Mikko A. J. Finnilä

BONE TOXICITY OF PERSISTENT ORGANIC POLLUTANTS
MIKKO A. J. FINNILÄ

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Abstract

Persistent organic pollutants (POPs), especially dioxin-like chemicals, have been shown to have adverse effects on skeleton and these effects are likely to be mediated via the aryl hydrocarbon receptor (AHR). In spite of the extensive research, the characteristics of developmental effects of POPs are poorly known and the role of AHR in POP bone toxicity and skeletal development in general.

In this project changes in bone morphology and strength as well as tissue matrix mechanics are studied by applying state of the art biomedical engineering methods. This allows understanding of the effects of dioxins exposure and AHR activity on the development and maturation of extracellular matrix in musculoskeletal tissues from a completely new perspective, and thereby improving the health risk assessment of POPs.

In the present study skeletal properties of rats exposed maternally to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), Northern Contaminant Mixture (NCM) and Aroclor 1254 (A1254) were studied for cross-sectional morphometric and biomechanical properties, and data were analysed with benchmark dose modelling. In addition, extracellular matrix properties were analysed using nanoindentation. Similar measurements were performed for adult wild-type and AHR-null mice after TCDD exposure. The same animals were also analysed for microstructural changes using micro-computed tomography and their bone cell activity was estimated from serum markers and gene expression.

Analyses show decreased bone length and cross-sectional properties with consequently decreased bone strength. On the other hand, an increased trabecular bone mineral density in response to NCM and A1254 was observed. In addition, bone matrix properties indicated delayed maturation or early senescence after maternal or adult exposure, respectively. The AHR is mainly responsible for bone toxicity of dioxin-like compounds and plays a role in bone development. This is likely due to disturbed bone remodeling as indicated by altered serum markers and gene expression. Overall these results indicate that POPs decrease bone strength, but the interpretation is difficult as there is more trabecular bone within cortical bone with compromised quality and increased porosity.

Keywords: aryl hydrocarbon receptor, biomechanics, bone growth, dioxins, microstructure, persistent organic pollutants, remodeling
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Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta, Biolääketieteen laitos, Anatomia ja solubiologia; Lääketieteellinen teknikka; Terveyden ja hyvinvoinninlaitos, Ympäristöterveyden osasto; Itä-Suomen yliopisto, Luonnonmateriaalien ja metätieteiden tiedekunta, Ympäristötieteellinen laitos; Cranfield University, Cranfield Defence and Security, Cranfield Forensic Institute

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Tiivistelmä

Tässä työssä tutkittiin luuston rakenteellisia ja mekaanisia ominaisuuksia niin perinteisillä kuin uusimilla biolääketieteen tekniikan menetelmissä. Tutkimuksen tavoitteena on saada uutta tietoa POP-altistuksen ja AHR-aktivisuuden vaikutuksista luuston kehitykseen ja luukudoksen ikääntymisprosesseihin, mikä edesauttaa kyseisten yhdisteiden riskinarviointia.


Asiasanat: aryylihiilivetyreseptorit, biomekaniikka, dioksiinit, hienorakenne, luun kasvu, luun uudismuodostus, pysyvät organamiset yhdisteet
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### Abbreviations and symbols

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>1,25(OH)2D3</td>
<td>1,25-dihydroxyvitamin D3</td>
</tr>
<tr>
<td>µCT</td>
<td>Micro-computed tomography</td>
</tr>
<tr>
<td>τ</td>
<td>Loading interval</td>
</tr>
<tr>
<td>ψ_e</td>
<td>Elastic (stored) energy</td>
</tr>
<tr>
<td>ψ_p</td>
<td>Plastic (absorbed) energy</td>
</tr>
<tr>
<td>δ</td>
<td>Plastic (absorbed) energy</td>
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<tr>
<td>ψ_e</td>
<td>Elastic (stored) energy</td>
</tr>
<tr>
<td>ψ_p</td>
<td>Plastic (absorbed) energy</td>
</tr>
<tr>
<td>A1254</td>
<td>Aroclor 1254</td>
</tr>
<tr>
<td>A&lt;sub&gt;creep&lt;/sub&gt;</td>
<td>Creep amplitude</td>
</tr>
<tr>
<td>AGEs</td>
<td>Advanced glycation end products</td>
</tr>
<tr>
<td>AHR</td>
<td>Aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>A&lt;sub&gt;p&lt;/sub&gt;</td>
<td>Projected contact area</td>
</tr>
<tr>
<td>B&lt;sub&gt;creep&lt;/sub&gt;</td>
<td>Initial creep rate</td>
</tr>
<tr>
<td>BD</td>
<td>Benchmark Dose</td>
</tr>
<tr>
<td>BDL</td>
<td>Benchmark Dose lower bound of the 95% confidence</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone mineral density</td>
</tr>
<tr>
<td>BMC</td>
<td>Bone mineral content</td>
</tr>
<tr>
<td>BV/TV</td>
<td>Trabecular bone volume fraction</td>
</tr>
<tr>
<td>Cat K</td>
<td>Cathepsin K</td>
</tr>
<tr>
<td>CA-AHR</td>
<td>Constitutively active AHR</td>
</tr>
<tr>
<td>Ct.A</td>
<td>Cortical bone area</td>
</tr>
<tr>
<td>Ct.Po</td>
<td>Cortical porosity</td>
</tr>
<tr>
<td>Ct.TMD</td>
<td>Cortical tissue mineral density</td>
</tr>
<tr>
<td>CTX</td>
<td>C-terminal telopeptides of type I collagen levels</td>
</tr>
<tr>
<td>DDT</td>
<td>Dichlorodiphenyltrichloroethane</td>
</tr>
<tr>
<td>DMA</td>
<td>Dynamic mechanical analysis</td>
</tr>
<tr>
<td>DXA</td>
<td>Dual-energy X-ray absorptiometry</td>
</tr>
<tr>
<td>E*</td>
<td>Complex modulus</td>
</tr>
<tr>
<td>E&lt;sub&gt;II&lt;/sub&gt;</td>
<td>Indentation modulus</td>
</tr>
<tr>
<td>E&lt;sub&gt;los&lt;/sub&gt;</td>
<td>Loss modulus</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
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<tr>
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<td>Estrogen receptor type β</td>
</tr>
<tr>
<td>E&lt;sub&gt;r&lt;/sub&gt;</td>
<td>Reduced (effective) modulus</td>
</tr>
<tr>
<td>E&lt;sub&gt;stor&lt;/sub&gt;</td>
<td>Storage modulus</td>
</tr>
</tbody>
</table>
\( F_{\text{max}} \)  Peak load
GD  Gestational day
HCB  Hexachlorobenzene
\( h_m \)  Peak displacement
\( h_c \)  Contact depth
\( H_{IT} \)  Universal (instrumented) hardness
Hyl  Hydroxylysine
\( I_p \)  Plasticity index
KO  Knock-out
K  Elimination constant
Lys  Lysine
M-CSF  Macrophage colony-stimulating factor
Msx2  Msh homeobox 2
NCM  Northern Contaminant Mixture
Ocn  Osteocalcin
OPG  Osteoprogerin
OPN  Osteopontin
OVX  Ovariectomy
PBB  Polybrominated biphenyls
PBDD  Polybrominated dibenzo-\( p \)-dioxins
PBDF  Polybrominated dibenzofurans
PCB  Polychlorinated biphenyls
PCDD  Polychlorinated dibenzo-\( p \)-dioxins
PCDF  Polychlorinated dibenzofurans
PFOA  Perfluorooctanesulfonic acid
PINP  N-terminal propeptide of type I collagen levels
PND  Postnatal day
Po.Th  Pore thickness
PPAR\( \gamma \)  Proliferator activating receptor subtype gamma
PTH  Parathyroid hormone
S  Contact stiffness
SMI  Structural model index
SOST  Sclerostin
\( t_{\frac{1}{2}} \)  Elimination half-life
TCDD  2,3,7,8-tetrachlorodibenzo-\( p \)-dioxin
TEQ  Toxic equivalent
TMD  Tissue mineral density
TNAP  Tissue non-specific alkaline phosphatase
TRACP-5b  Tartrate resistant acid phosphatase
Tb.N  Trabecular number
Tb.Sp  Trabecular separation
Tb.Pf  Trabecular pattern factor
Trab.BMD  Trabecular bone mineral density
VDR  Vitamin D receptor
WT  Wild type
$x_0$  Loading dose
$x_0$  Maintenance dose
List of original publications

The thesis consists of four original publications that are referred to in the text by their Roman numerals (I-IV). Additional yield and trabecular bone mineral density data related to studies II and III are also included.


*Equal contribution
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Original publications
1 Introduction

There is a huge range of different persistent organic pollutants (POP) including dichlorodiphenyltrichloroethane (DDT), polychlorinated and polybrominated biphenyls (PCB, PBB), dibenzo-p-dioxins (PCDD, PBDD) and dibenzofurans (PCDF, PBDF) present in nature. Although their production has been tightly regulated, due to their stability and long elimination half-life they are still present in biota and humans. PCDDs alone have 210 congeners of which 17 are extremely toxic. These compounds have spread around the world (Tuomisto et al. 1999). Humans are exposed to POP’s mainly via diet, especially meat, milk and fish products.

In particular fish consumption is problematic as fish is good source of vitamin D and omega-3-fatty acids. Sufficient vitamin D levels can prevent osteoporosis (Aro 2005) and decrease cancer incidence (Veldhuis et al. 2010), while omega-3-fatty acids may have a role in the prevention of cardiovascular diseases (Kris-Etherton et al. 2002). Thus, it would be tempting to recommend increased fish consumptions. However, such recommendations could potentially lead to developmental defects and cancer, as the margin of exposure between tolerable daily intake (TDI) and found concentrations of POP’s is very narrow in humans (European Commission 2000, WHO 2000). Therefore, better understanding of toxicity on the development and maturation of skeletal system is needed for more accurate risk assessment.

POP’s share some chemical characteristics as they are lipophilic and extremely persistent. Due to lipophilicity they accumulate in adipose tissue and can be transferred to mother’s milk. Therefore, alone for dioxins a child can get a significant proportion of mother’s body burden, about 20–25% in the case of dioxins (Tuomisto 2001). Moreover, only for this single group of chemicals, a huge spectrum of toxic effects has been recognized from mild biochemical changes to lethal wasting syndrome (Pohjanvirta & Tuomisto 1994). Most of the dioxin-induced effects are mediated via the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor. Osteoclasts (Ilvesaro et al. 2005), osteoblasts (Gierthy et al. 1994, Ilvesaro et al. 2005, Ryan et al. 2007), ameloblasts and odontoblasts (Sahlberg et al. 2002), and chondrocytes (Yang & Lee 2010) express AHR indicating potential for adverse effects of dioxin compounds in musculoskeletal tissues. Indeed, hypomineralization of molars has been observed in Finnish children exposed to background levels of POP’s via their mother’s milk (Alaluusua et al. 1999). This has been confirmed in animal models, where dioxin
disrupts the molar development in rats (Kattainen et al. 2001, Miettinen et al. 2002). In addition, dioxin accelerates eruption of the lower incisors and retards the eruption of molars (Miettinen et al. 2002). Furthermore, dioxin exposure decreases bone strength and bone geometry in adult rats (Jämsä et al. 2001). Later it was discovered that combined in utero and lactational exposure caused similar effects with decreased mineralization but with a dose less than one tenth compared to adults (Jämsä et al. 2001, Miettinen et al. 2005). Moreover, similar effects had been discovered on rats exposed to hexachlorobenzene (HCB) (Andrews et al. 1989, Andrews et al. 1990) and PCBs (Lind et al. 1999, Lind et al. 2000a, Lind et al. 2000b, Lind et al. 2004).


In spite of extensive research the characteristics and mechanisms of the developmental effects of dioxins are still insufficiently known. This project studies changes in bone morphology and strength as well as tissue matrix mechanics by applying state of the art biomedical engineering methodologies. This will allow us to understand the effects of dioxins exposure and AHR activity on the development and maturation of the extracellular matrix in musculoskeletal tissues from a completely new perspective, and thereby improve the health risk assessment of dioxin-like compounds.
2 Review of the literature

2.1 Bone

Bone is highly specialized mineralized connective tissue, which is mainly composed of hydroxyapatite, collagen and water contributing to hardness (Currey & Brear 1990, Zioupos 2005), toughness (Wang et al. 2001) and viscosity (Pathak et al. 2011), respectively. Besides varying matrix composition and material properties, bones come with multiple shapes and have locally varying ultrastructure both contributing to the overall strength of the bone (Chappard et al. 2011, Rho et al. 1999).

These properties are maintained in constant remodeling process (Robling et al. 2006), where osteoclasts resorb old bone matrix and osteoblasts lay new osteoid, which becomes more mineralized with a time. Additionally, if bone shape does not correspond to its mechanical demands, it is modelled to have optimal shape (Biewener & Bertram 1994, Plochocki et al. 2008, Sharir et al. 2011). Healthy bone is hard and tough, but changes at any hierarchical level can lead to decreased bone strength, fracture and loss of function (Rho et al. 1998).

