben exposure is associated with the obesity state. Xue et al. found a significantly positive correlation between urinary paraben metabolites (3,4-diydroxybenzoic acid (DHB)) and obesity rates among Indian children [97]. Whether the prenatal exposure to parabens is harmful to the developing endocrine system of the foetus is still unknown and needs investigation.

1.4.3. Socio economic factors and maternal stress

Next to prenatal internal or environmental factors, the maternal socio-economic status, including the educational status, family structure, living environment and household income has been identified as a risk factor for overweight development in children. Studies implicate that children living in poor neighbourhoods are at higher risk for overweight development, because they are less likely to join sport clubs and have less availability of healthy food and education [116-118]. But according to the cumulative risk theory usually not one single adverse factor, but the combination of adverse factors can increase obesity risk [119, 120].

Additionally, increased stress during infancy is a risk factor for overweight development [121, 122]. Reasons for parental stress are various and include precarious financial situations, poor neighbourhood, parenting stress and unfavourable family relationships that are also related to the socio-economic status. Especially maternal stress during pregnancy has been associated with increased risk for overweight development of their offspring. Presumably, permanently increased stress hormones during pregnancy can implement lasting alterations in foetal metabolism [123-125]. Maternal stress seems to alter the hypothalamic-pituitary-axis (HPA) signalling, exposing the foetus to an excess of glucocorticoids during the prenatal developmental phase, which might also contribute to prenatal growth restriction [123, 124]. Other studies focused on the impact of maternal stress and risky behaviour on children's overweight development at preschool and elementary school age. Studies found that particularly parenting stress does interact with parenting behaviour, like feeding practices, children's attendance at social events or transportation to sports [126-128]. In this context the mothers seem to play a pivotal role, as they often spend significantly more time in direct interaction with the child compared to the fathers, shaping the child's eating behaviour [129].

For example children's reduced consumption of fruits and vegetables has been associated to maternal stress [127, 128]. There is uncertainty whether the child must experience the stressor itself, but children's own experience may always exaggerate the experienced parental stress. Studies indicate that stress perceived by children themselves seems to alter their energy intake and food selection with a preference for sweet and high fat foods [127].
However, the majority of studies conducted so far focused on prenatal or postnatal stress exclusively while longitudinal maternal stress assessments in relation to children's weight development are rare [130, 131], which leads to an uncertainty whether pre- or postnatal maternal stress is contributing equally to children's overweight development.

1.5. Studying risk factors for obesity development

1.5.1. Epidemiological studies

Epidemiological studies are traditionally a good tool to investigate associations between socio-economic factors, living environment but also pollutant exposure and adverse health outcomes in medical sciences. Cross-sectional studies analyse population based data that has been collected at a specific point in time. Prospective cohort studies follow individuals over time after recruitment to collect longitudinal data about health outcomes and other relevant information. Longitudinal studies allow drawing connections between specific events of interest in the past and health outcomes later on. To study the impact of adverse prenatal exposures or events on infant's health outcomes, recruitment of participants must already start during pregnancy. Dependent on the study design, questionnaires are filled out during phone interviews or clinical visits by professionals or self-administrated by the participants in regular intervals. Clinical visits offer the possibility to get professional diagnoses of health outcomes of interest and in addition human samples (blood, urine, hair etc.) can be collected.

Anthropometric data about body weight and height, but also waist and hip circumference of participants are of particular interest in overweight and obesity research [132, 133]. Additional data about clinical outcomes like insulin tolerance and blood glucose level can add further information about the disease state in regard to the Edmonton staging system mentioned before. However, most studies recruit participants that already gained overweight or obesity and compare them with lean controls, rather than investigating weight development even before the disease has been manifested to identify risk factors.

Parts of this thesis are based on the LINA (**Li**festyle and Environmental Factors and their Influence on **N**ewborns **A**llergy Risk) study cohort, which is a prospective birth cohort that recruited 622 mothers (629 mother-child-pairs) during pregnancy between May 2006 and December 2008 in Leipzig, Germany. Starting in pregnancy and annually thereafter, participants attended clinical visits where blood and urine samples of mother and child (if

applicable) as well as anthropometric data of the child for weight development were collected. Additionally self-administrated questionnaires about housing conditions, lifestyle factors and health outcomes were assessed annually.

1.5.2. Obesity mouse models

Mouse models are often applied to study the complex inter-organic, endocrine and neurological interactions in obesity development. Under standardized housing and feeding conditions more detailed conclusions about cause-effect-relationships can be made in comparison to results derived from epidemiological studies. Specific mouse strains are more prone to obesity development. Two extreme mouse models for obesity development are ob/ob mice and db/db mice. ob/ob mice lack the gene that encodes leptin, an adipokine that signals satiety to the brain and lowers food intake [134]. ob/ob mice become super-obese even under normal caloric diet, but can be recovered by administration of leptin. db/db mice lack the gene that encodes the brain based leptin receptor but produce leptin normally [135, 136]. Next to obesity both mouse models show the related comorbidities like type-2-diabetes mellitus, dyslipidaemia and cardio-vascular diseases.

However, obesity can also be induced in other mouse strains by feeding a high caloric diet (HFD) or diet containing other obesogenic compounds, whereas interventions are usually conducted under low caloric diet (LFD) conditions [137]. These models give much more realistic insights into the effects of even small changes in environmental and social/behavioural factors on overweight development in otherwise healthy individuals.

1.5.3. In vitro cell culture models

To study adipogenesis *in vitro* several cell culture models with distinct advantages and disadvantages have been developed [138]. One of the most studied cell lines is the mouse embryonic fibroblast line 3T3 derived pre-adipocyte line 3T3-L1. Since introduced in 1975 by Green and Kehinde [139], 3T3-L1 cells have been extensively used to study the influence of endogenous and exogenous factors on adipocyte development. 3T3-L1 cells are easy to handle, to reproduce and to differentiate into mature adipocytes in a respectively short time of approximately 12 days. However, as 3T3 fibroblasts are derived of mouse tissue, effects cannot directly be assigned to human adipogenesis without restrictions [27]. Furthermore 3T3-L1 cells are pre-adipocytes, which have already undergone the first developmental steps from mesenchymal stem cells to adipocytes, missing out important information about early commitment to the adipocyte lineage.

To further extend mechanistic knowledge gain in this context, other cell culture models can be used or optimally combined with each other. The so called “gold standard” in that field are human primary MSCs, which have the capacity to differentiate into adipocytes, chondrocytes

and osteocytes. A differentiation mixture supplemented with dexamethasone (glucocorticoid receptor agonist), IBMX (intracellular calcium level regulation) and insulin is sufficient to differentiate MSC reproducibly into mature adipocytes over a period of about 14-20 days (see Chapter 1.3.1). MSC are usually isolated from umbilical cord blood, bone marrow or adipose tissue, with adipose tissue derived MSC having the highest differentiation potential towards adipocytes [140, 141]. Although MSC differentiation is physiologically an ideal model to study adipogenesis, differentiation time is longer, variation among experiments and donors is higher and efficiency is lower compared to pre-adipocyte model systems.

As this thesis aims to investigate prenatal exposure scenarios and their impact on early childhood overweight development, adipose derived human MSC are currently the most appropriate tool to do so. But it is important to mention that there are major differences between *in vitro* cell culture models and *in vivo* adipose tissue, like levels of secreted adipokines and expression of major early transcriptional marker like KLF4 [22].

1.5.4. Epigenetics

Next to the contribution of genetic factors to an increased risk for obesity development, epigenetic alterations become a focus of attention. Epigenetics investigates the heritable traits that cannot be explained by changes in the DNA sequence and may result from external environmental factors or be part of the normal developmental programming [142]. Epigenetics include cytosine methylation at CpG sites (a region at the DNA strand, where a cytosine is directly followed by a guanine in 5´-3´direction), RNA interference and histone modifications that change the accessibility of DNA sequences to the transcription machinery and can be summarized as the epigenome (see Figure 7).

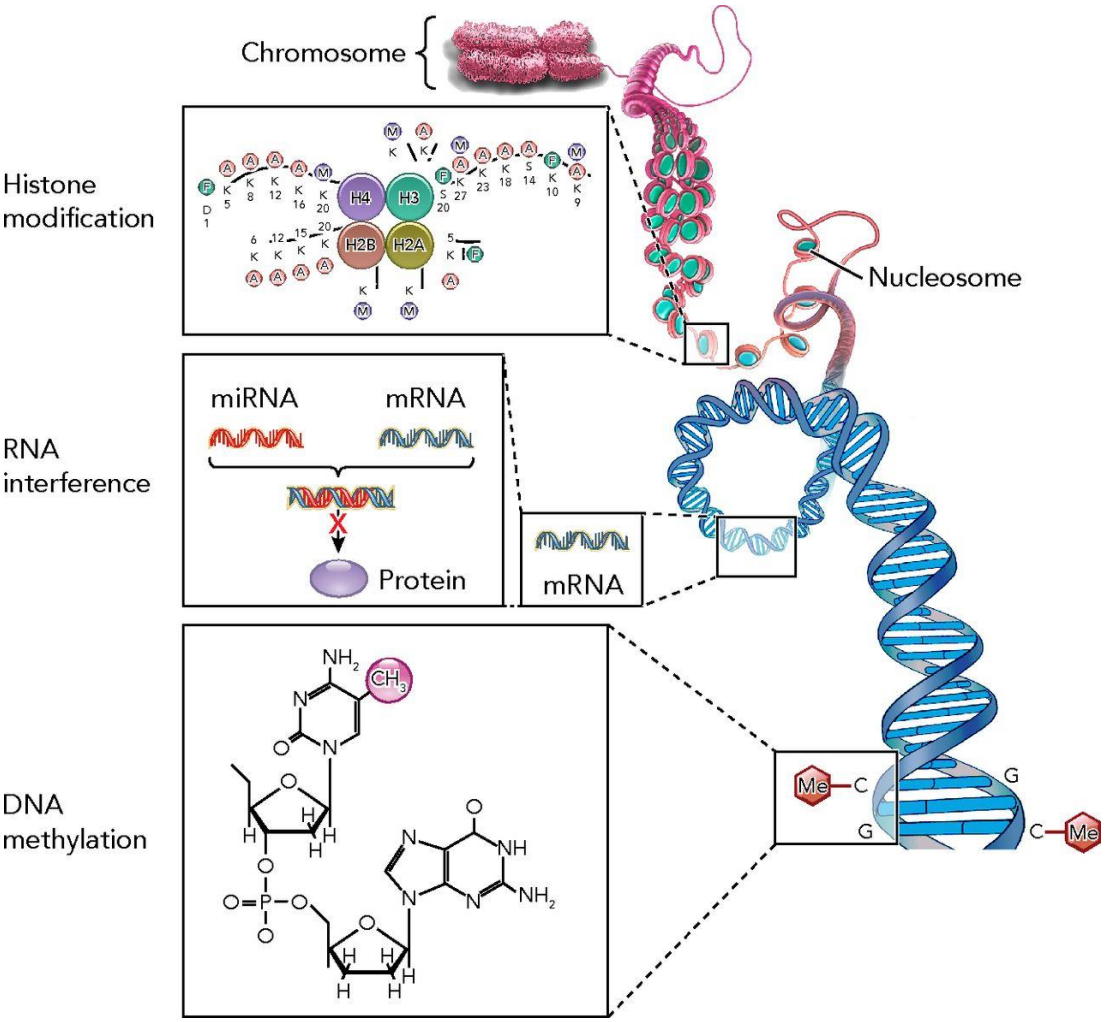


Figure 7: Schematic overview of the epigenome, including histone modifications, RNA interference and DNA methylation [143].

Epigenetic alterations are usually implemented during early development and can be caused by many factors, like environmental pollutants or an adverse foetal environment (prenatal starvation, gestational diabetes, chronic inflammation, smoking, stress etc.) [144-148]. There is evidence that the epigenome can influence risk for various diseases, like obesity and diabetes [149-151], asthma [152] and cancer [153]. So far the epigenome is not fully understood and research is necessary to draw functional mechanisms between already known connections.

1.6. Aim of this study

Obesity prevalence has risen all over the world over the last four decades and became one of the biggest health burdens in the 21th century. Special concerns are raised in regard of increasing overweight and obesity prevalence in children. In that context early priming in the perinatal period became of special interest within the last years in regard to childhood overweight development. During this highly sensitive period changes in maternal and environmental factors can implement tremendous alterations in the foetal and infant development that might even prolong until adulthood. Following this hypothesis, harmful triggers do not have to affect the born infant/child itself but might already be mediated and transferred via the maternal *in utero* conditions. In that context, this thesis aimed to investigate the impact of a broad spectrum of different potential risk factors in the pre- and early postnatal period on childhood overweight development within the epidemiologically prospective mother-child study LINA. In addition, relevant mechanistic aspects are considered in corresponding *in vitro* analyses.

Therefore this thesis was structured in 3 projects. **Project A** investigated the association of maternal inflammatory and anti-inflammatory cytokine levels during pregnancy with early childhood overweight development. It was known that maternal inflammation can impact children's weight but whether the anti-inflammatory Th2 cytokines IL4, IL5 and IL13, that are predominantly found in lean adipose tissue, do impact children's weight development has not been studied before. Therefore, project A focuses on the impact of the maternal Th2 status on children's body weight development

In project B the impact of prenatal exposure to the endocrine disrupting compounds BPA (project B_1) and parabens (project B_2) was investigated. BPA is one of the most studied EDCs so far but the mode of action in regard to obesity development is not sufficiently explained. Within **project B_1** a potential epigenetic link between prenatal BPA exposure and children's weight development was investigated. Further, the results obtained from the LINA study cohort were validated in *in vitro* experiments and supported by an *in vivo* mouse model.

Parabens are a relatively new class of EDCs and have not been extensively studies so far. Therefore the question of **project B_2** was to clarify a potential link between prenatal paraben exposure and increased risk for overweight development in children. Additionally the epidemiological results were supported by an *in vitro* adipogenesis model and a potential mechanism of paraben action was investigated.

Next to cytokine and chemical exposure, psychological stress can increase risk for overweight development in children and adults. The aim of **project C** was to differentiate between the impact of pre- and postnatal maternal perceived stress on children's weight development, whether that is mediated by stress hormones or behavioural changes and how maternal stress is associated with parameters of the socio-economic status.

Taken together, this thesis aims to add new information to the scientific community about the impact of early lifetime events on childhood overweight development by combining epidemiological observations with mechanistic *in vitro* assays.

2. MATERIAL AND METHODS

2.1. Material

	Cat. Number	Supplier
Media Supplements		
Adipocyte Differentiation Toolkit	PCS-500-050	LGC Standards, Wesel
D-PBS (ATCC)	30-2200	LGC Standards, Wesel
Gentamycin/Amphotericin B	50-0640	Life Technologies, Darmstadt
MSC Basal Medium (ATCC)	PCS-500-030	LGC Standards, Wesel
MSC Growth Kit (ATCC)	PCS-500-040	LGC Standards, Wesel
PenStrep (20ml, Life Technologies)	15140-148	Life Technologies, Darmstadt
Phenol Red (ATCC)	PCS-999-001	LGC Standards, Wesel
Trypsin Neutralisation solution (ATCC)	PCS-999-004	LGC Standards, Wesel
Trypsin/EDTA (ATCC)	30-2101	LGC Standards, Wesel
Kits		
AbsoluteIDQ p180 Kit	Biocrates LIFE Science AG, Innsbruck	
Adiponectin ELISA Kit, Human	KHP0041	Thermo Fischer, Ulm
BD CBA Human Soluble Flex Set system		Becton Dickinson, Heidelberg
EZ-96 DNA Methylation Kit	D5020S	Zymo Research, Orange, US
FastStart Universal Probe Master Mix	04913949001	Roche, Mannheim
Leptin ELISA Kit, Human	KAC2281	Thermo Fischer, Ulm
PreAmp Master Mix	4391128	Life Technologies, Darmstadt
QIAmp DNA Blood Mini Kit	51104	Qiagen, Hilden
Cell lines		
Adipose-derived mesenchymal stem cells; normal, human, donor 1 (59753760)	PCS-500-011	LGC Standards, Wesel
Adipose-derived mesenchymal stem cells; normal, human, donor 2 (60866843)	PCS-500-011	LGC Standards, Wesel

	Cat. Number	Supplier
Exposure chemicals		
BPA	239658	Sigma Aldrich, Hamburg
Ethylparaben	PHR1011-1G	Sigma Aldrich, Hamburg
LPS	1µg/ml, E.coli	Sigma Aldrich, Hamburg
Methylparaben	PHR1012-1G	Sigma Aldrich, Hamburg
n-Butylparaben	PHR1022-1G	Sigma Aldrich, Hamburg
n-Propylparaben	PHR1010-1G	Sigma Aldrich, Hamburg
PHA	50 µg/ml	Sigma Aldrich, Hamburg
Others		
96.96 Dynamic Array	101-0349	Fluidigm, San Francisco
E-Plate 96 View PET	2801047	OLS, Bremen
Formaldehyde	A0823	AppliChem
Infinium HumanMethylation450 BeadChip Array	WG-314-1002	Illumina, Sa Diego, USA
MassARRAY system	Sequenom/Agena Bioscience, Hamburg	
Oil Red	1.05230.0025	Merck, Schwalbach
UPL	Roche Applied Science, Mannheim	
xCELLigence 96 E VIEW	2801188	OLS, Bremen
Software		
IBM SPSS version 22	IBM Corps, USA	
STATISTICA for Windows, Version 12	Statsoft Inc, USA	
R version 3.3.1	R Foundation for Statistical Computing	
Instruments		
LightCycler 480	Roche Applied Science, Mannheim	
Synergy Microplate Reader	BioTek, Bad Friedrichshall	
xCELLigence MP station	2801006	OLS, Bremen

2.1.1. Cell culture media

Medium for human mesenchymal stem cell culture and differentiation		
Basal medium (BM)		
MSC Basal Medium		96.5 ml
MSC Growth Kit	L-Alanyl, L-Glutamine	1.2 ml
	MSC Supplement	2 ml
Penicillin / Streptavidin		0.1 ml
Gentamycin/Amphotericin B		0.2 ml
Adipocyte differentiation medium (ADIM)		
Adipocyte Differentiation Toolkit	ADM Supplement	1 ml
	adipocyte Basal Medium	15 ml
Adipocyte Maintenance Medium (ADMM)		
Adipocyte Differentiation Toolkit	ADMM Supplement	5 ml
	Adipocyte Basal Medium	85 ml

2.1.2. Primer for qPCR and Mass array

Gen	forward 5'→3'	reverse 5'→3'
human qPCR primer		
hGAPDH	CTCTCTGCTCCTCCTTTCGAC	TGAGCGATGTGGCTCGGCT
hPGK1	GACCGAATCACCGACCTCTC	AGCAGCCTTAATCCTCTGGT
hGUSB	GTCTGCGGCATTTTGTCGG	CACACGATGGCATAGGAATGG
hPPAR	TTACGCCTCGGTGTTTAGGG	TGGTCATTTCGTTAAAGGCTGA
hLEP	TTTCACACACGCAGTCAGTC	GTGGAGCCCAGGAATGAAGT
hADIPOQ	TGACCAGGAAACCACGACTC	AGGACCAATAAGACCTGGATCTC
hLPL	CCGCCGACCAAAGAAGAGAT	TAGCCACGGACTCTGCTACT
hCEBP	GGAGCAAATCGTGCCTTGTC	TTCTCTCATGGGGGTCTGCT
hFASN	GTCTTGAACTCCTTGGCGGA	AGGAAGATAGCCATGCCGAG
hIRS2	GTGAAAGAGTGAAGATCTGTCTGG	TTGCCTTGTTGGTGCCTCAT
hSREBF1	GCTCCCTAGGAAGGGCCGTA	AAGTGCAATCCATGGCTCCG
hGRα	CTATGCATGAAGTGGTTGAAAA	TTCAGCTAACATCTCGGG
hESR1	TGGCCCAGCTCCTCCTCATCCTC	AGTGGCTTTGGTCCGTCTCCTC
hMEST	ATCGGGTGATTGCCCTTGATT	GAAAGAAGGTTGATCCTGCGG
murine qPCR primer		
mGapdh	CCTGCTTCACCACCTTCTTGA	TGTGTCCGTCGTGGATCTGA
mMest	AGAGTGGTGGGTCCAAGTAGG	AAGCACAACTATCTCAGGGCT

2.2. Methods

2.2.1. Epidemiological studies – The LINA study cohort

The German prospective birth cohort LINA (**Li**festyle and Environmental Factors and their Influence on **N**ewborns **A**llergy Risk) was established by the Department of Environmental Immunology at the Helmholtz Centre for Environmental Research in Leipzig (PI Irina Lehmann) in collaboration with the Children`s hospital at the Municipal hospital "St. Georg" in Leipzig. The LINA study 622 pregnant women at the 34th week of gestation (resulting in 629 mother-child-pairs, 7 twin-pairs) between May 2006 and December 2008 in Leipzig, Germany. Information about lifestyle factors and health outcomes were collected annually by self-administrated questionnaires. Clinical parameters as well as blood and urine samples were collected during annual clinical visits by study assistants (see Figure 9). Study participation was voluntary and written informed consent was obtained by all participants. The study was approved by the Ethics Committee of the University of Leipzig (file ref 046-2006, 160-2008, 160b/2008, 144-10-31052010, 113-11-18042011, 206-12-02072012, #169/13-ff, #150/14-ff).

The present thesis comprises 3 LINA projects (A-C), which investigate the influence of different pre- and postnatal risk factors on childhood overweight development. Therefore different already available epidemiological data sets are included in each project. Project B is further supported by mechanistic *in vitro* studies (see summary in Figure 8).

		BMI z-scores	Maternal Cytokine Status	Prenatal BPA Exposure	Prenatal Paraben Exposure	Maternal Perceived Stress	Children's Metabolome	Epigenetics	*in vitro* studies
A	Maternal cytokine status during pregnancy may prime overweight development in children	✓	✓	-	-	-	✓	-	-
B_1	Prenatal exposure to Bisphenol A causes epigenetic changes at birth in the overweight associated gene MEST	✓	-	✓	-	-	✓	✓	✓
B_2	Prenatal exposure to Butylparaben increases risk for overweight development in children and alters adipogenesis in vitro	✓	-	-	✓	-	✓	-	✓
C	Impact of perinatal maternal stress on overweight development in preschool children	✓	-	-	-	✓	✓	-	-

Figure 8: Project overview with included data sets and additional mechanistic analyses.

Risk for overweight development was assessed up to the age of 6 years for projects A, B_1 and C, however only results for significant associations are displayed further on. For the most recent project B_2, also the complete data set for the year 8 assessment (ended January 2017) was available. Due to the longitudinal approach of the LINA study many measurements of diverse parameters (maternal immune status, metabolome, prenatal exposures, 450k DNA methylation status) were already conducted earlier and available for analyses. However, personal contributions include all final statistical analyses of their association with children's overweight development and mechanistic *in vitro* analyses. All corresponding methods are yet listed below with an indication of the data source and contribution by others. The personal contribution in the epidemiological parts of this thesis was mainly data analysis. *In vitro* adipogenesis was done without the contribution of others.

2.2.1.1. Anthropometric data

Children's body weight and height were assessed annually during clinical visits by a medical doctor or were obtained from regular preventive medical check-ups (U-examinations) asked for in questionnaires. At birth and the one-year follow-up children's length was measured horizontally; afterwards standing height was measured to the nearest 0.1 cm with an infantometer ("*Körpermessgerät nach Keller TypI / TypII*"). Body weight was measured to the nearest 0.1 kg. To adjust for children's age and gender, BMI z-scores were calculated according to the WHO reference data [154] in cooperation with Stefan Röder (Department of Environmental Immunology). Children with BMI z-scores <-1 were classified as underweight, children with BMI z-scores ≥-1 and <1 were classified normal weight and children with BMI z-scores ≥1 were classified as overweight.

2.2.1.2. Maternal cytokine measurement

To assess the maternal immune status during pregnancy, maternal cytokine level were measured by the group of Gunda Herberth in the Department of Environmental Immunology directly after collection of blood samples at the 34th week of gestation between May 2006 and December 2008. For the measurement of cytokine concentrations whole blood samples (500 µl) were incubated for 4 h at 37 °C with either lipopolysaccharide (LPS; 1µg/ml) or phytohemagglutinin (PHA; 50 µg/ml). LPS stimulation was used for the assessment of the inflammatory status (IL6, IL10, IL8 and TNFα), whereas PHA stimulation was implemented for the assessment of Th1 (IFNγ) and Th2 (IL4, IL5, IL13) cytokines of the adaptive immune system. Cytokine concentrations were measured by flow cytometry using a cytometric bead array (BD CBA Human Soluble Flex Set system) as described elsewhere [70].

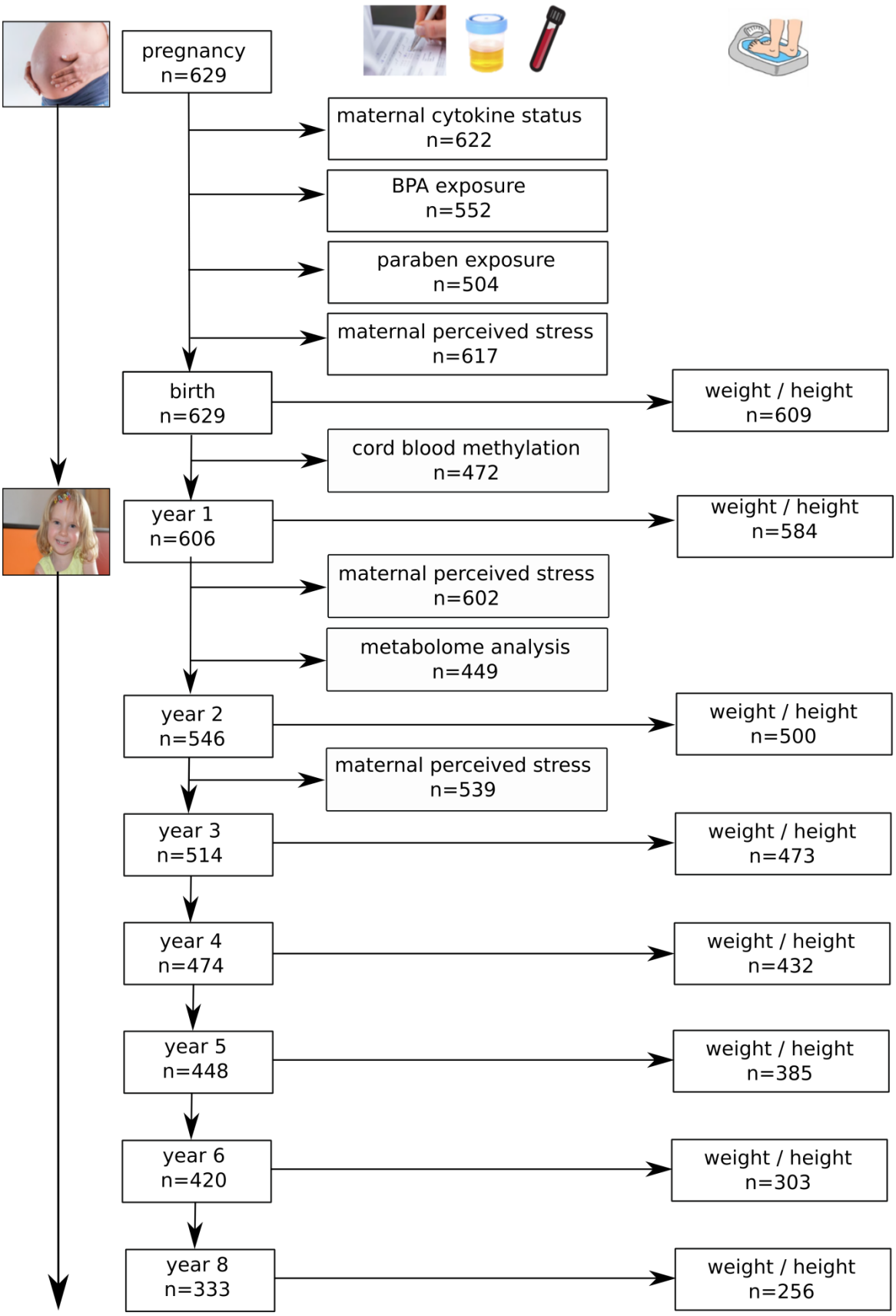


Figure 9: LINA study description. Overview of time points of questionnaire administration and sample collection (n=case numbers) as well as the assessment of parameters from the 34th week of gestation until children's age of 8 years that were used in the present thesis.

2.2.1.3. Metabolome analysis

Changes in the metabolism are often associated with diseases of the metabolic syndrome like obesity. To assess early priming a whole metabolome dataset for 449 LINA children at the age of 1 year was already provided by the group of Martin von Bergen in the Department of Molecular Systems Biology. Therein the endogenous metabolic profile (including 40 acylcarnitines, 21 amino acids, 14 biogenic amines, 76 phosphatidylcholines, 14 lysophosphatidylcholines, 15 sphingomyelins and hexose) was assessed.

Therefore blood sera were analysed using the AbsoluteIDQ p180 Kit as described in more detail elsewhere [155]. In brief, 10 µl of standards, 3 quality controls (QC) and plasma samples were pipetted onto a filter paper and nitrogen was used to dry the samples. Phenylisothiocyanate was applied to the dried samples prior to extraction of metabolites from the filter paper. Metabolites were detected using an Agilent 110 HPLC coupled to an API5000 triple quadrupole mass spectrometer. Blinded QC plasma samples were used to verify consistency of measurements between batches.

2.2.1.4. Assessment of environmental pollutant exposure in maternal urine

2.2.1.4.1. Assessment of bisphenol A (BPA)

To assess prenatal exposure to BPA and potential adverse effects on health, a measurement of BPA levels in maternal urine was already provided by the group of Martin von Bergen in the Department of Molecular Systems Biology. Urinary BPA quantification was carried out for 552 LINA samples using a multianalyte procedure also covering phthalate metabolites as described before [156].

In brief, 100 µl aliquots were spiked with $^{13}C_{12}$-labelled BPA, $^{13}C_4$ methylumbelliferone and unlabelled methylumbelliferone-glucuronide and deglucuronated with deglucuronidase/ arylsulfatase. Purification of samples was performed via solid phase extraction (SPE) on Isolute C18 columns via gravity flow. Analytes were eluted in 1.5 ml of 2 % FA in 90 % methanol, vacuum-dried at 45 °C, re-suspended in 100 µl 35 % acetonitrile and transferred to insert-containing auto-sampler vials, which were crimp-sealed. 10 µl aliquots of these extracts were analysed on a Dionex™ UltiMate™ 3000 UPLC System. Separation was achieved via reversed phase chromatography. Detection and quantification of analytes after elution and post-column infusion was achieved on a Q-Trap 5500 triple quadrupole mass spectrometer with electrospray ionisation at 350 °C and 4500 V in negative mode via previously established, scheduled SRMs. For BPA *m/z* = 227 to 212 and 227 to 139 da for $^{13}C_{12}$ BPA *m/z* = 239 to 224 and 239 to 139 da was used.

Absolute concentrations of BPA and the deglucuronated standard MeUmb were calculated with respect to the known, spiked-in concentrations of the isotopically labelled standards and previously obtained calibration curves, using dedicated software. Finally, concentrations were normalized against urinary creatinine concentrations [157]. All samples were analysed once with the described LC-MS/MS procedure.

