Antti Koskela

BONE AS A TARGET FOR PERSISTENT ORGANIC POLLUTANTS
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Abstract
Persistent organic pollutants (POPs) are ubiquitous and bioaccumulative man-made chemicals, resistant to chemical, biological and photolytic degradation and widely distributed to sediments, wildlife, and human. Many of these chemicals have adverse effects on a variety of targets, including the endocrine system, organogenesis and reproduction. Due to these effects and wide distribution, many of them are either banned or strictly controlled. However, because of persistency, they continue to interact with organisms globally.

Despite the existing knowledge of the adverse effects of POPs, the effects of many chemicals on bone tissue are still poorly known. In the present study, we investigated the adverse effects of three common POPs, including tributyltin (TBT), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and perfluorooctanoic acid (PFOA) on the skeletal system. In vitro models were used to study the effects of PFOA in mouse and in human, and the co-effects of TBT and TCDD on differentiating osteoblasts and osteoclasts of mice. An in vivo model for mice was used to study the developmental effects of maternal PFOA-exposure on pups among with morphometrical and biomechanical property analyses. Mass-spectrometry was used to study the presence of PFOA in bones both in mice and in human, the latter acquired from the bone bank held in the Oulu University Hospital, Finland. The bones were also analyzed with cone beam computer tomography and microcomputer tomography.

The results show that PFOA exposure in utero and during lactation leads to the accumulation of PFOA in bone, traceable even 17 months after exposure. PFOA exposure decreased the mineral density of the tibias and increased the medullary area. Nearly all of the human samples contained PFAS, including PFOA. PFOA also disturbed the differentiation of osteoblasts and with lower doses, increased bone resorption of osteoclasts both in mouse and human, the phenomenon being slightly stronger in mice. Co-exposure to TBT and TCDD led to decreased differentiation of osteoblasts and osteoclast, and the co-effect was partially synergistic in osteoblasts.

These results show disruption of bone development, bone cell differentiation, and PFAS accumulation in bone. Further studies are recommended to evaluate the co-effects of different POPs and the possible effects of long-term accumulation of POPs in bone and other tissues.

Keywords: accumulation, bone remodeling, bone toxicology, dioxin, microstructure, organotin, perfluoroalkylated substance
Koskela, Antti, Luu pysyyvien organisten ympäristömyrkkyyjen kohdekudoksena.
Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta; Medical Research Center Oulu; Terveyden ja hyvinvoinnin laitos; Itä-Suomen yliopisto
Oulun yliopisto, PL 8000, 90014 Oulun yliopisto

Tiivistelmä


Väitöskirja antaa lisätietoa POP-yhdisteiden vaikutuksista luun kehitykseen ja luuosojen erilaistumiseen sekä PFAS-yhdisteiden kertymisestä luuhun. Väitöksessä myös suositellaan lisätutkimuksia yhdisteenä eisteisvaikutuksista sekä pitkän aikavälin ympäristöksiä erilaistumisesta ja muissa kudoksissa.

Asiasanat: dioksiinin, kertyminen, luon remodellointi, luotuksikologia, mikrorakenteet, organotina, perfluoratut yhdisteet
“Data! Data! Data!” he cried impatiently. "I can't make bricks without clay.”
- *Sherlock Holmes*, in *The Adventure of the Copper Beeches* by Sir Arthur Conan Doyle

*To my parents*
Acknowledgements

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Oulu, December 2016

Antti Koskela
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>β-GP</td>
<td>Beta-glycerophosphate</td>
</tr>
<tr>
<td>µCT</td>
<td>Micro-computed Tomography</td>
</tr>
<tr>
<td>AHR</td>
<td>Aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>AHRR</td>
<td>Aryl hydrocarbon receptor repressor</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ANK</td>
<td>Progressive ankyloses</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>ARNT</td>
<td>Aryl hydrocarbon receptor nuclear translocator</td>
</tr>
<tr>
<td>ATRA</td>
<td>All-trans Retinoic Acid</td>
</tr>
<tr>
<td>Bdl</td>
<td>Below detection limit</td>
</tr>
<tr>
<td>BV</td>
<td>Bone Volume</td>
</tr>
<tr>
<td>BV/TV</td>
<td>Bone Volume/Tissue Volume</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone Mineral Density</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>CBCT</td>
<td>Cone-beam Computed Tomography</td>
</tr>
<tr>
<td>Col7a1</td>
<td>Collagen Type VII Alpha 1</td>
</tr>
<tr>
<td>CSF</td>
<td>Colony-stimulating factor</td>
</tr>
<tr>
<td>CTR</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CTR</td>
<td>Calcitonin receptor</td>
</tr>
<tr>
<td>CTSK</td>
<td>Cathepsin K</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DXM</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal-regulated Kinases</td>
</tr>
<tr>
<td>ERR</td>
<td>Estrogen-related Receptor</td>
</tr>
<tr>
<td>Fmax</td>
<td>Peak Load</td>
</tr>
<tr>
<td>FN1</td>
<td>Fibronectin-1</td>
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<tr>
<td>HA</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>hBM</td>
<td>Human Bone Marrow</td>
</tr>
<tr>
<td>hPB</td>
<td>Human Peripheral Blood</td>
</tr>
<tr>
<td>ITGAM</td>
<td>Integrin Alpha M</td>
</tr>
<tr>
<td>LHR</td>
<td>Luteinizing Hormone Receptor</td>
</tr>
</tbody>
</table>
LOAEL  Lowest-adverse-effect-observed level
LOQ     Level of Quantification
M-CSF   Macrophage Colony-stimulating Factor
MC3T3   Mouse Calvarial Clonal Preosteoblastic Cells
MSC     Mesenchymal stem cell
MMP     Matrix Metalloproteinase
MSX1    Msh Homeobox
NFATc1  Nuclear Factor of Activated T Cells c1
NOAEL   No-adverse-effect-observed level
OCN     Osteocalcin
OPG     Osteoprotegerin
OPN     Osteopontin
P4      Progesterone
PCDD    Polychlorinated dibenzo-p-dioxins
PFAS    Perfluoroalkylated substances
PFCs    Perfluorinated compounds
PFOA    Perfluorooctanoic acid
PFOS    Perfluorooctane sulfonate
PND     Postnatal Day
POP     Persistent Organic Pollutant
PPAR-γ  Peroxisome proliferator-activated receptor γ
PPRE    PPAR Response Elements
PTH     Parathyroid hormone
RANKL   Receptor Activator of Nuclear Factor Kappa-B Ligand
RAR     Retinoic Acid Receptor
RARE    Retinoic Acid Response Elements
ROB     Rat Calvarial Osteoblast-like Cells
ROI     Range of Interest
RPM     Rounds Per Minute
RT      Room temperature
RXR     Retinoid X receptor
SERM    Selective Estrogen Receptor Modulator
SIBLING Small Integrin-Binding Ligand
SOX9    SRY-Box 9
TBT     Tributyltin
TCDD    2,3,7,8-Tetrachlorodibenzo-p-dioxin
TEF     Toxic Equivalency Factors (TEF).
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor Beta</td>
</tr>
<tr>
<td>TMD</td>
<td>Tissue Mineral Density</td>
</tr>
<tr>
<td>TRAP</td>
<td>Tartrate-resistant acid phosphatase</td>
</tr>
<tr>
<td>TV</td>
<td>Tissue Volume</td>
</tr>
<tr>
<td>TWIST1</td>
<td>Twist Family BHLH Transcription Factor 1</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D Receptor</td>
</tr>
<tr>
<td>VOI</td>
<td>Volume of Interest</td>
</tr>
<tr>
<td>WNT</td>
<td>Wingless and Integration 1</td>
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</tbody>
</table>
List of original publications

This thesis is based on the following publications, which are referred to throughout the text by their Roman numerals I-III:


Contents

Abstract

Tiivistelmä

Acknowledgements 9

Abbreviations 11

List of original publications 15

Contents 17

1 Introduction 19

2 Review of the literature 21

2.1 Bone ........................................................................................................ 21

2.1.1 Bone development ........................................................................ 22

2.1.2 Bone modeling and remodeling .................................................... 22

2.1.3 Factors affecting the mechanical strength of bone ....................... 25

2.1.4 Lifetime of bone ........................................................................... 26

2.2 Persistent organic pollutants (POPs) ....................................................... 29

2.2.1 Dioxins and 2,3,7,8-Tetrachlorodibenzodioxin (TCDD) .............. 30

2.2.2 Tributyltin (TBT) .......................................................................... 32

2.2.3 Perfluoroalkylated substances ...................................................... 33

2.3 Bone toxicology ...................................................................................... 34

2.3.1 AHR in bone ................................................................................. 35

2.3.2 Retinoid receptors and PPARs in bone ......................................... 36

2.3.3 Effects on steroid hormones ......................................................... 37

2.3.4 Accumulation of xenobiotics in bone ........................................... 38

2.3.5 Analytics and biological end-points ............................................. 39

3 Aims of the study 41

4 Materials and methods 43

4.1 Chemicals (I, II, III) ............................................................................. 43

4.2 Study permissions and patients ............................................................... 43

4.3 Cell culture (I, II, III) ........................................................................... 44

4.3.1 C57BL mouse cells .................................................................. 45

4.3.2 MC3T3-E1 cells .................................................................... 46

4.3.3 Human bone marrow cells and peripheral blood samples ........ 46

4.4 qPCR studies (I, II) ............................................................................. 47

4.5 Quantification of osteoclast differentiation (I, II, III) ....................... 47

4.6 Mass-spectrometry (II, III) ................................................................. 48

4.6.1 Preparation of the samples........................................................... 48
4.6.2 LC-MS/MS Analysis ................................................................. 49
4.7 Microtomography (µCT; II, III) .................................................... 50
4.8 Whole bone biomechanical testing (II) ........................................... 51
4.9 Cone-beam computed tomography (III) ........................................... 52
4.10 Benchmark Dose Modeling (III) .................................................. 52
4.11 Statistical analyses .................................................................. 52
5 Results ...................................................................................... 53
5.1 Exposure effects in in vitro experiments ........................................ 53
  5.1.1 The co-effects of TBT and TCDD ........................................... 53
  5.1.2 The effects of PFOA exposure on differentiating bone cells .......... 55
  5.1.3 Benchmark Dose Calculations ............................................... 55
5.2 Developmental PFOA exposure in vivo experiments ..................... 56
  5.2.1 Body weight ........................................................................ 56
  5.2.2 Morphometric and biomechanical results ................................ 56
5.3 Mass-spectrometry studies .......................................................... 57
  5.3.1 Mouse femurs and tibias ...................................................... 57
  5.3.2 Human bone bank samples ................................................... 57
  5.3.3 Human cadaver tissue samples ............................................ 57
6 Discussion .................................................................................. 59
6.1 TBT affects bone cell differentiation ............................................. 59
6.2 TBT and TCDD show synergistic effects on differentiating bone cells .... 59
  6.3 Maternal exposure leads to PFOA accumulation in bone in mice ....... 61
6.4 PFAS-levels in human bone samples ............................................ 62
6.5 PFOA exposure affects bone in vivo and in vitro ........................... 62
6.6 PFOA's bone toxicity – mice vs. human ...................................... 64
6.7 Future aspects for bone toxicology studies ................................... 65
7 Conclusions ............................................................................... 67
References .................................................................................. 69
Original publications ...................................................................... 83
1 Introduction

During recent years, bone has been granted a more major role in human physiology, in addition to serving as a passive supporting structure and insertion site for skeletal muscles. In addition to the traditional aspects of bone functions, which include acting as a storage site for minerals and growth factors, recent studies have indicated an important role for bone, e.g. as a hormonal organ and in cross-talk with testosterone-producing Leydig cells of the testis, thus affecting male fertility (Ferlin et al. 2013, Karsenty & Oury 2014).

As new studies reveal the complex role of bone tissue in human physiology, an awareness of the presence of many persistent organic pollutants (POPs) in the environment, wildlife, and humans has also increased substantially. This has led to restrictions or prohibition of the use of certain of these hazardous environmental chemicals. Nevertheless, the problem persists especially in the developing countries, and currently many organisms have their own, individual mixture of different environmental chemicals.

Bone toxicology studies the effects of different environmental chemicals on the skeleton using a wide variety of methods, such as those based on computed tomography, biomechanical testing on long bones, in-vitro culture of bone resorbing osteoclasts and bone forming osteoblasts, and then assessment of multiple responses to exposure by microscopy quantification and gene analysis tools. Bone toxicology studies began in Finland at the end of the 1990s based on initial studies by dental researchers. Later collaboration of bone researchers and toxicologists was expanded from teeth to long bone studies (Alaluusua et al. 1999).

