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Significance of polymorphisms in human xenobiotic metabolising enzymes

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Stockholm 2003

Published and printed by Karolinska University Press

ISBN 91-7349-421-6

ABSTRACT

Cigarette smoke is the primary cause of lung cancer but urban air pollution and certain occupational exposures have also been found to elevate the incidence of lung cancer. These exposures contain polycyclic aromatic hydrocarbons (PAH), which can be metabolically activated to highly reactive compounds capable of binding to DNA and initiating the carcinogenic process unless they are eliminated. Interindividual variation in the capacity to activate and eliminate PAH is expected to modulate the individual response to PAH exposures. Different allelic variants (genetic polymorphisms) have been described for several xenobiotic metabolising enzymes including cytochrome P4501A1 (CYP1A1), glutathione transferase M1, P1 and T1 (GSTM1/P1/T1), microsomal epoxide hydrolase (mEH) and NAD(P)H:quinone oxidoreductase 1 (NQO1). In the biomonitoring and epidemiological studies presented in this thesis, susceptibility markers, in terms of well-characterised genetic polymorphisms in biotransformation enzymes, were included in order to improve the precision of carcinogen exposure and health risk estimates.

In the studies on lung cancer risk, 524 lung cancer patients and 530 healthy control subjects of Swedish origin constituted the study population. Lung cancer patients with the *CYP1A1(*1/*2A, *2A/*2A, *1/*2B), NQO1(*1/*2, *2/*2)* and *GSTM1(*O/*O)* variant genotypes were suggested to have an increased risk for certain histological subtypes. Stratification by cumulative smoking dose indicated that the risks associated with the *CYP1A1* and *GSTM1* variant genotypes were restricted to light smokers. In contrast, the *GSTT1*O/*O* genotype appeared to confer protection against lung cancer of all main histological subtypes in heavy smokers.

Genetic susceptibility markers were also applied in the studies of potroom workers in a Swedish aluminum production plant. Ninety-eight workers and 55 unexposed control subjects participated. No significantly elevated levels in any of the genotoxic markers used were found but urinary 1-hydroxypyrene (1-OHP) levels, the internal dose marker, correlated significantly with airborne PAH levels. The aluminum workers carrying the *CYP1A1* and *GSTM1* variant genotypes showed the highest levels of urinary 1-OHP, whereas those with the *GSTT1*O/*O* genotype had lower levels of 1-OHP in urine.

Thus, individuals with the *CYP1A1* and *GSTM1* variant genotypes appeared to have an increased susceptibility to PAH as shown by overrepresentation of these genotypes among smokers with certain lung cancer subtypes and the increased levels of urinary 1-OHP after occupational PAH exposure. Another interesting finding was that individuals with the *GSTT1*O/*O* genotype appeared to be less sensitive to PAH as they were underrepresented among patients of all main histological subtypes and had decreased levels of 1-OHP when occupationally exposed to PAH.

The trimodal distribution of GSTT1 activity measured with methyl chloride in erythrocytes was found to be explained by a recently identified *GSTT1* deletion polymorphism *(GSTT1*O)*. The concordance between the GSTT1 phenotypes and genotypes was very high. A novel polymorphism in the *GSTT1* gene *(GSTT1*B)* associated with the non-conjugator phenotype was subsequently identified. Further

characterisation indicated that this novel allele results in an unstable protein. By including analysis for the novel *GSTT1* polymorphism, the accuracy in predicting the GSTT1 phenotype was improved.

LIST OF PUBLICATIONS

I. Alexandrie AK, Ingelman-Sundberg M, Seidegård J, Tornling G and Rannug A. Genetic susceptibility to lung cancer with special emphasis on *CYP1A1* and *GSTM1*. A study on host factors in relation to age at onset, gender and histological cancer types. *Carcinogenesis* 1994; **15**:1785-90.

II. Alexandrie AK, Nyberg F, Warholm, M and Rannug A. Influence of *CYP1A1*, *GSTM1, GSTT1* and *NQO1* genotypes and cumulative smoking dose on lung cancer risk in a Swedish population. Manuscript.

III. Carstensen U, Hou SM, Alexandrie AK, Högstedt B, Tagesson C, Warholm M, Rannug A, Lambert B, Axmon A and Hagmar L. Influence of genetic polymorphisms of biotransformation enzymes on gene mutations, strand breaks of deoxyribonucleic acid, and micronuclei in mononuclear blood cells and urinary 8-hydroxydeoxyguanosine in potroom workers exposed to polyaromatic hydrocarbons. *Scand J Work Environ Health* 1999; **25**:351-60.

IV. Alexandrie AK, Warholm M, Carstensen U, Axmon A, Hagmar L, Levin JO, Östman C and Rannug A. *CYP1A1* and *GSTM1* polymorphisms affect the excretion of 1-hydroxypyrene in urine after PAH exposure. *Carcinogenesis* 2000; **21**:669-676.

V. Warholm M, Alexandrie AK, Monaghan G, Rane A and Rannug A. Genotypic and phenotypic determination of GSTT1 in a Swedish population. *Pharmacogenetics* 1995; **5**:252-254.

VI. Alexandrie AK, Rannug A, Juronen E, Tasa G and Warholm M. Detection and characterization of a novel functional polymorphism in the *GSTT1* gene. *Pharmacogenetics* 2002; **12**:613-619.

CONTENTS

LIST OF ABBREVIATIONS

AHH	aryl hydrocarbon hydroxylase
AHR	aryl hydrocarbon receptor
ARNT	AHR nuclear translocator
BP	benzo[*a*]pyrene
BPDE	benzo[*a*]pyrene-7,8-dihydrodiol-9,10 epoxide
CA	chromosome aberrations
CDNB	1-chloro-2,4 dinitrobenzene
COPD	chronic obstructive pulmonary disease
CYP450	cytochrome P450
EH	epoxide hydrolase
ELISA	enzyme-linked immunosorbent assay
EROD	ethoxyresorufin-O-diethylase
GST	glutathione transferase
HPLC	high performance liquid chromatography
HPRT	hypoxanthine phosphoribosyl transferase
mEH	microsomal epoxide hydrolase
MN	micronuclei
NQO1	NAD(P)H:quinone oxidoreductases
8-OHdG	8-hydroxydeoxyguanosine
1-OHP	1-hydroxypyrene
OR	odds ratio
PAH	polycyclic aromatic hydrocarbons
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PHA	phytohemagglutinin
RFLP	restriction fragment length polymorphism
ROS	reactive oxygen species
SCE	sister chromatid exchanges
SCGE	single cell gel electrophoresis
ST	sulphotransferase
TLC	thin layer chromatography
UGT	UDP-glucuronosyl transferase
XRE	xenobiotic response element

GENERAL BACKGROUND

INTRODUCTION

Genetic studies have identified some high penetrance genes involved in family cancers. Although such genetic traits may pose high individual risks, they are likely to account only for a minor part of all cancers [1]. Instead, most cancers in the general population probably result from the complex interactions of environmental and individual susceptibility factors over time [2,3]. Examples of the latter factors are age, sex and ethnicity. Other susceptibility factors comprise the low penetrance genes encoding enzymes involved in carcinogen metabolism and DNA repair. Allelic variants of such genes (genetic polymorphisms) have been associated with moderate risk increase for cancer. However, the proportion of cancer attributable to these variants may be large because they are common in the general population.

Polymorphisms in genes of xenobiotic metabolising enzymes are expected to modulate individual responses to genotoxic carcinogens. Incorporation of such susceptibility markers in epidemiological and biomonitoring studies may improve the precision of carcinogen exposure and health risk estimates.

LUNG CANCER

Environmental risk factors

Lung cancer is the most common cancer with more than one million deaths each year [4]. Cigarette smoking is the major determinant and accounts for about 85-90 % of the lung cancer deaths [4]. Exposure to environmental cigarette smoke has also been associated with increased risk for lung cancer although the risk is considerably lower than that of smoking [5]. Cigarette smoke contains several thousand compounds including over 60 established carcinogens [6]. Polycyclic aromatic hydrocarbons (PAH) and nitrosamines occur in relatively small quantities in cigarette smoke but belong to the most potent pulmonary carcinogens known. The most prevalent carcinogens are aldehydes and other volatile compounds such as benzene and butadiene [6].

Besides smoking, occupational exposures are probably the most important risk factors for lung cancer. Occupational exposure to asbestos, certain metals, coal tar and soot have been associated with increased risk for lung cancer [7]. Exposures in certain industries and occupations, including aluminum production, coke production and chimney sweeping, have also been found to elevate the incidence of lung cancer. Although several pulmonary carcinogens are present in these work environments, PAH are the most likely causes for the increased lung cancer incidence.

Other environmental risk factors for lung cancer include air pollution and dietary components. Residential exposure to radon and asbestos, [8,9] and urban air [10,11] have been correlated with higher lung cancer incidence. High fruit and vegetable consumption, particularly a diet rich in carotenoids and isothiocyanates possibly reduce

the risk of lung cancer [12,13]. A protective effect by alpha-tocopherol present in wheat and plant oils has also been reported [14]. High dietary intake of meat and fat is likely to increase the risk of lung cancer [15].

Susceptibility factors

The lung cancer risks from environmental exposures, including tobacco smoke, urban air, food, residential and certain occupational exposures, are strongly influenced by individual susceptibility factors [3]. For example, initiation of smoking at an early age is associated with enhanced risk of lung cancer [16] and female smokers are believed to be at higher risk for lung cancer than male smokers [17]. Furthermore, African-Americans are believed to be at higher risk for developing lung cancer than Caucasians [18].

Only one of ten lifetime smokers develops lung cancer implying that the differential risk for lung cancer may be explained by genetic susceptibility factors [4]. Several of the genes of enzymes involved in metabolic activation and detoxification of pulmonary carcinogens such as PAH are known to be polymorphic in humans. Interindividual differences in the ability to activate and detoxify carcinogens are expected to affect the risk of developing lung cancer.

