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MODULATION OF HORMONE SIGNALING BY CADMIUM: *FROM MOLECULAR MECHANISMS TO HEALTH IMPLICATIONS*

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Cover: Estradiol-3D-balls structure
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“Four things support the world: the learning of the wise, the justice of the great, the prayers of the good, and the valor of the brave”

(The last messenger)

Dedicated to my parents, grandparents and teachers…

ABSTRACT

Cadmium is a toxic metal classified as human carcinogen and ubiquitously found in our environment mainly from anthropogenic activities. Exposure to cadmium has been associated with increased risk of certain hormone-dependent cancers in humans, and the metal has been proposed to possess endocrine disruptive properties by mimicking the physiological actions of estrogens. However, the mechanisms behind these effects are unclear. The overall aim of this thesis was to provide mechanistic insights into the estrogenicity of cadmium that may have implications for the human health. To achieve this aim, investigations on the estrogen-like effects of cadmium as well as possible involvement of classical/non-classical estrogen receptor signaling was studied in mice, and these mechanisms were further scrutinized in cell-based models. Furthermore, associations of biomarker of cadmium exposure with endogenous circulating sex hormones were evaluated in a population-based study of women.

Results presented here indicate that exposure to cadmium does not affect the genomic estrogen response *in vivo* in mice, suggesting that classical estrogen signaling is not targeted by cadmium. However, some estrogen-like effects were observed in cadmium exposed mice, i.e. significant thickening of uterine epithelia, in the absence of uterine weight increase, and activation of ERK1/2 MAPKs in the liver. This suggests the existence of alternative signaling pathways modulated by cadmium. In addition, exposure to a wide dose range of cadmium, dose-dependently increased the expression of the endogenous genes *Mt1, Mt2, p53, c-fos,* and *Mdm2* in mouse liver, with *p53* being the most sensitive gene. However, phosphorylation of ERK1/2 was already induced at the lowest exposure level (0.5µg/kg body weight), rendering ERK1/2 a more sensitive marker of exposure than any change in gene expression. Furthermore, *in vivo* findings suggest that cadmium-induced effects are markedly concentration dependent: low-level exposure activates protein-kinases whereas high-level exposure turns on cellular stress responses. The data from *in vitro* studies indicate that cadmium at regular human exposure levels activates protein-kinase signaling through Raf-MEK-ERK/MAPKs, and we identified EGFR and GPR30 as the mediating receptors. This cadmium-induced activation of protein-kinases further leads to a disturbance in Mdm2/p53 balance, with a significant increase in the Mdm2/p53 ratio in the presence of genotoxic compounds, which in turn suggest that cadmium may disrupt stress response to genotoxins. In 438 postmenopausal women, a positive association was observed between the concentrations of cadmium in blood and testosterone in serum, while an inverse association was observed with estradiol. This may suggest that cadmium affects steroidogenesis.

In conclusion, data presented in this thesis collectively suggests that cadmium-induced estrogen-like effects do not involve classical estrogen receptor signaling but rather appear to be mediated via membrane-associated signaling. The activation/ transactivation of GPR30/EGFR-Raf-MEK-ERK/MAPKs and Mdm2 represent a general mechanism by which cadmium may exert its effects. Since EGFR, ERK and Mdm2 are all known key players in cancer promotion, cadmium-induced activation of these and disturbance in the estradiol/testosterone balance in women may have implications for the promotion/development of hormone-related cancers.

LIST OF PUBLICATIONS

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

I. **Ali I,** Penttinen-Damdimopoulou PE, Stenius U, Mäkelä SI, Berglund M, Åkesson A, Håkansson H, Halldin K. Estrogen-Like Effects of Cadmium *in vivo* do not appear to be mediated via the classical Estrogen Receptor transcriptional pathway. Environ Health Perspect, 2010, 118:1389-1394.

II. **Ali I,** Damdimopoulou P, Stenius U, Adamsson A, Mäkelä SI, Åkesson A, Berglund M, Håkansson H, Halldin K. Cadmium-induced effects on cellular signaling pathways in the liver of transgenic estrogen reporter mice. Toxicol Sci, 2012, 127(1):66-75.

III. **Ali I,** Damdimopoulou P, Stenius U, Halldin K. Cadmium at nanomolar concentrations activates MAPKs via EGFR, leading to disrupted Mdm2/p53 balance: A mechanism by which cadmium may increase cancer risk. 2013. (*Submitted for publication*)

IV. **Ali I,** Engström A, Vahter M, Skerfving S, Thomas L, Lidfeldt J, Samsioe G, Halldin K, Åkesson A. Associations between cadmium exposure and circulating levels of sex hormones in postmenopausal women. 2013. (*Submitted for publication*)

CONTENTS

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BSA	Bovine serum albumin
BMD	Benchmark dose
BMR	Benchmark response
$CdCl_2$	Cadmium chloride
CES	Critical effect size
CI	Confidence interval
DHEAS	Dehydroepiandrosterone sulfate
DNA	Deoxyribonucleic acid
EDCs	Endocrine Disrupting Chemicals
EE_2	Ethinyl estradiol
E_2	Estradiol
EFSA	European Food Safety Authority
EGFR	Epidermal growth factor receptor
ERE	Estrogen response elements
ERK	Extracellular signal-regulated kinases
ERs	Estrogen receptors
GPR30	G protein-coupled receptor 30
IARC	International Agency for Research on Cancer
ICPMS	Inductively coupled plasma mass spectrometry
i.p.	Intraperitoneal injection
JECFA	Joint Expert Committee on Food Additives
LOD	Limit of detection
MAPKs	Mitogen-activated protein kinases
mER	Membrane estrogen receptor
MT	Metallothionein
nER	Nuclear estrogen receptor
NOAEL	No observed adverse effect level
OECD	Organisation for Economic Co-operation and Development
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PI3K	Phosphoinositide 3-kinase
PTMI	Provisional tolerable monthly intake
PTWI	Provisional tolerable weekly intake
RNA	Ribonucleic acid
s.c.	Subcutaneous injection
S.D	Standard deviation
SERMs	Selective estrogen receptor modulators
SHBG	Sex hormone-binding globulin
siRNA	Small interfering RNA
TWI	Tolerable weekly intake
US-EPA	United States Environmental Protection Agency
WHILA	Women’s Health in Lund Area
WHO	World Health Organization

1 INTRODUCTION

Many natural and man-made chemicals pose a threat to human health and are a source of environment-related diseases. Despite preventive measures undertaken based on our understanding of the complex inter-relationship that exists between human health and the environment, the burden due to these diseases remains significant [1]. We are all exposed to pollutants from the air we breathe, the water we drink, and the food we eat which potentially may pose a threat to our health. In order to take effective preventive measures, efforts to identify the potential hazards posed by these environmental contaminants through toxicological and mechanistic studies, in parallel with observational population-based studies, are very vital to be able to establish a causal link between experimental results and the human diseases.

Several environmental pollutants are known to affect the endocrine system which may lead to an array of effects including reproductive, developmental, neurological, immunological, and carcinogenic effects [2]. A variety of chemicals with the ability to disrupt the endocrine system are suspected to play a role in the increased incidence of hormone-related cancers world-wide [2]. Hormone-related sporadic cancers of the breast, endometrium, ovary and prostate are all proposed to share a distinct mechanism of carcinogenesis [3, 4]. Cadmium has, besides its well-known nephrotoxic effects, been reported to possess endocrine disruptive properties [5] and was proposed as a potent metalloestrogen [6, 7] indicating a possible involvement in hormone-related cancers. All humans are, mainly via food, exposed to cadmium [8], and in a global setting, rather than acute exposure, the real challenge seems to be this chronic low level exposure [9]. The main focus of the studies in this thesis has been to investigate and characterize the estrogen-like effects of cadmium and to provide new knowledge on the molecular mechanisms behind these effects that may explain how cadmium at low doses increases hormone-related cancer risk.

2 BACKGROUND

2.1 CADMIUM

Cadmium is a toxic heavy metal that occurs ubiquitously in nature at relatively low levels [10]. Cadmium in soils is known to originate from geogenic (natural) and anthropogenic (industrial) sources [11]. Approximately 90% of the cadmium in our environment comes from anthropogenic sources, however the proportion differs significantly in those areas where much larger amounts of cadmium is present naturally in rocks containing lead-zinc minerals and in soils derived from some marine black shales [10, 12]. Considerable differences exist in the relative importance of anthropogenic sources for cadmium which include mainly phosphate fertilizers, ash from fossil-fuel combustion, waste from cement manufacture and metallurgical works, municipal refuse and sewage sludge, and atmospheric deposition [12]. The application of phosphate fertilizers and atmospheric deposition are significant sources of cadmium entry into arable soils in some parts of the world, whereas sewage sludge could also be an important source at the local level [12]. Among the anthropogenic sources, phosphate fertilizers (56%) and atmospheric deposition (40%) contributes the most [13]. However, due to strict regulations on the cadmium contents in phosphate fertilizers, the contribution to soil (from phosphate fertilizers) has been decreased. The presence of cadmium in soil results in an uptake of cadmium by plants, which is influenced by the pH of soil i.e. higher uptake at low pH levels [12]. Thus, whilst cadmium is present virtually in all foods, concentrations vary to a great extent depending on the type of food and the level of contamination in the soil [8].

2.1.1 Human Exposure

Cadmium exposure in humans occurs mainly through inhalation (e.g. via smoking) and ingestion (via food). Food crops grown on cadmium-contaminated soils or on soils naturally rich in this metal constitute a major source of non-occupational environmental cadmium exposure in non-smokers in most parts of the world [14-16]. Based on an estimation of cadmium intake, more than 80% of the food cadmium comes from cereals, vegetables and potatoes [17]. However, human exposure to dietary cadmium is strongly influenced both by cadmium levels in food and the consumption patterns. For instance, the fiber-rich foods that are often referred to as healthy foods, are considered to be significant source of dietary cadmium intake [18]. In humans, the average cadmium

intake from food varies between 8 and 25µg per day [17, 19-24] but is higher in contaminated areas like in Japan [25].

Besides dietary cadmium intake, tobacco smoking is an important source of non-occupational cadmium exposure. Due to the inherent genetic and physiological characteristics, the tobacco plant efficiently accumulates cadmium from soils [26]. Tobacco leaves contribute approximately 1-2 µg cadmium in each cigarette; however it may vary depending on type and brand of cigarette [27, 28]. Among smokers, roughly 10% of the cadmium content is inhaled and it is estimated that an individual smoking on an average 20 cigarettes per day will absorb about 1 µg cadmium daily [8]. There is some evidence of cadmium exposure from environmental tobacco smoke in children [29], although it doesn't seem to be a significant source of cadmium exposure in adults [30].

2.1.2 Absorption, Distribution, and Excretion

Dietary absorption of cadmium is favored by a deficiency of calcium, zinc, copper, iron and protein in the diet [19, 31-33]. Approximately 3–10% of ingested cadmium is absorbed from the gastrointestinal system and about 50% of inhaled cadmium is absorbed from the lungs. In blood, cadmium moves from plasma to the red blood cells where it binds mainly to metallothionein (MT) and to hemoglobin. MT is a class of small cysteine-rich proteins which exhibit high binding affinity to cadmium and zinc [34]. From blood, cadmium is transported to the liver, where it binds to albumin and stimulates the synthesis of MT.

The amount of cadmium stored in various organs varies considerably depending on MT concentration. The organs that store cadmium include the liver, kidney, testis, spleen, heart, lungs, thymus, salivary glands, epididymis, and prostate [34]. However, approximately 50% of the cadmium found in humans is stored in liver and kidney due to their high MT concentration. In humans, cadmium has a half-life of 10 to 30 years [8, 35], which leads to bioaccumulation of cadmium in the body by age.

MT plays an important role in the excretion of cadmium, probably due to its properties as a chelating agent [36, 37]. The excretion of cadmium occurs mainly through the liver and kidneys, but the excretion rate is normally very low. Approximately 0.005-0.1% of the total body burden of cadmium is excreted per day, mainly via urine. Such extremely

slow excretion rate of cadmium is due to the lack of an active biochemical mechanism for elimination coupled with renal reabsorption [35]. Brief details of cadmium transportation and distribution in the main compartments of human body are shown in **Figure 1** [38].

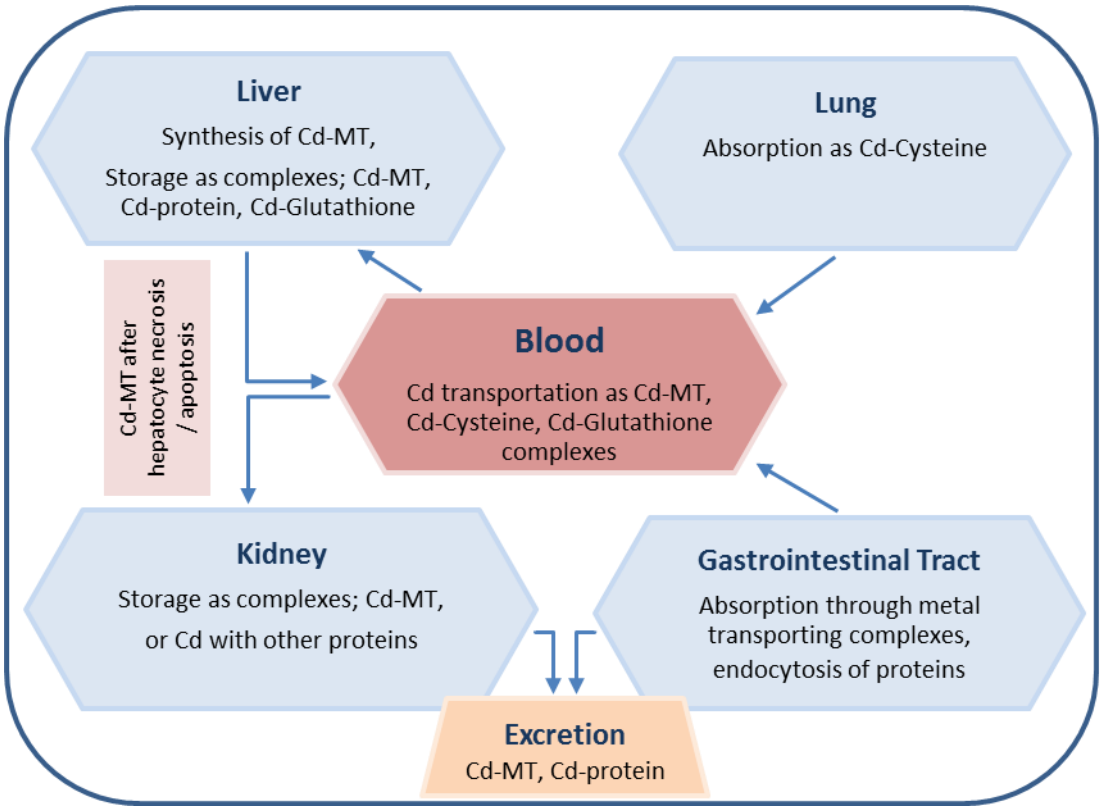

Figure 1: Distributional processing of cadmium in major compartments in human body

2.1.3 Biomarkers of Exposure

A biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological and/or pathogenic processes, or pharmacologic responses to a therapeutic intervention [39]. In epidemiology and toxicology, a biomarker can also be used to indicate exposure to various environmental substances, generally referred to as biomarker of exposure. In case of cadmium exposure, blood cadmium is considered to be the most valid biomarker of recent exposure and is usually assessed in the whole blood. In blood, cadmium is found in the erythrocytes bound to high- or low-molecular-weight. The half-life of cadmium in blood displays a fast component of 3 to 4 months and a slow component of 10 years after cessation of exposure [40]. However after long-term low-level exposure, cadmium in blood may serve as a good indicator of the cadmium body burden [35].

Urinary cadmium is often used as an indicator of whole body burden, attained throughout the life-span, and is directly proportional to the concentration in kidney [41, 42]. Ideally, urinary cadmium is assessed as the amount excreted over 24 hours but in practice spot urine sampling (often first voided morning urine) is commonly used. As spot urine samples vary in composition within and between individuals, some adjustments with creatinine concentrations or urinary density are made. The high correlation between blood cadmium and the urinary cadmium facilitates the interpretation of the vast majority of studies using urinary cadmium as the dose estimate [8]. Thus both biomarkers of exposure i.e. blood cadmium and urinary cadmium, are most likely good estimates of cadmium body burden in environmentally exposed populations. Generally, for non-smoking adults living in non-polluted areas, the concentrations of cadmium in the whole blood vary between 0.1 and 1.0 µg/L and those in urine varies between 0.6 and 1.2 µg/g creatinine [43].

2.1.4 Health Effects

Cadmium toxicity is believed to contribute to a large number of health conditions. Long-term cadmium exposure may give rise to renal, pulmonary, hepatic, skeletal and reproductive effects, as well as cancer [25]. The first report on human health effects in response to environmental cadmium exposure came out after the Second World War in Japan. Many women suffered with severe renal and skeletal damage, later referred to as itai-itai-disease (meaning "ouch-ouch" in Japanese), associated with high cadmium intake from rice grown on contaminated soil [8, 44]. However, in recent studies even low exposure has been clearly shown to be a risk factor for adverse effects on bones in the general population [18, 45-48].

The identification of adverse effect that occurs at the lowest exposure level, i.e. the critical effect, is very important for preventive measures. Many of the toxic effects of cadmium exposure stem from its accumulation in the kidneys [25], and renal tubular dysfunction is considered as the most critical effect that occurs with the increase in cadmium exposure [49]. The earliest signs of tubular dysfunction are decreased tubular reabsorption i.e. increased urinary excretion of low-molecular-weight proteins and intracellular tubular enzymes [49].

Cadmium has been suggested as an etiologic factor in certain human cancers. The International Agency for Research on Cancer (IARC) reevaluated the evidence for

carcinogenicity of cadmium in 2009 and reaffirmed that cadmium compounds are carcinogenic to humans [50]. This classification was based on sufficient evidence from studies in humans, including epidemiological and mechanistic studies. DNA repair inhibition and disturbance of tumor-suppressor proteins leading to genomic instability were considered as established mechanistic events. The evidence was classified as sufficient for lung cancer and limited for prostate and kidney cancer [50].