2.2.1.4.2. Assessment of Parabens

Next to BPA, prenatal paraben exposure is under suspicion to have adverse effects on children's development. Therefore also paraben concentrations in maternal urine were assessed by the group of Thorsten Reemtsma in the Department of Analytical Chemistry and described in detail by Schlittenbauer et al. [158]. In brief, preparation of 200 µl maternal urine samples was done by a combination of ultrasound-assisted enzymatic hydrolysis of glucuronide– and sulphate-paraben conjugates, followed by an extraction-free clean-up. Quantification was carried out by ultrahigh-performance liquid chromatography coupled to either triple-quadrupole (UPLC-QqQ) or time-of-flight (UPLC_QqTOF) mass spectrometry (Figure 10). Limits of quantification were at 0.5 µg/L for methylparaben, 0.21 µg/l for ethylparaben and 0.1 µg/l for propyl- and butylparabens.

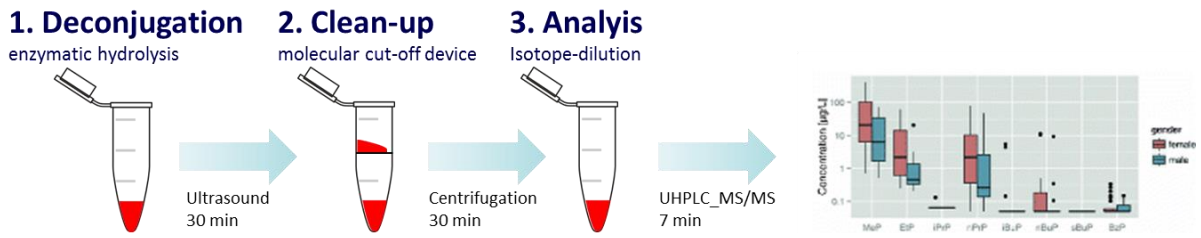


Figure 10: Schematic description of paraben level measurement in maternal urine. Adapted by Schlittenbauer et al. [158]

2.2.1.5. Assessment of cosmetic product application

The usage of cosmetic products might be a source of paraben exposure. Within the LINA study the usage of cosmetic products during pregnancy was assessed within questionnaires at the 34th week of gestation during the recruitment period 2006 to 2008 and processed later by Christiane Pfeiffer within a personal supervised Master's Thesis project. Participants were able to name up to 6 cosmetic products that were used on a daily basis. These cosmetic products were categorized in leave-on (crèmes, body lotion, make-up, facial cleansings (leave-on)) and rinse-off (toothpaste, facial cleansings (rinse-off), hairstyling, perfume and other) products. The qualitative content of parabens in the named cosmetic products was assessed between April and July 2016 via the TOXFOX app for iOS from the *Bund für Umwelt und Naturschutz Deutschland*, which contains information about the content of endocrine disrupting chemicals like parabens in a big online data base of cosmetic products.

Insufficiently indicated cosmetics that could not be assigned to a specific brand/product were excluded from further analyses. The analysis of cosmetic products was a personal contribution.

2.2.1.6. Maternal perceived stress assessment

Maternal perceived stress levels were assessed as a potential perinatal risk factor for childhood overweight development. It was measured at 3 time points (during pregnancy, the year 1 and year 2 follow-up) by the reduced and German translated perceived stress questionnaire (PSQ) that was originally designed by Levenstein et al. [159] and modified by Fliege et al. [160, 161]. The reduced PSQ assesses the individual's perception and emotional response to stress on 4 dimensions (demands, worries, lack of joy and tension). The last three dimensions reflect the internal stress load, whereas "demands" represents the perceived external stress load. The reduced PSQ is a 20-item questionnaire that includes 5 questions for each dimension. All items are scored on a 4-point scale according to their frequency of perception from (1) almost never to (4) occasionally. A total score was calculated by average the scored answers. The scores for the different dimensions and the total score were developed by Irina Lehmann from the Department of Environmental Immunology at the UFZ in close collaboration with Rosalind Wright from the Harvard School of Public Health in Boston, US. Complete information about perceived stress was available from 617 mother-child pairs at pregnancy, 602 mother-child pairs at year 1 and 539 mother-child pairs at year 2.

Furthermore, potential stressors were assessed within the annual questionnaires. Information about household members, household income and parental education were recorded once during pregnancy. Residential noise (commercial noise, noise from pedestrians, neighbours and restaurants/clubs), traffic (odours/exhausts and traffic noise) and poor living conditions (graffiti, vandalism, attempted break-ins and dirty streets) were assessed each year from pregnancy onwards. The frequency of occurrence was ranked on a 4 point scale with (1) almost never to (4) occasionally for each item and a mean score was calculated to assess total noise, traffic and poor living conditions. Parental divorce/separation was assessed retrospectively for the last 3 years at the 2 year follow-up.

The calculation of stress scores and assessment of stressors within the LINA questionnaires was a personal contribution.

2.2.1.7. Epigenetic analysis: 450k array data in the LINA cohort

Environmental pollutants can implement changes in the epigenome during early development that prolong until adulthood and may set the individuals on higher risk for disease development. To assess epigenetic alterations due to prenatal BPA exposure the

epigenome-wide DNA methylation was assessed by an Infinium HumanMethylation450 BeadChip (450k) array that was already provided by the group of Saskia Trump from the Department of Environmental Immunology and the group of Roland Eils at the German Cancer Reaserch Center (DKFZ) in Heidelberg. This array analyses the DNA methylation status in more than 450.000 CpG sites of the human genome and covers 99% of RefSeq genes with an average of 17 CpG sites per gene.

Genomic DNA was isolated from cord blood samples (n=472) by Mario Bauer and Beate Fink (Department of Environmental Immunology) using the QIAmp DNA Blood Mini Kit followed by bisulfite conversion using the EZ-96 DNA Methylation Kit according to the manufacturer's instructions. All samples subjected to DNA methylation analyses must have passed an initial quality control check. Genome-wide DNA methylation analyses was carried out in 0.5-1 µg genomic DNA by the Infinium HumanMethylation450 BeadChip array. Data was normalized using the SWAN (subset-quantile within array normalization) method of the minfi R package [162]. DNA methylation values (beta values (β)), were recorded for each locus in each sample. For statistical analyses β-values were transferred to M-values [163].

To adjust for potential differences in cell composition of whole blood samples, a similar approach as previously described was applied [164, 165]. In sum, cell type specific regions of differential methylation were derived from publically available data of FACS sorted cells [164] and resulting information on CD4+, CD8+ T-cells, natural killer cells, B cells and monocyte proportions were used as confounders in the subsequent regression analyses. All analysis of 450k data was conducted by Konrad Grützmann.

2.2.1.8. DNA methylation via MassARRAY

For the evaluation of results obtained by the 450k array analysis, a quantitative DNA methylation of the found target gene *mesoderm specific transcript (MEST)* was assessed via the MassARRAY system in cooperation with the group of Saskia Trump of the Department of Environmental Immunology and the group of Christoph Plass at the DKFZ Heidelberg. MassARRAY is a mass spectrometry based approach for targeted methylation assessment. Therefore DNA methylation analysis of the human *MEST* promoter in the LINA cohort at the time of birth and human *in vitro* derived adipocytes was performed using Sequenom's MassARRAY platform as described previously [166]. A PCR amplicon was designed on the reverse strand covering chr7:130,132,068-130,132,287 including cg17580798 (*hMEST* forward primer: aggaagagagTTTAGAGGTAGTTTTAGTTYGG, reverse primer: cagtaatacgactcactatagggagaaggctCCRCTACTAACCAACTCTAC with an annealing temperature of 52 °C). For further validation gDNA extracted from murine adipose tissue was also bisulfite converted using the EZ DNA Methylation kit and subjected to MassARRAY

analysis. Genome coordinates of the human *MEST* promoter were lifted over to the mouse genome assembly mm10 and corresponding primer pairs on the forward strand were designed (*Mest* forward primer: aggaagagagAGGAGGTTTGTGTTTTTAATG, reverse: cagtaatacgactcactatagggagaaggctCACCCACTTCTTTTCTACC, annealing temperature: 60 °C, amplicon coordinates: chr6:30,737,347-30,737,692).

Personal contributions included the primer design and assay preparation as well as final analysis in cooperation with Loreen Thürmann and the corresponding in vitro adipocyte culture. Mass spectrometry and initial quality control was done by the group of Christoph Plass.

2.2.1.9. Gene expression analysis of MEST in the LINA study cohort

Changes in DNA methylation must not consequently lead to changes in gene expression. Therefore gene expression of the *MEST* gene was performed by the group of Mario Bauer of the Department of Environmental Immunology as reported recently [167]. In brief, intron-spanning primer pairs were designed and UPL probes were selected by the Universal Probe Library Assay Design Center (http://qpcr.probefinder.com/organism.jsp.) (Table 1). For pre-amplification all primer pairs (final concentration, 50 nM), 5 µl of cDNA and 2x PreAmp Master Mix were pooled. The pre-amplification was carried out by 95°C for 10 min, followed by 14 cycles of 95 °C for 15 sec and 60 °C for 4 min on a LightCycler 480.

Table 1: Primer for gene expression analysis in LINA

Gene	Forward	reverse	UPL probes
hMEST	5'-ATCGTGGAAGCGCTTTG	5'-GACCAGATCGATTCTGCTTGTA	UPL50
hGAPD	5'-GCTCTCTGCTCCTCCTGTTC	5'-ACGACCAAATCCGTTGACTC	UPL60
hGUSB	5'-CGCCCTGCCTATCTGTATTC	5'-TCCCCACAGGGAGTGTGTAG	UPL57

qPCRs of 1:5 diluted with TE buffer pre-amplified templates were performed following manufacturer's instructions for UPL assays. Briefly, a 10x assay mix for each individual assay was prepared that included 2 µM of each forward and reverse primer, 1 µM UPL probe and 0.025% Tween-20; and 5 µl of the mix was loaded into the assay inlets of the 96.96 Dynamic Array. Also 5 µl of the following solution was dispensed into the sample inlets: 2.5 µl of PreAmp sample in 1.1x of FastStart Universal Probe Master Mix. The qPCR program consisted of 2 min at 50 °C, 10 min at 95 °C, followed by 35 cycles of 95 °C for 15 sec, 1 min at 60 °C and 70 °C for 5 sec. All reactions were performed in triplicates. Gene expression values were determined by using the 2-ΔΔCT method [168]. *GAPD* and *GUSB* were used as reference genes and normalized to the lowest measured value.

2.2.1.10. Statistical analysis for epidemiological studies

LINA study data was generally processed with STATISTICA for Windows, Version 12. Equal distribution of parameters in the analysed sub-cohorts and the entire LINA cohort was determined by the chi squared test. Since most analysed parameters were not normally distributed, tests for non-parametric data were used (Spearman correlation, Man-Whitney-U-Test). Log-transformation for normal distribution was applied before assigning non-normally distributed data to statistical models that require normal distribution (multiple regression, logistic regression, mediator-models). Longitudinal associations were assessed by applying a generalized estimating equation (GEE) model in IBM SPSS version 22 with BMI z-scores as dependent variable and unstructured correlation matrix. Mediator models were analysed using the PROCESS tool in IBM SPSS version 22, using model 4 with 1000 bootstrap samples to determine bias corrected bootstrap confidence intervals. All models were adjusted to weight related confounders, as indicated in the corresponding result sections. A principal factor analysis with oblique rotation was conducted on 10 questionnaire items assessing neighborhood strains to extract potential underlying scales. The extracted scales together with different socio-demographic-factors were assessed by linear regression for their impact on maternal perceived stress.

450k data were analysed and processed using the R packages minfi and qqman by the bio-informatics service. To determine differentially methylated CpG's [163] logistic regression models on M-values were adjusted to previously identified and available factors with an impact on cord blood methylation, including cell composition, the maternal vitamin D level [145], prenatal benzene exposure, maternal smoking [169] and maternal stress during pregnancy [144].

In general, *p*-values <0.05 were considered to be significant. Bonferroni post-hoc correction of *p*-values for large data sets was applied for metabolome, maternal stress and epigenome analyses.

2.2.2. In vitro studies

2.2.2.1. In vitro adipogenesis of human mesenchymal stem cells

Human adipose-derived mesenchymal stem cells were purchased from ATCC®, sub-cultured, frozen from passage 2 onwards and stored in liquid nitrogen until needed. MSCs were thawed and seeded in cell culture flasks at 5000 cells/cm^2 in MSC Basal Medium (BM) and maintained at 37 °C, 5 % CO_2 and 95 % humidity. Medium was changed every other day until MSCs reached 70 % confluency. For adipocyte differentiation MSCs of passages 3 to 7 were washed once with D-PBS and treated with Trypsin/EDTA. After 3 min at 37 °C and addition of the same volume of Trypsin neutralization solution, the cell suspension was transferred into a 15 ml tube and centrifuged at 1250 rpm for 10 min at room temperature

(RT). After discarding the supernatant, cells were re-suspended in BM and seeded at 9600 cells/cm^2 in a 96 well microelectrode plate from ACEA Biosciences Inc. (E-Plate 96 View PET, gold electrodes, 400 µm window for microscopy, well area 0.2 cm^2) for real-time monitoring with the impedance based xCELLigence SP or MP station (see Chapter 2.2.2.2).

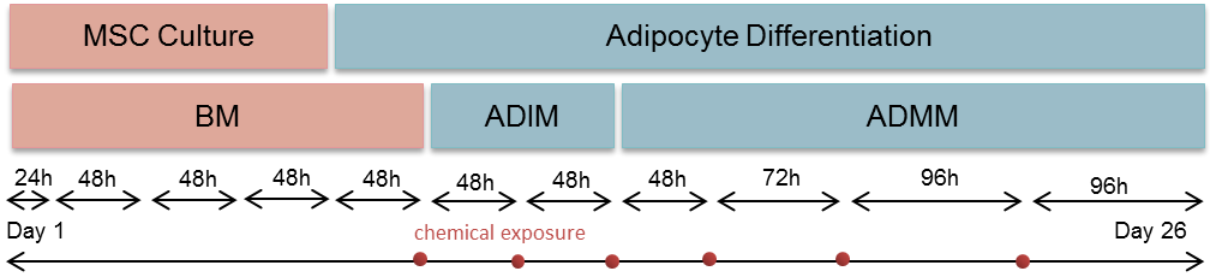


Figure 11: Adipocyte differentiation protocol of human mesenchymal stem cells.

MSCs were grown to 70 % confluency in the 96 wells when adipocyte differentiation was initiated after 48 h. For initiation cells were washed once with D-PBS and fed with Adipocyte Differentiation Initiation Medium (ADIM). ADIM was exchanged after 48 h and after another 48 h medium was changed to Adipocyte Maintenance Medium (ADMM). Further on, medium was exchanged every 48 h to 96 h according to the manufacturer's instructions (Figure 11). Adipocyte differentiation ended after a total of 12 days with ADMM.

Cells were treated with 10 or 50 µM BPA or 0.5, 1, 10 or 50 µM of either MeP, EtP, nPrP or nBuP (working solution in 0.05% ethanol) during the differentiation period (see "chemical exposure" in Figure 11). Pollutant exposure was refreshed at every medium change.

2.2.2.2. Real-time monitoring of adipogenesis with the xCELLigence System

The differentiation process was monitored in real-time with the impedance based xCELLigence System from Roche® on a microelectrode 96 well E-Plate (Figure 12). According to the manufacturer's instructions the system was initialized prior to cell seeding with 50 µl BM and equilibrated at 37°C for 30 min. The plate was placed into the MP working station in a humidified incubator at 37 °C and 5 % CO_2 and a background (blank) measurement was taken. Cells were seeded in triplicates or quadruplicates and allowed to settle for 30 min before starting the measurement. The growth rate was monitored every 10 min by electrical impedance measurements that were paused for media changes.

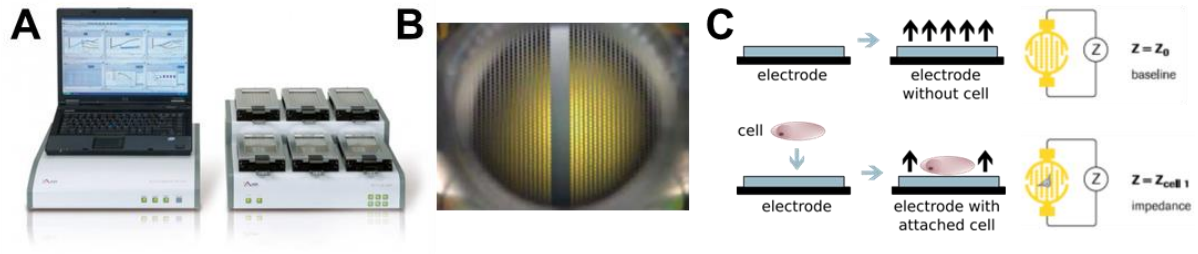


Figure 12: Impedance based real-time monitoring. (A) xCELLigence RTCA MP station. (B) Well of an E-Plate 96 View PET with gold electrodes and 400 µm window for microscopy. (C) Schematic figure of impedance measurement on microelectrodes on the well bottom of an E-Plate 96. Figures adapted by ACEA Biosciences [170].

2.2.2.3. Oil red staining for triglyceride storage

For oil red staining, differentiated adipocytes were fixed with 10 % formaldehyde for 1 min at RT, washed 3 times with PBS and stained with a filtered Oil Red O solution (0.3 % (w/v) in 60% iso-propanol) for 30-45 min at RT. Afterwards, wells were washed 3 times with aqua dest. and viewed under bright field conditions with an inverted microscope or red colour absorption at 510 nm was measured with the Synergy Microplate Reader.

2.2.2.4. Assessment of adipokines in cell culture supernatant via ELISA

Human adiponectin and leptin protein concentrations were assessed in cell culture supernatants of hMSC derived adipocytes by commercially available ELISA kits from Invitrogen according to the manufacturer's instructions. In brief, 100 µl of standard and undiluted samples were applied to pre-coated wells of a 96-well plate and incubated for 1-2 h at RT with 100 µl biotinylated anti-adiponectin /anti-leptin antibody. Afterwards wells were washed 4 times with washing buffer and 100 µl detection/ streptavidin working solution was added to each well and incubated for 0.5-1 h at RT. Wells were washed again for 4 times with washing buffer and 100 µl substrate solution/ stabilized chromogen were added to each well and incubated for 20-30 min at RT in the dark. Colour reaction was stopped with 100 µl stop solution and absorption was measured at 450 nm with the Synergy Microplate Reader.

2.2.2.5. Assessment of cytotoxicity

Cytotoxicity of exposure chemicals was assessed prior to adipocyte differentiation via a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyoltetrazoliumbromid) assay. Therefore, MSC were seeded in triplicates in a 96 well-plate and settled for 48 h. Afterwards basal medium was changed and cells were exposed to 0.5-100 µM of chemical solution (BPA, MeP, EtP, nPrP, nBuP) or a solvent control (0.05 % ethanol) for another 48 h. 10 µM MTT solution was added and incubated for 4 h at 37°C. To stop the reaction 100 µl stop solution was applied and incubated over night at 37°C. Extinction was measured at 570 nm with the Synergy

Microplate Reader. To assess long-term cytotoxicity of exposure chemicals, a MTT assay was also applied after adipocyte differentiation with exposure to 0.5-50 µM of chemical solutions.

2.2.2.6. Molecular biological analysis

2.2.2.6.1. RNA isolation and cDNA synthesis

RNA isolation was carried out with the MagNA Pure LC Isolation Kit and the MagNA automated pipetting robot from Roche according to the manufacturer's instructions. In brief, cells were harvested in 720 µl Magna Lysis Buffer and stored at -80 °C. For total RNA isolation frozen cell samples were thawed for 30 min and meanwhile the MagNA automated pipetting robot was set up. The program "external blood Hs isolation" was used and total RNA was eluted in 50 µl Elution Buffer. RNA concentration and purity was determined using a Tecan's Nanoquant infinite 200 system.

cDNA synthesis was carried out with 200 ng RNA template. 0.5 µl Random Hexamer Primer and 0.5 µl Oligo-dT-Primer were added to 10 µl RNA template and incubated for 5 min at 70 °C for primer annealing. Meanwhile the master mix with 4 µl AMV Reverse Transcriptase 5× Buffer, 3 µl Aqua dest., 2 µl dNTPs, 0.5 µl Recombinant RNasin Ribonuclease Inhibitor and 0.5 µl AMV Reverse Transcriptase for each probe was prepared and 10 µl master mix were added to each probe. cDNA synthesis took place at 42 °C for 1 h. Afterwards total cDNA was diluted 1:5 with aqua dest. and stored at -80 °C.

2.2.2.6.2. Gene expression analysis

Gene expression was assessed by real-time quantitative PCR using a SYBRgreen based assay. 4 µl 1:5 cDNA template were added to 8 µl PCR master mix containing 1.2 µl NH4-Buffer, 0.6 µl MgCl2, 0.96 µl dNTPs, 3.95 µl Aqua dest., 0.38 µl primer (each forward and reverse), 0.46 µl SYBRgreen and 0.06 µl Taq polymerase in a white 96-well microtiter plate. The plate was sealed, briefly centrifuged and the PCR was carried out in the Roche®LightCycler480 system. The PCR program is shown in Table 2.

Table 2: Protocol for real-time quantitative PCR

repeat	Step	temperature	time
1	initial denaturation	95 °C	10 min
40	denaturation	95 °C	10 sec
	annealing	60-58 °C	20 sec
	elongation	72 °C	20 sec
1	melting curve		

2.2.2.7. Reporter gene assays for receptor activation

To get a deeper mechanistic understanding on paraben action, activation of the oestrogen, androgen, progesterone, glucocorticoid receptor and PPARγ was conducted in the group of Beate Escher from the Department of Cell Toxicology at the UFZ. In brief, receptor activation and cell toxicology of parabens was asses using the GeneBLAzer® Beta-lactamase Reporter Technology which uses a Fluorescence Resonance Energy Transfer (FRET) substrate that generates ratiometric reporter response with minimal experimental noise and is described in detail elsewhere [171].

2.2.2.8. Statistical analysis for in vitro studies

In vitro study data was processed with GraphPad PRISM 7 for Windows. *P*-values were derived by Student's t-test when comparing two groups and by two-way ANOVA with appropriate post-hoc test (Dunnett, Tukey) when comparing multiple groups. *P*-values <0.05 were considered to be significant.

2.2.3. In vivo mouse study

For validation of results regarding prenatal BPA exposure in LINA, a transgenerational mouse model was applied by the group of Tobias Polte from the Department of Environmental Immunology. BALB/c mice were bred and maintained in the animal facility at the University of Leipzig and housed under conventional conditions with 23°C room temperature, 60% humidity and 12 h day/night rhythm. Mice were fed a phytoestrogen-free diet and received water ad libitum from custom build glass bottles to avoid BPA contamination. The Committee on Animal Welfare of Saxony approved animal protocols used in this study.

Dams were exposed to 5 µg/ml BPA via drinking water one week before mating until delivery of the offspring. Beginning one week after delivery pups were weighed two times per week and a mean weight per week was calculated for each mouse. At the end of the observation period (10 weeks) whole body composition (fat mass and lean mass) was determined in awake mice based on nuclear magnetic resonance technology using an EchoMRI700™ instrument (Echo Medical Systems, Houston, TX, USA) in the offspring of control and BPA exposed dams. Further, DNA-methylation analyses (MassARRAY) as well as gene expression analyses was performed in visceral fat tissue as described above in 10-week-old offspring (personal contribution).

3. RESULTS

3.1. General study characteristics of the LINA cohort study

The LINA study cohort included 629 mother-child pairs that were recruited during pregnancy and followed up annually since then. Although the study was originally designed to investigate the onset of allergic diseases, also many factors describing weight development have been assessed. So are information about birth outcomes as summarized in Table 3. There was an equal distribution of male and female children. Most mothers gave birth spontaneously (75 %) at 37-40 weeks of gestation (62 %) to children with a birth weight between 3000 g to 4000 g (69 %).

Table 3: General study characteristics of the total LINA cohort regarding birth outcomes and factors that may affect birth outcomes

	Entire LINA cohort n (%), n = 629[a]
Gender of the child	
Female	302 (48.0)
Male	327 (52.0)
Birth weight	
≤3000g	123 (19.6)
>3000-3500g	242 (38.5)
>3500-4000g	192 (30.6)
>4000g	71 (11.3)
Mode of delivery	
Spontaneous	471 (74.9)
C-section	132 (21.0)
Others	7 (1.1)
Gestational week at delivery	
< 37 weeks	25 (4.0)
37 – 40 weeks	389 (62.0)
>40 weeks	214 (34.0)
Smoking during pregnancy	
Never	534 (84.9)
Occasionally	47 (7.4)
Daily	48 (7.6)
Pregnancy IgE level	
<20 kU/L	268 (42.6)
20 – 100 kU/L	238 (37.8)
>100 kU/L	123 (19.6)

a – n may be different from total n due to missing data

Furthermore, different characteristics regarding individual prenatal body conditions, like for example pregnancy IgE level and maternal smoking were assessed. The majority of mothers

did not smoke during pregnancy (85 %) but about 8 % of mothers indicated to smoke on a daily basis.

Moreover, information about the socio-economic status was collected (see Table 4). The study was mostly composed of high educated families with an intermediate household income and stable family structure, which is associated with a high socio-economic status. Also high numbers for long breastfeeding duration (40.2 %) and a late introduction of solid food (52.7 %) point to a higher economic-status of the participants.

Table 4: General study characteristics of the total LINA cohort regarding the socio-economic status and early feeding behaviour

	Entire LINA cohort n (%), n = 629[a]
Parental school education [b]	
Low	16 (2.5)
Intermediate	144 (22.9)
High	469 (74.6)
Household income per month	
<2000€	240 (38.2)
2000€ - 4000€	308 (49.0)
>4000€	42 (6.7)
Separation/divorce [c]	
Yes	25 (4.0)
No	169 (26.9)
Breastfeeding exclusive	
1-3 month	87 (13.8)
1-6 month	155 (24.6)
1-12 month	253 (40.2)
Introduction of solid food	
1-3 month	23 (3.9)
4-6 month	251 (43.5)
7-12 month	305 (52.7)

[a] n may be different from 629 due to missing data

[b] low = 8 years of schooling ('Hauptschulabschluss`); intermediate = 10 years of schooling (`Mittlere Reife`); high = 12 years of schooling or more (`(Fach-)hochschulreife')

[c] assessed retrospectively at children's age of 3 years

3.1.1. Distribution of children's BMI z-scores in the LINA cohort

Children's weight and height were assessed each year during clinical visits or acquired from the regular preventive medical check-ups (U-examinations) asked for in the questionnaires. Due to the low number of obese children (BMI z-score ≥2) all overweight (BMI z-score ≥1) and obese children were classified together as overweight. Prevalence for overweight development between year 1-8 was 15.1 % with lowest prevalence at year 1 (8.7 %) and highest prevalence at year 2 (27.2 %) (Table 5). Average prevalence for underweight was 13.2 %, being lowest in year 3 (6.0 %) and highest in year 1 (22.3 %).

Table 5: Weight status distribution of children within the LINA study cohort. Total numbers of underweight (BMI z-score <1), normal weight (BMI z-score -1 to <1) and overweight/obese (BMI z-sore ≥1) children up to the age of 8 years

Year		**0**	**1**	**2**	**3**
	n (%)	n=609	n=584	n=500	n=473
Underweight		87 (14.3)	130 (22.3)	30 (6.0)	47 (9.9)
Normal weight		405 (66.5)	403 (69.0)	334 (66.8)	342 (72.3)
Overweight		117 (19.2)	51 (8.7)	136 (27.2)	84 (17.8)
Year		**4**	**5**	**6**	**8**
	n (%)	n=432	n=385	n=303	n=256
Underweight		43 (10.0)	49 (12.7)	45 (14.8)	43 (16.8)
Normal weight		335 (77.5)	291 (75.6)	223 (73.6)	170 (66.4)
Overweight		54 (12.5)	45 (11.7)	35 (11.6)	43 (16.8)

BMI z-scores were highly correlated throughout childhood (Table 6). However R-values for BMI z-scores increased with aging by about 10 % each year. Therefore the likelihood of keeping or gaining overweight increases continuously in the investigated time period and by the age of 3 years BMI z-scores manifests with more than 50 % of children will either keep their BMI z-score or gain more weight.

Table 6: Correlation Matrix of BMI z-scores during early childhood. Shown are Spearman correlation coefficients (R) with $p<10^{-4}$

BMI z-score at year	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**8**
0		0.27	0.27	0.32	0.26	0.28	0.25	0.21
1			0.64	0.62	0.62	0.54	0.53	0.43
2				0.77	0.71	0.64	0.56	0.50
3					0.82	0.74	0.72	0.64
4						0.86	0.82	0.70
5							0.87	0.80
6								0.86
8								

Next to this general study characterisation of the longitudinal overweight development, this thesis aimed to investigate the impact of a broad spectrum of different potential risk factors in the pre- and early postnatal period on childhood overweight development within different subgroups of the LINA study, as detailed described in project A, B_1, B_2 and C as followed.

3.2. Impact of the maternal cytokine status during pregnancy on overweight development in children and their metabolome (Project A)

The following results for project A have been published in Englich *et al.* (2017) "*Maternal cytokine status may prime the metabolic profile and increase risk of obesity in children."* in the *International Journal of Obesity*.

The maternal immune status during pregnancy has been shown to influence birth outcomes and weight development in children. However most recent studies focused on the impact of inflammatory immune markers like IL6, IFNγ and TNFα but did not investigate the effects of the maternal adaptive immune system. Therefore, alterations in the maternal adaptive immune system (including IL4, IL5 and IL13 level) during pregnancy and their influence on weight development in children up to 3 years after birth within the LINA study cohort is investigated in this project.

Personal contributions include the project idea and design, preparation of the initial manuscript, as well as all statistical analyses regarding the association of maternal cytokine status with children's BMI z-scores and children's metabolome parameters. Contributions of others include cytokine and metabolome analyses as indicated in the corresponding methods descriptions.

Complete data on maternal cytokine status during pregnancy, body weight development until year 3 and confounding variables was available for 407 children of the LINA study. There were no differences regarding gender, birth weight, birth season, gestational week at delivery, parental school education level, duration of breastfeeding, introduction to solid food, smoking during pregnancy and pregnancy IgE level between the total LINA cohort (n=629) and the analysed sub-cohort (n=407; see Table 7). Hence, all following analyses for project A were based on this sub-cohort. Underweight children were not considered for this analysis and only results comparing normal weight and overweight are presented.

3.2.1. Maternal cytokines and offspring BMI

Range and distribution of maternal blood cytokine levels of normal and overweight children are displayed in Table 8. Median values of IL4 and IL13 were significantly lower in the overweight group at year 1 and year 2, while IL13 and IFNγ levels were significantly lower in the overweight group at year 3. There was no difference in LPS-stimulated/ innate cytokine levels like IL6, IL10 and TNFα and no association of maternal cytokine level with BMI z-scores in later years was found (data not shown).