Histological subtypes

Lung cancer is classified according to different histological cell types. The four major subtypes, squamous cell carcinoma, small cell carcinoma, adenocarcinoma and large cell carcinoma, account for approximately 90 % of all lung carcinomas [19]. Squamous and small cell carcinomas are usually located centrally in the respiratory tract and are believed to derive from ciliated bronchial or bronchiolar epithelium [19]. Adenocarcinomas are usually located more peripherally in the lung and are likely to arise from Clara cells in cuboidal epithelium and type II pneumocytes in the alveolar epithelium [20]. The origin of the less commonly diagnosed large cell carcinomas is uncertain.

Cigarette smoking increases the incidence of all the major histological subtypes but squamous cell carcinoma and small cell carcinoma seem to be most strongly associated with smoking [19]. During recent decades, a change in the distribution of the histological subtypes has been observed. The proportion of squamous cell carcinoma decreases whereas the proportion of adenocarcinoma increases [21]. The reduction of tar and nicotine and the more frequent use of filtered cigarettes have been suggested to result in deeper inhalation of smoke. The changes in the tobacco blend have also resulted in increased amounts of nitrogen oxides and nitrosamines in smoke. Thus, the decrease in squamous cell carcinoma is possibly a result of the reduction of tar (PAH) exposure and the increase in adenocarcinoma may be related to higher exposure of the peripheral lung to nitrosamines [21].

POLYCYCLIC AROMATIC HYDROCARBONS

Sources, routes and carcinogenicity

PAH are widely distributed environmental pollutants and many of them are potent carcinogens. They constitute a wide class of structurally related compounds composed of two or more benzene rings that are formed during incomplete combustion of organic material. PAH are always found as complex mixtures of varying composition and concentration depending on their origin.

The general population is exposed to PAH primarily via food and air. High concentrations of benzo[a]pyrene (BP), one of the most carcinogenic PAH, have been found in grilled and barbecued meat (4 ngBP/g) and in certain cereals and greens (0.5 ng/g [22]. PAH in air derive from residential heating, vehicle exhaust and emission from certain industrial processes and power plants. In Europe, the BP concentrations in air are often below 1 ng/m^3 with peak concentrations up to 5 ng/m^3 near heavily trafficked routes [23]. Another source of PAH is cigarette smoke. The amount of BP in the mainstream smoke of one cigarette is 20-40 ng [24].

The highest PAH levels in air are found in the work environment. Heavy exposure to PAH has been found in certain industries such as aluminum production, coal gasification, coke production and in occupations such as chimney sweeping and road paving [7]. The main route for occupational PAH exposure is inhalation but in certain occupations skin uptake has been shown to be of importance. Examples of such occupations are the aluminum production and chimney sweeping. In western countries, the BP levels have often been found to range from 0.1-10 µg/m^3 in industries such as aluminum production, coal gasification and coke production [5]. Sweden has a permissible exposure limit for airborne BP in the work environment of 2 µg/m^3.

The first report of the carcinogenicity associated with PAH exposure is from 1775 when Sir Percival Pott attributed the high occurrence of scrotal cancer in sweeps to soot exposure. The carcinogenic agents in soot were identified as PAH in the early 1900s [25]. IARC has classified several different PAH mixtures and some occupational exposures in which high exposure to PAH occur as carcinogenic to humans. Based on a study of coke oven workers WHO has estimated the lung cancer risk following life long exposure to BP at a level of 1 ng/m^3 to 9 extra cancer cases per 100 000 exposed individuals [26]. A similar risk estimate has been calculated for workers in the aluminum production [27,28].

Mechanisms of PAH carcinogenesis

Certain structural properties are needed for PAH to be carcinogenic. Molecules with at least four aromatic rings arranged to form bay- or fjord regions are associated with high carcinogenic activity [29]. Examples of PAH with bay-region and fjord region are BP and dibenzo[a,l]pyrene, respectively. PAH with four benzo rings are mainly found in the gas phase but may also be found together with the more potent high molecular weight PAH in the particulate phase. To exert carcinogenic activity PAH must undergo

metabolic activation by primarily cytochrome P450 (CYP450) enzymes to form reactive electrophilic intermediates that are capable of damaging DNA.

The initial step in chemically induced cancer is believed to be the interaction between the carcinogen and DNA, which causes changes, often in form of DNA adducts. Genetic changes that escape DNA repair may be fixed as mutations following DNA replication. Most initiated cells are removed by apoptosis. Mutations in protooncogenes or tumour suppressor genes may result in dysregulation of cell growth and differentiation. In the following promotion phase the initiated cell is stimulated to proliferate and expand clonally to a (benign) tumour. The tumour progression towards malignancy is probably a result of accumulation of additional genetic damages. The carcinogenesis is strongly influenced by susceptibility factors that may involve altered expression of tumour suppressor genes, impaired ability to repair DNA and altered xenobiotic metabolism.

Certain PAH have high affinity for the aryl hydrocarbon receptor (AHR). This ligand activated transcription factor is involved in the complex regulation of genes controlling biotransformation, and growth and differentiation signal-transduction pathways [30]. AHR mediated induction of CYP450 leads to increased metabolic activation of PAH that may result in enhanced formation of DNA adducts and tumour initiation. The AHR mediated regulation of growth is believed to play an important role in tumour promotion. Thus, PAH act as both initiators and promoters in chemical carcinogenesis.

PAH metabolism, activation and DNA adduct formation

PAH need to undergo oxidation before they can be converted by various conjugation reactions into more water-soluble derivates that can be excreted. The PAH metabolism mediated by CYP450 and epoxide hydrolyse (EH) results in numerous oxidised metabolites including epoxides, dihydrodiols, phenols and quinones. Epoxides can be conjugated by glutathione transferases (GSTs). Quinones may be converted by NAD(P)H:quinone oxidoreductases (NQO1) to hydroquinones which together with phenols and dihydrodiols are conjugated by UDP-glucuronosyl transferases (UGTs) and sulphotransferases (STs).

Some of the CYP450 mediated reactions result in highly reactive PAH intermediates capable of binding to DNA if they escape from further detoxification reactions. The major pathway for metabolic activation is illustrated with BP in Fig 1 [31]. In the initial step, BP is oxidised to BP-7,8-epoxide. This reaction is mainly mediated by the CYP1A1 isoenzyme. The epoxide formed is further hydrated by EH, primarily the microsomal form (mEH), yielding a BP-7,8-dihydrodiol. A second oxidation by CYP450 results in formation of BP-7,8-dihydrodiol-9,10-epoxide (BPDE). Due to the stereoselectivity of CYP450 and mEH, four stereoisomeric forms of BPDE exist. The main metabolite appears to be (+)-*anti*-BPDE, a potent carcinogen that forms stable adducts predominantly with N^2 of deoxyguanosine and causes G to T transversions [32]. Several other PAH including the highly potent dibenzo[a,l]pyrene are activated

via the diol epoxide pathway and subsequently bind to the exocyclic amino group of deoxyguanosine and deoxyadenosine in DNA.

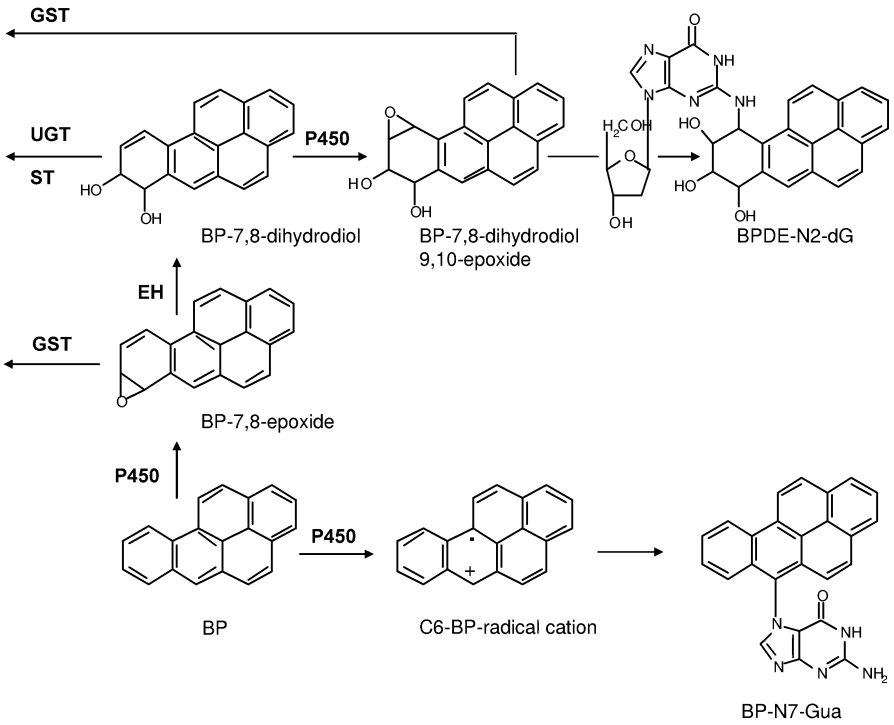

Figure 1. The diol epoxide- and the one-electron oxidation pathways for formation of DNA damage by BP.

The preferential binding sites for (+)-*anti*-BPDE in the nontranscribed strand of the p53 gene in bronchial epithelium cells coincide with the major mutation hotspots for G to T transversions in p53 in human lung tumour tissue [33]. Lung cancer patients with squamous cell carcinoma, the histological subtype which has been most strongly associated with smoking, have proportionally more G to T transversions in the p53 gene than patients with adenocarcinoma in the lung [34]. Bulky adducts including BPDE adducts are primarily repaired by nucleotide excision repair. In a recent study it was shown that the repair of BPDE adducts was slower in the non-transcribed strand of the p53 gene than in the transcribed strand [35]. The rate of DNA repair in the non-transcribed strand was slower at the major damage hotspots than at other damaged sites. Substantial interindividual variations of the DNA repair capacity of bulky DNA adducts have been observed in BP or BPDE treated lymphocytes from healthy subjects [36,37].

