CYP1A1 and CYP1B1 in human lung
PAH-bioactivation capacity, sex differences and steroid receptor mediated regulation

Thesis submitted for the degree of
Philosophiae Doctor
by
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List of papers

This project has resulted in the following papers, and will be referred to in the text by their roman numbering I, II and III:

Paper I
Heidi Uppstad, Steinar Øvrebo, Aage Haugen and Steen Mollerup. Importance of CYP1A1 and CYP1B1 in bioactivation of benzo[a]pyrene in human lung cell lines. Toxicology Letters 2010; 192: 221 - 228.

Paper II

Paper III
Heidi Uppstad, Tove Igeland, Kristine Haugen Anmarkrud, Aage Haugen and Steen Mollerup. Modulatory effect of sex steroid receptors on PAH metabolism genes in human lung cells. Manuscript.
Summary

Airway-exposure to polycyclic aromatic hydrocarbons (PAHs) increases the risk of lung cancer in humans. PAHs readily bind to the aryl hydrocarbon receptor (AHR), which is a ligand-inducible transcription factor that regulates transcription of cytochrome P450 (CYP) 1A1 and CYP1B1. CYP1A1 and CYP1B1 show similar, but not identical, substrate specificity toward various PAHs, and are responsible for bioactivation of most carcinogenic PAHs to reactive epoxide intermediates. PAH-epoxides generated by CYP1 can react with DNA and form PAH-DNA adducts. Epidemiological data have demonstrated an association between the formation of DNA adducts and an increased risk of lung cancer.

Clinical evidence has accumulated toward a notion of sex differences in the biology of lung cancer. Although not conclusive, studies indicate that women may be at greater risk of developing lung cancer from cigarette smoking than men. Furthermore, women are typically diagnosed at an earlier age than men are. After being diagnosed with lung cancer, however, women appear to display more favorable prognoses than men do. Hence, distinct clinical characteristics differ between men and women.

Although the lung is not considered a typical hormone responsive tissue, steroid receptors such as estrogen and androgen receptors have been reported to be expressed and active in lung cells. The rates and the extent of cellular processes such as metabolic activation of pro-carcinogens like PAHs may influence individual susceptibility to the tumorigenic effects associated with exposure. Studies have indicated that sex steroid receptors may modulate PAH-bioactivation in lung cells and therefore influence lung cancer susceptibility.

The purpose of this thesis was to study the individual roles of the PAH-bioactivation genes CYP1A1 and CYP1B1 in human lung, with specific focus on sex differences in expression and a possible modulatory effect of steroid receptors in gene regulation. First we wanted to establish the respective roles of CYP1A1 and CYP1B1 in bioactivation of prototype PAH benzo[a]pyrene (B[a]P). siRNA was used to knock down expression of CYP1A1 and CYP1B1, gene expression was measured by RT-qPCR and formation of B[a]P-metabolites by fluorescence-HPLC. In summary, the results revealed that the overall B[a]P-metabolism and
formation of $\text{B}[^{a}]\text{P}$-tetrol I-1 (a hydrolysis product of the ultimate $\text{B}[^{a}]\text{P}$-carcinogen BPDE I) in human lung cells are primarily dependent on $\text{CYP1A1}$. The formation of $\text{B}[^{a}]\text{P}$-tetrol I-1 may thus serve as an indicator of the current bioactivation capacity. Hence, the results show that CYP1A1 plays an important role in bioactivation of $\text{B}[^{a}]\text{P}$ in lung cells.

The next major objective was to elucidate whether previously reported in vivo sex differences in $\text{CYP1A1}$ expression and PAH-DNA adduct formation are intrinsic characteristics of lung cells. We therefore analyzed basal, cigarette smoke condensate (CSC)- and $\text{B}[^{a}]\text{P}$-induced gene expression of $\text{CYP1A1}$ and $\text{CYP1B1}$, CYP1 activity and formation of $\text{B}[^{a}]\text{P}$-DNA adducts in eleven non-small cell lung carcinoma (NSCLC) cell lines (five of female and six of male origin). Gene expression was measured by RT-qPCR, CYP1 enzyme activity was analyzed by EROD, and formation $\text{B}[^{a}]\text{P}$-adducts was measured by $^{32}$P-postlabelling. Significantly higher levels of basal, CSC- and $\text{B}[^{a}]\text{P}$-induced $\text{CYP1A1}$ gene expression, CSC-induced CYP1 activity and levels of $\text{B}[^{a}]\text{P}$-DNA adducts in $\text{B}[^{a}]\text{P}$-exposed cells were found in cell lines from women compared to cell lines from men. The results support the hypothesis that the female lung may be more susceptible to PAH than the male lung.

The last main focus was to examine the modulatory role of sex steroid receptors in regulation of $\text{CYP1A1}$ and $\text{CYP1B1}$ expression in human lung cells. Knockdown of gene expression was performed with siRNA, and gene expression was measured by RT-qPCR. Knockdown of estrogen receptor β ($\text{ER} \beta$) significantly reduced $\text{B}[^{a}]\text{P}$-induced expression of $\text{CYP1A1}$ and $\text{CYP1B1}$, whereas knockdown of androgen receptor ($\text{AR}$) significantly enhanced both CSC- and $\text{B}[^{a}]\text{P}$-induced expression of $\text{CYP1B1}$ in H2009 cells. ICI 182780, an antiestrogenic agent known to specifically degrade $\text{ER} \alpha$, increased the expression of $\text{CYP1A1}$ and $\text{CYP1B1}$, whereas dihydrotestosterone (DHT) repressed $\text{B}[^{a}]\text{P}$-induced expression of both genes in H2009. Together the results may indicate that the presence of $\text{ER} \beta$ may be necessary to maintain full $\text{B}[^{a}]\text{P}$-induced CYP1 expression, whereas the observed induction of $\text{CYP1A1}$ and $\text{CYP1B1}$ expression by ICI 182780 may indicate an opposite and thus suppressing role of $\text{ER} \alpha$ on CYP1 expression. The observed effects inflicted on CYP1 expression in $\text{AR}$ knockdown- and DHT exposure experiments coincide, and implicate a common mechanism. The results indicate that the expression of sex steroid receptors and exposure to sex steroid receptor ligands influence regulation of basal and induced expression levels of CYP1.
The importance of CYP1A1 in bioactivation of B[α]P, and the relationship between expression levels of \textit{CYP1A1} and elevated levels of B[α]P-DNA adducts in female cell lines compared to male cell lines, mutually strengthen one another. The present results also indicate a sex difference in PAH-bioactivation capacity of human lung adenocarcinoma cell lines, and together with previously published \textit{in vivo} data from our group, they support that the female lung may be more sensitive to PAH-exposure than the male lung. The final study showed how knockdown of \textit{ERβ} or \textit{AR}, respectively, and exposure to sex steroid receptor ligands had a significant impact on expression levels of \textit{CYP1} genes. These results support a role of sex steroid receptors in modulation of xenobiotic bioactivation in lung cells.

This thesis provides evidence indicating that female lung cells have higher PAH-bioactivation capacity than male lung cells, and that sex differences in expression of \textit{CYP1A1} may be mechanistically involved in these differences. Our results also indicate that regulation of important PAH-bioactivation genes is modulated by expression of sex steroid receptors. Further investigations of underlying interactions between existing signaling pathways and novel mechanisms behind sex differences in lung cancer biology should be granted necessary attention.
### Abbreviations and explanations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Explanation</th>
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<tbody>
<tr>
<td>4-OHT</td>
<td>4-hydroxytamoxifen</td>
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<tr>
<td>AHR</td>
<td>aryl hydrocarbon receptor</td>
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<tr>
<td>AHRR</td>
<td>aryl hydrocarbon receptor repressor</td>
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<tr>
<td>AI</td>
<td>aromatase inhibitor</td>
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<tr>
<td>AKR</td>
<td>aldo-keto reductase</td>
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<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
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<tr>
<td>ARE</td>
<td>androgen response element</td>
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<tr>
<td>ARNT</td>
<td>aryl hydrocarbon receptor nuclear translocator</td>
</tr>
<tr>
<td>BAG6</td>
<td>B-cell CLL/lymphoma 2 associated athanogene 6</td>
</tr>
<tr>
<td>B[a]P</td>
<td>benzo[a]pyrene</td>
</tr>
<tr>
<td>BPDE I</td>
<td>B[a]P-7,8-dihydrodiol-9,10-epoxide I</td>
</tr>
<tr>
<td>BTE</td>
<td>basic transcription element</td>
</tr>
<tr>
<td>CHRNA</td>
<td>cholinergic receptor nicotinic alpha</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
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<tr>
<td>CLPT1L</td>
<td>cleft lip and palate transmembrane protein 1-like protein</td>
</tr>
<tr>
<td>CSC</td>
<td>cigarette smoke condensate</td>
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<tr>
<td>CYP</td>
<td>cytochrome P450 monooxygenase</td>
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<tr>
<td>DEP</td>
<td>diesel exhaust particle</td>
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<tr>
<td>DHT</td>
<td>dihydrotestosterone</td>
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<tr>
<td>DNMT</td>
<td>DNA methyltransferase</td>
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<tr>
<td>DPN</td>
<td>diarylpropionitrile</td>
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<tr>
<td>E2</td>
<td>17β-estradiol</td>
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<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
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<tr>
<td>EH</td>
<td>epoxide hydrolase</td>
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<tr>
<td>ERα</td>
<td>estrogen receptor alpha</td>
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<td>ERβ</td>
<td>estrogen receptor beta</td>
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<tr>
<td>ERE</td>
<td>estrogen response element</td>
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<td>EROD assay</td>
<td>ethoxyresorufin-O-deethylase assay</td>
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<tr>
<td>GWAS</td>
<td>genome wide association study</td>
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HAH; halogenated aromatic hydrocarbon
HBEC; human bronchial epithelial cells
HDAC; histone deacetylase
HPLC; high-performance liquid chromatography
HRT; hormone replacement therapy
HSP90; heat shock protein 90
IARC; International Agency for Research on Cancer
ICI 182780; ERα antagonist (Fulvestrant, Faslodex)
In vitro; outside a living organism, in an artificial environment
In vivo; within a living organism
lncRNA; long non-coding RNA
LOI; loss of imprinting
miRNA; micro RNA
mRNA; messenger RNA
NNK; 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NSCLC; non-small cell lung carcinoma
OHF; hydroxyflutamide
p53; tumor protein p53
PAH; polycyclic aromatic hydrocarbon
ROS; reactive oxygen species
RR; relative risk
SERD; selective estrogen receptor degrader
siRNA; small interfering RNA
SNP; single nucleotide polymorphism
Sp1; stimulating protein 1
TCDD; 2,3,7,8-tetrachlorodibenzo-p-dioxin
TERT; telomerase reverse transcriptase
TSG; tumor suppressor gene
XAP2; aryl hydrocarbon receptor interacting protein
XRE; xenobiotic response element
1. Introduction

1.1 Lung cancer

1.1.1 Incidence, histology and mortality

Lung cancer is a global major health problem and the leading cause of cancer death across the world. Approximately 1.8 million new cases are diagnosed annually and current statistics reveal that lung cancer accounts for 12.9 % of all new cancers diagnosed, and 19.4 % of all cancer deaths registered (Ferlay et al. 2015, Fitzmaurice et al. 2015, Torre et al. 2016). Many developing countries experience a steep increase in lung cancer burden, in part due to rapid industrialization and increasing population growth (Hashim and Boffetta, 2014).

Typically, lung cancers are divided in two distinct histological types, which are further grouped into subcategories. The main histological types are non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), with prevalence rates of 86 % (NSCLC) and 14 % (SCLC). NSCLC typically originate from bronchial epithelial-cell precursors and are divided into squamous cell carcinoma, adenocarcinoma and large cell carcinoma. The distribution of histological types has changed over the past decades. Thus, the incidence of squamous cell carcinomas has decreased, while incidence of adenocarcinomas exhibits an increase, especially in women (Torre et al. 2016). SCLC usually originate from neuroendocrine-cell precursors, and are subcategorized into small cell carcinoma and combined small cell carcinoma (Travis et al. 2013).