Table 7: Study characteristics of the total study cohort and the analysed sub-cohort for project A

	Entire LINA cohort n (%), n = 629[a]	**Analysed sub-cohort** n (%), n =407	***χ²-test***[b]
Gender of the child			0.832
Female	302 (48.0)	203 (49.9)	
Male	327 (52.0)	204 (50.1)	
Birth weight			0.990
≤3000g	123 (19.6)	75 (18.4)	
>3000-3500g	242 (38.5)	162 (39.8)	
>3500-4000g	192 (30.6)	128 (31.4)	
>4000g	71 (11.3)	42 (10.3)	
Birth Season			0.806
Summer	346 (55.0)	222 (54.5)	
Winter	269 (42.8)	185 (45.5)	
Gestational week at delivery			0.997
< 37 week s	25 (4.0)	17 (4.2)	
37 – 40 weeks	389 (62.0)	252 (61.9)	
>40 weeks	214 (34.0)	138 (33.9)	
Smoking during pregnancy			0.374
Never	534 (84.9)	369 (90.7)	
Occasionally	47 (7.4)	24 (5.9)	
Daily	48 (7.6)	14 (3.4)	
Parental school education [c]			0.661
Low	16 (2.5)	5 (1.2)	
Intermediate	144 (22.9)	80 (19.7)	
High	469 (74.6)	322 (79.1)	
Household members			0.985
2	33 (5.5)	23 (5.7)	
3	365 (60.7)	251 (61.7)	
>4	203 (33.8)	133 (32.7)	
Breastfeeding exclusive			0.679
1-3 month	87 (13.8)	58 (14.2)	
1-6 month	155 (24.6)	115 (28.3)	
1-12 month	253 (40.2)	234 (57.5)	
Introduction of solid food			0.728
1-3 month	23 (3.9)	12 (2.9)	
4-6 month	251 (43.5)	159 (39.1)	
7-12 month	305 (52.7)	236 (58.0)	
Pregnancy IgE level			0.964
<20 kU/L	268 (42.6)	169 (41.5)	
20 – 100 kU/L	238 (37.8)	152 (37.3)	
>100 kU/L	123 (19.6)	86 (21.1)	

[a] n may be different from 629 due to missing data

[b] calculated using the chi squared test for cross relationship

[c] low = 8 years of schooling (‘Hauptschulabschluss`); intermediate = 10 years of schooling (`Mittlere Reife`); high = 12 years of schooling or more (`(Fach-)hochschulreife’)

Table 8: Distribution of maternal blood cytokine levels in normal and overweight children. Pregnancy cytokine concentrations (pg/l) are presented as median values with inter-quartile range in brackets for normal weight and overweight children.

	Year 1			Year 2			Year 3		
	normal weight (n=278)	overweight (n=35)		normal weight (n=274)	overweight (n=107)		normal weight (n=298)	overweight (n=68)	
PHA-stimulated (adaptive)									
IFNγ	428.0 (228.8,928.9)	334.8 (160.3,661.1)		432.0 (231.1,872.5)	366.6 (195.3,941.0)		**432.0 (231.1,883.6)**	**258.9 (171.5,804.3)**	*
IL4	**15.1 (10.7,22.1)**	**12.3 (9.4,14.7)**	**	**15.6 (11.2,21.7)**	**13.3 (9.4,19.0)**	*	15.0 (10.7,21.6)	13.1 (10.2,17.7)	
IL5	3.2 (1.5,4.0)	2.9 (1.5,3.7)		3.2 (2.1,4.0)	3.3 (1.5,4.0)		3.2 (1.5,4.0)	3.4 (1.5,3.9)	
IL13	**22.1 (14.8,34.3)**	**16.3 (13.9,22.7)**	*	**21.8 (15.4,34.3)**	**18.6 (13.3,27.4)**	*	**21.8 (14.8,34.6)**	**18.0 (13.6,25.4)**	**
LPS-stimulated (innate)									
TNFα	1729 (1296,2653)	2474 (1331,3454)		1761 (1235,2784)	1865(1409,3142)		1760 (1250,2809)	1952 (1400,2937)	
IL6	9809 (6937,12675)	8693 (6451,145957)		10023 (6771,12955)	9159(7458,12907)		10023 (6937,12821)	8400 (66676,12284)	
IL8	2912 (2250,4259)	3184 (2057,5387)		2857 (2223,4259)	3030(2144,4184)		2882 (2223,4150)	3072 (2162,4850)	
IL10	12.7 (6.7,24.3)	13.8 (7.9,33.5)		12.4 (6.5,22.8)	11.6(7.6,30.7)		12.0 (6.8,23.8)	15.1 (7.8,33.5)	

Significantly different values from Mann-Whitney U-test are presented in bold with *p<0.05 and **p<0.01

To adjust for weight related confounders, logistic regression models comparing normal and overweight children regarding maternal pregnancy cytokine level were applied (Figure 13A and Table S1A).

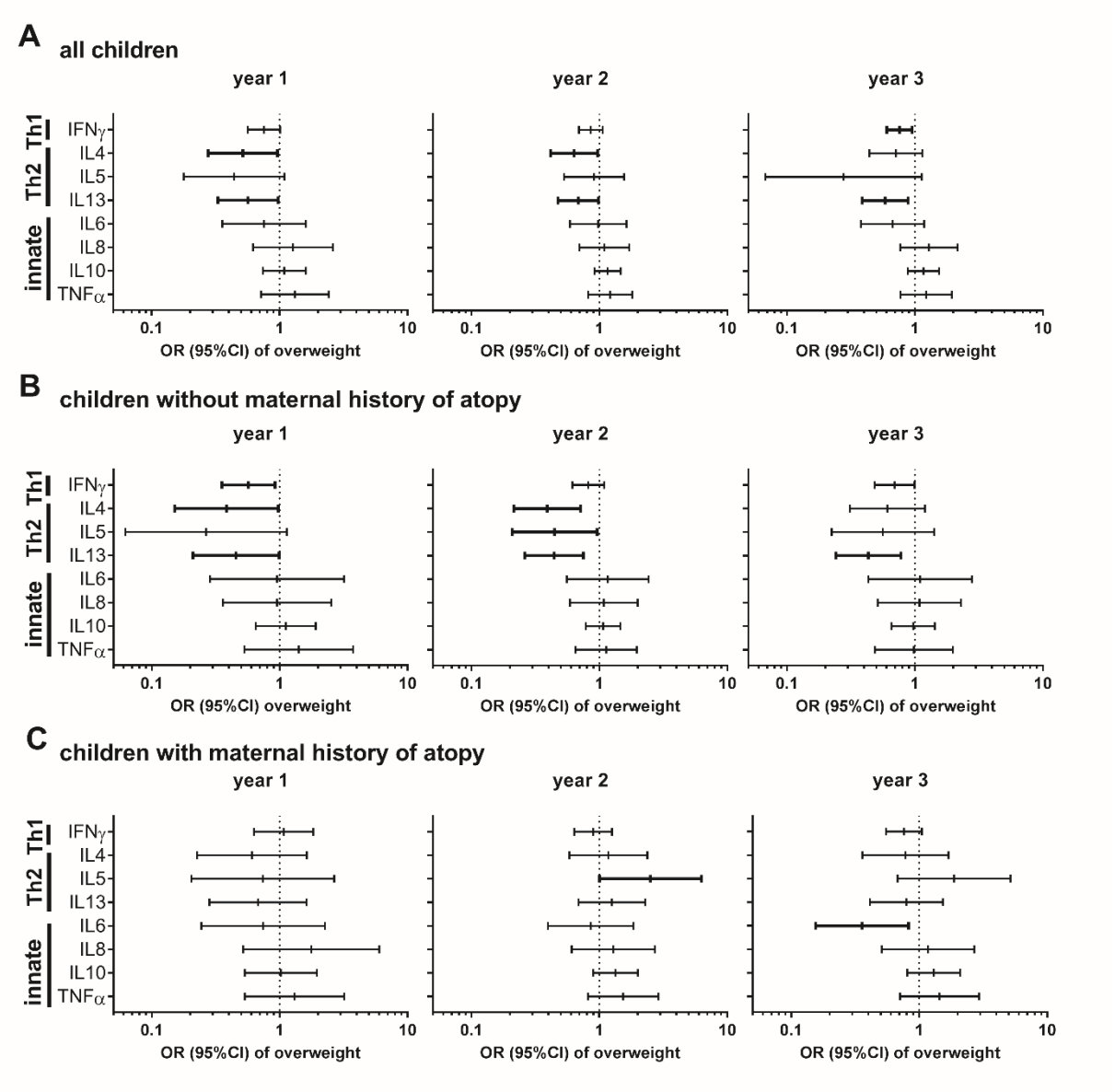


Figure 13: Effect of maternal cytokine concentrations during pregnancy on weight development in children. (A) Influence of prenatal cytokine exposure on overweight development until the age of 3 years. Shown are odds ratios with 95% confidence interval (OR, 95 % CI) in a logistic regression model adjusted for gender, birth weight, birth season, early delivery, smoking during pregnancy, parental school education, household members, breastfeeding, introduction of solid food and pregnancy IgE level. Significantly associations are presented in bold (p<0.05). (B) Logistic regression model only considering children without maternal history of atopy. (C) Logistic regression model only considering children with maternal history of atopy.

Models were adjusted to confounding parameters that have been associated to overweight development (birth weight, early delivery, smoking during pregnancy, parental school

education, household members, breastfeeding and introduction of solid food) and maternal cytokine status (birth season and pregnancy IgE level) in earlier studies. High maternal IL4 levels were associated with a reduced risk for overweight development at year 1 (adj.OR: 0.52, 95%CI (0.28,0.97)) and year 2 (adj.OR: 0.63, 95% CI (0.42,0.97)). High maternal IL13 levels reduced the risk for overweight development persistently from year 1 to year 3 (adj.OR year 1: 0.57, 95% CI (0.33,0.97), adj.OR year 2: 0.68, 95% CI (0.47,0.98), adj.OR year 3: 0.58, 95% CI (0.30,0.88)) and high IFNγ levels in pregnancy were associated with reduced risk for overweight development at year 3 only (adj.OR: 0.76, 95% CI (0.60,0.95)). There was no association of children's overweight development with maternal IL5, IL6, Il8, IL10 and TNFα levels.

As the maternal adaptive immune status is highly influenced by atopic diseases, it was differentiated between children with and without maternal history of atopy. Interestingly, the found association for IL4 and IL13 were only found in children without maternal history of atopy (Figure 13B and Table S1B) with the most persistent effect for high maternal IL13 level. High pregnancy IL13 levels in non-atopic mothers reduced the risk for overweight development in their children up to the age of 3 years (adj.OR year 1: 0.46, 95% CI (0.21,0.99), adj.OR year 2: 0.44, 95% CI (0.26,0.75), adj.OR year 3: 0.43, 95% CI (0.24,0.78)). Children of atopic mothers had an increased risk for being overweight at 2 and 3 years of age when exposed to higher maternal IL5 (adj.OR: 2.52, 95% CI (1.00,6.3)) and lower IL6 level (adj.OR: 0.36, 95% CI (0.16,0.82)), respectively (Figure 13C and Table S1C**Fehler! Verweisquelle konnte nicht gefunden werden.**).

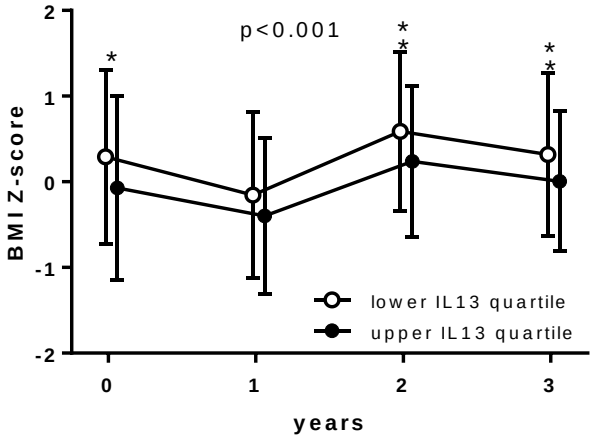


Figure 14: Weight distribution of children exposed to high (4th quartile) and low (1st quartile) prenatal IL13 levels from birth to age 3. Shown are BMI z-scores as mean ± SD with **p*<0.05, ***p*<0.01 derived from Student's t-test and *p*<0.001 between groups for the entire observation period from two-way ANOVA.

As the most dominant and long-term stable association was seen for IL13, the mean BMI z-score for children exposed to low (1th quartile) or high (4th quartile) maternal IL13 levels was calculated and found significantly lower from birth until the age of three years for children exposed to high maternal IL13 levels with p=0.015 (birth), p=0.007 (year 2) and p=0.012 (year 3), respectively (Figure 14). There was no association found in children age 4-6 years of age.

3.2.2. Maternal cytokines and offspring metabolic profile

Furthermore, the potential priming effect of maternal cytokines on infant's metabolic profile of the one-year-old children was assessed. Following correction for multi-testing (p<4.63*10^{-4}) a positive association between maternal IL13 and acylcarnitine levels of C10, C10:1, C12-DC, C3 and C4 was found, whereas maternal IL10 correlated negatively with acylcarnitines (C0, C10, C12-DC, C18:1, C3, C4) (Table 9).

Other metabolic parameters like amino acids, lyso-phosphatidylcholines (lysoPC), phosphatidylcholines (PC), sphingolipids (SM) and sugars were not correlated to maternal cytokine levels (Figure S1).

3.2.3. Offspring BMI and metabolic profile

In addition to the association of maternal IL13 levels with acylcarnitines at year 1, increased BMI z-scores at year 1 were associated with low levels of acylcarnitines at the same age (C10:1 p=0.0472; C14:1 p=0.0021; C18:1 p=0.0005; C2 p=0.0217). Acylcarnitines C0 and C2 at year 1 were also negatively correlated with the BMI z-score at year 2 (C0 p=0.005 C2 p=0.020). There was no association of acylcarnitines at year 1 with the BMI z-score at year 3.

Further, a potential mediating effect of acylcarnitines on overweight development in response to low maternal IL13 levels in the prenatal period was addressed. When acylcarnitines were introduced in the logistic regression model as a confounding factor, the association between low maternal IL13 and overweight development remained significantly with no significantly effect for the acylcarnitines. Applying a mediator analysis (bootstrapping mediator analysis with the PROCESS tool in SPSS) a mediating effect of acylcarnitines (C10:1, 95% CI (-0.197,0.107); C14:1, 95% CI (-0.140,0.064); C18:1, 95% CI (-0.272,0.011) and C2, 95% CI (-0.114,0.010)) could not be approved.

Table 9: Correlation of maternal cytokine levels during pregnancy and acylcarnitines of children at year 1. Shown are Spearman correlation coefficients (R) and *p*-values for (A) PHA stimulated cytokines and (B) LPS stimulated cytokines

(A)	**PHA stimulated (adaptive)**							
acylcarnitines	**IFNγ**		**IL4**		**IL5**		**IL13**	
	R	***p*-value**	**R**	***p*-value**	**R**	***p*-value**	**R**	***p*-value**
C0[a]	0.10	$6.34x10^{-2}$	0.15	$8.85x10^{-3}$	-0.10	$7.33x10^{-2}$	0.18	$1.63x10^{-3}$
C10	0.15	$9.13x10^{-3}$	0.14	$1.16x10^{-2}$	-0.16	$3.75x10^{-3}$	0.23	**$4.51x10^{-5}$**
C10:1	0.11	$5.10x10^{-2}$	0.15	$7.28x10^{-3}$	-0.17	$3.14x10^{-3}$	0.22	**$9.36x10^{-5}$**
C12-DC[b]	0.11	$4.73x10^{-2}$	0.18	$1.23x10^{-3}$	-0.17	$2.48x10^{-3}$	0.29	**$2.38x10^{-7}$**
C14:1	0.10	$7.01x10^{-2}$	0.03	$6.06x10^{-1}$	-0.15	$8.88x10^{-3}$	0.12	$3.75x10^{-2}$
C18:1	0.10	$8.88x10^{-2}$	0.15	$7.19x10^{-3}$	0.01	$8.24x10^{-1}$	0.19	$6.19x10^{-4}$
C2	0.15	$6.22x10^{-3}$	0.11	$5.84x10^{-2}$	0.04	$4.57x10^{-1}$	0.10	$7.47x10^{-2}$
C3	0.08	$1.58x10^{-1}$	0.16	$3.98x10^{-3}$	-0.07	$2.20x10^{-1}$	0.22	**$1.10x10^{-4}$**
C4	0.10	$6.78x10^{-2}$	0.17	$2.39x10^{-3}$	-0.13	$1.76x10^{-2}$	0.24	**$1.18x10^{-5}$**
(B)	**LPS stimulated (innate)**							
acylcarnitines	**IL6**		**IL8**		**IL10**		**TNFα**	
	R	***p*-value**	**R**	***p*-value**	**R**	***p*-value**	**R**	***p*-value**
C0[a]	0.10	$6.34x10^{-2}$	-0.01	$8.90x10^{-1}$	-0.23	**$2.74x10^{-5}$**	-0.14	$1.35x10^{-2}$
C10	0.15	$9.13x10^{-3}$	0.04	$4.54x10^{-1}$	-0.19	$5.87x10^{-4}$	-0.01	$9.04x10^{-1}$
C10:1	0.11	$5.10x10^{-2}$	0.04	$4.40x10^{-1}$	-0.18	$1.38x10^{-3}$	-0.05	$3.75x10^{-1}$
C12-DC[b]	0.11	$4.73x10^{-2}$	-0.01	$8.17x10^{-1}$	-0.29	**$1.12x10^{-7}$**	-0.07	$2.21x10^{-1}$
C14:1	0.10	$7.01x10^{-2}$	0.03	$5.48x10^{-1}$	-0.11	$5.68x10^{-2}$	0.04	$5.04x10^{-1}$
C18:1	0.10	$8.88x10^{-2}$	-0.09	$1.21x10^{-1}$	-0.23	**$3.01x10^{-5}$**	-0.11	$4.19x10^{-2}$
C2	0.15	$6.22x10^{-3}$	0.11	$5.20x10^{-2}$	0.00	$9.37x10^{-1}$	-0.10	$8.28x10^{-2}$
C3	0.08	$1.58x10^{-1}$	-0.02	$7.15x10^{-1}$	-0.22	**$7.95x10^{-5}$**	-0.19	$8.85x10^{-4}$
C4	0.10	$6.78x10^{-2}$	0.01	$9.25x10^{-1}$	-0.24	**$1.88x10^{-5}$**	-0.12	$3.53x10^{-2}$

Significantly different values are presented in bold with $p<4.63x10^{-4}$

a – free carnitine

b - Dodecanedioylcarnitine

3.3. Impact of prenatal exposure to Bisphenol A on children's epigenome at birth with regard to overweight development (Project B_1)

The following results for project B1 have been submitted for publication by Junge & Englich *et al*. as "*MEST* mediates the impact of prenatal bisphenol A exposure on long-term body weight development".

Next to the maternal immune system, external factors like environmental pollutants with hormone-like activity (so called endocrine disrupting chemicals; EDCs) can alter foetal priming and increase risk for children's overweight development through various pathways. BPA is one of the most studied EDCs in regard to overweight but mechanistic investigations for foetal priming are rare and focus mainly on transcription factor activation. But since there is growing evidence that epigenetic mechanisms can contribute to prenatal programming of diseases, the potential impact of BPA on children's DNA-methylation pattern in relation to increased risk for overweight development was assessed in project B_1 in a three dimensional setup, including epidemiological data, *in vitro* and *in vivo* analyses with the subsequent contributions.

Personal contributions include the manuscript preparation; statistical analyses including mediator and GEE models for associations of prenatal BPA exposure, DNA methylation, gene expression and children's BMI z-scores in LINA; assistance in MassARRAY preparation and final analysis for human and murine samples; conducting the *in vitro* adipogenesis model with all associated analyses; assessment of gene expression in murine samples and analysis of all data resulting from the mouse model. Contributions of others include the measurement of urinary BPA, DNA methylation assays and data preparation (450k and MassARRAY) and conduction of the mouse experiments, as indicated in the corresponding methods sections.

The analysed sub-cohort of the LINA study included 408 children for which complete data on prenatal BPA exposure and cord blood methylation status were available. General characteristics of the study participants (gender, birth weight, gestational week at delivery, smoking during pregnancy, parental school education, household members, breastfeeding and introduction to solid food) of the sub-cohort (n=408) were not different from the total LINA cohort (n=629) as shown in Table 10. Median urinary BPA concentrations at pregnancy were 12.7 ng/mg creatinine. Low BPA exposure was defined as < 7.6 ng/mg creatinine (<25 %; 1st quartile) and high BPA exposure as ≥ 15.9 ng/mg creatinine (≥75 %, 4th quartile).

Table 10: Study characteristics of the total study cohort and the analysed sub-cohort for project B_1

	Entire LINA cohort n (%), n = 629[a]	**Analysed sub-cohort** n (%), n = 408	***X²-test*[b]**
Gender of the child			0.966
Female	302 (48.0)	197 (48.3)	
Male	327 (52.0)	211 (51.7)	
Birth weight			0.941
≤3000g	123 (19.6)	68 (16.7)	
>3000-3500g	242 (38.5)	157 (38.5)	
>3500-4000g	192 (30.6)	129 (31.6)	
>4000g	71 (11.3)	54 (13.2)	
Gestational week at delivery			0.834
< 37 week s	25 (4.0)	10 (2.5)	
37 – 40 weeks	389 (62.0)	255 (62.5)	
>40 weeks	214 (34.0)	143 (35.0)	
Smoking during pregnancy			0.833
Never	534 (84.9)	358 (87.7)	
Occasionally	47 (7.4)	23 (5.6)	
Daily	48 (7.6)	27 (6.6)	
Parental school education[c]			0.969
Low	16 (2.5)	8 (2.0)	
Intermediate	144 (22.9)	96 (23.5)	
High	469 (74.6)	304 (74.5)	
Household members			0.932
2	33 (5.2)	20 (4.9)	
3	365 (58.0)	257 (63.0)	
>4	203 (32.3)	129 (31.6)	
Breastfeeding exclusive			0.968
1-3 month	112 (17.8)	69 (16.9)	
1-6 month	190 (30.2)	121 (29.7)	
1-12 month	254 (40.4)	172 (42.2)	
Introduction to solid food			0.897
1-3 month	23 (3.7)	11 (2.7)	
4-6 month	251 (39.9)	156 (38.2)	
7-12 month	305 (48.5)	205 (50.2)	
urinary BPA concentration at pregnancy			0.263[d]
median [ng/mg creatinine]	12.7	12.7	
IQR[e] [ng/mg creatinine]	7.5-16.0	7.6-15.9	

[a] n may be different from 629 due to missing data

[b] calculated using the chi squared test for cross relationship

[c] low = 8 years of schooling ('Hauptschulabschluss`); intermediate = 10 years of schooling (`Mittlere Reife`); high = 12 years of schooling or more (`(Fach-)hochschulreife')

[d] *p*-value derived by Student's t-test between group means

[e] IQR: inter quartile range (25the to 75th percentile)

3.3.1. Prenatal BPA exposure and cord blood DNA methylation

Using bisulfite converted gDNA from cord blood, genome-wide changes in DNA methylation were evaluated by applying a Illumina Infinium HumanMethylation450 BeadChip array.

Differentially methylated CpG sites were computed using an adjusted regression model (see Chapter 2.2.1.10) for high (4th quartile) versus low (1st quartile) prenatal BPA exposure. Two CpGs passed the threshold for Bonferroni correction (Figure 15A and Table 11), including a hypo-methylated CpG (cg17580798) in the *MEST* promoter (chr7:130132199, *p*=7.99E-08) and cg06914197 (chr12:6586920, intergenic, *p*=2.42E-08) that is not annotated to a certain gene.

Table 11: Epigenome wide association study comparing children prenatally exposed to high vs. low BPA. Shown are significantly CpGs observed in cord blood that passed Bonferroni correction

CpG	Chromosome	Position	Region	Host gene	*p*-value [a]	Δ β[b]
cg06914197	12	6586920	intergenic		2.42E-08	-1.2%
cg17580798	7	130132199	promoter	*MEST*	7.99E-08	-1.8%

[a] *p*-values are derived from an adjusted regression model
[b] methylation differences are shown as Δ methylation values (β)

Further analyses focused on cg17580798 since *MEST* is the imprinted *mesoderm specific transcript*, a member of the alpha/beta hydrolase superfamily and reported to control the initial phase of early adipose tissue expansion by regulating adipocyte size [172]. Although cg17580798 is located in the first intron of *MEST*, ENCODE histone modification data suggests that it is a promoter region. That indeed this region is potentially transcriptionally regulating, is supported by its overlap with a DNase 1 hypersensitivity cluster (Figure 15B).

MEST promotor methylation around cg17580798 was validated by MassARRAY (see Figure 15C) in cooperation with the group of Saskia Trump of the Department of Environmental Immunology and the group of Roland Eils at the University of Heidelberg, Institute of Pharmacy and Molecular Biotechnology, and Bioquant Center. The MassARRAY amplicon included 24 CpG sites of which 13 CpG sites passed the quality control and were summarized as total promotor methylation. BPA exposure was associated with total promotor methylation (adj.MR: 0.88, 95% CI (0.80, 0.97), *p*=0.010), as well as methylation of cg17580798 only (adj.MR: 0.90, 95% CI (0.82, 0.99), *p*=0.033), with a methylation difference between low and high BPA exposure of 2.6 % and 2.3 % respectively.

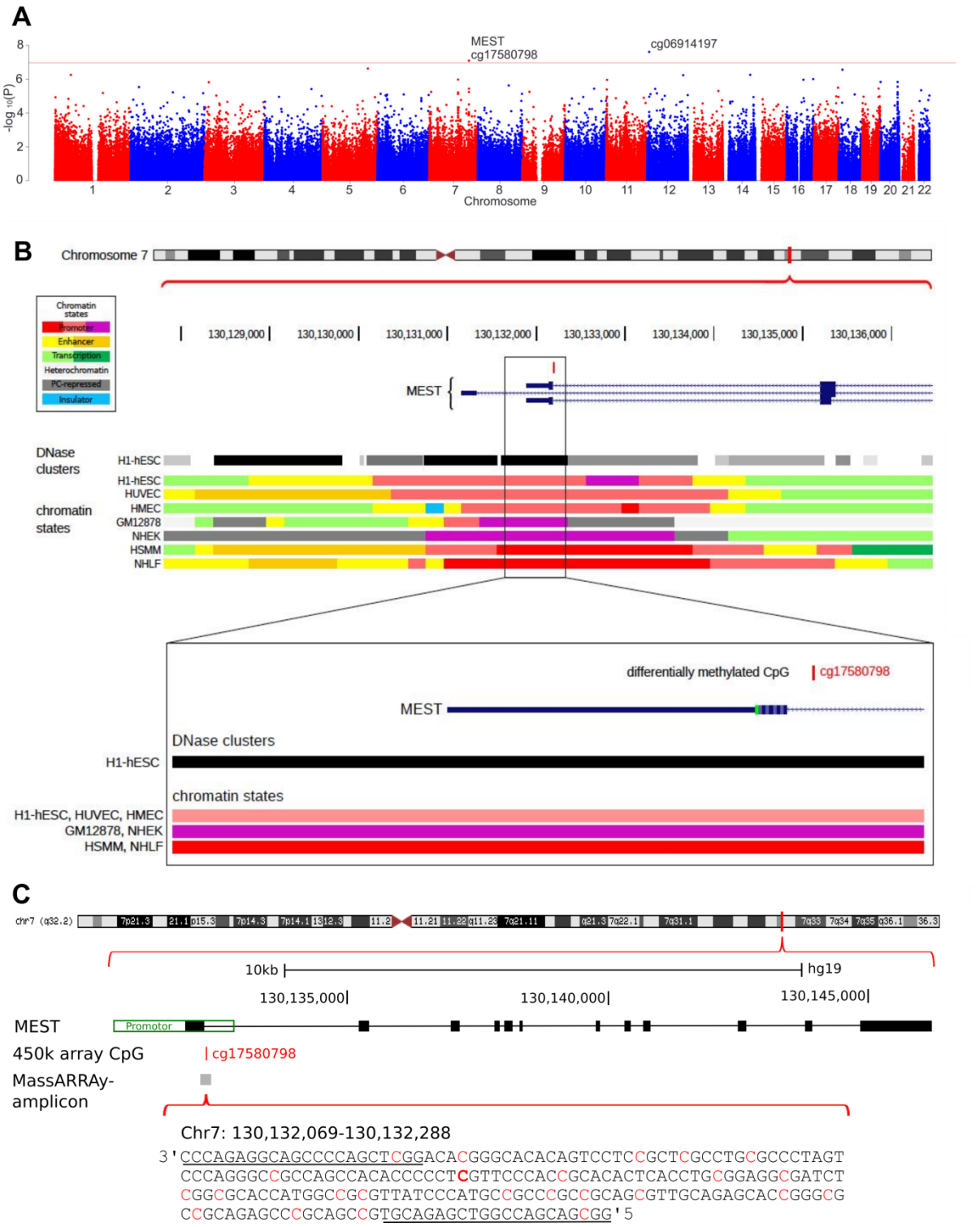


Figure 15: Epigenome wide analysis and *MEST* methylation assessment. (A) Manhattan-Plot from 450K array comparing children prenatally exposed to high vs. low BPA. Shown are significant CpGs observed in cord blood that passed threshold for Bonferroni correction (red threshold line). (B) ENCODE histone modification data suggest that this CpG is in a promoter region, although located in the first intron of *MEST*. In addition, the overlap with a DNase 1 hypersensitivity cluster indicates that this region is potentially transcriptionally regulating. (C) Location of the *MEST* CpG from 450K array which is localized in the *MEST* promotor region on chromosome 7 and designed MassARRAY amplicon within the promotor region. In bold is highlighted the CpG site of interest (cg17580798).

3.3.2. Impact of BPA on MEST promotor methylation and MEST expression in cord blood

MEST expression was measured in 408 cord blood samples of the LINA cohort. Complete information about *MEST* methylation status, *MEST* expression and prenatal BPA exposure was available for 356 children. High prenatal BPA exposure (≥75%, upper quartile UQ) was associated with a decrease in *MEST* promotor methylation at birth (Figure 16A). Further, decreased *MEST* promotor methylation in high BPA (≥Mean) exposed children was associated with an increase in *MEST* RNA expression (Figure 16B). There was no direct effect of prenatal BPA exposure on cord blood *MEST* expression. However, applying a mediator model using PROCESS in SPSS, prenatal BPA exposure was indirectly linked to *MEST* expression via *MEST* promotor methylation (ab=0.47, 95% CI (0.07, 1.24)) (Figure 16C and Table 12). Furthermore, *MEST* expression at birth was positively correlated with BMI z-scores at birth (adj.MR: 1.13, 95% CI (1.02, 1.26), *p*=0.024).