BP may also be activated via the one-electron oxidation pathway which generates radical cations that may form adducts to DNA (Fig 1) [38]. Such adducts are mainly unstable and are easily removed through depurination leaving apurinic sites. BP radical

cations may also be transformed via labile phenols to quinones. BP quinones, almost invariable found after BP metabolism, are reduced by NQO1 to hydroquinones. Redox-labile hydroquinones can react with molecular oxygen to form semiquinones and generate reactive oxygen species (ROS). These radicals are responsible for the formation of 8-hydroxydeoxyguanosine (8-OHdG) and DNA strand breaks.

Individuals vary in their ability to activate and eliminate carcinogens. Part of this variability is explained by polymorphisms in xenobiotic metabolising enzymes. Several of the *CYP450* genes encoding enzymes involved in activation of PAH exist as allelic variants. Polymorphisms in the genes of *mEH*, *GSTs* and *NQO1* have also been described. It is likely that polymorphisms in enzymes involved in PAH metabolism are important determinants of individual susceptibility to DNA adduct formation. Increased metabolic activation and deficient detoxification are believed to increase the risk for developing cancer.

PAH disposition and metabolism in lung

At lower levels of PAH exposure, the major part of all inhaled PAH is believed to be deposited on the thin alveolar epithelium [39,40]. The majority of these PAH are within a few minutes absorbed into the blood and subsequently most likely metabolised by the liver. The remaining part of the inhaled PAH is deposited on the tracheabronchial epithelium. PAH are absorbed into blood much slower from the thick bronchial epithelium than from the thin alveolar epithelium, due to the lipophilic properties of PAH. Thus highly lipophilic PAH such as BP (and BPDE) are retained in the bronchial epithelium and consequently the PAH concentration may locally become very high even at lower exposure levels. According to Gerde *et al*, the PAH retained in the bronchial epithelium is most likely metabolised at the site of entry [39,40]. At higher exposure levels, the capacity of the bronchial epithelium to dissolve and metabolise PAH may become saturated. Under these circumstances, the PAH metabolism is accomplished by the liver that may deliver activated PAH metabolites to the lung. Squamous and small cell carcinomas are usually located centrally in the respiratory tract and are believed to derive from ciliated bronchial or bronchiolar epithelium. Thus, the site of origin for these lung carcinomas appears to coincide with the area with high local concentrations of PAH.

Lung tissue consists of more than 40 cell types which display various profiles and expression levels of the enzymes involved in activation and elimination of PAH. CYP1A1 is the major enzyme responsible for PAH activation in lung but other CYP450 isoenzymes active towards PAH such as CYP1A2, CYP1B1 and CYP3A4 are also expressed in the lung [41-43]. Examples of other relevant enzymes present in lung are mEH and NQO1 [44,45]. GSTP1 and to a lesser extent GSTT1 and GSTM1 have also been found in lung tissue [41].

Although the major part of all inhaled PAH is metabolised in the liver the metabolic capacity of lung to activate and eliminate PAH most likely influences the aetiology of lung cancer. Interindividual susceptibility to lung cancer may partly be explained by

differences in the expression of the enzymes involved in PAH metabolism between individuals.

BIOMARKERS

Introduction

Biomarkers are thought to reflect disease risk in individuals because they indicate exposure of a causative agent or represent an early stage in the development of disease. In addition certain biomarkers allow assessment of individual susceptibility. Polymorphisms in genes controlling metabolism of the causative agent or DNA repair are examples of susceptibility markers modifying the relationship between exposure and disease at several levels. The incorporation of biomarkers along the continuum from exposure to disease is shown in Fig 2. Biomarkers are usually defined as markers of exposure, markers of effect and markers of susceptibility.

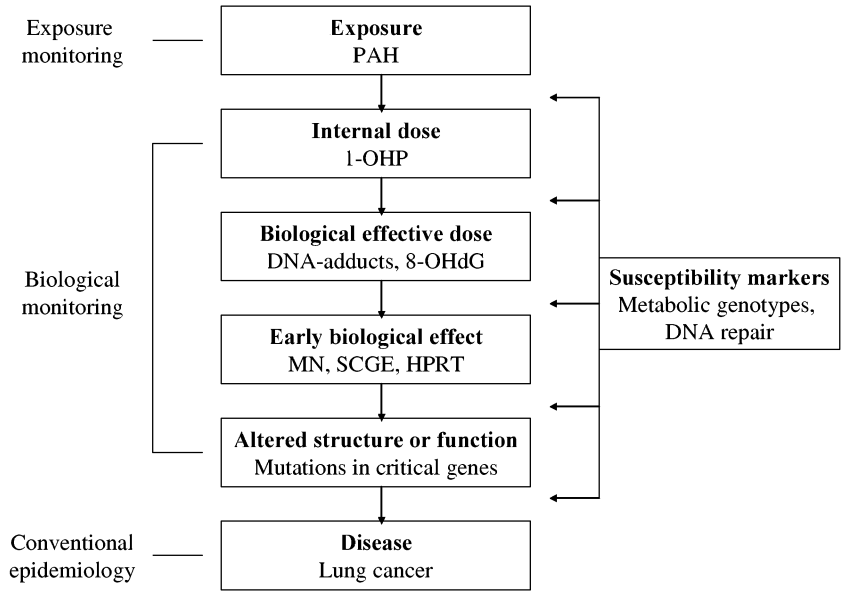

Figure 2. Markers of exposure, effect and susceptibility in molecular epidemiological research.

Markers of exposure

Markers of exposure may be the parent molecule itself or more often a metabolite in body fluids that provides the internal dose measure. Pyrene is one of the most abundant compounds in PAH mixtures but is not carcinogenic. Pyrene is metabolised via CYP1A1 to 1-hydroxypyrene (1-OHP) and is readily excreted in urine as the corresponding glucuronide [46]. 1-OHP has gained an established position as a biomarker for monitoring occupational exposures to PAH [47].

Markers of effect

DNA adducts are expected to reflect the biologically effective dose. Samples from target tissue of PAH are often not available and therefore white blood cells have often been used as surrogate tissue. In recent studies, the aromatic DNA adduct levels in mononuclear blood cells were reported to correlate significantly to the DNA adduct levels in lung tissue [48]. Elevated levels of DNA adducts in white blood cells have been reported in smokers and PAH exposed [49]. The formation of carcinogen DNA adducts is thought to be a crucial step in chemically induced carcinogenesis. Thus, it is expected that individuals with high levels of DNA adducts are at higher risk of developing cancer. A prospective study showed that smokers who had elevated levels of aromatic DNA adducts in white blood cells were approximately three times more likely to be diagnosed with lung cancer 1-13 years later than smokers with lower adduct levels [50]. Presence of PAH DNA adducts are routinely investigated with the ^{32}P-postlabelling method with TLC which is sensitive, but unable to distinguish adducts from individual PAH. The separation of ^{32}P-postlabelled DNA adducts with HPLC increases the possibility to detect specific DNA adducts upon exposure to complex mixtures such as PAH [51]. A HPLC method with fluorescence detection has been developed which enable the separate analysis of stereoisomers of BPDE [52].

Oxidative DNA damage occurs as a consequence of attack by oxygen species generated as a by-product of cellular metabolism. Oxygen radicals are involved in formation of 8-OHdG and DNA breaks. Several potent carcinogens including BP increase the level of 8-OHdG in animal tissue [53]. 8-OHdG adducts in urine or leukocytes DNA have been used as indicators of oxidative DNA damage in humans. Smokers and PAH exposed workers have increased mean 8-OHdG levels in white blood cells [54,55]. DNA strand breaks is caused by most genotoxic chemicals and is used as a general marker of DNA damage. The “comet assay” or the single cell gel electrophoresis (SCGE) is commonly used in epidemiological studies to estimate the level of DNA breaks in white blood cells. In one study of PAH exposed workers [55], but not in another [56], an elevated levels of the percentage of DNA migrating into the comet tail (indicating the presence of breaks) was observed. The level of DNA adducts and DNA breaks is ultimately controlled by the process of DNA repair and the extent to which this varies between individuals has yet to be established.

Occasionally DNA damages escape the efficient repair mechanisms and may result in chromosomal alterations such as chromosome aberrations (CA), sister chromatid exchange (SCE) and micronuclei (MN). CA are structural alterations, breaks and

rearrangements in chromosomes, usually observed in metaphase preparations. CA have been used as an indicator of early biological effect. In two large studies, increased frequencies of CA in peripheral lymphocytes were correlated with later development of cancer [57]. SCE and MN have been widely used since they are more easily monitored than CA. SCE represent symmetrical exchanges of DNA segments between sister chromatids that generally do not result in alteration of the chromosome morphology. MN are small additional nuclei formed by exclusion of chromosome fragments or of whole chromosomes lagging behind the main nucleus at the end of mitosis. Thus the MN analysis indirectly reflects chromosome breakage or impairment of the mitotic spindle. SCE and MN are also used as markers of early biological effect but whether they predict cancer risk is not fully understood.

The most commonly studied gene for detection of somatic gene mutations *in vivo* is the HPRT (hypoxanthine phosphoribosyl transferase) gene for which a simple selection assay is present. As this gene is X linked, a single mutational event in a cell is sufficient to render it HPRT-deficient. Cells deficient for HPRT survive in a selective medium (6-thioguanine) that kills wild type cells. In foundry workers, the HPRT mutation frequency was correlated with airborne levels of PAH and with PAH-DNA adducts in lymphocytes [58]. Smokers generally have an elevated HPRT mutant frequency [59].