Thus it is important to understand the link between modifications in matrix properties due to changes in cellular activity and overall strength. In this work the focus is in the effects of persistent organic pollutants on bone structure and strength at various hierarchical levels and the role of the AHR in these effects in vivo.

2.2 Bone tissue formation and (re)modeling

Bone development starts with condensation of mesenchymal stem cells followed by either of the two distinct ossification pathways. Most of the flat bones are formed by intramembranous ossification and rest by endochondral bone formation (Zelzer & Olsen 2003). In intramembranous ossification, cells differentiate directly to osteoblasts and start producing bony structures. The endochondral bone formation begins with the condensation of mesenchymal stem cells to cartilaginous anlagen. Primary ossification center will appear in the diaphyseal part of anlagen, where a collar of bone is formed. Later secondary ossification centers are formed inside the epiphyseal parts of bone surrounded by cartilaginous structure growth plate and articular cartilages.
Thereafter bone is modelled (growth and shape adaptation) and remodelled to maintain tissue quality (Fig. 1). These processes are regulated by various peptides and cytokines reflecting bone health, and they can be analyzed from blood samples. Due to slow turnover of bone tissue the changes in remodeling process have to be long lasting in order to ultimately cause alterations in physical properties of bone that could have any clinical significance.

In order to produce bone, mesenchymal stem cells need to differentiate towards osteoblastic phenotype as indicated by expression of Runt-related transcription factor 2, distal-less homeobox 5 and msh homeobox 2 (Msx2). Further dedication is indicated by expression of osterix, and β-catenin, T cell factor/lymphoid enhancer-binding factor 1 (Robling et al. 2006). Once the osteoblastic phenotype has been reached, cells start to produce type I pro-collagen, (Fig 1E), the main component of organic matrix, and tissue non-specific alkaline phosphatase (TNAP), a mineralization catalyst. Besides producing mineralization regulators (TNAP, Phospho1, Progressive ankylosis protein, nucleotide pyrophosphatase/phosphodiesterase, Fetuin-A) (Harmey et al. 2004, Szweras et al. 2002), osteoblasts produce paracrine regulators of remodeling (FasL, osteoprotegerin) and even endocrine regulators osteocalcin (Ocn), osteopontin (OPN) (Karsenty & Oury 2012) demonstrating that skeleton is part of endocrine system and endocrine disturbing chemicals (EDC) have potential skeletal manifestations.

To achieve amazing mechanical properties, possessed by bones, both collagen synthesis and matrix mineralization are orchestrated in a complex way. Collagen has to be assembled in triple helical form and packed into fibrils. Assembly of pro-collagen to triple helical form requires post-translation modification by collagen prolyl 4-hydroxylase (C-P4H) to result in a biopolymer stable at body temperature (Myllyharju & Kivirikko 2004). Upon the release respective proteinases cleave N and C terminal propeptides, while rest assembles as part of fiber in extracellular space (Fig 1F). Cleaved propeptides especially procollagen type I N-terminal peptide (PINP) measured from serum and urine are used as markers of bone formation (Suvanto-Luukkonen et al. 1997). Finally, to ensure optimal (post-yield) mechanical properties of collagen molecules, covalent intermolecular crosslinks are formed (Saito & Marumo 2010), while mineralization gives strength and stiffness to bone matrix (Fig 1G and Fig 1H). Optimal mineralization requires action of various mineralization promoters, and inhibitors to give hardness required from load bearing skeletal structures and to protect cells from being petrified with mineral dissipates as well as other organs.
from ectopic calcification (Kornak 2011). Most important ones for mineralization are TNAP and Phospho1 (Huesa et al. 2011, Yadav et al. 2011), regulating pyrophosphate/phosphate ratio and hydroxyapatite nucleation.

Some of the matrix producing osteoblasts undergo apoptosis (Karsdal et al. 2002), while some are trapped inside the matrix and start maturing to osteocytes (Fig 1E, G, A). Although bone matrix has good mechanical properties it will suffer from loading induced micro-cracking, which have to be remodelled (rebuild) (Fig 1B) in order to avoid complete fracture of whole bone. Both remodeling and modeling are most likely controlled by osteocytes, cells that are mechano-sensors as well as markers of bone quality. Osteocytes will increase production of nitric oxides (Zaman et al. 1999) and prostaglandins (Rawlinson et al. 1991) and decrease sclerostin (SOST) (Robling et al. 2008) as response to increased loading enhancing bone formation. In cases where loading causes damage bone osteocytes have been reported to secrete Macrophage colony-stimulating factor (M-CSF), and Receptor Activator for Nuclear Factor κ B Ligand (RANK-L) (Kurata et al. 2006), a surprisingly similar process as observed in unloading of a bone (O’Brien et al. 2013). Microfractures in the bone matrix as such do not probably play a role in this process (Rumpler et al. 2012). Osteocyte apoptosis leads to recruitment of pre-osteoclasts and enhancement of osteoclastogenesis, under a canopy (Fig 1C) that covers whole remodeling site, coupling bone resorption and formation (Andersen et al. 2009).

Osteoclastogenesis requires presence of M-CSF and RANK-L to drive differentiation of pre-osteoclasts to a more mature cellular phenotype (Teitelbaum 2000). Osteoblasts participate to osteoclastogenesis through their membrane bound RANK-L, that can be blocked by osteoprogenin (OPG), hence a direct contact between osteoblastic/stromal cells with pre-osteoclastic cells is required for osteoclastogenesis in vivo. Mature osteoclasts bind to bony surface by forming a sealing zone via αvβ3 integrin and start releasing bone matrix degrading Cathepsin K (Cat K) in acidic medium (Boyle et al. 2003). Degradation is further continued in intracellular vesicles containing tartrate resistant acid phosphatase (TRACP 5b) in addition to Cat K (Väääräniemi et al. 2004). These transcytotic vesicles are transported to the functional secretory domain in the basolateral membrane (Salo et al. 1997), where degradation products are released and can enter circulation and urine where they can be detected.
Fig. 1. Bone remodeling process. A) In a steady state the osteocytic network is active and SOST signals other bone cells to remain inactive. B) Damage causes local apoptosis of osteocytes, C) leading to formation of a canopy recruitment site for both hematopoetic and mesenchymal stem. Concurrent presence of both stem cells types is required for osteoclast activation, since osteoblastic RANKL binds to the RANK receptor of pre-osteoclasts activating a fusion process resulting in mature multinuclear osteoclast. D) Mature osteoclast secretes TRACP-5b and during resorption collagen telopeptides, both are released to circulation. When the damaged site is removed pre-osteoblasts start producing RANKL inhibiting and OPG stopping further fusion of osteoclasts. Then pre-osteoblasts E) start proliferating and further differentiating to osteoblasts that fill the resorption cavity F) with collagenous matrix by producing type I pro-collagen that is delivered to extracellular space, where propeptides are cleaved and remaining polymer is added to the fiber. G) Some osteoblasts are trapped inside unmineralized matrix called, osteoid, which is later mineralized H) with crystals growing around collagen fibers.
2.3 Bone composition and extracellular matrix physics

Although the skeleton appears to be inert organ, the well-controlled remodeling is the key for maintaining tissue quality at the level required to survive daily activities without plastic deformation or even fracturing.

The backbone of all the skeletal matrices is type I collagen. This heterodimeric fibrillar collagen is composed of two α1(I) chains and one α2(I) chain. During the processing in the endoplasmic reticulum various post-translational modifications takes a place. Upon the release collagen fibrils line up with 40 nm spacing and overlap 27 nm with fibril layers above giving the familiar periodic 67 nm cross-striated pattern. Here lysyl oxidase forms reactive aldehydes from lysine (Lys) and hydroxylysine (Hyl) residues in telopeptides (Gelse et al. 2003). These hydroxy-allysines and allysines bind to helical Lys and Hyl resulting in reducible immature crosslinks. Mature pyridinoline and pyrrole cross-links are formed when second telopeptic aldehyde binds to divalent complex (Eyre & Weis 2013). At this point collagen starts to mineralize. Senescence of bone introduces another type of crosslinks, non-enzymatic AGEs (advanced glycation end products). Generally the collagen content (Saito & Marumo 2010) seems to follow a similar pattern of overall bone mass (Haapasalo et al. 1996, Isaksson et al. 2010). As could be expected the amount of immature collagen cross-links decline rapidly while the mature cross-links and especially AGEs increase with age (Saito & Marumo 2010). Accumulated evidence until now suggests that the enzymatic cross-links improve the mechanical competence of collagen network while AGEs reduce it (Nalla et al. 2006, Nyman et al. 2007, Oxlund et al. 1995, Saito & Marumo 2010, Vashishth et al. 2001, Zimmermann et al. 2011) by increasing bone plasticity and microcracking (Zimmermann et al. 2011).

The early form of bone is called osteoid and is composed mainly of collagen. The positively charged regions in collagen fibers provide optimal sites for nucleation of the plate-like mineral crystals (2–5 nm thick, 5–25 nm wide and 15–55 nm long) (Nudelman et al. 2010), but the activity of TNAP is required for sufficient extravascular mineralization (Yadav et al. 2011). Intrafibrillar crystals account only about 20% of bone mineral, while the majority lies in interfibrillar space (McNally et al. 2012). These interfibrillar crystals are about 5 nm thick, 50 nm wide and 40–200 nm long having most likely plate-like shape (McNally et al. 2012). Bone mineral has been shown to grow becoming more elongated with age (Su et al. 2003, Tong et al. 2003). The main mineral in bone tissue is structurally similar to the naturally occurring hydroxyapatite (HA), however due to extensive
specific surface (100–200 m²/g) (Boivin & Meunier 2003) apatite in bone is unstable and often substituted with available impurities (Boskey 2003). The mineralization increases as function of tissue age (Turunen et al. 2011, Turunen et al. 2013) while the water content decreases (Mueller et al. 1966). This also leads to fundamental idea between the relationship of material properties and mineralization level, which should follow each other, where an increase in the mineral content increases both the modulus and hardness (Currey & Brear 1990, Riggs et al. 1993b, Zioupos 2005, Zioupos & Rogers 2006), decreases the plasticity index and reduces both viscoelasticity (i.e. decreased storage modulus / increased loss modulus), and the overall creep strain magnitude, last with a concomitant increase in the early creep strain rate (Zioupos 2005, Zioupos & Rogers 2006). As bone is formed in physiological fluid and phosphate is a strong acid, a part of it is transformed to hydrogen phosphate, which is then detectable in young bone tissue. However, with maturing bone tissue the quantity of hydrogen phosphate decreases and in tissue senescence natural physiological buffer, carbonate, is substituted in hydroxyapatite (Legros et al. 1987).

The final major component in this three-phase composite is water. It is well known that water is critical for mechanical performance of bone and thus hydration state should been hold close to native during all material testing. However, only recently it was suggested that water participates to organization of mineral crystals (Wang et al. 2003) and in amorphous layers could act as re-usable glue that allows some strain between crystals and even when broken could be easily re-formed (Duer & Veis 2013, Wang et al. 2013). Indeed a presence of sacrificable bonds in bone has been suggested (Thompson et al. 2001) and those could be the water between the plates.

Since collagen has Young’s modulus of 5GPa (Strasser et al. 2007, van der Rijt et al. 2006, Wenger et al. 2007, Yadavalli et al. 2010) or possibly much lower (Grant et al. 2008), is too flexible to maintain shape under daily compressive loading. On other hand Young’s modulus of pure mineral is about 150 GPa (Vanleene et al. 2012) and would fracture at low energies. For example the fracture toughness of enamel (Imbeni et al. 2005) (the most mineralized tissue in body) is about a quarter of bone (Nalla et al. 2005). In the healthy bone these material types are present in volume fractions of 62.6%, 28.3% and 11.3%, for mineral, organic and water respectively (Ding et al. 1997, Ding et al. 2012, Li & Aspden 1997), this combination results to unique mechanical properties of bone, where collagen conducts most of strain energy (Gupta et al. 2006) and mineral provides hardness. Such material possesses minimal weight (density) and still
provides a sufficient strength and toughness for skeletal structures (Ritchie 2011). Bone material is also heterogeneous increasing its energy dissipation capability (Tai et al. 2007). Although the matrix composition and material behaviour plays a role in overall mechanical performance of bone these changes are often buried under overall quantity of the various micro- and macrostructure.

### 2.4 Bone morphology

When moving from nanostructure towards higher hierarchical structural levels first there are lamellae, where collagen fibrils run in parallel to each other within the sheet. They contribute to mechanical properties of bone through their organization. For example in race horse radius, lamellae (collagen) are organized according to loading environment, where longitudinal orientation is found at cranial segment and oblique to transverse orientation in the caudal segment of cortical bone (Riggs et al. 1993a, Riggs et al. 1993b). Longitudinally orientated collagen can better resist tension while the transverse orientation is designed for compression (Riggs et al. 1993b) matching the strain during the pacing (Riggs et al. 1993a).