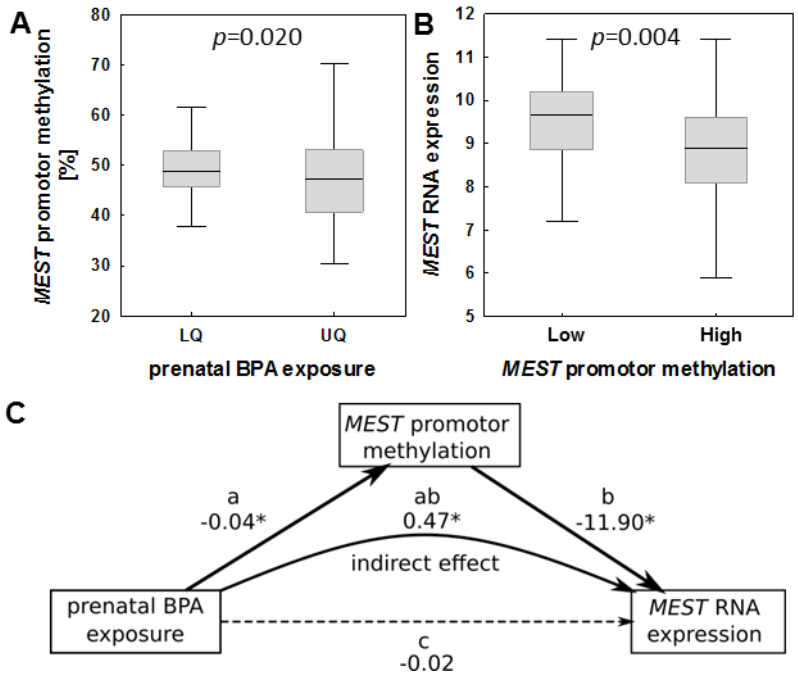


Figure 16: Association of BPA with *MEST* promotor methylation and *MEST* expression in cord blood (A) *MEST* promotor methylation in cord blood of low (<25%; lower quartile LQ, n=138) and high (>75%, upper quartile UQ, n=138) BPA exposed children. (B) *MEST* expression in cord blood of children with low (<Mean, n=55) and high (>Mean, n=45) *MEST* methylation (shown for high BPA exposed children (>75%, UQ)). Boxes represent the 25%-75% distribution with median-values. Spreads indicate the non-outlier range. *p*-value from Mann Whitney U-tests. (C) Mediator model for the association of prenatal BPA exposure with cord blood *MEST* methylation status and cord blood *MEST* expression. Model is adjusted for gender of the child, smoking during pregnancy, parental school education, solid food introduction, household members and early delivery. Shown are effect sizes with * *p*<0.05.

Table 12: Mediator model for the association of prenatal BPA exposure with cord blood *MEST* DNA methylation and expression (according to Figure 16C).

parameter	effect size	95% CI	*p*-value
a	**-0.04**	**-0.63,-0.48**	**0.011**
b	**-11.90**	**-21.34,-2.45**	**0.014**
c (direct effect)	-0.02	-0.41,0.37	0.911
ab (indirect effect)	**0.47**	**0.07,1.24**	<0.05*

* significantly *p*-value derived according to 95% CI range

3.3.3. Association of MEST methylation and BPA induced risk for childhood overweight development

Furthermore, the relevance of BPA associated changes in *MEST* promotor methylation for later BMI z-score development of the child was investigated. Therefore, a mediator analysis, adjusted for weight related confounders (gender of the child, smoking during pregnancy, parental school education, solid food introduction, gestational week at delivery, number of household members and early delivery (<37 week of gestation)), was applied to assess the impact of prenatal BPA exposure on children's BMI z-scores at year 1, which might be mediated by neonatal *MEST* promotor methylation. The effect of prenatal BPA exposure on BMI z-scores indeed was mediated by *MEST* promotor methylation in cord blood (ab=0.29, 95% CI (0.04,1.09), Figure 17A and Table 13).

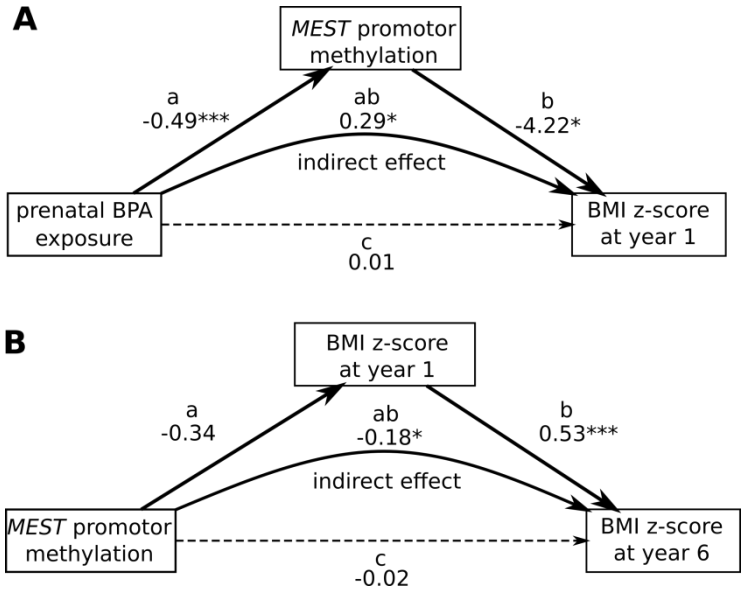


Figure 17: Mediator models (A) for the association of prenatal BPA exposure with the *MEST* DNA methylation status and children's BMI z-score at year 1 (B) for the association of cord blood MEST DNA methylation status with children's BMI z-score at year 1 and at year 6. Models were adjusted to gender of the child, smoking during pregnancy, parental school education, solid food introduction, household members and early delivery. Shown are effect sizes with * $p<0.05$; *** $p<0.001$.

Table 13. Mediator model for the association of prenatal BPA exposure with cord blood MEST DNA methylation and children's BMI z-scores at year 1 (according to Figure 17A).

parameter	effect size	95% CI	*p*-value
a	**-0.49**	**-0.62,-0.36**	**<0.001**
b	**-4.22**	**-8.18,-0.26**	**0.037**
c (direct effect)	-0.01	-0.19,0.17	0.934
ab (indirect effect)	**0.29**	**0.03,1.09**	<0.05*

* significantly *p*-value derived according to 95% CI range

Table 14. Mediator model for the association of cord blood MEST DNA methylation and children's BMI z-scores at year 1 and year 6 (according to Figure 17B).

parameter	effect size	95% CI	*p*-value
a	-0.34	-0.75,0.08	0.113
b	**0.53**	**0.40,0.67**	**<0.001**
c (direct effect)	-0.07	-0.46,0.32	0.716
ab (indirect effect)	**-0.18**	**-0.51,-0.06**	<0.05*

* significantly *p*-value derived according to 95% CI range

In addition, the impact of cord blood *MEST* promotor methylation on BMI z-scores at year 6 was mediated by the BMI z-scores at year 1 (ab=-0.18, 95% CI (-0.51,-0.57), Figure 17B and Table 14). There was no gender difference for *MEST* mediated changes in BMI z-scores.

3.3.4. Longitudinal association of MEST expression and BMI z-score development

The longitudinal impact of altered *MEST* expression at birth due to prenatal BPA exposure was calculated using an adjusted GEE model including BMI z-scores and *MEST* expression at birth and year 6 as well as weight related confounders (see above). A longitudinally higher *MEST* expression at birth and year 6 was positively correlated longitudinally with BMI z-scores at birth and year 6 (adj.RR: 1.03, 95% CI (1.00, 1.07), *p*=0.021)).

3.3.5. Impact of BPA exposure on early metabolic priming

As it was already shown in project A for the maternal cytokine status during pregnancy that the children's metabolome can be influenced by prenatal exposures, it was reasonable to look at BPA induced changes in the children's metabolome. After correction for multi-testing increased prenatal BPA exposure correlated negatively with acylcarnitine levels at year 1 (Table 15). As already shown in chapter "*3.2.3 Offspring BMI and metabolic profile"* acylcarnitines at year 1 were negatively correlated with the BMI z-score at year 2. However, *MEST* promotor methylation and expression in cord blood did not correlate with acylcarnitine levels at year 1. Therefore, the BPA-acylcarnitine association seemed not to be mediated by BPA induced hypo-methylation of *MEST*. It could rather be an independent mode of action or just picture the altered metabolome status in 1 year-old overweight children.

Table 15: Correlation of prenatal BPA exposure, *MEST* promotor methylation and *MEST* expression at birth with acylcarnitine levels in 1 year-old children. Shown are Spearman correlation coefficients (R) and *p*-values

acylcarnitines	**BPA** (n=350)		***MEST* promotor methylation** (n=261)		***MEST* expression** (n=332)	
	R	*p*-value	R	*p*-value	R	*p*-value
C0[a]	-0.25	**1.49×10^{-6}**	0.09	0.01×10^{1}	0.11	0.05×10^{1}
C10:0	-0.40	**6.86×10^{-15}**	0.05	0.04×10^{1}	0.11	0.04×10^{1}
C10:1	-0.40	**9.35×10^{-15}**	0.09	0.02×10^{1}	0.11	0.04×10^{1}
C12-DC[b]	-0.43	**2.95×10^{-17}**	0.08	0.02×10^{1}	0.10	0.08×10^{1}
C14:1	-0.24	**4.47×10^{-6}**	0.08	0.02×10^{1}	0.05	0.34×10^{1}
C18:1	-0.22	**2.29×10^{-5}**	0.08	0.02×10^{1}	0.05	0.41×10^{1}
C2	-0.06	0.03×10^{1}	-0.02	0.08×10^{1}	0.05	0.41×10^{1}
C3	-0.27	**3.86×10^{-7}**	0.03	0.07×10^{1}	0.05	0.39×10^{1}
C4	-0.35	**2.48×10^{-11}**	0.11	0.01×10^{1}	0.08	0.13×10^{1}

a – free carnitine
b - Dodecanedioylcarnitine

3.3.6. In vivo mouse model: impact of prenatal BPA exposure on weight development

Additional validation of these findings was done in a cross-generational mouse model by the group of Tobias Polte in the animal facilities of the University of Leipzig. Mice offspring of BPA exposed mothers were followed up until 10 weeks after delivery. Offspring weight was assessed twice a week, beginning one week after delivery, and compared to unexposed control animals (Figure 18A). Prenatally BPA exposed offspring mice had a significantly higher weight over the entire observation period compared to unexposed control mice (p=0.004, derived by 2-Way-ANOVA). There was no gender difference in BPA-dependent weight development. Furthermore lean mass and fat mass were assessed at 10 weeks, with BPA exposed mice showing a 53 % higher fat mass than control mice (p=0.013) (Figure 18B). *Mest* methylation and expression was assessed at 10 weeks in visceral fat tissue samples. *Mest* methylation was reduced by 7 % in BPA exposed mice (p<0.001, Figure 18C) with a corresponding increase in *Mest* expression by 2.1-fold in BPA exposed mice (p=0.022, Figure 18D).

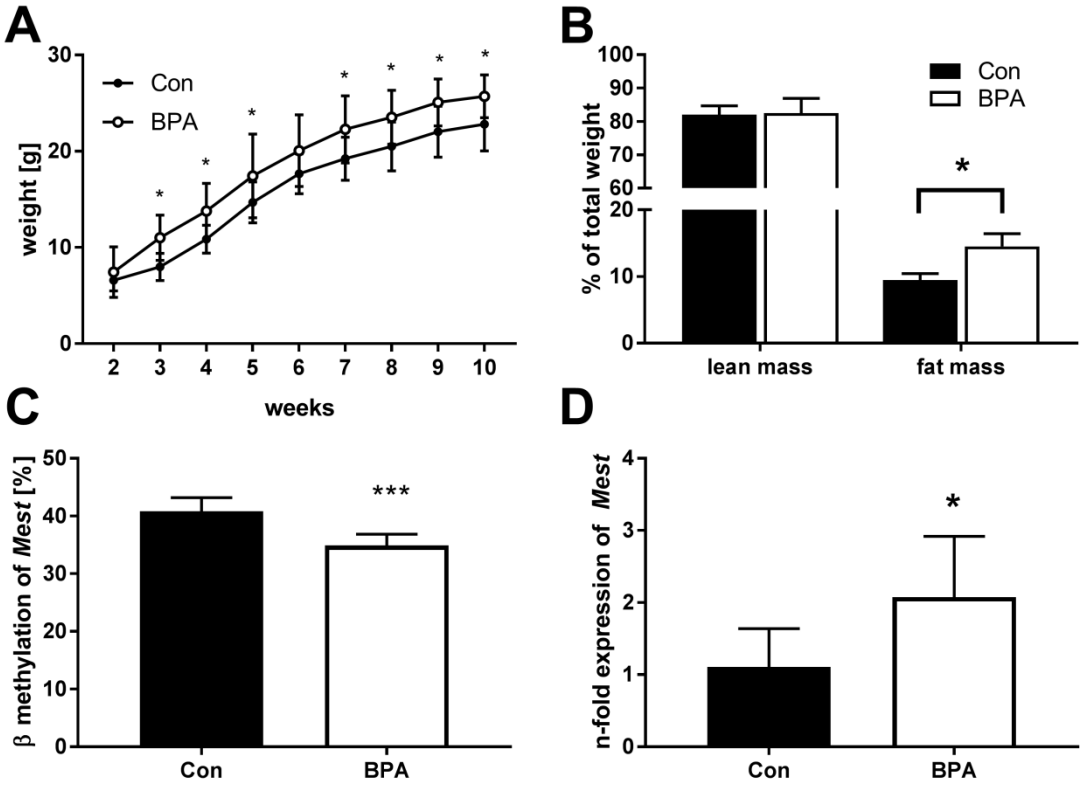


Figure 18: Effect of prenatal BPA exposure assessed in a murine *in vivo* model. (A) Impact of prenatal BPA exposure on weight development in the offspring. Shown are mean ± SD from n≥9/group and *p*-values are derived from ANOVA. (B) Differentiation of offspring weight at 10 weeks (n=3) in lean and visceral fat mass (C) Targeted MassArray for *Mest* methylation analysis in visceral fat tissue of 10-week-old offspring after prenatal BPA exposure compared to control (n≥3) (D) *Mest* expression analysis in fat tissue of 10-week-old offspring after prenatal BPA exposure compared to controls (n≥7). **p*<0.05 and ****p*<0.001.

3.3.7. In vitro model: impact of BPA exposure on adipocyte differentiation

To mechanistically assess a direct impact of BPA exposure on adipocyte development, a human mesenchymal stem cell (MSC) differentiation assay was conducted. Differentiation of human MSC to adipocytes was monitored in real time using the impedance-based xCELLigence System. 10 µM or 50 µM BPA were applied during the entire differentiation period and compared to a solvent control (EtOH, 0.05 %). BPA caused a dose-dependent decrease in cell index values after the differentiation initiation period compared to unexposed controls (Figure 19A). Significance was reached from day three onwards for 50 µM and from day five onwards for 10 µM BPA until the end of the observational period. Oil Red O staining of triglycerides in lipid droplets showed significantly more droplets for 50 µM BPA (*p*<0.001; Figure 19B,C) but not for 10 µM BPA compared to the unexposed control cells.

mRNA analyses of adipocyte specific genes after 17 days of differentiation in the presence of 50 µM BPA revealed a significantly up-regulation of *PPARγ* (2.2±1.15 fold, *p*=0.005), its target gene *LPL* (4.4±2.6 fold, *p*=0.029); *SREBF1* (1.8±0.4 fold, *p*=0.005), its target gene *IRS2* (1.9±0.6 fold, *p*=0.015), and *ESR1* (8.4±3.8 fold, *p*=0.006). For 10 µM BPA a significantly increase in gene expression was detected for *LPL* (1.9±0.3 fold, *p*=0.002), *SREBP1* (1.5±0.1 fold, *p*<0.001) and *FASN* (1.9±0.7 fold, *p*=0.046, Figure 19D).

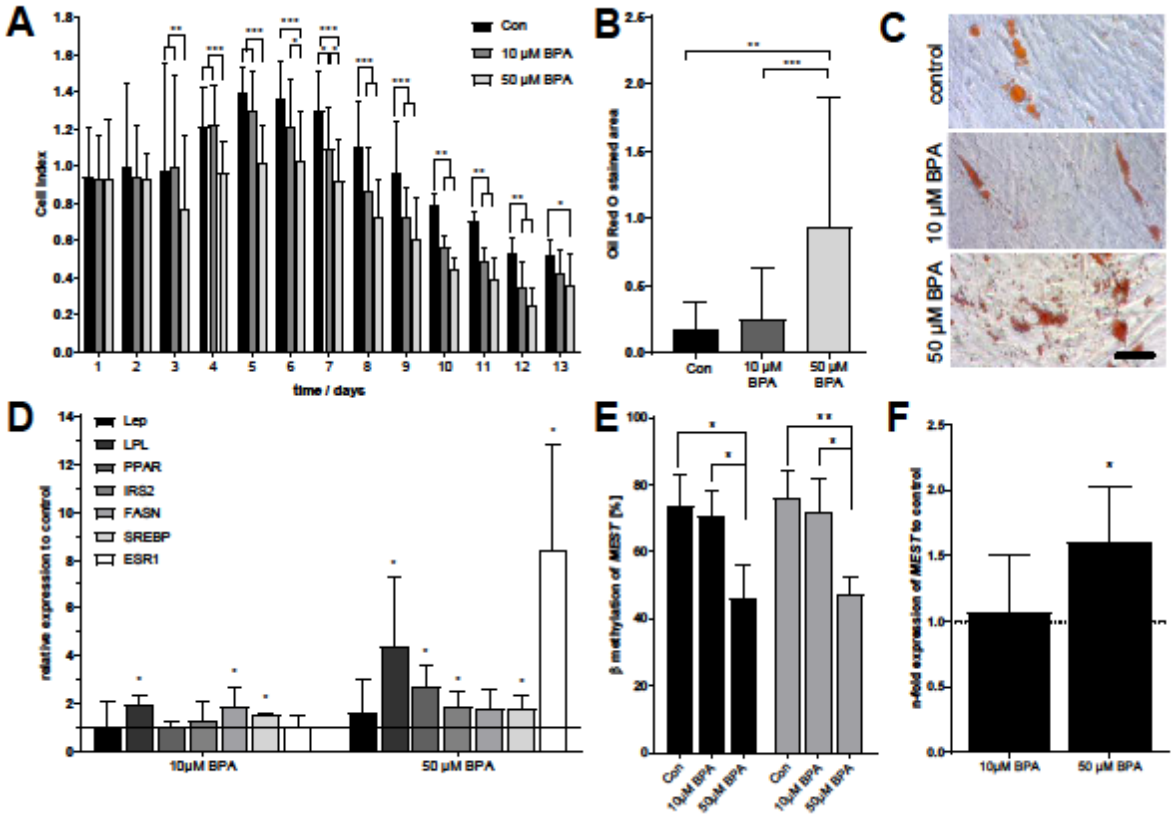


Figure 19: *In vitro* adipocyte differentiation from human MSCs. Exposure to BPA (10 µM, 50 µM) compared to solvent control (EtOH 0.05%) (A) Real time monitoring of cell differentiation (xCELLigence: normalized cell index) over 17 days (mean ± SD, n=4) (B) Quantification of Oil Red O stained area (mean±SD, n≥20 from one experiment) (C) Exemplary histological Oil Red O staining of adipocytes containing red coloured lipid droplets (black bar=100 µm) (D) qPCR data of genes involved in adipogenesis (n≥3) normalized to EtOH control and *GAPDH* (*LEP*=leptin, *LPL*=lipoprotein lipase, *PPARγ*= peroxisome proliferator activated receptor gamma, *IRS2*=insulin receptor substrate 2, *FASN*=fatty acid synthase, *SREBP1*=sterol receptor element binding factor 1, *ESR1*=estrogen receptor alpha) (E) Targeted MassArray of *MEST* DNA methylation analysis for the total MassArray amplicon or the specific CpG cg17580798 (n=3) (F) qPCR data of *MEST* (n≥3, normalized to EtOH control and *GAPDH*); **p*<0.05, ***p*<0.01, ****p*<0.001 from Student's t-test/ANOVA

To follow up on the hypothesis that *MEST* mediates fat tissue expansion, also *MEST* methylation and expression was measured in differentiated adipocytes as shown in Figure

19E and F. *MEST* promotor methylation (total and cg17580798) was decreased by 28 % after exposure to 50 µM BPA compared to the control. In accordance *MEST* expression was significantly increased in adipocytes exposed to 50 µM BPA (1.6±0.4 fold, p=0.027, Figure 19E-F).

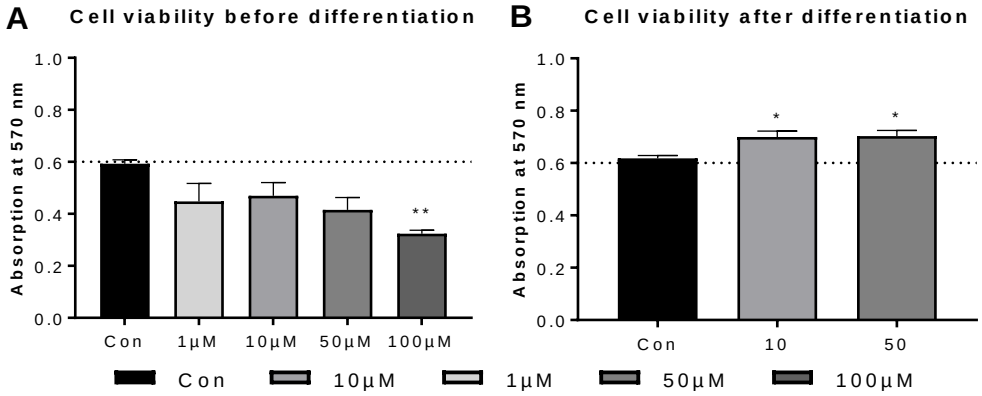


Figure 20: Cell viability after exposure to BPA. Shown is the mean±SEM. absorption at 570 nm after a MTT assay with *p<0.05, **p<0.01 (A) Cell viability of human mesenchymal stem cells after exposure to BPA for 48 h. (B) Cell viability of human mesenchymal stem cell derived adipocytes after exposure to BPA over the entire differentiation period of 17 days.

The *in vitro* results were not influenced by any cytotoxic effects of BPA, as can be seen from the performed MTT assay (Figure 20A). There was no significantly change in cell viability up to 50 µM BPA, although cells exposed to 100 µM BPA showed a significantly lower cell viability after 48 hours (p=0.007). After exposure to BPA over the entire differentiation period, cell viability was slightly increased (1.1-fold to control) by 10 µM (p=0.016) and 50 µM BPA (p=0.013) (Figure 20B).

3.4. Impact of prenatal exposure to parabens on overweight development in children and adipogenesis *in vitro* (Project B_2)

Another group of chemicals that is under suspicion to possess endocrine disrupting properties but has not been intensively studied so far, especially in the context of obesity, are parabens. Therefore project B_2 aims to investigate the impact of prenatal paraben exposure on childhood overweight development and adipogenesis in general. A manuscript for publication is currently in preparation with the subsequent contributions.

Personal contributions include the manuscript preparation; statistical analyses of associations between urinary paraben concentrations, maternal cosmetic product usage and children's BMI z-scores; and *in vitro* assays. Contributions of others include urinary paraben measurements and reporter gene assays as indicated in the corresponding methods sections.

In project B_2 a set of 9 parabens was measured in 504 maternal pregnancy urine samples of the LINA study. For 498 participants valid paraben concentrations above the detection limit were obtained for 5 parabens (methyl-, ethyl-,n-propyl-, i-butyl- and n-butylparaben). Highest median paraben levels were detected for methylparaben (MeP, 138.7 µg/l), followed by n-propylparaben (nPrP, 30.2 µg/l) and ethylparaben (EtP, 14.5 µg/l) (see Figure 21). n-butylparaben and i-butylparaben showed the lowest concentrations with 6.6 µg/l and 1.8 µg/l, respectively.

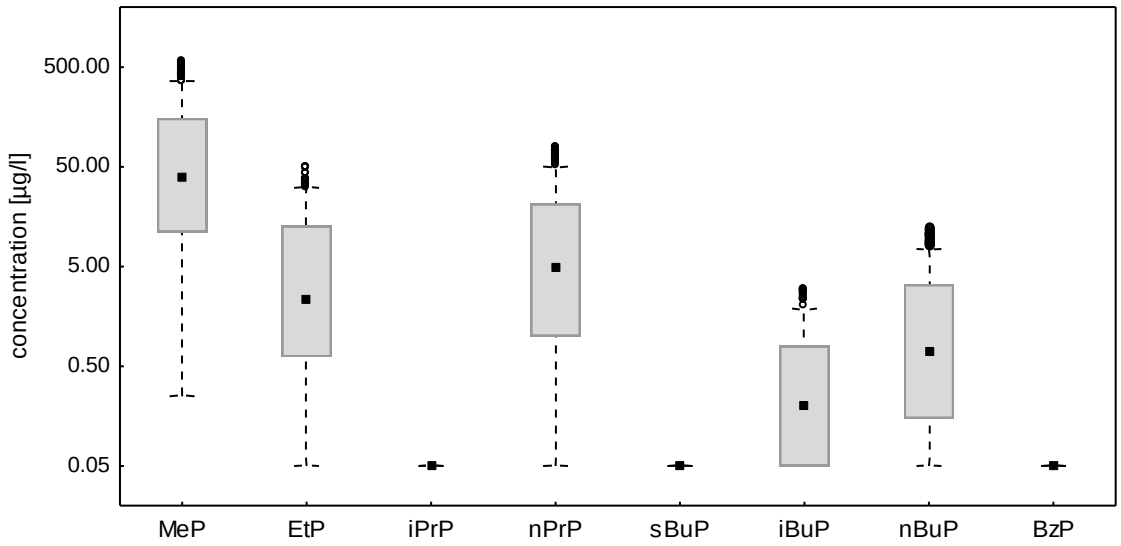


Figure 21: Urinary paraben concentrations at the 34th week of gestation. Boxes represent the 25%-75% distribution with median-values. Spreads indicate the non-outlier range. n=498

3.4.1. Cosmetic products as a source of paraben exposure

Parabens are usually used as preservatives in cosmetic products and highly processed food. Within the LINA study the usage of cosmetic products during pregnancy was assessed by questionnaires. Indicated cosmetic products were analysed according to their paraben content with the TOXFOX app from the *Bund für Umwelt und Naturschutz Deutschland* (app download with available online database version April 2016) and categorised in leave-on and rinse-off products. Since exposure time is obviously very short and TOXFOX indicated only 31 out of 381 rinse-off products as paraben containing (see Figure 22), only leave-on products with a high exposure time and high body area coverage were considered for further analyses.

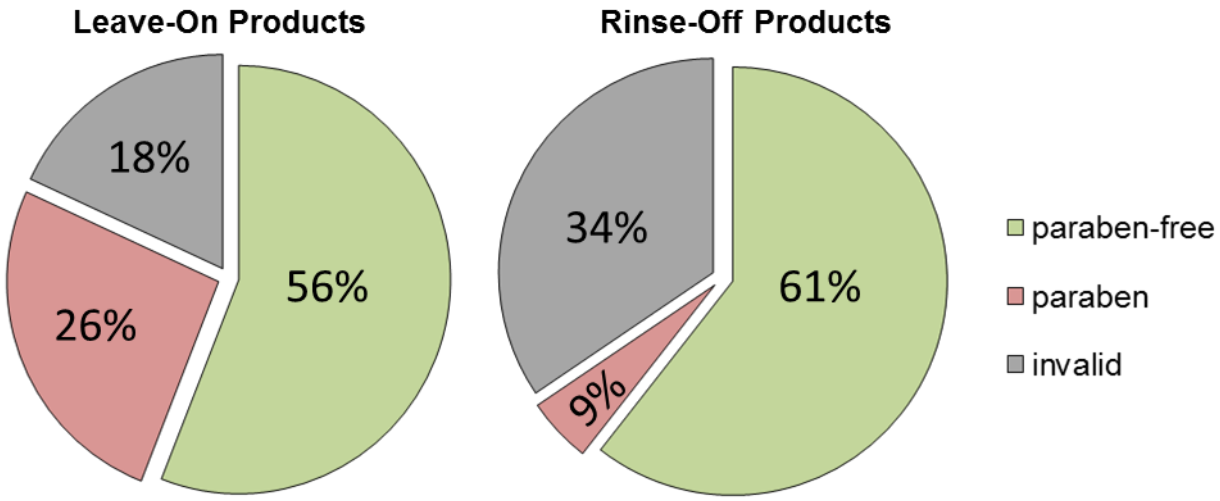


Figure 22. Distribution of paraben containing cosmetic product application in the LINA study. Percentage of participants (n=629) that named at least one leave-on or rinse-off cosmetic product that contains parabens or used paraben-free products only. Insufficiently indicated cosmetics that could not be assigned to a specific brand/product were excluded from further analyses (invalid).

Valid data on the application of paraben containing or paraben-free leave-on products during pregnancy was available from 515 participants. Out of these, 31 % used at least one cosmetic leave-on product that contained parabens, whereas 74 % used paraben-free leave-on products only.

When comparing urinary paraben concentrations, mothers that used paraben containing leave-on products had up to 3.0-fold higher concentrations for MeP, EtP, nPrP and nBuP compared to mothers using paraben-free leave-on products, as shown in Table 16.

Table 16: Urinary paraben concentrations in regard to cosmetic product application. Median-values with 25%-75% distribution of urinary paraben concentrations of mothers using paraben-free cosmetic products (leave-on) and using cosmetic products (leave-on) that contain parabens. *p*-values are derived by Mann-Whitney U-Test.

	Leave on products without parabens (n=276)			**Leave on products with parabens** (n=138)			*p*- value
	Median	<25%	>75%	Median	<25%	>75%	
MeP	28.05	6.62	133.20	68.80	17.00	167.20	**0.0018**
EtP	1.89	0.51	9.88	2.90	0.79	18.10	**0.0466**
nPrP	3.20	0.72	14.60	7.40	1.50	25.80	**0.0025**
iBuP	0.10	0.05	0.70	0.20	0.05	0.94	0.1875
nBuP	0.41	0.10	2.70	1.24	0.30	4.55	**0.0004**

Interestingly, a significantly positive association of urinary paraben concentrations and maternal age at delivery was found. Mothers aged 31 or older (31 was the average age of mothers within the LINA cohort) had up to 2.6-fold higher concentration of the analysed parabens (see Table 17).

Table 17: Urinary paraben concentrations in regard to maternal age at delivery. *p*-values are derived by Mann-Whitney U-Test.

	≤31 years (n=249)			**>31 years** (n=249)			*p*- value
	Median	<25%	>75%	Median	<25%	>75%	
MeP	26.40	8.60	126.55	57.90	12.10	185.30	**0.0048**
EtP	1.56	0.53	6.23	3.40	0.87	19.30	**0.0002**
nPrP	4.10	0.90	13.70	6.20	1.25	31.40	**0.0279**
iBuP	0.10	0.05	0.50	0.26	0.05	1.20	**0.0000**
nBuP	0.40	0.10	1.90	1.02	0.20	5.90	**0.0002**

3.4.2. Impact of prenatal paraben exposure on longitudinal overweight development in children

Next to showing the potential source of parabens, the impact of prenatal paraben exposure on children's weight development up to the age of 8 years was assessed. Analyses were performed in adjusted (gender of the child, smoking during pregnancy, parental school education, gestational week at delivery, existence of siblings, breast feeding duration (not for models with birth weight as outcome) and age of the mother at birth) logistic regression models comparing overweight (ever in the observation period))and non-overweight (never

overweight in the observation period) children. Results for birth only (n=482), for *early infancy* (year 1-2; n=375) or for *later infancy* (year 3-8; n=199) are shown in Figure 23. Later infancy was defined according to Körner *et al*. [173], who state that a longitudinal overweight stabilising effect after the age of 3 years is expected.

