Environmental Contaminants and Obesity

MONIKA RÖNN
Abstract

Obesity is a worldwide problem affecting both children and adults. Genetic, physiological, environmental, psychological, social and economic factors interact in varying degrees, influencing body weight and fat distribution and the progress of obesity. Moreover, some anthropogenic chemicals have proven to be endocrine disrupting chemicals (EDCs) with the potential to interfere with different actions of hormones in the body. EDCs may thereby disrupt homeostasis, modifying developmental, behavioral and immune functions in humans and animals, and also promoting adiposity. Because hormones generally act at low concentrations, small changes in the endocrine system may lead to extensive effects. Based on data from experimental and epidemiological studies this thesis elucidates the relationship between a large number of environmental contaminants and obesity.

The experimental studies demonstrated that fructose supplementation in the drinking water resulted in unfavorable metabolic alterations such as a higher liver somatic index (LSI), an increase in plasma triglycerides and increased plasma levels of apo A-I. Fructose in combination with exposure to bisphenol A (BPA) increased liver fat content and plasma levels of apo A-I in juvenile female Fischer 344 rats. The experimental studies also showed that the retro-peritoneal fat, which in rats is a distinct fat depot easy to distinguish and dissect, correlated well with the measurements of total fat mass analyzed with MRI, and could therefore be used as a substitute for total fat mass in rats.

The epidemiological studies showed that circulating levels of persistent organic pollutants (POPs) were related to fat mass measured by DXA. OCDD, HCB, TNC, DDE and the less chlorinated PCBs were positively related to fat mass, while the more highly chlorinated PCBs showed a negative association. Further, circulating levels of BPA were positively associated with levels of the hormones adiponectin and leptin, but negatively related with ghrelin, hormones which are involved in the regulation of hunger and satiety. However, serum BPA levels were not related to measures of fat mass in the elderly individuals in the PIVUS cohort.

This thesis concludes that environmental contaminants such as BPA and POPs most likely are contributors, along with genetic, social and behavioral factors, to the development of obesity.

Keywords: Fischer 344, rat, obesity, adipose tissue, persistent organic pollutants, POPs, bisphenol A, BPA, pesticides, dioxin, PCB, DDT, apo A-I, adiponectin, leptin, ghrelin

Monika Rönn, Department of Medical Sciences, Occupational and Environmental Medicine, Akademiska sjukhuset, Uppsala University, SE-75185 Uppsala, Sweden.

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List of Papers

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


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Abbreviations

AhR        Aryl hydrocarbon receptor
Apo A-1    Apolipoprotein A-1
AT         Adipose tissue
BAT        Brown adipose tissue
BDE        Brominated diphenyl ether
BMI        Body mass index
BPA        Bisphenol A
DDE        Dichlorodiphenyldichloroethylene
DDT        Dichlorodiphenyltrichloroethane
DES        Diethylstilbestrol
DOHaD      Developmental origin of health and disease
DXA        Dual-energy X-ray absorptiometry
EDC        Endocrine disrupting chemical
ELISA      Enzyme-linked immunosorbent assay
HCB        Hexachlorobenzene
HRGC/HRMS  High resolution chromatography coupled with high resolution mass spectrometry
LOD        Level of detection
LSI        Liver somatic index
MRI        Magnetic resonance imaging
NHP        Non human primate
OC         Organochlorine
OCDD       Octachlorodibenzo-p-dioxin
OVX        Ovariectomized
PCBs       Polychlorinated biphenyls
PIVUS      Prospective investigation of the vasculature in Uppsala seniors
POPs       Persistent organic pollutants
RIA        Radioimmunoassay
SAT        Subcutaneous adipose tissue
TBT        Tributyltin
TDI        Tolerable daily intake
TNC        Transnonachlordane
VAT        Visceral adipose tissue
WAT        White adipose tissue
WHO        World Health Organization
1 Introduction

Overweight and obesity are defined as abnormal or excessive fat accumulation that may impair health. These conditions are growing worldwide and are affecting both children and adults. Once considered a high-income country problem, overweight and obesity are now affecting low- and middle-income countries as well. Obesity is a risk factor for non-communicable diseases e.g. diabetes, cardiovascular diseases, chronic respiratory disease and some forms of cancer, but must not be ignored as a disease per se, influencing the quality of life for millions of people.

The prevalence of overweight and obesity has risen dramatically since the 1970s and reached epidemic proportions in a relative short time period. The etiology of obesity is complex and not yet fully understood. Genetic, physiologic, environmental, psychological, social and economic factors interact in varying degrees, influencing body weight and fat distribution and the progress of obesity. Hitherto interventions to counteract obesity have focused mainly on how to change habits of overconsumption of energy-dense food and of a sedentary lifestyle. But the trend of obesity is striking in various populations and age groups with different lifestyles. Thus factors influencing the balance between energy intake and energy expenditure need to be further explored. In this regard a strong proposition is to investigate the contribution of environmental contaminants on this balance, as they may act as obesity promoting factors, i.e. obesogens [1].

The previous century was pervaded by inventions and the development of a welfare society. It was also a century with a prosperous chemical industry. New compounds and new materials flooded the market, and little attention was paid to the potential long-term negative effects of products made to last, or for pesticides fighting the spread of malaria and agricultural pests. Today all humans worldwide are exposed to man-made chemicals already in the uterus, and continuously every day of their life course. The consequences have not yet been fully revealed, but we have learned that persistency is not always a desirable chemical property and that the impact on the environment and humans is not always easy to foresee. However, we do know that not all those chemicals are safe, and there is a great deal of research showing that exposure to certain chemicals poses a risk of negative effects on health and reproduction in both humans and wildlife.

Of particular interest for metabolic related diseases and obesity, which is the endocrine-related disease dealt with in this thesis, are endocrine disrupt-
ing chemicals (EDCs). Such chemicals interfere with different actions of hormones in the body. EDCs may thereby disrupt homeostasis, modifying developmental, reproductive, neurological, behavioral and immune functions in humans and animals [2-4]. Because hormones generally act at low concentrations, small changes in the endocrine system may lead to extensive effects. Dose-response curves for EDCs are sometimes non-monotonic and often sigmoidal, rendering the traditional toxicological paradigm “the dose makes the poison” invalid, since low-dose EDC health effects “cannot be predicted by the effects observed at high doses” [5]. Other types of endocrine dose-response curves are the U-shaped curve, where effects are seen at very low and very high concentrations, and the inverted U-shape curve, where the main effect is seen in a specific intermediate interval [5].

It is also important to consider the definition of low-dose effects and low-dose studies of EDCs, which is not an easy task, because there are actually multiple definitions. A first definition by the National Toxicology Program (NTP) (2001) was that 1) effects occurring in the range of typical human exposure, or 2) at doses lower than those typically used in standard testing protocols, i.e. doses below those tested in traditional toxicology assessments [6]. Other definitions include 3) any dose below the lowest observed effect level or lowest observed adverse effect level (LOAEL) or 4) a dose administered to an animal that produces blood concentrations of that chemical in the range of what has been measured in the general human population. This is also referred to as an environmentally relevant dose [5]. This is in contrast to traditional toxicology assessments where the endpoints have been more or less immediately measurable as adverse effects, ignoring more subtle or indirect health effects that may be observed a long time after the exposure and sometimes also in another stage of life.

1.1 Fetal programming – regulation later in life

During the fetal and neonatal period organs and neural systems develop, which makes it a sensitive period for deviations from the normal processes. Altered endocrine regulation in these periods may affect the future endocrine program. The hypothesis of fetal programming, i.e. that conditions during fetal life affect adult life, emerged from an epidemiological study where coronary heart disease in different parts of England and Wales paralleled previous death rates among newborn babies [7]. Further studies revealed the association between low birth weight and coronary heart disease. The result has been replicated in other studies on men and women in Europe, North America and India [8]. The fetal origins hypothesis (also called the Barker theory after its discoverer) has advanced, and today the developmental origin of health and disease (DOHaD) is a well-established concept.
Mothers’ use of certain pharmaceuticals during pregnancy offers the most striking proof of such effects that can be identified in their children. Diethylstilbestrol (DES), which is a synthetic, non-steroidal estrogen, was given to pregnant women during a longer time period, from about 1940 to 1970 to reduce the risk of miscarriages and other pregnancy complications. Instead, it caused a rare form of vaginal cancer in the girls born to exposed mothers years later [9]. Since the discovery of the increased cancer risk in girls, follow-up studies have indicated an array of complications in both girls and boys exposed to DES in utero [10], and even in the next generation [11, 12]. The DES scandal revealed that fetal exposure to EDCs may result in long-term effects and affect health during the whole life. Today DES is used as a model compound when studying EDCs. **Figure 1** illustrates an unexposed mouse compared with a mouse neonatally exposed to DES (1 µg/kg maternal bw/day) at 6 months of age. **Figure 2** is a photograph showing the difference in size between a control mouse and a neonatally DES-treated mouse at 4 months of age.

**Figure 1.** Images of control and DES-treated mice as generated by piximus densitometry. Image captured with PIXImus™ mouse densitometry at 6 months of age. Images are representative of control (A) and DES-treated (B) mice. Note that DES-treated mice are significantly larger.

**Figure 2.** Representative photograph of control and DES-treated mice. Photograph of 4-month-old mice showing the difference in body size of a control mouse (lower panel) and a neonatally DES-treated mouse (upper panel).

Figure 1 and figure 2 are reprinted from Reproductive Toxicology, Vol 23/3, Retha R. Newbold, Elizabeth Padilla-Banks, Ryan J. Snyder, Terry M. Phillips, Wendy N. Jefferson, Developmental exposure to endocrine disruptors and the obesity epidemic, Pages 290-296, (2007), with permission from Elsevier.
It took years to understand the connection between DES and adverse health outcomes despite the long-term prescription of DES to pregnant women and substantial health effects in the offspring. This casts light on a number of difficulties and the great challenge in identifying potentially harmful substances epidemiologically.

1. The outcome may differ depending on when in life, or when during a certain sensitive period the exposure occurs. The sensitive prenatal period consists of developmental steps where exposure might be harmful during a certain period, sometimes short, but not during others.
2. The outcome may differ in a dose-dependent manner.
3. The outcome may manifest itself many years later.
4. The outcome may vary from subtle to severe, and thus the connection between exposure and the outcome might be indistinct and difficult to uncover.
5. In diseases with multifactorial etiology, as diabetes and obesity, the proportion of the total risk of developing these conditions from a specific exposure is difficult to establish.
6. Prospective epidemiological studies take years to conduct, and for environmental pollutants there are no adequate controls that have not been exposed.

Knowing this, the importance of combining epidemiological studies with experimental testing of potentially harmful substances in vitro and in animal models is obvious.

1.2 Factors influencing body weight and fat distribution

A positive energy balance where the energy intake is larger than the expenditures, continuing for months and years, will result in overweight and obesity. Adipose tissue contains approximately 7 000 kcal/kg, which equals about 20 kcal/day (no more than a bite) for a year. Given the differences in activity and environmental conditions from day to day, it would be very hard to calculate the exact amount of food to balance the expenditures with that degree of accuracy. But even if regulation on a daily basis lacks precision, a balance with near parity of intake and expenditure most often appears over 1-2 weeks due to support from sophisticated, as yet not fully explored, control systems regulating the homeostasis of bodyweight [13]. However, several hormones and gastrointestinal and neuro-peptides have been identified as important key players in these systems and in central mechanisms localized in the hypothalamus.
There are various environmental factors suspected of interfering with the control of energy balance and homeostasis under investigation. For example, chronic stress is associated with overweight and obesity. This has been shown in epidemiological studies [14] as well as in experiments with rats and non-human primates (NHP) [15]. Suggested factors in chronic stress that affect obesity are sustained glucocorticoid and neuropeptide Y production [16]. Sleep quality and sleep deprivation are associated with alteration in weight homeostasis and demand for food through endocrine mechanisms [17, 18]. Another hypothesis is that adenovirus 36 infection is leading to oxidative stress and obesity, including childhood obesity [19-21]. Intestinal microbiota may influence weight gain, depending on the proportions of different bacterial phyla [22, 23]. The kind, not only the amount, of energy may matter, e.g. the types of fats and carbohydrates, and ratios thereof, fiber content and dietary patterns [24]. Further, some environmental contaminants may promote an increase in fat mass by interfering with the intricate balance between energy intake and expenditure, and how energy is stored if intake exceeds the need [25-28]. Yet another interesting consideration is how these and other factors influencing obesity interact with each other [29]. The balance between caloric intake and expenditure is definitely a major player in this multifactorial disease, but it is not the only one. It is time to investigate those other plausible contributors beyond the energy equation, in a physiological perspective [30-32].