Markers of susceptibility

Markers of susceptibility, such as polymorphisms in genes encoding enzymes involved xenobiotic metabolism and DNA repair, modulate individual responses to genotoxic carcinogens. Incorporation of such susceptibility markers in epidemiological and biomonitoring studies may improve the precision of carcinogen exposure and health risk estimates. Some of the susceptibility markers (i.e. genetic polymorphisms in xenobiotic metabolising enzymes) that have been extensively studied for their influence on tumour occurrence and on biomarkers of exposure and effect are reviewed below. The common denominator for the enzymes selected are their involvement in metabolism of cigarette smoke and PAH.

Cytochrome P450-CYP1A1

CYP450 constitute a large group of enzymes involved in the metabolism of numerous endogenous compounds and foreign chemicals including carcinogens and drugs [60]. They are mainly expressed in the liver but many isoenzymes are also highly expressed in other tissues including the lung, kidney and gastrointestinal tract [61]. The xenobiotic metabolising CYP450 enzymes have broad and to some extent overlapping substrate specificities. They introduce a functional group to the substrate that subsequently can be conjugated by various enzymes to a more water-soluble compound. Although most CYP450 mediated reactions serve to detoxify xenobiotics, they can also activate certain compounds to reactive electrophilic metabolites that possess mutagenic and toxic activity [61]. PAH are known to be metabolised by the CYP1A1, CYP1B1, CYP1A2 and CYP3A4 isoenzymes [62-64]. Many of the xenobiotic metabolising CYP450 are inducible and polymorphic, properties that can

affect the extent of metabolic activated substrates and thus modulate the risk of developing disease.

CYP1A1 is the major enzyme responsible for metabolic activation of PAH. This enzyme is predominately expressed in extrahepatic tissues including lung, lymphocytes and placenta at very low constitutive levels [65]. After induction by PAH and other similar planar compounds, CYP1A1 is also found in the liver [65]. The CYP1A1 induction is primarily regulated at the transcriptional level through the AHR pathway [66]. The unliganded AHR exists in complex with heat shock protein hsp90 within the cytoplasm. Upon ligand binding, the AHR dissociates from hsp90 and enters the cell nucleus. The ligand activated AHR interacts with the AHR nuclear translocator (ARNT) protein to form a complex that followed binding to the xenobiotic response elements (XRE) in the upstream region of *CYP1A1* up-regulates the transcription.

The oxidation of BP to the phenolic metabolite 3-OHBP, usually referred to as aryl hydrocarbon hydroxylase (AHH) activity, in 3-metylcholanthrene induced lymphocytes was found to be trimodally distributed, suggesting that the AHH inducibility is determined by a single diallelic locus [67]. *CYP1A1* or the genes regulating *CYP1A1* transcription were suggested as possible candidate genes for the trimodal distribution since most of the AHH activity has been attributed to CYP1A1. Until date, no polymorphism has been identified that convincingly explain the observed variation in AHH activity. Nevertheless, AHH activity has been positively correlated with CYP1A1 mRNA levels in lymphocytes [68]. Early studies found a correlation between the high AHH inducibility phenotype and increased risk of lung cancer in smokers [69] but others have failed to reproduce these findings [70,71]. CYP1A1 inducibility measured by the more specific ethoxyresorufin-O-diethylase (EROD) assay has also been associated with lung cancer [72,73]. By using the EROD assay, inducibility in mitogen activated lymphocytes was found to be a good marker for lung tissue CYP1A1 inducibility [74]. Pulmonary AHH and EROD activities have been positively related to intensity of immunohistochemical stained lung tissue from smoking lung cancer patients [75]. Furthermore, a positive correlation between pulmonary AHH activity and the level of BPDE adducts in lung from smoking lung cancer patients has been observed [76,77]. In contrast, the AHH level in lymphocytes was not associated with the level of BPDE adducts in lung microsomes [76]. A significant correlation between CYP1A1 mRNA levels and aromatic DNA adducts in lung has been reported [78].

Two closely linked polymorphisms in *CYP1A1*, the 3801T>C substitution in the 3´non coding region that creates a *Msp*I restriction site and the 2455A>G substitution in exon 7 resulting in an amino acid change (Ile462Val), have been extensively studied in relation to CYP1A1 inducibility and lung cancer risk [79,80]. The allele lacking both mutations has been designated *CYP1A1*1*, while the variant allele with only the *Msp*I RFLP (restriction fragment length polymorphism) has been designated *CYP1A1*2A*. The *CYP1A1*2B* allele carries both the *Msp*I RFLP and the Ile462Val polymorphism whereas the in Caucasians extremely rare allele *CYP1A1*2C* only has the Ile462Val polymorphism. The prevalence of the variant *CYP1A1* genotypes is higher in Asians than in Caucasians [81].

The functional significance of these polymorphisms has not been convincingly shown. The variant *CYP1A1* alleles have been associated with higher CYP1A1 inducibility in lymphocytes in some studies [82-84] but not in all [72,85]. The *CYP1A1* polymorphisms appeared not to have any significant influence on lung microsomal EROD activity [86]. Expression studies showed that the CYP1A1 Val462 and Ile462 variants had similar catalytic activity [87,88] but may differ in stereoselectivity [89]. A growing number of studies have also linked the polymorphisms in the *CYP1A1* gene to increased susceptibility to DNA damage. Smokers, lung cancer patients and PAH exposed individuals with variant *CYP1A1* alleles have been associated with higher levels of PAH DNA adducts in white blood cells and lung tissue [90-93]. These alleles have also been correlated with increased occurrence of p53 mutations in lung tumour tissue [94] although another group was not able to reproduce this finding [95]. Additional polymorphisms in *CYP1A1* and in genes involved in the regulation of *CYP1A1* have been described but the effects of these on CYP1A1 inducibility and lung cancer risk remain to be further evaluated.

Microsomal epoxide hydrolase-mEH

Several distinct forms of epoxide hydrolyses have been identified in humans. These enzymes catalyse the hydrolysis of reactive epoxides to their corresponding dihydrodiols. mEH is inducible and expressed in all tissues studied [96]. The enzyme is involved in the metabolism of steroid epoxides but several epoxides of carcinogens and drugs are also known substrates. The substrates of mEH are often specific to this enzyme, with little or no activity of the other EH. Most of mEH activity results in a detoxification but exceptions exist. For example, mEH is involved in the metabolic activation of PAH including BP, a reaction that is highly stereoselective and favours the formation of the most carcinogenic isomer of BPDE [97].

A number of polymorphisms within the *mEH* gene have been identified but only two have been found to alter the enzyme activity [98]. The Tyr113His exchange in exon 3 results in decreased mEH activity whereas the His139Arg substitution in exon 4 increases the mEH activity, probably by affecting the protein stability [98]. Although mEH plays an important role in detoxification of epoxides, evidence proving that these *mEH* polymorphisms contribute to inter-individual differences in susceptibility to carcinogens is not fully elucidated.

Glutathione transferase-GSTM1, GSTP1 and GSTT1

Glutathione transferases (GSTs) comprise a multigene family encoding enzymes that catalyse the conjugation of glutathione to a wide variety of compounds with an electrophilic centre [99]. GSTs play an important role in the cellular defence since they are involved in the detoxification of many carcinogens and environmental pollutants and facilitate their excretion. Certain GSTs appear also to have a role in protection against oxidative stress [100]. GSTs have very broad and often overlapping substrate specificities. Similar to the CYP450 enzymes, the GST isoenzyme profile varies between different tissues and cells. Some GST are substrate inducible, an important

property that allows the cell to adapt to changes in exposure levels. A number of other functions have also been attributed to these enzymes. For example, some GSTs influence cell proliferation by regulating kinase pathways [101,102]. They also act as intracellular binding proteins of some non-substrates including bilirubin [99]. GSTA1, GSTM1 and GSTP1 are involved in detoxification of PAH. They were all found to catalyse glutathione conjugation to various PAH diol epoxides but with different catalytic efficiencies and enatioselectivities [103]. Polymorphisms have been identified in several GSTs and those in the genes of *GSTM1*, *GSTP1* and *GSTT1* have been extensively studied in relation to cancer.

GSTM1 is expressed at high levels in the liver but has also been found in several other tissues including the lung [41,100]. Three allelic variants have been described in *GSTM1*. The functional *GSTM1*A* and *GSTM1*B* alleles, differing by a single base in exon 7 (Lys173Asn), appear to have similar catalytic activities [104]. The *GSTM1*O* allele is a result of a gene deletion and as a consequence the enzyme is missing [105]. The frequency of the GSTM1 null phenotype (*GSTM1*O/*O*) is in general higher in Caucasians and Asians (50%) than in Africans (25%) [81]. The GSTM1 null phenotype has been related to increased levels of PAH-DNA adducts and various cancers [92,93,100,106].

The GSTP1 enzyme is widely expressed and is the major GST in lung. Two partly linked polymorphisms, one resulting in an Ile105Val change in exon 5 and the other in an Ala114Val change in exon 6, have been detected in the *GSTP1* gene [107,108]. Since residue 105 is part of the binding site for electrophilic substrates, the Ile105Val change may result in altered enzyme efficiency for some substrates. In expression studies, the Ile105 variant metabolised 1-chloro-2,4 dinitrobenzene (CDNB) more efficiently than the Val105 variant [108,109]. Lung tissues from individuals homozygous for the Ile105 allele had the highest activity towards CDNB followed by individuals heterozygous and homozygous for the Val105 allele [110]. *In vitro* studies revealed that the Val105 variant had higher activity towards various diol epoxides of PAH including +-*anti*-BPDE than the Ile105 variant [111]. On the other hand the Val105 allele has been associated with higher levels of bulky DNA adducts and increased risk of lung cancer [112,113].