Exposure to MeP, EtP and nPrP did not increase risk for overweight development over the whole observation period. However, long chain iBuP and nBuP did increase risk for overweight at birth (iBuP: adj.OR: 1.57, 95% CI (1.18,2.09); nBuP: adj.OR: 1.63, 95% CI (1.21,2.19)) and in later infancy (iBuP: adj.OR:1.51, 95% CI (1.05,2.16); nBuP: adj.OR: 1.46, 95% CI (1.00,2.13)). There was no effect on overweight development in early infancy or over the entire observation period (year 0-8).

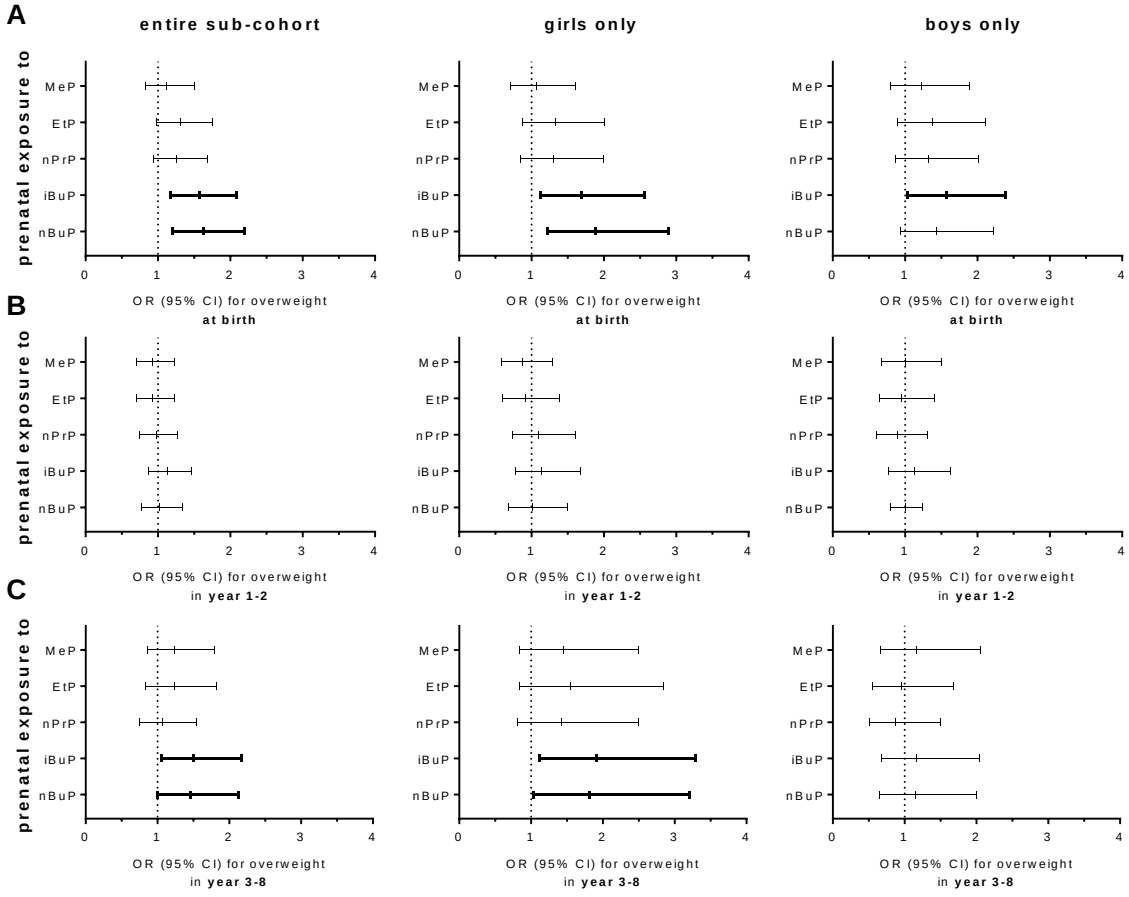


Figure 23: Effect of prenatal paraben exposure on weight development in children. Shown are odds ratios (OR) for overweight development for an increase of paraben exposure per tertile. with 95% confidence intervals (95% CI) derived from adjusted logistic regression models for the entire sub-cohort and stratified for girls and boys only. Significantly associations are presented in bold (p<0.05) (A) Impact of prenatal paraben exposure on birth weight (n=481). (B) Impact of prenatal paraben exposure on overweight development in year 1-2 (n=375). (C) Impact of prenatal paraben exposure on overweight development in year 3-8 (n=199).

According to the significantly effects of paraben exposure and BMI z-score development at birth and in later infancy, a sub-cohort for further analyses was defined with complete available data for prenatal paraben exposure, BMI z-scores at birth and later infancy as well as relevant confounders (n=199). The analysed sub-cohort showed an equal distribution for all general study characteristics compared to the entire LINA cohort except the presence of siblings (Table 18).

Table 18: Study characteristics of the total study cohort and the analysed sub-cohort for project B_2

	Entire LINA cohort n (%), n = 629[a]	**Analysed sub-cohort** n (%), n = 199	***χ²-test*[b]**
Gender of the child			0.497
Female	302 (48.0)	105 (52.8)	
Male	327 (52.0)	94 (47.2)	
Birth weight			0.967
≤3000g	123 (19.6)	38 (19.1)	
>3000-3500g	242 (38.5)	73 (36.7)	
>3500-4000g	192 (30.6)	61 (30.7)	
>4000g	71 (11.3)	27 (13.6)	
Gestational week at delivery			0.981
< 37 week s	25 (4.0)	8 (4.0)	
37 – 40 weeks	389 (62.0)	126 (63.3)	
>40 weeks	214 (34.0)	65 (32.7)	
Maternal age at pregnancy			0.301
< 31 years	305 (48.5)	111 (55.8)	
≥ 31 years	324 (51.5)	88 (44.2)	
Smoking during pregnancy			0.741
Never	534 (84.9)	175 (87.9)	
Occasionally	47 (7.4)	14 (7.0)	
Daily	48 (7.6)	10 (5.0)	
Parental school education[c]			0.651
Low	16 (2.5)	2 (1.0)	
Intermediate	144 (22.9)	41 (20.6)	
High	469 (74.6)	156 (78.4)	
Siblings			**<0.05**
Yes	414 (66.6)	69 (34.7)	
No	208 (33.4)	130 (65.3)	
Breastfeeding exclusive			0.960
Never	26 (4.1)	7 (3.5)	
1-3 month	112 (17.8)	34 (17.1)	
1-6 month	190 (30.2)	66 (33.2)	
1-12 month	254 (40.4)	92 (46.2)	

[a] n may be different from 629 due to missing data

[b] calculated using the chi squared test for cross relationship

[c] low = 8 yrs of schooling (‘Hauptschulabschluss`); intermediate = 10 yrs of schooling (`Mittlere Reife`); high = 12 yrs of schooling or more (`(Fach-)hochschulreife’)

To picture the different effect of prenatal paraben exposure in early and later infancy from another perspective, BMI z-scores of low (<1st tertile) and high (≥3rd tertile) prenatally

paraben exposed children are displayed in Figure 24 over the complete longitudinal time window. As seen in the regression models, there was no difference in BMI z-scores for MeP, EtP and nPrP in the entire sub-cohort. But children exposed to high iBuP or high nBuP concentrations showed higher BMI z-scores from birth to age 8 (p<0.0001, derived by 2-Way ANOVA). Also in confirmation with the regression models, differences were visually smaller in year 1 and year 2 and seemed to stabilize from year 3 onwards.

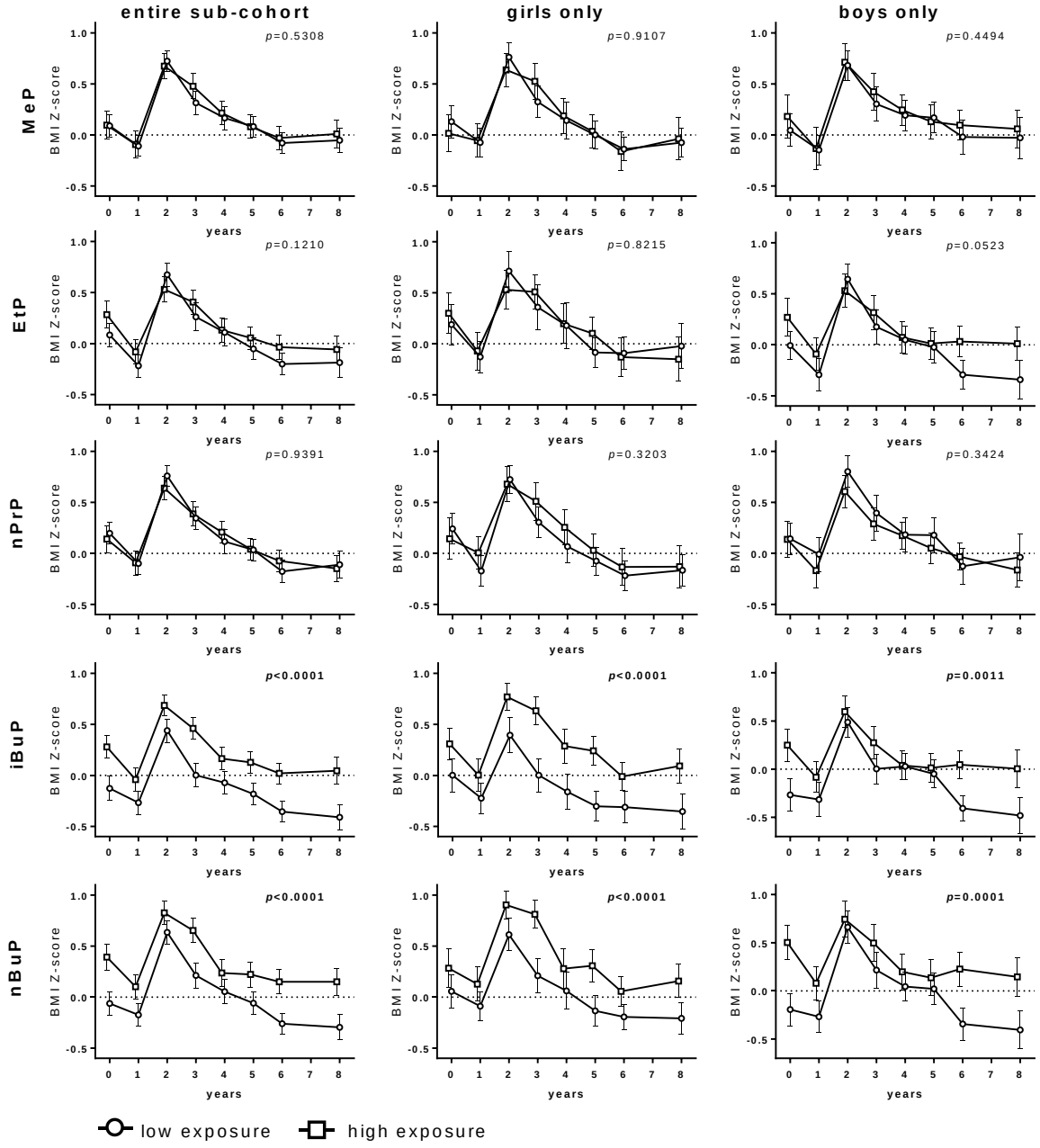
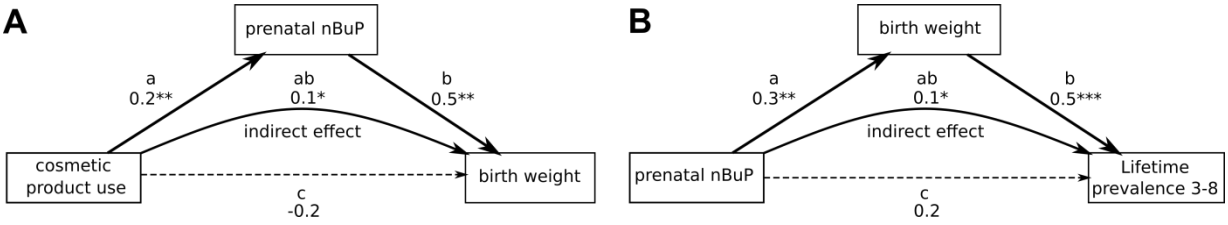


Figure 24: BMI z-scores of children exposed to low (<1th tertile) and high (>3rd tertile) levels of parabens during pregnancy. Shown are mean ± SEM BMI z-scores of the entire sub-cohort, as well as stratified mean±s.e.m. BMI z-scores for girls and boys only. *p*-values were derived from 2-Way ANOVA.

Further, analyses were performed for boys and girls separately. Indeed, a gender difference in susceptibility to prenatal BuP exposure was visible, with stronger effects in girls compared to boys. This gender difference in vulnerability to prenatal paraben exposure was also seen in the logistic regression models: an increased risk for overweight at birth and later infancy was only seen for girls (*birth*: iBuP: adj.OR: 1.70 95% CI (1.12, 2.56); nBuP: adj.OR: 1.88, 95% CI (1.22, 2.90); *later infancy*: iBuP: adj.OR: 1.91, 95% CI (1.11, 3.28); nBuP: adj.OR: 1.82, 95% CI (1.03, 3.21)). Only iBuP slightly increased risk for overweight in boys at birth (adj.OR: 1.57, 95% CI (1.03, 2.38)) but did not have an impact on overweight development in later infancy (Figure 23).

To consider interacting effects of cosmetic use, paraben measurements and weight development, a mediator model was applied. The maternal usage of cosmetic leave-on products that contained parabens was indirectly increasing BMI z-scores at birth of children, which was mediated by an increased prenatal nBuP exposure (Figure 25A).



C

parameter	effect size	95% CI	*p*-value
Model **A** (n=405)			
a	**0.25**	**0.08,0.42**	**0.004**
b	**0.55**	**0.22,0.88**	**0.001**
c (direct effect)	-0.18	-0.75,0.39	0.533
ab (indirect effect)	**0.14**	**0.04,0.31**	**<0.05***
Model **B** (n=176)			
a	**0.26**	**0.08,0.44**	**0.004**
b	**0.52**	**0.16,0.89**	**0.005**
c (direct effect)	0.16	-0.25,0.58	0.440
ab (indirect effect)	**0.14**	**0.03,0.32**	**<0.05***

* significantly *p*-value derived according to 95% CI range

Figure 25. Association of cosmetic product application during pregnancy with prenatal nBuP concentrations and weight development of children. Adjusted mediator models derived by the PROCESS tool in SPSS with effect sizes and p-values with *p<0.05, **p<0.01.Models were adjusted to gender, gestational week at delivery, siblings, age of the mother at deliver, parental educational status, smoking during pregnancy and breast feeding duration (only model B). (A) Indirect impact of the application of cosmetic products containing parabens during pregnancy on BMI z-scores at birth that was mediated by prenatal nBuP exposure (tertile). (B) Indirect impact of prenatal nBuP exposure (tertile) on the lifetime prevalence for overweight, that was mediated by BMI z-scores at birth. (C) Model parameter for A and B with effect sizes, 95% confidence intervals (95% CI) and exact *p*-values.

Furthermore, the impact of prenatal nBuP exposure on overweight development in later infancy was mediated by increased BMI z-scores at birth (Figure 25B). Effect sizes were summarized in Figure 25C. Again, these mediated associations were only seen in girls compared to boys (data not shown).

3.4.3. Impact of paraben exposure on early priming

Maternal urinary paraben concentrations were not associated with DNA methylation patterns at birth or metabolic parameters, like acylcarnitines of the child at 1 year of age. Therefore parabens might act via a different mode of action. The group of Beate Escher from the Department for Cell toxicology assessed the receptor activation of parabens for the androgen, oestrogen, glucocorticoid and progesterone receptor and PPARγ within a high-throughput reporter gene assay. Receptor activation by parabens could only be detected for the oestrogen receptor with intensity increasing by chain length (see Table 19). PPARγ activation could not be detected due to high cell toxicology within these assays (data not shown).

Table 19: IC_{10} for cytotoxicity and EC_{10} for activation of the oestrogen responses by parabens

	MeP	EtP	nPrP	nBuP
Cytotoxicity				
IC_{10} (M)	3.68E-04	5.20E-04	6.37E-05	8.39E-06
Std. Error	0.118	0.1144	0.068	0.079
Activation of ERα				
EC_{10}	2.14E-04	1.53E-05	3.09E-06	2.12E-06
Std. error	6.84E-06	5.89E-07	1.92E-07	7.45E-08

3.4.4. Impact of paraben exposure on adipocyte development in vitro

In accordance to project B_1 the direct impact of paraben exposure on adipocyte development was assessed in a human mesenchymal stem cell differentiation assay. Differentiation of human MSC to adipocytes was monitored in real-time using the impedance-based xCELLigence system (Figure 26). During the entire differentiation period 0.5 µM, 1 µM, 10 µM and 50 µM of MeP, EtP, nPrP and nBuP were applied and compared to a solvent control (EtOH, 0.05 %).

There was a significantly decrease of cell index values in the early differentiation period (day 1-6) for all investigated parabens. Only for nPrP a significantly decrease was also found from day 6 onwards. However, in regard to the impedance based cell index, no clear distinction between the parabens could be made.

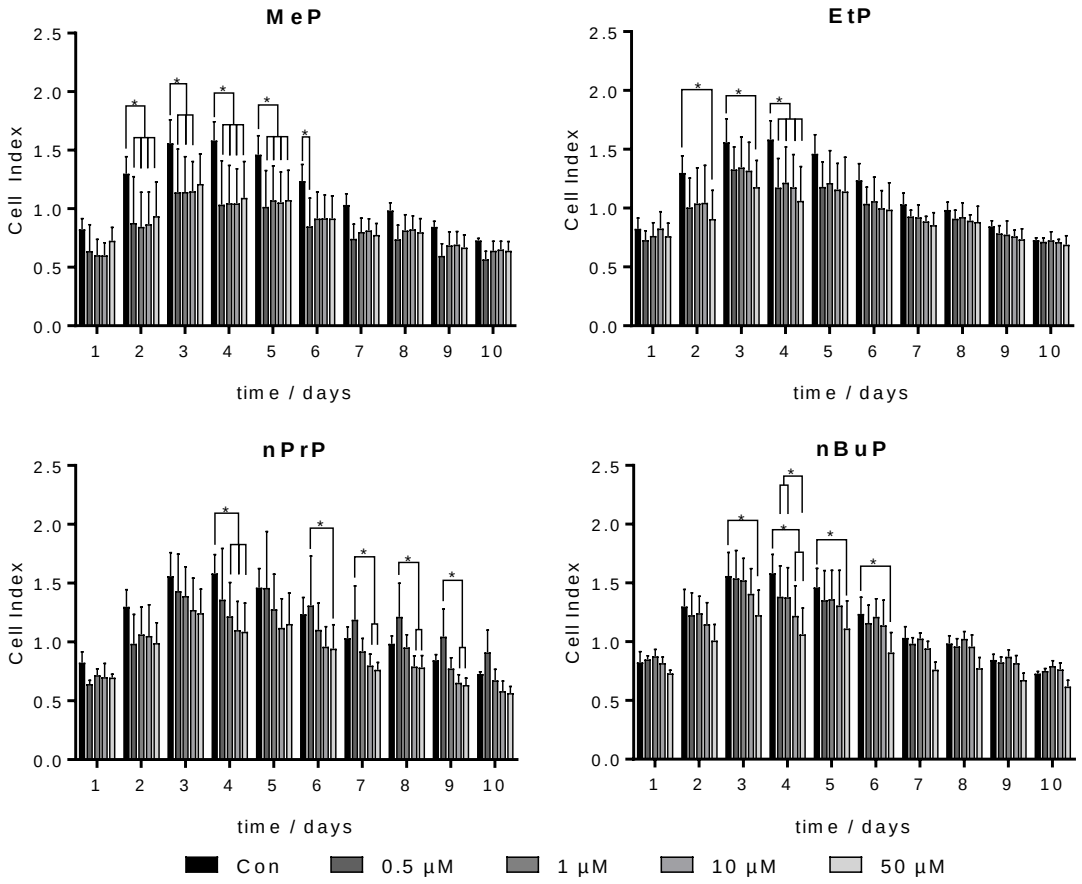


Figure 26: Real-time monitoring of adipocyte differentiation from human MSCs with exposure to 0.5-50 µM parabens. Shown are normalised cell index values (xCELLigence System) over 10 days of differentiation (mean ± SEM, n=3), Significant *p*-values (**p*<0.05) were derived from 2-Way ANOVA.

Gene expression analysis at day 10 of differentiation showed no differences in expression of the transcription factors *PPARγ* and *SREBP1* as well as of *ESR1* and *IRS2* (Figure 27). However, another important transcription factor in adipogenesis *CEBPα* was significantly upregulated in 50 µM nPrP (1.9-fold to control, *p*=0.032) and in 50 µM nBuP (2.5-fold to control, *p*<0.001). Also *ADIPOQ* as a marker for adipogenesis was upregulated under 50 µM EtP (2.3-fold to control, *p*=0.033), nPrP (2.4-fold to control, *p*=0.014) and nBuP (4.2-fold to control, *p*<0.001) exposure. *LPL* as a marker for lipid metabolism was upregulated for 50 µM EtP (2.2-fold to control, *p*=0.021) and nBuP (4.1-fold to control, *p*<0.001). Interestingly, *LEP* expression was significantly down-regulated for all parabens with the most persistent effect for long chain nPrP and nBuP with a significantly down-regulation even at 0.5 µM (nPrP: 0.22-fold to control, p=0.001; nBuP: 0.15-fold to control, p<0.001).

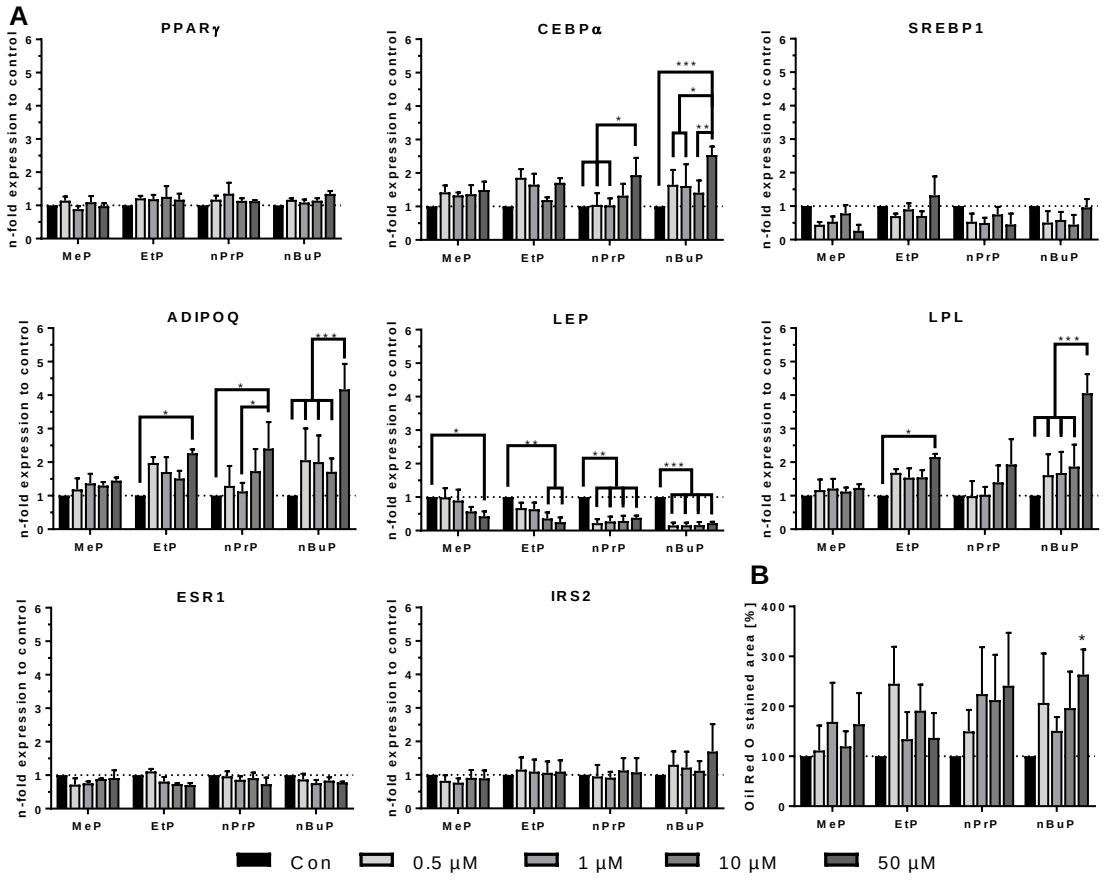


Figure 27: Adipogenic gene expression and triglyceride storage under paraben exposure. Human mesenchymal stem cells were differentiated to adipocytes for 10 days under exposure to 0.5-50 µM of either methylparaben (MeP), ethylparaben (EtP), n-propylparaben (nPrP) or n-butylparaben (nBuP).Shown are means ± SEM of n=3 experiments. Significantly differences were derived by 2-Way ANOVA with **p*<0.05, ***p*<0.01,****p*<0.001. (A) Gene expression of the transcription factors peroxisome proliferator-activated receptor gamma (*PPARγ*), CCAAT/enhancer-binding protein alpha (*CEBPα*), sterol regulatory element-binding proteins1 (*SREBP1*), adipocytokines adiponectin (*ADIPOQ*) and leptin (*LEP*), lipoprotein lipase (*LPL*) and receptors oestrogen receptor α (*ESR1*) and insulin receptor substrate 2 (*IRS2*). (B) Triglyceride storage of adipocytes assessed via Oil Red O staining.

The amount of triglyceride storage was increased in trend by 50 µM of nPrP and significantly by nBuP (nPrP: 2.4-fold to control, *p*=0.094; nBuP: 2.6-fold to control, *p*=0.043). MeP and EtP were not significantly affected by paraben exposure, as can be seen in Figure 27B.

Additionally to the gene expression data, the secretion of adiponectin and leptin into the cell culture supernatant was assessed to validate the unexpected adipocytokine expression after exposure to nPrP and nBuP. MeP was also used in the experimental setup as a no/weak effect paraben control with respect to gene expression of adiponectin and leptin. Secreted adiponectin levels significantly increased after exposure to nBuP in a dose dependent

manner (Figure 28A), which is in line with the gene expression results. There were no effects for nPrP and only a minor increase for 0.5 µM MeP. Secreted leptin levels decreased in a dose dependent manner after exposure to nPrP and nBuP, with a significantly effect at 50 µM (nPrP: 0.5-fold to control, p=0.025; nBuP: 0.5-fold to control, p=0.035) (Figure 28B). As expected, there was no effect for MeP exposure.

To check whether the results were affected by cytotoxicity a MTT assay was applied before and after differentiation of MSCs (Figure 28C and D). Cytotoxicity could not be detected, instead cell viability was increased for 0.5 µM MeP, nPrP, nBuP and 1 µM nBuP before differentiation and for 10 µM MeP and 1 µM EtP after differentiation.

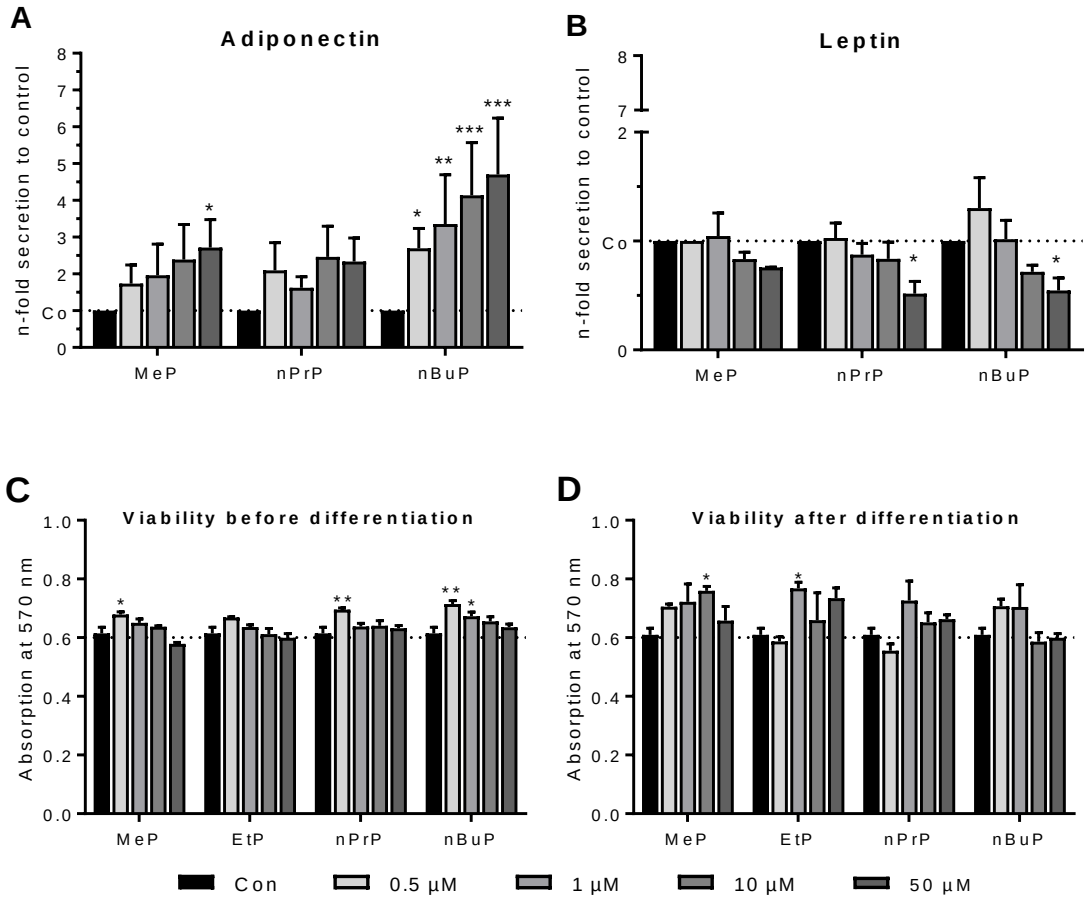


Figure 28: Impact of paraben exposure on adipocytokine secretion and cell viability of human mesenchymal stem cell derived adipocytes. Shown are means ± SEM for n=3 experiments. Significantly differences were derived by 2-Way ANOVA with *p<0.05, **p<0.01, ***p<0.001. (A) Adiponectin levels in cell culture supernatant with regard to the control. (B) Leptin levels in cell culture supernatant with regard to the control. (C) Cell viability of human mesenchymal stem cells after exposure to parabens for 48 h. Shown is the absorption at 570 nm after a MTT assay. (D) Cell viability of human mesenchymal stem cell derived adipocytes after exposure to paraben for 10 days. Shown is the absorption at 570 nm after a MTT assay.

3.5. Impact of perinatal maternal stress on overweight development in preschool children (Project C)

The following results have been submitted for publication as Englich et. al. ““*Longitudinal maternal stress assessment in LINA: Stress characteristics and impact on weight development in early infancy”.*

In addition to the early priming by endocrine disruptors or maternal health, also psychological priming takes place in the sensitive perinatal time window. Especially maternal stress during pregnancy but also later on is under suspicion to impact children’s weight development. However longitudinal maternal stress assessments in the perinatal time window in combination with longitudinal weight assessment of the child have not been investigated so far. Therefore project C aimed to distinguish between the impact of prenatal maternal stress (that may be mediated by maternal stress related hormones) and postnatal maternal stress (that may be associated with the implementation of adverse behaviour patterns) on children’s weight development.

