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Bone toxicity induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and the retinoid system: A causality analysis anchored in osteoblast gene expression and mouse data

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ABSTRACT

Dioxin exposures impact on bone quality and osteoblast differentiation, as well as retinoic acid metabolism and signaling. In this study we analyzed associations between increased circulating retinol concentrations and altered bone mineral density in a mouse model following oral exposure to 2,3,7,8-tetrachlordibenzo-p-dioxin (TCDD). Additionally, effects of TCDD on differentiation marker genes and genes involved with retinoic acid metabolism were analysed in an osteoblast cell model followed by benchmark dose-response analyses of the gene expression data. Study results show that the increased trabecular and decreased cortical bone mineral density in the mouse model following TCDD exposure are associated with increased circulating retinol concentrations. Also, TCDD disrupted the expression of genes involved in osteoblast differentiation and retinoic acid synthesis, degradation, and nuclear translocation in directions compatible with increasing cellular retinoic acid levels. Further evaluation of the obtained results in relation to previously published data by the use of mode-of-action and weight-of-

Abbreviations: AHR, aryl hydrocarbon receptor; AHRKO, AHR knockout; AHRR, aryl-hydrocarbon receptor repressor; ALP, alkaline phosphatase; ANOVA, analysis of variance; AOP, Adverse Outcome Pathway; ATRA, all-*trans* retinoic acid; BMD, bone mineral density; CAR, constitutive androstane receptor; cDNA, complementary DNA; CED, critical effect dose; CEDL, critical effect dose lower bound of the one-sided 95 % confidence interval; CEDU, critical effect dose upper bound of the one-sided 95 % confidence interval; CES, critical effect size; CORA, 9-*cis*-4-oxo-1314-dihydroretinoic acid; CRABP2, cellular retinoic acid-binding protein type 2; CRBP1, cellular retinol binding protein type 1; CYP, cytochrome P450; decaBDE, decabromodiphenyl ether; DMSO, dimethyl sulfoxide; DXA, dual energy X-ray absorptiometry; FBS, fetal bovine serum; HBCD, hexabromocyclododecane; IUL, *in utero* and lactational; KE, key event; KER, KE relationships; LRAT, lecithin:retinol acetyltransferase; MEM, Minimum Essential Medium; MIE, molecular initiating event; MOA, Mode of Action; OCN, osteocalcin; PCB, polychlorinated biphenyl; PCDD, polychlorinated dibenzo-*p*-dioxins; PCDF, polychlorinated dibenzofurans; pentaBDE, pentabromodiphenyl ether; PFOA, perfluorooctanoic acid; PLS, partial least square; PND, post-natal day; POP, persistent organic pollutants; Ppia, peptidylprolyl isomerase A; pQCT, peripheral quantitative computed tomography; PXR, pregnane X receptor; RALDH1, retinal dehydrogenase type 1; RALDH3, retinal dehydrogenase type 3; RAR, retinoic acid receptor; RAS, Retinoic Acid Syndrome; RDH10, retinol dehydrogenase 10; REOH, retinol; REPA, retinyl palmitate; RNA, ribonucleic acid; RT-PCR, reverse transcription polymerase chain reaction; RUNX2, Runt-related transcription factor 2; RXR, retinoid X receptor; SMI, structural model index; TBBPA, tetrabromobisphenol A; TCDD, 23,7,8-tetrachlorodibenzo-p-dioxin; VIP, Variable Importance in the Projection; WOE, Weight of Evidence.

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evidence inspired analytical approaches strengthened the evidence that TCDD-induced bone and retinoid system changes are causally related and compatible with an endocrine disruption mode of action.

1. Preface

Here, we report on an experimental study designed to determine the influence of sustained over-activation of the aryl hydrocarbon receptor (AHR) on osteoblast differentiation and bone properties in relation to the retinoid system as a specific segment in the toxicology of endocrine disruption. Results are relevant to most, if not all, tissues over the life-course since it is dealing with cross-talk between the global cellular regulator AHR and the retinoid system. Furthermore, high translation across species could be expected since both AHR and retinoid system is evolutionary well conserved. An important intention of the study is to support ongoing regulatory initiatives dedicated to science-based incorporation of the retinoid system into chemical safety evaluation programs in different domains of human and wildlife health as recently reviewed [1–3]. To this end, the original data presented in this study is further evaluated in relation to previously published data by the use of mode-of-action (MOA) and weight-of-evidence (WOE) inspired analytical approaches in order to derive additional regulatory-relevant insights. The experimental background, study design, and results are therefore presented and evaluated in comprehensive, yet narrative, and partly review-style Introduction and Discussion sections of this article, with the aim to provide the broader contexts of bone, AHR, and retinoid biology as a common background to the interpretation and conclusions of the presented original data for readers with varying interests and familiarity levels in fields as broad as toxicology, pharmacology, nutrition, and environmental medicine.

1.1. Introduction

Bone is the hard and dense connective tissue that forms most of the adult skeleton. There are more than 200 bones in an adult person, which makes up about 10–15 % of the body weight. Bones allow movement, provides body structure and define features, while at the same time they protect soft tissues. They also serve as the space for blood cell production in the bone marrow compartment of the skeleton. In addition, bones store minerals and lipids, and are, in this regard, metabolically highly active also on the whole-body level. A dedicated remodelling process serves to maintain bone cell homeostasis and at the same time continuously renew the skeleton for normal growth and adaptation, as well as for injury and fracture healing processes. It has been known for long that retinoids (vitamin A) play fundamental roles in bone formation and regeneration over the life-course, including bone tissue development during fetal and early postnatal life (reviewed by [4–6]).

Nevertheless, it took until the 1980ies before it was demonstrated that these effects of retinoids are mediated by retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which bind different forms of retinoic acids (reviewed in [7,8]). RARs function as heterodimers with RXRs, and as such regulate the transcription of target genes. RXRs are also heterodimerization partners of several other nuclear receptors, thereby playing a central role in the endocrine and metabolic systems with the potential to affect multiple signaling pathways [9,10]. Furthermore, data is accumulating in support of a close collaboration between the retinoid system and AHR both in biology and toxicology (reviewed [11]), for example, in the mutual control of multiple genes, which are involved with retinoic acid metabolism and signalling, phase I and II enzyme activities, and cell differentiation (Table S1, [106–124,126–133]). AHR is a well described ligand-activated transcriptional factor with important roles in bone cell differentiation [12] and remodelling [13], and with a suggested cross-talk with RAR, as demonstrated in hard tissue experimental models [14,15].

The mandatory role of retinoic acid signaling in skeletogenesis [5] as well as in bone maintenance (remodelling) over the life-course are now well established based on both experimental and clinical studies [16,8, 17,150]. It is also well known that the metabolic machinery for retinoic acid synthesis and degradation is present both in osteoblasts and osteoclasts (reviewed in [8,18]). Retinoids in the blood circulation, are derived from dietary vitamin A, which first have passed the gut and liver compartments, as illustrated in Fig. 1. Circulating retinoids represents a separate compartment of the complex retinoid machinery with roles to facilitate transport between cells, tissues, and organs and to sustain overall retinoid homeostasis on the organism level [19,20].

1.2. Dietary vitamin A and bone

A large number of studies both in humans and in experimental models have shown that both too high and too low intake of dietary vitamin A are associated with bone abnormalities, spanning from fetal skeletal impairments (reviewed in [17]) to increased bone fracture risk at older age (reviewed in [150]). Early data in dogs fed a vitamin A-deficient diet from weaning describe severe bone overgrowth resulting in a general thickening and coarsening of the bones, cramped and subsequently degenerated cranial and optic nerves, and smaller spinal canals [21]. These findings in dogs have been confirmed and extended to include observations in other species under vitamin A-deficiency conditions, as well as in bone cell studies (reviewed in [6,22]). Taken together, these early studies linked vitamin A-deficiency to the occurrence of bone in parts of the body where it is not normally present, such as the inner ear, failure of resorption of previously formed bone resulting in thickening of bone and diminution of bone cavities, and a shift towards an increase in osteoblast numbers and activity in vitamin A-deficiency situations. More recent studies using genetically modified mouse models have shown that, already at the age of 8 weeks, lack of the retinoic acid synthesizing enzyme retinal dehydrogenase 1 (RALDH1) results in increased cortical bone thickness and density, which is accompanied with micro-architectural changes suggestive of increased osteoblastic activity [23]. Likewise, a defect in the retinal synthesizing enzyme retinol dehydrogenase 10 (RDH10) was associated with reduced whole embryo retinoic acid concentrations, and skeletal face and limb defects, which could be rescued by retinoic acid supplementation [24].

On the other hand, dietary vitamin A excess results in altered long bone geometry, including reduced diameter, in rodent studies [25–28], reduced cortical thickness and cortical area [29], and lower mechanical strength [29,30] with harder and more brittle bone tissue [26]. Further, increase of trabecular bone content (Li et al., 1989), as well as reduced skull bone thickness [27], have been associated with excess vitamin A intake. Chronic moderately elevated to high vitamin A intakes have resulted in thin fragile bones [29]. Vitamin A excess also decreases longitudinal bone growth possibly via inhibition of growth plate chondrogenesis and bone matrix synthesis [16]. It has been proposed that a shift in the bone remodelling balance in favour of bone resorption may be one of the underlying mechanisms in vitamin A-excess situations [25].

1.3. AHR-mediated bone toxicity and the retinoid system

During the last decades, it has been shown in experimental studies, that exposure to the ubiquitous environmental food contaminant and potent AHR ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which is known to interfere with the retinoid system through multiple types of interactions (reviewed in [31–34]), also impacts on rodent bone properties in terms of altered geometrical, densitometrical, and biomechanical

characteristics [35–41]. Exposure to dioxin is also associated with reduced bone length in rats [39]. Concerning bone geometry, investigators have observed reduced cross-sectional, trabecular, and/or cortical area after exposure to TCDD [39]. Biomechanical studies have indicated reduced torsional strength and stiffness in bones exposed to TCDD [39]. Further, studies in bone cells have demonstrated inhibitory effects by TCDD on the differentiation of both bone forming osteoblasts [42–48], and bone resorbing osteoclasts [45]. An experimental TCDD-exposure study in rats, which analysed both bone properties [37] and retinoid system parameters [49,50], found an elevation of circulating retinol concentrations at the same exposure levels where alterations of bone properties, especially on bone geometry, were observed. Likewise, in mice, TCDD induced bone changes, which showed several similarities to bone properties to those observed following vitamin A excess, such as increased trabecular bone mineral density in combination with decreased trabecular bone area, as well as harder and stiffer trabecular bone matrix [38]. The cortical thickness was reduced and the cortical bone mineral density was lower due to increased cortical porosity [38]. Furthermore, on the molecular level it has been demonstrated that ablation of the retinaldehyde dehydrogenase 3 (*Raldh3*) gene, which synthesize retinoic acid from retinal, blocks TCDD-induced cleft palate in mice [15], suggesting that alteration of palate development, which include palatal osteogenesis, by TCDD is dependent on retinoic acid signalling. Together, these data suggest that retinoic acid metabolism and/or signalling might be causally involved in TCDD-induced bone toxicity.

1.4. Study aims

The current study aims were therefore to identify and quantify retinoic acid related biochemical and molecular events, which could be linked to the reported impacts of TCDD on osteoblast differentiation and retinoic acid metabolism, and to further analyse the strength of the identified associations between TCDD-induced bone toxicity and retinoid system disturbances in the AHRKO mouse model. It was an additional aim of the current study to support the initiative by the Organisation for Economic Co-operation and Development (OECD) testing guidelines programme to develop a Detailed Review Paper on the retinoid system in relation to the complex regulatory problem of endocrine disruption [10] as reviewed by Grignard et al. [1]. To this end, we extended the study aim to further evaluate and discuss the original new data in the broader context of current regulatory initiatives and current knowledge in AHR, retinoid, and bone biology. A narrative systems biology approach inspired by the MOA, Adverse Outcome Pathway (AOP), and WOE frameworks ([51–54];Becker et al., 2015 [55–59];) was used with the goal to address the hypothesis that TCDD-induced bone and retinoid system changes are causally related and compatible with an endocrine disruption mode of action. In addition, study results were compared with recent findings in two comprehensive and data-rich reports, which were addressing circumstances and potential uses of the retinoid system in predictive toxicology. One of the reports, which is part of the OECD report on the retinoid system (www.oecd.org/chemicalsafety/testing/draft-review-paper-retinoid-pathway-signaling.pdf),

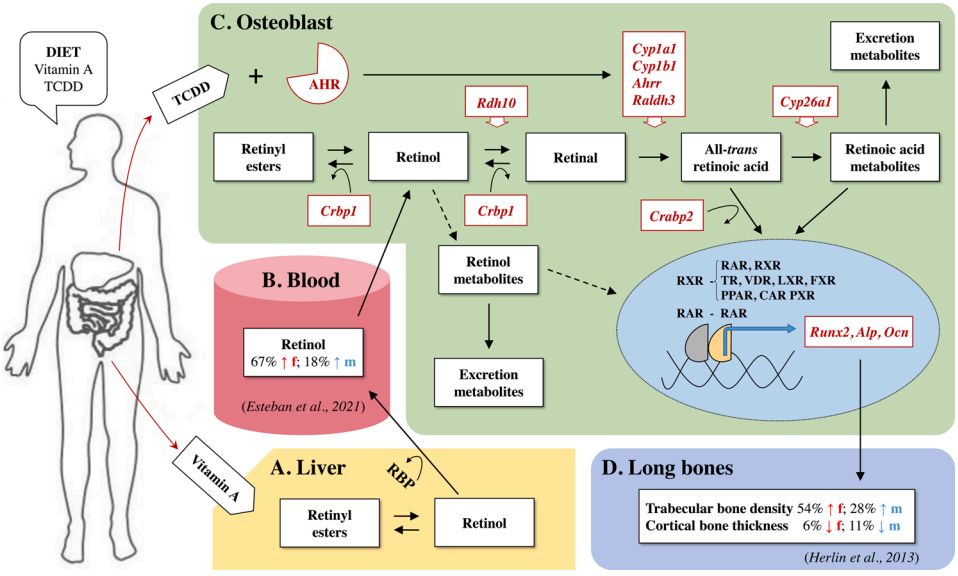

Fig. 1. Schematic overviews of the retinoid system and the study design with a focus on the genes selected for analyses in the MC3T3-E1 osteoblast differentiation assay. Briefly, dietary vitamin A is absorbed and further processed in the gut for chylomicron transfer via lymph to reach the liver (A). Next further transfer, metabolism, distribution, and release of the correct retinoid form and concentration into the blood circulation (B) and transport to target cells, such as, in this case, the osteoblast (C), and target tissues, such as, in this case, long bones (D) take place. The primary role of retinoids in circulation is to facilitate transport between cells, tissues, and organs, and contribute to sustain overall retinoid homeostasis on the organism level. Once in the target cell the local machinery for retinoic acid homeostasis and functionality will determine the fate of the retinoid molecule. Largely, options are storage as retinyl esters in lipid droplets or stellate cells, or metabolism for transcriptional or degradation/elimination purposes. The metabolic and transcriptional retinoid machinery, as visualized in the target cell compartment of this figure (C), is largely comparable between cell types and tissues, even though some cell types, such as the stellate cells, which are present in most tissues, have unique properties in terms of accumulating and releasing retinyl esters upon demand. Furthermore, the liver together with adipose tissues are unique in terms of hosting retinyl ester depots, which continuously turnover, and yet, stored amounts may represent lifetime needs. For the transcriptional part of the retinoid machinery it is likely that cell type and even cell stage specificity are important features in phenotypic control. TCDD, which is also dietary derived in most instances, will reach target tissues and cells, largely by diffusion between lipid compartments. Binding of TCDD to AHR will occur in the cytosol and a complex process regulates translocation of liganded AHR into the nucleus [154,155]. Published data on circulating retinol concentrations [11] and data on long bone properties [38] generated from the same mouse experiment, are depicted in compartments B and C, respectively, of this figure. Genes selected to follow changes in retinoic acid metabolism, included the synthesizing or degrading enzymes RDH10, RALDH3, CYP1A1, CYP1B1 and CYP26A1, as well as the binding protein CRBP1, and the nuclear retinoic acid translocator CRABP2. Analyses of *Alp*, *Ocn* and *Runx2* expressions were performed to follow the osteoblast differentiation process, while analyses of *Ahr*, *Ahrr*, and *Cyp1a1* expressions were performed to confirm that the AHR signaling system is functional in the MC3T3-E1 model system. For abbreviations used in the figure see the Abbreviation list. The represented percentages indicate alterations in the different observed parameters in relation to the controls without exposure to TCDD, with "f" referring to females and "m" to males.

identified inhibition of two retinoic acid metabolizing enzymes as molecular initiating events (MIE) in an embryofetal skeletal dysmorphogenesis model [60]. The other report identified the tipping point concentration of all-trans retinoic acid in a human stem cell differentiation model [61].

2. Materials and methods

2.1. Chemicals

TCDD (UFA Oil Institute, Ufa, Russia) was 99 % pure and dissolved in corn oil (Sigma Chemicals, St. Louis, MO). All other chemicals were of analytical grade and commercially obtained.

2.2. Osteoblast cell culture experiment

2.2.1. Cell conditions and TCDD-exposure

The murine osteoblastic cell line MC3T3-E1 (RRID:CVCL-0409) was maintained in a basic medium containing αMEM supplemented with 10 % fetal bovine serum (FBS), 100U penicillin/mL, and 100 μg streptomycin/mL. Mineralization medium consisted of basic medium supplemented with 10 mM β-glycerol phosphate (Sigma, USA) and 50 μg ascorbic acid/mL (Wako Chemicals GmbH, Germany). The cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cells were seeded in 6-well plates (45 000 cells/well) and allowed to attach. Next day TCDD dissolved in DMSO, or DMSO alone (final concentration of DMSO was 0.1 %) was added. The cells were exposed to TCDD at doses of 100 fM, 10 pM, 1 nM, 10 nM, and 100 nM. Fresh exposure medium was supplied every 3rd day. The first day of exposure was defined as day 0 and cells were sampled at day 20, which is a time point when all stages in osteoblast differentiation can be monitored via marker gene expression [44]. Each exposure was carried out in triplicates.

2.2.2. Gene selection and expression analysis

Genes were selected to trace and quantify molecular events in AHR and retinoid biology in relation to the osteoblast differentiation process under normal and TCDD-induced conditions as illustrated in Fig. 1. Genes, involved in retinoic acid endocrinology and metabolism, included the retinoic acid synthesizing or degrading enzymes retinol dehydrogenase 10 (*Rdh10*), *Raldh3*, and the cytochrome P450 (CYP) enzymes *Cyp1a1*, *Cyp1b1*, and *Cyp26a1*, as well as the cellular retinol binding protein (*Crbp1*), and the cellular retinoic acid binding protein (*Crabp2*), which is translocating retinoic acid from the cytosol to the nucleus. Detection and quantification analyses of Runt-related transcription factor 2 (*Runx2*), alkaline phosphatase (*Alp*), and osteocalcin (*Ocn*) expressions were performed to follow the osteoblast differentiation process, while analyses of *Ahr*[2], the AHR repressor (*Ahrr*), and *Cyp1a1* and *Cyp1b1* gene expressions were performed to confirm that the AHR signaling system is functional in the MC3T3-E1 model system. Of note is that both CYP1A1 and CYP1B1 are implicated in catalyzing the retinoic acid synthesizing step [62–65], and, are at the same time, well established marker genes for AHR activation [66,80]. Functional characterization of the selected genes is provided in Table S1. RNA was isolated from pelleted cells using E.Z.N.A Total RNA Kit (Omega Biotek, USA) as per the manufacturer's protocol. cDNA was generated using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA), and used for quantitative RT-PCR analyses. Gene expression levels were analysed using Maxima SYBR Green/ROX qPCR Master Mix (ThermoFisher Sci, Germany), and 7500 Real-Time PCR System (Applied Biosystems, USA). Target quantity was determined from a standard curve obtained from the primers. Expression levels were normalized against the levels of peptidylprolyl isomerase A (*Ppia*). Negative controls were included in each run.

[2] Dose level of 10 nM TCDD was not analysed for Ahr expression (Figure S3D).

2.2.3. Dose-response modelling

A family of exponential models were fitted to the gene expression data in order to describe the dose-response relationships by using the package Proast (RIVM, the Netherlands) in R version 3−5-0 (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria) for benchmark dose modelling. The Akaike Information Criterion (AIC) was used to select the best model according to EFSA guidance [78]. Then, the max response was calculated for each significant dose-response relationship and the critical effect size (CES) was defined as max response/2 (i.e. 50 % of the max response for each gene). The critical effect dose (CED) at the CES was calculated, as well as both lower and upper bounds of the one-sided 95 % confidence interval (CEDL and CEDU, respectively). The plots for continuous endpoints generated by the package Proast are reported with the following abbreviations CES, CED, CEDL and CEDU which are equivalent to BMR (benchmark dose response), BMD (benchmark dose), BMDL and BMDU (lower and upper bounds of the one-sided 95 % confidence interval of the BMD) [78].

2.3. Mouse association study data base and ethical permit

The mouse data base used in the association study consisted of tibia geometry, densitometry and mechanical property variables derived from pQCT (peripheral computer tomography), uCT (microCT) and biomechanical measurements [38], as well as circulating concentrations of retinol and retinoic acid [11] from the same animals. These data streams were derived from the same animal experiment, which was performed with wildtype ($Ahr^{+/+}$) and $Ahr^{-/-}$ mice in a C57BL/6 J background obtained from The Jackson Laboratory (Bar Harbor, ME; USA) and maintained using heterozygous breeding. The study design has been described in detail [11,38]. Briefly, the mice were 8–12 weeks old at the start of TCDD-treatment and were randomized by body weight into treatment groups of 6 males and 6 females per genotype. To rapidly achieve the kinetic steady state, the total oral dose of TCDD (200 μg/kg by gavage) was divided into one loading dose (40 μg/kg) and 9 maintenance doses (18 μg/kg), in weekly intervals, during a treatment period of 10 weeks. Control mice were given pure corn oil and otherwise identical treatment. The ethical permit for the animal study was approved by the Animal Experiment Committee of the University of Kuopio (license No. 05-42).

2.4. Descriptive statistics and regression analysis

2.4.1. Descriptive statistics and analysis of variance (ANOVA)

All data are expressed as mean ± standard deviation. Differences between groups of mice were determined by two-way ANOVA followed by the Mann-Whitney *U* test (SPSS 14, SPSS Inc., Chicago, IL, USA). One-way ANOVA followed by Dunnets *post hoc* test was performed for pairwise multiple comparisons of means in the osteoblast experiments. Significance was considered for values of $p < 0.05$.

2.4.2. Multivariate regression analysis

Multivariate regression analyses were performed by the Partial least square (PLS) method using R software version 2.9.2 (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria) as previously described [67]. For the osteoblast data, multivariate regression analysis was performed between TCDD exposure, significant explanatory variables, i.e. the metabolic gene expression markers *Cyp1a1*, *Cyp1b1*, *Cyp26a1*, *Ahrr*, *Raldh3*, and *Crabp2*, and significant osteoblast differentiation markers i.e. *Runx2*, *Alp*, and *Ocn*. Multivariate regression analysis was also performed based on the previously published mouse bone [38] and circulating retinoid concentrations [11]. The analysis was performed between TCDD exposure, the explanatory variables (i.e. AHR genotype, body weight gain, and circulating retinoid concentrations), and the bone geometry, densitometry, and mechanical property variables. All entered variables were modelled simultaneously for each individual animal to show the interrelationship of the variables

as they are organized in quadrants in the circle of correlations. Separate PLS models were developed for female and male mice in order, to visualize potential similarities or differences in response to TCDD between genders. Separate PLS models were also developed for circulating retinol alone, and for circulating retinol and retinoic acid together in the same analysis. The overall PLS results are represented as projections along the main component t1, i.e. the horizontal axis (abscissa), and along the secondary component t2, i.e. the vertical axis (ordinate), respectively. These two components were selected in the models according to the equivalent root-mean-square error [68].

2.5. Data integration inspired by MOA, AOP, and WOE frameworks

A MOA-scheme was developed based on the osteoblast gene expression dose-response and PLS data together with functional data on the analysed genes, which is available from the open literature as summarized in Table S1. The quantitative gene expression data, which included CEDs with associated CEDL-CEDU ranges and ratios, as well as maximum gene response values, were used to guide in the process to identify and define molecular and biochemical events, which in a sequential manner could be assigned roles in linking the direct chemical interaction of TCDD with AHR to the altered osteoblast differentiation, and then to skeletal measurements in the literature. First, well established knowledge defined the initial MOA-events, i.e. linking the direct chemical interaction of TCDD with AHR through high-affinity ligand-binding [69–71], followed by sustained AHR over-activation, i.e. the MIE, which initiates a broad cascade of gene transcription modulations [72–76]. With the goal to identify key events (KEs) with associated quantitative KE relationships (KERs) occurring subsequent to the MIE, the affected genes were next grouped in terms of their known roles and functions in retinoic acid metabolism, retinoic acid translocation to the nuclear compartment, and in osteoblast differentiation (Table S1). Complementary to that, the target genes were grouped along the TCDD-dosing scale of the cellular MOA-scheme according to their CEDL-CED ranges (Table 1). Noteworthy, by means of comparing CEDs and CEDLs, the BMD methodology allows to rank the different biological responses in terms of potency of the test compound (Sand et al., 2008), i.e. TCDD in this osteoblast cell culture experiment. To derive additional mechanistic knowledge from the gene expression data, we also applied the toxicological tipping point concept [61,77] to distinguish between reversible and potentially adverse events in the cellular MOA-evaluation. The cellular MOA-scheme was then extended to include the mouse bone and circulating retinoid data observed in this study, as well as additional published bone and associated retinoid data from experimental, clinical and population studies with the help of AOP and WOE approaches. Focus of the AOP approach was to identify a cellular gene expression event, which could be regarded as an indicator of an irreversible shift in cell destiny in response to the TCDD exposure. Focus of the WOE analysis was to evaluate original data from the current study in the broader context of the published literature with the aims to identify evidence in support of the causal involvement, or not, of the retinoid system in the TCDD-induced postnatal bone phenotype, and furthermore, to address the evidence for an endocrine disruption mode of action, including the concerns that insults early in life may manifest later in life, and that wildlife impacts can be associated to current background contaminant exposures. To this end, investigative yet narrative literature searches were carried out in PubMed to identify studies where bone status was related to tissue retinoid concentrations, and to exposure to dioxins or related contaminants. Four different searches were performed to capture studies in rodents exposed in postnatal life, in rodents exposed both *in utero* and during lactation, in human cohorts or populations, and in wild-life situations. Search, terms such as “vitamin A”, “retinol”, “retinoids”, “retinol content” and “hypervitaminosis A” were used to cover the retinoid aspect of the search. For bone status, terms such as “bone”, “bone health”, “cell bone”, “cell bone differentiation” were combined. For contaminant exposures, the terms “pcb”, “pops”, “dioxin” and “tcdd” were used to capture different categories of persistent organic pollutants (POPs). The search for effects in wild-life situations required additional specific terms, such as “wildlife”, “fish” or “wild animals”. All these key words and search parameters were combined using the advanced tool on the PubMed website (https://pubmed.ncbi.nlm.nih.gov) in order to identify as many publications as possible on each of the four searches.

3. Results

3.1. Osteoblast experiment

3.1.1. Dose-response analyses of individual genes

All analysed marker genes were endogenously expressed in the osteoblast cultures (Figures **S**1-**S**3), and clear TCDD dose-response relationships could be established for the majority of genes (Figs. 2–4). The quantitative benchmark dose modelling was used to describe dose-response relationships of the mRNA expression of the individual genes described in Table 1 and Figure S4. The maximum response as well as the critical effect dose (CED) along with its lower and upper bounds of the one-sided 95 % confidence interval (CEDL and CEDU, respectively) were calculated. Thus, the CED represented the dose, which caused a change in the mRNA expression, which corresponds to half of the maximum response [critical effect size (CES) = max response / 2] of the mRNA expression of each target gene. The CED is also known as benchmark dose (BMD), CES as benchmark dose response (BMR) and CEDL and CEDU as BMDL and BMDU, respectively [78].

Dose-related repression of *Cyp26a1* and *Crabp2* expressions were observed (Fig. 2A, B) with CED-values around 9 pM TCDD and maximum responses close to 60 % (Table 1). *Raldh3* was dose-dependently increased to a maximum of 80 % at a much higher CED of 743 pM TCDD (Table 1; Fig. 2C). The corresponding CEDL-values for these three genes were 0.9, 4.8, and 19.1 pM TCDD, respectively (Table 1). A narrow CEDL-CEDU range i.e. the 95 % confidence interval around the CED, was observed for *Crabp2* expression, while broader ranges were observed for *Cyp26a1* and *Raldh3* expressions (Table 1; Figure S5). Although expressed in the osteoblast cultures, there was no effect of TCDD exposure on *Crbp1* and *Rdh10* mRNA expressions (Figure S1E-F) and consequently no dose-response relationship could be established for these genes (Table 1, Fig. 2E,F). These data suggest that TCDD had no impact on the retinoid storage process of osteoblastic cells, or that osteoblasts themselves are devoid of such a capacity. Alternatively, as mRNA responses often are very time sensitive, it cannot be excluded that a response of these genes to TCDD-exposure would have been seen at protein and/or enzyme activity levels.

Expressions of the cell differentiation marker genes *Alp* and *Ocn* were dose-dependently repressed by TCDD with CEDs of 13.9 and 17.4 pM, respectively, with corresponding CEDL-values close to 3 pM TCDD for both genes (Table 1, Fig. 3A, B). Maximum gene expression repressions were 71 and 60 %, respectively for *Alp* and *Ocn* (Table 1). In contrast, *Runx2* was induced by TCDD-exposure to a maximum of 40 % with a CED of 8706 pM and a CEDL of 2270 pM TCDD (Table 1, Fig. 3A). CEDL-CEDU ranges for the cell differentiation gene expressions were most narrow for *Alp*, followed by *Ocn* and *Runx2* (Table 1; Figure S5). Expressions of the AHR activation marker genes *Ahrr* (Fig. 4A), *Cyp1b1* (Fig. 4B) and *Cyp1a1* (Fig. 4C) were dose-dependently induced by TCDD with CEDs of 9.2, 9.5, and 13.4 pM, respectively (Table 1). Corresponding CEDLs were 7.1, 3.6, and 12.5 pM TCDD, and maximum responses were 1190 % (12-fold), 455 % (4-fold), and 5334 % (53-fold), respectively (Table 1). Narrow CEDL-CEDU ranges were observed for all three of these highly inducible genes (Table 1; Figure S5). *Ahr* expression was not affected by TCDD exposure (Figure S3D), and thus the *Ahr* gene expression data was not suitable for dose-response modelling (Fig. 4D). Plotting gene CEDs versus maximum gene expression responses derived from Table 1 revealed three distinct gene clusters (Fig. 5). The typical AHR activation genes *Cyp1a1*, *Ahrr*, and *Cyp1b1* clustered together based on high maximum induction responses (4.5 to 53-fold) and low

Table 1
Critical effect dose (CED) at half maximum response for the effects of TCDD on gene expression in osteoblastic cells as determined by benchmark dose analyses[a,b].

Gene	Model	CED (pM)	CEDL (pM)	CEDU (pM)	CED/ CEDL	CEDU/ CEDL	Max. Response (%)	Fold change
Cyp26a1	E5	9.3	0.9	61.7	10.5	68.6	−57	0.4
Crabp2	E5	9.8	4.8	17.8	2.1	3.7	−58	0.4
Raldh3	E5	743.1	19.1	862.0	38.9	45.1	80	1.8
Alp	E5	13.9	3.5	51.6	4.0	14.7	−71	0.3
Con	E5	17.4	3.1	88.1	5.7	28.4	−60	0.4
Runx2	E3	8706	2270	67,200	3.8	29.6	41	1.4
Ahrr	E5	9.2	7.1	11.0	1.3	1.5	1190	12.9
Cyp1b1	E5	9.5	3.6	14.7	2.7	4.1	455	5.6
Cyp1a1	E5	13.4	12.5	35.1	1.1	2.8	5334	54.3

CED is also known as benchmark dose (BMD), CES as benchmark dose response (BMR) and CEDL and CEDU as BMDL and BMDU, respectively [78].
[a] CEDL, critical effect dose at lower bound of the one-sided 95 % confidence interval.
[b] CEDU, critical effect dose at upper bound of the one-sided 95 % confidence interval.

CEDs (9.2–13.4 pM TCDD). *Raldh3* and *Runx2* clustered based on low maximum induction responses (41 and 80 %, respectively) and high CEDs (743 and 8706 pM TCDD), while clustering based on repression responses (in the range 57–80 %) and low CEDs (in the range 9.3–13.9 pM TCDD) included *Alp*, *Ocn*, *Cyp261a1*, and *Crabp2*. In summary, TCDD-induced gene expression data include repression of *Cyp26a1*and *Crabp2* in combination with inductions of *Ahrr*, *Cyp1b1,* and *Cyp1a1*. The seemingly coordinated changes in these genes were preceded, or occurred, on a dose-level, in parallel with the *Alp* and *Ocn* repressions (Table 1, Figure S5). At slightly higher TCDD-doses also induction of *Raldh3* expression occurred, while the proliferation marker *Runx2* was affected at much higher TCDD-doses. The combined and seemingly co-ordinated changes in these retinoic acid regulating genes are suggestive of an impact of TCDD on the cell dynamics, which is in place to maintain retinoic acid within its homeostatic and physiological range.

3.1.2. PLS regression analyses

The explanatory variables entered in the osteoblast PLS analyses based on their VIP-values were TCDD-dose and expressions of *Ahrr*, *Cyp1a1*, *Crabp2*, *Raldh3*, *Cyp26a1*, and *Cyp1b1* (Figure S6). The PLS model (Figure S7) explained 88 % of the variance of the explanatory variables and 80 % of the variance of the osteoblast differentiation marker genes. For the osteoblastic cells, the decreased expressions of *Alp* and *Ocn* were associated with decreased expressions of *Cyp26a1* and *Crabp2* (similar projections in both component t1 and t2). Further, the expressions of *Alp* and *Ocn* (abscissa to the left of component t1) were negatively associated to the expression of *Raldh3* and *Cyp1b1* (abscissa to the right of component t1). The expression of *Runx2* responded similarly as the expression of *Raldh3* (similar projections in both component t1 and t2). Further, the expression of *Runx2* (abscissa to the right of component t1) was associated with decreased expressions of *Cyp26a1* and *Crabp2* (abscissa to the left of component t1). The expression of *Runx2* (upper part of component t2) was also associated with the induction of *Cyp1b1* (lower part of component t2). Thereby, these PLS-results support a role of *Raldh3* as a mediator both in the repression of *Alp* and *Ocn* expression and in the induction of *Runx2* expression, although *Alp* and *Ocn* were affected at much lower TCDD-doses as compared to *Runx2*.

3.1.3. Cellular MOA analysis

An AOP-inspired cellular MOA scheme was developed to visualize relationships between the analysed genes, which were affected by TCDD exposure in this study (Fig. 6). The scheme was organized to include direct chemical interaction of TCDD with AHR (Fig. 6A) followed by sustained AHR activation (Fig. 6B), which in turn is known to result in a broad cascade of quantifiable gene transcription modulations [49,31,50, 79,80]. By grouping the analysed genes along the combined TCDD dosing and cell differentiation scale according to their dose-response data (Table 1) and their known physiological functions and interrelationships (Table S1) (in the MOA scheme, it became clearly visble that the moderately induced expressions of the retinoic acid synthesising enzyme *Raldh3* (Fig. 6C) and the proliferation marker gene *Runx2* (Fig. 6E) were vastly preceded by the moderately repressed expressions of the retinoic acid degrading enzyme *Cyp26a1* (Fig. 6C), and the nuclear retinoic acid translocator *Crabp2* (Fig. 6D) with CEDs of 9.3 and 9.8 pM TCDD, respectively, as well as the highly induced expressions of the AHR activation marker genes *Ahrr, Cyp1b1* and *Cyp1a1* (Fig. 6C). Next, the MOA scheme revealed that repression of *Alp* and *Ocn* expressions were affected at doses of TCDD, which were comparable to or lower than the doses, which affected expressions of *Cyp26a1, Crabp2*, and the AHR activation marker genes, respectively, and clearly at much lower TCDD-doses than those required for the induction of *Runx2* and *Raldh3* expressions (Fig. 6C–E). Concerning the nuclear compartment, only one marker gene from the retinoid system was analysed, i.e. *Crabp2*, which, comparable to *Cyp26a1,* was repressed by TCDD-induced AHR over-activation at the low end of the tested doses (Fig. 6D).

Together with current knowledge on the physiological function and molecular regulation of the analysed genes (summarized in Table S1) and their interrelationships (Figs. 1, S4) the results from the gene expression analysis are compatible with a successive TCDD-induced increase in cellular retinoic acid concentration.[3] In addition, repression of *Crabp2* expression is expected to result in decreased nuclear retinoic acid concentration with an expected impact on retinoic acid mediated gene transcription (Table S1; Figure S4). On these grounds, the MOA-scheme was further developed to reflect the expected successive increase in retinoic acid concentrations in the cytosol compartment (Fig. 6C) along the TCDD dosing-scale and the expected decrease of retinoic acid transfer into the nuclear compartment already at the lower part of the tested dose-range (Fig. 6D). The expected impact on the intracellular retinoic acid concentrations was next indicated in the cell differentiation compartment of the MOA-scheme (Fig. 6E) as a “driving force” of the cell differentiation process; a process, which is known to be conditioned by numerous endogenous molecules, including retinoic acid.

Furthermore, at a certain point on the TCDD-dosing scale, it seems from the gene expression data as indicated in the MOA-scheme that cellular retinoic acid homeostasis is no longer maintained but a tipping point, as indicated by the dashed vertical pink-coloured line in Fig. 6, is reached. The cellular retinoic acid concentration may, at this point and beyond, fail to fulfil its normal metabolic and/or transcriptional functions. Instead, at and beyond this point of TCDD-dosing, when retinoic acid homeostasis is no longer maintained, or it is transferred to a revised level of homeostasis or stress, the cell differentiation process can be assumed to be at or on its way to be overwhelmed and thus irreversibly

[3] The indicated retinoic acid changes in retinoic acid cellular concentrations (Fig. 8C-E) are expected based on the obtained gene expression results and established knowledge on roles and functions of the analysed genes, and their interdependencies (Table S1).

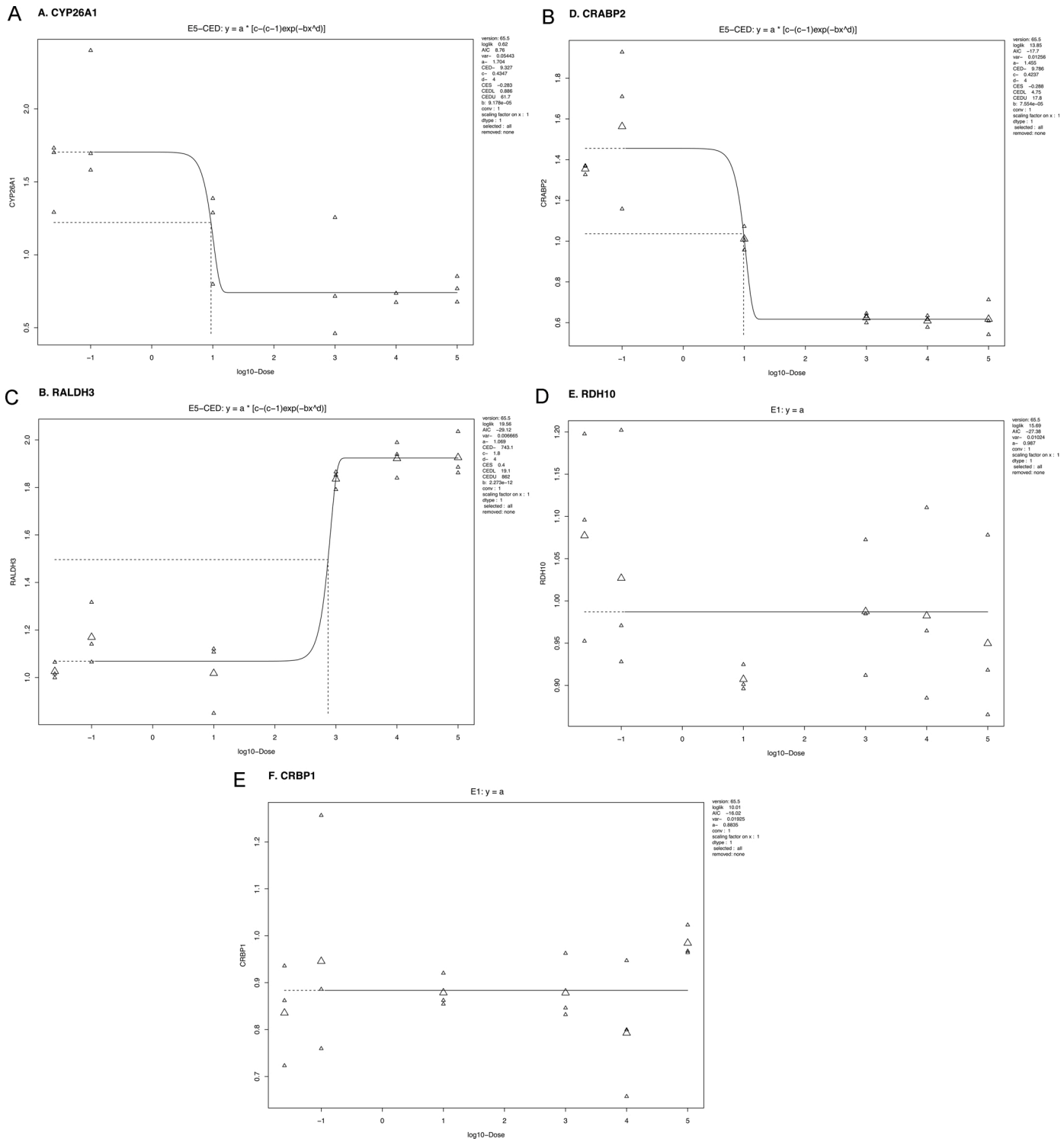

Fig. 2. Dose-response relationships with associated model parameters for the expression of **A.** *Cyp26a1*, **B.** *Crabp2*, **C.** *Raldh3*, **D.** *Rdh10* and **E.** *Crbp1* following exposure to TCDD. The small triangles are individual effect measurements and the larger triangles are group means. The horizontal dotted line indicates a critical effect size (CES) of max response / 2 in the mRNA expression of the target genes. CES is also known as benchmark dose response (BMR) [78]. The CES is used to calculate the critical effect dose (CED), which corresponds with the vertical dotted line, along with the lower and upper bounds of the one-sided 95 % confident interval of the CED (CEDL and CEDU, respectively). CED is also known as benchmark dose (BMD) and CEDL and CEDU as BMDL and BMDU, respectively [78]. The null model (E1) was selected for *Rdh10* (Fig. 2D) and *Crbp1* (Fig. 2E) expressions and hence no significant dose-response relationships were found, but instead horizontal relationships were established [78].

turned into a non-physiological or toxicologically adverse state for that cell model. Indeed, the repressed *Alp* and *Ocn* expressions in combination with the induced *Runx2* expression suggest that the osteoblasts, in this study, are turned into the proliferation mode instead of the maturation and/or mineralization modes, as the TCDD-dose is increasing and the toxicological tipping point is reached.

In this study it was expected that *Cyp1a1* induction would be responsible for or be directly involved in initiating the gene cascade

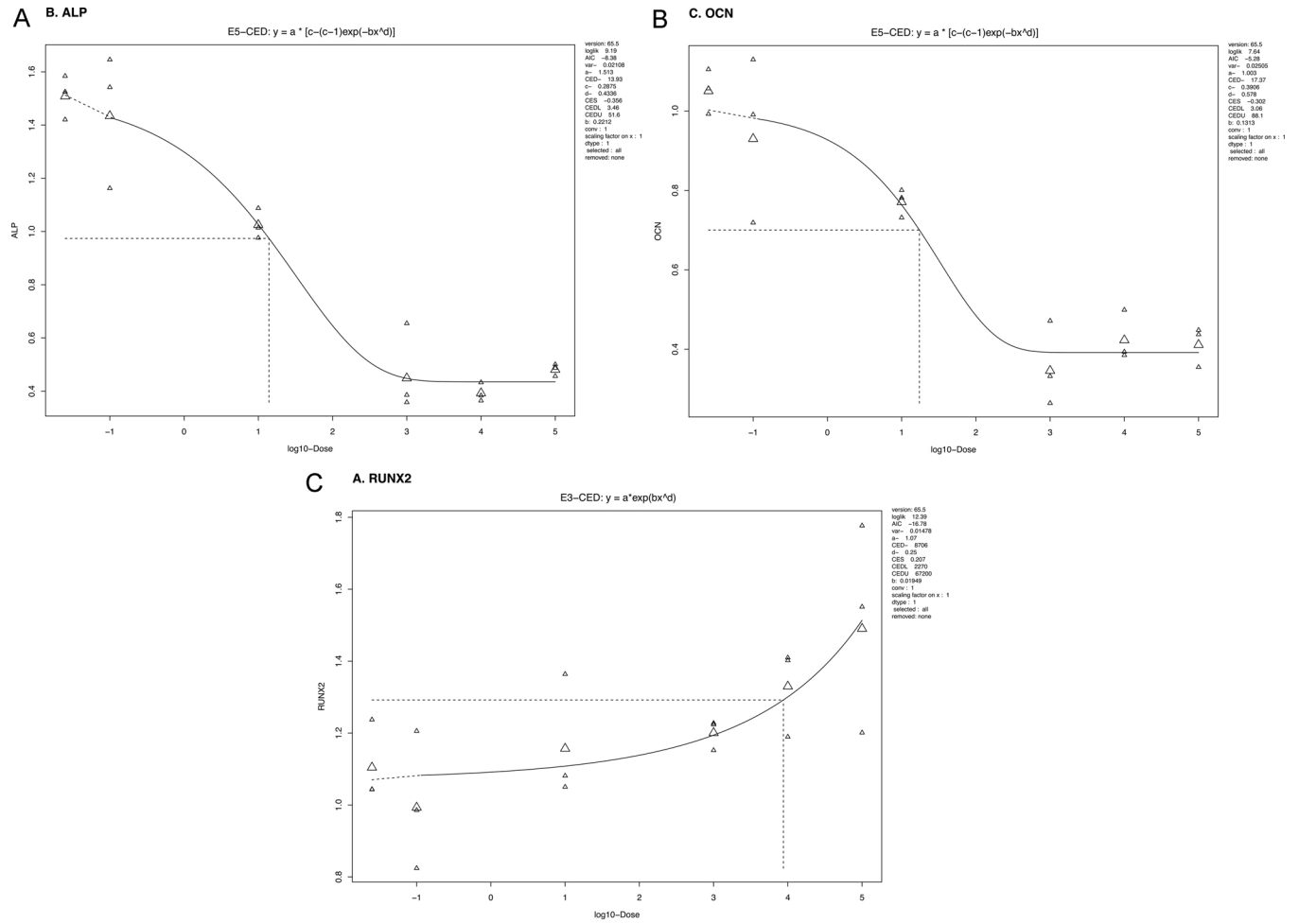

Fig. 3. Dose-response relationships with associated model parameters for the expression of **A.** *Alp*, **B.** *Ocn*, and **C.** *Runx2* following exposure to TCDD. The small triangles are individual effect measurements and the larger triangles are group means. The horizontal dotted line indicates a critical effect size (CES) of max response / 2 in the mRNA expression of the target genes. CES is also known as benchmark dose response (BMR) [78]. The CES is used to calculate the critical effect dose (CED), which corresponds with the vertical dotted line, along with the lower and upper bounds of the one-sided 95 % confident interval of the CED (CEDL and CEDU, respectively). CED is also known as benchmark dose (BMD) and CEDL and CEDU as BMDL and BMDU, respectively [78].

following upon AHR-activation by TCDD. A more detailed inspection of the dose-response curves (Figs. 4, S3) together with the curve parameters (Table 1), however, suggests that *Cyp1a1* is only moderately induced, compared to its max response at the lower TCDD-doses, when several of the other analysed genes are already induced or repressed to their max response. For example, as visualized in Figs. 6 and S5, induction of *Ahrr* expression occur at somewhat lower TCDD-doses as compared to *Cyp1a1* induction *(CEDL = 7.1 vs 12.5 pM TCDD)*, which can be understood as an attempt of AHRR to keep AHR activation at a low physiological rather than high toxicological level, and thereby limit the extent to which CYP1A1 may contribute to enhanced retinoic acid synthesis at the lower TCDD-doses: At some point of TCDD-dosing, however, the activation of CYP1A1 may over-ride the induced repression of AHR-activation by AHRR (Table 1, Figure S5). Therefore, the induced *Cyp1a1*-expression in this experiment may occur to its full potential only once the cytosolic cellular retinoic acid concentration has increased sufficiently to activate *Cyp1a1* also via it's RARE (Table S1).

Thus, *Cyp1a1* may not be the gene, which initiates the gene cascade following upon AHR-activation by TCDD. Instead, CYP1A1 together with AHRR may play key roles to avoid that cellular retinoic acid is reaching too high levels and thereby in a coordinated manner protect the cellular retinoic acid metabolizing machinery from reaching a or the tipping point. Such a "protection and/or gatekeeping"-mechanism may be difficult to maintain when AHR activation is high and persistent beyond an evolutionary adapted physiological range as can be the case when exposure is to high doses of high-affinity ligands such as TCDD. It is also likely that induction of *Raldh3* expression may represent or contribute to the tipping point-type of response, suggesting that induction of *Raldh3* expression may be seen as an adverse biomarker on the cellular level. Likewise, induction of *Runx2* expression at a low nM TCDD-dose could be considered as an adverse cellular response beyond the cellular tipping-point for normal cell differentiation homeostasis. From the dose-response relationship compared to the other genes, it seems that the *Runx2* response is dependent of several previous molecular and biochemical events, and that its alteration needs a considerably higher TCDD exposure and a preceding cellular homeostasis disturbance. Although it is likely that the homeostatic tipping point for cellular retinoic acid concentrations is controlled by one or more of the analysed genes i.e. *Cyp26a1*, *Crabp*, *Cyp1b1*, *Ahrr*, *Cyp1a1*, and *Raldh3* it is not possible based on the available data to pinpoint the exact sequential roles and interrelationships between these genes based on data in this study. It seems from the combined MOA and dose-response analysis, as visualized in Fig. 6, that repression of *Cyp26a1* and *Crabp* may play roles in the parallel initiating events in the cytosol and nucleus following upon AHR activation by TCDD, and that *Cyp1a1* and *Raldh3* may play a role in defining the tipping point. It was also an aim of the osteoblast experiment to estimate TCDD dose-levels, which, in a

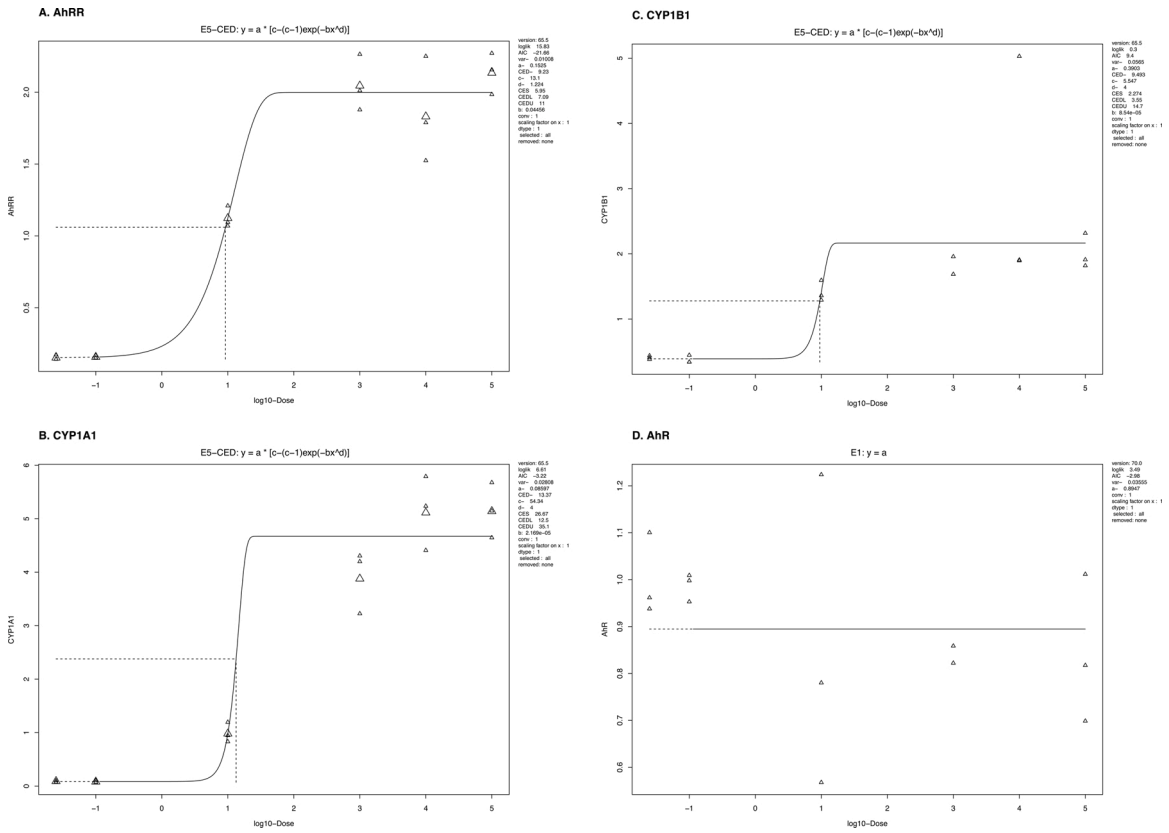

Fig. 4. Dose-response relationships with associated model parameters for the expression of **A.** *Ahrr*, **B.** *Cyp1b1*, **C.** *Cyp1a1* and **D.** *Ahr* following exposure to TCDD. The small triangles are individual effect measurements and the larger triangles are group means. The horizontal dotted line indicates a critical effect size (CES) of max response / 2 in the mRNA expression of the target genes. CES is also known as benchmark dose response (BMR) [78]. The CES is used to calculate the critical effect dose (CED), which corresponds with the vertical dotted line, along with the lower and upper bounds of the one-sided 95 % confident interval of the CED (CEDL and CEDU, respectively). CED is also known as benchmark dose (BMD) and CEDL and CEDU as BMDL and BMDU, respectively [78]. The null model (E1) was selected for *Ahr* expression and hence no significant dose-response relationship was found; instead a horizontal relationship was established [78].

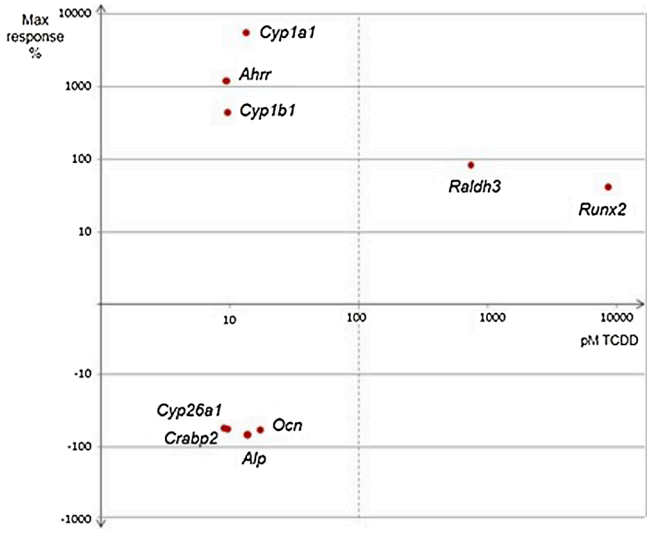

Fig. 5. Maximum gene expression response (%) and the critical effect dose (CED) for individual genes versus the applied TCDD dose range (pM) in the osteoblast differentiation assay. CED was defined as the dose which caused a change in the mRNA gene expression of max response / 2. CED is also known as benchmark dose (BMD) [78].

quantitative manner, could characterize identified critical gene expression changes, and the expected associated changes in cellular retinoic acid concentrations. The robust gene expression data set, which was generated over a large TCDD dose-range made it possible to identify among the analysed genes those, which were affected at the lower or higher TCDD-doses, and further to develop the MOA-inspired scheme (Fig. 6) to identify those genes, which were associated with each other under the influence of TCDD-exposure. On these grounds, we propose that the estimated CED-values can be utilized as one starting point to quantify key event relationships (KERs) and thereby form the basis for developing health and safety assessment guidance values.

Taken together with the MOA analysis the gene expression data suggest that TCDD-induced AHR-activation in the osteoblast differentiation model initiates two parallel chains of events. One, which impacts on the mRNA expression of several metabolic enzymes that are in control of cytosolic retinoic acid concentrations and homeostasis (Fig. 6C), and another, which impacts on translocation of retinoic acid into the nucleus by repressing *Crabp2* expression, and thereby controlling its availability for normal gene transcription (Fig. 6D). It can be assumed that these two event-chains are intertwined by one or more regulatory feed-back loops which, would remain to be identified by additional experiments. The obtained gene expression results are thereby compatible with the possibility that TCDD-induced modulation of intracellular retinoic acid homeostasis and/or availability for gene transcription in the nucleus can impact on the osteoblast differentiation process (Fig. 6C).

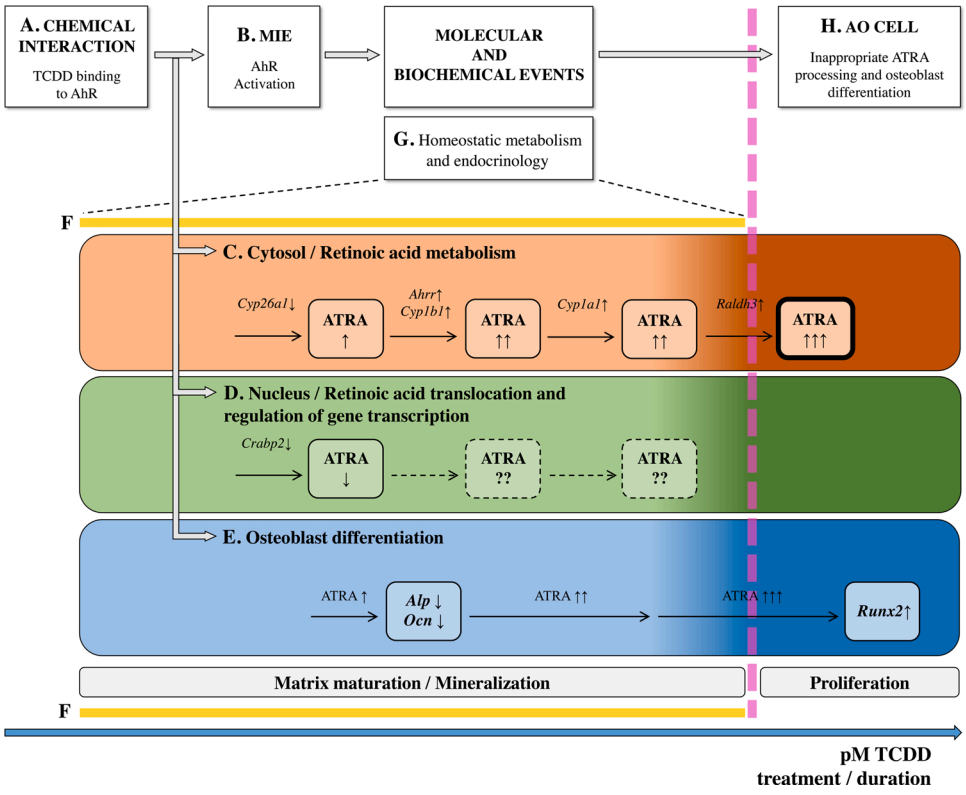

Fig. 6. Cellular mode of action (MOA) scheme for TCDD-induced modulation of osteoblast differentiation.[1,2] The cellular MOA scheme was developed to define likely sequences of molecular and biochemical events occurring upon TCDD-exposure of the osteoblast cultures. First, well established knowledge defined the initial events linking the direct chemical interaction of TCDD with AHR through high-affinity ligand-binding **(A)**, followed by sustained AHR over-activation, which is inducing a broad cascade of gene transcription modulations **(B)**. Next, the affected genes were grouped according to their known role and functions (Table S1) in retinoic acid metabolism **(C)**, retinoic acid translocation to the nuclear compartment **(D)**, and role in osteoblast differentiation **(E)**. Those genes were then placed according to their alterations along the TCDD dosing-scale (blue line) and according to their association with each other under the influence of TCDD-exposure. Based on the quantitative gene expression results (Table 1, Figures S4 and S5) and the known role and functions of the analysed genes in AHR, TCDD, retinoic acid, and bone biology (Table S1) the scheme was further developed to reflect the expected successive increase in retinoic acid concentrations in the cytosol compartment **(C)** along the TCDD dosing-scale and the expected decrease of retinoic acid transfer into the nuclear compartment already at the lower part of the dose-range **(D)**. Furthermore, the expected impact of the observed gene modulations on the intracellular retinoic acid concentrations were also indicated in the cell differentiation compartment **(E)** as a driving force of the cell differentiation process; a process, which is known to be conditioned by numerous endogenous molecules, including retinoic acid. The yellow horizontal lines **(F)** indicate the area on the TCDD-dosing scale, which is embracing cellular retinoic acid homeostasis **(G)**. The pink vertical dashed line represents the presumed tipping point, i.e. the irreversible shift in cell destiny from the osteoblast maturation/mineralization state to the proliferation state. When evaluated within the MOA and AOP contexts of this investigation, we considered this cellular consequence of TCDD exposure as the adverse outcome in this osteoblastic model (AOcell) **(H)**. The dashed line also represents the TCDD exposure level, which induces the tipping point change in intracellular retinoic acid concentration, as indicated by the bolded ATRA-box, by modulating key components in the enzyme and binding protein machinery required to maintain retinoic acid homeostasis. Arrows up or down represent increases and decreases, respectively, in the corresponding gene expression measurements or cellular ATRA concentrations. ??-marks indicate that current knowledge on retinoic acid concentrations in the nuclear compartment is absent. For abbreviations used in the figure see the Abbreviation list.

[1]Of note, the indicated retinoic acid concentrations (Fig. 8C-E) were not measured by chemical analytical methods in this study. Instead, indicated changes in directions and concentrations are expected based on the obtained gene expression results and established knowledge on roles and functions of the analysed genes, and their interdependencies (Table S1).

[2]Although the term AOcell does not conform to the regulatory established AOP parlance, we anyway introduced the term in this figure in order to illustrate how organ disease and pathologies of regulatory relevance, as exemplified by bone density and geometry parameters in this study, can be linked to measurable irreversible events on the cellular level.

3.2. Mouse association study

3.2.1. PLS regression analyses

The explanatory variables entered in the mouse PLS analyses based on their VIP-values were TCDD-dose, AHR genotype, body weight gain and circulating retinol concentrations, versus a handful of bone parameters in female and male mice separately (Figure S8). The applied regression models explained 77 % and 78 % of the variance of explanatory variables i.e. AHR genotype, body weight gain, and retinol in circulation for female and male mice, respectively, and 52 % and 37 % of the variance of the bone parameters for female and male mice, respectively (Fig. 7). For female mice (Fig. 7A), the higher circulating retinol concentration was associated with increased trabecular bone mineral density, trabecular number, and trabecular bone fraction in wild-type mice exposed to TCDD (similar projections in component t1). The higher circulating retinol concentration (abscissa to the left of component t1) was also associated with reduced structural model index (SMI) and cortical bone mineral density (abscissa to the right of component t1) in TCDD-exposed female wild-type mice. Female *Ahr*$^{-/-}$ mice (lower part of component t2) showed reduced cortical bone mineral density, as well as lower circulating retinol concentrations (similar projections in the upper part of component t2). For male wild-type mice (Fig. 7B), the higher circulating retinol concentration was associated with increased trabecular bone mineral density, as well as slight increase in total area, periosteal circumference, endosteal circumference and energy absorption capacity (similar projection in component t1). Further, the higher circulating retinol concentration (abscissa to the right of component t1) was associated with decreased cortical bone mineral density (abscissa to the left of component t1) in the male wild-type mice. The male *Ahr*$^{-/-}$ mice (upper part of component t2) showed reduced energy absorption

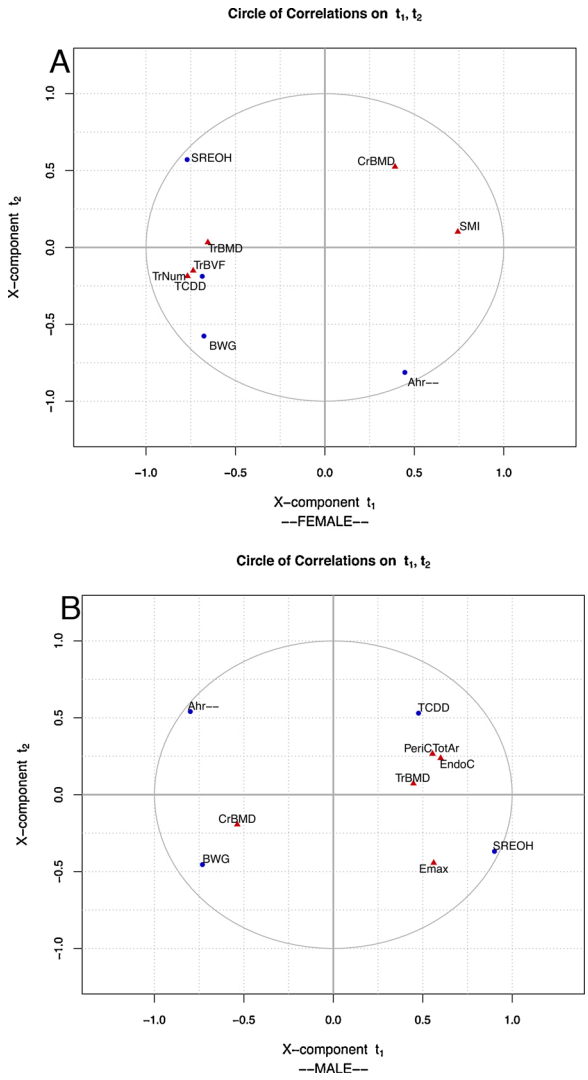

Fig. 7. Partial least square regression circles of correlations on components t1 and t2 for tibia parameters by variables for TCDD-exposure, AHR genotype, body weight gain, and serum retinol concentrations in female (A) and (B) male mice. Explanatory variables are displayed as blue circles and tibia variables as red triangles. The significance of each *X-variable* in the model is represented by Variable Importance in the Projection (VIP) values (Figure S6). For females, the fraction of cumulative explained variance of explanatory variables (R^2Xcum) were 0.43 and 0.77 for component t1 and t2, respectively. For corresponding result variables (R^2Ycum) fractions in females were 0.45 and 0.52. For males, the R^2Xcum fractions were 0.55 and 0.78 for component t1 and t2, respectively, and the corresponding R^2Ycum fractions were 0.30 and 0.37. Tibia data are derived from Herlin et al., [38] and retinol data from Esteban et al., [11] [This issue]. Abbreviations used in Fig. 7A for female mice: TCDD, TCDD-exposure; AhR, AHR-genotype: BWG body weight gain; SREOH, serum retinol concentration; CrBMD cortical bone mineral density; TrBMD, trabecular bone mineral density; TrNum, trabecular number, TrBVF, trabecular bone volume fraction; SMI, structural model index. Additional abbreviations used in Fig. 7B for male mice: TotAr, total area; Peri C, periosteal circumference; EndoC, endocortical circumference; Emax, maximal energy absorption capacity.

capacity as well as lower circulating retinol concentrations (similar projections in the lower part of component t2).

3.3. Narrative literature searches in support of the MOA/AOP and WOE analyses

As illustrated in Table S2, the literature searches provided a small range of experimental contaminant exposure studies in which both bone and retinoid effects were evaluated in parallel, as well as two studies, which analysed bone but not retinoids (Table S2A, B). In all those studies, the link between contaminant exposure and results on bone and retinoid parameters, respectively, was well defined in terms of exposure compound(s) and doses, and, furthermore, the exposure-effects links were specifically addressed in all the studies (Table S2A, B). In contrast, the corresponding link between exposures and outcomes in the two captured wildlife studies was less well described; largely the exposure parameters were limited to background exposure data only, i.e. polluted area or not (Table S2D). The search among human cohort and population publications identified no single study, which addressed contaminant exposure, as well as retinoid and bone endpoints in the same, or parallel studies (Table S2C). Nevertheless, since it is well known that human beings are exposed to contaminants, such as dioxin and related POPs, from the earliest stages of life, and, furthermore, accumulate these compounds over the life course, it was decided to tabulate those few studies, which analysed both bone and retinoid end-points in the same study population, despite the lack of contaminant background exposure information in study design or data evaluation. Likewise, three human studies, which include POP-exposure information are listed in Table S2C despite of lacking information on bone or retinoid data. Taken together, the search results revealed that POP exposures can be related to changes in bone parameters as well as tissue retinoid concentrations in coherent manners across the different categories of study models, and thereby supported the planned use of the literature search results in the WOE analysis.

4. Discussion

It has long been known that retinoids play important roles in bone tissue homeostasis, while more recent studies have shown that AHR has a role in bone biology, and that sustained over-activation of AHR by TCDD impact on bone cell activity and tissue properties. Although published data are compatible with a causal relationship between TCDD-induced bone and retinoid system alterations, there is need of more molecular and biochemical evidence to support this hypothesis. Only when there is sufficient mechanistic data available can the correlative information be made useful in a sustainable manner for preventive measures and regulatory decisions in diverse knowledge domains such as pharmacology, toxicology, and food safety, to the benefit of bone health and society at large. To feed in to this gap of mechanistic knowledge, in this study, we used a combined whole animal and cell culture experimental study design and evaluated the obtained results by the use of a systems biology approach inspired by the MOA, AOP, and WoE frameworks, which allow for discussing the study results in a further integrated manner.

4.1. Mouse bone and circulating retinol

In the mouse study we applied PLS-analysis to identify significant preclinical associations between previously published data [11,38] on circulating retinoid concentrations and data on tibia geometry, density, microarchitecture and mechanical properties from wildtype and AHRKO mice. We revealed that the TCDD-induced increase in trabecular bone mineral density and decrease in cortical bone mineral density were associated with elevated circulating retinol concentrations in male and female wildtype mice (Figures 7 and S8). Through the PLS association analyses, we showed that in TCDD-exposed female wildtype mice, the elevated circulating retinol concentrations were associated with increased trabecular number and bone tissue volume and with decreased structural model index. In male wildtype mice, moderately significant associations were found between elevated circulating retinol concentrations and increases in periosteal and endosteal circumferences, and in total area.

The tibia [38] and retinoid [11] findings as well as the strength of the associations (Figs. 7, S8), were largely comparable in male and female mice. We were also able to reveal through the PLS-analysis, that the endogenously low circulating retinol concentrations in AHRKO mice

[11] were associated with reduced cortical bone mineral density in female mice (Figs. 7A, S8A) and reduced energy absorption capacity in male bone (Figs. 7B, S8B). The identified associations extend previous findings from the same mouse experiment [36,38] and strengthen the hypothesis of a causal relationship between the observed TCDD-induced changes in bone properties and circulating retinol concentrations, which has been proposed both in experimental [81] and human studies (reviewed by [82]). The mouse results are also in line with associations between excess vitamin A intake and observations of harder and more brittle bone [26], increase of trabecular bone content [83], and lower mechanical strength [29,30] in rodent studies. It can be argued that the observed TCDD-induced elevation in circulating retinol concentrations, *per se* are mediating the associated bone tissue modulations observed in the study. It can also be argued that elevated circulating retinol concentration could be reflecting a corresponding elevation of retinol and/or retinoic acid in bone tissue itself; a circumstance supported by preliminary data from mouse femur (Figure S9, [125]). Considering the complexity of the retinoid system, however, it is also reasonable to assume that the TCDD-induced increase in circulating retinol can be a marker for disrupted overall retinoid homeostasis in the organism, which in turn might be a consequence of the observed gene expression changes and the expected and associated changes in cellular retinoic acid metabolism and gene regulation. To further investigate these possibilities, we next analysed the obtained gene expression and mouse data in the broader context of the published literature by using a narrative WOE-inspired approach.

4.2. Data integration – from MOA to WOE

Based on the detailed MOA analysis it is reasonable to assume that the measured osteoblast gene expression changes and the expected changes in cellular retinoic acid concentrations (Fig. 6) converge into the next levels of biological organization (Fig. 8). As indicated, beyond the cellular level, i.e. the osteoblast, the next levels of biological organization from the perspective of bone health include bone tissue, which is consisting of several distinct cell types in contact with peripheral blood, the skeleton with many individual types of bone as represented by tibia, one of the long bones assessed in this study, the organism where all organs are integrated and acting in a coordinated physiological manner, and finally the population level. Conceptually the WOE-analysis, was populated with data from published studies derived in the narrative literature searches (Table S2). On the experimental level we located a range of rodent studies, which applied environmental background exposure concentrations of TCDD or related contaminants

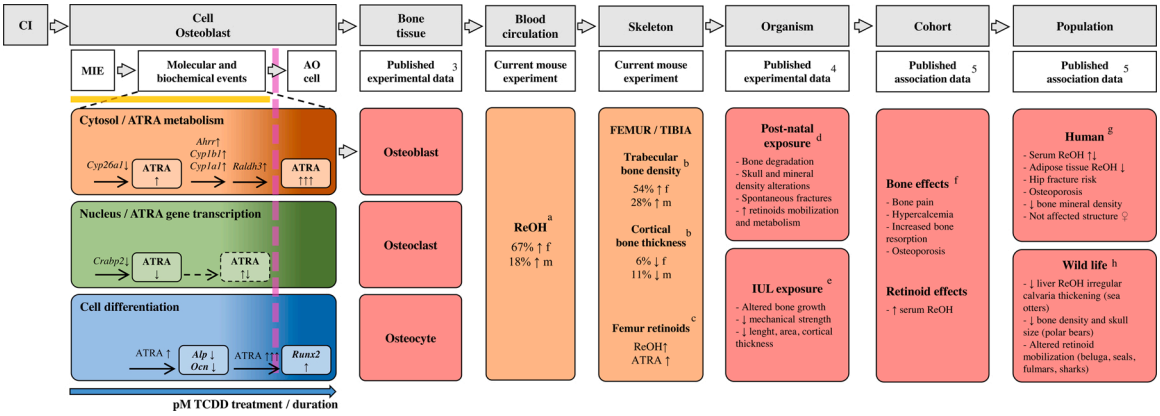

Fig. 8. Data integration scheme inspired by the MOA, AOP, and WOE approaches.[1,2] This data integration scheme builds on the MOA analysis presented in Fig. 6 and the assumption that the observed gene expression changes and the expected changes in cellular retinoic acid concentrations converge into the next levels of biological organization. The aim of the scheme is to visualize evidence and/or coincidences in support of the overall hypothesis that high-affinity AHR ligand-induced bone and retinoid system changes are causally related, and of equal relevance for different levels of biological organization, and, furthermore, is compatible with an endocrine disruption mode of action that may be equally relevant to human and wildlife populations.

[1]This WOE-analysis is a narrative evaluation of selected data; it is not based on a systematic review.

[2]For abbreviations and symbols used in the figure see the Abbreviation list and Figure 6, respectively.

[3]Bone tissue, which in addition to osteoblasts, consists of several additional types of specialized bone cells e.g. osteoclasts and osteocytes, assemble these cells in an environment of connective tissues and blood in circulation. Within the tissue these different bone cells communicate and collaborate intimately with each other, while at the same time assuring own integrity and characteristics. Although of different phenotypes and specialized functions these types of bone cells also share major commonalities such as for example hosting complete AHR [12,45,134,135] and retinoic acid machineries [136,18] and the close contact with circulating blood and the numerous number of endogenous molecules, including retinoids, vitamin D and steroid hormones, that are contained in this compartment. There are also distinctions between cell types within the bone tissue level of organization, for example in terms of the exact set up and responsiveness to endogenous molecules or external stressors such as high-affinity AHR-ligands and other POP contaminants. There is ample data supporting the key role of retinoic acid in the differentiation process of all these cell types [137–140] while there is less published work on the role of AHR in bone biology. Likewise, the impact of environmental contaminants on bone cell differentiation is less well studied.

[4]Presented data are from studies where bone, retinoids, and high affinity AHR-exposures were included in the same experiment. The more detailed bone and retinoid data, which are summarized in this figure, can be found in Table S2.

[5]Presented data from studies, which relate POP and/or high affinity AHR-exposures with bone effects or vitamin A-status alteration with bone effects, although they do not reflect an explicit interconnection between all three mentioned factors, so although plausible and supported by related evidences, it is part of the main discussion. The more detailed bone and retinoid data, which is summarized in this figure, can be found in Table S2.

[a][11].

[b][38].

[c]Preliminary results (personal communication).

[d][81,49,50,86,141,142,156,27,37,88,143–146].

[e][40,145,146,87,85,143,147,67,148–152].

[f][88,89].

[g][90,82,89,91–94,153].

[h][100,99].

individually or in combination to reflect real-life dietary exposure situations (Table S2A, B), in addition to data from the current mouse study. On the population level we located human and wild-life studies, which addressed bone conditions in relation to circulating retinoid concentrations or external retinoid exposures on the one hand, or to elevated POP-exposures via diet or geographical locations on the other hand (Table S2C, D).

4.2.1. Experimental findings

We could not locate any published experimental studies, which analysed the impact of TCDD or related compounds on the retinoid system in intact bone models. Thus, the current mouse study is the only such study, which is integrated in the WOE-scheme. Instead, as indicated in Fig. 8 and the associated Table S2A, B, the number of studies, where circulating and/or soft tissue retinoid concentrations and long bone parameters have been determined in parallel in the same animal experiment (although sometimes published in separate publications), is growing. These investigations often have regulatory toxicology study features, such as inclusion of human relevant dosing schemes with multiple orally provided dose-levels as well as sufficient, or at least minimal acceptable, numbers of animals in the study groups, and including both male and female study subjects. Many of these studies were using a combined *in utero* and lactational exposure design with postnatal follow-up time-points stretching into adult age as indicated in Figure 8. These studies are of importance from the perspective that they allow for the possibility to address regulatory aspects of later life effects due to early life exposures to compounds with endocrine disruption mode of actions. Although our mouse experiment was a single dose experiment, and not a dose-response study with TCDD, the obtained results tie in well with previous regulatory toxicology guideline type of studies in rats, where it has been well established that TCDD and other related POPs induce changes in bone parameters following prenatal as well as postnatal or adult exposure situations (Table S2A, B). Exposure levels in these long-term repeated-dose studies are of relevance for human, farm animal, and wildlife populations as these contaminants occur in everyday food commodities, such as fish, meat, dairy products, and mother's milk [84].

Apart from the preliminary data presented from the current mouse experiment (Figure S9), no one of the studies evaluated for the WOE analysis (Fig. 8, Table S2A, B) reported on retinoid concentrations in intact bone itself. Instead, many of the evaluated experimental studies reported on exposure-related changes in circulating retinoid concentrations and in tissues such as liver and kidneys. These data suggests that there is a systemic impact on the overall retinoid metabolism, which is characterized by markedly reduced hepatic retinyl ester concentrations, which are dose and compound-related, in addition to variations in tissue concentrations of retinol and retinoic acids. Further support for a systemic effect on the retinoid system comes from the observations that renal retinyl ester concentrations in contrast to hepatic concentrations are markedly elevated, and that, in rats, there is a sex difference in the renal retinyl ester response. Moderate increases in circulating retinol and retinoic acid concentrations have been reported in parallel to changes in bone geometry and mechanical properties in studies with TCDD [49,37,50]; Table S2) and the dioxin-like PCB126 ([81]; Table S2) in adult rats.

Many of the evaluated studies included careful dose-response analyses for each end-point and showed that retinoid effects may occur at comparable or somewhat lower doses of TCDD as compared to bone effects in these same studies Table S2A, B). The same results were revealed when exposure to TCDD occurred in adulthood [49]; [37,50] or at early life stages [85,67]. These results are in line with the MOA-analysis based on the osteoblast gene expression pattern, which is suggesting that the retinoic acid metabolizing enzymes and *Crabp2* were affected at lower TCDD-doses as compared to the induced expression of *Runx2*, i.e. the indicator of irreversible osteoblast perturbation (Table 1; Figs. 5, S5) of this experimental model. When exposed to PCB180 at early life stages (Alarcon et al., 2021) or to HBCD in adulthood [86] or at early life stages [87] lower doses were required to impact on tissue retinoid concentrations as compared to bone parameters. This observation may suggest that there is a difference in response pattern between the two endpoints depending on MIE-features of the analysed compound. So far, there is no reporting on association analyses between the bone and retinoid endpoints beyond this parallel reporting of bone and retinoid data from these individual experiments. Such additional statistical analyses to capture several related animal experiments can be fruitful as they provide efficient tools to trace, describe, and judge variations in pooled datasets. Despite the lack of such additional association analyses, it can nevertheless be concluded, that the evaluated experimental data base (Fig. 8; Table S2A, B) provides support for a correlative relationship between retinoid and bone observations in adult exposure situations as well as in situations of *in utero* and lactational exposures. Noteworthy, those associations are mechanistically consistent with the presented osteoblastic gene expression data, including the condition that the impact of TCDD on the retinoid system occurs at doses, which are much lower than the doses causing frank quantifiable changes in bone quality and functional parameters. Furthermore, the available database suggest that bone and retinoid system changes are not limited to impacts from AHR ligand compounds since they are also seen in exposure studies including chemicals, which are known to predominantly act *via* CAR and/or PXR such as PCB180 (Table S2B). For the future it will be interesting to find out if exposure to perfluorooctanoic acid (PFOA) impact, not only on bone (Table S2B), but also on the retinoid system. PFOA is known to bind and activate the peroxisome proliferator-activated receptors (PPAR), which heterodimerize with RXRs. Although not detailed in Table S2, it can be concluded from references in this table, that male and female responses are largely comparable, although not identical, and that responses generally occur at lower doses after in *in utero* and lactational exposure situations as compared to exclusively postnatal exposures.

4.2.2. Cohorts and population findings

On the population level, there were no human studies (Table S2C), which analysed the impact of environmental exposures to TCDD or related compounds on skeletal outcomes in relation to the retinoid system. Instead, there is a small and growing number of human cohort and population studies, which reported on fracture risk or other bone failures in relation to changes in dietary vitamin A-intake or circulating retinol concentrations (Table S2C; Fig. 8). A meta-analysis, which was based on eight intake studies (283 930 participants) and four prospective blood retinol concentration studies (8725 participants), concluded that high retinol intake is consistently associated to increased hip fracture risk [82]. Furthermore, dose-response meta-analysis revealed a U-shaped relationship between retinol in blood and risk of hip fracture with a narrow window of optimal benefits to bone health [82]. Altogether, the studies included in the meta-analysis shows clear links between retinol intake as well as its presence in circulation, with an increased risk of osteoporosis and hip fracture [82]. Furthermore, none of these studies addressed the potential for confounding due to background exposure to environmental contaminants though variables such as smoking, alcohol, and medications were included [88–91];Wu et al. [92]. Likewise, several small human studies analysed effects of dioxin-like compounds on bone mineral density (BMD) with variable results [93–96]. Positive and negative associations between bone mineral density and blood concentrations of individual congener were reported. Several bone mineral density, strength, and size indices in the Seveso Women's Health Study were positively associated with TCDD-exposure, and the associations were stronger when exposure had started before the age of 5 years [93]. Authors concluded that study results do not support the hypothesis of adverse effects on adult bone health by postnatal TCDD exposure; rather it seems that increasing TCDD concentrations in blood are associated with "better" bone. However, it needs to be considered that increased bone mineral density, as measured by dual-energy X-ray absorptiometry (DXA) or peripheral quantitative computer tomography (pQCT), does not

necessarily mean stronger and healthier bone in all situations. For example, increased trabecular bone mineral density, which was observed in a mouse study following exposure to TCDD, was accompanied by altered bone micro-structure [38]. The trabecular bone matrix had become harder and stiffer [38], suggesting imbalanced bone remodeling and less flexible bone. Thus, in that case, TCDD-exposure seemed to result in lower bone tissue quality despite of the apparently higher bone mineral density. Consistent with these skeletal findings in humans are results from a study in polar bears from East Greenland and Svalbard, which identified an increase in scull bone mineral density with increasing exposure to persistent organic pollutants [97]. Likewise, a small sample of Lake Apopka alligators, which were exposed to contaminated water environments, exhibited increased trabecular bone mineral density of femur and tibia compared to clean water alligators from the same area [98]. No retinoid analyses were included in those studies, instead monitoring of wildlife health in relation to the contamination situation in the Arctic region include coordinated observations on skeletal bone and retinoid concentrations in soft tissues and circulation of several species mainly in marine fauna (beluga, seals and sharks) and birds (fulmars) [99] and in sea otters [100]. Table S2D). According to the evidences, those marine mammals living under significant background contaminant exposures, showed variable alterations in the liver, kidney and serum retinol concentrations, together with a decrease in bone density and skull size, in the specific case of polar bears.

4.3. Regulatory support

Original data of the current study was entered into MOA/AOP and WOE-inspired schemes with the aim to facilitate practical use of the new research information among public health authorities and organizations in environmental medicine acting in different fields of chemicals safety. Furthermore, the MOA/AOP and WOE-schemes efficiently disclose obvious data gaps, and thereby serve the purpose to help identify areas where knowledge building is urgent. In this context it is important and interesting to note that, although the MOA/AOP and WOE- schemes of this study focuses on outcome measurements at later life stages and include the population level, the results, nevertheless, tie in well with the example AOPs for skeletal anomalies manifested during embryofetal development as presented in an extensive review on skeletal dysmorphogenesis linked to disruption of retinoid signaling [60]. The developmental example adverse outcomes in this article, i.e. cleft palate and hemivertebra, were associated to inhibitions of *Cyp26a1* and *Raldh2* enzyme activities, respectively, which were identified as the putative MIEs followed by estimations of increased and decreased endogenous retinoic acid concentrations, respectively, as the first putative KEs in the proposed AOPs for these developmental outcomes. In turn, hyper and hypoactivation of retinoic acid receptors followed as the next KEs.

Although it is not possible to directly compare between the hypothesized increasing endogenously formed retinoic acid concentrations in the osteoblast differentiation assay of the current study (Fig. 6) and the tipping point concentration of 17 ± 11 nM externally added retinoic acid as established in the human stem cell differentiation assay [61], it is anyway interesting to note that the CED-values for almost all the responding genes in the osteoblast study were in the low pM TCDD-range; only *Runx2* expression, i.e. the indicator-gene for an irreversible osteoblast perturbation, had a CED-value in the low nM-range of TCDD. It is also interesting to note that the retinoic acid concentration in mouse femur was increased following 10 weeks of TCDD-dosing (total dose of 200 ug/kg body weight), however, these preliminary data (Figure S7A) cannot be directly compared to the derived tipping point concentration of 17 ± 11 nM all-trans retinoic acid added to the stem cell model. Future studies, using dynamic study design, modern HPLC analytical equipment and tissue extraction methodologies are needed, in addition to computational data-driven models, to determine precise endogenous retinoic acid concentrations and changes related to various types of exposures. Staining techniques, such as LacZ, can also be useful to capture relative endogenous retinoic acid concentration variations between cells as well as subcellular localizations in various organs in response to external exposures.

4.4. Strengths and weaknesses

A major strength of the current study is the robust gene expression data, which was generated over a large TCDD dose-range to define detailed dose-response relationships for individual genes, and association analyses between the different categories of gene markers. All gene expression data undergoing dose-response modelling were captured within the broad range of TCDD-doses utilized in the study, meaning that the CEDL-CEDU ranges as well as the CEDs modeled by the benchmark dose methodology were within the experimental dose-range, and study focus was on impact of TCDD on the endogenous metabolism and processing of retinoic acid. An additional strength of this manuscript is the appropriate and representative selection of AHR battery and retinoid system genes, which, although small in numbers, embraced and described well the complete biological window of osteoblast differentiation, as well as the impact of TCDD-exposure on this process. Additional studies are required to further strengthen the initiated causality analysis. Repeated osteoblast experiments, where additional time-points and a broader selection of genes are being analysed, as well as corresponding analyses also in osteoclast and osteocyte cultures, will provide knowledge in support of a more detailed causality analysis. For example, a broader selection of metabolic as well as functional genes, can in future experiments with different toxicology exposure scenarios provide additional mechanistic insights, not the least, in the cross-talk area between AHR and RAR on the one hand, and between CAR/PXR and RXR on the other hand. Follow-up of observations in this study can be further strengthened by additional analysis of the effect of TCDD on phase I/II enzymes of relevance of retinoic acid metabolism in the osteoblasts. We did not include measurements of *Cyp2b* or *Cyp3a,* which are established retinoic acid degrading enzymes (Figs. 1, S4), or other such general and high capacity degradation and/or elimination enzymes, nor markers for cytotoxicity or other types of oxidative stress reactions. However, it is known from the literature that TCDD induces multiple oxidation and glucuronidation enzymes in dose-related manners [49,50,79]. It is also well known that TCDD-dosing results in increased concentrations of excreted retinoids in feces, bile and urine [101–105], thus providing further support to the assumed cellular increase in TCDD-induced degradation and elimination of cytosolic retinoic acid. A weakness of the current study is the narrative instead of systematic approach applied in the MOA/AOP and WOE-analyses. Since the literature on retinoids in health and disease on the evolutionary, physiological as well as molecular levels is very large and captures more or less all organ systems and functions, there is room and needs for many types of systematic reviews to clarify the role of retinoids in complex toxicological matters, such as endocrine disruption. In this context, it is, nevertheless, a strength of this study, that it is providing a solid and well-contextualized precedent, which lays foundations for future systematic elaborations to reach fully explanatory MOA, AOP, and WOE schemes for many diverse health issues in toxicology, environmental medicine and public health where the retinoid system may play fundamental, yet understudied molecular roles.

4.5. Summary and concluding remark

In conclusion, this study established robust associations between increased circulating retinol concentrations and bone mineral density parameters in tibia of TCDD-exposed mice. The study also identified gene expression profiles, which suggest that TCDD induces dose-related increases in cytosolic retinoic acid concentrations in parallel to osteoblast transformation from the maturation/mineralization state to the proliferation phenotype as judged from the dose-related repression of *Alp* and *Ocn* expressions at low pM doses of TCDD, and induction of

Runx2 expression at nM TCDD concentrations. Evidence supporting the scenario of increased retinoic acid concentrations in the cytosol, include repression of *Cyp26a1*, and inductions of *Ahrr, Cyp1b1* and *Cyp1a1* expressions at low pM TCDD-doses, and induction of *Raldh3* expression at somewhat higher doses. In addition, the observed, immediate repression of *Crabp2* in the osteoblast assay at low TCDD-doses is suggestive of a decrease in the transfer of retinoic acid to the nuclear compartment. A decrease, which in turn indicates an impact on the transcriptional activity of genes that are directly responding to retinoic acid and those, which are regulated via RARs, and possibly also those regulated via AHR. Among those genes are *Alp* and *Ocn*, which were repressed at the same or slightly higher TCDD-doses than those which repressed *Cyp26a1* and *Crabp2*. Further result evaluation by MOA/AOP and WOE inspired analytical approaches strengthened the evidence that TCDD-induced bone and retinoid system changes are causally related and compatible with an endocrine disruption mode of action of relevance for different levels of biological organization, and of equal relevance to human and wildlife populations. Given the central role of the retinoid system in cell homeostasis, transcriptional regulation, and extensive hormonal cross-talk, over the life-course, and in more or less all tissues and organs, the presented data lend full support to ongoing regulatory initiatives to address this evolutionary conserved and dietary derived pathway in toxicological sciences at large.

Declaration of Competing Interest

The authors report no declaration of interest

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.reprotox.2021.07.013.

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