1.3 Adipose tissue as an endocrine organ

Adipose tissue (AT) consists of about 80% fat and 20% water, protein and minerals. AT is often thought of negatively as a depot of superfluous energy, indicative of an indulgent lifestyle, but it is so much more. In fact, AT has an important function as an endocrine organ, in addition to being an energy reservoir, protecting and insulating the internals and the body. AT is involved in such different tasks as reproduction and regulation of metabolism. It consists of different cell types with different functions, and AT composition differs between brown adipose tissue (BAT) and white adipose tissue (WAT), which is further subdivided to subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) with different locations and properties. VAT is localized within the abdominal cavity, and an increase in VAT is associated with increased risk of insulin resistance and cardiovascular diseases [33]. Visceral adipocytes also produce more pro-inflammatory cytokines than subcutaneous adipocytes. SAT is localized in the hypodermis, a fat layer under the skin. Subcutaneous adipocytes are the main source of the hormones leptin and adiponectin, and SAT is thus associated with more benign plasma lipid profiles [34].
Hormones involved in the control of hunger and satiety

Two of the investigated hormones in this thesis are the adipocyte-derived adipokines, adiponectin and leptin, and the third is the gut hormone ghrelin.

Adiponectin is a hormone exclusively secreted from adipose tissue in adults, with the exception of secretion also from the placenta during pregnancy. The plasma concentration of adiponectin is high relative to other hormones, comprising as much as 0.01% of total serum proteins in humans [35]. Adiponectin increases insulin sensitivity in the body and low levels of adiponectin constitutes a strong marker for future development of type-2 diabetes [36]. Adiponectin levels are negatively associated with fat mass.

Leptin is mainly secreted from adipose tissue, but also from the placenta, ovaries, pituitary, bone marrow, cells in the stomach (gastric chief cells, P/D1 cells), mammary epithelial cells and liver. Leptin was identified in 1995 and quickly carried great expectations to find a cure for obesity by exogenous addition of this hormone. It is a hormone with central hypothalamic effects regulating energy balance through suppressing food intake and stimulating energy expenditure [37]. In this regard leptin acts as a satiety hormone, and indeed, leptin supplementation is a cure for obesity, but only in leptin-deficient individuals [38], which the vast majority of obese people are not. In the other way, leptin levels are associated with fat mass, and circulating leptin concentrations grow higher with increased fat mass. Hence, leptin sensitivity seems to be negatively related to the amount of fat mass.

Ghrelin is mainly secreted from cells in the stomach and is thus not adipocyte-derived. The circulating levels of ghrelin are higher during fasting and lower after feeding. The levels are also generally higher in lean individuals and lower in overweight or obese individuals. Ghrelin stimulates food intake through receptor binding in the hypothalamus but is also involved in other physiological functions such as growth hormone release and gastric emptying. Recent research also indicates a role for ghrelin in the regulation of blood glucose homeostasis [39].

There are many more hormones and peptides involved in the regulation of energy homeostasis, but they are not analyzed within the scope of the present thesis.
1.4 Environmental contaminants acting as obesogens

There are various sources of environmental contaminants with different original fields of application; most of them are man-made as a solution to a specific problem, but with undesired side effects creating new problems. Pesticides are examples in this category, made to be toxic to certain organisms and deliberately spread in the environment. Modern materials with desirable qualities, like plastics and textiles treated with flame retardants, are not intended to be environmentally toxic, but end up and linger in the ecosystem for a long time, if not handled properly. Industrial pollution from combustion and industrial byproducts, like dioxins, is undesired but almost impossible to avoid. Pharmaceuticals may also pose a threat to the environment. They are often designed to withstand digestion in the gut and are slowly degraded in the environment as well. When such long-life pharmaceuticals leave the body or are discarded, they still may exhibit physiological potency outside the intended place of action.

Some of these contaminants have proven to be obesogenic in both experimental and epidemiological studies by influencing weight regulation and homeostasis in an obesity-promoting way. Our knowledge about chemicals with metabolic effects is not new. There are scientific reports from the 1970s showing such effects, although at that time it was not regarded as an adverse effect [40, 41]. On the contrary, the reported signs of increased weights and fat mass were interpreted as signs of well being, and the negative consequences were not fully understood. Another example is the previously mentioned estrogenic pharmaceutical DES, used to treat complications during pregnancy, which also became a commercial product as a growth enhancer for livestock. It was approved for use in cattle by the U.S. Food and Drug Administration in 1954, but then banned for all use in cattle production by the same body in 1979 [42]. However, DES is still used for this purpose in some parts of the world [43], and estrogenic implants of estradiol/trenbolone and zeranol for use in livestock were approved in U.S. in the mid 1990s [42].

Obesogens have been revealed in epidemiological studies, animal studies and in in vitro studies, sometimes as a secondary finding when exploring e.g. reproduction disorders. The chemicals may pursue actions in several ways and on diverse levels – as agonists or antagonists in receptor binding, promoters, inhibitors, and may modify events on a molecular or behavioral level, while the most significant mode of action is not established. The role of chemical exposure in the development of obesity seen in experimental studies often lacks its counterpart in the epidemiological literature, and most animal studies are descriptive, thus not providing any specific mode of action.
1.4.1 Persistent organic pollutants (POPs)

POPs are a group of carbon-based chemicals sharing following properties:

- Persistent; remain intact for many years
- Bioaccumulate; accumulate in tissues of living organisms
- Biomagnifies; are found at higher concentrations at higher trophic levels in the food chain
- Become widely distributed throughout the environment due to natural processes involving transport in water, air and soil
- Are toxic to both humans and wildlife

Consequently, these substances spread globally and are found even where they never have been produced or even used, thus exposing most humans, including those living in the most remote areas, such as the Arctic. Effects of POPs can be both acute and chronic and include cancer, reproductive disorders, disruption of the immune system, and metabolic disorders such as diabetes and obesity. Some POPs may act as endocrine disruptors, altering the hormonal system. By endocrine mechanisms POPs may affect exposed individuals, as well as their offspring, and induce adverse developmental effects. As POPs pose a threat to the environment and human health some of the POPs with the most deleterious health effects have been prohibited or restricted (The Stockholm Convention: http://chm.pops.int/).

**Dioxins**

Dioxins is an umbrella term for extremely toxic, structurally and chemically related, polychlorinated compounds. The most toxic is the dioxin 2,3,7,8-tetrachlorodibenzo *para* dioxin (TCDD). Dioxins are unwanted byproducts of industrial processes but may also result from e.g. volcanic eruptions. These chemicals act via the aryl hydrocarbon receptor (AhR) as a ligand modulated transcription factor, but may also act in non-genomic pathways [44].

Dioxin exposure can cause various health effects. In a study including people exposed to high levels of TCDD after the explosion in a chemical plant in Seveso in 1976, Warner et al. (2013) found an increased prevalence of metabolic syndrome associated with TCDD among women who were ≤ 12 years of age at the time of the explosion [45]. In paper III in the present thesis, the dioxin octachlorodibenzo-*p*-dioxin (OCDD) associated positively with fat mass [46].

An experimental study by Kim et al (2012) [47] showed that the primary effect of TCDD on gene expression in a human model of preadipocytes and adipocytes was the induction of the inflammatory pathway. The gene regula-
tion was mediated via the AhR. The study also showed that mice exposed to 10 µg TCDD/kg bw exhibited an increased gene expression of several cytokines, as well as the number of macrophages in AT. Low-grade inflammation is known to be involved in the pathogenesis of metabolic diseases, and the in vivo and in vitro studies by Kim et al. suggest a possible mechanism of pollutant contribution to these diseases.

PCBs
PCBs are a group of compounds consisting of 209 different congeners chlorinated with one to ten chlorine atoms, most often manufactured as mixtures and under many names, e.g. Aroclor. A higher degree of chlorination increases their lipophilicity, while compounds with a lower degree of chlorination are more volatile, properties important for how they spread and persist in the environment. PCBs have high flash points and are virtually fire resistant. They have been used as dielectric and exchange fluids and as softeners in joint-sealing compounds in buildings. The congeners differ in structure and some PCBs possess dioxin-like properties.

The relationship between concentrations of PCBs in core blood and BMI of children between 1 and 3 years of age was shown in a study by Verhulst et al. (2009) [48]. In paper III in the present thesis, the more highly chlorinated compounds were negatively related to fat mass, but the less chlorinated congeners were positively related to fat mass [46]. A negative correlation between the PCB congeners 138, 153, 170 and 180 and measures of fat mass was also seen in a study by Dirinck et al. (2010) [49].

In mice, perinatal administration of PCB-126 resulted in altered body composition in the offspring without any significant change in body mass. Female offspring had increased fat mass but lower percentage lean mass compared with controls, while male prenatally exposed offspring had reduced lean mass expressed in grams, but no change in fat mass. In females these differences were dose dependent, but not in males [50].

DDT
The Nobel Prize in Medicine 1948 was awarded to Paul Müller for his discovery of the insecticidal properties of dichlorodiphenyltrichloroethane (DDT), a contact poison against arthropods (insects, arachnids, crustaceans). DDT was considered a miracle pesticide, killing the vectors carrying malaria and yellow fever, while harmless to humans. However, when Rachel Carson wrote Silent Spring 1960 [51] it was revealed that pesticides like DDT were devastating for wildlife. Since then the controversy between saving human lives in the struggle against malaria or protecting the environment has been going on, but research also shows that DDT is not without harm for humans [52]. DDT may act as a xenoestrogen, and is linked to cancer [53] and diabetes [54]. In the 1970s DDT was banned in many countries, but is still
used in some areas where the benefits are considered to outweigh the hazard, though with caution.

DDT refers to the isomer $p,p'$-DDT, but the insecticide also contains 15-20% of the isomer $o,p'$-DDT, and some smaller fractions of other compounds. In the body DDT is biotransformed to its metabolite dichlorodiphenyldichloroethylene (DDE), also present in isomers, mainly $o,p'$-DDE and $p,p'$-DDE. $o,p'$-DDT and $o,p'$-DDE are estrogenic and bind to ER [55], while $p,p'$-DDE, the main metabolite of DDT, is antiandrogenic and binds to the androgen receptor [56].

DDT exposure, most often measured as the DDE concentration, has been positively associated with BMI in several studies [46, 57-59]. In a study by Warner et al. (2013) the association between in utero exposure, measured as $o,p'$-DDT, $p,p'$-DDT, and $p,p'$-DDE concentrations in maternal serum, and odds of obesity at 2, 3.5, 5 and 7 years in Mexican-American children were explored. The exposure was non-significantly related with increased odds of obesity at 7 years of age, but with increasing age a significant trend toward a positive association between exposure and odds of obesity was observed [60]. A continued follow-up would be most interesting, maybe revealing a time scale associated with exposure to DDT for development of obesity later in life.

Experimental studies have also shown obesity-promoting effects of DDT/DDE. In a study from 2011 by Howell and Mangum exposure of mature NIH3T3-L1 adipocytes to DDE increased basal fatty acid uptake over a 24 h period and significantly increased the release of leptin, resistin and adiponectin. These results may indicate a DDE-induced obesity-promoting adipocyte dysfunction [61].

