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GENDER DIFFERENCES IN CHEMICAL CARCINOGENESIS

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To my Family

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ABSTRACT

Gender differences in cancer incidence and mortality is a regular finding in epidemiological studies. In addition to reproductive organs this pattern is also seen in non-reproductive organs, with men being the most affected gender for the majority of cancer-sites. The underlying reasons for the observed disparity are not known, but can partly be explained by differences in exposures, lifestyle factors and biological factors such as hormones and metabolism. Exposure to carcinogenic chemicals is one of the risk factors for cancer, however little is known about gender-specific sensitivity to carcinogens. The overall aim of this thesis was to investigate gender differences in susceptibility to chemical carcinogens and the underlying mechanisms.

In the National Toxicology Program (NTP) database detailed technical reports from 2-year bioassays on male and female rats exposed to the same concentration of chemical in well-controlled environments is publicly available. In the first paper, 477 chemicals tested on rats were evaluated for possible gender differences in the carcinogenic effect. The analysis of NTP bioassays showed that male rats were more affected than females. In a total of 278 carcinogens, 201 showed statistically significant gender differences in at least one non-reproductive organ. 69 carcinogens induced male-specific tumors and 19 induced female-specific tumors. Male-specific tumors included for example mesothelioma, kidney-, skin- and pancreas tumors, while female-specific tumors included neoplasms in pituitary, bone marrow and lymphoid tissues, lung and urinary bladder. The study further showed that genotoxicity was more common among male-specific carcinogens, compared to female-specific carcinogens.

Based on the results from the NTP study eight male-specific pancreatic carcinogens were studied in more detail in the second study. To find common mechanisms that could clarify the male-specific effect of these carcinogens, the published literature on the eight chemicals was analyzed using a text-mining tool, CRAB. This analysis proposed inflammation as a common mechanism for these carcinogens. In *in vitro* studies it was found that all eight carcinogens increased the levels of the inflammatory protein Autotaxin (ATX), in parallel with increased invasiveness. Testosterone further increased ATX levels, alone and in combination with carcinogens. These data suggests that ATX may be a target for carcinogens that promote pancreatic tumor development.

In the third study, the role of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), Polychlorinated biphenyl (PCB) and estradiol on benzo(*a*)pyrene (BaP)-induced apoptosis and p53 signaling was investigated. The results showed that BaP induced apoptosis increased nuclear p53 and phosphorylation of FOXO3a. The apoptotic effect of BaP was attenuated by pretreatment of TCDD, PCB or estradiol, leading to a further increase in nuclear p53 and decreased levels of phosphorylated FOXO3a. FOXO3a dephosphorylation was further showed to be essential for the attenuated apoptosis and nuclear trapping of p53, which resulted in restoration of BaP-induced apoptosis. The data suggests an interaction between p53 and FOXO3a, which leads to an attenuated BaP-induced apoptosis in cells co-exposed to TCDD, PCB153 or estradiol. This study also reflects the effect of estradiol as a modulator of the toxic response caused by carcinogens.

In conclusion, the results of this thesis show that male rats are more sensitive to chemical carcinogens compared to female rats. The data further suggests interactions between hormones and carcinogens that could be important for the cellular response to carcinogens.

LIST OF PUBLICATIONS

I. **Kadekar S**, Peddada S, Silins I, French JE, Högberg J, Stenius U. *Gender differences in chemical carcinogenesis in National Toxicology Program 2-year bioassays. Toxicol Pathol. 2012 Dec;40(8):1160-8.*

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AMN	2-amino-5-nitrophenol
ATX	Autotaxin
BaP	Benzo(*a*)pyrene
BA	Benzyl acetate
CRAB	Cancer Risk Assessment and Biomedical text mining tool
CPD	Carcinogenic Potency Database
CTA	Cell Transformation Assays
ChlA	Chlorendic acid
ABS	Chromosome aberration
CPN	Chronic progressive nephropathy
CA	Cinnamyl anthranilate
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CYP2A6	Cytochrome P450 2A6
DBP	Dibenzo(*a*,*l*)pyrene
ENPP2	Ectonucleotide pyrophosphatase/phosphodiesterase family member
FACS	Fluorescence-activated cell sorter
FOXO3A	Forkhead Box O3a
IHOP	Information Hyperlinked over Proteins
IL-6	Interleukin-6
IARC	International Agency for Research on Cancer
LPA	Lysophosphatidic acid
MER	2-mercaptobenzothiazole
MOA	Mode Of Action
SMAD	Mothers against decapentaplegic homolog (Drosophila)
NTP	National Toxicology Program
NNK	Nicotine-derived nitrosaminoketone
NDL-PCBs	Non-dioxin-like polychlorinated biphenyls
NFKB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
PUMA	p53 upregulated modulator of apoptosis
PanIN	pancreatic-intraepithelial-neoplasia
PBDE	Polybrominated diphenyl ethers
PCB	Polychlorinated biphenyl
PAH	Polycyclic aromatic hydrocarbons
PP2A	Protein phosphatase 2 A
Rb	Retinoblastoma
ROX	Roxarsone
SAL	Salmonella mutagenesis assays
SGK	Serine/threonine-protein kinase
STT	Short Term Tests
STAT3	Signal transducer and activator of transcription 3

SIRT1	Sirtuin 1
SCE	Sister chromatid exchange
TCP	1,2,3-trichloropropane
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TDI	2,4- and 2,6-toluene diisocyanate
TSH	Thyroid stimulating hormones
TGF	Transforming growth factors
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor

1. INTRODUCTION

Cancer is a group of diseases which are characterized by uncontrolled growth and spread of aberrant cells. Cancer is caused by a variety of factors that include inherited mutations, immune conditions, infections and chemicals, to name a few. These factors can act together or in a sequential manner in the development of cancer. Most of the cancer types are known to develop in people of age 55 years and above (American Cancer Society, 2012). Cancer statistics show that the life time risk of cancer for women is 30% and for men it is 50% (Hsu, 2010). The 5-year overall survival rate is currently increasing and is at around 67% for all cancer types combined in e.g. USA (American Cancer Society, 2012) .

2. HALLMARKS OF CANCER

The process of tumor growth and its metastatic spread involves several essential alterations, and these alterations have been structured into 10 "hallmarks of cancer" by Hanahan and Weinberg (Hanahan and Weinberg, 2000, Hanahan and Weinberg, 2011). These changes include: 1) Sustaining proliferative signaling (e.g. cancer cells produce growth factor receptor ligands on their own and respond through the expression of receptors, resulting in an autocrine proliferative signaling). 2) Evading growth suppressors, (e.g. cancer cells inactivate tumor suppressors like Rb and p53, which leads to circumvention of programs that regulate cell proliferation negatively). 3) Resisting cell death, (e.g. cancer cells attenuate apoptosis by e.g. loss of p53 function). 4) Enabling replicative immortality, (as cancer cells maintain immortality through telomerase activity). 5) Inducing angiogenesis, (e.g. cancer cells initiate neovasculature which is necessary for their sustenance by VEGF). 6) Activating invasion and metastasis, (e.g. epithelial cancer cells lose their attachment to the basement membrane by the loss of E-cadherin (Okada et al., 2005) and cancer cells undergo epithelial-mesenchymal transition to invade other tissues) (Hanahan and Weinberg, 2000). Four additional emerging hallmarks include avoiding immune destruction, deregulating cellular energetics, genome instability and tumor promoting inflammation (Hanahan and Weinberg, 2011). Genome instability involves deficiencies in genome repair and maintenance resulting in mutations which are necessary for tumor progression. Tumor promoting inflammation is an enabling character due to its ability to acquire other hallmark capabilities (Hanahan and Weinberg, 2011). Macrophages are known to play a role in tumor initiation, progression and metastasis (Qian and Pollard, 2010). Chronic

inflammation has been found to increase the risk of cancer development (de Visser et al., 2006, Grivennikov et al., 2010).