The GSTT1 enzyme is expressed in high levels in the liver and in many extrahepatic tissues including the lung [114]. This enzyme is involved in the metabolism of mono- and dihalomethanes, butadiene epoxides, ethylene oxide and other commonly used industrial chemicals. Monohalomethanes, butadiene and ethylene oxide are also constituents in tobacco smoke. These compounds are detoxified by the GSTT1 enzyme but other substrates, for example dihalomethanes such as dichloromethane, are activated [115]. Although the GSTT1 enzyme has low activity towards the major suspected carcinogens in tobacco smoke, PAH, it is possible that the activation of other carcinogens mediated by this enzyme is of more importance for smoking related cancer. The interindividual variation in GSTT1 activity towards halomethanes, with some individuals lacking activity, [116,117] was found to be trimodally distributed [118]. The GSTT1 null phenotype (*GSTT1*O/*O*) is a result of a deletion of the entire *GSTT1* gene [119]. The frequency of the GSTT1 null phenotype is in general lower in

Caucasians (10-25%) than in Africans and Asians (20-65%) [81,115]. Some studies have suggested that individuals with the GSTT1 null phenotype may be more susceptible to genotoxic damage and certain cancers than individuals with the gene [115].

NAD(P)H:quinone oxidoreductase1-NQO1

NQO1 is an obligate two-electron reductase that converts quinones to hydroquinones, which subsequently may be conjugated to facilitate their excretion [120]. Some of the NQO1-mediated reactions are considered as activating since they result in the formation of redox-labile hydroquinones. In reaction with molecular oxygen redox-labile hydroquinones form semiquinones and generate ROS. NQO1 is inducible by a wide range of chemicals including PAH and has been shown to compete with P450 reductase in protecting the cell from mutagenicity of BP quinones [121].

A NQO1 polymorphism (609C>T) resulting in replacement of proline for serine in residue 187 has been associated with loss of enzyme activity [122]. The lack of activity in individuals homozygous for the variant *NQO1*2* allele appears to be due to lack of protein. An accelerated degradation of the mutant protein by the ubiquitin/proteasomal pathway was suggested as the mechanism behind this finding [122]. The frequency of the homozygous variant *NQO1* genotype varies from 4% in Caucasians to 22% in Asians [123]. Previous studies on the *NQO1* polymorphism and lung cancer have not yielded consistent results [123].

Other polymorphic enzymes

Several other enzymes involved in metabolism of PAH are polymorphically expressed. Examples of such enzymes are CYP1A2 and CYP1B1 [124,125] and myeloperoxidase [126]. Some enzymes involved in conjugation of PAH metabolites such as UGTs and STs have also been found to be polymorphic [127]. Allelic variants of DNA repair genes including the nucleotide excision and base excision repair genes (XPD and XRCC1) have been identified [128].

Significance of genetic polymorphisms

The significance of genetic polymorphisms in relation to biomarkers of exposure and effect and in relation to lung cancer is discussed further in section 2 "the present study".

THE PRESENT STUDY

AIMS OF THE STUDY

The aim of the present study was to evaluate the significance of genetic polymorphisms in human xenobiotic metabolising enzymes.

More specifically, the aims were to;

- evaluate whether genetic polymorphisms in *CYP1A1*, *GSTM1*, *GSTT1* and *NQO1* influence individual susceptibility to smoking related lung cancer

- study if genetic polymorphisms in *CYP1A1*, *mEH*, *GSTM1*, *GSTP1* and *GSTT1* modify associations between external PAH exposures and biomarkers of exposure and effect

- determine the correlation between the GSTT1 phenotypes and common *GSTT1* genotypes and explore the genetic basis for a novel *GSTT1* allele associated with lack of activity

MATERIALS AND METHODS

Study populations

Paper I-II

Patients with lung cancer and chronic obstructive pulmonary disease (COPD) and healthy controls subjects were entered into the study between 1991 and 2000. They were all of Swedish origin and lived in the southern and middle parts of Sweden. The lung cancer cases were recruited from eight different hospitals. One of these hospitals also recruited the COPD patients. At two of the hospitals, the main part of the patients completed a questionnaire about smoking habits whereas such information was available to a lesser extent for the patients recruited at the other six hospitals. The control subjects included laboratory workers, welders and chimney sweeps. In addition, elderly subjects visiting one of the hospitals for ordinary health controls were recruited. Smoking history gathered via interview was available for most of the healthy controls.

Paper III-IV

Male potroom workers from a Swedish primary aluminum Söderberg type of production plant constituted the PAH exposed study group. The male referents were blue-collar workers (mail carriers and city council employees) from the same town as the aluminum smelters and they had not been occupationally exposed to PAH during the preceding 5 years. All subjects were interviewed with respect to medical and occupational history, smoking habits, alcohol consumption and use of personal protection devices.

Paper V-VI

The population consisted of healthy subjects of which most were ethnic Swedes. A group of Saamish subjects was included in the later study.

Ethical approval

The studies were approved by the Ethics Committees of Karolinska Institutet, Stockholm, the University of Lund or the University of Umeå.

Sampling and isolation of DNA and RNA

Paper I-II and V-VI

DNA was prepared from frozen peripheral blood collected in tubes containing EDTA or citrate. After lysis and protein digestion, the samples were subjected to extraction by a modified salt-out procedure or with phenol-chloroform and precipitated with ethanol. Total RNA was isolated from peripheral blood mononuclear cells (PBMC) collected in CPT® tubes (Becton-Dickinson) using a commercial kit (Qiagen RNeasy kit).

Paper III-IV

All sampling were done within an 8-week period. Blood samples were collected in the morning in CPT® tubes (Becton-Dickinson) for lymphocyte preparation or in vacutainer tubes containing citrate for DNA preparation. DNA was isolated using a commercial kit based on solid phase, anion-exchange chromatography (QIAGEN Genomic tips 100/G). Urine samples from the potroom workers were collected before and after work. For the controls, urine samples were collected before work and for five subjects also after work.

Methods

Genotyping, paper I-VI

Genotyping were performed by using different PCR based methods, of which some were previously published and some were developed within the projects. Single nucleotide polymorphisms were analyzed by either PCR-RFLP or by allele-specific PCR. Deletion polymorphisms were analyzed for presence or absence of amplified fragments, mostly together with an internal standard in multiplex PCR.

Phenotyping, paper I and V-VI

For phenotyping of GSTM1, whole blood was lysed and incubated for 15 min with reduced glutathione and tritiated *trans*-stilbene oxide. The reaction was terminated by extraction with hexanol and the radioactive glutathione conjugates of the aqueous phase were determined. For determination of GSTT1 phenotype, 2 ml cytosols from lysed erythrocytes was pipetted into head space vials with addition of 0.125 M sodium phosphate buffer and 10 mM glutathione in a total volume of 5 ml. The vials were incubate at 37°C for 30 min, while being rotated. Thereafter, 1.7 ml air from the gas phase of each vial was withdrawn and substituted by methyl chloride, to an initial concentration of 1000 ppm. Decline in concentration of methyl chloride was followed for 3h. At 0, 30, 60, 120, 180 min, 0.3 ml of the gas phase was withdrawn and the concentration of methyl chloride remaining was analysed by gas chromatography. As a crude measure of the hemoglobin content of the blood samples the absorbance at 415 nm was determined. In addition, GSTT1 phenotyping were performed by using ELISA and Western blot to determine presence or absence of the GSTT1 protein.

RT-PCR, PCR and direct sequencing, paper VI

For screening of potential polymorphisms in the *GSTT1* gene, total RNA from PBMC was treated with DNaseI and cDNA was generated using reverse transcriptase and random hexamers or oligo dT_{20}. PCR primers were designed to amplify the *GSTT1* cDNA but also certain parts of the *GSTT1* gene. Amplified fragments were screened for integrity and purified with a commercial kit. The subsequent DNA sequencing was performed at the Karolinska Institutet Core Facility.

Methods for measuring internal dose and genotoxicity, paper III-IV

For analysis of urinary 1-OHP, urine was submitted to enzymatic hydrolysis followed by a purification step. Samples were analysed by HPLC with fluorescence detection. The concentration of 1-OHP was adjusted for creatinine.

Presence of urinary 8-OHdG adducts was determined by automate coupled column HPLC and electrochemical detection.

DNA strand breaks were analyzed by the SCGE assay. Mononuclear cells embedded in agarose gel on glasses were lysed with detergents. The unwinding of DNA was achieved in an alkaline solution during the following electrophoresis. The cells were stained with ethidium bromide. Cell nuclei with DNA damage has an increased level of single strand DNA outside the nucleus. The cells were identified in a fluorescence microscope connected to a computer with an image analysing program.

For analysis of MN, isolated mononuclear leukocytes were incubated in medium with fetal calf serum using phytohemagglutinin (PHA) as mitogen. The different lymphocyte cell types were separated by magnetic attraction. The cells were smeared on slides and stained. MN were analysed in activated lymphocytes with preserved cytoplasm.

To determine the frequency of cells that carry HPRT inactivating mutations, the T-cell clonal assay was used. Briefly, lymphocytes were incubated in medium with fetal calf serum and human serum using PHA as mitogen. The mutant selection was performed by adding 6-thioguanine to the medium.

RESULTS

Genetic polymorphisms in relation to lung cancer (paper I-II).

Two closely linked *CYP1A1* polymorphisms were early indicated as risk factors for lung cancer in the Japanese population [79,80]. Subsequent studies in Caucasian populations were not able to confirm these findings [129,130]. A *GSTM1* deletion polymorphism was also early related to an increased risk of lung cancer [131]. Although some of the following studies confirmed this association, the risk was considerably lower than that preliminary reported [132,133]. We decided to evaluate the influence of the *CYP1A1* and *GSTM1* polymorphisms on lung cancer risk in the Swedish population.

In the study presented in paper I, 296 lung cancer patients, 79 COPD patients and 329 healthy controls were genotyped for the two *CYP1A1* polymorphisms and the *GSTM1* deletion polymorphism. The frequencies of the variant *CYP1A1* genotypes were similar in healthy control subjects, lung cancer patients and COPD patients. However, the frequencies of the heterozygous *CYP1A1* genotypes (m1/m2, Ile/Val) were twice as high in cases diagnosed with squamous cell carcinoma at "a younger age" (≤65 yrs) than in the healthy controls but the differences were not statistically significant.

The proportion of individuals with the GSTM1 null phenotype was similar in lung cancer patients, COPD patients and controls. The GSTM1 null phenotype was over-represented among cases with adenocarcinoma and small cell carcinoma, a tendency that was even more pronounced in those who had received their diagnoses at a younger age. Although the GSTM1 null phenotype was less frequently found in cases with squamous cell carcinoma, a marked sex difference in the distribution of the GSTM1 phenotypes was found in this group. The frequency for the GSTM1 null phenotype was significantly higher in women than in men. Individuals with the variant *CYP1A1* genotypes combined with the GSTM1 null phenotype were indicated to be at increased risk for contracting squamous cell carcinoma earlier than those with other combinations.

As the *CYP1A1* and *GSTM1* polymorphisms appeared to have a greater impact on lung cancer risk at younger age, we hypothesized that this was indirectly due to the lower smoking dose. The effects of these polymorphisms may be outweighed at higher age because of a higher cumulative smoking dose. In the second study (paper II), we further evaluated this hypothesis by analyzing smoking data more efficiently in an extended study population consisting of 524 lung cancer cases and 530 healthy controls. In addition, the influence of *GSTT1* and *NQO1* polymorphisms on lung cancer susceptibility was studied. Both these polymorphisms are functional and results in a total lack of gene product.

In agreement with the first study, we found no significant differences in the distributions of *CYP1A1* and *GSTM1* genotypes between the lung cancer cases overall and the healthy controls. The frequencies of the *GSTT1* and *NQO1* genotypes were similar in cases and controls. However, stratification by histological subtypes and different smoking variables revealed some associations between these genotypes and

lung cancer. In smokers, the variant *CYP1A1* and *NQO1* genotypes were suggested to be associated with increased risk for squamous cell carcinoma, whereas the GSTM1 null phenotype was associated with a higher risk for small cell carcinoma. In contrast, the GSTT1 null phenotype appeared to confer protection against lung cancer of all main histological subtypes.

Stratification by cumulative smoking dose indicated that the risks associated with the variant *CYP1A1* genotypes and the GSTM1 null phenotype were restricted to light smokers (≤21 packyrs). Unexpectedly, the GSTT1 null phenotype also appeared to be a possible risk factor in light smokers, but in heavy smokers this genotype conferred protection for lung cancer overall. Due to the limited number of light smokers among the cases, we were not able to analyse histological subtypes simultaneously with smoking dose.

Although the frequency of variant *CYP1A1* genotypes combined with the GSTM1 null phenotype was elevated in cases diagnosed with squamous cell carcinoma at younger age also in the second study, the difference was not statistically significant.

Genetic polymorphisms in relation to biomarkers of internal dose and effect (paper III-IV).

Epidemiological studies have shown that aluminum (potroom) workers are at increased risk of lung and bladder cancer and IARC has classified the exposure in aluminum production as carcinogenic. Although several pulmonary carcinogens are present in these work environments, exposure to PAH represents a prominent risk factor. Thus, we evaluated whether the present lower PAH exposure in a Swedish aluminum production plant still represents an increased risk by studying biomarkers of exposure and genotoxic effect. We also investigated whether polymorphisms in genes of xenobiotic metabolising enzymes could explain inter-individual variations in these biomarkers. Ninety-eight potroom workers and 55 unexposed control subjects participated in the study.

Air monitoring and biomarker of exposure

The median concentration of 22 airborne particulate PAH in the breathing zone after a full workday was 100-fold higher in samples from the potroom workers (15.8 $\mu g/m^3$) compared to those from the control subjects (0.11 $\mu g/m^3$). The airborne level of the abundant, but not carcinogenic PAH congener pyrene, was highly correlated with levels of total PAH and BP. The potroom workers had a 30-fold higher median level of the pyrene metabolite 1-OHP in their urine than the controls in samples collected immediately after work. The excretion of 1-OHP was found to correlate significantly with airborne exposures to pyrene, BP and total PAH. As the inter-individual variation in 1-OHP levels was substantial, univariate- and multivariate regression analyses were used to find variables that could explain part of the variation found.

Smoking controls had significantly higher urinary 1-OHP levels than non-smoking controls, but no such difference was found in the potroom workers. Potroom workers using respiratory protection devices most of the time had significantly lower levels of 1-OHP in urine than workers using protection equipment less frequently or not at all.

Potroom workers with GSTT1 null phenotype had borderline significant lower level of urinary 1-OHP compared to those with the intact *GSTT1* genotype. The exposed subjects with the rare variant *CYP1A1* (Ile/Val) genotype had a level of 1-OHP that was almost twice as high compared to those with the common *CYP1A1* genotype but the differences was not significant. A similar but significant difference was observed in the control group. The highest 1-OHP levels were observed in subjects with the variant *CYP1A1* (Ile/Val) genotype who were also of the GSTM1 null phenotype. This observation was evident for both the potroom workers and the controls.

Multivariate analyses showed that the interindividual variation in the 1-OHP levels could to some degree be explained by differences in airborne particulate associated PAH, use of personal respiratory protection devices and polymorphisms in the *CYP1A1*, *GSTM1* and *GSTT1* genes. Urinary 1-OHP was found to be a sensitive indicator of recent PAH exposure and may to some extent reflect interindividual variations in susceptibility to PAH.

Biomarkers of genotoxic effect

Several biomarkers were used to investigate whether present level of PAH exposure in the aluminum production plant results in genotoxic damages.

The concentration of urinary 8-OHdG, a general marker of oxidative damage, did not differ between potroom workers and control subjects. Furthermore, we could not detect any association between the excretion of 8-OHdG and airborne levels of PAH, internal dose of PAH measured as 1-OHP or smoking in the potroom workers. In analyses including both the potroom workers and controls, the subjects with the GSTM1 null phenotype had a higher level of 8-OHdG in urine compared to the subjects with at least one copy of the *GSTM1* gene. This effect was only seen in non smokers. A similar increase in the concentration of 8-OHdG in urine of non-smokers was observed in subjects with at least one the variant *GSTP1* Val105 allele.

The frequency of DNA strand breaks measured with the SCGE assay in PBMC of the potroom workers did not differ from that found in the controls. DNA strand breaks were not related to any of the exposure measures. In the total group however, individuals with the variant *CYP1A1* (*MspI*) genotypes showed increased median tail inertias compared to those with the common *CYP1A1* genotype.

Potroom workers had similar frequencies of MN in peripheral CD4+ and CD8+ lymphocytes as the referents. The MN frequencies in the CD8+ lymphocytes were significantly correlated with total airborne PAH but only in potroom workers with the common *mEH* exon 3 (Tyr/Tyr) genotype.

No significant difference in the HPRT mutant frequency was observed between potroom workers and control subjects. The smokers had a higher HPRT mutant frequency than the non-smokers. Moreover, the subjects with the variant *mEH* exon3 (His/His) genotype had a significantly higher HPRT frequency as compared with the subjects carrying the other *mEH* genotypes.

In summary, despite the 100-fold difference in the external PAH exposure and the 30-fold difference in the internal exposure between potroom workers and controls, none of the biomarkers of effect employed differed significantly between these groups. The lack of correlations between biomarkers of effect and all exposure measures remained, with one exception, also when the different genotypes were considered. However, the biomarker of internal dose, 1-OHP, was correlated with the external PAH level and the interindividual variation in levels of 1-OHP was partly explained by the *CYP1A1*, *GSTM1* and *GSTT1* polymorphisms.

Polymorphisms in the *GSTT1* locus (paper V-VI)

An interindividual variation in GST activity towards halomethanes, with approximately 40% of the individuals lacking activity, was reported in 1989 [134]. In a previous study including 208 healthy subjects, we found that the GSTT1 activity towards methyl chloride was trimodally distributed, with one group lacking activity [118]. In a study including 16 subjects, Pemble and coworkers showed that the null phenotype coincided with absence of the *GSTT1* gene [119]. We wanted to verify that the *GSTT1* deletion polymorphism correlated with lack of GSTT1 activity in a larger study population than that previously studied.

In almost all study subjects (268/270), a concordance between lack of GSTT1 activity and homozygousity for the *GSTT1* deletion was found. Studies of two families further supported the conclusion that interindividual variation in GSTT1 activity is determined by a single locus with one allele deleted. The frequency of the GSTT1 null phenotype was 10% in our material. This figure was considerable lower than those reported from the previous small studies [119,134].

A remarkable finding was that two of the 270 subjects appeared to have an intact *GSTT1* gene, but nevertheless lacked GSTT1 activity. Since only the *GSTT1* cDNA sequence was known and the protein was reported to be absent in white blood cells, we made no attempt to determine the genetic bases for the observed discrepancy at that time. Later, we found that *GSTT1* mRNA actually was present in lymphocytes. By sequencing of cDNA from individuals with a discrepancy between *GSTT1* genotype and phenotype, we found that a single nucleotide polymorphism resulting in a replacement of threonine for proline in residue 104 was responsible for the missing GSTT1 activity. The novel *GSTT1* polymorphism was confirmed by sequencing PCR products from genomic DNA. The lack of activity was indicated to be a result of absence of GSTT1 protein using immunochemical methods.

By combining the analysis for the novel single polymorphism with two different analyses for the deletion polymorphism, the accuracy in predicting all three GSTT1

phenotypes was improved from 96 to 99%. The frequency of the novel *GSTT1* allele (*GSTT1*B*) was only 1% in ethnic Swedes but was slightly more common in Swedish Saamis.

DISCUSSION

Lung cancer susceptibility

Numerous studies have investigated whether the *CYP1A1* polymorphisms modify the risk of lung cancer and they have reached conflicting conclusions. Studies on Asians [79,80] have in general showed a stronger association than studies on Caucasians [129,130] and African-Americans [135], possibly due to the higher prevalence of the variant *CYP1A1* alleles in Asians. Most studies in Caucasians have not been large enough to make up for the low frequencies of these alleles. Another possibility for the discrepancies observed between the ethnic groups is the differences in linkage between the two *CYP1A1* polymorphisms. The *CYP1A1 Msp*I polymorphism appears to be linked to the Ile462Val polymorphism more frequently in Asians than in Caucasians and rarely in Africans [136], suggesting the *Msp*I polymorphism alone to be of less importance for lung cancer susceptibility. The significance of the *CYP1A1 Msp*I polymorphism has also been questioned since it is located outside the coding region of the gene and consequently does not affect the amino acid sequence of the enzyme. Studies examining the significance of the *CYP1A1* polymorphisms have not been conclusive [86]. Although, the Val462 and the Ile462 variants appeared to have similar catalytic efficiencies *in vitro* [87,88], a recent study found that the Val462 variant was associated with increased formation of the highly carcinogenic +-*anti*-BPDE isomer [89]. The Val462 allele has also been correlated with higher inducibility of *CYP1A1* in lymphocytes [137] and increased levels of PAH-DNA adducts in white blood cells and lung tissues [91,93].

In agreement with our results, an early meta-analysis restricted to non-Asians suggested a small increased risk among light smokers associated with the variant *CYP1A1* genotypes [138]. An elevated risk for squamous cell carcinoma associated with these genotypes was also found. A recent meta-analysis found little evidence for an association of *CYP1A1* polymorphisms with lung cancer risk overall. However, histological subtypes or smoking dose were not taken into consideration in that study.

Studies on *GSTM1* deletion polymorphism in relation to lung cancer in smokers have also yielded conflicting results. Several meta-analyses of *GSTM1* polymorphism and lung cancer risk have been published [138-141] of which the latest also included a pooled analysis of individual data [139]. Although the meta-analyses suggested only a modest increased risk for lung cancer associated with the GSTM1 null phenotype, the majority of the included studies showed an odds ratio (OR) above unity. The association appeared to be more pronounced in Asians than in Caucasians and concerned all three main histological subtypes. As the prevalence of the *GSTM1* deletion polymorphism appears to be similar in Caucasians and Asians [81] the higher risk estimate found in Asians is probably explained by other factors than differences in *GSTM1* genotype frequencies between these populations. A possible explanation is the higher prevalence of the variant *CYP1A1* genotypes in Asians. Thus, the GSTM1 null phenotype will be more commonly found in combination with the variant *CYP1A1* genotypes in Asians than in Caucasians. The influence of combined variant *CYP1A1* genotypes and GSTM1 null phenotype has been implicated from our own studies as

well from studies of DNA adducts in white blood cells from smokers and occupationally PAH exposed subjects [93].
In our study, ever smokers with the GSTM1 null phenotype were at increased risk for small cell carcinoma but also the point estimates for squamous cell carcinoma and adenocarcinoma were above unity. The only meta-analysis addressing the effect of smoking, suggested, in an analysis restricted to Asians, that the association between GSTM1 null phenotype and lung cancer risk was stronger in heavy smokers than in light smokers [138]. We found an opposite association with an increased risk for lung cancer associated with the GSTM1 null phenotype in light smokers. Similar results have been reported by other groups [142-144]. Thus, it has been speculated that the effect of some genotypes is more pronounced at low doses [145]. Some of the inconsistency in the results between studies may also be explained by differences in the definition of light and heavy smokers.

The pooled analysis of the effect of the *GSTM1* polymorphism on lung cancer risk included original data of about 9500 lung cancer cases and controls from the International Collaborative Study on Genetic Susceptibility to Environmental Carcinogens (GSEC) [139]. The adjusted OR for lung cancer associated with the GSTM1 null phenotype was borderline significant with a value of 1.1 and a 95% confidence interval of 1.0-1.2. Adjustment for the effects of age, gender and ethnicity was suggested to explain the slightly attenuated lung cancer risk estimate in the pooled analysis compared to that of the meta-analysis. In agreement with our study, ever smokers with the GSTM1 null phenotype were associated with small cell carcinoma in the pooled analysis.

The role of the *GSTT1* deletion polymorphism and lung cancer susceptibility has also been examined in several recent studies [13,115,146-150]. In agreement with most of these studies, we found no significant association between the *GSTT1* polymorphism and lung cancer overall. On the other hand, the ORs for all the major histological subtypes associated with the GSTT1 null phenotype were below unity. This result was strengthened in an analysis of ever smokers only. This apparent protective effect by the GSTT1 null phenotype has also been observed in other studies [147,149]. Stratification by cumulative smoking dose showed that the protective effect by the GSTT1 null phenotype was restricted to heavy smokers. Surprisingly, the GSTT1 null phenotype was suggested to be a risk factor for lung cancer in light smokers. An association between the GSTT1 null phenotype and increased risk for head and neck cancer in light smokers has previously been reported [151]. However, in a recent study on the *GSTT1* polymorphism and lung cancer in another Swedish population an opposite result was observed, i.e. the GSTT1 null phenotype was associated with increased risk in heavy smokers [148]. Compared to our study, the study by Hou *et al* may have better validity due to stricter epidemiological design, but suffer from less precision due to the limited number of heavy smokers.

The GSTT1 enzyme is known to have a dual function by being involved in both detoxification and activation of carcinogens [115]. As the GSTT1 enzyme has low activity towards the major pulmonary carcinogens, PAH, it is possible that activation of other carcinogens by this enzyme is of importance for lung cancer susceptibility. Thus,

the protective role by the GSTT1 null phenotype on lung cancer risk in heavy smokers observed in our study may be explained by decreased activation of carcinogens. Another explanation may be related to dietary intake of isothiocyanates. It has been suggested that conjugation and elimination of these anti-carcinogenic compounds by glutathione transferases result in an attenuation of their protective effects on lung cancer [13,146]. A third possibility is that GSTT1 influenced cell proliferation by regulating kinase pathways as has been suggested for GSTP1 and GSTM1 [101,102]. The influence of the glutathione transferase polymorphisms on these complex pathways is not known.

The overall impression of previous studies on lung cancer and *NQO1* polymorphism is that the non functional variant *NQO1* allele is associated with increased risk in Caucasians whereas the inverse association has been found in Asians [150,152-158]. We found that the variant *NQO1* genotypes were more common in patients with squamous cell carcinoma. The only study on Caucasians with sufficient number of cases to undertake an analysis of histological subtypes also showed that the variant *NQO1* genotypes were slightly over-represented among patients with squamous cell carcinoma [152]. In the two studies on Asians that take histological subtypes into account, the variant *NQO1* genotypes were instead underrepresented among adenocarcinoma patients [150,156]. However, in the study on Asians, that also included patients with other histological subtypes than adenocarcinoma, the frequency of the variant *NQO1* genotypes were as in Caucasians slightly elevated in patients with squamous cell carcinoma [156]. Thus, the role of NQO1 in lung cancer appear to vary with different histological subtypes.

In a large study on Caucasians, the association between the *NQO1* polymorphism and lung cancer risk was found to differ depending on smoking status, smoking duration and smoking intensity [152]. Due to the limited number of individuals with complete smoking information we were not able to perform such analyses in our material. However, we did not find support for an interaction between the cumulative smoking dose and *NQO1* polymorphism on lung cancer risk.

Our study benefits from consisting of a homogenous population. This is of importance, since ethnic-specific effects on lung cancer susceptibility related to polymorphism in genes of xenobiotic metabolising enzymes have been shown to exist. The large sample size in our study allowed us to divide the material by histological subtypes and smoking status. After the material was restricted to smokers, associations between the genetic polymorphisms and certain histological subtypes were found, showing the necessity to evaluate the histological subtypes separately. When the cumulative smoking dose was considered, the modification of lung cancer risk by some of the polymorphisms was restricted to light smokers. Due to the numerous comparisons performed in the study and the small number of light smokers, caution in interpretation of the results is however required.

The inconclusive results from many studies with regard to genetic polymorphism and susceptibility to lung cancer point to the future need of more rigorously designed studies and larger study populations. Detailed information on participant characteristics

including ethnicity, age, gender, smoking habits, food intake, occupation and tumour site is required to obtain valid results. Biological plausibility of the enzyme selected, functional significance of the genetic polymorphism and the tissue- or cell specific regulation and expression of the gene are other factors of importance. To compensate for small study populations, meta-analyses have become attractive alternatives to study the relation between genetic polymorphism and cancer. Although the meta-analyses achieve sufficient statistical power to detect an average effect across the studies included, it is in general difficult to control for confounding or to take effect modification across studies into account in this type of analysis. Furthermore, studies on interaction between genes appear to be important for identification of subjects at-risk, but analysis of gene-gene interactions requires large study populations and consequently most of the studies so far published suffer from shaky results. Finally, although genetic polymorphisms are associated with a moderate risk increase for cancer, the proportion of cancer attributable to these variants may be large because the variants are common in the general population.

Biomarkers

A rapidly growing number of studies have evaluated the influence of genetic polymorphisms on biomarkers of dose and effect. Many of these studies including our own have focused on individuals that are occupationally exposed to high levels of PAH. Surprisingly, few of these studies had data on the airborne level of PAH in the work environment and therefore they were not able to analyse the correlation between external exposure and biomarkers of dose and effect. Many studies have instead used biomarkers of internal dose such as 1-OHP to reflect the current exposure levels.

In the large collaborative study described in paper III-IV, the airborne levels of BP measured by personal monitoring were within the range of what has been found in other modern aluminum production plants [5]. Although the median BP concentration in air was below the occupational exposure limit in Sweden some of the aluminum workers were heavily exposed and exceeded limit. The potroom workers showed significantly increased levels of 1-OHP in urine and the 1-OHP levels were weakly but significantly correlated to the total PAH levels. Since there was a substantial interindividual variation in 1-OHP levels observed, metabolic polymorphisms were suggested to explain part of this variation. Indeed, the variant *CYP1A1* (Ile462Val) genotype and the GSTM1 null phenotype were associated with elevated levels of urinary 1-OHP. Furthermore, the GSTT1 null phenotype was related to decreased levels of 1-OHP, suggesting a protective role of this genotype. In agreement with our results, Brescia and co-workers reported that the proportion of individuals with combined variant *CYP1A1* (Ile462Val) genotypes and GSTM1 null phenotype was higher in coke oven workers with high levels of urinary 1-OHP than in those with low levels of 1-OHP [159]. Also, Chinese and Korean coke oven workers with the variant *CYP1A1* (Ile462Val) genotypes had higher levels of urinary 1-OHP than those with the common *CYP1A1* genotype [160,161]. In incineration workers, multivariate analyses showed that the GSTM1 null phenotype was positively associated with the levels of 1-OHP [162]. Furthermore, the 1-OHP excretion was higher in smokers with the GSTM1

null phenotype than in those with the *GSTM1* gene [163]. The surprising observation from our study, that the GSTT1 null phenotype was associated with lower 1-OHP levels has been seen also in other studies. Smokers with the GSTT1 null phenotype had significantly lower 1-OHP levels in urine than smokers with the gene present [163]. Korean coke oven workers with the GSTT1 null phenotype also had a decreased level of 1-OHP compared to those with the *GSTT1* gene, but the difference was not significant [161]. Several other studies could not find evidence for any influence of *CYP1A1*, *GSTM1* and *GSTT1* polymorphisms on the excretion of 1-OHP [55,56,162].

No differences in the levels of MN, DNA strand breaks, 8-OHdG and HPRT mutations were found between the potroom workers and controls subject and none of these biomarkers of effect were correlated to any exposure measures. However, some associations between certain metabolic genotypes and these biomarkers were found and have to be evaluated further. Very few positive findings have been published from studies on potroom workers. Thus, no significant increases in the levels of CA, SCE, MN or DNA strand breaks have been reported [164-166]. The assays for HPRT mutations and urinary 8-OHdG have not previously been employed in studies of potroom workers. Taken together the results indicate that the biomarkers of early biological effect employed are not suitable for biological monitoring of present day occupational exposure in aluminum production plant. However, some previous studies have shown increased levels of DNA-adducts in aluminum workers [167,168]. In an earlier publication on the cohort in the present study, total PAH-DNA adducts in PBMC were analysed using a ^{32}P-postlabelling/TLC technique [169]. No significant differences in the levels of PAH-DNA adducts were, however, found between the potroom workers and controls subjects. In order to increase the resolution, the samples were reanalysed with an alternative method using ^{32}P postlabelling/HPLC. In that analysis, a higher proportion of the potroom workers were observed to have high levels of one individual DNA adduct compared to the control subjects [170].

Although the high PAH exposure in the aluminum production plant did not lead to increases in markers of early biological effect (MN, SCGE, HPRT, 8-OHdG), the markers of internal exposure dose (1-OHP) and the biological effective dose (PAH-DNA adducts) were elevated. Thus adverse health effects cannot be excluded at exposure levels that are found in modern aluminum production plants.

GSTT1 polymorphism

The trimodal distribution of GSTT1 activity strongly suggested the activity to be determined by a single locus. The three groups were distinct, showing one group of individuals with high activity towards methyl chloride, one group with intermediate activity and one group with no activity. Although a strict gene dosage effect was seen, the interindividual variation within each group was substantial. This suggests that other factors than the deletion polymorphism identified by Pemble *et al,* also influence the GSTT1 activity [170]. Studies on human GSTT1 inducibility are few, but the expression of GSTT1 was enhanced by dietary intake of crouciferous vegetables [171].

The proportion of non-conjugators in our study population, confirmed by genotyping for the *GSTT1* deletion polymorphism, was considerable lower than those reported from the previous small studies on Caucasians. For almost all subjects a concordance between lack of GSTT1 activity and homozygosity for the *GSTT1* deletion was found. Later studies have indicated that there are differences in the occurrence of the GSTT1 null phenotype, not only between the major races, but also between different Caucasian populations [81]. Interestingly, also other Scandinavian populations studied had a lower frequency of the GSTT1 null phenotype than the rest of the Caucasian population taken together.

In the extended study population, three individuals were identified with what appeared to be an intact *GSTT1* gene but nevertheless they lacked GSTT1 activity. Sequencing revealed that they carried a single nucleotide substitution altering the amino acid residue 104 from threonine to proline (Thr104Pro). Like the previously identified *GSTT1*O* allele, the novel *GSTT1*B* allele co-segregated with the non-conjugator phenotype and had no detectable level of immunoreactive protein. No crystal structure is currently available for GSTT1 but a homology model for human GSTT1 and the crystal structure of human GSTT2 suggest that the novel Thr104Pro polymorphism in GSTT1 resides in the middle of α–helix 4 [172,173]. Proline is rarely found in the middle of an α-helix since this amino acid is prevented from making hydrogen bonds to the preceding turn of the helix with disturbances in helix structure as a result. The homology model predicted the dimer interface of GSTT1-1 to be similar to that of GSTT2-2 up to residue 101 [172]. In the following more divergent region the model suggested a hydrogen bond between Arg107 and residue Thr104B instead of the salt link between Arg107 and Asp104B found in GSTT2-2. Thus replacing Thr104 with Pro possibly also alter the binding between the two subunits in the dimer. In either case, the introduction of a proline at residue 104 most likely distorts the conformation and decreases the stability of the protein. The secondary structure prediction analysis also indicates less protein stability by showing a lower confidence in prediction for α-helix 4 for GSTT1*B than for functional GSTT1*A protein. Further evidence for the importance of residue 104 comes from expression studies showing that replacement of Thr104 to Ala results in very low GSTT1 activity towards dichloromethane [174]. Although the novel *GSTT1* allele was rare in the Swedish population, the allele is of interest as it co-segregates with the non-conjugator phenotype. It remains to be determined whether *GSTT1*B* allele is present in other major ethnic groups.

CONCLUSIONS

The influences of genetic polymorphisms in *CYP1A1*, *GSTM1*, *GSTT1* and *NQO1* on lung cancer susceptibility were more pronounced after smoking variables and histological subtypes were taken into consideration. Ever smokers with the variant *CYP1A1* and *NQO1* genotypes were suggested to be at increased risk for squamous cell carcinoma whereas those with the GSTM1 null phenotype were found to be at higher risk for small cell carcinoma. Interestingly, in ever smokers the GSTT1 null phenotype appeared to confer protection against lung cancer of all main histological subtypes. The increased risk associated with the variant *CYP1A1* genotypes and the GSTM1 null phenotype seemed to be of importance at lower cumulative cigarette doses whereas the protective effect of the GSTT1 phenotype was found after higher smoking doses.

We found that the *CYP1A1*, *GSTM1* and *GSTT1* polymorphisms also influenced the levels of the internal PAH dose marker, urinary 1-OHP. The highest levels of urinary 1-OHP were found in subjects carrying both the variant *CYP1A1* genotype and the GSTM1 null phenotype. This was evident among the heavily PAH exposed aluminum workers but also among the unexposed control subjects, showing that the influence of the *CYP1A1* and *GSTM1* polymorphisms occurs already at low exposure levels. Aluminum workers with the GSTT1 null phenotype had decreased level of 1-OHP in urine. The variance in levels of 1-OHP in the aluminum workers that could be explained by metabolic genotypes seemed to be of the same order of magnitude as that explained by differences in exposure, showing the necessity to take metabolic genotypes into account.

Our studies on lung cancer patients and aluminum workers showed that individuals with the variant *CYP1A1* genotypes and the GSTM1 null phenotype had an increased susceptibility to PAH and that the impact of these genotypes was apparent already at lower PAH exposure. In contrast, subjects with the GSTT1 null phenotype appeared to be more protected against PAH exposure. The agreement in results between the studies and the biological plausibility of the susceptibility markers used, strengthen our findings that genetic polymorphisms do modify the metabolism of PAH and lung cancer risk.

The trimodal distribution of GSTT1 activity in erythrocytes was explained by the recently identified *GSTT1* deletion polymorphism. The concordance between the GSTT1 phenotypes and genotypes was very high. A novel polymorphism in the *GSTT1* gene associated with the non-conjugator phenotype was identified. Further characterisation indicated that this novel allele results in an unstable protein. By including analysis for the novel *GSTT1* polymorphism, the accuracy in predicting the GSTT1 phenotype was improved. The novel allele was found to be rare in the Swedish population but may be more common in other populations. Naturally occuring alleles such as the novel *GSTT1* allele, that obviously have a phenotypic effect, can also provide useful insight into protein structure and function.

ACKNOWLEDGEMENTS

First of all, I want to express my gratitude to my supervisor *Agneta Rannug* for her guidance, encouragement and support over the years.

I also wish to express my sincere thanks to:

Margareta Warholm for sharing her knowledge with me and always having "an open door".

Rainer Tuominen, Yudan Wei, AnnaKarin Nilsson, Ping Huang and *Margareta Ståhlberg* for contributing to the nice atmosphere in the lab.

Inger Johansson for her support and help in the later part of my work .

Friends and colleagues at the Institute of Environmental Medicine and the National Institute for Working Life.

Fredrik Nyberg, Janeric Seidegård, Magnus Ingelman Sundberg and Göran Tornling for fruitful collaboration in the lung cancer studies.

Lars Hagmar and Anna Axmon, for productive collaboration in several occupational studies.

Other co-authors of the publications included in this work: *Ulrica Carstensen, Kari Hemminki, Sai-Mei Hou, Benkt Högstedt, Masayoshi Ichiba, Erkki Juronen, Bo Lambert, Jan Olof Levin, Christer Tagesson, Gunnar Tasa and Conny Östman.*

My *parents* for your support and belief in me.

My husband *Lennart*, for his love and support, and my sons, *Gustav* and *Eric,* for being the very best in my life.

The National Institute of Working Life for the financial support during the years.

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