Cumulative exposure models estimate that the average latency period for lung cancer is approximately 30 years, but few patients receive their diagnosis at an early stage of their disease. In fact, approximately 50 % of the diagnosed cases are classified as advanced or metastatic (Quint, 2007). Unfortunately, the expected overall 5-year survival rate is 16 % for lung cancer patients, and the need for sensitive and specific diagnostic tools, as well as novel treatments to combat the disease is urgent (Ferlay et al. 2015; Sharma et al. 2016). An essential component required to provide these tools, is the increased understanding of underlying molecular mechanisms in lung carcinogenesis.
1.1.2 Lung cancer etiology and lung carcinogens

An immense amount of evidence is reaffirming tobacco smoking as the major cause of lung cancer. Globally, 85 % of lung cancers in men and 47 % of lung cancers in women are designated the act of tobacco smoking (Youlden et al. 2008). Moreover, the establishment of a causal association between secondhand tobacco smoke and lung cancer is firmly founded, and expected to account for 1.6 % of all lung cancers diagnosed (Boffetta, 2006). The aerosol emerging from the mouthpiece of a cigarette is composed of approximately 4,800 different chemical compounds and typically contains 1,000 particles/ml. Among these compounds are known carcinogens such as polycyclic aromatic hydrocarbons (PAHs), tobacco-specific nitrosamines, 1,3-butadiene, ethyl carbamate, ethylene oxide, nickel, chromium, cadmium, polonium-210, arsenic, and hydrazine (Hecht, 1999).

Occupational exposures are responsible for a considerable fraction of lung cancer incidences (Hashim and Boffetta, 2014). The most important occupational lung carcinogens include mixtures of PAHs, heavy metals, radon, crystalline silica and asbestos. More specifically, occupations such as welding and painting are associated with increased risk of lung cancer. Additionally, uranium miners and nuclear plant workers also have an increased risk of lung cancer due to the exposure to radioactive particulate mass (Algranti et al. 2010). It is also worth mentioning, that for most known occupational carcinogens, synergisms with tobacco smoking has been reported (IARC Monographs Vol 83, 2004).

Asbestos are mineral fibers considered to be strong lung carcinogens, and a serious occupational health hazard worldwide. Today, the use of asbestos is in most countries either abandoned or controlled, but it still poses a significant public health threat (Lin et al. 2007). Asbestos can act on its own, or synergistically with tobacco smoke to induce lung cancer (Markowitz, 2015). For individuals exposed to asbestos, the relative risk of lung cancer is three times higher than for non-exposed individuals (van Loon et al. 1997). Mechanistically, asbestos-induced carcinogenesis is considered to occur through the creation of chronic inflammation, involving oxidative stress and induction of DNA damage (Benedetti et al. 2015).
Cumulative exposure to emissions rich in various PAHs present in urban air poses an environmental lung cancer risk (Vineis and Husgafvel-Pursiainen, 2005). Urban air is typically polluted by vehicle emissions such as diesel exhaust particles (DEP) and high concentrations of carcinogenic PAH may be adsorbed on these particles (Ono-Ogasawara and Smith, 2004). DEP are linked to an elevated risk of lung cancer through mechanisms involving oxidative stress and oxidation of DNA (Hesterberg et al. 2012; Risom et al. 2005). The proportion of lung cancers attributable to urban air pollution in Europe is estimated to be 11% (Boffetta, 2006).

A considerable fraction of environmental exposure to lung carcinogens comes from arsenic in drinking water and food (Martinez et al. 2011). More than 200 million people live in regions with naturally elevated levels of arsenic in their drinking water (Naujokas et al. 2013), and the main cause of death after chronic arsenic intake is lung cancer (Hubaux et al. 2012). Arsenic exposure contributes synergistically with other lung carcinogens, such as exposure to tobacco smoke (Ferreccio et al. 2013).

Another highly relevant lung carcinogen is radon, a naturally occurring gas usually found in igneous rock and soil and originating from the decay of uranium. Radon itself subsequently decays further to generate radioactive alpha and beta emitting particles, which may damage DNA both directly and through generation of free radicals (Ruano-Ravina et al. 2009; Samet et al. 2009). Radon is a Class I human carcinogen (IARC Monographs Vol 43, 1988) and studies have shown that domestic and/or occupational inhalation exposure can cause lung cancer (Brauner et al. 2012). In the case of non-occupationally exposed never-smokers, studies have revealed that 1/3 of lung cancer deaths might be linked to indoor radon (Lubin et al. 1995).

1.1.3 Lung cancer epidemiology and smoking habits are tightly linked

Smoking behavior is the most influential factor in risk of developing lung cancer. It reflects on the trends in lung cancer incidence as well as mortality (IARC Monographs Vol 83, 2004). The first so-called modern cigarette was presented in the early 20th century, and its arrival led to a steep increase in tobacco use, in particular among male inhabitants of the Western world. In the
1940’s, women also began their tobacco smoking habits. The trend in the United States shows that the overall habit of cigarette smoking has peaked and declined (Figure 1).

![Smoking prevalence by sex in USA (1900 - 2010)](image)

Figure 1. Smoking prevalence by sex in USA (1900 - 2010). Modified from (Thun et al. 2012).

Although authorities all over the world have focused on tobacco control issues involving education on the harmful effects of smoking, along with tax increases and smoking restrictions, smokers still exist. Unfortunately, the global image highlights the fact that many developing countries are experiencing a considerable increase in tobacco use following a relative increase in wealth among the inhabitants (O’Connor et al. 2010). It is projected that if existing trends remain unchanged, many countries will not manage to reach their goals concerning tobacco control and it is therefore estimated that approximately 1 billion people will remain current smokers in year 2025 (Bilano et al. 2015). On the bright side, many developed countries have succeeded in reducing the adult smoking prevalence to less than 25% (Ng et al. 2014). Statistics Norway (SSB) reports that in 2016 only 13% of inhabitants in Norway smoke on a daily basis (Statistics Norway, Smoking Habits, 2016).

Despite a steady decline in smoking prevalence in recent decades primarily in developed countries (Bray et al. 2002), one can spot significant differences in the prevalence among different groups of inhabitants. Education, occupational status and income are all inversely
associated with smoking (Laaksonen et al. 2005). Another important demographic feature is that the epidemic most likely will subsist among women in some high-income countries as part of their self-realization process (Bilano et al. 2015). In the United States, almost all female smokers started their consumption of cigarettes in their teens, and even though they are well aware of the harmful effects, smoking has become a way to express independence and equality with their grown-up counterparts. The tobacco companies execute extensive marketing toward young girls for example by developing cigarette brands specifically designed for women (Jemal et al. 2008).

1.2 Lung carcinogenesis

1.2.1 Stages in carcinogenesis

Continuing discoveries in cancer research constantly renew the knowledge on the complex networks of biological mechanisms involved in carcinogenesis. Hanahan and Weinberg proposed six hallmarks of cancer, which comprise the most important biological capabilities acquired during the multistep development of human tumors (Hanahan and Weinberg, 2000). In 2011, these authors added two more emerging hallmarks of cancer, altogether comprising; sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming of energy metabolism and evading immune destruction (Hanahan and Weinberg, 2011).
A historical and less detailed conceptual model, explicitly described by C.C. Harris in 1991, focus on three main stages of carcinogenesis defined as initiation, promotion and progression (Harris, 1991). The initiation stage of carcinogenesis is characterized by irreversible genetic changes, which predispose susceptible normal cells to impending malignant transformation. An initiated cell is not neoplastic, but it has acquired several successive genotypical and phenotypical changes. Such changes may typically be mutations in DNA-repair genes, oncogenes and tumor-suppressor genes. Cell proliferation and inhibition of apoptosis are essential for this stage, as they both contribute to clonal expansion of initiated cells.

The promotion stage is characterized by increased cell proliferation in susceptible tissues. The increased proliferation will in turn contribute to a fixation of mutations that may enhance alterations in genetic expression (Ochieng et al. 2015). Promotion changes DNA in both genetic and epigenetic ways (Hanahan and Weinberg, 2000). In contrast to initiation, promotion is a potentially reversible stage. Representative agents acting in the promotion stage of lung carcinogenesis mostly belong to chemical categories like reactive oxygen species (ROS), redox
active xenobiotics/metals, phorbol esters, polycyclic aromatic compounds, peroxisome proliferators and endocrine disruptors (Hanahan and Weinberg, 2000).

Progression is the last stage of carcinogenesis, and here the neoplastic phenotype is acquired through both genetic and epigenetic mechanisms (Oliveira et al. 2007). Progression is irreversible and brings genetic instability, uncontrolled growth, invasion, metastization and altered biochemistry to the affected cells (Oliveira et al. 2007; Pitot and Dragan, 1991). In this stage, cell proliferation operates independently from surrounding stimuli (Lutz, 2000). Angiogenesis is essential for progression, although the attainment of an angiogenic phenotype has been found to precede the development of malignancy (Hawighorst, 2001). Agents involved in progression of lung carcinogenesis include both genotoxic and non-genotoxic, promoting compounds.

Figure 3. Chemical carcinogenesis divided into three steps. Modified from (Harris, 1991; Oliveira et al. 2007).
1.2.2 Lung cancer susceptibility

It has long been recognized that people differ in their susceptibility toward disease. Although different individuals may experience similar environmental and/or occupational exposures, time- and dose-wise, they do not necessarily have the same risk of developing a disease. Susceptibility usually appears as a consequence of genetic predisposition, including mutations, single nucleotide polymorphisms (SNPs) and epigenetic alterations. Their main features are their influence on gene expression or genetic stability. Epidemiological data have revealed that some individuals are predisposed to lung cancer, and susceptibility toward tobacco smoke carcinogens has been debated (Perera and Weinstein, 1982). Epidemiological and pedigree studies suggest that lung carcinogenesis in smokers depend on the combined effects of age, smoking, impaired lung function and genetic factors (Young et al. 2009). Eighty-five – 90 % of all NSCLC patients are smokers or former smokers, although only 15 % of lifetime smokers develop NSCLC (Villeneuve and Mao, 1994). Many genes can interfere with the carcinogenic potential of tobacco smoke and the most prominent susceptibility genes are typically involved in carcinogen-metabolism, DNA-repair and cell cycle control (Spitz et al. 2003).

1.2.3 Genetic susceptibility to lung cancer

Recent large scale multi-cohort genome wide association studies (GWAS) have revealed several chromosomal regions as lung cancer susceptible loci. In Caucasian smoking populations, the chromosomal regions 15q25, 5p15 and 6p21 have been reported to harbor genes associated with elevated risk of lung cancer (Wei et al. 2015; Yang et al. 2013). In never smoking females, however, other specific chromosomal regions were highlighted as susceptibility loci (Wei et al. 2015; Yang et al. 2013).

15q25 contains neuronal nicotinic acetylcholine receptor (nAChr) subunits (cholinergic receptor, nicotinic alpha 3 and 5) CHRNA3 and CHRNA5 genes. SNPs in these genes can modulate lung cancer risk indirectly through smoking behavior, or through the direct anti-apoptotic or proliferative effects of nicotine (Marshall and Christiani, 2013).
SNPs in cleft lip and palate transmembrane protein 1-like protein (\textit{CLPTL1L}) and telomerase reverse transcriptase (\textit{TERT}) on locus 5p15 have been reported to increase the risk of lung cancer and other cancers. The \textit{CLPTL1L} gene is presumed to have an essential role regarding apoptosis in lung cells (McKay \textit{et al.} 2008). \textit{TERT} is important in maintenance of telomere length, and short telomere length has been associated with a significantly increased risk of lung cancer (Ma \textit{et al.} 2011). SNPs in telomere maintenance genes such as \textit{TERT} are therefore considered to play a role in lung cancer susceptibility (Choi \textit{et al.} 2009; Hosgood \textit{et al.} 2009).


\textbf{1.2.4 Epigenetic alterations}

At present, epigenetic alterations are considered as important as genetic changes in carcinogenesis. Among the epigenetic mechanisms involved in lung cancer are changes in DNA methylation, histone modification, nucleosome repositioning, and altered regulation by noncoding RNAs (miRNA, lncRNA) (Robertson, 2005).

Methylation of DNA and acetylation of histones influence gene expression by regulating chromatin structure and accessibility for transcription factors. Different mechanisms of DNA hypomethylation can result in lung carcinogenesis. The first one involve either retrotransposons or activation of repetitive elements (SINE or LINE) with subsequent microsatellite instability. The second mechanism is transcriptional activation and overexpression of oncogenes, and the third is through the loss of imprinting (LOI) (Brena and Costello, 2007; Ehrlich, 2002; Herman and Baylin, 2003). LOI is important in carcinogenesis because it can result in dual allele activation and increased gene expression of oncogenes. LOI can also result in lack of expression of tumor-suppressor genes (TSGs) and DNA repair genes due to lost imprinting and thus increased accessibility to responsive elements that suppress normal transcription (Jeclinic and Shaw, 2007). In conjunction with changes in DNA methylation, histone modifications such as
acetylation and deacetylation may influence gene expression, DNA replication, DNA repair and DNA recombination, and thus lead to cancer (Glozak and Seto, 2007; Kouzarides, 2007).

DNA may become modified through hydroxymethylation of cytosine. Tet methylcytosine dioxygenases typically catalyze the conversion of 5-methylcytosine to 5-hydroxymethylcytosine, and this is believed to be an intermediate state leading to hypometylation (Guibert and Weber, 2013). Chemical exposure to metals, persistent organic pollutants and PAH, can activate Tet methylcytosine dioxygenases, and indirectly contribute to hypometylation of DNA (Ruiz-Hernandez et al. 2015).

Furthermore, target mRNAs can be repressed or degraded by ncRNA, such as miRNA. miRNA can control a wide variety of cellular processes such as proliferation and apoptosis which often show irregularities in human cancer (Calin and Croce, 2006; Motoyama et al. 2009; Vasudevan et al. 2007), and several miRNAs become silenced by CpG hypermethylation in cancer cells (Bueno et al. 2008). This system shows great complexity, since one mRNA can be the target of many miRNA’s, and one miRNA can target many mRNA’s.

Figure 4. Potential genetic and epigenetic pathways linking environmental exposure to health effects. Modified from (Bollati and Baccarelli, 2010).
1.2.5 Epigenetics in lung cancer

Recent data suggest that smoking directly alters and causes aberrant expression of epigenetic modulators and their downstream targets during initiation and progression of lung cancer (D'Alessio and Szyf, 2006; Izzotti et al. 2009). Furthermore, in a study by Watanabe et al. (2012) fifty-five miRNA were reported epigenetically silenced in NSCLC patients with a smoking history (Watanabe et al. 2012).

Several recent studies suggest that epigenetic changes detected in sputum, bronchoalveolar lavage and saliva may serve as biomarkers for lung cancer (Langevin et al. 2015). A growing number of hypermethylated TSG promoters and single gene hypomethylations that develop during early stages of lung carcinogenesis have been identified (Brzezianska et al. 2013). Detection of such alterations using non-invasive diagnostic tools could contribute to earlier detection of lung cancer (Langevin et al. 2015).

Epigenetic alterations are not only important in diagnostics, but also as therapeutic targets in lung cancer. Considering treatment, histone deacetylase- (HDAC) and DNA methyltransferase- (DNMT) inhibitors could principally restore TSG expression in lung cancer patients. In the clinic, both types of drugs have been tested in treatment of NSCLC, however none of them are currently approved (Lawless et al. 2009; NIH, National Cancer Institute, cancer drugs approved by the Food and Drug Administration (FDA) for lung cancer, 2016).

1.2.6 Lung cancer and sex differences

Trend analyses show that lung cancer still is the most common cancer in men worldwide. Globally, approximately 1.2 and 0.6 million new cases of lung cancer are diagnosed in males and females each year, respectively. The incidence rate in women is thus generally lower, but nonetheless, lung cancer is the fourth most frequent cancer and the most common cause of death from cancer among women in developed regions (Bray et al. 2013; Ferlay et al. 2015; Patel, 2005).
It has been debated for several decades whether there are sex differences in lung cancer risk. Several case-control studies have shown that women could be at greater risk of developing lung cancer from cigarette smoking than men (Novello and Baldini, 2006; Patel et al. 2004; Ramchandran and Patel, 2009). A recent study of patients diagnosed with lung adenocarcinoma, concluded that female smokers were significantly younger than their male counterparts regarding onset of the disease (Nagy-Mignotte et al. 2011). Another recent report showed that women were overrepresented amongst NSCLC patients under 40 years (Thomas et al. 2015). Furthermore, an elevated incidence of lung cancer in female never-smokers compared to male never-smokers has been described (Koo and Ho, 1990; Stockwell et al. 1990; Wakelee et al. 2007) which at least in some parts of the world may be associated with exposure to domestic cooking oil- and coal vapors (Gao et al. 1987).

In contrast, prospective studies have not been as conclusive regarding sex differences in risk of developing lung cancer (Bain et al. 2004; Henscke et al. 2006), and a study by De Matteis et al. (2013) supports this notion and reports no significant sex difference in risk of lung cancer (De Matteis et al. 2013). Discussions regarding study designs, risk estimate measures, model of interactions and potential confounders (e.g., depth of inhalation, tobacco type) that are
preferable or should be taken into consideration, still withstand (De Matteis et al. 2013), and the matter of sex differences in risk of lung cancer remains controversial.

A number of hypothesis regarding underlying reasons for a possible sex difference in human lung cancer risk have been proposed. One suggestion is that the combination of exposure to environmental risk factors and genetic differences may account for unequal RR for developing lung cancer (Kiyohara and Ohno, 2010; Kreuzer et al. 2002; Siegfried, 2001; Stabile and Siegfried 2003). Other possible explanations have focused on under-reporting of smoking habits and a potentially greater exposure to passive smoking among women (Haugen, 2002; Patel, 2005;).

Although increased risk of lung cancer among women is debatable, women appear to have improved survival rates in every stage of lung cancer, with women over the age of 60 years having a survival advantage over men and younger women (Nakamura et al. 2011; Patel et al. 2004). Additionally, women are more likely than men to be diagnosed with adenocarcinoma compared with other histological lung cancer subtypes, and in Asian populations, women with lung adenocarcinoma seem to have a higher incidence of tumors with EGFR mutations. These lung tumors are more responsive to EGFR tyrosine kinase inhibitors, and this may account for a significant fraction of the survival advantage observed at least in Asian women (Marquez-Garban et al. 2007; Mitsudomi, 2014). To sum up, epidemiological data indicate that lung tumor biology differs between males and females; however, the matter of sex differences in risk of lung cancer is still not completely sorted out. This is an important area of research, which would benefit from greater dedication.

1.2.7 Animal studies and sex differences in lung cancer biology

Sex differences in lung cancer risk are supported by animal studies. One study reported lower lung tumor incidence and reduced tumor size in androgen receptor (Ar) knockout mice co-exposed to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane (NNK) and benzo[a]pyrene (B[a]P) compared to wild-type mice receiving the same treatment (Yeh et al. 2012). Moreover, female A/J mice were confirmed to be more susceptible to NNK-induced lung carcinogenesis than male mice. In males, lung carcinogenesis was increased by castration, whereas in females,
malignant transformation of lung proliferative lesions tended to be inhibited by ovariectomy. These results suggested that NNK-induced lung carcinogenesis is inhibited by testosterone and accelerated by E2 (Ninomiya et al. 2013), confirming that female A/J mice are more susceptible to NNK-induced lung carcinogenesis than males. These findings indicate the possibility that sex hormones play important roles in determining sex differences in lung carcinogenesis in the A/J mice initiated by NNK (Ninomiya et al. 2013).

1.3 Biotransformation of PAH in tobacco smoke

1.3.1 Chemistry, sources and biotransformation of PAH

PAHs are formed by incomplete combustion of organic material, and typical sources comprise urban and industrial air pollution, tobacco smoke and diet (Bostrom et al. 2002). The most prominent PAH exposure routes for humans are inhalation, digestion and skin absorption (Bostrom et al. 2002). Most PAHs are nonpolar organic molecules consisting of two or more benzene rings arranged in different configurations. Most PAHs are insoluble in water, and their aqueous solubility decreases logarithmically as the molecular mass increases. Consequently, PAHs tend to persist in the environment (Johnsen et al. 2005).

Airway-exposure to PAH increases the risk of lung cancer in humans, and IARC has listed thirteen PAHs as human carcinogens or potential carcinogens, comprising benz[a]anthracene, benzo[b]fluoranthene, benzo[j]fluoranthene, B[a]P, dibenz[a,h]anthracene, 7H-dibenzo[c,g]carbazole, dibenzo[a,h]pyrene, dibenzo[a,i]pyrene, indeno[1,2,3-cd]pyrene,

Tobacco smoke is a significant source of PAH. Cigarettes with regular tar content typically contain approximately 100 ng of total PAHs per gram of tobacco, and a smoker may thus daily inhale ~0.26 μg of B[a]P per pack of 20 cigarettes (Grimmer et al. 1988; Piccardo et al. 2010).

PAHs can diffuse passively across cell membranes due to their lipophilic nature. After entering the cells they usually remain as inert procarcinogens unless bioactivated by endogenous enzymes. Biotransformation of PAH encompasses several metabolic enzymes and includes at least three different pathways. Prominent enzymes present in the first pathway are cytochrome P450 1A1 (CYP1A1), CYP1B1 and epoxide hydrolase (EH) (Shimada and Fujii-Kuriyama, 2004). The second pathway is referred to as the CYP-peroxidase pathway and is catalyzed by a completely different battery of enzymes (Moorthy et al. 2015), while the third pathway is called the aldo-keto reductase pathway and the main enzymes involved are aldo-keto reductase 1A1 (AKR1A1) and AKR1C1-1C4 (Palackal et al. 2001, 2002).

B[a]P is the most extensively studied PAH, and it often serves as a reference for the carcinogenic potential of other PAHs (Bostrom et al. 2002). B[a]P has the prospective of being bioactivated to the ultimate carcinogen B[a]P-diolepoxide I (BPDE I). BPDE I binds to DNA and forms predominantly covalent (+) trans adducts at the N2 position of guanine, and strong evidence from molecular epidemiologic studies show that lung tissue in smokers have significantly higher PAH–DNA adduct levels than lung tissue in non-smokers (Dipple, 1995; Perera et al. 1987; Wolfe et al. 2004).
PAHs exhibit great variability regarding their carcinogenic potential (Deutsch-Wenzel et al. 1983; Grimmer et al. 1988). Furthermore, exposure often includes mixtures of several different PAHs. In a study conducted by Tarantini et al. (2011) different compounds present in PAH-mixtures were shown to affect each other’s potential carcinogenicity both synergistically and antagonistically, making the evaluation of cancer risk for PAHs difficult to establish (Tarantini et al. 2011).

**1.3.2 Aryl hydrocarbon receptor (AHR)**

The AHR is a Class E basic helix-loop-helix protein with the highest expression found in liver, adipose tissue, and bronchial epithelial cells (Tsay et al. 2013). The human gene encoding the AHR is localized to chromosome position 7p15, and the full-length protein consists of 848 amino acids and weighs 96147 Da. The mRNA sequence corresponding to the full-length protein consists of 11 exons and at least two additional splice variants have been identified (National Center for Biotechnology Information, PubMed Gene ID: 196, 2016).

The AHR is a transcription factor normally found in a cytosolic inactive protein complex comprising a heat shock protein 90 (HSP 90) dimer, co-chaperone p23 and hepatitis B virus X-associated protein 2 (XAP2). When hydrophobic ligands such as PAHs enter the cell by simple diffusion, they can readily bind to the receptor. Ligand binding results in phosphorylation of two protein kinase C sites on the AHR adjacent to nuclear localization sequences. Subsequently, the AHR complex translocates to the nucleus where the receptor dissociates from its chaperone complex and dimerizes with the AHR nuclear translocator (ARNT). The AHR/ARNT
heterodimer is the active transcription factor able to recognize xenobiotic response elements (XREs) in promoter regions of AHR responsive genes and thereby alter their expression levels (Esser and Rannug, 2015).

AHR has many physiological effects in the lungs, including altered cell proliferation, differentiation, cell-cell adhesion and xenobiotic metabolism (Chiba et al. 2011; Wong et al. 2010). Furthermore, recent studies have implicated AHR in the development of lung cancer (Tsay et al. 2013) AHR is highly expressed in lung cancer patients, and AHR-agonists induce lung cancer cell growth through activation of multiple pathways (Chuang et al. 2012; Portal-Nunez et al. 2012; Wang et al. 2009). Overall, activation of AHR may cause a number of downstream effects, which influence tumorigenesis, inflammation, formation of DNA-adducts and cell proliferation (Tsay et al. 2013). Some of the genes regulated by the AHR are cytochrome P450 (CYP) 1A1, CYP1A2, and CYP1B1 (Shimada and Fujii-Kuriyama, 2004). However, the extent of AHR/ARNT-activation is both tissue-specific and dependent upon co-regulators present in different cell types (Whitlock, 1999).

It is apparent, however, that the AHR binds not only toxicants like PAHs, but also endogenous compounds, dietary flavonoids, phytochemicals and many pharmaceuticals (Denison and Nagy, 2003; Esser and Rannug, 2015; Safe et al. 2013). Consequently, it has been proposed that this receptor in fact may prove to be an important drug target (Safe et al. 2013).

1.3.3 Cytochrome P450 1A1 and 1B1 (CYP1A1 and CYP1B1)

The CYP1A1 and CYP1B1 enzymes belong to the cytochrome P450-superfamily, which catalyzes a variety of reactions, including metabolism of xenobiotics and biosynthesis of endogenous compounds essential to the cells. CYP1A1 and CYP1B1 catalyze the incorporation of one atom of molecular oxygen into the substrate and they typically convert hydrophobic compounds to more polar metabolites that may be readily excreted (Hukkanen et al. 2002).

The CYP1A1 gene is located on chromosome 15q24.1, and the full-length protein consists of 512 amino acids and weighs 58165 Da. The mRNA sequence corresponding to the full-length
variant of the protein consists of seven exons. At least two additional splice variants of the protein have been identified in human leukocytes (Bauer et al. 2007; National Center for Biotechnology Information, PubMed Gene ID: 1543, 2016). The CYP1B1 gene contains three exons and is located on chromosome 2p22.2. This protein consists of 543 amino acids and has a molecular mass of 60846 Da. Four transcript variants of CYP1B1 have been identified. (National Center for Biotechnology Information, PubMed Gene ID: 1545, 2016).

CYP1A1 and CYP1B1 are mainly expressed in extrahepatic organs. In human lung, the enzymes are highly expressed in alveolar type I and II cells, ciliated columnar bronchoalveolar epithelial cells, and alveolar macrophages (Hukkanen et al. 2002). Both enzymes typically localizes to the endoplasmic reticulum. CYP1A1 is highly inducible by PAHs and halogenated aromatic hydrocarbons (HAHs) via AHR-mediated gene transcription. CYP1A1 metabolizes PAHs, but also endogenous compounds such as arachidonic acid, eicosapentaenoic acid and bilirubin are metabolized by CYP1A1 (National Center for Biotechnology Information, PubMed Gene ID: 1543, 2016; Rifkind, 2006; Shrenk, 1998). CYP1B1 expression is also highly inducible by PAHs through the AHR, and in addition to its role in metabolism of several procarcinogens such as PAH, the CYP1B1 enzyme is central in the bioactivation of 17β-estradiol to 4-hydroxyestradiol (National Center for Biotechnology Information, PubMed Gene ID: 1545, 2016; Shimada and Guengerich, 2006). This catechol is considered important in breast and uterine carcinogenesis. Quinone intermediates derived from 4-hydroxyestradiol may react with DNA and form highly mutagenic adducts (Cavalieri et al. 2000).

Several studies have shown that CYP1A1 and CYP1B1 are responsible for bioactivation of most carcinogenic PAHs to epoxide intermediates (Shimada and Fujii-Kuriyama, 2004). CYP1A1 and CYP1B1 show similar, but not identical, substrate specificity regarding various PAHs (Nebert et al. 2004; Shimada et al. 2001). Analyses of human lung tissue have showed significantly higher expression of CYP1A1 in both smokers and ex-smokers compared to never-smokers. Similar results were found in cells from bronchoalveolar lavage and bronchial biopsies in smokers versus non-smokers (Mollerup et al. 2006; Thum et al. 2006). CYP1B1 expression in human lung displays a similar pattern (Kim et al. 2004). The expression of CYP1A1 is associated with increased lung cancer risk (London et al. 2000; San Jose et al. 2010; Vineis et al. 2003; Yang et al. 2004) and CYP1B1 is often overexpressed in tumor tissues, and
is therefore be considered to be a potential histopathological tumor marker (McFadyen and Murray, 2005).

1.3.4 Metabolism of B[a]P catalyzed by CYP1A1 and CYP1B1

The lipophilic nature of PAHs initiate cellular enzymatic metabolism to enhance clearance and excretion of the compounds. Metabolism of the prototype PAH B[a]P catalyzed by CYP1A1 and CYP1B1 illustrates how procarcinogenic PAH may become bioactivated during this process (Shimada and Fujii-Kuriyama, 2004).

The major B[a]P-metabolites formed by CYP1A1 and CYP1B1 are B[a]P-4,5-epoxide, B[a]P-7,8-epoxide and B[a]P-9,10-epoxide. The next enzymatic step catalyzed by EH forms the respective dihydrodiols B[a]P-(−)-trans-4,5-dihydrodiol, B[a]P-(−)-trans-7,8-dihydrodiol, and B[a]P-(−)-trans-9,10-dihydrodiol. Five B[a]P-phenols; 1-OH-B[a]P, 3-OH-B[a]P, 6-OH-B[a]P, 7-OH-B[a]P, and 9-OH-B[a]P are frequently formed during metabolism of B[a]P, usually by spontaneous rearrangement of B[a]P-dihydrodiols. These phenols can further be converted to their corresponding quinones; the most common being B[a]P-1,6-quinone, B[a]P-3,6-quinone, and B[a]P-6,12-quinone (Gelboin, 1980). The primary B[a]P-epoxides formed can be conjugated with glutathione by glutathione-S-transferases, and B[a]P-phenols and dihydrodiols can be conjugated to water-soluble compounds by either sulfate- or glucuronide conjugation.

B[a]P-7,8-dihydrodiol and B[a]P-9,10-dihydrodiol have the ability to become activated to highly reactive dihydrodiol-epoxides, also catalyzed by CYP1A1 and CYP1B1. The epoxide groups formed are either cis or trans, some which will be hydrolyzed by EH to yield B[a]P-tetrols. If not hydrolyzed, B[a]P-7,8-dihydrodiol-9,10-epoxide and B[a]P-9,10-dihydrodiol-7,8-epoxide can readily react with protein or DNA and form adducts. The B[a]P-trans-7,8-dihydrodiol-9,10-epoxide I is often referred to as the ultimate B[a]P-carcinogen, abbreviated BPDE I. BPDE I is highly mutagenic due to the bay-region of the molecule. This region provides an area of steric hindrance for detoxifying enzymes and the epoxide is very susceptible to nucleophilic attack from DNA and proteins. The mutagenic potential of B[a]P-9,10-
dihydrodiol-7,8-epoxide III (BPDE III) is low compared to BPDE I (Conney, 1980; Gelboin, 1980; Wei et al. 1991).

Figure 7. Metabolic pathways of B[a]P. Modified from (Hecht, 1999; Poirier, 2004).

1.3.5 B[a]P-DNA adduct formation

When BPDE I reacts with DNA, it preferentially becomes covalently linked to the exocyclic amino groups of deoxyguanosine and form adducts (Dipple, 1995; Wolfe et al. 2004). If the BPDE-DNA-adducts remain unrepaired, they can cause a miscoding during the replication process. The result may be transversion mutations and if the mutation occurs in a critical region of important genes, like oncogenes and tumor suppressor genes, the carcinogenesis process may thus be initiated (Dong et al. 2004; Greenblatt et al. 1994). Several studies have shown that DNA-adduct formation is associated with tumor induction in a dose-related fashion (Poirier, 2004; Poirier and Beland, 1992; Swenberg et al. 2008). Furthermore, epidemiological data have demonstrated a significant association between the formation of DNA-adducts and an increased risk of lung cancer (Kyrtopoulos, 2006, Veglia et al. 2008).
1.4. Sex steroid receptors and lung cancer

1.4.1 Estrogen receptors in lung

Estrogen receptor α (ERα) was discovered by Elwood Jensen in the late 1950s, whereas estrogen receptor β (ERβ) was discovered several decades later in 1996 (Jensen and Jacobson, 1960, Kuiper et al. 1996). The ERα gene (ESR1) is localized to chromosome 6q25.1. The gene corresponding to the full-length protein consists of eight exons. This protein contains 595 amino acid and has a molecular weight of 66216 Da. Twelve distinct splice variants have been identified (National Center for Biotechnology Information, PubMed Gene ID: 2099; Weickert et al. 2008). The ERβ gene (ESR2) also contains eight exons and is localized to chromosome 14q23.2. The corresponding full-length protein contains 535 amino acids with a molecular weight of 59216 Da. To date, at least six different splice variants of the receptor have been identified (National Center for Biotechnology Information, PubMed Gene ID: 2100, 2016).

The ERs are ligand inducible transcription factors and members of the nuclear steroid receptor superfamily. They typically mediate cellular responses to estrogens and share a high degree of sequence homology except for their NH2-terminal domains. Furthermore, they have similar affinities for 17β-estradiol (E2) and bind the same estrogen response elements (ERE) on DNA (Heldring et al. 2007). ERα and ERβ can in some cases, however, show opposite actions. This has been explained by the receptors’ individual capabilities to adopt a multitude of response states dependent on the bound ligand, with subsequent unique receptor conformational changes and recruitment of distinct coactivators and corepressors to the receptor-transcription complex in a cell-specific manner (Heldring et al. 2007; Liu et al. 2002).

Ligand binding to ER are followed by hetero- or homodimerization. In the nucleus the dimers bind to EREs on DNA and recruit co-activators to help induce target gene expression (Heldring et al. 2007). Alternatively, ERs can interact with transcription factors already bound to the DNA, such as activator protein-1 (AP-1) and stimulating protein 1 (Sp1). In fact, several
estrogen responsive genes do not contain classical EREs, but instead they often contain ERE half-sites, AP-1- and Sp1-sites or combinations of the two (O’Lone et al. 2004).

ERα and ERβ exhibit tissue- and cell-type specific expression, but both receptors are expressed in histologically normal lung, lung tumor tissue, normal bronchial epithelial cells (HBECs) and lung tumor cell lines (Kawai et al. 2005; Mollerup et al. 2002; Stabile et al. 2002). ERα is, however, primarily reported in cytoplasm in human lung tissue (normal and tumor), whereas in HBECs, both receptors were predominantly found in cytoplasm (Ivanova et al. 2009; Stabile et al. 2002). Higher expression levels of both ERα and ERβ have been demonstrated in tumor tissue compared with normal lung (Stabile et al. 2011).

Ivanova et al. (2009) reported that the antiestrogens 4-hydroxytamoxifen (4-OHT) and ICI 182780, respectively, inhibited proliferation of HBECs, and thus indicated endogenous ERs to be transcriptionally active (Ivanova et al. 2009). Moreover, E2-mediated transcription and proliferation in lung tumor cell lines were blocked by exposure to antiestrogens, providing evidence that ERs in lung cancers are functional and transcriptionally active (Hershberger et al. 2005; Shen et al. 2010). In addition, mouse lung was reported to be an estrogen-responsive tissue, with a 5-fold increase in luciferase activity in the lungs of transgenic mice upon E2-treatment (Ciana et al. 2001). Studies in ER knockout mice have revealed a sexual dimorphism in mouse lung. More specifically, in female mice the expression of ERs was required for formation of alveoli of appropriate size and number, whereas in male mice, the receptors had a smaller effect on alveolar dimensions (Massaro and Massaro, 2006).

1.4.2 Hormone replacement therapy (HRT) and antiestrogen treatment

The first two reports of a possible correlation between the use of hormone replacement therapy (HRT) and increased risk of lung cancer were proposed in the late 1980s (Adami et al. 1989; Wu et al. 1988). The evidence of an elevated lung cancer risk in smokers as a consequence of HRT was further strengthened in a small case–control study conducted by Taioli and Wynder in 1994 (Taioli and Wynder, 1994). Newer reports support the notion that HRT-use (combined estrogen and progesterone for > 5 years) elevates the risk of lung cancer and negatively affects prognosis and overall survival (Chlebowski et al. 2009; Ganti et al. 2006; Slatore et al. 2010).
There are, however, studies that do not support an association between HRT-use and increased risk of lung cancer. In a case-control study, self-reported use of HRT in lung cancer patients and healthy age-matched controls suggested an association between the use of HRT and a decreased risk of lung cancer (Schabath et al. 2004). Likewise, in a study by Rodriguez et al. (2008), HRT-use in postmenopausal women and lung cancer incidence by smoking status was reported inversely correlated (Rodriguez et al. 2008). Additionally, the Women’s Health Initiative clinical trials demonstrated no increase in the risk of lung cancer in postmenopausal women treated with HRT (Schwartz et al. 2015), and a recently published study comprising 0.6 million female never-smokers found that current use of HRT in postmenopausal participants did not increase the risk of lung cancer (Pirie et al. 2016). The conflicting results emerging from these studies have emphasized the need for knowledge regarding the specific type of HRT used and the time and duration of use relative to lung tumor development (Siegfried and Stabile, 2014). In that respect, further mechanistic studies on ER and HRT in lung carcinogenesis and lung tumor biology are warranted. Evidently, there are highly complex interactions between multiple factors that may modulate the possible effect of HRT-use on lung cancer risk.

The role of estrogen in lung carcinogenesis has been reinforced by the observation that the use of antiestrogens is associated with decreased lung cancer mortality. Women diagnosed with breast cancer between 1980 and 2003 in the Geneva Cancer Registry, received antiestrogen treatment for their disease, and the epidemiological data showed that the women who received antiestrogen treatment had a 5-fold reduction of lung cancer mortality compared with the expected rates of the general population (Bouchardy et al. 2011). An additional study performed by Lother et al. (2013) also found a significant decrease in lung cancer mortality among women who were treated with antiestrogens both prior to and after their lung cancer diagnosis (Lother et al. 2013).

A recent clinical study reported that the inclusion of antiestrogens (tamoxifen) to standard chemotherapy regimens in treatment of NSCLC is promising. NSCLC patients who received chemotherapy with inclusion of tamoxifen had significantly higher median survival (13.1 months) compared to patients that received chemotherapy only (9.5 months) (Kadzhoian and Shevchenko, 2014, only abstract in english). Moreover, the aromatase enzyme that is essential
for the conversion of testosterone to estrogen, is active in normal lung tissue, lung cancer cell lines and lung tumors (Mah et al. 2007; Marquez-Garban et al. 2009; Weinberg et al. 2005). Estrogen stimulate growth in lung tumor cell lines and high aromatase activity has been detected in lung tumors (Mah et al. 2007; Marquez-Garban et al. 2009). Both aromatase inhibitors and antiestrogens are therefore considered potential anticancer drugs in NSCLC treatment (Lother et al. 2013; Weinberg et al. 2005). Clinical and epidemiological data thus suggest an increased recognition of estrogens in lung carcinogenesis.

1.4.3 A reciprocal association between the signaling pathways of ERs and AHR

Several studies have reported a crosstalk between ER and AHR signaling pathways, but the molecular mechanisms behind this interaction are not fully understood. Most studies support a theory where ER is required for complete AHR-activity. This is partially based on the lack of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced CYP1A1 expression in ER-negative cells that was restored by transient expression of ERα (Jana et al. 1999b, Thomsen et al. 1994).

ERα has been shown to indirectly impact on gene expression of CYP1A1 in estrogen-dependent tissues such as breast and endometrium (Ricci et al. 1999; Thomsen et al. 1994). Moreover, a study by Matthews et al. (2007), showed that stable knockdown of ERα in HC11 mouse mammary cells, was followed by significant downregulation of induced Cyp1a1 expression (Matthews et al. 2007). In addition, the antiestrogen 4-hydroxytamoxifen (4-OHT) has been shown to alter the transcriptional activity of AHR and induce expression of both CYP1A1 and CYP1B1 in breast cancer cell lines. Likewise, the antiestrogen ICI 182780 was reported to induce CYP1A1 expression (DuSell et al. 2010).

Only a few studies have analyzed the ER-AHR crosstalk in lung cells. Normal bronchial epithelial cells transfected with an ERα adenoviral constructs, showed increased constitutive and cigarette smoke extract-induced mRNA and protein expression of CYP1B1, and chromatin immunoprecipitation assay (ChIP) confirmed ERα bound to the CYP1B1 promoter. ERα transfection did not alter the CYP1A1 mRNA levels, but increased the protein levels (Han et al. 2005). In contrast, transient overexpression of ERα in immortalized bronchial epithelial cells (BEAS-2B and BEP2D) had no effect on either basal or TCDD-induced expression of CYP1A1.
and \textit{CYP1B1}. Likewise, exposure of these cell lines to E2 did not influence constitutive or TCDD-induced levels of \textit{CYP1A1} and \textit{CYP1B1} (Berge \textit{et al.} 2004), indicating that the crosstalk may be cell and/or tissue specific. \textit{In vivo}, female ER\textbeta{} knockout mice, but not male mice were protected against development of lung tumors after \textit{in utero} exposure to the polycyclic hydrocarbon dibenzochrysene, indicating that ER\textbeta{} plays a role in lung carcinogenesis in females (Benninghoff and Williams, 2013).

One possible mechanism for the observed crosstalk was proposed by Wormke \textit{et al.} (2003). In this study TCDD was reported to induce an interaction between the AHR and ER\textalpha{}, while exposure to E2 did not induce such interactions. The study suggested a mechanism were TCDD induces formation of a nuclear AHR-complex which coordinately recruits ER\textalpha{} and the proteasome complex, resulting in degradation of both receptors (Wormke \textit{et al.} 2003).

The similarities in molecular actions of ER and AHR, suggest that the two receptors may compete for coactivators necessary for recruitment of the transcription complex to the promoter regions of their respective target genes. Competition for common coactivators may thus provide another explanation for the interaction between the two signaling pathways (Monostory \textit{et al.} 2009).

Besides being under the regulatory control of the AHR, the \textit{CYP1B1} gene promoter contains an ERE that ER can bind to and initiate transcription upon exposure to E2 (Tsuchiya \textit{et al.} 2004). Furthermore, the antiestrogens 4-OHT and ICI 182780 have been shown to effectively inhibit E2-induced \textit{CYP1B1} expression in breast cancer cell lines, and when not co-exposed with E2, 4-OHT exhibited an agonistic function, upregulating \textit{CYP1B1} expression (Tsuchiya \textit{et al.} 2004). This proposes a different mode of action, other than an ER-AHR crosstalk, responsible for estrogenic effects on \textit{CYP1B1} expression.

\textbf{1.4.4 Androgen receptor in lung}

The androgen receptor (AR) is the main mediator of male sex hormones. It is a zinc-finger transcription factor that belongs to the superfamily of nuclear receptors. The \textit{AR} gene is
localized to the X-chromosome (Xq12), and the mRNA corresponding to the full-length protein contains 9 exons. The full-length protein has a molecular weight of 98989 Da and consists of 919 amino acids. To date, at least seven splice variants have been identified (National Center for Biotechnology Information, PubMed Gene ID: 367, 2016; Watson et al. 2010).

In the absence of ligand, the AR resides in the cytoplasm associated with heat-shock and other chaperone proteins. Once androgens bind to the AR, a conformational change takes place, chaperone proteins dissociate and nuclear localization sequences become exposed (Davey and Grossmann, 2016). In the nucleus, homodimers of AR form and bind to androgen response elements (AREs) in the promoter regions of target genes, to which they recruit various coregulatory proteins to facilitate transcription (Tan et al. 2015).

Both human and murine lung show expression of AR, principally in type II pneumocytes and bronchial epithelium (Mikkonen et al. 2010; Wilson and McPhaul, 1996). Several studies have shown that lung tumors express AR, and Mikkonen et al. (2010) reported functionality of the receptor in the human adenocarcinoma cell line A549 that exhibited androgen-dependent gene expression (Mikkonen et al. 2010; Rades et al. 2012). These findings confirmed earlier documented presence of AR in human lung and in NSCLC cell lines (Kaiser et al. 1996).

Androgens are well known regulators of lung development, specifically they delay lung maturation in males compared to females (Carey et al. 2007; Rodriguez et al. 2001). The AR has been implicated to act as a low penetrance cancer susceptibility gene in different tissues, in addition to prostate cancer where it plays a prominent role (Ferroa et al. 2002). Furthermore, in vitro studies have shown that AR may play a stimulatory role in lung cancer metastasis (Chang et al. 2014). Although NSCLC cell lines express AR, and expression levels have been reported to correlate with progression of NSCLC (Yan et al. 2008), only limited reports exist on AR’s role in lung carcinogenesis.
1.4.5 A reciprocal association between the signaling pathways of AR and AHR

The effect of TCDD and other potent AHR-agonists’ on AR transcriptional activity has been extensively studied. TCDD has been reported to decrease androgen-induced PSA expression in LNCaP cells, suggesting that TCDD might disrupt AR transcriptional activity in prostate cancer cells (Jana et al. 1999a). In lung cells, B[a]P has been shown to reduce both constitutive and testosterone induced expression of AR in NSCLC cell line H1355 and in immortalized human bronchial epithelial cell line BEAS-2B. Additionally, exposure to a carcinogenic metabolite of B[a]P (B[a]P-7,8-dihydrodiol-9,10-epoxide) in H1355 cells and human lung fibroblasts WI-38, was shown to significantly repress AR expression in both cell lines (Lin et al. 2004).

Studies in knockout mice revealed that Ahr expression was required for maintaining the inhibitory effect of TCDD on AR-driven gene transcription (Lin et al. 2002), which suggests a potential crosstalk between AHR and AR. Further support of such a crosstalk was found by Barnes-Ellerbe et al. (2004), who reported that AHR directly inhibited AR-activity, and that elevated levels of AHR-ARNT alone were adequate to downregulate AR-function (Barnes-Ellerbe et al. 2004). It has however also been suggested, that AP-1 is at least partially responsible for the antiandrogenic action of PAHs (Kizu et al. 2003).

AR has been shown to interact with the main modulator of CYP1 expression, the AHR, upon exposure to the antiandrogen hydroxyflutamide (OHF) in hepatocellular carcinoma cell lines (Koch et al. 2015). Sanada et al. (2009) showed that dihydrotestosterone (DHT) repressed 3-methylcolantrene (3-MC) induced expression of CYP1 genes in LNCaP and T47D cells. This inhibitory effect of DHT was abolished by knockdown of AR, and a significantly facilitated formation of AR-AHR complex in 3-MC treated cells was reported (Sanada et al. 2009). Furthermore, testosterone treatment in immature pigs in vivo significantly decreased the hepatic expression of Cyp1al and Cyp1a2 in both sexes (Kojima et al. 2008). However only limited data exist on AR’s influence on CYP1 expression in lung. Although evidence toward a reciprocal association between the signaling pathways of AHR and AR exists, further elucidation of molecular mechanisms are necessary.
2. Aims of the study

The aim of this thesis was to clarify associations between PAH-bioactivation, sex differences in susceptibility to PAH and regulatory involvement of steroid receptors. A more detailed outline of the aims included:

- To establish the individual roles of *CYP1A1* and *CYP1B1* in bioactivation of PAH in human lung cells.

- To elucidate whether sex differences in capacity to bioactivate PAH are intrinsic characteristics of human lung cells.

- To examine the modulatory role of sex steroid receptors in regulation of *CYP1A1* and *CYP1B1* expression in human lung cells.
3. Summary of papers

Paper I

Importance of CYP1A1 and CYP1B1 in bioactivation of benzo[a]pyrene in human lung cell lines.

In paper I, the individual roles of CYP1A1 and CYP1B1 in bioactivation of benzo[a]pyrene (B[a]P) were analyzed in immortalized human bronchial epithelial cell line BEP2D and NSCLC cell line H2009. siRNA-technique was used to knockdown expression of CYP1A1 and CYP1B1. Gene expression was measured with RT-qPCR (and normalized to the expression of β-actin), and formation of B[a]P-metabolites in cell culture medium was analyzed by fluorescence-HPLC. Several metabolites of B[a]P were formed in both cell lines. Knockdown of CYP1A1 in BEP2D cells significantly reduced the formation of B[a]P-tetrol I-1 (hydrolysis product of the ultimate B[a]P carcinogen B[a]P-7,8-dihydrodiol-9,10-epoxide I; BPDE I) by 86 %, whereas knockdown of CYP1B1 significantly reduced the formation by 35 %. When both CYP1A1 and CYP1B1 were knocked down the reduction of B[a]P-tetrol I-1 formation was 98 % in BEP2D cells, and similar results were found for H2009 cells. Three other B[a]P-tetrols (also hydrolysis products of B[a]P-7,8-dihydrodiol-9,10-epoxide) were measured, following a similar pattern, although at lower concentrations. Knockdown of CYP1A1 caused higher levels of both B[a]P-cis-7,8-dihydrodiol and B[a]P-trans-7,8-dihydrodiol compared to control cells indicating a considerable role for CYP1B1 in the formation of B[a]P-7,8-dihydrodiol. Significantly higher concentrations of unmetabolized B[a]P were found in cell culture media where both CYP1A1 and CYP1B1 were knocked down. In conclusion, in this paper we showed that the overall B[a]P-metabolism and formation of B[a]P-tetrol I-1 in human lung BEP2D and H2009 cells is primarily dependent on CYP1A1.

Paper II

Sex differences in susceptibility to PAH is an intrinsic property of human lung adenocarcinoma cells.

In paper II, we examined if sex differences in gene expression of important polycyclic aromatic hydrocarbon (PAH) metabolizing enzymes and ability to bioactivate B[a]P are intrinsic characteristics of NSCLC cell lines. Basal, cigarette smoke condensate (CSC)- and B[a]P-
induced gene expression was measured by RT-qPCR and normalized to the expression of \( \beta\text{-actin} \) (arbitrary units). CYP1-enzyme activity after exposure to CSC or B[a]P, respectively, was analyzed by EROD and formation B[a]P-adducts was measured by \(^{32}\text{P}\)-postlabelling in 11 adenocarcinoma cell lines (5 of female and 6 of male origin). Significantly higher levels of \( CYPIA1 \) gene expression were found in lung adenocarcinoma cell lines from women compared to cell lines from men: basal expression (median = 4.40 versus median = 0.23), B[a]P-induced (median= 73.64 versus median = 1.51) and CSC-induced (median = 208.78 versus median = 29.81). No significant sex differences in \( CYPIB1 \) expression were observed in either control cells or B[a]P-/CSC-exposed cells. EROD activity after treatment with CSC was significantly higher in cell lines of female origin compared to those of male origin. Furthermore, significantly higher levels of B[a]P-DNA adducts were found in B[a]P-exposed lung adenocarcinoma cell lines from women (median = 403.6 adducts/10\(^8\) nucleotides) compared to cell lines from men (median = 24.2 adducts/10\(^8\) nucleotides). No significant sex differences in gene expression of \( AHR, ARNT \) or \( AHRR \) were detected. In conclusion, in this paper we showed a significant sex difference in B[a]P-bioactivation capacity in lung adenocarcinoma cell lines of female origin compared to cell lines of male origin. These results confirm previously published data showing sex differences in \( CYPIA1 \) gene expression and hydrophobic DNA-adducts in female compared to male human lung tissue.

Paper III

**Modulatory effect of sex steroid receptors on PAH metabolism genes in human lung cells.**

In paper III, we studied if sex steroid receptors and their ligands could influence expression and induction of PAH bioactivating genes in human lung cells \textit{in vitro}. Gene expression of estrogen receptors (\( ER\alpha \) and \( ER\beta \)) and androgen receptor (\( AR \)) mRNA was measured by RT-qPCR in nine non-small cell carcinoma (NSCLC) cell lines and three immortalized bronchial epithelial cell lines (HBEC). Gene expression was normalized to the expression of \( \beta\text{-actin} \). The effect of siRNA-mediated knockdown of \( ER\beta \) and \( AR \) on basal, CSC- and B[a]P-induced gene expression of \( CYPIA1 \) and \( CYPIB1 \), respectively, was tested in the NSCLC cell line H2009. Furthermore, we examined how sex steroid receptor agonists/antagonists modulated gene expression of \( CYPIA1 \) and \( CYPIB1 \) in H2009. All NSCLC cell lines, with exception of H23 (negative for \( ER\alpha \)) and H838 (negative for \( AR \)), showed detectable expression levels of \( ER\alpha \),
ERβ and AR. However, large interindividual differences in expression levels were evident between the cell lines. When analyzed by sex of the donors of the NSCLC cell lines, no significant differences in expression were apparent. All three HBEC cell lines showed detectable expression of ERα, ERβ and AR. Knockdown of ERβ significantly reduced B[a]P-induced expression of CYP1A1 (56 %) and CYP1B1 (22 %) whereas knockdown of AR significantly enhanced both CSC- and B[a]P-induced expression of CYP1B1 by 78 % and 64 %, respectively. Exposure to ICI 182780, which is a selective ERα protein degrader, induced expression of CYP1A1 and CYP1B1 by 3-fold. Exposure to the androgen dihydrotestosterone (DHT) significantly reduced B[a]P-induced expression of CYP1A1 by 56 % and CYP1B1 by 52 % when compared to cells that only received B[a]P. Thus, knockdown of ERβ and exposure to the ERα specific selective estrogen receptor degrader (SERD) resulted in contradictory effects on CYP1 expression, which may indicate that the two receptors influence transcription in opposite directions. ERβ may be necessary for optimal induction of CYP1 upon PAH-exposure, whereas ERα could be hypothesized to play a suppressing role on CYP1 expression. The observed alterations inflicted on CYP1 expression in AR knockdown- and DHT exposure experiments coincide, suggesting a common mechanism. In conclusion, in this paper we showed that sex steroid receptors might play a role in lung carcinogenesis through the modulation of xenobiotic bioactivation in lung cells.
4. Discussion

In lung cancer etiology, exposure to polycyclic aromatic hydrocarbons (PAHs) plays a central role (Veglia et al. 2008). The sources of inhalation exposure are mainly designated cigarette smoke, urban air and certain occupational environments (Bostrom et al. 2002; Hashim and Boffetta, 2014; Hecht, 2003). The ultimate carcinogenic potential of PAHs generally depend on enzymatic bioactivation yielding highly reactive intermediates that may react with DNA and form PAH-DNA adducts (Shimada and Fujii-Kuriyama, 2004). Cytochrome P450 (CYP) 1A1 and CYP1B1 are important PAH-metabolizing enzymes in human lung, and a clear association between CYP1 expression, formation of PAH-DNA adducts and lung carcinogenesis has been established (Moorthy et al. 2015; Veglia et al. 2008). PAHs that enter lung cells can readily bind to the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor. The expression of CYP1A1 and CYP1B1 in human lung is highly inducible by PAH-exposure through the action of AHR (Shimada and Fujii-Kuriyama, 2004).

Distinct clinical characteristics and unequal lung cancer mortality rates between men and women have suggested the presence of sex differences in tumor biology and risk of lung cancer (Burns and Stabile, 2014). Both epidemiological data and animal studies support the notion that lung tumor biology differs between males and females (Amr et al. 2008; Kiyohara and Ohno, 2010; Marquez-Garban et al. 2007; Ninomiya et al. 2013; Patel et al. 2004; Ramchandran and Patel, 2009; Wakelee et al. 2007; Yang et al. 2005). Although not conclusive, studies indicate that women may be at greater risk of developing lung cancer from cigarette smoking than men (Novello and Baldini, 2006; Patel et al. 2004; Ramchandran and Patel, 2009). Furthermore, women are typically diagnosed at an earlier age than men are (Nagy-Mignotte et al. 2011; Thomas et al. 2015). The rates and the extent of cellular processes such as metabolic activation of procarcinogens, detoxification, DNA-adduct formation and DNA-repair may influence individual susceptibility to the tumorigenic effects associated with PAH-exposure (Benhamou et al. 2002). In that respect, significantly higher CYP1A1 gene expression and levels of PAH-DNA adducts have been reported in female lung cancer patients compared to male patients suggesting a higher susceptibility to PAH among women (Mollerup et al. 1999, 2006).
It has been hypothesized that steroid receptors may play a role in the possible sex differences in lung cancer risk and susceptibility to PAH (Townsend et al. 2012). Both estrogen receptors (ERα and ERβ) (Ivanova et al. 2009; Mollerup et al. 2002; Stabile et al. 2002) and androgen receptor (AR) (Mikkonen et al. 2010; Wilson and McPhaul, 1996) are expressed and functional in human lung cells. Furthermore, in vitro studies have indicated interactions between the signaling pathways of AHR and ERs resulting in altered gene expression of CYP1 genes (Matthews et al. 2007; Ricci et al. 1999; Thomsen et al. 1994). A reciprocal association between AHR and AR has also been suggested by several studies (Lin et al. 2002; Lin et al. 2004). Although the extent and the mechanisms involved are not fully understood, sex steroid receptors may thus be implicated in the regulation of CYP1 genes and metabolic activation of PAH in lung cells.

The major topic of this thesis is associations between PAH-bioactivation, sex differences in susceptibility to PAH and regulatory involvement of steroid receptors in human lung cells. The first paper is designated the respective roles of CYP1A1 and CYP1B1 in bioactivation of B[a]P. The second paper is assigned the elucidation of whether previously reported in vivo sex differences in PAH-DNA adduct formation and CYP1A1 expression are intrinsic characteristics of human lung cells. The last paper is based on an analysis of sex steroid receptors’ potential incursion on CYP1 expression in lung cells.

4.1. The individual roles of CYP1A1 and CYP1B1 in bioactivation of benzo[a]pyrene in human lung cells

Benzo[a]pyrene (B[a]P) is the most thoroughly studied prototype PAH and is often present at high concentrations in PAH polluted environments (Conney, 1982; Kleiner et al. 2004; Pelkonen and Nebert, 1982). CYP1A1 and CYP1B1 enzymes are considered to play important roles in bioactivation of B[a]P to B[a]P-dihydrodiol-epoxides (BPDEs). These electrophilic intermediates, in particular BPDE 1, have the capacity to react with DNA and form B[a]P-DNA adducts, which are considered molecular dose-indicators predictive of cancer risk (Veglia et al. 2008). Individual differences in the ability to bioactivate lung carcinogens like PAH and formation of DNA adducts may be a determining factor in lung cancer susceptibility (Kouri et
Therefore it was important to establish the respective roles of CYP1A1 and CYP1B1 in bioactivation of B[a]P in human lung cells.

In Paper I we showed how siRNA-mediated knockdown of CYP1A1 reduced the formation of B[a]P-tetrol I-1 (hydrolysis product of B[a]P-7,8-dihydrodiol-9,10-epoxide I; BPDE I) by 86 %, whereas knockdown of CYP1B1 reduced the formation by 35 % in BEP2D (immortalized human bronchial epithelial) cells. When both CYP1A1 and CYP1B1 were knocked down the reduction of B[a]P-tetrol I-1 formation was 98 %, with similar results in H2009 non-small cell lung carcinoma (NSCLC) cells. The three other B[a]P-tetrols measured (B[a]P-tetrol I-2, II-1 and II-2) followed a similar pattern, although at much lower concentrations. The highest concentrations of unmetabolized B[a]P were found in cells where both CYP1A1 and CYP1B1 were knocked down. To sum up, in BEP2D and H2009 cells the overall B[a]P-metabolism and formation of B[a]P-tetrol I-1 are primarily dependent on CYP1A1. However, the results also indicated a significant role for CYP1B1 in the formation of B[a]P-7,8-dihydrodiol (B[a]P-7,8-DHD), a precursor of BPDE I.

An early study by Gautier et al. (1996) analyzed the metabolism of B[a]P and B[a]P-7,8-DHD in the presence of human microsomal epoxide hydrolase and either CYP1A1, 1A2, 2C8, 2C9, 2C18, 2C19, 2D6 or 3A4 expressed in Saccharomyces cerevisiae (Gautier et al. 1996). CYP1A1 was shown to have the highest turnover numbers for the formation of all B[a]P metabolites (Gautier et al. 1996), and this is in agreement with the observed significance of CYP1A1 in B[a]P-metabolism reported in Paper I. Further support of a causal relationship between CYP1A1 expression and bioactivation in lung cells was demonstrated in normal lung tissue from lung cancer patients where expression of CYP1A1 and levels of PAH-related DNA adducts showed a strong, significant correlation (overall Spearman ranked correlation coefficient: $r^2=0.66$) (Mollerup et al. 2006). Likewise, Hodek et al. (2013) demonstrated that in rat liver and intestine, increased activity of CYP1A1 significantly enhanced the bioactivation and B[a]P-DNA adduct formation upon oral exposure to B[a]P (Hodek et al. 2013).

Paper I indicated a significant role for CYP1B1 in the formation of B[a]P-7,8-DHD. This coincides with data from Shimada et al. (2001) who showed that CYP1B1, together with
epoxide hydrolase, catalyzed the conversion of B[a]P to B[a]P-7,8-DHD at much higher (10-fold) rate than CYP1A1 in a cell free system of microsomes (Shimada et al. 2001).

In contrast to observations in Paper I, studies in knockout mice orally exposed to B[a]P reported that Cyp1a1 may have a more prominent role in detoxification than bioactivation. Cyp1a1-/- mice given oral B[a]P died within 30 days, whereas Cyp1a1 wild-type mice survived throughout the one-year long experiment. Furthermore, B[a]P-DNA adducts were significantly higher in internal organs of Cyp1a1-/- mice compared to wild-type mice, despite of a significantly lower B[a]P-metabolism and slower clearance (Uno et al. 2004, 2006). The authors concluded that in vivo, Cyp1a1 have a protective role against orally ingested PAH. The elevated level of adducts found in Cyp1a1-/- mice was explained by reduced phase II conjugation due to the lack of coupling between phase I and phase II enzymes in the knockout models (Nebert et al. 2004).

Studies in Ahr knockout mice support the notion that the distributions and levels of B[a]P and B[a]P-protein adducts are clearly dependent on the route of exposure (Godschalk et al. 2000; Sagredo et al. 2006, 2009). Sagredo et al. (2006) reported that orally exposed Ahr knockout mice had higher levels of B[a]P and protein adducts than intraperitoneal or skin- treated knockout mice (Sagredo et al., 2006). Furthermore, in adduct-analysis of skin topically applied with B[a]P, the Ahr wild-type mice showed significantly higher levels of protein adducts compared to Ahr -/- mice. In contrast, analysis of skin distal to the B[a]P-application site revealed significantly lower levels of protein adducts in Ahr wild-type mice compared to Ahr -/- mice (Sagredo et al. 2009). These results coincide with an important role of liver and skin in a first-pass effect that reduces the systemic uptake of B[a]P and thus subsequent formation of adducts in wild-type mice (Nebert, 1989). Our results reveal a central role of CYP1A1 in bioactivation of B[a]P in human lung cells, which differs from studies in Cyp1a1 knockout mice (Uno et al. 2004, 2006). However, the reliability of our results is ensured by studies reporting that differences in target organ toxicity can be designated the route of administration used in the respective experiments (Nebert et al. 2004; Routledge and Shand, 1979; Sagredo et al. 2006, 2009).
In Paper I CYP1B1 was designated a minor role in bioactivation of B[a]P in human lung cells compared to CYP1A1. In other studies however, a PAH-bioactivating role of Cyp1b1 has been strengthened. Studies in Cyp1b1/- mice showed that they exhibited increased protection against 7,12-dimethylbenz[a]anthracene toxicity and dibenzo[a,l]pyrene induced tumors compared to Cyp1b1 wild-type mice (Buters et al. 2002; Page et al. 2003). Furthermore, exposure to dibenzo[def,p]chrysene (DBC) in Cyp1b1 knockout mice resulted in significantly reduced adduct formation in lung when compared to exposed wild-type mice. Cyp1b1 is thus indicated as the primary Cyp1 enzyme involved in bioactivation of DBC to DBC-diolepoxide (Harper et al. 2015). Together, these data illustrate the distinct substrate specificities of CYP1A1 and CYP1B1 toward different PAHs. The respective roles of CYP1A1 and CYP1B1 in bioactivation of different PAHs depend on the chemical structure of the compound (Murray et al. 2001; Shimada et al. 2001; Shimada and Fujii-Kuriyama, 2004). A possible reason for the enzymes’ respective preferences has been argued to be related to differences of hydrogen bond donor/acceptor side chains in the two enzymes putative active site regions (Lewis, 1999). Studies conducted in cell free systems however, indicate that CYP1A1 and CYP1B1 have relatively similar substrate specificities toward various PAHs. The enzymes exhibit the same activity in metabolism of PAH-dihydrodiols such as 7,12-DMBA-3,4-dihydrodiol, DB[a,l]P-11,12-dihydrodiol, 5-methylchrysene-1,2-dihydrodiol and B[a]A-3,4-dihydrodiol (Shimada et al. 2001).

In Paper I, cells transfected with either CYP1A1 or a combination of CYP1A1 and CYP1B1 siRNA showed the highest levels of unmetabolized B[a]P in culture media. When interpreting the results one should however bear in mind that the measured quantities of B[a]P and B[a]P-metabolites, are not an absolute measure due to low recovery during preparation on Sep-Pak C18 cartridges. Nevertheless, the result reveal that the overall metabolism of B[a]P was significantly reduced in cells with downregulated expression levels of CYP1A1. This underlines the importance of CYP1A1 in degradation of B[a]P in human lung cells. Both cell lines, immortalized human bronchial epithelial cells and NSCLC cells, showed similar metabolic profiles and similar effects upon transfection with CYP-siRNA. Together with results from other in vitro and in vivo studies, Paper I provides evidence that CYP1A1 plays an important role in bioactivation of PAH and thus show a stimulating effect on PAH-induced lung carcinogenesis.
4.2 Sex differences in expression of CYP1A1 and capacity to form B[a]P-DNA adducts in human lung cells

Smoking is considered the main causative agent regarding development of lung cancer, and among the potential human carcinogens present in cigarette smoke are ten different PAHs (IARC Monographs Vol 83, 2004). The majority of PAHs present in cigarette smoke have the ability to induce CYP1 expression by ligand-activation of the transcription factor AHR, and CYP1 enzymes are essential in bioactivation of PAH (Shimada and Fujii-Kuriyama, 2004). It has been hypothesized that sex differences in lung cancer tumor biology may partially originate from different abilities to bioactivate procarcinogenic PAHs. Differences in PAH bioactivation could affect susceptibility to PAH, and this hypothesis is strengthened by studies where the expression levels of CYP1A1 and the levels of aromatic/hydrophobic DNA adducts were reported to be significantly higher in the lungs of female smokers compared to the lungs of male smokers. In addition, a strong and significant correlation between CYP1A1 expression and levels of formed PAH-DNA adducts was found, irrespective of sex and smoking status (Mollerup et al. 2006).

In Paper II we verified that previously reported sex differences in CYP1A1 expression and PAH-DNA adduct formation in human lung also are preserved in NSCLC cell lines. More specifically, Paper II showed significantly higher levels of basal, CSC- and B[a]P-induced CYP1A1 gene expression, CSC-induced CYP1 activity and levels of formed B[a]P-DNA adducts in lung adenocarcinoma cell lines from women compared to cell lines from men. In contrast, no significant sex difference in expression levels of CYP1B1, AHR, ARNT or AHRR mRNA were identified. These results are in agreement with previous studies conducted on normal lung tissue from current smokers, where females had a significantly higher median level of CYP1A1 mRNA and significantly higher levels of PAH-DNA adducts compared to currently smoking males (Mollerup et al. 2006).

In Paper II, the observation of high CYP1A1 expression, together with high levels of B[a]P-DNA adducts and high CYP1 enzyme activity, mutually support one another. This association is reinforced by previous studies where coherence between CYP1A1 activity and pulmonary PAH-associated DNA adductions was reported (Bartsch et al. 1992 a,b). Furthermore, current smoke exposure is considered a major determinant of CYP1A1 induction in human lung, and
levels of CYP1A1 expression and DNA-adducts have been shown to significantly correlate in current smokers (McLemore et al. 1990; Mollerup et al. 1999). Likewise, formation of B[a]P-DNA adducts was reported to correlate with CYP1A1 protein expression, but not with expression levels of CYP1B1 protein in normal mammary epithelial cells and breast cancer cell line MCF-7 (Divi et al. 2014).

The degree of interindividual variability in basal expression of CYP1A1 and CYP1B1 and the large differences in CYP1A1 inducibility between the cell lines in the present study coincide with data from lung tissue (Mollerup et al. 2006). Coherence with in vivo data, also applies to the lack of observable sex differences in gene expression of CYP1B1 in both Paper II and Mollerup et al. (2006).

An increased induction of CYP1A1 mRNA expression and CYP1 activity upon exposure to CSC, compared to B[a]P, was observed in Paper II. The CSC used in our experiments has been extracted from the particulate phase of mainstream smoke from Kentucky reference cigarettes 2R4F, and contains at least 13 different PAHs (Roemer et al. 2014). The yield of CSC per cigarette is 26.1 mg, and analysis show that this comprises 8.9 mg tar per cigarette, which makes up 1/3 of its constituents (Richter et al. 2010). Additionally, the CSC contains a complex composition of other compounds, and halogenated aromatic hydrocarbons (HAH) present in CSC may contribute to the observed elevated induction of CYP1A1 in comparison to B[a]P (Wilson et al. 2008).

Several animal studies support the notion of a sex difference in Cyp1a1 expression. In mature Meishan pigs, the hepatic gene expression of Cyp1a1 was reported to be higher in females than in males, but not in castrated males. No such sex difference was reported in immature pigs, but treatment with testosterone markedly decreased the expression levels of Cyp1a1 in both sexes (Kojima et al. 2008). Furthermore, recent studies in mice found that Cyp1a1 gene expression was significantly higher in homogenates from female whole embryos compared to male embryos (Macak-Safranko et al. 2011; Penaloza et al. 2014). Also in rat kidney, throughout the whole life cycle, female rats showed in average 4-fold higher expression of Cyp1a1 than male rats (Kwekel et al. 2013).
The mechanisms behind the observed sex difference in \textit{CYP1A1} expression observed both \textit{in vivo} (Mollerup \textit{et al.} 1999, 2006) and \textit{in vitro} (\textbf{Paper II}) are not clear, although several mechanisms have been proposed. One possible explanation for the observed sex differences in expression of \textit{CYP1A1} could be related to the AHR. However, we were not able to detect any sex differences in measured expression of \textit{AHR} mRNA in \textbf{Paper II}, and the levels of \textit{AHR} mRNA were relatively homogeneous across all the cell lines. \textbf{Paper II} shows significant correlations between expression levels of \textit{AHR} mRNA and \textit{CYP1A1} mRNA, and between \textit{AHR} mRNA and \textit{CYP1B1} mRNA. These results confirm that AHR plays an important role in transcriptional regulation of \textit{CYP1}.

DNA-methylation status of gene promoter regions modulate the accessibility for transcription factors and thus expression of genes. Sex distinct patterns of DNA-methylation in the promoter of \textit{Cyplal} have been reported. The gene expression of \textit{Cyplal} was reported higher in both embryonal and adult lung cells from female mice compared to male mice. Four distinct sites in the promoter region of \textit{Cyplal} were methylated in female mice, whereas in males only three sites showed methylation (Penaloza \textit{et al.} 2014). When exposed to E2, the methylation status was altered, and the sex difference in \textit{Cyplal} gene expression diminished between female and male mice. This may indicate that E2 normally helps maintain the sex difference in gene expression since only female mice usually become exposed to E2 (Penaloza \textit{et al.} 2014). In contrast, quantitative analysis of \textit{CYP1A1} enhancer DNA methylation levels in normal lung tissue from NSCLC patients revealed no such sex differences (Tekpli \textit{et al.} 2012). The results, however, indicated that low levels of methylation were associated with high mRNA expression of \textit{CYP1A1}. The study also reported that the methylation levels were significantly higher in never-smokers indicating that smoking may alter DNA-methylation status of this gene.

A hypothesis of the involvement of steroid hormones in lung tumorigenesis and sex differences in risk of lung cancer has been proposed (Haugen, 2002; Rivera, 2013). This hypothesis may be extended to the observed sex differences in \textit{CYP1A1} gene expression and PAH-DNA adduct formation \textit{in vitro} (\textbf{Paper II}) and \textit{in vivo} (Mollerup \textit{et al.} 1999, 2006). Accordingly, the main objective of \textbf{Paper III} was to analyze whether sex steroids and their receptors could influence \textit{CYP1} expression in lung cells.
To sum up, the importance of CYP1A1 in bioactivation of B[a]P reported in Paper I, and the relationship between expression levels of CYP1A1 and elevated levels of B[a]P-DNA adducts in female cell lines compared to male cell lines reported in Paper II, mutually strengthen one another. Combined, the in vitro data from Paper I and Paper II lend support to the previously published in vivo data (Mollerup et al. 1999, 2006), and strengthen the hypothesis that female lung may be more sensitive to PAH-exposure than male lung. The vast majority of present knowledge on biochemical processes and signaling pathways in human cells have been obtained in cell lines with no knowledge on or attention to their sex origin (Shah et al. 2014). The significant sex differences in gene expression, enzyme activity and DNA adduction observed in Paper II, encourage the notion that the sex of cell lines used during in vitro studies should be considered to the same extent as in the case of in vivo studies.

4.3 Sex steroid receptors’ impact on CYP1 regulation in lung cells

Evidence has accumulated toward sex differences in the biology of lung cancer, presented by distinct clinical characteristics and unequal mortality rates. Experimental studies indicate that sex steroid receptors may play a role in the pathogenesis of lung cancer, although the exact role of sex steroids and their receptors remains unclear (Burns and Stabile, 2014). In Paper III we studied the modulatory effect of ER and AR on PAH metabolism in human lung cells through siRNA-mediated receptor knockdown and exposure to a panel of sex steroid receptor ligands.

In Paper III we report that ERα, ERβ and AR mRNA are expressed in both NSCLC and human bronchial epithelial (HBEC) cells. This coincides with earlier gene expression analyses of sex steroid receptors in lung tissue and lung cell lines (Ivanova et al. 2009; Mikkonen et al. 2010; Mollerup et al. 2002; Stabile et al. 2002). Furthermore, we show that knockdown of ERβ and AR influence the mRNA levels of CYP1A1 and CYP1B1 in the human NSCLC cell line H2009. However, the two receptors seem to modulate the expression levels in divergent directions. More specifically, siRNA-mediated ERβ knockdown significantly downregulated B[a]P-induced gene expression of both CYP1A1 and CYP1B1, whereas AR knockdown significantly upregulated both CSC- and B[a]P-induced gene expression of CYP1B1. Exposure to sex steroid receptor ligands also modulated the expression levels of CYP1A1 and CYP1B1. The ERα
specific degrader ICI 182780 induced gene expression of both CYP1A1 and CYP1B1, whereas exposure to dihydrotestosterone (DHT) significantly decreased B[a]P-induced mRNA levels of CYP1A1 and CYP1B1 in H2009 cells. Together these observations support the hypothesis of a role of sex steroid receptors in modulation of CYP1 expression.

The NSCLC cell lines analyzed in Paper III showed no sex difference in expression levels of ERα, ERβ or AR. This is in agreement with former studies where no sex difference in gene expression of ER has previously been reported in human lung (Ivanova et al. 2009, 2010; Mollerup et al. 2002; Stabile et al. 2002). However, Dougherty et al. (2006) reported an increased transcriptional activity of ER present in NSCLC cell lines of female origin compared to cell lines of male origin, and the cellular localization of the sex steroid receptors may play a role in their transcriptional activity and hence their functionality (Dougherty et al. 2006; Ivanova et al. 2009, 2010; Kawai, 2014). Regarding expression of the AR, at present little or no information on sex differences in expression and/or activity in human lung exists.

In Paper III, ERβ and AR were chosen as knockdown targets based on their expression levels in lung cells and their previously described importance in lung development and maturation (Patrone et al. 2003; Rodriguez et al. 2001). Furthermore, exclusion of ERα knockdown in this study was decided based on previous transfection experiments with ERα in lung cells conducted by our group, which showed no alterations on the expression levels of neither CYP1A1 nor CYP1B1 upon overexpression of ERα (Berge et al. 2004).

Knockdown of ERβ mRNA resulted in subsequent downregulation of B[a]P-induced CYP1A1 and CYP1B1 gene expression. Downregulation of CYP1B1 may be related to presence of an active estrogen responsive element (ERE) in the CYP1B1 promoter region (Tsuehiya et al. 2004). Likewise, Penaloza et al. (2014) identified an ERE in the promoter region of Cyp1a1 in mice, indicating that ERs also directly interact with transcriptional initiation of Cyp1a1 expression (Penaloza et al. 2014). Limited amounts of available ERβ after siRNA-treatment may thus inhibit normal transcription of CYP1A1 and CYP1B1. Additionally, a basic transcription element (BTE) has been reported to control the constitutive expression of CYP1A1 (Yanagida et al. 1990). The BTE binds Sp1, a transcription factor known to directly interact with ER forming an ER-Sp1 complex (Imataka et al. 1992; Safe and Kim, 2004). Thus, a
decrease in $ER\beta$ may reduce binding to this BTE, and hence reduce normal expression levels of $CYP1A1$.

We found that ICI 182780 induced expression of $CYP1A1$ and $CYP1B1$ in the NSCLC cell line H2009. ICI 182780 degrades ER$\alpha$ (Yeh et al. 2013), and simultaneously induces expression of $CYP1A1$ and $CYP1B1$. The presence of ER$\alpha$ may thus contribute in the opposite direction as ER$\beta$, suppressing $CYP1$ expression. ICI 182780 may also function as an AHR-agonist. Several synthetic ER ligands, including ICI 182780, possess the ability to activate AHR and significantly enhance expression of $CYP1A1$ in MCF-7 breast cancer cells (DuSell et al. 2010).

Upregulated levels of B[a]P-/CSC-induced $CYP1B1$ gene expression in cells pretreated with AR siRNA, may be related to the presence of several steroidogenic factor-1 (SF-1) responsive elements in the upstream enhancer region of $CYP1B1$ (Zheng and Jefcoate, 2005). The AR has been reported to suppress transcription of genes regulated by SF-1, involving a mechanism where AR directly binds to already DNA-bound SF-1 in the promoter region of the distinct genes, and inhibits transcription initiation (Jorgensen and Nilson, 2001).

In **Paper III**, DHT repressed B[a]P-induced gene expression of both $CYP1A1$ and $CYP1B1$. The observed alterations inflicted on $CYP1$ expression in AR knockdown- and DHT exposure experiments coincide, and implicate a common mechanism for the modulatory effect on $CYP1$ expression. The mode of action may involve whether or not an AR-SF-1 interaction takes place in the promoter region of $CYP1B1$. DHT-bound AR may translocate to the nucleus where it will bind directly to SF-1 proteins already present on the promoter of $CYP1B1$, and elicit the observed reduced induction. The mechanism underlying DHT-induced repression of $CYP1A1$ is unknown. However, a possible involvement of activator protein-1 (AP-1) in the anti-androgenic effects of AHR-agonists have been suggested. More specifically, this mechanism is suggested to involve AHR-stimulated $AP-1$ expression and AP-1 is known to inhibit binding of AR to androgen responsive elements (AREs) by protein-protein interaction with the receptor (Kizu et al. 2003).
Animal studies have indicated that 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung carcinogenesis may be inhibited by testosterone (Ninomiya et al. 2013). These data, together with reports showing the importance of androgens in lung development and maturation (Rodriguez et al. 2001) and results presented in Paper III, support that lung tissue is responsive to androgen exposure.

Several studies have reported that the signaling pathways of ER and AHR may interact in a crosstalk (DuSell et al. 2010; Jana et al. 1999b; Matthews et al. 2007; Ricci et al. 1999; Thomsen et al. 1994; Wormke et al. 2003). In normal bronchial epithelial cells, Han et al. (2005) reported increased constitutive and cigarette smoke extract-induced mRNA and protein expression of CYP1B1 in cells transfected with ERα-adenoviral constructs. In addition, ERα was confirmed to bind to the CYP1B1 promoter. Still, ERα transfection did not alter the CYP1A1 mRNA levels, but increased CYP1A1 protein levels (Han et al. 2005). A reciprocal influence has also been reported between the AR and AHR, but only limited data exist (Lin et al. 2002; Lin et al. 2004). A theory of competition for common nuclear receptor coactivators necessary for recruitment of the transcription initiation complex to the promoter regions of their respective target genes has been proposed as an underlying mechanism for such crosstalks. Nuclear receptor coactivator-1 (NCOA1), CREB binding protein (CBP/p300) and Sp1 are all regulatory proteins involved in transcription initiation mediated by several nuclear receptors including AHR, ERs and AR (Esser and Rannug, 2015, Heldring et al. 2007). A rivalry for common coactivators may provide an explanation for the observed alterations on AHR transcriptional activity (Monostory et al. 2009).

Cancer susceptibility as a consequence of sex steroid receptor expression and exposure to sex steroids has been indicated in studies conducted on Ar knockout mice. The results revealed that exposure to NNK-B[a]P contributed to lower lung tumor incidence and reduced tumor size when compared to wild-type mice receiving the same treatment (Yeh et al. 2012). Additionally, female A/J mice were confirmed to be more susceptible to NNK-induced lung carcinogenesis than male mice (Ninomiya et al. 2013). NNKs like PAHs, are bioactivated by CYP1A1, and in male mice, lung carcinogenesis was significantly increased by castration, whereas in females, malignant transformation of lung proliferative lesions tended to be inhibited by ovariectomy (Ninomiya et al. 2013).
In conclusion, Paper III showed that knockdown of ERβ or AR and exposure to sex steroid receptor ligands, respectively, have a significant impact on CYP1 gene expression. The results support a role of sex steroid receptors in modulation of xenobiotic bioactivation in lung cells. These mechanisms could also be speculated to play a role in the previously reported higher levels of CYP1A1 expression and PAH-DNA adduction in both female NSCLC patients and NSCLC cell lines derived from women compared to their male counterparts (Mollerup et al. 1999, 2006, Paper II). However, further clarification of mechanisms behind the modulatory effects is warranted. The matter of sex differences in risk of lung cancer is still not completely sorted out. Of particular relevance for this thesis, a specific mechanistic linkage between steroids and sex differences in CYP1A1 expression and formation of PAH-DNA adducts is still incomplete and should be further examined.
5. Conclusions and future perspectives

The work presented in this thesis has provided new insight regarding regulation of PAH bioactivation in lung cells. In conclusion, we have:

✓ Established that CYP1A1 plays a major role in bioactivation of B[a]P in lung cells.
✓ Verified that significantly higher levels of CYP1A1 expression and significantly higher levels of B[a]P-induced DNA adducts is an intrinsic capacity in lung cell lines from women compared to cell lines from men.
✓ Showed that sex steroid receptors may modulate the expression of important PAH-metabolism genes in lung cells.

Data provided by this thesis indicate that female lung cells have higher PAH-bioactivation capacity than male lung cells, and that sex differences in expression of CYP1A1 may be mechanistically involved in these differences. However, our results warrant further exploration, and the need for clarification of molecular mechanisms in the matter of differences in lung tumor biology between women and men is invoking.

Tobacco smoking is still recognized as one of the world’s most important health threats. The epidemic of smoking is rising amongst women globally, and female smoking is predicted to double between 2005 and 2025 (Amos et al. 2012). Young urbanized women are often among the first to adopt cigarette smoking in low-income countries, and a link between gender empowerment and smoking has been observed (Hitchman and Fong, 2011). Airway exposure to PAH increases the risk of lung cancer in humans, and lung cancer risk partially depend on the proximity to PAH-emission sources (including tobacco smoking) and individual susceptibility (Shen et al. 2014). An increased susceptibility to PAH amongst women is especially important to clarify in order to implement these findings in anti-smoking campaigns specifically directed toward girls and women (Amos et al. 2012).

Furthermore, sex differences are often inadequately addressed in risk assessment. If and how xenobiotic metabolism is influenced by gender is not always predictable given the current state of knowledge. Differences in gene expression between males and females should be further explored to develop a broader understanding of sex-specific toxicity and carcinogenesis (Vahter et al. 2007). It is estimated that the risk of lung cancer could be underestimated by nearly 50 %
if individual susceptibility is not taken into consideration in risk assessments (Shen et al. 2014). Furthermore, results from this thesis demonstrate that it is important to consider sex of the cell lines when trying to identify and understand the complexity of cellular mechanisms. Male and female cells are not the same (Dewing et al. 2003; Pollitzer, 2013; Shah et al. 2014).
6. References


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