3. CHEMICAL CARCINOGENESIS

The first known reported description of a chemical compound acting as a carcinogen was in 1567 by Paracelsus. He described the wasting disease of miners and proposed that it was caused by exposure to arsenic sulphide (Hohenheim, 1567) – later the disease of the miners was shown to be lung cancer. In 1761 it was shown through the observation of John Hill that nasal cancer was more common in people using snuff compared to the general population (Hill, 1761). One of the first experimental works to identify the role of chemicals in carcinogenesis was done in 1915 by Katsusaburo Yamagiwa and Koichi Ichikawa. They applied coal tar to ears of rabbits, which resulted in epithelial tumors (Yamagiwa K, 1918). The first experimental evidence to show a single chemical as a carcinogen was done by Dr. Heiger et al. in 1933. They showed evidence of Benzo(*a*)pyrene (BaP) in coal tar as an active ingredient in causing tumors (Cook et al., 1933). Others groups working around the same time showed that 2-naphthylamine and 2-acetylaminofluorene could cause tumors in dogs and rats (Hueper, 1938, Wilson et al., 1941), which has been confirmed in many studies since. Later studies have shed light on the mechanism of action of several carcinogens (Verma et al., 2012, Weiss et al., 2007).

Carcinogenesis is the process which leads to the formation of tumors. Based on findings in animal models carcinogenesis has been divided into three operational steps: initiation, promotion and progression. The initiation step was early recognized to involve a change in the genome of the cell induced by a variety of stimuli including physical, chemical and biological factors (e.g. chemicals such as BaP or UV radiation) (Weisburger et al., 1983). The initiation stage is followed by promotion, which results in the clonal expansion of initiated cells. The final stage of progression is characterized by genomic instability along with physical changes in the cell. Many carcinogens are known to act as initiators or as promoters of carcinogenesis (Boyland, 1985, Fujiki et al., 2013). Other carcinogens are complete carcinogens and function both as initiator and promoter (Weston A, 2000).

3.1 MODE OF ACTION

The term *"mode of action"* (MOA) is defined as a sequence of key events and processes leading to cancer development. It is defined by the U.S. Environmental Protection Agency (EPA) as the "sequence of key events and processes starting with interaction of an agent with a cell, proceeding through operational and anatomical changes, and resulting in cancer formation" (EPA, 2005). Carcinogens act through different MOA and they are commonly broadly classified into two different MOA categories: genotoxic and nongenotoxic (Williams, 2008, Melnick et al., 1996). Classifying carcinogens based on their MOA can support cancer risk assessment in several ways (Dellarco and Baetcke, 2005).

3.1.1 Genotoxic mode of action

A genotoxic MOA means that the carcinogen affects the DNA directly and damages it. For example, a genotoxic MOA may involve the binding of the carcinogen covalently to the DNA (Weisburger and Williams, 1983) causing DNA adducts, which eventually may lead to mutations. Other examples of DNA damage include chromosomal aberrations and breaks (Luch, 2005). Mutagenicity specifically involves induction of mutations at a chromosome or at a gene level and mutations are persistent, inheritable changes. Not all genotoxic effects result in mutations as cellular repair mechanisms are efficient (Christmann and Kaina, 2013). Some carcinogenic chemicals are reactive and may bind DNA directly. Other carcinogenic chemicals must be metabolized by cellular enzymes to form electrophilic products that has the capacity to bind to DNA (Gonzalez et al., 1998).

3.1.2 Non-genotoxic mode of action

Chemicals acting by a non-genotoxic or indirect genotoxic MOA do not bind to or affect cellular DNA directly. Carcinogens working through a non-genotoxic MOA induce effects such as oxidative stress, inflammation, chronic cell injury, immunosuppression and stimulation of cell proliferation (Silva Lima and Van der Laan, 2000, Benigni et al., 2013). Epigenetic effects may include changes in the methylation status of DNA and proteins without affecting the DNA sequence. Indirect MOA includes changes in cellular processes like basic transport processes, metabolic handling, aryl hydrocarbon receptor activation, gap junction inhibition and TNF alpha pathway activation (Hattis et al., 2009). Mitogenic effects could be followed by

spontaneous genetic changes due to errors in DNA replication or DNA damage by electrophiles generated during cellular metabolism (Gonzalez et al., 1998). The knowledge of different mechanisms of non-genotoxic carcinogens are less established compared to the mechanisms of genotoxic carcinogens e.g. the definition of non-genotoxicity as described in the scientific literature may vary (Melnick et al., 1996).

3.2 GENDER DIFFERENCES IN CANCER STATISTICS

Epidemiological studies have shown that cancer incidences are higher in men than women (Edgren et al., 2012, Jemal et al., 2009). Part of the reason for this disparity could be due to differences in occupational exposure of chemicals, as well as physiological differences, including hormones and metabolism. An evaluation of IARC's international cancer statistics has shown that men have higher cancer incidences than women (Ferlay et al., 2013). Out of 35 cancer sites, 32 sites showed higher cancer incidences in men compared with women. The sites with the highest cancer incidence in men were larynx and hypopharynx. The three sites which had a higher cancer incidence in women were thyroid, gall bladder and anus (Edgren et al., 2012). Another epidemiological study conducted in the US population suggested that men have 1.8 times higher cancer incidence compared to women. Some of the cancer types with higher cancer incidence in men were Kaposi sarcoma, mesothelioma, cancer of lip, larynx, hypopharynx, urinary bladder, esophagus, tonsil, oropharynx, and other urinary organs. The organs with higher cancer incidence in women were breast, thyroid, gallbladder and anus (Cook et al., 2009). One study shows that even in childhood boys are at a higher risk of developing cancer, the male to female ratio for all types of childhood cancer was 1.2 (Dorak and Karpuzoglu, 2012). Cancer statistics in the US also shows higher cancer incidence and mortality in men for almost all types of cancer and furthermore, the probability of being diagnosed with cancer is also higher in men (Uppstad et al., 2011, Harris et al., 1987). However, trends in some cancer types have shown changes recently, e.g. lung cancer incidences are increasing in non-smoking women, although women still tend to have a higher survival rate compared to men (Serke et al., 2013).

Several reasons have been suggested for the observed gender differences in cancer incidence, e.g. the role of genetic and epigenetic effects (Dorak and Karpuzoglu, 2012). Another reason is the ability of women to mount a stronger immune response (Lleo et al., 2008). Hormones could also play a role (Dorak and Karpuzoglu, 2012). However, some studies discuss that differences

in hormones, the immune response and genetic factors alone cannot explain the observed gender disparity. One hypothesis is that gender-specific biological factors could be involved, which can modify the susceptibility to carcinogens (Edgren et al., 2012).

In *in vitro* studies using human cell lines some carcinogens have shown differences in affecting cancer-related endpoints. For example, BaP has been shown to induce higher levels of DNA adducts in female human lung cancer cells compared to cells of male origin, which could be a result of higher expression of CYP1A1 in the cells of female origin (Uppstad et al., 2011). Differences in the metabolism between individuals, including males and females could be due to both genetic and environmental factors (Wogan et al., 2004).

3.3 CHEMICAL RISK FACTORS

Around 200 - 300 new chemicals are introduced yearly in Europe alone (Hartung and Daston, 2009) and around 80 000 chemicals are in use in US (NTPb, 2012). Humans are exposed to chemicals in their daily life, through their occupation and lifestyle (Harris et al., 1987). Epidemiological studies have estimated that around 30% to 40% of all cancer cases are primarily due to environmental factors, which include carcinogens (Doll and Peto, 1981, Danaei et al., 2005, Loeb et al., 1984). However, the exact percentage of cancer cases caused by environmental factors is difficult to estimate. The President's Cancer Panel concluded that the actual number of cancers caused by environmental factors have been grossly underestimated (PCP, 2009).

The International Agency for Research on Cancer (IARC) has classified approximately 110 environmental factors or agents, which include chemicals, complex mixtures, physical and biological agents, as carcinogenic to humans (group1) (IARC, 2013).The classification is based on available scientific literature, including epidemiological studies, animal studies, animal testing, cell studies, short term tests etc. Environmental factors are selected for review based on the evidence of human exposure and suspicion of carcinogenicity. They are classified into different groups based on the strength of evidence. The different groups are group 1 (carcinogenic to humans); group 2A (probably carcinogenic to humans); group 2B (possibly carcinogenic to humans); group 3 (not classifiable as to its carcinogenicity to humans); group 4 (the agent is probably not carcinogenic to humans) (IARC, 2006).

3.3.1 CANCER RISK ASSESSMENT AND BIOMEDICAL TEXT MINING

The risk assessment procedure is commonly described by steps such as: hazard identification, dose-response assessment, exposure assessment and risk characterization (Hsu, 2010). The risk assessment process of a suspected chemical carcinogen thus involves assessment of its carcinogenic hazard to humans *per se*, and at what exposure levels these effects occurred. By combining the hazard information and human exposure conditions the risk could be assessed (EPA, 2005).