**Organotin compounds**

Organotin compounds include a variety of organic derivates of tin with different properties. The triorganotins are toxic with endocrine disrupting (ED) properties. They are used as bactericides, fungicides and have been used as marine anti-biofouling paint to prevent growth of organisms on ships. That use is banned as of 2003 by the International Maritime Organization for reasons of environmental protection. The major commercial application of organotin compounds is in the stabilization of PVC plastics. It is mainly dibutyltin, monobutyltin, monoo- and dioctyltin that are used. They are less toxic than triorganotins, but dibutyltin may be linked to auto-immune related diseases and disturbance of metabolic functions [62]. Tributyltin (TBT) is obesogenic and an ED model chemical. TBT has sex differentiating properties, exhibited as imposex, a superimposition of male sex characteristics (penis and a vas deferens) on to a functionally normal female reproductive anatomy of some gastropod species [63]. TBT is an activator of the nuclear hormone receptors retinoid X receptor (RXR) and PPARγ, promotes adipocyte diffe-
rentiation, and might thus contribute to development of obesity and metabolic diseases [64-66].

An experimental study by Chamorro-García et al. (2013) showed that prenatal exposure of TBT produced transgenerational effects on fat depots in female mice. TBT exposure increased most WAT depot weights, adipocyte size and number, and reprogrammed mesenchymal stem cells toward the adipocyte lineage at the expense of bone. It also induced a phenotype resembling nonalcoholic fatty liver disease. These effects persisted through at least the F3 generation [67].

1.4.2 Plastic associated chemicals (PACs)

The PACs are generally not persistent and bioaccumulative like the POPs, nor are they acutely toxic in environmentally relevant doses, but they are ubiquitous, with ED properties, and the environment and humans are constantly exposed to them. PACs also have the capacity to adsorb organic pollutants and thus act in concert with POPs, contributing to dispersal and ingestion in various organisms [68, 69].

BPA

BPA is a high production volume chemical used as a component in different types of plastics and the monomer building block to make polycarbonate plastic. It is also used in epoxy resins lining food and beverage containers, and as a color developer for the printing dye in thermal cash receipts. It was first recognized for its estrogenic properties in the 1930s [70], but a more potent estrogenic compound (DES) was developed in the late 1930s, and thus BPA was never used as a pharmaceutical. Today BPA is considered an endocrine disruptor [71, 72]. Human studies report BPA exposure in almost all investigated individuals. The diet has been regarded as the main source of exposure, but this assumption is made on limited data and needs to be further investigated [73].

Because BPA exposure generally is well below the tolerable daily intake (TDI) at 50 µg/kg and day, as established by the U.S. Environmental Protection Agency (EPA) and the European Food Safety Authority (EFSA), it has been considered safe for humans. However, results from animal studies show that BPA causes effects even at exposure levels below the current TDI, and that the dose response curves are not necessarily monotonic [74]. Animal studies also indicate that the time of exposure is important [75, 76].

After ingestion, which probably is the major but not the only route of exposure [73, 77], BPA is conjugated in the liver during first pass metabolism to form the major metabolite BPA glucuronide [78] and then cleared from the blood via excretion in the kidneys. Thus, the unconjugated and active form of BPA is present in the body only for a short time. BPA is fat soluble (log k_{ow} 3.32) but probably does not bioaccumulate to any significant extent
due to the high excretion rate. However, a study by Nahar et al. (2013) shows higher hepatic concentration of free BPA in fetuses than in their mothers, indicating some possibility of fetal accumulation [79]. The glucuronidated form of BPA may also be deconjugated to active BPA in the fetus. Expression of β-glucuronidase, which deconjugates glucuronide conjugates, has been found in fetal liver cells [80].

Numerous studies have shown associations between levels of BPA in urine and/or serum and obesity. A recent study shows higher odds of obesity with increasing quartiles of BPA concentration measured in urine for children aged 6 to 18 years in the National Health and Nutritional Examination Survey (NHANES) 2003-2010 [81]. The relationship between urinary levels of BPA and obesity is also seen in adults in the NHANES 2003-2008 [82]. In an epidemiological study by Li et al. (2013) high urine BPA level was associated with overweight among females aged 9-12 years. The association was not seen in males [83].

Sex differences in response to BPA exposure have also been seen in animal studies. Rubin et al. (2001) showed that rats perinatally exposed to BPA had an increased bodyweight compared with controls, and the low dose increased body weight more than the higher dose in the offspring. However, the difference in weight gain between low- and high-dose exposed groups was greater for females than males [84]. Also in a study by Somm et al. (2009) female rats seem to be more susceptible to prenatal exposure to BPA than male rats. The females exposed to BPA had an increased body weight, whether fed standard chow or a high-fat diet, while the males only showed an increase in body weight compared with controls if fed the high-fat diet. Interestingly, no change in food intake was observed in rats on either standard chow or high-fat diet compared with controls [85].

Several experimental studies have shown effects of BPA on obesity and various variables related to obesity. In in vitro studies BPA has been shown to increase gene expression of adipogenic transcription factors in 3T3-L1 preadipocytes [86], and enhance adipocyte differentiation and lipid accumulation in a dose-dependent manner [87]. In a study by Miyawaki et al. (2007), female and male mice exposed to BPA perinatally and through the lactation period via the dams had higher body weights and increased adipose tissue weights, compared with controls. Exposed females had higher cholesterol levels, and males had higher triacylglycerol levels than controls [88]. In paper II in the present thesis, liver fat content and plasma levels of apolipoprotein A-I (apo A-I) were higher in rats exposed to BPA in fructose-supplemented drinking water, compared with fructose controls. However, no significant increase in weight gain or fat mass were seen after ten weeks [89]. In paper IV associations between circulating levels of BPA and levels of the hormones adiponectin, leptin and ghrelin were seen, but no association with measures of obesity in the PIVUS cohort exposed mainly as adults.
Phthalates

Phthalates are used as plasticizers, adding flexibility and durability to plastic products, but also to a variety of other products as e.g. coatings of pharmaceutical tablets, emulsifying agents, stabilizers, adhesives and glues, modeling clay, paints and textiles. As phthalate-containing products break down, the phthalates leak out into the environment. Due to the wide dispersal of such products most people are exposed, and metabolites of several phthalates are commonly found in the urine of analyzed individuals. Phthalates are known PPARγ agonists, affecting lipid metabolism and adipocytes in experimental studies [90, 91].

An epidemiological study by Lind et al. (2012) showed positive associations in women between measures of fat mass and circulating concentrations of mono-isobutyl phthalate (MiBP) and mono-methyl phthalate (MMP) [92]. The phthalates function mainly as anti-androgens and are EDCs associated with e.g. breast cancer. A shorter anogenital distance in males is also indicative of an anti-androgen effect associated with phthalate exposure [93]. In a cross-sectional study by Stahlhut et al. (2007) with participants from the National Health and Nutrition Examination Survey (NHANES) 1999–2002, four phthalate metabolites were associated with increased waist circumference in males [94].

In an experimental study Manikkam et al. (2013) exposed Sprague Dawley gestating F0 generation female rats to daily intraperitoneal injections of vehicle or a mixture of PACs (BPA, DEHP and DBP) in two different doses. The incidence of adult onset disease was evaluated in F1 and F3 generation rats, and transgenerational inheritance of obesity was seen in the F3 generation female descendants to the lower dose of plastic-exposed dams [95].

1.4.3 Other possible obesogens

There are other chemicals with known or potentially obesogenic effects. Chemicals that bind to the RXR or PPARγ, like tributyltin and phthalates, may promote adipocyte differentiation and alter adipose tissue, which could promote obesity and metabolic diseases. This is seen for the thiazolidinediones, PPARγ agonists used as pharmaceuticals in the treatment of type-2 diabetes to increase insulin sensitivity, increase glucose utilization and decrease gluconeogenesis. A study by Kolak et al. (2007) showed that treatment with the thiazolidinedione rosiglitazone altered expression of genes involved in fatty acid uptake and storage and structural proteins [96].

Perfluoroalkyl acids, e.g. perfluorooctanoate (PFOA), are persistent compounds used in non-stick coatings and various industrial applications. PFOA is found in tissues of humans and wildlife worldwide. In a study performed near a fluoropolymer production facility, the highest levels were found in children aged 2-5, and in the oldest age group, age > 60 years [97]. In a prospective epidemiological study, levels of PFOA measured in maternal
serum from gestational week 30 were positively associated with BMI and waist circumference in female offspring at 20 years of age [98]. *In utero* PFOA exposure of CD-1 mice (0.01-0.3 mg/kg maternal bw) increased body weight and serum leptin and insulin levels in post-pubertal female offspring. PPARγ is a potential mediator in these low dose effects but needs to be further evaluated [99].

Organophosphates are insecticides with possible obesogenic activity. In a study by Meggs and Brewer (2007), long-term exposure to the organophosphate chlorpyrifos in female Long Evans rats caused a progressive increase in weight, associated with increased fat mass [100]. Prenatal exposure to chlorpyrifos has also been shown to cause excess weight gain in male Long Evans rats [101].

Atrazine, an herbicide, acts on a functional structure in herbs similar to mitochondria in animals and thus might contribute to development of obesity by causing mitochondrial dysfunction. In fact, rats chronically exposed to a low dose of atrazine exhibited decreased basal metabolic rate and increased body weight, intra-abdominal fat and insulin resistance without changing food intake or physical activity level [102]. Further, atrazine is an endocrine disruptor that induce aromatase, the enzyme that convert testosterone to estrogen, leading to excess estrogen production and feminization of male vertebrates [103]. Atrazine is banned in the European Union, but is the most widely used herbicide in the United States.

Hexachlorobenzene (HCB) is a fully chlorinated hydrocarbon that is highly soluble in fat, oils and organic solvents, but almost insoluble in water. It has mainly been used as a fungicide but has been banned for use in the United States since 1966, and globally under the Stockholm Convention on Persistent Organic Pollutants, signed in 2001. However, it is also a by-product when making other chlorine-containing products, thus still adding to the environment. HCB was related to increased fat mass in the PIVUS study, presented in paper III in this thesis [46]. Another study showed that levels of HCB in maternal serum during pregnancy was associated with rapid growth (0-6 months) and overweight (14 months) in children [104]. Also the levels of HCB in cord blood have been shown to be associated with increased BMI at the age of 6.5 years. Children in the higher exposure group in that study had an increased risk of overweight and obesity [105]. Concentrations of hexachlorobenzene in mother’s milk were related to lower birth weight and small for gestational age (SGA) in babies born to past or current smokers [106]. Further, nicotine found in tobacco products is also a risk factor for low birth weight. Nicotine crosses the placental barrier, and the levels are higher in fetal circulation than in maternal circulation. Several epidemiological studies have reported that children prenatally exposed to maternal smoking have lower birth weights than unexposed children, but catch up in weight at different ages, and have an increased risk of developing overweight and obesity (reviewed in Somm et al. 2008) [107].
Brominated flame retardants are used in plastics in electronic products and in textile products like furniture, with the electronics industry accounting for the greatest consumption. Exposure to polybrominated diphenyl ethers (PBDEs) flame retardants has been associated with altered thyroid function [108] and prenatal exposure with lower birth weights [109]. The thyroid produces hormones involved in the control of the basal metabolic rate and therefore may influence the development of obesity. However, the results regarding PBDEs are inconclusive and need to be further explored.

1.5 Methods for measuring obesity

The World Health Organization (WHO) categorizes obesity according to the body mass index, i.e. weight in relation to squared height (kg/m²). Having a BMI ≥ 30 is defined as obesity, and a BMI ≥ 25 is defined as overweight. In 2009-2010, 35.5% of adult men and 35.8% of adult women in the US were obese [110] and WHO confirms that there are more than 1.4 billion overweight adults globally. Of these nearly 500 million were obese (2008). More than 40 million children under the age of five were overweight in 2011. In this context the BMI is a satisfactory measure. It is non-invasive, quick and cheap, making it possible to screen a lot of people, and for most people the BMI correlates well with fat mass, though not for e.g. muscular individuals. However, BMI does not give any information about fat content, neither its mass nor its distribution. This is important missing information, because abdominal fat poses a greater health risk than fat elsewhere, and because VAT poses a greater health risk than SAT [33], indicating the need for more sophisticated methods than BMI. An alternative is to measure waist circumference (WC) with a tape measure or using the sagittal measure, where the abdominal height is measured with the person in a supine position, which also are non-invasive and cheap methods to estimate fatness by assessing the degree of abdominal fat mass. However, total fat mass is probably also of importance for health outcomes, and the combination of BMI and WC or sagittal measure is an option to consider, and already in use in some studies [26, 57, 111].