Despite many efforts made during the 21st century, numerous research questions still need to be answered. This project opens a field for studying the effects of co-exposure to different environmental chemicals in bone and also shows how bone acts as a reservoir for POPs and how perfluoroalkylated substances affect bone.
2 Review of the literature

2.1 Bone

The human skeleton in the adult is formed by over two hundred individual bones (Gray 1918). Bone is a heterogeneous composite material that consists 60-70% of a mineral part formed by hydroxyapatite (HA) and an organic part that consists of approximately of 90% collagen I, 5% non-collagenous proteins, 2% lipids by weight, and water, the relative proportions of which vary with age, health and gender (Boskey 2013). The anatomical structure of a typical long bone is presented in Fig. 1. In addition to providing mechanical support, cover to soft tissues, and offering lever sites for skeletal muscle action, bone tissue has a major role in maintaining blood calcium levels and supporting hematopoiesis throughout the life of an individual (Harada & Rodan 2003). To maintain its capability in these multiple functions, bone undergoes constant remodeling where bone units are replaced by the coupled, strictly-controlled action of bone-resorbing osteoclasts and bone forming osteoblasts in accordance with the feedback of mechanical loading and ion-homeostasis (Rodan 1997). Failure in this resorbing-forming cycle can lead to various bone diseases, such as osteoporosis or osteopetrosis (Yasuda 2013).

![Fig. 1. Typical structure of a long bone. The figure is a microCT-derived 3D-reconstruction of a mouse femur. Compact bone is surrounded by a periosteum, and encloses an endosteum.](image_url)
2.1.1 Bone development

There are two distinguishable types of bone development, intramembranous ossification in the flat bones of the skull (the calvaria, the maxilla, and the palate) and in the outer surfaces of long bones, and endochondral ossification in most other bones of the skeleton, including the axial and appendicular skeletal elements and the base of the skull (Yang 2009). In the latter, an initial cartilage template formed by chondrocytes is converted into bone by locally recruited osteoclasts and osteoblasts, while chondrocytes undergo hypertrophy, calcification and apoptosis. In the end, all the remaining cartilage except that of articular surfaces, is replaced by bone.

Intramembranous ossification lacks the cartilage template formation, and mesenchymal precursor cells from the neural crest and paraxial mesoderm form a sheet-like structure, from which they differentiate directly into bone-forming osteoblasts (Erlebacher et al. 1995, Salazar et al. 2016). Chondrocytes express specific molecular markers, such as aggrecan and α1(II) collagen, which distinguishes them from those that reside at the perichondrium. After the formation of cartilaginous templates, the innermost chondrocytes further differentiate into hypertrophic chondrocytes, which then become surrounded by calcified extracellular matrix (ECM) that then drives the chondrocytes into an apoptotic pathway. Cartilaginous ECM containing type X collagen is initially replaced by bone ECM, rich in type I collagen, and initially osteoblasts replace chondrocytes and cartilage with bone (Karsenty & Wagner 2002).

2.1.2 Bone modeling and remodeling

Already in 1965 Hattner, Epker and Frost established the foundation for the bone homeostasis hypothesis. Their study showed that approximately 3% of adult bone is formed directly over the underlying old bone (modeling), and that 97% of bone is formed after the resorption activity of osteoclasts (remodeling) (Hattner et al. 1965). The modeling ensures the correct shaping of a growing bone and tends to decline with age. From a clinical perspective, it is also likely to offer an explanation for the observed increase in bone mineral density in osteoporotic patients after continuing use of osteoporotic drugs, such as teriparatide, when the osteoclast-action and their coupled action with the osteoblasts is damped (Teti 2011).

In remodeling, the resorption-formation cycle starts from the action of highly specialized, multinuclear osteoclasts, which resorb a certain amount of bone on the
surface of trabeculae, or dig cone-shaped tunnels around Haversian canals in cortical bone. This resorption cycle lasts about three weeks and is followed by bone formation by osteoblasts lasting over several months (Rodan 1997).

Osteoclasts differentiate from bone marrow monocyte/macrophage lineage cells, as multiple cells fuse together to form multinuclear osteoclasts. The precursor cells are affected by macrophage colony-stimulating factor (M-CSF), which enhances the proliferation and survival of osteoclast precursor cells. The receptor activator of nuclear factor-κB ligand (RANKL) secreted by osteoblasts stimulates this process and, furthermore, stimulates the differentiation, resorption activity and also the survival of mature osteoclasts (Kim & Kim 2016). Due to their common origin with different immune cells and the role osteoclasts have as phagocytic cells in the bone and as immunomodulators in the pathogenesis of bone loss, they can also be considered as immune cells (Sharaf-Eldin et al. 2016). In addition, mature functional immune cells have been found residing in the bone marrow, and a feedback regulation between osteoclasts and activated T-cells via T-cell-derived IFN-γ and CD40-ligand (CD40L) has quite recently been found (Li et al. 2014).

Osteoprotegerin (OPG), a member of the TNF receptor superfamily, is also secreted by osteoblasts in response to anabolic agents, such as estrogens and BMPs. OPG acts as an inhibitor for osteoclast differentiation by disrupting the interaction between RANKL and RANK by blocking RANKL from binding to its receptor, thus inhibiting bone resorption (Simonet et al. 1997, Udagawa et al. 2000).

Mature osteoclasts typically have a sealing zone and a ruffled border, which is formed when intracellular vesicles containing the enzymes and transmembrane proteins fuse. The sealing zone attaches the osteoclast to the extracellular bone matrix, especially to osteopontin (OPN) and bone sialoprotein, where it forms a tight bond towards the resorption compartment that allows the osteoclast to generate lysosomes with low pH and proteases (Teitelbaum 2006). The ruffled border can be divided further into the outer “fusion zone”, which allows vesicular fusion to insert ion transporters into the ruffled border and discharge lysosomal enzymes, and the inner “uptake zone”, which takes up the degradation products from the resorption area (Cappariello et al. 2014). Resorption is achieved, as protons are released via V-H+-ATPase along with the 2Cl⁻/1H⁺ antiporter (Supanchart & Kornak 2008). Secretion continues as the lytic enzymes TRAP (tartrate-resistant acid phosphatase) and pro-CATK (pro-cathepsin K) are exported into the resorption pit, and the degradation products, mainly collagen fragments, calcium and phosphate, are processed inside the osteoclast and released into the
circulation. Mature osteoclasts typically express TRAP, CTR (calcitonin receptor) and \( \beta \)-integrin (Boyle et al. 2003).

Bone resorption is then followed by bone formation by osteoblasts, originally differentiated from mesenchymal stem cells (MSCs). Multifunctional signaling cytokines and bone morphogenetic proteins (BMPs) have a major role in the process. BMPs belong to a large growth factor-beta (TGF-\( \beta \)) family, and have a role in every step in bone formation from differentiation of osteoblasts to bone repair, as well as a major role in organ development (Rahman et al. 2015). Many BMPs in knockout animal models have been studied, and the effects vary from BMP6’s minor defects to BMP7’s multiple skeletal, kidney and eye defects, as reviewed by Zhao and colleagues (2003). There is also variability in the localization of BMPs. For example, BMP2 is expressed in periosteal and osteogenic zones and near cartilage, while BMP4 is expressed in the perichondrium (Fakhry et al. 2013). Interestingly, the overexpression of TRAP, mostly expressed by osteoclasts, has been shown to induce the differentiation and activation of osteoblasts (Gradin et al. 2012).

The other important regulator family for osteoblasts is the Wnt-family (Wingless and integration 1), of which many members induce canonical pathways. Wnt10b may be particularly important for bone development, as it is expressed in the bone marrow in osteoblast progenitors, and its overexpression has been shown to lead to increased bone and accelerated osteoblastogenesis in vitro, whereas the lack of it induces the loss of trabecular bone (Bennett et al. 2005, Zhong et al. 2012). In addition, Movérare-Skrtic and colleagues (2014) showed that Wnt16-deficient mice developed spontaneous fractures due to low cortical thickness and high cortical porosity, underscoring the importance of Wnt16. Osteoblast-derived Wnt16 also inhibited human and mouse osteoclastogenesis by acting on osteoclast progenitors and increasing the expression of OPG.

As mature osteoblasts start to form new bone, collagen type I along with non-collagenous proteins, such as proteoglycans, tissue nonspecific alkaline phosphatase (ALP), small integrin-binding ligand (SIBLING) proteins, Glα-containing proteins, such as matrix Glα protein and osteocalcin (OCN), and lipids, are secreted to form the initial osteoid, which is then finished as hydroxyapatite is incorporated into the new deposit (Raggatt & Partridge 2010). ECM mineralization occurs only if osteoblasts express both collagen type I along with ALP, but nucleotide pyrophosphatase phosphodiesterase, and ANK (progressive ankylosis) are also involved in optimizing the ECM concentration of inorganic phosphate,
which allows the mineralization phase to proceed (Harmey et al. 2004, Murshed et al. 2005).

Initially, osteoblasts either become lining cells on the bone surface, undergo apoptosis, or are trapped inside their secreted ECM and form an osteoid, becoming osteocytes (Prideaux et al. 2016). During further maturation of the osteocytes, the osteoid is mineralized and further embedded within the mineralized bone matrix (Franz-Odendaal et al. 2006). Mature osteocytes are interconnected via long cell processes, which reside in channels in the bone matrix, which allows them to pass nutrients and oxygen, but also signalling molecules to each other (Bonewald 2007). After becoming mature osteocytes, the cells are not passive, but rather have an active role in bone remodeling. They produce many inhibitors of the WNT-family, such as sclerostin, which is a negative regulator of bone formation by inhibiting Wnt-receptor interaction, which in turn leads to decreased osteoblast differentiation (Prideaux et al. 2016). Mechanical stimulation decreases sclerostin expression, which makes it possible to induce bone formation in sites with increased mechanical loading (mechanosensing) (Robling et al. 2008). Sclerostin has also become an interesting target for osteoporosis treatment, and anti-sclerostin romosozumab has shown potential for increasing bone formation and bone mineral density (BMD) in a phase II study (McClung et al. 2014).

### 2.1.3 Factors affecting the mechanical strength of bone

To provide optimal fracture resistance, bone must be stiff enough to withstand deformation when loaded, and simultaneously be sufficiently flexible to absorb energy during impact loading (Currey 2001). The forces applied to the skeleton are typically a combination of compression or tension with bending and torsional loading, among which the resistance to bending and torsional loading are particularly important, as in the appendicular skeleton the highest stresses are caused by these loading modes (Bouxsein 2005). An important factor affecting the mechanical strength of bone is bone’s capability to constantly adapt to environmental changes and forces through the bone remodeling discussed earlier, which causes changes in bone size and shape. Many diseases can cause disturbances in this balance and thus either increase the fracture risk or cause other problems as bone turnover is positive.

The biomechanical properties can be divided into intrinsic (material) and extrinsic (structural) properties, where the former is the sum of the size, bone mass, shape and macrostructure of the bone, whereas in the latter bone composition,
microstructure and microdamage are the main factors (Forestier-Zhang & Bishop 2016). The hierarchical structure of the bone from the molecular to the macroscopic level and the individual and joint effects of these steps impact the biomechanical endurance of bone (Bouxsein 2005).

Collagen I is stiffened by calcium hydroxyapatite, and an increase in tissue mineral density increases the stiffness but decreases flexibility. The composition and the amount of collagen cross-linking also influence function, as the triple helix of collagen I enhances the strength in tension, and the cross-links in collagen stabilize the helices. Again, too few cross-links lead to separation of the helices, while too many decrease the energy absorption ability (Seeman & Delmas 2006).

When considering the macroscopic features of bone, trabecular bone is thought to sustain compressive load along its main trabecular orientation, and is shown to be much weaker in response to shearing forces (Sanyal et al. 2012). The main determinants in trabecular bone sustainability are the density and the microstructural arrangement of the trabecular network (Keaveny et al. 2001). In cortical bone, the thickness of the cortex, cortical cross-sectional area and moment of inertia along with the microstructural properties, such as porosity, are the main factors affecting the strength of cortical bone. Cortical bone is loaded mainly by bending moments causing tensile strain in which the orientation of collagen fibrils have an important role. Thus in long bone, cortical bone couples with trabecular bone to endure mechanical loading in many directions (Augat & Schorlemmer 2006).

Bone mineral density (BMD) has been shown to be a good indicator of bone strength, and decreased BMD correlates to increased fracture risk in the hip and vertebrae (Kanis 2002). BMD is also genetically influenced, as the BMD of young adults relates well to BMD measurements of their parents (Jouanny et al. 1995). In genome-wide association (GWAS) studies, WNT-polymorphism, especially WNT5B, has been linked to peak BMD and body composition; macroautophagy-related genes have also been linked to BMD (Zhang et al. 2010, Zheng et al. 2016).

### 2.1.4 Lifetime of bone

Nutritional status, especially sufficient vitamin D intake, physical activity and early onset of walking in childhood are associated with enhanced bone strength, which contributes to an individual’s adulthood bone health (Pekkinen et al. 2012, Ireland et al. 2014). The peak bone mass is reached in the mid-twenties, and is maintained at a steady-state until approximately 50 years of age, after which bone turnover
turns negative and leads in postmenopausal women and elderly men to bone loss, osteopenia, and osteoporosis. This imbalance is the main reason for most bone diseases with low bone mass (Seeman & Delmas 2006, Weaver et al. 2016).