2.1.5 Health Risk Assessment

Human health risk assessment is a process to estimate the nature and probability of adverse health effects in humans who may be exposed to chemicals in contaminated environmental media, now or in the future [51]. In order to protect human health in response to cadmium exposure, a health based guidance value of 7 µg/kg body weight per week [provisional tolerable weekly intake (PTWI)] was established by the Joint FAO/WHO Expert committee on Food Additives (JECFA) and endorsed by the Scientific Committee on Food [52]. Based on this guidance value, 70 µg corresponds to a tolerable daily intake of cadmium for an average person with 70 kg weight. Although available data on cadmium intake in the general population indicated that most individuals are below PTWI (7 µg/kg body weight), several international bodies recognized that the margin between the established PTWI and the actual weekly intake of cadmium was very small and in some populations may be non-existent [43]. However, in 2009 The European Food Safety Authority (EFSA) performed a risk assessment on cadmium and established a new tolerable weekly intake (TWI) of 2.5 µg/kg body weight [43]. This new TWI was set to keep 95% of the population below the "reference point" of critical urinary cadmium concentrations i.e. 1 µg/g creatinine at the age of 50 years. Thus in order to remain below this reference point, the average daily dietary cadmium intake should not exceed 0.36 µg/kg body weight. Later in 2010, JECFA reviewed its previous evaluation and established a provisional tolerable monthly intake (PTMI) of 25 mg/kg body weight which corresponds to weekly intake of 5.8 µg/kg body weight, which is based on the reference point of 5.4 µg/g creatinine [53]. Regardless of the inconsistencies between the two risk assessments, EFSA concluded that the TWI established in 2009 i.e. 2.5 µg/kg body weight, should be maintained in order to ensure the high level of protection of consumers from the effects on kidney [54].

2.2 HORMONE-RELATED CANCERS

The concept that hormones can increase the incidence of human cancers is mainly developed for four hormone-related cancers: breast, endometrium, ovary and prostate [55, 56]. Estrogens, progesterone and androgens affect the cell proliferation and thus increase the risk of accumulation of random genetic errors [57]. Estrogen is an established key risk factor in the etiology of breast, endometrium and ovarian cancers [3, 58] and may play a role in the development of prostate cancer [59].

2.2.1 Estrogens and Cancer

Breast cancer develops as a result of abnormal proliferation of epithelial cells in the mammary glands [60]. Estrogens and progestins are required in the normal growth and development of mammary gland and also serve as growth factors for the majority of breast cancers [61-65]. In addition to estrogen, high testosterone levels have also been associated with increased risk of breast cancer among postmenopausal women, in a meta-analysis of nine prospective studies [66].

Endometrial cancer is the most common gynecological cancer, affecting mostly post-menopausal women [67-69]. Endometrial cancers originate from the lining of the uterus (endometrium) and most of these are endometrioid adenocarcinomas, which are estrogen driven [70]. Ovarian cancer is another common female genital tract cancer and is included in the category of gynecological cancers [71]. More than 90% of ovarian cancers are classified as "epithelial" and are believed to arise from the surface (epithelium) of the ovary. Estrogens have been suggested as potential factor to stimulate the proliferation of these epithelium cells [72]. Larger prospective cohort and case-control studies have suggested increased risk of epithelial ovarian cancer, particularly with use of estrogen for long duration [72-75].

2.3 STEROID HORMONES

Steroid hormones are derivatives of cholesterol that are synthesized by a variety of tissues and regulate many physiological processes [76]. Steroid hormones are grouped into androgens, estrogens, glucocorticoids, mineralocorticoids and progestins [77]. All these classes of steroid hormones are structurally similar and arise from a common series of pathways and are distinguished by their actions on one or more specific steroid hormone receptors [77]. Biosynthesis of these hormones requires a battery of oxidative enzymes located in both mitochondria and endoplasmic reticulum [78]. The detailed pathways involved in the biosynthesis of steroid hormone in humans are shown in **Figure 2**.

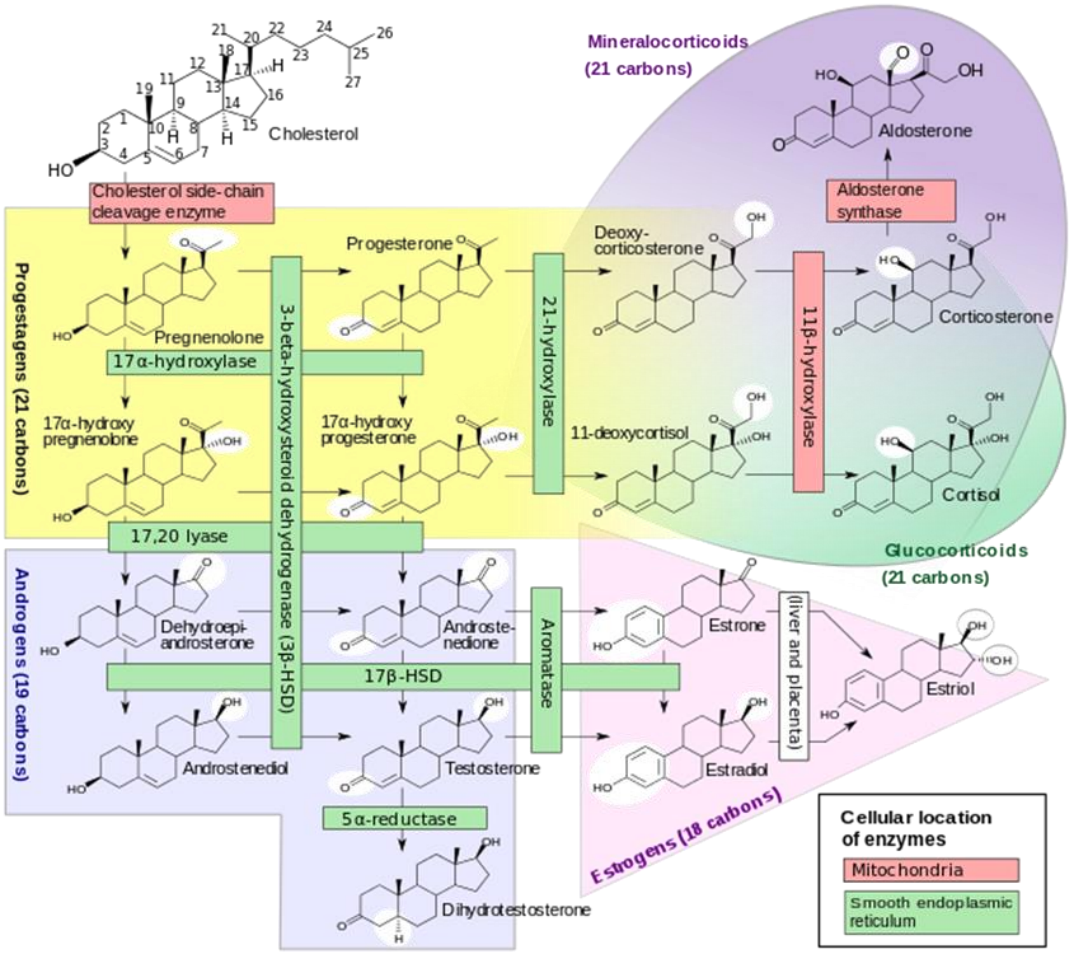

Figure 2: The pathways of human steroidogenesis

2.3.1 Androgens in women

Testosterone is a biologically potent androgen with specific receptors and target tissues. In women, testosterone is secreted into the circulation by both the adrenals and the ovaries [79]. Testosterone is also formed within the target tissue from circulating dehydroepiandrosterone sulfate (DHEAS). During the reproductive years, approximately 25% of circulating testosterone originates from the ovaries, about 25% from the adrenal glands and about 50% from peripheral androstenedione conversion. In aging women, circulating testosterone remains stable well into the age of about 80 years. Although ovarian volume decreases by about 30%, the ovarian stroma, driven by high undulating menopausal gonadotropins, secretes testosterone in increasing abundance. Testosterone production thus decreases little after menopause, even if adrenal androgen production of DHEAS and androstenedione declines substantially [80, 81].

2.3.2 Estrogens

Estrogens are synthesized in a variety of tissues during reproductive years [82]. One of the main functions of estrogens is to promote the growth and differentiation of sexual organs and other tissues related to reproduction. The major estrogens include estradiol, estrone and estriol. Estradiol [17β-estradiol (E_2)] is the best characterized member of this family produced predominantly in the ovaries by conversion of testosterone into estradiol by aromatase (CYP19) and plays an important role in mammalian physiology. Furthermore, studies have revealed that considerably lower levels of estrogens are also synthesized at multiple discrete sites throughout the body including mesenchymal cells of adipose tissue, osteoblasts and chondrocytes in bone, the vascular endothelium, aortic smooth muscle and many regions in the brain [83], where they may have highly localized effects [82]. For instance, it acts on the mammary glands to stimulate the ductal growth, on the endometrium to regulate the growth and cyclic changes, and on the ovarian follicles to promote granulosa cell differentiation [84]. Thus local synthesis of estradiol is important for the non-reproductive functions, such as in the maintenance of bone density and cardiovascular protection, particularly in post-menopausal women.

2.3.3 Mechanism of Estrogen action

Estrogen Receptor (ER) signaling is a complex biological pathway that controls and regulates a variety of functions, such as cell proliferation, apoptosis, invasion, and angiogenesis, and also serves as a major survival pathway driven by estrogen [85]. The molecular basis of estrogen action begins with the interaction of the hormone with the ERs which are zinc finger transcription factors [86]. The classical mode of action of estrogen, also referred to as genomic activity, is mediated by transcriptional activation of the nuclear ER (nER) i.e. ERα and ERβ [87, 88]. Upon binding to an estrogen, ER dimerizes and attracts a complex of co-regulators to specific DNA binding sites present in the promoter regions of target genes [89, 90]. Thus transcriptional activation of ER modulates and alters the expression of hundreds of genes important for normal cellular functions [91]. However, selective modulation of ERs by chemicals that may or may not structurally resemble estradiol can lead to selective agonist or antagonist activity in estrogen target tissues [92]. These actions of selective estrogen receptor modulators (SERMs) can be explained based on 1) differential ER expression in a target tissue 2) differential ER conformation on ligand binding 3) differential expression and binding of co-regulator proteins to the ER [93-95], 4) differing binding affinities to ERα and ERβ. ERs can also function as co-regulators where they bind to other transcription factors such as AP-1 (activator protein-1) and Sp1 (specificity protein 1) at their specific sites on DNA [86, 96]. Thus, as a regulator, ER serves a fine-tuning mechanism by inducing or reducing the transcriptional activity of other receptors [97].

In addition to its genomic activity, estrogen also generates rapid cellular responses that cannot be explained by its nuclear actions alone, but by the existence of signals generated from cytoplasmic and cell surface receptors. This is generally referred to as non-genomic activity [87, 98]. Non-genomic estrogen responses involve a series of events depending on cell type and include activation of protein-kinases and phosphatases, increases in ion fluxes across the membranes and the generation of nitrogen oxide (NO) [99, 100]. Membrane-initiated estrogen stimuli are known to modulate gene transcription via several mechanisms including phosphorylation and activation of transcription complexes [100, 101]. Furthermore, the ER signaling pathway is also regulated by membrane receptor tyrosine kinases such as G protein-coupled receptor 30 (GPR30), insulin like growth factor-1-receptor (IGF-1R), and epidermal growth factor receptor (EGFR), and also by activation of various protein kinase

cascades, including mitogen activated protein kinases (MAPKs), extracellular signal-regulated kinases (ERK), Src and phosphatidylinositol-3 kinase (PI3K) [102-105]. These membrane kinase activated signaling pathways sometimes also referred to as ligand-independent receptor activation, eventually resulting in the phosphorylation of ER as well as its co-activators and co-repressors at multiple sites to influence their specific functions [103, 106-109]. A simplified scheme showing the mechanism of actions of estrogens by different pathways [84, 98, 99] is given in **Figure 3**.

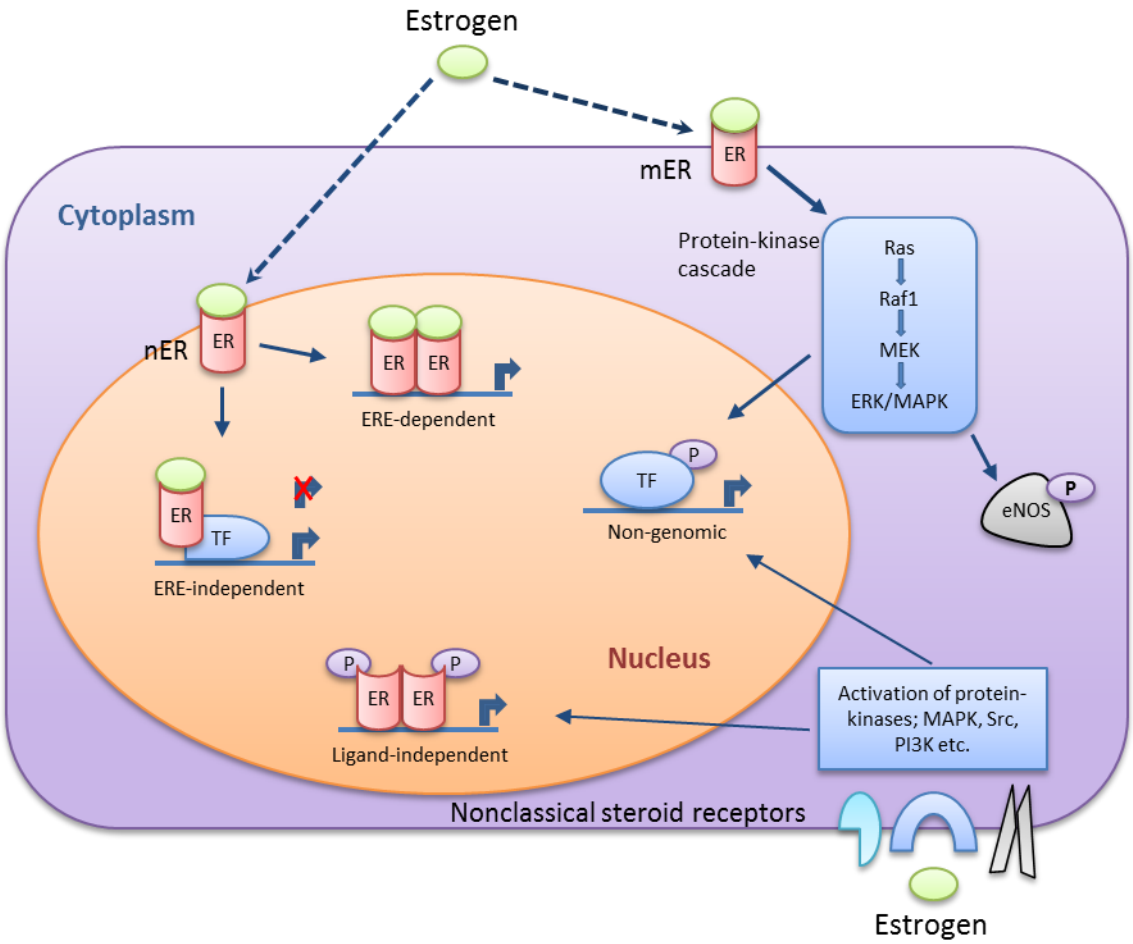

Figure 3. A simplified scheme showing the mechanism of estrogen actions by different pathways i.e. membrane versus nuclear estrogen receptor signaling within a generic cell. No specific cell type is shown to display all of them.

2.4 ENDOCRINE DISRUPTION

All biological networks are complex in nature with multiple levels of regulation, fine tuning capabilities, redundancy and evolvability. In order to perform normal biological functions, these features allow cells to adopt to cellular stresses, toxins and potentially hostile environments [85]. The endocrine system is one of the major homeostatic control systems that together with the metabolic machinery regulates and maintains the normal biological functions in the face of constantly changing environment [110]. According to the scientific statement of Endocrine Society, any exogenous substance either natural or synthetic that alters the function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub)populations is termed as an endocrine disruptor [111]. Endocrine disruptors are generally referred to as "Endocrine Disrupting Chemicals" (EDCs) [2].

The inception of research focusing on the potential of environmental chemicals to alter endocrine physiology can be traced back in the mid-1990 [112, 113]. A significant impact was made by the release of a book entitled *"Our Stolen Future: Are We Threatening Our Fertility, Intelligence and Survival?-A Scientific Detective Story"* written by Colborn and colleagues in 1996. This led to a great deal of attention and concerns about EDCs, their mechanisms of toxicity, the issue of cumulative exposures, as well as the introduction of new concepts and initiatives to modernize chemical screening and testing programs [114]. Due to the central role of estrogens in the reproductive system, environmental estrogens have been suggested as an underlying cause for the reproductive disorders of wild animals and consequently, are proposed as potential risk factors of hormone-related diseases and cancers in the Western world [5, 115].

2.4.1 Environmental Estrogens

A number of chemicals in our environment demonstrate estrogen-like activity when tested in biological systems [116]. These chemicals, sometimes structurally similar and sometimes different from the physiological estrogen 17β-estradiol (**Figure 4**), are able to mimic physiological actions of natural estrogens and are therefore referred to as environmental estrogens. Environmental estrogens are mainly categorized into phytoestrogens and xenoestrogens. Phytoestrogens are naturally occurring estrogenic compounds that are found in a variety of plants such as beans, seeds, and grains. These compounds exhibit mixed weak estrogen agonist and antagonist activity [117],

Phytoestrogens are classified into isoflavones, coumestans, and lignans. Early ecological and epidemiological studies showing that Asian populations that consumed diets rich in phytoestrogens have a lower incidence of breast cancer have raised the hypothesis that phytoestrogens may be effective chemopreventive agents protecting from breast cancer [118]. However, most epidemiological studies have not been able to show a protective effect [119]. In comparison to phytoestrogens, xenoestrogens are synthetic chemical contaminants in the environment and represent a structurally diverse group of hydrocarbons, however most lack the phenanthrene ring structure of estradiol [120]. While phytoestrogens are considered as protective for breast cancer, xenoestrogens are thought to increase the risk of breast cancer by mimicking the effects of estrogens. Metalloestrogens have been identified as a new sub-class of xenoestrogens [121], which contains all those inorganic metal ions that exhibit estrogenic properties [5]. These include the heavy metals and metalloids; cadmium, aluminum, antimony, arsenite, barium, cobalt, copper, chromium, lead, mercury, nickel, nitrite, selenite, tin, uranium and vanadate [122].

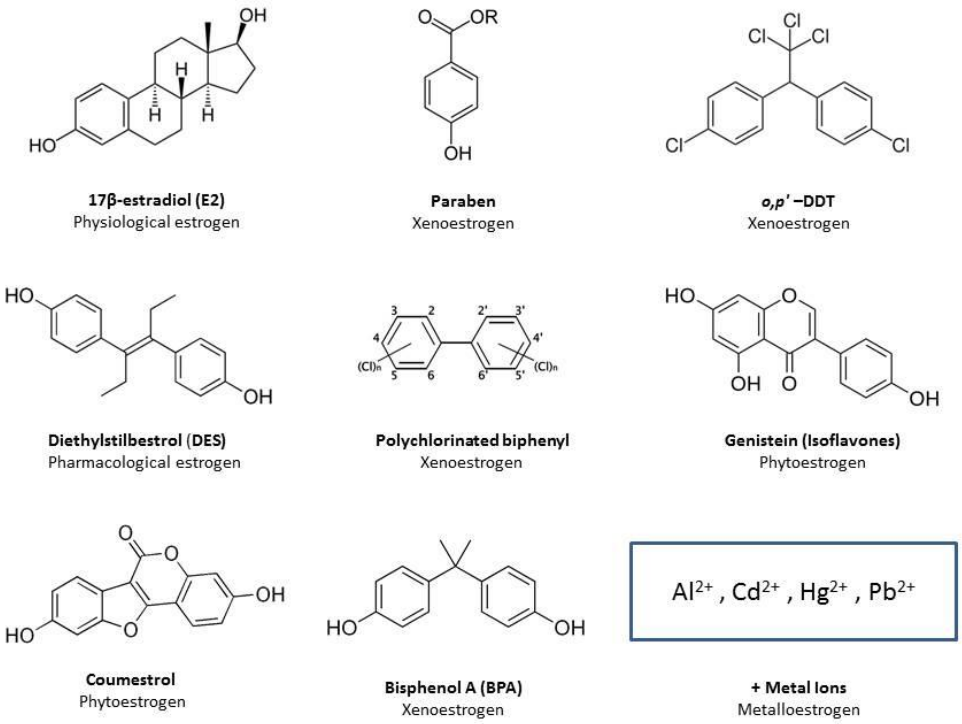

Figure 4. Diversity in the chemical structure of environmental estrogens in comparison to physiological estrogen 17β-estradiol.

2.4.2 Metalloestrogen Cadmium

Research on endocrine modulatory effects of cadmium started over a decade ago, when this metal was shown to imitate estrogens [7, 123]. In 2003, the first *in vivo* report on the estrogenic effects of cadmium in ovariectomized female Sprague–Dawley rats was published reporting several well-characterized estrogenic responses on uterus and mammary gland after a single intraperitoneal (i.p.) injection of 5 µg $CdCl_2$/kg body weight. Effects observed include increase in uterine wet weight, hyperplasia and hypertrophy of the endometrial lining and induction of uterine progesterone receptor expression and complement C3 gene expression as well as induction of milk protein synthesis in mammary glands [6]. In this study, cadmium treatment was initiated three weeks after ovariectomy at postnatal day 28 and the dose of cadmium selected corresponding to the human dietary PTWI of 7 µg/ kg body weight established by the World Health Organization [52]. Later on several reports emerged on this issue, able to reproduce similar effects in rodents, but at almost 100-fold higher concentrations of cadmium [124-128].

For instance, in female CD-1 Swiss albino mice, i.p injection of 3000 µg $CdCl_2$/kg body weight, 5 days per week for two weeks, could produce effects on the development of mammary gland and the uterus [126]. Similarly, in female overiectomized Wistar rats, an i.p. injection of 120 or 1200 µg Cd^{2+}/kg body weight per day for three consecutive days caused an increase in uterine weight accompanied by an increased thickness of luminal epithelium cells and endometrium [124]. Furthermore, Liu et al. (2010) studied the effects of cadmium on uterotrophic end points in ovariectomized Sprague Dawley rats after an i.p. injection of 6.4, 32, 160 or 800 µg $CdCl_2$/kg body weight per day for three consecutive days. Increased uterine wet weight, endometrial thickness and endometrial stromal thickness were observed only at 800 µg $CdCl_2$/kg body weight [128].

Experimental studies on the estrogenicity of cadmium conducted in rodents showed wide differences in the doses of cadmium used and the routes of administration. In this regard, Höfer at al. (2009) made an effort and demonstrated that there was a pronounced difference in the tissue levels of cadmium and hormonal potency (estrogenicity) after short- and long-term oral cadmium exposure at several dose levels and after a single i.p. injection. For instance, in comparison to i.p. injection, an oral exposure to cadmium by gavage feeding or in drinking water did not show any effects

on uterine wet weights or epithelial thickness [125]. Later on in a follow-up study, the same group revealed that the expression of ERβ both at mRNA and protein levels was significantly down-regulated in rat intestine after oral cadmium exposure and that the response was similar to ethinyl estradiol. As the intestinal tissue is known to express ERβ predominantly, the data suggested that cadmium modulates intestinal tissue homeostasis via ERβ-mediated mechanism and that oral administration of cadmium results in the down-regulation of ERβ [129]. In addition, mRNA expression and proliferating cell nuclear antigen (PCNA as an index for cell proliferation) was also decreased after long-term administration of cadmium and ethinyl estradiol [129]. However, in an *in vitro* experimental setting, some reports documented no estrogenic activity by using Yeast and E-Screen estrogenicity assays [130-132].

In addition to the estrogen mimicking effects of cadmium, it has also been reported that cadmium mimics the effects of androgen on the prostate gland and induces the expression of androgen regulated genes through binding to the androgen receptor [133].

2.4.3 Cadmium and Hormone-related Cancers

EDCs are believed to be a potential risk factor behind the increasing incidence of hormone-related cancers in the industrialized world [2, 134]. The presence of ER plays an essential role in the growth, differentiation and prognosis of human breast cancer [135]. Two third of all breast cancers are ER-positive and thus estrogen dependent [136]. Cadmium, due to its estrogen mimicking properties, has been hypothesized to play a role in the etiology of the disease. Recent observations in epidemiological studies support this hypothesis and have shown that cadmium exposure is a potential risk factor for hormone-related cancers- in particular breast, endometrial and prostate cancer [15, 16, 30, 137-141]. Cadmium concentrations analyzed in human studies focusing on associations between cadmium and risk of breast and endometrial cancer are given in **Table 1**.

Table 1: Description of studies on cadmium concentrations evaluated in humans

Study	Sample size	Cadmium concentrations		Methodology
Antila et al. (1996) [140]	43 cases breast cancer 32 controls	Breast fat	3.2-87 µg/g 0.1-160µg/g	GFAAS
McElroy et al. (2006) [30]	246 cases breast cancer 254 controls	Urinary	0.02-4.55 µg/g crea (range) 0.08-2.64 µg/g crea (range)	ICP-MS
Gallagher et al. (2010) [138]	100 cases breast cancer 98 controls	Urinary	0.58µg/g crea 0.41µg/g crea	ICP-MS
Saleh et al. (2011) [141]	50 cases breast cancer 150 control	Blood	5.87±1.53µg/L 3.81±1.9µg/L	AAS
Jackson et al. (2008) [142]	61 cases endometriosis 1362 control	Urinary	0.53µg/L (CI; 0.44-0.64) 0.42 µg/L (CI; 0.41-0.44)	GFAAS
Nasiadek et al. (2005) [143]	15 uterine myomas 28 uterine nonlesion	Uterine	0.09±0.04 µg/g wet tissue 0.15±0.09µg/g wet tissue	AAS
Nasiadek et al. (2011) [144]	53 uterine myomas	Uterine Blood	0.05±0.05 µg/g 1.48±0.81µg/L	GFAAS
Heilier et al. (2006) [145]	119 cases endometriosis 25 control	Urinary Blood	0.04-14.6 µg/g (range) 0.4µg/L	GFAAS
Itoh et al. (2008) [146]	54 cases endometriosis 74 controls	Urinary	0.34-0.76 µg/g crea (range) Similar to cases	ICP-MS

AAS, atomic absorption spectroscopy; CI, 95% confidence interval; Crea, creatinine; GFAAS, graphite furnace atomic absorption spectroscopy; ICP-MS, inductively coupled plasma mass spectrometry.

2.4.3.1 Cadmium and breast cancer

McElroy and colleagues conducted a population-based case-control study of 246 women, aged 20-69 years, with breast cancer and 254 age-matched control subjects among pre- and postmenopausal women, in order to assess the risk of breast cancer in relation to cadmium concentrations in urine [30]. The findings in this study demonstrated a multivariable-adjusted odds ratio of 2.29 (95% CI: 1.3-4.2) comparing the highest quartile of urinary cadmium versus the lowest. Based on this study, the absolute risk difference is 45 (95% CI: 0-77) per 100,000 given an overall breast cancer rate of 124 per 100,000 [30]. Another study, conducted on a two case-control samples (100 and 98 samples, respectively) from United States with a purpose to examine the association between urinary cadmium and breast cancer, showed increased odds ratios [2.69 (95% CI: 1.07-6.78) and 2.50 (95% CI: 1.11-5.63), respectively] comparing the highest quartile of urinary cadmium with the lowest [138]. Furthermore, a cross-sectional study was conducted in order to assess the association between cadmium exposure (urinary cadmium) to mammographic density, a strong marker of breast cancer risk [139]. The

findings of this study demonstrate that each 2-fold increase in urinary cadmium, was associated with higher odds of 1.29 (95% CI: 0.82-2.02) in the upper tertile [139].

More recently, Julin and colleagues examined the association between cadmium exposure from diet and the risk of overall and ER-defined breast cancer within a population-based prospective cohort of 55,987 postmenopausal women [15]. In this study, dietary cadmium intake [energy-adjusted mean (µg/day) = 15 ± 3.2, which is below the established TWI of 2.5 µg/kg body weight] was positively associated with overall breast cancer tumors, with a relative ratio of 1.21 (95% CI: 1.07-1.36), comparing the highest tertile with the lowest. Interestingly, both dietary cadmium exposure and vegetables/whole grain consumption were associated with the risk of breast cancer, but in opposite directions i.e. dietary cadmium was associated with increased risk and vegetables/whole grain consumption with decreased risk. The findings from this study suggest the role of dietary cadmium in the development of breast cancer in postmenopausal women [16]. However, further research is warranted on the impact of environmental cadmium (in particularly cadmium from diet) on breast cancer risk in specific populations and on identifying the underlying molecular mechanisms.

2.4.3.2 Cadmium and endometrial cancer

Association between cadmium intake from diet and endometrial cancer incidence was evaluated in the same population-based prospective cohort of postmenopausal women as the study on breast cancer by Julin and colleagues (dietary cadmium exposure: 15 µg/day, of which 80% from cereals and vegetables) [137]. Findings from this study demonstrated that cadmium intake was significantly associated with increased risk of endometrial cancer in all women. In analysis stratified by body mass index, smoking status and postmenopausal hormone use, a 2.9-fold increased risk (95% CI; 1.05-7.79) of endometrial cancer associated with long-term cadmium intake via diet was observed in lean, never smoking, non-users of postmenopausal hormones.

Apart from the evidence on cadmium exposure and estrogen dependent cancers, the epidemiological findings on cadmium and benign gynecological diseases showed mixed results [142-146]. For instance, Jackson and colleagues reported a positive association between high blood cadmium concentrations and endometriosis in premenopausal women in USA [142], while Heilier et al and Itoh et al did not find any

association between urinary cadmium concentrations and endometriosis, in Belgium [145] and in Japan [146], respectively. However, in a study by Nasiadek and colleagues, blood cadmium was positively associated with the expression of ER in uterus and negatively associated with serum estradiol levels in women. These findings suggest the metalloestrogenic effects of cadmium on uterus and involvement in the etiology of uterine myomas [144].

2.4.4 Mechanism of Cadmium action

Multiple distinct mechanisms, including interaction with cellular proteins and DNA repair processes as well as tumor suppressor and signal transduction proteins and epigenetics alterations have been proposed in cadmium-induced carcinogenicity [147, 148]. However, when it comes to cadmium and hormone-related cancers, endocrine disruption was proposed as an additional mechanism [149].

The endocrine system has evolved to function when unbound physiologically active ligands (hormones) are present at extremely low doses [150]. Because of the shared receptor-mediated mechanisms, EDCs that mimic the natural hormones have been proposed to follow the same rules and therefore have biological effects in the same low dose range [151, 152]. Similarly, EDCs that influence in any way the production, metabolism, uptake, or release of hormones may have effects at low doses, because even small changes in hormone concentration can have biologically important consequences [152-154]. EDCs can act through various mechanisms including classical nuclear receptors, but also through estrogen-related receptors, membrane-bound estrogen-receptors, as well as interaction with targets in the cytosol that results in the activation of Src/Ras/ERK pathway [134].

Studies conducted on cadmium so far have focused on the hypothesis that, similar to estrogen, cadmium binds and activates the ERα and thus alters the transcription and translation of estrogen regulated genes. Mechanistically, *in vitro* studies have shown that cadmium interacts with the hormone binding domain of nuclear ERα, with high affinity in a non-competitive manner [7, 123, 155]. Furthermore, the induction of the expression of estrogen target genes, the activation of cytoplasmic kinases, and the proliferation of estrogen responsive cell lines [156-161], suggests that cadmium promotes an agonistic conformation of the ER and activates both genomic and non-genomic estrogen signaling. Activation of cadmium-induced non-genomic estrogen signaling

through the membrane bound estrogen receptors such as GPR30 and membrane ER, suggests that cadmium as metalloestrogen could exert its effects through different pathways in the absence of classical/genomic ER signaling [158, 162, 163]. While data on the detrimental effects of cadmium is accumulating, the information on the molecular mechanism is lagging behind. Keeping in view the need-to-know basis, this thesis work is an effort to characterize the molecular mechanism behind the proposed endocrine modulatory effects of cadmium with special focus on estrogen-like effects.

3 THE PRESENT STUDY

3.1 AIMS

The overall aim of this thesis was to explore and evaluate the estrogenicity of cadmium and the molecular mechanism involved, at the exposure levels relevant to humans.

The specific objectives linked to each study are as follow;

- To explore whether potential estrogen-like effects of cadmium are caused by modulation of the transcriptional activity of nuclear estrogen receptors *in vivo*. (**Papers I & II**)

- To investigate the effects of cadmium on the cellular signaling pathways *in vivo* over a large dose range and to evaluate the sensitivity of these effects using benchmark dose analysis. (**Paper II**)

- To elucidate the molecular mechanisms responsible for cadmium-induced ERK1/2 activation at nanomolar exposure levels. (**Paper III)**

- To assess the associations between blood and urinary cadmium and the circulating levels of sex hormones in postmenopausal women. (**Paper IV**)

4 COMMENTS ON METHODOLOGY

This section provides an overview of the methodology used in the thesis. Detailed description of the techniques used in the studies can be found in the publications and manuscripts included in this thesis.

4.1 STUDY DESIGN AND SAMPLING

4.1.1 Uterotrophic Bioassay

The study design of **Papers I & II** follows, with some modifications, the Organization for Economic Co-operation and Developments (OECD) guideline 440 "Uterotrophic Bioassay in Rodents" [164]. This guideline originates from bioassays conducted in the 1930's, was first standardized in 1962, and has since undergone an extensive validation program by OECD including intra- and interlaboratory studies to show the relevance and reproducibility of this bioassay with a potent reference estrogen, weak estrogen receptor agonists, strong estrogen receptor agonists and a negative reference chemical [164, 165]. However, little effort has been directed at the antiestrogenicity component of the assay. The current TG440 protocol is directed to the screening of estrogen agonist activity and does not involve any antiestrogen control. In **Papers I & II**, the focus was on the widely reported estrogen agonist type of activity of cadmium and the OECD guideline was chosen to investigate this aspect. Most of the study parameters are derived from this guideline, including the positive reference estrogen ethinyl estradiol (EE_2), the subcutaneous route of administration, and the time point of sacrifice. However, some additional parameters, which were not a requirement under OECD guidelines, were also included.

4.1.2 Estrogen Reporter Mice

Transgenic ERE-luciferase mouse is a well-documented, *in vivo* model of choice for detection of estrogen agonist activity. The estrogen-responsive reporter gene construct (3xERE-TATA-Luciferase) consists of trimerized ERE coupled to a minimal TATA-box that drives the expression of the luciferase gene. The sensitivity of this mouse model with different types of ER ligands (i.e. weak, strong and partial agonists) can be evaluated based on widely published available literature. The mouse model responds well to strong ligands such as steroidal estrogens (E_2 and E_2 dipropionate) and estrogenic pharmaceuticals (DES) [166-168]. However, also weaker ER ligands,

such as environmental chemicals (BPA) and phytoestrogens (enterolactone) have been successfully detected in this model [167, 168]. For example, the IC_{50} value of enterolactone in ERα binding assays is in the μM order of magnitude, suggesting very weak affinity [168, 169]. In contrast, cadmium is suggested to have as high similar affinity for the ERα as E_2 [7]. In addition to pure compounds administered as such, the reporter mouse model also responds to administration of soy, naturally containing precursors of phytoestrogens, incorporated into the diet [170]. Immature ERE-luciferase female mice **(Papers I & III)** and adult ERE-luciferase male mice (**Papers II & III**) were used in this thesis work.

4.1.3 Treatment, Asphyxiation and Tissue collection

Immature female (**Paper I**) and adult male mice (**Paper II**), randomly selected for each treatment group, received cadmium chloride ($CdCl_2$) dissolved in sterile phosphate buffer saline (PBS) at 5, 50 and 500 μg/kg body weight per day and at 0.5, 5, 50 and 500 μg/kg body weight per day, respectively for three consecutive days via subcutaneous (s.c.) injections under the skin in the neck. Control animals were injected with sterile PBS. EE_2 dissolved in corn oil (50 μg/kg body weight) was used as positive control. In anticipation of estrogen type of activity, the vaginal opening as a marker of precocious puberty was observed before asphyxiation. On the fourth day of the experiment, animals were euthanized by carbon dioxide asphyxiation and weighed. Subsequently, whole blood was drawn by heart puncture and tissues were collected into micro centrifuge tubes, snap frozen in liquid nitrogen, and then stored at -80°C until analysis. Organ weights were recorded for uterus, testis, liver and kidneys. For cadmium analysis, whole blood, one kidney and a piece of liver were taken separately into sterile acid washed micro-centrifuge tubes and kept on dry ice before storage at -80°C. One horn of the uterus from each female and one testis from each male was fixed and preserved for histological evaluation.

4.1.4 Cell Lines

In **Paper III**, human hepatocellular carcinoma (HepG2), human breast cancer (MCF-7) and human endometrial carcinoma (ECC-1) cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, USA). HepG2 and the endometrial carcinoma ECC-1 cells were selected because findings from **Papers I & II** indicate that $CdCl_2$ has biological activity in the liver and the endometrium. The breast

cancer cell line MCF-7 was selected due to its extensive use in cadmium studies by others before. HepG2 cells were cultured on collagen coated dishes in minimal essential medium with Earle's salt and L-glutamine, supplemented with sodium pyruvate, non-essential amino acids, penicillin/streptomycin and 10% heat inactivated fetal bovine serum. MCF-7 cells were cultured in Dulbecco's modified Eagle's medium, supplemented with penicillin/streptomycin and 10% heat inactivated fetal bovine serum. ECC-1 cells were cultured using RPMI-1640 culture medium supplemented with penicillin/streptomycin and 5 % heat inactivated fetal bovine serum.

4.1.5 Women's Health in Lund Area (WHILA)

A population-based cohort study of all women 50–59 years of age (n = 10,766) with the participation rate 64% (n = 6,917), in the Lund area, in southern Sweden, started in December 1995 [171]. The general objective of this cohort was to assess the health profile of these women under the health screening program [172]. All women received the invitation together with a basic questionnaire containing questions on previous and present diseases, drug treatment, smoking- and alcohol habits, education, physical activity, working status, parity, months of lactation and menopausal status [171]. In June 1999, when 1,160 subjects remained to be examined, the study was extended to include health aspects of cadmium exposure; the participation rate was 71% (n = 820) women (**Figure 5**). Morning first-voided urine samples were obtained from 813 women and blood sample from 792 [173]. All samples were collected during 8-months from June 1999 through January 2000 [173]. Informed consent was obtained and the ethics committee at Lund University approved the study (**Paper IV**).

4.1.6 Ethical permissions

In **Papers I-III**, all the animal experiments were performed at the animal department of the University of Turku, Turku, Finland, under the license numbers 1592/05 and ESLH-2007-03984.

In **Paper IV**, the population-based study was performed under License number LU 174-95 and oral informed consent was obtained from all women participating in the study.

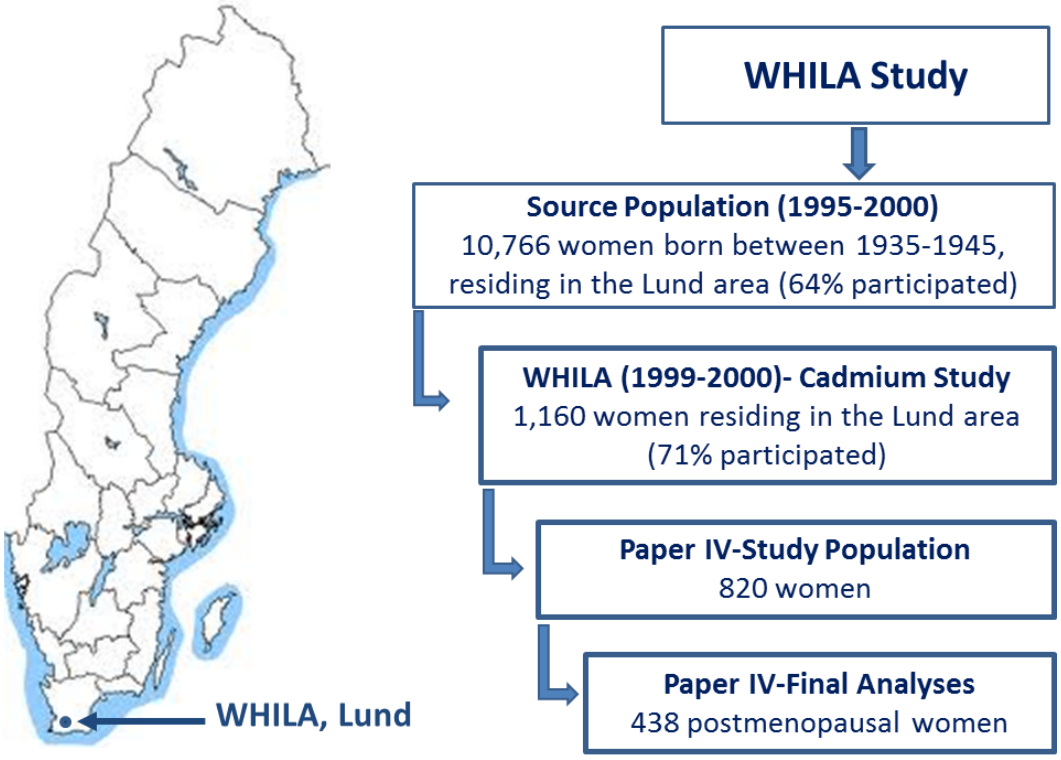

Figure 5. Women's Health in the Lund Area (WHILA), source populations and study populations for **Paper IV**.

4.2 ANAYTICAL TECHNIQUES

4.2.1 Cadmium Analysis

Inductively coupled plasma mass spectrometry (ICP-MS) is an analytical technique which is capable of detecting metals and several non-metals at very low concentrations i.e. one part in 10^{12} (part per trillion). In principle the sample is ionized with the inductively coupled plasma and the ions are separated and quantified by using mass spectrometer. ICP-MS was used in **Papers I & II** to detect and quantify cadmium concentrations in target tissues. Approximately 0.5 g of sample was digested in 2 mL nitric acid and 3 mL of deionized water, at 250^{o}C for 30 min in the autoclave and then subjected to ICP-MS. The analytical quality control was checked by using reference samples for each tissue and was satisfactory. The limit of detection (LOD) was 0.004 ng/g calculated as mean of digested blanks + 3 standard deviations (S.D). All the measured concentrations were above the calculated LOD.

In **Paper IV**, cadmium concentrations in urine and blood samples were also measured using ICP-MS [173]. The analyses were performed at the Department of Occupational and Environmental Medicine, Lund University, Sweden. The LODs for urinary and blood cadmium were 0.31 and 0.12 µg/L, respectively. Urinary cadmium concentrations were adjusted for urinary creatinine (µg/g creatinine) as well as urinary density (mean urinary density of 1.015 g/mL in WHILA).

4.2.2 Luciferase Reporter Gene Assay

Reporter genes are used as indicators to study gene expression as well as cellular events coupled to gene expression both *in vivo* and *in vitro*. Luciferase-based reporter gene assays are widely used in cell biology due to their ultrasensitive detection capacity and wide dynamic range. These assays involve placing a genetic regulatory element upstream of a luciferase gene and then transferring the resulting reporter construct into animal cells, plant cells or bacteria through transfection, transformation or injection. Expression of the luciferase reporter gene is then measured to quantify the activity of the regulatory element (cis-acting) or proteins (trans-acting) in the biological pathway affected by the target element. Luciferase activity assays were used in **Papers I & II** in order to detect the expression of the reporter gene in both reproductive and non-reproductive tissues. Luciferase activity was measured in the tissue homogenate using BioThema's Luciferase Assay – kit and luminescence was recorded with a Victor^2 1420 Multilabel – analyser. In principle, firefly Luciferase catalyzes luciferin oxidation

using ATP-Mg^{2+} as a co-substrate that results in generation of flashes of light. The assay also incorporates coenzyme A (CoA) for improved kinetics, allowing greater enzymatic turnover and resulting in increased light intensity that is nearly constant for at least 1 minute. Furthermore, total protein contents in each sample were measured using Bradford assay reagents in comparison to bovine serum albumin (BSA) standards on a spectrophotometer.

4.2.3 Quantification of Gene Expression

The gene expression in the cells of all organisms is regulated by turnover of gene transcripts (mRNA). The amount of expression of a target gene in a cell can be measured by the number of copies of an mRNA transcript of that gene. Amplification of a gene transcript is important in order to robustly detect and quantify gene expression from small amounts of RNA in a sample. The polymerase chain reaction (PCR) is a commonly used method for amplifying DNA [174], however for mRNA-based PCR the RNA sample is first reverse-transcribed to cDNA with reverse transcriptase. In **Paper II** real-time PCR was used for relative quantification of specific mRNAs and internal reference genes (also known as control house-keeping genes). RNA extraction from tissue sample was made according to the protocol provided by OMEGA Biotek, E.Z.N.A. ® total RNA Kit (VWR International AB, Karlskoga, Sweden). Briefly, 10 mg of liver tissue was homogenized in lysis buffer and applied to HiBind RNA spin columns and total RNA was extracted. The purity of RNA was determined by NanoDrop 1000 spectrophotometer. Approximately 2 µg of total RNA was reverse transcribed into cDNA by using High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, USA). Following the first strand cDNA synthesis, PCR amplification was carried out using the Maxima SYBR Green qPCR Master Mix (2X) kit (FERMENTAS GMBH, Baden- Württemberg, Germany) according to the manufacturer's instructions. Quantification was done using a 7500 real time PCR System (Applied Biosystems, Foster City, USA).

4.2.4 Histology

Histology was used in **Papers I & II** to study the anatomical changes in uterus and testis respectively. Briefly, paraffin embedded sections of uterine and/or testicle tissues were mounted on microscopic slides (MENZEL-GLAZER, SuperFrost® Plus, Germany) followed by clearing, rehydration and staining with hematoxylin and eosin.

All sections were examined under light microscope (DIAPLAN, Leitz, Wetzlar, Germany) and photos were taken for computerized image analysis.

4.2.5 Cell Viability and Cell Proliferation

The MTT assay is a colorimetric assay for measuring the activity of cellular enzymes that reduce the tetrazolium dye MTT, to its insoluble formazan, giving a purple color [175]. The assay measures cellular metabolic activity via NAD(P)H-dependent cellular oxidoreductase enzymes and may, under defined conditions, reflect the number of viable cells (cell proliferation). Tetrazolium dye assays can also be used to measure cytotoxicity (loss of viable cells) or cytostatic activity (shift from proliferative to resting status) of potential medicinal agents and toxic materials. Cell proliferation was measured in **Paper III.** Briefly cells were incubated with the medium containing MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma–Aldrich, St. Louis, MO) for 4 hours followed by Dimethyl sulfoxide (DMSO) that solubilizes the insoluble purple formazan product into colored solution. The absorbance of colored solution was measured at 570-620 nm by using Spectrophotometer.

4.2.6 Small Interfering RNA Transfections

Small/short interfering RNA (siRNA) plays many roles in cell biology and in particular it is involved in the RNA interference (RNAi) pathway, where it interferes with the expression of specific genes with complementary nucleotide sequence. siRNA is used as an experimental strategy to efficiently knock-down the mRNA expression of particular genes and thereby abolishing the expression of selected proteins. This method was employed in **Paper III** to selectively and efficiently silence the expression of EGFR and GPR30. Cells were transfected the day after plating with siRNA pools targeted against EGFR and/or GPR30 (Santa Cruz Biotechnology, Heidelberg, Germany) using Lipofectamin transfection reagent for 48 hours according to the manufacturer's protocol. In all experiments a scrambled siRNA was included as negative control and the transfection efficiency was detected by protein expression using western blot.

4.2.7 Western Blot Analyses

Western blotting is a conventional analytical technique used to detect and quantify proteins in a sample based on their binding to a specific antibody. Western blot analysis can detect specific proteins from a mixture of a great number of proteins [176]. This

technique was used in **Papers I-III**. Briefly, total protein contents were quantified in the target tissue homogenates and/or in cell lysate by using Coomassie Plus™ Protein Assay Reagent (Pierce, Täby, Sweden). Samples containing equal proteins were subjected to SDS-PAGE to separate the proteins based on their molecular weights and then transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA), and subsequently probed with primary antibodies and then secondary antibodies. Cdk2 was used as a loading control in all experiments. The results were visualized by the ECL detection kit (Amersham™ GE Healthcare Bio-sciences AB, Uppsala, Sweden) and the densitometric analysis was made with Image J version 1.34s software.

4.2.8 Quantification of Serum Concentrations of Hormones

In **Paper IV**, quantification of total cholesterol, androstenedione, testosterone, estradiol and SHBG were made by various techniques described elsewhere [177]. Briefly total serum cholesterol was quantified in conjunction with the sampling/clinical examinations, on capillary whole blood using a Cholestech LDX instrument (Cholestech Corporation, Hayward, CA, USA). ELISA techniques were employed for the determination of serum concentrations of androstenedione and SHBG by using commercially available monoclonal antibodies (DRG Diagnostics Instrument GmbH, Marburg, Germany). Testosterone and estradiol concentrations in serum were measured by KRYPTOR1-Testosterone and KRYPTOR1-Estradiol 17β (estradiol) kits respectively, designed for KRYPTOR Compact fully automated random-access immunoassay system (Thermo Scientific, BRAHMS AG, Germany) [177]. LOD and CV were as follow: Androstenedione, 0.15 nmol/L and 5.1%; testosterone, 0.15 nmol/L and 6.4%; estradiol, 3.5 pmol/L and 6.0%; SHBG, 4.0 nmol/L and 3.0%, respectively. A testosterone index and an estradiol index were defined as testosterone/SHBG*100 and estradiol/SHBG*100, respectively. This index was calculated to consider potential differences between free and protein-bound steroids [178].

4.3 STATISTICAL ANALYSIS

Student pair t-test and one-way ANOVA was employed in order to compare the effect among different treatment groups. Where statistically significant ($p \leq 0.5$), Dunnett's multiple comparison tests was applied to make *post hoc* comparisons between the means of control and treatment groups (**Papers I-III**). All the statistical analyses were performed using Graph Pad prism using version 5.04 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com

In **Paper IV**, ANOVA and Kruskal Wallis Test was used to assess the differences between groups and Spearman's rank correlation coefficient (r_s) to assess the univariate associations for continuous variables. In multiple linear regression models, each sex hormone related variable was evaluated in relation to cadmium exposure (blood and urinary cadmium), potential confounders (factors associated with both cadmium and sex hormones) and effect modifiers (factors associated with sex hormones). The criterion for covariates to be entered in the multivariable-adjusted models was $p \leq 0.25$ based on univariate assessments. All tests were two sided, and statistical evaluation was performed using SPSS (version 19; SPSS Inc., Chicago, IL, USA).

4.3.1 Benchmark Dose Modeling

The current risk assessment practice assumes that, apart for oncogenicity, a threshold dose exists for the effects i.e. toxicologically significant effects are not likely to occur below the threshold dose. Two approaches can be used to define this threshold dose. The traditional approach determines the toxicity threshold as the no observed (adverse) effect level (NOAEL) i.e. the highest tested dose or concentration of a substance, at which no such adverse effect is found in exposed test organisms where higher doses or concentrations resulted in an adverse effect. However, in recent practices the benchmark dose (BMD) approach has been proposed as an alternative to the NOAEL, for setting limit values for non-cancer health effects [179]. The main advantage of BMD over NOAEL is that it is not limited to only one experimental dose, instead it involves fitting a mathematical model to the entire dose-response dataset for a particular endpoint, and it allows the model to estimate the threshold dose corresponding to a certain critical effects size (CES) or benchmark response (BMR). Thus the derived BMD refers to the central estimates of the dose-response above the background for a particular continuous variable. The choice of CES/BMR depends on non-adverse but biologically relevant change in the response for a particular endpoint, and EFSA

recommended a CES of 5% as a default value to start with [43]. However, this effect level might not be relevant from a biological point of view for certain end points like effects on enzymes and changes in gene- and protein expression. Therefore, other CES levels (i.e. 10%, 20%, 30% or more) can also be used [180]. In **Paper II** BMD analysis was performed in order to determine the sensitivity of all those end points where a dose response effect was observed. A schematic diagram showing the differences between NOAEL, LOAEL and BMD on dose-response curve is given in **Figure 6**.

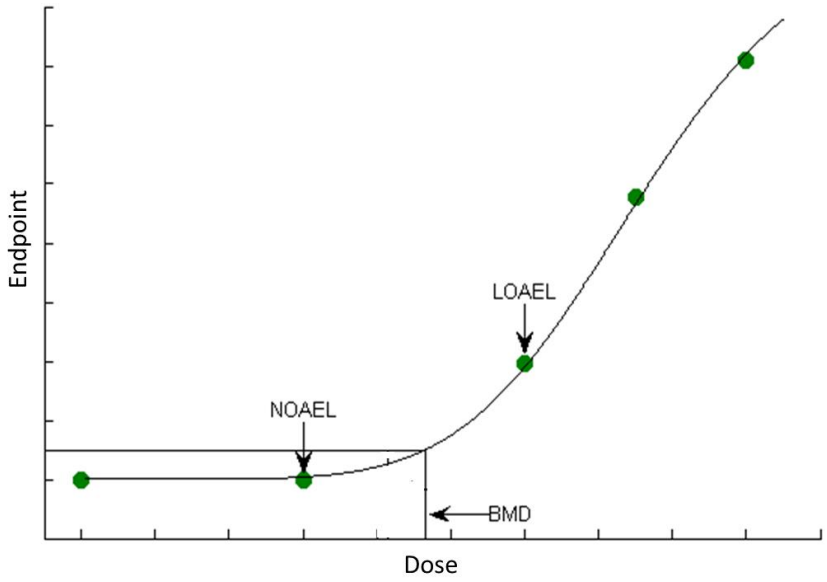

Figure 6: Schematic dose-response curve showing BMD, NOAEL and LOAEL

5 RESULTS AND DISCUSSIONS

5.1 ESTROGEN-LIKE EFFECTS OF CADMIUM AND THE ESTROGEN RECEPTOR SIGNALING

Estrogenicity of cadmium was investigated by analyzing the ER-regulated ERE-dependent Luciferase reporter gene expression in both female and male mice (**Papers I & II**). The uterine epithelial height and wet weight, and the advancement of vaginal opening as a marker of precocious puberty was analyzed in immature female mice (**Paper I**).

Results from **Papers I & II** demonstrate that exposure to $CdCl_2$ had no effect on the ERE-dependent reporter gene expression in either reproductive or non-reproductive tissues. These findings are in line with the previous reports demonstrating that cadmium cannot trigger the agonist conformation of the ER [130, 132, 155]. Furthermore, we observed a significant dose-dependent increase in the height of uterine epithelium after $CdCl_2$ exposure (**Figure 7**), suggesting estrogen-like activity of cadmium. This is in line with previous reports [6, 124, 125]. However, uterine wet weight (the hallmark of estrogen activity) was not affected after $CdCl_2$ exposure. Both of these markers i.e. uterine wet weight and uterine epithelium height are used for evaluating the nature of estrogen-induced responses. For instance, uterine growth response is regulated by the classical ER signaling [181, 182], whereas uterine epithelium responds well to estrogen treatment even in the absence of classical ER signaling [182]. The increase in uterine epithelium height along with the absence of effects on uterine wet weight and on reporter gene expression, suggests that the classical ER-mediated ERE-dependent signaling is not involved in cadmium-induced estrogen-like effects. Thus in the absence of classical ER signaling, the non-classical ER signaling (or some other signaling altogether) may be considered as an alternative mechanism in cadmium-induced effects.

Estrogen signaling not only leads to modulation of transcription in the cell nucleus, but also triggers rapid membrane-associated signaling events in the cell. Interestingly, it appears as if environmental estrogens have distinct effect profiles when tested for non-genomic and genomic effects as a function of time and concentration *in vitro* [183, 184]. Because rapid phosphorylation of ERK1/2 MAPK is an indicator of membrane

associated ER signaling, cadmium-induced phosphorylation of ERK1/2 in the liver (**Paper I & II**) may suggest activation of non-classical ER signaling [124, 158, 159].

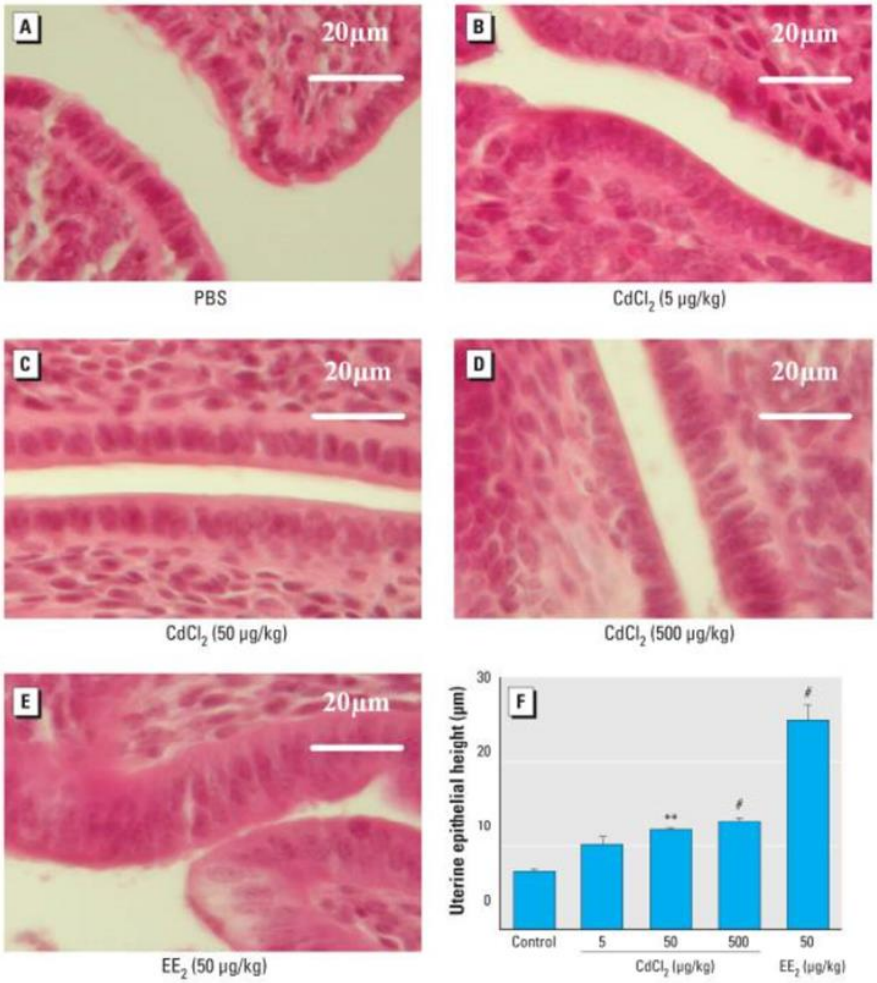

Figure 7: Height of the uterine luminal epithelium (µm) after s.c. exposure to $CdCl_2$, EE2, or PBS for 3 consecutive days. (A–E) Representative photomicrographs of uterine tissue sections stained with hematoxylin and eosin (40×). (F) Quantitative evaluation of the uterine epithelium height (µm). Reprinted from Ali et al (2010) **Paper 1**.

The ERK1/2 MAPK is a major cellular signaling pathway activated by mitogens to promote proliferation. The typical MAPK inducer i.e. insulin like growth factor has been shown to promote proliferation in uterine epithelia in mice [185]. Like-wise, it seems plausible that significant thickening of uterine epithelia in response to cadmium exposure (**Paper I**) might be mediated through MAPK activation. However, it remains to be studied whether cadmium activates MAPK through ER located at cell membrane or through some other receptors, such as GPR30 and/or EGFR [163]. As uncontrolled proliferation in turn could contribute to the initiation of cancer, we further investigated

the observed low dose effect of cadmium on MAPK and the possible involvement of membrane associated receptor signaling in **Papers II & III**.

Our data on the estrogen-like effects of cadmium (**Papers I & II)** shows that cadmium neither promotes uterine weight gain, nor does it induce ERE-dependent Luciferase reporter gene expression in any tested tissues (14 different tissues tested, males and females). These results strongly suggest the lack of agonistic interaction of cadmium with ER, at least via the classical ERE-mediated pathway. Furthermore, the recent biosensor-based profiling of cadmium and ER interaction suggest that cadmium does not bind to the same ligand binding pocket of the ERα as steroidal estrogens [155], and thus support the idea that cadmium is not a "true estrogen", and most likely exerts a share of its effects through other additional pathways.

5.2 CADMIUM-INDUCED EFFECTS ON CELLULAR SIGNALING PATHWAYS

Based on findings from **Paper I**, the study design in **Paper II** was modified by extending the scope from "disruption of estrogen signaling" to a more general concept of "disruption of cellular signaling". Several additional molecular markers including activation of MAPKs, general cellular stress responses and various transcriptional end points as well as an extra low dose group was added. The extra low dose level in this study i.e. 0.5 µg/kg body weight corresponds to a total dose of 1.5 µg/kg b.w. in the 3-day exposure, which represents well the established TWI of 2.5 µg/kg by EFSA for life-long dietary cadmium exposure in humans [54]. Furthermore, the sensitivity of observed molecular markers was evaluated based on benchmark dose (BMD) analysis.

In **Paper II**, exposure to a wide dosage range of $CdCl_2$ showed distinct response profiles depending on the dose. For instance, we observed the activation of MAPKs at low doses, whereas the inductions of transcriptional responses were found at relatively higher doses. Both MAPK p38 and p53 are well known cellular stress sensors. The activation of these can lead to delay in cell cycle, and cellular senescence or apoptosis. We observed cadmium-induced increases in the expression of MAPK p38 and p53 both on mRNA and protein levels suggesting that cadmium may induce cellular stress. Also it is well known that DNA damage can cause the accumulation of p53 in the cell nucleus. We further investigated the DNA damage marker, histone-variant H2AX in the mouse liver. However, we did not detect any changes in the phosphorylation of

H2AX (ser 139) suggesting that the concentrations of cadmium, or treatment time, used in our studies are not sufficient to cause detectable genotoxic stress. Furthermore, not only at higher doses but also at lower doses $CdCl_2$ clearly affected the mitogenic kinases and cellular stress signaling. In contrast to that we observed significant inductions in the expression of traditional markers of metal exposure i.e. Mt1 and Mt2, as well as other transcriptional responses only at higher doses of $CdCl_2$. These observations suggest that the observed differences between the low- and high-dose cadmium responses could depend on the diverging interactions between the non-genomic and genomic signaling. However, due to a limitation of single time point observations in our study, it is hard to speculate if cadmium at the higher tested doses stimulated a strong initial MAPK activation that could lead to a subsequent transcriptional response.

In addition to our observations on the traditional effect markers, in **Paper II** we also evaluated the sensitivity of cadmium-induced effects on the cellular-signaling pathways by applying the BMD modeling approach. As one of the criteria of BMD modeling, we could only choose the effect markers where a dose-response effect was observed. Interestingly, among all the tested responses, p53 was found to be the most sensitive effect with the lowest BMDL followed by *Mt2*, *Mt1* and *c-fos*. However, the BMDL for p53 was still substantially higher than the effective dose causing ERK1/2 MAPK activation. Furthermore, a collective analysis of our observations revealed that activation of p38 and p53 are the most similar response patterns observed between $CdCl_2$ and EE2 after 3-days exposure. These findings may suggest that stimulation of stress sensors could be a common denominator in cadmium- and estrogen-induced effects, which leads to generation of hypotheses i.e. selective modulation of ERs is involved in the mode of action of cadmium, and that additional signaling pathways and receptors are likely to be involved too.

5.3 CADMIUM AND ACTIVATION OF EGFR-RAF-MEK-ERK/MAPK PATHWAYS

In **Paper III**, we further extended our investigation to dissect the role of additional signaling pathways and the associated receptors in cadmium-induced effects. Traditional toxicological studies on the carcinogenicity of cadmium in cell models used concentrations that greatly exceed the levels as compared to blood levels in general human populations. While considering the exposure levels in general human

populations and cadmium response profiles in our *in vivo* settings (**Papers I & II**), we selected low nanomolar concentrations of cadmium to investigate the involvement of different protein-kinases in cadmium-induced effects using human cell lines i.e. HepG2, MCF-7 and ECC-1 (**Paper III**).

Our findings clearly showed that low nanomolar concentrations of $CdCl_2$ promoted MAPK/ERK signaling in all three different human cell lines. Interestingly, this activation of MAPK/ERK signaling was not affected by pure antiestrogen ICI, suggesting that the involvement of ERs in the low dose effects of cadmium is minor, if any. A similar antiestrogen insensitive cadmium-induced ERK1/2 activation has been reported by others before, however at much higher concentrations [158, 163]. In addition to activation of ERK1/2, the activation of its upstream partners i.e. Raf and MEK were also observed after $CdCl_2$ exposure, both in mouse tissue and in human cell lines (**Paper III**). The ERK1/2 MAPK signaling pathway serves as a central signal integrator and thereby contributes significantly to the central cellular decisions on proliferation, differentiation, death and/or malignant transformation. A variety of factors, such as steroids, growth factors, cytokines, and carcinogens have been shown to affect the ERK1/2 MAPK pathway [186]. Our findings on cadmium-induced activation of the Raf-MEK-ERK1/2 MAPK pathway (**Papers I-III**) suggest that cadmium is an additional factor in the activation of this pathway. Since, ERK1/2 MAPK is commonly deregulated in human cancers [187], it could therefore represent a target for the carcinogenic effects of cadmium.

Protein tyrosine kinases, including EGFR, have been proposed as targets of metal ions. In addition to cadmium, also other metals like arsenic, cobalt, lead and nickel have been suggested to strongly bind to the C-terminal cysteine containing motif of several protein tyrosine kinases [188]. We further explored the role of cell surface receptors in the biological activity of cadmium by using specific receptor inhibitors and siRNA knockdown technique. The membrane receptor GPR30 has been previously suggested as a mediator of cadmium effects in cells [163], and in our findings it was only observed in two out of three cell lines. In contrast, the inhibition and knockdown of EGFR had more pronounced effects, implying that it could be a more general target of cadmium. Apart from being a vital signal transductor in normal cell physiology, EGFR is also an oncogene and a target for chemotherapy. Thus inappropriate activation of EGFR, for instance in our case by cadmium, could

increase the risk of cancer. Keeping in view the fact that cadmium is a reactive ion with high affinity towards thiol groups in proteins, and less than 5 % of cadmium that enters the cell can reach the nucleus [189], a model that explains the activity of cadmium without requiring transport to the nucleus, such as activation of cell surface receptors i.e. EGFR and/or GPR30, appears plausible and physiologically relevant.

The activation of upstream signaling cascade i.e. ERGR/GPR30-Raf-MEK-ERK1/2 MAPK by cadmium in our experimental models and the suggested role of cadmium in cancer promotion by others before, led us to further investigate the phosphorylation of Mdm2, a ubiquitin ligase regulating the protein level of the tumor suppressor p53 [190]. We observed cadmium-induced activation of Mdm2 at ser-166 at nanomolar concentrations in all three human cell lines, which was reversed by a MEK inhibitor, suggesting that Mdm2 activation occurs downstream of the MAPK-ERK pathway. MAPK/ERK mediated phosphorylation of Mdm2 has been documented previously and was associated with increases in p53 degradation [191]. Alterations in the delicate balance of cell survival and cellular senescence through Mdm2/p53 could lead to undesirable outcomes, such as escape from the cell cycle control and uncontrolled cell proliferation. Therefore in **Paper III**, we tested the effect of cadmium on Mdm2/p53 response in HepG2 cells. We found that at low concentrations, cadmium alone did not alter the cellular content of p53, however its presence affected the cellular response to other known genotoxic compounds i.e. cells treated with 5-fluorouracil, benzo(*a*)pyrene and etoposide in the presence of cadmium displayed significantly higher pMdm2/p53 ratio than those treated only with the genotoxic compounds. Interestingly, cadmium-induced sensitization of the cells was similar to all three genotoxic compounds which stabilize p53 through different mechanisms, suggesting that cadmium-induced Mdm2 activation does not involve the genotoxic stress signaling. A similar mechanism has been previously proposed for the dioxin TCDD, which has been shown to disrupt the p53 response through the activation of Mdm2 [192]. Since, cadmium is already known to render cells more sensitive to genotoxic stress [193], our findings thus suggests that this sensitization occurs through the activation of MAPK/ERK and Mdm2.

A collective analysis of our observations including the lack of classical ER genomic activity (**Papers I & II**), activation of MAPK/ERK pathways and cellular stress signaling (**Papers I-III**) and the involvement of membrane receptors in response to

cadmium exposure (**Paper III**), suggests that cadmium-induced effects occur through the activation of Raf-MEK-ERK MAPKs via the membrane associated receptors EGFR/GPR30. A schematic model that represents the proposed mechanism of cadmium-induced effects is shown in **Figure 8**.

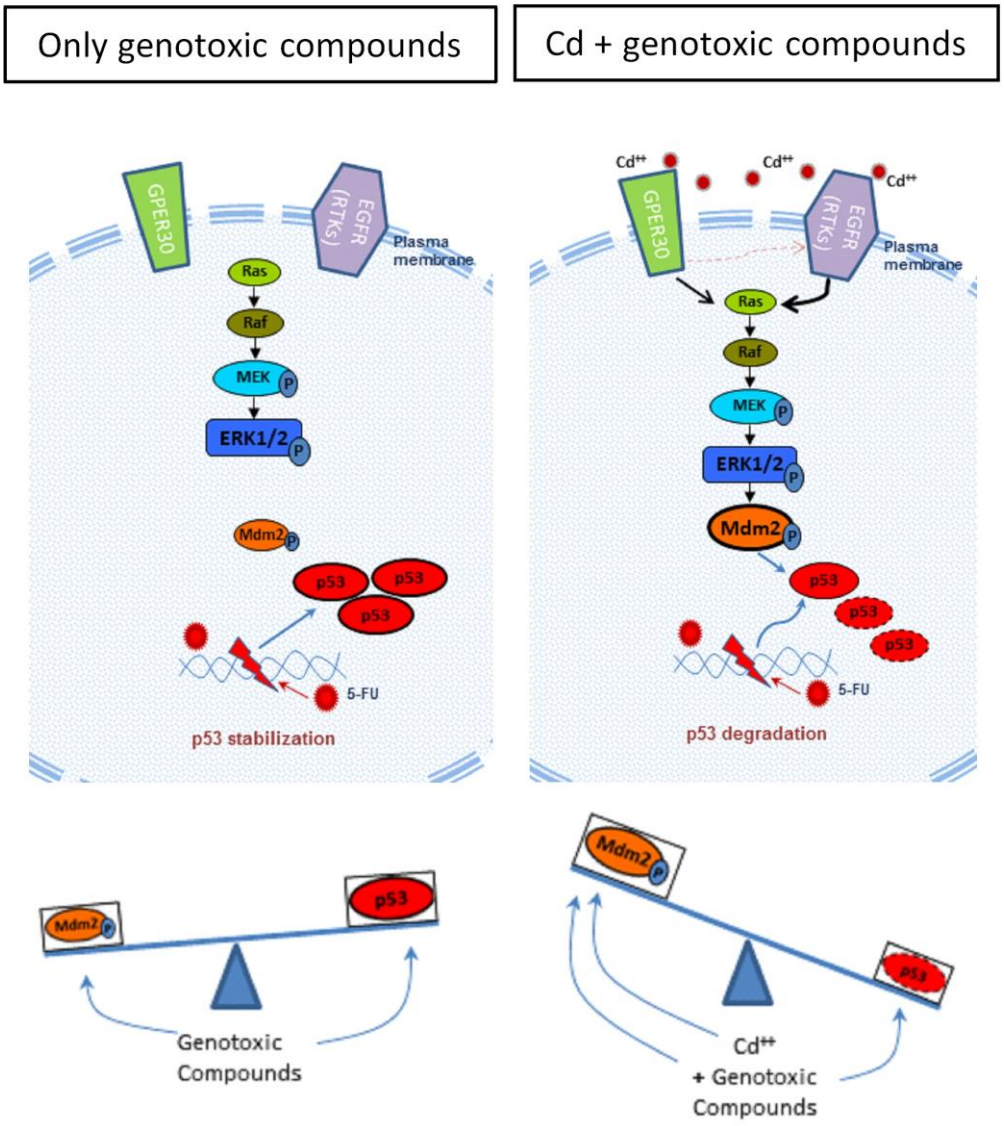

Figure 8. Proposed model for how Cadmium modulates the Mdm2/p53 response by activating Raf-MEK-ERK1/2 MAPKs signaling via EGFR (from **Paper III**)

5.4 CADMIUM AND ENDOGENOUS LEVELS OF SEX HORMONES IN WOMEN

In addition to the mechanistic studies on cadmium-induced estrogen-like effects in experimental models, our objective in **Paper IV** has been to assess the associations between cadmium exposures (blood and urinary cadmium) and serum concentrations of androstenedione, testosterone, estradiol and SHBG in a population-based study of women. Brief summary of the main findings is given in **Table 2.**

Table 2: Summary of associations between blood cadmium and serum concentrations of testosterone and estradiol using multiple linear regression models.

Parameters[a]	**Blood Cadmium** ***(nmol/L)***		
	Regression coefficient (95% CI)	*P* value	R^2
Serum Testosterone ***(nmol/L)*** (Adjusted for smoking and alcohol)	+0.020 (+0.001; +0.039)	<0.05	0.03
Serum Estradiol ***(pmol/L)*** (Adjusted for age)	-0.026 (-0.051; -0.001)	<0.05	0.02
Estradiol /testosterone ratio[b] (Adjusted for age, smoking and alcohol)	-0.063 (-0.097; -0.029)	<0.001	0.05

95% CI, 95% confidence interval; R^2, explained adjusted variance for the total model; [a] Ln (dependent variable)/Ln2; [b] *(pmol)/(nmol).*

Our findings based on multivariable-adjusted regression analysis showed a low to moderate but statistically significant positive association between blood cadmium and serum testosterone levels after controlling for smoking and alcohol consumption (P = 0.04). A similar association was also observed in a previous study with urinary cadmium [194]. Theoretically, there are two possibilities that could explain the increases in testosterone levels in response to cadmium exposure: 1) an increase in SHBG levels may result in the increases concentrations of total testosterone in serum, 2) cadmium exposure may interfere with the mechanism that maintains the estrogen-androgen balance. We observed a significant positive association between blood cadmium and SHBG in univariate analysis (P = 0.001). However, this association became non-significant after controlling for BMI, smoking and alcohol consumption (P = 0.114). Furthermore, no association was found between SHBG and serum testosterone levels suggesting that an increase in SHBG is not associated with the increase in testosterone levels. In order to explore further if cadmium exposure poses an insult to the mechanism that maintains the estrogen-androgen balance, we

performed analyses on blood cadmium and serum levels of estradiol. Interestingly, we observed a significant inverse association between blood cadmium and serum estradiol levels (P = 0.041), which was also significant with estradiol index (P = 0.005). These findings suggest that cadmium exposure interferes with the mechanism that maintains estrogen-androgen balance.

Cadmium exposure has been shown to inhibit the lutenizing hormone (LH)-induced ovarian aromatase activity (conversion of testosterone to 17β-estradiol) and P450 arom gene expression *in vitro* [195]. To indirectly investigate this aspect of LH-induced aromatase activity in relation to cadmium exposure, we modeled the estradiol/testosterone ratio against blood cadmium in our study population. Interestingly, we observed a significant inverse association between blood cadmium and estradiol/testosterone ratio (P <0.001), which suggests that cadmium may interfere with the LH-induced P450 aromatase activity and thereby disturb the estradiol/testosterone balance. These findings represent a possible explanation of the mechanism by which cadmium exposure poses insult to the estrogen-androgen balance. Further investigations are however warranted to clarify this.

The observed associations in **Paper IV** were more pronounced with blood cadmium than with urinary cadmium, which is probably because the former mainly reflects recent exposure and is the active cadmium present in the blood and liver, while the latter is an integrated measure of long-term exposure mainly reflects the cadmium accumulation in the kidney.

Cadmium has been shown as a potential risk factor of endometrial and breast cancer among women in recent epidemiological studies. Furthermore, a meta-analysis of nine prospective studies among postmenopausal women, suggests 1.4-fold increases risks of breast cancer with the doubling of testosterone levels [66]. Testosterone production by the ovaries decreases a little bit after menopause, the levels are however relatively constant. In contrast to that, a dramatic decrease in estradiol production leads to decreases in the estradiol/testosterone ratio to a great extent in postmenopausal women [80, 81]. The exposure to cadmium in the study population is low, especially in comparison to occupational settings, but also to the general population [8]. However, the observed increases in the levels of testosterone and decreases estradiol/testosterone ratio associated with cadmium exposure in our study,

suggest that cadmium at low exposure levels may contribute to breast-cancer through interference with the estrogen-androgen balance.

6 CONCLUSIONS

Based on our findings from both experimental models (*in vivo* and *in vitro*) and human data, it was concluded that;

- Cadmium does not activate classical ER signaling. Instead, it affects other cellular signaling pathways that can produce physiological effects reminiscent of bona fide estrogen stimulation.

- Cadmium-induced effects on cellular signaling pathways are markedly concentration dependent i.e. low concentrations stimulate protein-kinases while higher concentrations induce cellular stress responses.

- Cadmium activates Raf-MEK-ERK1/2 MAPKs signaling mainly through the cell surface receptors EGFR/GPR30. This represents a previously uncharacterized mechanism in cadmium-induced effects that operate at low nanomolar levels in cell culture systems.

- Cadmium-induced activation of EGFR/GPR30-Raf-MEK-ERK1/2 MAPKs leads to the phosphorylation of Mdm2 (ser-166), a negative regulator of the tumor suppressor p53, and thus compromise p53 response upon co-treatment with genotoxic compounds.

- Cadmium-induced sensitization of cells to genotoxins via Mdm2/p53 balance at low 10^{-8} M range and cadmium-associated alterations in the estrogen-androgen balance in postmenopausal women, may suggest that cadmium at physiological concentrations may play a role in the promotion/development of hormone-related cancers.

7 FUTURE PERSPECTIVES

Cadmium induces cell proliferation, inactivates negative growth stimuli such as the tumor suppressor protein p53, and provokes resistance towards apoptosis [147]. The combination of these multiple mechanisms may give rise to a high degree of genomic instability in cadmium-adapted cells, relevant not only for tumor initiation, but also for later steps in tumor development. Future research is needed to clarify the relevance of these interactions for cadmium at low exposure conditions in humans. Furthermore, it would be important in future studies to address the possible effect of cadmium and other metalloestrogens on additional cell surface tyrosine kinases.

DNA methylation plays an intricate role in the regulation of gene expression. Recent research on cadmium-induced carcinogenicity has shown epigenetic alterations (both hyper and hypo methylation) in response to low level cadmium exposure suggesting that DNA methylation may be an additional mechanism in cadmium-induced carcinogenicity [148]. Further investigations are needed to explore the role of epigenetics and the mechanism behind cadmium-induced alterations in DNA methylation.

Furthermore, over the last 10 years, it has been established that EDCs can work together to produce additive effects, even when combined at low doses that individually do not produce observable effects. As humans are exposed to hundreds of chemicals including cadmium, further studies are required in order to investigate the additive effects of cadmium in mixtures.

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9 REFERENCES

1. WHOs. Preventing Disease through Healthy Environments, in Towards an estimate of environmental burden of disease, A. Prüss-Üstün and C. Corvalán, Editors. 2006: Geneva, Switzerland. . p. 1-19. http://www.who.int.
2. WHO/UNEPs. State-of-the-science of Endocrine Disrupting Chemicals, in Inter-Organization Programme for the Sound Management of Chemicals (IOMC). 2012: Geneva, Switzerland. p. 1-250.
3. Persson, I. Estrogens in the causation of breast, endometrial and ovarian cancers - evidence and hypotheses from epidemiological findings. J Steroid Biochem Mol Biol, 2000. **74**(5): p. 357-64.
4. Brawley, O.W. Hormonal prevention of prostate cancer. Urol Oncol, 2003. **21**(1): p. 67-72.
5. Safe, S. Cadmium's disguise dupes the estrogen receptor. Nat Med, 2003. **9**(8): p. 1000-1.
6. Johnson, M.D., Kenney, N., Stoica, A., Hilakivi-Clarke, L., Singh, B., Chepko, G., Clarke, R., Sholler, P.F., Lirio, A.A., Foss, C., Reiter, R., Trock, B., Paik, S., and Martin, M.B. Cadmium mimics the in vivo effects of estrogen in the uterus and mammary gland. Nat Med, 2003. **9**(8): p. 1081-4.
7. Stoica, A., Katzenellenbogen, B.S., and Martin, M.B. Activation of estrogen receptor-alpha by the heavy metal cadmium. Mol Endocrinol, 2000. **14**(4): p. 545-53.
8. Järup, L. and Åkesson, A. Current status of cadmium as an environmental health problem. Toxicol Appl Pharmacol, 2009. **238**(3): p. 201-8.
9. Thévenod, F. and Lee, W.-K.s, Toxicology of Cadmium and Its Damage to Mammalian Organs, in Cadmium: From Toxicology to Essentiality. , A. Sigel, H. Sigel, and R.K.O. Sigel, Editors. 2013, Springer Netherlands. p. 415-490
10. Thornton, I. Sources and pathways of cadmium in the environment. IARC Sci Publ, 1992(118): p. 149-62.
11. Satarug, S., Baker, J.R., Urbenjapol, S., Haswell-Elkins, M., Reilly, P.E.B., Williams, D.J., and Moore, M.R. A global perspective on cadmium pollution and toxicity in non-occupationally exposed population. Toxicology Letters, 2003. **137**(1-2): p. 65-83.
12. IPCSs. CADMIUM. ENVIRONMENTAL HEALTH CRITERIA 134. 1992, World Health Organization: Geneva, Switzerland. http://www.inchem.org/documents/ehc/ehc/ehc134.htm.
13. Pan, J., Plant, J.A., Voulvoulis, N., Oates, C.J., and Ihlenfeld, C. Cadmium levels in Europe: implications for human health. Environ Geochem Health, 2010. **32**(1): p. 1-12.
14. Satarug, S. and Moore, M.R. Adverse health effects of chronic exposure to low-level cadmium in foodstuffs and cigarette smoke. Environ Health Perspect, 2004. **112**(10): p. 1099-103.
15. Julin, B., Wolk, A., Bergkvist, L., Bottai, M., and Åkesson, A. Dietary cadmium exposure and risk of postmenopausal breast cancer: a population-based prospective cohort study. Cancer Res, 2012. **72**(6): p. 1459-66.
16. Julin, B., Wolk, A., Johansson, J.E., Andersson, S.O., Andren, O., and Akesson, A. Dietary cadmium exposure and prostate cancer incidence: a population-based prospective cohort study. Br J Cancer, 2012. **107**(5): p. 895-900.
17. Olsson, I.M., Bensryd, I., Lundh, T., Ottosson, H., Skerfving, S., and Oskarsson, A. Cadmium in blood and urine - Impact of sex, age, dietary intake, iron status, and former smoking - Association of renal effects. Environmental Health Perspectives, 2002. **110**(12): p. 1185-1190.
18. Järup, L. and Alfven, T. Low level cadmium exposure, renal and bone effects--the OSCAR study. Biometals, 2004. **17**(5): p. 505-9.
19. Berglund, M., Akesson, A., Nermell, B., and Vahter, M. Intestinal absorption of dietary cadmium in women depends on body iron stores and fiber intake. Environ Health Perspect, 1994. **102**(12): p. 1058-66.

20. Larsen, E.H., Andersen, N.L., Moller, A., Petersen, A., Mortensen, G.K., and Petersen, J. Monitoring the content and intake of trace elements from food in Denmark. Food Addit Contam, 2002. **19**(1): p. 33-46.
21. Llobet, J.M., Falco, G., Casas, C., Teixido, A., and Domingo, J.L. Concentrations of arsenic, cadmium, mercury, and lead in common foods and estimated daily intake by children, adolescents, adults, and seniors of Catalonia, Spain. J Agric Food Chem, 2003. **51**(3): p. 838-42.
22. MacIntosh, D.L., Spengler, J.D., Ozkaynak, H., Tsai, L., and Ryan, P.B. Dietary exposures to selected metals and pesticides. Environ Health Perspect, 1996. **104**(2): p. 202-9.
23. Thomas, K.W., Pellizzari, E.D., and Berry, M.R. Population-based dietary intakes and tap water concentrations for selected elements in the EPA region V National Human Exposure Assessment Survey (NHEXAS). J Expo Anal Environ Epidemiol, 1999. **9**(5): p. 402-13.
24. Ysart, G., Miller, P., Croasdale, M., Crews, H., Robb, P., Baxter, M., de L'Argy, C., and Harrison, N. 1997 UK Total Diet Study--dietary exposures to aluminium, arsenic, cadmium, chromium, copper, lead, mercury, nickel, selenium, tin and zinc. Food Addit Contam, 2000. **17**(9): p. 775-86.
25. Nordberg, G.F., Nogawa, K., Nordberg, M., and Friberg, L.T. Cadmium. In: Nordberg, G.F., Fowler, B.A., Noredberg, M., Friberg, L.T. (Eds.), Handbook on the Toxicology of metals Third Edition ed. 2007: Academic press. 446-486.
26. Chaney, R.L., Ryan, J.A., Li, Y.M., and Brown, S.L.s, Soil cadmium as a threat to human health, in Developments in Plant and Soil Sciences, M.J. McLaughlin, B.R. Singh (Eds.),. 1999, 85Kluwer Academic Publishers, Dordrecht. p. 219-256
27. Watanabe, T., Kasahara, M., Nakatsuka, H., and Ikeda, M. Cadmium and Lead Contents of Cigarettes Produced in Various Areas of the World. Science of the Total Environment, 1987. **66**: p. 29-37.
28. Elinder, C.G., Kjellstrom, T., Lind, B., Linnman, L., Piscator, M., and Sundstedt, K. Cadmium exposure from smoking cigarettes: variations with time and country where purchased. Environ Res, 1983. **32**(1): p. 220-7.
29. Willers, S., Gerhardsson, L., and Lundh, T. Environmental tobacco smoke (ETS) exposure in children with asthma - relation between lead and cadmium, and cotinine concentrations in urine. Respiratory Medicine, 2005. **99**(12): p. 1521-1527.
30. McElroy, J.A., Shafer, M.M., Trentham-Dietz, A., Hampton, J.M., and Newcomb, P.A. Cadmium exposure and breast cancer risk. J Natl Cancer Inst, 2006. **98**(12): p. 869-73.
31. Åkesson, A., Berglund, M., Schutz, A., Bjellerup, P., Bremme, K., and Vahter, M. Cadmium exposure in pregnancy and lactation in relation to iron status. Am J Public Health, 2002. **92**(2): p. 284-7.
32. Kippler, M., Lonnerdal, B., Goessler, W., Ekstrom, E.C., Arifeen, S.E., and Vahter, M. Cadmium interacts with the transport of essential micronutrients in the mammary gland - a study in rural Bangladeshi women. Toxicology, 2009. **257**(1-2): p. 64-9.
33. Reeves, P.G. and Chaney, R.L. Nutritional status affects the absorption and whole-body and organ retention of cadmium in rats fed rice-based diets. Environ Sci Technol, 2002. **36**(12): p. 2684-92.
34. Waalkes, M.P. and Klaassen, C.D. Concentration of Metallothionein in Major Organs of Rats after Administration of Various Metals. Fundamental and Applied Toxicology, 1985. **5**(3): p. 473-477.
35. Järup, L., Berglund, M., Elinder, C.G., Nordberg, G., and Vahter, M. Health effects of cadmium exposure--a review of the literature and a risk estimate. Scand J Work Environ Health, 1998. **24 Suppl 1**: p. 1-51.
36. Zalups, R.K. and Ahmad, S. Molecular handling of cadmium in transporting epithelia. Toxicol Appl Pharmacol, 2003. **186**(3): p. 163-88.
37. Nordberg, M., Jin, T., and Nordberg, G.F. Cadmium, metallothionein and renal tubular toxicity. IARC Sci Publ, 1992(118): p. 293-7.

38. Godt, J., Scheidig, F., Grosse-Siestrup, C., Esche, V., Brandenburg, P., Reich, A., and Groneberg, D.A. The toxicity of cadmium and resulting hazards for human health. J Occup Med Toxicol, 2006. **1**: p. 22.
39. McCarthy, J.F. and Shugart, L.R. Biomarkers of environmental contamination. 1990: Chelsea, MI (US); Lewis Publishers, United States.
40. Järup, L., Rogenfelt, A., Elinder, C.G., Nogawa, K., and Kjellstrom, T. Biological half-time of cadmium in the blood of workers after cessation of exposure. Scand J Work Environ Health, 1983. **9**(4): p. 327-31.
41. Orlowski, C., Piotrowski, J.K., Subdys, J.K., and Gross, A. Urinary cadmium as indicator of renal cadmium in humans: an autopsy study. Human & Experimental Toxicology, 1998. **17**(6): p. 302-306.
42. Åkerström, M., Barregard, L., Lundh, T., and Sallsten, G. The relationship between cadmium in kidney and cadmium in urine and blood in an environmentally exposed population. Toxicology and Applied Pharmacology, 2013. **268**(3): p. 286-293.
43. EFSA. Cadmium in food - Scientific opinion of the Panel on Contaminants in the Food Chain. The EFSA Journal, 2009. **980**: p. 1–139.
44. Hagino, N. and Kono, M. A study on the cause of itai-itai-disease. in Proc 17th Mtg Japanese Soc of Clinical Surgeons (in Japanese). 1955.
45. Engström, A., Michaelsson, K., Suwazono, Y., Wolk, A., Vahter, M., and Akesson, A. Long-Term Cadmium Exposure and the Association With Bone Mineral Density and Fractures in a Population-Based Study Among Women. Journal of Bone and Mineral Research, 2011. **26**(3): p. 486-495.
46. Alfven, T., Elinder, C.G., Carlsson, M.D., Grubb, A., Hellstrom, L., Persson, B., Pettersson, C., Spang, G., Schutz, A., and Jarup, L. Low-level cadmium exposure and osteoporosis. Journal of Bone and Mineral Research, 2000. **15**(8): p. 1579-1586.
47. Engström, A., Michaelsson, K., Vahter, M., Julin, B., Wolk, A., and Åkesson, A. Associations between dietary cadmium exposure and bone mineral density and risk of osteoporosis and fractures among women. Bone, 2012. **50**(6): p. 1372-8.
48. Thomas, L.D.K., Michaelsson, K., Julin, B., Wolk, A., and Akesson, A. Dietary Cadmium Exposure and Fracture Incidence Among Men: A Population-Based Prospective Cohort Study. Journal of Bone and Mineral Research, 2011. **26**(7): p. 1601-1608.
49. Bernard, A. Renal dysfunction induced by cadmium: biomarkers of critical effects. Biometals, 2004. **17**(5): p. 519-23.
50. IARC. A review of human carcinogens—Part C: metals, arsenic, dusts, and fibres. The Lancet Oncol, 2009. **10**: p. 453-54.
51. US-EPA. Human Health Risk Assessment. 2012; Available from: http://www.epa.gov/risk_assessment/health-risk.htm.
52. FAO/WHO (1972) Evaluation of certain food additives and the contaminants mercury, lead, and cadmium (Sixteenth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series.
53. WHOs. Safety evaluation of certain food additives and contaminants, in WHO Food Additive Series. 2011: Geneva, Switzerland. http://www.who.int.
54. EFSA. Statement on tolerable weekly intake for cadmium EFSA Journal, 2011. **9**(2): p. 19.
55. Henderson, B.E., Ross, R.K., Pike, M.C., and Casagrande, J.T. Endogenous Hormones as a Major Factor in Human Cancer. Cancer Research, 1982. **42**(8): p. 3232-3239.
56. Henderson, B.E. and Feigelson, H.S. Hormonal carcinogenesis. Carcinogenesis, 2000. **21**(3): p. 427-433.
57. Feigelson, H.S., Ross, R.K., Yu, M.C., Coetzee, G.A., Reichardt, J.K., and Henderson, B.E. Sex steroid hormones and genetic susceptibility to breast and prostate cancer. Drug Metab Rev, 1998. **30**(2): p. 421-34.
58. Brekelmans, C.T. Risk factors and risk reduction of breast and ovarian cancer. Curr Opin Obstet Gynecol, 2003. **15**(1): p. 63-8.
59. Harkonen, P.L. and Mäkela, S.I. Role of estrogens in development of prostate cancer. J Steroid Biochem Mol Biol, 2004. **92**(4): p. 297-305.

60. Sainsbury, J.R., Anderson, T.J., and Morgan, D.A. ABC of breast diseases: breast cancer. BMJ, 2000. **321**(7263): p. 745-50.
61. Key, T.J.A. and Pike, M.C. The Role of Estrogens and Progestagens in the Epidemiology and Prevention of Breast-Cancer. European Journal of Cancer & Clinical Oncology, 1988. **24**(1): p. 29-43.
62. Badve, S. and Nakshatri, H. Oestrogen-receptor-positive breast cancer: towards bridging histopathological and molecular classifications. J Clin Pathol, 2009. **62**(1): p. 6-12.
63. Pasqualini, J.R., Chetrite, G., Blacker, C., Feinstein, M.C., Delalonde, L., Talbi, M., and Maloche, C. Concentrations of estrone, estradiol, and estrone sulfate and evaluation of sulfatase and aromatase activities in pre- and postmenopausal breast cancer patients. J Clin Endocrinol Metab, 1996. **81**(4): p. 1460-4.
64. Castagnetta, L.A., LoCasto, M., Granata, O.M., Polito, L., Calabro, M., LoBue, A., Bellavia, V., and Carruba, G. Estrogen content and metabolism in human breast tumor tissues and cells. Basis for Cancer Management, 1996. **784**: p. 314-324.
65. Chlebowski, R.T., Hendrix, S.L., Langer, R.D., Stefanick, M.L., Gass, M., Lane, D., Rodabough, R.J., Gilligan, M.A., Cyr, M.G., Thomson, C.A., Khandekar, J., Petrovitch, H., McTiernan, A., and Investigators, W. Influence of estrogen plus progestin on breast, cancer and mammography in healthy postmenopausal women - The Women's Health Initiative Randomized trial. Jama-Journal of the American Medical Association, 2003. **289**(24): p. 3243-3253.
66. Key, T., Appleby, P., Barnes, I., and Reeves, G. Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. J Natl Cancer Inst, 2002. **94**(8): p. 606-16.
67. Purdie, D.M. and Green, A.C. Epidemiology of endometrial cancer. Best Practice & Research in Clinical Obstetrics & Gynaecology, 2001. **15**(3): p. 341-354.
68. Oldenburg, C.S., Boll, D., Nicolaije, K.A.H., Vos, M.C., Pijnenborg, J.M.A., Coebergh, J.W., Beijer, S., van de Poll-Franse, L.V., and Ezendam, N.P.M. The relationship of body mass index with quality of life among endometrial cancer survivors: A study from the population-based PROFILES registry. Gynecologic Oncology, 2013. **129**(1): p. 216-221.
69. Nicolaije, K.A., Ezendam, N.P., Vos, M.C., Boll, D., Pijnenborg, J.M., Kruitwagen, R.F., Lybeert, M.L., and van de Poll-Franse, L.V. Follow-up practice in endometrial cancer and the association with patient and hospital characteristics: a study from the population-based PROFILES registry. Gynecologic Oncology, 2013. **129**(2): p. 324-31.
70. Kaaks, R., Lukanova, A., and Kurzer, M.S. Obesity, endogenous hormones, and endometrial cancer risk: a synthetic review. Cancer Epidemiol Biomarkers Prev, 2002. **11**(12): p. 1531-43.
71. Piek, J.M.J., van Diest, P.J., and Verheijen, R.H.M. Ovarian carcinogenesis: An alternative hypothesis. Ovarian Cancer: State of the Art and Future Directions in Translational Research, 2008. **622**: p. 79-87.
72. Lukanova, A. and Kaaks, R. Endogenous hormones and ovarian cancer: epidemiology and current hypotheses. Cancer Epidemiol Biomarkers Prev, 2005. **14**: p. 98-107.
73. Rodriguez, C., Patel, A.V., Calle, E.E., Jacob, E.J., and Thun, M.J. Estrogen replacement therapy and ovarian cancer mortality in a large prospective study of US women. JAMA, 2001. **285**(11): p. 1460-5.
74. Risch, H.A. Estrogen replacement therapy and risk of epithelial ovarian cancer. Gynecologic Oncology, 1996. **63**(2): p. 254-257.
75. Purdie, D.M., Bain, C.J., Siskind, V., Russell, P., Hacker, N.F., Ward, B.G., Quinn, M.A., and Green, A.C. Hormone replacement therapy and risk of epithelial ovarian cancer. Br J Cancer, 1999. **81**(3): p. 559-63.
76. Rhoades, R. and Pflanzer, R., eds. Endocrine control mechanisms. Human Physiology. 4 Ed. ed. 2003, Pacific Grove: Thomson Learning.
77. Miller, W.L. Molecular biology of steroid hormone synthesis. Endocr Rev, 1988. **9**(3): p. 295-318.

78. Hanukoglu, I. Steroidogenic enzymes: structure, function, and role in regulation of steroid hormone biosynthesis. J Steroid Biochem Mol Biol, 1992. **43**(8): p. 779-804.
79. Davis, S.R. Androgens. Menopause: Biology and Pathobiology., ed. K.J. In Lobo RA, Marcus R, eds. . 2000, New York: Academic Press.
80. Meldrum, D.R., Davidson, B.J., Tataryn, I.V., and Judd, H.L. Changes in circulating steroids with aging in postmenopausal women. Obstet Gynecol, 1981. **57**(5): p. 624-8.
81. Rohr, U.D. The impact of testosterone imbalance on depression and women's health. Maturitas, 2002. **41 Suppl 1**: p. S25-46.
82. Nelson, L.R. and Bulun, S.E. Estrogen production and action. J Am Acad Dermatol, 2001. **45**(3 Suppl): p. S116-24.
83. Simpson, E.R. and Davis, S.R. Minireview: Aromatase and the Regulation of Estrogen Biosynthesis—Some New Perspectives Endocrinology, 2001. **142**(11): p. 4589-4594.
84. Wierman, M.E. Sex steroid effects at target tissues: mechanisms of action. Advances in Physiology Education, 2007. **31**(1): p. 26-33.
85. Osborne, C.K. and Schiff, R. Mechanisms of endocrine resistance in breast cancer. Annu Rev Med, 2011. **62**: p. 233-47.
86. Nilsson, S., Makela, S., Treuter, E., Tujague, M., Thomsen, J., Andersson, G., Enmark, E., Pettersson, K., Warner, M., and Gustafsson, J.A. Mechanisms of estrogen action. Physiol Rev, 2001. **81**(4): p. 1535-65.
87. Zhang, D. and Trudeau, V.L. Integration of membrane and nuclear estrogen receptor signaling. Comp Biochem Physiol A Mol Integr Physiol, 2006. **144**(3): p. 306-15.
88. Katzenellenbogen, B.S. Estrogen receptors: bioactivities and interactions with cell signaling pathways. Biol Reprod, 1996. **54**(2): p. 287-93.
89. Enmark, E. and Gustafsson, J.A. Oestrogen receptors - an overview. J Intern Med, 1999. **246**(2): p. 133-8.
90. Klinge, C.M. Estrogen receptor interaction with estrogen response elements. Nucleic Acids Research, 2001. **29**(14): p. 2905-2919.
91. Frasor, J., Stossi, F., Danes, J.M., Komm, B., Lyttle, C.R., and Katzenellenbogen, B.S. Selective estrogen receptor modulators: discrimination of agonistic versus antagonistic activities by gene expression profiling in breast cancer cells. Cancer Res, 2004. **64**(4): p. 1522-33.
92. Riggs, B.L. and Hartmann, L.C. Selective estrogen-receptor modulators -- mechanisms of action and application to clinical practice. N Engl J Med, 2003. **348**(7): p. 618-29.
93. Jordan, V.C. Selective estrogen receptor modulation: concept and consequences in cancer. Cancer Cell, 2004. **5**(3): p. 207-13.
94. McKenna, N.J. and O'Malley, B.W. An issue of tissues: divining the split personalities of selective estrogen receptor modulators. Nat Med, 2000. **6**(9): p. 960-2.
95. McKenna, N.J. and O'Malley, B.W. Combinatorial control of gene expression by nuclear receptors and coregulators. Cell, 2002. **108**(4): p. 465-74.
96. Kushner, P.J., Agard, D.A., Greene, G.L., Scanlan, T.S., Shiau, A.K., Uht, R.M., and Webb, P. Estrogen receptor pathways to AP-1. J Steroid Biochem Mol Biol, 2000. **74**(5): p. 311-7.
97. Smith, C.L. and O'Malley, B.W. Coregulator function: a key to understanding tissue specificity of selective receptor modulators. Endocr Rev, 2004. **25**(1): p. 45-71.
98. Lösel, R. and Wehling, M. Nongenomic actions of steroid hormones. Nat Rev Mol Cell Biol, 2003. **4**(1): p. 46-56.
99. Heldring, N., Pike, A., Andersson, S., Matthews, J., Cheng, G., Hartman, J., Tujague, M., Strom, A., Treuter, E., Warner, M., and Gustafsson, J.A. Estrogen receptors: How do they signal and what are their targets. Physiological Reviews, 2007. **87**(3): p. 905-931.
100. Björnström, L. and Sjoberg, M. Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. Molecular Endocrinology, 2005. **19**(4): p. 833-42.

101. Madak-Erdogan, Z., Kieser, K.J., Kim, S.H., Komm, B., Katzenellenbogen, J.A., and Katzenellenbogen, B.S. Nuclear and extranuclear pathway inputs in the regulation of global gene expression by estrogen receptors. Molecular Endocrinology, 2008. **22**(9): p. 2116-27.
102. Prossnitz, E.R., Arterburn, J.B., Smith, H.O., Oprea, T.I., Sklar, L.A., and Hathaway, H.J. Estrogen signaling through the transmembrane G protein-coupled receptor GPR30. Annu Rev Physiol, 2008. **70**: p. 165-90.
103. Schiff, R., Massarweh, S.A., Shou, J., Bharwani, L., Mohsin, S.K., and Osborne, C.K. Cross-talk between estrogen receptor and growth factor pathways as a molecular target for overcoming endocrine resistance. Clinical Cancer Research, 2004. **10**(1): p. 331s-336s.
104. Maggiolini, M. and Picard, D. The unfolding stories of GPR30, a new membrane-bound estrogen receptor. J Endocrinol, 2010. **204**(2): p. 105-14.
105. Olde, B. and Leeb-Lundberg, L.M. GPR30/GPER1: searching for a role in estrogen physiology. Trends Endocrinol Metab, 2009. **20**(8): p. 409-16.
106. Wu, R.C., Smith, C.L., and O'Malley, B.W. Transcriptional regulation by steroid receptor coactivator phosphorylation. Endocr Rev, 2005. **26**(3): p. 393-9.
107. Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., Metzger, D., and Chambon, P. Activation of the Estrogen-Receptor through Phosphorylation by Mitogen-Activated Protein-Kinase. Science, 1995. **270**(5241): p. 1491-1494.
108. Levin, E.R. Plasma membrane estrogen receptors. Trends Endocrinol Metab, 2009. **20**(10): p. 477-82.
109. Li, Y., Wang, J.P., Santen, R.J., Kim, T.H., Park, H., Fan, P., and Yue, W. Estrogen Stimulation of Cell Migration Involves Multiple Signaling Pathway Interactions. Endocrinology, 2010. **151**(11): p. 5146-5156.
110. Nelson, R.J.s, Endocrine mediation of homeostatic processes, in An introduction to behavioral endocrinology (3rd ed.). . 2005, Sinauer Associates. : Sunderland, MA, US. p. 822.
111. Diamanti-Kandarakis, E., Bourguignon, J.P., Giudice, L.C., Hauser, R., Prins, G.S., Soto, A.M., Zoeller, R.T., and Gore, A.C. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. Endocr Rev, 2009. **30**(4): p. 293-342.
112. Colborn, T., Saal, F.S.V., and Soto, A.M. Developmental Effects of Endocrine-Disrupting Chemicals in Wildlife and Humans. Environmental Health Perspectives, 1993. **101**(5): p. 378-384.
113. Colborn, T., Dumanoski, D., and Myers, J.P. Our Stolen Future, Are We Threatening Our Fertility, Intelligence and Survival?—A Scientific Detective Story 1996, New York: Penguin Books USA.
114. Marty, M.S., Carney, E.W., and Rowlands, J.C. Endocrine disruption: historical perspectives and its impact on the future of toxicology testing. Toxicological Sciences, 2011. **120 Suppl 1**: p. S93-108.
115. McLachlan, J.A. Environmental signaling: What embryos and evolution teach us about endocrine disrupting chemicals. Endocrine Reviews, 2001. **22**(3): p. 319-341.
116. Olsen, C.M., Meussen-Elholm, E.T., Hongslo, J.K., Stenersen, J., and Tollefsen, K.E. Estrogenic effects of environmental chemicals: an interspecies comparison. Comp Biochem Physiol C Toxicol Pharmacol, 2005. **141**(3): p. 267-74.
117. Yildiz, F. Phytoestrogens in Functional Foods. 2005: Taylor & Francis Ltd. 336.
118. Sarkar, F.H. and Li, Y. Mechanisms of cancer chemoprevention by soy isoflavone genistein. Cancer Metastasis Rev, 2002. **21**(3-4): p. 265-80.
119. Peeters, P.H., Keinan-Boker, L., van der Schouw, Y.T., and Grobbee, D.E. Phytoestrogens and breast cancer risk. Review of the epidemiological evidence. Breast Cancer Res Treat, 2003. **77**(2): p. 171-83.
120. Cheek, A.O., Vonier, P.M., Oberdorster, E., Burow, B.C., and McLachlan, J.A. Environmental signaling: a biological context for endocrine disruption. Environ Health Perspect, 1998. **106 Suppl 1**: p. 5-10.

121. Martin, M.B., Reiter, R., Pham, T., Avellanet, Y.R., Camara, J., Lahm, M., Pentecost, E., Pratap, K., Gilmore, B.A., Divekar, S., Dagata, R.S., Bull, J.L., and Stoica, A. Estrogen-like activity of metals in MCF-7 breast cancer cells. Endocrinology, 2003. **144**(6): p. 2425-36.
122. Darbre, P.D. Metalloestrogens: an emerging class of inorganic xenoestrogens with potential to add to the oestrogenic burden of the human breast. Journal of Applied Toxicology, 2006. **26**(3): p. 191-197.
123. Garcia-Morales, P., Saceda, M., Kenney, N., Kim, N., Salomon, D.S., Gottardis, M.M., Solomon, H.B., Sholler, P.F., Jordan, V.C., and Martin, M.B. Effect of cadmium on estrogen receptor levels and estrogen-induced responses in human breast cancer cells. J Biol Chem, 1994. **269**(24): p. 16896-901.
124. Zhang, W., Yang, J., Wang, J., Xia, P., Xu, Y., Jia, H., and Chen, Y. Comparative studies on the increase of uterine weight and related mechanisms of cadmium and p-nonylphenol. Toxicology, 2007. **241**(1-2): p. 84-91.
125. Höfer, N., Diel, P., Wittsiepe, J., Wilhelm, M., and Degen, G.H. Dose- and route-dependent hormonal activity of the metalloestrogen cadmium in the rat uterus. Toxicol Lett, 2009. **191**(2-3): p. 123-31.
126. Alonso-Gonzalez, C., Gonzalez, A., Mazarrasa, O., Guezmes, A., Sanchez-Mateos, S., Martinez-Campa, C., Cos, S., Sanchez-Barcelo, E.J., and Mediavilla, M.D. Melatonin prevents the estrogenic effects of sub-chronic administration of cadmium on mice mammary glands and uterus. Journal of Pineal Research, 2007. **42**(4): p. 403-410.
127. Pillet, S., D'Elia, M., Bernier, J., Bouquegneau, J.M., Fournier, M., and Cyr, D.G. Immunomodulatory effects of estradiol and cadmium in adult female rats. Toxicological Sciences, 2006. **92**(2): p. 423-432.
128. Liu, J., Huang, H.L., Zhang, W.C., and Li, H. Cadmium-Induced Increase in Uterine Wet Weight and Its Mechanism. Birth Defects Research Part B-Developmental and Reproductive Toxicology, 2010. **89**(1): p. 43-49.
129. Höfer, N., Diel, P., Wittsiepe, J., Wilhelm, M., Kluxen, F.M., and Degen, G.H. Investigations on the estrogenic activity of the metallohormone cadmium in the rat intestine. Arch Toxicol, 2010. **84**(7): p. 541-52.
130. Silva, E., Lopez-Espinosa, M.J., Molina-Molina, J.M., Fernandez, M., Olea, N., and Kortenkamp, A. Lack of activity of cadmium in in vitro estrogenicity assays. Toxicol Appl Pharmacol, 2006. **216**(1): p. 20-8.
131. Vetillard, A. and Bailhache, T. Cadmium: an endocrine disrupter that affects gene expression in the liver and brain of juvenile rainbow trout. Biol Reprod, 2005. **72**(1): p. 119-26.
132. Guevel, R.L., Petit, F.G., Goff, P.L., Metivier, R., Valotaire, Y., and Pakdel, F. Inhibition of rainbow trout (Oncorhynchus mykiss) estrogen receptor activity by cadmium. Biol Reprod, 2000. **63**(1): p. 259-66.
133. Martin, M.B., Voeller, H.J., Gelmann, E.P., Lu, J., Stoica, E.G., Hebert, E.J., Reiter, R., Singh, B., Danielsen, M., Pentecost, E., and Stoica, A. Role of cadmium in the regulation of AR gene expression and activity. Endocrinology, 2002. **143**(1): p. 263-75.
134. De Coster, S. and van Larebeke, N. Endocrine-disrupting chemicals: associated disorders and mechanisms of action. J Environ Public Health, 2012. **2012**: p. 713696.
135. Johnston, S.R. New strategies in estrogen receptor-positive breast cancer. Clinical Cancer Research, 2010. **16**(7): p. 1979-87.
136. Yaghjyan, L. and Colditz, G.A. Estrogens in the breast tissue: a systematic review. Cancer Causes & Control, 2011. **22**(4): p. 529-540.
137. Åkesson, A., Julin, B., and Wolk, A. Long-term dietary cadmium intake and postmenopausal endometrial cancer incidence: a population-based prospective cohort study. Cancer Res, 2008. **68**(15): p. 6435-41.
138. Gallagher, C.M., Chen, J.J., and Kovach, J.S. Environmental cadmium and breast cancer risk. Aging (Albany NY), 2010. **2**(11): p. 804-14.
139. Adams, S.V., Newcomb, P.A., Shafer, M.M., Atkinson, C., Bowles, E.J., Newton, K.M., and Lampe, J.W. Urinary cadmium and mammographic density in premenopausal women. Breast Cancer Res Treat, 2011. **128**(3): p. 837-44.

140. Antila, E., Mussalo-Rauhamaa, H., Kantola, M., Atroshi, F., and Westermarck, T. Association of cadmium with human breast cancer. Sci Total Environ, 1996. **186**(3): p. 251-6.
141. Saleh, F., Behbehani, A., Asfar, S., Khan, I., and Ibrahim, G. Abnormal blood levels of trace elements and metals, DNA damage, and breast cancer in the state of Kuwait. Biol Trace Elem Res, 2011. **141**(1-3): p. 96-109.
142. Jackson, L.W., Zullo, M.D., and Goldberg, J.M. The association between heavy metals, endometriosis and uterine myomas among premenopausal women: National Health and Nutrition Examination Survey 1999-2002. Human Reproduction, 2008. **23**(3): p. 679-687.
143. Nasiadek, M., Krawczyk, T., and Sapota, A. Tissue levels of cadmium and trace elements in patients with myoma and uterine cancer. Human & Experimental Toxicology, 2005. **24**(12): p. 623-30.
144. Nasiadek, M., Swiatkowska, E., Nowinska, A., Krawczyk, T., Wilczynski, J.R., and Sapota, A. The Effect of Cadmium on Steroid Hormones and Their Receptors in Women with Uterine Myomas. Archives of Environmental Contamination and Toxicology, 2011. **60**(4): p. 734-741.
145. Heilier, J.F., Donnez, J., Verougstraete, V., Donnez, O., Grandjean, F., Haufroid, V., Nackers, F., and Lison, D. Cadmium, lead and endometriosis. Int Arch Occup Environ Health, 2006. **80**(2): p. 149-53.
146. Itoh, H., Iwasaki, M., Nakajima, Y., Endo, Y., Hanaoka, T., Sasaki, H., Tanaka, T., Yang, B., and Tsugane, S. A case-control study of the association between urinary cadmium concentration and endometriosis in infertile Japanese women. Science of the Total Environment, 2008. **402**(2-3): p. 171-175.
147. Hartwig, A. Mechanisms in cadmium-induced carcinogenicity: recent insights. Biometals, 2010. **23**(5): p. 951-60.
148. Brocato, J. and Costa, M. Basic mechanics of DNA methylation and the unique landscape of the DNA methylome in metal-induced carcinogenesis. Critical Reviews in Toxicology, 2013. **43**(6): p. 493-514.
149. Byrne, C., Divekar, S.D., Storchan, G.B., Parodi, D.A., and Martin, M.B. Metals and breast cancer. J Mammary Gland Biol Neoplasia, 2013. **18**(1): p. 63-73.
150. Eick, G.N. and Thornton, J.W. Evolution of steroid receptors from an estrogen-sensitive ancestral receptor. Mol Cell Endocrinol, 2011. **334**(1-2): p. 31-8.
151. Sheehan, D.M. Activity of environmentally relevant low doses of endocrine disruptors and the bisphenol A controversy: initial results confirmed. Proc Soc Exp Biol Med, 2000. **224**(2): p. 57-60.
152. Welshons, W.V., Thayer, K.A., Judy, B.M., Taylor, J.A., Curran, E.M., and vom Saal, F.S. Large effects from small exposures. I. Mechanisms for endocrine-disrupting chemicals with estrogenic activity. Environ Health Perspect, 2003. **111**(8): p. 994-1006.
153. Hayes, T.B., Anderson, L.L., Beasley, V.R., de Solla, S.R., Iguchi, T., Ingraham, H., Kestemont, P., Kniewald, J., Kniewald, Z., Langlois, V.S., Luque, E.H., McCoy, K.A., Munoz-de-Toro, M., Oka, T., Oliveira, C.A., Orton, F., Ruby, S., Suzawa, M., Tavera-Mendoza, L.E., Trudeau, V.L., Victor-Costa, A.B., and Willingham, E. Demasculinization and feminization of male gonads by atrazine: consistent effects across vertebrate classes. J Steroid Biochem Mol Biol, 2011. **127**(1-2): p. 64-73.
154. Vandenberg, L.N., Colborn, T., Hayes, T.B., Heindel, J.J., Jacobs, D.R., Jr., Lee, D.H., Shioda, T., Soto, A.M., vom Saal, F.S., Welshons, W.V., Zoeller, R.T., and Myers, J.P. Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. Endocr Rev, 2012. **33**(3): p. 378-455.
155. Fechner, P., Damdimopoulou, P., and Gauglitz, G. Biosensors Paving the Way to Understanding the Interaction between Cadmium and the Estrogen Receptor Alpha. Plos One, 2011. **6**(8).
156. Brama, M., Gnessi, L., Basciani, S., Cerulli, N., Politi, L., Spera, G., Mariani, S., Cherubini, S., d'Abusco, A.S., Scandurra, R., and Migliaccio, S. Cadmium induces mitogenic signaling in breast cancer cell by an ERalpha-dependent mechanism. Mol Cell Endocrinol, 2007. **264**(1-2): p. 102-8.

157. Choe, S.Y., Kim, S.J., Kim, H.G., Lee, J.H., Choi, Y., Lee, H., and Kim, Y. Evaluation of estrogenicity of major heavy metals. Sci Total Environ, 2003. **312**(1-3): p. 15-21.
158. Liu, Z., Yu, X., and Shaikh, Z.A. Rapid activation of ERK1/2 and AKT in human breast cancer cells by cadmium. Toxicol Appl Pharmacol, 2008. **228**(3): p. 286-94.
159. Zang, Y., Odwin-Dacosta, S., and Yager, J.D. Effects of cadmium on estrogen receptor mediated signaling and estrogen induced DNA synthesis in T47D human breast cancer cells. Toxicol Lett, 2009. **184**(2): p. 134-8.
160. Wilson, V.S., Bobseine, K., and Gray, L.E. Development and characterization of a cell line that stably expresses an estrogen-responsive luciferase reporter for the detection of estrogen receptor agonist and antagonists. Toxicological Sciences, 2004. **81**(1): p. 69-77.
161. Siewit, C.L., Gengler, B., Vegas, E., Puckett, R., and Louie, M.C. Cadmium promotes breast cancer cell proliferation by potentiating the interaction between ERalpha and c-Jun. Mol Endocrinol., 2010. **24**(5): p. 981-92.
162. Thomas, P. and Dong, J. Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: A potential novel mechanism of endocrine disruption. Journal of Steroid Biochemistry and Molecular Biology, 2006. **102**(1-5): p. 175-179.
163. Yu, X., Filardo, E.J., and Shaikh, Z.A. The membrane estrogen receptor GPR30 mediates cadmium-induced proliferation of breast cancer cells. Toxicol Appl Pharmacol, 2010. **245**(1): p. 83-90.
164. OECDs. OECD guideline for the testing of chemicals. Test No. 40. Uterotrophic Bioassay in Rodents: A short-term screening test for oestrogenic properties, O.f.E.C.-o.a. development), Editor. 2007.
165. OECDs. Detailed Background Review of the Uterotrophic Bioassay, OECD Series on Testing and Assessment, No. 38, O.f.E.C.-o.a. Development, Editor. 2003.
166. Lemmen, J.G., Arends, R.J., van Boxtel, A.L., van der Saag, P.T., and van der Burg, B. Tissue- and time-dependent estrogen receptor activation in estrogen reporter mice. J Mol Endocrinol, 2004a. **32**(3): p. 689-701.
167. Lemmen, J.G., Arends, R.J., van der Saag, P.T., and van der Burg, B. In vivo imaging of activated estrogen receptors in utero by estrogens and bisphenol A. Environ Health Perspect, 2004b. **112**(15): p. 1544-9.
168. Penttinen, P., Jaehrling, J., Damdimopoulos, A.E., Inzunza, J., Lemmen, J.G., van der Saag, P., Pettersson, K., Gauglitz, G., Makela, S., and Pongratz, I. Diet-derived polyphenol metabolite enterolactone is a tissue-specific estrogen receptor activator. Endocrinology, 2007. **148**(10): p. 4875-86.
169. Mueller, S.O., Simon, S., Chae, K., Metzler, M., and Korach, K.S. Phytoestrogens and their human metabolites show distinct agonistic and antagonistic properties on estrogen receptor alpha (ERalpha) and ERbeta in human cells. Toxicol Sci, 2004. **80**(1): p. 14-25.
170. Penttinen-Damdimopoulou, P.E., Power, K.A., Hurmerinta, T.T., Nurmi, T., van der Saag, P.T., and Makela, S.I. Dietary sources of lignans and isoflavones modulate responses to estradiol in estrogen reporter mice. Mol Nutr Food Res, 2009. **53**(8): p. 996-1006.
171. Lidfeldt, J., Nerbrand, C., Samsioe, G., Schersten, B., and Agardh, C.D. A screening procedure detecting high-yield candidates for OGTT. The Women's Health in the Lund Area (WHILA) study: a population based study of middle-aged Swedish women. Eur J Epidemiol, 2001. **17**(10): p. 943-51.
172. Samsioe, G., Lidfeldt, J., Nerbrand, C., and Nilsson, P. The women's health in the Lund area (WHILA) study--an overview. Maturitas, 2010. **65**(1): p. 37-45.
173. Åkesson, A., Lundh, T., Vahter, M., Bjellerup, P., Lidfeldt, J., Nerbrand, C., Samsioe, G., Stromberg, U., and Skerfving, S. Tubular and glomerular kidney effects in Swedish women with low environmental cadmium exposure. Environ Health Perspect, 2005. **113**(11): p. 1627-31.
174. Livak, K.J. and Schmittgen, T.D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. Methods, 2001. **25**(4): p. 402-408.

175. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods, 1983. **65**(1-2): p. 55-63.
176. Hirano, S.s, Methods in Molecular Biology, in Nanotoxicity. 2012, Springer. p. 87-97.
177. Khatibi, A., Agardh, C.D., Shakir, Y.A., Nerbrand, C., Nyberg, P., Lidfeldt, J., and Samsioe, G. Could androgens protect middle-aged women from cardiovascular events? A population-based study of Swedish women: The Women's Health in the Lund Area (WHILA) Study. Climacteric, 2007. **10**(5): p. 386-392.
178. Shelley, J.M., Green, A., Smith, A.M., Dudley, E., Dennerstein, L., Hopper, J., and Burger, H. Relationship of endogenous sex hormones to lipids and blood pressure in mid-aged women. Ann Epidemiol, 1998. **8**(1): p. 39-45.
179. Sand, S., Victorin, K., and Filipsson, A.F. The current state of knowledge on the use of the benchmark dose concept in risk assessment. Journal of Applied Toxicology, 2008. **28**(4): p. 405-421.
180. Slob, W. Dose-response modeling of continuous endpoints. Toxicol Sci, 2002. **66**(2): p. 298-312.
181. Ahlbory-Dieker, D.L., Stride, B.D., Leder, G., Schkoldow, J., Trolenberg, S., Seidel, H., Otto, C., Sommer, A., Parker, M.G., Schutz, G., and Wintermantel, T.M. DNA Binding by Estrogen Receptor-alpha Is Essential for the Transcriptional Response to Estrogen in the Liver and the Uterus. Molecular Endocrinology, 2009. **23**(10): p. 1544-1555.
182. O'Brien, J.E., Peterson, T.J., Tong, M.H., Lee, E.J., Pfaff, L.E., Hewitt, S.C., Korach, K.S., Weiss, J., and Jameson, J.L. Estrogen-induced proliferation of uterine epithelial cells is independent of estrogen receptor alpha binding to classical estrogen response elements. Journal of Biological Chemistry, 2006. **281**(36): p. 26683-26692.
183. Silva, E., Kabil, A., and Kortenkamp, A. Cross-talk between non-genomic and genomic signalling pathways - Distinct effect profiles of environmental estrogens. Toxicology and Applied Pharmacology, 2010. **245**(2): p. 159-+.
184. Bulayeva, N.N. and Watson, C.S. Xenoestrogen-induced ERK-1 and ERK-2 activation via multiple membrane-initiated signaling pathways. Environmental Health Perspectives, 2004. **112**(15): p. 1481-1487.
185. Winuthayanon, W., Hewitta, S.C., Orvisb, G.D., Behringer, R.R., and Korach, K.S. Uterine epithelial estrogen receptor α is dispensable for proliferation but essential for complete biological and biochemical responses. Proceedings of the National Academy of Sciences, 2010. **107**(45): p. 19272–19277.
186. Watson, C.S., Jeng, Y.J., and Kochukov, M.Y. Nongenomic signaling pathways of estrogen toxicity. Toxicol Sci, 2010. **115**(1): p. 1-11.
187. Dhillon, A.S., Hagan, S., Rath, O., and Kolch, W. MAP kinase signalling pathways in cancer. Oncogene, 2007. **26**(22): p. 3279-90.
188. Ahmadibeni, Y., Hanley, M., White, M., Ayrapetov, M., Lin, X., Sun, G., and Parang, K. Metal-binding properties of a dicysteine-containing motif in protein tyrosine kinases. Chembiochem, 2007. **8**(13): p. 1592-605.
189. Schwerdtle, T., Ebert, F., Thuy, C., Richter, C., Mullenders, L.H., and Hartwig, A. Genotoxicity of soluble and particulate cadmium compounds: impact on oxidative DNA damage and nucleotide excision repair. Chem Res Toxicol, 2010. **23**(2): p. 432-42.
190. Lahav, G., Rosenfeld, N., Sigal, A., Geva-Zatorsky, N., Levine, A.J., Elowitz, M.B., and Alon, U. Dynamics of the p53-Mdm2 feedback loop in individual cells. Nat Genet, 2004. **36**(2): p. 147-50.
191. Malmlöf, M., Roudier, E., Högberg, J., and Stenius, U. MEK-ERK-mediated phosphorylation of Mdm2 at Ser-166 in hepatocytes. Mdm2 is activated in response to inhibited Akt signaling. J Biol Chem, 2007. **282**(4): p. 2288-96.
192. Pääjärvi, G., Viluksela, M., Pohjanvirta, R., Stenius, U., and Högberg, J. TCDD activates Mdm2 and attenuates the p53 response to DNA damaging agents. Carcinogenesis, 2005. **26**(1): p. 201-8.

193. Meplan, C., Mann, K., and Hainaut, P. Cadmium induces conformational modifications of wild-type p53 and suppresses p53 response to DNA damage in cultured cells. J Biol Chem, 1999. **274**(44): p. 31663-70.
194. Nagata, C., Nagao, Y., Shibuya, C., Kashiki, Y., and Shimizu, H. Urinary cadmium and serum levels of estrogens and androgens in postmenopausal Japanese women. Cancer Epidemiol Biomarkers Prev, 2005. **14**(3): p. 705-8.
195. Das, S. and Mukherjee, D. Effect of cadmium chloride on secretion of 17beta-estradiol by the ovarian follicles of common carp, Cyprinus carpio. Gen Comp Endocrinol, 2013. **181**: p. 107-14.