Impedance scales can be used to measure the weight and the amount of body fat in various segments of the body, and also get information about the proportions of body fat, muscle and bone mass. As obesity is defined as excess of body fat regardless of body weight, the impedance scale make it possible and cost-effective to find thin persons with a high degree of visceral fat, as well as muscular persons with a high BMI who are not obese.

Even more accurate measurements are possible with dual-energy X-ray absorptiometry (DXA), which calculates the fat mass from a two dimensional picture. The disadvantage is a higher cost, and the availability of whole body scan DXA equipment is often restricted to central hospitals. The scan-
ning procedure will expose the individual to a smaller amount of x-ray compared with computed tomography (CT), another technique using electromagnetic radiation. Magnetic resonance imaging (MRI) and CT are the most advanced techniques today for measurements of body composition. MRI calculates the fat volume from a two- or three-dimensional image, depending on the protocol, and gives information about the distribution of VAT and SAT (Figure 3). These techniques are also the most expensive. Another disadvantage is that some people find it difficult to stay in the MRI-scan, which requires an immobile position in a closed space. CT is not used to any great extent, although it is fast and accurate, but the cost is very high, and the ionizing radiation used in the process is higher than that for other methods and thus adds to the risk of cancer.

Figure 3. Pictures from the Prospective Investigation of the Vasculature in Uppsala Seniors study (PIVUS) showing abdominal cross-sections from two persons having a minor difference in waist circumference, but a fundamental difference in fat distribution in this area (fat seen as white color). In the left picture the visceral adipose tissue (VAT) dominates, while in the right picture the subcutaneous adipose tissue (SAT) dominates.
2 Aims

The aim of this thesis was to explore possible relationships between environmental contaminants and obesity in humans and experimentally in rats.

In specific this was addressed by performing the following studies:

I The first study had two aims. The first aim was to develop a rodent model for studying adipose tissue in an environment of modest caloric excess, mimicking a modern lifestyle. The second aim was to develop a method for assessing total, subcutaneous, visceral adipose tissue and lean tissue volumes using a clinical 1.5T MRI scanner.

II The second study tested the hypothesis that exposure to BPA in combination with fructose could affect fat mass or liver fat content in juvenile rats. As a secondary aim, we investigated whether outcomes related to obesity or liver-related variables were affected by fructose supplementation alone.

III The third study evaluated the relations between plasma levels of POPs and obesity, measured as fat mass by DXA, in the population-based Prospective Investigation of the Vasculature in Uppsala Seniors study (PIVUS). The aim was to test if the analyzed POPs were positively related to fat mass.

IV The fourth study analyzed the associations between serum levels of BPA and obesity, using the PIVUS cohort. Fat mass and fat distribution were analyzed together with circulating levels of hormones involved in the control of hunger and satiety to explore possible relationships with BPA exposure.
3 Materials and methods

The following is a summary of materials and methods used in this project. The dissertation comprises two animal studies and two epidemiological studies. The epidemiological studies are based on the same cohort from the Prospective Investigation of the Vasculature in Uppsala Seniors study (PIVUS).

3.1 Animals, exposure and analysis (paper I and II)

The animal studies were approved by the Uppsala Animal Ethical Committee and followed the guidelines laid down by the Swedish legislation on animal experimentation (Animal Welfare Act SFS1998:56) and European Union legislation (Convention ETS123 and Directive 86/609/EEC).

3.1.1 Paper I

The first animal study was carried out with 24 female Fischer 344 rats, 4 weeks old at arrival. They were housed in groups of six in special wire cages (length, width, height: 59 x 38 x 43 cm) in order to provide enough space for them to move around freely. Glass bottles were used to reduce contamination from plastic materials. After the one-week acclimatization period preceding the ten-week intervention the animals were divided into two groups with 12 in each. The exposed group was given water containing fructose (5% for the first 7 weeks and 20% for the last 3 weeks), and the control group was given water without fructose. Food and liquid intake were measured by weighing (grams of pellet/water/fructose solution per cage) during the entire experiment.

3.1.2 Paper II

The second animal study (paper II) was carried out with 60 female F 344 rats, 3 weeks old at arrival. They were housed three rats/cage. To minimize background BPA exposure Polysulfone IV cages and glass water bottles were used. The rats were fed a standard pellet diet, RM1 (ad lib.), which is a natural ingredient diet with a low level of phytoestrogens (100-200 µg/g) [112, 113]. During the two-week acclimatization period preceding the ten-
week intervention all animals were given water to drink, and during the intervention water or 5% fructose solution. At 5 weeks of age the rats were assigned to five groups (12 rats/group): water control (W), fructose control (F), low dose BPA (0.025 mg/L), medium dose BPA (0.25 mg/L) or high dose BPA (2.5 mg/L). Three stock solutions of BPA in ethanol (2.5 mg/L, 25 mg/L and 250 mg/L) were diluted 1:100 in 5% fructose solution to prepare BPA exposure solutions.

To avoid unnecessary stress no cage-mates were separated, but the cages were allocated to the different groups to achieve equality in weights in all groups. Food and liquid consumption in each cage were determined once a week (for details of exposure and consumption, see Table 1).

**Table 1.** Exposure to bisphenol A, liquid and food consumption (RM1 *ad lib.*) and energy intake during the ten-week study of juvenile female Fischer 344 rats given water or a 5% fructose solution or bisphenol A (BPA) — 0.025, 0.25 or 2.5 mg/L — dissolved in a 5% fructose solution. Time period (week) with highest/lowest BPA exposure (µg/kg/day) is given within brackets. Food and liquid consumption are measured per cage (4 cages/group; 3 rats/cage) and all values are given as the calculated mean/rat. N=12/group, w=week (paper II).

<table>
<thead>
<tr>
<th>Exposure group</th>
<th>Control</th>
<th>Control</th>
<th>BPA 0.025 mg/L</th>
<th>BPA 0.25 mg/L</th>
<th>BPA 2.5 mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid</td>
<td>water</td>
<td>5% fructose solution</td>
<td>5% fructose solution</td>
<td>5% fructose solution</td>
<td>5% fructose solution</td>
</tr>
<tr>
<td>BPA Exposure, mean w 1-10^a</td>
<td>—</td>
<td>—</td>
<td>5.1</td>
<td>54.3</td>
<td>487.3</td>
</tr>
<tr>
<td>BPA Exposure highest^a</td>
<td>—</td>
<td>—</td>
<td>5.6 (w 2)</td>
<td>61.6 (w 3)</td>
<td>595.3 (w 2)</td>
</tr>
<tr>
<td>BPA Exposure lowest^a</td>
<td>—</td>
<td>—</td>
<td>4.6 (w 9)</td>
<td>46.3 (w 6)</td>
<td>400.3 (w 9)</td>
</tr>
<tr>
<td>Liquid, mean w 1-10^b</td>
<td>11.5</td>
<td>28.3</td>
<td>28.1</td>
<td>30.1</td>
<td>24.7</td>
</tr>
<tr>
<td>Liquid w 1^b</td>
<td>11.6</td>
<td>20.8</td>
<td>20.4</td>
<td>21.8</td>
<td>19.8</td>
</tr>
<tr>
<td>Liquid w 10^b</td>
<td>10.8</td>
<td>32.0</td>
<td>33.0</td>
<td>36.8</td>
<td>29.4</td>
</tr>
<tr>
<td>Food, mean w 1-10^b</td>
<td>10.2</td>
<td>8.7</td>
<td>8.5</td>
<td>8.3</td>
<td>8.7</td>
</tr>
<tr>
<td>Food w 1^b</td>
<td>10.8</td>
<td>10.1</td>
<td>10.0</td>
<td>9.6</td>
<td>9.3</td>
</tr>
<tr>
<td>Food w 10^b</td>
<td>10.0</td>
<td>8.3</td>
<td>7.9</td>
<td>7.8</td>
<td>8.3</td>
</tr>
<tr>
<td>Fructose energy mean w 1-10^c</td>
<td>0</td>
<td>5.7</td>
<td>5.6</td>
<td>6.0</td>
<td>4.9</td>
</tr>
<tr>
<td>Food energy mean w 1-10^c</td>
<td>28.9</td>
<td>24.6</td>
<td>24.1</td>
<td>23.5</td>
<td>24.6</td>
</tr>
<tr>
<td>Energy tot mean w 1-10^c</td>
<td>28.9</td>
<td>30.3</td>
<td>29.7</td>
<td>29.5</td>
<td>29.5</td>
</tr>
</tbody>
</table>

*a* µg/kg/day; *b* g/rat and day; *c* kcal /rat/day
3.1.3 Papers I and II

In both studies body weights were measured at the beginning of the study, thereafter weekly and finally immediately before the rats were euthanized. The last concurrent weight, when all animals were alive, was used to compare weights between the groups. It was not possible to scan all on the same day, therefore the weight before euthanization was used in the comparison to the body weights estimated by MRI.

Before the MRI exam performed in both studies, the rats were anesthetized with Ketalar 90 mg/kg bw (Pfizer, New York, NY) and Rompun 10 mg/kg bw (Bayer, Leverkusen, Germany). Immediately after the scanning they were sacrificed by exsanguinations from the abdominal aorta while still under anesthesia. The liver and the left retro-peritoneal fat pad (Figure 4) were dissected and weighed. The liver weight was used to calculate the liver somatic index: (liver weight/bodyweight)*100 (LSI).

Standard laboratory techniques were used for the analyses of cholesterol and triglycerides. Analysis of apolipoprotein A-I (apo A-I) was done with western blotting.

Figure 4. Illustration of the location and shape of the left retro-peritoneal fat pad (rWAT) (see arrow). The rWAT depots are separated from any organ and enclosed in a thin membrane (paper I and II).
3.2 Participants and analyses (papers III and IV)

The PIVUS study was approved by the Ethics Committee of Uppsala University, and all the participants gave their informed consent. The PIVUS cohort is the data source in both papers.

Eligible for the study were all men and women aged 70 living in the community of Uppsala, Sweden, years 2001-2004. Using the Swedish population register individuals was randomly chosen and a total of 1,016 participated, yielding a participation rate of 50.1%. They were all investigated in the morning after an overnight fast, and no medication or smoking was allowed after midnight. Venous blood samples were taken and then stored at -70°C until analysis. Basic characteristics for height, weight, fat mass and fat distribution, are given in Table 2, where also energy intake, alcohol consumption and levels of the hormones adiponectin, leptin and ghrelin are included.

Table 2. Basic characteristics, energy intake, alcohol consumption and levels of the hormones adiponectin, leptin and ghrelin in males and females. Mean and standard deviation (SD) are given together with minimum and maximum values. Median is given together with the inter quartile rate (IQR) for the hormones (papers III and IV).

<table>
<thead>
<tr>
<th>Variables</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Tot. fat mass&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.5</td>
<td>8.2</td>
</tr>
<tr>
<td>Fat leg&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.3</td>
<td>2.6</td>
</tr>
<tr>
<td>Fat trunk&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.2</td>
<td>5.1</td>
</tr>
<tr>
<td>VAT&lt;sup&gt;b&lt;/sup&gt;</td>
<td>119</td>
<td>63</td>
</tr>
<tr>
<td>SAT&lt;sup&gt;c&lt;/sup&gt;</td>
<td>189</td>
<td>80.3</td>
</tr>
<tr>
<td>Height&lt;sup&gt;d&lt;/sup&gt;</td>
<td>176</td>
<td>6.4</td>
</tr>
<tr>
<td>Weight&lt;sup&gt;e&lt;/sup&gt;</td>
<td>83.8</td>
<td>12.6</td>
</tr>
<tr>
<td>BMI&lt;sup&gt;e&lt;/sup&gt;</td>
<td>27.1</td>
<td>3.6</td>
</tr>
<tr>
<td>Energy&lt;sup&gt;f&lt;/sup&gt;</td>
<td>2056</td>
<td>531</td>
</tr>
<tr>
<td>Alcohol&lt;sup&gt;f&lt;/sup&gt;</td>
<td>8.9</td>
<td>9.1</td>
</tr>
</tbody>
</table>

|                      | Median | IQR   | N   | Median | IQR   | N   |
| Adiponectin<sup>h</sup> | 4.8    | 3.0—7.2 | 429 | 8.2    | 5.0—12.0 | 454 |
| Leptin<sup>i</sup>     | 6.4    | 4.1—9.8 | 429 | 17.0   | 11.0—25.1 | 454 |
| Ghrelin<sup>i</sup>    | 5.6    | 4.3—7.2 | 407 | 6.1    | 4.5—7.7  | 432 |

<sup>a</sup>(kg); <sup>b</sup>Visceral adipose tissue (cm²); <sup>c</sup>Subcutaneous adipose tissue (cm²); <sup>d</sup>(cm)<br><sup>e</sup>Body Mass Index (kg/m²); <sup>f</sup>(kcal/day); <sup>g</sup>(g/day); <sup>h</sup>(µg/mL); <sup>i</sup>(ng/mL)
Fat mass and fat distribution were determined by dual-energy X-ray absorptiometry (DXA) in 890 individuals, and by magnetic resonance imaging (MRI) in 287 individuals.