Vitamin D is essential in bone homeostasis from early childhood throughout life. Its active form, 1,25-dihydroxyvitamin D \([1,25(OH)_{2}D]\), functions through a nuclear transcription factor, the vitamin D receptor (VDR) (Brumbaugh & Haussler 1975). Among multiple targets, vitamin D increases intestinal calcium absorption and the production of fibroblast growth factor-23 (FGF23) in osteoblasts and osteocytes. FGF23 might act as a counter-regulatory hormone to the active form of vitamin D in the case where PTH is suppressed by vitamin D, and thus maintains the phosphate balance (Liu et al. 2006). The phosphate balance is crucial to bone homeostasis in children, as a failure in adequate vitamin D consumption can lead to hypophosphatemia, and through that to hypertrophic chondrocytes in the growth plate and initially, to rickets (Tiosano & Hochberg 2009). Overall, the action of vitamin D in bone varies, as 1, 25-dihydroxyvitamin D directly inhibits osteoblastic differentiation, but in fully matured osteoblasts, it stimulates the formation of mineralized matrix and possibly inhibits the functioning of bone-resorbing osteoclasts through specific pathways in osteoblasts and osteocytes (Gardiner et al. 2000).

Primary osteoporosis typically occurs in postmenopausal women and elderly men due to estrogen or calcium deficiency, and/or aging-related processes, such as oxidative stress and apoptotic mechanisms especially in the longest-lived bone cells, osteocytes. This leads eventually to filling of the canaliculae with mineralized connective tissue, and macroautophagy (Hendrickx et al. 2015, Rivadeneira & Makitie 2016). Bone mass is continuously lost and the skeletal microarchitecture deteriorates as a result of accelerated osteoclastogenesis and bone resorption, which increases the risk of bone fractures even with low-energy traumas. Osteoporotic fractures are linked with increased morbidity and mortality, which makes it a significant public health issue (Henriksen et al. 2011, Duong et al. 2016). The overall number of cells expressing RANKL has been shown to be increased in postmenopausal women compared with premenopausal or estrogen-treated women, which indicates that the changed bone marrow microenvironment favors osteoclastogenesis in osteoporosis. In addition, the bone marrow cells isolated from estrogen-treated women have been shown to have decreased differentiation-potential towards osteoclastogenesis (Eghbali-Fatourechi et al. 2003, Clowes et al. 2009).Interestingly, also the number of osteoblasts is increased, probably at least in part due to the coupling of bone formation to bone resorption, but the bone
formation rate is not sufficient to restore the bone loss (Han et al. 1997). In men, low androgen levels are associated with increased bone remodeling and bone loss, but also decreased aromatization of testosterone causing reduced levels of estrogen also has a role (Katznelson et al. 1996). The main drugs used to treat osteoporosis are categorized as inhibitors of bone resorption, such as bisphosphonates, the RANK-ligand antagonist denosumab, stimulators of bone formation, such as parathyroid hormone –recombinant teriparatide, and selective estrogen receptor modulators (Duong et al. 2016). Together with possible pharmaceutical therapy, strength and balance training are very effective for preventing fractures in older women, as shown in a randomized clinical trial conducted in Finland by Uusi-Rasi and colleagues (2015).

As discussed earlier, estrogen has a major role in bone homeostasis and decreasing estrogen levels with bone loss, both in trabecular and cortical bone, are strongly connected (Manolagas et al. 2013). There are two estrogen receptors, ER-α and ER-β, and androgens bind to the androgen receptor (AR). The receptors not only serve as receptors for their ligands, but also function in the absence of the ligands and regulate the production of their ligands (Beato & Klug 2000). Another member of the nuclear hormone receptor superfamily, estrogen-related receptor (ERR)-α, has no known ligand and might have a role in influencing early osteoblastogenesis by favoring the differentiation of osteoblasts at the expense of adipocytes, but also the opposite has been proposed, as reviewed by Gallet and Vanacker (2010).

The effects of estrogen and estrogen receptor activation on bone have been studied in various mouse models. In female mice lacking ER-α, the loss of trabecular bone was observed while the trabecular bone in males was intact. The same study also showed the induction of apoptosis and upregulation of Fas ligand (FasL) expression in wild type osteoclasts by estrogen. However, the reducing effect seen in trabecular bone in mice lacking ER-α was not seen in cortical bone (Martin-Millan et al. 2010). In mature osteoblasts and osteocytes, the lack of ER-α increases the apoptosis of both types of bone cells, but in various mouse models, bone mass in total did not change, which suggests that the induction of apoptosis alone cannot cause bone loss and that the effect of estrogen on osteoclast differentiation and action is the main mechanism in bone sparing (Manolagas et al. 2013). Furthermore, it has been found that trabecular bone formation is regulated by ER-α in osteocytes in male mice, and by ER-α in osteoclasts in female mice (Windahl et al. 2013). Interestingly, osteopontin-deficient mice have been shown to be resistant to ovariectomy-induced bone resorption, so OPN, which is an
important regulator for bone cell attachment to bone surface, probably has an essential role in bone loss caused by estrogen depletion (Yoshitake et al. 1999).

2.2 Persistent organic pollutants (POPs)

Persistent organic pollutant (POP) is a general status given to certain stable environmental chemicals that are resistant to degradation and ubiquitous in soils, sediments, air, wildlife and humans. They are typically lipophilic, and easily become stored in fat tissue and bioaccumulate in the food chain, and many also have the ability to enter the gas phase and thus travel long distances in air (Jones & de Voogt 1999). The United Nations Environment Program (UNEP) initially listed 12 POPs, “the dirty dozen”, by the Stockholm Convention in 2001, that are considered of immediate concern (Table 2).

Table 2. The 12 initial POPs under the Stockholm Convention (http://chm.pops.int).

<table>
<thead>
<tr>
<th>Chemical Category</th>
<th>Chemical</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldrin</td>
<td>Pesticide</td>
<td></td>
</tr>
<tr>
<td>Chlordane</td>
<td>Pesticide</td>
<td></td>
</tr>
<tr>
<td>Dichlorodiphenyltrichloroethane (DDT)</td>
<td>Pesticide</td>
<td></td>
</tr>
<tr>
<td>Dieldrin</td>
<td>Pesticide</td>
<td></td>
</tr>
<tr>
<td>Endrin</td>
<td>Pesticide</td>
<td></td>
</tr>
<tr>
<td>Heptachlor</td>
<td>Pesticide</td>
<td></td>
</tr>
<tr>
<td>Hexachlorobenzene</td>
<td>Pesticide; Industrial chemical; By-product</td>
<td></td>
</tr>
<tr>
<td>Mirex</td>
<td>Pesticide</td>
<td></td>
</tr>
<tr>
<td>Topaphene</td>
<td>Pesticide</td>
<td></td>
</tr>
<tr>
<td>Polychlorinated biphenyls (PCBs)</td>
<td>Industrial chemical; By-product</td>
<td></td>
</tr>
<tr>
<td>Polychlorinated dibenzo-p-dioxins (PCDDs)</td>
<td>By-product</td>
<td></td>
</tr>
<tr>
<td>Polychlorinated dibenzofurans (PCDFs)</td>
<td>By-product</td>
<td></td>
</tr>
</tbody>
</table>

Since the original listing, new POPs have been added in addition to the original twelve and the list has been organized into annex A (elimination), B (Restriction), and C (Unintentional production). Due to the vast amount of man-made environmental chemicals, not all adverse effects-causing chemicals are on the list. The list is being updated regularly (Stockholm Convention on Persistent Organic Contaminants). This thesis focuses on the environmental chemicals 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), tributyltin (TBT) and perfluorooctanoic acid (PFOA), and the chemical structures of these POPs are presented in Fig. 2.
2.2.1 Dioxins and 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)

Polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyls (PCBs) are jointly called dioxin-like compounds. They have similar chemical structures and toxic effects, but the toxic potency varies according to toxic equivalency factors (TEF). They are lipophilic and have very low water solubility, so they easily enter the food chain and accumulate in fatty tissues (Fernandez-Gonzalez et al. 2015). Global temporal and spatial contamination of PCDDs, PCDFs and PCBs in human milk, a core source for biomonitoring of dioxins, have recently been reviewed by van den Berg and colleagues (2016). The highest levels of PCDDs and PCDFs are mostly associated with industrialization through combustion and industrial processes, but also some less-industrialized countries from Asia, Africa and South-America had similar high levels, whereas PCBs in the same countries were low. In addition, according to the same authors’ risk-benefit assessment, the benefits of breastfeeding outweigh the toxicological disadvantages of possible POP exposure.

Dioxins are not produced for industrial use, but rather are unwanted and often unavoidable by-products of numerous activities, such as incomplete combustion processes, pesticide production and the metallurgical industry (EPA 2004). They are widely spread in air, water, soil, and sediments (Birch et al. 2007, Chi et al. 2010, Friedman et al. 2012), and a major source for human exposure is proposed to be fish in the Nordic countries and milk and meat products in Central Europe (Shin et al. 2016). In Finland, the Baltic Sea as an environment has been identified as a concerning source for dioxins (Peltonen et al. 2014). Species-dependent variability of toxic effects among dioxins is also of importance (Fernandez-Gonzalez et al. 2015).
Of the wide dioxin-family, TCDD is the most potent model compound, often termed as the most toxic man-made chemical with a TEF-value of 1.0. Internationally agreed TEF-values indicate the potency of all dioxin-like congeners in relation with TCDD (Van den Berg et al. 1998). Because dioxin-like compounds typically exist as complex mixtures with a variable congener profile, the concentrations are made comparable by presenting them as toxic equivalents (TEQ). The joint effect of dioxin-like compounds follows additivity and therefore TEQ of a mixture is the sum of the concentrations of each congener multiplied by its individual TEF.

TCDD is ubiquitously present, persistent, lipophilic and has therefore the potential to accumulate in the food chain. It has been shown to cause a wide variety of adverse toxic effects. Most of the data are derived from animal experiments, but some epidemiological exposure studies on accidentally and occupationally exposed cohorts have been conducted. TCDD is a known human carcinogen as stated by the National Toxicology Program (NTP) and the International Agency for Research on Cancer (IARC 1997). An old study by Kociba and colleagues (1978) showed, that a 2-year dietary exposure of rats to TCDD increased the incidence of hepatocellular carcinomas and squamous cell carcinomas of the lung, but also reduced the risk for developing tumors in certain organs, such as in the uterus and mammary glands. The odds ratio of soft tissue sarcoma was below 1 in humans exposed to background concentrations of dioxins (Tuomisto et al. 2004). However, a critical review of the multiple cohorts showed controversial evidence of cancer risk with TCDD in US herbicide producers, the Seveso accident cohort and among Vietnam veterans (Boffetta et al. 2011). Other toxic effects of TCDD exposure include suppression of both humoral and cell-mediated immunity (Kerkvliet 1995, Kerkvliet 2002), abnormalities of tooth development in rodents (Alaluusua et al. 1993, Kattainen et al. 2001) as well as in humans after the Seveso accident (Alaluusua et al. 2004), and a variety of other types of developmental toxicity (Birnbaum & Tuomisto 2000).

Toxic and biochemical effects of TCDD and other dioxins are mostly mediated by AHR, which is capable of binding hundreds of different xenobiotics, but also endogenous ligands, such as 6-formylindolo[3,2-b]carbazole (FICZ), which has been shown to have a very high-affinity to AHR. The conservation of AHR during evolution and its presence in most cell types might indicate an important role in a wide variety of functions, e.g. fertility, immunity and hematopoiesis (Okey 2007, Wincent et al. 2012). It is also notable, that not all ligands evoke toxicological pathways, and the main reasons for this have been thought to be the differences in
AHR-affinity, the persistency of dioxin-like compounds and the involvement of AHR-independent pathways (Denison et al. 2011). In the absence of a ligand, AHR resides in the cytosol and complexes with certain chaperone proteins. To be released from the chaperones after ligand binding, AHR has to go through dimerization with the AHR nuclear translocator (ARNT, also known as hypoxia-inducible factor 1β, HIF1β). The AHR-ARNT complex then binds to the proper regulatory regions of DNA and activates the target genes (Gu et al. 2000, Okey 2007).

Perhaps the most visible manifestation of dioxin intoxication at high dose exposure is chloracne, where acne-like eruption of comedones, cysts and pustules occur as a response to a non-inflammatory alteration of keratinization in the pilosebaceous units, observed in humans, rabbits, monkeys, and in nude mice (Saurat & Sorg 2010, Saurat et al. 2012). The onset is usually in the face after a delay of 2-4 weeks from initial contact with high doses of dioxins. Non-infectious abscesses may occur and later form scars. The patients are otherwise in good health. Improvement of the skin condition occurs slowly, usually around 2-3 years, though in some individuals the condition has been visible even 15 years after the initial exposure. The specific cellular and molecular mechanisms in chloracne are still unknown, although aryl hydrocarbon receptor (AHR) activation rather than downregulation has been proposed (Panteleyev & Bickers 2006, Forrester et al. 2014).