Personal contributions include the manuscript preparation in cooperation with KM. Junge and S. Trump and all statistical analyses.

The analyses in project C were based on the sub-cohort of 498 mother-child pairs for which a complete perceived stress questionnaire (PSQ) assessment including pregnancy, year 1 and year 2 as well as weight related confounders were available. General study characteristics (gender, gestational week at delivery, mode of delivery, birth weight, household members, breast feeding, smoking during pregnancy, parental education, household income and parental divorce/separation) were equally distributed between the analysed sub-cohort (n=498) and the entire LINA cohort (n=629) (Table 20).

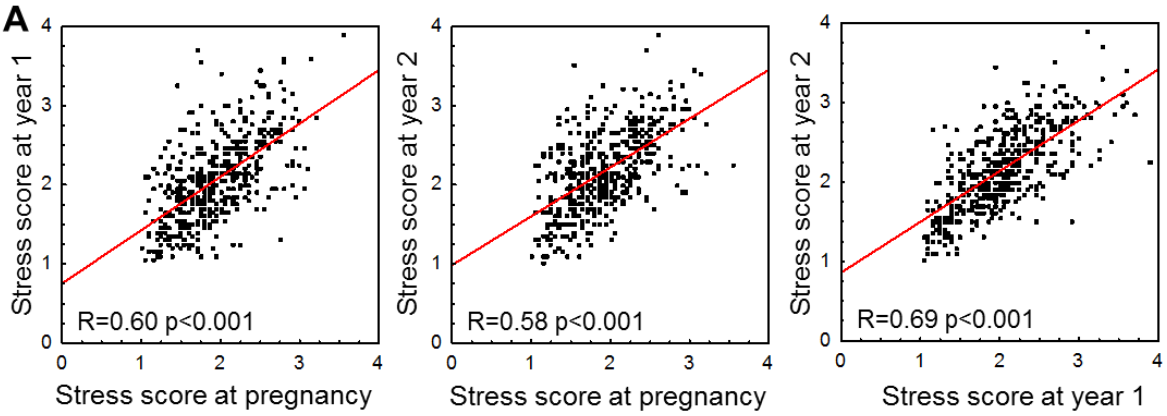
Maternal perceived stress levels were assessed during pregnancy as well as the 1 and 2 year follow-up using the reduced German version of the PSQ scoring the answers on a scale from (1) hardly ever to (4) usually [161, 174]. Median, minimum, maximum, lower and upper quartiles of maternal stress values are shown in Figure 29B. Maternal median stress levels were lowest in pregnancy with 1.85 and increased subsequently to a median score of 2.15 at the 2-year follow-up. Pre-and postnatal maternal stress levels were highly correlated with each other (Figure 29A).

Table 20: Study characteristics of the total study cohort and the analysed sub-cohort for project C

	Entire LINA study cohort n (%), n=629[a]	**Analysed sub-cohort** n (%), n=498[a]	**χ^2-test**
Gender			0.810
Male	332 (52.8)	253 (50.8)	
Female	297 (47.2)	245 (49.2)	
Gestational week at delivery			0.951
<37 weeks	25 (4.0)	16 (3.2)	
37-40 weeks	389 (61.8)	308 (61.8)	
>40 weeks	214 (34.0)	174 (34.9)	
Mode of delivery			0.979
Spontaneous	471 (74.9)	387 (77.7)	
C-section	132 (21.0)	104 (20.9)	
Others	7 (1.1)	7 (1.4)	
Birth weight			0.996
<3000g	123 (19.6)	92 (18.5)	
≥3000g – 3500g	242 (38.5)	195 (39.2)	
≥3500g – 4000g	192 (30.5)	151 (30.3)	
≥4000g	71 (11.3)	60 (12.0)	
Household members			0.887
2	33 (5.2)	26 (5.2)	
3	365 (56.6)	300 (60.2)	
≥4	203 (32.3)	196 (39.4)	
Breastfeeding			0.515
1.-3. month	112 (17.8)	87 (17.5)	
1.-6. month	268 (42.6)	166 (33.3)	
1.-12. month	254 (40.4)	226 (45.4)	
Parental education [b]			0.697
Low	16(2.5)	6 (1.2)	
Medium	144(22.9)	101 (20.3)	
High	468(74.4)	391 (78.5)	
Household income per month			0.648
<2000€	240 (38.2)	172 (34.5)	
2000€ - 4000€	308 (49.0)	171 (34.3)	
>4000€	42 (6.7)	35 (7.0)	
Separation/divorce [c]			0.973
yes	25(4.0)	23 (4.6)	
no	169(26.9)	158 (31.7)	
Prenatal ETS exposure[d]			0.243[e]
Median [µg/g creatinine]	2.0	1.85	
<25% , >75% [µg/g creatinine]	0.8,5.6	0.75,4.95	

a – n may be different from total n due to missing data
b – low = 8 yrs of schooling (‘Hauptschulabschluss`); medium = 10 yrs of schooling (`Mittlere Reife`); high = 12 yrs of schooling or more (`(Fach-)hochschulreife’)
c – parental separation/divorce in the last 3 years from children’s age 3 years
d – ETS = environmental tobacco smoke (urinary cotinine level at pregnancy)
e – *p*-value derived from Student’s t-test between group means

A



B

	Median	Min	Max	< 25%	> 75%
Pregnancy	1.85	1.00	3.55	1.55	2.25
Year 1	2.00	1.05	3.90	1.70	2.40
Year 2	2.15	1.00	3.90	1.85	2.50

Figure 29: Maternal perceived stress levels during pregnancy, year 1 and year 2. Stress levels were assessed with the reduced Perceived Stress Questionnaire (PSQ) scored (1) hardly ever to (4) usually. (A) Spearman correlation of maternal perceived stress levels from pregnancy, year 1 and year 2. (B) Description of maternal perceived stress levels with median, min, max and quartile boundaries, n=498.

By applying an adjusted GEE model (adjusted for gestational week at delivery, mode of delivery, breast-feeding duration, exposure to environmental tobacco smoke (urinary cotinine level during pregnancy and gender, if applicable), a significantly effect of maternal perceived stress levels at year 1 on children's longitudinal BMI z-score development until the age of 5 years (adj. β: 0.23, 95% CI (0.08-0.37), p = 0.002) was observed (Figure 30A). There was no effect of maternal stress during pregnancy or year 2 on children's BMI z-scores (Table S2). By stratifying the GEE model for maternal stress at year 1 for gender we could show that only BMI z-scores of girl's were positively affected by maternal stress (adj. β: 0.30, 95% CI (0.11-0.49), p = 0.002) (Figure 30B), whereas BMI z-scores of boys were unaffected (Table S3). This gender-specific effect was not based on different study characteristics between boys and girls as can be seen from Table S4. As the maternal perceived stress level during the first year after birth had the most prominent effect on BMI z-scores, further analyses on were focused on this early postnatal period.

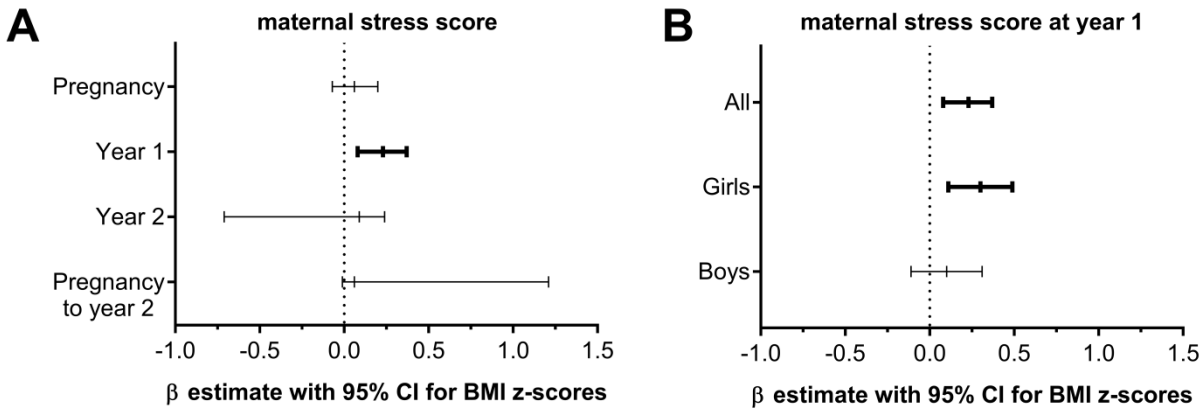


Figure 30: Adjusted effect of maternal perceived stress levels on longitudinal BMI z-scores in preschool children. β estimates and 95 % confidence intervals (CI) from longitudinal GEE models for the effect of (A) maternal stress at pregnancy (n=498), year 1 (n=491), year 2 (n=473) and total stress from pregnancy to year 2 (n=473) on children' BMI z-score development up to year 5. (B) Gender stratified GEE model for the effect of maternal perceived stress at year 1 on BMI z-score development in Girls (n=241) and Boys (n=250) until year 5. Significantly associations are presented in bold ($p \leq 0.05$).

3.5.1. Influence of different stress dimensions on BMI z-score development

Further the contribution of different stress dimensions assessed by the PSQ at year 1 ("demands", "tension", "worries" and "lack of joy"), which are summarized as the total stress score, to children's overweight development was calculated. Median, minimum, maximum, lower (<25%) and upper quartiles (≥75%) of maternal stress dimensions are given in Table 21. Median scores for "tension" and "demands" (2.20) scored highest, followed by "lack of joy" (2.00) and "worries" (1.60).

Table 21: Characteristics of maternal perceived stress scores of the four different stress dimensions at year 1. Stress dimensions were assessed with the reduced perceived stress questionnaire (PSQ) scored 1 (almost never) to 4 (usually) with 5 questions each, n=498.

	median	min	max	<25%	>75%
Worries	1.60	1.00	4.00	1.20	2.00
Tension	2.20	1.00	4.00	1.80	2.60
Lack of joy	2.00	0.80	3.80	1.60	2.40
Demands	2.20	1.00	4.00	1.80	2.60

GEE models adjusted for the mentioned weight-related confounders are summarized in Table 22. After Bonferroni-correction for multiple testing, the stress dimensions "tension", "lack of joy" and "demands" showed a significantly positive association with children's BMI z-scores, with "tension" showing the best model fit (QIC) and highest β-estimate.

Table 22: Effect of the different stress dimensions on the longitudinal BMI z-score development in preschool children

		β estimate[a]	95% CI	*p*-value[b]	QIC[c]
Entire cohort	(n=491)				
Worries		0.14	0.01 – 0.27	0.039	2051.99
Tension		**0.19**	**0.07 – 0.31**	**0.003**	**2041.97**
Lack of Joy		**0.15**	**0.04 – 0.27**	**0.009**	2045.80
Demands		**0.19**	**0.05 – 0.33**	**0.006**	2048.45
Girls only	(n=241)				
Worries		**0.24**	**0.06 – 0.41**	**0.009**	1005.93
Tension		0.22	0.05 – 0.39	0.014	1013.26
Lack of Joy		**0.23**	**0.08 – 0.38**	**0.002**	**1003.31**
Demands		**0.24**	**0.05 – 0.43**	**0.012**	1012.31
Boys only	(n=250)				
Worries		-0.01	-0.18 – 0.18	0.963	1056.08
Tension		0.13	-0.03 – 0.29	0.108	1048.00
Lack of Joy		0.06	-0.11 – 0.23	0.503	1054.36
Demands		0.11	-0.08 – 0.31	0.247	1053.93

a - Estimates derived from general estimation equations (GEE) for BMI z-scores (birth to age 5) as dependent variable, adjusted for gestational week at delivery, mode of delivery, pregnancy cotinine levels and breastfeeding duration.
b - Bonferroni adjusted significance level, $p \leq 0.0125$
c - Quasi- Akaike Information Criterion (QIC) for model selection

As seen for the total stress score the different dimensions of maternal stress only showed an association to girl's BMI z-scores, whereas in boys none of the stress dimensions were related to their BMI z-scores. "Worries", "lack of joy" and "demands" were significantly associated with higher BMI z-scores in girls, from which "lack of joy" showed the best model fit (QIC).

3.5.2. Influence of maternal stress on early maternal feeding behaviour

The particular impact of maternal perceived stress during the first year of life, a time window in which children are dependent on the maternal feeding behaviour, led to the assessment of the impact of the duration of breastfeeding and the time-point of solid-food introduction on children's BMI z-scores. However, both parameters, evaluated in 3-month-intervals during the first year of life, were not related to pre- and postnatal maternal perceived stress within the LINA study (Table 23).

Table 23: Influence of maternal stress at year 1 on breastfeeding duration and introduction to solid food. Breastfeeding and introduction to solid food was assessed in 3-month-intervals during the first year of life. Estimates derived from general estimation equations (GEE) for BMI z-scores (age 1 to age 5) as dependent variable

	β estimate	**95% CI**	***p*-value**
breastfeeding duration	-0.01	-0.10 – 0.08	0.798
introduction to solid food	-0.03	-0.15 – 0.09	0.622

3.5.3. Multiple stressors contribute to maternal stress perception

Since there was a clear impact of maternal stress on children's weight development, potential *stressors* contributing to the perceived maternal stress level were identified. Based on questionnaire information potential contextual features (e.g. neighbourhood quality, educational level, low income) were assessed, which might contribute to the overall perception of stress in the mothers.

Based on 10-items from the questionnaires assessing the neighbourhood quality a principal factor analysis was conducted. Three factors with eigenvalues over Kaiser's criterion of 1 were identified, which in combination explained 58.1% of the variance. Table 24 shows the factor loadings after rotation. The items that clustered on the same factor loadings suggested the factors represented by "poor living conditions", burdens due to "traffic", and exposure to "residential noise", respectively. All three of these factors showed a significantly association to the maternal stress level at year 1(Figure 31,Table S5). Of the socio-demographic factors analysed only low household income contributed significantly to the overall stress perceived by the mothers. Additionally information about parental separation or divorce during the first 3 years was available from 189 participants and associated with an increased maternal stress level at year 1 (β: 0.33, 95% CI (0.14-0.55), $p = 0.003$).

Table 24: Summary of exploratory factor analysis results of questionnaire items assessing the residential environment (n=495). Factor loadings over 0.4 appear in bold

ITEMS	**ROTATED FACTOR LOADINGS**		
How often did you experience the following impairments in your neighborhood?[a]	Poor living conditions	Traffic	Residential Noise
Vandalism	**0.828**	-0.026	0.023
Graffiti	**0.826**	-0.091	0.008
Attempted break-ins/thefts	**0.479**	0.091	-0.078
Dirty streets (e.g. waste, dog excrements)	**0.453**	0.147	0.175
Odours/exhausts	0.001	**0.797**	-0.028
Traffic noise	0.009	**0.793**	0.002
Commercial noise	0.006	0.362	0.080
Noise from pedestrians	-0.091	0.002	**0.885**
Noise from neighbours	0.116	0.016	**0.417**
Noise from restaurants/clubs	0.042	0.018	0.176
Eigenvalues	3.35	1.43	1.03
% of variance	33.5	14.3	10.3
α	0.74	0.76	0.57

a - Answered on a four-point scale from hardly ever (0) to usually (3)

The impact of these stressors on the different stress dimensions however was quite different. While “worries”, “tension” and “demands” were similarly positively associated with the factors of poor neighbourhood quality, “lack of joy” was not affected (Table S6). However “lack of joy” was positively associated with a “low household income”, as was an increase in “worries”. A low educational level and the number of household members, which were not associated to the overall maternal stress level, were significantly associated with the stress dimensions “lack of joy” and “demands”.

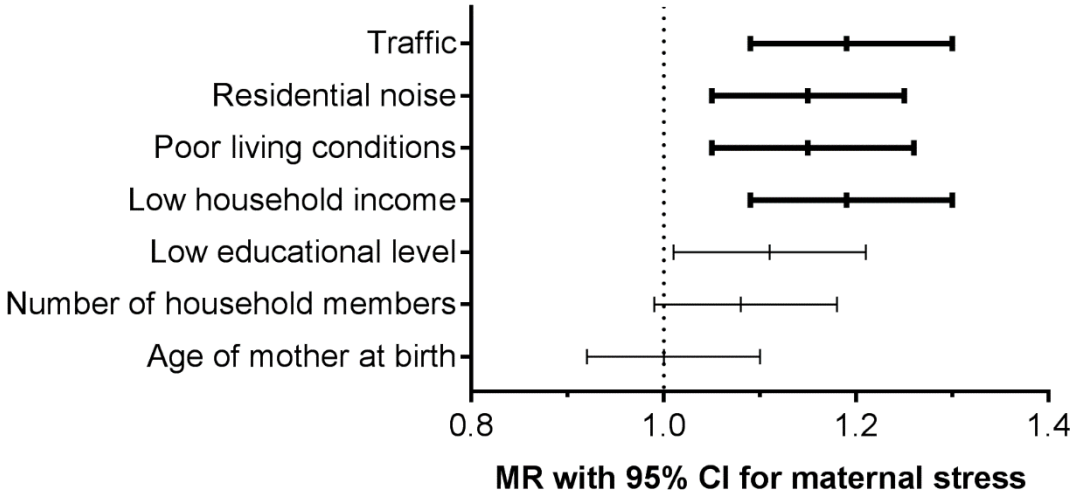
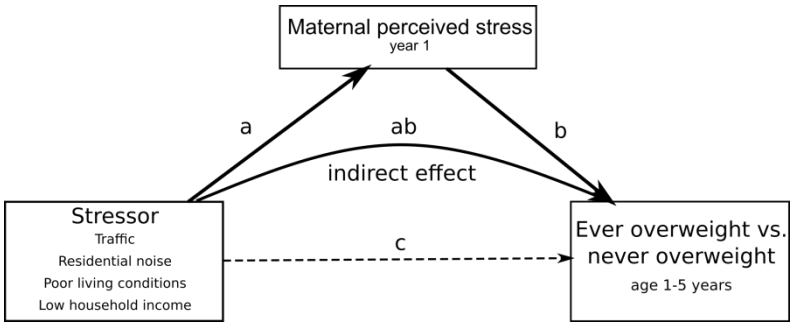


Figure 31: Effects of different stressors on maternal perceived stress. Mean ratios and 95 % confidence intervals for the effect of shown stressors on maternal perceived stress levels at year 1 were calculated from multiple regression models. Significantly associations after Bonferroni correction are depicted in bold, p ≤ 0.007.

3.5.4. Impact of stressors on weight development

A mediation analysis showed that neighbourhood strains (noise, traffic, poor living environment) did not affect girl's BMI z-score development directly. However they had an indirect effect on BMI z-scores mediated by maternal perceived stress (Figure 32).



Stressor	n	**a** (95%CI)	*p*-value	**b** (95%CI)	*p*-value	direct effect **c** (95%CI)	*p*-value	indirect effect **ab** (95%CI)
Traffic	191	0.20(0.09-0.30)	**0.001**	0.63(0.06-1.21)	**0.031**	0.04(-0.37-0.46)	0.884	**0.12(0.01-0.30)**
Residential noise	189	0.19(0.05-0.33)	**0.007**	0.71(0.14-1.29)	**0.015**	0.04(-0.51-0.58)	0.891	**0.14(0.02-0.35)**
Poor living conditions	189	0.17(0.03-0.31)	**0.019**	0.84(0.26-1.43)	**0.005**	-0.58(-1.16-0.01)	0.052	**0.14(0.02-0.35)**
Low household income	180	0.05(0.01-0.09)	**0.029**	0.78(0.21-1.35)	**0.008**	-0.11(-0.27-0.05)	0.191	**0.04(0.01-0.09)**

Figure 32. Summary of mediation analysis. Stressors themselves had no direct effect on girl's BMI-z-scores but rather mediated their effect by their contribution to the maternal perceived stress level. Confidence intervals for the indirect effects were derived for 5000 bootstrap samples. Confidence intervals do not cross 0 indicating significance of the indirect effect and are presented in bold.

4. DISCUSSION

Obesity is a multifactorial disease that often originates in early childhood and adolescents. Despite the common knowledge that the 20th century lifestyle with a high caloric food intake and a sedentary behaviour contribute to an increased prevalence for overweight and obesity, both cannot explain the fast raise in obesity rates all over the world [12]. Therefore environmental, socio-economic and genetic factors gained more and more attention in the scientific community to unravel the roots of this epidemic. Especially early priming in the perinatal period became of interest in regard to childhood overweight development. During this highly sensitive period changes in maternal and environmental factors can implement tremendous alterations in the foetal and infant development that might even prolong until adulthood. Following this hypothesis, harmful triggers don't have to affect the infant/child itself but might already be mediated and transferred via the maternal *in utero* conditions.

In this context the aim of the present study was to investigate pre- and perinatal risk factors for childhood obesity with regard to maternal health (project A), environmental exposure to pollutants (project B_1 and B_2) and maternal stress (project C) in the epidemiological, prospective birth cohort LINA and to support the results with mechanistic *in vitro* studies, if applicable.

Epidemiological data in general can examine multiple health effects due to a certain exposure scenarios, with the participating subjects being defined according to their exposure levels and followed over time for outcome occurrence. However, general descriptive characteristics of the study participants need to be considered in case of data interpretation and comparison with other reference studies. Within the LINA study, data from more than 600 mother-child pairs was collected from pregnancy until primary school age within regular annual clinical visits and through self-administrated questionnaires. As often observed in cohort studies the majority of LINA participants can be considered of a high socio-economic status (SES) with a high educational level (75%), intermediate household income (49%), comparably low pregnancy smoking rates (15%) [175, 176] and a high percentage of mothers given birth spontaneously (75%). This shift is due to the fact that families with a high SES show more motivation to join studies, to fill out intense questionnaires or to attend regular clinical visits, compared to families with a low SES [177]. Thus the group that is on highest risk for overweight development might consequently often be inadequately represented in longitudinal studies.

According to the study outcome of interest, most babies had a normal birth weight between 3000 g and 4000 g (69 %) but already 11 % of new-borns had a birth weight of more than 4000 g and 20 % were less than 3000 g, setting these babies on higher risk for overweight development [178]. The overall prevalence for overweight (including obesity) up to the age of 8 years was 15 %, which is in line with the prevalence for overweight assessed by the large German KIGGS Wave 1 study (15 %), collecting data from more than 4000 children age 4-10 years between 2009 and 2012 [179]. Data collected from the CrescNet data base in the East German area in 2008 revealed an overweight prevalence of 11 % in children age 4-8 years [180]. But this lower prevalence might be due to higher cut-offs (90^{th} percentile) for overweight compared with the WHO cut-offs (85^{th} percentile; used in LINA). BMI z-scores at birth were highly correlated with all investigated children's BMI z-scores up to the age of 8 years, which is in line with previous studies [181-183] and indicates that early priming impacts long-term weight development.

That highlights the high impact of alterations during sensitive windows in development, when epigenetic marks are implemented, metabolic priming takes place and organ development (including brain, liver and adipose tissue) is in progress. One of these sensitive windows is the prenatal phase, when the foetus is highly dependent on the maternal environment. But certainly correlations of BMI z-scores improve noticeable from year 3 onwards, indicating that stable overweight development is manifested in this period, as also proposed by large observational studies like the CrescNet data base [173, 184].

First indications that maternal health and nutrition during pregnancy can influence the prevalence for overweight development in their children originate from the large Dutch famine study [67]. Exposure to famine during gestation was associated with an increased risk for the development of a metabolic syndrome with insulin tolerance, overweight and coronary heart disease. However, besides the maternal nutritional status, little is known about the impact of prenatal and early postnatal exposures on children's weight development.

4.1. Maternal cytokine status during pregnancy may prime overweight development in children and their metabolome (project A)

One prenatal factor that might influence infant's body composition is the maternal cytokine status at pregnancy. So far, there is only conflicting data on the impact of the maternal inflammatory immune status on children's weight development and there were no studies investigating particularly the association of the maternal adaptive immune system and children's longitudinal body weight. Therefore the aim of project A was to investigate the

influence of maternal Th1 and Th2 blood cytokine levels on children's overweight development, which was recently published in the *International Journal of Obesity* [185].

The present data indicated that especially low levels of maternal IL4 and IL13 were related to an increased risk for overweight development in children up to the age of 3 years. Both cytokines have anti-inflammatory functions in adipose tissue and are representatives of the lean Th2 phenotype of adipose tissue in adults [56, 186]. Mechanistic studies found that IL4 may directly be linked to adipogenesis by inhibiting terminal differentiation of pre-adipocytes *in vitro* via a STAT6 mediated pathway [77]. However, there is evidence from mouse studies that both IL4 and IL13 may not be able to cross the blood-placenta barrier [187], which makes a direct effect on adipose tissue development highly unlikely. Nevertheless, cytokines can influence the nutrition release from the placenta by binding interleukin receptors at the placenta surface [188], thereby indirectly changing the foetal environment and along going development [189].

Since a direct impact of maternal IL4 and IL13 on organ development (for example adipose or brain tissue) is unlikely, the metabolic profile might be changed due to altered foetal nutrition. The metabolic profile comprises information on metabolic processes including for example disease-triggering changes of lipid metabolism in obesity and may thereby help to understand underlying pathophysiological mechanisms in the onset of the disease. In fact a positive association of maternal IL13 levels, and children's acylcarnitine levels, as well as an inverse association of acylcarnitine levels and children's BMI z-scores was found. Although increased acylcarnitine levels have been reported for adults suffering from obesity and T2DM [190], data for overweight children under the age of 5 years are sparse [191-194]. Acylcarnitines transport fatty acids into the mitochondria for β-oxidation, a process that is often impaired in obese subjects and accompanied by a reduced oxidatively mitochondrial capacity resulting in increased acylcarnitine levels [195]. Acylcarnitines are released from muscle or adipose tissue in order to prevent intracellular carnitine-related cytotoxicity, leading to increased blood carnitine levels [196]. In that context especially the ratio of acetylcarnitine and free-carnitine is discussed as a marker of lipid metabolism dysfunction. Certainly the observed ratios for C2:C0 and C4:C3 were not affected by either maternal cytokine levels nor by children's weight status (data not shown). However, infantile mitochondria are often able to compensate for increased fatty acid metabolism, potentially resulting in overcompensation with decreased overall acylcarnitine levels in overweight children [197]. Although this indicates a mediating role of acylcarnitines on maternal IL13 induced overweight development, the present data does not support this hypothesis. One explanation could be that acylcarnitine levels may be altered as a secondary marker due to a different lipid

metabolism in patients with obesity and just picture children's overweight state with no causal direct effect of IL13.

Furthermore it was observed that only children without maternal history of atopy are prone to overweight development when exposed to low maternal IL4 and IL13 levels compared to children with maternal history of atopy. The hypothesis that atopic mothers have higher intrinsic IL4 and IL13 levels due to their disease state and therefore do not reach the low overweight associated cytokine concentrations could not be approved within this project.

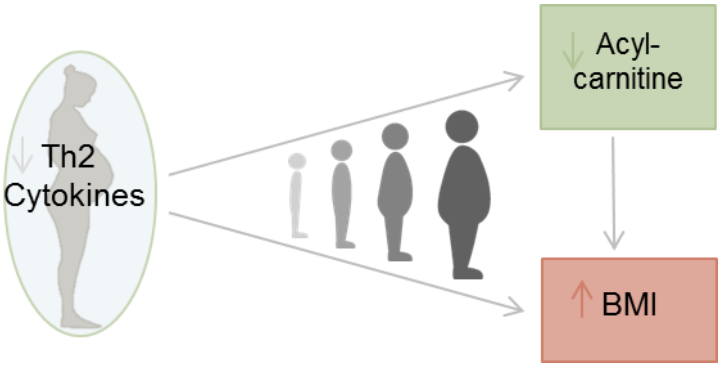


Figure 33: Summary scheme for the association of maternal cytokine level, children's weight development and metabolome

Taken together the results indicate an association of the maternal immune status during pregnancy with children's longitudinal overweight development as well as overweight related metabolic alterations in acylcarnitines, as summarized in Figure 33.

4.2. Exposure to endocrine disruptors may lead to increased risk for overweight development

Besides the intrinsic exposure to maternal inflammatory and metabolic factors, the foetus is also exposed to external factors like environmental pollutants that are able to cross the placenta-blood barrier. Some environmental pollutants possess the ability to mimic hormonal activity and therefore are classified as endocrine disruptors (EDCs). According to the obesogen hypothesis, some of these endocrine disruptors are able to disrupt pathways that are related to adipocyte development, hunger and appetite regulation or weight development in general [198].

For example, there are many obesogenic EDCs like TBT/TPT, DES or BPA that have been reported to enhance adipocyte development and increase risk for overweight in rodent and human studies, but a conclusively substantiated mechanism for overweight promotion has not been reported yet. A reasonable explanation would be that many EDCs don't act via one receptor only, but interfere with many endocrine pathways.

Some members of the organotin class of persistent organic pollutants, like tributyltin (TBT) and triphenyltin (TPT), have been shown to be highly potent PPARγ-RXR agonists [199, 200], thereby driving lipid biosynthesis and storage, favouring adipocyte development *in vitro* and modulate pro-adipogenic gene expression. Organotins are used as anti-fouling paints, plasticizers and fungicides on food. One European study found TPT at 0.09 to 0.67 ng/ml blood but only occasional trace amounts of TBT [201]. However, human exposure to organotins is not well studied so far.

Far more EDCs act via sex steroid dysregulation by mimicking oestrogen or androgen activity. Oestrogen replacement therapies in menopausal women are generally protective against weight gain [202] but studies indicate that exposure to oestrogens at critical periods of foetal development can have an obesogenic effect due to a foetal oestrogenisation syndrome [203]. Rodent experiments showed that prenatal exposure to diethylstilbestrol (DES), a potent oestrogen replacement reagent, yielded in weight gain along with elevated levels of leptin, adiponectin, triglycerides and altered insulin signalling in female offspring mice [204]. Male mice did not become obese, reflecting the often observed gender difference in EDC response. But other potentially oestrogenic compounds like parabens are far less studied and many endocrine disruptors are yet to be identified.

One highly investigated EDC that has been reported to act on various pathways is BPA. BPA is an oestrogenic compound that was found to accelerate pre-adipocyte cell differentiation by up-regulation of adipogenic gene expression [103, 104, 205], to modulate the insulin dependent PI 3-kinase/Akt pathway [103] and to affect hunger and appetite regulation by changing neuropeptide Y expression in the midbrain [206]. Recent studies also indicate that BPA might affect epigenetic priming during the very sensitive prenatal time window [146, 207, 208]. Whether these observations are linked to the obesity promoting features of BPA was investigated within project B_1 of this thesis and is discussed in the following section.

4.2.1. MEST mediates the impact of prenatal bisphenol A exposure on long-term body weight development (project B_1)

BPA is one of the most studied endocrine disruptors and even so conflicting studies exist, there is a broad consensus that BPA exposure during pregnancy and early infancy may lead to adverse health outcomes. Thus BPA is already banned from consumer products in most western countries. However, studies that report novel mechanisms for BPA induced risk for overweight development are rare but would help to identify other chemicals as harmful to human health.

After oral administration BPA is rapidly bio-transformed to glucuronidated BPA in the liver via UDP-glucuronosyltransferase (UGT) and is eliminated by urinary excretion within 24 h [209, 210]. Interestingly, studies in rats suggest that BPA metabolism might change during pregnancy due to alterations in UGT isoforms and expression levels [211]. In addition, decreased UGT levels in foetal liver can lower the excretion capacity for BPA, making the foetus even more vulnerable to environmental pollutant EDC exposure [212-214].