Persistent organic pollutants (POPs) were measured in stored plasma samples collected at baseline. A total of 21 POPs were measured: 16 polychlorinated biphenyls (PCBs) congeners, 3 organochlorine (OC) pesticides, 1 octachlorodibenzo-p-dioxin (OCDD), and 1 brominated diphenyl ether (BDE) congener. Analyses were performed using Micromass Autospec Ultima high resolution chromatography coupled to a high resolution mass spectrometry (HRGC/HRMS) system based on the method by Sandau et al. (2002) [114] with some modifications. Two OC pesticides (trans-chlordane and cis-chlordane) with a detection rate of <10\% were not included in the final analysis.

Serum samples were analyzed for levels of BPA (ALS Environmental Canada) using an isotope liquid chromatograph/tandem mass spectrometer (API4000LC-MS/MS). The detection limit (LOD) was 0.2 ng/mL. BPA in serum was detectable in 98 percent of both females and males.

On average two years following the baseline investigation, fat mass and lean mass in different body compartments were estimated by DXA. The primary outcome was total body fat mass, but as a secondary outcome the fat distribution was explored, since it has repeatedly been shown that abdominal fat is more harmful than fat located subcutaneously in other parts of the body [115]. The bias associated with DXA fat measurement is systematic, with an underestimation of fat content for lean subjects and an overestimation of fat content among obese subjects, but these inaccuracies amount to less than 2\% of the variation [116].

MRI of the abdominal region was performed in 287 randomly selected individuals, in addition to the DXA measurements. Visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) areas (cm\(^2\)) were manually segmented from a single 10 mm thick axial steady-state-free precession slice as previously described [117].

Adiponectin and leptin in plasma were analyzed with double-antibody radioimmunoassays (RIA). Total coefficient of variation (CV) for leptin was 4.7\% at both low (2–4 ng/ml) and high (10–15 ng/ml) levels, and for adiponectin the total CV was 15.2\% at low (2–4 μg/ml) and 8.8\% at high (26–54 μg/ml) levels. Serum ghrelin was analyzed with an enzyme-linked immunosorbent assay (ELISA). The total CV for the assay was 5\%.

The participants were asked at baseline to answer a questionnaire about their medical history, educational level, exercise habits, and smoking habits. The dietary intakes of 359 men and 358 women were assessed by a 7-day food diary.
3.3 Statistics

3.3.1 Experimental studies (papers I and II)

In the first study unpaired two-tailed $t$ tests were used to test differences between the groups. Pearson correlation coefficients ($R^2$) were used to measure linear correlations. The reproducibility of the VAT segmentation was measured by coefficient of variation defined by standard deviation of the two measurements divided by their mean. The statistical analyses were performed using Prism 5 by GraphPad Software Inc.

In the second study differences between the fructose control group and the three BPA plus fructose exposed groups were evaluated by factorial ANOVA. When the three BPA groups were analyzed vs. the fructose control group one by one, a Bonferroni adjustment for 3 tests was used and $p<0.0167$ considered significant ($p=0.05/3=0.0167$). In the secondary analysis, when the water control group was compared with the fructose control group $p<0.05$ was considered significant. StatView was used for calculations.

3.3.2 Epidemiological studies (papers III and IV)

In the third and fourth study all variables were evaluated regarding non-normality, and variables with a skewed distribution, such as plasma triglycerides, BPA, hormone levels, and all POPs, were ln-transformed. Since men and women normally have different distributions for leptin and adiponectin, with higher levels in women, the levels for those adipocytokines were transformed to the SD scale in men and women separately.

Three models were tested. In the first model, we adjusted for sex only. In the second model, adjustments were performed for sex, height, and lean mass by DXA. In the third model, additional adjustment was performed for smoking, exercise habits, education level, total daily energy intake, and alcohol consumption. Since data on dietary intake was missing in 155 subjects, we used multiple imputations (20) in order not to lose these subjects in the third model.

PIVUS – POPs (paper III)

The POPs were lipid-standardized against the sum of plasma cholesterol and triglycerides. In separate linear regression models for the different POPs, the POPs were related to indices of fat mass measurements by DXA. POP levels were also divided into quintiles to evaluate potential non-linear relations with fat mass. In this latter analysis, quintile 1 was used as the reference.

To evaluate how the individual POPs interfere in their relation to total fat mass, all POPs were entered as independent variables together with sex in a multiple regression model with total fat mass as dependent variable.
Introducing a quadratic term for the POPs along with the original POP variable was used to assess non-linearity in the relationships between POPs and fat mass.

For all POPs and for all outcomes, interactions between the POP levels and sex were evaluated in the first model. If there was an improved fit of the model with an interaction term (P<0.05), separate sex-specific analysis was performed.

STATA 11 was used for calculations, with P<0.05 regarded as statistically significant.

**PIVUS – BPA (paper IV)**

In linear regression models, BPA was related to indices of fat mass as well as to the three hormones evaluated.

To screen for non-linear relationships, the quadratic term of BPA was added to the linear regression models.

For all outcomes, interactions between the BPA level and sex were evaluated in the first model. If there was an improved fit of the model with an interaction term (P<0.05), separate sex-specific analysis was performed.

STATA 12 was used for calculations, with P<0.05 regarded as statistically significant.
4 Results and discussion

4.1 Quantification of total and visceral adipose tissue in fructose-fed rats using water-fat separated single echo MRI (paper I)

4.1.1 Results

In this study it was found that rats in the fructose group consumed substantially more liquid than rats in the control group during the whole study. The liquid consumption was highest week 8–10, after the fructose concentration was changed from 5% to 20%, but at the same time the chow intake decreased in this group. Energy intake was fairly constant, but the amount of energy from fructose rose from about 12 to 50% during the last three weeks (Table 3).

The weight gain was significantly greater in rats exposed to fructose than in controls (p<0.05), and the main difference in weight gain took place during the first seven weeks. The mean fat pad weight was 27% greater in the fructose group (p<0.001) and the ratio fat pad/body weight was 21% greater, compared with the control group (p<0.001). Liver weights and LSI were also greater (p=0.001 and p=0.012, respectively) in the fructose-exposed group.

Regarding the circulating markers, the most pronounced effects were seen for triglycerides and HDL associated protein apo A-I. The levels of triglycerides and apo A-1 were 30% (p=0.034) and 21% (p=0.005) higher, respectively, in the fructose-exposed individuals than in the controls (Table 4).
Table 3. Body weight, weight gain (mean ± SD), food (RM1, \textit{ad lib}) and liquid consumption (mean/cage) in female Fischer 344 rats given water or a 5% fructose solution (week 1-7), and then a 20% fructose solution (week 8-10). Food and liquid consumption were measured per cage and all values are given as the calculated mean/rat. \(N=12/\text{group (individual measures)}, N=2\) represents 2 cages with 6 animals in each cage (paper I).

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>(N)</th>
<th>Fructose-exposed</th>
<th>(N)</th>
<th>(p)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight week 10 (^a)</td>
<td>159.8 ± 6.0</td>
<td>12</td>
<td>166.7 ± 6.20</td>
<td>12</td>
<td>0.010</td>
</tr>
<tr>
<td>Weight gain week 1-7 (^a)</td>
<td>88.7 ± 5.6</td>
<td>12</td>
<td>94.3 ± 6.21</td>
<td>12</td>
<td>0.028</td>
</tr>
<tr>
<td>Weight gain week 8-10 (^a)</td>
<td>12.0 ± 2.9</td>
<td>12</td>
<td>11.5 ± 2.02</td>
<td>12</td>
<td>0.66</td>
</tr>
<tr>
<td>Weight gain week 1-10 (^a)</td>
<td>100.6 ± 5.5</td>
<td>12</td>
<td>105.8 ± 6.35</td>
<td>12</td>
<td>0.042</td>
</tr>
<tr>
<td>Food week 1-7 (^b)</td>
<td>9.2 ; 9.5</td>
<td>2</td>
<td>9.1 ; 9.4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Food week 8-10 (^b)</td>
<td>8.5 ; 8.5</td>
<td>2</td>
<td>5.3 ; 5.8</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Food week 1-10 (^b)</td>
<td>9.0 ; 9.2</td>
<td>2</td>
<td>8.1 ; 8.4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Liquid week 1-7 (^b)</td>
<td>12.7 ; 9.7</td>
<td>2</td>
<td>17.7 ; 18.7</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Liquid week 8-10 (^b)</td>
<td>10.5 ; 9.4</td>
<td>2</td>
<td>22.4 ; 18.2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Liquid week 1-10 (^b)</td>
<td>12.1 ; 9.6</td>
<td>2</td>
<td>19.0 ; 18.5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Food energy week 1-7 (^c)</td>
<td>26.1 ; 26.9</td>
<td>2</td>
<td>25.7 ; 26.6</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Food energy week 8-10 (^c)</td>
<td>24.0 ; 24.1</td>
<td>2</td>
<td>15.0 ; 16.5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Food energy week 1-10 (^c)</td>
<td>25.4 ; 25.9</td>
<td>2</td>
<td>22.1 ; 23.2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Fructose energy week 1-7 (^c)</td>
<td>—</td>
<td>2</td>
<td>3.53 ; 3.74</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Fructose energy week 8-10 (^c)</td>
<td>—</td>
<td>2</td>
<td>17.5 ; 14.5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Fructose energy week 1-10 (^c)</td>
<td>—</td>
<td>2</td>
<td>8.0 ; 7.3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Total energy week 1-7 (^c)</td>
<td>26.1 ; 26.9</td>
<td>2</td>
<td>29.2 ; 30.3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Total energy week 8-10 (^c)</td>
<td>24.0 ; 24.1</td>
<td>2</td>
<td>32.5 ; 31.0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Total energy week 1-10 (^c)</td>
<td>25.4 ; 25.9</td>
<td>2</td>
<td>30.1 ; 30.5</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) (g)
\(^b\) (g/rat/day)
\(^c\) (kcal/rat/day)
Table 4. Final body weight, adipose tissue volumes, liver and circulating markers in female Fischer 344 rats at sacrifice. The rats were given water (controls) or a 5% fructose solution (week 1-7) and then a 20% fructose solution (week 8-10). Values for adipose and lean tissue volumes are calculated from the MRI measurements (paper I).