2.2.2 Tributyltin (TBT)

Tributyltin, the most toxic substance in the organotin family, is a widespread chemical, and due to its usage as an antifouling agent in ships, boats and aquaculture nets, it is a common contaminant in marine and freshwater environments. Its first toxicological effects were observed in the 1970s, as the number of marine molluscs decreased in the coastal areas, and female gastropods showed signs of an imposex effect, where male sex characteristics occurred in female gastropods (Antizar-Ladislao 2008). Eventually, the effect was linked to TBT and increased testosterone levels in the animals (Smith 1981).

Due to the high presence in the marine environment, TBT toxicology has been studied extensively in marine species, but in mammals, TBT has been shown to affect the immune system by disrupting cell mobility, phagocytosis and lysosomal enzyme activity, as well as to disrupt embryonal development, alter neuronal

In water, TBT is slowly released from paint, and typically adheres quickly to bed sediments because of its high specific gravity and low solubility (Konstantinou & Albanis 2004, Landmeyer et al. 2004). Because of its persistence, bioaccumulation potential in the food chain and high toxic potency, TBT has been legally restricted in most developed countries since the 1980s and early 1990s (European Commission 2003). Nevertheless, due to high prevailing concentrations in sediments around the world and slow degradation rate under anaerobic conditions, there is still a chance of human exposure, as the estimation for persistence is tens of decades (Gadd 2000, EFSA 2004). Another issue is the continuing usage of organotins in material and wood preservatives.

Organotin toxicity is strongly related in interactions with nuclear hormone receptor pathways, as TBT has a high affinity to both the retinoid X receptor (RXR) and the peroxisome proliferator-activated receptor \( \gamma \) (PPAR-\( \gamma \)), and it is also an inhibitor of the cytochrome P450 (CYP) enzyme aromatase, which is responsible for converting testosterone to estradiol (Grun & Blumberg 2006, le Maire et al. 2009).

### 2.2.3 Perfluoroalkylated substances

Perfluoroalkylated substances (PFAS) are fluorinated substances, which are amphiphilic, which means that one part of the structure is lipophilic and other hydrophilic. The most common substances are perfluorooctanoic acid (PFOA) and perfluorooctanoic sulfonic acid (PFOS). In the past, they have been widely used in a variety of industries, including surfactant and polymer industries, where they are used in fire-extinguishing foams and as textile stain repellents, because of their surface tension-lowering properties (EFSA 2008, Buck et al. 2011). They are widely distributed in the environment, wildlife and humans, and according to certain reports, extensive use of PFOA and PFOS, for example, in fire-fighting foams has resulted in local contamination of ground water supplies in Germany and Sweden (Weiss O et al. 2012).

Toxicological effects of PFOA include the induction of neonatal mortality, targeting especially the lungs, but also reproductive toxicity, liver enlargement and spleen atrophy have been reported, as reviewed by Post and colleagues (2012). Due to the wide range of toxicological effects and ubiquitous distribution, PFOA and PFOS have been added to the Annex B of the Stockholm Convention on Persistent
Organic Contaminants, and in 2013, the European Chemical Agency (EHCA) proposed classification of PFOA as a substance of very high concern (SVHC) (ECHA 2013).

In a manner similar to TBT, PFOA is also an agonist of nuclear receptors, as it binds to the PPAR receptor family, especially PPAR-α, but also partially PPAR-γ and PPAR-β/δ (Maloney & Waxman 1999, Vanden Heuvel et al. 2006, Takacs & Abbott 2007). These nuclear receptors have been shown to contribute among other targets to lipid metabolism, energy homeostasis and cell differentiation. PPAR-γ is involved more in regulation of energy storage, whereas PPAR-α and PPAR-δ are involved in energy expenditure (Evans et al. 2004, Poulsen et al. 2012).

### 2.3 Bone toxicology

This chapter reviews first the current knowledge of the signaling routes of TCDD, TBT, and PFASs in bone, and then focuses on the endocrinological effects of the selected POPs and how they alter bone homeostasis indirectly. Finally, bone as an active storage for POPs is reviewed and considered. Some known human tissue concentrations of the three POPs are presented in Table 3.

#### Table 3. Concentrations of TBT, TCDD, and PFOA in various healthy human populations in different tissues, shown in mean (min-max). The units are µg/l for TBT and PFOA, and pg/g for TCDD.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Adipose tissue</th>
<th>Blood</th>
<th>Breast milk</th>
<th>n</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBT</td>
<td>USA</td>
<td>8.18 (&lt;1-85)</td>
<td>-</td>
<td>22</td>
<td>(Kannan et al. 1999)</td>
</tr>
<tr>
<td></td>
<td>Finland</td>
<td>&lt;LOQ</td>
<td>-</td>
<td>300</td>
<td>(Rantakokko et al. 2008)</td>
</tr>
<tr>
<td>TCDD</td>
<td>Germany</td>
<td>7.2 (1.5-18)</td>
<td>3.6 (0.9-9.7)</td>
<td>112</td>
<td>(Beck et al. 1994)</td>
</tr>
<tr>
<td></td>
<td>Japan</td>
<td>0.51 (0.1-2.0)</td>
<td>-</td>
<td>10</td>
<td>(Choi et al. 2003)</td>
</tr>
<tr>
<td></td>
<td>Sweden</td>
<td>&lt;0.05 (&lt;0.04-0.06)</td>
<td>-</td>
<td>9</td>
<td>(Ericson Jogsten et al. 2010)</td>
</tr>
<tr>
<td></td>
<td>Ghana</td>
<td>0.66-1.31</td>
<td>-</td>
<td>42</td>
<td>(Wittsiepe et al. 2015)</td>
</tr>
<tr>
<td></td>
<td>Vietnam</td>
<td>-</td>
<td>0.2 (0.12-0.25)</td>
<td>25</td>
<td>(Tue et al. 2014)</td>
</tr>
<tr>
<td>PFOA</td>
<td>Germany</td>
<td>(0.7-8.7)</td>
<td>(&lt;0.15-0.25)</td>
<td>47</td>
<td>(Fromme et al. 2010)</td>
</tr>
<tr>
<td></td>
<td>Spain</td>
<td>-</td>
<td>0.15 (0.021-0.9)</td>
<td>20</td>
<td>(Llorca et al. 2010)</td>
</tr>
</tbody>
</table>

LOQ = Level of Quantification
2.3.1 AHR in bone

The presence of AHR in bone has been known since the first studies of the effects of TCDDs on bone were published by Jämsä and colleagues (2001). In the study, TCDD was shown to inhibit the tibial growth of two rat strains and reduce the biomechanical strength of the tibia, especially in the more dioxin-sensitive Long-Evans rat strain. In addition, maternal exposure to TCDD has been shown to alter bone development of the offspring, including decreased bone length, cross-sectional area of the cortex, and bone mineral density, and also weakened biomechanical properties of the long bones (Miettinen et al. 2005). In the same year, rat calvarial osteoblast-like cells (ROB cells) and mouse calvarial clonal preosteoblastic cells (MC3T3-E1) were shown to express mRNAs for both AHR and ARNT (Naruse et al. 2002, Ilvesaro et al. 2005).

Multiple studies on the effects of AHR agonists in vitro have been conducted. Differentiating osteoblasts and osteoclasts have been shown to be very sensitive targets of dioxin toxicity, as TCDD even at very low doses has the ability to interfere in every distinct temporal period during the differentiation of osteoblasts via AHR signaling, and also to reduce the resorption capability of mouse and rat osteoclasts (Korkalainen et al. 2009). TCDD-exposure has been also shown to cause hardened bone matrix, thinner and more porous cortical bone, and more compact trabecular bone in wild type mouse, while in the AHR knockout animals, the manifestations have been shown to be much milder. These effects are at least partly more visible in female mice (Herlin et al. 2013, Wejheden et al. 2010). In utero and lactational exposure to TCDD has been shown to delay bone matrix maturation in rats (Finnilä et al. 2010). In rheumatoid arthritis, high expression of AHR has been shown to correspond with the degree of bone erosion in arthritic mice, as well as bone loss and inhibition of osteoblast proliferation and differentiation. The ERK (extracellular signal-regulated kinases) pathway probably has a major role in dioxin-induced bone toxicology (Yu et al. 2014).

In a study by Herlin and colleagues, AHR knockout mice showed alteration of both cortical and trabecular bone tissue properties, which shows that AHR is probably involved in normal bone development. Other AHR ligands, such as a quercetin analogue from a medicinal plant, Himalayan elm, have the ability to stimulate osteogenic differentiation and survival via the AHR pathway without affecting the differentiation or activity of osteoclasts or adipocytes (Sharan et al. 2011).
AHR has also been shown to rapidly down-regulate the expression of osteopontin in an osteoblastic cell line, UMR-106 (Wejheden et al. 2006), which could weaken the bone loss preventing the effect of osteopontin (Yoshitake et al. 1999).

2.3.2 Retinoid receptors and PPARs in bone

Retinoic acid receptors (RARs) and retinoid X receptors (RXRs) are the main receptors and mediators of retinoid effects, but they also mediate the effects of many other substances, including tributyltin (Bastien & Rochette-Egly 2004, Grun & Blumberg 2006). The RAR and RXR receptor families are produced by separate genes. They both have at least two different isoforms, which have their own specific functions. RARs dimerize with RXRs and ATRA binds itself to the heterodimer, which then binds to retinoic acid response elements (RAREs) in the target genes and activates them (Bastien & Rochette-Egly 2004). It is also notable from the environmental chemical perspective, that RXR can form heterodimers with PPAR, another receptor family that binds both TBT and PFOA, and activate PPAR response elements (PPRE) in target genes (Al Tanoury et al. 2013).

A fraction of retinoids is also delivered by chylomicrons, which carry many fat-soluble substances in the circulation. A study by Niemeyer and colleagues (2008) demonstrated that, after liver, bone is the second most important organ for the clearance of chylomicron remnants from the circulation, and that osteoblasts contribute in a large degree to this uptake process. The study also showed a direct impact of osteocalcin carboxylation and secretion in mice in vivo.

The toxic effects of TBT on bone were reported for the first time in 2004, as Tsukamoto and colleagues administered subcutaneously 1 mg/kg body weight of TBT to pregnant mice and noticed a delay in ossification of the supraoccipital bone in fetuses. In addition, in in vitro experiments TBT exposure inhibited rat calvarial osteoblast differentiation and mineralization in a dose-dependent manner, as the activity of ALP, OCN, and the degree of calcium secretion decreased. TBT also inhibits osteoclast differentiation in mouse monocytic RAW264.7 cells at concentrations of 3-30 nM, and the mechanism was confirmed to be via RAR/RXR. One hypothesis for osteoclast differentiation inhibition is the repression of RANKL-induced AP-1 activation via RAR/RXR (Yonezawa et al. 2007).

Furthermore, TBT increases adipogenesis at the cost of osteoblast differentiation following in utero exposure, cellular lipid content and the expression of adipogenic genes via PPAR-γ (Kirchner et al. 2010, Watt & Schlezinger 2015).
PPAR-γ deficient embryonic stem cells can differentiate into osteoblasts spontaneously, but cannot differentiate into adipocytes, and PPAR-γ heterozygous mice have higher bone mass due to increased osteoblast number and bone formation. The study also concluded that already differentiated osteoblasts and osteoclasts functioned normally, so the haploinsufficiency had more effect on the differentiation process in bone marrow progenitor cells (Akune et al. 2004). Consistently, PPAR-γ suppression by WNT/β-catenin activation also increases osteoblastogenesis by activating osteoblastogenic transcription factors (Kang et al. 2007).

PPAR-α, the main receptor for PFOA, is also expressed in cells of the hematopoietic and mesenchymal lineages in bone, though less than PPAR-γ (Giaginis et al. 2007). The changes in bone phenotype of PPAR-α null mice is not that distinct as with PPAR-γ null animals, as bone mass is not altered and bone cortical area did not differ from control animals. Also, MSCs extracted from bone marrow from the mice have the potential to differentiate into both osteoblasts and adipocytes. However, in male animals, the medullar volume is significantly larger (Wu et al. 2000). PPAR-α also regulates bone marrow myeloid cell commitment toward the B cell lineage, and some PPAR-α agonists, such as bezafibrates, stimulate rodent osteoblast differentiation and inhibit human osteoclast development (Yang & Gonzalez 2004, Chan et al. 2007, Still et al. 2008).