The lamellae run as sheets on periosteal as well endosteal bone envelopes. These parallel sheets form individual trabeculae but when wrapped around each other they form circular osteons, where the trabeculae and the osteons provide the next higher structural level. Furthermore the osteons buried between endosteal and periosteal bone borders form the first macrostructure, cortical bone, which forms surrounding layer over all the bones. The osteons form either Haversian or Volkmann’s canals depending whether they run along (Haversian) or across (Volkmann) the bone axis. These canals contain blood vessels and form vascular network of bone, a critical transportation system. However, they also increase the porosity of bone and as such reduce bone strength (Riggs et al. 1993b, Zimmermann et al. 2011). Osteons are remodeled by bone multicellular units where osteoclasts resorb some of the primary osteon making space for secondary osteons. These primary osteon remnants have become more mineralized and thus if their amount increases due to reduced remodeling or insufficient filling of cavity the whole bone may become brittle (Akkus et al. 2003). If the resorption surpass the bone formation the cortical porosity increases (Idelevich et al. 2011), as happens with aging (Chen et al. 2010, Zebaze et al. 2010, Zimmermann et al. 2011), even to such an extent, where endocortical bone starts to resemble trabecular bone (Zebaze et al. 2010). This reduces bone toughness by limiting
capacity of the crack-bridging, one of the bone toughening mechanisms (Zimmermann et al. 2011). One possible mechanism for increased porosity is decline in osteocyte density with age (Busse et al. 2010) reducing bone quality control. Furthermore observation of hypermineralized occlusion within these lacunas (Busse et al. 2010) could be indication of cell death that could increase the bone resorption as described previously.

Another microstructure is the individual trabeculae. Generally the trabeculae have lower modulus and hardness compared to cortical bone (Lewis & Nyman 2008, Szabo et al. 2011). Same applies for their mineralization (Tjhia et al. 2012) and mineral:matrix ratios (Szabo et al. 2011), while the link to material properties has been difficult to show (Szabo et al. 2011, Tjhia et al. 2012, Zebaze et al. 2011). This could indicate that the material properties are more related to orientation at lower structural levels and collagen properties (Szabo et al. 2011) or alternatively it could be that nanoindentation results are affected by osteocyte lacunas under the measurement point. The trabeculae can take various shapes from spheres to plates. The dominant shape on the other hand depends on anatomical location (Turunen et al. 2013) and possibly prevailing pathology (Salmon 2004). The study of individual trabeculae is not simple and thus most of the data arises from studying the second macrostructure trabecular bone. When studying trabecular bone specimens, it has been possible to show that mechanical properties follow mostly logical pattern where thicker trabeculae with shorter separation and higher number of trabeculae increase trabecular bone volume fraction, often giving a plate-like structure with low fragmentation gives high load capacity and stiffness (Mitra et al. 2005, Teo et al. 2006). This is further supported by studying fracture regions showing absence of plate-like structures, trabecular orientation parallel to loading direction or high trabecular bone specific surface or thickness are not present in fractured areas (Tassani & Matsopoulos 2014). Also increased microdamage accumulation in rod-like trabeculae has been reported (Karim & Vashishth 2011). Somewhat surprisingly connectivity has low correlation to mechanical properties (Teo et al. 2006) and seems not to provide any resistance against fractures (Karim & Vashishth 2011).

Bones come in variety of shapes and the relative amounts of trabecular and cortical bone varies between bones. Even though much effort in this review has been spent on bone “quality” aspect and its relation to bone strength, the overall quantity has huge impact on strength. Maybe for the idea that “the bigger the stronger“ has shifted the diagnostic focus on the bone quantity meters as they are relatively simple and still clinically relevant. On the other hand, dual energy x-ray
based bone mineral density (BMD) may not be sufficient to predict fracture risk (Pulkkinen et al. 2011) and for this reason the bone qualitative properties are clinically relevant.

Fig. 2. Finite element models of tube-like cortical structures with constant volume and varying structure. The endosteal perimeter is defined as the periosteal diameter on left. This decreases cortical thickness but still increases bending resistance. For example 17% increase in diameter increases inertia by 52% and stiffness by 30%.

Also the gain of strength only by increasing size would require more energy from movement. Most likely for this reason, most of the long bones are built according to principle that same amount of material, when placed further away of mass center gives most resistance against bending and torsion through increased inertia (Fig. 2). For this reason many morphological variables have good correlation to mechanical properties (Jämsä et al. 1998, Lind et al. 2001). However, the bones are not perfect tubes and their shape is fine tuned to correspond to loading activities already during embryogenesis (Sharir et al. 2011).

2.5 Persistent organic pollutants (POP)

Criteria for classification of chemicals as POPs they have to be (1) highly toxic to humans and the environment, (2) persistent in the environment, resisting biodegradation, (3) taken up and bio-accumulated in terrestrial and aquatic ecosystems and (4) capable of long-range, transboundary atmospheric transport and deposition. Table 1 presents different POPs with their sources and possible
indication of skeletal effects. In the Stockholm convention there are 12 compounds (including their congeners) listed, whose usage were agreed to be limited. More recently this list was extended in 2009.

It has been approximated that due to industrialization only the level of dioxin-like compounds were tripled in the nature from 1920 to 1970. Fortunately their levels seem to decline slowly. However, recently a wider nominator endocrine disturbing chemical (EDC) is used for these chemicals. It includes POPs and many more and potentially involves 800 chemicals (WHO 2013), many of them are potentially toxic to bone.

Humans are exposed to POPs mainly via diet, especially meat, fatty milk and fish products. Due to lipophilicity of these compounds they are distributed to tissues with high lipid content, they are able to cross the placenta and during lactation they mobilize readily into mother’s milk that has high lipid concentration. For dioxin-like compounds lactational exposure is quantitatively more significant than placental transfer (Hurst et al. 2000, Li et al. 1995, Miettinen et al. 2005). This results in relatively high exposures to infants, who in the case of dioxins can be exposed to 20–25% of the mother’s body burden (Tuomisto 2001). High prenatal and neonatal exposure during critical windows of sensitivity may lead to potentially permanent developmental defects (Tuomisto & Vartiainen 2004, WHO 2013).

The governmental actions for risk management of these chemicals have shown to be effective and the levels of several POPs have turned to slow decline. However, they still are significant global pollutants due their persistency. While the regulation of these chemicals seems to have achieved the wanted effects on their existence, the chemicals designed to replace them are on rise and often possess endocrine disturbing chemical-type activity. (WHO 2013)
Table 1. List of POPs and their usage (or source). Indication if compounds were used in A1254 or Northern Contaminant Mixture (NCM) and bone toxicity are given.

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<tr>
<td>Aldrin</td>
<td>Pesticide to treat seed and soil.</td>
<td>3 [1]</td>
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<tr>
<td>(Oxy)Chlordane</td>
<td>Pesticide and as an additive in plywood adhesives</td>
<td>3 [2]</td>
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<tr>
<td>Dichlorodiphenyltrichloroethane (DDT)</td>
<td>Pesticide</td>
<td>p,p'-DDE[^3] [3-4]</td>
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<tr>
<td></td>
<td></td>
<td>p,p'-DDT[^4]</td>
<td></td>
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<tr>
<td>Dieldrin</td>
<td>Pesticide (agriculture)</td>
<td>3</td>
<td></td>
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<td>Heptachlor</td>
<td>Pesticide (building materials)</td>
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<tr>
<td>Hexachlorobenzene (HCB)</td>
<td>A solvent for pesticides (e.g. in Ky-5)</td>
<td>3 [5-6]</td>
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<tr>
<td>Mirex</td>
<td>Pesticide</td>
<td>3</td>
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<tr>
<td>Polychlorinated biphenyls (PCB)</td>
<td>Dielectric, coolant, lubricant, plasticizers, pesticide extender, flame retardants, de-dusting agents, waterproofing, microscopy fixative, surgical implants.</td>
<td>PCB52[^1] [7-10]</td>
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<td>Polychlorinated dibenzofurans (PCDF)</td>
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<td>2,3,7,8-PCDF[^2]</td>
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<td>1,2,3,7,8-PeCDF[^2]</td>
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<td>2,3,4,7,8-PeCDF[^2]</td>
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<td>1,2,3,4,7,8-HexaCDF[^2]</td>
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<td>1,2,3,6,7,8-HexaCDF[^2]</td>
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<td>2,3,4,6,7,8-HexaCDF[^2]</td>
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<td>1,2,3,4,7,8,9-HexaCDF[^2]</td>
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<td>1,2,3,4,7,8,9,10-OCDF[^2]</td>
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<tr>
<td>Toxaphene</td>
<td>Pesticides</td>
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<tr>
<td>Chlordecone</td>
<td>Hexabromobiphenyl</td>
<td>γ-HCH (lindane) to control head lice and scabies.</td>
<td>β-HCH[^3] [14]</td>
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<tr>
<td>Hepta and heptabromobiphenyl ethers</td>
<td>Recycling and reuse of articles containing these compounds</td>
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<tr>
<td>Pentachlorobenzene</td>
<td></td>
<td>Various mainly for surfactant properties</td>
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<tr>
<td>Tetra- and pentabromobiphenyl ethers</td>
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<tr>
<td>Perfluoroctanesulfonic acid (PFOA), its salts</td>
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<tr>
<td>Perfluorooctanesulfon fluoride</td>
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2.6 POP effects on bone tissue


Fig. 3. A schematic summary on skeletal toxicity of POPs constructed according to average results of animal experiments reported. The left image presents cortical effects and right trabecular. Green and grey colors present controls and exposed animals respectively.


HCB was the first POP shown to affect skeletal properties of adult laboratory rats (Andrews et al. 1988, Andrews et al. 1989, Andrews et al. 1990). Later similar effects have been observed in adult rats after exposure to A1254 (commercial PCB mixture) (Andrews 1989) and individual PCB congeners (Lind et al. 1999, Lind et al. 2000a, Lind et al. 2000b, Lind et al. 2004). These studies report a similar general pattern of decreased bone length, periosteal and endosteal perimeter with decreased cortical bone area (Ct.A.), but increased cortical thickness (Fig. 3) with consequently decreased bone strength.
Also the most toxic POP, TCDD, reduces bone size and strength (Jämsä et al. 2001). Somewhat surprisingly, TCDD can have some short term effects on trabecular bone with reduced bone remodeling only 5 days after exposure to a single high dose (Lind et al. 2009). A similar phenotype was present in cortical bones of maternally exposed rat offspring exposed in utero / lactationally to very low dose of TCDD including reduced BMD (Miettinen et al. 2005). The amplitude of the effect was dependent on the AHR genotype of rats (Miettinen et al. 2005).

In both TCDD and PCB exposed rats a mild decrease in trabecular bone volume fraction has been observed histologically (Jämsä et al. 2001, Lind et al. 1999, Lind et al. 2004). On the other hand, when studied with pQCT increased trabecular BMD has been reported with PCB153 (Lundberg et al. 2006) and PCB126 (Alvarez-Lloret et al. 2009, Lind et al. 2000a), the later with decreased trabecular area. The increased trabecular BMD has been observed in tibias from American alligators (Alligator mississippiensis) (Lind et al. 2004).

Histologically PCB 126 has been shown to increase osteoid (Lind et al. 1999). Interestingly overall collagen and hydroxyproline contents were decreased with increased number of mature cross-links (Lind et al. 2000b). Compositionally this is reflected as increased organic and inorganic contents, but reduced water content (Lind et al. 1999) (Lind et al. 2000b). More recently PCB126 exposure in adult rats has been reported to reduce matrix mineralization, crystal size and crystallinity (Alvarez-Lloret et al. 2009). In acute TCDD toxicity decreased monohydrogen phosphate levels have been reported indicating more mature bone tissue (Lind et al. 2009).

2.7 Nuclear receptors and bone toxicity of POPs

POPs are considered to be endocrine disrupting chemicals and some of them have been shown to be able to interfere with nuclear receptors. It has been shown that dioxin-like chemicals can bind to AHR (Okey et al. 2005); Tributyltin with retinoid-X-receptor and peroxisome proliferator activating receptor subtype gamma (PPARγ) (Grun & Blumberg 2006); and PFOA only with PPARγ and PPARα (Vanden Heuvel et al. 2006). Due the focus on mainly dioxin-like compounds brief explanations of AHR, estrogen and vitamin D signaling will follow focusing bone cell biology and skeletal phenotypes during the absence of ligand or receptor.
2.7.1 Aryl Hydrocarbon receptor

The most studied group of POPs, dioxins mainly mediated their effects via AHR. This member of the basic helix-loop-helix Per-Amt-Sim family of proteins binds specifically the “classical” AHR ligands dioxin-like compounds, polyaromatic hydrocarbons as well as a variety of structurally diverse “nonclassical” AHR ligands, such as benzimidazole drugs (e.g. omeprazole), indoles, flavones and midazoles. In addition to regulating xenobiotic metabolism and mediating toxic effects AHR has ubiquitous physiological functions and is involved at least in organogenesis, reproduction, neuronal differentiation, vascular development, cardiac function, cell cycle control, immunity and regulation in circadian rhythms (Pohjanvirta 2012).

As mentioned previously, the lipophilicity of these chemicals allows them to penetrate cell membranes and in cytoplasm these chemicals can bind to AHR. This causes release of receptor bound to hsp90 and other possible chaperons such as p60s-src. Then the new complex travels to nucleus and binds with aryl hydrocarbon nuclear translocator protein (ARNT) and starts reading the specific regulatory aryl hydrocarbon response elements (AHRE), which is also referred as dioxin-response element (DRE) (Lukinmaa et al. 2007, Okey et al. 2005, Okey 2007). Han/Wistar rats have been found to be a dioxin resistant rat line for their point mutation in AHR (Pohjanvirta et al. 1998).

As a curiosity it could be mentioned, that human AHR is highly homologous to guinea pigs, a species very sensitive to dioxin (Korkalainen et al. 2001) with lethal dose 50% (LD50) of 0.6–2.1 μg/kg (compared to hamsters and male H/W rats 3000 μg/kg). Considering varying ligand binding activity to the receptor between species and the functional responses from altered hormonal activity to changes in bone morphology after POP exposure are often non-monotonic (WHO 2013), translation of data is often difficult.

The AHR is also found at least in osteoblasts (Ilvesaro et al. 2005, Ryan et al. 2007) and osteoclasts (Ilvesaro et al. 2005, Korkalainen et al. 2009) further indicating capability of dioxins to disturb well-orchestrated symmetry of osteoblasts and osteoclasts during the remodeling process. Indeed, it can be seen in cell culture of calvarial osteoblasts that with normal cellular proliferation the mineral nodule formation is decreased (Gierthy et al. 1994) with reduced levels of alkaline phosphatase (ALP), collagen type I (Singh et al. 2000) OPN, Ocn and bone sialoprotein (Gierthy et al. 1994, Koskela et al. 2012, Ryan et al. 2007, Singh et al. 2000, Wejheden et al. 2006). Osteoblast function was possible to
rescue with AHR antagonists MNF (3′4′ methoxynitroflavone) (Ryan et al. 2007) or resveratrol (3,5,4′-trihydroxystilbene) (Singh et al. 2000). Furthermore TCDD does not have any effects on osteoblasts from AHR knockout mice $Ahr^{-/-}$ (Korkalainen et al. 2009).

The data on osteoclasts indicates disturbed osteoclastogenesis (Iqbal et al. 2013, Korkalainen et al. 2009), while evidence of weak activation of differentiated osteoclasts exists (Ilvesaro et al. 2005). Also the genetically modified mice with constitutively active AHR (CA-AHR) present a skeletal phenotype with increased osteoclastic activity. In the same model collagen synthesis was increased, while the ALP levels were decreased, all together causing reduced cortical BMD on larger cross-section (Wejheden et al. 2010). CA-AHR also caused reduction of trabecular area (with consequently decrease in trabecular BMC) in females while it increased trabecular BMD in males.

2.7.2 Estrogen Receptors (ER)

Some of the skeletal effects of POPs (Kuiper et al. 1998) or AHR binding chemicals (Ohtake et al. 2003) might be mediated by disrupted estrogen signaling. The effects of estrogens (and androgens) on the skeleton have been well studied and it is known that AHR signaling could interfere with ER signaling for which the following four possible mechanisms have been suggested: (1) increased metabolism of estradiol (E2), (2) reduced amount of ER, (3) suppressed E2 induced transcription and (4) competition of shared cofactors (Nilsson et al. 2001).

Estrogen affects bone remodeling by inducing osteoblastic production of Fas ligand (FasL) (Krum et al. 2008) that activates apoptosis in osteoclasts (Nakamura et al. 2007). Recently estrogen ablation has also been reported to increase osteocytic apoptosis in ovariectomy (OVX) models (Emerton et al. 2010, Huber et al. 2007). It is well known that OVX increases body mass (Banu et al. 2012, Tivesten et al. 2004), reduces trabecular bone fraction (Banu et al. 2012, Maimoun et al. 2012, Tivesten et al. 2004) and cortical area and thickness (Banu et al. 2012) with consequently bone strength reducing effect (Brennan et al. 2009, Väänänen & Härkönen 1996). At bone matrix level, the lack of estrogen decreases hardness of both cortical and trabecular compartments as well as elastic modulus and energy dissipation in trabecular bone (Brennan et al. 2009, Maimoun et al. 2012).

These OVX induced skeletal effects can be compensated with hormone replacement therapy by administrating selective estrogen receptor modulators
(Huber et al. 2007), bisphosphonates, parathyroid hormone (Brennan et al. 2009), estradiol or estradiols and androgens (Moverare et al. 2003, Tivesten et al. 2004).

ER type α is the main mediator of estradiol effects on bone (Sims et al. 2003). ERα has been shown to be important for skeletal maturation in both men (Smith et al. 1994) and women (Quaynor et al. 2013). Increased trabecular bone volume fraction and decreased cortical thickness has been reported in ERα null mice (Callewaert et al. 2009, Lindberg et al. 2001, Lindberg et al. 2002, Sims et al. 2002). In males these effects to require orchidectomy to appear (Lindberg et al. 2001, Lindberg et al. 2002). In ERα−/− also increased serum leptin concentration and body fat percentage has been observed (Lindberg et al. 2001). In ERβ−/− mice increased cortical bone quantity has been observed in adult females, but the null genotype is not enough to suppress the catabolic effects of OVX (Windahl et al. 1999) or aging (Ke et al. 2002).

2.7.3 Vitamin D Receptor

Vitamin D has been shown to have multiple health promoting effects from reducing vascular calcification and osteoporosis in epidemiological studies. 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) is the active form of the Vitamin D. It mainly stimulates calcium uptake in the intestine, but can activate bone resorption and inhibit bone formation, when calcium levels are low (Lieben & Carmeliet 2013a). In bone it has similar effect as parathyroid hormone (PTH), i.e. increased osteoclastogenesis and regulation of expression of osteoblastic genes. This effect might be because PTH stimulates CYP27B1 production and thus activates vitamin D signaling. This, on the other hand, stimulates fibroblast growth factor 23 production that acts as negative feedback for both PTH and CYP27B1 (Lieben & Carmeliet 2013b).

It has been shown that increased 1,25(OH)$_2$D$_3$ can increase cortical porosity and decrease bone formation and cortical thickness (Idelevich et al. 2011). On the other hand, it has been also shown that alfalcaldiol, a prodrug metabolized to 1,25(OH)$_2$D$_3$, could prevent bone loss in OVX model by inhibiting osteoclastogenesis (Shibata et al. 2002).

In a vitamin D receptor (VDR) knock-out model the mice have been shown to suffer from alopecia, hypercalcaemia, infertility and rickets with epiphyseal widening and thinner cortex. In the same mice the number of osteoclasts was similar with wild type mice, while osteoblasts were rather active, but unable to mineralize (Yoshizawa et al. 1997). However, skeletal phenotype can be rescued
by restoring mineral homeostasis (Amling et al. 1999). Indeed recently it has been shown, that ablation of VDR in the intestine can cause thinner cortex, increase cortical porosity and lead to decreased trabecular bone volume fraction, while osteocytic VDR-KO have relatively normal bone phenotype (Lieben et al. 2012) and osteoblastic VDR-KO even increased bone mass (Yamamoto et al. 2013).

Interestingly, it has been shown that maternal dioxin exposure increases renal CYP27B1 activity leading to increased active 1,25(OH)2D3 in serum with decreased BMD, cortical thickness and expression of bone formation marker genes with increased the amount of osteoid (Nishimura et al. 2009). On other hand, adult exposure to dioxin-like PCB126 has been reported to decrease 25-OH vitamin-D levels with altered bone mineralization (Alvarez-Lloret et al. 2009). Early studies on POPs, have indeed shown elevated serum D3 together with PTH and cholesterol after HCB exposure in adult rats as well (Andrews et al. 1988). Two later reports showed increased bone density with decreased bone volume (ostesclerotic changes) (Andrews et al. 1989) and increased bone strength, cortical area with decreased medullar volume (Andrews et al. 1990). However, later only changes in PTH levels were observed in HCB exposed rats (Andrews et al. 1990).
3 Aims of the study

The main goal of this thesis is to understand the effects of POPs on bone strength and morphology, and the role of AHR in bone toxicity and skeletal development. In this thesis, bone was studied at multiple hierarchical levels to test the hypothesis that POPs affect not only bone quantity, but also bone quality. This is important for environmental health risk assessment. Four individual subworks have been build around the following hypotheses:

1. Developmental TCDD exposure interferes with development of bone material properties, which causes bones to be weaker.
2. Exposure to NCM, the mixture of chemicals present in the Canadian Arctic (including heavy metals), decreases bone size and strength at dose levels detected in the present arctic population.
3. PCB mixture A1254 causes skeletal changes and this response is proportional to TCDD exposure estimated via chemical TEQ of A1254.
4. The AHR has a major role in TCDD induced bone toxicity and bone homeostasis.
4 Materials and methods

4.1 Chemicals

Details of the chemical for studies I and IV as well as II and III have been described elsewhere by Miettinen et al. (Miettinen et al. 2005) and Chu et al. (Chu et al. 2008), respectively. Northern Contaminant Mixture (NCM) used in study II was reconstructed according to the profile of chemicals found in 130 maternal blood samples of Canadian Arctic populations (Butler Walker et al. 2003, Van Oostdam et al. 1999). It consisted of 14 PCB congeners, 12 organochlorine pesticides and MeHg. Technical grade A1254 (AccuStandard, New Haven, CT) was used in study III. Concentrations of individual PCB and PCDF congeners in dosing solution and the corresponding concentrations of toxic equivalent (TEQ) was estimated according to van den Berg et al. (Van den Berg et al. 2006).

4.2 Study permissions

Study protocols were reviewed and approved by either the Animal Experiment Committee of the University of Kuopio (Kuopio, Finland) No. 03–04 in study I and No. 05–42 for study IV, or the Canadian Council Animal Care guidelines and Health Canada’s Institutional Animal Care Committee No. 01045 for studies II and III.

4.3 Experimental designs

4.3.1 Maternal TCDD exposure

A single intragastric dose of 0.01; 0.03; 0.1; 0.3; and 1.0 µg TCDD/kg or corn oil (vehicle) was administered to pregnant Sprague-Dawley rats at the previously detected most sensitive time point on gestational day (GD) 11 (Miettinen et al. 2005). This is two days before the condensation of mesenchymal cells starts in the rat skeleton (Hebel & Stromberg 1986). All the dose groups were used in study III for dose modelling but only the highest dose was examined in study I. This section was done based on observation that exposure of pregnant rats to 1.0 µg TCDD/kg body weight (bw) is the smallest dose able to significantly decrease bone geometry and mechanical strength without maternal toxicity (Miettinen et al. 2005).
Also in study I only females were selected since they have been demonstrated to be more sensitive to dioxin exposure than males. Litters were weaned on postnatal day (PND) 28 and bones were collected on PND 35 and PND 70. As the elimination half-life of TCDD in adult female Sprague-Dawley rats is about 26 days (Li et al. 1995), the lactational exposure continued until weaning, and TCDD is present in tissues of the offspring on PND 35 and to lesser extent also on PND 70.

4.3.2 Maternal NCM exposure

In study II in order to evaluate the impact of perinatal NCM exposure on bone development in rat offspring, pregnant Sprague-Dawley dams were administered corn oil (as vehicle), or NCM at dose levels of 0.05, 0.5, or 5 mg/kg bw from the GD 1 until weaning on PND 23 via Teddy Graham cookie (Nabisco Ltd., Toronto, ON) as previously described (Chu et al. 2008). Animals were sacrificed PNDs 35, 77 and 350 following sample collections.

4.3.3 Maternal A1254 exposure

For study III pregnant Sprague-Dawley dam rats were administered corn oil (as vehicle), or A1254 (15 mg/kg bw/day, equivalent to 0.123 µg TEQ/kg bw/day) from the GD 1 until weaning on PND 23 via cookies and bone samples were collected as in NCM study.

4.3.4 Role of AHR in skeletal development and TCDD toxicity

In study IV Ahr<sup>−/−</sup> and Ahr<sup>+/+</sup> mice in a C57BL/6J background (Schmidt et al. 1996) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained using heterozygous breeding. At the start of the treatment the mice were 8–12 weeks old, and randomized by body weight into treatment groups of 6 males and 6 females per genotype.

TCDD was mixed in corn oil using a magnetic stirrer and ultrasonicated for 20 min before administering by oral gavage at 4 ml/kg, whereas controls were given pure corn oil. To rapidly achieve the kinetic steady state (0), the total dose of 200 µg/kg was divided into one loading dose (x<sub>0</sub>; 40 µg/kg) and 9 maintenance doses (x<sub>0</sub>;18 µg/kg), in weekly intervals (τ), which were calculated according using first-order kinetics (equation 1) and assuming 100% bioavailability (Gibaldi 40
To calculate elimination constant ($K$) elimination half-life ($t_{1/2}$) of 10 days was used (Birnbaum 1986).

\[ x_0^* = x_0 \left( \frac{1}{1 - e^{-Kt}} \right) \]  
\[ K = \ln \left( \frac{2}{t_{1/2}} \right) \]  

At the end of the treatment period of 10 weeks (one week after the last maintenance dose) the mice were anesthetized with $\text{CO}_2/\text{O}_2$ (70/30%). Blood samples were drawn from the left ventricle using Venoject needles (Terumo) and blood collection tubes, and the mice were killed by exsanguination.

4.4 Bone sample preparation

Bones were dissected and cleaned of soft tissue. Femoral and tibial lengths were measured from distal point of condylus medialis to caput femoris or from medial condyle to the medial malleolus, respectively using an electronic sliding caliper (IP65, Sylvac SA, Crissier, Switzerland). Bones were then stored in Ringers solution at $-20^\circ \text{C}$ until analysis.

After mechanical testing, bones were air dried in room temperature for 24 h before embedding in polyester resin (KLEER-SET type FF Resin and hardener kit, MetPrep Ltd, Coventry, United Kingdom) for nanoindentation measurements. A metallurgical diamond saw was used to cut a 1mm thick slice from the tibial
cortical bone close to the fracture surface from mechanical testing (publication I Figure 1 A.) in study I. In study IV two blocks were prepared. The first block was prepared by cutting the whole block in two from the middle of diaphysis (about 50% of length). The proximal half was used for cortical bone characterization, while to study trabecular bone an additional cut was made 15% of length proximally from femoral condyles. All surfaces were prepared by grinding on sand papers using water to cool and lubricate the sample. Grit size was successively decreased and finalized by polishing on cloth with a small amount of 0.05 µm γ-alumina suspension. Before indenting bones were cleaned using an ultrasonicator.

4.5 Peripheral Quantitative Computed Tomography (pQCT)

All bones were scanned using a pQCT system (Stratec XCT Research SA+ with software version 5.50R, Norland Stratec Medizintechnik, GmbH, Birkenfeld, Germany). System stability and calibration was performed with a phantom provided by the manufacturer. Transverse tomographic slices were collected perpendicular to the long axis of the bone with a voxel size of 0.07×0.07×0.5 mm³. A scan to represent cortical bone properties was collected at 50% of bone length. Representative slice from metaphyseal bone was collected at 15% from condyles.

The manufacturer’s software was used for numerical analysis. To analyze cortical bone a density value of 710 mg/cm³ was used for thresholding. For trabecular bone periosteal surface was segmented by thresholding images by 400 mg/cm³. Inside of the segmented bone, trabecular and subcortical bone were separated at 200 mg/cm³, the lower pixel values representing trabecular bone.

4.6 Microtomography (μCT)

In μCT imaging recent guidelines for the assessment of bone microstructure in rodents using micro–computed tomography were followed (Bouxsein et al. 2010). The source voltage was set to 49 kV with 200 µA current in micro computed tomography (μCT) device (SkyScan 1172, SkyScan, Kontich, Belgium). An aluminium filter with thickness of 0.5 mm was used to reduce the beam hardening effect and noise was reduced by using frame averaging of three frames. Tibias were imaged wrapped in parafilm and vertebrae were scanned in 1 ml PCR tubes using respective isotropic voxel sizes of 4.7 µm and 9.9 µm.
Images were reconstructed in NRecon using ring artefact correction of 10 and beam hardening of 40%. The cross-sectional datasets were analyzed with the CTAn (version 1.12.0.0+) software provided with the device. Analysis of the trabecular region of interest (ROI) in tibia was 0.19 mm (40 slices) from the distal end of the growth plate towards the metaphysis, and extended 1.64 mm (350 slices). In vertebrae, ROI was 0.10 mm (10 slices) after distal growth plate and extended over the whole medullar space until 0.10 mm (10 slices) to proximal growth plate. The cortical ROI was 2.44 mm (520 slices) from the growth plate and extended 0.94 mm (200 slices) further. ROI for trabecular bone was separated from the cortical bone with a freehand drawing tool while a bitwise operation ROI-shrinks wrap function was used for cortical bone analyses. Tissue mineral density was calibrated against hydroxyapatite (HA) phantoms provided by the manufacturer. A global threshold value was used to separate bone from the surrounding matter. Thresholded images were despeckled and analyzed. Cortical bone analyses were performed in three steps. First overall morphology was analyzed with medullary area included in ROI. For cortical porosity and tissue mineral density (TMD) analyses, thresholded image was selected as ROI using bitwise operation and original images were reloaded for density analysis and thresholded with appropriate threshold revealing pores.

4.7 Whole bone biomechanical testing

The mechanical behavior of bones were evaluated in three-point-bending tests for femoral (studies II and III) and tibial diaphysis (studies I-IV) or in site specific axial loading of the femoral neck (studies II and III) using a universal mechanical testing machine (Instron 3366, Instron Corp., Norwood, MA, USA) as described previously (Jämsä et al. 1998, Miettinen et al. 2005, Peng et al. 1994). In diaphyseal tests bones were positioned horizontally with the anterior surface upwards on the supports with a span length of 13 mm or 6.5 mm for rat and mouse bone, respectively. For the testing of the femoral neck the proximal half was placed in a plastic holder with anatomically shaped holes for femurs changing diameter every 0.1 mm, and load was applied axially on the femoral head using concave loading cup. In all experiments load was applied with constant crosshead speed of a 0.155 mm/s until failure, and load-deformation curves were recorded. Stiffness was calculated as a slope of the linear part of the force-displacement data and maximal force, deformation and energy absorption
were defined. Additionally for study IV, yield parameters were defined from the
point where the fitted slope separated from the measured.

4.8 Nanoindentation of bone tissue

Material properties of bone extracellular matrix were analyzed with
nanoindentation testing using a Berkovich diamond tip, equipped in CSM-
NanoHardnessTester system controlled with Indentation v.3.75 software (CSM
Instruments SA; 2034 Peseux, Switzerland). Two different indentation protocols
were used denoted as quasistatic and dynamic loading modes. Indenter properties
have been presented in more detail earlier (Zioupos 2005, Zioupos & Rogers
2006). Creep and static material properties were analyzed from a quasistatic
loading protocol with 60 s or 15 s loading and unloading phase and 60 s or 30 s
hold phase at peak load of 10 mN for rat (study I) and mice (study IV) bone,
respectively. Viscoelastic properties were analyzed from dynamic loading
protocol consisting of 1.00 mN vibration amplitude at 1.5Hz superimposed on the
loading signal applied with 0.05 N/Ns until depth of 750 nm. The following
parameters were defined from every measurement: peak load \( F_{\text{max}} \), peak
displacement \( h_{\text{m}} \), contact depth \( h_{c} \), projected contact area \( A_{p} \) and contact stiffness
\( S \).

The \( A_{p} \) (equation 3) was defined from area function calibrations by using a
series of 110 indentations at different loads and was approximated by a
polynomial function for the contact depth \( h_{c} \)

\[
A_{p}(h_{c}) = 24.5 + \sum_{n=0}^{4} C_{n} \cdot h_{c}^{n/2}.
\]  

(3)

Using this relationship as well as the Oliver Pharr method (Oliver & Pharr 1992)
to calculate \( S \), where a power law fitted polynomial between 98% and 40% of
\( F_{\text{max}} \) from unloading phase data was defined, well established parameters were
defined as in equation 4 for universal (instrumented) hardness \( H_{IT} \), in GPa)
and in equation (5) indentation modulus \( E_{IT} \) (Oliver & Pharr 1992). \( H_{IT} \)
was calculated as peak force ratio to projected area (equation 4):

\[
H_{IT} = \frac{F_{\text{max}}}{A_{p}(h_{c})} \quad (4)
\]
The reduced (effective) modulus \((E_r)\) was calculated according equation (6) (Oliver & Pharr 1992). The reduced modulus was used to determine the indentation modulus \(E_{IT}\) as

\[
E_{IT} = \left[ \frac{1}{E_i} \left( 1 - \frac{v_i^2}{E_i} \right) \right] \left( 1 - v_s^2 \right)
\]

where \(v\) is the Poisson ratio, \(i\) refers to indenter, and \(s\) to sample, respectively. These values used were: \(v_i = 0.07\), \(v_s = 0.3\) and \(E_i = 1141\) GPa.

The quasistatic protocol employed hold phase necessary for stabilizing viscosity and presence of “nose” in unloading phase (Ebenstein & Pruitt 2006). During the hold at maximal load the material creeps and this can be analyzed from consequent deformation as following:

\[
dh/h = A \ln [Bt + 1]
\]

where \(h\) is the indentation depth (nm), \(t\) is the time (s) and \(A, B\) are constants representing creep amplitude and initial creep rate, respectively. The initial 30 s of hold phase time-deformation data was fitted to equation (7) (Chudoba & Richter 2001) using a numerical approximation code based on least square fitting in the SigmaPlot for Windows (v.10) or Matlab (Mathworks) (10,000 iterations, tolerance 0.0001, starting values \(A = 0.02; B = 1\)) (Zioupos & Rogers 2006).

The ‘plasticity index’ \((I_p)\) was defined from the dynamic protocol. The plastic response of bone was calculated from load penetration data according to Briscoe et al. (Briscoe et al. 1998) using equation 8 for \(I_p\). Total energy, a sum of energies absorbed in plastic \((\psi_p)\) and elastic \((\psi_e)\) modes, was defined by numerical integration under the loading trace. The ratio of the energies absorbed plastically over the total energy input was calculated by:

\[
I_p = \frac{\psi_p}{\psi_p + \psi_e}
\]

The viscoelastic behaviour of bones was also analyzed from the dynamic protocol because both storage \((E_{stor})\) and loss modulus \((E_{los})\) can be calculated within each cycle in the loading phase. We used the dynamic mechanical analysis (DMA) option available in v.3.75 of the CSM software. The modulus values from DMA
for $E_{\text{tot}}$ and $E_{\text{loss}}$ vary with the depth of indentation and came to some stable value for penetration depths of 100nm (Zioupos 2005, Zioupos & Rogers 2006) or more.

In the study I, approximately seven indentations with both protocols were performed on anterio-medial section of cortical bone as indicated in Fig. 5.

Fig. 5. Polished bone surface in nanoindentation instrument. White crosses indicate locations for 16 indentations with quasistatic (1–8) and dynamic (9–16) protocols. Scale bar shows 200 µm.

Approximately twelve indentations on anterior segment of cortical (Fig. 6) and eight indentations on trabecular bone (Fig. 6) were performed with both dynamic and quasistatic loading protocols. Indentations were performed on control and dioxin exposed $Ahr^{-/-}$ and $Ahr^{+/+}$ mice of both genders.

Fig. 6. Indentations positioned in A) anterior segment of femoral cortex and B) various trabeculae over the transverse cross-sections. Specific care was taken to measure individual trabeculae using both loading protocols. Scale bars shows 50 and 200 µm in A and B, respectively.
4.9 Serum markers for bone remodeling

Bone formation and resorption markers were measured in study IV from serum samples by analyzing N-terminal propeptide of type I collagen levels, (PINP) (Immunodiagnostic Systems, Boldon, UK) and C-terminal telopeptide of type I collagen levels (CTX), RatLaps™ (Immunodiagnostic Systems, Boldon, UK) in enzyme immunoassays following the manufacturers’ introductions. Measurements were performed in duplicates for both markers. To estimate if bone remodeling is in balance the PINP:CTX ratio was calculated (Chopin et al. 2008).

4.10 Osteogenesis PCR array

The mouse osteogenesis PCR array (The Mouse Osteogenesis RT² Profiler PCR Array, PAMM-026A, SABiosciences, a Qiagen Company) was performed using pooled samples of three animals per group in study IV. Humeri were homogenized using Ultra-Turrax T8 homogenizer, followed by RNA extraction using Trizol (Life Technologies, Carlsbad, CA, USA) and RNeasy Mini kit (Qiagen, Hilden, Germany). 1 µg of pooled RNA was generated into cDNA using RT² First Strand Kit (SABiosciences). cDNA samples were mixed with RT² qPCR Master Mix (SABiosciences) and distributed in every well on PCR array plate. This array profiled the expression of 84 genes related to osteogenic differentiation (skeletal development, bone mineral metabolism, cell growth and differentiation, extracellular matrix proteins, cell adhesion proteins, transcription factors and regulators). The array also contained controls for RT reaction and PCR reaction as well as a genomic DNA control. Applied Biosystems 7000 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) was used to determine Ct-values of each well. The fold-changes in Ct-values were calculated using SABiosciences web-based data analysis program.

4.11 Statistical analyses and dose response modelling

Statistical comparison between groups were conducted using SPSS Statistical Software (SPSS Inc. Chicago, USA) (Study I ver. 16.0 Study II and III and Study IV PASW18.0). Distributions of variables were studied using Kolmogorov Smirnov (variations distributed equally around mean) test or by P-P plot and boxplot methods. In studies II and III variables were log-transformed for statistical comparison between groups or tested using the Mann-Whitney test. All
data were also tested with Levene test for homogeneity (deviations with equal width). If homogeneity test failed for log-transformed data, Kruskal-Wallis one way ANOVA rank sum test was used, followed by Mann-Whitney U test, with compensation for multiple comparisons (p≤0.017) (studies II and III) or assumption of equal variances for t-test was not used (studies I and IV). In cases in which all requirements were fulfilled, groups were compared with Student’s t-test (studies I, IV) or one-way analysis of variance (ANOVA) and Dunnett post hoc test when ANOVA was significant. Data were evaluated in one-way or three-way repeated measures ANOVA, if one of the variables had degrees of freedom two or higher. Repeated measures ANOVA was performed to assess differences over age, treatment, sex; and interactions “age x treatment”, “age x sex”, “treatment x sex”, and “age x treatment x sex”.

Relationships between bone mineralization and nanomechanical parameters were evaluated from Pearson’s correlations, and analysis of covariance (ANCOVA) was performed with SigmaPlot for Windows (version 10) (SYSTAT Software, Point Richmond, CA) to evaluate if these relations differ between groups. SigmaPlot was also used for generating graphs in publication I. Graphs in this thesis were made with GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA).

Finally PROAST (version 20.2) plugin in R-software (version 2.7) was used in dose-response modelling. Dose-response relations were studied by fitting mathematical models to data using the Benchmark Dose (BD) method (Slob 2002) allowing more accurate estimation of toxic doses than traditional “no observed adverse effect level”. A family of nested models was fitted to morphological and mechanical variable after NCM and TCDD exposures in studies II and III, respectively. The BD was read from a fitted model at 5% response level in agreement with European Food Safety Authority’s (EFSA) guidelines for continuous endpoints (European Food Safety Authority 2009). Modelling was performed only for all age groups in study II but only for PND 35 in study III. Both genders were implemented into models simultaneously. The likelihood ratio test was used as a formal criteria to select one member from a family of nested models including minimal number of fitted parameters, but still having the ratio between BD and BD at the lower bound of the 95% confidence interval (BDL) over 10.

In study III TCDD dose was read from the fitted dose-response curve at measured effect size for A1254 exposure. These “TCDD equivalents” were
compared against chemically calculated toxic equivalent (TEQ) to test if bone effects of A1254 are mediated by TCDD like compounds. 

$$TE_q = \Sigma (C_i \times TEF_i)$$ (9)

These theoretical chemically calculated TCDD TEQs (equation 9) for A1254 were based on concentrations (C) of PCBs and PCDFs reported previously by Rushneck et al. (Rushneck et al. 2004) and Kodavanti et al. (Kodavanti et al. 2001) and their toxic equivalency factor (TEF) (Van den Berg et al. 2006).
5 Results

5.1 Bone size

Developmental exposure to persistent organic pollutants decreased bone length (studies I-III). Because no statistically significant gender-by-treatment or gender-by-treatment-by-age interactions were detected for bone geometry or density data, male and female offspring were combined for NCM and A1254. TCDD, NCM and A1254 decreased tibial length by 4.2%, 5.2% and 7.4% in PND 35 rats, respectively. However, only after TCDD exposure effects remained significant at PND 70, while bone length was the same or close to controls in rats exposed to NCM and A1254, although measured at PND 77. Similarly to previous reports in TCDD, NCM and A1254 effects were not present after one year. On the other hand, adult exposure to TCDD neither caused changes in tibial length nor vertebral height or width (study IV).

5.2 Bone morphology and mineralization

5.2.1 Cortical bone

Besides reducing overall bone length, TCDD, NCM and A1254 reduced cross-sectional cortical bone size. Cortical bone morphology was measured in 2D with pQCT (studies I - III) and in 3D with µCT (study IV). TCDD, NCM and A1254 all decreased cross-sectional parameters at PND 35 (Fig. 7). A1254 decreased cross-sectional Ct.A. at most by 16% (p < 0.01), while both TCDD and NCM decreased Ct.A. by 14% (p < 0.01 and p < 0.05, respectively). These changes were due to decreased periosteal and endosteal circumferences by 7% and 5% for TCDD; 7% and 7% for NCM; as well as 6% and 5% for A1254 respectively. This was further seen as decreased cortical thickness (Ct.Th) by 8%, 7% and 14% for TCDD, NCM and A1254, respectively. The cross-sectional data from pQCT suggested decreased mechanical strength, with decreased polar moment of inertia (J) (~27%) and moment of resistance ~23%) for TCDD, and decreased J by 31% and 29% and polar strength strain index by 22% and 23% for NCM and A1254, respectively.

In developmental studies I-III, only TCDD caused changes in cross-sectional bone properties at PND 70 (publication I, table I), while no significant treatment-
related changes were observed for these parameters at PND 77 for NCM or A1254. There were no detectable changes in any of the studies I-III at PND 350. However, it should be pointed out that exposure had terminated at PND 28 for TCDD and terminated PND 23 for NCM and A1254.

In study IV, pQCT derived diaphyseal parameters indicated statistically significantly decreased Ct.Th in TCDD exposure in $Ahr^{+-}$ females as well as reduced Ct.Th and Ct.A in $Ahr^{-/-}$ females.

Fig. 7. Representative cross-sectional images from PND 35 control, NCM (0.05, 0.5 and 5 mg/kg) and A1254 exposed male and female rats. A1254 caused visually detectable changes, with similar magnitude compared to 5mg/kg of NCM. These results might be due to low density areas at endosteal side of NCM exposed rats, which are not included in the thresholded images.
High-resolution μCT showed also decreased Ct.Th in TCDD-treated \( \text{Ahr}^{+/+} \) females and control \( \text{Ahr}^{-/-} \) females (Fig. 8) as well as reduction in Ct.Th cortical thickness in males. Additionally, cortical volume was decreased in TCDD exposed \( \text{Ahr}^{+/+} \) males and in females with AHR ablation. In TCDD exposed \( \text{Ahr}^{+/+} \) males the medullary volume was also smaller. Decreased size of anterior crest (Fig. 8) and reduced eccentricity in TCDD exposed wild types and control knockout females were seen.

Fig. 8. The upper four images present variations in cortical bone morphology and density in tibias of female control and TCDD exposed AHR wild type (\( \text{Ahr}^{+/+} \)) and knockout (\( \text{Ahr}^{-/-} \)). Lower four pictures are 3D representations of cortical pores extrapolated from the upper pictures.
Study I showed observable difference in cortical porosity (Figure 1. in supplementary material of publication I). A detailed analysis on cortical porosity was performed on µCT data (study IV) as described in paragraph 4.6. These results (Fig. 8) indicated that dioxin exposure increases cortical porosity (Ct.Po) by 13% in TCDD exposed $Ahr^{+/+}$ females mainly by increasing pore thickness (Po.Th) of 32 µm in controls to 43 µm in TCDD exposed (p < 0.0001). Po.Th was also increased by 16% at statistically significant level in control $Ahr^{-/-}$ females but it was not sufficient to cause statistically significant changes in Ct.Po. However, TCDD exposed $Ahr^{-/-}$ males had reduced number of pores (23%) with increased spacing (4 µm) and decreasing Ct.Po by 23%.

It is possible to estimate bone mineralization level from both pQCT and µCT data, where µCT produces estimation of tissue density (tissue mineral density - TMD) while pQCT returns volumetric density (bone mineral density - BMD) including porosity as well as overall content of mineral in bone (bone mineral content - BMC).

In study II exposure to NCM did not cause any statistically significant changes in cortical BMD (Ct.BMD). In study III A1254 decreased tibial Ct.BMD by 3%. Consistent changes were neither observed at PND 77 nor at PND 350. Both maternal and adulthood exposure to TCDD as well as AHR ablation decreased Ct.BMD. In study I developmental exposure to TCDD caused reduction in cortical BMC (Ct.BMC) being 16 % lower at PND 35 and 11 % lower at PND 70 in the exposed animals compared to controls. Ct.BMD was reduced by 0.9 % at PND 70 (p < 0.05). In study IV, TCDD exposed $Ahr^{+/+}$ and control $Ahr^{-/-}$ females Ct.BMD was decreased by 3% in both. However µCT derived mineralization parameters showed a trend of increased Ct.TMD in TCDD exposed $Ahr^{+/+}$ (not statistically significant). In control $Ahr^{-/-}$ males, Ct.TMD was decreased by 2% (p < 0.05), while it was increased by 4% (p < 0.005) after TCDD administration to $Ahr^{-/-}$ males.

**5.2.2 Trabecular bone**

Trabecular bone mineral density (Trab.BMD) was decreased at the lowest dose of NCM at PND 35 in males, while there was significant dose dependent increase in Trab.BMD in males at PND 77 at doses of 0.5mg/kg and 5mg/kg (Fig. 9). An even higher increase was detected in Trab.BMD in males exposed to A1254 at this time point. Neither NCM nor A1254 altered trabecular bone parameters in females at any studied time point.

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Adult exposure to TCDD in *Ahr*+/+ increased pQCT derived Trab.BMD in both females and males by 54% and 28%, respectively. Also in AHR ablating females Trab.BMD was increased by 16%.

Trabecular bone morphology was further studied from proximal tibiae and 4th lumbar vertebrae (Fig. 10) using µCT. In tibia, TCDD exposure increased the trabecular bone volume fraction (BV/TV) by 58% and 122%, respectively in both male and female *Ahr*+/+ mice. This was mainly caused by increased trabecular number (Tb.N) (by 56% and 113%, in males and females, respectively) and decreased trabecular separation (Tb.Sp) (by 17% and 25%, respectively). The trabecular pattern factor (Tb.Pf) was decreased (by 68% and 58%, respectively), indicating higher connectivity of trabecular bone. A related parameter, structural model index (SMI), was decreased (by 45% and 34% in males and females, respectively), indicating more plate-like trabeculae rather than sphere-like structure.

Similarly in vertebrae in both genders of *Ahr*+/+ mice, the BV/TV of lumbar vertebrae was increased by TCDD-exposure (by 25% and 39%, respectively) (Fig.
Further, increased SMI and decreased Tb.Pf indicated again trabeculae in TCDD-exposed mice to be more plate-like trabecular structure and connected in both genders.

AHR ablation increased BV/TV (23% and 41%, in males and females respectively) as compared to control $Ahr^{+/+}$ mice. Again this was mainly due increased Tb.N (22% and 48% in males and females, respectively), and decreased Tb.Sp (by 11% and 21%, respectively). Increased SMI and decreased Tb.Pf suggested again that the trabecular mesh has more plate like structures with higher connectivity in female $Ahr^{-/-}$ mice.

A similar pattern of increased connectivity and more plate-like trabecular morphology was observed in the vertebral trabecular bone of female $Ahr^{-/-}$ mice compared to the $Ahr^{+/+}$ mice, as indicated by decreased Tb.Pf (by 40%) and SMI (by 26%). No differences in vertebral trabecular bone structure were seen between male $Ahr^{-/-}$ and $Ahr^{+/+}$ mice. TCDD exposure did not affect the tibial or vertebral trabecular microstructure in $Ahr^{-/-}$ mice.

**Fig. 10.** A coronal view of vertebrae, where segmented trabecular bone is presented in green in otherwise grey colored vertebral bodies of control and TCDD exposed $Ahr^{+/+}$ and $Ahr^{-/-}$ mice. Upper pictures are females and lower males. There is evident increase in BV/TV in TCDD exposed $Ahr^{+/+}$ mice causing related changes in trabecular bone morphology (plate like trabeculae which are more closely packed).
### 5.3 Mechanical properties

Table 2. Mean ± SD of the biomechanical properties in femoral diaphysis of Sprague-Dawley offspring maternally exposed to NCM and A1254.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Control</th>
<th>0.05 mg/kg</th>
<th>0.5 mg/kg</th>
<th>5 mg/kg</th>
<th>A1254</th>
</tr>
</thead>
<tbody>
<tr>
<td>37 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>17</td>
<td>17</td>
<td>18</td>
<td>20</td>
<td>16</td>
</tr>
<tr>
<td>k (N/mm)**</td>
<td>57.5 ± 17.3</td>
<td>71.4 ± 18.8</td>
<td>68.4 ± 24.1</td>
<td>43.8 ± 10.9</td>
<td>49.9 ± 14.9</td>
</tr>
<tr>
<td>Dmax (µm)</td>
<td>715.9 ± 139.1</td>
<td>695.6 ± 226.5</td>
<td>660.2 ± 149.4</td>
<td>756.1 ± 144.1</td>
<td>781.5 ± 207.9</td>
</tr>
<tr>
<td>Fmax (N)**</td>
<td>25.4 ± 4.6</td>
<td>29.6 ± 4.3</td>
<td>28.9 ± 6.7</td>
<td>21.9 ± 3.6</td>
<td>22.4 ± 5.2</td>
</tr>
<tr>
<td>Emax (mJ)</td>
<td>11.5 ± 3.1</td>
<td>13.6 ± 5.7</td>
<td>12.3 ± 5.0</td>
<td>10.7 ± 3.3</td>
<td>10.7 ± 3.6</td>
</tr>
<tr>
<td>Dy (µm)</td>
<td>257.0 ± 89.6</td>
<td>226.0 ± 61.2</td>
<td>257.2 ± 90.6</td>
<td>283.3 ± 95.0</td>
<td>287.6 ± 78.4</td>
</tr>
<tr>
<td>Fy (N)*</td>
<td>13.9 ± 4.6</td>
<td>16.3 ± 3.0</td>
<td>16.2 ± 4.6</td>
<td>13.1 ± 4.1</td>
<td>13.3 ± 3.7</td>
</tr>
<tr>
<td>Ey (mJ)</td>
<td>1.9 ± 1.1</td>
<td>1.9 ± 0.7</td>
<td>2.2 ± 1.4</td>
<td>2.0 ± 1.1</td>
<td>1.9 ± 0.8</td>
</tr>
<tr>
<td>77 days</td>
<td>18</td>
<td>18</td>
<td>19</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>N</td>
<td>18</td>
<td>18</td>
<td>19</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>k (N/mm)</td>
<td>348.8 ± 53.7</td>
<td>322.9 ± 73.9</td>
<td>372.2 ± 40.0</td>
<td>348.0 ± 62.2</td>
<td>351.2 ± 42.4</td>
</tr>
<tr>
<td>Dmax (µm)</td>
<td>642 ± 98.5</td>
<td>567.3 ± 236.9</td>
<td>656.3 ± 112.3</td>
<td>600.4 ± 169.2</td>
<td>674.4 ± 114.0</td>
</tr>
<tr>
<td>Fmax (N)</td>
<td>114.5 ± 14.3</td>
<td>123.3 ± 16.8</td>
<td>126.1 ± 15.0</td>
<td>114.2 ± 14.9</td>
<td>119.3 ± 17.2</td>
</tr>
<tr>
<td>Emax (mJ)</td>
<td>48.3 ± 9.3</td>
<td>54.4 ± 12.4</td>
<td>54.0 ± 14.2</td>
<td>49.5 ± 7.5</td>
<td>55.6 ± 15.6</td>
</tr>
<tr>
<td>Dy (µm)**</td>
<td>185.0 ± 35.6</td>
<td>314.0 ± 213.9</td>
<td>176.1 ± 31.7</td>
<td>232.5 ± 144.5</td>
<td>181.6 ± 31.2</td>
</tr>
<tr>
<td>Fy (N)</td>
<td>62.8 ± 12.7</td>
<td>78.5 ± 24.0</td>
<td>65.2 ± 10.5</td>
<td>67.1 ± 16.6</td>
<td>63.5 ± 7.6</td>
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<tr>
<td>Ey (mJ)*</td>
<td>6.0 ± 1.1</td>
<td>14.4 ± 14.3</td>
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<td>18</td>
<td>19</td>
<td>19</td>
<td>17</td>
<td>20</td>
</tr>
<tr>
<td>k (N/mm)</td>
<td>301.0 ± 47.8</td>
<td>374.0 ± 101.7</td>
<td>283.8 ± 54.2</td>
<td>345.4 ± 97.2</td>
<td>344.9 ± 82.9</td>
</tr>
<tr>
<td>Dmax (µm)</td>
<td>500.0 ± 148.7</td>
<td>556.7 ± 154.5</td>
<td>542.6 ± 161.9</td>
<td>550.0 ± 135.4</td>
<td>502.3 ± 144.6</td>
</tr>
<tr>
<td>Fmax (N)</td>
<td>113.7 ± 25.2</td>
<td>145.3 ± 48.2</td>
<td>111.7 ± 30.5</td>
<td>136.0 ± 44.8</td>
<td>132.3 ± 29.4</td>
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<td>Emax (mJ)</td>
<td>39.1 ± 24.8</td>
<td>55.1 ± 36.4</td>
<td>37.7 ± 20.8</td>
<td>49.4 ± 26.8</td>
<td>38.8 ± 18.5</td>
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<tr>
<td>Dy (µm)</td>
<td>265.2 ± 89.9</td>
<td>244.8 ± 41.1</td>
<td>263.3 ± 44.5</td>
<td>252.7 ± 46.8</td>
<td>271.4 ± 41.0</td>
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<tr>
<td>Fy (N)</td>
<td>76.5 ± 24.5</td>
<td>89.2 ± 23.1</td>
<td>73.6 ± 15.5</td>
<td>89.1 ± 30.5</td>
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<td>Ey (mJ)</td>
<td>11.7 ± 9.8</td>
<td>11.4 ± 3.4</td>
<td>10.2 ± 3.4</td>
<td>12.0 ± 5.5</td>
<td>12.5 ± 3.5</td>
</tr>
</tbody>
</table>

Treatment effects on stiffness (k) as well as yield (y) and maximal (max) force (Fy, Fmax), deformation (Dy, Dmax), and energy (Ey, Emax). NCM treatment effect as indicated by MANOVA *p < 0.05, **p < 0.005, *** p < 0.001 and with Tukey’s HSD post hoc test †p < 0.05. A1254 treatment effect as indicated by independent Student’s t-test *p < 0.05.

In maternally TCDD exposed rat offspring the tibial yield force was decreased by 28% and maximal bending force decreased by 16 % at PND 35. At PND 70 stiffness and maximal bending force were decreased by 14% and 20%, respectively.
In NMC and A1254 exposed offspring both females and males responded similarly as indicated by non-significant treatment-by-gender interaction, thus biomechanical data from males and females were combined. At PND 35 MANOVA indicated significant treatment effect for stiffness and both maximal and yield force (Table 2). However, group-wise comparison indicated that there was no significant change between any of the dose groups compared to controls. Later at PND 77 MANOVA indicated significant treatment effect for yield energy and force. Post-hoc group-wise comparison showed that these parameters are increased at the dose of 0.05 mg/kg (Table 2). At PND 350 none of the biomechanical parameters showed treatment response for NCM. In the NCM exposed offspring the maximum force and energy absorption of the femoral neck were affected at every time point but group-wise comparison revealed significant reduction only for maximum force at PND 35 with highest dose (data not shown).

Also for A1254 the MANOVA test did not show gender difference for biomechanical properties and thus data was combined. Similarly to NCM, stiffness and both maximal force and energy absorption of the femoral diaphysis showed decreasing trend at PND35, but this reduction was not statistically significant (Table 2). These parameters were not affected at PND 77 or PND 350 but rather overcompensated by age. For the femoral neck stiffness and both maximum force and energy absorption were significantly decreased by 22%, 15% and 24% respectively at PND 35 offspring. Again, no significant effects were observed at PND 77 or PND 350 (data not shown).

5.4 Intrinsic matrix properties

As described previously, intrinsic matrix properties partly define overall mechanical behavior of bone as well as provide cues to cell activation or differentiation. Here we probed these properties using nanoindentation in maternally TCDD exposed rats (study I) as well as in control and TCDD exposed AHR knockout mice with corresponding wild type counterparts (study IV).

In study I, the bone material properties were not different between exposed and control animals at PND 35 or PND 70. However, when we compared the bone properties at PND 35 to those at PND 70 within groups significant differences were seen. The normal maturation pattern in the control group showed significant changes in the intrinsic properties measured from dynamic loading protocol between PND 35 and PND 70, where the plasticity index \( I_p \) decreased by 5.3% (\( p < 0.05 \)), while the complex modulus \( E^* \), storage modulus \( E_{stor} \) and the
universal hardness $H_{IT}$ increased respectively by 12%, 13%, and 26%. In the TCDD-exposed animals, there were no significant changes in these parameters of the bone maturation process between PND 35 and PND 70 and especially plasticity remained almost the same at PND 35 and PND 70.

As it is known that mineral fraction is related to intrinsic properties (Oyen et al. 2008, Zioupos 2005), we investigated the relationship between nanomechanical data and the mineral content in controls and TCDD exposed animals separately. There was significant increase between both indentation hardness and complex modulus and mineral content, while plasticity decreased, and this was the case in both controls and TCDD bones. Analysis of covariance (ANCOVA) indicated that the slopes for control and TCDD exposed animals were not different between $E_{Stor}$, $H_{IT}$, and $I_p$ vs. BMC ($F_{1,26}(E_{Stor}) = 0.014$; $F_{1,26}(H_{IT}) = 1.071$; $F_{1,26}(I_p) = 1.371$).

As these parameters are novel, and how they should be measured and analyzed is still under debate, we examined the interrelationships between the various nanomechanical parameters. There were significant correlations between the plasticity index $I_p$ and overall creep strain amplitude A ($R = 0.58$, $p < 0.001$), early rate creep strain B ($R = -0.53$, $p < 0.01$), and hardness value $H_{IT}$ ($R = 0.74$, $p < 0.001$).

In study IV, TCDD exposure in male $Ahr^{+/+}$ mice increased the dynamic hardness of cortical bone (by 9%) and the static hardness, as well as both static and dynamic elastic modulus, of trabecular bone (by 12%, 13% and 28%, respectively), while the creep amplitude of trabecular bone was decreased (by 10%). Furthermore increased static hardness (by 9%), as well as both static and dynamic elastic modulus (8% and 7%) of cortical bone were observed in female $Ahr^{+/+}$ mice after TCDD exposure.

AHR ablation lead to increased (5%) static hardness of cortical bone in unexposed (controls) males, while the plasticity index was 2% decreased ($p < 0.05$). The trabecular bone matrix properties did not differ between the genotypes in males. In female $Ahr^{-/-}$ mice, the trabecular bone had decreased static and dynamic elastic modulus (by 14% and 10%, respectively) as compared to wild type counterparts, while females did not show any differences in cortical bone matrix properties. TCDD exposure did not affect cortical or trabecular bone matrix properties of $Ahr^{-/-}$ mice.
5.5 Serum markers

Serum markers were evaluated in study IV. TCDD exposure decreased the PINP/CTX ratio by 43% (p < 0.001) in Ahr<sup>+/+</sup> female mice, while in males this was present only as a trend. AHR ablation increased bone resorption as indicated by increased levels of CTX (by 60% (p < 0.05) in female Ahr<sup>-/-</sup> mice compared to corresponding Ahr<sup>+/+</sup> mice, while the PINP/CTX ratio did not differ between the genotypes. TCDD treatment neither affected the PINP nor CTX levels, nor the ratio, in either gender Ahr<sup>-/-</sup> mice.

![Bone remodeling balance as PINP/CTX ratio for controls and TCDD exposed for wild type (WT) and knockout (KO) females (white) and males (grey). *** p < 0.05.](image)

5.6 Gene expression

Of the 84 osteogenesis-related genes for which the expression was analyzed, 20 genes were altered (fold change ≥2) by TCDD-exposure of Ahr<sup>-/-</sup> mice. Only three of these were up-regulated and they were associated with skeletal development (Tnf) and extracellular matrix proteins (Itgam, Mmp8). Remaining 17 genes were down-regulated and associated with bone mineral metabolism (Bmp5, Cdhl1, Col4a2), skeletal development (Bmp2, Bmp5, Dmp1, Fgfr2, Phex, Sost1, Vdr, Ahsg), extracellular matrix proteins (Cstk, Dmp1, Fgfr2, Mmp2, Sost1, Tgfb2), cell growth and differentiation (Fgf1), transcription factors and regulators (Msx1) and cell adhesion molecules (Col12a1).
Control AHR ablated mice differed for two genes as compared with wild type controls. One up-regulated was associated with extracellular matrix proteins and down-regulated with skeletal development (Ahsg). The down-regulated gene was lower also both in TCDD-exposed Ahr−/− and Ahr+/− mice.

For Ahr−/− mice, TCDD-exposure altered the expression of only five genes. Three of these were up-regulated and associated with skeletal development (Tfip11, Col2a1), bone mineral metabolism (Col2a1) and extracellular matrix proteins (Col10a1), while the two down-regulated genes were associated with skeletal development (Ahsg) and with cell growth and differentiation (Vegfb).

5.7 Dose modelling

For NCM the pQCT and biomechanical variable with highest mean square values got the lowest BDs indicating highest sensitivity. At PND 35 the 5% decrease was estimated to 0.8 and 1.2 mg/kg bw/d for maximal breaking force and stiffness of femoral diaphysis, while for the femoral the neck maximal breaking force corresponding dose was 1.4 mg/kg bw/d. Also the strength estimates from pQCT got low BD values being 0.9 and 1.1 mg/kg bw/d for polar moment of inertia and polar strength strain index. Interestingly, the tibial length and morphological values had higher BD estimates being 3.2, 1.6, 2.2 and 4.8 mg/kg bw/d for tibial length, cortical area, cortical thickness and periosteal circumference, respectively. All BD values had BD/BDL within the well tolerable range of 1.2–1.5. It is possible to extract also maximal response from BD analyses. Generally maximal response decrease with age (publication II, table 2B), while BD values first decreased and then increased at PND 77 and PND 350 respectively, while the BD/BDL were highest at PND 77 compared to other age points.

The TEQ for the used A1254 was 0.123 µg/kg bw/d with major contributions from dioxin-like PCB congeners 126, 118 and 105 by 46%, 29%, and 12%, respectively. Thus in this experiment the total dose of A1254 was estimated to 5.289 µg/kg bw. When the measured responses of the various bone parameters to this dose of A1243 were compared against dose models of classical TCDD exposure, the equivalents settled between 0.83–1.22µg/kg bw giving a range of 16 – 23% against the theoretical TEQ (publication III, table 4).
6 Discussion

The main goal of this study was to better understand the effects of POPs in bone strength and morphology and the role of AHR in bone toxicity and skeletal development at multiple hierarchical levels, thus bringing new mechanistic data for environmental health risk assessment. This is crucial since according to the WHO (WHO 2000) developmental defects are the most sensitive toxic endpoint of dioxin-like compounds, and at current exposure levels the safety margin for these effects is very narrow and simultaneous exposure to a wide variety of different chemicals emphasize the potential significance of mixture effects (e.g. additivity or synergism) that are dismissed when studying single chemicals (WHO 2013).

6.1 Novel methods in bone toxicology

Part of the research novelty arises in applying recent biomedical engineering methodologies in such an exciting field as environmental toxicology. The added scientific value is visually clear when comparing pQCT and µCT images to each other of the same sample Fig. 12.

Although pQCT provides a better picture of the skeletal changes than DXA and captures some key features as with µCT (total area, cortical area) (Schmidt et al. 2003) the method has problems separating the endosteal border of bone thus leading to potential underestimation of BMD with increased trabecular bone volume fraction and medullary area. Also the cortical porosity is mixed in cortical BMD due to partial volume effects, while µCT can provide separate estimates of TMD and cortical porosity.

Where traditional mechanical testing can be used to quantify mechanical properties of whole bone, nanoindentation characterizes the bone material properties, describing the bone matrix quality. These qualitative properties contribute to overall bone strength and can make bones brittle, harder and stiffer. It was noticed that these properties are inter-related and appear to follow BMC. Unfortunately by the time of study I, we did not have access to µCT equipment, and we cannot compare TMD in that study.
Fig. 12. Measurement positions indicated in scout view for both cortical and trabecular bone for pQCT (two yellow dotted lines) and µCT (between two dotted black lines). The µCT imaging provides a more complete picture of changes based on morphological information from longer length that can be used for 3D analyses and visualization.

Even if we utilized latest technologies to show effects of TCDD on intrinsic properties of extracellular bone matrix and true 3D volumetric analysis of trabecular bone and cortical porosity, there are still some improvements that should be implemented in future studies. The technical limitation in our nanoindentation studies was the use of dried samples, which has been often the case (Lewis & Nyman 2008), partly due to equipment requirements, partly due to the practical difficulty in preparing moist bone specimens without embedding, especially from animals as small as mice. However, it has been shown, that strontium ranelate induced improvements in material properties were more pronounced in samples stored in a physiological solution as compared to dried samples, where the effect disappeared (Ammann et al. 2007). Thus, it is tempting to speculate that the effects observed in this study are more likely an underestimation of real effects that should be probed in physiological conditions.
Further, the porosity analyses here were performed from images with resolution of 5 µm, although it has recently been suggested that a resolution of as low as 2 µm should be used for murine vascular structures (Palacio-Mancheno et al. 2014). The same study demonstrated that osteocyte lacunas could be quantified and visualized at resolutions of 1µm or better.

### 6.2 Model compound and genetic model studies

Study I showed delayed maturation after maternal TCDD exposure. More specifically, bone matrix became more ductile, softer and less able to store energy than control bones. Based on the correlation between intrinsic material properties and mineralization parameters we suspect these changes are mainly due to decreased mineralization. However, the role of the organic part cannot be excluded since these changes are also supported by notification of previously reported decrease in water and collagen content (Lind et al. 2000b). In this case the increased mature cross-links could not provide improved strength and the role of hydroxyproline in bone strength is still unknown. On the other hand, these results are also supported by previously reported increased amount of osteoid (Lind et al. 1999), while the time course is surprising. In humans ERα inactivation has been shown to delay bone maturation (Quaynor et al. 2013, Smith et al. 1994).

The toxic effects of TCDD and other dioxin-like compounds are mostly mediated through AHR signaling. Thus, the role of AHR was studied in normal skeletal development and dioxin toxicity at various hierarchical levels. Bone remodeling was increased in Ahr−/− mice and they presented a skeletal phenotype with softer and more elastic bones. Interestingly, also the bones of CA-AHR mice were less stiff and more bendable, which most likely reflects to material level alterations as they had also increased periosteal circumference (Wejheden et al. 2010). Ahr−/− also showed increased trabecular bone volume fraction and the number of trabeculae were higher in these mice, similar to another recent report (Iqbal et al. 2013). The previous studies have indicated reduced osteogenesis by RANK-L, BaP (Iqbal et al. 2013) and less active osteoblastic cells (Korkalainen et al. 2009, Ryan et al. 2007) in absence of AHR in vitro. However, in our Ahr−/− mice high serum CTX indicated increased osteoclastic resorption, which might increase osteoblastic activity through coupling. Furthermore, dioxin administration had hardly any effects on the bone properties of the Ahr−/− mice.
In Ahr+/+ mice, dioxin exposure caused increased cortical porosity and a strong anabolic effect on trabecular bone formation. Here porosity analysis consisted mainly of blood vessels (Haversian and Volkmann channels), but might include some collagen fibers from tendon attachment at lateral side. However, still it would be logical that osteoclastic resorption would take place in close proximity to blood vessels and with reduced bone formation the filling of cavity would not be complete. These effects appeared only in females. Additionally, females showed reduced maximal and yield strengths. Reduced yield strength indicates increased microfracture susceptibility and is probably due the increased hardness and reduced elasticity of tissue. Generally these results indicate early onset of skeletal senescence further supported by an observed reduced remodeling ratio.

Similarly to TCDD exposed Ahr+/+ mice, increases in trabecular bone volume fraction and decreased cortical thickness have been reported in ERα−/− mice (Lindberg et al. 2001, Lindberg et al. 2002, Sims et al. 2002). Furthermore there is evidence that AHR activation could cause ERα (Beischlag & Perdew 2005) and ERβ (Ruegg et al. 2008) transrepression supporting the view that phenotype could be due ER inactivation (but not antiestrogenic). However, it should be pointed out that there are other animal models with similar skeletal phenotype e.g. IL11α1−/− and IL6−/− (Sims et al. 2005). In PCB126 exposed adult female rats an increase in expression of ER mRNAs has been reported with no effect on the ER content (Lind et al. 2004).

### 6.3 Towards studies with naturally occurring POP mixtures

As most toxicological studies have been previously based on exposure to model compounds, such as TCDD and individual PCB congeners, here we studied two mixtures that resemble more of the existing POP body burden of human populations (Van Oostdam et al. 2005). The used mixtures NCM and A1254 included 28 and 21 separate chemicals, respectively, while they did not include the most potent PCDD/Fs. Still used mixtures appeared to have skeletal toxicity compatible with the profile of bone changes elicited by perinatal TCDD and / or PCB exposure described in chapter Table 1. Also the both mixtures increased Trab.BMD later at PND 77.

Effects of A1254 were somewhat lower than what could be estimated via TEQ estimation based on the assumption of additivity of effects of individual chemicals. Recently it has been suggested that various types of POPs might have
even synergistic effects (Koskela et al. 2012). This will set a huge challenge for chemical risk assessment, since there are over 800 chemicals that could potentially disturb the endocrine system (WHO 2013) and thus the skeletal system.

In the population of the Canadian Arctic, 2- to 10-fold higher levels of environmental contaminants have been shown than in general populations living in regions further south (Van Oostdam et al. 1999). Recently it has been shown that POP intake in these communities exceeds the Canadian provisional tolerable daily intake, and in one of these communities, 7% of young women exceed the Health Canada tolerable serum level of 5 μg/L total PCB as A1254 (AMAP 2009). However, in population based studies the correlation between skeletal properties and POP burden has been mild (Paunescu et al. 2013b) or absent (Paunescu et al. 2013a) in both Canada and Greenland (Cote et al. 2006).

6.4 **Translation of findings to human health**

There have been a few known major chemical accidents or incidents that have heavily increased POP exposure of affected populations. In Turkey the use of HCB for treating grain in 1955–1959 resulted in HCB-exposure via contaminated food and caused a *porphyria cutanea tarda* epidemic in the affected population (Peters et al. 1987). Various skeletal manifestations have been observed within this population including arthritis and osteomyelitis soon after exposure (Dean 1961), small hands, peripheral arthritis with osteoporosis and joint space narrowing even decades after exposure (Gocmen et al. 1989, Peters et al. 1987).

Later in 1968 in northern Japan, ingestion of PCB and PCDF contaminated rice oil (Yusho) lead to fetal PCB syndrome including abnormal calcification of skull and feet malformation with dark pigmentation, gingival hyperplasia and exophthalmic edematous eyes (Yamashita & Hayashi 1985). A recent study showed a lack of association between serum PCB, PCQ, PCDF and BMD and CTX (Yoshimura et al. 2009). However, there has been about 120-fold decrease in serum TEQ between 1969 and 2007 (Masuda 2009).

Finally, a major incident took place in Seveso, Italy on 10th of July 1976, where an explosion in a chemical factory resulted in a release of up to 30 kg of TCDD, spread over the surrounding 18 km² area. It has been recently reported that DXA bone scans did not reveal adverse effects on bone health 32 years later in women who were < 20 years old during the explosion (Eskenazi et al. 2014).
There are reports showing relationships between exposure to various POPs and altered skeletal parameters. DDE (Beard et al. 2000) and PCB28 have been associated with decreased skeletal mass, while PCB28 and γ-HCH with increased mass (Glynn et al. 2000). Often, when adjusting with confounding factors the effect dilutes and is no longer statistically significant. Thus the skeletal toxicity of POPs has been mild or difficult to detect.

However, the socioeconomic burden of reduced bone strength arises from the fractures rather than osteoporosis per se. Increased fracture incidence was found in fishermen and their wives exposed to POP through dietary intake of fatty fish from the Baltic sea (Alveblom et al. 2003). This causality was not present in a questionnaire based study on the following year (Wallin et al. 2004). Thus the attempts to correlate clinical findings with POP exposure levels for predicting fracture risk are necessary. DXA based BMD has been the gold standard for fracture risk assessment, but evidence shows that BMD is not a sufficient tool to indicate patients at risk of fracture (Schuit et al. 2004, Stone et al. 2003). Also the currently used ultrasound parameters mainly reflect the overall bulk properties (Gluer 2007). Furthermore, the animal models in these studies present often with weaker and smaller bones, however with increased trabecular bone fraction. This indicates that POPs decrease bone strength, but in such a way that they are difficult to diagnose due to increased amount of trabecular bone inside cortical bone with poorer quality and smaller dimensions. For these reasons it is not surprising that in some studies no statistically significant associations between BMD/ultrasound and POP were found (Beard et al. 2000, Cote et al. 2006, Eskenazi et al. 2014, Glynn et al. 2000, Hodgson et al. 2008, Paunescu et al. 2013a, Paunescu et al. 2013b, Rignell-Hydbom et al. 2009, Wallin et al. 2005). Therefore in future more advanced diagnostic tools such as high resolution pQCT should be used to study skeletal properties in POP exposed populations to elucidate fracture risk in adult population. Some studies showed weak association with POP burden and skeletal properties but they disappeared after various adjustments for all possible confounding factors (Cote et al. 2006, Glynn et al. 2000, Rignell-Hydbom et al. 2009, Wallin et al. 2005). Age alone is such a strong predictor of skeletal properties. In our NCM study the age explained about 200 times higher correlation to radiological and biomechanical parameters compared to treatment. However, it should be realized that the treatment was relatively short as NMC exposure was stopped at PND 23, whereas the whole study period extended over 360 days.
However, it should be pointed out that the skeletal manifestations were dependent on exposure timing. In adults TCDD exposure leads to decreased diaphyseal bone size, increased porosity and brittleness of tissue. The maternal exposure, on the other hand, delayed maturation of bone tissue increasing a risk of green stick fractures. This implies that early POP exposure might increase bone ductility and later brittleness. Interestingly, in all studies increased trabecular bone quantity was observed. Nevertheless, in all cases POP exposure decreased bone strength and it would be surprising if this would not be the case in humans as well.
7 Conclusions

The main goal of the thesis was to understand the effects of POPs on bone strength and morphology, and the role of AHR in bone toxicity and skeletal development. Based on the results of this study, it can be concluded that:

1. Developmental TCDD does not alter bone matrix properties at the level that they would contribute to decreased bone strength. However, TCDD exposure was shown to delay bone matrix maturation.
2. Exposure to the NCM decreases bone size and strength at relevant but relatively high dose levels. NCM was observed to increase Trab.BMD.
3. PCB mixture A1254 causes similar changes as TCDD exposure leading to shorter, thinner and weaker bones in juvenile rats. Again as in the NCM study Trab.BMD was observed to be increased in adult animals after discontinued exposure.
4. The AHR receptor has a major role in TCDD induced bone toxicity and bone homeostasis. A new insight was gained for cortical porosity and tissue senescence as well as increased trabecular bone quantity.
5. In general the opposite effects on trabecular and cortical bone compartments demonstrate a need for a new view when analyzing radiological data from humans.
References


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Original publications


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1237. Jansson, Miia (2014) The effectiveness of education on critical care nurses’ knowledge and skills in adhering to guidelines to prevent ventilator-associated pneumonia

1238. Turunen, Sanna (2014) Protein-bound citrulline and homocitrulline in rheumatoid arthritis : confounding features arising from structural homology


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BONE TOXICITY OF PERSISTENT ORGANIC POLLUTANTS