<table>
<thead>
<tr>
<th></th>
<th>Controls (mean ± SD)</th>
<th>Fructose-exposed (mean ± SD)</th>
<th>N</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>160.9 ± 5.76</td>
<td>167.8 ± 6.25</td>
<td>12</td>
<td>0.010</td>
</tr>
<tr>
<td>Estimated body weight, MRI (g)</td>
<td>119 ± 4.9</td>
<td>122 ± 4.9</td>
<td>9</td>
<td>0.20</td>
</tr>
<tr>
<td>Total Adipose Tissue (cm$^3$)</td>
<td>23.6 ± 2.1</td>
<td>28.8 ± 5.3</td>
<td>9</td>
<td>0.017</td>
</tr>
<tr>
<td>Visceral Adipose Tissue (cm$^3$)</td>
<td>12.6 ± 1.64</td>
<td>15.7 ± 3.1</td>
<td>8</td>
<td>0.019</td>
</tr>
<tr>
<td>Subcutaneous Adipose Tissue (cm$^3$)</td>
<td>11.0 ± 1.1</td>
<td>13.2 ± 2.4</td>
<td>8</td>
<td>0.031</td>
</tr>
<tr>
<td>Lean Tissue (cm$^3$)</td>
<td>92.5 ± 4.03</td>
<td>91.0 ± 1.98</td>
<td>9</td>
<td>0.35</td>
</tr>
<tr>
<td>Fat pad (g)</td>
<td>0.67 ± 0.069</td>
<td>0.85 ± 0.10</td>
<td>12</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fat pad/bodyweight ratio (%)</td>
<td>0.42 ± 0.043</td>
<td>0.51 ± 0.057</td>
<td>12</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>4.70 ± 0.10</td>
<td>5.24 ± 0.37</td>
<td>12</td>
<td>0.001</td>
</tr>
<tr>
<td>Liver/bodyweight ratio (%)</td>
<td>2.9 ± 0.18</td>
<td>3.1 ± 0.19</td>
<td>12</td>
<td>0.012</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>2.6 ± 0.18</td>
<td>2.6 ± 0.18</td>
<td>12</td>
<td>0.92</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.15 ± 0.36</td>
<td>1.50 ± 0.39</td>
<td>12</td>
<td>0.034</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>0.74 ± 0.050</td>
<td>0.81 ± 0.051</td>
<td>12</td>
<td>0.003</td>
</tr>
<tr>
<td>Apo A-I (optical density, %)</td>
<td>8.66 ± 1.07</td>
<td>10.46 ± 1.61</td>
<td>12</td>
<td>0.005</td>
</tr>
</tbody>
</table>

4.1.2 Discussion

The results support the model set up as suitable for studies of obesity, and how different environmental factors may affect weight regulation in an environment of modest caloric excess.

From this study it can be concluded that fructose supplementation results in unfavorable metabolic alterations as a higher LSI, increase in plasma triglycerides and also increased apo A-I.

The MRI method allows assessment of adipose tissue volumes and distribution in living rats. Similar MRI methods have been used in studies in humans, which facilitates translational research.

The retro-peritoneal fat, which is a distinct fat depot easy to distinguish and dissect, correlated well to the measurements of total fat mass analyzed with MRI in female Fischer 344 rats, and could therefore be used as a substitute for total fat mass in rats.
4.2 Bisphenol A exposure increases liver fat in juvenile fructose-fed Fischer 344 rats (paper II)

4.2.1 Results

In the second animal study the same model was used as in the first study, with some minor modifications, the most important one being the addition of BPA exposure. No differences between the four fructose-fed groups were seen regarding the initial body weight recorded prior to the intervention. Neither the weight at the time of termination of the experiment nor the weight gain during the intervention differed across the four groups. No differences were found across the four groups regarding the weight of the fat pad, and MRI showed no differences in total or visceral adipose tissue volumes across the four groups.

However, MRI revealed greater fat infiltration in the liver of BPA-exposed rats than in the fructose-fed controls. Rats exposed to the medium or to the high dose of BPA had a higher liver fat content than rats from the fructose control group. The lowest dose of BPA did not significantly influence liver fat content (Figure 5).

![Figure 5](image)

*Figure 5. Liver fat content (%, mean ± SEM) in juvenile female Fischer 344 rats given water, 5% fructose solution or bisphenol A (0.025, 0.25 or 2.5 mg/L) dissolved in 5% fructose solution for ten weeks. Water; n=12, Fructose; n=11, BPA 0.025 mg/L; n=12, BPA 0.25 mg/L; n=12 and BPA 2.5 mg/L; n=12 (paper II).
Rats exposed to the medium or the high dose, but not the lowest dose of BPA showed significantly higher levels of plasma apo A-I, when compared with rats in the fructose control group (Figure 6). Plasma cholesterol and plasma triglycerides were not significantly altered by the BPA exposure. Neither was blood glucose at week 9, or ASAT and ALAT altered by BPA exposure.

Of all variables studied, only plasma triglycerides and LSI were significantly increased by fructose feeding alone when compared to the water-fed control.

Figure 6. Apolipoprotein A-I (INT/mm², mean±SEM) in juvenile female Fischer 344 rats given water, 5% fructose solution or bisphenol A (0.025, 0.25 or 2.5 mg/L) dissolved in 5% fructose solution for ten weeks. Water; n=12, Fructose; n=12, BPA 0.025 mg/L; n=11, BPA 0.25 mg/L; n=8 and BPA 2.5 mg/L; n=9 (paper II).

4.2.2 Discussion

These results did not reveal any evidence that BPA exposure in the range 5-500 ug/kg/day would increase fat mass in juvenile female fructose-fed F 344 rats. However, the observed increase in liver fat infiltration, detected by MRI in parallel with increase in LSI, at dosages close to TDI, and elevated plasma A-I levels are findings that warrant further investigation.
The increase in liver fat infiltration appeared at the middle dose, but was not further increased at the highest BPA dose. This finding confirms a previous in vivo study on mice by Marmugi et al. (2012) [118], using the same dose range of BPA as in the present study. The Marmugi study showed an impact on the hepatic transcriptome, particularly on genes involved in lipid synthesis and that various transcription factors followed a non-monotonic dose-response curve. In addition, also in line with the Marmugi study, the most significant effects were observed within one magnitude around the current TDI.

There are three possible sources of increases in liver fat: i) de novo lipid synthesis, ii) decreased degradation or iii) increased transport of cholesteryl esters to the liver. According to our data the most likely mechanisms behind the lipid accumulation in the liver are a combination of de novo lipid synthesis and increased transport of cholesteryl esters to the liver. The individual contribution from fructose and BPA can only be postulated, but considering the higher LSI in the fructose group in the first study and further increase accompanied by the increase of plasma apo A-I after BPA exposure, we suggest that fructose is the main contributor to the de novo lipid synthesis while BPA is the main contributor to the increased reverse transport. The decrease in plasma apo A-I and thereby LSI at the highest BPA dose may be a negative feedback response to apo A-I synthesis, but this needs to be further investigated.

Another interesting finding was the increase in plasma apo A-I. This is the dominating protein in high-density lipoproteins (HDL), and by its interaction with lecithin-cholesterol acyltransferase (LCAT) a crucial component in the cholesterol transport to the liver. The increased expression of apo A-I may result in BPA elimination from the plasma together with cholesteryl esters via the Scavenger Receptor Class B-I (SR-BI) in the liver. The interaction between apo A-I and SR-BI may thereby result in non-endocytotic hepatocytic uptake of hydrophobic compounds, such as cholesteryl esters and also possibly BPA.

There are at least four different possibilities regarding the mechanisms of elevated plasma apo A-I levels in response to BPA exposure: i) induced apo A-I gene expression by BPA, as has been reported regarding aspirin [119], ii) increased apo A-I expression in response to (pro)-inflammatory effects caused by BPA, iii) that BPA, due to its structural similarities to cholesterol is in fact recognized as free cholesterol and iv) that BPA causes estrogenic effects on apo A-I gene expression [120].
4.3 Circulating levels of persistent organic pollutants associate in divergent ways to fat mass measured by DXA in humans (paper III)

4.3.1 Results

The first epidemiological study analyzed the relationship between POPs and fat mass and fat distribution. The highest median concentrations of analyzed POPs were 325 ng DDE/g lipid, followed by 250 ng PCB 153/g lipid and 215 ng PCB 180/g lipid.

In regression analysis using the POPs as continuous variables, the lipid-standardized plasma concentrations of OCDD, the PCBs 74, 99, 105, and 118, the pesticides hexachlorobenzene (HCB), transnonachlordane (TNC), and DDE, were all positively related to total fat mass. For example, subjects in the fifth quintile for PCB 105 showed a mean fat mass that was 4.8 kg more than subjects in the first quintile. On the other hand, the PCBs 126, 153, 156, 169, 170, 180, 189, 194, 206, and 209 were negatively related to fat mass. For example, regarding PCB 194, subjects in the fifth quintile showed a mean fat mass that was 10.8 kg less than subjects in the first quintile (see Figure 7 for a comprehensive view of the relationships).

Following further adjustment for smoking, physical activity, educational level, height, lean mass, energy intake, and alcohol consumption, these results remained significant. Additional adjustments for either total fat intake, saturated fat intake or total carbohydrate intake did not change the results in any substantial way. Almost identical results were obtained if wet weight values for the POP concentrations were used instead of lipid-normalized values.

The interaction terms between the PCBs 153, 156, 157, 170, 180, 194, 206, and 209 and sex were significant. When stratifying the analysis by sex regarding the relationships between these POPs and total fat mass, it was found that the negative relationships were more pronounced in women than in men, with a regression coefficient that was 2-3 times greater compared to the males.

For the PCBs 74, 105, 118, 153, 156, 169, 170, 180, 189, 194, 206 and 209, the quadratic terms for these PCB congeners were significant, suggesting non-linear relationships. When the POPs were divided into quintiles, a possible low-dose effect for only PCB74 could be found, with the main increase in fat mass seen already in the second quintile of PCB74.

Body weight was measured at baseline and also at the time of the DXA measurement. The mean change in body weight between these measurements was -0.5 kg (SD 4.2). Excluding subjects that were not weight-stable, defined as either a reduction or a gain of at least 3 kg or more between the measurements, resulted in a subsample of 617 weight-stable subjects.
Figure 7. An overview of the relationships between individual POPs given as the mean differences (and 95 confidence intervals) in fat mass between the first quintile of each POP and the quintile that showed the greatest difference vs. the first quintile following adjustment for sex. PCB=polychlorinated biphenyl, OCDD=octachlorodibenzo-p-dioxin, HCB=hexachlorobenzene, TNK=transnonachlordane, DDE=dichlorodiphenyldichloroethylene (the main metabolite of the pesticide DDT), BDE=brominated diphenyl ether (paper III).

When the analysis was repeated in that subsample, the relationships between POP levels and fat mass described in Table 3 were essentially unaltered compared to when all available subjects were analyzed. The relationships between the POPs and trunk fat or leg fat were generally very similar, and similar to the relationships described above for total fat mass. However, one difference from the above was a significant positive relationship between trunk fat mass and the PCBs 74, 99, and 138 found in men but not in women. BDE 47 showed no significant relationships to any of the fat mass variables.
4.3.2 Discussion

We found multiple associations between levels of POPs and fat mass. There was a consistent pattern with generally highly chlorinated compounds showing a negative association, while the OC pesticides and the less chlorinated compounds generally showed a positive association. This indicates the importance of separate measurements for different PCBs, not just using the sum, since they differ in chemical structure and effect.

The opposite associations for different PCBs has been reported once before, where PCB 118, representing a dioxin-like PCB, showed a positive relationship to BMI, while the sum of marker PCBs (PCB 138, 153, 180) showed a negative relationship with BMI [121]. Looking further into these relationships, the common divider between these two behaviors of the PCBs is the number of chlorine atoms included in the molecule. No such division could be seen for other characteristics of the PCBs in our study, such as whether or not they were planar or “dioxin-like”. Obviously, the degree of chlorination of the PCB molecule could change its chemical properties. One characteristic that is highly dependent on the degree of chlorination is the lipophilicity of the compound and thereby its storage in fat tissue and its half-life in the body. For most of the highly chlorinated PCBs the effect was more pronounced in women than in men, which could represent differences in how the compounds are stored in the tissues. The highly chlorinated compounds have a significantly prolonged elimination period compared with the low-chlorinated PCBs. Whether this difference in toxicokinetics could lead to opposite associations between the plasma levels of the compounds and fat mass needs to be further evaluated. Another possible explanation for the phenomenon noted in the present study might be different agonist/antagonist actions on receptors involved in fat mass control.