There is evidence that BPA exposure interferes with DNA methyltransferase 3a/ 3b (DNMT2A/ DNMT3B) expression in mice, that specifically affect the *de novo* methylation of imprinted genes during development [215] and may lead to alterations in the epigenetic code and increase risk for disease development. Moreover BPA might interfere with folate metabolism as studies show that folate supplementation during pregnancy can recover BPA induced epigenetic changes [216]. Previous studies associated epigenetic alterations related to BPA exposure with an increased risk of carcinogenesis in rodent models of hepatic and prostate cancer [217-219]. But so far, no data on BPA-induced epigenetic modifications leading to overweight development exist.

Project B_1 provides evidence that prenatal BPA exposure causes a hypo-methylation in the human *MEST* promotor, resulting in increased *MEST* expression and increased risk for overweight development in children. To support the epidemiological data, *MEST* methylation and expression after BPA exposure was also investigated in an *in vitro* adipogenesis model and an *in vivo* mouse study.

MEST is a paternally imprinted gene that encodes a member of the α/β hydrolase fold family with currently unknown function. However, *MEST* expression has been described to be associated with obesity [147, 220, 221], adipocyte size [172] and pre-adipocyte development [222] in mouse and human studies. One of these studies found that *Mest* knock-out mice showed reduced body weight and less obesity. Further, *Mest* expression has been associated with variable obesity in mice and is attenuated by a positive energy balance [222]. They found that high-gainers showed increased *Mest* expression even after one week of high fat diet and propose that *Mest* expression level may foreshadow food metabolism capacity in mice [223-225].

In addition, prenatal BPA exposure was already linked to epigenetic modifications of the *Mest* gene in a murine study, with BPA induced hypo-methylation of the *Mest* promotor region in murine oocytes [226]. Within project B_1 it was shown for the first time that also in humans prenatal BPA exposure is related to DNA methylation changes in the *MEST* promotor. Further, it was already suggested that the *MEST* methylation status is associated with increased obesity risk in humans. In addition, results from project B_1 indicate that a

hypo-methylation of the *MEST* promotor region may link prenatal BPA exposure to overweight development in the offspring. In line with this hypothesis, *MEST* expression was associated to an increased BMI z-score of children on a longitudinal scale.

MEST is predominantly expressed in mesenchymal tissue and also in mesenchymal stem cells, which are the source of adipose tissue; but not in blood cells. Since cord blood, in which the epigenetic analysis in LINA was performed, contains a sizeable number of MSC's it can be assumed that the observed hypo-methylation relates to an altered DNA-methylation in the cord-blood MSC fraction (which fits to the approximate percentage of the MSC fraction in cord blood and the observed delta methylation). Unfortunately, this could not be approved within the LINA study, due to limited cell availability. Thus, an *in vitro* model was applied to analyse the impact of BPA on MSCs.

After adipocyte differentiation from BPA exposed human MSCs a hypo-methylation of the *MEST* promoter region and an enhanced *MEST* expression supported the hypothesis that BPA induces *MEST* activation in human MSCs, which corroborates a role of *MEST* in BPA induced adipogenesis. In agreement with other studies BPA increased both *ESR1* and *PPARG* expression along with downstream targets like *LPL* [227, 228]. Further this thesis is about the first to describe a dose dependent difference in cell index values of real-time impedance monitoring of adipocyte differentiation after BPA exposure. Adipocyte differentiation can be monitored by impedance based monitoring tools revealing a decrease in cell index values following differentiation through massive morphological changes from MSCs to adipocytes [229, 230]. A BPA induced decrease in cell index values may therefor indicate an enhanced adipogenesis, which might be due to the concomitantly observed activation of *MEST* and *PPARG,* crucial for adipocyte commitment.

Further, *Mest* hypo-methylation was in line with enhanced *Mest* expression in visceral fat tissue and increased body weight of prenatally with BPA exposed mice in an associated study that supports our hypothesis as well as recent studies on *MEST* function in adipose tissue.

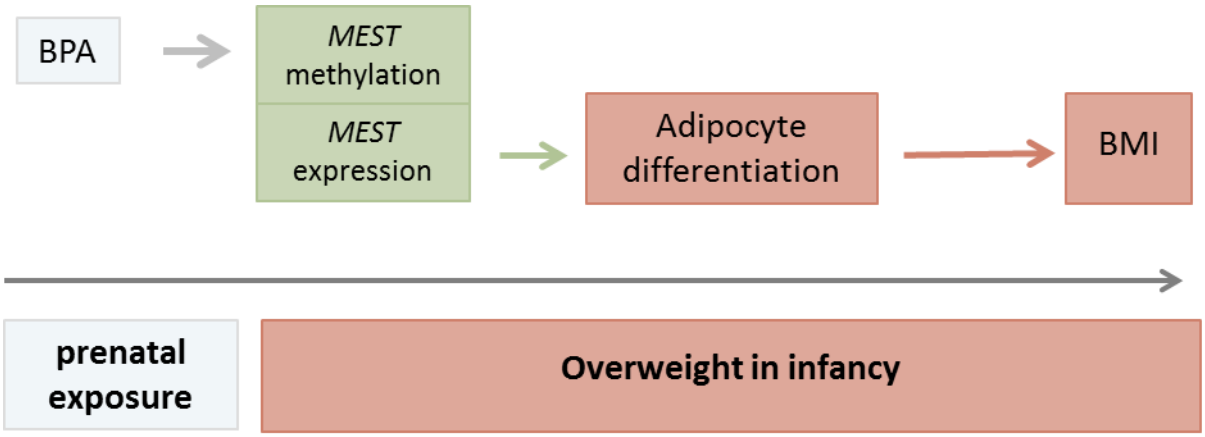


Figure 34: Summary scheme: Results overview indicating the influence of prenatal BPA exposure on MEST methylation and expression that mediates is associated with adipocyte differentiation and overweight development in infant offspring.

To conclude, project B_1 combines epidemiological data with *in vitro* and *in vivo* experimental models to demonstrate that prenatal BPA exposure seems to be a contributing factor in the development of overweight in early childhood by implementing epigenetic changes in the obesity related gene *MEST*, as summarized in Figure 34.

4.2.2. Prenatal exposure to parabens increases risk for overweight development in children and alters adipogenesis in vitro (Project B_2)

Another group of industrial chemicals that was just recently reported to possess endocrine disrupting properties but has not been extensively studied so far are parabens. Parabens are currently still authorized for the use as preservatives in cosmetic products by the Scientific Committee on Consumer Safety (SCCS). However, the committee's opinion is based on a few studies in rodents and artificial cell culture models with toxicological read-outs at high paraben concentrations. Similar to BPA, parabens are weak oestrogenic compounds with oestrogen-activity increasing with chain length. Hence, parabens are suspected to have harmful effects within the human body even at nontoxic concentration levels. An influence on overweight development, especially when exposure starts during the early prenatal time window seems to be reasonable, especially after some very exemplary epidemiological hints from the literature [16]. Therefore the aim of this project B_2 was to investigate the influence of a prenatal exposure to parabens on overweight development in children on a longitudinal scale.

Paraben concentrations were measured in 504 urine samples of pregnant mothers within the LINA study cohort that were collected between 2006 and 2008 in Leipzig, Germany. Three parabens (iPrP, sBuP and BzP) could not be detected in the analysed samples. Paraben concentrations for MeP, EtP, nPrP, iBuP and n-BuP were considerably higher than reported for women in studies from Canada (2013), Sweden (2014) and Germany (2016) with mean

values for BuP that ranged from 0 µg/l to 0.3 µg/l [231, 232] for example, compared to 1.8 µg/l for nBuP in LINA. But it has to be considered that these studies collected their samples more than 5 years later than the LINA study and after the first evaluation of parabens by the SCCS or other chemical safety authorities took place. Therefore it is likely that paraben concentrations in cosmetic products were significantly reduced within this time period and as a consequence thereof human exposure decreased. Moreover the awareness of endocrine disruptors was raised in recent years and many cosmetic products are advertised as paraben-free now.

As parabens are frequently used in cosmetic products, it can be assumed that cosmetics are a principal source of human paraben exposure. This assumption could be approved within the LINA cohort. Mothers that used cosmetic leave-on products that contain parabens on a daily basis had significantly higher urinary paraben concentrations. Even so the actual number of paraben containing products might be underestimated due to limited data on cosmetic product composition 8-10 years ago (with presumably higher paraben content). Another potential source of paraben exposure would be the consumption of convenience products and other highly processed foods but unfortunately food consumption during pregnancy was not sufficiently assessed within the LINA study. Interestingly, paraben concentrations are also correlated with maternal age at delivery. Parabens are absorbed by the skin after application and hydrolysed to PHBA and paraben conjugates in the liver before excreted via the kidneys. In humans also free paraben species can be detected in blood and urine samples when paraben load is high. However, either for metabolized or free paraben species a bioaccumulation has not been reported so far and seems not to account for the higher paraben levels in older woman Therefore it can be assumed that older women do either apply cosmetic products more often or have a lower capacity to metabolise parabens. A reduced efficiency for paraben metabolism can also be assumed for foetuses, new-borns and infants with immature drug metabolising enzymes, as was already discussed for BPA. Hence, higher body fluid levels of free paraben species may set children on higher risk for adverse outcomes.

With respect to the project hypothesis, high prenatal paraben levels, particularly butylparaben levels, were indeed associated with higher BMI z-scores in children of the LINA study up to year 8. Adjusted regression models suggested an increased risk for overweight development at birth and later infancy, when exposed to high butylparaben levels during prenatal development. Parabens with shorter chain length (MeP, EtP, nPrP) did not have an effect on children’s BMI z-scores. In confirmation, increased nBuP concentrations were identified as mediator of maternal cosmetic product application and increased BMI z-scores at birth.

This is in line with results from the *in vitro* adipogenesis model. The most significantly and consistent changes in adipogenic gene expression for *CEBPα*, *ADIPOQ* and *LPL* as well as adipocytokine secretion of adiponectin and leptin were observed for nBuP. For real-time monitoring no consistent changes, as seen for BPA, could be observed. However, nBuP was shown to have the highest oestrogenic activity of the investigated parabens, with an EC_{10} for activation of oestrogen receptor α (ERα) of 2 µM. Although other studies also report the activation of the glucocorticoid receptor [113, 233] or even PPARγ [111], this could not be approved within this project. However, cytotoxicity kicked in at rather low concentrations in the applied reporter gene assays and may have mask activation of PPARγ and GRα.

The fact that girls were more vulnerable to prenatal BuP exposure than boys also seems to be reasonable in the context of ERα activation. Gender differences in susceptibility are often observed phenomena for EDCs but so far no convincing explanation exists. It seems reasonable that the oestrogen activity of many EDCs accounts for some variation in susceptibility to EDCs and that early sensitisation to high oestrogen levels has a higher impact on female metabolism. Studies in rodents show that exposure to oestrogens is causing a foetal oestrogenisation syndrome, as was already discussed. A study by Newbold et al. also reported that female mice treated with DES for only 5 days in the neonatal period had increased body weight compared to the control beginning at 2 months of age, which was maintained until adulthood [204]. But certainly nBuP exposure did not change *ESR1* expression in the present *in vitro* adipogenesis model.

Further hints for a mechanistic link between prenatal BuP exposure and overweight development were derived from the *in vitro* studies in project B_2. It was observed that leptin expression and secretion was significantly decreased after exposure to butylparaben, whereas adiponectin levels increased in a dose-dependent manner. Leptin and adiponectin are adipocytokines that are almost exclusively produced by adipocytes and secreted to the periphery. During adipogenesis expression of both adipocytokines usually increases and only mature adipocytes produce and secret leptin and adiponectin. Increased expression of adiponectin as well as LPL and CEBPα indicates an increased adipogenesis after exposure to nBuP, therefore someone would expect an associated increase in leptin levels. But somehow leptin production seems to be disrupted in nBuP exposed adipocytes. The result may be an inadequately increased feeling of hunger and appetite *in vivo*, as leptin production is no longer proportional to body fat mass. Evidence for this hypothesis comes from a yet unpublished mouse study, where they see that dams exposed to nBuP have an increased food intake compared to controls (data not shown). However, further research is necessary to proof this theory.

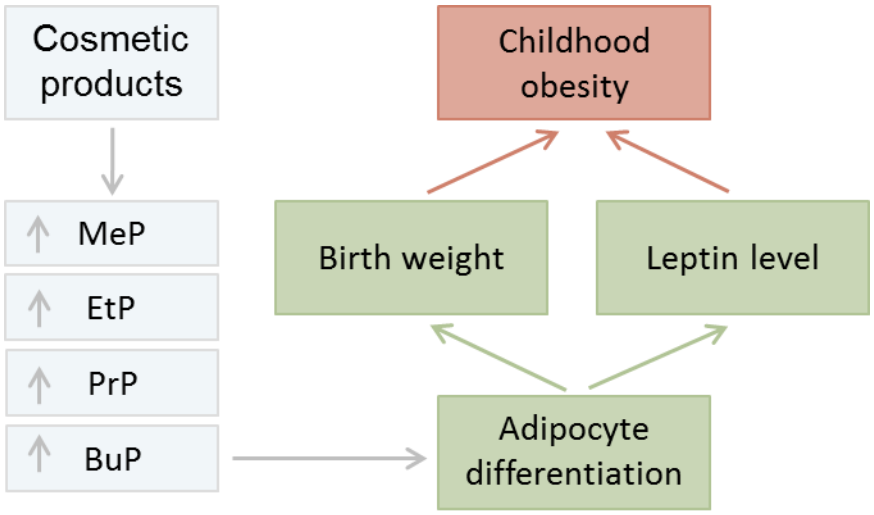


Figure 35: Summary scheme for the impact of prenatal paraben exposure on adipocyte development and childhood obesity prevalence

To summarize, human exposure to parabens is at least partly due to cosmetic product consumption that contain parabens as preservatives. Increased prenatal exposure to butylparaben increases risk for overweight development in children at birth and later infancy (year 3-8). A possible mechanism would be an increased appetite and related food intake due to disruptions in leptin metabolism (Figure 35).

4.2.3. Relevance of concentrations used in in vitro studies

For the risk assessment of parabens and BPA, concentrations between 0.5 and 50 µM of each compound were tested. Those concentrations were chosen after a literature review to make a comparison of studies possible. Further there was no cytotoxicity detected at the applied concentrations. However, when compared to concentrations found in human samples, these concentrations seemed rather high. Especially concentrations for BPA found in human urine samples of the LINA study (12 ng/mg creatinine, equals 40 nM) are 1000-fold lower compared to the applied concentrations *in vitro*. Certainly BPA is rapidly metabolised within approximately 24 hours after exposure. Maternal spot urine samples were collected in the morning hours after at least 8 hours of sleep without food or water consumption. Therefore the detected BPA concentrations may be underestimated when considering a total BPA load of the day. Unfortunately, the availability of repeated total day urine samples is very limited and therefore a longitudinal estimation of BPA exposure was not possible within the LINA cohort study design.

Urinary concentrations of parabens vary for different paraben species with 0.9 µM for MeP and 4 nM for nBuP in LINA. However, it must be considered that only free or glucuronidated parabens have been detected. Paraben species that were metabolised to PHBA could not be detected in maternal urine. Furthermore, local paraben concentrations after the application of

cosmetic products can yield much higher values. Therefore it can be assumed that internal paraben concentrations are much higher but so far there are no assessments of e.g. adipose tissue or amniotic fluid paraben concentrations available. Bioaccumulation is not reported for both compounds and therefore must not be taken into account.

4.3. Longitudinal maternal stress and its impact on weight development in early childhood

As already discussed, obesity is a multifactorial disease with several causes. So far prenatal exposures to maternal cytokines and pollutants have been discussed to alter metabolic or epigenetic priming of the child. But additionally behavioural priming takes places in early infancy. Especially high maternal stress is under suspicion to favour adverse behaviour in children, setting them on higher risk for overweight development. However, only a few longitudinal studies exist so far [119, 121, 234] and none of them is investigating the pre- and early postnatal time to address the question, whether increased risk for overweight development results from stress hormones during pregnancy and breastfeeding or the implementation of behavioural structures during the first years of life. Therefore the present thesis investigates whether and how maternal stress in the highly vulnerable early pre- and postnatal period is related to overweight development in preschool children.

In line with previous studies, the present results indicate a longitudinal association between maternal perceived stress and weight development of the child. There is evidence that maternal stress during pregnancy can alter the placental environment via an increased glucocorticoid exposure [125, 235], thereby potentially influencing the metabolic priming [236, 237] as discussed before. However, a longitudinal impact of prenatal maternal stress on children's BMI z-scores could not be observed within the LINA study. Therefore it is likely that this effect may be superimposed by other factors like parenting practices, mother-child interactions or other environmental factors.

But importantly maternal stress during the first year of life was identified as a persistent predictor of overweight development up to the age of 5 years. That is in line with other studies, suggesting that toddlers (1-3 years) are more vulnerable to maternal stress than infants (<1year) [130], potentially via their own stress perception. Of particular interest during the first year of life in regard to overweight development is the breastfeeding behaviour of the mother. While maternal stress can negatively affect initiation of breastfeeding, its influence on breastfeeding duration is less clear. There are a few hints indicating a shorter duration of breastfeeding in relation to maternal stress [127]. However, there was no evidence for alterations in breastfeeding duration as well as time of solid food introduction as possible factors for overweight development within the analysed LINA cohort.

Next to the excluded direct mother-child-transfer of stress mediators via the breastmilk, literature suggests that maternal stress may lead to obesity-promoting feeding styles and feeding attitudes, which in turn could establish and manifest long time eating/weight-related behavioural patterns in the child [238, 239]. Studies show that maternal stress is not only associated with lower motivation for food preparation but also seems to diminish fruit and vegetable consumption in the children [240, 241]. Further on, this is associated with an increased sedentary behaviour of the child and less motivation of the mother to take their children to sport events [242, 243].

Surprisingly maternal stress at year 2 was not associated with increased BMI z-scores within the LINA cohort. That observation was unexpected, since maternal stress levels were highest for the year 2 assessment. This might be due to the fact, that the majority (68%) of children started day care during their second year of life, thereby spending a significantly amount of time away from their mother as their primary care taker.

Moreover this study shows that a gender disparity in stress vulnerability was already seen in very early childhood. An increased risk for overweight development was only found in girls of stressed mothers, whereas boys were unaffected. A gender disparity in stress perception, processing and coping mechanisms is well known in adults and adolescents [244, 245] but is less often reported in early childhood. Particularly girls have been described to respond to stress by impulsive eating or requesting sweet and high fat foods [119, 127]. In line, a study by Suglia et al. reported that girls experiencing high cumulative stress were at a higher risk for obesity development, whereas boys were not affected [119].

Further on the influence of different stress dimensions (“demands”, “worries”, “tension” and “lack of joy”) on overweight development was assessed. Especially, high scores for “worries” and “lack of joy” increased the risk for overweight development in girls, whereas there was no association found in boys. This observation is supported by previous studies. Female children experiencing anxiety or depression have an increased BMI [245]. Other studies suggest that maternal depression can promote overweight development in females but not males at age 18 [246, 247].

In addition it was of interest, which stressors contribute to maternal stress throughout the years. The urban environment provides several sources of stressors [248]. Noise exposure is a well-described example that waas already studied for its impact on prenatal/postnatal growth [249-251] and associated to adiposity and metabolic outcomes in adults [252-254]. Three potential stressors were identified, covering among others different sources of noise, which were all related to the quality of the living environment (burden due to traffic, residential noise and poor living conditions). While these stressors similarly influenced the

overall maternal perceived stress, and the three stress dimensions “worries”. “tension”, and “demands”; “lack of joy” was only affected by a low household income and low educational level. The only other stressor with a significantly impact on perceived maternal stress was a low household income, which increased not only “lack of joy” but also “worries”. These stressors are likely related to a low socioeconomic status of the families, which is known to be strongly associated with obesity in the Western world but also in developing countries adopting a Western lifestyle [116-118].

Interestingly, the stressors themselves did not have a direct effect on girl’s BMI z-scores but rather mediated their effect through their impact on the maternal perceived stress level. As the maternal perceived stress is most likely a cumulative account of these stressors and potentially also covers others stress-related factors, single stressors might not have a sufficient predictive power. This phenomenon is known as the cumulative risk theory, stating that a combination of adverse effects within a family can increase the obesity risk, whereas unfavorable social factors in isolation often do not [255] (as summarized in Figure 36).

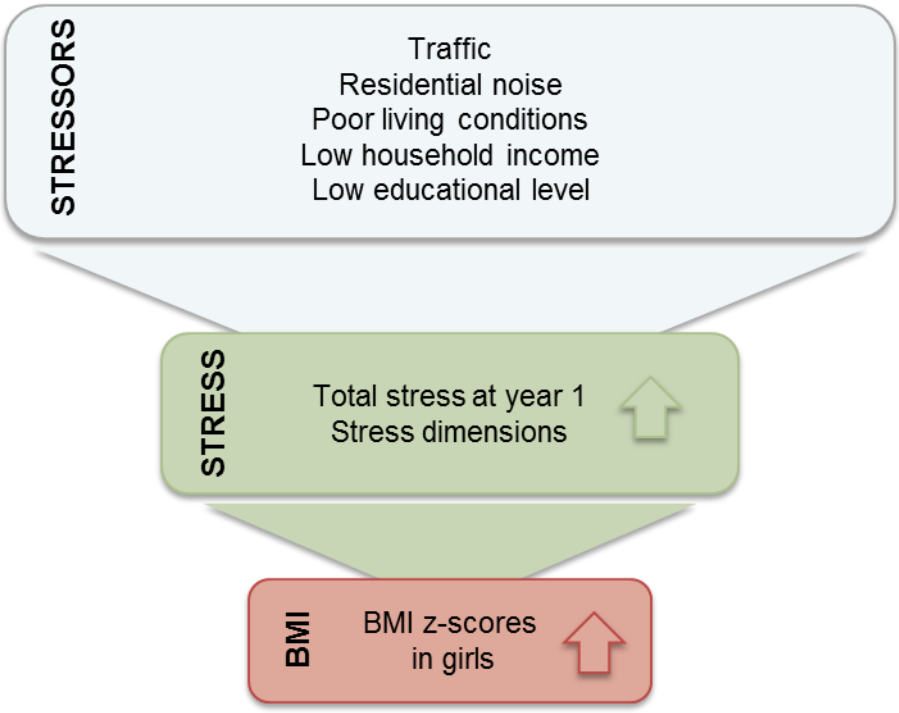


Figure 36: A summary scheme of the influence of perceived maternal stress level on overweight development in preschool children with all contributing stressors according to the cumulative risk theory.

To summarize, perceived maternal stress during the first year had a consistent and long-term effect on the development of overweight particularly in girls.

4.4. Strength and shortcomings of the present thesis

Three perinatal risk factors for childhood overweight development were identified within this study that cover maternal health during pregnancy, exposure to environmental pollutants and maternal perceived stress (see final summary Figure 37).

All four projects used a longitudinal, epidemiological approach and assessed risk for overweight development in children from birth up to the age of 6 years (8 years for project B_2). Longitudinal approaches have the advantage that conservative conclusions about causality can be drawn from the results, compared to the rather associating character of one point analyses within cross-sectional cohort studies. For example, this study is about the first to show the relevance of prenatal exposure to butylparaben in case of triggering obesity development. It can be recommended, that authorities should not only consider restricting paraben supplementation in childcare products (as it already was recommended for propyl- and butylparaben by the SSC in 2013) but should also direct their attention to pregnant women as a group on high risk; either for themselves or the unborn foetus. Moreover, the results drawn from that thesis should encourage other research groups to more deeply use their epidemiological data and investigate the effects of EDCs on human health. Scientific results showing either known or generally new substances as harmful chemicals, especially in terms of childhood health, will hopefully put pressure on authorities and industry to ban these EDCs from consumer products.

Furthermore the impact of maternal health and perceived stress on children's weight development elucidates that prevention or intervention studies should even start before birth and pay more attention to the whole family, rather than just focusing on the child or a single person.

Another strength of this thesis is the combination of epidemiological and *in vitro* studies. Therefor epidemiological observations could be validated and supported by possible underlying mechanisms for the impact of BPA and paraben exposure on adipogenesis.

However, obesity is a multifactorial disease and it is not possible to take all aspects of obesity development into consideration when doing analyses within one cohort study. Especially in early childhood factors like genetics, diet, child care, physical activity, psychological aspects, diseases and many more can influence weight development and may also superimpose prenatal priming. Unfortunately, the LINA study did not qualitatively assess childhood food intake, child's physical activity, child's psychological aspects and genetic background (including maternal weight before pregnancy) until now. Thus these factors could not be taken into consideration for infant overweight risk assessment.

Also foetal exposure to chemicals was only assessed once in maternal spot urine samples at pregnancy. To get a full idea of human exposure, some would have to collect whole day urine samples over a couple of days, but that is usually not feasible in big cohort studies. In addition to the prenatal conditions, it would further be interesting to assess the exposure to chemicals after birth and elucidate the health effect of children that are continuously exposed to high concentrations of EDCs by themselves.

Besides it was not possible to distinguish between overweight and obese children within the LINA study, as obesity rates were quiet low in the very young children (about 3%, which equals less than 20 children). Overweight was defined by using the WHO reference data with slightly lower cut-offs (85^{th} percentile for overweight) compared to big German studies like KIGGS [18] and Kromeyer-Hauschild (90^{th} percentile for overweight) [256]. However, the Kromeyer-Hauschild study (data assessment 1985-1999) does not include representative data for children under 2 years of age and the KIGGS study was done in the early 2000^{th} (2003-2006), when the obesity epidemic has already started. The WHO data was collected between 1997-2003 for children age 0-2 years and in the 60^{th} and 70^{th} for children > 2 years in different countries in Europe, North America, South America and Asia. In summary, children may have been classified as overweight on a slightly lower BMI z-score compared to other German based studies.

Taken together this study is of interest for the scientific community, because of its longitudinal approach starting already in pregnancy and following up children to the age of 8 years (at the time of the thesis). It adds new information for the understanding of EDC exposure and function and for understanding the impact of maternal cytokine and perceived stress levels on children’s overweight development.

5. SUMMARY

Obesity is a multifactorial disease that often originates in early childhood and adolescents. Despite the common knowledge that the 20th century lifestyle with a high caloric food intake and a sedentary behaviour contribute to an increased prevalence for overweight and obesity, both cannot explain the fast increase in obesity rates all over the world. Therefore environmental, socio-economic and epigenetic factors gained more and more attention in the scientific community to unravel the roots of this epidemic.

Within the last years early priming in the perinatal period became of special interest in regard to later overweight development. During this highly sensitive period changes in maternal and environmental factors can implement tremendous alterations in the foetal and infant development that might even prolong until adulthood. Following this hypothesis, harmful triggers do not have to affect the born infant/child itself but might already be mediated and transferred via the maternal *in utero* conditions. In that context, this thesis aimed to investigate the impact of a broad spectrum of different potential risk factors (including maternal health, environmental exposure to pollutants/endocrine disrupting chemical (EDCs) as well as maternal stress) in the pre- and early postnatal period on childhood overweight development within the epidemiological, prospective mother-child study LINA. In addition, relevant mechanistic aspects are considered in corresponding *in vitro* analyses.

One prenatal factor that might influence infant's weight development is the maternal cytokine status. So far, there is only conflicting data on the impact of the maternal inflammatory immune status on children's weight development and there were no studies investigating particularly the association of the maternal adaptive immune system and children's longitudinal weight gain. Within project A it was shown that a decreased maternal Th2 cytokine level of IL13 increased risk for overweight development up to the age of 3 years. As IL13 is not able to cross the blood-placenta barrier, a direct influence on adipocyte development is highly unlikely. But instead this study suggested that decreased maternal IL13 level alter infant lipid metabolism via their acyl-carnitine levels and thereby potentially setting the child on higher risk for overweight development.

Next to the maternal immune status and associated *in utero* signals that could be transferred to the foetus, also external environmental chemicals to which the mother was exposed to during pregnancy were assessed within that thesis. As seen in project B_1, prenatal exposure to BPA, the highly prevalent EDC and most important component in the manufacture of polycarbonate plastics, led to a hypo-methylation and increased expression of the *mesoderm*

specific transcript (*MEST*), a gene that was recently recognized as associated to high weight gain and adiposity in human and murine studies. In fact, *MEST* methylation was mediating BPA-induced increase in BMI z-scores of children up to the age of 6 years. These results were supported by an *in vitro* adipogenesis model of human mesenchymal stem cells. BPA treated adipocytes showed a hypo-methylation of the *MEST* promotor region and increased *MEST* expression along with increased adipogenesis.

Moreover this thesis showed in project B_2 for the first time that prenatal exposure to another EDC, the long chain butylparaben increases risk for overweight development in 3-8 year old children whereas there was no association of lower chain-length parabens with children's BMI z-scores. This effect was seen in girls only when separated for gender. The applied *in vitro* adipogenesis model supported these epidemiological observations. After exposure to nBuP adipocytes showed higher adipogenic gene expression and increased triglyceride storage. However, leptin expression and secretion were significantly reduced in exposed cells. Whether an altered leptin metabolism of adipocytes after exposure to nBuP and subsequently a potentially modified hunger/appetite regulation via the brain may lead to a higher risk for overweight development, is currently under investigation in a cross-generational mouse study.

In addition to the maternal immune status or environmental chemical exposures, behavioural priming takes places in early infancy. Especially high maternal stress is under suspicion to favour adverse behaviour in children, setting them on higher risk for overweight development. However, only a few longitudinal studies exist so far and none of them is investigating the pre- and early postnatal time to address the question, whether increased risk for overweight development results from transferred stress hormones during pregnancy and breastfeeding or may be the implementation of maternal behavioural structures during the first years of the child's life. Project C looked at associations of pre- and postnatal maternal perceived stress with childhood BMI z-scores on a longitudinal scale. Therefore it was possible to outline that maternal perceived stress in the children's first year of life has the most tremendous impact on children's overweight development up to year 5. It was proposed that adverse behavioural patterns due to maternal stress may be implemented during that time. Again this association was only seen in girls, when stratifying for gender.

Taken together this thesis identified different perinatal risk factors that seem to contribute to the cumulative risk for childhood overweight development. All four projects used a longitudinal epidemiological approach and assessed risk for overweight development in children from birth up to the age of 8 years in combination with hypothesis driven mechanistic *in vitro* and *in vivo* analyses. Because of this highly broad and qualitative experimental setting results could and should be used to raise public awareness: For example, this thesis

is about the first to show the relevance of prenatal exposure to butylparaben in case of triggering obesity development. Therefore, it should be recommended, that authorities do not only consider restricting paraben supplementation in childcare products but should also direct their attention to pregnant women as a group on high risk; either for themselves or the unborn foetus. Furthermore, results shown in this thesis highlight the aspect that the status of maternal health as well as her individual stress perception should intensively be considered when strategies for early childhood overweight prevention are developed. Based on the present data early prevention should therefore start already before birth and needs to include the whole family.

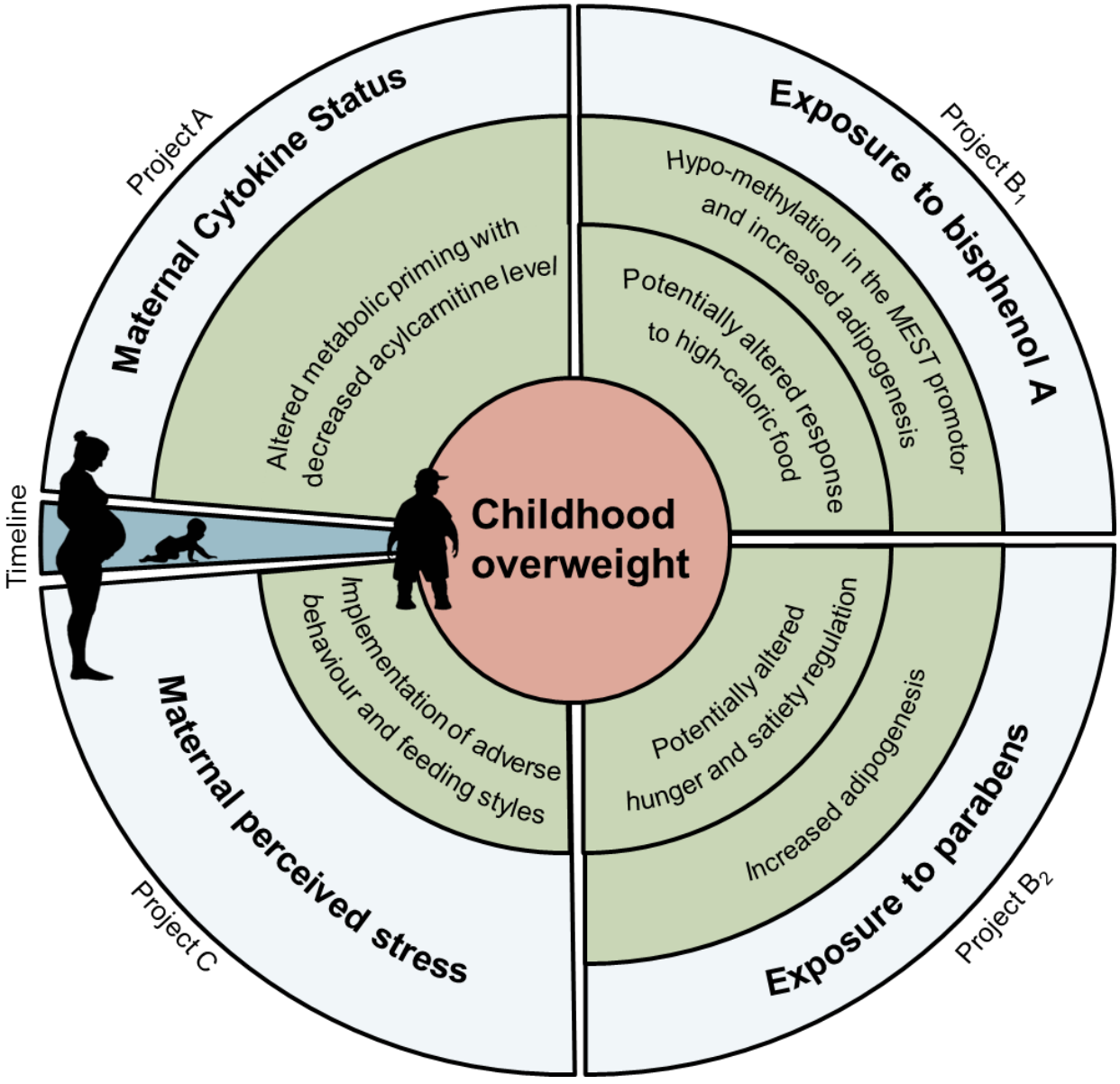


Figure 37. Summary figure to highlight the processed projects and results within the present thesis.

ZUSAMMENFASSUNG

Übergewicht und Fettleibigkeit sind komplexe metabolische Erkrankungen, welche ihren Ursprung oftmals schon in der frühen Kindheit und Jugend haben. Trotz des Wissens, dass der Lebensstil des 20ten Jahrhunderts mit der damit einhergehenden hoch-kalorischen Ernährung und reduzierten Bewegung zum Anstieg der Prävalenz für Übergewicht beigetragen hat, kann dies allein nicht die weltweit drastisch steigende Prävalenz von Übergewicht und Fettleibigkeit erklären. Stattdessen sind vor allem in den letzten Jahren Faktoren wie Umwelt, sozio-ökonomischer Status und Epigenetik in den Fokus der Wissenschaft gerückt, um die Wurzeln dieser Epidemie zu ergründen.

Von besonderem Interesse ist dabei die frühe embryonale und postnatale Prägung und deren Auswirkung auf die spätere Entstehung von Übergewicht. Während dieser hoch sensitiven Entwicklungsphasen können äußere Einflussfaktoren immense Veränderungen in der embryonalen und kindlichen Entwicklung bewirken, welche sich zum Teil bis in das Erwachsenenalter auswirken können. Folgt man dieser Hypothese, so muss der Fötus den schädlichen Faktoren nicht einmal selbst ausgesetzt sein, sondern kann deren schädliche Wirkung bereits über die mütterliche *in utero* Umgebung übertragen bekommen. In diesem Zusammenhang war es das Ziel dieser Dissertation, den Einfluss mehrerer potentieller Risikofaktoren (mütterlicher Immunstatus, Exposition mit Umweltschadstoffen/endokrinen Disruptoren (EDC's) und mütterlichem Stress) in der prä- und postnatalen Entwicklungsperiode auf die Ausbildung von kindlichem Übergewicht in der prospektiven Mutter-Kind-Studie LINA zu untersuchen. Zusätzlich wurden die epidemiologischen Beobachtungen durch mechanistische *in vitro* Versuche verifiziert.

Ein pränataler Faktor, welcher als potentieller Risikofaktor für kindliches Übergewicht diskutiert wird, ist der mütterliche Immun- bzw. Zytokinstatus während der Schwangerschaft. Bisher existieren nur widersprüchliche Studien über den Einfluss inflammatorischer (Th1) Zytokine und kindlichem Übergewicht, aber keine Studie adressierte bislang den Zusammenhang zwischen dem mütterlichen adaptiven (Th2) Immunsystem und der kindlichen Gewichtsentwicklung. Im **Projekt A** dieser Dissertation konnte diesbezüglich gezeigt werden, dass vor allem reduzierte Level des mütterlichen Th2 Zytokins IL13 mit einem erhöhten Risiko für kindliches Übergewicht bis zum 3. Lebensjahr assoziiert war. Da jedoch IL13 nicht in der Lage ist die Blut-Plazenta-Schranke zu überwinden, ist ein direkter Einfluss auf die Differenzierung von Fettzellen nicht anzunehmen. Allerdings konnte in dieser Dissertation gezeigt werden, dass ein reduzierter mütterlicher IL13 Spiegel mit einem

veränderten Lipid-Metabolismus (verminderte Acylcarnitine) im Kleinkind assoziiert war und dadurch möglicherweise das Risiko für die Entwicklung von Übergewicht gesteigert wurde.

Neben dem mütterlichen Immunstatus wurde in dieser Arbeit auch der Einfluss von pränataler Chemikalienbelastung aus der Umwelt auf die Entstehung von kindlichem Übergewicht untersucht. Im **Projekt B_1** konnte gezeigt werden, dass eine Exposition mit Bishphenol A (BPA), einem weit verbreiteten EDC und wichtigen Bestandteil bei der Herstellung von Plastikartikeln, während der Schwangerschaft zu einer Hypo-Methylierung und damit einhergehenden gesteigerten Expression des *mesoderm specific transcript* (*MEST*) Genes im Nebelschnurblut führte. Das Gen *MEST* wurde erst kürzlich mit einer raschen Gewichtzunahme und Adipositas in humanen und murinen Studien assoziiert. Tatsächlich vermittelte die verminderte *MEST* Methylierung einen BPA-induzierten Anstieg der BMI z-scores von Kindern bis zum 6. Lebensjahr. Diese Ergebnisse wurden zudem von einem humanen Adipogenese Modell unterstützt. Mit BPA exponierte Adipozyten zeigten neben einer Hypo-Methylierung im *MEST* Promotor und einer gesteigerten Expression auch eine einhergehende vermehrte Adipogenese.

Im **Projekt B_2** dieser Dissertation wurde zudem zum ersten Mal der Einfluss einer pränatalen Exposition mit einer weiteren Klasse an EDCs, den Parabenen, auf die kindliche Gewichtsentwicklung untersucht. Es wurde gezeigt, dass eine erhöhte Exposition mit dem langkettigen Butylparaben zu einem erhöhten Risiko für Übergewicht in 3-8 jährigen Kindern führt. Parabene mit einer kürzeren Kettenlänge hatten hingegen keinen Einfluss auf die Gewichtsentwicklung der Kinder. Zudem wurde hier ein klarer Geschlechterunterschied in der Empfindlichkeit gegenüber Butylparaben festgestellt, wobei ausschließlich Mädchen ein erhöhtes Risiko für die Entwicklung von Übergewicht bei erhöhter Butylparaben-Exposition aufwiesen. Auch hier konnte im *in vitro* Modell eine vermehrte Adipogenese von mesenchymalen Stammzellen nach Parabenexposition beobachtet werden, einhergehend mit gesteigerter Genexpression von Adipozyten-spezifischen Genen und erhöhter Triglycerideinlagerung. Interessanterweise wurde zugleich auch eine verringerte Expression und Sekretion von Leptin, einem zentralen Regulator von Hunger und Sättigung, in exponierten Zellen beobachtet. Inwieweit ein veränderter Leptin-Metabolismus exponierter Adipozyten und eine damit einhergehende Fehlregulation von Appetit und Hunger im Gehirn zu dem erhöhten Risiko für kindliches Übergewicht führen könnte, wird derzeit in einem generations-übergreifenden Mausmodell untersucht.

Zusätzlich zur frühen Prägung des kindlichen Metabolismus durch innere oder äußere Einflussfaktoren, beginnt auch die Manifestation von Übergewichts-relevanten Verhaltensmustern bereits sehr zeitig im Leben. Vor allem mütterlicher Stress steht hier unter Verdacht zur Entstehung von ungünstigen Verhaltensweisen beizutragen und damit

kindliches Übergewicht zu fördern. Allerdings gibt es bislang nur wenige longitudinale Studien auf diesem Gebiet, schon gar nicht mit Fokus auf das hochsensible perinatale Zeitfenster, in denen der Einfluss von prä- und postnatalem mütterlichen Stress auf die Entstehung von Übergewicht im Kindesalter untersucht wurde. Im **Projekt C** dieser Dissertation wurde deshalb epidemiologisch analysiert ob pränataler mütterlicher Stress, vermittelt über Stresshormone, oder postnataler mütterlicher Stress, vermittelt durch die frühe Prägung von Ernährungsmustern oder Verhaltensweisen, einen Einfluss auf die longitudinale Gewichtsentwicklung von Kindern im Vorschulalter hat. Hierbei konnte ausschließlich ein erhöhter postnataler Stresslevel der Mutter während des ersten Lebensjahres des Kindes mit einem longitudinal erhöhten BMI z-score der Kinder assoziierten werden. Das Stillverhalten wurde durch den postnatalen Stress der Mutter widererwartend nicht beeinflusst. Demnach wurde geschlussfolgert, dass während dieser Zeit ungünstige Verhaltensweisen durch die Mutter vermittelt oder/und vom Kind erlernt werden, welche eine spätere Gewichtszunahme begünstigen könnten. Dieser Zusammenhang basierte ebenfalls auf Beobachtungen in den Mädchen.

Zusammengefasst konnten im Rahmen dieser Dissertation jenseits der klassisch adressierten Parameter „ungünstige Ernährung“ und „wenig körperliche“ Aktivität, mehrere prä- und postnatale Risikofaktoren identifiziert werden, welche in Übereinstimmung mit der Kumulativen Risiko-Theorie zu einer Entstehung von kindlichem Übergewicht beizutragen scheinen. Alle 4 Projekte verfolgten dabei einen longitudinalen Ansatz, in welchem prä- und postnatale Risikofaktoren für eine kindliche Übergewichtsentwicklung bis zu einem Alter von 8 Jahren erfasst wurden. Zudem wurden Hypothesen-getriebene *in vitro* Modelle zur mechanistischen Validierung eingesetzt. Aufgrund dieses breit aufgestellten und qualitativen Studiendesigns/Setups können und sollten die erhaltenen Ergebnisse öffentlichkeitswirksam genutzt und diskutiert werden. Hervorzuheben ist dabei die im Rahmen dieser Dissertation zum ersten Mal gezeigte übergewichtsrelevante Risikoerhöhung durch eine pränatale Belastung mit Butylparaben. Dies sollte zum Anlass genommen werden, dass die zuständigen Behörden nicht nur ein Verbot von Parabenen in Kinderprodukten veranschlagen, sondern zudem auch gezielt die Produktpalette von schwangeren Frauen einschließen. Des Weiteren zeigt diese Arbeit, dass sowohl der mütterliche Zytokinhaushalt als auch mütterlicher Stress wichtige übergewichtsrelevante Einflussgrößen sind, die bei Präventionsmaßnahmen unbedingt einbezogen werden sollten. Basierend auf den vorliegenden Daten sollten darüber hinaus - insbesondere in Hoch-Risikofamilien - Präventionsmaßnahmen bereits vor der Geburt des Kindes beginnen und das gesamte Familienumfeld einschließen.

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7. SUPPLEMENTARY MATERIAL

7.1. Supplementary figures

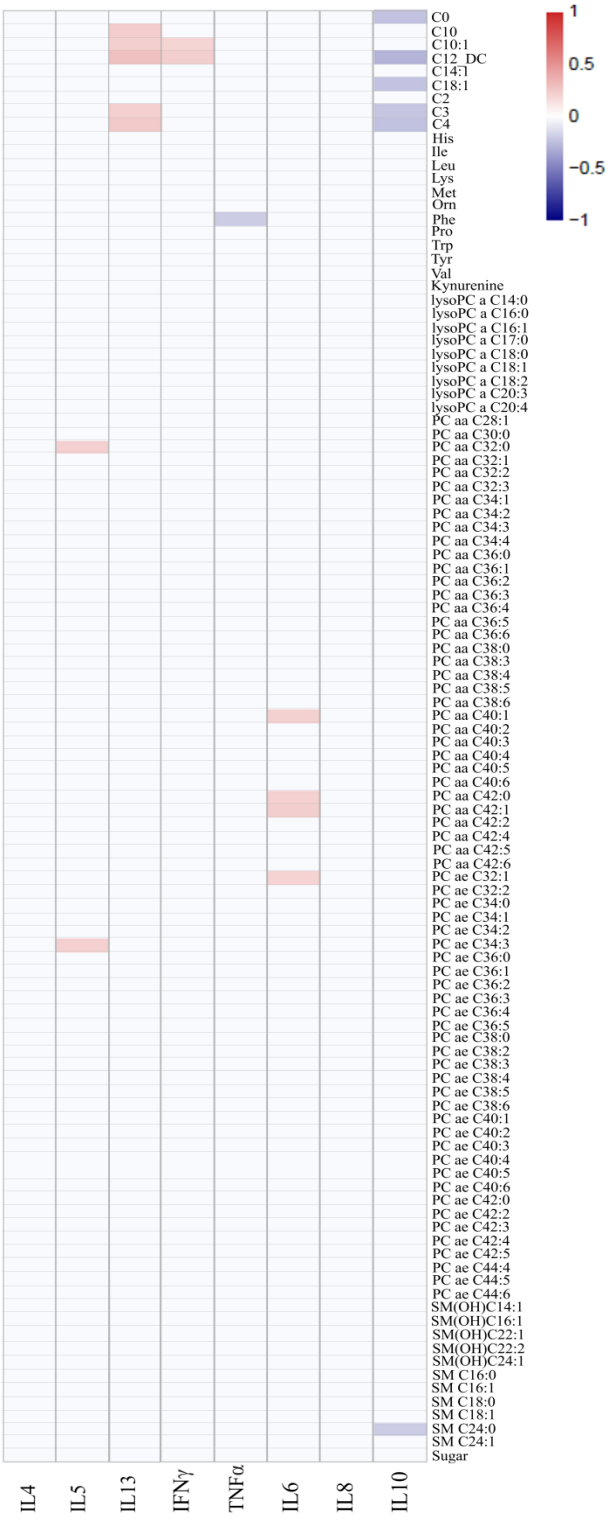

Figure S1: Heat map of metabolic changes in children due to prenatal cytokine exposure. The heat map present correlations between prenatal cytokine concentrations and blood metabolite concentrations in 1 year old children (Spearman correlation coefficient R, red to blue). Only significantly values after Bonferroni correction (p<4.63x10^{-4}) are presented.

7.2. Supplementary Tables

Table S1: Relationship between maternal cytokine concentrations in pregnancy and overweight development in children. Logistic regression model adjusted for gender, birth weight, birth season, early delivery, smoking during pregnancy, parental school education, household members, breastfeeding, introduction of solid food and pregnancy IgE level. Significantly differences between normal weight and overweight children are presented in bold (p<0.05).

	Year 1		**Year 2**		**Year 3**	
	OR(95% CI)[a]	*p*-value	OR(95% CI)[a]	*p*-value	OR(95% CI)[a]	*p*-value
Entire sub-cohort		**n=313**		**n=381**		**n=366**
IL4	**0.52(0.28,0.97)**	**0.038**	**0.63(0.42,0.97)**	**0.033**	0.71(0.44,1.15)	0.158
IL5	0.44(0.18,1.09)	0.075	0.91(0.53,1.56)	0.725	0.98(0.52,1.85)	0.961
IL13	**0.57(0.33,0.97)**	**0.039**	**0.68(0.47,0.98)**	**0.039**	**0.58(0.39,0.88)**	**0.011**
IFNγ	0.76(0.57,1.01)	0.059	0.86(0.69,1.06)	0.151	**0.76(0.60,0.95)**	**0.017**
TNFα	1.32(0.72,2.42)	0.372	1.21(0.81,1.81)	0.341	1.22(0.77,1.94)	0.388
IL6	0.76(0.36,1.60)	0.464	0.979(0.59,1.63)	0.934	0.67(0.38,1.18)	0.164
IL8	1.27(0.62,2.61)	0.510	1.09(0.70,1.71)	0.701	1.29(0.77,2.15)	0.336
IL10	1.09(0.74,1.60)	0.662	1.16(0.91,1.46)	0.224	1.17(0.88,1.54)	0.282
No maternal history of atopy		**n=168**		**n=198**		**n=191**
IL4	**0.38(0.15,098)**	**0.043**	**0.39(0.21,0.71)**	**0.002**	0.61(0.31,1.20)	0.149
IL5	0.28(0.07,1.13)	0.071	**0.45(0.21,0.96)**	**0.037**	0.56(0.22,1.41)	0.217
IL13	**0.46(0.21,0.99)**	**0.045**	**0.44(0.26,0.75)**	**0.002**	**0.43(0.24,0.78)**	**0.005**
IFNγ	**0.57(0.35,0.92)**	**0.019**	0.82(0.62,1.09)	0.165	**0.69(0.49,0.99)**	**0.0452**
TNFα	1.41(0.52,3.76)	0.488	1.13(0.65,1.96)	0.664	0.98(0.49,1.98)	0.960
IL6	0.95(0.28,3.19)	0.937	1.16(0.56,2.43)	0.687	1.10(0.43,2.79)	0.841
IL8	0.95(0.36,2.53)	0.925	1.08(0.59,2.00)	0.796	1.08(0.51,2.29)	0.831
IL10	1.11(0.65,1.92)	0.686	1.07(0.78,1.45)	0.675	0.97(0.66,1.43)	0.874
Maternal history of atopy		**n=142**		**n=180**		**n=172**
IL4	0.61(0.23,1,63)	0.317	1.18(0.58,2.38)	0.647	0.78(0.36,1.69)	0.528
IL5	0.74(0.20,2.67)	0.640	**2.51(1.00,6.30)**	**0.047**	1.87(0.68,5.16)	0.224
IL13	0.68(0.28,1.62)	0.378	1.25(0.69,2.28)	0.458	0.79(0.41,1.53)	0.488
IFNγ	1.08(0.63,1.83)	0.788	0.89(0.64,1.26)	0.518	0.76(0.55,1.05)	0.092
TNFα	1.31(0.53,3.20)	0.555	1.54(0.82,2.90)	0.180	1.44(0.71,2.93)	0.311
IL6	0.74(0.24,2.26)	0.596	0.86(0.40,1.85)	0.690	**0.36(0.16,0.83)**	**0.015**
IL8	1.76(0.52,6.02)	0.358	1.29(0.61,2.72)	0.508	1.17(0.51,2.69)	0.708
IL10	1.02(0.53,1.96)	0.947	1.34(0.90,2.00)	0.152	1.30(0.81,2.09)	0.278

[a] odds ratio (OR) adjusted for all confounders with 95 % confidence interval (CI)

Table S2: **Impact of maternal perceived stress levels during pregnancy, year 1 and year 2 on longitudinal BMI z-score development in preschool children (birth-age5).** Estimates were derived from general estimation equations (GEE) for BMI z-scores (birth to age 5) as dependent variable, adjusted for gestational week at delivery, mode of delivery, pregnancy cotinine levels and breastfeeding duration (not for pregnancy stress levels).

		β estimate	**95% CI**	***p*-value**
Pregnancy	(n=498)	0.06	-0.07 – 0.20	0.372
Year 1	(n=491)	**0.23**	**0.08 – 0.37**	**0.002**
Year 2	(n=473)	0.09	-0.71 – 0.24	0.283
Pregnancy to year 2	(n=473)	0.06	-0.01 – 1.21	0.078

Table S3: **Gender disparity in susceptibility to maternal stress-related weight development in preschool children age 1-5 years**. Estimates were derived from general estimation equations (GEE) for BMI z-scores (birth to age 5) as dependent and maternal stress during the first year of life as independent variable.

	β estimate	**95% CI**	***p*-value**
Entire cohort (n=491)			
raw	**0.22**	**0.08 – 0.37**	**0.003**
adj.[a]	**0.23**	**0.08 – 0.37**	**0.002**
Girls only (n=241)			
raw	**0.30**	**0.11 – 0.49**	**0.002**
adj.[a]	**0.30**	**0.11 – 0.49**	**0.002**
Boys only (n=250)			
raw	0.14	-0.07 – 0.35	0.194
adj.[a]	0.10	-0.11 – 0.31	0.333

a - Adjusted for gestational week at delivery, mode of delivery, pregnancy cotinine levels and breastfeeding duration.

Table S4: Comparison of gender-related study characteristics of the analyzed LINA sub-cohort.

	girls only n (%), n=245 [a]	**boys only** n (%), n=253 [a]	**χ^2-test**
Week of gestation at birth			0.747
<37 weeks	7 (2.9)	9 (3.6)	
37-40 weeks	158 (64.5)	150 (59.3)	
>40 weeks	80 (32.6)	94 (37.1)	
Mode of delivery			0.962
spontaneous	191 (80.0)	196 (77.5)	
C-section	51 (20.8)	53 (20.9)	
others	3 (1.2)	4 (1.6)	
Birth weight			0.35
<3000g	51 (20.8)	41 (16.2)	
≥3000g – 3500g	104 (42.4)	91 (36.0)	
≥3500g – 4000g	69 (28.2)	82 (32.4)	
≥4000g	21 (8.6)	39 (15.4)	
Household members			0.871
2	12 (4.9)	14 (5.6)	
3	152 (62.3)	148 (58.7)	
≥4	80 (32.8)	90 (35.7)	
Breast feeding			0.958
1. -3. month	43 (18.5)	44 (17.8)	
1.-6. month	78 (33.6)	88 (35.6)	
1.-12. month	111 (47.8)	115 (46.6)	
Education [b]			0.822
low	4 (1.6)	2 (0.8)	
medium	52 (21.2)	49 (19.4)	
high	189 (77.1)	202 (79.8)	
Household income			0.844
<2000€	83 (36.1)	89 (37.2)	
2000€ - 4000€	132 (57.4)	130 (54.4)	
>4000€	15 (6.5)	20 (8.4)	
Separation/divorce [c]			0.832
yes	11 (12.2)	12 (13.2)	
no	79 (87.8)	79 (86.8)	
Pregnancy cotinine level [d]			0.567 [e]
median [µg/g creatinine]	1.88	1.80	
<25%, >75%	0.72, 5.13	0.80, 4.82	

a – n may be different from total n due to missing data
b – low = 8 yrs of schooling (‘Hauptschulabschluss`); medium = 10 yrs of schooling (`Mittlere Reife`); high = 12 yrs of schooling or more (`(Fach-)hochschulreife’)
c – Parental separation/divorce in the last 3 years from children’s age 3 years
d – ETS = environmental tobacco smoke (urinary cotinine level at pregnancy)
e – *p*-value derived from Student’s T-test between group means

Table S5::Association of different stressors with maternal stress levels at year 1 (n=489). Standardized Mean ratios and Confidence Intervals (95% CI) were derived from linear regression

	Mean Ratio	**95% CI**	***p*-value[a]**
Traffic	**1.19**	**1.09 – 1.30**	**8.65E-5**
Residential noise	**1.15**	**1.05 - 1.25**	**0.0026**
Poor living conditions	**1.15**	**1.05 – 1.26**	**0.0017**
Low household income	**1.19**	**1.09 – 1.30**	**8.72E-5**
Low educational level	1.11	1.01 – 1.21	0.023
Number of household members	1.08	0.99 – 1.18	0.092
Age of the mother at birth	1.00	0.92 – 1.10	0.964

a - Bonferroni adjusted significance level, $p \leq 0.007$

Table S6: Associations of different stressors on the four different stress dimensions at year 1 (n=489). Given are β values with 95% confidence intervals and *p*-values from standardized linear regression models.

	β (95% CI) *p*-value[a]						
	Traffic	Residential Noise	Living Conditions	Household Income	Low Educational Level	Household Members	Age of mother at birth
Worries	**0.19 (0.08-0.23), 2.79E-5**	**0.12 (0.04-0.23), 0.006**	**0.16 (0.09-0.29), 2.88E-4**	**0.26 (0.05-0.10), 5.21E-9**	0.13 (0.06-0.29), 0.003	0.02 (-0.05-0.08), 0.648	-0.05 (-0.02-0.01), 0.243
Tension	**0.19 (0.09-0.25), 3.14E-5**	**0.13 (0.05-0.26), 0.005**	0.11 (0.03-0.26), 0.012	0.12 (0.01-0.07), 0.007	0.08 (-0.02-0.24), 0.086	0.10 (0.01-0.16), 0.032	0.01 (-0.01-0.02), 0.780
Demands	**0.15 (0.05-0.18), 0.001**	**0.14 (0.06-0.24), 0.002**	**0.18 (0.10-0.29), 8.95E-5**	0.04 (-0.01-0.04), 0.338	-0.01 (-0.12-0.10), 0.884	**0.15 (0.04-0.17), 0.001**	0.09 (0.001-0.02), 0.038
Lack of Joy	0.09 (-0.002-0.14), 0.056	0.08 (-0.01-0.18), 0.086	0.04 (-0.06-0.15), 0.357	**0.19 (0.03-0.08), 3.57E-5**	**0.15 (0.08-0.31), 0.001**	0.003 (-0.07-0.07), 0.950	-0.04 (-0.02-0.01), 0.343

a - Bonferroni adjusted significance level, $p \leq 0.002$

Curriculum Vitae

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2013-2014	Stem Cell Scientist, National Institute of Biological Standard and Control (NIBSC), London, UK
2011-2013	Master of Science (Biochemistry), University of Leipzig (1.4, A), Germany Master Thesis: "Development and characterization of human neuronal precursor cells derived from pluripotent stem cells" Prof. A. Robitzki
2008-2011	Bachelor of Science (Biochemistry), University of Leipzig (1.9, B), Germany Bachelor Thesis: “Isolation of keratin-associated proteins” Prof. T. Magin
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List of Publications

Leppert B & Junge KM, Röder S, Borte M, Stangl GI, Wright RJ, Hilbert A, Lehmann I, Trump S. "*Maternal perceived stress and children's BMI: longitudinal impact and influencing factors."* BMC Pub Health. 2018, in Review

Junge KM & **Leppert B**, Jahreis S, Wissenbach DK, Feltens R, Grützmann K, Thürmann L, Bauer T, Ishaque N, Schick M, Bewerunge-Hudler M, Röder S, Bauer M, Schulz A, Borte M, Landgraf K, Körner A, von Bergen M, Stangl GI, Trump S, Eils R & Polte T & Lehmann I. (2018) *"MEST mediates the impact of prenatal bisphenol A exposure with long-term weight development*" Clin Epigenetics;10:58. doi: 10.1186/s13148-018-0478-z. PMID:29721103

Englich B, Herberth G, Rolle-Kampczyk U, Trump S, Röder S, Borte M, Stangl GI, von Bergen M, Lehmann I, Junge KM.(2017) "*Maternal cytokine status may prime the metabolic profile and increase risk for obesity in children*." Int J Obes (Lond). 9:1440-1446 doi: 10.1038/ijo.2017.113. PMID: 28487553

Seidel D, Jahnke HG, **Englich B**, Girard M, Robitzki AA.(2017) *"In vitro field potential monitoring on a multi-microelectrode array for the electrophysiological long-term screening of neural stem cell maturation."* Analyst. 30;142(11):1929-1937. doi: 10.1039/c6an02713j. PMID: 28484750

Seidel D, Obendorf J, **Englich B**, Jahnke HG, Semkova V, Haupt S, Girard M, Peschanski M, Brüstle O, Robitzki AA.(2016) *"Impedimetric real-time monitoring of neural pluripotent stem cell differentiation process on microelectrode arrays."* Biosens Bioelectron. 86:277-86. doi: 10.1016/j.bios.2016.06.056. PMID: 27387257

List of Poster and Oral Presentations

Strunz S & **Englich B** (2017) *"Maternal Exposure to n-Butylparaben increases body weight in offspring."* 24th European Congress on Obesity, Porto, Portugal – shared oral presentation

Englich B, Schlittenbauer L, Pfeifer C, Borte M, Stangl GI, Reemtsma T, Lehmann I, Junge KM. (2017) *„Prenatal Paraben Exposure Impacts Infant Overweight Development and in-vitro Adipogenesis"* – 24th European Congress on Obesity, Porto, Portugal – Poster

Englich B, Junge KM, Seiwert B, Pfeifer C, Röder S, Borte M, Stangl GI, Reemtsma T, Lehmann I (2017) *„Pretty Women and super-size babies? Is cosmetic use in pregnancy priming infant's obesity?"* HIGRADE PhD Conference, Leipzig, Germany - Poster

Englich B & Junge KM, Wissenbach D, Feltens R, Röder S, Borte M, von Bergen M, Lehmann I. (2015) *"Prenatal Bisphenol A (BPA) exposure favors body weight increase in early childhood"* 31. Jahrestagung der Deutschen Adipositas-Gesellschaft e.V., Berlin, Germany - Poster

Declaration under Oath/ Eidesstattliche Erklärung

I declare under penalty of perjury that this thesis is my own work entirely and has been written without any help from other people. I used only the sources mentioned and included all the citations correctly both in word or content.

Ich erkläre an Eides statt, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst, keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Date /Datum	Signature of the applicant/ Unterschrift des Antragstellers

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