An early step in risk assessment is to evaluate all scientific evidence of carcinogenicity for the chemical of interest. This is commonly done manually by searching and analyzing published biomedical literature (Korhonen et al., 2009). However, biomedical research has been increasing exponentially the past 10 years (Moses et al., 2005) and resulted in vast amount of biomedical literature. For example, PubMed, the major database for biomedical literature, has more than 2,8 million publications related to cancer out of 23 million currently present citations (PubMed, 2013). This large amount of publications makes finding relevant literature for risk assessment highly time-consuming if done manually. Text mining is a computer aided process which is very useful in obtaining relevant data from the available extensive amount of text and could support researchers to make literature searches easier. Computational techniques like machine learning, natural language processing and knowledge discovery are used by current text mining tools to unearth relevant information in the biomedical literature (Zhu et al., 2013).

3.3.1.1 THE CRAB AND OTHER TEXT-MINING TOOLS

One example of a text mining tool is CRAB; a text mining-based tool developed to support the process of literature review and knowledge discovery in cancer risk assessment (Korhonen et al., 2012).

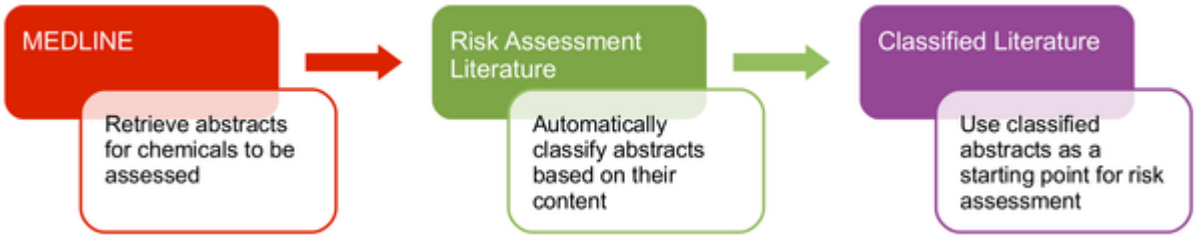

Figure 1. An overview of CRAB text mining tool (Korhonen et al., 2012).

CRAB can be used to analyze scientific abstracts from PubMed on chemicals of interest and to classify the abstracts according to an extensive taxonomy using supervised machine learning technology (figure 1). The taxonomy has two main parts, the first part regards the scientific evidence for carcinogenic activity (figure 2) and the second part concerns carcinogenic mode of action (figure 3). Based on the literature, the CRAB tool displays a publication profile for a chemical or a group of chemicals. The publication profile can also be used for hypothesis generation that deserves experimental studies (Korhonen et al., 2012).

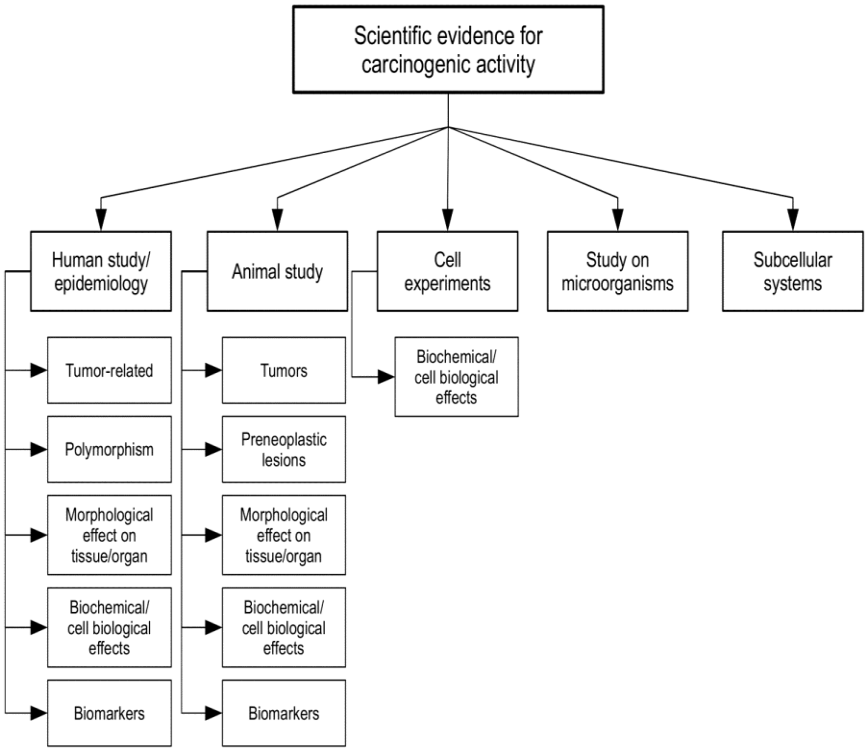

Figure 2. The "***Scientific Evidence for Carcinogenic Activity***" taxonomy (Korhonen et al., 2012).

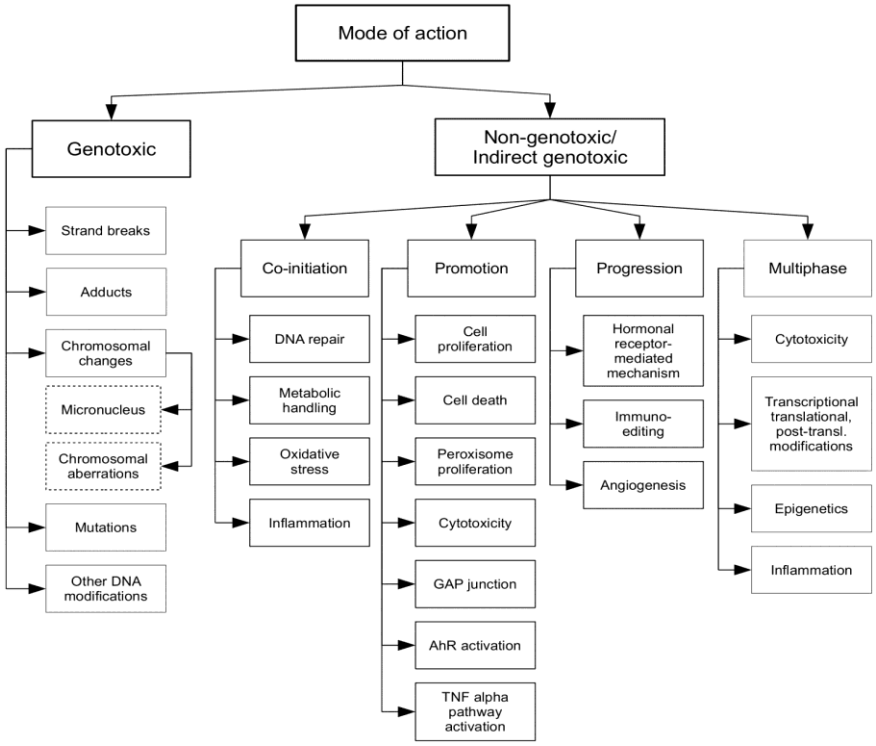

Figure 3. The **"*Mode of Action*"** taxonomy (Korhonen et al., 2012).

There are a number of other text mining tools available. Chilibot (Chilibot, 2007) can be used for finding relevant relationships between proteins or genes. Information Hyperlinked over Proteins (iHOP) can be used to form a web of relevant genes and proteins from PubMed abstracts (Hoffmann and Valencia, 2004). Medscan is another tool which could be applied to biomedical literature for clinical information and analysis of cellular signaling pathways (Novichkova et al., 2003).

3.4 ANIMAL MODELS

One of the ways to evaluate the carcinogenicity of a chemical is to test it in an animal model (Corpet and Pierre, 2005). The rodent bioassay seems to be the ideal model to study chemical carcinogenesis as it roughly predicts similar effects in humans. Models in rodents could be used to screen chemicals for carcinogenic effects but also to study more detailed mechanisms of the carcinogenic process (Corpet and Pierre, 2005, Roe, 1998). Mice and rats are the two most common animals used in cancer bioassays, where rats may serve as a better model compared to

other animals as rats develop more epithelial tumors, the most common tumor type of tumors in humans (Anisimov et al., 2005). Rodents often develop multiple neoplastic sites in response to carcinogens and sometimes in rodent-specific tissues, like the zymbal gland, which cannot be directly compared with humans. Despite some differences, the human anatomy is quite similar to the rodent anatomy (Maronpot et al., 2004). The overall cancer risk is similar in both rodents and humans at a time point after the female reproductive period (Anisimov et al., 2005). Animal models are particularly useful because suspected chemicals can be tested in a controlled environment. All known human carcinogens are carcinogenic to rodents and one third of human carcinogens were identified in rodents first (Maronpot et al., 2004). Most of the major human cancer types have been reproduced in animals through induction by carcinogens (Saffiotti, 1980). Rodent models have been criticized for reasons such as the use of high doses in cancer bioassays (Ames and Gold, 2000) and too long or too short time exposure of chemicals (Davies et al., 2000, Blakey et al., 1985). Although not all animal carcinogens are proven human carcinogens, results from testing in animals may support risk assessments and also provide means to study molecular mechanisms that could be similar in the development of human cancer (Huff and Haseman, 1991, Hoenerhoff et al., 2009).