In this cross-sectional study, the age of the participants (70 years) could influence the results in various ways. Endocrine function generally declines with age because hormone receptors become less sensitive and levels of hormones change [122], which perhaps alleviates the impact of endocrine disruptors. On the other hand, there is a positive relationship between age and plasma concentration of POPs due to bioaccumulation. In contrast to younger people, the PIVUS cohort were probably not exposed to POPs to any great extent during their fetal period or early childhood, which may be of importance if the effects differ depending on when the individuals are exposed.
4.4 Bisphenol A is related to circulating levels of adiponectin, leptin and ghrelin, but not to fat mass or fat distribution in humans (paper IV)

4.4.1 Results

The second epidemiological study investigated relationships between serum concentrations of BPA and measures of fat mass and fat distribution and between BPA and circulating levels of adiponectin, leptin and ghrelin. In a regression analysis using BPA as a continuous variable, adjusted for sex, there were no significant associations with any of the fat-mass-related measures. Following further adjustment for height, lean mass, smoking, exercise habits, education level, energy intake, and alcohol consumption, these results remained (Table 5). The interaction between BPA concentration and sex was not significant for any of the fat measures studied.

Serum concentrations of BPA adjusted for sex were positively associated with adiponectin (p<0.001) and leptin (p=0.018) levels. These results remained significant (p<0.001 and p=0.009, respectively) after further adjustment for height, lean mass, smoking, alcohol consumption, physical activity, energy intake, and education level. The results for adiponectin and leptin were also independent of adjustment for fat mass (p<0.001 for both). Introducing a quadratic term suggested a non-linear relationship for BPA vs. adiponectin (p<0.001), but not vs. leptin. As can be seen in Figure 8 the effects of BPA regarding adiponectin levels were seen even at rather low levels of BPA.
Figure 8. Relationship between bisphenol A (BPA, ln-transformed) and adiponectin (SD-scale). A quadratic term for BPA was used in the model to search for non-linear relationship. The predicted values and the 95% CI are given at each 0.5 interval for BPA (paper IV).

Serum concentrations of BPA adjusted for sex were negatively related to ghrelin (p<0.001). Following adjustment for height, lean mass, smoking, alcohol consumption, physical activity, energy intake, and educational level, these results remained (p<0.001). Introducing a quadratic term for BPA in the models did not reveal any significant non-linear effects. The results were independent of adjustment for fat mass.

The interaction term between BPA and sex was significant for ghrelin (p=0.04), revealing a stronger relationship between BPA and ghrelin for women, with a regression coefficient about twice as high as for men (-0.12, p=0.002 vs. -0.06, p<0.001). The relationship between BPA and ghrelin levels was very similar in subjects with a fat mass above the median compared to those below the median (linear regression coefficient -0.09, p=0.0001 for both strata).
Table 5. Relationships between BPA concentration in serum and fat mass and levels of hormones related to fat mass, hunger and satiety. Relationships are given for BPA as a continuous variable (regression coefficient using ln-transformed values) (paper IV).

<table>
<thead>
<tr>
<th>Variable</th>
<th>N</th>
<th>Model 1: sex adjusted</th>
<th></th>
<th>Model 2: body stature adjusted</th>
<th></th>
<th>Model 3: multiple adjusted</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Beta (95% CI)</td>
<td>p-value</td>
<td>Beta (95% CI)</td>
<td>p-value</td>
<td>Beta (95% CI)</td>
<td>p-value</td>
</tr>
<tr>
<td>Total fat</td>
<td>845</td>
<td>-113 (-744 ; 518)</td>
<td>0.73</td>
<td>-188 (-759 ; 383)</td>
<td>0.52</td>
<td>-130 (-703 ; 442)</td>
<td>0.66</td>
</tr>
<tr>
<td>Fat leg</td>
<td>845</td>
<td>-33.0 (-253 ; 187)</td>
<td>0.77</td>
<td>-54.2 (-267 ; 158)</td>
<td>0.62</td>
<td>-35.0 (-251 ; 181)</td>
<td>0.75</td>
</tr>
<tr>
<td>Fat trunk</td>
<td>845</td>
<td>-54.0 (-417 ; 309)</td>
<td>0.77</td>
<td>-96.0 (-422 ; 230)</td>
<td>0.56</td>
<td>-65.5 (-390 ; 259)</td>
<td>0.69</td>
</tr>
<tr>
<td>Fat trunk/leg</td>
<td>845</td>
<td>-0.001 (-0.038 ; 0.035)</td>
<td>0.94</td>
<td>-0.002 (-0.037 ; 0.034)</td>
<td>0.93</td>
<td>-0.0003 (-0.037 ; 0.036)</td>
<td>0.99</td>
</tr>
<tr>
<td>SAT\textsuperscript{d}</td>
<td>268</td>
<td>2.23 (-9.50 ; 14.0)</td>
<td>0.71</td>
<td>0.248 (-11.0 ; 11.5)</td>
<td>0.97</td>
<td>1.17 (-9.99 ; 12.3)</td>
<td>0.84</td>
</tr>
<tr>
<td>VAT\textsuperscript{e}</td>
<td>268</td>
<td>0.756 (-6.18 ; 7.70)</td>
<td>0.83</td>
<td>-0.766 (-7.26 ; 5.73)</td>
<td>0.82</td>
<td>-0.279 (-6.70 ; 6.14)</td>
<td>0.93</td>
</tr>
<tr>
<td>VAT/SAT ratio</td>
<td>268</td>
<td>-0.017 (-0.044 ; 0.01)</td>
<td>0.22</td>
<td>-0.017 (-0.045 ; 0.01)</td>
<td>0.22</td>
<td>-0.0157 (-0.044 ; 0.012)</td>
<td>0.27</td>
</tr>
<tr>
<td>Adiponectin\textsuperscript{f}</td>
<td>844</td>
<td>0.169 (0.104 ; 0.235)</td>
<td>&lt;0.001</td>
<td>0.167 (0.096 ; 0.238)</td>
<td>&lt;0.001</td>
<td>0.164 (0.093 ; 0.236)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Leptin\textsuperscript{g}</td>
<td>844</td>
<td>0.08 (0.014 ; 0.146)</td>
<td>0.018</td>
<td>0.09 (0.021 ; 0.159)</td>
<td>0.010</td>
<td>0.0918 (0.023 ; 0.160)</td>
<td>0.009</td>
</tr>
<tr>
<td>Ghrelin\textsuperscript{h}</td>
<td>834</td>
<td>-0.09 (-0.12 ; -0.064)</td>
<td>&lt;0.001</td>
<td>-0.096 (-0.127 ; -0.065)</td>
<td>&lt;0.001</td>
<td>-0.10 (-0.13 ; -0.07)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

\textsuperscript{a} p-value adjusted for sex
\textsuperscript{b} p-value adjusted for sex, height and lean mass
\textsuperscript{c} p-value adjusted for sex, height, lean mass, smoking, exercise habits, education level, total daily energy intake, and alcohol consumption
\textsuperscript{d} subcutaneous adipose tissue
\textsuperscript{e} visceral adipose tissue
\textsuperscript{f} SD ln adiponectin
\textsuperscript{g} SD ln leptin
\textsuperscript{h} ln ghrelin
4.4.2 Discussion

The serum levels of BPA were comparable with the levels in studies by others, as reviewed by Olsén et al. (2012) [123]. Both free BPA and its metabolites, mainly its glucorinated form, were included in the measured concentration. Although it is free BPA that evinces biological activity, the measurement of both free BPA and its metabolites yields a more reliable measure of exposure, since free BPA is rapidly glucoronidated.

Numerous studies, both experimental and epidemiological, have shown an association between BPA and obesity and other metabolic effects, such as insulin resistance and dyslipidemia [71, 88, 124-127]. In our study the BPA concentrations in serum were associated with levels of adiponectin, leptin and ghrelin, hormones known to be involved in the regulation of hunger and satiety [128, 129]. Leptin and adiponectin regulate energy intake in the long term and ghrelin also in the short term [130]. Circulating levels of leptin and adiponectin are generally higher in women, and sex steroids including estrogens are partly responsible for these differences [131].

BPA concentrations in serum were not associated with total or regional body fat measures by DXA or MRI, but obesity is a multifactorial disease [132] and the progress is rather slow. These features regarding etiology and time-course of obesity development will make it difficult to establish a relationship between BPA in serum and fat volume measured at one time point in this elderly cohort, mainly exposed as adults. Hormone levels on the other hand respond very fast to changes in the environment and therefore a relationship between hormone levels and a single measurement of BPA is more likely to be revealed.
5 Summary

Based on data from experimental and epidemiological studies this thesis elucidates the relationship between a large number of environmental contaminants and obesity.

- The retro-peritoneal fat, which is a distinct fat depot easy to distinguish and dissect, correlated well to the measurements of total fat mass analyzed with MRI in female Fischer 344 rats, and could therefore be used as a substitute for total fat mass in rats.

- Fructose supplementation in the drinking water created an environment of modest caloric excess, mimicking a modern lifestyle, and resulted in unfavorable metabolic alterations such as a higher LSI, an increase in plasma triglycerides and also increased plasma levels of apo A-I.

- Exposure to BPA in combination with fructose increased liver fat content and plasma levels of apo A-I in juvenile female Fischer 344 rats.

- Circulating levels of POPs were related to fat mass measured by DXA. OCDD, HCB, TNC, DDE and the less chlorinated PCBs were positively related to fat mass, while the more highly chlorinated PCBs showed a negative association.

- Circulating levels of BPA were positively associated with levels of the hormones adiponectin and leptin, but negatively related with ghrelin, hormones which are involved in the regulation of hunger and satiety. Serum BPA levels were however not related to measures of fat mass in elderly individuals.

This thesis concludes that environmental contaminants most likely are contributors, along with genetic, social and behavioral factors, to the development of obesity.
6 General Discussion

The combination of overeating and a sedentary lifestyle is the presumed key pathogenetic mechanism behind obesity. The contrary, dieting and exercise, have been, and still are, consequently the main tools to combat superfluous kilos. Ironically the importance of lifestyle factors, such as healthy food and exercise, has never been as emphasized as much as during the last 40 years. This is about the same time period in which the global rise in overweight and obesity has assumed epidemic proportions. Concurrent with the obesity epidemic there has been a worldwide exponential increase of human exposure to synthetic chemicals. It is clear that some of these chemicals possess endocrine-disrupting properties. As obesity is a disease involving endocrine mechanisms, including hormones and growth factors controlling adipose tissue metabolism, adipogenesis, and centrally mediated appetite and food intake, there could be a link between environmental contaminant exposure and increased prevalence of obesity.

In the study of contaminants as obesogens, in doses reflecting common environmental exposure, the expectation is not any overt and immediate health effects, but mild abnormalities in regulatory pathways. If a change in energy homeostasis occurs, it may start a slow process that might become manifest after years of continuous exposure, or follow the developmental origin of health and disease (DOHaD) principle, with exposure during critical time windows that may lead to manifestation many years later. The most well-studied sensitive windows are during the prenatal and neonatal periods, but childhood, puberty and menopause are also phases in life with substantial bodily rearrangements that are potentially sensitive to disrupters. It is also possible that times of heavy stress, chronic stress or disease increase susceptibility to endocrine disruption.

There are thousands of anthropogenic chemicals used and dispersed in the environment. Most of them have not been investigated regarding health effects, and our knowledge about interactions of the mixture that comprise the real exposure is minimal. New legislation (Reach 2010) requires the registration of chemicals but only for those chemicals that are produced or imported in amounts of at least 1 metric ton/year and are carcinogenic, mutagenic or reprotoxic. Chemicals that are very toxic to aquatic environments need to be registered if the amount produced or imported exceeds 100 metric tons/year. Other chemicals need to be registered only if the amount produced or imported exceeds 1000 metric tons/year. To register a chemical does not mean
that it is tested and found safe, but the registration is regarded as a first step towards a safer society.

Our exposure to many POPs peaked in the 1970s, but when the negative impact on the environment and human health was uncovered, efforts were made to remove the most threatening compounds. The Stockholm Convention on Persistent Organic Pollutants identified twelve chemicals or chemical groups (the “Dirty Dozen”) for priority action. Since then international agreements have been made, and the banning of some of the most deleterious chemicals has resulted in decreasing levels of those compounds in the environment. Unfortunately, new ones have been introduced, e.g. per- and polyfluoroalkyl substances (PFASs). These are both oil and water repellent and have a very wide field of application.