Despite these anabolic effects on bone that certain PPAR-α agonists have, a negative association between PFASs, including PFOS and PFOA, and bone mineral density of various bone sites in U.S. women has been observed in an epidemiological study consisting of 1914 participants (Khalil et al. 2016). PFOA exposure may also alter the BMP2 pathway, as a low dose (10 mg/kg egg weight) of PFOA has been shown to decrease the amount of mRNA of BMP2 in chicken embryos (Jiang et al. 2013). BMP2 has an essential role in bone regeneration, and lack of BMP2 results in significant delays in ossification and an altered skeletal phenotype, which worsens with age in mice where fracture healing is clearly compromised. Proliferation of osteoprogenitor cells decreases and the differentiation into fully functioning osteoblasts is clearly reduced (Tsuji et al. 2006, Zhao et al. 2009).

### 2.3.3 Effects on steroid hormones

PFAS are well-known endocrine disruptors. Exposure to perfluorooctanoate has been shown to cause a dose-dependent increase in Leydig cell adenomas and
decrease the serum and testicular interstitial fluid testosterone levels and increase serum estradiol levels in male CD rats (Biegel et al. 1995). In older men, hypogonadism is associated with a low BMD and an increased bone fracture risk (Kaufman & Vermeulen 2005).

TCDD decreases testosterone levels by reducing the expression levels of the P450scc-gene and luteinizing hormone receptor (LHR) in wild-type rats exposed at an adult age (Fukuzawa et al. 2004). In a study by Pesonen and colleagues (2006), in utero and lactational exposure of TCDD increased the serum progesterone (P4) level at PND14 without altering body weight, FSH or E2 levels in female Sprague-Dawley rats. In the same study, ex vivo cultures of the ovaries showed a decrease in E2 secretion. In the same animals, TCDD interfered with the regulation of testicular steroidogenesis in male offspring and decreased steroidogenesis in 19.5-day-old fetal rat testis (Adamsson et al. 2009). An interesting study was also conducted by Nishimura and colleagues (2009), where 15 µg/kg bodyweight of TCDD exposure in mouse pups lead to impaired bone mineralization in the tibia, but also to an increase in vitamin D synthesis, which the authors concluded to be an important factor in the decrease of osteoblastic activity. At the same time, no difference between the number of osteoclasts or the expression levels of RANK, RANKL or OPG mRNA in exposed or control groups were found.

TBT’s hormonal disruption potential has long been known since the finding of the imposex effect due to increased testosterone secretion in female gastropods (Smith 1981). Most of the studies of the endocrinological and hormonal effects have focused on aquatic organisms, such as zebrafish, rockfish, rainbow trout and molluscs, whereas mammals have been studied less frequently. However, exposure to low doses of TBT (0.05-50 µg/kg) for 30 days significantly decreased spermatogenesis and ER-α and ER-β receptor levels in male mice (Chen et al. 2008). The serum estrogen level was significantly decreased and P4 levels increased in female Wistar rats treated daily with 100 ng/kg of TBT for 15 days (Rodrigues et al. 2014).

2.3.4 Accumulation of xenobiotics in bone

Many metals, such as lead, aluminium and manganese have been known to accumulate in bone, and an interesting linear correlation between manganese concentration in bone and in the central nervous system, especially in striatum, hippocampus and CSF, after chronic oral exposure in rats has been shown (O'Neal et al. 2014). In the same study, the average half-life of manganese in a rat skeleton
was equivalent to 8.5 years in a human skeleton. Therefore, under certain conditions it has been proposed that bone can act as a long time reservoir for foreign substances, and probably under increased bone resorption some xenobiotics may be released to some degree into the systemic circulation.

Studies of environmental chemical concentrations in bone are limited. Perez and colleagues (2013) analysed 21 PFAS from twenty Spanish cadavers. The samples, which consisted of brain, liver, lung, rib bone, and kidney, were taken 24 h after the time of death, and all of the samples contained at least two of the investigated compounds. In contrast to lung, bone contained the lowest amounts of PFASs, but the highest amounts of PFOA, while the PFOS was absent. The mean PFOA concentration in bone was 60.2 ng/g ww, range Bdl-234 ng/g ww. For comparison, liver samples contained an average of 13.6 ng/g of PFOA, lung 29.2, kidney 2.0 and brain was below the detection limit. To our knowledge, no other studies of the accumulation of POPs in bone have been conducted.

### 2.3.5 Analytics and biological end-points

POPs analyses require precise methods and knowledge due to the low concentrations that are typically within the picogram-nanogram level. Mass spectrometry (MS), especially combined with either gas chromatography (GC) or liquid chromatography (LC), techniques used for physical separation of substances before mass analysis has grown in popularity in laboratories due to its high level of sensitivity and specificity, and the possibility of analyzing multiple analytes simultaneously even from complex mixtures. LC-MS/MS is routinely used in many fields, such as therapeutic drug monitoring, neonatal screening and toxicology (Himmelsbach 2012).

Mesenchymal stem cells are commonly used in *in vitro* studies because of their easy obtainability and differentiation capability towards different cell lineages, including bone-forming osteoblasts. Common biopsy locations for MSCs are bone marrow from the iliac crest or the head of the femur (Pittenger *et al.* 1999), but in addition, adipose tissue is another source (Panes *et al.* 2016). Our laboratory uses bone marrow –derived MSCs for bone-focused studies, but considering the fat-accumulation potential of many POPs, further studies extending the focus to other tissues and cells, such as adipose tissue –derived MSCs outside bone deserve attention.

In addition to bone formation analyses, in our experience and that of others, quantification of bone resorption by bone marrow- and the activity of peripheral
blood derived osteoclasts is a highly sensitive marker for bone homeostasis evaluation, as mature osteoclasts are highly specialized end-point cells with very few cellular competitors; other cells have a very limited capability for bone resorption (Teitelbaum 2006).
3 Aims of the study

Bone as a target tissue for different environmental chemicals, such as dioxins, perfluorinated compounds and organotins, has attracted little interest during the last decades. The toxicity of these chemicals is well known for many specific organs, tissues and cell types, but bone tissue as such has been left to a narrow interest group among researchers. The hypothesis of this study was that POPs can accumulate in bone tissue and have adverse effects on bone alone and in combination, and can therefore contribute to bone loss and osteoporosis both directly through bone cell alteration, and indirectly via hormonal alterations. Therefore, the wider goal of this study was to produce new information and knowledge about the effects of common environmental chemicals on bone tissue.

The specific aims were:

1. To develop an in vitro model to investigate the co-effects of different environmental chemicals on bone.
2. To investigate the co-effects of TBT and TCDD on differentiating bone cells.
3. To study the concentrations of PFOA in bones of mice and humans.
4. To study the effects of in utero and lactational exposure of PFOA on 17 week old mouse long bone histomorphometry and biomechanical properties after a long period of exposure.
5. To study, whether PFOA affects the differentiation of mouse and human osteoblasts and osteoclasts.
6. To study, if PFOA concentrations in bone tissue samples correlate with bone architecture parameters measured with micro-CT.
4 Materials and methods

4.1 Chemicals (I, II, III)

TBT (Tributyltin chloride) and PFOA were purchased from Sigma and TCDD from Ufa-Institute (Ufa, Russia). TCDD was over 99% pure as assessed by gas chromatography-mass spectrometry. Methanol and ammonium acetate for mass-spectrometry studies were obtained from J.T. Baker (Deventer, the Netherlands). N-methylpiperidine was from Sigma-Aldrich (St. Louis, MO, USA), and both PFOA standard and mass-labeled PFOA (1,2,3,4-13C4, used as an internal standard) were from Wellington Laboratories Inc. (Guelph, Ontario, Canada). All POPs were dissolved in DMSO (Sigma) and the DMSO concentration was less than 1% in in vitro experiments.

4.2 Study permissions and patients

The animal experiment protocols for study I were reviewed and approved by the Animal Experiment Committee of the University of Kuopio. The in vivo part of study II was conducted in accordance with the rules of the Swedish animal protection legislation and was approved by the local Animal Ethics Committee (Stockholms Norra Djurförsökssetiska Nämnd), and the in vitro experiments were approved by the Animal Experiment Committee of the University of Oulu (permission number 027/11).

Human bone marrow samples were collected from patients operated for hip osteoarthrosis (III). All patients gave their written informed consent according to the Declaration of Helsinki and the Ethical Committee of Oulu University Hospital had approved the study protocol. Bone marrow samples were then used to acquire stromal mesenchymal stem cells to be differentiated into osteoblasts, and monocytes/precursor cells into human osteoclasts.

The cadaver biopsies in study III were taken from a 46-years old female, who had given a written consent before dying of kidney carcinoma. Anonymous femoral head samples (n=19) were acquired from the clinical bone bank held in Oulu University Hospital, Oulu, Finland. Special National Supervisory Authority for Welfare and Health (Valvira) granted permission for use of the aged cadaver and live donor bone collection of clinically unusable specimens for research purposes.
(Decision 8.5.2009, Diary number 2240/05.01.00.06/2009). The only donor information available from the tissue bank was age and sex.

### 4.3 Cell culture (I, II, III)

Cells for *in vitro* experiments were obtained either from C57BL mouse bone marrow (I and II), the MC3T3-E1 cell line (II) or from human bone marrow and peripheral blood (III). The basic medium contained α-MEM (Sigma-Aldrich, St. Louis, MO, USA), 10% fetal bovine serum (FBS) and penicillin-streptomycin and was used in the beginning of each experiment before introducing exposure medium. Experimental designs for the *in vitro* experiments are listed in Table 4.

**Table 4. Exposure-study designs for *in vitro* studies.**

<table>
<thead>
<tr>
<th>Study</th>
<th>TCDD</th>
<th>TBT</th>
<th>PFOA</th>
<th>Monoexposure</th>
<th>Co-exposure</th>
<th>Cell source</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.01-1.0</td>
<td>0.1-10</td>
<td>-</td>
<td>x</td>
<td>x¹</td>
<td>Mouse (primary)</td>
</tr>
<tr>
<td>II</td>
<td>-</td>
<td>-</td>
<td>0.1-200</td>
<td>x</td>
<td>-</td>
<td>Mouse (cell line)</td>
</tr>
<tr>
<td>III</td>
<td>-</td>
<td>-</td>
<td>0.1-200</td>
<td>x</td>
<td>-</td>
<td>Human (primary)</td>
</tr>
</tbody>
</table>

¹10 nM TBT + 1 nM TCDD

Control medium contained DMSO alone (final concentration 0.1%). Half of the medium in each experiment was changed every 3-4 days and the experiments lasted 12 days for osteoclast cultures, and for osteoblast cultures 14 days or 5 weeks for mouse and human, respectively, unless otherwise stated. Cell sources and differentiation medium compositions are presented in Table 5.
Table 5. Differentiation media and cell sources used. The substances were solved to alfaMem medium.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Mouse</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C57BL (I, II)</td>
<td>MC3T3 (II)</td>
</tr>
<tr>
<td>Osteoblasts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dxm</td>
<td>10 nM</td>
<td>10 nM</td>
</tr>
<tr>
<td>β-GP</td>
<td>10 mM</td>
<td>10 mM</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>50 µg/ml</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td>Osteoclasts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M-CSF</td>
<td>10 ng/ml</td>
<td>-</td>
</tr>
<tr>
<td>RANKL</td>
<td>30 ng/ml</td>
<td>-</td>
</tr>
<tr>
<td>TGF-β</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dxm</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

hBM = Human Bone Marrow, hPB = Human Peripheral Blood, Dxm = dexamethasone, β-GP = Beta-glycerophosphate, M-CSF = Macrophage Colony-stimulating Factor, RANKL = Receptor Activator of Nuclear Factor Kappa-B Ligand, TGF-β = Transforming Growth Factor Beta.

**4.3.1 C57BL mouse cells**

Bone marrow stromal cells were acquired from femurs and tibias of two 10-12 week old C57BL mice. The mice were first sacrificed by CO2 asphyxiation and the hind limbs were dissected free from the body and adhering tissues. The bones were transferred to a laminar flow hood in 37 °C 1xPBS, after which they were briefly soaked in 70% ethanol. The bone ends were then excised and the bone marrow content was collected by centrifugation. The rest of the marrow cavity was then flushed into a 50 ml tube with a 25 G syringe and α-MEM. The cell pellet was homogenized by pipetting gently up and down, and then centrifuged at 500 G for 5 min in RT. The cell pellet was resuspended in α-MEM to a final volume of 10 ml of cell suspension, the cells were counted using a hemocytometer, and differentiation medium was introduced.

The next day, the exposure medium was changed. MTT-tests were done on the 1st, 14th and 21st day to assess the viability of the cells. On the 7th and 10th days expression of the differentiation markers alkaline phosphatase (ALP) and osteocalcin (OCN) was measured. On the 10th day the degree of mineralization was measured using a Calcium Detection Kit (Abcam, Cambridge, MA) according to the manufacturer's instructions, and ALP activity per milligram of protein was measured as described earlier (Leskelä et al. 2003).
For osteoclast differentiation, the cells were placed on bovine bone slices in differentiation medium. The next day, either medium containing TBT and TCDD (I) or PFOA (II) was added.

### 4.3.2 MC3T3-E1 cells

The murine calvarial osteoprogenitor cell line MC3T3-E1 (subclone IV, ATCC. LCG Promochem) cells used in study II were passaged three times to acquire a total of 12 million cells. The cells were then detached by using 10x Tryple Select (Gibco) medium, and differentiation medium was introduced.

Exposure and the measurement of differentiation followed the same principles as those of the C57BL mouse cell experiments.

### 4.3.3 Human bone marrow cells and peripheral blood samples

In study III, the exposure medium was changed every 4th day and the cell morphology was observed daily under the microscope to confirm the normal cell morphology and monitor the differentiation process towards an osteoblast lineage. During differentiation, the cells started to secrete microscope-detectable calcium after 14 days. ALP activity was measured at the 3rd week and the amount of calcium secreted at the 5th week.

Human osteoclasts were differentiated from bone marrow and peripheral blood samples (III). Human bone marrow samples from the femoral collum and trochanteric region were taken during total hip arthroplasty from a 44-year old male and a 64-year old female, who had given written permission to take and study the samples. Approximately 5 ml of bone marrow was taken, and the bone marrow sample was first maintained in α-MEM (Corning Life Sciences, Tewksbury, MA) containing 10% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin and 24 mM Hepes buffer (Sigma-Aldrich, St. Louis, MO) at +37°C (5% CO2, 95% air) for 1-2 days. After this pre-culture, the media containing the non-adherent mononuclear cells were collected and the isolation was continued with Ficoll-Paque Premium (GE Healthcare, Little Chalfont, UK) following the manufacturer’s protocol. Isolated monocytes were then seeded on 96-well plates (300 000 cells/plate) with a 0.28 mm² human bone slice on the bottom of each well. The next day the medium was replaced with osteogenic differentiation medium.

A peripheral blood sample was taken from a healthy 45-year old volunteer from the cubital vein. After the Ficoll-Paque treatment described earlier, the isolated
monocytes were then seeded in the same manner on 96-well plates containing bone slices, and the differentiation medium was changed the next day. The medium was changed every 3-4 days, and culture lasted for 10 days.

4.4 qPCR studies (I, II)

For gene expression studies, RNA was isolated from the pelleted cells using an RNeasy Mini Kit and RNase free DNase (Qiagen). cDNA was generated with an Omniscript RT Kit (Qiagen, Hilden, Germany) using random hexamers (Roche, Mannheim, Germany) and used as a template for quantitative PCR analysis. The expression levels of alkaline phosphatase (ALP) and osteocalcin as well as the reference gene GAPDH were analyzed using Power SYBR Green PCR Master Mix and Applied Biosystems 7000 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Standard curves were generated using isolated and purified PCR products produced with the same primers designed for quantitative PCR. The PCR products were purified from the agarose gels using a Wizard SV Gel and PCR Clean-Up System (Promega), and the concentrations were determined spectrophotometrically by NanoDrop (Thermo Scientific, Wilmington, DE, USA). PCR primers were designed using Primer Express software from Applied Biosystems that allowed the use of universal thermal cycling parameters. The following mouse primers were used: ALP (NM-007431), ggacggtgaacgggaaaat and cttctccaccgtgggtctca; mouse GAPDH (NM-008084), gtagctcctcagggcaaa and ggtctcgctcctggaagatg; mouse OCN (NM-001032298), gcggccctgagtctgaca and gcgccggagtctgttcacta. The PCR reaction was initiated with an incubation step of 10 min at 95°C to activate AmpliTaq Gold DNA Polymerase. This was followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. A dissociation curve was run to confirm the absence of non-specific amplification. Negative controls containing all the components of the reaction mixture but with water replacing the template were included in each run. The expression levels were related to mRNA concentrations of the housekeeping gene GAPDH to normalize the amount of cDNA in the PCR reactions.

4.5 Quantification of osteoclast differentiation (I, II, III)

To evaluate the differentiation of hematopoietic precursor cells to osteoclasts, bone slices having osteoclasts were stained with a TRACP-kit (Sigma) at +37°C according to the manufacturer’s instructions. The quantification procedure was
similar both in mouse and human osteoclasts. The nuclei were stained with the DNA-binding Hoechst 33258 fluorochrome (Sigma) for 10 min at room temperature. TRACP-positive cells having three or more nuclei were considered as osteoclasts and were counted from bone slices using a fluorescence microscope (Zeiss AX10; Carl Zeiss Ltd., Hertfordshire, England) with a 20 ×/NA 0.5 objective (Zeiss).

After counting the osteoclasts, the cells were removed from the bone slice by gentle scraping and the slices were dyed with peroxidase-conjugated WGA-lectin (WGA; 20 μg/ml) for 30 min at room temperature. Slices were washed 3 times with PBS and counterstained with a diaminobenzidine (DAB) kit according to the manufacturer's instructions (Invitrogen, CA, USA). The total area of the resorption pits was measured by acquiring images of each bone slice with a fluorescence microscope (Zeiss AX10), 10 ×/NA 0.25 Objective (Zeiss) and QImaging Retiga 4000R camera with a digital image analyzer (MCID Core 7.0, Ontario, Canada). Range of interests (ROIs) were drawn for each resorption pit and analyzed with FIJI (ImageJ) software (Schindelin et al. 2012).

4.6 Mass-spectrometry (II, III)

4.6.1 Preparation of the samples

In study II with PFOA-exposed mouse bones, after removal of the bone marrow by flushing, femurs and tibias were placed in a laminar hood overnight for drying. The next day the bones were powdered in a liquid nitrogen mill (Cryomill MM400, Retsch, Germany) for 5 minutes to avoid heating the samples. Samples were pre-frozen in a plastic tube, which was placed in liquid nitrogen for 10 s prior to powdering. Control groups were powdered first to avoid possible contamination, and the equipment was thoroughly cleaned with water and 70-% ethanol between samples. Tibias and femurs of each group were pooled together in order to obtain enough powdered sample for each group. Pooled samples were then placed in standard 50 ml polyethylene falcon tubes for further investigations. In study II, only PFOA was analyzed.

In study III, human femoral heads were cut with an electrical saw, as shown in Fig. 3. The cortical part of the bone was left out, so that the sample consisted only of trabecular bone and bone marrow. Bone marrow was then removed by centrifuging the sample at 1000 RPM for 5 min and taken as a separate sample. The
residual bone marrow was then removed by applying hexane treatment and further centrifuging the sample at 300 RPM for 5 min, then incubating the sample for 1 h, and finally the sample was washed with 1xPBS. After drying in the laminar hood, the sample was powdered in a liquid nitrogen mill described earlier for 2 min to avoid heating of the samples. In addition to PFOA, concentrations of PFNA, PFDA, PFUnA, PFDoA, PFTrA, PFTeA, PFHxS, PFHpS, PFOS and PFDS were also analyzed.

Fig. 3. 3D-reconstruction of one of the human femoral bone samples showing the biopsy location for trabecular bone. The lower right corner photograph is a trabecular bone specimen with bone marrow removed. Scale bar presents 1.0 cm.

PFOA was extracted from the homogenized bone powder in test tubes with 2 ml of 20 mM ammonium acetate in methanol. After mixing for 10 min at 2500 rpm with a Vibramax 110 (Schwabach, Germany), the sample was centrifuged with an Eppendorf 5810 (Hamburg, Germany) at 2500 g for 10 min. The supernatant was removed and the extraction procedure was repeated. The supernatants were combined and allowed to evaporate to dryness under nitrogen before being reconstituted to 0.3 mL with 60% aqueous methanol (v/v). The samples were filtered (0.2 µm syringe filter, Pall Life Sciences, Ann Arbor, MI) and stored at –20 ºC until LC-MS/MS analysis.

4.6.2 LC-MS/MS Analysis
Quantitative PFOA analysis was performed using liquid chromatography negative ion electrospray tandem mass spectrometry (LC-ESI-MS/MS). A 10 µL aliquot of the sample was separated on a 30mm × 2.1mm (3.5 µm) Waters XBridge C18 column. To eliminate possible contamination of PFOA from inner LC parts, a
Waters XBridge C18 trap column (50mm × 2.1mm, 5µm) was installed between the pump and the injector switching valve. The LC separation was performed in a gradient elution at a flow rate of 0.24 mL/min using 10% aqueous methanol with 0.001% N-methylpiperidine as eluent A and HPLC grade methanol with 0.010% N-methylpiperidine as eluent B. Chromatograms were recorded by single reaction monitoring (SRM). The SRM for PFOA was the 413 Da –> 369 Da. PFOA was quantified using a calibration curve. The other PFASs were analyzed accordingly. Concentrations of PFASs are reported as ng/g of homogenized sample.

4.7 Microtomography (µCT; II, III)

µCT imaging analyses of the mouse long bones (II) followed published guidelines (Bouxsein et al. 2010). Prior to scanning the bone specimens were allowed to thaw at +8 °C in PBS for 12 h. The bone was wrapped in a PBS-moistened tissue paper and inserted into a plastic tube, with the proximal end pointing upwards. This container was then placed into the chamber of the µCT device (SkyScan 1174, Bruker MicroCT, Kontich, Belgium).

Projection images were acquired by scanning each bone with an image pixel size of 6.73 µm. X-rays were generated with a voltage of 50 kV and filtered with a 0.5 mm aluminum filter to reduce the beam hardening effect. One projection was collected every 0.5° over 360° rotation with an exposure time of 4000 ms. All image processing was performed with software provided by the manufacturer. The regions of interest (ROIs) were drawn for cortical and trabecular bone. For both femur and tibia the growth plate in the knee was used as a reference. To generate volumes of interest ROIs were drawn over 3.365 mm and 1.346 mm for cortical and trabecular bone, respectively. ROI drawings were started at 0.1346 mm and 1.685 mm for trabecular and cortical bone, respectively, as schematically shown in Fig. 4. In order to analyze the morphology of both bone compartments, the threshold was optimized individually for trabecular and cortical bone. In cortical bone analysis the 3D shrink-wrap function was used to capture both peri- and endosteal surfaces. For bone mineral density (BMD) and tissue mineral density (TMD) measurements, calcium hydroxyapatite phantom rods with a diameter of 2.0 mm and densities of 0.25 and 0.75 g/cm³ were scanned and reconstructed to calibrate grey scale images to density.

In study III, ten femoral head samples were scanned using the microCT device (Skyscan 1176, Bruker) inside a plastic container put into the chamber of the device. Projection images were acquired by scanning each sample with an image pixel size
of 34.84 µm. X-rays were generated with a voltage of 65 kV and filtered with a 1.0 mm aluminum filter to reduce the beam hardening effect. One projection was collected every 0.7° over 360° with an exposure time of 100 ms. ROIs were drawn in the same manner as with the samples studied with CBCT (see below).

Fig. 4. Anterior view of volume rendered models of the right femur (A) and the tibia (B) showing a schematic presentation of Volume of Interests (VOIs) of cortical and trabecular bone for PFOA-exposed mice.

4.8 Whole bone biomechanical testing (II)

After µCT imaging the biomechanical properties of the mouse bones were measured by the 3-point bending method using an electromechanical loading system (Instron 3366, Instron Corp., Norwood, MA, USA) controlled with Bluehill 2 (version 2.6). The biomechanical testing of midshafts of femurs and tibias was conducted as explained in a study by Jämsä and colleagues (2001) with a span length of 5.5 mm. The axial loading of the femoral neck was done as described in a study by Peng and colleagues (1999). The velocity of the load cell was 0.155 mm/s for all the loadings.
Biomechanical parameters were then acquired from the load-deformation curves; stiffness was defined as the slope of the linear part of the curve, breaking force as the maximum force ($F_{\text{max}}$) in the curve and toughness as the area under the curve from the origin to the point of fracture.

4.9 Cone-beam computed tomography (III)

Human bone bank samples were scanned with cone beam computed tomography (CBCT) using a 451-row cone CT scanner (Scanora 3D, Soredex, PaloDEx Group Oy, Tuusula, Finland) with a field of view (FOV) of 60 mm×60 mm, voxel size of 0.133 mm, and exposure time of 4.5 s. The parameters were a peak tube voltage of 85 kVp and a tube current of 15 mA. The captured images were reconstructed using a high-spatial frequency reconstruction algorithm. The reconstructed images were then re-oriented similarly and new cross-sectional images were produced with DataViewer software (Bruker MicroCT, Kontich, Belgium). The regions of interests (ROIs) were drawn for trabecular bone inside the femoral head with a small margin, and the threshold was optimized for trabecular bone. Finally, the trabecular part was analyzed with CTAn software (Bruker MicroCT, Kontich, Belgium).

4.10 Benchmark Dose Modeling (III)

Dose responses were analyzed using the benchmark dose (BMD) method, PROAST version 38.9 in R software. Based on the likelihood ratio test, the best-fitted models were selected among the exponential model families. Benchmark doses (critical effect dose) and their lower and upper 95%-confidence bounds were calculated at a 50% change in response compared with unexposed control.

4.11 Statistical analyses

In cell culture, microCT and biomechanical loading experiments, in normally distributed data assessed by Kolmogorov-Smirnov test and histogram, one-way ANOVA was used with the posthoc-test LSD. In a non-normally distributed data, a Mann-Whitney U-test and Kruskal-Wallis H-test were used. In study III, correlations between bone parameters and PFAS concentrations were analyzed using Pearson’s correlation.
5 Results

5.1 Exposure effects in in vitro experiments

In mouse bone marrow–derived stromal cells and MC3T3-E1 cells differentiated towards osteoblasts (I, II, respectively) mineralization of the extracellular matrix became detectable by microscopy typically after 6 days and later even without magnification. According to MTT-tests used to assess the viability of the cells, 100 nM of TBT reduced cell proliferation at day 6 by about 50% as compared to control cells, and therefore TBT-concentrations of 0.1-10 nM were used. In the case of PFOA, the mouse cells went through apoptosis in the two highest concentrations, 100 and 200 µM. 1 nM TCDD was based on earlier studies and did not alter the morphology or the viability of the cells, and the same mouse cell model has previously shown tolerance even for the 100 nM concentration.

In human bone marrow–derived stromal cells directed towards osteoblasts (III), proliferation and differentiation were slower compared to the mouse cells, as expected. Mineralization of the extracellular matrix was observed microscopically after 2 weeks and macroscopically after 3 weeks. The cells did not show direct toxic effects even to the highest concentration of PFOA, 200 µM.

5.1.1 The co-effects of TBT and TCDD

Study I investigated the co-effects of TBT and TCDD on differentiating osteoblasts and osteoclasts. The mRNA expression of alkaline phosphatase was significantly lowered after 6 days in the highest concentrations of TBT and TCDD, and in the combination group. The decrease seemed to be similar in all groups, the mixture group showing a slightly larger effect than the others. On day 10, only the combination group showed a significantly decreased level of alkaline phosphatase. The co-effect was greater than the sum of the effects of individual exposures; therefore, this response was clearly synergistic.

After 6 days of exposure, the mRNA expression of osteocalcin was significantly reduced in cells exposed to the highest concentration of TCDD both alone and in combination with TBT, while all TBT concentrations, when used alone, significantly increased it. At the mineralization stage (day 10), when the basal expression of osteocalcin has been shown to be highest, only the middle concentration of TBT increased the osteocalcin level. The highest concentration
tended to decrease it, albeit not significantly. The highest concentration of TCDD clearly reduced the amount of osteocalcin mRNA and in the mixture group the reduction was even greater, thus revealing a synergistic effect.

The mRNA expressions of aryl hydrocarbon receptor (AHR) and AHR repressor (AHRR) were measured in order to determine how treatments with TBT and TCDD affect the AHR signaling pathway. As expected, TCDD concentration-dependently induced the expression of AHRR, which negatively regulates AHR function and consequently inhibited the expression of AHR. TBT, however, reduced both the mRNA levels of AHR and AHRR at the highest concentration. The combined treatment with TBT and TCDD decreased the amount of AHR mRNA to approximately the same level as the individual exposures. For AHRR, the TBT-induced decrease antagonized the TCDD-induced increase and as a net result no significant change was seen. Prevention of the induction of AHRR expression by TBT potentially increases the AHR mediated effects of TCDD. However, expression of CYP1A1 in differentiating osteoblasts proved to be so low that levels could not be reliably analyzed.

The osteogenesis PCR-array analysis indicated completely different gene expression patterns for TBT and TCDD. Interestingly, the combined exposure affected many genes that the individual exposures alone did not affect, which furthermore indicates a synergistic effect for osteogenesis. TCDD alone both up- and down-regulated genes for extracellular proteins as well as those participating, e.g. in calcium ion binding and homeostasis, skeletal development, and cell growth and differentiation. In addition, the combined treatment with TBT and TCDD affected other genes involved in bone mineral metabolism, cell adhesion and transcriptional regulation.

In osteoclast cultures, the general appearance and number of nuclei of osteoclasts treated with TBT alone were not affected, whereas both TCDD concentrations decreased the number of nuclei significantly. The decrease was equal in cultures exposed to 1 nM TCDD alone and in combination with 10 nM TBT. Both concentrations of TCDD and the highest concentration of TBT significantly decreased the resorption pit area as compared to the control group. Cells exposed to both 10 nM TBT and 1 nM TCDD showed the greatest decrease in the area resorbed. This reduction was additive, since the combined effect was not greater than the sum of the individual effects.
5.1.2 The effects of PFOA exposure on differentiating bone cells

We exposed differentiating osteoblasts and osteoclasts to PFOA-concentrations of 0.1-200 µM (II, III). Study II focused on mouse cells, study III on human-derived cells. The two highest concentrations in study II, 100 and 200 µM, caused apoptosis detectable by microscopy, and significantly decreased ALP activity at day 7, while the corresponding ALP mRNA expression level remained unaffected both at 7 and 10 days of exposure. The amount of calcium peaked at 1 and 10 µM, and then drastically decreased to the control level at PFOA doses of 100 and 200 µM. The same effect was observed in OCN mRNA levels at day 7, but at day 10 the differences diminished except for the last group, which was significantly decreased.

The number of osteoclasts in study II was increased by exposure to PFOA concentration-dependently, and the increase reached statistical significance at 10 µM and above. The highest increase was observed at 200 µM, the concentration that already tended to inhibit resorption activity. Low PFOA concentrations stimulated the resorption activity, which peaked at 1.0 µM, but higher concentrations reduced it and at 200 µM the area of resorption pits was already below that of controls.

In human-derived cells (III), PFOA exposure did not affect the ALP activity or the amount of calcium. According to MTT-tests, cells tolerated the exposure well even at the highest concentration, 200 µM, and after 3 weeks, cell viability even peaked significantly at 100 µM, but this effect decreased after 5 weeks, where cell viability was non-significantly reduced in all PFOA exposed groups.

The number of osteoclasts of bone marrow origin decreased non-significantly as the concentration of PFOA increased, whereas in osteoclasts of peripheral blood origin, the number increased and peaked significantly at 1.0 and 10 µM, and then dropped to zero at the highest concentration (200 µM). In both types of osteoclasts, the resorption area increased dose-dependently and peaked either at 1.0 µM or at 10 µM.

5.1.3 Benchmark Dose Calculations

The EC50-concentration for human osteoclast resorption activity was 0.49 µM, whereas the EC50 in mouse osteoclast resorption activity was calculated as 0.27 µM. Due to biological variation, no benchmark dose could be reliable modeled for the number of osteoclasts in human, but in mouse data, the response was clearly more sensitive as the EC50 was 0.001 µM.
5.2 Developmental PFOA exposure in vivo experiments

5.2.1 Body weight

The mean body weight of the developmental PFOA-exposed group was consistently higher than the control mice throughout the lifetime of the animals, and the differences reached statistical significance \( p<0.05 \) at the last two time points, 13 months and 17 months, where the differences were 9.9% and 7.8% above the control values, respectively.

5.2.2 Morphometric and biomechanical results

In femoral cortical bone, the total cross-sectional area inside the periosteal envelope showed a 6.8% increase in the PFOA 17 month group compared to the corresponding control \( p<0.05 \). In addition, peri- and endosteal perimeters in this group were 3.2% \( p<0.05 \) and 5.2% higher \( p<0.01 \), respectively, compared to controls. There was no difference in femoral cortical bone area between the age groups, but the marrow area was 10.0% greater in the PFOA 17 month group compared to the corresponding control \( p<0.05 \). The polar moment of inertia was 9.6% greater in the PFOA exposed group \( p<0.05 \), although there were no differences in femoral mineral densities. In the tibias of 17-month-old mice, the total area inside the periosteal envelope was 4.9% higher in the PFOA exposed group \( p<0.05 \), as were the periosteal perimeter \( 3.5%, p<0.05 \) and polar moment of inertia \( 10.2%, p<0.01 \). In addition, the mineral density of tibias was 2.5% lower compared to the control \( p<0.05 \). In contrast to femurs, tibial medullary area, and endosteal perimeter were essentially the same between groups.

There were no significant differences in any trabecular parameters of femurs or tibias. Both femurs and tibias showed the same biomechanical properties regardless of the group, although there was a trend for increasing maximum force \( (F_{\text{max}}) \) in both exposed groups compared to controls.
5.3 Mass-spectrometry studies

5.3.1 Mouse femurs and tibias

In study II, femurs and tibias of mice were pooled in four groups to obtain enough powdered bone for mass-spectrometry analyses. The concentrations of PFOA were 4-5 times higher in exposed groups compared to the corresponding controls, as at 13 and 17 months of age, the concentrations were 0.73 vs. 3.0 and 0.64 vs. 3.7 ng/g ww, respectively.

5.3.2 Human bone bank samples

Femoral heads were obtained from 6 female patients, and 13 male patients operated for total hip arthroplasty. The mean age was 63 years with a range of 50-79 years. Bone samples and bone marrow samples were analyzed separately. All of the samples contained perfluorinated substances, PFOS and PFOA being the most prominent substances. PFOA was prominent in bone marrow, whereas PFOS was quite evenly distributed between bone marrow and trabecular bone.

5.3.3 Human cadaver tissue samples

Eight bone biopsies and five soft tissue biopsies were taken one week after time of death from a deceased 46-years old female, who had died from kidney carcinoma. The femur, tibia and fibula did not contain either PFOA or PFOS, but PFNA was present in all three long bones. The lung and liver contained the highest concentrations of PFASs, whereas bone marrow from the femur and whole blood contained no PFASs.
6 Discussion

6.1 TBT affects bone cell differentiation

TBT exposure decreased mRNA expression of alkaline phosphatase, typically secreted during matrix maturation, and the expression of osteocalcin, typically expressed in the onset of mineralization, which was increased at lower concentrations, but tended to decrease at the highest concentration. Thus TBT clearly affected the differentiation of osteoblasts from mouse-derived bone marrow stromal cells.

When compared to other models, the rat ROB cell model for osteoblast differentiation was less sensitive to TBT because cell viability was not affected at concentrations up to 100 nM (Tsukamoto et al. 2004), which in our model clearly reduced cell proliferation and viability. Therefore 10 nM was the highest concentration used in our experiments. In previous studies with rat cell models, lower TBT concentrations seemed to decrease osteocalcin expression (Tsukamoto et al. 2004, Korkalainen et al. 2008), which suggests species-specific differences in sensitivity. Our finding on TBT-induced disruption of osteoblastic differentiation is also in line with the reduced ossification observed in rat fetuses after in utero exposure to TBT (Adeeko et al. 2003).

It is also noteworthy, that in our model, TBT alone did not affect the differentiation of osteoclasts, but the highest concentration of 10 nM, significantly decreased the resorption activity.

6.2 TBT and TCDD show synergistic effects on differentiating bone cells

Our cell model for studying the co-effects of two different environmental chemicals proved to be very sensitive both to dioxins and TBT. The results indicated that TCDD alone significantly inhibits osteoblast differentiation in vitro, since the characteristic markers for matrix maturation and mineralization stages of osteoblastic differentiation were suppressed. The expression of both alkaline phosphatase and osteocalcin mRNAs decreased concordantly with our earlier study (Korkalainen et al. 2009)

The combined exposure to both TBT and TCDD resulted in synergistic effects on the differentiation of osteoblasts. The reductions in the mRNA levels of alkaline
phosphatase and osteocalcin at the mineralization stage exceeded the sum of the individual exposures. Moreover, at 10 days only co-exposure was able to reduce the expression of alkaline phosphatase.

The PCR-array studies were done to further study this observed co-effect. The data showed that TBT and TCDD evoked completely distinct gene expression patterns, thus indicating different mechanisms of toxicity. TBT mainly down-regulated, TCDD both up and down-regulated, and the co-exposure mainly down-regulated the expression of genes related to osteogenesis. The co-exposure mostly suppressed the expression of genes that play roles in skeletal development, particularly in ossification (Enam, Phex) and cartilage condensation (Sox9), which suggests that both early and late stages of bone formation are likely to be affected.

In addition, altered gene expression was also seen among genes encoding extracellular proteases (Ctsk, Mmp9), their inhibitors (Col7a1) and other extracellular matrix proteins (Csf3, Fn1, Itgam). Also, some transcription factors and regulators (Msx1, Sox9, Twist1) were affected after the co-exposure. Most importantly, the PCR-array results indicate that at the molecular level, the joint effect of these two chemicals is not driven by the specific effects of either of them, but represents a unique response to the combination. In addition, combined exposure leads to clearly increased alteration of the expression of genes as compared with TCDD or TBT alone, or with the sum of the alterations after individual exposures. Overall, the qualitatively and quantitatively different gene expression pattern is consistent with the observed disruption of bone formation and is strongly indicative of a synergistic mode of interaction.

The expressions of AHR and AHRR were studied to monitor the functioning of the AHR signaling pathway on the observed co-effects. We found that TBT antagonized the induction of AHRR by TCDD. The likely consequence is less feedback inhibition in the AHR pathway and aggravation of AHR-mediated toxic effects. Low expression levels of CYP1A1 in differentiating osteoblasts did not allow us to use this marker gene for monitoring the AHR pathway, which would have been interesting to study.

Dioxins can also interfere with Vitamin A status by disturbing several steps of the retinoid signaling pathway, such as retinoid-specific receptors, enzymes, and their binding proteins (Nilsson & Håkansson 2002). At the retinoid receptor level TCDD inhibits the transcription of RAR-β (Weston et al. 1995) and the binding of trans-retinoic acid to RAR, possibly due to an altered ligand binding domain of RAR subsequent to interaction with the AHR or AHR/ARNT complex (Lorick et al. 1998). AHR can also activate RAR-α by acting as an antagonist of the silencing
mediator of retinoid and thyroid receptors (SMRT) (Widerak et al. 2006). This is a potential mechanism of cross-talk that could explain the possible amplification of RAR-dependent TBT signaling by TCDD. A linkage between AHR and RAR and the TCDD altered RA-response has also been confirmed (Toyoshiba et al. 2004). In addition, an interaction between AHR and PPAR may also play a role as the AHR ligand Sudan III was shown to inhibit the function of PPAR-α (Shaban et al. 2004). This could provide another mechanism for the co-effects observed.

TCDD inhibited the differentiation of osteoclasts as the area resorbed and the number of nuclei were significantly decreased already at the lowest concentration of TCDD (10 pM). Similarly, exposure to 10 nM TBT interfered with osteoclast differentiation resulting in decreases in both the number of osteoclasts and in the area of bone resorbed. Our findings are in accordance with those of Yonezawa and colleagues (2007), who reported that TBT and triphenyl tin (but not monobutyltin and dibutyltin) at non-cytotoxic nanomolar concentrations inhibited the formation of TRACP+ and multinuclear osteoclast-like cells derived from the mouse monocytic RAW264.7 cells, and the area of resorption pits. Because the aromatase inhibitors aminoglutethimide and letrozole did not inhibit osteoclastogenesis even at 100 µM concentration, it was considered unlikely that inhibition of aromatase would play a role in this effect. Moreover, the study by Yonezawa and colleagues with RAR and RXR agonists and antagonists revealed that TBT suppresses osteoclastogenesis via an RAR-dependent signaling pathway; TBT (similarly with RAR agonist) was shown to inhibit the RANKL-induced expression of nuclear factor of activated T cells c1 (NFATc1), the key regulator of osteoclastogenesis. Thus, both TCDD and TBT seem to interfere with the RAR-dependent pathway, and we found that their combined effect on osteoclast differentiation is additive.

6.3 Maternal exposure leads to PFOA accumulation in bone in mice

Maternal exposure of mice to PFOA during pregnancy and lactation resulted in accumulation of the compound in bones of the offspring. The elimination was so slow that elevated concentrations were detectable even at the age of 17 months, practically the whole lifetime of a mouse.

The data extend the earlier findings on the distribution of PFOS and PFOA to bone (Borg et al. 2010, Bogdanska et al. 2011) and demonstrate the slow elimination from the bone. There are no data on the distribution of PFOA within the bone, but whole-body autoradiography studies with 35S-labeled PFOS indicated localization mainly to the bone marrow and not to the mineralized bone.
(Bogdanska et al. 2011). Therefore, bone marrow derived pre-osteoblasts and pre-osteoclasts are potential targets of PFOA, which highlight the value of these cell types in further characterization of the in vivo findings.

### 6.4 PFAS-levels in human bone samples

After concluding that mouse bone can act as a storage site for PFOA, we studied human bone bank samples to analyze the levels of multiple PFASs, including PFOA, in humans. Nineteen patients who had undergone a total hip arthroplasty operation gave their femoral head with a written consent to the bone bank of The University Hospital of Oulu. Bone marrow was separated from each sample and analyzed separately from trabecular bone to determine the distribution of PFASs in bone.

All of the samples contained perfluorinated substances. The most prominent were PFOS and PFOA, and both were detected from both dry bone material and bone marrow samples. The average PFOA concentrations from bone and bone marrow in our samples were 0.15 and 0.44 ng/g ww, respectively. In a Spanish population, rib bone samples from twenty cadavers contained on average 60.2 ng/g ww of PFOA (Perez et al. 2013), which might indicate a difference between the Finnish and Spanish population exposure profile. Due to the anonymous nature of the bone bank, no other information, e.g. occupation of the donors, is available.

From occupational exposure studies, firefighters have been shown to yield higher serum concentrations compared to other occupations, probably due to exposure to fire-fighting foams used in safety-drills and other activities (Shaw et al. 2013). In the study by Shaw and colleagues, the average serum concentration for a firefighter was 7.0 ng/g, clearly exceeding those of our study population concentrations (III, Table 3).

### 6.5 PFOA exposure affects bone in vivo and in vitro

We observed mild bone-increasing effects in femurs and decreased mineral density of tibias in both age groups in PFOA-exposed pups. The mice showed significantly increased overall cross-sectional periosteal and medullary areas, and periosteal and endosteal perimeters were also increased. Bone area, however, was not affected. In tibias, the overall cross-sectional area and cortical bone area in the 17-month-old group followed the same trend as in the femurs of the corresponding age.

The mild effects seen here are probably explained to some degree by increased body weight and thus increased load on the weight-bearing long bones, but the
effects of PFOA on bone cell differentiation cannot be excluded. Increased bone resorbing activity at low concentrations is likely to represent increased activation frequency, as indicated by decreased mineral density of tibias. Tibias seemed to be more sensitive to PFOA than femurs, and thus decreased mineral density of cortical bone was observed in tibias, while the increase in bone volume seen in femurs was absent. This might indicate increased bone resorption in the exposed animals. The increase in bone volume was not reflected in the biomechanical properties, although a non-significant increase (possibly due to the small number of animals) especially in the maximum force was observed.

Due to changes in microCT morphometry, we studied the possible effects of PFOA on differentiating mouse and human osteoblasts and osteoclasts. In mice, low PFOA concentrations (0.1-10 µM) stimulated osteoblast differentiation as indicated by increased expression of OCN mRNA and increased amounts of calcium. The mineralization step in osteoblast differentiation seems to be the PFOA responsive period as these parameters are specific markers of mineralization. On the other hand, ALP, the marker of the matrix maturation step was not affected at these concentrations. In human osteoblasts, the same concentrations did not affect cell differentiation or bone formation activity.

The differentiation of osteoclasts proved to be especially sensitive to PFOA. The number of osteoclasts in all exposed groups increased, and the resorption activity increased significantly at low concentrations, but the effect diminished as the concentration increased. This indicates that PFOA concentrations in the range of 0.1-10 µM stimulate the bone resorbing activity of osteoclasts by possibly enhancing osteoporotic effects on bone, whereas higher concentrations decreased cell viability disturbing the cellular homeostasis in general.

Noteworthy is that PFOS increased cell membrane fluidity and decreased mitochondrial membrane potential at concentrations of 30-100 µM (Hu et al. 2003). No similar study has been carried out with PFOA, but due to its similar amphiphilic properties it is likely that PFOA has the same potential for membrane disruption. In addition, the inhibitory effects on both osteoclasts and osteoblast differentiation seem to occur within the range of concentrations interfering with membrane integrity. Altered membrane function may also affect the organization of membrane fusion of mononuclear cells when multinucleated osteoclasts are formed.

To study the effects of PFOA exposure on human osteoclasts, we used two sources to acquire precursor cells, bone marrow and peripheral blood. In human bone marrow derived precursor cells, the cells differentiated to osteoclasts showed a non-significant decrease in the number of osteoclasts and significantly increased
resorption activity, which peaked at 1 µM and then decreased as the concentration increased. Osteoclasts differentiated from peripheral blood precursor cells significantly increased in number and in resorption activity up to 10 µM and then drastically decreased. The main difference between the two sources is the larger presence of stromal mesenchymal stem cells in the bone marrow sample, since not all the mesenchymal stem cells attach to the bottom of the flask, and thus they reside with the floating cells. In peripheral blood, stromal mesenchymal stem cells are also present, but to a much lesser degree. This can also explain the difference in responses to PFOA exposure seen in osteoclast number. It is noteworthy that LOAEL (lowest-observed-adverse-effect level) in peripheral blood –obtained and differentiated osteoclasts was lower than the mean concentration of the Spanish population (41.4 ng/g vs. 60.2 ng/g, respectively) (Perez et al. 2013). The results are also combined in study III, in Table 3.

When considering other factors of PFOA’s known toxicity in maternally exposed mice, the maternal dose of PFOA did not cause general toxicity in dams or in the offspring. This is important to notice, since neonatal mortality has been considered as one of the most sensitive endpoints of PFOA toxicity. The lower confidence limit of the benchmark dose (BMDL5) of PFOA for neonatal mortality was the maternal dose of 1.09 mg/kg/day (Lau et al. 2006). In our study pregnant dams received 0.3 mg/kg/day from GD1 throughout pregnancy, and no mortality among the newborn pups was observed. However, exposed mice gained more weight than the corresponding controls throughout the experiment, which suggests an increased amount of adipose tissue. In fact, PFOA has been shown to increase adipocyte differentiation (Bastos Sales et al. 2013), and, in accordance with our findings, female offspring of CD-1 mice exposed in utero to a maternal dose of 0.01-0.3 mg PFOA/kg/day for 17 days of pregnancy had a significantly increased body weight up to the age of 40 weeks (Hines et al. 2009). In addition to increased body weight the PFOA-exposed female offspring in the present study showed decreased physical activity and a mild decrease in motor coordination (Onishchenko et al. 2011). These changes are likely to secondarily contribute to the bone effects, especially the decreased mineral density observed in tibias.

6.6 PFOA’s bone toxicity – mice vs. human

Mouse osteoclasts seem to be slightly more sensitive to PFOA when compared with human osteoclast resorption activity, according to BMD calculations from the resorption activity. Unfortunately, human data could not provide a reliable BMD.
for the number of osteoclasts, but in mouse data, BMD for a number of cells was 0.001 µM, clearly more sensitive than the BMD of resorption activity in mice. According to the data, PFOA affects the cell number at lower doses than it affects the resorption capability of the cells.

6.7 Future aspects for bone toxicology studies

The observation that PFASs can accumulate in bone is important (II, III). As discussed earlier, a linear correlation between bone and hippocampal concentration of metals has been observed. It is possible that bone under normal or pathological conditions can release stored xenobiotics into the blood stream through increased bone resorption, for example in osteoporosis, pregnancy or breastfeeding, and from the blood stream to other target organs. Studying the concentrations of POPs from multiple sites, including bone, and observing possible correlations between the sites and pathological conditions, would provide more information on this hypothesis.

The ability of PFOSs to disrupt cell membrane permeability should be studied further in mesenchymal stem cells and expand the study also to PFOA, since membrane fusion is an important step in the differentiation process of osteoclasts, where mono-nuclear cells fuse to form multinuclear osteoclasts, in many cases of consisting of tens of nuclei. This disrupting potential can also be part of the toxicity of complex mixtures of chemicals, as it can increase the influx of other chemicals into the cells.

To study co-effects of three or more compounds on different types of cells and thus expand the investigation towards real life situations, in vitro experimental settings should be optimized further to minimize the number of cells, work and space in the laboratory required.
7 Conclusions

The main goal of this thesis was to investigate widely the effects of three common, toxic environmental POP chemicals on bone tissue and the presence of PFASs in bone tissue. Based on the results of this study, it can be concluded that:

1. The \textit{in vitro} osteoblast and osteoclast differentiation models developed in this thesis can be used to study the joint effects of environmental chemicals.
2. Combined exposure to TBT and TCDD evoked a unique gene expression profile in mouse osteoblasts, and differentiation was synergistically disturbed. In osteoclasts, bone resorption activity was additively decreased.
3. Bone was a target tissue for PFOA both \textit{in vivo} and \textit{in vitro}, and maternal exposure during pregnancy results in PFOA accumulation in bone of the offspring.
4. Developmental exposure of PFOA caused its presence in bones until old age. PFOA was also capable of causing mild alterations in bone morphometry and decreased bone mineral density, as well as stimulating the bone resorption activity of osteoclasts.
5. Various PFASs, including PFOA, accumulate in human bone. It was observed both in bone tissue and bone marrow, and PFOA also stimulated the bone resorption activity of osteoclasts in human-derived cells.
6. All human bone and bone marrow samples contained perfluorinated compounds, of which PFOS and PFOA were the most prominent. Levels were low compared to the animal models and other human population studies.
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Original publications


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