3.4.1 NATIONAL TOXICOLOGY PROGRAM (NTP)

A number of databases are available where information regarding test results from cancer bioassays can be found. The NTP is an interagency program in the US, which is designed to test and grade agents of human health concerns by applying methods of toxicology and molecular biology (figure 4) (NTPb, 2012). From 1976 to 2012 NTP has tested 579 chemicals for their carcinogenic potential.

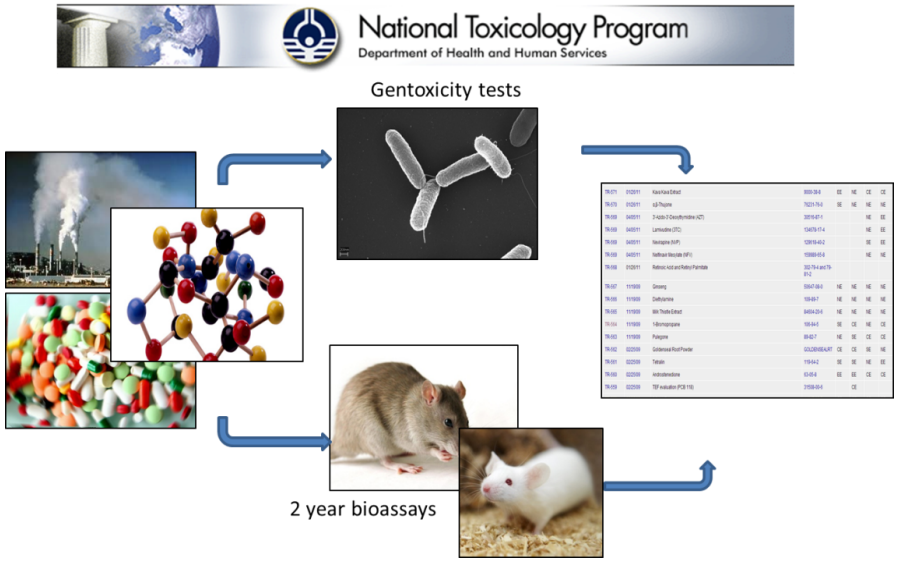

Figure 4. Schematic view of NTP testing strategies (NTPa, 2012).

In addition to bioassays in rats and mice, chemicals are also tested for their genotoxic potential in different animal cells lines and in bacteria. The agents tested by NTP are mainly chemicals, but the effects of other agents such as magnetic fields have also been investigated (NTP, 1999). Chemicals are selected for testing based on the extent of human exposure, on suspicion of toxicity (NTP, 1993) or because of toxicological data gaps. Chemicals are usually tested on rats and mice for 2 years. Short-time toxicity tests, like 14-day studies, are conducted to identify potential target organs and for setting doses for the 13-weeks toxicity tests. The 13-weeks toxicity tests are conducted to pre-determine doses for the 2-year bioassays and to study the induction of micronuclei. The route of exposure selected should be as similar as possible to the human exposure situation and may include inhalation, exposure through drinking water and dermal application. Other route of exposure includes gavage and intraperitoneal injections. A complete necropsy is performed on all treated and control animals that complete the 104-week exposure. Necropsies are also conducted on animals which die before two years. All the tissues, including muscles and rat-specific organs like the zymbal and harderian gland, are required for the complete histopathology. Organs are embedded, sectioned and stained with hematoxylin and eosin for histopathologic evaluation (NTP, 2010). For each chemical tested a detailed technical report is compiled and published in the NTP database. These reports include detailed information about the tests, test results and NTP's conclusions regarding the carcinogenicity of the chemical. Each chemical is given

an evidence level for each sex and for each species. Four carcinogenic evidence levels are used; clear evidence (CE), some evidence (SE), equivocal evidence (EE) and no evidence (NE). These evidence levels refer to the strength of the experimental evidence and not to the potency of the chemical. Clear evidence is given to chemicals which cause dose-related increases in a combination of benign and malignant neoplasms or dose-related increases of malignant or benign neoplasms. Some evidence is given to chemicals which cause an increase in benign and malignant neoplasms, but the response is lower than that of clear evidence. Equivocal evidence is given to chemicals which show a marginal increase in neoplasms. No evidence is given to chemicals which do not show any neoplasms (NTPb, 2013). Non-neoplastic effects are also described in the reports. All data from the 2-year bioassays are available in the NTP database (NTPa, 2013).

The carcinogenic potency database (CPD) is another database which has information on tested chemicals (Carcinogenic Potency Database, 2012).This database holds the information regarding approximately 1500 chemicals and their carcinogenic potential and includes information from tests conducted by the NTP.

3.4.2 CHEMICAL CARCINOGENESIS IN ANIMAL AND GENDER DIFFERENCES

Many carcinogens have shown gender differences in causing tumors in animals. For example, ethenzamide, an antipyretic analgesic has been shown to induce liver tumors in both male and female mice. It causes dose-dependent increases of liver tumors in male mice, an effect not seen in female mice (Naito et al., 1986). Another example is neonatal exposure of 4-aminobiphenyl, which causes liver tumors in males but not in female mice (Wang et al., 2012). Carcinogens ability to cause different responses in male and female animals has often been referred to differences in hormones between sexes. For example, azaserine has been shown to induce more pancreatic tumors in male rats than in female rats. In addition, castrated male and female rats had higher amount of atypical acinar cell nodules when treated with testosterone (Lhoste et al., 1987). Another example, the chemical 4-nitroquinoline 1-oxide, has been shown to induce tongue carcinomas in male and female rats, a response which was enhanced in both male and female rats treated with testosterone (Haque et al., 2007). Diethylnitrosamine-induced hepatocellular carcinomas in mice are heavily dependent on interleukin-6 (IL-6). Exposure to

diethylnitrosamine induced higher levels of IL-6 in male mice compared to female mice and consequently caused more hepatocellular carcinomas in male mice. This study further showed that estrogen reduced the levels of IL-6 induced by diethylnitrosamine, thereby suggesting a hormonal influence on the observed gender difference (Naugler et al., 2007). Azoxymethane, a colon carcinogen in male and female rats also induced kidney tumors in female rats only. The influence of hormones was considered important for the development of the female-specific renal tumors, as estrogen receptors were found in kidney tumor cells in female rats but not in normal kidney cells (Kobaek-Larsen et al., 2004).

3.5 *IN VITRO* SYSTEMS

In vitro cell systems can be used to study endpoints related to the carcinogenic process induced by carcinogens. Such analyses include e.g. assays for genotoxicity, cell transformation, cell cycle effects and apoptosis (Breheny et al., 2011). *In vitro* genotoxicity assays or short term tests (STT) are conducted to identify genotoxic chemicals. The four most widely used STT are the Ames Salmonella (SAL) mutagenesis assay, chromosome aberration (ABS) and sister chromatid exchange (SCE) induction tests and mutation tests in different cell lines (Tennant et al., 1987, Zeiger, 2010). In addition to study the effects of single chemicals STTs have also been useful in the identification of mutagenicity of complex mixtures (Tennant et al., 1987). *In vivo* genotoxicity assays are also available such as the mammalian erythrocyte micronucleus test in rodents. However, this assay is known to be insensitive and to give results contradicting those obtained in salmonella tests (Benigni et al., 2010). Comet assay is conducted to study DNA damage both *ex vivo* and *in vitro* (Zeiger, 2010). Cell transformation assays (CTA) are developed to study the carcinogenic potential of a chemical. Other *in vitro* systems to detect endpoints related to carcinogenicity include the angiogenesis assays, where potential carcinogens are studied to observe whether they induce cellular processes involved in angiogenesis, in cell invasion and in migration. For example, nicotine has been found to promote tumor cell migration *in vitro* in human lung cancer cell lines (Zhang et al., 2007).

3.6 CANCER TYPES DISPLAYING GENDER DIFFERENCES

As mentioned previously regarding cancer statistics (3.2), there are gender disparities in cancer incidences (Jemal et al., 2009, Edgren et al., 2012). Prostate cancer, which is the most common cancer type in men, and breast cancer, which is the most common cancer type in women are

obvious examples. Other cancer types which show gender differences in incidences and mortality rates are kidney, urinary bladder, skin, lung, thyroid, pancreatic cancer, mesothelioma and lymphoma (Jemal et al., 2009).

Lung cancer incidences are higher in men than women (Jemal et al., 2009). However, regarding lung cancer caused by tobacco smoke, one study suggests that women who smoke are more at risk for developing lung cancer compared to smoking men (Powell et al., 2013). One reason for the higher incidence of women who smoke contracting lung cancer could be due to higher expression of CYP2A6 in women, because higher CYP2A6 activity could lead to increased metabolism of nicotine-derived carcinogens (Benowitz et al., 2006).

Malignant mesothelioma is a form of cancer which is highly linked to asbestos exposure. The incidence rates of mesothelioma are much higher in men compared to women, and a recent study showed 4 times higher rates in men than in women (Robinson, 2012). A higher exposure of asbestos in men, due to occupational exposure, could explain the difference (Frost, 2013, Skammeritz et al., 2011). However, in studies on asbestos-exposed men and women the incidence of malignant mesothelioma is still shown to be higher in men than women (Frost, 2013). Incidences of thyroid cancer are higher in women compared to men (Jemal et al., 2009). The causative agents for thyroid cancer are still not completely known, however, radiation, thyroid stimulating hormones (TSH) and environmental carcinogens are thought to affect the development of thyroid cancer in humans (Pellegriti et al., 2013). Animal studies have shown that polyhalogenated aromatic hydrocarbons, especially polybrominated diphenyl ethers (PBDEs), can cause thyroid cancer by affecting the thyroid function (Zhang et al., 2008).

3.6.1 PANCREATIC CANCER

Pancreatic cancer has a high mortality rate compared to the incidence rate; the 5-year survival rate is less than 5% (Jemal et al., 2009). It is an especially aggressive tumor type which shows early metastasis (Stelow et al., 2010). Several studies have shown that the incidence of pancreatic cancer is higher in men than in women (Zhou et al., 2010, Lau et al., 2010, Levi et al., 2003). Recent studies indicate that high alcohol intake and red meat consumption are risk factors for pancreatic cancer among men only (Larsson and Wolk, 2012, Stolzenberg-Solomon et al., 2007, Michaud et al., 2010). Hereditary pancreatic cancer accounts for 10% of pancreatic cancer incidences and tobacco smoke presents a strong risk factor for men under the age of 50

with a hereditary pancreatic cancer background (Rulyak et al., 2003). Risk factors for pancreatic cancer also include diabetes and chronic inflammation of pancreas (pancreatitis) (Lowenfels and Maisonneuve, 2006, Koorstra et al., 2008) and people with pancreatitis are 17 times more susceptible to pancreatic cancer than people without (Farrow and Evers, 2002). Exposure to environmental factors is one of the reasons for chronic inflammation in humans, which can also lead to tumor formation. Important proteins involved in the development of pancreatic cancer through pancreatitis are NFkB and STAT3 (Surh et al., 2010), which are also activated in other cancer types (Aggarwal et al., 2009).

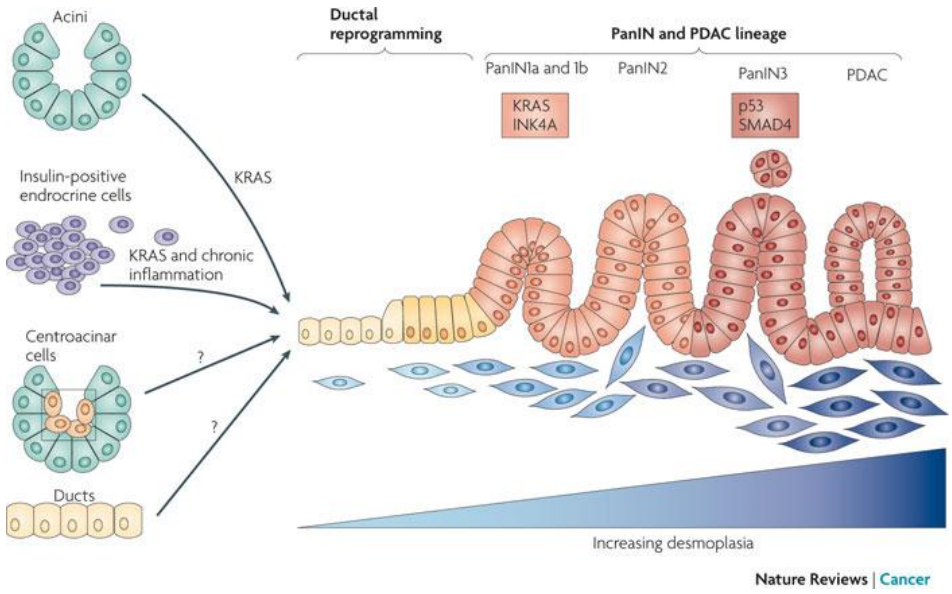

Figure 5. Schematic overview of the development of pancreatic ductal adenocarcinoma (Morris et al., 2010).

As shown in figure 5, one of the most common mutations is the KRAS mutation, which is found in more than 95% of pancreatic tumors (Morris et al., 2010). Other prominent mutations in pancreatic cancer are TP53, CDKN2A (INK4A) and SMAD4. These mutations are responsible for the formation of pancreatic-intraepithelial-neoplasia (PanIN), which is the initial stage in the development of pancreatic tumors. Pancreatic cancer progresses through the

formation of PanIN1A to PanIN3 (Gungor et al., 2013). One of the inflammatory proteins which are highly expressed in pancreatic cancer is the lysophospholipase autotaxin (ATX) (figure 6) (Komachi et al., 2009). ATX regulates the levels of lysophosphatidic acid (LPA).

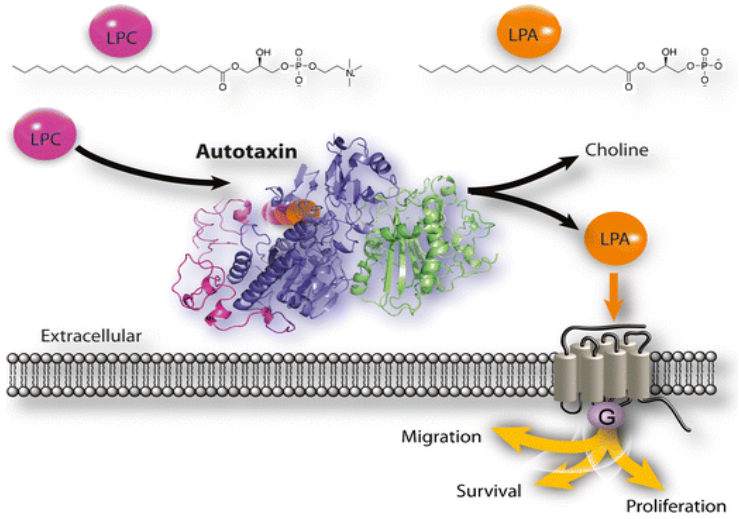

Figure 6. A simplified scheme of ATX functions (Houben and Moolenaar, 2011).

LPA is a phospholipid affecting cells through the activation of LPA receptors (Choi et al., 2010), and the ATX-LPA axis is involved in cellular processes like cell motility and stimulation of cell proliferation, prevention of apoptosis, cell migration, cytokine and chemokine secretion (Nakanaga et al., 2010). Other effects ascribed to the ATX-LPA axis are blood vessel formation, lymphocyte homing and neuropathic pain (Okudaira et al., 2010). The ATX-LPA axis has been suggested to be involved in pancreatic cancer development by stimulating migration of pancreatic cancer cells (Komachi et al., 2009) and also in the progression of other cancer types (Liu et al., 2009, Yu et al., 2008). Levels of ATX have been shown to be increased in serum of patients with pancreatic cancer, compared to patients with cancer in esophagus, stomach and colorectum (Nakai et al., 2011). ATX initiates tumor cell invasion through Matrix metallo protease 9 (MMP9), which is functional in the extracellular matrix (Mroczko et al., 2009). MMP9 has been shown to be involved in cancer metastasis and in the progression of pancreatic tumors (French et al., 1994), thus its expression is associated with pancreatic tumor metastasis (Mroczko et al., 2009).

Several chemicals have been shown to induce pancreatic tumors in rats, and azaserine is one example (Rao, 1987). Animal studies have indicated that hormones could play a role in

chemically-induced pancreatic cancer. For example, testosterone treatment increased the number of azaserine-induced pancreatic tumors in both male and female rats, while addition of estradiol decreased the number of tumors (Lhoste et al., 1987, Sumi et al., 1989). It should however be mentioned that rat pancreatic exocrine tumors exhibit an acinar phenotype, whereas human pancreatic cancer commonly exhibit a ductal phenotype, so the relevance of the rat model has been questioned (Maronpot et al., 2004, Sistare et al., 2011).

3.7 PAH AND DIOXINS

Polycyclic aromatic hydrocarbons (PAH) and dioxins are common persistent environmental pollutants which have carcinogenic capabilities (Mastrangelo et al., 1996, Mandal, 2005). BaP is one of the most carcinogenic and most well-studied of all PAHs (Boysen and Hecht, 2003). The mechanism by which BaP causes tumors is through the formation of DNA adducts and induction of mutations (Boysen and Hecht, 2003), which leads to transformation of normal cells to carcinogenic cells. BaP is known to affect p53 by inducing mutations in the gene rendering p53 inactive, subsequently leading to tumor development through proliferation of DNA damaged cells in mice (Ruggeri et al., 1993). In addition to inducing mutations, BaP is also known to induce apoptosis in various human and animal cell lines (Kim et al., 2007, Chen et al., 2003) through p53 (Xiao and Singh, 2007, Huc et al., 2006).

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is another ubiquitous environmental carcinogen. It is the most well-studied dioxin. TCDD is a very powerful tumor promoter and is also known to act as an endocrine disruptor by binding to the Aryl hydrocarbon receptor and triggering of downstream signaling pathways (Birnbaum, 1994). One possible carcinogenic mechanisms of TCDD is to induce mitochondrial dysfunction. This results in increased intracellular calcium levels and activation of NFkB, an inflammatory response protein. This has been shown to further result in resistance to apoptosis and an increased invasiveness in a non-invasive mouse cell line (Biswas et al., 2008). Other studies show anti-apoptotic effects of TCDD (Park and Matsumura, 2006, Davis et al., 2001). TCDD is also known to downregulate p53 levels through Mdm2 in HepG2 cells and to decrease the cell's ability to handle genotoxic agents (Paajarvi et al., 2005).

3.7.1 FOXO GENES

The human Forkhead Box (FOX) genes codes for transcription factors. There are subfamilies ranging from A to P (Katoh, 2004). The FOXO subfamily of transcription factors is involved in different cellular processes like proliferation, apoptosis, and differentiation binding to SMAD3 and SMAD4 transcription factors in a TGF beta-dependent manner (van der Vos and Coffer, 2008). Some chemicals are known to regulate FOXO3a. For example, the nicotine-derived nitrosaminoketone (NNK), a human lung carcinogen, has been shown to inactivate FOXO3a (Blake et al., 2010). Furthermore, non-dioxin-like polychlorinated biphenyls (NDL-PCBs) and PAHs are known to influence the phosphorylation status of FOXO3a (Al-Anati et al., 2010). Hormones like estrogen has also shown to affect FOXO3a by up-regulation of FOXO3a expression in prostate cancer cell lines (Dey et al., 2013). FOXOs have been suggested to act as tumor suppressors as they have been found inactivated in several cancer types. FOXO and p53 show similarities in that post-translational modifications like phosphorylation, acetylation and ubiquitination regulate their transcriptional activity (Zhang et al., 2011b). A study showed that activation of p53 leads to inactivation of FOXO3a by phosphorylation through the serine/threonine-protein kinase SGK (You et al., 2004). Unphosphorylated FOXO3a is known to push the cells towards apoptosis through the proapoptotic protein Bim and the cell cycle inhibitor p27kip. This is prevented by phosphorylation of FOXO3a by Akt (Dijkers et al., 2002). Oxidative stress is known to cause deacetylation of FOXO3a by the deacetylase SIRT1, which causes attenuation of FOXO3a-dependent apoptosis. Oxidative stress also leads to interaction between p53 and FOXO3a (Brunet et al., 2004). Overall, p53 and FOXO3a together protect cells from stress conditions by functionally interacting with each other (Zhang et al., 2011a). The phosphorylation status of FOXO3a regulates the localization of FOXO3a and the nuclear to cytoplasmic shuttling of phosphorylated FOXO3a is conducted by 14-3-3, an adapter protein (Tzivion et al., 2011), Dephosphorylation of FOXO3a is carried out by the phosphatase PP2A and results in nuclear localization of FOXO3a (Singh et al., 2010, Liu et al., 2012).

4. THE PRESENT STUDY

4.1 Aims of the present study

The overall aim of the thesis was to investigate gender differences in chemical carcinogenesis and to identify the molecular mechanisms that might explain such gender differences.

The specific aims were as follows:

- To study gender differences in the susceptibility to chemical carcinogens in rats using results from 2-year bioassays from the National Toxicology Program.
- To investigate the cellular mechanisms of eight pancreatic carcinogens identified as male-specific in rat bioassays.
- To study the *in vitro* mechanisms of apoptosis upon exposure to a combination of carcinogens and estradiol.

5. MATERIALS AND METHODS

The National Toxicology Program (NTP) database

477 2-year bioassays conducted by NTP in male and female rats were included in the analysis in paper 1. Assays conducted in only one gender and inadequate experiments were excluded. In eighty-six percent of the assays Fisher 344 rats were used. Other rat breeds used were Sprague-Dawley and Wistar. Tumors in male and female reproductive organs like penis, testis, epididymis, vas deferens, seminal vesicles, ejaculatory ducts, prostate and bulbourethral glands, vulva, clitoral glands, clitoris, vagina, uterus, ovary, fallopian tubes, and mammary glands were not included in the analysis. All other tumor types along with the number of rats with tumors were compared statistically between male and female rats. An overall analysis was performed based on the evidence levels of the chemicals regardless of the organs they affected, which also included reproductive organs. In addition, standard analysis of variance (ANOVA) was conducted on the results from the chemical bioassays. Male and female tumor-bearing rats were compared initially with their respective control (i.e. male tumor-bearing animals with male controls) and then compared with same-type tumor bearing rats of the opposite gender.

Cancer Risk Assessment and Biomedical (CRAB) tool analysis

In paper 2, we used the CRAB tool (Korhonen et al., 2012, Korhonen et al., 2009) to analyze the literature in PubMed for eight rat male specific carcinogens. The tool classifies literature abstracts according to a taxonomy for carcinogenic "mode of actions" (MOA). The MOA taxonomy covers the current understanding of processes leading to carcinogenesis. The tool automatically analyzes PubMed abstracts according to the evidence they provide for the different MOAs. By comparing the publication profiles of different chemicals, shared properties of apparently unrelated substances can be identified. Patterns in data can be identified by comparing publication profiles of different chemicals or groups of chemicals. Such patterns can also support hypothesis generation for further studies to identify potential MOAs of chemicals. The tool classifies PubMed literature into two distinct MOAs; genotoxic and non-genotoxic. These two MOAs are further divided into different sub-categories. For example, the sub-categories for the genotoxic MOA part of the taxonomy include strand breaks, adducts, chromosomal changes and mutations, while the non-genotoxic MOA part includes categories such as DNA repair, oxidative stress, inflammation, cell proliferation and cell death amongst

others. The tool assigns abstracts to one or more MOA classes and generates a publication profile for a single chemical or a group of chemicals. The results are displayed as percent (or mean percent) of the total number of MOA abstracts. Thus, the tool shows the distribution of PubMed abstracts according to the MOA taxonomy. The eight carcinogens analyzed in paper 2 were 1,2,3-trichloropropane, 2-amino-5-nitrophenol, 2-mercaptobenzothiazole, benzyl acetate, chlorendic acid, cinnamyl anthranilate, roxarsone, 2,4-and 2,6-toluene diisocyanate. The distribution pattern of abstracts of the eight pancreatic carcinogens was compared with the distribution pattern of six well-known genotoxic and non-genotoxic carcinogens.

Cell cultures

Three different human pancreatic cancer cell lines were used in paper 2; Panc-1, Miapaca-2 and Capan-2. All the three cell lines are derived from male subjects and are KRAS mutants. Panc-1 and Miapaca-2 cell lines are p53 mutant, while capan-2 has wild-type p53. Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin-streptomycin (0.1% serum for 48 hours for starvation) was used to culture the cell lines. Human hepatocellular carcinoma HepG2 cell lines were used in paper 3. The cells were grown in minimal essential medium with Earle's salts and L-glutamine and supplemented with sodium pyruvate (1 mM), non-essential amino acids, (100 IU/100 mg/ml) penicillin/streptomycin, and 10% inactivated fetal bovine serum.

Intracellular Ca^{2+}measurements

Calcium measurements were conducted using Panc-1 cells exposed to eight different pancreatic carcinogens. Cells were incubated with 5 µM Fura-2AM for 30 minutes at 37°C. Fura-2AM bound to free calcium was measured at 340 nm. Cells were suspended in Krebs–Ringer buffer containing 125 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5 mM $NaHCO_3$, 25 mM HEPES, 6 mM glucose, and 2.5 mM probenecid (pH 7.4). Fura-2AM-loaded cells were maintained at 25°C for 90 min before fluorescence measurements at 340 nm and 380 nm.

Western blotting

Cells washed with PBS were lysed in IPB-7 containing protease inhibitors. Conditioned media was obtained by using Amicon Ultra-50K filters. The samples were subjected to SDS-PAGE and thereafter blotted onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules). The protein bands were subsequently probed using antibodies. Proteins were visualized by chemiluminiscence procedure (Amersham Biosciences). The Western blot results were then analysed with NIH Image 1.42 software.

RNA purification and Real-Time RT-PCR

RNA was extracted using the RNeasy Mini Kit (Qiagen). cDNA was generated by High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Quantification of gene expression was performed in duplicates using HotStart-IT® SYBR® Green qPCR Master Mix (USB) with the detection on an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems).

Cell Invasion assay

8-μm pore size Transwell Biocoat Control inserts (Becton Dickinson) were used for the invasion assay. The inserts were coated with matrigel, a reconstituted basement membrane containing 60% laminin, 30% collagen IV, and 8% entactin. When the tested cells digest the matrigel, it performs a cellular process depicting tumor cell metastasis which involved detachment of cells from basement membrane. Panc-1 cells were incubated for 48 hours with pancreatic carcinogens, before being seeded onto the insert. The cells were fixed with methanol and stained with Toluidine Blue (Merck). Stained cells on the insert were counted.

Immunocytochemical staining

Cells were washed with PBS and fixed in 3.7% formaldehyde. After blocking the cells were incubated with monoclonal antibodies at 4°C overnight. Secondary polyclonal antibodies were added. The slides were mounted using DAPI.

Annexin V-FITC/PI stained fluorescence-activated cell sorter (FACS analysis)

Cells were harvested through trypsinization, and washed with cold PBS. Annexin V-solution (annexin V buffer, annexin V-FITC, propidium iodide) was added to the cells and incubated for 10 min at room temperature in the dark. Thereafter annexin V buffer was added to each sample, and the samples were analyzed by FACS (Becton Dickinson).

Small interfering RNA transfection

HepG2 cells were transfected with FoxO3a siRNA (human) (Cell Signaling Technology, Beverly, MA) for 48 hours. Protein levels were detected by Western blotting.

6. RESULTS AND DISCUSSION

Gender differences in human cancer incidences have been observed in cancer statistics. However, the reasons for the observed gender differences are not known (Edgren et al., 2012). To further study gender differences in chemical carcinogenesis, we evaluated the NTP database, which publishes reports on results from 2-year bioassays in mice and rats. NTP conducts bioassays in rats and mice and toxicity tests in cell lines to show carcinogenic hazard. Nominated chemicals are possible public health hazards due to the extent of human exposure. We analyzed the 2-year rat bioassays and genotoxicity tests. Chemicals investigated by NTP are tested under similar conditions and importantly, both male and female rats were used in most bioassays and under similar and well controlled conditions. The same rat strains were used for most chemicals.

As shown in Table 1 (in paper 1) a higher number of carcinogens affected male rats than female rats. The number of carcinogens affecting male rats alone was 68 and the numbers of carcinogens affecting female rats were 19. When the evidence level was used as an indicator of carcinogenicity, an overall analysis showed that male rats were classified 1.69 times more often at a higher evidence level of carcinogenicity than female rats. These data clearly indicates that male rats are more susceptible than female rats to chemical carcinogens. Even when considered at an organ level more carcinogens caused tumors in male rats than in female rats. The organs which were prominent in displaying male-specific tumors in rats were pancreas; (79% of all pancreatic tumor-inducing carcinogens), kidney (64% of all kidney tumor-inducing carcinogens) and skin (58% of all skin tumor-inducing carcinogens). In humans, men exhibit a higher cancer incidence level than women (Edgren et al., 2012) and the same cancer types which were prominent for male rats were also higher in men. These included organs such as pancreas, kidney and skin. The weighted averages of the percentage of carcinogens inducing tumors in different organs were favorably inclined towards male rats compared to female rats.

When we studied whether these organ-specific carcinogens induced tumors in other sites in the same or in the other gender we found that for male carcinogens the weighted average was 55% and for female carcinogens it was 59%. For male carcinogens that induced tumors in other sites in the other gender weighted average was 46% and for female carcinogens it was 66%. This data suggests that most carcinogens were not site-specific in inducing tumors and that the effects were not a consequence of gender dimorphisms in xenobiotic metabolism. These results

also suggest that the observed gender difference could be due to more basic gender-specific factors like the effect of hormones on the mode of action of carcinogens.

Carcinogens which were classified as positive in salmonella assays by the NTP were 1.4 times more common among the carcinogens which induced tumors exclusively in male rats. One exception was the group of lung carcinogens where a higher number of female lung carcinogens were genotoxic compared to male lung carcinogens. The five sites that exhibited the highest female-specific tumors were pituitary (80% of pituitary tumor-inducing carcinogens), bone marrow and lymphoid tissues (leukemia and lymphomas; 47% of leukemia and lymphomas-inducing carcinogens), lung (33% of lung tumor-inducing carcinogens), and thyroid (40% of thyroid tumor-inducing carcinogens). Hormonal effects could play a role in this observed gender difference, although more studies are necessary to understand better the underlying reasons. As mentioned in the introduction, there are examples of chemicals' carcinogenic effects being affected by hormones. For example, hormones could affect cellular levels of oxidative stress (Marin et al., 2010, Sawada et al., 1998), which could have an impact on the sensitivity towards carcinogens. For instance, estrogen has been shown to decrease oxidative stress in women through the regulation of NADPH oxidases (Miller et al., 2007). Hormonal effects on oxidative stress levels have been discussed in relation to women's increased life span (Vina et al., 2011).

Not all tumor types in rats can be compared to human tumor sites. For example, kidney tumors in male rats are partly caused by chronic progressive nephropathy (CPN) which is not equivalent to any condition in humans. CPN in male rats is caused by alpha-2-microglobulin binding to test chemicals. This may result in the accumulation of non-degraded test chemicals in kidney tubular cells and leading to promotion of kidney tumor through CPN (Hard et al., 2009). The expression of alpha-2-microglobulin is androgen dependent (Chatterjee et al., 1989). However, incidences of kidney cancer are still more common in men than in women (Jemal et al., 2009). For lung cancer, epidemiological studies show that women are more susceptible to tobacco smoke than men and women also tend to have higher levels of DNA adducts (Ramchandran and Patel, 2009). In study 1 we found that more female-specific lung carcinogens were genotoxic compared to the male-specific lung carcinogens. This is in line with data from human cells which shows that female human lung cancer cells have higher levels of smoking-induced bulky/hydrophobic DNA adducts than male human lung cancer cells (Uppstad et al., 2011). In conclusion, our data shows that gender differences in chemical

carcinogenesis are common in rats, with male rats being more susceptible, and this is similar to the higher cancer incidences observed in men compared to women. In future experiments interactions between hormones and carcinogens should be studied *in vitro* to further investigate the underlying mechanisms.

In paper 2 we studied eight rat pancreatic carcinogens tested by the NTP: 1,2,3-trichloropropane (TCP), 2-amino-5-nitrophenol (AMN), 2-Mercaptobenzothiazole (MER), benzyl acetate (BA), chlorendic acid (ChlA), cinnamyl anthranilate (CA), roxarsone (ROX) and 2,4- and 2,6-toluene diisocyanate (TDI). These chemicals were selected for the study as they were male-specific and did not induce pancreatic tumors in female rats. We theorized that even though these pancreatic carcinogens were structurally different, their mode of action could be similar in causing pancreatic tumors in male rats. In order to test this, we first searched for carcinogenic evidence of these chemicals by conducting a PubMed literature analysis using the text mining-based CRAB tool. The publication profiles for the pancreatic carcinogens as a group showed that 46% of the abstracts were classified in the non-genotoxic part of the MOA taxonomy and 39% in the genotoxic part of the MOA taxonomy. This result suggests that the pancreatic carcinogens were a mix of genotoxic and non-genotoxic chemicals. A group of well-studied genotoxic chemicals and a group of well-known non-genotoxic chemicals were also analyzed by the tool, and the publication profiles of the three groups of chemicals were compared. A more detailed evaluation of sub-categories within the non-genotoxic MOA part of the taxonomy showed that the inflammation category had the highest percentage of abstracts: 10% of total MOA abstracts and this sub-category also differed most between the pancreatic carcinogens and the two groups of control chemicals.

We studied the effect of BA, CA, ChlA and TDI in the Panc-1 pancreatic cancer cell line. TDI has previously been shown to increase intracellular calcium in human neuroblastoma SH-SY5Y cells (Liu et al., 2006). We wanted to study whether the other carcinogens also had a similar effect on calcium levels. Calcium assays indicated that BA, CA, ChlA and TDI increased intracellular calcium. The use of the P2X7 receptor inhibitor KN62 along with the carcinogen did not result in increased intracellular calcium levels, suggesting a role of this receptor in the upregulation of calcium levels. The increase in calcium levels was also reflected in the activation of a calcium-dependent phosphatase calcineurin. Calcineurin, which is an inflammatory marker, was activated by TCP, ChlA and BA. Substrates of calcineurin include NFAT, a transcription factor, which is involved in the inflammatory responses activated by

calcineurin through dephosphorylation (Seifert et al., 2009). We further analyzed the inflammatory response genes IL8, TNFα, TGFβ and ENPP2, transcribed by NFAT, by Real Time PCR at 6 and 24 hours. Most of the chemicals activated the genes at the 6 hour time-point and some at 24 hours. The majority of carcinogens seemed to upregulate ENPP2. ENPP2 codes for a protein named autotaxin (ATX), an extracellular lysophosholipase involved in cell migration (Nam et al., 2000). ATX was upregulated by all the carcinogens except AMN, either in intracellular or extracellular regions, at 24 hours. ATX upregulation by CA was inhibited by the P2X7 receptor inhibitor, suggesting P2X7 could be involved. ATX levels are known to be increased in the serum of pancreatic cancer patients (Nakai et al., 2011) and it is also known to be involved in the migration of pancreatic cancer cells (Komachi et al., 2009). ATX induced cell migration through lysophoshatidic acid (LPA). LPA is known to bind LPA1-4 receptors leading to cell migration and invasion (Okudaira et al., 2010). Cell migration requires the function of several different proteins, e.g. matrix metalloproteases (MMPs). MMPs degrade the extracellular matrix and help the cells to detach from the basement membrane, which leads to cell migration (Nabeshima et al., 2002). MMP9 is a collagenase that is activated by ATX and is upregulated in pancreatic cancer cells. We studied MMP9 levels and found that most of the carcinogens increased MMP9 protein levels. In invasion assays we found that ATX and MMP9 were involved in cell migration and invasion of cancer cells, and that all carcinogens, except TDI, increased the invasive capability of the cells. In order to confirm the role of ATX in carcinogen-induced cell migration, we used an inhibitor of ATX lysophospholipase activity called HA130. HA130 was used with ROX and ChlA in invasion assays, the results showed that the cell invasion decreased by the addition of the ATX inhibitor and suggests that ATX is involved in carcinogen-induced cell invasion. As the carcinogens tested were male-specific in the NTP analysis (study 1), the role of testosterone was studied on the carcinogens' abilities to induce ATX. Cells were pre-incubated with testosterone before exposure to CA. The results showed that testosterone combined with CA increased intracellular ATX levels and also prevented CA-induced toxicity. These results suggest that testosterone could be involved in increasing ATX levels and preventing the death of cancer cells. Overall, our data suggests that the pancreatic carcinogens act by inflammatory-related mechanisms and stimulate invasion through ATX induction.

Humans are exposed to mixtures of polycyclic aromatic hydrocarbons (PAHs), dioxins and polychlorinated biphenyls (PCBs) in the environment. In spite of this, most studies on these compounds have been conducted using on single chemicals instead of mixtures. In paper 3 we

studied the combined effects of PAHs with PCBs, TCDD and estradiol on HepG2 cells. The specific polycyclic aromatic hydrocarbons studied were benzo[*a*]pyrene (BaP) and dibenzo[*al*]pyrene (DBP) and the PCB studied was PCB153. A previous study had shown that PCB101 exposure lead to trapped p53 in the nucleus and attenuated apoptosis induced by BaP, which subsequently resulted in reduced phosphorylation of FOXO3a (Al-Anati et al., 2010). FOXO3a is transcription factor involved in a variety of cellular processes (Salih and Brunet, 2008). We further studied if the phosphorylation of FoxO3a was affected by TCDD, PCB153 and estradiol. Pre-incubation with TCDD, PCB153 and estradiol before treatment with BaP and DBP resulted in increased nuclear p53 and reduced phosphorylated FOXO3a. These results were confirmed by western blotting, fractionation and immunostaining of cells. The pretreatments also resulted in the attenuation of apoptosis induced by BaP, which was confirmed by FACS and analysis of apoptotic markers like cleaved caspase-3 and PUMA by western blotting. The role of FOXO3a in the nuclear accumulation of p53 and attenuated apoptosis was investigated by inhibition of PP2A. PP2A is a phosphatase involved in the dephosphorylation of FOXO3a (Singh et al., 2010). The inhibition of PP2A resulted in an increase of phosphorylated FOXO3a. Furthermore, BaP-induced apoptosis, which was attenuated by TCDD, PCB153 and estradiol, was restored and the BaP-induced accumulation of nuclear p53 was also reduced by the inhibition of PP2A. To further study the role of FOXO3a in this process we investigated whether silencing of FOXO3a would lead to trapping of p53 in the nucleus. Silencing of FOXO3a by siRNA led to an increase in the number of cells with nuclear p53 induced by BaP. These results further support the role of FOXO3a in the nuclear accumulation of p53. Another protein involved in the translocation of p53 from the nucleus to the cytoplasm is 14-3-3. This protein is also involved in the binding of FOXO3a and PP2A and is a part of the complex exporting FOXO3a and p53 from the nucleus (Tzivion et al., 2011). We used an inhibitor of 14-3-3, R18, to study the effect on p53 localization. We found that the levels of nuclear p53 were increased in cells treated with R18. These results show that apart from FOXO3a, 14-3-3 is also involved in the nuclear accumulation of p53. Overall, we show that FOXO3a is central to TCDD, PCB153 and estradiol-mediated attenuation of BaP-induced apoptosis. The toxicity caused by the combination of chemicals is important to study as it may reflect a more realistic exposure scenario than single exposures, and the toxic responses may differ. A better understanding about cellular effects caused by mixtures is not only valuable for basic research but may also support and improve chemical risk assessments of combined exposures. This study also reflects the effect of estradiol as a modulator of the toxic response caused by carcinogens.

7. CONCLUSIONS

Several studies have shown that men have higher cancer incidences than women. Hormones could play a role in this observed gender difference. Chemicals are one of the risk factors for cancer and some chemical carcinogens have shown gender differences in causing tumors in rodent studies and on cancer-related endpoints in human cell lines. In this thesis gender differences in susceptibility to chemical carcinogens were investigated. The analysis of the NTP database showed that there was a clear gender difference in the susceptibility to chemical carcinogens in rats and among the analyzed carcinogens the majority caused more tumors in male rats than in female rats. This result also reflects findings from human studies. The pancreas was one organ which was more affected by carcinogens in male rats than in females. A number of pancreatic male-specific carcinogens were studied more in detail and it was found that the pancreatic carcinogens induced inflammatory markers in several pancreatic cancer cell lines, and some effects were augmented by testosterone. The results suggest that the studied pancreatic carcinogens may share a common MOA. The effect of estradiol was also investigated in combination with the carcinogen benzo(a)pyrene (BaP) *in vitro*. The results showed that the toxic response caused by the carcinogens was modulated by estradiol.

Overall we show that chemical carcinogens exhibit gender differences in rats and this suggests that gender differences in susceptibility to chemical carcinogens also may affect humans. The data shows that hormones could play a role in the gender differences observed in chemical carcinogenesis.

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