Today there is also an increased awareness of potential environmental threats from less persistent but ubiquitously present compounds, like those in plastics. A chemical that has been proven to be a threat to human health may be phased out, or as in the case for BPA, restricted when it comes to use in certain products, e.g. baby bottles, targeting a vulnerable subgroup in the population. However the problem may remain if it is replaced with new less thoroughly investigated ones, e.g. bisphenol S (BPS), which has replaced BPA and has not yet proven to be harmful.

Obesity is no longer a problem of wealth, but strikes on all levels: thin people are fatter, fat people are fatter, rich and poor people are fatter [133]. It is necessary to find new tools to fight obesity, and they must include efforts to find the reasons and answers to why unhealthy amounts of fat tissue have become a common body constituent in many populations. The most promising treatment of severe obesity today is surgery, which cannot be regarded as a solution for the millions of affected individuals. It is necessary to find out if the exposure to environmental contaminants contributes to the obesity epidemic and other non-communicable diseases, and if so, to take action.

Tools to use in this scenario are political will, regulation, and legislation, but not least new methods to develop safe chemicals and products. New chemicals should be designed with hazard risks in mind from an early point in the process. This is possible with cooperation between different scientific disciplines such as chemistry, environmental toxicology and human health, which could generate test protocols to go with the process. An example of this is the Tiered Protocol for Endocrine Disruption (TiPED), which consists of five testing tiers ranging from broad in silico evaluation up to specific cell- and whole-organism-based assays [134]. Interventions that facilitate reuse of products, recycling of materials, and more efficient waste management in the society are additional interventions to decrease our exposure to environmental contaminants.
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8 Svensk sammanfattning: Miljögifter och fetma

I nästan hela världen är övervikt och fetma ett växande problem som drabbar såväl barn som vuxna av båda könen. Förutom att övervikt och fetma ofta leder till fysiskt och psykiskt lidande och en försämrad livskvalitet innebär det också en ökad risk för en rad kroniska sjukdomar, såsom diabetes typ 2, kardiovaskulära sjukdomar, högt blodtryck och vissa cancerformer.

Ett ökat välstånd med lättillgänglig och energität mat och en livsstil med begränsad fysisk aktivitet har varit den allmänt accepterade förklaringen till ökningen av övervikt och fetma som observeras framförallt i västvärlden under de senaste decennierna. Utan positiv energibalans, dvs. ett energiintag som är större än energiåtgången, ökar ingen i vikt. Samtidigt är ett finjusterat kroppseget styr- och reglersystem för kroppsvikt och fettansättning en förutsättning för att kroppen ständig ska vara försedd med lagom mycket energi.

Fettväv har tidigare betraktats som passiva energidepåer, men idag vet vi att så inte är fallet. Ämnesomsättning och aptitreglering styrs av hormoner och signalsubstanser vars ursprung i vissa fall är just fettceller. Det är dessutom uppenbart att det finns en ärftlighet för övervikt [135]. Detta gör problematiken med övervikt och fetma mycket komplex och svårare att lösa än genom att enbart räkna kaloriintag och kaloriförbrukning. En bättre förståelse av fettcellernas biologi och deras samverkan med det neuroendokrina systemet och med miljön är nödvändig för att kunna begränsa fetmaepidemin.

som kan förklara hur endokrinstörande miljongifter kan ge just sådana obesogena effekter [27]. Även epidemiologiska studier tyder på att det finns ett samband mellan exponering för miljongifter och fetma. En studie visar t ex att barn med högre koncentrationer av hexaklorobenzen, mätt i navelsträngsblod, hade ett högre BMI när de var 6,5 år än de som hade lägre koncentrationer vid födseln [105]. För att kunna dra slutsatser om orsakssamband krävs prospektiva humanstudier och dessa är tyvärr få [137]. Syftet med denna avhandling var att med experimentella och epidemiologiska studier undersöka eventuella samband mellan exponering för miljongifter och fetma.

8.1 Studie 1

Den första studien var en metodstudie. Frågeställningen var hur tillväxt och fettinlagring hos råtta påverkas av fruktosintag.

Juvenila honrätter (Fischer 344) fick antingen vatten eller en fruktoslösning att dricka under 10 veckor. Syftet med fruktoslösningen var att efterlikna en modern livsstil med ett lätt överflöd av kalorier i form av kolhydrater. Lösningen innehöll 5% fruktos under de första sju veckorna, och därefter höjdes koncentrationen till 20% under de sista 3 veckorna. Djuren utfördades med standardfoder ad libitum (fri tillgång) och åtgången av både foder och vätska registrerades löpande. Försöket avslutades med att råttorna skannades med magnetisk resonanstomografi (MRT) (kallas också magnetkamera) som ger en beräkningsbar bild av fettmängd och fettdistribution i kroppen.

Viktökningen var större för råttorna som fått fruktoslösning än de som enbart drack vatten. Den största skillnaden ågås under de första sju veckorna när lösningen innehöll 5%. En hos rätter väl avgränsad fettedepå (se figur 4), som dissekerades ut efter avlivningen och leveroomatiskt index (LSI) var också större. Även apolipoprotein A-I (apo A-I) och plasmatriglycerider var högre i gruppen som fått fruktoslösning.

Rätterna som fick fruktoslösning drack mer än de som fick vatten. När koncentrationen ökades från 5% till 20% under de tre sista veckorna ökade konsumtionen ytterligare, men samtidigt åt de mindre och det totala kaloriintaget förblev tämligen konstant.

8.2 Studie 2

Den andra studien var ett djurförsök där 60 juvenila honrätter (Fischer 344) delades in i 5 grupper om 12 djur i varje. Djuren utfördades med vätska och standardfoder ad libitum. För att efterlikna en modern livsstil med ett mättligt överflöd av energi innehöll vattnet till 4 av grupperna 5% fruktos.
Under tio veckor, från 5 till 15 veckors ålder exponerades 3 grupper också för testsubstansen bisfenol A (BPA) löst i etanol (vehikel, 1% av den färdiga lösningen) i fruktoslösningen enligt:

Grupp 1: Vatten och vehikel  
Grupp 2: Fruktoslösning 5% och vehikel  
Grupp 3: Fruktoslösning 5% och BPA 0,025 µg/ml  
Grupp 4: Fruktoslösning 5% och BPA 0,25 µg/ml  
Grupp 5: Fruktoslösning 5% och BPA 2,5 µg/ml


De exponerade grupperna visade ingen skillnad i vikt eller i fettmängd jämfört med kontrollgrupperna. Däremot var det skillnad i mängden fett i lever. Grupperna som exponerats för mellandosen och den högre dosen hade ett högre fettinnehåll i lever än kontrollgruppen. Leverarna från låg- och mellandosgruppen var större (ett högre LSI) jämfört med kontrollen. Koncentrationen apo A-I i plasma var högre i grupperna som fått BPA i mellandos eller den högre dosen.

I en jämförelse mellan gruppen som fått vatten och gruppen som endast fått fruktoslösning skiljde sig endast plasmatriglycerider och LSI åt. Plasmatriglycerider ökade i fruktosgruppen och det gjorde även LSI.

8.3 Studie 3

Den tredje studien var en human tvärsnittsstudie där associationen mellan plasma-koncentrationerna av vissa persistenta organiska miljögifter (POPar) och fetma undersöks.

Epidemiologiskt material från PIVUS studien (The Prospective Investigation of the Vasculature in Uppsala Seniors) analyserades statistiskt. De variabler som analyserade var body mass index (BMI: vikt/längd²), magomfång (mätning med måttband), total mängd fett och visceral fett, så kallat bukfett, mätt med Dual energy X-ray absorptiometry (DXA).

De miljögifter som analyserades i plasma var: PCB-kongenerna 74, 99, 105, 118, 126, 153, 156, 157, 169, 170, 180, 189, 194, 206 och 209, pesticiderna hexaklorbensen (HCB), transnonakloridan (TNC) och DDE som är en
metabolit till DDT, och dioxinen OCDD. Av dessa hade DDE den högsta mediankoncentration (325 ng/g lipid) följt av PCB 153 (250 ng/g lipid) och 180 (215 ng/g lipid).

Regressionsanalys visade att plasmakoncentrationerna av OCDD, PCB 74, 99, 105 och 118, HCB, TNC och DDE var positivt associerade till den totala fettmassen. Till exempel hade personerna med de högsta halterna av PCB 105 i genomsnitt 4,8 kg mer fett än de som hade lägst halter. Det omvända förhållandet gällde för PCB 126, 153, 156, 157, 169, 170, 180, 189, 194, 206 och 209. Personerna med de högsta halterna av PCB 194 hade i genomsnitt 10,8 kg mindre fett än de som hade lägst halter. En förklaring till detta kan vara att de mer högklorerade PCBerna har en högre fettlösighet än de med färre kloratomer och därför är starkare bundna i fettväven. Då kommer koncentrationen som uppmätts i blodet vara låg, men den totala mängden av dessa PCBer i kroppen kan ändå vara hög.

Vägdes också faktorer som rökning, fysisk aktivitet, utbildningsnivå, längd, fettfri massa, energiintag och alkoholkonsumtion in i den statistiska modellen kvarstod resultatet ovan med undantag för PCB 74 och PCB 99. Ytterligare justering för total fettkonsumtion, intag av mättat fett eller kolhydrater påverkade inte nämnvärt resultatet. Det spelade inte heller någon roll om vävtvikt eller lipidnormaliserade värden användes i analyserna.

Hos män sågs ett samband mellan PCBerna 74, 99 och 138 och ökad mängd bukfett som inte sågs hos kvinnor.

8.4 Studie 4

Den fjärde studien grundar sig på samma kohort som den tredje studien. Sambandet mellan serumkoncentrationen av BPA och fetma undersöktes, och även sambandet mellan BPA och plasmakoncentrationerna av adipokininerna leptin och adiponektin och hormonet ghrelin som produceras i magssäcken.

Variabler för fettmängd var BMI, total mängd fett och visceralt fett, så kallat bukfett, mätt med DXA och MRT.

Regressionsanalys visade inget samband varken mellan serumkoncentrationen av BPA och BMI, eller mängden fett som uppmättes med DXA eller MRT. Sambandet mellan serumkoncentrationen av BPA och koncentrationen av adiponektin och leptin var positivt men negativt för ghrelin. Resultatet kvarstod när modellen justerades för kroppskonstitution, rökning, motionsvanor, utbildningsnivå, energiintag och alkoholkonsumtion.
8.5 Sammanfattning av studierna

Den första djurstudien visade att modellen med ett måttligt kaloriöverskott i form av fruktos fungerar för att studera viktregering och utvecklingen av fetma. Råttorna som exponerats för fruktos via dricksvattnet hade ökat mer i vikt, hade mer fett, högre LSI och högre koncentration av apo A-I i plasma än råttorna i kontrollgruppen. Modellen användes i den andra djurstudien och då med fruktos i kombination med BPA. Råttor som exponerats för fruktos och BPA hade ett högre LSI, mer fett i leveren och högre koncentration av apo A-I i plasma än råttorna i kontrollgruppen. I den andra studien observerades inga skillnader varken i vikt eller i fettmängd mellan exponerade djur och kontroller.

De epidemiologiska studierna visade en korrelation mellan plasma-koncentrationen av vissa POPar och fettmängd och mellan BPA och hormonnivåerna av adiponektin, leptin och ghrelin i blodet som har betydelse för hunger och mättnad. Vissa av POParna, t ex PCB 105 och DDE, visade ett positivt samband med fettmängd. Andra, t ex den högklorerade PCB 194, visade ett negativt samband med fettmängd.

Sammantaget visar studierna att det kan finnas ett samband mellan exponering för miljögifter och förändringar i de system som reglerar fettinlagring, ämnesomsättning och aptit.
9 References


Table 1: Summary of selected studies on dietary exposure to bisphenol-A.


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A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine.